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Effect of photoperiod on the regulation of wheat vernalization genes *VRN1* and *VRN2*

Jorge Dubcovsky[†,*], Artem Loukoianov[†], Daolin Fu, Miroslav Valarik, Alexandra Sanchez and Liuling Yan[†]
*Department of Plant Sciences, Mail Stop 1, University of California, One Shields Avenue, Davis CA, 95616-8780, USA (*author for correspondence; e-mail jdubcovsky@ucdavis.edu); [†]These authors contributed equally to this work*

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Abstract

Wheat is usually classified as a long day (LD) plant because most varieties flower earlier when exposed to longer days. In addition to LD, winter wheats require a long exposure to low temperatures (vernalization) to become competent for flowering. Here we show that in some genotypes this vernalization requirement can be replaced by interrupting the LD treatment by 6 weeks of short day (SD), and that this replacement is associated with the SD down-regulation of the *VRN2* flowering repressor. In addition, we found that SD down-regulation of *VRN2* at room temperature is not followed by the up-regulation of the meristem identity gene *VRN1* until plants are transferred to LD. This result contrasts with the *VRN1* up-regulation observed after the *VRN2* down-regulation by vernalization, suggesting the existence of a second *VRN1* repressor. Analysis of natural *VRN1* mutants indicated that a *CArG*-box located in the *VRN1* promoter is the most likely regulatory site for the interaction with this second repressor. Up-regulation of *VRN1* under SD in accessions carrying mutations in the *CArG*-box resulted in an earlier initiation of spike development, compared to other genotypes. However, even the genotypes with *CArG* box mutations required LD for a normal and timely spike development. The SD acceleration of flowering was observed in photoperiod sensitive winter varieties. Since vernalization requirement and photoperiod sensitivity are ancestral traits in Triticeae species we suggest that wheat was initially a SD–LD plant and that strong selection pressures during domestication and breeding resulted in the modification of this dual regulation. The down-regulation of the *VRN2* repressor by SD is likely part of the mechanism associated with the SD–LD regulation of flowering in photoperiod sensitive winter wheat.

Abbreviations: LD, long day (16 h light); PAR, Photosynthetically Active Radiation; RNAi, RNA interference; SD, short day (8 h light)

Introduction

One critical trait in the adaptation of temperate grasses to cold winters is the requirement of long exposures to low temperatures (vernalization) to accelerate flowering. The vernalization requirement in temperate grasses is mainly controlled by allelic variation at the vernalization genes *VRN1* and *VRN2* (Yan *et al.*, 2003; Yan *et al.*, 2004b; Jensen *et al.*, 2005; vonZitzewitz *et al.*, 2005), which are different from the Arabidopsis genes with the same name (Gendall *et al.*, 2001; Levy *et al.*, 2002). The wheat and barley vernalization gene *VRN1* is similar to the related meristem identity genes *AP1, FUL*,

and *CAL* in Arabidopsis (Yan *et al*., 2003), which are responsible for the initiation of the transition between the vegetative and reproductive apices. Although the function of this gene is conserved, its regulation seems to differ between wheat and Arabidopsis. Whereas most of the natural allelic variation in vernalization requirement in wheat and barely is associated with mutations in the *VRN1* promoter and first intron (Yan *et al*., 2003; Yan *et al*., 2004a; Fu *et al*., 2005; vonZitzewitz *et al*., 2005), in Arabidopsis this natural variability, is associated to nonfunctional or weak *FRI* and *FLC* alleles rather than to mutations in *AP1, FUL*, or *CAL* (Gazzani *et al*., 2003).

A more significant difference between the vernalization pathways in Arabidopsis and the temperate cereals was found between the vernalization genes involved in flowering repression. The central repressor in the vernalization pathway of Arabidopsis is the MADS-box gene *FLC* (Michaels and Amasino, 1999a), whereas in the temperate cereals is the Zinc finger – CCT domain transcription factor (*ZCCT1*) *VRN2* (Yan *et al*., 2004b). The wheat *VRN2* gene has no clear homologues in Arabidopsis, and the same is true from the Arabidopsis *FLC* in wheat (Yan *et al*., 2004b). These results suggest that the vernalization pathways of these two species (or at least the last regulatory steps of these pathways) evolved independently (Yan *et al*., 2004b).

Mutations in the regulatory regions of *VRN1* are associated with a dominant spring growth habit (*Vrn1*), whereas loss-of function point mutations at the *VRN2* locus (*vrn2a* allele) or complete gene deletions (*vrn2b* allele) are associated with a recessive spring growth habit in diploid wheat (*Triticum monococcum* L.) and barley (*Hordeum vulgare* L.) (Yan *et al*., 2004b; Dubcovsky *et al*., 2005). Down-regulation of *VRN2* by vernalization is followed by the up-regulation of *VRN1* in winter varieties from both species. These two vernalization genes show strong epistatic interactions suggesting that they are part of the same regulatory pathway (Takahashi and Yasuda, 1971; Tranquilli and Dubcovsky, 2000; Dubcovsky *et al*., 2005). In spring lines homozygous for the non-functional *vrn2* alleles, allelic differences in *VRN1* have no effect on flowering time. On the other hand, mutations in the promoter (Yan *et al*., 2003, 2004a) or the first intron (Fu *et al*., 2005) in *VRN1* eliminate or reduce the effect of *VRN2* and result in a spring growth habit (Tranquilli and Dubcovsky, 2000; Dubcovsky *et al*., 2005). These dominance and epistatic interactions suggested a model in which *VRN2* is a direct or indirect repressor of *VRN1* (Yan *et al*., 2003, 2004a). This model was also supported by the observation that the reduction of the *VRN2* transcript levels in transgenic winter plants of hexaploid wheat 'Jagger' (*T. aestivum* L.) by RNA interference (RNAi::*VRN2*) (Yan *et al*., 2004b) results in the up-regulation of *VRN1* and the elimination or reduction of the vernalization requirement.

In some photoperiod-sensitive winter varieties of hexaploid wheat the vernalization requirement can be eliminated or greatly reduced by exposing the plants to short days (SD) for several weeks and then transferring them back to long days (LD) (McKinney and Sando, 1935; Krekule, 1964; Evans, 1987). This phenomenon has not been described in Arabidopsis but is common in winter grasses from the subfamily *Festucoideae* (Heide, 1994). We show here that the SD replacement of vernalization in wheat is associated to the down-regulation of the vernalization gene *VRN2* by SD. In addition, we identify a putative regulatory site in the *VRN1* promoter responsible for differences in the regulation of this gene by SD.

Materials and methods

Plant materials

The *T. monococcum* lines used in this study are listed in Table 1 with their growth habit and genotypes. The transgenic RNAi::*VRN2* lines of hexaploid wheat Jagger were described before (Yan *et al*., 2004b).

We produced three different mapping populations using G3116 as the winter parent to confirm that the spring growth habit in *T. monococcum* accessions PI 503874 (*Vrn1f*), PI 326317 (*Vrn1g*), and PI 306540 (*Vrn1h*) was associated with variation at the *VRN1* locus. Two additional F_2 populations were developed from the crosses between spring line DV92 (*vrn1 vrn2a*) with spring lines PI 266844 (*Vrn1f*, *Vrn2*) and PI 326317 (*Vrn1g vrn2b*) to study the effect of the *Vrn1f* and *Vrn1g* mutations on the transcription of *VRN1* under SD. In the PI 266844 × DV92 population, we first selected homozygous *vrn2a* plants (spring) using

Table 1. Genotypes of *T. monococcum* lines used in this study.

VRN1 allele	*VRN2* allele	Habit	Accession
Vrn1f CArG 1-bp deletion & insertion in intron 1	*Vrn2*	Spring	PI 10474, PI 191096, PI 191097, PI 192063, PI 266844, PI 393496, PI 503874*
Vrn1g CArG 34-bp deletion	*vrn2b* deletion	Spring	PI 326317*, PI 349049, PI 418582
Vrn1h Insertion in intron 1	*Vrn2*	Spring	PI 306540*, PI 94743
vrn1	*vrn2a* CCT mutation	Spring	DV92*, PI 277140
vrn1	*vrn2b* deletion	Spring	PI 286068, PI 323437
vrn1	*Vrn2*	Winter	G3116**, PI 355516, PI 355534.

* GenBank accession numbers: DV92 (AY188331), G3116 (AY485969), PI 306540 (DQ146423), PI 326317 (DQ146422), and PI 503 874 (DQ146421).
** G3116 is the only accession of wild *T. monococcum* ssp. *aegilopoides*. All other accessions are cultivated *T. monococcum* ssp. *monococcum*.

molecular markers, to eliminate differences due to growth habit. We used 7 to 10 plants per genotype.

Molecular markers used in the genetic studies

The polymorphic 1-bp deletion (*Vrn1f*) was converted into a degenerate CAPS marker (dCAPS, Michaels and Amasino, 1999b) using primers VRN1F_F (5′-ACAGCGGCTATGCTCCAG AC-3′) and VRN1F_R (5′- GGAGGATGGCCA GGCCAAATC -3′). The second nucleotide at the 3′ end of the reverse primer (underlined T) was mutated to generate a *Taq*I restriction site for the *vrn1* allele that was absent in the *Vrn1f* allele. The polymorphic 34-bp deletion (*Vrn1g*) was tested using primers VRN1G_F (5′-ACAGCGGCTATG CTCCAG-3′) and VRN1G_R (5′-TATCAGGTG GTTGGGTGAGG-3′) flanking this deletion. Linkage between spring growth habit and the 493-bp insertion in the first intron of PI 306540 (*Vrn1h Vrn2)* was tested using primers VRN1INT1_F (5′-ATGAAACTCCGGCATGA AGA-3′) and VRN1INT1_R (5′-CAAAATAAA GCCGGCAAGC-3′) flanking the repetitive element insertion. *VRN2* alleles were determined with molecular markers developed before (Yan *et al.*, 2004b).

Photoperiod and vernalization conditions

LD conditions were 16 h of light and SD conditions were 8 h of light in all experiments. Unvernalized plants were grown at 18–25 °C at Photosynthetically Active Radiations (PAR) of 200–270 μE m^{-2} s^{-1}. Vernalization experiments were performed for 6 weeks at 4 °C under both SD and LD (PAR 150–190 μE m^{-2} s^{-1}).

Transcription profiles

RNA samples were extracted using the TRIZOL method (INVITROGEN) from leaves as described before (Yan *et al.*, 2003). Transcript levels of *VRN1* and *VRN2* in *T. monococcum* were determined using TaqMan systems (Yan *et al.*, 2003, 2004b). ACTIN and UBIQUITIN TaqMan systems were used as endogenous controls (Yan *et al.*, 2003).

VRN2 transcript levels in hexaploid wheat were determined using a SYBR GREEN® system designed on the basis of the A genome sequence of *VRN2*, using primers TaVRN2-F (5′-AGCCCA CATCGTGCCATTT-3′) and TaVRN2-R (5′-AC-CTCATCACCTTCGCTGCT-3′). The primers for the *ACTIN* SYBR GREEN® system were SYBR-Actin-F (5′-ACCTTCAGTTGCCCAGCAAT-3′) and SYBR-Actin-R (5′-CAGAGTCGAGCACAA TACCAGTTG-3′). The difference in slopes between *VRN2* and *ACTIN* systems was −0.01 passing the efficiency test (difference < 0.1). The efficiency of the *VRN-A2* SYBR GREEN and *ACTIN* primers was higher than 99% based on six 2-fold dilutions tested in triplicate.

All quantitative PCR experiments were performed in an ABI7000. The $2^{(-\Delta\Delta^{C}T)}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous controls.

Results

Regulation of VRN2 *transcript levels and flowering time by photoperiod*

Exposure of plants of photoperiod sensitive winter accessions of diploid wheat G3116 (Figure 1A)

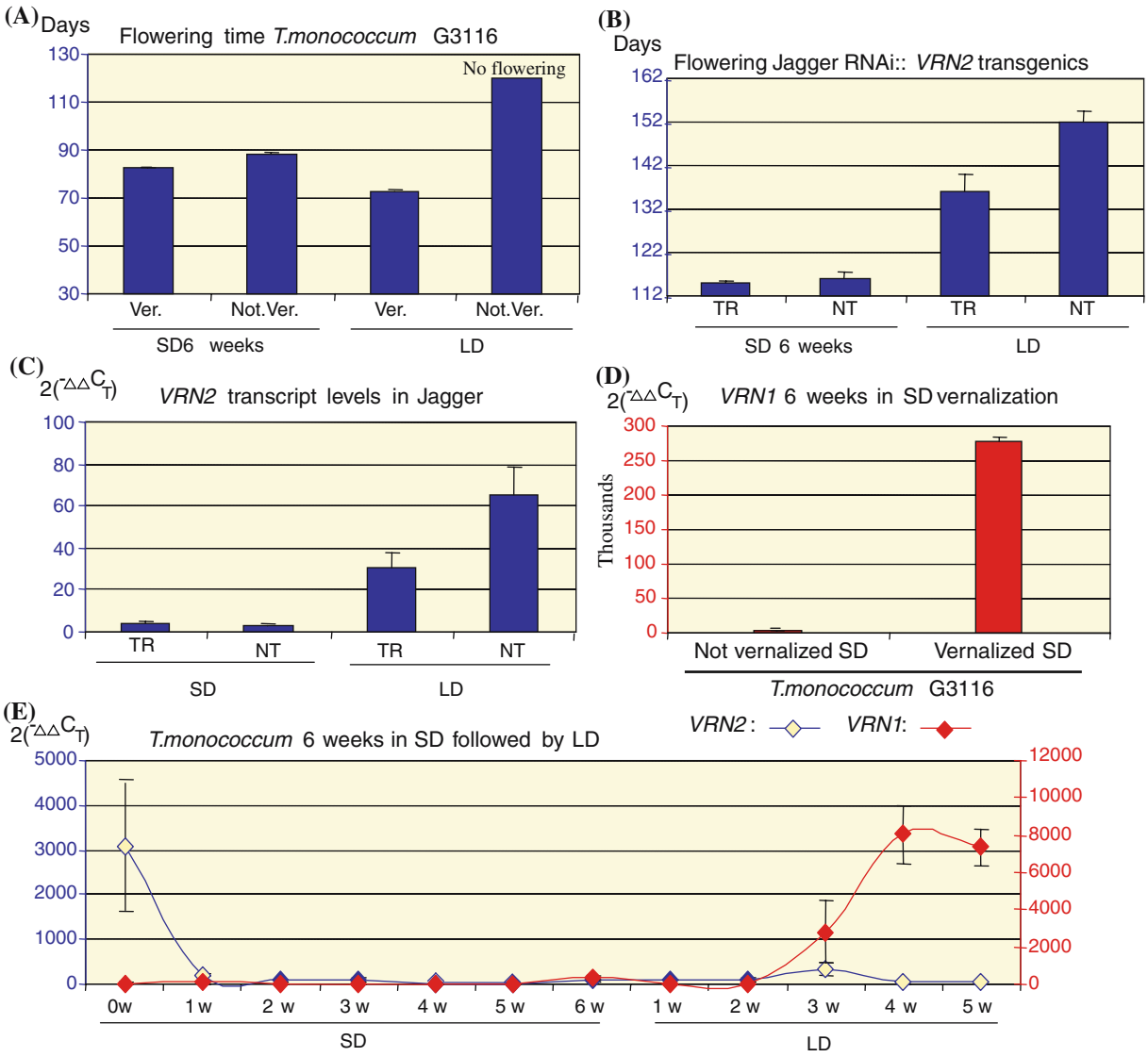

Figure 1. (A) Days to heading in winter *T. monococcum* accession G3116 under continuous LD or 6 weeks SD followed by LD. "Ver." indicates plants vernalized for 6 weeks at 4 °C whereas "Not ver." indicates non-vernalized plants. Values are averages of 5 plants ± SE of the means. LD non-vernalized plants were discarded after 120 days without flowering. (B) Days to heading in *RNAi::VRN2* transgenic plants of hexaploid wheat 'Jagger' (TR) and the non-transgenic controls (NT). LD and SD indicate similar conditions to those described in 'A'. (C) *VRN2* transcript levels in the same plants described in 'B'. (D) Comparison of *VRN1* transcript levels in vernalized and unvernalized plants of *T. monococcum* G3116 grown under SD. (E) Time course transcription profiles of *VRN1* (red line, filled diamonds) and *VRN2* (blue line, open diamonds). Initial samples (0 week) were collected immediately before transferring unvernalized G3116 plants grown under LD for 7 weeks to SD (20 °C). Plants were grown under SD for 6 weeks and samples were collected every week (1st week to 6th week). Finally, plants were transferred to LD for 5 weeks (1st week to 5th week). Note the delay in *VRN1* transcription until the transfer to LD. (C–G) Units for the Y-axis are linearized values using the $2^{(-\Delta\Delta C)}{}_T$ method, where C_T is the threshold cycle (Livak and Schmittgen, 2001). ACTIN was used as endogenous control in C (SYBR GREEN) and UBIQUITIN in D and E (TaqMan).

and hexaploid wheat Jagger (Figure 1B) to 6 weeks of SD at non-vernalizing temperatures (20 °C) followed by the transfer of the plants to LD (referred hereafter as SD–LD) resulted in a significant acceleration of flowering in both species. The SD–LD experiment in hexaploid wheat

also included transgenic RNAi::*VRN2* Jagger plants with reduced levels of *VRN2* (Yan *et al.*, 2004b). The differences in flowering time observed between transgenic and non-transgenic Jagger plants under LD disappeared after the SD–LD treatment (Figure 1B) suggesting that *VRN2* was involved in the SD replacement of vernalization.

The characterization of *VRN2* transcript levels at the end of the SD treatment supported the previous hypothesis. In both transgenic and non-transgenic Jagger plants the *VRN2* repressor was down-regulated by SD (Figure 1C), eliminating the effect of the down-regulation of *VRN2* by RNAi. A time course of transcript levels of *VRN2* during a SD–LD experiment revealed a rapid down-regulation of this gene by SD in diploid winter wheat G3116 (Figure 1E). The *VRN2* gene is also down-regulated by vernalization under LD (Yan *et al.*, 2004b) suggesting that this gene might play an important role in the integration of the SD and vernalization signals.

In spite of the rapid down-regulation of *VRN2* by SD observed in G3116 plants grown at room temperature, up-regulation of *VRN1* transcripts was not observed until the plants were transferred to LD (Figure 1E). This result differed from the up-regulation of *VRN1* transcripts in the same genotype after vernalization, both under SD (Figure 1D) or LD (Yan *et al.*, 2004b). The lack of *VRN1* up-regulation in the absence of *VRN2* suggests the existence of a second *VRN1* repressor, different from *VRN2*. *VRN1* transcript levels were up-regulated when G3116 plants were transferred to LD after 6 weeks at SD (Figure 1E), but not when the plants with the same genotype were maintained at continuous LD (Yan *et al.*, 2003), suggesting the possibility that the regulation of this second repressor by photoperiod requires the previous down-regulation of *VRN2*.

To identify the *VRN1* regulatory sites that interact with this second repressor we studied the transcript levels of *VRN1* under SD in *T. monococcum* lines carrying different combinations of natural *VRN1* and *VRN2* mutants (Table 1). To facilitate the discussion of the differences in transcript levels observed among these lines, we first present a detailed characterization of the sequence differences among these lines and the genetic evidence confirming the presence of dominant *Vrn1* alleles in these lines.

Sequence characterization of different dominant Vrn1 *alleles*

The comparison of the complete sequence of three dominant *Vrn1* alleles with the recessive *vrn1* allele from accession DV92 (AY188331) and with partial sequences from other cultivated winter accessions of diploid wheat (Table 2) showed a limited number of polymorphisms, as expected from the recent origin of cultivated *T. monococcum* (Heun *et al.*, 1997). The 15.7-kb region studied here for each allele includes the complete gene, the promoter region (2.3-kb) from the end of a 67-kb block of repetitive elements (AY188331) to the start codon and a 718-bp region after the stop codon.

Figure 2 shows the location of the 1-bp deletion in *T. monococcum* accession PI 503874 (*Vrn1f*, GenBank DQ146421) and the 34-bp deletion in accession PI 326317 (*Vrn1g*, GenBank DQ146422) relative to the putative *CArG*-box (bold letters). The *VRN1* promoter of accession PI 306540 (*Vrn1h*, GenBank DQ146423) exhibits two unique polymorphisms, but both of them far from the start codon (Table 2). The *Vrn1h* and *Vrn1f* alleles have a 493-bp insertion of a repetitive element differing only in one 9-bp indel (Figure 2 underlined). PCR analysis with primers VRN1INT1_F and _R confirmed that the seven accessions carrying the *CArG*-box 1-bp deletion all have an intron insertion similar to PI 503874 (Table 1).

The most likely location of the regulatory region within the *VRN1* intron 1 was previously delimited to a 4-kb region by overlapping several independent deletions (Fu *et al.*, 2005). The repetitive element found in the intron of the *Vrn1f* and *Vrn1h* alleles is inserted 200-bp upstream from this critical region. However, it has been shown in Arabidopsis that the effect of the insertion of a repetitive element in the first intron of *FLC* has significant effects on transcription in regions beyond the insertion site (Liu *et al.*, 2004). Therefore, this intron insertion is still a candidate for the determination of the spring growth habit associated with the *Vrn1h* allele. However, we cannot rule out the other two exclusive mutations in the promoter of *Vrn1h* (Table 2) as a cause of the spring growth habit.

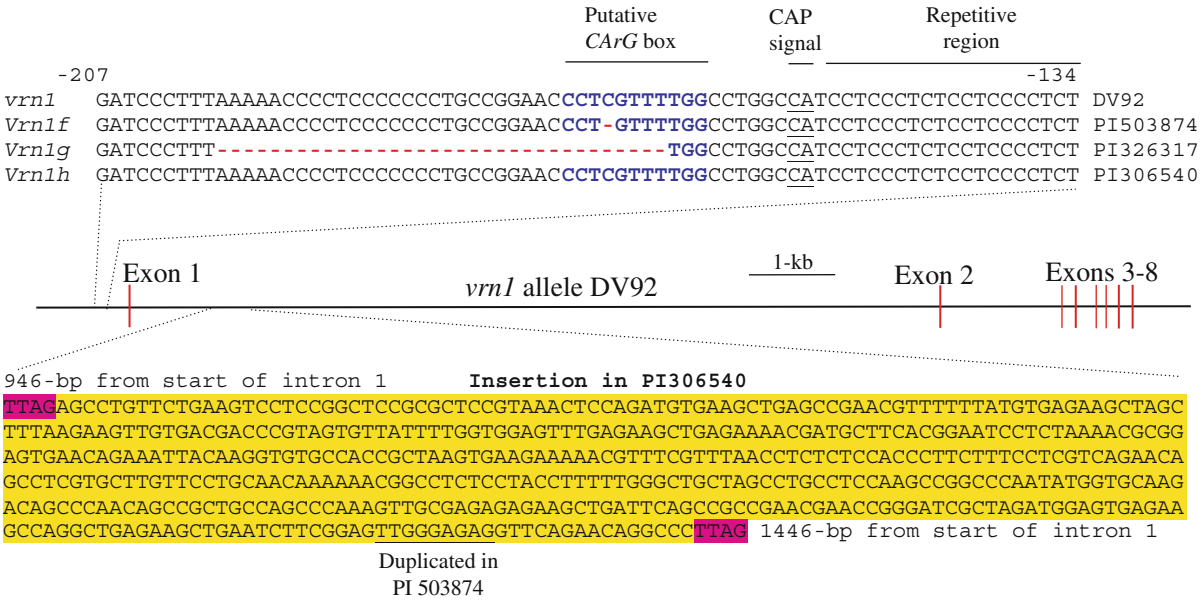

Figure 2. Sequence differences among *VRN1* alleles. Top: 74-bp segment of the *VRN1* promoter showing the location of the putative *CArG* box in blue and the CAP signal for transcriptional initiation underlined. Middle: schematic representation of the *VRN1* gene in *T. monococcum*. Bottom: repetitive element inserted in *VRN1* first intron in PI 306540 (*Vrn1h*) and PI 503874 (*Vrn1f*).

Genetic analyses of the dominant Vrn1 *alleles*

The presence of dominant *Vrn1* alleles in spring *T. monococcum* accessions PI 326317 (*Vrn1g*), PI 503874 (*Vrn1f*), and PI 306540 (*Vrn1h*) was confirmed in three different mapping populations including G3116 as the winter parent. All F_1 hybrids had spring growth habit confirming that the alleles present in these three lines were dominant for spring growth habit. In each population we tested the linkage between growth habit and polymorphisms at the *VRN1* locus in greenhouse experiments (20–25 °C) under LD conditions.

Linkage between spring growth habit and the 1-bp deletion/intron one insertion found in PI 503874 (*Vrn1f Vrn2)* was confirmed in a population of 144 F_2 individuals from the cross PI 503874 × G3116. We observed 110 spring and 34 winter plants, fitting a 3:1 segregation for a single dominant gene ($P = 0.70$). The dCAPS marker for the 1-bp deletion showed that all the spring plants were homozygous or heterozygous for the *Vrn1f* allele whereas all winter plants were homozygous for the recessive *vrn1* allele, confirming that the PI 503874 has a dominant *Vrn1* allele.

Linkage between spring growth habit and the 34-bp deletion found in PI 326317 was investigated in a population of 71 F_2 individuals (PI 326317 × G3116). In addition to the 34-bp deletion in the *CArG* box, PI 326317 has a recessive *vrn2b* allele and therefore this population was expected to segregate for both *VRN1* and *VRN2*. The observed segregation of 55 spring and 16 winter plants agreed with the expected 13:3 segregation for one dominant (*Vrn1g*) and one recessive (*vrn2b*) allele for spring growth habit ($P = 0.41$). All 16 winter plants were homozygous for the recessive *vrn1* allele (absence of the 34-deletion) confirming the presence of a dominant *Vrn1* allele in PI 326317.

Linkage between spring growth habit and the 493-bp insertion in the first intron of PI 306540 (*Vrn1h Vrn2)* was confirmed in a population of 150 F_2 individuals from the cross PI 326317 × G3116. The observed 115 spring and 35 winter plants agreed with the expected 3:1 segregation for one dominant gene for spring growth habit ($P = 0.64$). All the winter plants were homozygous for the absence of the intron one insertion (*vrn1* allele) confirming the presence of a dominant *Vrn1* allele in PI 306540. Out of the 25 earliest flowering lines 21 were homozygous for the *Vrn1h* allele (the other four were heterozygous) suggesting the existence of some dosage effect for the *Vrn1h* allele.

Table 2. Haplotype variation at the *VRN1* gene in cultivated *T. monococcum* (15.7-kb).

Acc. No.	DV92*	PI 355516	PI 355534	PI 503874*	PI 326317*	PI 306540*
GeneBank	AY18833	–	–	DQ146421	DQ146422	DQ146423
Growth habit	Spring	Winter	Winter	Spring	Spring	Spring
SD induc. *VRN1*	No	No	No	Yes	Yes	No
VRN1 allele	*vrn1*	*vrn1*	*vrn1*	*Vrn1f*	*Vrn1g*	*Vrn1h*
VRN2 allele	*vrn2a*	*Vrn2*	*Vrn2*	*Vrn2*	*vrn2b*	*Vrn2*
Prom. −1377[a]	G	G	G	G	G	A
Prom. −978	wild	wild	wild	wild	wild	9-bp del.
CArG-box	wild	wild	wild	1-bp del.	34-bp del.	wild
Int. 1 +950	wild	wild	wild	502-bp ins.	wild	493-bp ins.
Int. 1 +6447	T	G	T	G	G	G
Int. 2 +192	T	C	C	C	C	C

Sequence coordinates are based on the DV92 sequence.
[a] Within a foldback repetitive element located −1,026 to −1,424-bp upstream of start codon.

Regulation of VRN1 *transcript levels under SD in lines carrying different vernalization alleles*

Analysis of the *T. monococcum* lines carrying mutations in the different *VRN1* regulatory regions (Tables 1 and 2) under SD pointed to the promoter *CArG*-box as the most likely interaction site of the second *VRN1* repressor. After six weeks at SD, lines carrying a mutated *CArG*-box (1-bp deletion: *Vrn-1f* allele, or 34-bp deletion: *Vrn-1g* allele) exhibited significantly higher *VRN1* transcript levels than the spring lines carrying the dominant *Vrn-1h* allele or recessive *vrn-1* alleles in combination with different non-functional *vrn2* alleles (Figure 3A). A similar result was observed in an independent experiment including additional *T. monococcum* lines for the different allelic combinations (Figure 3C).

The association between the *Vrn1 CArG*-box mutations and the SD induction of *VRN1* at room temperature was confirmed in two F_2 populations (Figure 3B). F_2 plants carrying the mutated *CArG*-box (*Vrn1f* or *Vrn1g*) showed significantly higher *VRN1* transcript levels than the F_2 plants homozygous for the recessive *vrn1* allele (Figure 3B). Comparison of the complete *VRN1* gene and promoter sequence among these alleles confirmed that except for the *CArG*-box, none of the other polymorphisms were associated with the acceleration of *VRN1* transcripts under SD (Table 2).

The earlier up-regulation of *VRN1* in plants carrying *CArG*-box mutations was also reflected in faster apex development. After 6 weeks under SD, the apices of the *T. monococcum* F_2 plants without the *CArG*-box mutations were still in the vegetative stage whereas the apices of the plants with the *CArG*-box mutations were at the double-ridge stage (Figure 3D). Two weeks after transferring the plants to LD, the apices of the plants without the *CArG*-box mutation were just initiating their differentiation whereas those with the *CArG*-box mutations were already at the terminal spikelet stage (Figure 3E).

The impact of the different alleles on the apices response to photoperiod was confirmed in an independent experiment, in which lines with the *vrn1* (G3116), *Vrn1f* (PI 503874) and *Vrn1h* (PI 306540) alleles were grown for 10 weeks under SD and then divided in two groups and exposed to different photoperiod treatments. The first group was exposed to SD for additional 4 weeks whereas the second group was transferred to LD for 4 weeks (Figure 4). Control winter plants (G3116) maintained for 14 weeks at LD showed no sign of apex differentiation (Figure 4A), whereas spring plants (*Vrn1f* and *Vrn1h*) were already heading after 14 weeks at LD (not shown). The plants with the recessive *vrn1* allele (Figure 4B) or the dominant *Vrn1h* allele (Figure 4C) showed limited induction of apex differentiation after 14 weeks under SD. On the contrary, the plants carrying the *Vrn1f* allele showed a clear apex differentiation into the early stages of spike development. Plants with the *vrn1* (Figure 4E) and *Vrn1h* alleles (Figure 4F) moved for 4 weeks to LD have started their spike development, but were significantly behind the plants with the *Vrn1f* allele (Figure 4G). The *Vrn1f* plants under SD–LD were also significantly more advanced in their reproductive development

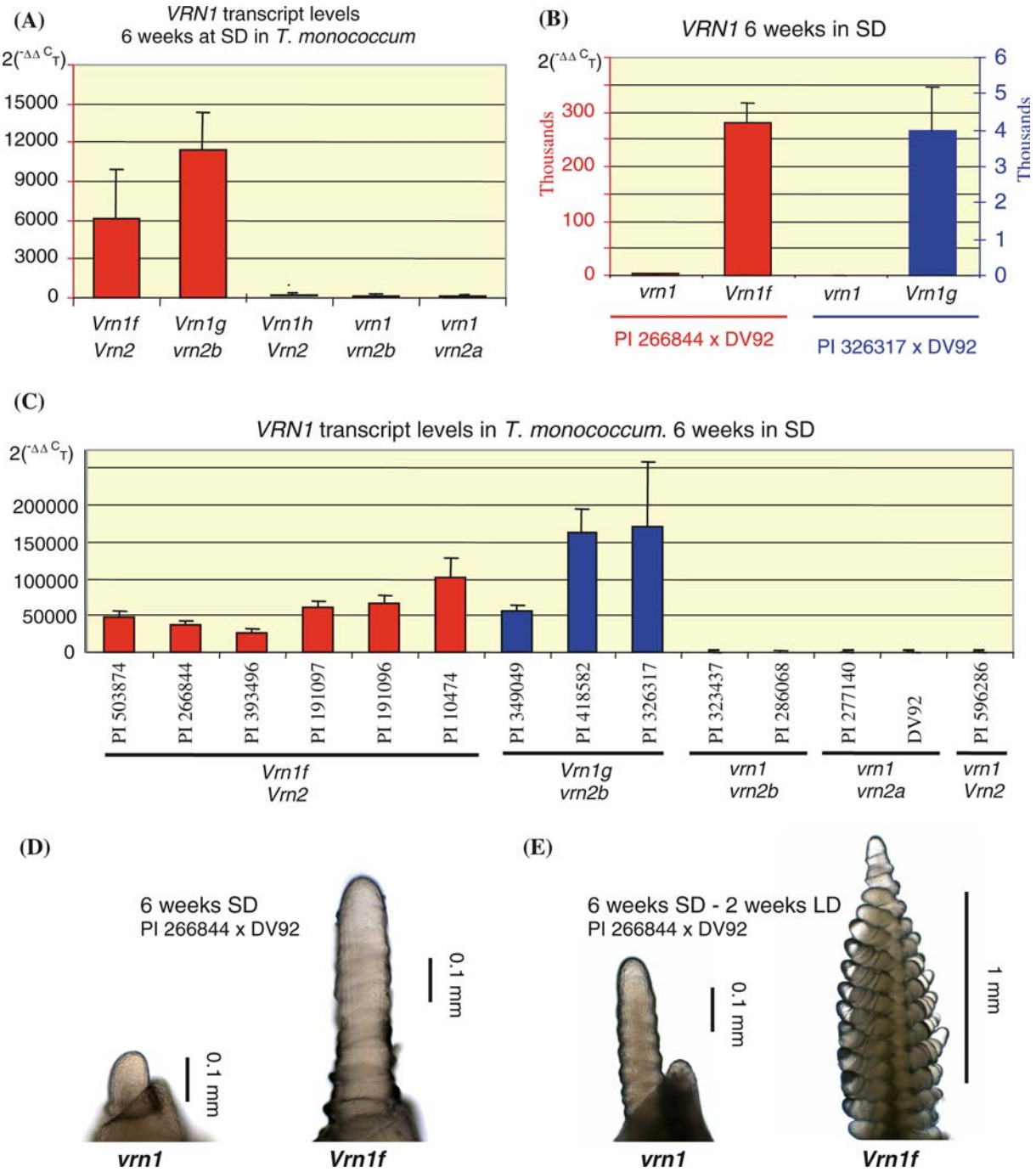

Figure 3. (A) *VRN1* transcript levels in *T. monococcum* spring lines carrying *Vrn1f* (PI 503874, 1-bp deletion), *Vrn1g* (PI 349049, 34-bp deletion), *Vrn1h* (PI 306540, intron one insertion), and recessive *vrn1* alleles combined with recessive *vrn-2b* (PI 323437) and *vrn2a* (DV92) alleles. (B) Comparison of homozygous *vrn1* and *Vrn1* F_2 plants from the cross between DV92 (*vrn1 vrn2a*) with PI 266844 (*Vrn1f*, *Vrn2*) or PI 326317 (*Vrn1g vrn2b*). We first selected plants homozygous for the recessive *vrn2* allele using molecular markers to avoid differences in growth habit. (C) *VRN1* transcript levels in additional *T. monococcum* lines carrying different vernalization alleles. (A–C) Units for the Y-axis are linearized values using the $2^{(-\Delta\Delta C)}{}_T$method, where C_T is the threshold cycle (Livak and Schmittgen, 2001). TaqMan system for ACTIN (A) and UBIQUITIN (B and C) were used as endogenous controls. (D–E) Apex development in plants of *T. monococcum* F_2 plants from the cross between PI 266844 (*Vrn1f*) × DV92 (*vrn1*). Five homozygous lines with and without the 1-bp deletion in the *CArG*-box were selected within the homozygous *vrn2a* lines (spring growth habit). Apexes were observed (D) after 6 weeks under SD (8 h of light) and (E) 2 weeks after transferring the plants back to LD.

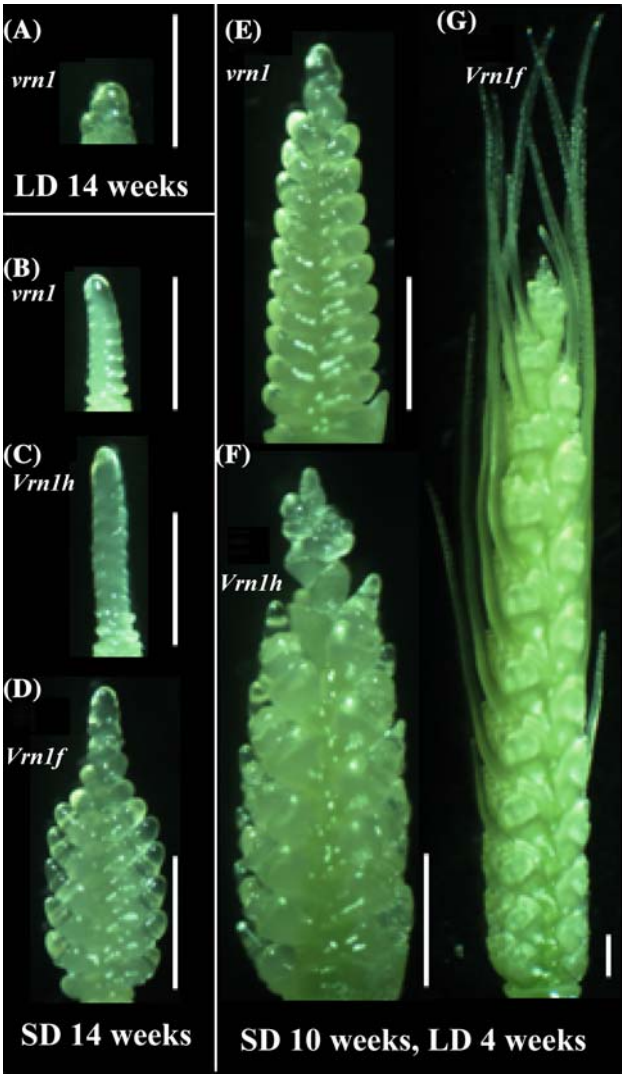

Figure 4. Comparison of apex development in *T. monococcum* plants. (A–B and E) Winter G3116 (*vrn1*); (C and F) PI 306540 (*Vrn1h*); (D & G) PI 266844 (*Vrn1f*). (A) Continuous LD, vegetative stage. Under the same conditions lines with the dominant *Vrn1h* and *Vrn1f* alleles had already headed (not shown). (B, C, D) Continuous SD. (E, F, G) 10 weeks at SD and 4 weeks at LD (SD–LD). Note the more advanced development of the inflorescence in *Vrn1f* relative to *vrn1* and *Vrn1h* in both SD and SD–LD. White bars represent 0.5 mm.

than the *Vrn1f* plants of the same age kept under continuous SD (Figure 4D).

Discussion

Effect of mutations in the VRN1 *promoter on its regulation by photoperiod*

The absence of up-regulation of *VRN1* after the down regulation of *VRN2* by SD at room temperature indicated the existence of a second *VRN1* repressor, different from *VRN2*. The *VRN1* haplotype analysis, including the complete promoter region, suggests that the mutations affecting the *CArG*-box are the most likely regions associated with the interactions between *VRN1* and this second SD repressor (Table 2). The lines carrying the 1-bp mutation in the *CArG*-box also have a repetitive element inserted in the first intron providing an alternative putative regulatory site. However, the line with the *Vrn1h* allele that has

the same intron insertion but no *CArG*-box mutation, showed no increase of *VRN1* transcripts under SD. In addition, the lines with the *Vrn1g* allele (34-bp deletion) that have no insertion in the first intron show increased levels of *VRN1* transcripts under SD (Figure 3A). These results indicate that the intron one insertion does not affect the site regulating this response to SD. On the contrary, all the genotypes showing the 1-bp or 34-bp in the *VRN1* promoter *CArG*-box (Figure 3C), and the F_2 lines carrying either of these alleles showed a significant increase of *VRN1* transcripts under SD (Figure 3B). Taken together, these results suggest that the *CArG*-box is a critical regulatory site for the response of *VRN1* to SD. *CArG*-boxes are generally recognized by MADS-box genes, so it is possible that the second repressor is a MADS-box gene.

Allelic variation for the insertion within the *VRN1* first intron does not seem to be important in the up-regulation of *VRN1* under SD. The intron one site seems to be more important in the regulation of *VRN1* by *VRN2*. A comparison of two different *VRN1-VRN2* epistatic interaction studies suggested that a large *Vrn-H1* intron one deletion in barley has larger epistatic effects on *VRN2* (Dubcovsky *et al*., 2005) than a 20-bp deletion adjacent to the *VRN-*A^m*1 CArG*-box in *T. monococcum* (Tranquilli and Dubcovsky, 2000). However, the fact that mutations in either of these sites are sufficient to accelerate flowering under LD suggests the existence of interactions between these two mechanisms in the regulation of *VRN1* (Yan *et al*., 2004a; Dubcovsky *et al*., 2005; Fu *et al*., 2005). The presence of two interacting regulatory sites has been also described in the MADS-box *FLC* gene in Arabidopsis (Sheldon *et al*., 2002). Mutations in the regulatory regions located in *FLC* promoter or first intron are sufficient to induce a spring growth habit in Arabidopsis, a similar result to that reported for the wheat *VRN1* MADS-box gene.

Effect of CArG-*box mutations on apex development*

The earlier induction of the meristem identity gene *VRN1* under SD in the spring lines carrying the *CArG*-box mutations was associated with an earlier differentiation of the reproductive apices compared to spring plants with the dominant *Vrn1h* allele (intron insertion). These differences in apex development can affect the adaptability of wheat to different environments. Plants showing SD induction of *VRN1* and early apex differentiation might be favorable in regions with mild winters that do not pose a significant risk for the delicate floral meristems. In agreement with this hypothesis most of the plants carrying these mutations were collected from temperate regions such as Spain, Portugal, Israel, U.K. (accession PI 10474 was, however, from Germany), whereas those carrying the *Vrn1h* allele were collected in regions subjected to severe winters such as Romania and Russia.

Although the acceleration of *VRN1* transcription under SD in genotypes with *CArG*-box mutations resulted in an earlier differentiation of the apices, the spike development under continuous SD (Figure 4D) proceed slowly compared with that in plants transferred to LD (Figure 4G). This result suggests that that there is an additional regulatory point after the induction of *VRN1*, which requires LD to proceed with a normal and timely reproductive development. The faster apex development under LD than under SD (in the absence of *VRN2*) provides a possible explanation for the slightly earlier flowering of the G3116 plants after LD vernalization than after SD vernalization (Figure 1A). It is important to point out here, that the *T. monococcum* accessions used in this study are photoperiod sensitive and that the responses described here might be different in photoperiod insensitive plants.

SD replacement of vernalization

Using a positional cloning approach we demonstrated before that the *ZCCT1* gene is *VRN2* and showed that deletions or mutations in this gene were responsible for natural differences in growth habit (Yan *et al*., 2004b, Dubcovsky et al. 2005). We also demonstrated that the down-regulation of *VRN2* by RNAi produced a significant acceleration of flowering in transgenic Jagger plants relative to the non-transgenic controls (Yan *et al*., 2004b). Therefore, the down-regulation of *VRN2* by SD described here is also expected to result in the acceleration of flowering and the elimination of the vernalization requirement. The convergence of photoperiod and vernalization signals at the *VRN2* gene, provides a possible explanation to the interchangeability of SD and vernalization

treatments in winter wheats, and likely in other temperate cereals. Krekule (1964) analyzed the responses of different wheat varieties to the SD replacement of vernalization and concluded that this response was restricted to photoperiod sensitive winter varieties.

Since photoperiod sensitivity and vernalization requirement are ancestral traits in wheat and barley (Kihara, 1958; Halloran, 1967; Evans and Blundell, 1994; Yan *et al*., 2003, 2004b; Karsai *et al*., 2004), the dual SD–LD induction of flowering is likely an ancestral trait in these crop species. This dual flowering induction requirement is also characteristic of most winter grasses of the subfamily *Festucoideae* (Heide, 1994). Therefore, it is likely that the strong selective pressures applied to the Triticeae crops during domestication and modern breeding accelerated the loss of the dual SD–LD flowering induction in a large number of varieties (Yan *et al*., 2003, 2004b; Karsai *et al*., 2004; Dubcovsky *et al*., 2005; Fu *et al*., 2005; vonZitzewitz *et al*., 2005). The absence of SD flowering induction in numerous modern wheat varieties has led to the general classification of wheat as LD plant. However, it would be more correct to refer to this species as SD–LD plant as suggested before by other researchers (McKinney and Sando, 1935; Evans, 1987). The down-regulation of the *VRN2* repressor by SD reported here is likely part of the mechanism associated with this SD–LD classification.

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