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Journal

Molecular Breeding, 15(4)

ISSN

1380-3743

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Publication Date

2005-05-01

DOI

10.1007/s11032-005-0084-6

Peer reviewed

Molecular Breeding (2005) 15: 395–407
DOI: 10.1007/s11032-005-0084-6

Molecular characterization of the allelic variation at the *VRN-H2* vernalization locus in barley

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Received 2 August 2004; accepted in revised form 3 January 2005

Key words: Allelic variation, Barley, Induced mutants, Vernalization, *VRN-2 ZCCT*

Abstract

VRN-2 is a dominant repressor of flowering that plays a central role in the vernalization pathway of wheat. This gene is a zinc-finger/CCT domain transcription factor (*ZCCT-1*) down-regulated by vernalization. In barley, there are three related and linked *ZCCT* genes designated *VRN-H1a, b*, and *c*. A deletion of these three genes was observed in six putative spring induced mutants from winter barley variety 'Chikurin Ibaraki I'. However, analysis of additional molecular markers demonstrated that these deletions were originated by outcrossing with a spring barley variety carrying the natural *ZCCT* deletion. Hybridization of Southern blots from 84 barley varieties with a wheat ZCCT1 probe showed three or more fragments in 23 winter varieties but none in 60 out of the 61 *vrn-H2* spring varieties. The *vrn-H2*-spring barley variety 'Fan' showed only the *ZCCT-Hb* gene, suggesting that this gene is not sufficient to determine winter growth habit in barley. We also show in this study, that the effect of *VRN-H2* on flowering time completely disappears in the presence of the dominant *Vrn-H1* allele in a Morex × *Hordeum spontaneum* segregating population. A different result was described before for the epistatic interactions between the orthologous genes in diploid wheat. We discuss the possible causes of these differences.

Introduction

Variation in flowering time in barley is mainly due to variation in genes regulated by day length (photoperiod) or long-period exposures to low temperatures (vernalization) (Laurie et al. 1995). Particularly important for fall-sawn barley varieties are the vernalization genes, which prevent flowering during winter.

Vernalization requirement in barley is mainly controlled by three loci: *VRN-H1*, *VRN-H2*, and *VRN-H3* (Takahashi and Yasuda 1971). Comparative RFLP mapping between wheat and barley has shown that the *VRN-1* and *VRN-2* genes from these species are orthologous. The *VRN-H1* gene has been mapped on the long arm of chromosome 5H in barley, in the same region as the *VRN-A1*, *VRN-B1*, and *VRN-D1* in wheat (Dubcovsky et al. 1998; Barrett et al. 2002; Galiba et al. 1995; Laurie et al. 1995; Iwaki et al. 2002; Yan et al. 2003). The *VRN-H2* gene has been mapped in the distal part of chromosome arm 4HL in barley (Laurie et al. 1995) and in the distal part of chromosome arm $5A^mL$ in diploid wheat, in a region translocated from chromosome arm $4A^mL$ (Devos et al. 1995; Laurie et al. 1995; Dubcovsky et al. 1998; Yan et al. 2004). *Vrn-H3* has been reported only in barley, and was mapped on chromosome 1H (Takahashi and Yasuda 1971).

Of these three genes, *VRN-H1* and *VRN-H3* are dominant for spring growth habit whereas *VRN-H2* is dominant for winter growth habit. The analysis of

the epistatic interactions among these genes showed that the alleles for spring growth habit (*Vrn-H1*, *vrn-H2*, and *Vrn-H3*) are epistatic to the alleles for winter growth habit (Takahashi and Yasuda 1971). Therefore, the allelic combination *vrn-H1 Vrn-H2 vrn-H3* is the only one that results in a winter growth habit (Takahashi and Yasuda 1971).

We recently cloned the *VRN-2* gene in wheat and showed that it plays a central role in the determination of winter growth habit in both diploid and hexaploid wheat. We named this gene *ZCCT* because of its putative zinc finger and CCT domain (**C**O, **C**O-like, and **T**OC1) (Yan et al. 2004). Two *ZCCT* genes were linked to the *VRN-2* locus, but allelic variation, expression profiles, and transgenic studies demonstrated that the *ZCCT-1* gene was the one responsible for the variation in vernalization requirement. RNA interference of the *ZCCT-1* gene in transgenic hexaploid winter wheat accelerated flowering more than 40 days relative to the non-transgenic control (Yan et al. 2004). In the same study, we reported the sequences for two barley *ZCCT* genes, and designated them *ZCCT-Ha* (GenBank AY485977) and *ZCCT-Hb* (GenBank AY485978). It was not possible to establish the correspondence between the two barley and two wheat *ZCCT* genes because the duplication of these genes occurred close to the time of divergence between wheat and barley (Yan et al. 2004).

The objectives of this study were to provide a detailed molecular characterization of the *ZCCT* genes in barley, establish their linkage with the *VRN-H2* locus, and characterize induced and natural mutants at this locus. We hypothesized that this information might provide valuable clues to identify *VRN-H2* among the three *ZCCT* genes present in barley. We also wanted to use this molecular information to develop perfect markers for growth habit in barley.

Materials and methods

Mapping studies

We mapped the *ZCCT* deletion and other markers linked to the *VRN-H2* and *VRN-H1* loci in 91 F_2 plants from the cross between spring *H. vulgare* L. variety Morex and the winter *H. spontaneum* Koch (OSU6, PBI004-7-0-015). Dr. A. Kleinhofs (Department of Crop and Soil Science, Washington State University) kindly provided the seeds for this experiment. Seeds were planted in the greenhouse in December 2000 under natural light conditions and F_3 seeds were harvested for progeny test. The requirement for vernalization in barley is not absolute, and even plants with winter growth habit eventually flower. Therefore, the days from sowing to emergence of the first head (heading time) was recorded for each F_2 plant and analyzed using a quantitative approach.

Since vernalization genes *VRN-H1* and *VRN-H2* are known to show strong dominance (Takahashi and Yasuda 1971), the heterozygous and homozygous dominant classes were merged in a single group for the statistical analysis. A 2×2 factorial ANOVA was performed using SAS Version 8.0 (SAS Institute 2001). Mapping of the RFLP markers in the *VRN-H2* region was done with the computer program Mapmaker 3.0 (Lander et al. 1987). Genetic distances were calculated using the Kosambi function (Kosambi 1943).

Induced barley mutant lines

Eleven barley mutants induced by ionizing radiations and chemicals in a barley six-rowed, hulled winter cultivar 'Chikurin Ibaraki I' (Ukai and Yamashita 1981) were kindly provided by the National Institute of Agrobiological Resources of Japan (Table 1).

According to their vernalization requirements, Ukai and Yamashita (1981) classified these lines into 4 types. The *V1* type mutants had a spring growth habit, whereas the other three types (*V2, V3*, and *V4*) had a winter growth habit. *V2* and *V3* type mutants have a reduced vernalization requirement, whereas *V4* has a small increase in vernalization requirement relative to the original variety (Ukai and Yamashita 1981). Seeds of the original cultivar Chikurin Ibaraki I were not available. We also included in these comparisons the spring barley Iwate Mensury C that is the type variety for the recessive *vrn-H2* allele (Yasuda 1972).

Germplasm screening

The 61 spring barley accessions classified by previous genetic analysis as homozygous for the

Table 1. Early maturing mutants used in this experiment. Flowering status was recorded after 106 days in the greenhouse without vernalization.

Mutant Line	Mutagenesis method	Flowering at UC Davis without vernalization
V1 spring type mutants		
Ea 4*	gamma-ray	Flowered
Ea 6	gamma-ray	Flowered
Ea 7a	gamma-ray	Flowered
Ea 14a	Ethylenimine	Flowered
Ea 14b	Ethylenimine	Flowered
Ea 23a	gamma-ray	Flowered
Ea 23b	gamma-ray	Not flowered
V2 winter type mutants		
Ea 7b	gamma-ray	Not flowered
Ea 31	gamma-ray	Not flowered
V3 winter type mutants		
Ea 5	gamma-ray	Not flowered
V4 winter type mutants		
Ea 8	gamma-ray	Not flowered

*Lines with different numbers correspond to independent M1 lines (first generation of mutagenized plants). Lines with similar numbers and different letters correspond to lines with different flowering times selected from the same M1 line.

recessive *vrn-H2* allele and the 23 winter accessions (dominant *Vrn-H2* allele) from diverse geographical locations were kindly provided by the Research Institute for Bioresources, Okayama University (Table 2). Information for growth habit and *VRN-H2* alleles was obtained from the Okayama University Catalogue of Barley Germplasm (Takahashi 1983).

Vernalization tests were performed using winter varieties Dairokkaku and Iwate Mensury A1 as winter controls and Iwate Mensury C as a spring control. Five plants for each of the selected lines were planted in June 2001 and were vernalized for four weeks in a cold chamber at 4 °C under 16-h light. Sixteen days before the end of the vernalization period, a second group of seeds from the same varieties was germinated and transplanted to the pots in the greenhouse without vernalization. When vernalized and non-vernalized plants were at the same developmental stage, they were transferred together to a greenhouse at 20–25 °C under 16-h light, to determine the influence of vernalization on heading date.

Experimental procedures

Nuclear DNA isolation, Southern blotting, and hybridization procedures were as described before (Dvorak et al. 1988; Dubcovsky et al. 1994). DNAs from the eleven early-flowering barley induced lines (Table 1) and the three control varieties were digested with *Hin*dIII, *Dra*I, *Eco*RV, and *Eco*RI and screened with RFLP probes. DNAs from the F_2 population between the spring *H. vulgare* variety Morex and the winter *H. spontaneum* were digested with the restriction enzyme *Xba* I. All the molecular markers mapped in this study were polymorphic for this restriction enzyme and were mapped using the same set of filters, minimizing the possibility of mapping errors. DNAs from all the selected varieties (Table 2) were digested with *Dra*I, and *Eco*RV and screened with the RFLP probes listed below.

RFLP probes used in the mapping experiment

Genes *AP1* and *ZCCT-1* corresponding to the *VRN-1* and *VRN-2* genes in wheat (Yan et al. 2004; Yan et al. 2003) were used to screen for polymorphisms between Morex and *H. spontaneum*. The *VRN-1* gene was not polymorphic for the selected mapping enzyme (*Xba*I), and was replaced by the closely linked locus *Xwg644*, located only 0.1 cM from *VRN-1* in wheat (Yan et al. 2003). The wheat ZCCT1 probe was polymorphic and was used to map the *VRN-H2* gene. Additional markers previously mapped in the $VRN-A^m2$ region (*Xucw1 (=NUCELLIN)*, *Xucw2*, *Xucw22*, *Xmwg616* and

Table 2. Accessions selected from the Okayama University collection: 23 winter and 61 spring (excluding the Ea6 mutant included in the 62 spring accessions reported before by Yan et al. (2004)). The + sign in the *ZCCT* column indicates hybridization with 3–4 bands except in variety Fan that showed a single hybridization band. The – sign indicates absence of hybridization signal.

Varieties	Acc. No.	*ZCCT*	Varieties	Acc. No.	*ZCCT*
Winter varieties			**Spring Varieties Cont**		
Black Russian	A014	+	Sapporo Rokkaku	J303	–
Heine Escourgeon Noir	A031	+	Murasaki Hadka	J307	–
3895–2	B630	+	Asahi 5	J509	–
Chinniu 1	C038	+	Seijo 1	J515	–
Suchou 2	C046	+	Satsuki Nijo	J516	–
Ethiopia 508	E469	+	Goldenmelon	J517	–
Astara	I338	+	Nihon Beer 1	J519	–
J.B.S.21[a]	I006	+	Iwate Mensury C	J732	–
Kabul 10	I437	+	Hamjon Covered 1	K113	–
Ardabil 1	I639	+	Harumaki Harumugi 2	K122	–
Iwate Mensury A1	J131	+	Gunwi Covered 2	K362	–
Akimaki Chevalier[a]	J236	+	Jeomchon Covered 1	K366	–
Dairokkaku	J641	+	Icheon Naked	K396	–
Ongjin Covered 3	K101	+	Hwacheon Native	K711	–
Akimaki Osomugi	K728	+	Suncheon Native	K714	–
Harumaki Shokubimugi[a]	K420	+	Natsudaikon Mugi	K735	–
Yeoncheon Seungmaeg[a]	K698	+	Bimtakothi 5	N604	–
Thomje 5	N660	+	Bursa	T267	–
Ayas[a]	T568	+	Amaya	T268	–
Turkey 87	T629	+	Turkey 413	T438	–
Balkan 2	U025	+	Turkey 210	T670	–
Bohemian[a]	U641	+	Istanbul	T867	–
Tibilisi 7	U699	+	Ankara	T868	–
Spring Varieties			Rene	U029	–
Hadostreng	A626[b]	–	Kitzing	U041	–
Vladivostok	C001	–	Tiroler Imperial	U045	–
Sanchiang Poli	C004	–	Jubilee	U049	–
Chientao Lungching	C005	–	Prentice	U050	–
Harbin Native	C008	–	Tantalus	U054	–
Taonan	C009	–	Erhart Frederikson	U055	–
Fengtien Hsinmin	C305	–	Tammi	U059	–
Chiamssu	C307	–	Bulgaria 36–1-H	U315	–
Peking Naked	C315	–	Kindoku	U332	–
Pingchiang Chaotung	C603	–	Dometzkoer Paradies	U347	–
Yihsien	C615	–	Vega	U355	–
Tinghsien	C616	–	Russia 46	U376	–
Ethiopia 118	E040	–	Doitsu Harumaki	U629	–
Ethiopia 634	E511	–	Mehltau Res. Barin	U645	–
H.E. 3649	I317	–	Fan	U650	
Nissei	J208	–	Ymer	U658	+
Ko A	J215	–	Vankhuri	U659	–
Fuji Nijo	J220	–	Russia 54	U678	–
New Golden	J232	–			

[a] Varieties with winter growth habit in vernalization tests performed at UC Davis but classified as spring in the Okayama University catalogue. The variety Ayas showed only 22 days difference in flowering time between vernalized and unvernalized plants.
[b] The first letter of the Okayama University identification code indicates geographical origin. A = America, B = North Africa, C = P.R. China, E = Ethiopia, I = SW Asia, J = Japan, K = Korea, N = Nepal, T = Turkey, U = Europe.

*Xβ−Amy−*1) were used to construct a linkage map of the *VRN-H2* region (Dubcovsky et al. 1998; Yan et al. 2004).

The UCW probes were developed at UC Davis (Yan et al. 2004), the NUCELLIN probe was provided by F. Q. Chen (Chen and Foolad 1997), the *Xmwg616* by A. Graner (Gatersleben, Germany), and the *Xβ−Amy−*1 by A. Kleinhofs (Washington State University, USA). A few markers selected from chromosome regions not

linked to *VRN-H2* (*Xbcd351*, *XTri*, *XGsp*, *Xcdo393*, and *Xcdo1173*) were used to test the isogenic nature of the induced mutant lines. These last markers were provided by Mark Sorrells (Cornell, USA) and Sadiq Rahman (CSIRO, Australia).

Cloning of ZCCT-Hc

Hybridization of genomic DNA from *H. spontaneum* and other winter barley varieties with a wheat ZCCT1 probe showed three restriction fragments with different restriction enzymes, suggesting the presence of three related genes. All three bands were absent in spring variety Morex that has a major deletion in the *ZCCT* region (Yan et al. 2004).

To clone the third *ZCCT* gene, we designed conserved primers based on the consensus sequence between the *ZCCT-Ha* and *ZCCT-Hb*, and used them to amplify genomic DNA for winter barley variety Hayakiso. PCR products were cloned into pCR4TOPO using the TA cloning system. Multiple colonies were selected and sequenced using an ABI3730.

Results

Epistatic interactions between VRN1 and VRN2 loci

We characterized the 91 F_2 plants from the *H. vulgare* × *H. spontaneum* cross with probes WG 644 and ZCCT1. We then classified these lines into dominant and recessive classes for each locus and performed a 2 × 2 factorial analysis of variance for heading date (Table 3). Significant differences in heading date were detected for both *VRN-H1* ($p = 0.006$) and *VRN-H2* ($p = 0.0001$). All plants carrying a recessive *vrn-H1* allele and one or two dominant *Vrn-H2* alleles showed a significant delay in flowering time, indicating that this population was segregating for *VRN-H1* and *VRN-H2* but not for *VRN-H3*. Since *H. spontaneum* has the *vrn-H1 Vrn-H2 vrn-H3* alleles (winter growth habit) we inferred that the spring barley variety Morex has the *Vrn-H1*, *vrn-H2*, and *vrn-H3* alleles.

The factorial analysis of variance also showed a significant interaction between *VRN-H1* and *VRN-H2* ($p = 0.001$, Table 3). Therefore, the effect of each locus was analyzed within each class of the other locus by four one-way analyses of variance (Figure 1a–d).

No significant effects for the *VRN-H1* alleles were observed among the plants carrying the recessive *vrn-H2* allele ($p = 0.80$, Figure 1a), but highly significant differences were observed among the plants carrying the dominant *Vrn-H2* allele ($p < 0.0001$, Figure 1b). When the three allelic classes of the *VRN-H1* locus were analyzed within the F_2 plants carrying at least one dominant *Vrn-H2* allele, a significant dominance effect was detected (quadratic contrast: $p = 0.02$). Plants carrying one or two doses of the *Vrn-H1* allele flowered at similar times (68 and 64 days respectively) and much earlier than the plants carrying only the *H. spontaneum vrn-H1* alleles (103 days). The degreed of dominances was 0.804 (d/a), indicating almost complete dominance.

Conversely, no significant effects for the *Vrn-H2* alleles were observed among the plants carrying one or two doses of the dominant *Vrn-H1* allele ($p = 0.64$, Figure 1c), but highly significant differences were observed among the plants carrying the recessive *vrn-H1* allele ($p = 0.0002$, Figure 1d). When the three allelic classes of *VRN-H2* were analyzed within the plants carrying the *vrn-H1* allele, a significant dominance effect was detected

Table 3. Two by two factorial ANOVA for heading date in the *H. spontaneum* × Morex mapping population. The first and second factors were the *VRN-H1* and *VRN-H2* genotypes respectively. Heterozygous and homozygous dominant classes were grouped.

Source	DF	Type III SS	Mean Square	*F* Value	Pr > *F*
Model	3	12,954	4318	10.51	< .0001
VRN-H1	1	3298	3298	8.03	0.0059
VRN-H2	1	6853	6853	16.68	0.0001
*VRN-H1***VRN-H2*	1	4631	4631	11.27	0.0012
Error	75	30,807	411		
Total	78	43,763			

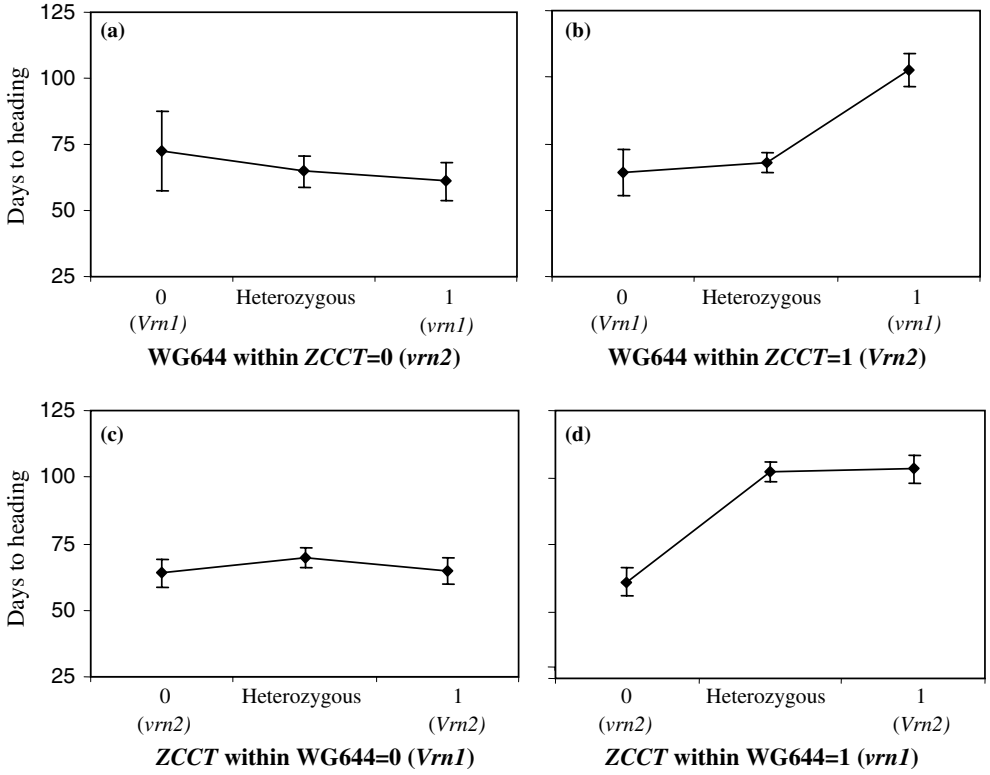

Figure 1. Effect of the *VRN-H1* and *VRN-H2* loci on flowering time in the Morex × *H. spontaneum* segregating population and their epistatic interactions. 0 = Morex allele (*Vrn-H1 vrn-H2*), 1 = *H. spontaneum* allele (*vrn-H1 – Vrn-H2*). a–b) Effect of VRN-H1 aneles within each VRN-WR class. c–d) Effect of VRN-H2 allets within each VRN-H1 class.

(quadratic contrast: ($p = 0.03$). Plants carrying one or two doses of the *Vrn-H2* allele flowered at the same time (103 and 102 days respectively) and much later than the plants homozygous for the recessive *vrn-H2* allele (61 days). The degree of dominance was 0.944 (d/a), indicating almost complete dominance.

RFLP barley map

Hybridization of *Xba*I digested DNAs from Morex and *H. spontaneum* with the *ZCCT-1* gene revealed the presence of three fragments in *H. spontaneum* and no signal in Morex indicating a deletion of all three *ZCCT* genes in the Morex genome. The absence of *ZCCT* bands was completely linked to the recessive *vrn-H2* allele (spring growth habit).

No recombinants were found between the deletion and molecular markers *Xucw1*, *Xucw2*, and *Xucw22* (Figure 2). The *Xmwg616* locus was mapped 2.8 cM proximal to the previous group of markers, whereas *Xβ-Amy-1* was mapped 6.2 cM distal to the same group, showing good colinearity with the *Triticum monococcum* map (Dubcovsky et al. 1998; Yan et al. 2004). This map confirmed that the *H. spontaneum* bands detected by the wheat ZCCT1 probe were allelic to the deletion observed in Morex. Therefore, we selected the wheat ZCCT1 probe to first characterize the induced and natural mutants for the *VRN-H2* locus.

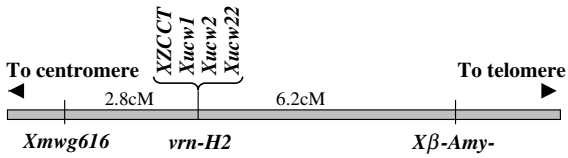

Figure 2. RFLP map based on the Morex × *H. spontaneum* segregating population.

Molecular characterization of barley mutants for VRN-H2

The unvernalized plants from the 11 induced mutants (Table 1) segregated into two groups. Plants from the first group, referred hereafter as the spring mutant lines, headed within 48 days after sowing. The spring control Iwate Mensury C flowered approximately two weeks after the early mutants. This first group included all *V1* type spring mutants (Ukai and Yamashita 1981) that have no vernalization requirement, with the exception of Ea23b (Table 1). Line Ea23b was classified as a spring type mutant by Ukai and Yamashita (1981) but in our conditions it failed to flower, and was classified as a winter type. The second group, referred hereafter as the winter mutant lines, included Ea23b from the *V1* type, and all *V2, V3*, and *V4* mutant lines. Under the greenhouse conditions at UC Davis, the winter mutant lines did not flower and were discarded two months after the heading date of the last of the spring mutant lines.

Hybridization of *Eco*RV digested DNAs from these 11 lines with the ZCCT1 probe detected three restriction fragments in the winter control variety Iwate Mensury A1 and in all the winter mutant lines (Figure 3a). All the spring mutant lines showed no hybridization signal with ZCCT1, but normal hybridization with other probes (Figure 3b–d) confirming that the absence of hybridization with the ZCCT1 probe was caused by a deletion in the barley genome and not by the absence of DNA or improper digestion with the restriction enzyme.

Large deletions were expected in the gamma ray mutants (Table 1). However, the deletion of all *ZCCT* genes in the EMS mutants *E14a* and *E14b* was unexpected, because EMS treatments usually generate point mutations (Swaminathan et al. 1962). To investigate this further, we characterized these lines with three additional markers from the *VRN-H2* region.

RFLP probe UCW2.1 hybridized with different restriction fragments than ZCCT1. This probe hybridized with three stronger *Eco*RV fragments in all winter mutants (Figure 3b) and winter variety Iwate MensuryA1, but with one fragment in the spring mutant lines and the control variety Iwate Mensury C (Figure 3b) and Morex (data not shown). Several copies with similar sequence to the UCW2.1 probe are interspersed around the *ZCCT* genes in wheat (AY485644), indicating that the deletion in the spring barley mutant lines that eliminated the *ZCCT* genes also eliminated several of the UCW2 copies. However, the presence of the same polymorphic band in all the spring mutants (Figure 3b) suggested an identical deletion, a very unlikely result for independent mutations.

Hybridization of the 11 mutant lines with a probe for the single copy gene *SNF2P*, located 13.1 kb distal to *ZCCT-1* in *Triticum monococcum* (Yan et al. 2004) confirmed that there was a problem with these mutant lines (Figure 3c). A polymorphism was detected between the spring and winter mutant lines, but no differences were detected within each of these groups. The RFLP fragment detected in the spring mutant lines were identical to those found in the spring control variety Iwate Mensury C (Figure 3c, last lane). Hybridization with a probe for the more proximal *NUCELLIN* gene showed similar results to those observed with the probe for the *SNF2P* gene. A polymorphism was observed between the spring and winter mutant lines, and no differences were detected within each of these groups.

Presence of polymorphic bands in the *VRN-H2* region suggested the introgression of a chromosome segment from a different barley variety rather than the presence of real mutations. To test the possibility of introgression of other chromosome segments in the mutant lines, 5 probes that mapped in other chromosomes were hybridized with the mutant lines. Three of the probes (CDO1173-1HL, CDO393-1HL, GSP-5HS) showed no polymorphism among the mutant lines, but were also not polymorphic between the mutant lines and the control spring variety Iwate Mensury C. Therefore, it was not possible to differentiate between lack of polymorphism with the two restriction enzymes tested and lack of introgressed segments in this region.

Barley cDNA probe BCD351–5HL showed a polymorphic *Eco*RV RFLP fragment in Iwate Mensury C compared to most of the mutant lines (Figure 3d). This RFLP fragment was absent in all mutant lines with the exception of the spring *V1* type mutant Ea14a that showed a fragment of similar mobility as Iwate Mensury C. Wheat cDNA probe for the *TRIPLET* gene, located on the short arm of homoeologous group 1 also showed *Eco*RV polymorphic restriction fragments between Iwate Mensury C and the mutant lines. Spring *V1* type

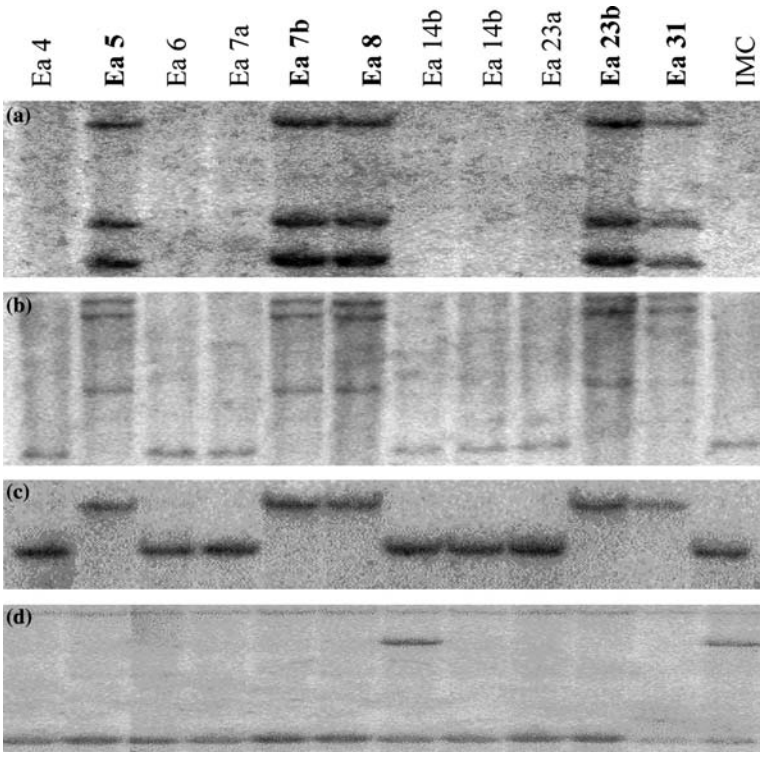

Figure 3. *Eco*RV digested DNAs from induced mutants *Ea4* to *Ea 31* (Table 1) and control variety Iwate Mensury C (IMC). Mutants with names indicated in bold have a winter growth habit and the others have a spring growth habit. Probes from (a) *ZCCT-1*, (b) *Xucw2*, (c) *XSNF2P*, and (d) *Xbcd351*.

mutant lines Ea4, Ea7a and Ea23a showed two polymorphic bands that were absent in other spring mutant lines, in the winter mutant lines and also in Iwate Mensury C. These results suggest that the recessive *vrn-H2* allele in all six spring mutants was not the result of the mutagenesis process but of the introgression of a chromosome segment from a spring variety carrying a *ZCCT* deletion similar to the one observed in the spring varieties Iwate Mensury C and Morex.

Molecular characterization of barley varieties

Since the ''induced mutants'' did not provide useful information to identify which of the barley *ZCCT* genes was critical for the determination of the winter growth habit, we decided to explore the allelic variation in the *ZCCT* genes among winter and spring barley varieties. Hybridization of *Dra*I digested DNAs from 23 winter barley varieties with the ZCCT1 probe revealed the presence of three to four bands of similar intensity (Table 2, Figure 4a). A different result was observed for the spring barley varieties previously classified as homozygous *vrn-H2*. Sixty out of the 61 *vrn-H2*-spring accessions showed no bands when hybridized with the ZCCT1 probe (Table 2, Figure 4a). The same membranes, when hybridized with RFLP probe UCW24 showed a single *Dra*I fragments in both spring and winter varieties (Figure 4b). This result confirmed that the absence of the *ZCCT* fragments in the spring varieties was generated by a deletion of the *ZCCT* genes and not by lack of DNA or incomplete DNA digestion in these lines.

The UCW24 probe includes the last two exons of gene *Tm_AY485644.3*, located 225 kb proximal to *ZCCT-2* in *T. monococcum* (Yan et al. 2004) indicating that the *ZCCT* deletion in barley did not extend to this gene in any of these accessions. The *SNF2P* gene was also present in the *vrn-H2*-spring varieties (data not shown) confirming that the deletions in the spring varieties occurred within

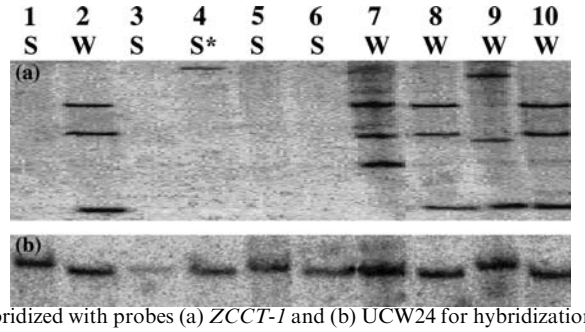

Figure 4. *Dra*I digested DNAs hybridized with probes (a) *ZCCT-1* and (b) UCW24 for hybridization control. Barley varieties from left to right: (1) Doitsu Harumaki; (2) Bohemian; (3) Mehltau Res. Barin; (4) Fan; (5) Ymer; (6) Vankhuri; (7) Black Russian; (8) Suchou 2; (9) Ethiopia 508 and (10) Astara. S indicates spring growth habit determined by the *vrn-H2* locus and W winter growth habit. An *indicates the spring variety Fan that shows a single band when hybridized with the ZCCT probe.

the interval flanked by *Xucw24* and *XSNF2P* loci. These two genes flank the *ZCCT* deletion in Morex BAC 615K1 (AY485643).

Spring barley variety 'Fan' was different from the other *vrn-H2*- spring barley varieties. *Dra*I digested DNA from this variety hybridized with a single fragment when hybridized with the ZCCT1 probe (Figure 4a). The presence of a single *ZCCT* copy in this variety was confirmed by digesting Fan DNAs with eight different restriction enzymes. Cloning and sequencing of a partial *ZCCT* gene from Fan showed that it was identical to the *ZCCT-Hb* gene from winter variety barley Dairokkaku (GenBank AY485978).

We crossed the spring variety Fan with *H. spontaneum* (winter growth habit). The F_1 plants had a winter growth habit, flowering almost two months later than Fan. This result indicated that Fan does not have the *Vrn-H1* or *Vrn-H3* alleles, and that its spring growth habit was determined by a recessive allele. Among the known vernalization genes in barley, only *vrn-H2* is recessive for spring growth habit. This result confirmed previous genetic studies that indicated that Fan carries the recessive *vrn-H2* allele (Takahashi 1983).

Cloning of ZCCT-Hc

PCR amplification of winter barley DNA with conserved primers for the *ZCCT-Ha* and *ZCCT-Hb* second exon yielded three different types of clones (223-bp excluding primers). Two were identical to previously sequenced *ZCCT-Ha* and *ZCCT-Hb*, but one showed a different sequence tentatively designated *ZCCT-Hc*. This last DNA sequence was less similar to *ZCCT-Ha* (84%) and *ZCCT-Hb* (83%) than these last two sequences were to each other (92%). *ZCCT-Ha* (84%) and *ZCCT-Hb* were also more similar to *T. monococcum ZCCT-1* (89%) than to *ZCCT-Hc* (83%).

Comparison of the CCT domains of these three sequences with the CCT domains from other grass genes (Griffiths et al. 2003) demonstrated that *ZCCT-Hc* belongs to the *ZCCT* group (Figure 5). Inclusion of *ZCCT-Hc* within the *ZCCT* cluster was confirmed using alternative clustering methods (Kumar et al. 2001): UPGMA (Figure 5), Neighbor Joining, Minimum Evolution and Maximum parsimony (data not shown).

To test if the *ZCCT-Hc* sequence corresponded to one of the three fragments observed in previous hybridizations (Figure 4), we used the *ZCCT-Hc* clone as a probe. *ZCCT-Hc* hybridized with the same restriction fragments detected by the wheat ZCCT1 probe in the winter varieties and with no bands in the spring varieties carrying the recessive *vrn-H2* allele (Yan et al. 2004). This result confirmed that *VRN-Hc* was closely linked to the *VRN-Ha* and *VRN-Hb* genes within the region deleted in the *vrn-H2* spring varieties.

Attempts to obtain the complete sequence of *ZCCT-Hc* using a specific primer for the second exon and conserved primers for the first exon were not successful, suggesting divergence of the *ZCCT-Hc* first exon. The partial sequence of *ZCCT-Hc* was deposited in GenBank under accession number AY687931.

Molecular marker for VRN-H2

We selected the *VRN-Ha* gene to develop a PCR marker because this gene was absent in all the

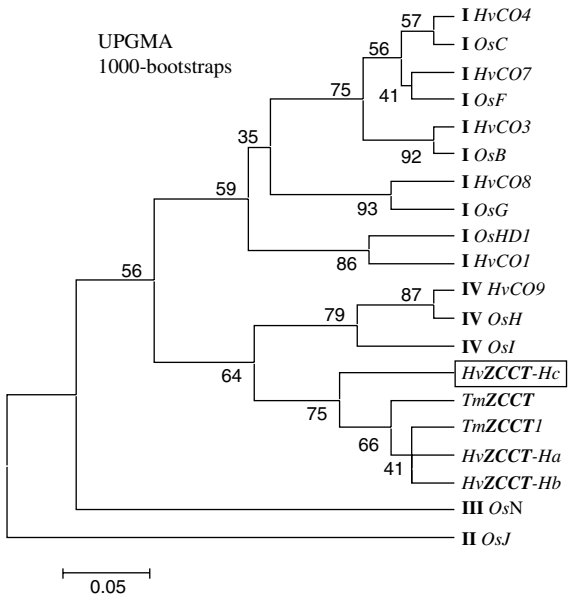

Figure 5. Sequence analysis of *ZCCT-Hc*. UPGMA cluster analysis of the amino acids from the CCT domain. Roman numbers indicate groups of CCT proteins (Griffiths et al. 2003). *Os = Oryza sativa*, *Hv = Hordeum vulgare*, *Tm = Triticum monococcum*. Note the presence of *HvZCCT-Hc* within the *ZCCT* group. Confidence values on the branches are based on 1000 bootstraps. This analysis was performed using MEGA2 (Kumar et al. 2001).

vrn-H2 spring varieties characterized in this study (including 'Fan'). In addition, *VRN-Ha* is slightly more similar than *VRN-Hc* to the wheat *ZCCT-1* gene that was previously shown to correspond to the *VRN-2* gene (Figure 5). Primers VRN-Ha-F (5′-GCCTCTTCTTCTTCCTCGAC-3′) and VRN-Ha-R (5′-ACTGGTACTCGTGCAGTGGG-3′) amplified a 208-bp fragment in all the varieties carrying the dominant *Vrn-H2* allele but did not produce any amplification product in the spring varieties carrying the recessive *vrn-H2* allele (Figure 6). PCR conditions included 1 cycle at 94 °C 5′, 38 cycles at 94 °C 30″, 55 °C 30″, 72 °C 30″; and a final extension step at 72 °C 7′.

Discussion

Barley VRN loci and their epistatic interactions

The molecular characterization of the F_2 plants from the cross between spring *H. vulgare* variety Morex and the winter *H. spontaneum* indicated

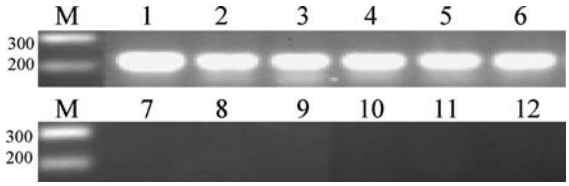

Figure 6. PCR marker for the *VRN-H1a* deletion. (1–6) Winter varieties (*Vrn-H2* allele), (7–12) Spring varieties (*vrn-H2* allele). (1) Ethiopia 508 (E469), (2) Akimaki Osomugi (K728), (3) Thomje 5 (N660), (4) Turkey 87 (T629), (5) Igri (PI406263), (6) *H. spontaneum* (OSU6), (7) Taonan (C009), (8) Sapporo Rokkaku (J303), (9) Russia 46 (U376), (10) Fan (U650), (11) Vankhuri (U659), (12) Morex (CIho 15773).

that the variety Morex carries the dominant *Vrn-H1* and recessive *vrn-H2* alleles. The presence of a recessive *vrn-H2* allele in Morex explains the absence of *ZCCT* genes in Morex BAC 615K1 between the colinear genes flanking the *ZCCT-1* and *ZCCT-2* in *T. monococcum* (Yan et al. 2004). No *ZCCT* gene was detected in the genomic DNA from Morex indicating that these genes were deleted and not translocated to another chromosome region. This deletion was allelic to the three RFLP fragments in the winter accession of *H. spontaneum* detected with the ZCCT1 probe.

The epistatic interactions between *VRN-1* and *VRN-2* in the Morex × *H. spontaneum* cross were similar but not identical to those previously observed in *T. monococcum* (Tranquilli and Dubcovsky 2000). In both species, no differences in heading time were detected between the plants carrying the dominant *Vrn-1* and recessive *vrn-1* alleles within the recessive *vrn-2* class in the segregating population. This result is in agreement with the model proposed by Yan et al. (2003, 2004). According to this model the *VRN-2* gene is a dominant repressor of flowering that regulates the transcription of *VRN-1*. In the absence of a functional *VRN-2* repressor (*vrn-2* class) the allelic variation at the *VRN-1* site that recognizes this repressor has no effect on flowering time in both barley and *T. monococcum*.

However, when the effect of the *VRN-2* allelic variation within the segregating population was studied within the dominant *Vrn-1* class, different results were obtained in the wheat and barley crosses. In barley, within the dominant *Vrn-H1* class, we found no significant differences in flowering time between the plants carrying the recessive *vrn-H2* or the dominant *Vrn-H2* alleles. On the

contrary, in the *T. monococcum* segregating population, highly significant differences in flowering time ($p < 0.0001$) were detected between the plants carrying the *Vrn-A*^{m}2 and *vrn-A*^{m}2 alleles within the dominant *Vrn-A*^{m}1 class. These result suggests that the recognition site of the *VRN-2* gene (or a gene regulated by *VRN-2)* within the *VRN-1* locus was eliminated or inactivated in the barley cross more effectively than in the *T. monococcum* cross.

In *T. monococcum*, the dominant *Vrn-A*^{m}1 allele was associated with a 20-bp deletion in the promoter region. Based on the differences in the epistatic interactions in these two crosses we hypothesized that the regulatory site affected in the dominant *Vrn-H1* allele in Morex would be different from the one observed in the *Vrn-A*^{m}1 allele in *T. monococcum* (Yan et al. 2003). A comparison of the Morex *Vnr-H1* and *H. spontaneum vrn-H1* alleles supports this hypothesis. Morex and *H. spontaneum VRN-H1* genes do not have sequence differences in the promoter but differ in a large deletion in intron one (Fu et al. 2005).

Molecular characterization of putative mutants for Vrn-H2

The molecular characterization of the induced mutants in the winter variety 'Chikurin Ibaraki I' showed that the segregation in vernalization requirement among these lines was not caused by the induced mutations but by the introgression of a chromosome segment from a *vrn-H2* spring barley variety.

The most likely explanation for these results is a cross-pollination event between a *vrn-H2* spring barley variety and 'Chikurin Ibaraki I'. This contamination in the seed sources used for the mutagenesis treatments resulted in the presence of heterozygosity in the mutagenized seed. The source of this pollination event seems to be a variety similar but not identical to Iwate Mensury C, based on the presence of common polymorphisms for most probes with the only exception of the *Triplet* locus. The plants heterozygous for the deletion segregated in the subsequent generation and the resulting spring lines were erroneously identified as vernalization mutants.

The pollen contamination hypothesis was further supported by the detection of segments from other chromosomes segregating among the mutant lines. These additional chromosome segments were independently and randomly fixed in the different mutant lines, and were the possible source of the differences in vernalization requirement observed in mutant types *V2, V3* and *V4*. The heterozygosity for additional segments of the spring variety explains the variation in flowering requirements observed in advanced generations of the mutant lines. These differences were used by Ukai and Yamashita (1981) to separate lines 7a and 7b, 14a and 14b and 23a and 23b.

In conclusion, these mutants were not real, and did not provide information for the identification of the *ZCCT* gene responsible for the winter growth. However, it is interesting that all the mutants that show no vernalization requirement have the *ZCCT* deletion, confirming the linkage of this deletion with the spring growth habit.

Molecular characterization of the barley varieties

A good correlation was observed between the presence of the *ZCCT* deletion and the presence of the recessive *vrn-H2* allele. None of the 23 winter varieties from different parts of the world showed this deletion. Sixty out of the 61 spring barley varieties previously classified by genetic studies as recessive *vrn-H2* (Takahashi 1983) showed complete absence of the *ZCCT* genes. The only exception was the spring variety Fan that showed a single *ZCCT* gene and that will be discussed separately below.

Varieties carrying the *ZCCT* deletion were collected from America, P.R. China, Ethiopia, SW Asia, Japan, Korea, Nepal, Turkey, and Europe suggesting that the *ZCCT* deletion occurred (or was selected) early during the domestication of barley and was then distributed across the world. An alternative explanation would be the recurrent occurrence of independent deletions in different geographic locations. The first hypothesis seems more likely since all these 60 accessions showed a simultaneous deletion of the three *ZCCT* copies and the deletion did not extend to the adjacent *Xucw24* and *XSnf2P* markers in the lines tested. A more detailed characterization of the borders of these deletions will be necessary to determine which of these hypotheses is correct.

Independent point mutations and deletions of the *ZCCT* genes were also found in several *T. monococcum* accessions throughout the geographic distribution of this species (Yan et al. 2004). The wide geographic distribution of the *T. monococcum* and barley *vrn-2* mutants suggests that the primitive farmers had the ability to identify and select these early mutants.

In wheat, natural mutations, expression studies, and transgenic experiments were used to demonstrate that the *VRN-2* gene was *ZCCT-1* and not *ZCCT-2*. Comparison with the barley *ZCCT* genes indicated that *ZCCT-Ha* and *ZCCT-Hb* were more similar to the wheat genes than *ZCCT-Hc*. This result suggests (but does not demonstrate) that *ZCCT-Ha* and *ZCCT-Hb* might be better candidates for *vrn-H2* than *ZCCT-Hc*. However, it was not possible to establish which of the first two barley genes was more similar to the wheat *ZCCT-1* gene (Yan et al. 2004).

The unique deletion observed in the barley variety Fan provided preliminary evidence to identify the *vrn-H2* gene in barley. The single *ZCCT* copy identified in Fan corresponded to the *ZCCT-Hb* gene. Since Fan has a spring growth habit and a recessive *vrn-H2* allele (and also recessive *vrn-H1* and *vrn-H3* alleles), we propose that the *ZCCT-Hb* gene is not critical for the determination of winter growth habit in barley. Additional experiments will be necessary to determine which of the other two barley *ZCCT* genes is responsible for the determination of winter growth habit in barley.

Finally, the PCR marker developed for *VRN-Ha* is completely linked to the deletion that originates the recessive *vrn-H2* allele, and therefore is a perfect marker for this locus. In the future this marker, in combination with perfect markers for the *VRN-H1* gene will be useful for breeding programs to determine the effect of different alleles or allele combinations for growth habit on flowering time and agronomic performance in different barley growing regions.

Acknowledgements

This research was supported by the United States Department of Agriculture CSREES NRI competitive grant 2003–00929 and IFAFS competitive grant 2001–04462.

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