Assaying natural variation in the barley *VRN1* gene

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ABSTRACT

The *VRN1* gene is the major determinant of vernalization requirement in barley and wheat. In varieties that require vernalization to flower, *VRN1* is expressed at low basal levels and is induced by exposure to low temperatures[3,6,8,11]. Deletions in the first intron of *VRN1* cause increased *VRN1* activity in the absence of cold treatment and reduce the vernalization requirement[3,8,11]. We assayed the extent of natural variation in the first intron of the *HvVRN1* gene amongst four thousand barleys from diverse geographical regions. Twelve alleles of *HvVRN1* containing deletions or insertions in the first intron were identified, including five alleles that have not been described previously. Comparison of the position and length of deletions in the first intron of *HvVRN1* has allowed us to identify a region that is required for repression of this gene prior to winter and a correlation between the position and length of deletions and *HvVRN1* expression levels. We also show that deletion of this region does not prevent induction of *HvVRN1* by low temperatures. We suggest that a specific region within the first intron of *HvVRN1* is required to maintain low levels of *HvVRN1* expression prior to winter but acts independently of the regulatory mechanisms that allow low-temperature induction of *HvVRN1* during winter.

INTRODUCTION

The *VRN1* gene is the major determinant of vernalization requirement in barley and wheat. In varieties that require vernalization to flower, *VRN1* is expressed at low basal levels and is induced by exposure to low temperatures[3,6,8,11]. Deletions in the first intron of *VRN1* cause increased *VRN1* activity in the absence of cold treatment and reduce the vernalization requirement[3,8,11]. We assayed the extent of natural variation in the first intron of the *HvVRN1* gene amongst four thousand barleys from diverse geographical regions.

METHODS AND MATERIALS

Plant materials and growth conditions

Barley lines were obtained from the Australian Winter Cereals Collection. Plants were grown in pots of soil in sunlit glasshouses under long days (16 hours light/8 hours dark) or short days (8 hours light/16 hours dark) with supplementary lighting when natural light levels dropped below 200 μE. Glasshouses had an average temperature of 19 °C. Experiments were terminated after 120 days of growth in short days and 100 days of growth in long days. For RNA extraction, plants were harvested at the middle of the light period. Heading date was measured as the day when the head first emerged from the sheath, and averaged for at least 3 plants. For low temperature treatment, plants were grown at 8 °C ± 4 °C.

DNA and RNA extraction

DNA for PCR genotyping was extracted from individual dry seeds in a 96 well format. Seeds were ground in an extraction buffer, (2% Cetyl trimethylammonium bromide, 2% polyvinylpyrrolidone, 0.02 M EDTA, 1.4 M NaCl, 100 mM Tris-Cl pH 8.0) using a ball bearing in a mix mill. Extracts were incubated at 65 °C for 30 minutes then cleared by centrifugation. DNA was precipitated with 1 volume of isopropanol and 0.1 volumes of 6 M ammonium acetate, then centrifuged. Pellets were washed in 70% ethanol and resuspended in 150 μl of TE buffer (10 mM Tris-Cl pH 8, 1 mM EDTA). Total RNA was extracted from whole seedlings, excluding root tissue, using the method of Chang et al.[1]

PCR genotyping

PCR was performed using Taq F1 DNA polymerase (Biotech). Plants were genotyped for *HvVRN2* (*HvZCCTb*) and *PPD-H1* as described previously[5,9].

Quantitative RT-PCR

An oligo(T) primer (T18[G/C/A]) was used to prime first strand cDNA synthesis from 5 μg of total RNA using the SuperScript III reverse transcriptase enzyme (Invitrogen) according to the manufacturer's instructions. A single reverse transcription reaction was performed for each RNA sample. The primers used for *HvVRN1* and *ACTIN* have been described previously[9]. Each primer pair amplifies cDNA specific DNA products. Quantitative RT-PCR (qRT-PCR) was performed on a Rotor-Gene 3000 Real-time Cycler (Corbett Research) using Platinum Taq DNA polymerase (Invitrogen). Cycling conditions were 2 minutes at 95 °C, 50 cycles of 10 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. This was followed by a melting-curve program (72 °C-95 °C with a 5 s hold at each temperature). Fluorescence data were acquired at the 72 °C step and during the melting-curve program. Expression levels of genes of interest relative to *ACTIN* were calculated using the Comparative Quantification analysis method (Rotogene-5, Corbett Research). Quantification for each primer set and cDNA template combination was performed in triplicate, and included a no-template control, to ensure results were not influenced by primer-dimer formation or DNA contamination. Data presented is the average and standard error from triplicate reactions on the same PCR run.

RESULTS

We screened a collection of four thousand barleys from diverse geographical regions for variation in the first intron of the *HvVRN1* gene. Twelve alleles, containing either insertions or deletions in the first intron, were identified. Seven of these alleles have been described previously [2,4,7,10] and 5 were new (Table 1).

Barley lines representing all 12 *HvVRN1* alleles were grown without vernalization in short or long days to determine the effect of these alleles on flowering time. To reduce the effect of other loci controlling flowering time, lines used in this study carry the wildtype alleles of *HvVRN2* and *PPD-H1*. In addition, lines that flowered early (<50 d) in short days were excluded, as they were assumed to carry variation at other loci affecting flowering time through activation of the daylength pathway (for example, active alleles of *FT1* (*VRN3*)). The lines carrying alleles *HvVRN1-1*, *HvVRN1-2*, *HvVRN1-3*, *HvVRN1-4*, *HvVRN1-5*, *HvVRN1-7*, *HvVRN1-8*, *HvVRN1-10*, *HvVRN1-11,* and *HvVRN1-12* flowered with an average of 50 days in long days. Lines carrying *HvVRN1-6* flowered later, with an average of 81 days. Lines carrying *HvVRN1-9* had a range of flowering times from 47 days to not flowering by 100 days. In the same conditions, lines carrying wildtype alleles of *HvVRN1* do not flower within 100 days.

Table 1. *HvVRN1* alleles carrying insertions or deletions in intron 1.

HvVRN1 allele	Exemplar variety	D/I[1]	Source[2]
HvVRN1-1	Morex	D	4
HvVRN1-2	OWB-D	D	4
HvVRN1-3	Triumph	D	10
HvVRN1-4	Calicuchima-sib	D	7
HvVRN1-5	Etu	D	2
HvVRN1-6	Express	D	2
HvVRN1-7	Varunda	I	2
HvVRN1-8	-	D	TS
HvVRN1-9	-	D	TS
HvVRN1-10	-	D	TS
HvVRN1-11	-	D	TS
HvVRN1-12	-	I	TS

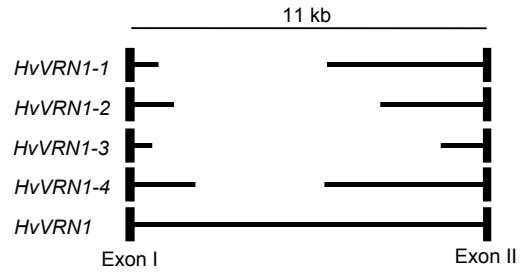
[1]D, deletion; I, insertion. [2]TS, This study.

Expression levels of the 12 *HvVRN1* alleles were assayed in whole plant tissue from 7 day old seedlings grown in long days. At this timepoint the apex was vegetative. *HvVRN1* expression levels were higher in lines carrying any of the deletion/insertion alleles than in plants carrying wildtype alleles of *HvVRN1*. Deletions or insertions in the *HvVRN1* first intron are sufficient to allow de-repression of *HvVRN1* and cause early flowering in the absence of exposure to low temperature.

There was a correlation between the position and length of deletions in the first intron and *HvVRN1* expression levels (Figure 1). Comparison of the position and length of deletions identified a critical region required for repression of *HvVRN1* in nonvernalized plants.

Induction of *HvVRN1* expression by cold could be mediated by a repressor that binds to the first intron of the *HvVRN1* gene. To test this hypothesis, plants carrying alleles of *HvVRN1* with deletions in intron 1 were examined for the response of *HvVRN1* expression to cold. This experiment included the *HvVRN1-3* allele (EF591642) which carries the largest intron 1 deletion (8.9 kb) (Figure 1A). Plants were grown at 19 ºC in short days until they reached the two leaf stage and then either maintained at 19 ºC or transferred to 8 ºC. Expression of *HvVRN1* was assayed when the plants were at the three leaf stage. Expression of *HvVRN1* was higher in plants grown at 8 ºC than plants grown at 19 ºC in all lines examined (Figure 1B). Deletion of regions of intron 1 required for repression of *HvVRN1* in non-vernalized plants does not prevent induction of *HvVRN1* expression by low temperatures.

A



B

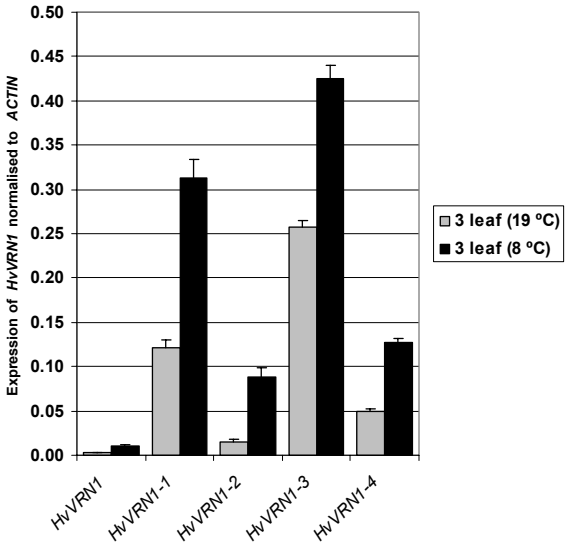


Figure 1. Deletion of a critical region within the *HvVRN1* first intron does not prevent induction of *HvVRN1* by low temperatures. A, Schematic

representation of intron 1 from the *HvVRN1* alleles for which data are presented. B, Relative expression levels of *HvVRN1* assayed by qRT-PCR and normalised to *ACTIN* in RNA from plants that were grown at 19 ºC in short days until they reached the two leaf stage and then either maintained at 19 ºC or transferred to 8 ºC. Expression of *HvVRN1* was assayed when the plants were at the three leaf stage.

DISCUSSION

We have identified sequence variation in the first intron of *HvVRN1* in diverse barley germplasm. Deletions and insertions in the first intron of the *HvVRN1* gene are correlated with *HvVRN1* expression levels and flowering time. *HvVRN1* alleles that produce different flowering times will be useful for breeding barley varieties adapted to different climatic conditions. We are crossing each *HvVRN1* allele identified in this study to an elite Australian barley variety in order to characterise the effect of each allele on flowering time in an isogenic background.

Deletion of critical regions in the *VRN1* first intron leads to de-repression of the *VRN1* gene in plants that have not been vernalized. It is thus possible that the *VRN1* first intron contains a binding site for a repressor of *VRN1* expression that is removed by low temperature treatment. If such a repressor responded to low temperatures in a quantitative manner, this would provide a mechanism by which *VRN1* expression is quantitatively induced by low temperature treatment. We have shown that deletion of a critical region within the *HvVRN1* intron which is required for repression of the gene in non-vernalized plants does not prevent induction of *HvVRN1* by low temperatures. We suggest that specific regions within the first intron of *VRN1* are required to maintain low levels of *VRN1* expression prior to winter but act independently of the regulatory mechanisms that allow low-temperature induction of *VRN1* during winter.

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