doi: 10.17221/16/2016-CJGPB

Marker Characterization of Vernalization and Low-temperature Tolerance Loci in Barley Genotypes Adapted to Semi-arid Environments

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Abstract

Benkherbache N., Tondelli A., Djekoune A., Francia E., Pecchioni N., Hassous L., Stanca A.M. (2016): Marker characterization of vernalization and low-temperature tolerance loci in barley genotypes adapted to semi-arid environments. Czech J. Genet. Plant Breed., 52: 157–162.

For the purpose of marker assisted selection (MAS), six Algerian cultivars and landraces developed locally or introduced from external breeding programs for semi-arid environments were marker characterized at *VRN-H1*, *VRN-H2* and *FR-H2* loci. As controls the lines Nure and Tremois were used. Markers available in the literature, known to discriminate efficiently the trait-associated alleles between the two cultivars Nure and Tremois, were used. This study revealed that the used Algerian cultivars carry a dominant (spring) allele for *VRN-H1*, while the *HvZCCT* marker generated a polymorphic profile for the second vernalization locus *VRN-H2*. No cultivars possessed the Nure allele conferring tolerance to low-temperatures at the *HvCBF4*. Three cultivars possessed the Tremois allele at *FR-H2*, while the remaining three presented different haplotypes.

Keywords: Algerian cultivars; growth habit; low-temperature; MAS; *VRN-H1*; *VRN-H2*; *FR-H2*

The phenotypic description of genotypes has always been the basis of improvement in plant breeding, essentially through agronomic traits related to yield. Consequently, phenotypic characters are often biased by environmental factors. Over the last two decades, molecular markers have proved useful for describing the allelic status at different loci across the genome in crop species. With the availability of a range of molecular markers and genetic maps, marker-assisted selection has become possible for traits governed by major genes and quantitative trait loci (QTL) (Francia *et al*. 2005; Collard *et al*. 2008).

Vernalization is known to be a period of exposure to low-temperatures, which allows the plant to move from the vegetative to the flowering stage (Ritchie & Smith 1991). Otherwise, flowering can be delayed for several months (Laurie 1997). The exposure of vernalization requiring plants to low-temperatures can reduce their vegetative phase by reducing the final number of leaves (Wang *et al*. 1995).

Dubcovsky *et al*. (1998) have standardized the nomenclature of the vernalization loci (*VRN*). Barley (*Hordeum vulgare* L.) has three vernalization genes *VRN-H1* (chromosome 5H), *VRN-H2* (chromo-

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some 4H) and *VRN-H3* (chromosome 7H). Yan *et al.* (2004), in their work on the *Triticeae*, suggested a model of epistatic interactions between *VRN2* and *VRN1*. *VRN2*, which is a zinc-CCT transcription factor (ZCCT1), is a dominant repressor of flowering through downregulation of *VRN1*. The *VRN1* in barley is similar to the *Arabidopsis* MADS-box gene *APETALA1* (*AP1*). *VRN2* transcription is repressed by vernalization and by short day allowing the expression of *VRN1* in winter varieties. A lack of low-temperature demand occurs in genotypes with deletions of the *ZCCT* gene (i.e. recessive *vrn2* allele), regardless of the allele at *VRN1*, as well as among genotypes with *VRN2* but lacking the target binding site for the repressor gene in the MADS box (dominant *Vrn1* allele). The relationship between the type of growth and genetics of vernalization revealed three types of barley (von Zitzewitz *et al.* 2005). The winter growth habit of barley requires the presence of a recessive *vrn-H1* allele, together with an active *Vrn-H2* allele (Cockram *et al.* 2007; Hemming *et al.* 2009). Vernalization induces *VRN-H1* under both short and long days, which then represses *VRN-H2*. Facultative types do not require vernalization because even if they have *Vrn-H1* alleles with the repressor binding site, they completely lack the *VRN-H2* gene encoding the repressor. Spring types do not need vernalization, because they have *vrn-H1* alleles which do not have a binding site for the repressor, the *VRN-H2* gene may or may not be present.

Several candidate genes involved in responses to cold stress and drought have been mapped (Tondelli *et al.* 2006). Two groups of genes are responsible for cold tolerance in barley. The first group includes the vernalization genes that delay flowering until the end of winter and thus protect the leaf primordia. The second group comprises a series of repeated CBF transcription factors in tandem (C-repeat binding factor) on the *FR2* (*FROST RESISTANCE 2*) locus. CBF transcription factors are known to be regulators of the *COR* (*COLD REGULATED*) genes which are induced by cold and confer resistance to low temperatures. The QTL for cold tolerance *FR-H1* coincides with *VRN-H1* (Hayes *et al.* 1993; Laurie *et al.* 1995; Francia *et al.* 2004). Francia *et al.* (2004) developed a molecular marker map for the Nure × Tremois mapping population, which was then used to detect and map QTLs both for traits related to frost tolerance and vernalization requirement.

The objective of the present study is the characterization of the allelic combinations of growth habit and cold tolerance loci in six Algerian cultivars using allele-specific molecular markers in order to understand the underlying factors governing adaptation of barley.

MATERIAL AND METHODS

Six barley cultivars were provided by the Technical Institute of Field Crops (ITGC, Setif, Algeria). They included local cultivars and introduced cultivars from foreign breeding programs. Tichedrett and Soufara 'S' are local six-row cultivars. Tissa and Rahma are two-row cultivars introduced from Syria (ICARDA). Bahia and Fouara are six-row lines selected by ITGC of Setif. Their pedigrees are Rebelle/Line 686 for Bahia and Deiralla106/Strain205//Gerbel.ICB85.1376.0AP.1AP.2AP for Fouara (Benmahammed *et al.* 2001; Bensemane *et al.* 2011). These cultivars were evaluated in these studies for their adaptation to the conditions of semi-arid regions (Benmahammed *et al.* 2001). They are resistant to biotic and abiotic stresses and used for animal feed as grain or green forage.

Two cultivars were used as positive controls in this study. They included Nure, a high yielding, cold tolerant, two-row feed-barley cultivar released by the Istituto Sperimentale per la Cerealicoltura Section of Fiorenzuola (Italy). It has a wide range of adaptability with the recessive genotype at *VRN-H1* and dominant at *VRN-H2* as typical winter growth habit. Tremois is a French two-row malting variety. It is cold susceptible and high yielding with the dominant genotype at *VRN-H1* and recessive *at VRN-H2*. Tremois has a typical spring growth habit based on a SNP (single nucleotide polymorphism) in the promoter region of the *VRN-H1* candidate gene *HvBM5A* and the deletion of the *ZCCT-Hc* gene at the *VRN-H2* locus on chromosome 4H (von Zitzewitz *et al.* 2005). Low-temperature tolerance is described by both *FR-H2* (*HvCBF4*) and *FR-H1*/*VRN-H1* (*HvBM5A*) genes.

DNA was isolated from the fresh leaf tissue of two-week old barley plants using Wizzard Magnetic 96 DNA Plant System (Promega, Madison, USA).

Thermal cycling profile for *HvBM5A, HvZCCT* and *HvCBF4* started by an initial denaturing step of 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR reaction was terminated by a final extension of 72°C for 7 min. The full list of primer names and sequences is listed in Table 1.

For *HvZCCT* and *HvBM5A*, PCR reactions of 25 µl contained 20 ng DNA, 10 × PCR buffer, 50 mmol

doi: 10.17221/16/2016-CJGPB

Table 1. The list of primer names, sequences and expected sizes of PCR products used in this study with the references cited

Primers names	Sequences	Marker essay	Size (bp)	References
HvBM5A	F CAGCCTCAAACCAGCTCTTC R AAACAACACCCAGGAGCAAC	CAPS *Nle*III[1]	465	Von Zitzewitz *et al.* (2005)
HvZCCT	F CACCATCGCATGATGCAC R TCATATGGCGAAGCTGGAG	STS	400	Von Zitzewitz *et al.* (2005)
HvCBF4	F ATGGACGTCGCCGACATC R TTAGCAGTCGAACAAATAGCT	ARMS	675	Drenkard *et al.* (2000)
	NURE F ACGAGGAGCAGTGGTTTAGA- TREMOIS F ACGAGGAGCAGTGGTTTAGC			Rozen and Skaletsky (1998)

F – forward; R – reverse; [1]restriction enzyme; CAPS – cleaved amplified polymorphic site; STS – sequence-tagged site; ARMS – amplification refractory mutation system

$MgCl_2$, 10 mmol dNTP, 10 µmol of each primer, and 5 U/µl of Taq polymerase. Digestion of CAPS-based (cleaved amplified polymorphic sequence) markers was performed with the *Nle*III restriction enzyme (RE). According to the manufacturer's instructions, 10 µl of PCR product were incubated for 2 h with 2 U of RE, 1X reaction buffer and 0.1 mg/ml of bovine serum albumin, and then separated on a standard 1.5% agarose gel. PCR reactions of *HvCBF4* were performed in a 30 µl final volume containing 100 ng of genomic DNA, 1 × PCR buffer, 1.8 µl of $MgCl_2$, 0.74 µl of each dNTP, 1.5 µl of forward and reverse primer, and 1 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, USA). DNA amplification products were separated in a 1.5% agarose gel. *HvCBF4* was sequenced after purification using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and sequenced with the Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Foster City, USA). The reaction mixture is composed of 1.5 ng/100 bp DNA, 0.3 µl of Big Dye, 2 µl buffer and 0.8 µl primers. This mixture was divided into two volumes, each containing either the forward or the reverse primer. Two sequencing reactions were performed with each primer and the results were compared to *HvCBF4* by alignment with the Gap4 STADEN software package (Ver. 2.0, 2010).

RESULTS AND DISCUSSION

PCR amplification using primers for the *HvBM5A* gene (*VRN-H1*) showed that all the genotypes are identical to Tremois (Figure 1). According to the model of Von Zitzewitz *et al.* (2005), the Nure genotype is the recessive homozygote for *vrn-H1* allele, while the remaining genotypes (including Tremois) carry a dominant allele *Vrn-H1* at the homozygous constitution.

The *HvZCCT* marker (*VRN-H2*) generated a polymorphic profile, which revealed that different alleles exist among genotypes selected in Algeria. In fact, four genotypes (Tichedrett, Fouara, Tissa and Bahia) carried the same Nure allele. On the contrary, two genotypes, Soufara 'S' and Rahma showed the same (spring) allele of Trémois (Figure 2).

According to Von Zitzewitz *et al.* (2005), the description of allele combinations at the *VRN-H1* and *VRN-H2* loci is sufficient to explain the barley vernalization requirement and growth habit. If we consider *A* the Nure allele and *B* the Tremois allele, Algerian genotypes may be therefore described as in Table 2.

The genotypes Rahma and Soufara 'S' carried the Tremois allele which is a spring-type. Tichedrett, Fouara, Tissa and Elbahia have the vernalization gene

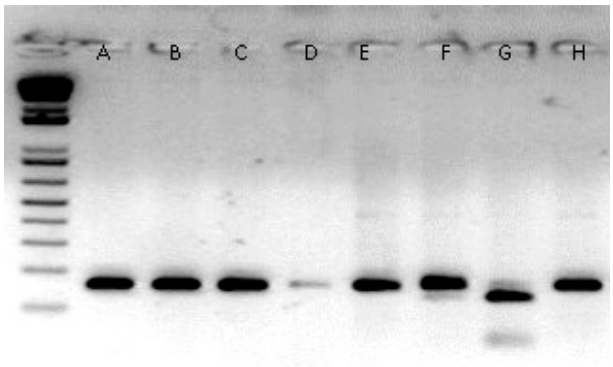

Figure 1. Polymorphism generated by the CAPS marker *HvBM5A* (*VRN-H1*) on six Algerian varieties (A – Tichedrett, B – Soufara 'S', C – Rahma, D – Fouara, E –Tissa, F – Bahia) compared with Nure (G) and Trémois (H)

doi: 10.17221/16/2016-CJGPB

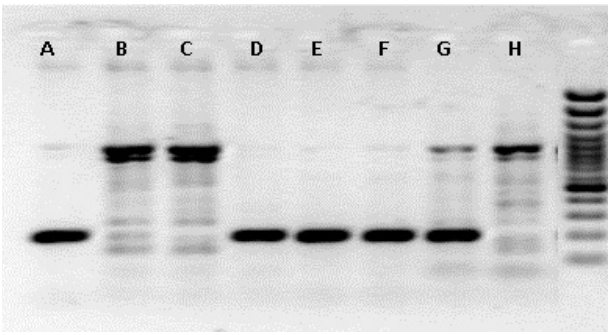

Figure 2. Polymorphism generated by the STS marker *HvZCCT* (*VRN-H2*) on six Algerian varieties (A – Tichedrett, B – Soufara 'S', C – Rahma, D – Fouara, E – Tissa, F – Bahia) compared with Nure (G) and Trémois (H)

Table 2. Growth habit in the time of flowering and genotypes at *VRN-H1* and *VRN-H2* loci of six Algerian barley varieties compared to Nure and Tremois

Variety	*HvBM5A* (*VRN-H1*)	*HvZCCT* (*VRN-H2*)	Types
Nure	*AA*	*AA*	winter
Tremois	*BB*	*BB*	spring
Tichedrett	*BB*	*AA*	spring
Soufara 'S'	*BB*	*BB*	spring
Rahma	*BB*	*BB*	spring
Fouara	*BB*	*AA*	spring
Tissa	*BB*	*AA*	spring
Bahia	*BB*	*AA*	spring

but do not have the repression site encoded by *VRN-H1*, so they do not need vernalization to flower. Algerian cultivars were identified as dominants at *VRN-H1/VRN-H2* with the expected spring growth habit.

In our study, the allele-specific CAPS marker designed for *HvCBF4* generated as expected a presence/absence polymorphism for each allele (Figure 3). We consider that only Rahma carries the Nure allele, while Tichedrett, Tissa and Bahia are identical to Tremois. For the other genotypes we could not identify any PCR product after many tests, which might indicate the absence of the gene in the two genotypes, or the presence of different *CBF4* alleles from Nure and Tremois. The presence of a third allele was verified by successful amplification and sequencing of *HvCBF4* by the amplification reaction used for sequencing.

The sequencing of *CBF4* generated three haplotypes, those formed by the group of Nure and group of Tremois, and a third allele (*C*) that was found identical in both Soufara 'S' and Fouara (Table 3).

HvCBF4 represents the best marker for the *FR-H2* QTL controlling resistance to cold, through the activation of the expression of *COR* genes (Francia *et al*. 2004) whose product leads to frost tolerance (Cattivelli *et al*. 2002; Shinozaki *et al*. 2003). *FR-H1* and *FR-H2* were identified in the doubled haploid population of Nure × Tremois and contribute 60–80% to the phenotypic variation against frost tolerance (Tondelli *et al*. 2006).

Nure is frost tolerant, contains *FR-H1* and *FR-H2* and is resistant to low temperatures (Francia *et al*. 2004). Tremois is considered less resistant because of the lack of one or more genes of *FR-H2* from the CBFs (Stockinger *et al*. 2007). The present results suggest Tichedrett, Tissa and Bahia are identical to Tremois, which is less resistant to cold compared to Nure as described above. Soufara 'S' and Fouara have different, newly identified alleles. This suggests that they might show different cold resistance levels, a hypothesis that needs to be verified experimentally.

CONCLUSION

These results are the first description of the growth habit of barley varieties, selected in Algeria. The tested markers allowed us to detect the signature of the classic phenotypic, no-MAS, selection program of barleys in Algeria for semi-arid environments. The characterization of their growth habit and low-temperature tolerance loci suggests spring growth

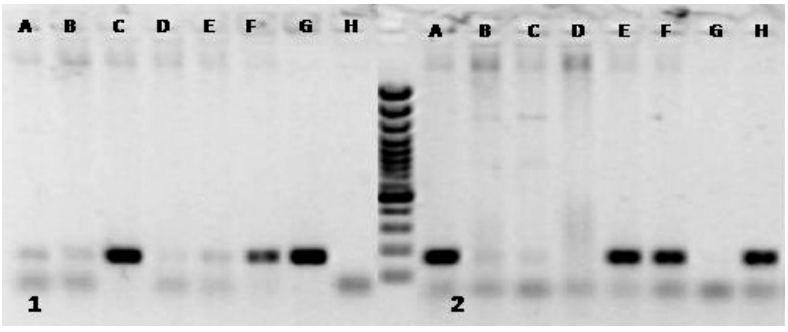

Figure 3. Allele-specific polymorphisms generated by the CAPS marker *CBF4* on six Algerian varieties compared to Nure (1) and Tremois (2); (A – Tichedrett, B – Soufara 'S', C – Rahma, D – Fouara, E – Tissa, F – Bahia, G – Nure, H – Trémois)

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Table 3. Haplotypes generated by *HvCBF4* and frost tolerance of six Algerian barley varieties compared to Nure and Tremois

Variety	Position (bp)					Frost tolerance alleles	
	93	300	494	531	haplotype	*FR-H2* (*HvCBF4*)	*FR-H1* (*HvBM5A*)
Nure	G	C	G	A	III	A	A
Tremois	G	C	G	C	I	B	B
Tichedrett	G	C	G	C	I	B	B
Soufara 'S'	C	T	C	G	II	C	B
Rahma	G	C	G	A	III	A	B
Fouara	C	T	C	G	II	C	B
Tissa	G	C	G	C	I	B	B
Bahia	G	C	G	C	I	B	B

G – guanine; C – cytosine; A – adenine; T – thymine

habit and susceptibility to cold for the *FR-H2* component. However, a third allele was identified for the *HvCBF4* marker designed on a causal candidate gene for part of the trait. Therefore, the two cultivars carrying this allele might be tested to verify their behaviour when subjected to cold winter stress. As already mentioned, the selection based on phenotyping can be improved by marker assisted selection, and the landrace Tichedrett is considered as a standard reference for all barley selection programs in these regions. Therefore, the description of both *FR-H1* (*VRN-H1*) and *FR-H2* (*HvCBF4*) loci of this and other cultivars could be exploited by MAS in populations derived from these genotypes.

Acknowledgements. This research was supported by the Genomics Research Centre of Fiorenzuola d'Arda in Italy and the Technical Institute for Field Crops (ITGC), Sétif, Algeria.

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Received for publication February 18, 2016
Accepted after corrections October 14, 2016

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