https://doi.org/10.17221/62/2020-CJGPB

Allelic variations at the *HvSNF2* and *HvBM5* loci are associated with the heading date and growth habit of barley (*Hordeum vulgare* L.) under a semi-arid climate

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Citation: Marzougui S. (2021): Allelic variations at the *HvSNF2* and *HvBM5* loci are associated with the heading date and growth habit of barley (*Hordeum vulgare* L.) under a semi-arid climate. Czech J. Genet. Plant Breed., 57: 76–79.

Abstract: The heading date and growth habit are key factors that regulate the transition from the vegetative to the reproductive stage in barley. In this study, we used PCR based markers to identify the allelic variations in the *Vrn-H1* (*HvMB5*) and *Vrn-H2* (*HvSNF2*) genes and to predict the heading date and growth habit of a collection of Tunisian barley assessed under a semi-arid climate. The allelic variation at *HvBM5* revealed two PCR fragments at 830 and 344 bp. Primer sets used to amplify the *HvSNF2* gene have resulted in different alleles size of 543, 623, and 700 bp. Different allelic combinations of *HVBM5* and *HvSNF2* were associated with the heading date and growth habit. The spring and early heading accessions were only characterised by the amplification of the *HvSNF2* fragment at 700 bp. All the winter accessions yielded the PCR product *HvBM5* at 830 bp, but the variation in the heading date was determined by the *HvSNF2* alleles. These DNA markers will be a powerful tool to predict the heading date and growth habit and can be used as markers for the assisted selection to speed up the national breeding programme.

Keywords: allelic combinations; days to heading; growth habit; long-day conditions; vernalisation; *Vrn-H* loci

Based on the growth habit, barley (*Hordeum vulgare* L.) can be classified as winter, spring or facultative. The molecular basis of the vernalisation response has been proposed as the epistatic interaction between three loci *Vrn-H1* (5H), *Vrn-H2* (4H) and *Vrn-H3* (1H) (Takashi & Yasuda 1971). The absence of allelic variants at the *Vrn-H3* locus reduces the genetic model to a two-locus epistatic model. The allelic variation at *Vrn-H1*is associated with a mutation in the promoter or deletions (~2.8 kb) within the first intron of the *HvBM5A* gene leads to the spring growth habit and a reduction in the vernalisation requirement (von Zitzewitz et al. 2005). A loss of function or deletion in the coding region of *Vrn-H2* (*ZCCT* transcription factor) leads to the recessive inheritance of the spring growth habit (Dubcovsky et al. 2005; Szucs et al. 2007). The *ZCCT* candidate gene in barley consists of three tightly linked genes (*ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc*) (Dubcovsky et al. 2005). The *Vrn-H2* allele structure was inferred by the codominant *HvSNF2* locus, which co-segregated with the ZCCT-H presence/deletion (Karsai et al. 2005)

In this study, we screened a collection of Tunisian barley accessions to predict the heading date and growth habit by using allele specific markers to identify the allelic variations of *Vrn-H1* (*HvBM5*) and *Vrn-H2* (*HvSNF2*). The phenotyping of the winter growth habit was scored based on no flowering tillers (Mohammadi et al. 2013; Taheripourfard et al. 2018) as they require a prolonged period of vernalisation.

Supported by a research grant from The Korea-Africa Food & Agriculture Cooperation Initiative (KAFACI), Korea and the Institution for Agricultural Research and Higher Education (IRESA), Tunisia

https://doi.org/10.17221/62/2020-CJGPB

This study was conducted at the El Kef research station located in the north-west of Tunisia, which characterised by a low and irregular rainfall distribution, especially during the vegetative and grain filling stages which affect the grain yield (Table S1 in the Electronic Supplementary Material (ESM)). The sowing date started from December 4th for all three cropping seasons. In 2017 and 2018, the heading date phenotype showed a variation ranging from 105 days to 120 days for the significantly early accessions and from 125 to 135 days for the significantly late heading. The winter accessions were characterised as late heading starting from 132 days. In 2019, all the accessions showed a delay in the heading date of 5 to 10 days except for the Durez accession, which showed an accelerated heading by a prolonged period of cold for three days.

Eight seeds from each accession (Table S2 in the ESM) were germinated and leaves were harvested at the three-leaf stage after 15 days of planting. The genomic DNA was extracted according to the manufacture's GRS Genomic DNA kit (Grisp, Portugal). The DNA quality and quantity were determined using a UV-Vis spectrophotometer and the visual comparison of 2% agarose gel electrophoresis.

PCR (polymerase chain reaction) amplification was performed in a 10 µL total volume consisting of 6 uL of GRS Hotstart Taq Mastermix (Grisp, Portugal), 0.25 µL of each primer (10 µM) and 1 µL of DNA (50 ng). The PCR product was then analysed on the 2% agarose gel. The DNA amplification was performed in a FastGene Ultra Cycler (96-well) (Nippon Genetics, Germany).

Three primer sets were used to assess the deletions in the three-gene cluster *Vrn-H2* locus that is, *ZCCT-Ha*, *ZCCT-Hb*, and *ZCCT-Hc* (Yan et al. 2006) (Table S3 in the ESM). The PCR amplification resulted in the amplification DNA fragments of 600 bp, 600 and 200 bp respectively. According to (von Zitzewitz et al. 2005), the *ZCCT-H* genes are present in each winter accession, but deleted from the facultative and spring accessions and the presence/absence of the tightly linked *ZCCT-H* gene family members on chromosome 4H perfectly correlates with the growth habit. Dubcovsky et al. (2005) suggested that the *ZCCT-Hb* gene is not sufficient to determine the winter growth habit in barley. However, Trevaskis et al. (2006) suggested that *ZCCT-Ha* and *ZCCT-Hb* may be sufficient for the winter growth habit as the daylength was shown to reduce their expression and then it is not required for the expression of *Vrn-H1*. Here, in three accessions, G16, G26 and Durez, *ZCCT-Hb* was found to be deleted; G16 and G25 were characterised as a facultative accession and Durez as a winter accession with an accelerated heading by a prolonged period of cold in 2019. Then it was suggested that the *ZCCT-H* genes are not sufficiently deterministic for the growth habit in this barley collection.

The primer sets HvSNF2.01F and HvSNF2.04R (von Zitzewitz et al. 2005) were used to amplify the *HvSNF2* gene located in the 4HL chromosome (Table S3 in ESM). The genotyping resulted in different allele sizes of 543, 623, and 700 bp. The 168-bp long insertion/deletion between the DNA sequences made it possible to use *HvSNF2* as the codominant marker. The PCR amplification using primers HvBM5.84F and HvBM5.85R resulted in PCR products of 437 bp in all the accessions indicating the presence of the vernalization critical region in the intron I region of the *Vrn-H1* gene. Testing for the presence or absence of the proposed 437 bp "vernalisation critical" region within intron 1 is not sufficient to predict spring growth habit. Therefore, this region is likely to be larger or more diffuse than previously proposed. It is possible that deletions of varying size result in a continuum of the vernalisation sensitivity, as suggested by (Szucs et al. 2007). An additional assessment using the primers HvBM5A-intronI-F3b and HvBM5A-intronI-R3b resulted in PCR products of 830 bp (allele *1A*) in seven barley accessions (Lemsi, G7, G29, the Reno cultivar, Durez landrace, the Hanover cultivar and G42), 344 bp (allele *5C*) in four accessions (G10, G25, G47 and G48) and deleted from the remaining accessions. According to Cockram et al. (2009), *1A* and *5C* are typical alleles for winter habit accessions (Figure S1 in the ESM).

The early heading accession (E) did not yield a PCR product for the winter alleles *1A* (830 bp) and *5C* (344 bp), but was determined by the *HvSNF2* allele 700 bp only with a heading date of 105 days. All typed accessions with the *HvSNF2* allele 543 bp were characterised as significantly late heading (SLH) (125 to 135 days). However, allele 623 bp was present in all the significantly early accessions SEH (120 days). The three-winter barley cultivar positive controls were characterised by the combination of the allele *1A* (830 bp) and the *HvSNF2* alleles. The winter accessions with the *830/543* alleles and *830/623* alleles showed a heading date of 132 days. However, the 830/700 bp winter accessions headed later at 140 to 146 days, but both responded differently to the prolonged cold period. T40 showed a delay in heading

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Table 1. Different allelic combinations associated with the heading date and growth habit of the 56 barley accessions

No. of accessions	*Vrn-H1* 437 bp	*Vrn-H1* 1A/5C alleles (bp)	*HvSNF2* alleles (bp)	*Vrn-H2*			Heading date (days)		Predicted growth habit	Observed growth habit
				ZCCT-Ha	*ZCCT-Hb*	*ZCCT-Hc*	2017, 2018	2019		
21	+	–	543	22 (+)	+	+	125–130	130–137	facultative	facultative
20	+	–	623	+	18 (+) 2 (–)	+	~120	125–130	facultative	facultative
4	+	-	543/623	+	+	+	~123	130–135	facultative	facultative
1	+	–	700	+	+	+	~105	122	spring	spring
3	+	*1A* (830)	543	+	+	+	132	140–142	winter	winter
1	+	*1A* (830)	623	+	+	+	132	137	winter	winter
2	+	*1A* (830)	700	+	1 (+) 1 (–)	+	140 146	146 143	winter	winter
2	+	*5C* (344)	543	+	+	+	125–130	130–135	facultative	facultative
1	+	*5C* (344)	623	+	+	+	123	133	facultative	facultative
1	+	*5C* (344)	543/623	+	+	+	123	137	facultative	facultative

+/–presence or absence of alleles; the presence of HvSNF2 700 bp allele delayed the heading; the fragments 830/543 bp resulted in a significantly late heading DNA fragment and the 700 bp allele characterises the spring growth habit with an early heading

by 6 days, and T45 showed an accelerated heading by 3 days. This vernalisation response may be due to the *ZCCT-Hb* gene deletion in the T45 accession.

Four accessions showed three different combinations of alleles including two with *5C* (344/543 bp), one with *5C* (623 bp) and one with *5C* (543/623 bp). Accessions having the *HvSNF2* 543 bp allele showed a significantly late heading (125/135 days). All the accessions were characterised as facultative. Based on the different allelic combinations, the presence of 830 bp at the *Vrn-H1* locus is considered as sufficient to determine the winter growth habit. The presence of the *HvSNF2* 700 bp allele delayed the heading. The alleles 830/543 bp resulted in a significantly late heading in the presence of the prolonged cold period. The DNA fragment at 700 bp characterises the spring growth habit with an early heading of 105 days, but is very sensitive to the vernalisation (Table 1). In this study, the amplification of the *HvMB5* 830 bp allele was sufficient to predict the winter growth habit, but the amplification of the vernalisation critical region (437 bp) and allele *5C* (344 bp) were not enough. The spring habit is characterised by an early heading, fully flowered tiller and the presence of the *HvSNF2* 700 bp allele. Our results suggested that different allele combinations at *HvSNF2* (*Vrn-H2*) and *HvBM5* (*Vrn-H1*) can be used to predict the heading date and growth habit under a semi-arid climate.

Acknowledgement: We thank the technical staff of the agricultural experimental research unit at EL Kef.

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Received: July 2, 2020
Accepted: November 20, 2020
Published online: January 20, 2021