Allelic Variations at *Dhn4* and *Dhn7* are Associated with Frost Tolerance in Barley

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Abstract: The sequences of the closely linked barley dehydrin genes *Dhn4* and *Dhn7* are both known to vary allelically. Here we associated allelic constitution at *Dhn4/7* with frost sensitivity across a panel of 30 diverse barley varieties. The combined presence of a 6 bp insertion in exon1 of *Dhn4* and a 30 bp deletion in exon1 of *Dhn7* was restricted to six-rowed winter and intermediate varieties characterised by relatively higher frost tolerance (12 genotypes; LT_{50} from –14.2°C to –15.6°C). The alternative combination was present with one exception (six-rowed winter variety Alissa) only in spring and two-rowed winter varieties (17 genotypes; LT_{50} from –10.0°C to –14.3°C). The genetic linkage between *Dhn4* and *Dhn7* identified e.g. in Dictoo and Morex varieties was verified by segregation analysis of F_2 plants from a cross between two genotypes carrying different allelic combination of *Dhn4* and *Dhn7* genes (two-rowed spring variety Akcent × six-rowed winter variety Okal). The potential of the former allelic combination as a marker for enhanced frost tolerance was tested in a sample of F_5 derivatives of a cross between the two-rowed winter type variety Monaco (Akcent allele combination) and the six-rowed winter type variety Okal. Plants with the Okal allele combination showed significantly higher frost tolerance than those with the alternative growth habit. The effect of ear type on frost tolerance was insignificant.

Keywords: abiotic stress; dehydrins; gene polymorphism; *Hordeum vulgare*

The expression of frost tolerance requires a number of physiological adaptations within the plant, both at the cellular and at the whole plant level, and thus its inheritance is unsurprisingly complex. Even the measurement of the trait is fraught with complications, and is variously evaluated either directly or indirectly (Prášil *et al.* 2007). In bread wheat, two major genes underlying the contrasting frost tolerance of a pair of varieties have been identified (Sutka & Snape 1989; Galiba *et al.* 1997), while in barley a quantitative trait locus (QTL) approach has defined a single genomic region responsible for field winter survival and the fructan content of the crown (Hayes *et al.* 1993). A less simple genetic model was obtained from controlled environment experiments, where nine freezing tolerance QTLs, distributed across four of the seven barley chromosomes, were identified (Tuberosa *et al.* 1997). More recently, the loci *Fr-H1* and *Fr-H2*, both involved in the determination of freezing tolerance, were mapped to the long arm of barley chromosome 5H (Skinner *et al.* 2006; Francia *et al.* 2007), and DNA-based genetic markers for these loci have been developed since then (Akar *et al.* 2009; Rapacz *et al.* 2010).

At the gene level, a number of candidate gene families have been proposed as being responsible

for stress tolerance (Cattivelli & Bartels 1990; Thomashow 1999; Yang *et al*. 2005). Among these, the dehydrins have been repeatedly associated with the plant response to dehydration or low temperature (Close 1996; Cambel & Close 1997; Xu *et al*. 2008). A distinctive feature of these proteins is their conserved lysine-rich domain designated the *K*-segment, which is predicted to form an amphipathic α-helix structure (Close 1997). The dehydrins appear to be important determinants of membrane interactions and/or protein stabilization (Close 1996; Bravo *et al*. 2003; Koag *et al*. 2003). The expression of some dehydrin (*Dhn*) genes is induced by the imposition of either drought or low temperature stress (Danyluk *et al*. 1998; Zhu *et al*. 2000; Kobayashi *et al*. 2004; Suprunova *et al*. 2004; Kosová *et al*. 2007). In barley, 13 *Dhn* genes have been identified to date (Van Zee *et al*. 1995; Choi *et al*. 1999; Choi & Close 2000; Rodriguez *et al*. 2005), and these are distributed across four of the seven barley chromosomes (*Dhn10* and *Dhn11* on 3H, *Dhn6* and *Dhn13* on 4H, *Dhn1*, *Dhn2* and *Dhn9* on 5H, and *Dhn3*, *Dhn4*, *Dhn5*, *Dhn7*, *Dhn8* and *Dhn12* on 6H) (Choi *et al*. 2000). The *Dhn* genes mapping to chromosomes 5H and 6H appear to be clustered (Pan *et al*. 1994; Campbell & Close 1997). Allelic variation at the sequence level has been demonstrated for most of these *Dhn* genes (Choi *et al*. 1999; Close *et al*. 2000; Lababidi *et al*. 2004), with *Dhn4* being the most variable (Choi *et al*. 1999). However, no correlation has been found to date between allelic constitution at any of the *Dhn* genes and phenotype in barley, although in cowpea such an association was drawn by Ismail *et al*. (1999). At the level of gene expression, in contrast, variation in sensitivity to abiotic stress has been demonstrated for a number of barley and wheat *Dhn* genes (Zhu *et al*. 2000; Suprunova *et al*. 2004; Kosová *et al*. 2008; Holková *et al*. 2009).

Here, we set out to identify the allelic variation at the sequence level in barley *Dhn* genes which have been implicated in frost tolerance. We have analyzed a set of barley germplasm known to show variation in their frost sensitivity, with a focus on *Dhn4* and *Dhn7*. The possibility to use this specific allelic variation for estimation of frost tolerance was studied in F_5 generation lines developed from crosses between selected winter barley varieties differing in their frost sensitivity.

MATERIALS AND METHODS

Germplasm panel and segregating populations

The germplasm panel consisted of 30 barley entries, of which eight were spring types (Akcent, Amulet, Atlas 68, Braemar, Diamant, Morex, Prestige and Sebastian), three had an intermediate vernalization requirement (Dicktoo, Kromir and Lunet) and the remaining 19 (Alissa, Babylone, Camera, Campill, Carola, Duet, Khutorok, Igri, Jolante, Kamil, Kromoz, Luran, Luxor, Merlot, Monaco, Okal, Reni, Tiffany and Vilna) were winter types. The seed was obtained from the Central Institute for Supervising and Testing in Agriculture (Brno, Czech Republic), Crop Research Institute (Prague, Czech Republic), Krasnodar Lukyanenko Research Institute of Agriculture and from the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). Characteristics of the examined varieties are given in Table 1.

The frost tolerant winter type variety Okal (LT_{50} −15.1°C) was crossed with the susceptible spring type variety Akcent (LT_{50} −10.9°C) and 34 randomly selected plants of $F_{2:3}$ generation were used for analyses. The second segregating population was obtained from the cross between Okal and Monaco (LT_{50} −3.4°C). In $F_{2:3}$ generation ten two-rowed and ten six-rowed segregants were randomly selected and bred to F_5 using a pedigree method. Frost tolerance evaluation was carried out in the lines of F_5 generation.

Isolation and amplification of DNA and sequencing of *Dhn4* and *Dhn7* alleles

Genomic DNA was extracted from 200 mg fresh leaf using the standard protocol provided with the Dneasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA was PCR amplified using a pair of primers (F1/R1) directed simultaneously to the first exon of *Dhn4* and *Dhn7*. The F1/R1 sequences were 5-GAGTACCAGGGACAGCAG and 5-CGAGCTGGAGCTGGAGCTGC. PCR employed reagents provided in the *Taq* PCR Core kit (Qiagen, Hilden, Germany), with the addition of 0.48mM of each primer and 50–100 ng genomic DNA. The PCR cycling regime consisted of an initial denaturation of 94°C/3 min, followed by 40 cycles of 94°C/90 s, 57°C/90 s and 72°C/90 s, and terminated by a final extension of 72°C/10 min. The resulting amplicon

was separated by electrophoresis either through 7.5% denaturing polyacrylamide gels and visualized by silver staining (Blum *et al*. 1987), or through 1.5% agarose gels followed by ethidium bromide staining.

The detection of mutations in Dhn4/Dhn7 in F_2 plants was done with the F1/R1 primers. The specific products were identified by electrophoresis through 7.5% denaturing polyacrylamide gels and visualized by silver staining (Blum *et al*. 1987).

The full-length sequences of the *Dhn4* alleles present in Akcent, Okal, Monaco and Luran, and Akcent and Okal, respectively, were derived from the amplicons generated from genomic DNA using the PCR primers suggested by Choi *et al*. (1999). The PCR cycling regime consisted of an initial denaturation step of 94°C/3 min, followed by 35 cycles of 94°C/90 s, 66°C/30 s and 72°C/90 s, and terminated by a final extension step of 72°C/10 min.

Table 1. Allelic variation at *Dhn4/7* and frost tolerance of 30 barley entries

No.	Variety	Origin	Year of registration/ accession No.	Growth habit	Ear type	LT_{50}		Type of polymorphic sequence
						°C	SD	
1	Sebastian	DNK	2005	spring	2-rowed	–10.0	0.31	A
2	Braemar	GBR	2006	spring	2-rowed	–10.5	0.27	A
3	Diamant	CZE	03C0600166	spring	2-rowed	–10.7	0.17	A
4	Amulet	CZE	1995	spring	2-rowed	–10.9	0.26	A
5	Prestige	FRA	2002	spring	2-rowed	–11.2	0.28	A
6	Akcent	CZE	1992	spring	2-rowed	–11.2	0.30	A
7	Morex	USA	03C0601603	spring	2-rowed	–11.5	0.32	A
8	Atlas 68	USA	HOR9592	spring	6-rowed	–11.5	0.25	A
9	Camera	GBR	2001	winter	2-rowed	–13.0	0.21	A
10	Duet	GBR	2000	winter	2-rowed	–13.1	0.18	A
11	Monaco	FRA	1995	winter	2-rowed	–13.4	0.13	A
12	Igri	DEU	01C050751	winter	2-rowed	–13.4	0.25	A
13	Jolante	DEU	2000	winter	2-rowed	–13.5	0.32	A
14	Reni	DEU	2001	winter	2-rowed	–13.7	0.24	A
15	Babylone	FRA	1997	winter	2-rowed	–13.7	0.08	A
16	Vilna	NLD	2001	winter	2-rowed	–13.8	0.11	A
17	Alissa	DEU	2001	winter	6-rowed	–13.9	0.30	A
18	Tiffany	DEU	1999	winter	2-rowed	–14.3	0.32	A
19	Campill	DEU	2005	winter	6-rowed	–14.2	0.16	O
20	Kromoz	CZE	1992	winter	6-rowed	–14.3	0.32	O
21	Kromir	CZE	1995	intermediate	6-rowed	–14.3	0.26	O
22	Luran	CZE	1998	winter	6-rowed	–14.4	0.09	O
23	Merlot	DEU	2002	winter	6-rowed	–14.5	0.31	O
24	Khutorok	RUS	2005*	winter	6-rowed	–14.7	0.22	O
25	Carola	DEU	2001	winter	6-rowed	–14.9	0.23	O
26	Okal	CZE	1992	winter	6-rowed	–15.1	0.16	O
27	Luxor	CZE	1996	winter	6-rowed	–15.2	0.15	O
28	Kamil	CZE	1993	winter	6-rowed	–15.2	0.25	O
29	Dicktoo	USA	HOR3113	intermediate	6-rowed	–15.3	0.36	O
30	Lunet	CZE	1990	intermediate	6-rowed	–15.6	0.22	O

*The variety was developed at the Krasnodar Lukyanenko Research Institute of Agriculture

LT_{50} – median lethal temperature; SD – standard deviation; type of polymorphic sequence: O – Okal, A – Akcent

The Akcent and Okal *Dhn7* alleles were sequenced, based on primers 5-GCCAAGTGAGGAAGACAACC and 5-CCGGCACCTCTTAACTTTC, *de novo* designed from the sequence of the Dictoo *Dhn7* allele (GenBank AF043092). PCR reaction was initiated at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min 30 s, 55°C for 1 min 30 s, and terminated at 72°C for 10 min. The amplicons were purified using a PCR purification kit (Qiagen, Hilden, Germany).

Determination of frost tolerance

Barley grains were germinated at 20°C in the dark for two days, potted into soil and exposed for 21 days to a 16 h day/8 h night regime at 17°C under an irradiance of 350 µmol/m^2/s. The seedlings were cold hardened for further 21 days by dropping the temperature to 3°C. Then they were removed from the soil, divided into five groups of 8–10 plants each, and exposed to –4°C in the dark for three days. Then, each group was chilled for further 24 h at either –9, –11, –13, –15 or –17°C, after which they were restored to +20°C, repotted, and left to recover for further 21 days at this temperature. Both the cooling and the thawing rate was 2°C/h according to Prášil *et al*. (2007). At the end of the recovery period, the survival percentage for each entry within each freezing treatment was calculated, and LT_{50}, with its associated standard error, was obtained according to Janáček and Prášil (1991).

RESULTS

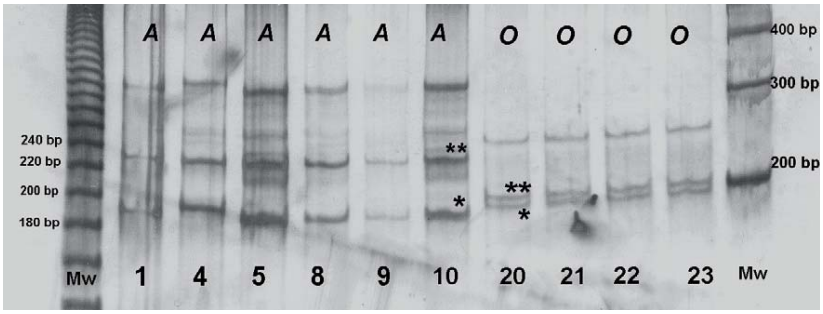
Frost tolerance of the examined barley varieties

The level of the frost tolerance as reflected by LT_{50} varied from –10.0°C to –15.6°C, with the least negative LT_{50} levels detected for the two-rowed spring types, and the most negative for the six-rowed winter and intermediate types (Table 1).

Dhn4 and *Dhn7* allele sequences

The *Dhn4* and *Dhn7* amplicons were of different lengths, which allowed their electrophoretic discrimination. The agarose gel profile of Akcent consisted of two fragments, while that of Okal showed only a single one (Figure 1b). On PAGE, however, both products were resolved in both Akcent and Okal (Figure 1a). The profiles included fragments of the sizes expected for *Dhn4* and *Dhn7*, but also other products which could not be identified. A Southern hybridisation experiment demonstrated that many of these latter products are homologous to the *Dhn* specific probe used (data not shown). The first exons of both *Dhn4* and *Dhn7* genes were sequenced from Okal/Luran/Monaco/Akcent and Okal/Akcent, respectively. At a location 18 bp downstream of the *Dhn4* ATG translation start codon, the 6 bp insertion present in Dicktoo was also present in Luran and Okal, but like in Morex, not in either Akcent or Monaco (Figure 2).

(a)



(b)

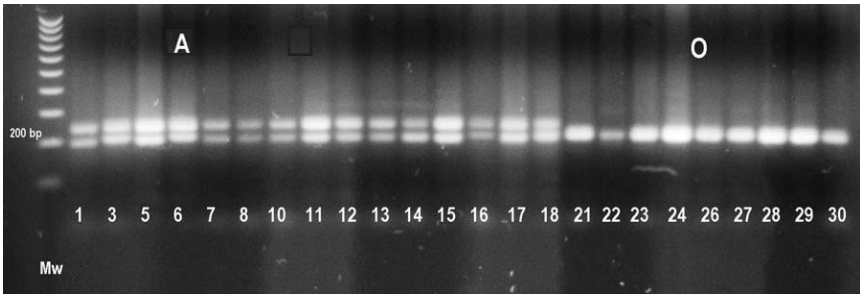


Figure 1. F1/R1 amplicon separated by denaturing PAGE (a) and AGE (b); 1–30 refer to accession numbers listed in Table 1; A – fragment typical of Akcent; O – fragment typical of Okal, *alleles of *Dhn4*, **alleles of *Dhn7*

Table 2. Amplicon sizes (bp) following PCR with the primer pair F1/R1

Gene	Varieties			
	Morex	Akcent	Okal	Dicktoo
Dhn4	198	198	204	204
Dhn7	240	240	210	210

Within *Dhn7*, a 30bp deletion present in Dicktoo was shared by Okal, but not by Akcent (or Morex) (Figure 3). These polymorphisms were also recognizable from the PAGE profiles of these amplicons (Figure 1). The expected primer sequences were present in all the sequenced *Dhn4* and *Dhn7* alleles (Figures 2 and 3), and the *Dhn4* and *Dhn7* amplicon sizes are given in Table 2.

These results have already been partially published in the special issue of the Kvasný průmysl devoted to the results of the Project No. RC1M0570 (Holková *et al.* 2010).

Frost tolerance and allelic constitution at *Dhn4/7*

The groups of entries showing a comparable level of frost tolerance tended to share the same *Dhn4*, *Dhn7* alleles (Table 1). The Okal type was present in the six-rowed winter and intermediate entries which all have a relatively good level of frost tolerance, and was not present in any of the spring types. The Akcent type was shared by all

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DICKTOO GB -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAGCAGCAGCACGGCCGCGTCGACGAGTAC
OKAL       -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAGCAGCAGCACGGCCGCGTCGACGAGTAC
LURAN      -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAGCAGCAGCACGGCCGCGTCGACGAGTAC
MONACO     -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAG------CACGGCCGCGTCGACGAGTAC
AKCENT     -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAG------CACGGCCGCGTCGACGAGTAC
MOREX GB   -CGGCAGCGCAAGATGGAGTACCAGGGACAGCAG------CACGGCCGCGTCGACGAGTAC
            ********************************      **********************

DICKTOO GB  GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGA
LURAN       GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGA
OKAL        GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGA
MONACO      GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGA
AKCENT      GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGA
MOREX GB    GGCAACCCTGTGGCCGGACATGGCGTCGGCACCGGCATGGGAACGCACGGCGGCGTCGGC
            ************************************************************

DICKTOO GB  ACTGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
OKAL        ACTGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
LURAN       ACTGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
MONACO      ACCGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
AKCENT      ACTGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
MOREX GB    ACTGGCGCGGCCGCCGGTGGGCATTACCAGCCCATGAGGGACGAGCACCAGACTGGCCGT
            ** ********************************************************

DICKTOO GB  GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
OKAL        GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
LURAN       GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
MONACO      GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
AKCENT      GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
MOREX GB    GGGATCCTGCACCGCTCCGGCAGCTCCAGCTCCAGCTCGGTGCGTATATACTGTCCTTGC
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Figure 2. Alignment of sequences from *Dhn4* exon1 in six barley varieties; Dicktoo and Morex sequences, recovered from GenBank (AF043090 and AF181454, respectively), shown in italics. Location of the F primer given by Choi *et al.* (1999) is underlined, and those of F1 and R1 are highlighted

Table 3. Marker segregation in the F_2 generation

Genotype of parents	Occurrence in F_2		
	Ak (FFSS)	H (FfSs)	Ok (ffss)
Ak × Ok (FFSS × ffss)	4	4	7
Ok × Ak (ffss × FFSS)	10	2	7

Ak – Akcent, H – heterozygote, Ok – Okal; F, f – alleles of *Dhn4*; S, s – alleles of *Dhn7*

the spring entries, by the two-rowed winter entries and by Alissa, a six-rowed winter type. The mean LT_{50} of carriers of the Okal allelic combination (–14.8°C) (all winter varieties) was > 1°C below that of winter type carriers of the Akcent allelic combination (–13.6°C) (Figure 4).

The F_2 individuals derived from the Akcent × Okal cross were used to confirm the close linkage between *Dhn4* and *Dhn7* reported by Pan *et al.* (1994) for Dictoo × Morex, and Francia *et al.* (2004) for Nure × Tremois. As shown in Table 3, only parental homozygotes or double heterozygotes were detectable among the 34 segregants tested.

The effect of *Dhn4*/*7* allele on frost tolerance was tested in winter × winter (Okal type × Akcent type) F_5 lines obtained from 20 $F_{2:3}$ plants (Okal × Monaco cross). Since the six-rowed ear barley was associated with higher frost tolerance than the two-rowed type (Prášil *et al.* 2007), the ear type was also taken into consideration. Even if no six-rowed winter variety of Akcent type allelic variation was detected among the set of barley entries (Table 1), five F_5 lines were six-rowed Akcent type genotypes. The lines were divided into four groups on the basis of their *Dhn4*/7 allelic combination (Okal type or Akcent type, regardless of ear type) and their ear type (two- *vs* six-rowed, regardless of allelic combination). From each seed bulk five replications of 8–10 plants

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DICKTOO GB -ATGGAGTACCAGGGACAGCAGCAGCACGGCCAGGCGACCAACCGCGTCGACGAGTACGGT
OKAL       -ATGGAGTACCAGGGACAGCAGCAGCACGGCCAGGCGACCAACCGCGTCGACGAGTACGGT
AKCENT     -ATGGAGTACCAGGGACAGCAGCAGCACGGCCAGGCGACCAACCGCGTCGACGAGTACGGT
MOREX GB   -ATGGAGTACCAGGGACAGCAGCAGCACGGCCAGGCGACCAACCGCGTCGATGAGTACGGT
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DICKTOO GB  AACCCGGTTGCCGGACACGGCGGTGGCACCGGCATGGGC----------------------
OKAL        AACCCGGTTGCCGGACACGGCGGTGGCACCGGCATGGGC----------------------
AKCENT      AACCCGGTTGCCGGACACGGCGTCGGCACCGGCATGGGCGCGCACGGCGGCGTCGGCACC
MOREX GB    AACCCGGTTGCTGGACACGGCGTAGGCACCGGCATGGGCGAGCACGGCGGCGTCGGCACC
            ********** **********  ***************

DICKTOO GB  ---------GCGCACGGCGGCGTCGGCACCGGTGCGGCCGCTGGTGGTCATTTCCAGCCG
OKAL        ---------GCGCACGGCGGCGTCGGCACCGGTGCGGCCGCTGGTGGTCATTTCCAGCCG
AKCENT      GGCATGGGCGCGCACGGCGGCGTCGGCACCGGTGCGGCCGCTGGTGGGCATTTCCAGCCG
MOREX GB    GGGATGGGCGCGCACGGCGGCGTCGGCACCGGTGCGGCCGCTGGTGGGCATTTCCAGCCG
                    ************************************ ************

DICKTOO GB  ACGAGGAAGGAACACAAGGCCGGCGGGATCCTGCAGCGCTCCGGCAGCTCAAGCTCCAGC
OKAL        ACGAGGAAGGAACACAAGGCCGGCGGGATCCTGCAGCGCTCCGGCAGCTCAAGCTCCAGC
AKCENT      ACGAGGAAGGAGCACAAGGCCGGCGGGATCCTGCAGCGCTCCGGCAGCTCAAGCTCCAGC
MOREX GB    ACGAGGAAGGAGCACAAGGCCGGCGGGATCCTGCAGCGCTCCGGCAGCTCAAGCTCCAGC
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DICKTOO GB  TCG         243
OKAL        TCG
AKCENT      TCG
MOREX GB    TCG
            ***
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Figure 3. *Dhn7* exon1 in four varieties; Dicktoo and Morex sequences, recovered from GenBank (AF043092 and AF181457, respectively), shown in italics. The locations of the F1 and R1 primers are highlighted; bold letters mark point mutations

Table 4. Variation in frost tolerance among the F_5 progeny of the cross Okal × Monaco, expressed both as LT_{50} and mean percentage of plant survival following freezing treatment

Ok × Mo F_5 families		Average of survival (%)	LT_{50} (°C)	SD
Type of polymorphic sequence	A	53.5	-13.3^{b*}	0.21
	O	60.3	-14.0^{a}	0.18
Type of ear	2-rowed	54.0	-13.4^{b}	0.28
	6-rowed	58.0	-13.8^{ab}	0.24

SD – standard deviation; *different letters denote statistically significant differences at $P < 0.05$

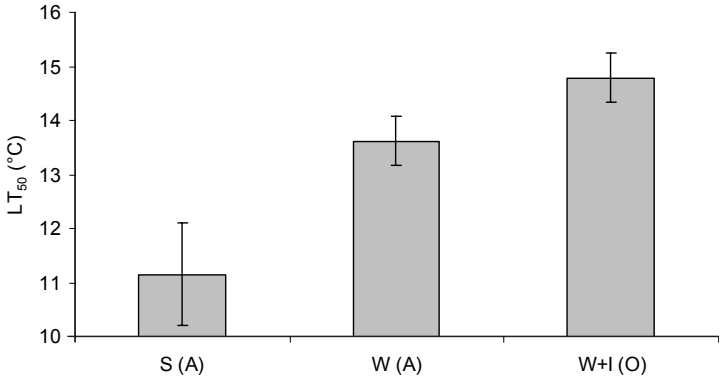

Figure 4. Evaluation of the frost tolerance level of spring (S), winter (W) and intermediate (I) barley varieties with A/O allele; frost tolerance is expressed as $LT_{50} \pm SD$

were grown and used for the determination of frost tolerance. Their response to frost stress is summarized in Table 4. A significant difference in the mean survival of plants was obtained between Okal and Akcent types (60.3% *vs* 53.5%), which was reflected by an LT_{50} difference of ~0.7°C. The poorer frost tolerance of Akcent type plants was further tested by χ^2 analysis of the number of survivors/non-survivors. This showed a statistically significant effect for the *Dhn4/7* allele ($P < 0.01$), but an insignificant one for ear type ($P = 0.92$).

DISCUSSION

Allelic variation at the DNA sequence level has been documented among barley *Dhn* genes, but the relationship between *Dhn* allele and abiotic stress phenotype remains unclear. Here, we set out to determine whether such variation at *Dhn4* and *Dhn7* (mapping to barley chromosome 6H) can be associated with variation in frost sensitivity. We could show that there was sequence variation within the first exon of both genes. The question is whether these different allelic sequences translate into a substantial effect on the gene product function. In cowpea, the perfect genetic correlation between the allele constitution at a *Dhn* gene and chilling tolerance during emergence (Ismail *et al*. 1999) suggests that such a functional relationship is possible. In barley, the most common *Dhn* allelic variants involve a combination of changes in the number of copies of Φ-segments (tandemly repeated domains lying between consecutive K-segments) present, and single nucleotide polymorphisms, some of which induce a change in peptide residue (Choi *et al*. 1999). Lababidi *et al*. (2004) identified sequence variation in *Dhn* genes across 14 barley varieties, but the length polymorphisms was detected only at *Dhn3*. A six residue (18 bp) insertion downstream of a *Dhn3* Y-segment was present in the variety Zambaka but absent in the variety Tadmor; these two varieties differ markedly from one another with respect to their drought tolerance. Note that the 30 bp deletion in the Okal *Dhn7* allele is also located downstream of the Y-segment, so this type of mutation and its intragenic position may negatively impact on the functioning of a stress defence pathway by compromising the functionality of the *Dhn* product.

Genetic linkage between *Dhn3*, *Dhn4*, *Dhn5* and *Dhn7* has been demonstrated by their partial co-segregation in the Dictoo × Morex mapping

population (Choi *et al*. 2000) as well as in Nure × Tremois (Francia *et al*. 2004). The genetic linkage between *Dhn4* and *Dhn7* was verified in our segregating population. Among a limited number of Akcent × Okal F_2 plants we were unable to recover any *Dhn4/Dhn7* recombinants. Thus these two sequence polymorphisms can be regarded as a single "marker" sequence.

The barley varieties Dicktoo and Morex showed contrasting levels of frost tolerance in our experiments, but no report of any allelic variation between them at the *Dhn* genes mapping to chromosome 5H was available until then. They do, however, show allelic variation at *Dhn4* and *Dhn7*, neither of which has been associated with a differential response to cold stress to date (Zhu *et al*. 2000). The product of *Dhn5* (which maps within the same cluster as *Dhn4/7*) has been shown to be more highly expressed in cold tolerant entries (Ping *et al*. 2000; Zhu *et al*. 2000; Kosová *et al*. 2008). Several overlapping QTL have also been identified which underlie variation in traits related to water status and drought tolerance (Teulat *et al*. 2003). The co-localisation of such QTL with the *Dhn* genes suggests a role for these genes in controlling the plant water status when challenged with drought (Campbell & Close 1997; Cattivelli *et al*. 2002).

We have demonstrated the existence of a significant association between the level of frost tolerance and the allelic constitution at *Dhn4/7*. The preponderance of the Okal allele among the six-rowed entries may be either linked to a higher level of frost tolerance or due to the fact that two-rowed and six-rowed genotypes tend to form different genetic pools (Zhang *et al*. 2009). The polymorphism of *Dhn* gene sequences on the 6H chromosome, specifically of *Dhn4* and *Dhn7*, has been demonstrated in several populations of wild barley of different geographic origins (Morrell & Clegg 2007). In Central Europe six-rowed barley varieties are usually winter feed types. The development of a six-rowed ear is genetically determined by the allelic status at the *Vrs1/vrs1* locus, located on chromosome 2H. The relationship between ear type and frost sensitivity may be the outcome of possible linkage between *vrs1* and the frost sensitivity genes *cor14b* and *blt14*, both of which were mapped to the long arm of 2H (Komatsuda *et al*. 2007).

Our results suggest that the Okal allele at *Dhn4/7* is associated with enhanced frost tolerance in winter barley. If it is to be exploited for indirect selection purposes, of course it will need to be validated across a much larger sample of germplasm; this evaluation will need to improve the precision of frost tolerance phenotyping at the same time. In particular, due to the absence of genetic correlation between frost tolerance and ear type, there is every prospect for improving the level of frost tolerance of two-rowed winter varieties.

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