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FR-H3: a new QTL to assist in the development of fall-sown barley with superior low temperature tolerance

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Abstract Fall-sown barley will be increasingly important in the era of climate change due to higher yield potential and efficient use of water resources. Resistance/tolerance to abiotic stresses will be critical, and foremost among the abiotic stresses is low temperature. Simultaneous gene discovery and breeding will accelerate the development of agronomically relevant fall-sown barley germplasm with resistance to low temperature. We developed two doubled haploid mapping populations using two lines from the University of Nebraska (NE) and one line from Oregon State University (OR): NB3437f/OR71 (facultative × facultative) and NB713/OR71 (winter × facultative). Both were genotyped with a custom 384 oligonucleotide pool assay (OPA). QTL analyses were performed for low temperature tolerance (LTT) and vernalization sensitivity (VS). The role of *VRN-H2* in VS was confirmed and a novel alternative winter allele at *VRN-H3* was discovered in the Nebraska germplasm. *FR-H2* was identified as a probable determinant of LTT and a new QTL, *FR-H3*, was discovered on chromosome 1H that accounted for up to 48 % of the phenotypic variation in field survival at St. Paul, MN, USA. The discovery of *FR-H3* is a significant advancement in barley LTT genetics and will assist in developing the next generation of fall-sown varieties.

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Introduction

Fall-sown barley is a relatively new crop to the higher latitudes. Current varieties lack sufficient low temperature tolerance (LTT) for extensive production in these target environments. Selecting for improved LTT in a crop that has undergone intensive selection for the past ~10,000 years within a spring growth habit framework is a challenge and a necessity in a time of climate change. Fall-sown barley is higher yielding than spring-sown barley in regions that receive ample winter precipitation. Fall-sown barley conserves scarce water resources in regions where irrigation is required and can provide important ecosystem services as a winter cover crop. The challenge is to ensure that the crop survives LT stresses.

LTT is the critical component of "winter-hardiness". Two other associated traits are photoperiod sensitivity (PS)

and vernalization sensitivity (VS) (Hayes et al. 1993). Maximum vegetative stage LTT requires cold acclimation, a process in which many cold-responsive genes are up- and down-regulated (Fowler and Thomashow 2002). LTT is reduced with the vegetative to reproductive transition (Galiba et al. 2009). The associations of PS and VS with LTT are due to their involvement in the timing of this transition (Fowler et al. 2001; von Zitzewitz et al. 2005). PS delays the vegetative to reproductive transition under short days and promotes the transition under long days (Pan et al. 1994; Cuesta-Marcos et al. 2008). VS delays the transition until a sufficient number of "cold units" are achieved. VS can be satisfied, in target northern hemisphere environments, as early as December, but there is still considerable risk of LT injury after VS is satisfied. PS, in contrast, will maintain plants in a vegetative stage until the risk of LT injury is past.

PS, VS, and LTT define the three growth habits in barley (winter, facultative, and spring). As defined by von Zitzewitz et al. (2011), winter types have varying degrees of LTT and PS, and they have VS. Facultative types have varying degrees of LTT, they do not have VS, and for maximum LTT they should have short PS (flowering suppressed under short photoperiods). Spring types do not have LTT, they are not vernalization sensitive, and if they have short PS it is usually not of agronomic importance since they are grown under long photoperiod conditions. Reduced sensitivity to the induction of flowering by long photoperiods can be advantageous for spring types, because it increases the growing period. A key point is that facultative and winter types show comparable variation in LTT: VS is not a prerequisite for maximum LTT (Rizza et al. 2011).

VS is determined by the epistatic interactions between alleles at three loci: VRN-H1 (5H), VRN-H2 (4H), and VRN-H3 (7H). Winter genotypes are Vrn-H2_/vrn-H1vrn-H1/vrn-H3vrn-H3 and all other allele combinations do not have VS (Szucs et al. 2007). Facultative types have a winter allele at the VRN-H1 locus and a complete deletion of the VRN-H2 locus (Karsai et al. 2005; von Zitzewitz et al. 2005; Szucs et al. 2007). The determinant of VRN-H1 is HvBM5A, a MADS-box floral meristem identity gene which promotes flowering after a period of cold temperatures (von Zitzewitz et al. 2005). The size of deletions in the critical region of the first intron of VRN-H1 causes phenotypic variation in the degree of VS (Fu et al. 2005; von Zitzewitz et al. 2005; Szucs et al. 2007). The determinant of VRN-H2 is a zinc finger-CCT domain transcription factor which encodes a flowering repressor that is down-regulated by short days and the expression of VRN-H1 (Yan et al. 2004). In barley, there are three ZCCT genes at this complex locus (ZCCT-Ha, ZCCT-Hb, and ZCCT-Hc) (Dubcovsky et al. 2005; Karsai et al. 2005). ZCCT-Ha

is the candidate for VRN-H2 (Dubcovsky et al. 2005). VRN-H3 is an ortholog of the Arabidopsis Flowering Locus T (FT) gene (Trevaskis et al. 2007; Hemming et al. 2008; Kikuchi et al. 2009). HvFT1 is the candidate gene for VRN-H3. Mutations in the first intron of HvFT1 have been proposed to differentiate between dominant and recessive VRN-H3 alleles (Yan et al. 2006). SNPs have also been reported in the promoter to better differentiate between dominant and recessive alleles (Cuesta-Marcos et al. 2010). Dominant alleles are associated with a lack of VS and may also lead to very early flowering (Yan et al. 2006). The expression of HvFT1 is dependent on day length: long days induce expression, which promotes flowering (Turner et al. 2005). The two main determinants of photoperiod sensitivity are PPD-H1 (candidate gene HvPRR7) and PPD-H2 (candidate gene HvFT3). The variation at the PPD-H1 locus is due to changes in the amino acid sequence in the CCT domain with the recessive allele conferring insensitivity to long photoperiod conditions (Turner et al. 2005). Variation at PPD-H2 is caused by a complete or partial deletion of the gene leading to sensitivity to short photoperiods (Faure et al. 2007, Kikuchi et al. 2009).

There are two principal LTT QTL reported in the Triticeae. The first LTT QTL reported in barley was in the 'Dicktoo' × 'Morex' population (Hayes et al. 1993) and corresponds to what was subsequently designated as Frost Resistance-1 (FR-H1) (Galiba et al. 1995; Francia et al. 2004). Frost Resistance-2 (FR-H2) was discovered subsequently (Francia et al. 2004; Skinner et al. 2005; Galiba et al. 2009). In barley, FR-H1 and FR-H2 are approximately 30 cM apart on chromosome 5H. FR-H1 cosegregates with VRN-H1; however, it has not yet been shown if this is due to pleiotropic effects of VRN-H1 or to the effects of a tightly linked gene (or genes) (Francia et al. 2007). Underlying FR-H2 are two physically linked clusters of more than 11 C-repeat binding factor (CBF) genes (Francia et al. 2004; Skinner et al. 2005; Galiba et al. 2009). A polymorphism in HvCBF4 accounted for 31 % of the variation in LTT based on controlled freeze tests (Francia et al. 2004). Phenotypic variation in LTT associated with FR-H2 may be due to copy number variation in one or more CBF genes (Knox et al. 2010).

Genome-wide association mapping (GW-AM) validated the importance of FR-H1 and FR-H2 (von Zitzewitz et al. 2011) and confirmed that maximum LTT is achieved with specific alleles at each of the two loci. The question remains: is LTT due only to FR-H1 and FR-H2 or are there other undiscovered determinants of this critical trait?

In order to advance our understanding of LTT genetics and to further the improvement of winter six-row malting barley, we developed two doubled haploid (DH) populations from crosses between germplasm originating in the Pacific Northwest of the US (Oregon) and the Upper

Midwest (Nebraska). The Oregon parent (OR71) was considered to have moderate LTT and the Nebraska parents (NB3437f and NB713) were hypothesized to have high LTT based on the climatic profiles at each of the breeding program locations. The DH populations were phenotyped for LTT and VS. QTL analyses were performed using each of the two populations and the pooled data of the two half-sib populations, when appropriate.

Materials and methods

Germplasm

NB3437f (NB92711/P-954) and NB713 (P-954/Pennco) were chosen as donors of LTT. Based on long-term records, Nebraska germplasm accessions have shown some of the best LTT in the Uniform Barley Winterhardiness Nursery (UBWHN), conducted by the USDA-ARS in cooperation with State Agricultural Experiment Stations since 2003. The "f" designation in NB3437f indicates facultative growth habit, because in the NB3437 seed lot we found that <1 % of plants grown without vernalization flowered under long day conditions. A report on this finding and subsequent characterization of the NB3437f germplasm at the genotype and phenotype levels is presented in Supplemental Figure 1. OR71 (Stab 47/Kab 51-7) was developed by the Oregon State University breeding program. This selection had shown average levels of LTT in the UBWHN and in trials throughout the Pacific Northwest of the USA. The Oregon and Nebraska parental lines were not tested in the UBWHN at the same time. Therefore, we conducted a preliminary controlled environment freeze test on the parental lines, using the protocol described in "Phenotyping", prior to undertaking characterization of the DH populations (Supplemental Table 1).

The two doubled haploid mapping populations, consisting of 111 and 124 lines, were derived via anther from the F1 of NB3437f/OR71 and NB714/OR71 as described in Cistué et al. (2003). OR71 and NB3437f are facultative (VRN-H2 deletions) and we therefore expected the DH progeny to be facultative. NB713 is vernalization sensitive. Therefore, we expected the DH progeny of NB713/OR71 to segregate for VS.

Phenotyping

The two DH populations and parents were phenotyped for final leaf number (FLN) and flowering time (FT) under greenhouse conditions in vernalized and unvernalized treatments. Each DH line and parent was replicated twice within each treatment. The vernalized treatment consisted of planting seed directly into moist soil and maintaining the

experiment in a growth chamber, without light, at 4 °C for 6 weeks. Seedlings were then transferred to a greenhouse maintained at 18 ± 2 °C day and night. Supplemental light was used to provide a photoperiod of 16 h light/8 h dark. After 1 week of acclimation, seedlings were transplanted to 13.5 cm pots filled with Sunshine Professional Growing Mix. For the unvernalized treatment, seed was sown directly into 13.5 cm pots under the same greenhouse conditions. As we expected NB713/OR71V− to segregate for VS, we allotted a longer time frame for this population in the greenhouse: it was planted on 10 April 2009. Both NB3437f/OR71 V+ and NB713/OR71 V+ were planted on 13 October 2009 and NB3437f/OR71 V− was planted the next day. FLN was assessed as the total number of leaves on the main stem of each plant. FT was recorded as the number of days from planting to the appearance of awns 1–2 cm out of the boot (for unvernalized plants) and as the number of days from transfer to the greenhouse (for vernalized plants). The vernalized and unvernalized experiments were terminated 100 days after planting/transfer. Plants that did not flower were assigned an FT value of 150.

For measurement of FT under field conditions (FTF), the two populations were grown in field trials at the Oregon State University Hyslop Farm near Corvallis, OR (COR). The NB3437f/OR71 population was sown in the fall of 2008 and the NB713/OR71 population in the fall of 2009. Each DH line and the parents were planted in two-row plots, 1 m in length. FTF was recorded as the number of days after 1 January when 50 % of the heads in a plot were 50 % emerged from the boot.

LTT was assessed in field experiments and in a controlled freeze test. The field experiments were conducted at St. Paul, MN (SPMN), Lincoln, NE (LNE), Fort Collins, CO (FCCO) and Fairfield, MT (FMT). LNE, FCCO, and FMT trials were planted in fall 2009. The SPMN trial was planted in fall 2010. Each DH line and parent was grown in single row plots using two replications. LTT was rated as the percentage of plants in each plot surviving the winter. The controlled freeze tests were carried out at the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár, Hungary (MRI). This involves a stringent protocol including two hardening phases before reaching the lowest temperature of −13.5 °C. The number of plants that survived for each entry was expressed as percentage of the number of plants before freezing. This process is further described by Skinner et al. (2006).

Genotyping

The parental lines were genotyped using Barley OPA 1 and Barley OPA 2 and the data are available from the T3 database (http://triticeaetoolbox.org/; verified 13 June

2012). Accession designators changed since the genotype data were uploaded for all accessions. Therefore, data for NB3437f need to be accessed as NB03437f, data for NB713 as P-713 and data for OR71 as Stab 47/Kab 51-7. Genotype data can be retrieved by entering the appropriate accession designator in the "Quick Search" box. For genotyping the DH populations, genomic DNA was extracted from a single plant of each DH line and each parent using ∼100 mg of leaf tissue collected from greenhouse-grown plants using the Qiagen DNeasy Plant Maxi Kit (Qiagen, Valencia, CA, USA). A custom 384 oligonucleotide pool assay (OPA) was designed based on polymorphisms identified between parents using Barley OPA 1 and Barley OPA 2 (Close et al. 2009). SNPs represented in the custom OPA (Supplemental Table 2) were selected to maximize polymorphism, provide complete genome coverage, and to focus on specific genome regions/genes. Genotyping was performed using an Illumina BeadXpress at the James Hutton Institute (Invergowrie, Scotland). For quality assurance purposes, two markers were developed and assayed "in-house" based on two of the SNPs targeted in the custom 384-OPA (2_0653, and 3_0162). These markers were generated by identifying a restriction site polymorphism in the sequence flanking each of the SNPs, based on the OPA SNP sequence data obtained from the barley HarvEST database (http://harvest.ucr.edu; verified 9 November 2011). The NB3437f/OR71 population was genotyped "in-house" for *PPD-H2*. Genotyping protocols are provided in Supplemental Table 3.

Linkage mapping

JoinMap 4 (Van Ooijen 2006) was used for de novo construction of three linkage maps: NB3437f/OR71 (220 SNPs), NB713/OR71 (217 SNPs) and a combined map of the two populations created using only the 157 markers polymorphic in both of the populations. A minimum LOD score of 4 was used in the formation of linkage groups. The maximum likelihood mapping algorithm was used to determine marker order in each linkage group. Recombination frequencies were converted to centiMorgans using Haldane's mapping function as implemented in JoinMap4.

QTL analysis

QTL analyses were performed for 11 traits using each of the DH populations and the combined population using composite interval mapping (CIM) as implemented in Windows QTL Cartographer 2.5 (Wang et al. 2010). Up to seven cofactors were chosen using the CIM standard model with forward selection and backward elimination, 0.1

probabilities into and out of the model, a walk speed of 1 cM, and a scan window of 10 cM. Experiment-wise significance ($\alpha = 0.05$) likelihood ratio test (LR) thresholds for QTL identification were determined with 1,000 permutations and expressed as LOD (LOD = 0.217 LR). Multiple interval mapping (MIM) was used to evaluate epistatic interactions between QTL.

Allele effects for markers that were not assigned a position in the linkage map were estimated using PROC MIXED with the maximum likelihood (ML) method in SAS v9.1 (SAS Institute Inc.,2006). The cofactors and thresholds determined using QTL cartographer were used in calculating the significance of these unmapped markers. The proportion of variance explained by a marker at a position (R^2) was calculated using SAS PROC GLM as $R^2 = (MS_1 - MS_2)/MS_3$ where MS_1 is the mean square error including only the cofactors, MS_2 is the mean square error including the cofactors and the marker being tested, and MS_3 is the total mean square for the trait. These unmapped markers are addressed in detail in "Results" and "Discussion" sections. Broad-sense heritability values were estimated with SAS PROC VARCOMP using the following formula: $H^2 = \sigma_G^2/(\sigma_G^2 + \frac{\sigma_e^2}{r})$ where σ_G^2 represents the genetic variance, σ_e^2 the residual variance, and r the number of replicates per genotype.

Results

Linkage maps

Linkage maps are shown in Supplemental Figures 2, 3, and 4. Orders and distances of markers in the two population linkage maps, and in the combined map, were as expected for chromosomes 1H, 2H, 3H, 4H, and 6H. The chromosome 7H maps were as expected, except for three markers in the short arm that showed segregation distortion and did not map as expected with the rest of the linkage group in NB713/OR71. In both populations, markers in chromosome 5H did not map as expected in several regions. Based on the parental genotyping with BOPAs 1 and 2, from which the custom 384 OPA was designed, we expected a ∼24 cM region of monomorphism in the vicinity of *VRN-H1*. However, rather than a single gap of this approximate distance in each of the two mapping populations, we found five discrete linkage groups in NB3437f/OR71 and two linkage groups in NB713/OR71 (Fig. 1). As is apparent in this figure, linkage distances on 5H were also much greater in each of the two populations than in the consensus map of Close et al. (2009). Most markers adjacent to gaps, or areas of apparently high recombination, also showed segregation distortion.
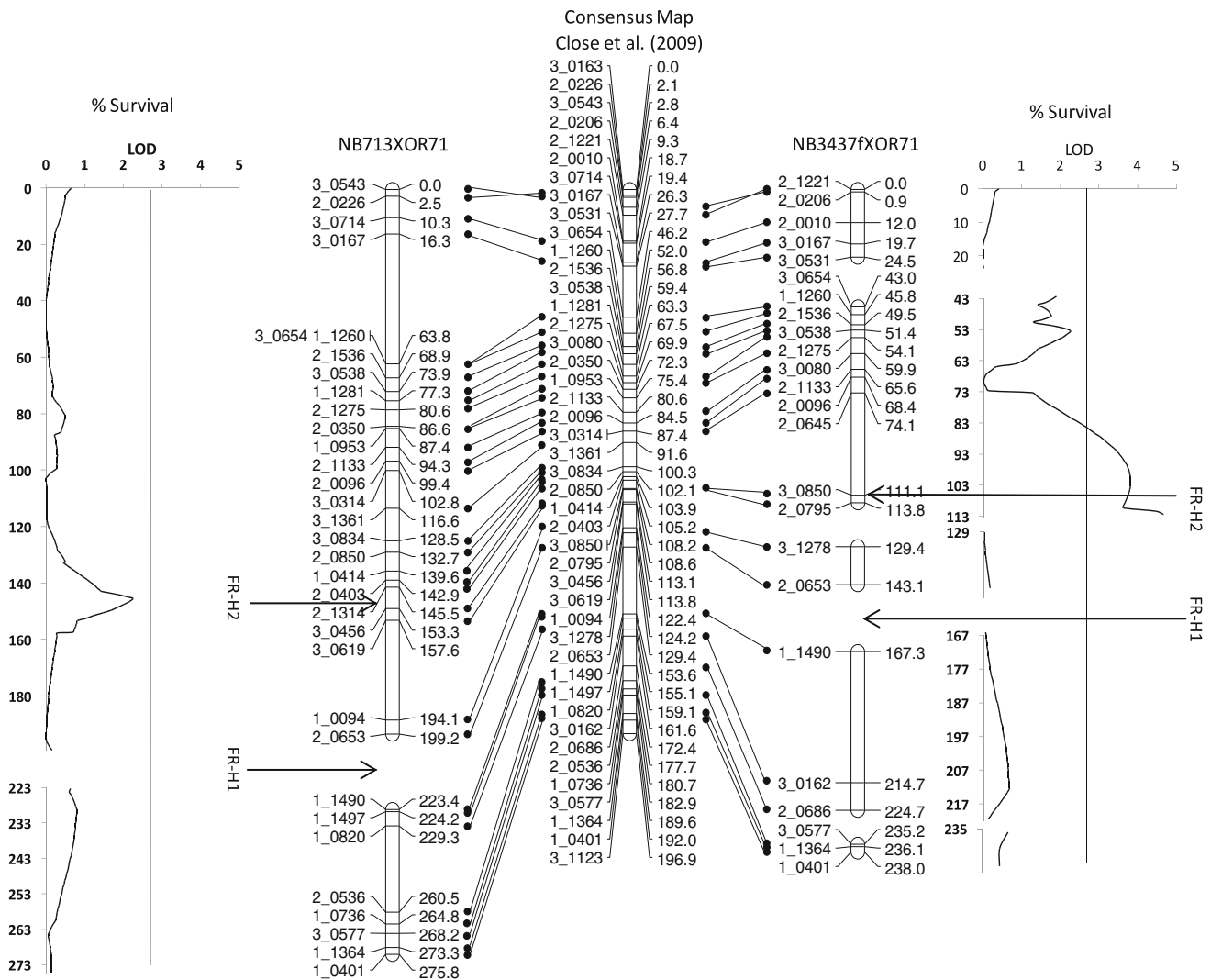
Fig. 1 Linkage maps of chromosome 5H in the NB3437f/OR71 and NB713/OR71 doubled haploid (DH) mapping populations (*left* and *right panels*) and the 5H quantitative trait locus (QTL) scans for low temperature tolerance (LTT) as measured in a controlled freeze test at the Martonvasar Research Institute aligned with the 5H consensus map published by Close et al. (2009) (*center panel*). Single nucleotide polymorphism (SNP) marker names are indicated on the left of each linkage group. Distances are given in Haldane cM on the right. The distances on the two DH maps are continuous with distances between unlinked groups (gaps) based on the consensus map

Low temperature tolerance

The survival of the two populations varied greatly between environments. No DH line or parent survived at FMT. Nearly all lines in the NB713/OR71 population survived at FCCO. There was differential survival in the remaining tests. Phenotypic frequency distributions were diverse (Fig. 2, Supplemental Figure 5), ranging from nearly bi-modal (NB3437f/OR71-SPMN and NB713/OR71-SPMN considering the 100 % surviving lines vs. "others") to nearly normal (both populations, MRI). In the MRI and SPMN trials, LTT patterns for the parents fit expectations: the Nebraska germplasm had superior LTT. In the LNE trials, the Oregon and Nebraska parents had similar levels of survival and there were positive and negative phenotypic transgressive segregants. In FCCO, OR71 was superior to NB3437f and the same as NB713. Overall, the two populations (one facultative and the other segregating for VS) had similar means, ranges, and distributions. Within the NB713/OR71 population, there were no patterns of LTT survival for vernalization-sensitive versus facultative progeny (Supplemental Figure 6).

Based on prior reports, the most likely region for LTT QTL in barley is at *FR-H1* and/or *FR-H2* (reviewed by von Zitzewitz et al. 2011). However, due to the 5H linkage map issues addressed in the prior section, it is not possible at the current level of map resolution to unequivocally state that one or both of these loci are, or are not, determinants of LTT in this germplasm. As shown in Fig. 1, using the MRI data, there is a marker/trait association coincident with
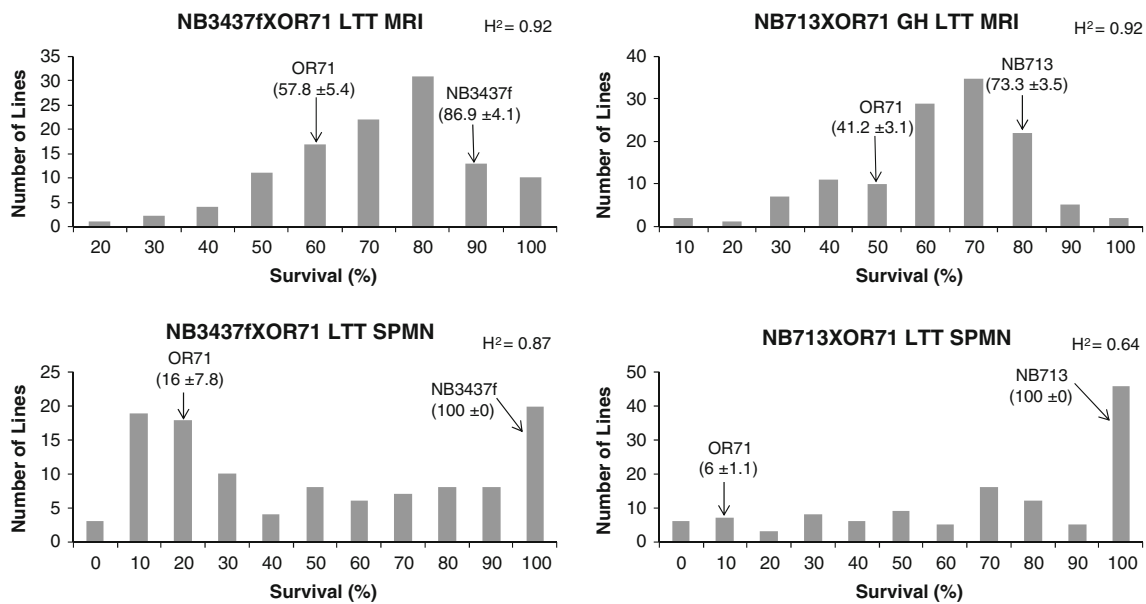
Fig. 2 Phenotypic frequency distributions for low temperature tolerance (LTT) evaluated in the NB3437f/OR71 and NB713/OR71 doubled haploid populations in a controlled freeze test at the Martonvasar Research Institute (MRI) and under field conditions in St. Paul, MN (SPMN). Standard deviations are shown for the parents. Broad-sense heritability (H^2) is also noted

FR-H2 in NB713/OR71, but it does not reach the significance threshold. In this population, there is a significant effect at SNP 3_0850, with NB713 contributing the favorable allele based on an adjusted single locus test (Table 1). This marker maps to the *FR-H2* region in the consensus map, but was highly distorted and did not coalesce with the 5H markers in the NB713/OR71 population. In NB3437f/OR71, there are significant effects for markers that map to the *FR-H2* region in the consensus map, SNP 3_0850 and 2_0795.

The largest effect QTL, which we designate as *FR-H3*, was detected in the SPMN and MRI data on the short arm of chromosome 1H. The best estimates of the position of this QTL are based on the individual populations because of the higher density of markers, whereas the best estimates of effects are based on the combined population due to number of individuals. Considering each of the populations and data sets separately, the 2-LOD confidence intervals overlap for both populations at MRI and one of the populations at SPMN. However, the confidence intervals for the two populations at SPMN are contiguous. Therefore, we believe that all peaks represent the same QTL. The maximum LOD in the combined population is 22.9 for the SPMN data and 8.6 for the MRI data (Fig. 3). The Nebraska parents contributed the favorable allele at this QTL in the combined population and in each of the two populations. Additional QTL information is shown in Table 2. Most other QTL were environment- and/or

population specific. Considering all environments and populations, QTL were detected on all chromosomes except 2H and 6H. Assuming QTL with overlapping confidence intervals (as well as the SPMN QTL) to represent the effects of the same gene or genes, a total of eight distinct QTL were detected and at all but one of these environments (LNE) the Nebraska parent contributed the favorable allele.

Vernalization sensitivity

Evaluation of the two DH populations under greenhouse conditions allowed for assessment of VS (the unvernalized treatment) and flowering time per se (the vernalized treatment). We used two measures of the phenotype, FLN and FT. The latter is simpler to measure, but the former is reported to be more accurate (Cuesta-Marcos et al. 2008; Baga et al. 2009; Sasani et al. 2009). The populations behaved as expected: NB3437f/OR71 did not segregate for VS and NB713/OR71 did segregate for VS. All DH lines, and the parents, in the facultative × facultative population (NB3437f/OR71) flowered within 95 days of planting in the unvernalized treatment and had a maximum FLN value of 17. The phenotypic frequency distributions for the vernalized and unvernalized treatments are very similar, with flowering times, on average, 10 days earlier in the vernalized treatment (Supplemental Figure 7). Vernalization accelerates FT in facultative germplasm, although not

Table 1 Summary of QTL detected in the NB3437fXOR71 (A) and NB713XOR71 (B) double haploid mapping populations

Trait	QTLNo.	Chromosome A	Chromosome B	Peak position A	Peak position B	2-LOD CI A	2-LOD CI B	Closest marker[a] A	Closest marker[a] B	LOD A	LOD B	R² A	R² B	Additive effect A	Additive effect B	LOD threshold A	LOD threshold B	MIM R² A	MIM R² B
LTT-MRI	1	1H	–	2.8	–	0–7	–	2_0502	–	3.5	–	0.07	–	–4.7	–	2.8	2.8	0.4	0.2
	2	1H	1H	52.4	75.1	41–60	66–81	1_0938	3_0350	6.0	4.0	0.13	0.09	–6.3	–5.3				
	3	–	4H	–	186.9	–	185–187	–	3_0006	–	3.2	–	0.08	–	–5.7				
	4	5Hb	–	70.1	–	43–70	–	2_0795	–	4.6	–	0.11	–	–6.1	–				
	5	–	7Hb	–	162.9	–	160–166	–	1_0078	–	3.4	–	0.08	–	–4.9				
	6[b]	–	5H	–	NA	–	NA	–	3_0850	–	4.7	–	0.10	–	–7.2				
LTT-SPMN	1[c]	1H	1H	47.7	42.0	44–52	21–56	3_1467	2_0749	23.2	9.0	0.48	0.27	–26.1	–17.5	2.9	2.9	0.6	0.3
	2	3H	–	0.0	–	0–4	–	3_0192	–	3.3	–	0.04	–	–8.3	–				
LTT-NE	1	–	4H	–	55.6	–	47–77	–	3_0864	–	3.9	–	0.16	–	3.9	2.6	2.7	0.0	0.1
LTT-CO	1	–	5Ha	–	90.4	–	86–97	–	1_0953	–	3.2	–	0.10	–	–6.0	2.7	2.7	0.0	0.1
FT-F	1	1H	–	95.9	–	90–103	–	PPD-H2	–	12.8	–	0.18	–	2.7	–	2.9	2.9	0.8	0.8
	2	–	1H	–	67.6	–	48–86	–	3_1467	–	4.9	–	0.05	–	–1.4				
	3	–	1H	–	200.2	–	197–200	–	2_0915	–	3.1	–	0.03	–	1.1				
	4	3H	–	0.0	–	0–7	–	3_0192	–	4.3	–	0.05	–	–1.4	–				
	5	6H	–	80.3	–	70–86	–	3_1253	–	4.0	–	0.05	–	1.4	–				
	6	7H	7Hb	40.5	39.8	35–48	36–43	1_0232	1_0576	35.1	23.4	0.64	0.36	5.2	4.2				
FT-GHV+	1	–	3H	–	139.1	–	135–143	–	1_0381	–	3.2	–	0.06	–	2.7	2.8	2.8	0.6	0.5
	2	–	4H	–	186.9	–	175–187	–	3_0006	–	4.4	–	0.08	–	–3.2				
	3	–	6H	–	64.3	–	62–69	–	2_0058	–	3.4	–	0.06	–	–2.7				
	4	7H	7Hb	38.5	39.8	33–49	36–47	1_0232	1_0576	16.2	14.0	0.47	0.31	7.3	6.4				
FT-GHV–	1	–	4H	–	177.6	–	174–180	–	2_1210	–	119.0	–	0.96	–	–43.3	2.9	3	0.6	1.0
	2	7H	7Hb	39.5	43.7	34–48	43–47	1_0232	1_0576	23.1	3.3	0.56	0.01	6.6	3.7				

Table 1 continued

Trait	QTL No.	Chromosome		Peak position		2-LOD CI		Closest marker[a]		LOD		R²		Additive effect		LOD threshold		MIM R²	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
FLN-GHV+																			
	1	1H	–	61.1	–	52–65	–	2_0956	–	4.1	–	0.05	–	–0.4	–	2.9	2.8	0.7	0.6
	2	–	3H	–	141.0	–	133–153	–	1_1154	–	7.1	–	0.11	–	0.6				
	3	3H	–	191.8	–	183–192	–	1_0283	–	3.9	–	0.05	–	–0.3	–				
	4	–	4H	–	5.5	–	3–7	–	1_0319	–	3.0	–	0.05	–	0.4				
	5	–	4H	–	186.9	–	181–187	–	3_0006	–	11.2	–	0.20	–	–0.7				
	6	5Hb	–	10.4	–	0–11	–	2_1275	–	5.9	–	0.08	–	–0.4	–				
	7	–	6H	–	54.7	–	53–70	–	2_0720	–	4.7	–	0.07	–	–0.4				
	8	7H	7Hb	39.5	49.0	33–49	37–63	1_0232	3_0143	19.5	9.9	0.40	0.18	1.0	0.7				
FLN-GHV–																			
	1	3H	–	37.7	–	36–41	–	1_0281	–	3.1	–	0.07	–	0.3	–	2.9	2.8	0.8	0.7
	2	–	4H	–	184.5	–	180–187	–	3_0889	–	28.1	–	0.56	–	–1.6				
	3	6H	–	28.9	–	17–37	–	3_1485	–	4.4	–	0.10	–	0.4	–				
	4	7H	7Hb	39.5	47.0	33–51	36–54	1_0232	3_0143	11.8	6.1	0.37	0.07	0.8	0.6				

QTL alignment was based on overlapping 2-LOD confidence intervals. Refer to Supplemental Figures 1 and 2 for detail. Negative additive effect denotes higher value allele contributed by the Nebraska parent

CI confidence interval, *MIM* multiple interval mapping

Environments and treatments are coded as follows: *LTT* low temperature tolerance, *FT* flowering time, *FLN* final leaf number, *MRI* Martonvasar Research Institute, *SPMN* St. Paul, MN, USA, *LNE* Lincoln, NE, USA, *FCCO* Fort Collins, Colorado, USA, *F* field, *GH* greenhouse, *V+* vernalized, *V–* unvernalized

[a] Pilot OPA name

[b] Marker was not included in map; marker/trait association was performed using SAS Proc Mixed with the same cofactors as QTL analysis

[c] The 2- LOD CI do not overlap; however, they are contiguous and believed to represent the same QTL

required (Distelfeld et al. 2009; Greenup et al. 2009; Trevaskis 2010). FLN values ranged from 8 to 15 in the vernalized treatment and from 10 to 17 in the unvernalized treatment (Fig. 4). FLN values were the same for the two parents with vernalization and differed by two leaves in the unvernalized treatment. As with FT, there were positive and negative phenotypic transgressive segregants with both treatments. Comparison of flowering time in the field is confounded by the year effect, since the two populations were tested in different years. Overall, both populations showed a range of flowering times, spanning nearly 1
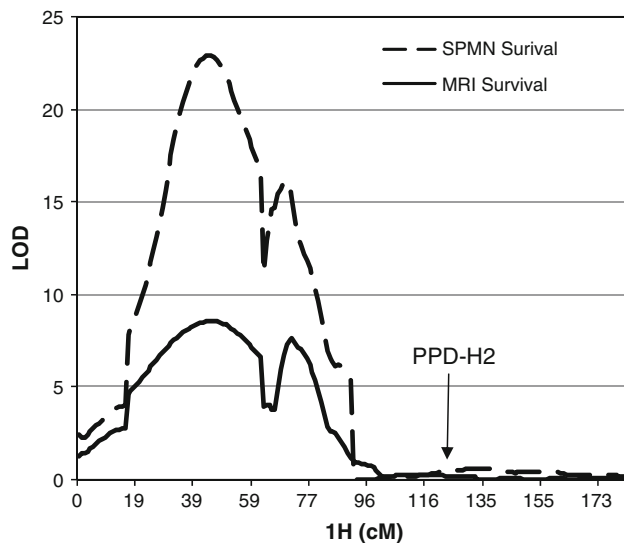
Fig. 3 Quantitative trait locus (QTL) scans on chromosome 1H for low temperature tolerance (LTT) at St. Paul, MN (SPMN) and LTT from the Martonvasar Research Institute (MRI) freeze test. Composite interval mapping with a 20 cM scan window was used for this analysis, which is based on the combined map of two doubled haploid mapping populations: NB3437f/OR71 and NB713/OR71. Inferred position of *PPD-H2* is shown

month. There were positive and negative phenotypic transgressive segregants in both populations, and OR71 was later than either of NB3437f or NB713.

VS in NB713/OR71 was determined by *VRN-H2* (Supplemental Figure 8; Table 1). Expressed as QTL effects for FT in the unvernalized treatment, segregation of alleles at this locus had an LOD of 119 and an R^2 of 0.96. NB713 contributed the larger value (e.g., later flowering, higher FLN) allele. The corresponding values for FLN were LOD 28, and R^2 of 0.56. For FLN, there was also a QTL on chromosome 7H, at the position of *VRN-H3*, with an LOD of 6.1 and R^2 of 0.07, where OR71 contributed the larger value allele. The *VRN-H3* effect was also significant for FT in NB713/OR71 (unvernalized) with an LOD of 3.3 and it was highly significant for both FT and FLN in the unvernalized treatment for NB3437f/OR71. In this population, OR71 also contributed the later flowering and higher FLN alleles. With vernalization, the primary determinant of FT per se, and FLN, in both populations was *VRN-H3*. VS itself was not affected by *VRN-H3*; however, FT and FLN were. The later flowering, and higher FLN allele, was contributed by OR71. Additional QTL were detected on chromosomes 3H, 4H, 5H, and 6H. The 4H QTL is coincident with *VRN-H2*.

Under field conditions, *VRN-H3* had the largest effect on FT, accounting for a 5-day additive effect, with OR71 contributing the later flowering allele. The magnitude and sign of this *VRN-H3* effect reflects the results obtained under greenhouse conditions. *PPD-H2*, on chromosome 1H, had a significant effect, but only in NB3437f/OR71. The phenotype data were corroborated by genotyping: the NB713/OR71 population is monomorphic for *PPD-H2*. OR71 contributed the later flowering allele. Other lesser effect QTL were detected on chromosomes 3H, 6H, and 7H (short arm, not coincident with *VRN-H3*).

Table 2 Summary of QTL detected in the half-sib combined mapping population constructed using 157 markers in common between the NB3437f/OR71 and NB713/OR71 doubled haploid populations

Trait	Chromosome	Peak position	2-LOD CI	Closest marker[a]	LOD	R^2	Additive effect	LOD threshold
LTT-MRI	1H	45.2	26–63	1_0764	8.6	0.19	−8.3	2.6
	5Hb	1.0	0–2	2_0795	15.9	0.21	−8.1	
LTT-SPMN	1H	44.2	36–52	1_0764	22.9	0.64	−28.9	2.7
FT-F	7H	75.3	71–81	1_0232	26.0	0.36	5.1	2.7
FT-GHV+	7H	76.3	70–82	1_0232	24.7	0.39	7.0	2.7
FLN-GHV+	7H	75.3	67–82	1_0232	18.8	0.32	0.9	2.7

Only QTL which are coincident in both populations are shown. Negative additive effect denotes higher value allele contributed by the Nebraska parent

Environments and treatments are coded as follows: *LTT* low temperature tolerance, *FT* flowering time, *FLN* final leaf number, *MRI* Martonvasar Research Institute; *SPMN* St. Paul, MN, USA, *F* field, *GH* greenhouse, *V+* vernalized

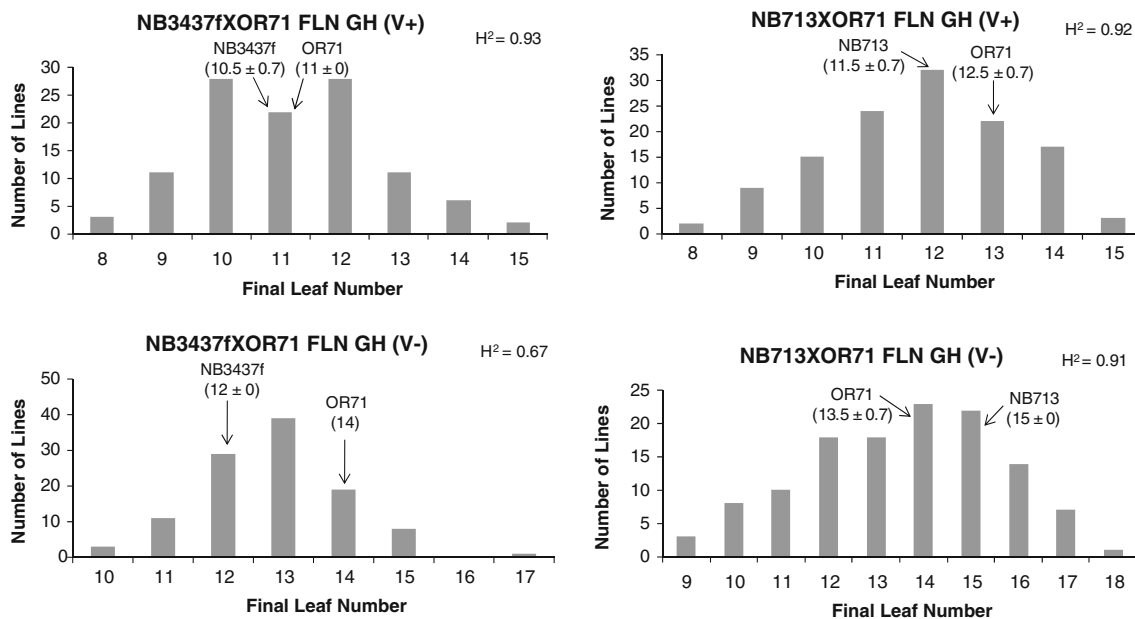*CI* confidence interval, *MIM* multiple interval mapping

[a] Pilot OPA name

Fig. 4 Phenotypic frequency distributions for final leaf number (FLN) evaluated for NB3437f/OR71 and NB713/OR71 doubled haploid populations. FLNs were measured with and without vernalization (V+, V–) under greenhouse conditions. Standard deviations are shown for the parents when replicated. Broad-sense heritability (H^2) is also noted

Discussion

The FR-H3 QTL is a determinant of LTT

Phenotyping LTT is a challenge due to the difficulty in encountering field environments that allow accurate measures of differential survival. In this project, for example, we had nearly complete survival at FCCO and complete mortality at FMT. The lowest temperatures were quite similar for the field tests: SPMN = −27 °C; LNE = −26 °C; FCCO = −25 °C; and FMT −32 °C. The MRI freeze test temperature was −13.5 °C. The absolute low temperature is just one of many factors in winter survival: acclimation conditions, snow cover, soil moisture, and plant growth stage are all very important factors. The MRI-controlled environment test and the SPMN winter field survival data sets proved to be most informative in assessing LTT. These two data sets provided the greatest differential in survival and also had the highest heritabilities (0.92 and 0.78, respectively). Controlled freeze tests are very expensive ($90 per line) and therefore the principal constraint to using this approach is cost. However, controlled tests cannot reflect the full complexity of field survival. To date, SPMN has been a very informative environment. It provided the best estimates of *FR-H3* in this project and allowed for separation of *FR-H1* and *FR-H2* effects in previous research (von Zitzewitz et al. 2011).

The superior LTT of NE germplasm was generally recognized, but the genetic basis was unknown. Understanding the genetic basis of a complex phenotype is a prerequisite for effective use of molecular breeding tools. Our discovery of *FR-H3* QTL will make it possible to efficiently use this unique germplasm. The only previous report of a LTT QTL on chromosome 1H was in the Dicktoo × Morex population. Dicktoo, like NB3437f, is facultative and it also originated in the Upper Midwest. However, the 1H QTL in Dicktoo × Morex is likely a pleiotropic effect of *PPD-H2* and is distal to *FR-H3*. *FR-H3* had a very large effect in SPMN, accounting for an additive effect of 29 % in the combined population. Additional research will be necessary to confirm the precise genome coordinates of this QTL and to proceed with isolating the gene (or genes) responsible.

When we developed these populations, we did not expect to find a QTL at *FR-H1*. All parents have a degree of LTT and are monomorphic for all available BOPA 1 and BOPA 2 SNP markers within 5 cM of *VRN-H1/FR-H1* ($n = 27$ markers). Even if there were contrasting alleles at *FR-H1*, we would not be able to detect them at the current level of marker resolution. We hypothesized that we would detect a QTL at *FR-H2*, based on the presence of marker polymorphisms in the region and the difference in LTT between OR71 and the Nebraska parents. There are indications of an LTT QTL in the region, but the fragmentation of the 5H linkage map precludes definitive characterization. It is curious that linkage map construction was straightforward in all regions of the genome except for the one region, on 5H, known to contain genes determining LTT. Determining the cause(s) of fragmentation is an exciting challenge that will be addressed through localized

sequencing, cytogenetic characterization, and high-density mapping. Chromosome rearrangements (duplication or inversion) are possible, but would be expected to lead to recombination "suppression" rather than "expansion". Furthermore, no confirmed inversions or duplications have been detected in the hundreds of molecular marker-based linkage maps constructed over the past 20 years in cultivated × cultivated and cultivated × ancestral barley crosses. Copy number of the CBF genes has been hypothesized to be related to the degree of LTT in barley (Stockinger et al. 2007), and a complex locus of multiple CBF genes underlies FR-H2. However, localized gene duplication would not be expected to cause issues in low-density linkage mapping. Heterozygosity in the 5H region in one or both parents is possible, but no cases of heterozygosity were detected in the genotyping of the parents or the DH progeny.

Many minor LTT QTL were also found in addition to FR-H2 and FR-H3, suggesting that this complex trait has major and minor determinants. Detecting, quantifying, and accumulating the essential, but minor-effect alleles is likely beyond the scope of QTL mapping and marker-assisted selection. These strategies will be effective in fixing favorable alleles at FR loci (FR-H1, FR-H2, and FR-H3) and genomic selection should be more effective for accumulating the minor alleles.

VRN-H2 determines VS, and VRN-H3 affects FT in this germplasm

The role of VRN-H2 in determining vernalization sensitivity was confirmed: when grown under unvernalized conditions, all progeny of the facultative × facultative cross (NB3437f × OR71) flowered in a time frame comparable to that of a spring barley grown under the same conditions. The parents and all progeny are VRN-H2 deletions. In the winter × facultative cross (NB713 × OR71), the parents behaved as expected and the progeny segregated for vernalization sensitivity. Further validation of the role of VRN-H2 in vernalization sensitivity was the discovery of the facultative line, NB3437f, in the bulk seed of NB3437. NB3437f flowered at the same time as spring barley checks, without vernalization, and carried the VRN-H2 deletion. All non-flowering plants in the bulk seed lot tested positive for the Vrn-H2 dominant allele (Supplemental Figure 1). The effect of VRN-H3 on FT was unexpected. Variation in VRN-H3 was first reported by Takahashi and Yasuda (1971) and most recently by Casas et al. (2011). The Nebraska germplasm has exotic ancestry: the variety Wong, released in Jiangsu, China in 1941 (http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1434053; verified 9 November 2011) figures in the pedigrees of both NB713 and NB3437f from which the variation could be

attributed. The early flowering allele at VRN-H3 traces to the Nebraska parents in both populations. In the currently accepted vernalization model (Yan et al. 2004; Trevaskis et al. 2007), the recessive allele at vrn-H3 is necessary for VS. Very early flowering is the phenotype of the dominant allele at Vrn-H3. Since NB713 is vernalization sensitive, its "earlier" allele is most likely an alternative recessive allele. If this alternative earlier flowering allele is triggered only under long days, as is the case of winter alleles (Faure et al. 2007), then the earlier allele would not increase the risk for low temperature damage due to a precocious vegetative to reproductive transition during the short days of winter.

LTT and vernalization sensitivity are uncoupled

Several lines of evidence point to the "uncoupling" of VS and LTT in this germplasm. Overall, LTT values were the same in both populations, and in the population segregating for VS there were lines with high survival in both the VS and non-VS categories. However, a QTL in the region of VRN-H2 was detected in the NB713 × OR71 population under the controlled freeze test conditions of the Martonvasar phytotron. A possible explanation is that under controlled freeze tests, the acclimation regime and timing of the freeze occurred at a point in plant development when VS was still effective in delaying the vegetative to reproductive transition and thus ensuring maximum LTT. Finally, the NB3437f variant persisted in the heterogeneous mixture that is NB3437 under the low temperature field selection conditions customary for the University of Nebraska breeding program. Under controlled environment conditions, NB3437f has an LTT value comparable to NB713 (Supplemental Table 1).

Prospects for LT-tolerant fall-sown barley

Fall-sown barley offers numerous advantages in many production environments. Likewise, within the fall-sown class, facultative types offer advantages to both breeders and producers. A pre-requisite to realizing these advantages is sufficient LTT as well as agronomic performance (i.e., yield and resistance to biotic stresses) and grain that commands a premium in the marketplace. We have shown, with this research, that gene discovery and breeding can be coupled to generate new knowledge and useful facultative germplasm with good LTT. The biparental QTL mapping described in this report, together with the results of the genome-wide association mapping, are expected to effectively mine a multitude of favorable alleles from the Oregon × Nebraska germplasm for MAS and genomic selection (GS). In this way, we hope to contribute to realizing the potential of fall-sown malting barley. This

market class is likely to be of increasing importance in an era of increasingly volatile climate change.

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