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Allelic variation at *Fr-H1/Vrn-H1* and *Fr-H2* loci is the main determinant of frost tolerance in spring barley

Alessandro Tondelli[a,*], Donata Pagani[a], Iman Naseh Ghafoori[a,b], Mehdi Rahimi[a,c], Reza Ataei[a,d], Fulvia Rizza[a], Andrew J. Flavell[e], Luigi Cattivelli[a]

[a] *Consiglio per la Ricerca e la sperimentazione in Agricoltura, Genomics Research Centre, Fiorenzuola d'Arda, Italy*
[b] *Department of Agronomy and Plant Breeding, University of Tehran, Tehran, Iran*
[c] *Department of Biotechnology, Graduate University of Advanced Technology, Kerman, Iran*
[d] *Department of Production and Plant Breeding Engineering, Imam Khomeini International University, Qazvin, Iran*
[e] *Division of Plant Sciences, University of Dundee at JHI, Invergowrie, Dundee, UK*

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ABSTRACT

Frost tolerance is the main component of winter hardiness for cereals and is largely dependent on the plant's ability to cold acclimate by means of a slow adaptive response induced by low, non-freezing temperatures. In the present work, a Genome-Wide Association mapping study has been performed, looking for the genetic determinants of frost tolerance in a panel of European spring 2-row barley cultivars. Frost damage was evaluated by measuring chlorophyll fluorescence (F_v/F_m) and membrane integrity (Electrolyte Leakage) and considerable variation for this trait was discovered. Genetic association of the trait was sought with >5000 gene-based mapped SNP markers using a Mixed Linear Model approach. A simple genetic architecture was revealed for frost tolerance in this population, with a principal role for the *Fr-H1/Vrn-H1* and *Fr-H2* loci already detected in previous linkage and association mapping studies. Allelic richness might exist at these loci, not only between winter and spring barley, but also within the spring germplasm, thus offering the opportunity for future development of new spring barley lines with improved resilience in the face of emerging climate change.

1. Introduction

The ability of temperate cereals such as barley and wheat to survive over winter is largely dependent on the degree of frost tolerance acquired at the vegetative stage. Maximum tolerance is reached by induction of a coordinated and complex genetic network that follows exposure to low but not freezing temperatures, a process known as cold acclimation or hardening (Thomashow, 1999; Pecchioni et al., 2012). A range of methodologies have been developed for assessing the ability of barley and wheat plants to cold acclimate (Prášil et al., 2007; Rizza et al., 2011). Accurate evaluation of plant survival is usually carried out in fields during vegetative growth or under controlled conditions. Alternatively, rapid and simplified experimental systems, like those based on chlorophyll fluorescence (Rizza et al., 2001), can be used for fast and precise monitoring of frost tolerance in large populations required for quantitative genetic analysis of the trait (Francia et al., 2004). Damage to cellular integrity, as in the case of freezing stress, leads to a decrease in the maximum quantum yield of the photosystem II (PSII), as it can be assessed by measuring the ratio of variable (F_v) to maximal (F_m) chlorophyll fluorescence in the dark adapted state (F_v/F_m parameter; Rizza et al., 2001). In barley, it has been observed that these alternative methods are strongly correlated with both winter survival and measurement of regrowth after stress (traditional LT_{50} experiments), when plants are analyzed at the first leaf stage (Rizza et al., 1994, 2011).

Both family-based and population-based genetic approaches have been used to dissect the genetic architecture of barley cold acclimation and frost tolerance. Two *Frost Resistance* (*Fr*) loci playing crucial roles in the determination of the trait and accounting for a large proportion of the observed phenotypic variance have been repeatedly mapped 30 cM apart on the long arm of the chromosome 5H (Hayes et al., 1993; Francia et al., 2004; Skinner et al., 2006; von Zitzewitz et al., 2011). The *Frost resistance-H2* (*Fr-H2*) locus overlaps with a cluster of C-repeat binding factors (*CBF*) genes, whose products are known to activate a set of cold responsive (*COR*) genes, with a direct role in protecting the plant cells from frost injury (Francia et al., 2007; Tondelli et al., 2011). Sequencing of genomic

* Corresponding author at: Consiglio per la Ricerca e la sperimentazione in Agricoltura, Genomics Research Centre, via San Protaso 302, Fiorenzuola d'Arda, Italy. Tel.: +39 0523983758; fax: +39 0523983750.
E-mail address: alessandro.tondelli@entecra.it (A. Tondelli).

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clones encompassing the *Fr-H2* locus in two frost-hardy barleys ('Nure' and 'Dicktoo') and two frost-sensitive barleys ('Tremois' and 'Morex') suggested that an increase in the copy-numbers of *HvCBF2* and *HvCBF4* might be the causal functional polymorphism underlying frost tolerance (Stockinger et al., 2007; Knox et al., 2010). However, a candidate gene association mapping study of 216 barley accessions linked allelic variation at *HvCBF14* with frost tolerance (Fricano et al., 2009).

The *Frost resistance-H1* (*Fr-H1*) locus most probably represents a pleiotropic effect of *HvBM5A*, a MADS-box gene which is the candidate for the major vernalization gene *Vrn-H1* (Yan et al., 2003; von Zitzewitz et al., 2005). Temperatures that induce cold acclimation also satisfy the vernalization requirement of barleys with winter growth, allowing them to switch from vegetative to the reproductive growth phase. Moreover, it has been observed that levels of frost tolerance decrease after vernalization (Limin et al., 2007; Galiba et al., 2009) and in wheat the extent of cold-regulated gene expression is influenced by allelic variation at *Vrn-A1* (Dhillon et al., 2010). The interconnection between frost tolerance, vernalization requirement and photoperiod sensitivity has led to the definition of three different genotypic classes for barley, namely winter, facultative and spring. Winter and facultative types are both frost resistant, but they differ in sensitivity to vernalization. Rizza et al. (2011) have presented experimental evidence that supports a faster acclimation capacity in facultative vs. winter barley genotypes. Interestingly, a positive allelic contribution to frost acclimation from a facultative barley line has been observed for the *Frost resistant-H3* (*Fr-H3*) QTL that has been recently mapped on the short arm of barley chromosome 1H (Fisk et al., 2013). The underlying gene for this locus has not yet been cloned. Spring barleys are also able to cold acclimate, despite showing higher LT_{50} temperatures compared to winter or facultative genotypes (Limin et al., 2007). Notably, the spring barley cultivar 'Morex' contributes a favorable allele at a frost resistance QTL on chromosome 4HS in the 'Dicktoo' (facultative) × 'Morex' biparental population (Skinner et al., 2006), reinforcing the idea that frost resistance genes segregate in un-adapted genetic material.

Association mapping approaches have the advantage over biparental studies of capturing multiple significant genetic effects that segregate in diverse germplasm. For instance, barley association scans have facilitated the identification of several genes responsible for simple inherited traits (Ramsay et al., 2011; Comadran et al., 2012). Genome-Wide Association (GWA) scans have also been carried out for the genetic dissection of frost tolerance in barley in germplasm panels containing winter, facultative and spring genotypes, where major developmental and phenological genes such as *Vrn-H1* were segregating (Fricano et al., 2009; von Zitzewitz et al., 2011; Visioni et al., 2013). In the present manuscript we have focused on a more compact diversity panel of European spring 2-row barley cultivars only, aiming to identify new variation in frost tolerance, together with underlying genetic determinant(s) that can be used to improve the level of tolerance in spring 2-row barley. As a consequence of the global climate change, the average winter temperature is raising (Brunet et al., 2007), and in some regions (e.g. southern Europe) it might occasionally not be sufficient to fulfill the vernalization requirement of standard winter barley cultivar. Given this scenario, spring cultivars with significant frost tolerance could be exploited as autumn-sown crop to improve barley yield stability.

2. Materials and methods

2.1. Plant material and SNP genotyping

A panel of 200 spring 2-row (S2r) cultivars of *Hordeum vulgare* was used in the present work. The panel contains old and modern barley cultivars collected from different European countries, thus representing the diversity and evolution of spring barley in the 20th century in Europe (Tondelli et al., 2013). The lines were genotyped using an Illumina™ iSelect marker set comprising 7864 gene-based SNPs, most with known genetic position on the 7 barley chromosomes (Comadran et al., 2012; Tondelli et al., 2013). The population structure, genomic distribution of genetic diversity and linkage disequilibrium for this germplasm has also been determined (Tondelli et al., 2013). Following preliminary screening of frost tolerance in the whole S2r set, the 46 best and 50 worst performing genotypes were selected for further phenotypic characterization (see Section 2.2). Within this sub-set, markers with more than 5% missing value and non segregating SNPs were filtered out, resulting in 5880 markers for the analysis of population structure. The software PAST (Hammer et al., 2001) was used for UPGMA cluster analysis, based on a similarity matrix of the genotypes calculated from simple matching of the SNPs. Genetic stratification was also assessed by means of the software STRUCTURE 2.3 (Pritchard et al., 2000), considering the admixture model for the ancestry of individuals and correlated allele frequencies. Burn-in and Markov Chain Monte Carlo values were set to 20,000 and 10,000, respectively, with K values ranging from 1 to 10. The best K value was defined by the ΔK method of Evanno et al. (2005). A SNP 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 were analyzed as reported by von Zitzewitz et al. (2005).

2.2. Evaluation of frost tolerance

Frost tolerance can be assessed by chlorophyll fluorescence analysis of samples from growth chamber experiments (Rizza et al., 2011). Due to the space limitation in the growth chamber and the high numbers of lines under evaluation, a preliminary screening was carried out on the whole set of 200 S2r barley genotypes, with single plants for each genotype and the entire experiment was replicated six times. Plantlets were grown in 2 cm × 2 cm pots for one week at 20/15 °C (day/night), 10 h photoperiod, and 200 μmol m^{-2} s^{-1} light intensity, and then acclimated for 4 weeks at 3/1 °C (day/night) under the same light conditions. Plants at the first leaf stage were subjected to frost treatment in the dark. Gradual freezing of the plants occurred at a rate of 2 °C h^{-1}, down to a temperature of −3 °C, where plants were kept for 16 h. Subsequently, temperature was lowered at the same rate to the minimum temperature of −11 ± 1 °C, where plants were kept for 16 h. Finally, the temperature was gradually raised to 1 °C at 2 °C h^{-1}. Decrease in the maximum quantum efficiency of photosystem II (PSII) was used as an indicator of frost-induced damage. The ratio of variable (F_v) to maximal (F_m) chlorophyll fluorescence in the dark adapted state (F_v/F_m parameter) was measured using a PAM-2000 fluorometer (Walz, Germany), both before and immediately after the freezing treatment and after a period of 24 h of recovery under the same growth conditions. Further phenotypic characterization of a sub-set of 96 selected genotypes used a more discriminating temperature of −12 ± 1 °C and unchanged growing and hardening conditions. For this study four experiments were performed, each with two randomized complete blocks.

The extent of membrane damage after freezing was also determined on the above subset of 96 S2r lines by Electrolyte Leakage (EL), following the same conditions of growth, hardening and freezing stress (−12 ± 1 °C) described above. Four different experiments were performed; for each experiment, 4 plants per genotype were arranged in 2 randomized complete blocks with 2 plants per pot. After frost treatment, the four plants were pooled for measuring the rate increase in ion release, according to Rizza et al. (1994). Leaf-segments were placed in a vial containing 15 ml of de-ionized water, degassed under vacuum for 20 min and stirred at 25° C for

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