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Copy number variation at the HvCBF4–HvCBF2 genomic segment is a major component of frost resistance in barley

This is a pre print version of the following article:

Original
Copy number variation at the HvCBF4–HvCBF2 genomic segment is a major component of frost resistance in barley / Francia, Enrico; Morcia, Caterina; Pasquariello, Marianna; Mazzamurro, Valentina; Milc, Justyna Anna; Rizza, Fulvia; Terzi, Valeria; Pecchioni, Nicola. - In: PLANT MOLECULAR BIOLOGY. - ISSN 0167-4412. - 92:1(2016), pp. 161-175.

Availability:
This version is available at: 11380/1111652 since: 2017-03-31T10:18:50Z

Publisher:

Published
DOI:10.1007/s11103-016-0505-4

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(Article begins on next page)

18 September 2017

Plant Molecular Biology

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--Manuscript Draft--

Manuscript Number:	
Full Title:	Copy Number Variation at the HvCBF4-HvCBF2 genomic segment is a major component of frost resistance in barley
Article Type:	Original Article
Keywords:	barley; frost resistance; FR-H2 locus; CBF genes; CNV; RT-qPCR
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Funding Information:	Fondazione Cassa di Risparmio di Carpi (Project GENOMORE) — Prof. Nicola Pecchioni
	Fondazione Cassa di Risparmio di Modena (Project FROSTMAP) — Dr. Enrico Francia
Abstract:	A family of CBF transcription factors plays a major role in reconfiguring the plant transcriptome in response to low-freezing temperature in temperate cereals. In barley, more than 13 HvCBF genes map coincident with the major QTL FR-H2 suggesting them as candidates to explain the function of the locus. Variation in copy number (CNV) of specific HvCBFs was assayed in a panel of 41 barley genotypes using RT-qPCR. Taking advantage of an accurate phenotyping that combined Fv/Fm and field survival, resistance-associated variants within FR-H2 were identified. Genotypes with an increased copy number of HvCBF4 and HvCBF2 (at least 10 and 8 copies, respectively) showed greater frost resistance. A CAPS marker able to distinguish the CBF2A, CBF2B and CBF2A/B forms was developed and showed that all the higher-ranking genotypes in term of resistance harbour only CBF2A, while other resistant winter genotypes harbour also CBF2B, although at a lower CNV. In addition to the major involvement of the HvCBF4-HvCBF2 genomic segment in the proximal cluster of CBF elements, a negative role of HvCBF3 in the distal cluster was identified. Multiple linear regression models taking into account allelic variation at FR-H1/VRN-H1 explained 0.434 and 0.550 (both at $p < 0.001$) of the phenotypic variation for Fv/Fm

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Title: Copy Number Variation at the *HvCBF4–HvCBF2* genomic segment is a major component of frost resistance in barley

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Number of words: 10,545

Number of tables: 3

Number of figures: 4

Supplementary materials: 2

Abstract

A family of CBF transcription factors plays a major role in reconfiguring the plant transcriptome in response to low-freezing temperature in temperate cereals. In barley, more than 13 *HvCBF* genes map coincident with the major QTL *FR-H2* suggesting them as candidates to explain the function of the locus. Variation in copy number (CNV) of specific *HvCBF*s was assayed in a panel of 41 barley genotypes using RT-qPCR. Taking advantage of an accurate phenotyping that combined F_v/F_m and field survival, resistance-associated variants within *FR-H2* were identified. Genotypes with an increased copy number of *HvCBF4* and *HvCBF2* (at least 10 and 8 copies, respectively) showed greater frost resistance. A CAPS marker able to distinguish the *CBF2A*, *CBF2B* and *CBF2A/B* forms was developed and showed that all the higher-ranking genotypes in term of resistance harbour only *CBF2A*, while other resistant winter genotypes harbour also *CBF2B*, although at a lower CNV. In addition to the major involvement of the *HvCBF4-HvCBF2* genomic segment in the proximal cluster of *CBF* elements, a negative role of *HvCBF3* in the distal cluster was identified. Multiple linear regression models taking into account allelic variation at *FR-H1/VRN-H1* explained 0.434 and 0.550 (both at $p < 0.001$) of the phenotypic variation for F_v/F_m and field survival respectively, while no interaction effect between CNV at the *HvCBF*s and *FR-H1/VRN-H1* was found. Altogether our data suggest a major involvement of the *CBF* genes located in the proximal cluster, with no apparent involvement of the central cluster contrary to what was reported for wheat.

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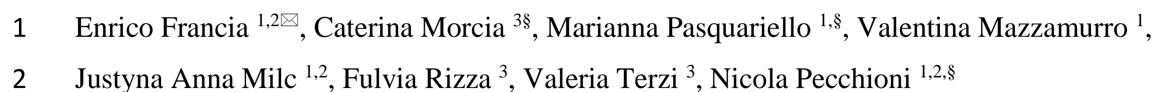

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Acknowledgments

This work was partially supported by Fondazione Cassa di Risparmio di Modena, Progetto di Ricerca Internazionale "FROSTMAP – Physical mapping of the barley *Frost resistance-H2* (*Fr-H2*) locus". Funding from "GENOMORE" project of the Fondazione Cassa di Risparmio di Carpi is also acknowledged. The authors are grateful to Dr. Franz W. Badeck (PIK – Potsdam, Germany) for his critical suggestions during statistical analysis and to Mrs. Donata Pagani (CRA-GPG, Fiorenzuola, Italy) for the excellent technical assistance during phenotypic analysis, and to Dr. Ilja Tom Prášil (Crop Research Institute, Prague, Czech Republic) for providing some barley varieties.

Abstract

A family of CBF transcription factors plays a major role in reconfiguring the plant transcriptome in response to low-freezing temperature in temperate cereals. In barley, more than 13 *HvCBF* genes map coincident with the major QTL *FR-H2* suggesting them as candidates to explain the function of the locus. Variation in copy number (CNV) of specific *HvCBF*s was assayed in a panel of 41 barley genotypes using RT-qPCR. Taking advantage of an accurate phenotyping that combined F_v/F_m and field survival, resistance-associated variants within *FR-H2* were identified. Genotypes with an increased copy number of *HvCBF4* and *HvCBF2* (at least 10 and 8 copies, respectively) showed greater frost resistance. A CAPS marker able to distinguish the *CBF2A*, *CBF2B* and *CBF2A/B* forms was developed and showed that all the higher-ranking genotypes in term of resistance harbour only *CBF2A*, while other resistant winter genotypes harbour also *CBF2B*, although at a lower CNV. In addition to the major involvement of the *HvCBF4-HvCBF2* genomic segment in the proximal cluster of *CBF* elements, a negative role of *HvCBF3* in the distal cluster was identified. Multiple linear regression models taking into account allelic variation at *FR-H1/VRN-H1* explained 0.434 and 0.550 (both at $p < 0.001$) of the phenotypic variation for F_v/F_m and field survival respectively, while no interaction effect between CNV at the *HvCBF*s and *FR-H1/VRN-H1* was found. Altogether our data suggest a major involvement of the *CBF* genes located in the proximal cluster, with no apparent involvement of the central cluster contrary to what was reported for wheat.

Keywords: barley; frost resistance; *FR-H2* locus; *CBF* genes; CNV; RT-qPCR

Abbreviations: *FR*: frost resistance loci, CBF/DREB: C-Repeat Binding Factor/Dehydration Responsive Element Binding Factor proteins, CRT/DRE: C-RepeaT/Dehydration Responsive Elements, *VRN*: vernalization requirement genes and loci, CNV: Copy Number Variation, CNVs: Copy Number Variants.

73 **Introduction**

74

75 Low freezing temperature is one of the primary limiting factors that affect yield potential of fall-
76 sown crops such as barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.). In
77 temperate cereals serious damages to plant cells and tissues are caused by intracellular or
78 extracellular ice formation during the freeze events (Levitt 1980), and cold winters with large
79 temperature fluctuations may result in severe winterkill. In view of possibly higher and faster
80 temperature variations, expectedly due to climate change, improving fast resilience to frost is
81 fundamental for minimizing the so-called yield gap (the gap between potential and actual yield),
82 increasing yield stability, and guaranteeing crop sustainability (Rizza et al. 2011; Visioni et al.
83 2013). During the last 10 years, barley has been extensively used as a model system for genetic
84 dissection of low temperature tolerance in fall-sown cereals (Pecchioni et al. 2012). There is
85 indeed abundant variation for this trait within the primary gene pool and an ever expanding set
86 of genomics tools is available, from high-resolution maps (Poland et al. 2012) to the structured
87 whole-genome context describing the barley gene-space (International Barley Genome
88 Sequencing Consortium 2012). As in other members of the Triticeae, barley shows also genetic
89 variation for growth habit, which is broadly classified as 'winter' or 'spring'. In general, winter
90 genotypes are low-temperature tolerant, vernalization requiring, and photoperiod sensitive;
91 whereas, spring ones have minimal low temperature tolerance capacity, do not require
92 vernalization, and are typically insensitive to short day photoperiod. These combinations of
93 characters have a recognized role in winterhardiness, i.e. the capacity of a plant to survive the
94 winter, and the genetic basis of each component trait has been extensively reviewed (Kosová et
95 al. 2008; Galiba et al. 2013). According to the epistatic interaction between the two major genes
96 that determine vernalization requirement in Triticeae, i.e. *VRN-1* and *VRN-2*, a third growth
97 habit classification, 'facultative', has been proposed (Tranquilli and Dubcovsky 2000; von
98 Zitzewitz et al. 2005). Facultative barleys represent thus a subclass of the winter genotypes that
99 are low-temperature tolerant, have winter (recessive) alleles at *VRN-H1*, but lack the repressor
100 encoded by *VRN-H2*.
101 Phenotypic evaluations of plant survival after exposure to natural or artificial freezing events
102 display a wide range of ability to acclimate and cope with frost in small grain cereals (Limin
103 and Fowler 2006; Akar et al. 2009; Rizza et al. 2011). Freezing resistance in fact depends on a
104 period of cold acclimation that involves a highly integrated physiological response and in which
105 the expression of structural, regulatory, and developmental genes is finely modulated (for a
106 review, see Galiba et al. 2013). In the Triticeae – as in Arabidopsis – such transcriptome
107 reconfiguration acts through major "regulatory hubs" and relies on the action of CBF/DREB
108 (hereafter CBF) proteins, fundamental components of the frost resistance regulon (Thomashow
109 2010). The *CBF* genes are intron-less and encode AP2/ERF-type transcription factors that
110 recognize the CRT/DRE regulatory element in the promoters of many CBF-targeted COld

111 Regulated (*COR*) and Dehydrin (*DHN*) genes. The corresponding COR and DHN proteins
112 operate as effector components of the stress response, and are involved in protecting the cell
113 against the harmful effects of frost (Thomashow 2010). Overexpression of specific *CBF*s from
114 wheat and barley in the susceptible barley cultivar 'Golden Promise' accelerates *COR* transcript
115 accumulation during cold acclimation and results in an improved frost resistance (Morran et al.
116 2011; Jeknić et al. 2014). The *CBF* gene family in temperate cereals is particularly large and
117 complex, with up to 25 different members classified into ten groups that share common
118 phylogenetic origin and similar structural features (Skinner et al. 2005; Badawi et al. 2007).
119 Another common characteristics of the genome of grasses is the organization of *CBF*s in
120 syntenic clusters of tandemly duplicated paralogs that belong to the phylogenetic subgroups
121 CBF3/CBFIII and CBF4/CBFIV (Badawi et al. 2007; Tondelli et al. 2011). In barley,
122 quantitative genetic studies have demonstrated that a large part of the observed phenotypic
123 variation in frost resistance is explained by two major loci (QTL), *FR-H1* and *FR-H2*, located
124 approximately 25 cM apart on chromosome 5H (Francia et al. 2004; von Zitzewitz et al. 2011;
125 Tondelli et al. 2014). *FR-H1* overlaps the *VRN-H1* vernalization response locus; *FR-H2*
126 segregates with a cluster of at least 13 *CBF*s, co-localizes with QTL influencing COR protein
127 accumulation, and correlates with mRNA levels of the *CBF* genes within the locus (Francia et
128 al. 2004, 2007; Stockinger et al. 2007). Recent investigation of the signal transduction pathway
129 leading to cold acclimation in the frost tolerant cultivar 'Nure' indicates a diversity in regulation
130 between different CBF factors (Marozsán-Tóth et al. 2015). Such induction pattern specificity is
131 in accordance with phylogenetic relationships (Tondelli et al. 2011) and suggests functional and
132 regulatory specialization of the *CBF* elements in addition to their structural separation at *FR-H2*.
133 High-resolution mapping in barley and diploid wheat was used by Francia et al. (2007) and
134 Miller et al. (2006) to identify informative recombinant events within *FR-H2* and *FR-A^{m}2* loci,
135 respectively. In diploid wheat (*Triticum monococcum* L.), Knox et al. (2008) resolved the
136 cluster of *TmCBF* genes into three clusters at *FR-A^{m}2*, referred to as proximal (containing
137 *TmCBF2*, *4*, *9*, *17*), central (*TmCBF14*, *15*, *12*) and distal (*TmCBF16*, *13*, *3*, *10*). Recently,
138 Pasquariello et al. (2014) physically mapped, sequenced and assembled ca. 1.5 Mb of sequence
139 that harbour the entire *HvCBF* cluster in the barley reference cultivar 'Morex'. Representing
140 more than 90% of the target genomic region, and with a general survey of Repetitive Elements,
141 this sequence can serve as a useful genomic tool for structural and evolutionary comparisons of
142 *FR-H2* in germplasm accessions. In fact, even though mutations within individual CBF-coding
143 sequences were firstly proposed as causative of frost resistance variation in diploid wheat (Knox
144 et al. 2008), a differential expansion of the gene cluster together with copy number variation
145 (CNV), has been put forward as a structural explanation for the functional role played by the
146 *CBF*s at *FR-2* in different genotypes. Evidences in favour of this hypothesis start to accumulate
147 in barley and hexaploid wheat (Knox et al. 2010; Pearce et al. 2013; Jeknić et al. 2014; Zhu et
148 al. 2014).

149 Copy number variants (CNVs) are unbalanced changes in the genome structure and represent a
150 large category of genomic structural variants, which include by definition insertions, deletions
151 and tandem or interspersed duplications (Alkan et al. 2011). In the wake of the human genetics
152 research, plant structural variation has increasingly been recognized not only as a common
153 feature and evolutionary force of genomes but also as a genetic mechanism regulating relevant
154 adaptive traits (Zmieńko et al. 2013; Francia et al. 2015). Evidences of structural variation at the
155 *FR-2* locus in Triticeae entail CNV of some *CBF* genes, however apparently involving different
156 regions of the cluster in barley and wheat. In barley, involvement of the proximal cluster of
157 *CBF* elements in the resistance effect of *FR-H2* has been highlighted by sequencing genomic
158 clones. The two frost hardy genotypes 'Nure' and 'Dicktoo', which have a winter allele at *VRN-*
159 *H1*, harbour two *CBF2* paralogs (*2A* and *2B*) and multiple copies of the *HvCBF4B-HvCBF2A*
160 genomic segment, whereas the two non-frost hardy spring cultivars 'Tremois' and 'Morex',
161 which have a spring allele at *VRN-H1*, harbour only single paralogs of *HvCBF4* and *HvCBF2*
162 (Knox et al. 2010). Such CNV correlates with a quantitative difference in gene expression in
163 tolerant genotypes vs. susceptible ones under short days (Stockinger et al. 2007). The absence of
164 *HvCBF2B* and the presence of a single copy of *HvCBF2A* and *HvCBF4B* in the 'Morex'
165 genome was confirmed by sequencing the entire BAC-based physical map of *FR-H2*
166 (Pasquariello et al. 2014), while a novel *HvCBF2C* was reported in this genotype.
167 A large multi-gene deletion at the central cluster of the *FR-B2* locus, which included *CBF-B12*,
168 *CBF-B14* and *CBF-B15*, is associated with reduced frost resistance in both tetraploid durum (*T.*
169 *turgidum* ssp. *durum*) and hexaploid (*T. aestivum*) bread wheat (Pearce et al. 2013). Increased
170 copy number of the central cluster has also been observed, and this was frequently associated
171 with haplotype variation at *VRN-A1* and with higher *TaCBF-A14* transcript levels (Zhu et al.
172 2014). Taken together, the expression and genetic data suggest that the central cluster plays a
173 critical role in frost resistance in wheats.
174 The recent availability of a complete *FR-H2* genomic sequence in the barley reference genotype
175 'Morex' (Pasquariello et al. 2014) enables a thorough CNV survey at this locus. Several well-
176 established methods have been applied for validation or replication of targeted CNV at either
177 the single or multiple-locus scale. Owing to D'haene et al. (2010) RT-qPCR based copy number
178 assessment may serve as the method of choice for screening of relevant genes and their
179 surrounding genomic landscape. Accordingly, our objectives were to (i) determine variation in
180 copy number of *HvCBF* genes at the *FR-H2* locus using RT-qPCR, (ii) identify resistance-
181 associated CNVs at specific clusters of *HvCBF* genes, (iii) unravel whether the effect of *FR-H2*
182 is linked to a single gene variation or to a sort of "*CBF*-number game", and its dependency from
183 the allelic state at *VRN-H1/FR-H1*.
184
185
186

187 **Materials and methods**

188

189 *Plant material*

190 A barley germplasm panel together with a set of bi-parental $F_{5:6}$ segregating lines were used in
191 this study. The germplasm panel consisted of 41 six- and two-row type varieties, with different
192 growth habit and origin (Supplementary Table 1). The majority of genotypes originated from 11
193 European countries while ten were from Australia (1), Syria (2), Turkey (5) and USA (2).
194 Cultivars identified in different field and laboratory experiments as highly tolerant to freezing
195 were included in the panel, moreover some of them were already characterized for adaptation to
196 Mediterranean environments (Rizza et al. 2011; Visioni et al. 2013). In addition, four 'Nure'
197 (resistant) x 'Tremois' (susceptible) recombinants were used. They represent a subset of $F_{5:6}$
198 progeny derived from F_2 recombinants within the *HvCBF* cluster used by Francia et al. (2007)
199 and Pasquariello et al. (2014) for fine mapping the *FR-H2* genomic region. Two additional
200 Doubled Haploid lines –NT-42 and NT-64– from a 'Nure' x 'Tremois' DH population (Francia
201 et al. 2004), harbouring reciprocal alleles at *FR-H2* and *FR-H1/VRN-H1* QTLs were tested as
202 control genotypes.

203

204 *DNA extraction*

205 Leaves from two-week-old plants of each genotype were selected and manually ground in liquid
206 Nitrogen. 300-mg of grinded material was mixed with 860 µl of extraction buffer (10 mM Tris-
207 HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% SDS) and 100 µl of 5 M guanidinium chloride.
208 After incubation at 60°C for 3 h, samples were centrifuged for 10 min at 14,000 rpm. RNase (5
209 µl at 500 µg $µl^{-1}$) was added to 500 µl of the supernatant, and the preparation was incubated at
210 37°C for 10 min to digest contaminant RNA. The extracted DNA was purified according to the
211 Wizard® Genomic DNA Purification Kit (Promega) and eluted with 55 µl of buffer (10 mM
212 Tris-HCl, pH 9.0). DNA concentration was measured at 260 and 280 nm using a Nanodrop ND-
213 1000 spectrophotometer (Thermo Scientific).

214

215 *Copy number profiling using real-time quantitative PCR*

216 Sequences of barley *CBFs* available from GenBank were used for primer pairs selection with
217 Primer Express Software v3.0 (Life Technologies), and according to stringent parameters
218 (reviewed by D'haene et al. 2010) a total of 13 sequence-specific amplicons were obtained
219 (Supplementary Table 2). Eleven out of 13 primer pairs targeted *HvCBF* genes residing at *FR-*
220 *H2* (GenBank KF686739; Pasquariello et al. 2014). Primers were designed using the sequences
221 available in GenBank for the cultivars 'Morex', 'Nure' and 'Tremois' to enable amplification of
222 either A and B forms for *CBF2*, *4*, *10* and *15*. Primers for both *CBF2A* and *CBF2B* were not
223 able to amplify *CBF2C* form identified in 'Morex' (Pasquariello et al. 2014) due to significant
224 sequence differences. Two gene-specific amplicons were designed on *HvNUD* (GenBank

225 AP009567) and *HvSAMDC* (GenBank AK252992) and were assumed to amplify control
226 (reference) segments typically known as single copy genes per haploid genome. The absence of
227 secondary structures in the region in which the primers anneal were verified *via* MFOLD
228 (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dnaform1.cgi) using the theoretical
229 melting temperature of primers and the appropriate Na^{+} and Mg^{2+} concentrations as in the used
230 SYBR Green kit. Each primer pair and the corresponding amplicon sequence were checked for
231 homology also against 'Morex' KF686739 to verify their specificity and uniqueness. In
232 addition, an empirical validation of the assays was performed using four-fold dilution series of
233 'Nure' genomic DNA. Using the direct method of calibration dilution curve and slope
234 calculation, real-time qPCR efficiencies were determined and data are summarized in
235 Supplementary Table 2.
236 RT-qPCR was carried out in a total of 25 µl consisting of 12.5 µl of SYBR Green PCR Master
237 Mix (QuantiFast SYBR Green PCR kit, Qiagen), 2 µl of both the forward and the reverse primer
238 (10 µM) (Sigma-Aldrich), 5 µl of template DNA solution, and 3.5 µl of water. PCR was
239 performed on a 7300 Real Time PCR System (Applied Biosystems) using the following cycling
240 protocol: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s and 60°C for 1 min.
241 A dissociation curve was included to confirm amplification of single gene products.
242 For copy number calculation, quantification cycle (Cq) values were imported into qbasePLUS
243 v2.4 (http://www.qbaseplus.com). The $2^{-\Delta\Delta Cq}$ method was used to determine CNV at the target
244 gene assays by normalizing to the single-copy reference genes *HvNUD* and *HvSAMDC*. The
245 genotype 'Morex' was set as the reference sample so that ΔΔCq calibrated each ΔCq value to
246 the 'Morex' (*c*) copy number for the target according to the formula:

247 $$\Delta\Delta Cq = (\Delta Cq - \Delta Cq, c) \pm \sqrt{s.e.m._{\Delta Cq}^{2} + s.e.m._{\Delta Cq,c}^{2}}$$

248 where *s.e.m.* is the standard error of the mean. Amplification efficiencies of both the target and
249 the reference assays were assumed, and since the reference sample harbours two copies of the
250 target segment per diploid genome, Copy Number Relative Quantity (CNRQ) values were
251 derived from:

252 $$CNRQ = 2 \times 2^{-\Delta\Delta Cq}$$

253 (see Weaver et al. 2010 for further details).
254 To verify the presence/absence of the different forms of *CBF2* (Knox et al. 2010) in the
255 genomes of the barley accessions tested in this study, a novel CAPS (Cleaved Amplified
256 Polymorphic Sequences) protocol was developed to discriminate between *CBF2A*, *2B* and *2A/B*.
257 A specific primer pair (Sigma-Aldrich): CBF2-F (5'-CCAAGCTCGCGTAGTAGGAC-3') and
258 CBF2-R (5'-AGCTCCAGCTCTCCAACTCA-3') was designed on the sequences of 'Nure'
259 *CBF2A* (DQ480160) and *CBF2B* (DQ445244), 'Tremois' *CBF2* (DQ445249), and 'Morex'
260 *CBF2A* (DQ445239) using Primer3 software. Amplification via standard PCR was performed in
261 25 µl volume containing 20 ng of template DNA, 1x PCR buffer, 1 mM $MgCl_2$, 0.2 mM of

262 dNTPs, 0.2 μM of each primer and 1 unit of DreamTaq™ polymerase (Thermo Scientific). The
263 PCR profile was set at: initial denaturation at 94°C for 5 min, followed by 33 cycles of 94°C for
264 30 s, 57°C for 30 s and 72°C for 30s, and the final extension at 72°C for 7 min. The PCR was
265 performed on a 2700 GeneAmp PCR system (Applied Biosystem) and produced a fragment of
266 667 bp for all three sequences. PCR products were then double-digested with *Mlu*CI and *Mlu*I
267 enzymes (New England Bio Labs). The amplicons for *CBF2A* and *CBF2B* had a single
268 restriction site revealed by *Mlu*I and *Mlu*CI digestion, respectively; while *CBF2A/B* amplicon
269 harboured both restriction sites. The samples were electrophoresed for 3 h at 50 V on 1.5%
270 agarose gels and produced two bands of 555 bp and 112 bp for *CBF2A* form, two bands of 537
271 e 130 bp for *CBF2B* form, and three bands of 425 bp, 130 bp and 112 bp for *CBF2A/B* form.
272
273 *Assessment of frost resistance*
274 Frost resistance assessment of the 41 genotypes was obtained by chlorophyll fluorescence
275 analysis in three independent growth chamber experiments (namely, 'F_v/F_m-exp1', '-exp2' and
276 '-exp3'). Seedlings were grown during 1 week at 20/15°C (day/night), 10 h photoperiod, and
277 200 μmol m−2 s−1 photosynthetic photon flux density (PPFD) at leaf level, supplied by cool
278 white fluorescence lamps, and then acclimated during 4 weeks at hardening temperature 3/1°C
279 (day/night) and the same light conditions. Subsequently, plants were subjected to freezing
280 treatments at a minimum temperature of −14°C for experiments 'F_v/F_m-exp1' and 'F_v/F_m-exp2',
281 and of −13°C for experiments 'F_v/F_m-exp3'. The freezing treatments, for 18 h, were conducted
282 in the dark in a VT3050V test cabinet (Vötsch) and freezing resistance quantified by measuring
283 chlorophyll fluorescence of the last fully expanded leaf with a PAM-2000 fluorometer (Walz).
284 Each experiment was arranged in a randomized complete block design (RCBD) with six
285 replicates, and F_v/F_m values were recorded after a recovery period of 24 h at 20/15°C. In
286 addition to F_v/F_m measurements, three sets of field-laboratory assessments of winter survival
287 (%) were derived for the genotypes from a previous study conducted during three consecutive
288 years (namely, 'Field 2006/07', 'Field 2007/08' and 'Field 2008/09'), see Rizza et al. (2011) for
289 details.
290 Testing of F5:6 progeny derived from F_2 'Nure' x 'Tremois' recombinants was conducted in
291 this study in five independent frost resistance experiments conducted in growth chamber
292 between 2008 and 2011. Phenotyping was performed according to the protocol described above
293 and plants were subjected to a freezing treatment at minimum temperatures ranging from −11°
294 to −13°C. A validation experiment in field condition on the same plant material was conducted
295 at Fiorenzuola d'Arda (Italy, 44°55'N, 9°53'E) during year 2012/13, and minimum temperature
296 recorded was −7°C. Each entry was sown in plots of two 1m-long rows in a RCBD with two
297 replicates and winter survival assessed on the basis of a visual score scale from 1 (all plants
298 killed) to 9 (all plants survived).
299

300 *Statistical analysis*

301 For investigating either simple association or cause-and-effect relationships between CNV at
302 barley *CBF*s and frost resistance traits, correlation and linear regression statistical tests were
303 applied. All data were analyzed through the software GenStat 17th Edition (Payne 2014), while
304 charts and graphs were obtained with SygmaPlot v10 (Systat). Initially, correlations were
305 computed for any pair of quantitative variables (i.e. CNRQ values, F_v/F_m and field survival);
306 then, the "All-subsets regression" procedure was used to efficiently fit all possible regression
307 models explaining F_v/F_m and field survival data. Selection of the more effective *CBF* variable(s)
308 was based on the convergence of three different statistics: 1) the adjusted R-squared, 2) the
309 Mallows Cp criterion, and 3) the Akaike Information Criterion. Once the best subsets of
310 different *CBF*s were identified, simple and multiple linear regression analyses (namely, SLR
311 and MLR) were used to estimate the amount of phenotypic variance accounted for by the fitted
312 models. Regressions with groups (namely, gSLR and gMLR) were also performed to compare
313 how the model changed across the allelic states of *VRN-H1*. In this case the genotypes were
314 classified as 'winter/facultative' or 'spring' according to von Zitzewitz et al. (2005). For each
315 analysis Levene's test was carried out to assess the assumption of equality of variances of
316 standardized residuals.

317 Linear mixed models and estimation of variance components using the method of residual
318 maximum likelihood (REML) was applied to compare frost resistance levels of $F_{5:6}$
319 recombinants with the two parents 'Nure' and 'Tremois' and the two DH lines NT-42 and NT-
320 64. Genotypic class (Genoclass), experiment (Exp) and Genoclass x Exp treatments were fitted
321 as fixed terms in the REML variance components analysis, while replicates were set as random
322 terms. Approximate least significant differences ($LSD_{0.05}$) of REML means were then used for
323 multiple comparisons.

324

325 **Results**

326

327 *Survey of copy number variation at the FR-H2 locus*

328 With the aim of determining the copy number of barley *CBF* genes at *FR-H2*, highly specific
329 RT-qPCR assays were used, scattered along this genomic region. Effectiveness of the probes
330 was verified through a preliminary *in silico* analysis and, when blasted against the reference
331 'Morex' sequence – GenBank KF686739, nine out of 11 *CBF* primer pairs retrieved a unique
332 position in the locus, thereby confirming the desired specificity for the target genes
333 (Supplementary Table 2). On the other hand, the amplicon designed on *HvCBF6* could amplify
334 two different regions of 'Morex' *FR-H2*; the target sequence downstream *HvCBF6* (position
335 1,088,950–1,089,050), and an identical sequence downstream *HvCBF6-pseudo* (position
336 1,051,128–1,051,228). The assay for *HvCBF13* hit several sequences other than the intended
337 target (data not shown) and was excluded from further analysis. According to the relative

physical and genetic position of the *HvCBF* genes (Pasquariello et al. 2014), the designed assays spanned an approximate distance of 1,089 kb. The inter-probe space between two consecutive amplicons ranged in size from 14 to 438 kb (for the *HvCBF4–HvCBF2* and *HvCBF2–HvCBF14* intervals, respectively); while the average probe density was 97.9 kb. For CNV calculation the copy number was fixed as 2 for 'Morex' (reference genotype) for all genes apart from *HvCBF6* were the copy number in 'Morex' was fixed as 4 as this primer pair targeted both the gene and its pseudogene. Copy number relative quantity (CNRQ) data revealed that *HvCBF4* and *HvCBF2* in the proximal cluster of *Fr-H2* displayed extensive CNV, with an average of 4.38 and 4.00 copies per diploid genome, respectively (Table 1). Such variability was evident from the much higher coefficient of variation of the two genes (64% and 78%, respectively) compared to the other target genes (ranging from 15.2% for *CBF15* to 28.0% for *HvCBF10*). Conversely, the two reference genes *HvNUD* and *HvSAMDC* showed a difference <0.5 in CNRQ mean (1.82 and 2.22, respectively) and the smallest coefficient of variation (9.8% and 9.3%, respectively). If we consider winter/facultative and spring genotypes separately, the copy number of both *HvCBF4* and *HvCBF2* was double in the former group, namely 6.20 vs. 2.81 and 5.60 vs. 2.61 copies per diploid genome, respectively (Table 1). Covariance between *HvCBF4* and *HvCBF2* is also graphically illustrated by Fig. 1, where a positive relationship between the numbers of copies of the two genes was observed along with a clear separation of the spring types from the winter and facultative ones.
Relationships among CNRQ values at the ten assayed *HvCBF*s were explored by pairwise correlation analysis, and the strongest association (r = 0.89***) was once again observed between *HvCBF4* and *HvCBF2* in the proximal cluster of *FR-H2* locus. Other positive association were evident, although at lower values of correlation coefficient. In particular, *HvCBF9* CNRQ correlated with the genes belonging to the central cluster (*HvCBF15* and *HvCBF12*) and to the distal cluster (*HvCBF16*, *HvCBF3*, *HvCBF10* and *HvCBF6*), as well as *HvCBF12* copy number correlated with CNRQ of *HvCBF15* and of all the genes in the distal cluster (Supplementary Table 3).

CNV at specific HvCBF genes is associated with frost resistance

To identify resistance-associated CNVs at specific *HvCBF*s, relationships among phenotypic data were firstly explored by correlation. Phenotypic records obtained from three consecutive experiments of freezing survival in controlled conditions (F_v/F_m) were more interrelated than field-laboratory assessments of winter survival (%) from three consecutive trials with mean r coefficient 0.77 and 0.48, respectively. Frost resistance data were then averaged separately for F_v/F_m and for field survival score, and used for investigating relationship of phenotypic values to CNV effects of the single *CBF*s (Table 1). Both traits showed high positive correlation with CNRQ variation at *HvCBF4* and *HvCBF2* in the proximal cluster (r ranging from 0.56*** to 0.42*). A lower yet significant negative correlation was observed between *HvCBF3* CNV and

376 frost resistance traits. No other statistical association between CNV at the assayed genes and
377 frost resistance was found (Table 1). The association between frost resistance and copy number
378 variation at *HvCBF4* and *HvCBF2* is well illustrated by the scatter plots in Fig. 2 and Fig. 3
379 respectively. In both cases, when linear regression fits were plotted separately for the
380 winter/facultative and spring accessions, genotypes with increased copy number of *HvCBF4*
381 and *HvCBF2* similarly showed greater frost resistance in the two breeding groups.
382 Owing to Knox et al. (2010) 'Nure' harbours two forms of *HvCBF2*, *CBF2A* and *CBF2B*,
383 'Morex' only *CBF2A* while 'Tremois' has a single *CBF2* gene in the form of a *CBF2A/B*
384 paralog (*CBF2B*-like in its 5' region and *CBF2A*-like in its 3' region). To verify the presence and
385 absence of the different *HvCBF2* forms in the genomes of all the genotypes tested, a CAPS
386 protocol was developed that could discriminate between the *2B, 2A* and *2A/B* forms
387 (corresponding to sequences cloned in 'Nure', both in 'Morex' and 'Nure', and in 'Tremois',
388 respectively). Fig. 4 shows the discrimination of the *HvCBF2* forms obtained using the
389 *Mlu*Ci/*Mlu*I double digestion assay. As expected, the digestion produced bands corresponding
390 to *CBF2A* for 'Morex', to *CBF2A/B* for 'Tremois' while for 'Nure' the results confirmed the
391 presence of both *HvCBF2A* and *HvCBF2B.* All accessions used in this study were genotyped by
392 the *HvCBF2*-discriminating CAPS marker. Considering the growth habit classification in
393 winter, facultative and spring types according to von Zitzewitz et al. (2005), a significant
394 difference in CNV was observed between the average values obtained in the three groups
395 (Supplementary Table 4). Noteworthy, all facultative (including the highly resistant genotypes
396 'Lunet', 'Pamina' and 'U259') and the best ranking winter genotypes ('Bazant' and 'Cetin')
397 harboured only the *CBF2A* form. The majority (11 out of 14) of other winter types harboured
398 both the *CBF2A* and *CBF2B* forms, while the 22 tested spring genotypes harboured all the three
399 possible combinations. What should be stressed is that the fused *CBF2A/B* form was observed
400 mainly in spring (8 out of 9) genotypes and that this form had the lowest CNV respect other
401 *HvCBF2* forms. No genotype harbouring the only *CBF2B* form was observed in this study.
402
403 *Combination of HvCBF genes showing CNV at FR-H2 better explain frost resistance*
404 With the final aim of unravelling whether the effect of *FR-H2* is linked to CNV for a single
405 gene within the genomic region under study or to the involvement of multiple *HvCBF*s in the
406 cluster, single and multiple linear regression analysis models were applied. As a first step,
407 prediction of F_v/F_m and winter survival phenotypes through single linear regression confirmed
408 the major involvement of *HvCBF4* and *HvCBF2* and, to a lesser extent, of *HvCBF3*. The fitting
409 of the model also allowed to estimate the proportion of variance accounted for by either single
410 genes or by different combinations of copy number (i.e. sum and/or difference) of multiple
411 genes together. The effect of combined gene copy change at *HvCBF4*, *-2*, and *-3* was generally
412 associated to greater values of adjusted *R*-squared rather than taking CNV at the single genes

413 separately (Supplementary Table 5). A cumulative effect of the proximal cluster was confirmed
414 in barley, while again no significant effect of the central and distal clusters was observed.
415 Owing to Stockinger et al. (2007), Akar et al. (2009), and Zhu et al. (2014), a crosstalk between
416 vernalization and cold acclimation pathways through the interaction of *FR-1/VRN-1* and *FR-*
417 *2/CBF* loci should not be overlooked. Therefore, the allelic state at *VRN-H1* was included as
418 covariate in the regression models by classifying the accessions in winter/facultative or spring
419 types and setting the 'Tremois' (spring, susceptible) allele as reference. As expected, a greater
420 explanatory power of the model was observed in all cases, and the effect of combined gene copy
421 change at *HvCBF4*, *-2*, and *-3* was generally associated to larger values of adjusted *R*-squared
422 (0.403–0.414 and 0.371–0.445 for F_v/F_m and winter survival, respectively) rather than taking
423 CNV at the genes separately (Supplementary Table 5).
424 Generalized Linear Model regression analysis was further exploited using the "All subsets
425 regression" method, and this allowed identifying the best combinations of predictors explaining
426 the two frost resistance traits. The allelic variation at *FR-H1/VRN-H1* was also included in the
427 final multiple linear regression model with groups (gMLR), but no significant interaction effect
428 between CNV at the *HvCBF*s and *FR-H1/VRN-H1* were found (data not shown). For F_v/F_m, the
429 combined copy number of *HvCBF4* and *HvCBF2* (i.e. *SUM4+2*) together with variation at
430 *HvCBF3* in winter/facultative or spring genotypes could explain up to 0.434*** of the variance
431 accounted for by the fitted model (Table 2). Likewise, winter survival was better explained by
432 fitting copy number variation at *HvCBF4* and *HvCBF3*, and this resulted in an adjusted *R*-
433 squared of 0.550*** (Table 3). Overall, this data showed that genotypes more tolerant to frost
434 carried a higher copy number of the *HvCBF4–HvCBF2* segment in combination with a lower
435 CNV at *HvCBF3*, and that roughly 50% of the trait could be explained after the *VRN-H1* allelic
436 effect is fixed in the model.
437 During recent years, high-resolution mapping in barley was used by Francia et al. (2007) and
438 Pasquariello et al. (2014) to identify informative recombinants among *HvCBF* gene elements at
439 *FR-H2* in a 'Nure' x 'Tremois' (NxT) experimental population. Accordingly, in this work,
440 selected $F_{5:6}$ lines were tested to obtain an independent proof of the CNV involvement in frost
441 resistance observed in the barley collection. The recombinants were phenotyped both for the
442 photochemical capacity of photosystem II (F_v/F_m parameter) after freezing and for winter
443 survival in the field, and compared to the parents and to two NxT doubled haploid lines
444 harbouring alternative alleles at *FR-H1/VRN-H1* and *FR-H2/CBF* loci (Supplementary Fig. 1).
445 Results of restricted maximum likelihood (REML) ANOVA agreed with multiple linear
446 regressions. In particular, a recombinant line (Rec 15-12) carrying *HvCBF4* and *HvCBF2* from
447 'Nure' in combination with *HvCBF3* from 'Tremois' was more resistant in terms of F_v/F_m than
448 the three recombinants harbouring reciprocal alleles at the same loci. An equivalent trend was
449 observed in a one-year field trial experiment. Overall, these results confirmed the major role

450 played by the genes of the proximal cluster combined with a minor effect of a single element of
451 the distal cluster, while no effect was observed for the *HvCBF*s of the central cluster.
452
453 **Discussion**
454
455 In the present study, a multiple gene-specific CNV detection at the *Frost resistance-H2* (*FR-*
456 *H2*) locus was performed. The implementation of effective stress resistance mechanisms in
457 crops is expected to translate in improved productivity in challenging environments (Mickelbart
458 et al. 2015). Plant genomes show extensive variability such as single nucleotide polymorphisms
459 (SNP), presence/absence sequence elements, and different forms of structural variation (SV)
460 that includes copy number variation. CNV is complementary to SNP as a major source of
461 genetic variability and could account for an important part of the missing heritability of
462 complex traits (Manolio et al. 2009). For many crops, the recent availability of reference
463 genomes coupled with advances in comparative genomic hybridization and deep sequencing
464 techniques allowed successful discovery of SV. In maize and soybean, and more recently in
465 barley, a catalogue of CNVs has been reported (Swanson-Wagner et al. 2010; Jiao et al. 2012;
466 McHale et al. 2012; Muñoz-Amatriaín et al. 2013). However, to date only few CNV studies in
467 plants regarded association to characters as the majority have been merely structural
468 investigations. In crops a causal relationship between copy number and phenotypic variation
469 linked to single genomic regions has been firstly demonstrated for the barley *Bot1* boron
470 transporter gene (Sutton et al. 2007). Later on, the study of the soybean *Rhg1* cyst nematode
471 resistance locus (Cook et al. 2012), of the maize efflux transporter *MATE1* (Maron et al. 2013),
472 and the barley flowering time determinant *FT1* (Nitcher et al. 2013) showed that different
473 phenotypes in evolutionary distant plant species could be ruled by CNV gene dosage effects. A
474 similar phenomenon was also hypothesized for the *FR-H2* locus by (Knox et al. 2010). This
475 QTL in the Triticeae defines a region on the homeologous chromosome group 5 that harbour
476 several duplicated *CBF* genes and in barley reference cultivar 'Morex' the region entails a
477 genomic segment of ca. 1.5 Mb (Knox et al. 2010; Pearce et al. 2013; Pasquariello et al. 2014).
478 The physical structure of the locus suggests that this gene cluster is subject to dynamic
479 expansions and contractions potentially entailing for a more flexible response to cold stress
480 (Zhu et al. 2014). Accordingly, in the present study this was investigated through a CNV survey
481 in 41 barley varieties representing a broad genetic basis (Supplementary Table 1).
482 Although nowadays the methods of choice for genome-wide identification of CNVs are those
483 based on high-throughput platforms (Li and Olivier 2013), for targeted locus studies a RT-
484 qPCR could be preferred as amenable for analysis of a large number of samples and no need of
485 post-amplification labor processing. RT-qPCR presents also some constraints that should be
486 taken into consideration, such as a relative analysis method requiring a control with 2 copies of
487 the gene or region of interest, and results may fall between integers (e.g. copy number

488 measuring 1.3) making interpretation more difficult (Ma and Chung 2014). Here RT-qPCR was
489 adopted to determine how *HvCBF* gene copy number varies at *FR-H2* and, as expected, allowed
490 a thorough investigation of the copy number relative quantity of the different *CBF* genes (Table
491 1). No CNV was observed for the majority of the investigated genes, whereas *HvCBF4* and
492 *HvCBF2* in the proximal cluster of *FR-H2* displayed extensive CNV (Fig. 2 and Fig. 3). The
493 values obtained for *CBF10* should putatively attributed to the presence of *CBF10A* and *CBF10B*
494 forms in the tested collection as already suggested by Knox et al. (2010) who compared
495 'Morex' and 'Nure' lambda phage genomic sequences. On the other hand, in case of *CBF6* it
496 was not possible to design a primer pair that would no amplify the pseudogene due to the
497 sequence identity. In fact the observed copy number was double respect to the reference genes
498 (CNRQ 4.01 in Table 1).
499 Knox et al. (2010) stated that the genotypes harbouring the *vrn-H1* winter allele display two
500 distinct *CBF2* paralogs (2A and 2B), while genotypes carrying the *Vrn-H1* spring allele have
501 single copies of *CBF2* and *CBF4* paralogs. However, in the present work the results obtained
502 using a CAPS protocol for the whole collection revealed that not all the winter accessions do
503 harbour the *CBF2B* form identified in 'Nure' and 'Dicktoo' (Knox et al. 2010). Two winter
504 varieties, 'Cetin' and 'Bazant', have only the *CBF2A* form associated with a high degree of
505 CNV (7.6 in Supplementary Table 4), while *CBF2B* was observed together with *CBF2A* also
506 among the spring accessions ('Angela', 'Mellori', 'Ponente', 'Aldebaran', 'Tidone') with a
507 lower average CNV value of 5.6. This finding shows that, contrary to what stated by Knox et al.
508 (2010), the presence of *CBF2B* form is not able to discriminate winter from spring barleys. As a
509 general rule however it could be confirmed that the number of copies of both *HvCBF4* and
510 *HvCBF2* was double in winter/facultative vs. spring accessions, namely 6.20 vs. 2.81 and 5.60
511 vs. 2.61 copies per diploid genome, respectively (Table 1).
512 Several studies have revealed that the cereal *CBF* family is large and complex with at least 17
513 *CBF*s in barley (Skinner et al. 2005; Francia et al. 2007) and 13 in diploid einkorn wheat (Knox
514 et al. 2008). An even more complex organization of the family is observed in the hexaploid
515 wheat genome, which contains at least 65 *CBF* genes, 27 of which are paralogs with 1-3
516 homeologous copies for the A, B and D genomes (Mohseni et al. 2012). In addition, in the
517 Triticeae inter- and intra-specific variability has been observed in terms of presence/absence of
518 specific *CBF* paralogs that reside at *FR-2*. While some genes are conserved, some others seem
519 to be species-specific. For example, *CBF17* has never been isolated in barley and is not present
520 in the 'Morex' reference genome as well as orthologs of barley and *T. monococcum CBF13*,
521 *CBF16* have yet to be identified in rye and bread wheat (Skinner et al. 2006; Campoli et al.
522 2009; Pearce et al. 2013; Pasquariello et al. 2014). This large diversity in Triticeae seems to
523 represent an evolutionary adaptation to temperate climate habitats with a wide range of
524 temperature changes. The present study suggests that also as far as the CNV within the *FR-2*
525 locus is regarded, different clusters or subgroups of *CBF* paralogs are involved in barley and

526 wheat. In wheat, the copy number of *CBF-A14, CBF-A15 and CBF-A12* was found to be higher
527 in winter than in spring wheats revealing a crucial role of the central cluster (Dhillon and
528 Stockinger 2013; Zhu et al. 2014). Here the availability of the 'Morex' reference genome
529 allowed to obtain a much more comprehensive picture of the locus than the one obtained in
530 wheat by Zhu et al. (2014) where only some *TaCBF*s were investigated and showed that none of
531 those *CBF*s was affected by CNV in barley (Table 1). On the other hand, an increase in copy
532 number of two genes belonging to the proximal cluster reported by Knox et al. (2010) was fully
533 confirmed highlighting a very high number of copies in winter/facultative over spring genotypes
534 (Fig. 1).
535 Accurate phenotyping is an essential step for studying the implications of gene/genomic CNV in
536 the genetic architecture of underlying complex traits. Frost resistance data showed high positive
537 correlation with CNRQ variation at *HvCBF4* and *HvCBF2*. When linear regressions fits were
538 plotted separately for the winter/facultative and spring accessions, genotypes harbouring more
539 copies of *HvCBF4* and *HvCBF2* showed greater frost resistance (Fig. 2 and Fig. 3). In
540 particular, two winter ('Bazant' and 'Cetin') and three facultative ('Lunet', 'Pamina' and
541 'U259') genotypes that were highly resistant, ranked in the five top positions at both genes for
542 CNRQ. All of them harboured at least 10 copies of *HvCBF4* and at least 8 copies of *HvCBF2*
543 per diploid genome. Interestingly, these cultivars originated from different countries and did not
544 share any common ancestor (Supplementary Table 1). All accessions used in this study were
545 genotyped by the *HvCBF2*-discriminating CAPS marker and divided into winter, facultative and
546 spring types according to von Zitzewitz et al. (2005) highlighting a significant difference in
547 CNV between the average values obtained in the three groups (Supplementary Table 4).
548 Noteworthy, all facultative (including the highly resistant genotypes 'Lunet', 'Pamina' and
549 'U259') and the best ranking winter genotypes ('Bazant' and 'Cetin') harboured only the
550 *CBF2A* form, while other resistant winter genotypes harbouring also the *CBF2B* form had lower
551 CNV (4−6). As reported by Rizza et al. (2011) the groups of winter and facultative genotypes
552 showed the highest frost resistance when evaluated in standard freezing stress (e.g. −12 or
553 −14°C) in controlled conditions. Moreover, the interesting association of copy number of
554 *HvCBF4* and *HvCBF2*, physically tightly linked in a genomic region of only ca. 14 kb (Knox et
555 al. 2010; Pasquariello et al. 2014), and the presence of *CBF2A* form was highlighted in the
556 present work thus extending the molecular understanding of the phenomenon.
557 One of the working hypotheses of the present paper was that the frost resistance effect ruled by
558 *FR-H2* could depend on a sort of "*CBF*-number game" rather than to a genetic variation at
559 single gene element. Having obtained a global view of the locus highlighted not only interesting
560 relationships between paralogs within the proximal cluster, but also the involvement of the
561 distal one through the action of *HvCBF3*. This combined effect of gene copy change involving
562 *HvCBF4*, *-2*, and *-3* was generally associated to greater values of adjusted *R*-squared rather than
563 taking CNV at the single genes separately (Table 2 and Table 3). This result was confirmed

564 using recombinants of a ‘Nure’ x ‘Tremois’ experimental population. The line carrying the
565 *HvCBF4–HvCBF2* segment from ‘Nure’ in combination with *HvCBF3* from ‘Tremois’ was
566 significantly more resistant than recombinants harbouring reciprocal alleles at the same loci
567 (Supplementary Fig. 1). Noteworthy, self-regulated feedback loops for CBFs action, as already
568 reported in Arabidopsis (Novillo et al. 2011), could be involved also in Triticeae. In the present
569 study this is suggested by the negative influence of *HvCBF3* on frost resistance. No
570 involvement of the central cluster was detected, contrary to what was observed by Zhu et al.
571 (2014) for wheat.
572 Stockinger et al. (2007) hypothesized that *VRN-H1/FR-H1* is a regulatory locus affecting the
573 expression of multiple *CBF*s at the chromosomally linked *FR-H2* locus. Modulation of *CBF*
574 transcription at the promoter level exercised by the MADS box transcription factor *HvBM5*
575 (*VRN-H1*) has been recently hypothesized by Deng et al. (2015), in accordance with reduced
576 expression of *CBF2*, *-4* and *-9* during low-temperature treatment in lines with elevated *VRN-1*
577 activity (Dhillon et al. 2010). These differences had been proposed to putatively depend either
578 on a different modulation at promoter level between ‘Nure’ and ‘Tremois’ *CBF2* and *CBF4*
579 alleles, or a greater stability of the *CBF2* and *CBF4* mRNAs from the ‘Nure’ allele (Stockinger
580 et al. 2007). All the above findings suggested a qualitative link between frost resistance and
581 allelic state variation at *VRN-H1/FR-H1* and a quantitative connection to the expression of *CBF*
582 genes. On the other hand, in the present study the relationship between CNV at *FR-H2* and the
583 allelic state at *VRN-H1/FR-H1* was investigated for the first time in barley. When the allelic
584 state at *VRN-H1* was included as covariate in the regression models by classifying the
585 accessions in winter/facultative or spring types and setting the ‘Tremois’ (spring, susceptible)
586 allele as reference, the effect of combined gene copy change at *HvCBF4*, *-2*, and *-3* was
587 generally higher than taking CNV at the genes separately (Table 2 and Table 3).
588

589 **Conclusions**

590

591 Overall, our data show that genotypes more tolerant to frost carry higher copies of the
592 *HvCBF4–HvCBF2* segment in combination with lower copies of *HvCBF3*, and that increased
593 copy number of *CBF2A* was observed in highly resistant facultative and winter genotypes.
594 Roughly 50% of the trait could be explained after the *FR-H1/VRN-H1* allelic state is fixed in the
595 model. However when the allelic variation at *FR-H1/VRN-H1* was also included in multiple
596 linear regression with groups, no significant interaction effect between CNV at the *HvCBF*s and
597 *FR-H1/*VRN-H1 was found, contrary to what was reported in both winter and spring wheat
598 (Zhu et al. 2014). The results obtained in the present study suggest the additive effect of those
599 two loci, as firstly observed in barley by Francia et al. (2004), where no interaction effects were
600 found between *FR-H1* and *FR-H2*. Taken together the data show a major involvement of the
601 genes located in the proximal cluster, with no apparent involvement of the central cluster,

602 confirming that the combinatorial game of *HvCBF*s at *FR-H2* allows a more flexible response to
603 cold stress and suggest this gene cluster has shared ancestry generated by multiple duplication
604 events and subjected to dynamic expansions/contractions. It might be hypothesized that
605 duplication of different *CBF*s and putatively subsequent association of different clusters with
606 frost resistance took place in barley and wheat during their evolution preserving however the
607 functional role of the *FR-2* locus as a whole. The present work could represent a useful example
608 of targeted identification of specific genetic determinants of stress resistance that might serve as
609 potential candidates for precise introgression of superior alleles into breeding programmes.

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Tables

Table 1 CNV at each of the *HvCBF*s tested vs. phenotypic evaluation; genes are ordered according to Pasquariello et al. (2014)

	***HvCBF* target genes**										**Reference genes**	
	Proximal cluster			**Central cluster**			**Distal cluster**					
	9	***4***	***2***	***14***	***15***	***12***	***16***	***3***	***10***	***6***	***HvNUD***	***HvSAMDC***
CNRQ in the genotypes tested [a]												
Mean	2.15	4.38	4.00	2.04	2.06	1.90	1.90	1.47	3.25	4.01	1.82	2.22
Range	1.58	10.30	14.94	1.42	1.22	1.71	1.21	1.48	3.47	3.56	0.85	0.92
Lower Q	1.84	1.94	1.53	1.74	1.80	1.58	1.64	1.22	2.32	3.09	1.67	2.06
Upper Q	2.41	5.38	5.07	2.33	2.27	2.19	2.07	1.79	3.90	4.68	1.94	2.40
Std dev	0.43	2.80	3.12	0.36	0.31	0.45	0.30	0.38	0.91	0.96	0.18	0.21
CV%	19.9	64.0	78.0	17.8	15.2	23.6	16.1	25.64	28.0	24.1	9.8	9.3
CNRQ in Winter/Facultative vs. Spring genotypes [b]												
W/F mean	2.15	***6.20 a***	***5.60 a***	2.13	2.11	1.90	1.90	1.44	3.42	3.95	1.79	2.24
S mean	2.16	***2.81 b***	***2.61 b***	1.96	2.01	1.90	1.90	1.49	3.10	4.05	1.85	2.19
Pearson correlation CNRQ - frost resistance [c]												
F_v/F_m	-0.11	***0.56**** ***	***0.51**** ***	0.31	0.09	0.05	0.04	***-0.37****	-0.07	-0.05		
Winter Surv	-0.31	***0.56*****	***0.42****	0.39	0.16	-0.27	0.04	***-0.49****	-0.16	-0.25		

[a] : CNRQ is the copy number relative quantity; Range is maximum - minimum values; Lower Q (quartile) is the value *l* such that 25% of a sample are less than *l*, Upper Q (quartile) is the value *u* such that 25% of a sample are greater than *u*; CV% is coefficient of variation (Std dev/Mean x 100).
[b] : For each gene Student's *t*-test (p<0.05) was used to compare mean value of two sets of data; only significant differences are indicated.
[c] : *r* coefficient is calculated on 38 and 26 observations for F_v/F_m and winter survival data, respectively; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Table 2 Best multiple linear regression model for prediction of F_v/F_m by CNV at the *HvCBF*s and ANOVA for changes in the fitted model. Estimates of the parameters are derived in a model including the effect of allelic variation at *VRN-H1* ('Tremois' susceptible allele set as reference)

Explanatory variable	**Estimate β**	**s.e. β**	***t*-statistic**	***p*-value**
MLR with groups predicting F_v/F_m (n=38); adj-R^2 = 0.434 ($p < 0.001$)				
Constant	0.640	0.084	7.60	< 0.001
SUM4+2 [a]	0.007	0.003	1.98	0.056
HvCBF3	-0.082	0.048	-1.69	0.099
***VRN-H1* allele**	0.104	0.038	2.73	0.010

ANOVA for changes in the fitted model

Change	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F prob**
+ ***SUM4+2***	1	0.192	0.192	19.66	< 0.001
+ ***HvCBF3***	1	0.041	0.041	4.24	0.047
+ ***VRN-H1* allele**	1	0.073	0.073	7.44	0.010
Residual	34	0.332	0.332		
Total	37	0.638	0.638		

[a] : *SUM4+2* is the arithmetic sum of CNRQ values for *HvCBF4* and *HvCBF2*.

Table 3 Best multiple linear regression model for prediction of winter survival by CNV at the *HvCBF*s and ANOVA for changes in the fitted model. Estimates of the parameters are derived in a model including the effect of allelic variation at *VRN-H1* ('Tremois' susceptible allele set as reference)

Explanatory variable	**Estimate β**	**s.e. β**	***t*-statistic**	***p*-value**
MLR with groups predicting winter survival (n=26); adj-R^2 = 0.550 ($p < 0.001$)				
Constant	66.2	13.9	4.75	< 0.001
HvCBF4	2.4	1.0	2.35	0.028
HvCBF3	-23.0	7.9	-2.89	0.008
***VRN-H1* allele**	15.1	5.7	2.67	0.014

ANOVA for changes in the fitted model

Change	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F prob**
+ ***HvCBF4***	1	2826.9	2826.9	17.66	< 0.001
+ ***HvCBF3***	1	1392.3	1392.3	8.70	0.007
+ ***VRN-H1* allele**	1	1142.7	1142.7	7.14	0.014
Residual	22	3520.9	160.0		
Total	25	8882.8	355.3		

Figure Captions

Fig. 1 Relationship between *HvCBF4* and *HvCBF2* copy number. Genotypes with different growth habit (i.e. either winter, facultative or spring) are indicated with different symbols

Fig. 2 Relationship between *HvCBF4* copy number and frost resistance traits. Continuous and dashed lines are linear regression fits for genotypes harbouring either winter/facultative or spring allele at *VRN-H1* (dark vs. light symbols), respectively. (A) F_v/F_m, (B) winter survival, (C) ranking of genotypes according to *HvCBF4* CNRQ in the two *VRN-H1* groups

Fig. 3 Relationship between *HvCBF2* copy number and frost resistance traits. Continuous and dashed lines are linear regression fits for genotypes harbouring either winter/facultative or spring allele at *VRN-H1* (dark *vs*. light symbols), respectively. (A) F_v/F_m, (B) winter survival, (C) ranking of genotypes according to *HvCBF2* CNRQ in the two *VRN-H1* groups

Fig. 4 CAPS assay designed to distinguish between the different forms of *HvCBF2*. Double-digested amplicons are resolved using ethidium bromide stained gels. Only representative genotypes plus the reference cultivars 'Morex', 'Nure' and 'Tremois' are shown. Numbers on the right indicate the size (bp) of the fragments. MW, GeneRuler 1kb DNA Ladder (Thermo Scientific)

List of Online Resources

Online Resource 1:

Supplementary Table 1 List of the barley genotypes used for the study

Supplementary Table 2 Amplicons designed on target and reference genes used for RT-qPCR analysis

Supplementary Table 3 Matrix of correlations among copy number values at ten *HvCBF*s

Supplementary Table 4 Frost resistance vs. copy number of *HvCBF2* forms in facultative, winter and spring genotypes

Supplementary Table 5 Relationship between CNV of *HvCBFs* and frost resistance

Online Resource 2:

Supplementary Fig. 1 Graphic genotyping and frost resistance of 'Nure' x 'Tremois' $F_{5:6}$ recombinants in the *HvCBF* cluster at *Fr-H2*

Key message

CNV of *HvCBF4* and *HvCBF2* genes coupled with a negative role of *HvCBF3* play a major role in barley frost resistance conferred by the *Fr-H2* QTL.

Authors contribution

All the authors have participated in the work and none of them have any conflict of interest. In particular, Enrico Francia and Nicola Pecchioni conceived the study. Enrico Francia and Caterina Morcia designed sequence-specific amplicons on the tested genes, carried out RT-qPCR and copy number calculation. Marianna Pasquariello and Valentina Mazzamurro developed a novel CAPS marker and together with Justyna Milc performed CBF2A/B screening. Fulvia Rizza and Valeria Terzi performed the phenotypic characterization for frost resistance of the plant material. Nicola Pecchioni contributed to analysis and interpretation of data and participated in the discussion of results. The manuscript was written Enrico Francia and Justyna Milc.

Figure 1

Click here to download Line figure Figure1.tif

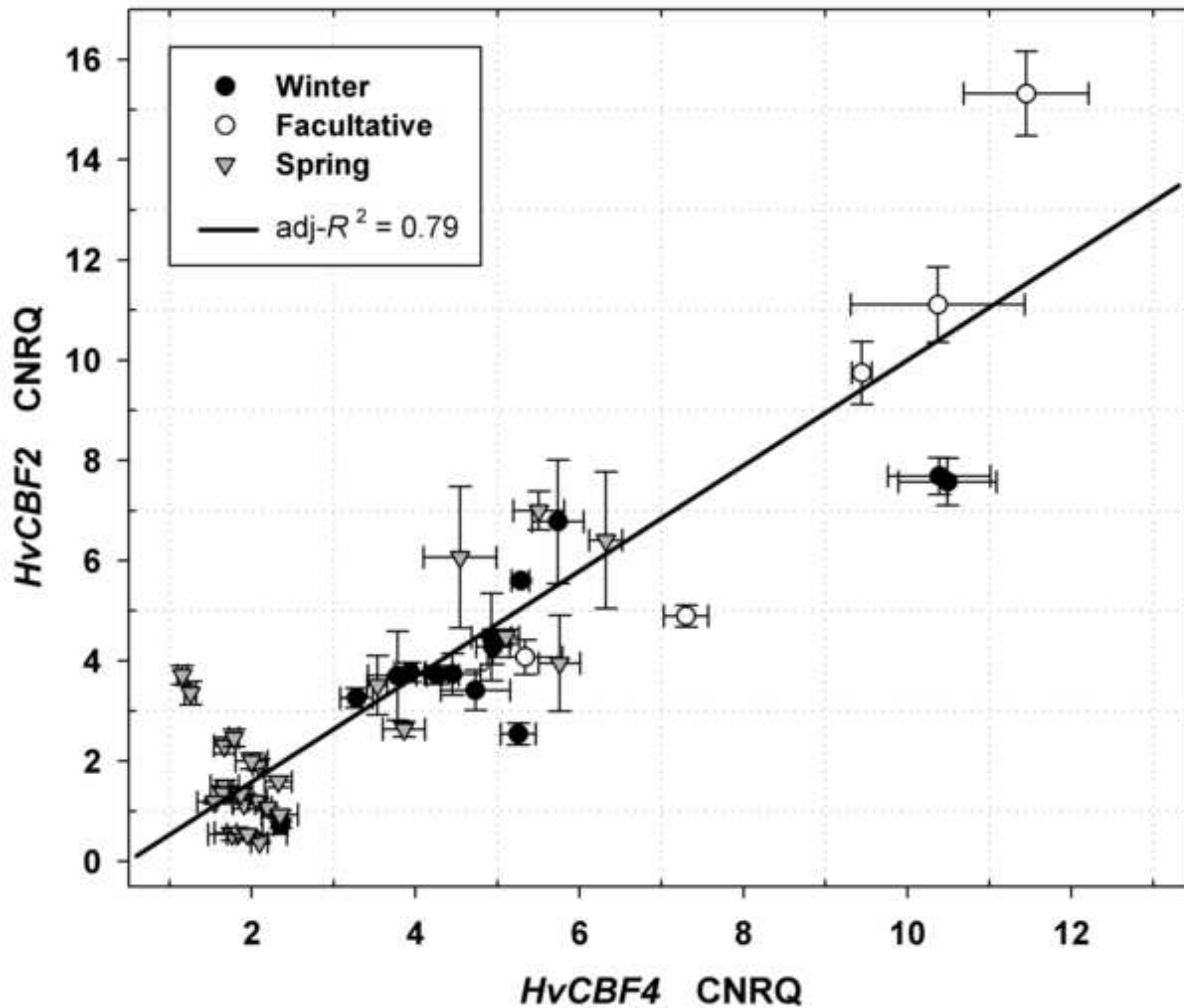

Figure 2

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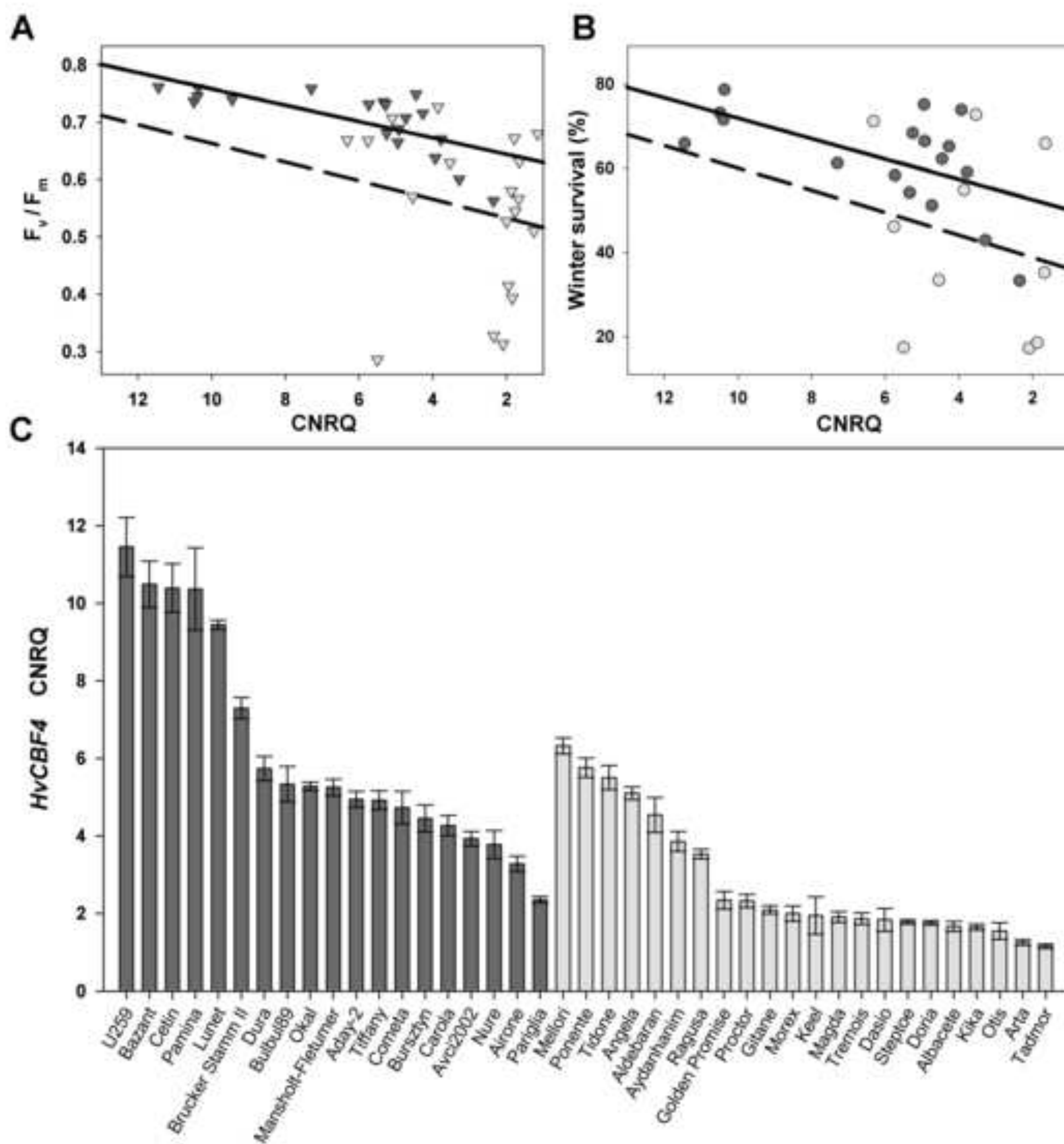

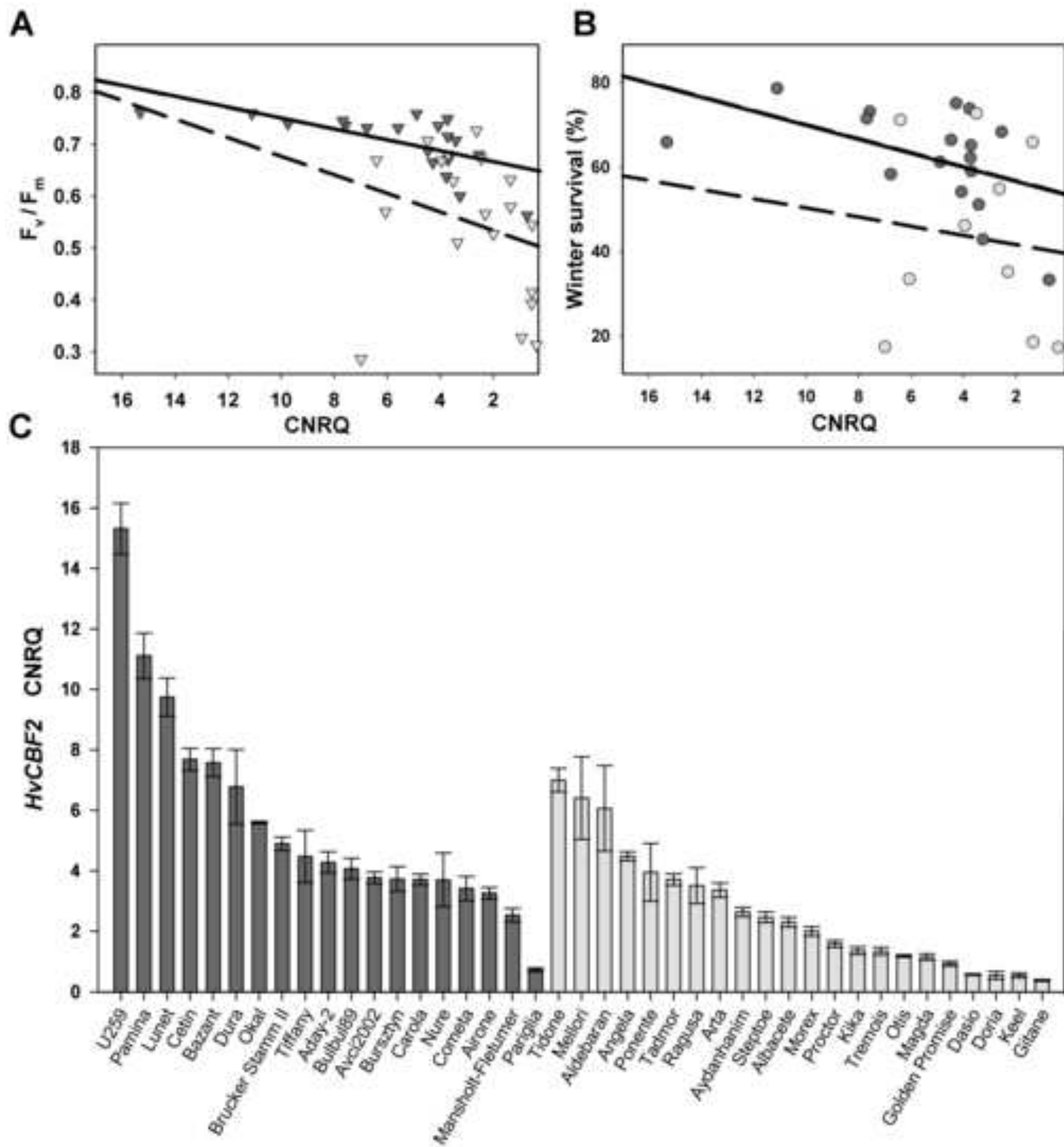
A
B
C
F_v / F_m
Winter survival (%)
CNRQ
HvCBF2 CNRQ
U259
Pamina
Lunet
Cetin
Bazant
Dura
Okal
Brucker Stamm II
Tiffany
Aday-2
Bulbul89
Avci2002
Bursztyn
Carola
Nure
Cometa
Airone
Mansholt-Fletumer
Pariglia
Tidone
Meltori
Aldebaran
Angela
Ponente
Tadmor
Ragusa
Arta
Aydanhanim
Steptoe
Albacete
Morex
Proctor
Kika
Tremois
Otis
Magda
Golden Promise
Dasio
Doria
Keel
Gitane

Figure 4

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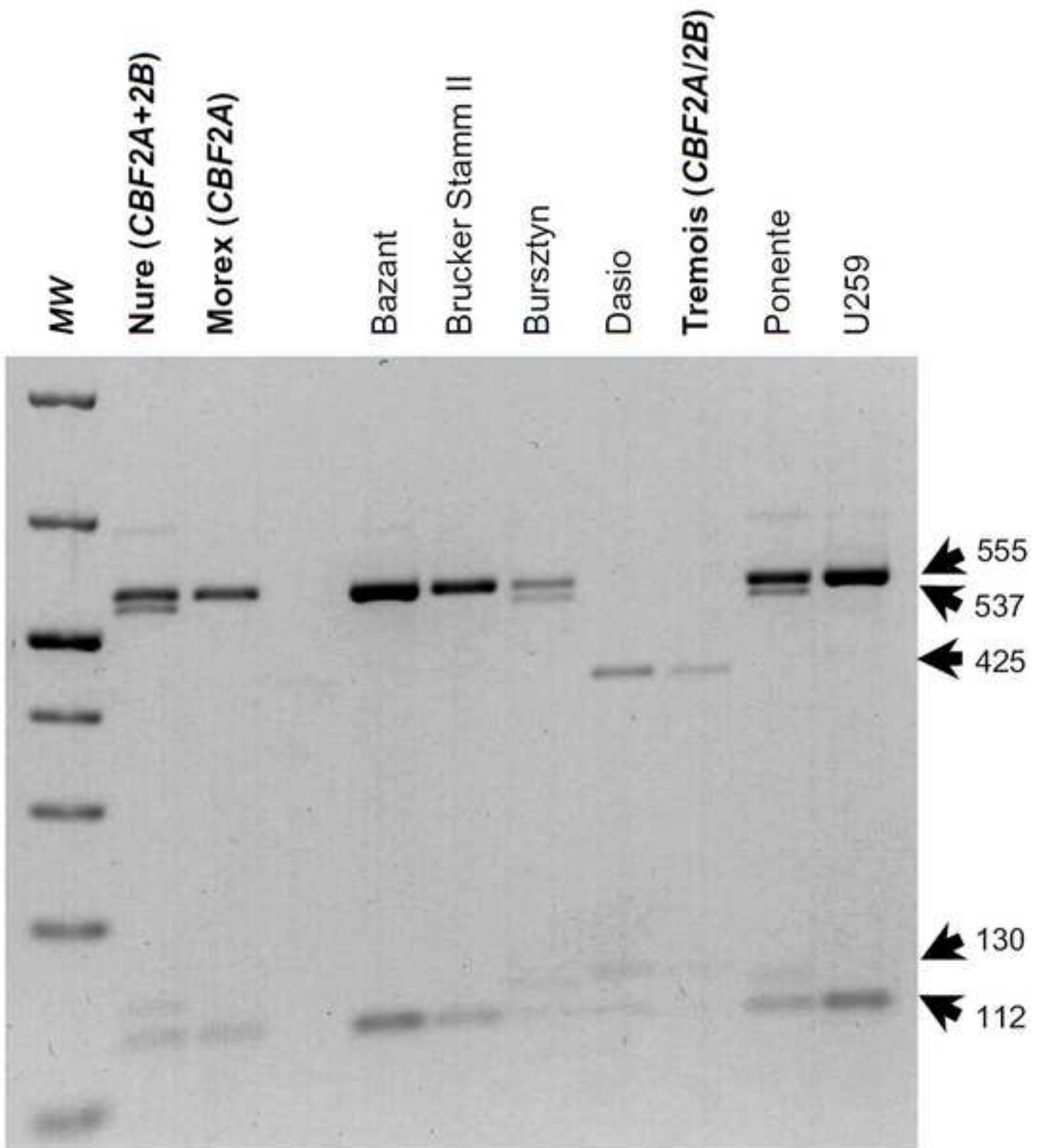

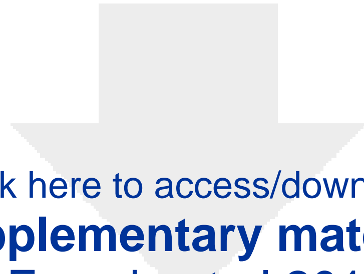

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Supplementary material 2

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