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Mapping regulatory genes as candidates for cold and drought stress tolerance in barley

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Abstract Cereal crop yield is greatly affected in many growing areas by abiotic stresses, mainly low temperature and drought. In order to find candidates for the tolerance genes for these stresses, 13 genes encoding for transcription factors and upstream regulators were screened by amplification and SSCP on six parental genotypes of three barley mapping populations ('Nure' × 'Tremois', 'Proctor' × 'Nudinka', and 'Steptoe' × 'Morex'), and mapped as newly developed STS, SNP, and SSCP markers. A new consensus function map was then drawn using the three maps above, including 16 regulatory candidate genes (CGs). The positions of barley cold and drought tolerance quantitative trait loci (QTLs) presently described in the literature were added to the consensus map to find positional candidates from among the mapped genes. A cluster of six Hv*CBF* genes co-mapped with the *Fr-H2* cold tolerance QTL, while no QTLs for the same trait were positioned on chromosome 7H, where two putative barley regulators of *CBF* expression, *ICE1* and *FRY1*, found by homology search, were mapped in this work. These observations suggest that *CBF* gene(s) themselves, rather than their two regulators, are at present the best candidates for cold tolerance. Four out of 12 drought tolerance QTLs of the consensus map are associated with regulatory CGs, on chromosomes 2H, 5H, and 7H, and two QTLs with effector genes, on chromosomes 5H and 6H. The results obtained could be used to guide MAS applications, allowing introduction into an ideal genotype of favourable alleles of tolerance QTLs.

Keywords Candidate genes · Stress tolerance · *CBF* · *ICE1* · *FRY1*

Abbreviations CG: Candidate gene · QTL: Quantitative trait locus · TF: Transcription factor · COR: Cold regulated gene · STS: Sequence tagged site · SNP: Single nucleotide polymorphism · SSCP: Single strand conformation polymorphism · CAPS: Cleaved amplified polymorphic sequence · ARMS: Amplification refractory mutation system

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Introduction

During their evolution, plants have acquired adaptive strategies to cope with abiotic stresses. Throughout domestication and breeding, man has driven the genetic improvement of crops towards high yields in different environments. These two facts notwithstanding, crop yield is greatly affected in many growing areas by abiotic stresses such as low temperature and drought. Among cereals, barley (*Hordeum vulgare* L.) can be considered a good genetic model for its tribe (*Triticeae*) to study plant response to adverse environmental conditions. Its inbreeding behaviour and diploidy make the genetic studies easy to perform; its wide range of adaptability, the availability of a wide range of genetic stocks, and the extended colinearity with other members of the tribe are additional advantages as a model (Hayes et al. 2003).

Two approaches have been pursued up to now to understand the biological bases of abiotic stress tolerance in plants: quantitative genetic and molecular genetic approaches. From the quantitative genetic point of view, the dissection of these complex traits has been made possible by the combined application of marker-mediated techniques and quantitative trait loci (QTL) analysis tools. This has led to the identification of QTL controlling barley resistance to cold and drought (Francia et al. 2004; Diab et al. 2004) as well as to other abiotic stresses. Different alleles of the gene(s) responsible for the phenotypic variation must obviously segregate in the chromosomal region underlying each QTL.

Employing the molecular genetic approach, analysis of plant response to stress (especially in the model plant *Arabidopsis thaliana* L.) resulted in the characterisation of several genes directly involved in stress perception, signal transduction inside the cell, and transcriptional regulation of stress-responsive 'effector' genes, as reviewed by Thomashow (1999), Xiong et al. (2002), and Yamaguchi-Shinozaki and Shinozaki (2005). Among the various abiotic stresses, the molecular responses to cold and drought have been the most studied in plants.

Single-gene expression studies and transcriptome profiling in response to drought and cold revealed the induction of 'effector' genes responsive to a single stress (e.g. barley *Cor14b* or *Arabidopsis Rd22*), and genes whose expression changed in response to both drought and cold (e.g. barley *Dhn* genes or *Arabidopsis Rd29a*), suggesting the existence of crosstalk between distinct stress signalling pathways (Knight and Knight 2001; Shinozaki et al. 2003).

Several transcription factors (TFs) regulating stress-inducible expression of the above-mentioned effector genes *via* interaction with *cis*-acting elements in promoter regions have been isolated and characterised (Singh et al. 2002; Xue 2003; Maruyama et al. 2004; Vogel et al. 2005). In particular, TFs belonging to the C-repeat binding factor (CBF) family have been shown to have a prominent role in cold acclimation (Thomashow et al. 2001). Moreover, by means of mutant screening (Ishitani et al. 1997), many *Arabidopsis* mutants have been isolated with altered induction of stress-responsive genes, and most of the mutated genes have been cloned. Some of these genes function upstream of the above TFs, principally in the early events of the signal-transduction cascade (e.g. *FRY1*, Xiong et al. 2001) with some acting directly in the activation of TF expression (e.g. *ICE1*, Chinnusamy et al. 2003).

The candidate gene (CG) strategy shows promise in bridging the quantitative genetic with the molecular genetic approach to study the complex traits (Pflieger et al. 2001). Especially in plants with large genomes such as barley (5,000 Mb), the generation of molecular-linkage maps based on the CGs (molecular-function maps) can be a way to identify the genetic determinants of QTLs, in spite of the time-consuming fine mapping (Thornsberry et al. 2001; Palaisa et al. 2003). Moreover, transcriptional regulators are better candidates to give a measurable trait at the QTL level than effector genes because a single TF can coordinately regulate a gene pool leading to the phenotype (e.g. enhanced cold tolerance). The CG strategy has for example been applied in finding genes with a putative role in determining potato starch content (Chen et al. 2001), expressed sequences potentially involved in tomato fruit size and composition (Causse et al. 2004), and rice and barley ESTs potentially involved in drought tolerance (Nguyen et al. 2004; Diab et al. 2004).

In this study we report the development of a new 'function map' of barley that will serve as a useful genetic tool for understanding the molecular bases of tolerance to the two most studied abiotic stresses in cereals: cold and drought. In particular, our aims are: (1) the identification and mapping in barley of functional CGs encoding TFs and their upstream regulators implicated in cold and drought tolerance, and (2) the construction of a consensus map from three barley populations that contain the above CGs, previously mapped putative effector genes, and published QTLs of tolerance to cold and drought. Co-locations of CGs and QTLs are then discussed.

Materials and methods

DNA and plant material

Genomic DNAs extracted from (1) 136 'Nure' × 'Tremois' barley doubled haploid (DH) lines (Francia et al. 2004), (2) 113 'Proctor' × 'Nudinka' DH lines (Heun et al. 1991), and (3) 150 'Steptoe' × 'Morex' DH lines (Kleinhofs et al. 1993) were used for linkage analysis in the present study. Chromosome arm assignments were conducted using the wheat–barley (cv. 'Betzes' in cv. 'Chinese Spring') addition lines (Islam et al. 1981).

PCR conditions, SSCP analysis, and sequencing

Twenty-one barley CGs were selected from the literature on the basis of reported involvement in cold or drought stress responses. The amino acid sequences of four CGs from other plant species were submitted to tBLASTn analysis against the TIGR barley EST database (release 9.0) and the best putative orthologous barley TCs were found.

Nucleotide sequences of the CGs were downloaded from NCBI (http://.ncbi.nlm.nih.gov) and TIGR (http://www.tigr.org) websites. GenBank accession numbers are indicated in Table. 1 and 2; TIGR TC numbers are indicated in Table 1. A total of 29 specific PCR primer pairs were designed on the downloaded sequences (on the 3′-UTR region when possible), with length ranging 17–27 nucleotides, by using the software Primer3 (Rozen and Skaletsky 2000). Primers used to map Hv*CBF2B*, Hv*CBF10B,* and Hv*CBF12* were designed to flanking, non-coding sequences determined from bacteriophage λ

Table 1 New barley candidate genes for cold and drought stress tolerance found by homology search in other organisms (rice and *Arabidopsis*), and mapped in barley by linkage analysis

TC	TIGR tentative annotation	Putative orthologs			tBLASTn (TIGR)		Linkage group	Primer (5′-3′)	Mapping population[a]	Marker assay
		Protein (GenBank accession)	Function assignment	Reference	% Identities	E value				
TC146711	Homologue of UP|O04417 (O04417) Calcium dependent protein kinase, partial (86%)	OsCDPK7 (AB042550)	Ca-dependent protein kinase	Saijo et al. 2000	96	$2.8\ e^{-217}$	2H (Bin12)	F-atattatgtagcgccagaagtgc R-ttgtttgatgcg agataagacag	'SxM'	CAPS (*ClaI*)
TC138581	Homologue of UP|Q7XBE4 (Q7XBE4) Enolase, complete	AtLOS2 (AY054253)	Enolase	Lee et al. 2002	88	$4.0\ e^{-210}$	5H (Bin1)	F-aaggaccctaccgctcaaac R-gaagatgggcgttgaagtgtac	'N×T'	CAPS (*Alw26I*)
TC147474	Similar to UP|DPNP_ORYSA (Q40639) DPNPase, partial (97%)	AtFRY1 (AY034894)	Inositol polyphosphate-phosphatase	Xiong et al. 2001	60	$2.0\ e^{-100}$	7H (Bin4)	F-gaaggtatcctctctgcaatcg R-gactctacttcagcaccacaacc	'N×T'	STS
TC143232	Similar to (Q8RYL7) OSJNBa0052O12.3 protein, partial (33%)	AtICE1 (AY195621)	bHLH TF	Chinnusamy et al. 2003	65	$1.7\ e^{-50}$	7H (Bin7)	F-ctgagcaatgcaaggatgg R-gacacagggtagtgacatcagg	'PxN'	CAPS (*MnlI*)

[a]Indicates the DH population used for mapping: 'N×T' = 'Nure' × 'Tremois', 'PxN' = 'Proctor' × 'Nudinka', 'SxM' = 'Steptoe' × 'Morex'

genomic clones encompassing each of these genes (E.J.S., unpublished data). Amplifications were performed in a 15 µl final volume containing: 40 ng of genomic DNA as template, 1X Qiagen PCR buffer, 1.5 mM of $MgCl_2$, 1X Q-solution, 0.25 mM of each dNTP, 0.4 µM of each primer, and 1U of *Taq* DNA Polymerase (Qiagen). Reactions were incubated for 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. Fragments were checked by standard agarose gel electrophoresis and, when differing in size between parental genotypes, candidates were mapped as STS markers with the same amplification conditions.

To identify single nucleotide polymorphisms and short INsertion/DELetions, CGs resulting in unique, specific, but same-size bands in agarose gel were screened on the six parental genotypes by using the single strand conformation polymorphism technique (Orita et al. 1989) with minor modifications. Briefly, 5 µl of PCR product were mixed with 9.0 µl of formamide-dye (99.6% formamide, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), denatured for 4 min at 92°C and quickly cooled on ice; 5.0 µl of the sample were loaded onto a 10% non-denaturing polyacrylamide gel (acrylamide:bisacrylamide = 49:1, 16×14×0.15 cm) containing 0.6X TBE (90 mM Tris-borate, 4 mM EDTA, pH 8.3) and 5% glycerol, then subjected to electrophoresis in 0.6X TBE at constant power at 4W for 16–18 h at 16°C. DNA bands were visualised with silver staining by fixing the gel in a 10% ethanol, 0.5% acetic acid solution for 3 min, staining it in a 0.2% $AgNO_3$, 10% ethanol, 0.5% acetic acid solution for 5 min and developing it in a 3% NaOH, 0.1% formaldehyde solution for 20 min. PCR products showing polymorphisms were purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and sequenced on both strands to confirm their identities. Sequences were aligned using BioEdit 5.0.6 (Hall 1999) to identify SNPs and INDELs.

CG linkage mapping and chromosome arm assignment

New CAPS, ARMS-PCR, and SSCP markers were designed for linkage mapping, based on the SNPs found in sequenced CG PCR products. Depending on the polymorphism type, three different techniques were used to map the SNPs of the CGs. In five cases, inspection of the restriction enzyme cleavage sites with the software WebCutter (http://www.firstmarket.com/cutter) revealed a restriction enzyme cleavage polymorphism. These candidates were thus mapped as CAPS markers (Table. 1, 2). In three cases, the nucleotide sequence surrounding an SNP allowed the design of new allele-specific PCR primers (Table 2), and therefore they were mapped as ARMS-PCR markers. The remaining CG was genotyped directly as an SSCP marker (Table 2) with the same conditions that were used in the pre-screening step due to lack of opportunity for mapping by CAPS or ARMS-PCR.

Table 2 Transcription factors as candidate genes mapped either by linkage analysis (A) or by chromosome arm assignment with addition lines (B)

Gene	GenBank accession	Function assignment	Reference	Linkage group	Primer (5′-3′)	Mapping population[a]	Marker assay
A: Linkage mapping							
Hv*MYB2*	X70880	Myb TF	Wissenbach et al. 1993	1H (Bin6)	F-gaaggagcacaccaacaagg Nure_R-aagagtgttggcgatgtcg Tremois_F-cggcaacaagtaagcacg R-agagaccacctgcacgagac	'N×T'	ARMS
Hv*MYB4*	X99973	Myb TF	Wissenbach et al. 1993	2H (Bin9)	F-cagcgacgacaacaacaacc Proctor_R-cgggccggaaactcgtt Nudinka_F-cggagggattgctaacatgg R-gaaaagcggacagacagactcc	'P×N'	ARMS
Hv*ABI5*	AY156992	bZIP TF	Casaretto and Ho 2003	5H (Bin7)	F-cccgccacgtagtattaacc R-ctcgaacgtcagggagtagatg	'N×T'	SSCP
Hv*CBF2B*	AF442489	AP2 TF	E.J.S., unpublished data	5H (Bin9-10)	F-agatccagcgtgatccgata R-agcggtgacacagttggac	'N×T'	STS
Hv*CBF12*	–	AP2 TF	E.J.S., unpublished data	5H (Bin9-10)	F-tggcaagcataaagggctaa Nure_F-cataaagggctaattgcggtt Tremois_R-ggacttttgattttctactccg R-ccggccttctcatctatctg	'N×T'	ARMS
Hv*CBF3*	AF298231	AP2 TF	Choi et al. 2002	5H (Bin9-10)	F-tcccagatgattctgctctg R-ggctggatgtaaggggtatatg	'N×T'	STS
Hv*CBF6*	–	AP2 TF	J.S.S., unpublished data	5H (Bin9-10)	F-cgactcgagcaaccataacaag R-ccccaatttacaccatcacattac	'N×T'	CAPS (*KpnI*)
Hv*CBF10B*	–	AP2 TF	E.J.S., unpublished data	5H (Bin9-10)	F-tgtactactctactactccctccgttc R-tgtgctccttttttacggattg	'N×T'	STS
Hv*MYB1*	X70879	Myb TF	Wissenbach et al. 1993	5H (Bin9-10)	F-atgctctcttgctgctccat R-gtgccatctcctctgctctc	'N×T'	CAPS (*DraI*)
B: Chromosome arm assignment (wheat–barley addition lines)							
Hv*CBT1*	AJ002610	Calmodulin binding transporter	Schuurink et al. 1998	7HS	F-ggccatatacgcttcacgtt R-gtgggggactatgttgcatt	None	STS
Hv*SPY*	AF035820	OGT-related factor	Robertson et al. 1998	6HS	F-gctgggggtcaatataggtgttg R-cgcctttctctctgtttctacc	None	STS

[a]Indicates the DH population used for mapping: 'N×T' = 'Nure' × 'Tremois', 'P×N' = 'Proctor' × 'Nudinka'

Digestion of CAPS-based markers was performed with the enzymes shown in Table. 1 and 2. According to manufacturer's instructions, 10 µl of PCR product were incubated for 1.5 h with 2U of restriction enzyme, 1X reaction buffer and 0.1 mg/ml of bovine serum albumin, and then separated on a standard 2% agarose gel. For ARMS-PCR, amplification was as described above except that 60°C annealing temperature was used, and PCR products were separated on a standard 1.5% agarose gel.

Linkage analysis in the individual mapping populations was performed with the software MAPMAKER 3.0 (Lander et al. 1987) by adding the markers to the available 'Nure' × 'Tremois' ('N×T', Francia et al. 2004; von Zitzewitz et al. 2005), 'Proctor' × 'Nudinka' ('P×N', http://www.wheat.pw.usda.gov), and 'Steptoe' × 'Morex' ('S×M', http://www.wheat.pw.usda.gov) linkage maps. Hv*CBF4*, Hv*CBF8,* and Hv*WRKY38* CGs had been previously mapped by Francia et al. (2004) and Marè et al. (2004) in the 'N×T' population. The consensus map was then constructed by using the software JoinMap 2.0 (Stam and Van Ooijen 1995). The final marker order was compared with the order of each constituent map, and as main reference, with the barley BIN map (http://www.barleygenomics.wsu.edu/arnis/linkage_maps/maps-svg.html; Kleinhofs and Graner 2001). Cold and drought tolerance QTLs reported in literature (Hayes et al. 1993; Pan et al. 1994; Francia et al. 2004; Reinheimer et al. 2004; Teulat et al. 1998, 2001, 2002, 2003; Diab et al. 2004) were indicated on the consensus map. Only intervals above the LOD thresholds chosen by their respective authors were considered.

Two CGs that were not polymorphic (Hv*CBT1* and Hv*SPY*) after SSCP analysis were assigned to a specific barley chromosome arm by PCR amplification from wheat–barley addition lines using the sequence-specific primers reported in Table 2. PCR reactions and agarose gel electrophoresis were performed as described above.

Results

Homology search of CGs

A homology search in the barley TIGR gene index (HvGI) found four tentative consensus (TC) sequences representing new putative barley CGs for cold and drought stress tolerance based on similarity to characterised genes from other organisms. The four newly mapped TCs are indicated in Table 1, together with their

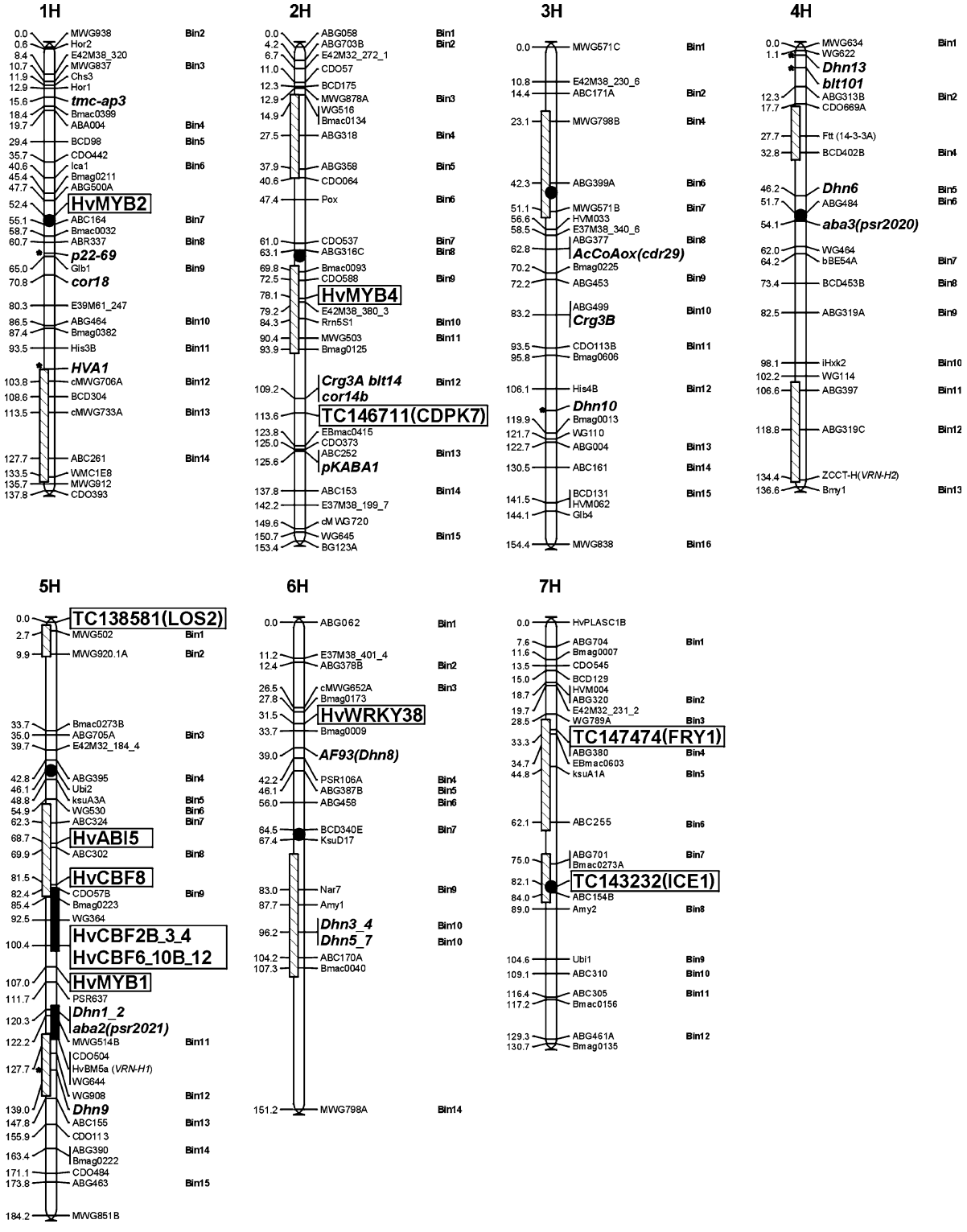

Fig. 1 Barley consensus function map. Distances are given in Kosambi cM and linkage groups are oriented with short arms at the top. Centromeres and BIN positions use the system of Kleinhofs and Graner (2001). Candidate genes either collected from GenBank accessions (Hv-) or obtained by homology searches (TC-) mapped in this study are boxed. *COR* and other abiotic stress-induced effector genes are in *bold italic*. An *asterisk* is present if their position was inferred from other maps, and no map distance (cM, top to end, left of the chromosomes) is given in these cases. *Solid* and *hatched* boxes inside chromosomes represent cold and drought tolerance QTLs, respectively

similarity to *Arabidopsis* or rice orthologous proteins. Polypeptides encoded by *TC146711* and *TC138581* showed high homology (provided as Electronic Supplementary Material: ESM Fig. S1), respectively, with the rice Ca^{2+} dependent protein kinase OsCDPK7, induced by cold and salt stresses (Saijo et al. 2000), and with the *Arabidopsis* enolase AtLOS2, regulating cold-responsive gene transcription (Lee et al. 2002).

More interestingly, *TC147474* and *TC143232* can be considered respectively the barley orthologs of the *Arabidopsis* genes At*FRY1* and At*ICE1*, two regulators of the expression of At*CBFs*. Putative orthology is strongly supported by the high E-value (Table 1) and by the amino acid sequence identity in the functional domains of the corresponding *Arabidopsis* and rice proteins (ESM Fig. S2). Although the *TC143232(ICE1)* sequence is truncated at the 5′ extremity, in the active domain, only one conservative substitution was found between the two proteins inside the existing aligned part of the DNA binding domain. No other barley sequences were found in the searched databases to complete *TC143232*.

CG mapping

The CG mapping strategy was applied to genes encoding TFs and upstream regulators in barley in order to find sequences associated with abiotic stress tolerance QTLs. A summary of the mapped CGs is presented in Table. 1 and 2.

Twenty-four PCR primer pairs designed to the 25 selected candidates successfully amplified from genomic DNA of the six parental genotypes ('Nure', 'Tremois', 'Proctor', 'Nudinka', 'Steptoe', and 'Morex'), and gave a unique PCR product. Markers developed for Hv*CBF2B*, Hv*CBF3*, Hv*CBF10B,* and *TC147474*(*FRY1*) were directly mapped as STS on the 'N×T' DH population on the basis of a length polymorphism between the parents. Identity of the amplified genes was also verified by sequencing in parentals. The remaining 20 amplicons were subjected to pre-screening for point mutations by SSCP analysis of the amplified products. Nine CGs (45%) revealed a clearly polymorphic SSCP pattern in at least one set of mapping parents, indicating the existence of SNPs which were confirmed by sequencing. As summarized in Table. 1 and 2, ten new candidates were mapped on the 'N×T' population, two on the 'P×N', and one on the 'S×M'. Two additional regulatory genes for which only chromosome arm assignment was given are reported in Table 2.

The consensus function map created by the software JoinMap 2.0 is composed of 202 loci, mainly RFLP and SSR markers (Fig. 1). Loci order is generally in accordance with previously published maps, as can be seen by the reported position of BINs. Only BIN1 of chromosome 1H, BIN3 and BIN5 of 3H, BIN3 of 4H, BIN10 of 5H, and BINs from 11 to 13 of 6H have not been covered by markers in our resulting consensus, whereas the position of centromeres was deduced from the published BIN map (Kleinhofs and Graner 2001).

As shown in Fig. 1, a total of 16 barley TFs and upstream regulators (highlighted in boxes) were genetically located on the barley genome, and the presence of a cluster of six *CBF* genes on chromosome 5H is noteworthy. In addition to the 16 regulatory candidates, 18 *COR* and abiotic stress-induced genes previously mapped in the three populations are shown in bold italics. Moreover, the position of six *COR* and abiotic stress-induced genes mapped on other populations was inferred by exploiting their relationships with common markers; an asterisk was placed alongside their positions, and no distances were reported on the map (Fig. 1).

Finally, through the relationships with markers common between the consensus and other linkage maps of barley, the position of cold and drought tolerance QTLs have been added to the consensus function map (Fig. 1). The QTLs were those reported by Hayes et al. (1993), Pan et al. (1994), Francia et al. (2004) and Reinheimer et al. (2004) for cold, and by Teulat et al. (1998, 2001, 2002, 2003) and Diab et al. (2004) for drought.

Discussion

In barley, more than 370,000 ESTs have been released (HvGI Release 9.0, September 15, 2004) and organised into more than 23,000 TC sequences. Among them, 132 TCs have been annotated by gene onthology (GO) as 'osmotic stress-', 'cold-', and 'water deprivation-' response gene products, representing an initial set of barley functional CGs to be studied, but many remain unclassified. In this work we demonstrated the feasibility of the approach using homology searches of *Arabidopsis* and rice stress-induced genes to find putative uncharacterised barley orthologs present in the TIGR TC database.

Single nucleotide polymorphisms are the most common type of sequence differences between alleles (Rafalski 2002). The present work has demonstrated the usefulness of a (low cost) workflow involving sequence retrieval and SNP marker development through SSCP pre-screening. A set of new PCR-based molecular markers from barley abiotic stress regulators, listed in Table. 1 and 2, was developed for linkage analysis and for association mapping studies. CAPS and ARMS-PCR markers proved very useful, as also observed by Chen et al. (2001) and Ye et al. (2001). In contrast, SSCP as markers *per se* turned out to be more time consuming and should only be used as a last choice.

Starting from 21 barley genes plus four TCs, in total 13 (52%) were genetically mapped. Because their role in regulating the biochemical and physiological pathways affecting the traits of interest was either previously demonstrated or suggested in the literature, and because of the predicted orthology of the TCs with genes of other

species (see Table. 1, 2 for references), all these genes and TC sequences were designated as 'functional candidates'. Recently, differentially expressed sequences tags related to drought tolerance have been mapped in barley by Diab et al. (2004). Nevertheless, the mapped ESTs were mostly 'effector' genes. Therefore the present work represents the first example of an integrated approach for mapping several regulatory CGs involved in cold and drought stress responses. Greater emphasis was given here to TFs and upstream regulators, rather than to structural genes. The hypothesis is that the trait of interest may be influenced by molecular variation in these regulatory genes more than in the structural ones (Morandini and Salamini 2003).

Transcription factors and other regulators of cold and drought-induced genes that were mapped are not randomly dispersed in the barley genome (Fig. 1). The most represented chromosome is 5H: 10 CGs at 5 loci, out of 16 CGs at 11 loci in total. This is also intriguing since most abiotic stress QTLs of the *Triticeae* are located on this chromosome (Cattivelli et al. 2002). Clustering of Hv*CBF* genes could be explained by local gene duplication, and selection pressure either for differently regulated or for a large *CBF* transcript pool could have been a driving force of the complexity of this gene family.

The cluster of six Hv*CBF* genes mapped to a single locus on 5H; in contrast, Skinner et al. (2005a, b) found two linked loci, separated by 0.5 cM, in the 'Dicktoo' × 'Morex' segregating population. Once verified the complete correspondence of sequence of each amplified Hv*CBF* with the Hv*CBFs* classified by Skinner et al. (2005a, b), the unresolved locus of the present work may be due to the more limited number of DH genotypes analysed (136 vs. 236 in Skinner et al. 2005b). On the other hand, the three Hv*MYB* genes were located on different chromosomes. The accessed effector genes were more uniformly distributed throughout the seven chromosomes, although 7H contained relatively few of them (Fig. 1).

CBFs and their regulators

The positions on the molecular consensus map of all previously reported QTLs for barley cold and drought tolerance revealed some interesting positional candidates (i.e. genes placed in regions containing QTL, Pflieger et al. 2001).

Two QTLs on the long arm of chromosome 5H were found to determine frost tolerance in the 'Nure' × 'Tremois' cross, named *Fr-H2* (proximal) and *Fr-H1* (distal), with similar and almost completely additive effects (Francia et al. 2004). One of these QTLs (*Fr-H1*) was in the same region of the genome as the previously identified QTL, with tolerance contributed by cultivar 'Dicktoo' (Hayes et al. 1993; Pan et al. 1994). It was demonstrated that Hv*CBF4* mapped to the *Fr-H2* QTL peak (Francia et al. 2004). The Hv*CBFs* are excellent functional CGs because several pieces of experimental evidence suggest that the cold responsive pathway based on CBF TFs is conserved between *Arabidopsis* and the *Triticeae*, and that a set of *COR* effector genes is transactivated by barley Hv*CBFs* (Xue 2002, 2003; Skinner et al. 2005a). In this report we show that there are at least five additional members of the barley CBF family located in this region of the genome (Fig. 1). If they, and not another gene, are demonstrated definitely to be the determinants of the *Fr-H2* QTL, this then raises the question as to whether one specific Hv*CBF* gene, or several Hv*CBFs*, is responsible for this effect in the 'Nure' × 'Tremois' population. Addressing this question will require a larger recombinant mapping population because all of these Hv*CBFs* cosegregate as one linked cluster in the 'N×T' DH mapping population.

Liu et al. (1998) demonstrated that the At*CBFs* were capable of imparting drought tolerance while Haake et al. (2002) showed that the induction of *Arabidopsis CBF4* occurred in response to osmotic stress, and that it too was capable of activating *COR* genes containing a C-repeat element in their promoter. Together these data indicate that the CBF TFs also play an important role in drought tolerance. Hv*CBF8* together with Hv*ABI5* maps to the vicinity of a QTL that controls osmotic potential. Nevertheless, we consider the latter CG to be a better positional candidate for this QTL because of its expression behaviour (see below).

Since At*FRY1* and At*ICE1* are regulators of *CBF* genes (Xiong et al. 2001; Chinnusamy et al. 2003), we wanted to test whether allelic variation at a regulator of a *CBF*, instead of at one/more *CBF* itself, could be responsible for barley tolerance to cold. The two barley orthologs of the above genes were mapped to chromosome 7H (Fig. 1), where no QTLs of cold tolerance were positioned. This suggests that allelic variation at these two regulators of *CBF* genes are not important for the trait, at least for the assays used and in the populations used to map the cold tolerance QTLs. Whereas the binding of AtICE1 to the promoter of At*CBF3* is directly involved in expression induction (Chinnusamy et al. 2003), At*FRY1* takes part in an early step of stress and ABA responses, regulating the turnover of the second messenger IP_3. This attenuation mechanism is critical to maintain resistance to abiotic stresses since *fry1* (loss of function) mutant plants seem to be compromised in tolerance to freezing, drought, and salt stresses (Xiong et al. 2001). In view of this, even if no QTLs for frost tolerance co-mapped with the putative barley At*FRY1* ortholog, *TC147474* might be a positional CG for the drought tolerance QTL located on chromosome 7H (Teulat et al. 2001).

Other positional CGs

No other obvious positional candidates for cold tolerance were identified among the regulatory CGs mapped. While the peak marker for *Fr-H1* on 5H was Hv*BM5A*,

the MADS-box gene controlling flowering after wintering (Fig. 1: *VRN-H1*; von Zitzewitz et al. 2005), it is still under debate as to whether it has a direct role in controlling cold tolerance. On one hand, Roberts (1990) and Limin and Fowler (2002) have postulated that 'winter' alleles at the *VRN-H1* locus of barley and at the *VRN-A1* locus of wheat would have a pleiotropic effect on the frost tolerance of cereals. On the other, it was demonstrated in wheat that *Fr-A1* and *VRN-A1* were distinct loci by linkage and physical mapping (Galiba et al. 1995; Sutka et al. 1999).

Osmotic adjustment (OA), relative water content (RWC), and carbon isotope discrimination (Δ) are different parameters used to measure plant reaction to drought conditions (Farquhar and Richards 1984; Schonfeld et al. 1988; Blum 1989), and it should be emphasised that 'drought tolerance' is too complex a trait to be represented by a simple measure. In barley, QTL analyses for these traits/parameters were performed by Teulat et al. (1998, 2001, 2002, 2003) and by Diab et al. (2004) in the segregating population deriving from the 'Tadmor' (drought tolerant) × 'Er/Apm' (susceptible) cross. For the above reason, only those loci consistently confirmed by multiple independent experiments were reported on our consensus function map (Fig. 1).

Hv*MYB4* (Wissenbach et al. 1993) maps on the long arm of chromosome 2H in a region where multiple QTLs for drought-related traits—i.e. RWC, OA, and water soluble carbohydrates—were discovered by Teulat et al. (2002) and Diab et al. (2004). The general involvement of MYB TFs in the induction of drought-responsive genes was demonstrated by At*Myb2* overexpression in transgenic plants, resulting in an improved osmotic stress tolerance (Abe et al. 2003). Although no data on Hv*MYB4* participation in stress response are available to date, the rice orthologous gene Os*MYB4* encodes for an MYB TF induced during exposure to low temperatures and other abiotic stresses (Vannini et al. 2004). Further experiments will be required to prove that Hv*MYB4* is a valid CG also for the control of barley drought tolerance.

Hv*ABI5* maps on the long arm of chromosome 5H, together with two overlapping drought tolerance QTLs controlling osmotic potential (Teulat et al. 2001). HvABI5 is a bZIP TF up-regulated by ABA and is responsible for ABA-dependent induction of the barley effector genes *HVA1* and *HVA22* (Casaretto and Ho 2003). It has also been shown that constitutive expression of *HVA1*, which encodes a group 3 LEA protein, can confer dehydration tolerance to transgenic rice plants (Chandra Babu et al. 2004). On chromosome 7H, an association exists between the two drought tolerance QTLs and the *TC147474(FRY1)* and *TC143232(ICE1)* CGs.

Effector genes were considered as less probable CGs for explaining the tolerance to the two abiotic stresses. Nevertheless, among the 24 assigned to the consensus map, only some dehydrins (Close 1996; Ismail et al. 1999) out of the 11 mapped could be considered as positional candidates. A cluster of 4 *Dhn* genes is located under a QTL for RWC and for OA on chromosome 6H, as already noted for *Dhn4* and *Dhn5* by Teulat et al. (2003), and the *Dhn9* gene maps to another drought QTL on the long arm of chromosome 5H (Fig. 1). Besides these, the locus containing the *Dhn1* and *Dhn2* (and *aba2*) genes is located under the cold tolerance QTL *Fr-H1* (Fig. 1).

These results represent a good example of the CG approach application for the dissection of complex phenotypes such as abiotic stress tolerances. Since a QTL is a region in the genome which exerts a major measurable effect corresponding to the trait of interest and could cover several genes, complementary validation experiments should be conducted to confirm the actual involvement of a co-segregating CG in the trait variation, including association mapping and expression studies (Pflieger et al. 2001), but above all, genetic transformation. A validated CG could then represent a very efficient molecular marker for MAS applications, as it should show no recombination with the QTL of interest. In the case of regulatory CGs, in fact, the integration of favourable alleles into a genotype should have the consequence of activating a cascade of molecular responses, resulting in a major effect on the phenotype. This MAS strategy would lead to improvement of barley as well as of other *Triticeae* species in abiotic stress tolerance and, consequently, in grain yield in limiting conditions.

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