Kobe University Repository : Kernel

PDF issue: 2025-12-04

A major quantitative trait locus for cold-responsive gene expression is linked to frost-resistance gene Fr-A2 in common wheat

Motomura, Yoichi
Kobayashi, Fuminori
Iehisa, Julio C. M.
Takumi, Shigeo

(Citation)
Breeding Science,63(1):58-67

(Issue Date)
2013-03

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90002723

Breeding Science 63: 58–67 (2013)
doi:10.1270/jsbbs.63.58

A major quantitative trait locus for cold-responsive gene expression is linked to frost-resistance gene *Fr-A2* in common wheat

Yoichi Motomura[1)], Fuminori Kobayashi[2)], Julio C. M. Iehisa[1)] and Shigeo Takumi*[1)]

[1)] *Graduate School of Agricultural Science, Kobe University*, 1-1 Rokkodai, Nada, Kobe, Hyogo 657-8501, Japan
[2)] *Plant Genome Research Unit, National Institute of Agrobiological Sciences*, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

Low temperature induces expression of *Cor* (cold-responsive)/*Lea* (late embryogenesis-abundant) gene family members through C-repeat binding factor (CBF) transcription factors in common wheat. However, the relationship between the genetic loci controlling cold-responsive gene expression and freezing tolerance is unclear. In expression quantitative trait locus (eQTL) analysis, accumulated transcripts of *Cor*/*Lea* and *CBF* genes were quantified in recombinant inbred lines derived from a cross between two common wheat cultivars with different levels of freezing tolerance. Four eQTLs controlling five cold-responsive genes were found, and the major eQTL with the greatest effect was located on the long arm of chromosome 5A. At least the 1D and 5A eQTLs played important roles in development of freezing tolerance in common wheat. The chromosomal location of the 5A eQTL, controlling four cold-responsive genes, coincided with a region homoeologous to a frost-tolerance locus (*Fr-A^{m}2*) reported as a *CBF* cluster region in einkorn wheat. The 5A eQTL plays a significant role through *Cor*/*Lea* gene expression in cold acclimation of wheat. In addition, our results suggest that one or more *CBF* copies at the *Fr-2* region positively regulate other copies, which might amplify the positive effects of the *CBF* cluster on downstream *Cor*/*Lea* gene activation.

Key Words: *Triticum aestivum* L., cold acclimation, freezing tolerance, recombinant inbred lines, transcript accumulation.

Introduction

Natural genetic variations provide useful traits for crop breeding. Diversity at the molecular level usually far exceeds phenotypic variation, described as phenotypic buffering or robustness (Delker and Quint 2011, Fu *et al.* 2009). Expression-level polymorphisms, which are natural variations in gene expression between accessions, are considered more sensitive to molecular diversity than phenotypic differences. Quantitative trait loci (QTLs) controlling differences in transcript accumulation are generally called expression QTLs (eQTLs) (Hansen *et al.* 2008). Most expression level polymorphisms are regulated in *trans* or in *cis* by multiple eQTLs (Delker and Quint 2011). Mapping of eQTLs is an efficient approach to identify genetic loci controlling complex crop traits such as seed development and disease resistance (Chen *et al.* 2010a, 2010b, Jordan *et al.* 2007).

Freezing tolerance, one of these complex traits, is acquired through the cold acclimation process in many overwintering plants from temperate regions (Skinner 2009, Thomashow 1999). A large number of genes with various functions are induced during cold acclimation (Laudencia-Chingcuanco *et al.* 2011, Rabbani *et al.* 2003, Seki *et al.* 2002). In particular, cold-responsive (*Cor*)/late-embryogenesis-abundant (*Lea*) genes are transcriptionally activated in cold acclimation, and the accumulated COR/LEA proteins lead to protection of the integrity of cell structures and functions from freezing damage (Kosová *et al.* 2010, Thomashow 1999). Most *Cor*/*Lea* genes, including *Wlt10*, *Wdhn13* and *Wcor14*, show differential expression levels in two wheat cultivars with contrasting levels of freezing tolerance under low temperature conditions (Kobayashi *et al.* 2004, Laudencia-Chingcuanco *et al.* 2011, Ohno *et al.* 2001, 2003, Tsvetanov *et al.* 2000). A functional *cis*-acting element, i.e., the CCGAC core motif known as a CRT (C-repeat)/DRE (dehydration-responsive element) sequence, is involved in cold-responsive transcriptional activation of the wheat *Cor*/*Lea* genes as well as *Arabidopsis COR15A*/*RD29A* (Baker *et al.* 1994, Kobayashi *et al.* 2008a, Takumi *et al.* 2003, Yamaguchi-Shinozaki and Shinozaki 1994). A family of transcription factors called CRT-binding factors (CBFs) or DRE-binding proteins (DREBs) regulates *Cor*/*Lea* gene expression through binding CRT/DRE elements in both *Arabidopsis* and wheat (Kobayashi *et al.* 2008a, Takumi *et al.* 2008, Thomashow 1999). Overexpression of the wheat CBF/DERB transcription factors increases freezing tolerance of transgenic plants (Kobayashi *et al.* 2008a, Morran *et al.* 2011, Takumi *et al.* 2008). The CBF regulon controls one of the important regulatory pathways in development of freezing tolerance in wheat (Winfield *et al.* 2010).

Communicated by H. Tsujimoto
Received September 11, 2012. Accepted October 27, 2012.
*Corresponding author (e-mail: takumi@kobe-u.ac.jp)

The wheat frost-resistance QTLs that are the most significant for freezing tolerance are *Fr-1*, which map to the long arm of chromosomes 5A and 5D (Galiba *et al.* 1995, Snape *et al.* 1997). *Fr-1* loci affect the expression of several *CBF* genes located in the *Fr-2* region (Kobayashi *et al.* 2005, Vágújfalvi *et al.* 2005). The map position of the wheat *CBF* gene cluster and an eQTL of *Cor14b* correspond to the *Fr-A^{m}2* locus on chromosome 5A of einkorn wheat, *Triticum monococcum* (Miller *et al.* 2006, Vágújfalvi *et al.* 2003). Similarly, in barley, *Hordeum vulgare*, multiple *CBF* copies constitute a gene cluster around a frost tolerance QTL, *Fr-H2*, on chromosome 5H (Francia *et al.* 2007, Fricano *et al.* 2009, Skinner *et al.* 2005, 2006). In the clustered *CBF* copies, *TmCBF12* and *HvCbf14* are candidates for wheat *Fr-A^{m}2* and barley *Fr-H2*, respectively (Fricano *et al.* 2009, Knox *et al.* 2008). In common wheat, *Triticum aestivum* L., chromosome 5A regulates freezing tolerance (Kocsy *et al.* 2010) and two loci on chromosome 5A determine the cultivar difference in cold-responsive expression of *Cor14b* (Vágújfalvi *et al.* 2000). QTLs involved in freezing tolerance were recently analyzed using 107 doubled haploid (DH) lines between Norster and Winter Manitou; a major QTL was found on chromosome 5A and weaker QTLs on chromosome 1D (Båga *et al.* 2007). At the 5A QTL, two *CBF* genes, *Cbf14* and *Cbf15*, co-localize, indicating that the major QTL on chromosome 5A coincides with *Fr-A2* (Båga *et al.* 2007). However, the relationship between the *Fr* loci and cold-responsive gene expression patterns remains unclear.

Two common wheat cultivars, *T. aestivum* 'Chinese Spring' (CS) and 'Mironovskaya 808' (M808), show differences in freezing tolerance; M808 is much more tolerant to freezing stress than CS (Kume *et al.* 2005, Ohno *et al.* 2001). In addition, a correlation between *Cor*/*Lea* gene expression patterns and freezing tolerance has been reported in the two cultivars (Kobayashi *et al.* 2004, Ohno *et al.* 2001, Takumi *et al.* 2003). Recently, a genetic linkage map was constructed using recombinant inbred lines (RILs) obtained from a cross between M808 and CS (Kobayashi *et al.* 2010). Here, we attempted to map eQTLs for wheat cold-responsive gene expression using the M808/CS RIL map and discussed the relationship between the identified eQTLs and freezing tolerance.

Materials and Methods

Plant materials

Two common wheat cultivars, M808 and CS, were used as parental accessions for the mapping population. The mapping population of 210 RILs was established at the F_7 generation by the single-seed descent method from an F_2 family derived from M808 and CS (Kobayashi *et al.* 2010). M808, reported to be one of the hardiest wheat cultivars (Veisz and Sutka 1990), was bred in the Mironovska Institute, Ukraine. Therefore, CS and M808 were used as freezing-sensitive and -tolerant cultivars, respectively (Ohno *et al.* 2001).

For seed germination, seeds from each line were imbibed in tap water for 5 h and kept overnight at 4°C. Imbibed seeds were placed in glass Petri dishes (90 mm in diameter and 20 mm in depth) containing a filter paper (82 mm diameter) wetted with distilled water, then incubated for 24 h at 20°C in darkness. Synchronously germinated seeds were transferred to pots containing soil and incubated at 23°C under short-day conditions (12/12 h light/darkness).

Quantitative RT-PCR analysis

To analyze gene expression patterns, 7-d-old seedlings were transferred to 4°C and grown for various time periods. Total RNA was extracted from leaves using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). First-strand cDNA was synthesized from DNase I-treated mRNA samples with oligo-dT primers using the high-fidelity ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan).

The transcript accumulation of each gene was detected by quantitative RT-PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) with the following gene-specific primer sets: 5′-CAGAGCC TCCCAGTTAGCAATG-3′ and 5′-CAGACGCTCATCAA GGAAGGAA-3′ for *Wlt10*, 5′-GGCGAGAAGAAGGGCG TCAT-3′ and 5′-GTGGTGGCTGTGGTGGCAT-3′ for *Wdhn13*, 5′-TTCTTCTTCCGTGCTGCTCG-3′ and 5′-TTTGCTCACATCCTCGACCG-3′ for *Wcor14*, 5′-AGTGGGTGTGCGGAGGTGC-3′ and 5′-GTCGGCGAA GTTGAGGCAG-3′ for *WCBF2*, 5′-AGTGGGTGTGCGA GGTGCG-3′ and 5′-GTCGGCGAAGTTGAGGCAG-3′ for *TaCBF12*, 5′-CATACGCCCTCACCAGTTT-3′ and 5′-CTCCGTCTCGCCACTACAG-3′ for *TaCBF15* and 5′-GGCTGGTTTTGCTGGTGACGAT-3′ and 5′-AATGAAG GAAGGCTGGAAGA-3′ for *Actin*. The *Actin* gene was used as an internal control. The rate of amplification was monitored using THUNDERBIRD SYBR qPCR mix (Toyobo) according to the manufacturer's protocol. The relative expression level was calculated as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in number of PCR cycles required to reach the log phase of amplification of the target gene relative to *Actin*; representative values were expressed relative to the transcript levels in CS samples obtained at 0 h.

QTL mapping

A linkage map of M808 and CS was previously constructed using 210 RILs (Kobayashi *et al.* 2010). The linkage map currently shows 410 loci of simple sequence repeat (SSR) markers and the total map length is 2,814.5 cM with an average spacing of 6.9 cM between markers. QTL analysis was carried out by composite interval mapping using Windows QTL Cartographer ver. 2.5 software (Wang *et al.* 2007) with the forward and backward method. An log-likelihood (LOD) score threshold of 2.5 was determined by computing 1,000 permutations. The percentage of phenotypic variation explained by a QTL for a trait and any additive effect were also estimated using the software.

Bioassay conditions for freezing tolerance

To assay freezing tolerance, 7-d-old seedlings grown at

23°C were frozen at −10 ± 1°C for 6 h in the dark after 4°C treatment for 5 days under short-day conditions (12/12 h light/darkness). More than 20 frozen seedlings for each line were thawed overnight at 4°C and transferred back to 23°C conditions. At 2 weeks after transfer, the number of surviving seedlings was recorded. The experiment was performed 3–5 times and the data were statistically analyzed by Student's *t*-test.

Results

Time-course expression analysis of wheat cold-responsive genes

Expression patterns of six cold-responsive genes, including three CBF transcription factor genes and three *Cor*/*Lea* genes, were quantitatively compared between wheat cultivars M808 and CS. Transcripts of two transcription factor genes, *WCBF2* and *TaCBF12*, were detected at low levels under unstressed conditions, and their levels increased within 3 h after exposure of wheat seedlings to low temperature (Fig. 1). The transcript levels reached a high plateau by 3 h or 6 h and then gradually decreased over 24 h in both M808 and CS. The transcript levels of *WCBF2* and *TaCBF12* were higher in M808 than in CS until 24 h of low-temperature treatment. The transient increase of *TaCBF15* transcripts was similarly observed only in M808 but not in CS. Transcripts of *Wlt10*, *Wdhn13* and *Wcor14* were observed at low levels under non-stress conditions, and their levels gradually increased in M808 and CS until 24 h or 72 h of low temperature treatment. Transcript accumulation levels of the *Cor*/*Lea* genes were higher in M808 than in CS. Significant differences ($P < 0.05$) of the transcript levels between M808 and CS were respectively observed 3 h and 72 h after exposure to low temperature in the three *CBF* genes and three *Cor*/*Lea* genes.

Identification of QTLs controlling cold-responsive gene expression

Total RNA was isolated from seedling leaves of 210 lines of the M808 × CS RIL population 3 h and 72 h after exposure to low temperature. Levels of accumulated transcripts of the three CBF transcription factor genes and the three *Cor*/*Lea* genes were respectively using RNA samples of seedlings treated at low temperature for 3 h and 72 h, respectively. The RILs showed a continuous distribution of estimated values for the six cold-responsive genes (Fig. 2). Correlation coefficients among the estimated values were compared (Table 1). Significantly positive correlations were observed among the expression levels of the three CBF transcription factor genes. Significant correlations were also detected between the *Wlt10* transcript levels and two other *Cor*/*Lea* transcript levels, whereas no correlation was found between the *Wcor14* and *Wdhn13* transcript levels. The transcript level of *TaCBF15* showed significantly positive correlations with the three *Cor*/*Lea* transcript levels and the *WCBF2* and *TaCBF12* transcript levels were significantly correlated to those of *Wlt10* and *Wdhn13*.

Using the genetic map between M808 and CS (Kobayashi *et al.* 2010), eQTLs for four of the five cold-

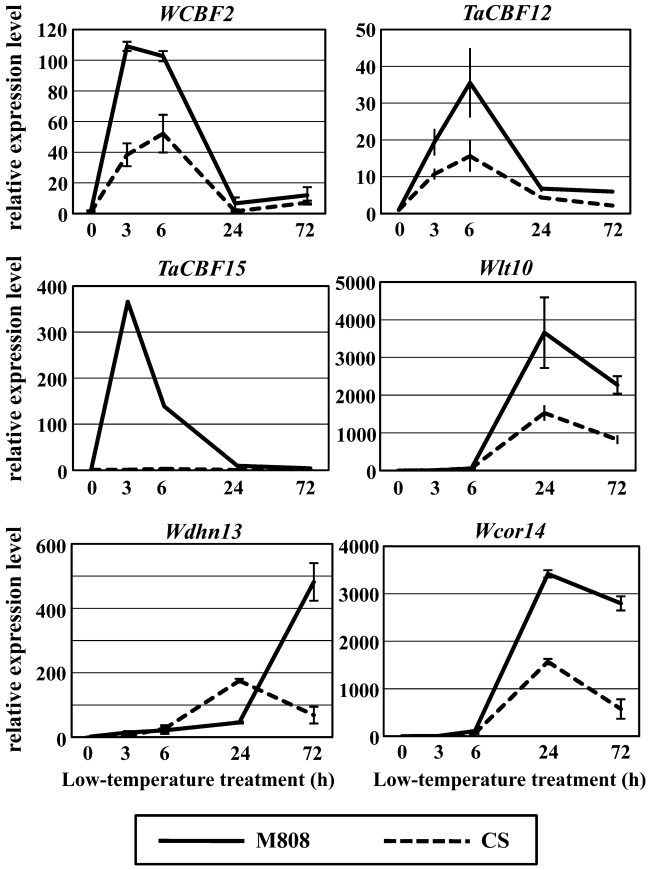


Fig. 1. Expression profiles of six cold-responsive genes during cold acclimation in leaves of M808 and CS as revealed by real-time RT-PCR analysis. Total RNA was extracted from seedling leaves under low-temperature conditions. *Actin* was used as internal control. Each transcript level is represented as the value relative to that of the CS level at 0 h; values are means with standard deviation.

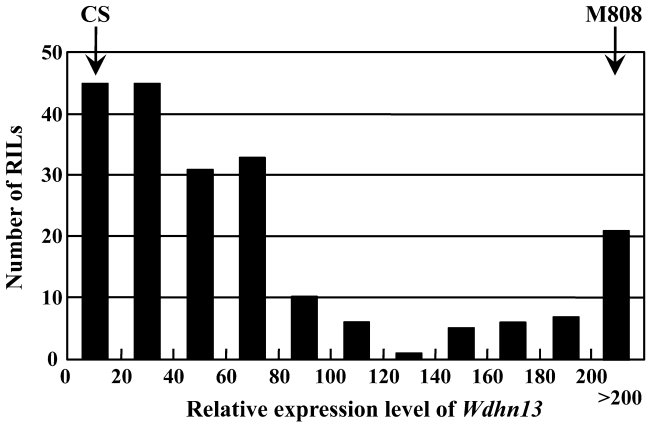


Fig. 2. Frequency distribution of the number of RILs at each relative accumulation level of the *Wdhn13* transcript. Each transcript level is represented as the value relative to that of the CS level at 72 h.

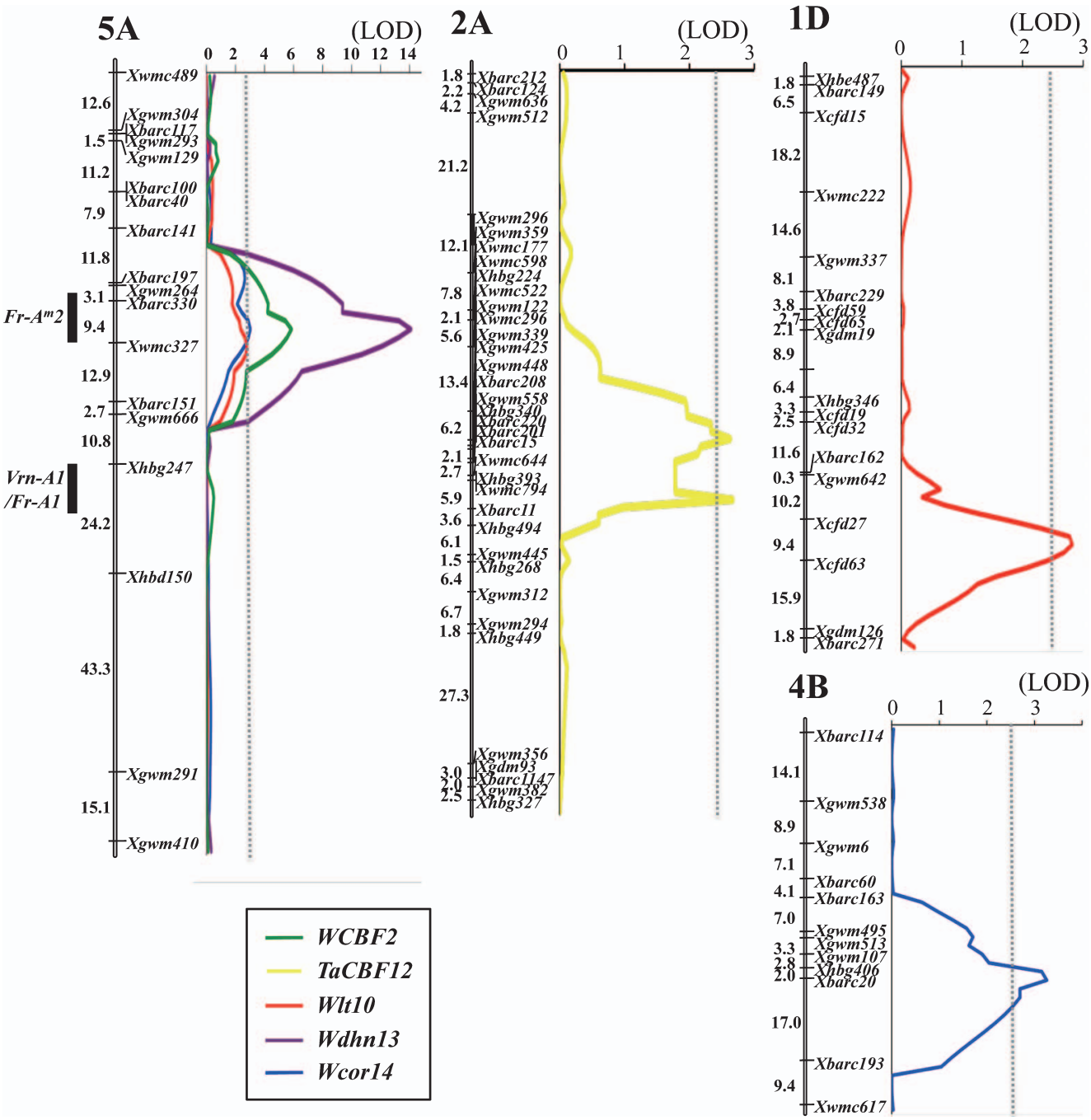


Fig. 3. Linkage maps and QTL-likelihood curves of LOD scores based on transcript accumulation of the five cold-responsive genes on chromosomes 1D, 2A, 4B and 5A. Genetic distances are represented in centimorgans on the left of each chromosome. The 2.5 LOD score threshold is indicated by a dashed line.

responsive genes (excluding *TaCBF15*) were detected based on transcript accumulation data for the 210 RILs. For the five cold-responsive genes found on chromosomes 1D, 2A, 4B and 5A, seven eQTLs showed significant LOD scores >2.5 ($P < 0.05$) (Fig. 3). An eQTL for *WCBF2* was found on chromosome 5A and an eQTL for *TaCBF12* was found on chromosome 2A. Three eQTLs for the three *Cor*/*Lea* genes were assigned to a similar chromosomal position on chromosome 5A. Chromosomes 1D and 4B included a single eQTL for *Wlt10* and *Wcor14*, respectively. A major eQTL with an LOD score of 14.1 was located on the long arm of chromosome 5A and contributed 24.5% of the variation in the *Wdhn13* transcript accumulation levels (Table 2). eQTLs at a similar position on chromosome 5A explained 5.6, 6.4 and 10.9% of the variation in the *Wlt10*, *Wcor14* and *WCBF2* transcript levels, respectively. The SSR markers *Xbarc330* and *Xwmc327* flanked these eQTLs at 9.4 cM intervals (Fig. 3). Other eQTLs on chromosomes 1D, 2A

Table 1. Correlation coefficients (R^2 values) among transcript accumulation of six cold-responsive genes in RILs of M808 and CS

	WCBF2	*TaCBF12*	*TaCBF15*	*Wlt10*	*Wdhn13*
TaCBF12	0.07221***				
TaCBF15	0.10777***	0.12063***			
Wlt10	0.02497*	0.05427***	0.03022*		
Wdhn13	0.06276***	0.03060*	0.02921*	0.03047*	
Wcor14	0.01183	0.00990	0.02520*	0.45736***	0.00696

The Pearson coefficient values were calculated based on the relative values of the transcript accumulation levels in the RILs. Significant correlations are indicated by asterisks (* $P < 0.05$, *** $P < 0.001$).

Table 2. Characteristics of identified eQTLs for five cold-responsive genes of common wheat

Gene	Chromosome	Vicinity marker	Position (cM)	LOD	Additive effect	Contribution (%)
WCBF2	5A	*Xbarc330*	49.8	5.86	−581	10.9
TaCBF12	2A	*Xwmc644*	83.6	2.67	2.39	5.6
TaCBF12	2A	*Xgwm448*	78.6	2.63	2.1	5.3
Wlt10	5A	*Xbarc330*	49.8	3.03	−5243	5.6
Wlt10	1D	*Xcfd27*	101	2.82	5106	5.3
Wdhn13	5A	*Xbarc330*	49.8	14.1	−51.2	24.5
Wcor14	5A	*Xbarc330*	49.8	2.79	−3467	6.4
Wcor14	4B	*Xhbg406*	48.8	3.26	−3410	6.1

and 4B contributed 5.3 to 6.1% of total variation in cold-responsive gene expression.

The mean values for the transcript levels of RILs carrying the M808 or CS allele at each eQTL showed that RIL groups carrying the M808 allele at the 5A or 4B eQTL accumulated more abundant transcripts of *Wlt10*, *Wdhn13*, *Wcor14* and *WCBF2* than the other RIL groups (Table 3). Similarly, the RIL groups carrying the CS-type 1D or 2A eQTL showed higher accumulation of *Wlt10* and *TaCBF12* transcripts. These results indicated that the M808 alleles at eQTLs on chromosomes 4B and 5A and the CS alleles at eQTLs on chromosomes 1D and 2A contributed to abundant accumulation of *CBF* and *Cor*/*Lea* gene transcripts in leaves under low-temperature conditions.

Effects of the identified eQTLs on freezing tolerance and cold-responsive gene expression

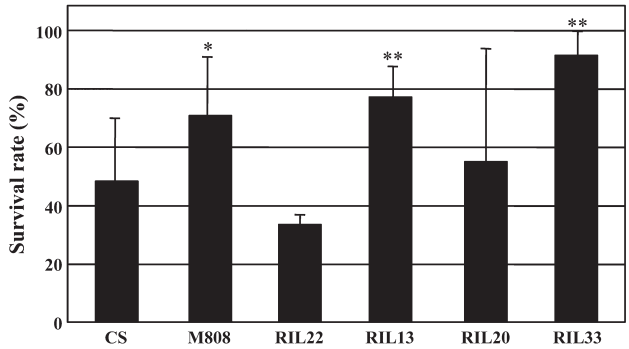
To evaluate the effect of the eQTLs for *Cor*/*Lea* genes on freezing tolerance, four of the 210 RILs were chosen based on their genotypes at the 1D, 4B and 5A eQTLs. RIL13, 20 and 33 were presumed to contain a highly accumulated allele for a *Cor*/*Lea* transcript at each of the three eQTLs (Fig. 4). RIL22 did not seem to contain any highly accumulated allele transcripts at these eQTLs, while M808 appeared to have two alleles with highly abundant transcripts and CS had one. M808, RIL13 and RIL33 showed significantly higher levels of freezing tolerance after cold acclimation than CS, whereas no significant difference in freezing tolerance was observed among CS, RIL20 and RIL22 (Fig. 4). These bioassay results for freezing tolerance indicated that the 1D and 5A eQTLs played important roles in development of freezing tolerance, and the M808 allele of the 5A eQTL contributed to the high freezing tolerance of M808.

Table 3. Differences in the relative expression of five cold-responsive genes in RILs with the M808 and CS alleles at the identified eQTLs

Gene	eQTL	Putative allele	Number of RILs	Mean value (Mean ± standard deviation)
WCBF2	5A***	M808	63	1806 ± 2453
		CS	69	492 ± 562
TaCBF12	2A*	M808	102	3.8 ± 4.6
		CS	88	6.1 ± 9.8
	2A	M808	49	3.8 ± 4.2
		CS	42	5.3 ± 7.1
Wlt10	5A	M808	63	24780 ± 24364
		CS	69	17743 ± 18249
	1D*	M808	81	18017 ± 18398
		CS	84	26486 ± 26409
Wdhn13	5A***	M808	63	130 ± 125
		CS	69	36 ± 36
Wcor14	5A*	M808	63	13734 ± 15285
		CS	69	8629 ± 9336
	4B*	M808	94	13520 ± 16266
		CS	105	8539 ± 10455

Student's *t*-test was used for statistical significance of the allelic difference (* $P < 0.05$, *** $P < 0.001$).

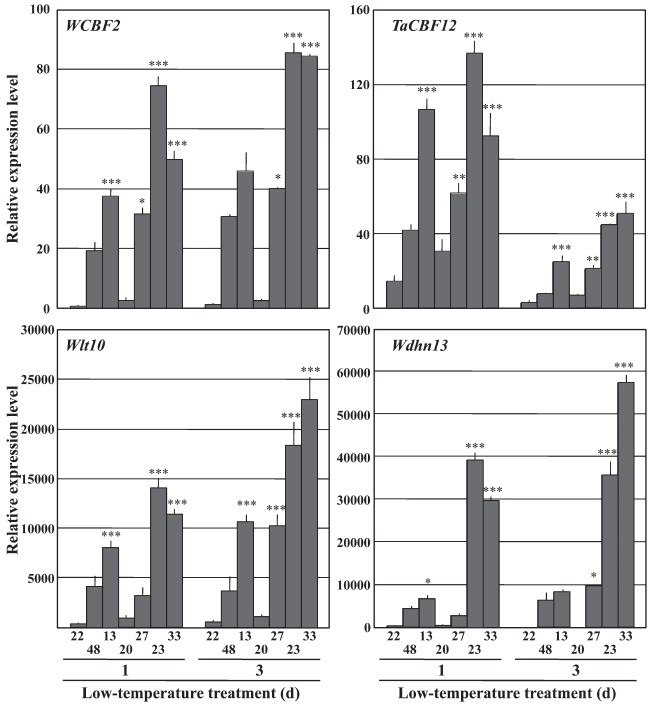
To study the effects of the 1D, 4B and 5A eQTLs on *CBF* and *Cor*/*Lea* gene expression during cold acclimation, seven RILs were chosen based on their genotypes at these eQTLs. The seven RILs were classified by allele type at SSR markers flanking the eQTLs (Fig. 5). Transcript levels of the four cold-responsive genes *WCBF2*, *TaCBF12*, *Wlt10* and *Wdhn13* among the seven RILs were compared by real-time RT-PCR analysis using seedling leaves treated at low



Putative genotype

locus	CS	M808	RIL22	RIL13	RIL20	RIL33
eQTL (1D)	CS	M808	M808	CS	M808	M808
eQTL (4B)	CS	M808	CS	CS	M808	CS
eQTL (5A)	CS	M808	CS	CS	CS	M808

Fig. 4. Freezing tolerance levels of the selected RILs after cold acclimation. Survival rates after −10°C treatment for 6 h are represented as means with standard deviations from three to five independent experiments. Postulated genotypes at the three eQTLs in the four RILs and parental cultivars are shown below the graph and alleles with positive effects are shaded. Student's *t* test was used to test for statistical significance (* $P < 0.05$, ** $P < 0.01$) compared with CS.



RILs	Putative genotype at the QTL region		
	1D	4B	5A
22, 48	M808	CS	CS
13	CS	CS	CS
20, 27	M808	M808	CS
23, 33	M808	CS	M808

Fig. 5. Expression of four cold-responsive genes in the selected RILs after 1 d and 3 d of low-temperature treatment. Transcript accumulation in the seven RILs was quantified as mean values with standard deviations relative to the *Actin* transcript by real-time RT-PCR. Putative genotypes at the three eQTLs in the seven RILs are shown below the graph and alleles with positive effects are shaded. Student's *t* test was used to test for statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) compared with RIL22.

temperature for 0 h, 3 h, 6 h, 1 d and 3 d. No obvious differences were observed in the gene expression levels among the RILs under untreated conditions (data not shown). At 1 and 3 d after low-temperature treatment, all four genes showed clear differences among lines in their expression levels (Fig. 5). Expression of the four cold-responsive genes was significantly higher in the RILs carrying the M808 allele at the eQTL on chromosome 5A (RIL23 and RIL33) than in the others. In RIL13, carrying the CS allele at the 1D eQTL, *WCBF2*, *TaCBF12* and *Wlt10* showed significantly higher expression after 1 and 3 d of low-temperature treatment than RIL22 and RIL48, with no alleles corresponding to high transcript accumulation at the three eQTLs. The *Wdhn13* expression level after 1 d of cold treatment was also significantly higher in RIL13 than in RIL22. The levels of expression of the four genes in RIL27 with the M808 allele at the 4B eQTL were significantly higher than in RIL22 after 3 d of low-temperature treatment, whereas no significant differences were detected in their expression in RIL20, which carried the same allele at the 4B eQTL. Thus, the 4B eQTL did not necessarily contribute to expression of the cold-responsive genes except for *Wcor14*. Thus, both the M808 allele at the 5A eQTL and the CS allele at the 1D eQTL contributed to the high expression levels of the cold-responsive genes in seedling leaves during cold acclimation. The effect on transcript accumulation was higher for the M808 allele at the 5A eQTL than for the CS allele at the 1D eQTL.

Discussion

Freezing tolerance is one of the most important traits for wheat breeding. We identified wheat loci controlling cold-responsive genes during cold acclimation on four wheat chromosomes, 1D, 2A, 4B and 5A, using real-time RT-PCR analysis. Previous reports showed that M808, one of the hardiest wheat cultivars (Veisz and Sutka 1990), exhibits a clear contrast with CS in level of freezing tolerance after cold acclimation (Kobayashi *et al.* 2004, Kume *et al.* 2005, Ohno *et al.* 2001). During cold acclimation, transcripts of several *Cor*/*Lea* genes accumulated more abundantly in seedling leaves of M808 than CS. However, our eQTL analysis showed that CS alleles on 1D and 2A contributed to abundant accumulation of cold-responsive gene transcripts in the leaves under low-temperature conditions. Cold induction of *Cor*/*Lea* gene expression is directly regulated by transcription factor genes including *CBF*s, *WDREB2*, *Wabi5* and *Wlip19* (Egawa *et al.* 2006, Kobayashi *et al.* 2008a, 2008b,

2008c, Kume *et al.* 2005, Takumi *et al.* 2008). *WDREB2* and *Wlip19* have been assigned to homoeologous group 1 chromosomes (Egawa *et al.* 2006, Kobayashi *et al.* 2008c) and *Wabi5* to homoeologous group 5 chromosomes in hexaploid wheat genomes (Kobayashi *et al.* 2008b). In barley, a hypothetical gene order has been proposed for each of the seven chromosomes based on conserved synteny among barley, *Brachypodium*, rice and sorghum (Mayer *et al.* 2011). Due to the conserved synteny between wheat and barley (Carollo *et al.* 2005), chromosomal localization of wheat genes can be assumed *in silico*. This so-called genome zipper analysis revealed the putative location of *WDREB2*, for which the barley ortholog is *HvDRF1* (AK249060.1) (Egawa *et al.* 2006), at a similar position to *Xcfd27* on the long arm of chromosome 1D (Supplemental Fig. 1). Thus, the chromosomal position of *WDREB2* seems to be proximal to the 1D eQTL. The barley *Wlip19* ortholog is NIASHv1140N16 in the full-length cDNA database (Matsumoto *et al.* 2011) and *Wlip19* was putatively assigned to the short arm of chromosome 1D (Supplemental Fig. 1). Since *Wlt10* was assigned to homoeologous group 2 chromosomes (Ohno *et al.* 2001), another unknown gene on chromosome 1D might act in *trans* as a positive regulator of the downstream *Cor*/*Lea* genes. The chromosomal location of the 1D eQTL we identified seems to coincide with a freezing tolerance QTL on chromosome 1D identified using the DH lines of two winter wheat cultivars (Båga *et al.* 2007). These results suggested that the 1D QTL might act as a positive regulator of the *Cor*/*Lea* gene expression during cold acclimation in common wheat.

The 5A eQTL identified in the present study showed large effects on the cold-responsive gene expression, particularly on the *Wdhn13* transcript accumulation, and contributed to development of freezing tolerance during cold acclimation, implying that the eQTL on chromosome 5AL plays a significant role through the *Cor*/*Lea* gene expression in wheat cold acclimation. The chromosomal region of the 5A eQTL corresponded to that of *Fr-A^{m}2* (Fig. 3), which was identified as a frost-tolerance locus in einkorn wheat (Vágújfalvi *et al.* 2003). In addition, the 5A eQTL location coincided with a freezing tolerance QTL on chromosome 5A previously reported using doubled haploid lines of two winter wheat cultivars (Båga *et al.* 2007). Genome zipper analysis showed that *Wabi5* is proximal to the *CBF* cluster on chromosome 5AL, indicating distinct localization to the 5A eQTL (Supplemental Fig. 2). In einkorn wheat, two loci on chromosome 5A are associated with *Cor14b* expression (Vágújfalvi *et al.* 2000); one is closely linked to *Fr-A1* and the other to the RFLP marker *Xpsr911* 35 cM proximal to the *Fr-A1* locus. *Fr-A1* (formerly *Fr1*) is a major QTL for frost and freezing tolerance in common wheat and is located near *Vrn-A1* (Galiba *et al.* 1995, Sutka and Snape 1989). Until now, *Fr-A1* has been considered a single locus related to freezing tolerance on chromosome 5A of common wheat (Sutka 2001), although *Fr-D1* (formerly *Fr2*) on chromosome 5D explains the cultivar difference in frost tolerance between CS and Cheyenne (Snape *et al.* 1997, Sutka 2001). Our eQTL study showed that the 5A eQTL are tightly linked to the homoeologous locus (*Fr-A2*) of *Fr-A^{m}2* but not to *Fr-A1*/*Vrn-A1* (Fig. 3), strongly suggesting that the cultivar difference in freezing tolerance after cold acclimation is generated by allelic differences at the *Fr-A2* locus between M808 and CS. Therefore, *Fr-A2* is one of two loci involved in freezing tolerance on chromosome 5A of both common wheat and einkorn wheat.

In einkorn wheat and barley, a *CBF* gene cluster with more than 10 copies was located on the *Fr-A^{m}2* and *Fr-H2* regions, respectively (Francia *et al.* 2007, Miller *et al.* 2006, Skinner *et al.* 2005, Vágújfalvi *et al.* 2003). Einkorn wheat orthologs of *TaCBF12* and *TaCBF15* are localized at the *Fr-A^{m}2* chromosomal region (Miller *et al.* 2006). Three homoeologous copies of *WCBF2*, reported as *TaCBFIVb-A20*, *TaCBFIVb-B20* and *TaCBFIVb-D20*, were found in the *Fr-2* regions of common wheat (Badawi *et al.* 2007). The 5A eQTL identified in the present study regulated not only the *Cor*/*Lea* genes in *trans* but also the *CBF* copies in *cis*. It was also reported that allelic difference of *Fr-H2* affects the expression levels of *CBF* genes at *Fr-H2* in barley (Stockinger *et al.* 2007). These results suggest that one or some of the *CBF* copies at the *Fr-2* region positively regulate other copies, which might amplify the positive effects of the *CBF* cluster on the downstream *Cor*/*Lea* gene activation. In our eQTL analysis, homoeologous copies of the cold-responsive genes were examined without any distinction, and thus sum of transcripts of three homoeologs in the A, B and D genomes was used for the eQTL identification. It was possible that the amplification effects at the 5A eQTL might affect other *CBF* copies around the homoeologous *Fr-B2* and *Fr-D2* regions as well as *Fr-A2* in the hexaploid wheat genomes.

M808 is a winter-type wheat cultivar, which means it contains recessive alleles at all three *Vrn-1* loci on homoeologous group 5 chromosomes. On the other hand, CS carries a dominant allele at *Vrn-D1* and recessive alleles at *Vrn-A1* and *Vrn-B1* (Fu *et al.* 2005). *Fr-1* is either tightly linked to *Vrn-1* or is tightly associated with the pleiotropic effects of *Vrn-1*; chromosomal relationship between *Fr-1* and *Vrn-1* is unknown in wheat and barley (Casao *et al.* 2011, Galiba *et al.* 1995, 2009, Snape *et al.* 1997). The winter-type allele of the *Fr-A1*/*Vrn-A1* region shows both a frost-tolerant and vernalization-requiring phenotype and allelic linkage at *Fr-A1* and *Vrn-A1* guarantees winter survival in winter-type wheat (Galiba *et al.* 2009, Kobayashi *et al.* 2005, Sutka 2001, Thomashow 1999). Thus, M808 and CS are considered to carry the same freezing-tolerant allele at the *Fr-A1* locus, which may have resulted in our failure to detect any eQTL at the *Fr-A1* region. In doubled haploid lines from a cross between two winter wheat cultivars with all three recessive *vrn-1* alleles, only *Fr-A2* was detected as a major freezing tolerance QTL on chromosome 5A (Båga *et al.* 2007). On the other hand, an allelic difference between M808 and CS is present at *Vrn-D1*. If the phenotypes of *Vrn-*

D1 and *Fr-D1* are due to pleiotropic effects of the same gene, an eQTL for cold-responsive genes should be observed at the *Vrn-D1*/*Fr-D1* region. However, we found no eQTL on chromosome 5D. This result supported the alternative hypothesis that *Fr-1* is independent but tightly linked to *Vrn-1*. Our previous study using Japanese wheat cultivars showed that allelic linkage between *Fr-1* and *Vrn-1* loci was not observed in the D-genome of common wheat (Ishibashi *et al.* 2007). In barley, two QTLs for low-temperature tolerance, *Fr-H1* and *Fr-H2*, were found on the long arm of chromosome 5H (Francia *et al.* 2004) and the *Vrn-H1*/*Fr-H1* genotype affects both the expression of *CBF* genes at *Fr-H2* and low-temperature tolerance (Chen *et al.* 2009, Stockinger *et al.* 2007). Thus, the barley *Vrn-H1*/*Fr-H1* and *Fr-H2* regions function to develop freezing tolerance through *Cor*/*Lea* gene expression during cold acclimation. In contrast to barley, the functions of *Vrn-A1*/*Fr-A1* and *Vrn-D1*/*Fr-D1* in regulation of cold-responsive gene expression remains unclear. Our eQTL analysis of cold-responsive gene expression using RILs between M808 and CS was useful for identification of some freezing tolerance-related loci, including *Fr-A2*. To elucidate the function of *Vrn-A1*/*Fr-A1* in common wheat, mapping populations should be generated from other cross combinations with contrasting alleles of *Vrn-A1*.

eQTL analysis requires marker genes with expression patterns associated with development of specific traits. In wheat breeding, complex traits such as drought stress and *Fusarium* tolerance are important targets. Marker genes with transcript accumulation showing clear cultivar differences after stress treatment should be selected to conduct eQTL analysis for these traits. Our study demonstrates the usefulness of eQTL analysis in identifying genetic loci controlling complex traits in common wheat. The two eQTLs on chromosomes 1D and 5A correspond to QTLs for freezing tolerance (Båga *et al.* 2007), indicating the usefulness of eQTL analysis.

Acknowledgements

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research (B) No. 21380005 and Grant-in-Aid for Challenging Exploratory Research No. 23658010) to ST.

Literature Cited

Badawi, M., J. Danyluk, B. Boucho, M. Houde and F. Sarhan (2007) The *CBF* gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal *CBF*s. Mol. Genet. Genomics 277: 533–554.

Båga, M., S.V. Chodaparambil, A.E. Limin, M. Pecar, D.B. Fowler and R.N. Chibbar (2007) Identification of quantitative trait loci and associated candidate genes for low-temperature tolerance in cold-hardy winter wheat. Funct. Integr. Genomics 7: 53–68.

Baker, S.S., K.S. Wilhelm and M.F. Thomashow (1994) The 5′-region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol. Biol. 24: 701–713.

Carollo, V., D.E. Matthews, G.R. Lazo, T.K. Blake, D.D. Hummel, N. Lui, D.L. Hane and O.D. Anderson (2005) GrainGenes 2.0. An improved resource for the small-grains community. Plant Physiol. 139: 643–651.

Casao, M.C., E. Igartua, I. Karsai, P.R. Bhat, N. Cuadrado, M.P. Gracia, J.M. Lasa and A.M. Casas (2011) Introgression of an intermediate *VRNH1* allele in barley (*Hordeum vulgare* L.) leads to reduced vernalization requirement without affecting freezing tolerance. Mol. Breed. 28: 475–484.

Chen, A., J. Reinheimer, A. Brulé-Babel, U. Baumann, M. Pallotta, G.B. Fincher and N.C. Collins (2009) Genes and traits associated with 2H and 5H regions controlling sensitivity of reproductive tissues to frost in barley. Theor. Appl. Genet. 118: 1465–1476.

Chen, X., C.A. Hackett, R.E. Niks, P.E. Hedley, C. Booth, A. Druka, T.C. Marcel, A. Vels, M. Bayer, I. Milne *et al.* (2010a) An eQTL analysis of partial resistance to *Puccinia hordei* in barley. PLoS ONE 5: e8598.

Chen, X., R.E. Niks, P.E. Hedley, J. Morris, A. Druka, T.C. Marcel, A. Vels and R. Waugh (2010b) Differential gene expression in nearly isogenic lines with QTL for partial resistance to *Puccinia hordei* in barley. BMC Genomics 11: 629.

Delker, C. and M. Quint (2011) Expression level polymorphisms: heritable traits shaping natural variation. Trends Plant Sci. 16: 481–488.

Egawa, C., F. Kobayashi, M. Ishibashi, T. Nakamura, C. Nakamura and S. Takumi (2006) Differential regulation of transcript accumulation and alternative splicing of a *DREB2* homolog under abiotic stress conditions in common wheat. Genes Genet. Syst. 81: 77–91.

Francia, E., F. Rizza, L. Cattivelli, A.M. Stanca, G. Galiba, B. Tóth, P.M. Hayes, J.S. Skinner and N. Pecchioni (2004) Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. Theor. Appl. Genet. 108: 670–680.

Francia, E., D. Barabachi, A. Tondelli, G. Laido, F. Rizza, A.M. Stanca, M. Busconi, C. Fogher, E.J. Stockinger and N. Pecchioni (2007) Fine mapping of a *HvCBF* gene cluster at the frost resistance locus *Fr-H2* in barley. Theor. Appl. Genet. 115: 1083–1091.

Fricano, A., F. Rizza, P. Faccioli, D. Pagani, P. Pavan, A. Stella, L. Rossini, P. Piffanelli and L. Cattivelli (2009) Genetic variants of *HvCbf14* are statistically associated with frost tolerance in a European germplasm collection of *Hordeum vulgare*. Theor. Appl. Genet. 119: 1335–1348.

Fu, D., P. Szücs, L. Yan, M. Helguera, J.S. Skinner, J. von Zitzewitz, P.M. Hayes and J. Dubcovsky (2005) Large deletions within the first intron in *VRN-1* are associated with spring growth habit in barley and wheat. Mol. Genet. Genomics 283: 54–65.

Fu, J., J.J.B. Keurentjes, H. Bouwmeester, T. America, F.W.A. Verstappen, J.L. Ward, M.H. Beale, R.C.H. de Vos, M. Dijkstra, R.A. Scheltema *et al.* (2009) System-wide molecular evidence for phenotypic buffering in *Arabidopsis*. Nat. Genet. 41: 166–167.

Galiba, G., S.A. Quarrie, J. Sutka, A. Morgounov and J.W. Snape (1995) RFLP mapping of the vernalization (*Vrn1*) and frost resistance (*Fr1*) genes on chromosome 5A of wheat. Theor. Appl. Genet. 90: 1174–1179.

Galiba, G., A. Vágújfalvi, C. Li, A. Soltész and J. Dubcovsky (2009) Regulatory genes involved in the determination of frost tolerance in temperate cereals. Plant Sci. 176: 12–19.

Hansen, B.G., B.A. Halkier and D.J. Kliebenstein (2008) Identifying the molecular basis of QTLs: eQTLs add a new dimension. Trends

Plant Sci. 13: 72–77.

Ishibashi, M., F. Kobayashi, J. Nakamura, K. Murai and S. Takumi (2007) Variation of freezing tolerance, *Cor*/*Lea* gene expression and vernalization requirement in Japanese common wheat. Plant Breed. 126: 464–469.

Jordan, M.C., D.J. Somers and T.W. Banks (2007) Identifying regions of the wheat genome controlling seed development by mapping expression quantitative trait loci. Plant Biotechnol. J. 5: 442–453.

Knox, A.K., L. Chengxia, A. Vágújfalvi, G. Galiba, E.J. Stockinger and J. Dubcovsky (2008) Identification of candidate *CBF* genes for the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. Plant Mol. Biol. 67: 257–270.

Kobayashi, F., S. Takumi, M. Nakata, R. Ohno, T. Nakamura and C. Nakamura (2004) Comparative study of the expression profiles of the *Cor*/*Lea* gene family in two wheat cultivars with contrasting levels of freezing tolerance. Physiol. Plant. 120: 585–594.

Kobayashi, F., S. Takumi, S. Kume, M. Ishibashi, R. Ohno, K. Murai and C. Nakamura (2005) Regulation by *Vrn-1*/*Fr-1* chromosomal intervals of CBF-mediated *Cor*/*Lea* gene expression and freezing tolerance in common wheat. J. Exp. Bot. 56: 887–895.

Kobayashi, F., M. Ishibashi and S. Takumi (2008a) Transcriptional activation of *Cor*/*Lea* genes and increase in abiotic stress tolerance through expression of a wheat *DREB2* homolog in transgenic tobacco. Transgenic Res. 17: 755–767.

Kobayashi, F., E. Maeta, A. Terashima and S. Takumi (2008b) Positive role of a wheat *HvABI5* ortholog in abiotic stress response of seedlings. Physiol. Plant. 134: 74–86.

Kobayashi, F., E. Maeta, A. Terashima, K. Kawaura, Y. Ogihara and S. Takumi (2008c) Development of abiotic stress tolerance via a bZIP-type transcription factor LIP19 in common wheat. J. Exp. Bot. 59: 891–905.

Kobayashi, F., S. Takumi and H. Handa (2010) Identification of quantitative trait loci for ABA responsiveness at seedling stage associated with ABA-regulated gene expression in common wheat. Theor. Appl. Genet. 121: 629–641.

Kocsy, G., B. Athmer, D. Perovic, A. Himmelbach, A. Szücs, I. Vashegyi, P. Schweizer, G. Galiba and N. Stein (2010) Regulation of gene expression by chromosome 5A during cold hardening in wheat. Mol. Genet. Genomics 283: 351–363.

Kosová, K., I.T. Prásil, P. Prásilová, P. Vítámvás and J. Chrpová (2010) The development of frost tolerance and DHN5 protein accumulation in barley (*Hordeum vulgare*) doubled haploid lines from Atlas 68 × Igri cross during cold acclimation. J. Plant Physiol. 167: 343–350.

Koyama, K., H. Hatano, J. Nakamura and S. Takumi (2012) Characterization of three *VERNALIZATION INSENSITIVE3-like* (*VIL*) homologs in wild wheat, *Aegilops tauschii* Cross. Hereditas 149: 62–71.

Kume, S., F. Kobayashi, M. Ishibashi, R. Ohno, C. Nakamura and S. Takumi (2005) Differential and coordinated expression of *Cbf* and *Cor*/*Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. Genes Genet. Syst. 80: 185–197.

Laudencia-Chingcuanco, D., S. Ganeshan, F. You, B. Fowler, R. Chibbar and O. Anderson (2011) Genome-wide gene expression analysis supports a developmental model of low temperature tolerance gene regulation in wheat (*Triticum aestivum L.*). BMC Genomics 12: 299.

Matsumoto, T., T. Tanaka, H. Sakai, N. Amano, H. Kanamori, K. Kurita, A. Kikuta, K. Kamiya, M. Yamamoto, H. Ikawa *et al.* (2011) Comprehensive sequence analysis of 24,783 barley full-length cDNAs derived from 12 clone libraries. Plant Physiol. 156: 20–28.

Mayer, K.F.X., M. Martis, P.E. Hedley, H. Simková, H. Liu, J.A. Morris, B. Steuernagel, S. Taudien, S. Roessner, H. Gundlach *et al.* (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell 23: 1249–1263.

Miller, A.K., G. Galiba and J. Dubcovsky (2006) A cluster of 11 *CBF* transcription factors is located at the frost tolerance locus *Fr-A^{m}2* in *Triticum monococcum*. Mol. Genet. Genomics 275: 193–203.

Morran, S., O. Eini, T. Pyvovarenko, B. Parent, R. Singh, A. Ismagul, S. Eliby, N. Shirley, P. Langridge and S. Lopato (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotech. J. 9: 230–249.

Ohno, R., S. Takumi and C. Nakamura (2001) Expression of a cold-responsive *Lt-Cor* gene and development of freezing tolerance during cold acclimation in wheat (*Triticum aestivum* L.). J. Exp. Bot. 52: 2367–2374.

Ohno, R., S. Takumi and C. Nakamura (2003) Kinetics of transcript and protein accumulation of a low-molecular-weight wheat LEA D-11 dehydrin in response to low temperature. J. Plant Physiol. 160: 193–200.

Rabbani, M.A., K. Maruyama, H. Abe, M.A. Kharn, K. Katsura, Y. Ito, K. Yoshiwara, M. Seki, K. Shinozaki and K. Yamaguchi-Shinozaki (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. 133: 1755–1767.

Seki, M., M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J. 31: 279–292.

Skinner, D.Z. (2009) Post-acclimation transcriptiome adjustment is a major factor in freezing tolerance of winter wheat. Funct. Integr. Genomics 9: 513–523.

Skinner, J.S., J. von Zitzewitz, P. Stucs, L. Marquez-Cedillo, T. Filichkin, K. Amundsen, E.J. Stockinger, M.F. Thomashow, T.H.H. Chen and P.M. Hayes (2005) Structural, functional, and phylogenetic characterization of a large *CBF* gene family in barley. Plant Mol. Biol. 59: 533–551.

Skinner, J.S., J. von Zitzewitz, L. Marquez-Cedillo, T. Filichkin, E.J. Stockinger, M.F. Thomashow, T.H.H. Chen and P.M. Hayes (2006) Mapping of barley homologs to genes that regulate low temperature tolerance in *Arabidopsis*. Theor. Appl. Genet. 112: 832–842.

Snape, J.W., A. Semikhodskii, L. Fish, R.N. Sarma, S.A. Quarrie, G. Galiba and J. Sutka (1997) Mapping frost resistance loci in wheat and comparative mapping with other cereals. Acta Agr. Hungarica 45: 265–270.

Stockinger, E.J., J.S. Skinner, K.G. Gardner, E. Francia and N. Pecchioni (2007) Expression levels of barley *Cbf* genes at the *Frost resistance-H2* locus are dependent upon alleles at *Fr-H1* and *Fr-H2*. Plant J. 51: 308–321.

Sutka, J. and J.W. Snape (1989) Location of a gene for frost resistance on chromosome 5A of wheat. Euphytica 42: 41–44.

Sutka, J. (2001) Genes for frost resistance in wheat. Euphytica 119: 167–172.

Takumi, S., A. Koike, M. Nakata, S. Kume, R. Ohno and C. Nakamura (2003) Cold-specific and light-stimulated expression of a wheat (*Triticum aestivum* L.) *Cor* gene *Wcor15* encoding a chloroplast-targeted protein. J. Exp. Bot. 54: 2265–2274.

Takumi, S., C. Shimamura and F. Kobayashi (2008) Increased freezing tolerance through up-regulation of downstream genes via the wheat

CBF gene in transgenic tobacco. Plant Physiol. Biochem. 46: 205–211.

Thomashow, M.F. (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Ann. Rev. Plant Physiol. Plant Mol. Biol. 50: 571–599.

Tsvetanov, S., R. Ohno, K. Tsuda, S. Takumi, N. Mori, A. Atanassov and C. Nakamura (2000) A cold-responsive wheat (*Triticum aestivum* L.) gene *wcor14* identified in a winter-hardy cultivar 'Mironovska 808'. Genes Genet. Syst. 75: 49–57.

Vágújfalvi, A., C. Crosatti, G. Galiba, J. Dubcovsky and L. Cattivelli (2000) Two loci on wheat chromosome 5A regulate the differential cold-dependent expression of the *cor14b* gene in frost-tolerant and frost-sensitive genotypes. Mol. Gen. Genet. 263: 194–200.

Vágújfalvi, A., G. Galiba, L. Cattivelli and J. Dubcovsky (2003) The cold-regulated transcriptional activator *Cbf3* is linked to the frost-tolerance locus *Fr-A2* on wheat chromosome 5A. Mol. Genet. Genomics 269: 60–67.

Vágújfalvi, A., A. Aprile, A. Miller, J. Dubcovsky, G. Delugu, G. Galiba and L. Cattivelli (2005) The expression of several *Cbf* genes at the *Fr-A2* locus is linked to frost resistance in wheat. Mol. Genet. Genomics 274: 506–514.

Veisz, O. and J. Sutka (1990) Frost resistance studies with wheat in natural and artificial conditions. *In*: Panayotov, I. and S. Pavlova (eds.) Proceedings of the international symposium on cereal adaptation to low temperature stress, Albena, Bulgaria, pp. 12–17.

Wang, S., C.J. Basten and Z.B. Zeng (2007) Windows QTL cartographer 2.5. Department of Statics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

Winfield, M.O., C. Lu, I.D. Wilson, J.A. Coghill and K.J. Edwards (2010) Plant responses to cold: transcriptome analysis of wheat. Plant Biotech. J. 8: 749–771.

Yamaguchi-Shinozaki, K. and K. Shinozaki (1994) A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high salt stress. Plant Cell 6: 251–264.