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| A major quantitative trait locus for cold-responsive gene expression is linked to frost-resistance gene Fr-A2 in common wheat |
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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-A2) 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 (Dellker 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 (Dellker 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 Wht10, Whd13 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, Morrán 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).
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-A2 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 *HvChf14* are candidates for wheat Fr-A2 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, *Chf14* and *Chf15*, 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 responsive gene expression patterns remains unclear.

Two common wheat cultivars, *T. aestivum* ‘Chinese Spring’ (CS) and ‘Mironovskaya 808’ (M808), show differences in 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$_1$ 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'-CAGAGCCCTCCAGTTAGCAATG-3' and 5'-CAGACGCTCTCACTCAGGAAGGAA-3' for *Wtt10*, 5'-GGCGAAAGGGCCGCGTAT-3' and 5'-GTTGGTGCGTGGGCAT-3' for *Wdh1n13*, 5'-TCTCTTTCTCGTCCAGTCTGC-3' and 5'-TTCTATACATCCTCTGACC-3' for *Wcor14*, 5'-AGTCGGGTGTAGGAGAGTGG-3' and 5'-GTGGTGGCTGTGGTGGCAT-3' for *TaCBF12*, 5'-CATACTGCCCTCACAGTTT-3' and 5'-CTCCGTCTCAGCCTAAGC-3' for *TaCBF15* and 5'-GCTCGCTGATTGAGGACGAG-3' and 5'-GTGAAGCGA-3' for *WCBF2*, 5'-AGTGGGTGTGGTCGGAATCGTGCAG-3' and 5'-GGAGAGATGAGGAGACG-3' for *TaCBF2*, 5'-CATACTGCCCTCACAGTTT-3' and 5'-CTCCGTCTCAGCCTAAGC-3' for *TaCBF15* and 5'-GCTCGCTGATTGAGGACGAG-3' and 5'-GTGAAGCGA-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 $\Delta Ct$ 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 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-
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
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 1. Correlation coefficients ($R^2$ values) among transcript accumulation of six cold-responsive genes in RILs of M808 and CS

| Gene   | eQTL  | Chromosome | Vicinity marker | Position (cM) | LOD  | Additive effect | Contribution (%) |
|--------|-------|------------|----------------|--------------|------|----------------|-----------------|
| WCBF2 | 5A*** | 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    | 5A         | Xbarc330       | 49.8         | 3.03 | $-$5243        | 5.6             |
| Wlt10 | 1D    | 1D         | Xcf27          | 101          | 2.82 | 5106           | 5.3             |
| Wdhn13 | 5A    | 5A         | Xbarc330       | 49.8         | 14.1 | $-$51.2        | 24.5            |
| Wcor14 | 5A    | 5A         | Xbarc330       | 49.8         | 2.79 | $-$3467        | 6.4             |
| Wcor14 | 4B    | 4B         | Xhbg406        | 48.8         | 3.26 | $-$3410        | 6.1             |

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         | Xcf27          | 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             |

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
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, \textit{WCBF2}, \textit{TaCBF12} and \textit{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 \textit{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 \textit{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 \textit{Cor}/\textit{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 \textit{Cor}/\textit{Lea} gene expression is directly regulated by transcription factor genes including \textit{CBFs}, \textit{WDREB2}, \textit{Wabi5} and \textit{Wlip19} (Egawa et al. 2006, Kobayashi et al. 2008a, 2008b,
near frost and freezing tolerance in common wheat and is located freezing tolerance on chromosome 5A of common wheat. 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 Wli19 ortholog is NIAHSh1140N16 in the full-length cDNA database (Matsumoto et al. 2011) and Wli19 was putatively assigned to the short arm of chromosome 1D (Supplemental Fig. 1). Since Wli19 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 Wdm13 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-A2 (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 Cor/Lea 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 (Snapa et al. 1997, Sutka 2001). Our eQTL study showed that the 5A eQTL are tightly linked to the homoeologous locus (Fr-A2) of Fr-A2 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-A2 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-A2 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 (Casaos et al. 2011, Galiba et al. 1995, 2009, Snapa 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-
Expression QTL analysis of wheat cold-responsive genes

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-I is independent but tightly linked to Vrn-I. Our previous study using Japanese wheat cultivars showed that allelic linkage between Fr-I and Vrn-I 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 breeding, complex traits such as drought stress and Fusarium toleration requirement without affecting freezing tolerance. Mol. Breed. 28: 475–484.

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