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Line differences in *Cor*/*Lea* and fructan biosynthesis-related gene transcript accumulation are related to distinct freezing tolerance levels in synthetic wheat hexaploids

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ABSTRACT

In common wheat, cultivar differences in freezing tolerance are considered to be mainly due to allelic differences at two major loci controlling freezing tolerance. One of the two loci, *Fr-2*, is coincident with a cluster of genes encoding C-repeat binding factors (CBFs), which induce downstream *Cor*/*Lea* genes during cold acclimation. Here, we conducted microarray analysis to study comprehensive changes in gene expression profile under long-term low-temperature (LT) treatment and to identify other LT-responsive genes related to cold acclimation in leaves of seedlings and crown tissues of a synthetic hexaploid wheat line. The microarray analysis revealed marked up-regulation of a number of *Cor*/*Lea* genes and fructan biosynthesis-related genes under the long-term LT treatment. For validation of the microarray data, we selected four synthetic wheat lines that contain the A and B genomes from the tetraploid wheat cultivar Langdon and the diverse D genomes originating from different *Aegilops tauschii* accessions with distinct levels of freezing tolerance after cold acclimation. Quantitative RT-PCR showed increased transcript levels of the *Cor*/*Lea*, *CBF*, and fructan biosynthesis-related genes in more freezing-tolerant lines than in sensitive lines. After a 14-day LT treatment, a significant difference in fructan accumulation was observed among the four lines. Therefore, the fructan biosynthetic pathway is associated with cold acclimation in development of wheat freezing tolerance and is another pathway related to diversity in freezing tolerance, in addition to the CBF-mediated *Cor*/*Lea* expression pathway.

Introduction

Exposure of higher plants to low, nonfreezing temperatures leads to increased freezing tolerance levels, which is considered a plant adaptive process and is called cold acclimation. Cold acclimation involves drastic physiological, biochemical and metabolic changes in plant cells and tissues (Thomashow, 1999). Most of these alterations are regulated through changes in gene expression during cold acclimation. One of the mechanisms behind development of freezing tolerance is induction of the *Cor* (cold-responsive)/*Lea* (late-embryogenesis-abundant) gene family (Thomashow, 1999; Kobayashi et al., 2004).

In wheat and barley, major loci controlling freezing tolerance (*Fr-1* and *Fr-2*) have been assigned to the long arm of group 5 chromosomes (Galiba et al., 1995; Snape et al., 1997). *Fr-2* is coincident with a cluster of genes encoding C-repeat binding factors (CBFs) (Miller et al., 2006; Francia et al., 2007), which directly induce downstream *Cor*/*Lea* gene expression during cold acclimation (Takumi et al., 2008). The expression quantitative trait locus region for *Cor*/*Lea* and *CBF* genes on 5AL, which play important roles in development of freezing tolerance in common wheat (Motomura et al., 2013), coincides with a region homologous to a frost-tolerance locus (*Fr-A^{m}2*) reported as a *CBF* cluster region in einkorn wheat (Vágújfalvi et al., 2003; Miller et al., 2006). Allelic differences at *Fr-A2* might be a major cause of cultivar differences in extent of freezing tolerance in common wheat (Motomura et al., 2013). Large deletions in the *CBF* cluster at *Fr-B2* significantly reduce frost tolerance in tetraploid and hexaploid wheat (Pearce et al., 2013). Thus, the *CBF* clusters at *Fr-2* function during cold

Abbreviations: ABA, abscisic acid; CBF, C-repeat binding factor; Cor, cold responsive; 6-FEH, fructan 6-exohydrolase; 1-FFT, fructan:fructan 1-fructosyltransferase; Lea, late embryogenesis abundant; LT, low temperature; 6-SFT, sucrose:fructan 6-fructosyltransferase; 1-SST, 1-fructosyltransferrase; QTL, quantitative trait locus.

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acclimation and contribute to wheat cultivar differences in freezing tolerance. In barley, two quantitative trait loci (QTLs) for low-temperature (LT) tolerance, *Fr-H1* and *Fr-H2*, are 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 LT tolerance (Stockinger et al., 2007; Chen et al., 2009). 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 common wheat, the *Vrn-A1*/*Fr-A1* and *Vrn-B1*/*Fr-B1* chromosomal regions play a major role in developing freezing tolerance through regulation of *CBF*-mediated *Cor*/*Lea* gene expression (Kobayashi et al., 2005).

A lot of other genes, including *Wlip19* and *Wabi5* bZIP transcription factor genes (Kobayashi et al., 2008a,b), act in abscisic acid (ABA) signaling and contribute to cold acclimation and freezing tolerance in common wheat. These transcription factors bind to ABA-responsive elements in the promoters of *Cor*/*Lea* genes (Kusano et al., 1995; Choi et al., 2000; Kim et al., 2004). ABA sensitivity strongly affects the basal levels of freezing tolerance (Kobayashi et al., 2006, 2008c), and some QTLs on wheat chromosomes controlling ABA sensitivity at the seedling stage are also related to *Cor*/*Lea* gene expression and are putatively associated with freezing tolerance (Kobayashi et al., 2010). The QTLs for ABA sensitivity do not correspond to *Fr-1* and *Fr-2*, and the two *Fr* loci are postulated to act independently of ABA signal transduction pathways. Therefore, multiple signal pathways are involved during cold acclimation to develop freezing tolerance. Unknown components involved in multiple pathways might be involved in wheat LT and freezing tolerance.

In most LT-responsive transcription factor genes, the first up-regulation occurs within 1–4 h, which might correspond to the rapid response to LT. Maintenance of a high *CBF* transcript level in freezing tolerant cultivars might represent a long-term effect of cold acclimation (Kume et al., 2005), suggesting that effects of long-term LT treatment on gene expression profiles could be distinct from rapid changes in response to cold stress. The above-ground tissues of wheat plants wilt and wither under freezing conditions, whereas cold-acclimated seedlings of freezing tolerant wheat cultivars rapidly recover from freezing stress and develop new shoots from surviving meristems of the crown tissues (Ohno et al., 2001). Therefore, biologically important events in the development of freezing tolerance likely occur in the crown tissues. Freezing stress significantly alters gene expression profiles of more than 400 wheat genes, and some of the up-regulated genes encode kinases, phosphatases, calcium trafficking-related proteins and glycosyltransferases in the crown tissues of cold-acclimated plants (Skinner, 2009). This observation implies genetic variation among wheat cultivars in the ability to alleviate the damage to crowns exposed to freezing stress (Skinner, 2009). Thus, many genes besides *CBF* and *Cor*/*Lea* presumably participate in each step of developing freezing tolerance in the crown tissues.

Long-term stress conditions lead to higher soluble sugar concentrations and lower amounts of starch (Silva and Arrabaca, 2004). Fructans, soluble fructosyl polysaccharides, are storage carbohydrates in a large number of higher plants, and possibly function in membrane stabilization through formation of a fructan-lipid interaction under water stresses such as cold and drought (Valluru and Van den Ende, 2008; Livingston et al., 2009). Fructans accumulating in perennial grasses can be considered longer-term reserve carbohydrates for survival of the winter period (Yoshida et al., 1998). In wheat and barley, three enzyme families, sucrose:sucrose 1-fructosyltransferrase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT) and fructan:fructan 1-fructosyltransferase (1-FFT), synthesize graminian-type fructans consisting of β-2,6-linked fructosyl units with β-2,1 branches (Ritsema and Smeekens, 2003). The TaMYB13 transcription factor binds to the promoters of wheat *1-SST* and *6-SFT* genes and activates fructosyltransferase gene expression (Xue et al., 2011a). Snow mold resistant cultivars accumulate and maintain higher fructan levels in the crown tissues from autumn to the end of winter (Yoshida et al., 1998; Kawakami and Yoshida, 2002). Yoshida et al. (1998) also reported that fructan may increase freezing tolerance, although its efficiency is lower than that of monosaccharides and disaccharides in common wheat. Livingston III (1996) suggested that fructan is indirectly involved in freezing tolerance of oat and barley. Therefore, fructans surely play important roles in development of water stress tolerance, acting as anti-stress agents in overwintering plants (Kawakami and Yoshida, 2002, 2005; Livingston et al., 2009). Although fructan accumulation is associated with freezing tolerance in higher plants, its relation to wheat cultivar difference in LT and freezing tolerance is unknown.

Here, we studied gene expression profiling under long-term LT treatment to identify novel components involved in wheat signal pathways during cold acclimation and to elucidate the relationship between fructan accumulation levels and line differences in freezing tolerance using wheat synthetic hexaploids.

Materials and methods

Plant materials

In our previous study, tetraploid wheat accession *Triticum turgidum* ssp. *durum* (Desf.) Husn. cv. Langdon (Ldn) was used as the female parent, and crossed with 69 *Aegilops tauschii* Coss. accessions to artificially produce triploid wheat hybrids (Kajimura et al., 2011). Selfed seeds (F_2 generation), called synthetic wheats, from the triploid F_1 hybrids were obtained through unreduced gamete formation (Matsuoka and Nasuda, 2004; Matsuoka et al., 2013). In this study, we used five synthetic hexaploid wheat lines (F_3 generation) derived from five cross combinations between Ldn and five *Ae. tauschii* accessions, KU-2059, AE1090, KU-2109, PI476874 and IG48042 (Kajimura et al., 2011). These synthetic lines did not show any abnormal growth, such as hybrid necrosis, hybrid chlorosis or severe growth abortion (Mizuno et al., 2010). A synthetic wheat, Ldn/KU-2059, was used for transcriptome analysis, and four other synthetic lines, Ldn/AE1090, Ldn/KU-2109, Ldn/PI476874 and Ldn/IG48042, were used for comparative studies. These four synthetic wheat lines were selected based on preliminary observation of their winter growth in the 2011–2012 season (unpublished data). The selfed seeds (F_3 generation) were sown in November 2012, and the synthetic wheat lines were grown individually in pots in the field at Kobe University to confirm their growth phenotype.

Transcriptome analysis

A synthetic hexaploid wheat line, Ldn/KU-2059, was used for microarray analysis. The synthetic wheat plants were grown in a growth chamber with a 12 h photoperiod (110–120 μmol photons m^{-2} s^{-1}). Total RNA was extracted using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) from the youngest leaves of the synthetic line grown at normal temperature (23 °C) for 3 weeks and then at 4 °C for 12 weeks and from the crown tissues of the synthetic line grown at 23 °C for 3 weeks and then at 4 °C for 6 weeks. A KOMUGI 38 k oligonucleotide DNA microarray (Agilent Technologies, Santa Clara, CA) was supplied by the National BioResource Project (NBRP)-Wheat, Japan (https://www.nbrp.jp) for analysis. Detailed information on the 38 k microarray platform can be found in Kawaura et al. (2008) and the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) website under GPL9805. Hybridization of Cy3-labeled cRNA, washing and image scanning were performed