Molecular Characterization of Sucrose:Sucrose 1-Fructosyltransferase and Sucrose:Fructan 6-Fructosyltransferase Associated with Fructan Accumulation in Winter Wheat during Cold Hardening

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We isolated two cDNAs of winter wheat (Triticum aestivum L.), designated wft1 and wft2, which encoded sucrose:fructan 6-fructosyltransferase (6-SFT) and sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.100), respectively, which are involved in the synthesis of fructan in wheat. wft1 and wft2 were cloned by screening of a cDNA library with probed-cDNA fragments corresponding to plant fructosyltransferase and invertase. The identity of the clones was verified by functional characterization of recombinant proteins expressed in methylotrophic yeast, Pichia pastoris. Northern blotting showed that the level of wft2 transcripts increased from autumn to early winter in the crown tissues of all field-grown wheat cultivars examined. Higher levels of wft1 and wft2 transcripts were found in leaf tissues of snow mold-resistant cultivars, which accumulated more fructan than other cultivars. Our results showed that Wft1 and Wft2 were important in fructan accumulation during cold hardening of winter wheat.

Key words: fructan; sucrose:fructan 6-fructosyltransferase; sucrose:sucrose 1-fructosyltransferase; fructan accumulation; cold hardening; Triticum aestivum L.

Fructans, a class of water-soluble, fructose-based oligo- and polysaccharides, are major nonstructural carbohydrates in many plants including Asteraceae, Liliaceae, and Poaceae. Fructan is important in the temporary storage of assimilates instead of starch or sucrose.1–6 Fructans are classified into several forms depending on their glycosidic linkages. Dicotyledonous species such as Cichorium intybus and Helianthus tuberosus accumulate an inulin-type fructan, a linear β(2→1)-linked fructofuranosyl unit. Helianthus tuberosus contains a fructan polymer of a β(2→6)-linked fructofuranosyl unit and a β(2→1)-linked fructofuranosyl unit. Some cereals, including Triticum aestivum, have a branched-type fructan known as graminan with both β(2→1) and β(2→6) linkages.

Winter cereals that grow in northern regions, where low temperatures and a thick, snow cover persist in winter, must resist both freezing and snow molds.2–9 Accumulation of water-soluble carbohydrates in plant tissues during cold hardening is associated with freezing tolerance and is essential to winter survival.3,6 In particular, the storage carbohydrate fructan and its metabolism are related to the development of freezing tolerance in cereals.3–9 Fructan levels are associated with the overwintering ability of wheat cultivars that grow in regions where snow mold limits the survival of the plant.9,8 However, the physiological relevance of fructan accumulation to the overwintering ability of wheat is the role of the molecular mechanisms of fructan accumulation during cold hardening. The purpose of this study was to isolate and characterize wheat genes of enzymes involved in fructan synthesis associated with winter stresses.

Another purpose of this study was to compare different fructan-synthesis genes in a cereal plant. There has been much interest in the functional similarity and high degree of identity of amino acid sequences in fructosyltransferase and invertase.11,12 The cloning of cDNAs encoding fructosyltransferase would enable us to analyze their molecular relations and functional evolution. Two enzymes, sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) and fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100), are involved in the biosynthesis of fructan.13–15 1-SST transfers a fructosyl moiety from one sucrose to another, resulting in the formation of trisaccharide, 1-kestose, and glucose. 1-FFT elongates a fructosyl chain by transferring a fructosyl moiety from one fructan to another. Other enzymes, sucrose:fructan 6-fructosyltransferase (6-SFT), 16–18...
fructosyltransferases (6G-FT),\(^\text{17}\) and fructan:fructan 6'-fructosyltransferase (6G-FTT),\(^\text{18}\) are also important in fructan biosynthesis in plants. 1-SST and 6-SFT may contribute to the synthesis of the branched-type fructans known as graminins that are found in plants of the Poaceae family, such as wheat and barley.\(^\text{11,16}\) Sprenger \etal\(^\text{19}\) first reported the cloning of a plant gene of the fructan biosynthesis enzyme 6-SFT from \textit{Hordeum vulgare}. Since then, several genes encoding enzymes in fructan biosynthesis have been cloned from various plants: 1-SST from \textit{C. intybus};\(^\text{19}\) 1-SST and 1-FTT from \textit{Cynara scolymus}\(^\text{20}\) and from \textit{H. tuberosus};\(^\text{21}\) 1-SST and 6G-FTT from \textit{Allium cepa};\(^\text{18,23}\) and 1-SST from \textit{Festuca arundinacea};\(^\text{22}\) and 6-SFT from \textit{Agropyron cristatum}.\(^\text{12}\) We have identified cDNAs for 1-SST and 6-SFT from wheat, and this is the first report of cloning 1-SST and 6-SFT from the same plant.

### Materials and Methods

**Plant materials.** Seeds of five winter wheat cultivars (\textit{Triticum aestivum} L. cv. PI 173438, Norin 62, Chihokukomugi, Norstar, and Valuevskaya) were sown in the middle of September 1997 and 1998, and grown in a field of the National Agricultural Research Center for Hokkaido Region, Sapporo. Plant materials harvested at the dates indicated later were frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until use. The cultivars Valuevskaya and Norstar are highly freezing-tolerant but are susceptible to snow mold; the cultivars PI 173438 and Norin 62 are snow mold-resistant; and the cultivar Chihokukomugi is moderately freezing-tolerant and snow mold-resistant.\(^\text{9}\) Freezing tolerance (LT\(_{50}\)) of plants is killed by freezing) of PI 173438, Norin 62, Chihokukomugi, Norstar, and Valuevskaya in the field at early December were \(-19, -20, -20, -24\), and \(-24^\circ\text{C}\), respectively.

**Preparation and screening of a cDNA library of wheat.** Total RNA was extracted from crown tissues of the winter wheat cv. PI 173438 sampled in the middle of November 1997. Poly(A\(^+\)) RNA was purified with Oligotex-dT30 resins (Takara, Kyoto, Japan), and double-stranded cDNA was synthesized from the poly(A\(^+\)) RNA. A cDNA library was constructed with a ZAP Express XR library construction kit (Stratagene, La Jolla, CA).

The degenerate primers 5'-ATGAA(T/C)GA(T/C)CCNAA(T/C)GG-3' and 5'-CCNGTNGC(A/G)TT(A/G)TT(A/G)AA-3' (Fig. 1) were designed on the basis of the amino acid sequences MNDPNG and FNNATG, respectively, which were conserved among several plant invertases and fructosyltransferases (Fig. 2, regions B and D). Two DNA fragments of similar size (1.5 kbp) were amplified by PCR. The fragments were highly similar to a part of cDNA clones for enzymes related to fructan metabolism, and were used as hybridization probes. The cDNA library was screened by plaque hybridization. Nucleotide sequences of isolated clones were determined by an automated DNA sequencer (model 373S, Applied Biosystems, Foster City, CA) with Thermo sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The nucleotide sequences and deduced amino acid sequences were analyzed by DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

**Expression of recombinant proteins in a methylotrophic yeast.** The isolated cDNA was expressed in the methylotrophic yeast \textit{Pichia pastoris} (EasySelect \textit{Pichia} Expression Kit, Invitrogen, Carlsbad, CA), with the secretory expression vector pPICZ\(_{\text{a}}\)B by the method of Lüscher \etal\(^\text{20}\). DNA sequences corresponding to putative mature protein regions of Wft1 and Wft2 (Fig. 1), between nucleotides 196 and 1848 (wft1m) and between 322 and 1986 (wft2m), were amplified by PCR and translationally fused behind the alpha-factor signal sequence of pPICZ\(_{\text{a}}\)B. Transformation and culture of \textit{P. pastoris} strain X-33 was transformed by electroporation, with 10 \(\mu\)g of the PmeI-linearized vector with inserts, and transformants were selected on YPDS-Zeo agar plates. A 100-ml pre-culture medium (BMGY, pH 6.0) was inoculated with freshly prepared single colonies and incubated for 48 h at 30\(^\circ\text{C}\) with vigorous shaking (200 rpm). The cells were collected by centrifugation, transferred to 20 ml of an induction medium (BMMY, pH 6.0) and incubated at 28\(^\circ\text{C}\) under aerobic conditions. Every day, 100 \(\mu\)l of methanol was added to the culture medium. At 96 h after induction, the culture was centrifuged and the supernatant was obtained for the enzyme solution. A 10-ml portion of the medium was concentrated to 200 \(\mu\)l, diluted with 15 ml of 20 mM citrate-phosphate buffer (pH 5.0), and concentrated by ultrafiltration on a Vivaspin 20 concentrator (cut-off 10,000 Da, Vivascience, Lincoln, U.K.). The dilution and concentration process was repeated twice on Vivaspin and the final volume of enzyme solution was adjusted to 100 \(\mu\)l with a Microcon concentrator (Millipore, Bedford, MA).

**Enzyme assay and carbohydrate analysis.** The concentrated medium containing a recombinant enzyme was mixed with the same volume of 20 mM citrate-phosphate buffer (pH 5.0) containing 100 mM sucrose, 1-kestose, or a mixture of sucrose and 1-kestose (final concentration of the substrate, 50 mM each). The reaction mixture was incubated at 4\(^\circ\text{C}\) for 17 h. The reaction was stopped by the container...
Results

Molecular characterization of wheat fructosyltransferases

We isolated two clones that each contained one long open reading frame designated wft1 or wft2. Analysis of the deduced amino acid sequences of open reading frames of these clones showed that wft1 (DDJB accession no. AB029887) encoded a 616-amino acid polypeptide with 93\% identity to the 6-SFT of barley, and that wft2 (AB029888) encoded a 662-amino acid polypeptide with 73\% identity to tall fescue 1-SST (Fig. 1). The Wft1 protein was predicted to contain six putative N-glycosylation sites and to have an \( \text{pI} \) of 5.1, and the Wft2 protein was predicted to contain six putative N-glycosylation sites and to have an \( \text{pI} \) of 4.9. Identity between Wft1 and Wft2 proteins was 61\%.

Wft1 and Wft2 proteins were also predicted to contain conserved domains in various fructosyltransferases and invertases (Fig. 2). Comparison of the enzymes has shown that sequences of the signal peptide regions are less conserved than those of the mature protein regions. The mature protein of barley 6-SFT and carrot invertase start at the 68th codon and 116th codon, respectively. Comparison of N-terminal sequences of Wft1 and Wft2 with those of plant invertase and barley 6-SFT suggested that

Northern hybridization. Total RNA was isolated from leaf and crown tissues of wheat cultivars with TRizol reagent (Gibco-BRL, Rockville, MD). Northern hybridization was done by the standard protocol. Total RNA (15 µg) was electrophoresed on 1.5% (w/v) agarose gel, blotted onto a hybond N\+ membrane (Amersham Pharmacia Biotech) and hybridized with a 32P-labeled cDNA fragment corresponding to Wft1 or Wft2. The probed blot was washed twice with 0.1 × SSC and 0.1% SDS for 20 min at 65°C, and then used to expose X-ray film at −80°C.

being placed in boiling water for 3 min, and sugar products were analyzed by HPLC. For analysis of fructan oligomers with different glycosidic linkages, high performance anion exchange (HPAE) chromatography was done on a DX 300 chromatograph (Dionex, Sunnyvale, CA) with a Carbo Pac PA-1 anion-exchange column and a pulsed amperometric detector (PAD) as described by Shiomi et al. Peaks for glucose, fructose, sucrose, 1-kestose, 1,1-nystose, 1,1,1-kestopentaose, and 6-kestose were identified with authentic standards. Sugar content in reaction mixtures and wheat tissues was measured by HPLC with Shodex columns of a combination of KS-802 and KS-803 (Shodex, Tokyo), with the refractive index detector described by Yoshida et al.9)
Fig. 2. Comparison of Amino Acid Sequences of Highly Conserved Regions of Wft1 and Wft2 with Those of Plant Fructosyltransferases and Different Invertases.

Regions B and C contain consensus motifs in invertases for catalytic sites. Asp (D) in the sucrose-binding box NDPNG (region B) and Glu (E) in region C are involved in sucrose hydrolysis.11,27) Hv 6-SFT, barley 6-SFT (X83233); Fa 1-SST, tall fescue 1-SST (AJ297369); Ac 1-SST, onion 1-SST (AJ006066); Ac 6G-FFT, onion 6G-FFT (Y07838); Os INV, rice vacuolar invertase (AF276704); Zm INV, maize vacuolar invertase (U16123); Ta WINV, wheat cell-wall invertase (AF030420); Zm WINV, maize cell-wall invertase (AF043346); and Dc WINV, carrot cell-wall invertase (X69321).

Fig. 3. HPLC Analysis of Carbohydrates Generated from Sucrose in the Reaction Mixture with the Culture Media of P. pastoris Transformed with Empty Plasmid (A), wft1m-Plasmid (B) and wft2m-Plasmid (C).

The reaction was done with 50 mM sucrose at 4°C for 17 h, and then carbohydrates in the reaction mixtures were analyzed by HPLC using Shodex columns (KS-802 and KS-803) with a refractive index detector. F, fructose; G, glucose; S, sucrose; DP3, trisaccharide, DP4, tetrasaccharide.

the putative mature proteins of wft1 and wft2 might start downstream of codons 95 and 91, respectively (Figs. 1 and 2). Fructan probably is synthesized in vacuole,11) suggesting that the encoded protein should contain a vacuolar targeting signal. N-terminal regions of the putative mature proteins Wft1m and Wft2m were more similar to the corresponding sequences of vacuolar-type fructosyltransferases and invertases than to those of cell-wall type invertases (Fig. 2, region A). Several hydrophobic amino acids were found at the end of the C-terminal region of Wft2 (Fig. 1). The short hydrophobic sequence of the C-terminal region is necessary and sufficient for targeting of the plant vacuole.28,29) These results suggest that wft1 and wft2 clones encode vacuole-type fructosyltransferases or fructosidases.

**Functional characterization of recombinant Wft1 and Wft2 proteins**

The enzymatic activity of Wft1m and Wft2m encoded by wft1m and wft2m, respectively, were examined. The culture medium of P. pastoris transformed with empty plasmid (control) did not act on sucrose (Fig. 3(A)) or 1-kestose (data not shown), but fructosyltransferase activities were detected in the culture medium of P. pastoris with wft1m or wft2m (Figs. 3(B) and 3(C); Figs. 4 and 5). The recombinant proteins Wft1m and Wft2m were produced by the transformed P. pastoris. The reaction mixture of Wft1m contained mostly 1-kestose and 6-kestose when incubated with sucrose alone, and a large amount of kestotetraose (peak 7 in Fig. 4) when incu-
Molecular Characterization of Wheat Fructan-synthesis Genes

Fig. 4. HPAE Separation of Oligo-fructans Generated by Wft1m in Culture Medium of Transformed P. pastoris.

The reaction was done with 50 mM sucrose (B), a combination of 50 mM sucrose and 50 mM 1-kestose (E) or 50 mM 1-kestose (F) at 4°C for 17 h. A and D are chromatograms of the zero h-solutions for B and E, respectively. C indicates a chromatogram of standard sugars: 1, glucose; 2, fructose; 3, sucrose; 4, 1-kestose; 5, 6-kestose; 6, 1,1-nystose; 7, bifurcose (putative); 8, 1,1,1-kestopentaose. HPAE-PAD was done using DX 300 and PA-1 column (Dionex). A small amount of 1,1-nystose was included in 1-kestose substrate. Peaks without numbers could not be identified and the single peak marked with an asterisk included in the reaction medium was different from that of fructose.

Wft1m also produced much fructose when incubated with sucrose, indicating that Wft1m had potential invertase activity (Fig. 3(B)). However, Wft1m produced little fructose when incubated with 1-kestopentaose (Fig. 4(F)). Little fructose appeared when Wft2m was incubated with sucrose (Fig. 3(C)). Wft2m produced fructose when incubated with only 1-kestose as the substrate (Fig. 5(F)). These results indicate that Wft1m and Wft2m were 6-SFT and 1-SST, respectively.

Accumulation of wft1 and wft2 transcripts in winter wheat during cold hardening

In our previous report, we showed accumulation of fructan in leaf and crown tissues of winter wheat during the first stage of cold hardening by mid-November. In snow mold-resistant cultivars, the fructan levels continue to increase during the second stage of cold hardening, which lasts until snow cover is established. In contrast, freezing-tolerant cultivars accumulate mono- and disaccharides instead of fructan during the second stage of cold hardening. Table 1 shows different levels of fructan accumulation in field-grown wheat cultivars sampled at various dates. More fructan accumulated in the snow mold-resistant cultivars PI 173438 and Norin 62 than in the other cultivars in 1998. Fructan accumulated at considerable levels in all cultivars from mid-October to mid-November and then the accumulation rates decreased by mid-December. Figure 6 shows the expressions of wft1 and wft2 in wheat sampled at the same dates for sugar analysis. The levels of both gene transcripts were higher in crown tissues than in leaf tissues at every sampling date (data not shown). The levels of wft2 transcripts had increased in the crown tissues of all cultivars during cold hardening by December. However, the levels of wft1 and wft2 transcripts in leaf tissues increased from mid-October to mid-November and accumulation of both gene transcripts ceased or decreased by December in leaf tis-
Fig. 5. HPAE Separation of Oligo-fructans Generated by Wft2m in Culture Medium of Transformed *P. pastoris*.

The reaction was done with 50 mM sucrose (B), combination of 50 mM sucrose and 50 mM 1-kestose (E) or 50 mM 1-kestose (F) at 4°C for 17 h. A and D are chromatograms of the 0 h-solutions for B and E, respectively. C indicates a chromatogram of standard sugars: 1, glucose; 2, fructose; 3, sucrose; 4, 1-kestose; 6, 1,1-nystose. HPAE-PAD was done using DX 300 and PA-1 column (Dionex). A small amount of 1,1-nystose was included in 1-kestose substrate. Peaks without numbers could not be identified and the single peak marked with an asterisk included in the reaction medium is different from that of fructose.

<table>
<thead>
<tr>
<th>Cultivars (Resistance *)</th>
<th>Leaf</th>
<th></th>
<th></th>
<th></th>
<th>Crown</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Ref LT&lt;sub&gt;50&lt;/sub&gt; (°C)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Oct. 18</td>
<td>Nov. 15</td>
<td>Dec. 10</td>
<td></td>
<td>Oct. 18</td>
<td>Nov. 15</td>
<td>Dec. 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI 173438 (SR)</td>
<td>17 ± 9</td>
<td>109 ± 10</td>
<td>124 ± 4</td>
<td></td>
<td>27 ± 8</td>
<td>130 ± 1</td>
<td>130 ± 3</td>
<td></td>
<td>- 19</td>
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</tr>
<tr>
<td>Norin 62 (SR)</td>
<td>10 ± 4</td>
<td>76 ± 10</td>
<td>112 ± 12</td>
<td></td>
<td>26 ± 2</td>
<td>123 ± 19</td>
<td>147 ± 16</td>
<td></td>
<td>- 20</td>
<td></td>
</tr>
<tr>
<td>Chihokukomugi (M)</td>
<td>8 ± 6</td>
<td>56 ± 13</td>
<td>49 ± 7</td>
<td></td>
<td>22 ± 4</td>
<td>100 ± 9</td>
<td>91 ± 10</td>
<td></td>
<td>- 20</td>
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</tr>
<tr>
<td>Norstar (FT)</td>
<td>3 ± 1</td>
<td>45 ± 18</td>
<td>53 ± 11</td>
<td></td>
<td>9 ± 5</td>
<td>85 ± 21</td>
<td>89 ± 26</td>
<td></td>
<td>- 24</td>
<td></td>
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<tr>
<td>Valuevskaya (FT)</td>
<td>14 ± 16</td>
<td>49 ± 8</td>
<td>48 ± 6</td>
<td></td>
<td>22 ± 5</td>
<td>92 ± 8</td>
<td>87 ± 8</td>
<td></td>
<td>- 24</td>
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</tbody>
</table>

Values of fructan content represent the mean ± SD of three measurements.

* SR, snow mold resistant; M, moderately snow mold resistant; FT, freezing tolerant and snow mold susceptible, based on the published data from other laboratories.9) § Values of LT<sub>50</sub> (°C) of cultivars measured at early December 1994, based on data from our laboratories.9

Sues of almost all cultivars (Fig. 6). Snow mold-resistant cultivars contained higher levels of *wft1* and *wft2* transcripts than the other cultivars. In particular, the levels of *wft2* transcripts increased greatly in leaf tissues of the snow mold-resistant cultivar PI 173438 by December, but no such increases were noted in other cultivars after mid-November. The seasonal accumulation patterns of *wft1* and *wft2* transcripts roughly reflected fructan levels in different tissues from different cultivars.

**Discussion**

It is necessary to identify the precise catalytic functions of cloned genes of fructan-biosynthesizing enzymes and invertase because these enzymes have not only much similarity in their amino acid sequences but also catalytic similarities. Some invertases have 1-SST activity at high sucrose concentrations, and some fructosyltransferases involved in fructan synthesis have invertase activity (e.g., barley 6-SFT). Recently, a new technique for expressing secreted recombinant proteins in the culture medium...
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of the methylotrophic yeast *P. pastoris* has been used for the characterization of genes encoding sugar-metabolizing enzymes such as invertase and 6-SFT. Our results using this system indicate that both Wft1 and Wft2 encode fructan-synthesizing enzymes. The profiles of reaction products of recombinant Wft1m protein were similar to those of barley 6-SFT expressed by a similar methylotrophic yeast system. Likewise, the specific production of 1-kestose with low release of fructose when sucrose was the substrate, characterized with recombinant Wft2m protein, was similar to that for purified barley 1-SST and tall fescue 1-SST expressed by *P. pastoris*. Purified plant invertases do not produce 6-kestose and bifurcose and produce less 1-kestose than fructose when the sucrose concentration is 50 mM. On the basis of the results of these studies and our findings, we concluded that Wft1 and Wft2 were 6-SFT and 1-SST, respectively, of wheat. This report is the first of the cloning of 1-SST and 6-SFT from the same species.

In this study, the course of accumulation of *wft1* and *wft2* transcripts in field-grown wheat cultivars during cold hardening closely resembled that of fructan accumulation. A relationship between ambient temperatures and fructan levels in wheat cultivars during cold hardening was reported previously. In brief, winter wheat cultivars accumulate fructan during cold-hardening processes as long as the minimum temperature remains above 0°C (the first stage of cold hardening). However, differences become clearer between sugar accumulation patterns of freezing-tolerant cultivars and those of snow mold-resistant cultivars after mid-November (the second stage of cold hardening) when freezing temperatures prevail in wheat fields in Sapporo, located in the north part of Japan. From mid-November, mono- and disaccharide contents increase in freezing-tolerant and moderately freezing-tolerant cultivars, but snow mold-resistant cultivars continue to accumulate fructans. In this study, we showed that increases in *wft1* and *wft2* transcripts in leaf and crown tissue of wheat cultivars grown until November 15, (i.e., until the end of the first stage of cold hardening) were related to the accumulation of fructan in wheat tissues. Our results indicated that the increased expression of *wft2* encoding 1-SST, which produces 1-kestose (a first step in fructan synthesis), might reflect much accumulation of fructan in the crown tissues in the first stage, because there was no major change in the level of *wft1* transcripts in wheat crown tissues throughout cold hardening. In contrast, the accumulation of *wft1* and *wft2* transcripts in leaves was different in different wheat cultivars from November 15 to December 10. This result indicates that there is differential expression of genes between freezing-tolerant cultivars and snow mold-resistant cultivars in response to freezing temperatures during the sec-

### Table 1: Northern Blot Analysis of *wft1* and *wft2* Transcripts in Leaf and Crown Tissues of Winter Wheat Cultivars, cv. PI 173438, Norin 62, Chihokukomugi, Norstar, and Valuevskaya, Sampled at October 18 (1), November 15 (2) and December 10 (3), 1998.

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>PI 173438</th>
<th>Norin 62</th>
<th>Chihokukomugi</th>
<th>Norstar</th>
<th>Valuevskaya</th>
</tr>
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<tbody>
<tr>
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</table>

### Fig. 6: Northern Blot Analysis of *wft1* and *wft2* Transcripts in Leaf and Crown Tissues of Winter Wheat Cultivars, cv. PI 173438, Norin 62, Chihokukomugi, Norstar, and Valuevskaya, Sampled at October 18 (1), November 15 (2) and December 10 (3), 1998.

mRNA levels in crown tissues were so high that exposure time was shorter for crown-blots than for leaf-blots. The 25S rRNA bands stained with ethidium bromide are shown as a loading control at the bottom.
ond stage of cold hardening.

Several studies reported conversion of fructan to simple sugars, glucose and fructose, and to sucrose, in winter cereals exposed to the temperature of −3°C, all of which sugars were deposited intercellularly.27,38 Livingston and Henson39 showed that apoplastic sugars, including low-molecular-weight fructan, and the activity of β-fructosidases, such as invertase and fructan exohydrolase, increased in the second phase of cold hardening in winter oat. Freezing-tolerant wheat cultivars may increase in hardness in response to sub-zero temperatures by converting fructan to mono- and disaccharides, all of which have a cryoprotective function. In addition, the levels of wft1 and wft2 transcripts decreased in the leaf tissues of the freezing-tolerant cultivars Norstar and Chihokukomugi in December, so the synthesis of fructan in the freezing-tolerant wheat cultivars may be down-regulated at the level of transcription in the second stage of cold hardening, since the supply of simple sugars in tissues may improve the freezing tolerance of the plant. In contrast with freezing-tolerant cultivar, the persistent increases in the transcript levels of both genes at higher levels in the snow mold-resistant cultivars PI 173438 and Norin 62 in December presumably resulted in the accumulation of large amounts of fructan, which may be advantageous for snow mold resistance with respect to the energy supply.40 Thus, our results are consistent with the idea that Wft1 and Wft2 are an important in fructan accumulation of winter wheat during cold hardening.

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