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Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing

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15 *Abbreviations:* BAP, benzylaminopurine; CaMV, cauliflower mosaic virus; 2,4-D, 2,4-dichlorophenoxyacetic acid; 1-FFT, fructan-fructan 1-fructosyltransferase; 6G-FFT, fructan-fructan 6G-fructosyltransferase; HPLC, high performance liquid chromatography; 6-SFT, sucrose-fructan 6-fructosyltransferase; 1-SST, sucrose-sucrose 1-fructosyltransferase; PFD, photosynthetic flux density; PCR, 20 polymerase chain reaction; RT-PCR, reverse transcription-PCR.

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Abstract

The accumulation of fructan in grasses during autumn is linked to winter hardiness. Genetic manipulation of the accumulation of fructan could be an important molecular breeding strategy for the improvement of winter hardiness in grasses. We produced transgenic perennial ryegrass (*Lolium perenne*) plants that overexpress wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose-fructan 6-fructosyltransferase (6-SFT) and sucrose-sucrose 1-fructosyltransferase (1-SST), respectively, under the control of CaMV 35S promoter using a particle bombardment-mediated method of transformation. Significant increases in fructan content were detected in the transgenic perennial ryegrass plants. A freezing test using the electrical conductivity method indicated that transgenic plants that accumulated a greater amount of fructan than non-transgenic plants have increased tolerance on a cellular level to freezing. The results suggest that the overexpression of the genes involved in fructan synthesis serves as a novel strategy to produce freezing-tolerant grasses.

Keywords: Freezing tolerance; Fructan; *Lolium perenne*; Transgenic plants

1. Introduction

Fructan, a polymer of fructose and a major component of nonstructural carbohydrates, is accumulated in many plants, especially C3 temperate grasses [1].

5 The accumulation of fructan in temperate grasses and winter wheat during autumn is linked to both acclimation and resistance to attacks by snow molds [2]. Snow mold-resistant cultivars of temperate grasses accumulate higher levels of fructan and metabolize them at slower rates than susceptible cultivars [3]. Thus, fructan accumulation is associated with winter hardiness in temperate grasses. It has been

10 concluded that at least four enzymes are involved in fructan synthesis in higher plants [4]. These enzymes are sucrose-fructan 6-fructosyltransferase (6-SFT), sucrose-sucrose 1-fructosyltransferase (1-SST), fructan-fructan 1-fructosyltransferase (1-FFT) and fructan-fructan 6G-fructosyltransferase (6G-FFT). Following the cloning of cDNA of barley (*Hordeum vulgare*) 6-SFT [5], genes encoding a range

15 fructosyltransferases were isolated from several plants: 6-SFT from wheatgrass (*Agropyron cristatum*) [6], big bluegrass (*Poa secunda*) [7] and winter wheat (*Triticum aestivum*) [8]; 1-SST from chicory (*Cichorium intybus*) [9], globe artichoke (*Cynara scolymus*) [10], onion (*Allium cepa*) [11], Jersalem artichoke (*Helianthus tuberosus*) [12], dandelion (*Taraxacum officinale*) [13], tall fescue (*Festuca arundinacea*) [14],

20 winter wheat [8], and perennial ryegrass (*Lolium perenne*) [15,16]; 1-FFT from globe artichoke [17] and Jersalem artichoke [12]; and 6G-FFT from onion [18].

Many transgenic plants carrying genes encoding the bacterial fructan polymerase, levansucrase, were made. For example, transgenic Italian ryegrass (*Lolium*

multiflorum) expressing a *SacB* gene, which was isolated from *Bacillus subtilis*, and accumulating fructans was produced recently [19]. Transgenic plants carrying genes encoding plant-derived fructosyltransferase were also made. For instance, barley 6-SFT gene was introduced into tobacco (*Nicotiana tabacum*) and chicory [20],
5 Jerusalem artichoke 1-SST gene and/or 1-FFT gene was introduced into sugarbeet (*Beta vulgaris*) [21] and petunia (*Petunia hybrida*) [12] and globe artichoke 1-SST gene and/or 1-FFT gene was introduced into potato (*Solanum tuberosum*) [10].

In some reports of transgenic plants carrying genes encoding the bacterial fructosyltransferase, the plants' tolerance of environmental stresses was tested. For
10 example, the tobacco plants carrying *SacB* showed increased drought tolerance under drought stress conditions [22] and increased cold tolerance [23], and those carrying *levU*, also a levansucrase gene, showed increased osmotic stress tolerance [24]. Also, the sugarbeet carrying *SacB* had enhanced resistance to drought [25]. Although Schellenbaum *et al.* [26] reported changes in the fructan accumulation of transgenic
15 tobacco plants carrying barley 6-SFT gene under drought stress, there has been no report on the improvement of tolerance to cold stress in transgenic plants carrying plant-derived fructosyltransferase genes.

Perennial ryegrass is one of the most important temperate grasses in the world because of its particularly high digestibility combined with its good tolerance to
20 grazing and adequate seed production [27]. However, in general, perennial ryegrass has less winter hardiness than other temperate grasses, such as timothy (*Phleum pratense*) and orchardgrass (*Dactylis glomerata*), because of its greater susceptibility to snow mold diseases and freezing [28].

Recently, Kawakami and Yoshida [8] isolated two cDNAs of winter wheat, designated *wft1* and *wft2*, which encode 6-SFT and 1-SST, respectively. These cDNAs are involved in the synthesis of fructan during cold acclimation. The expression of these genes was increased during cold acclimation in winter wheat cultivars. In this
5 study we obtained transgenic perennial ryegrass plants carrying *wft1* and *wft2* in order to improve winter hardiness. Significant increases in both fructan content and freezing tolerance on a cellular level were detected in the transgenic perennial ryegrass plants.

2. Materials and Methods

2.1 Callus induction

5 Embryogenic calli of diploid perennial ryegrass (cv. 'Riikka') were used for transformation. The calli were induced from seeds on callus induction medium (IM: based on MS medium supplemented with 30 g/l sucrose, 500 mg/l casamino acid, 5 mg/l 2,4-D and 3 %(w/v) gellan gum, pH adjusted to 5.8 before autoclaving) [29] under dark conditions (25°C).

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2.2 Transformation of perennial ryegrass by particle bombardment

Transformation of perennial ryegrass plants were performed essentially as described by Spangenberg *et al.* [30] except that calli were used as materials for
15 transformation in the present study.

Plasmids pE7133-SFT and pE7133-SST were constructed using pE7133-GUS [31] by replacing the GUS gene which was regulated by CaMV 35S promoter with coding region of *wft1* (accession No. AB029887) and *wft2* (accession No. AB029888), respectively (Fig. 1A, B). These plasmids were used for transformation by particle
20 bombardment. pGreen0229 [32] containing bialaphos-resistant gene (*bar*) regulated by NOS (nopalinsynthetase) promoter (Fig. 1C) was also used. These three plasmids were co-bombarded at every bombardment.

Six- to ten-week-old calli were transferred for osmotic pretreatment to osmotic

medium (OM0.3: based on IM containing 0.3M sorbitol and 0.3M mannitol). After three hours of pre-treatment with OM0.3, the calli were bombarded with gold particles coated with plasmids using a particle gun (GIE-III, Science TANAKA, Ishikari, Hokkaido, Japan). For bombardment, gold particles were coated with plasmid DNA according to *Finer et al.* [33]. Parameters for bombardment (i.e., bombardment pressures, bombardment distances, and DNA amount) were adjusted beforehand by a transient assay using a reporter gene. After bombardment, the calli were kept on the osmotic medium for 24 hours, and then transferred to IM.

10 2.3 Selection of transgenic plants by bialaphos

One week after the bombardment, the calli were transferred to bialaphos selection medium (based on IM containing 2 or 3 mg/l bialaphos) and grown for 4 weeks with a fortnightly subculture. Regeneration was performed on regeneration medium (based on MS medium supplemented with 30 g/l sucrose, 500 mg/l casamino acid, 0.1 mg/l 2,4-D, 1 mg/l BAP and 3% (w/v) gellan gum, and with its pH adjusted to 5.8 before autoclaving). Calli were grown for between 3 and 12 weeks with 3-weekly subculture under 16-hour light and 8-hour dark conditions. All of the cultures performed at 25°C.

20 Regenerated small plants were transferred to root formation medium (based on 1/2 MS medium supplemented with 15 g/l sucrose, 250 mg/l casamino acid and 3% (w/v) gellan gum, pH adjusted to 5.8 before autoclaving) and the regenerated plants were transferred to soil. The regenerated plants were grown at 22/18°C (day/night),

under 14-hour daylight and 100 to 110 $\mu\text{mol}/\text{m}^2\text{s}$ PFD conditions.

2.4 DNA isolation and PCR analysis

5 Total DNA was extracted from the leaves of perennial ryegrass plants following the CTAB method [34]. PCR was carried out in a total volume of 30 μl using 500 ng total DNA as a template and using *rTaq*TM (TaKaRa, Tokyo, Japan) as the DNA polymerase. To amplify transgenes *wft1* or *wft2*, a primer specific to CaMV 35S promoter (35SP-1: 5'-TATCTCCACTGACGTAAGGG-3') and a primer specific to the
10 coding region of *wft1* (INV-Tail-SFT2: 5'-CATCCACGGTAGTACATCAG-3') or a primer specific to the coding region of *wft2* (SST-Rev1: 5'-ACGATTTGGGGCTCCCAC-3') were used. PCR cycling conditions were 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for *wft1* and 96°C for 1.5 min, 55°C for 1 min, and 72°C for 1 min for *wft2*. These cycles were repeated 28 times, and the
15 reaction mixture was then further incubated at 72°C for 5 min.

2.5 RNA extraction and RT-PCR analysis

Total RNA was extracted from leaves of perennial ryegrass essentially by the
20 method of Chomczynski and Sacchi [35] using a guanidine-HCl extraction buffer. DNA was removed by treatment with DNaseI. The cDNA synthesis from total RNA and the PCR that followed were performed according to the method of Tominaga *et al.* [36]. 1 μg of total RNA was used as a template for cDNA synthesis, and the

poly(dT)-adapter primer (B26:

5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT-3') was used as a primer for reverse transcription. The cDNA synthesis was carried out in a total volume of 20 µl, and 1 µl of the cDNA solution was used for PCR. Two *wft1*-specific primers (SFT-For1: 5'-CGCCGTGGCCGAGCAAAC-3' and SFT-RT-Rev1: 5'-CCATATTGGAGAGCTGGTTG-3') were used to amplify the *wft1* transcripts. A *wft2*-specific primer (SST-For3: 5'-GCCTCCGCCATTGAGGC-3') and an adapter primer (B25: 5'-GACTCGAGTCGACATCGA-3') were used to amplify *wft2* transcripts. The transcripts of ubiquitin activating factor gene were amplified by two specific primers (Lp ubiquitin For: 5'-CACCGGAAGTAGACGGATAC-3' and Lp ubiquitin Rev: 5'-CACCAAGACATGACGTGGAC-3') to act as a control for RT-PCR.

2.6 Southern hybridization

The products of PCR and RT-PCR were separated by electrophoresis on a 0.8 % (w/v) agarose gel in Tris-Borate-EDTA buffer. After the products of PCR and RT-PCR were blotted onto a membrane (Hybond-N+™, Amersham Biosciences Corp., Piscataway, NJ USA), hybridization was performed using AlkPhos Direct™ Labelling and Detection Systems (Amersham Biosciences Corp.). The labelling of probes, hybridization, and washing of the membrane were performed according to the protocol of AlkPhos detection systems.

2.7 Sugar extraction and determination

For the extraction of sugars, leaves of transgenic and non-transgenic plants were sampled three times every 2 weeks. Total water-soluble sugars were extracted from finely chopped leaves in boiling deionized water for 1 hour. Total sugars were measured by HPLC with Shodex columns of KS-802 and KS-803 combined (Shodex, Tokyo, Japan), using the refractive index detector as described by Yoshida *et al.* [3].

2.8 Freezing test

Leaf blades from transgenic and non-transgenic plants that were cold-acclimated (6°C/2°C day/night, 8-hour daylight and 30 to 40 $\mu\text{mol}/\text{m}^2\text{s}$ PFD) for 3 weeks were used for a test of freezing tolerance. Approximately 1.5 cm-cut leaf blades were placed into a 1.5 ml tube with ice-cold 100 μl -deionized water and frozen by a programmed freezer (MPF-1000, EYELA, Tokyo, Japan). The temperature was kept at -2°C for 16 hours and then dropped 1°C per hour to -12°C. As the temperature decreased, samples were taken at -4°C, -8°C and -12°C. After 1.5 cm-cut leaf blades in tubes were thawed slowly at 4°C for 16 hours, 900 μl -deionized water was added to each tube, and the samples were incubated at 4°C for 24 hours. The value of electrical conductivities of the resulting solution was measured using a conductance meter (Twin Cond, HORIBA, Kyoto, Japan). A comparative value for 100 % leakage was obtained by freezing these samples at -80°C for 24 hours after measuring the conductivity. The level of electrolyte leakage was calculated as the percentage of the conductivity before treatment at -80°C over that after treatment at -80°C.

3. Results

3.1 Plant transformation

5 The transformation of perennial ryegrass plants with the *wft1*, *wft2* and *bar* genes was performed by a particle bombardment-mediated method. The putative transformants obtained were selected on MS medium supplemented with 2 or 3 mg/l bialaphos. The presence or absence of transgenes in the plants selected by bialaphos-resistance was examined by PCR amplification of the transgene sequences, 10 which was followed by Southern blot analysis of the PCR products (Fig. 2). For PCR, primers specific to the sequences of CaMV 35S promoter and *wft1* or *wft2* genes were used. Signals of the expected sizes (0.56 kb and 0.75 kb for *wft1* and *wft2*, respectively) were detected in the analysis. Additional bands were detected in some putative transgenic perennial ryegrass plants. These could be due to non-specific 15 amplification by PCR. 1,488 calli were used for particle bombardment-mediated transformation, and 134 bialaphos-resistant green shoots were regenerated from 20 calli that were selected by bialaphos. DNA analysis was done on 71 of the 134 bialaphos-resistant plants. Among these 71 bialaphos-resistant plants, 27 plants carried *wft1*, 6 carried *wft2* and 4 carried both genes. No morphological 20 abnormalities were observed in the transgenic plants (Fig. 3).

3.2 Determination of sugar and fructan contents by HPLC

The glucose, fructose, sucrose and fructan contents of the plants transgenic for *wft1* and/or *wft2* as well as those of the non-transgenic control plants were determined by HPLC (Fig. 4). The fructan contents of the transgenic plants “7-2”, “7-3”, “9-1”, “10-1” and “14-1” with *wft1* or *wft2* were three to fifteen-fold greater than those of non-transgenic plants (Fig. 4A). There were significant differences in fructan content between all the plants transgenic for either *wft1* or *wft2* (“7-2”, “7-3”, “9-1”, “10-1” and “14-1”), and the non-transgenic plants. However, no significant difference in fructan content was detected between plants transgenic for both *wft1* and *wft2* (“2-3” and “6-9”) and the non-transgenic plants (Fig. 4A).

The sucrose, glucose and fructose contents were also measured (Fig. 4B). No difference in sucrose content was found between the transgenic plants and the non-transgenic plants, while significant differences in the glucose and fructose contents were detected between the transgenic plants that accumulated a large amount of fructan and the non-transgenic plants.

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3.3 Analysis of transgene transcription

Total RNA was extracted from the leaves of transgenic and non-transgenic perennial ryegrass. The presence of the transcripts of transgenes was analyzed by RT-PCR. To confirm the specific amplification by RT-PCR, products of RT-PCR were further analyzed by Southern hybridization (Fig. 5). Transcripts of *wft1* were detected in “7-3” and “14-1” plants that accumulated a large amount of fructan but were not detected in non-transgenic and “2-3” plants that accumulated less fructan (Fig.

5A). Also, transcripts of *wft2* were detected in “7-2”, “9-1” and “10-1” plants that accumulated a large amount of fructan, but were not detected in non-transgenic plants and “2-3” plants (Fig. 5C). The transcripts of the ubiquitin activating factor gene were equally amplified from every cDNA sample, suggesting that cDNA synthesis was
5 done equally from each mRNA sample (Fig. 5B, D).

3.4 Test of freezing tolerance

The comparison of freezing tolerance between transgenic (“7-2”, “7-3”, “9-1” and
10 “10-1”) and non-transgenic plants was carried out using the electrical conductivity method, also known as the ion leakage method, which has been used extensively to evaluate freezing tolerance through measuring the release of cellular electrolytes after freezing [37] (Fig. 6). While there was no difference in electrolyte leakage between the plants after they were treated at -4°C , the electrolyte leakages for transgenic plants
15 were lower than for non-transgenic plants after the treatments at -8°C and -12°C . This result indicated that transgenic plants that accumulated high levels of fructan have increased tolerance on a cellular level to freezing.

4. Discussion

We introduced the wheat fructosyltransferase genes, *wft1* and *wft2*, regulated by CaMV 35S promoter, into perennial ryegrass for the purpose of improving its winter
5 hardiness. We succeeded in obtaining transgenic perennial ryegrass plants that carry the wheat fructosyltransferase genes and accumulate greater fructan content than non-transgenic perennial ryegrass plants (see Fig. 4). Accumulation of transgene mRNA was detected in the transgenic perennial ryegrass plants (see Fig. 5).
10 However, the levels of mRNA and fructan contents in plants transgenic for both *wft1* and *wft2* were lower than those in plants transgenic for either *wft1* or *wft2*. This can be attributed to the fact that multi-copies of transgene may induce homology-dependent gene silencing because two transgenes have the same promoter, and moreover, sequence-homology is very high between *wft1* and *wft2*.

Previously, bacterial fructan synthase (levansucrase) genes were introduced into
15 non-fructan accumulating higher plants, such as tobacco [22-24,38-41], potato [40,42-44], maize (*Zea mays*) [45] and sugarbeet [24] for the purpose of analyzing fructan metabolisms and cellular localizations as well as the regulatory mechanisms of fructan accumulation. In these reports, some transgenic tobacco and sugarbeet plants were shown to be tolerant to environmental stresses, such as drought and cold [22-25].
20 Although the fructan content and stress tolerance were increased, the transformants expressing bacterial fructan synthesis genes often exhibited a number of aberrant phenotypes such as stunting, leaf bleaching, necrosis, inhibition of development, reduction in starch accumulation and chloroplast agglutination [46]. For example,

SacB-transgenic Italian ryegrass plants, which were made for the analysis of the regulation and role of fructan metabolism, were stunted and had narrower leaves [19]. A number of developmental aberrations seem to be associated with the expression of levansucrase activity. Moreover, contrary to the expectations of Ye *et al.* [19], who performed the study, the total amount of fructan in the transgenic Italian ryegrass plants was decreased. Ye *et al.* [19] considered this phenomenon to be due to the activity of native fructan exohydrolase (FEH). However, transformants carrying plant-derived fructan synthase (fructosyltransferase) genes did not exhibit the phenotypic aberrations that were caused by the expression of bacterial levansucrase genes [46]. Because no aberrant development was observed in the present study (Fig. 3), we can conclude that plant-derived fructosyltransferase would probably be more suitable for the genetic transformation of intrinsically fructan-accumulating plants such as grasses than bacterial levansucrase. One likely explanation for this is that plant-derived fructosyltransferase genes such as *wft1* and *wft2* encode signal peptides that direct fructosyltransferases to the vacuole, whereas bacterial levansucrase genes do not encode these sorting signals. Because we used the entire sequences of the coding regions of plant-derived fructosyltransferase genes, *wft1* and *wft2*, for transformation, fructans are most likely accumulated in vacuoles in the transgenic perennial ryegrass plants.

Recently, fructan metabolism-related genes encoding 6-SFT, 1-SST, 1-FFT and 6G-FFT have been isolated and characterized in plants [5-10,12-14,17,18]. In perennial ryegrass, 1-SST gene has been isolated and characterized [15,16]. Some of these genes were introduced to non-fructan-accumulating plants, such as tobacco

[20,26], potato [10,47], petunia [12] and sugarbeet [21] and naturally-fructan-accumulating chicory [17,19] for the purpose of analyzing fructan metabolisms. Though plant-derived fructosyltransferase genes have been introduced into some dicotyledonous plants, they have not yet been introduced into monocotyledonous plants that accumulate fructan intrinsically. To our knowledge, this is the first report regarding the introduction of plant-derived fructosyltransferases into monocotyledonous plants.

Kawakami and Yoshida [8] examined whether the expression of endogenous fructosyltransferases and fructan content are up-regulated in wheat during cold acclimation. They were able to demonstrate that the gene expression of *wft1* and *wft2* and the fructan content all increased during cold acclimation in leaf and crown tissues of winter wheat. These results suggest that the accumulation of fructan at a high level coincides with cold tolerance in plants, and that this accumulation can be achieved by the overexpression of plant-derived fructosyltransferase genes. Based on this assumption, in this study, we produced perennial ryegrass plants that express *wft1* or *wft2* isolated from wheat. The cellular level of freezing tolerance of the perennial ryegrass plants transgenic for *wft1* or *wft2* was tested by the electrical conductivity method, whose estimations correlated with the winter survival of crops [37,48,49]. We found that these plants showed increased tolerance on a cellular level to freezing compared with non-transgenic plants (see Fig. 6) in addition to increases in fructan content (see Fig. 4). Our present results thus demonstrate that the accumulation of fructan increased tolerance on a cellular level to freezing in perennial ryegrass. We were not able to find other reports on increasing tolerance to freezing in transgenic

plants that carried plant-derived fructosyltransferase genes. The accumulation of fructan is correlated with the resistance to snow mold diseases [3]. Further study is necessary to confirm the resistance to snow molds in transgenic plants.

In view of these circumstances, the isolation of fructan metabolism-related genes
5 will enable us to genetically dissect fructan biosynthesis in grasses. This capability will be useful for elucidating the regulation and role of fructan metabolism in the process of promoting the winter hardiness of plants and predicting the physiological consequences of genetic manipulation.

Fructan is produced as the main soluble storage carbohydrate form by grass
10 species during their growing seasons. The increased level of soluble carbohydrates appears to improve the nutritional value by animals of these grasses, particularly during the summer when grasses suffer a great decline in digestibility [50]. Transgenic plants that accumulate more fructan may also be useful for improving forage quality in breeding programs.

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Figure legends

Fig. 1. Plasmid maps of constructs used for transformation. A. Plasmid pE7133-SFT, containing *wft1* regulated by CaMV 35S promoter (P35S). B. Plasmid pE7133-SST, containing *wft2* regulated by CaMV 35S promoter. C. Plasmid pGreen0229 containing bialaphos-resistant gene (*bar*) regulated by nopaline synthase promoter (Pnos). The arrows indicate the position of primers used for PCR analysis of the transgene presence. E7, 5'-upstream enhancer sequence of CaMV 35S promoter [30]; Ω , 5'-untranslated sequence of TMV; I, first intron of a gene for phaseolin; Tnos, nopaline synthase terminator; *lacZ*, β -galactosidase gene.

Fig. 2. Southern blot analysis of the PCR products of transgenes amplified from regenerated plants that were selected by bialaphos. Fragments of *wft1* (panel A) or *wft2* (panel B) were amplified by PCR from DNA samples of regenerated plants (lanes 1-12) and a non-transgenic control plant (lane C). PCR was also performed without adding template DNA to the reaction mixture to obtain a negative control of PCR (lane N) or was performed using cloned *wft1* or *wft2* sequences as positive controls of PCR (lane P). These DNAs were hybridized with a *wft1*-specific probe (panel A) or a *wft2*-specific probe (panel B) after being separated by electrophoresis and blotted to a membrane. Lane M indicates the DNA size marker (Φ X174/ *Hae*III digest). Arrows indicate the positions of the PCR products of *wft1* or *wft2*. The locations of the designed primers are shown in Fig. 1.

Fig. 3. Phenotype of transgenic perennial ryegrass plants. A. Regenerated plants selected by bialaphos. B. Heading of transgenic perennial ryegrass containing *wft1* (“7-3” plant; right side) and non-transgenic perennial ryegrass (left side). C. Spikes of transgenic perennial ryegrass (“7-3” plant; two spikes on the right side) and non-transgenic perennial ryegrass (two spikes on the left side).

Fig. 4. Sugar contents of the leaves of transgenic and non-transgenic plants. The amounts of fructan (panel A) and glucose, fructose and sucrose (panel B) were measured by HPLC. “C1” and “C2” are non-transgenic plants regenerated from calli, and “C3” is a plant grown from a seed. The transgenic plants “7-3” and “14-1” contain *wft1*; “7-2”, “9-1” and “10-1” contain *wft2*; “2-3” and “6-9” contain both genes. The data for the transgenic plants were analyzed for significant differences from the data for the non-transgenic plants by using the *t* test. *Asterisks* indicate; *, P<0.05; **, P<0.02; ***, P<0.01 and ****, P<0.001.

Fig. 5. Analysis of transcripts of transgenes in transgenic perennial ryegrass plants by RT-PCR followed by Southern hybridization of the RT-PCR products. Transcripts of *wft1* (panel A) and *wft2* (panel C) were amplified by RT-PCR from transgenic plants that contain *wft1* (“7-3” and “14-1”), *wft2* (“7-2”, “9-1” and “10-1”) or both genes

(“2-3”), as well as from non-transgenic plants (NC). These cDNAs were hybridized with a *wft1*-specific probe (panel A) or a *wft2*-specific probe (panel C) after being separated by electrophoresis and blotted to a membrane. The reactions were carried out with reverse transcriptase (a) and without reverse transcriptase (b) as genomic DNA contamination control. Ethidium bromide-staining of the RT-PCR products of ubiquitin activating factor gene was used for a positive control (panels B and D). Arrows indicate the positions of the RT-PCR products of *wft1* (panel A), *wft2* (panel C) and ubiquitin activating factor gene (panels B and D).

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Fig. 6. Electrolyte leakage after the freezing treatment in the leaves of transgenic perennial ryegrass. The tolerance on a cellular level to freezing of transgenic (“7-2”, “7-3”, “9-1” and “10-1”) and non-transgenic (“C1” and “C2”) perennial ryegrass was determined using the electrical conductivity method. The leaf blades were treated at 15 -2°C for 16 hours in 1.5 ml tubes with ice-cold 100 μl -deionized water, and then the temperature was dropped 1°C per hour to -12°C . Samples were taken from these tubes when the temperature reached -4°C , -8°C and -12°C . The level of electrolyte leakage was calculated based on a value of the electrical conductivity.

Fig. 1. Hiroshi HISANO *et al.*

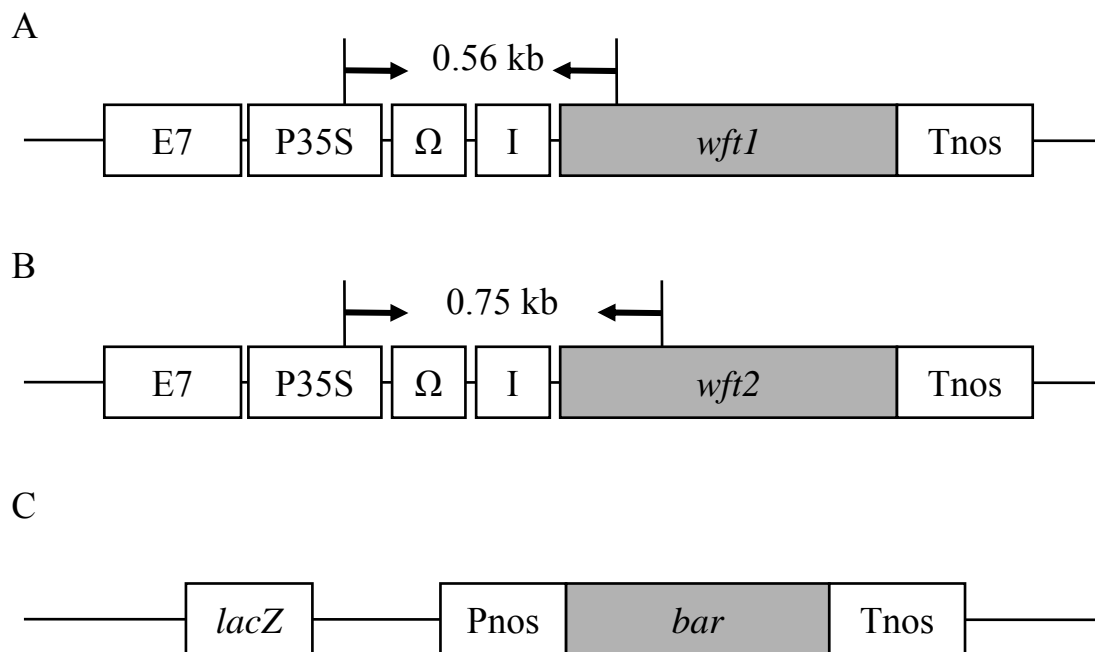


Fig. 2. Hiroshi HISANO *et al.*

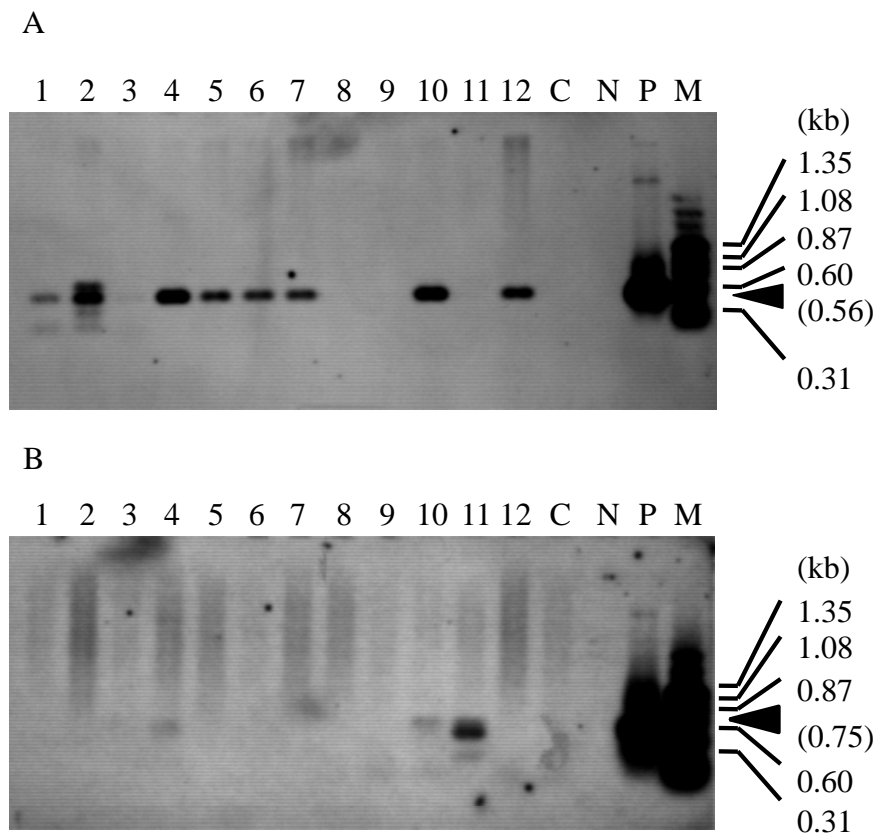


Fig. 3. Hiroshi HISANO *et al.*

A



B



C



Fig. 4. Hiroshi HISANO *et al.*

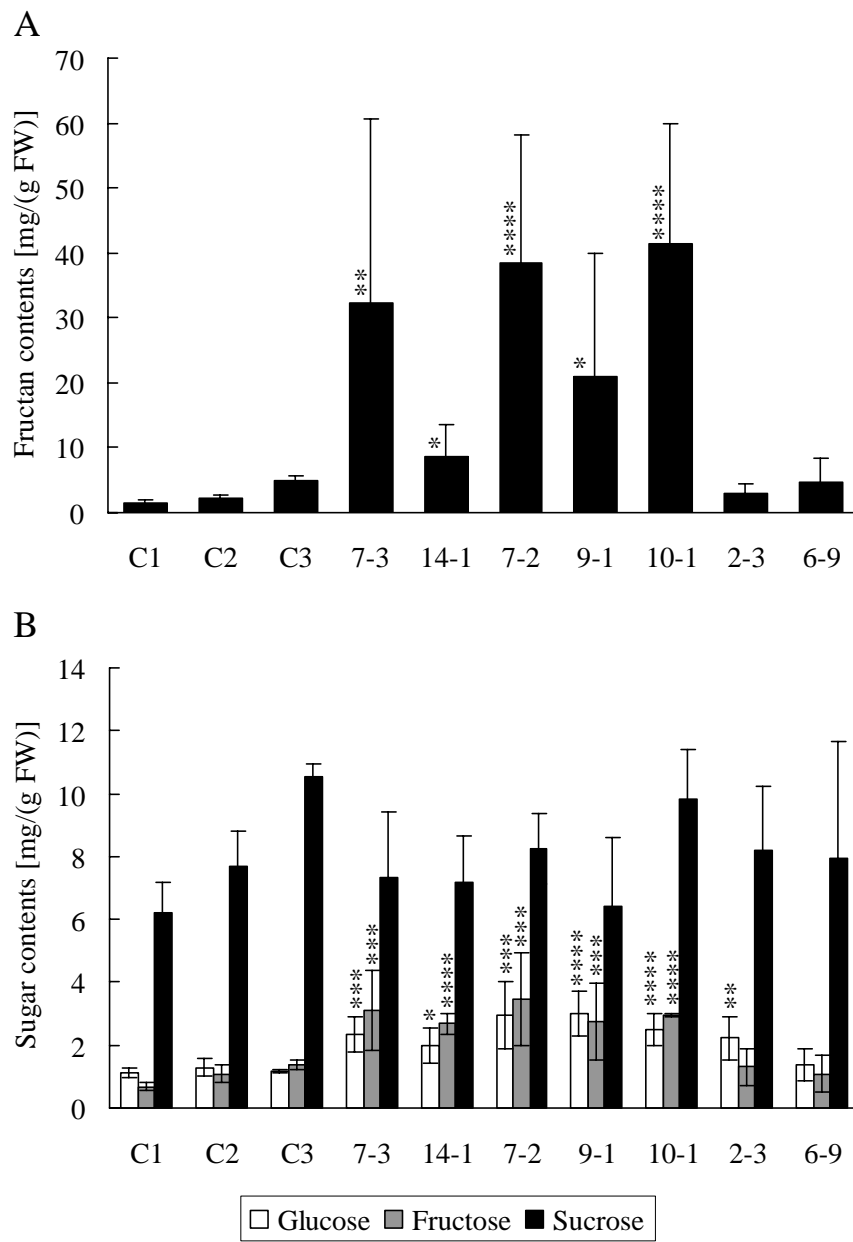


Fig. 5. Hiroshi HISANO *et al.*

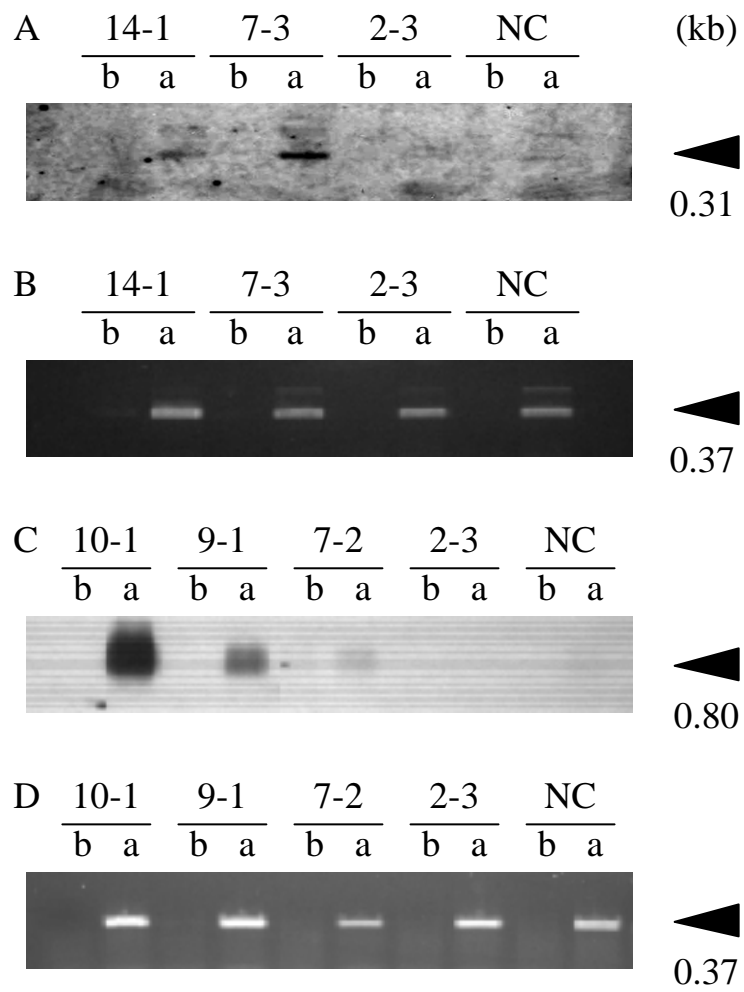


Fig. 6. Hiroshi HISANO *et al.*

