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ORIGINAL ARTICLE

Comparative study of transgenic *Brachypodium distachyon* expressing sucrose:fructan 6-fructosyltransferases from wheat and timothy grass with different enzymatic properties

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Abstract Fructans can act as cryoprotectants and contribute to freezing tolerance in plant species, such as in members of the grass subfamily Pooideae that includes Triticeae species and forage grasses. To elucidate the relationship of freezing tolerance, carbohydrate composition and degree of polymerization (DP) of fructans, we generated transgenic plants in the model grass species *Brachypodium distachyon* that expressed cDNAs for sucrose:fructan 6-fructosyltransferases (6-SFTs) with different enzymatic properties: one cDNA encoded PpFT1 from timothy grass (*Phleum pratense*), an enzyme that produces high-DP levans; a second cDNA encoded wft1 from wheat (*Triticum aestivum*), an enzyme that produces low-DP levans. Transgenic lines expressing *PpFT1* and *wft1* showed retarded growth; this effect was particularly notable in the *PpFT1* transgenic lines. When grown at 22 °C, both types of transgenic line showed little or no accumulation of fructans. However, after a cold treatment, *wft1* transgenic plants accumulated fructans with DP = 3–40, whereas *PpFT1* transgenic plants accumulated fructans with higher DPs (20 to the separation limit). The different compositions of the accumulated fructans in the two types of transgenic line were correlated with the differences in the enzymatic properties of the overexpressed 6-SFTs. Transgenic lines expressing *PpFT1* accumulated greater amounts of mono- and disaccharides than wild type and *wft1* expressing lines. Examination of leaf blades showed that after cold acclimation, *PpFT1* overexpression increased tolerance to freezing; by contrast, the freezing tolerance of the *wft1* expressing lines was the same as that of wild type plants. These results provide new insights into the relationship of the composition of water-soluble carbohydrates and the DP of fructans to freezing tolerance in plants.

Keywords *Brachypodium* · Degree of polymerization · Freezing tolerance · Fructan · Fructosyltransferase · Timothy · Wheat

Abbreviations

DP	Degree of polymerization
DTT	Dithiothreitol
FEH	Fructan exohydrolase
1-FFT	Fructan:fructan 1-fructosyltransferase
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
HPLC	High-performance liquid chromatography
LE	Ligand exchange
RI	Refractive index
SE	Size exclusion
1-SST	Sucrose:sucrose 1-fructosyltransferase
6-SFT	Sucrose:fructan 6-fructosyltransferase
PFT	Transgenic line expressing PpFT1
WFT	Transgenic line expressing wft1
WT	Wild type

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Introduction

Fructans are soluble fructose polymers derived from sucrose that are synthesized and assimilated as storage carbohydrates during photosynthesis in many plant species. The structures of fructans vary widely among plant species (Vijn and Smeekens 1999) and this variation is exemplified in plants belonging to the tribes Poeae and Triticeae (family Poaceae, subfamily Pooideae). In the Poeae, timothy grass (*Phleum pratense*), orchard grass (*Dactylis glomerata*), and big blue grass (*Poa secunda*) accumulate a simple levan comprising linear β(2,6)-linked fructose units with a terminal glucose (Chatterton et al. 1993; Bonnett et al. 1997; Chatterton and Harrison 1997; Cairns et al. 1999). However, Triticeae species, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), produce mixed levans, called graminans, composed of branched-type fructans containing β(2,6)- and β(2,1)-linked fructose residues (Carpita et al. 1989; Bonnett et al. 1997). The degree of polymerization (DP) of fructans also differs among plant species (Vijn and Smeekens 1999). The DPs of the major components of wheat fructan range from 3 to 20, while some varieties accumulate fructans with a higher DP during the winter (Yoshida and Tamura 2011). By comparison, the DP of the fructans found in timothy grass may be 90 in leaf tissue (Cairns et al. 1999) and as high as 260 in the stem base (Grotelueschen and Smith 1968). The variation in structure and DP of fructans arises from differences in the substrate specificities and the combined actions of different fructosyltransferases. In timothy grass, levans with high DPs are thought to be produced by sucrose:fructan 6-fructosyltransferase (6-SFT) which transfers fructosyl units from sucrose to a target sucrose or fructan molecule with a β(2,6)-linkage (Cairns et al. 1999; Tamura et al. 2009). In wheat, graminans are produced by the combined activities of 6-SFT, sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) (Kawakami and Yoshida 2002, 2005). A cDNA for a protein with 6-SFT activity has been identified in timothy grass as *PpFT1* (Tamura et al. 2009) and in wheat as *wft1* (Kawakami and Yoshida 2002). Comparison of the activities of recombinant PpFT1 and Wft1 enzymes identified quite different enzymatic properties as follows: (i) When sucrose is an only substrate, PpFT1 mainly synthesizes a β(2,6)-linked, linear fructan series, whereas Wft1 preferentially uses 1-kestose (β(2,1)-linked fructan with DP = 3 produced by sub-activity of Wft1) as an acceptor and generates branched fructans (β(2,1)- and β(2,6)-linked) in addition to a β(2,6)-linked, linear fructan series. (ii) PpFT1 has a higher affinity for high-DP fructans as acceptors than Wft1; thus, PpFT1 produces longer fructans than Wft1. (iii) PpFT1 has a lower substrate affinity for sucrose than Wft1 (Kawakami and Yoshida 2002; Tamura et al. 2009).

During cold acclimation before winter, fructans are accumulated in Triticeae and forage grass species. Later, they may be used as an energy source under snow during winter. In addition to their role as carbohydrate storage molecules, fructans have been suggested to have a role as a cryoprotectant and to directly or indirectly contribute to freezing tolerance at the cellular level (Valluru and Van den Ende 2008; Livingston et al. 2009). Currently, several possible mechanisms for fructan activity against freezing damage have been proposed: membrane protection, reduction in water potential, freezing point depression, and scavenging of reactive oxygen species (ROS) (Gaudet et al. 1999; Valluru and Van den Ende 2008; Livingston et al. 2009; Van den Ende and Valluru 2009; Keunen et al. 2013). Recently, it is hypothesized that small fructans act as phloem-mobile signaling compounds under stress (Van den Ende 2013). Cryoprotectant activity is thought to differ among fructans with different structures and different DPs. For example, Cacela and Hincha (2006) found increased protection of dry model membranes with an increase in the DP of inulin (a β(2,1)-linked, linear fructan) from 2 to 5. However, fructans with DPs between 7 and 17 from oat and rye do not provide cryoprotection of liposomes, whereas smaller fructans do have a protective activity (Hincha et al. 2007). Vereyken et al. (2003) reported that a bacterial levan with a DP of approximately 125 has a protective activity in liposomes during air-drying. Dionne et al. (2010) reported that freezing tolerance in bluegrass ecotypes was correlated with the amount of higher DP fructans rather than with lower DP fructans. Moreover, simple sugars such as glucose and sucrose, which are accumulated before winter and are the products of hydrolysis of fructans, are also reported to be involved in freezing tolerance and play crucial roles in ROS homeostasis (Valluru and Van den Ende 2008; Livingston et al. 2009; Bolouri-Moghaddam et al. 2010).

The in vivo physiological roles of fructan can be investigated using transgenic plants. Thus, transgenic tobacco plants overexpressing Bp6-SFT from *Bromus pictus* and 1-SST from *Lactuca sativa* accumulate fructans and show increased freezing tolerance compared to wild type plants that do not accumulate fructans (Li et al. 2007; del Viso et al. 2011). Hisano et al. (2004) generated transgenic *Lolium perenne*, a species that normally accumulates fructans, that overexpressed *wft1* (6-SFT cDNA) and *wft2* (1-SST cDNA) from wheat; they found an increase in fructan accumulation and freezing tolerance at the cellular level.

In this study, we sought to examine the relationship of freezing tolerance, carbohydrate composition and the DP of fructans. We generated two different types of transgenic line of the model species *Brachypodium distachyon* by inserting the 6-SFT cDNA *PpFT1* from timothy grass or *wft1* from wheat. *B. distachyon* is closely related to timothy

grass and wheat but does not have fructosyltransferases (Li et al. 2012). The inserted gene was expressed and the freezing tolerance of the two transgenic types was compared with regard to their accumulation of fructans with different structures and DPs, and to the levels of water-soluble carbohydrates.

Materials and methods

Plant materials

A *PpFT1* cDNA (AB436697, Tamura et al. 2009) or a *wft1* cDNA (AB029887, Kawakami and Yoshida 2002) coding an enzyme that works as sucrose:fructan 6-fructosyltransferase (6-SFT) in plant cells with different properties described in the introduction was inserted into the Ti-based vector pMLH7133 (Mitsuhara et al. 1996), downstream of the maize ubiquitin promoter (Christensen and Quail 1996) that was substituted for the CaMV 35S promoter to ensure a high level of expression of the gene in grass. The resulting plasmids were used for transformation of the *B. distachyon* diploid inbred line 'Bd21-3' by the *Agrobacterium*-mediated method using a previously reported protocol (Vogel and Hill 2008) except that *Agrobacterium* strain EHA101 was used. Regenerated T_0 plants with hygromycin resistance were screened for the presence and expression of the transgene. T_1 generation lines were screened by an analysis to detect fructans. For the *wft1* expressing lines, homozygous T_3 lines were used for subsequent analyses. For the *PpFT1* expressing lines, homozygous plants could not be generated; therefore, only transgenic plants from T_3 heterozygous lines were used after PCR confirmation of the genotype. Plants were grown in cell pots (3.5 × 3.5 cm) under normal temperature conditions of 10 h light, 100 μmol m^{-2} s^{-1} PFD, and 22 °C for 7 weeks or under cold acclimation conditions of 8 h light, 75 μmol m^{-2} s^{-1} PFD, 22 °C for 8 weeks and 4 °C for 5 weeks. Plant height and fresh weight were measured after growth under normal temperature conditions.

Carbohydrate extraction and analysis

Total water-soluble carbohydrates were extracted from 20 to 50 mg of fully expanded leaves by boiling for 1 h in 500 μl–1 ml deionized water. After filtration, the qualitative analysis of carbohydrate profiles in tissues was analyzed by high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) (DX-500, Dionex) with a Carbo Pac PA-1 anion-exchange column (Dionex) as described by Tamura et al. (2009). Glucose, fructose, sucrose, 1-kestotriose (Wako Chem.) and 6-kestotriose (Iizuka et al. 1993) were used for known standards to identify peaks. Products of the Wft1 and PpFT1 recombinant enzyme generated by *Pichia pastoris* with sucrose (Tamura et al. 2009) were used as standards for polymerized fructans. Extracted sugar solutions were treated with mild acid or fructan exohydrolase (FEH) to degrade fructans. Mild acid treatment was performed using 0.06 N HCl at 70 °C for 1 h. Recombinant enzyme solution of Pp6-FEH1 produced by *P. pastoris* prepared as in Tamura et al. 2011 (0.1 mg/ml) was incubated with the extracted sugar solution at 30 °C for 13 h in 20 mM citrate–phosphate buffer (pH 5.2). The quantitative measurements of carbohydrate contents were performed according to the method as described by Yoshida et al. (1998). The filtrated carbohydrate solution was measured by high-performance liquid chromatography (HPLC) using Shodex KS-802 and KS-803 columns with the size exclusion (SE) and ligand exchange (LE) separation mode (Showa Denko), and an L-2490 refractive index (RI) detector (Hitachi). Propylene glycol (1 mg ml^{-1}) was used as the internal standard to control the extraction efficiency.

Assay of fructosyltransferase activities in crude enzymatic extracts

Crude enzymatic extracts were prepared using 20 mM citrate–phosphate buffer (pH 5.2) containing 1 mM dithiothreitol (DTT) and 0.2–1.0 g of shoots from plants grown under normal temperature conditions. Following centrifugation at 8,000*g* for 15 min, proteins in the resulting supernatants were concentrated by precipitation with 70 % ammonium sulfate. The pellets were dissolved in 0.1–0.5 ml of 20 mM citrate–phosphate buffer (pH 5.2) with 1 mM DTT and desalted using a Biospin-30 column (Bio-Rad). Twenty microliters of enzymatic solution, including ca 0.7 μg μl^{-1} of protein was incubated with 20 μl of a solution containing 2 M sucrose, 0.2 % bovine serum albumin, 20 mM citrate–phosphate buffer, and 1 mM DTT at 37 °C for 4 h. The reaction was terminated at 95 °C for 3 min. The enzymatic products were analyzed by HPAEC-PAD as described above.

Freezing tolerance assay

Fully expanded leaf blades from each plant were used to test freezing tolerance. Two or three leaf blade segments of approximately 2 cm were placed on ice produced by freezing 100 μl deionized water in a 1.5-ml tube. The temperature was held at −2.5 °C for 16 h and then decreased by 1 °C per hour to a minimum temperature of −12 °C using a programmed freezer (MPF-1000, Eyela, Tokyo, Japan). As the temperature decreased, samples were taken at each temperature. Deionized water (1.2 ml) was added to each tube, and the samples were incubated with shaking at room

temperature for 4 h. Electrical conductivities of the resulting solutions were measured using a conductance meter (Twin Cond B-173, Horiba, Kyoto, Japan). Electro leakage was estimated as the ratio of electro conductivity at each freezing temperature to that at −80 °C.

Results

Two lines of *B. distachyon* overexpressing *PpFT1* and three lines overexpressing *wft1* were analyzed in this study. Under normal conditions (22 °C), the growth rate of all five transgenic lines was slower than that of the wild type (WT) plants as evidenced by reductions in plant height and in fresh weight (Fig. 1). The *PpFT1* expressing lines were smaller than *wft1* expressing lines (Fig. 1).

The components of the water-soluble carbohydrates that accumulated in the leaf blades of transgenic and non-transgenic lines were qualitatively and quantitatively analyzed. Under normal temperature conditions, the qualitative analysis by HPAEC-PAD detected 6-kestotriose (β(2,6)-linked fructan with DP = 3) only in *wft1* transgenic plants, whereas fructans with DP >4 could not be unambiguously identified in any transgenic plant or in the WT (Fig. 2). The quantitative HPLC analysis revealed significantly higher oligosaccharide amounts in *wft1* plants as compared to WT and *PpFT1*. However, these levels were low as compared to those of glucose, fructose and sucrose (Table 1). Polysaccharides corresponding to polymerized fructans were not detected also in this quantitative analysis. The average amount of sucrose in transgenic lines expressing *PpFT1* was slightly lower than in WT and *wft1* transgenic lines (Table 1). To investigate the carbohydrates in the cold-acclimated plants, leaves of plants treated at 4 °C

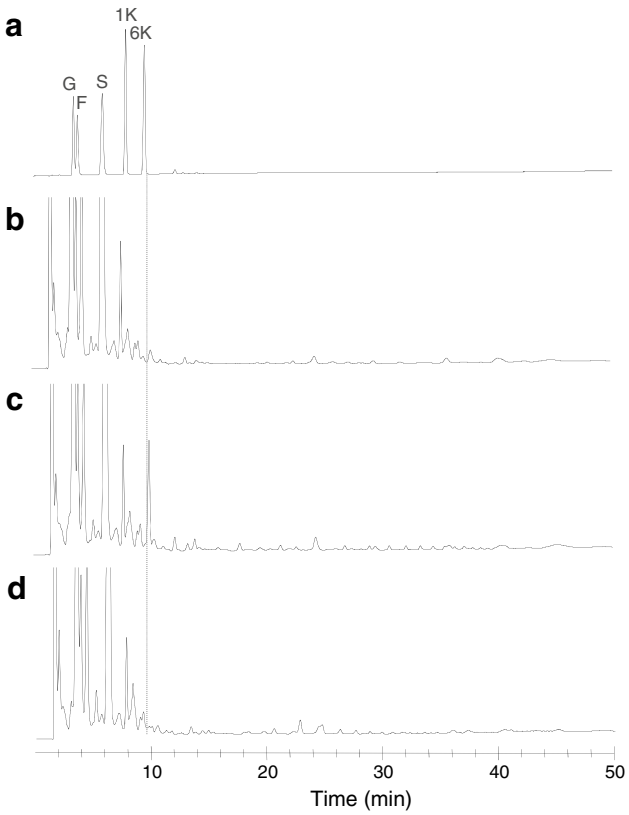

Fig. 2 Anion exchange HPLC (HPAEC-PAD) analysis of water-soluble carbohydrates from the leaf blades of transgenic and wild type *B. distachyon* grown under normal temperature conditions. Extracted solutions from fully expanded leaves of wild type (**b**), plants of a transgenic line expressing *wft1,* WFT1-20 (**c**) and plants of a transgenic line expressing *PpFT1*, PFT1-31 (**d**) were analyzed with a standard (**a**). Abbreviations for each sugar peak are *G* glucose, *F*, fructose, *S* sucrose, *1K* 1-kestotriose, *6K* 6-kestotriose; The *numbers* indicate the putative DPs of β(2,6)-linked and linear fructan oligomers

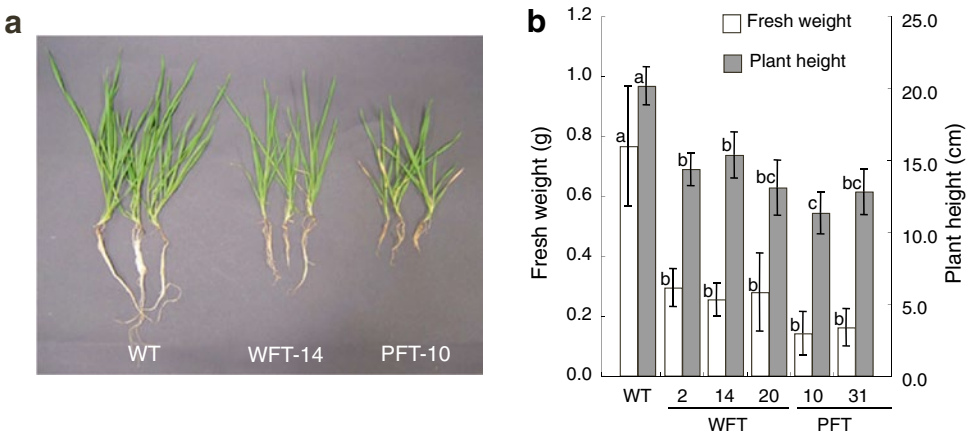

Fig. 1 **a** Phenotypes of transgenic *B. distachyon* expressing *wft1* and *PpFT1* driven by the maize ubiquitin gene promoter. Plants were grown at 22 °C, under 10 h light for 7 weeks. *WT* wild type, *WFT wft1* expressing transgenic plant, *PFT PpFT1* expressing transgenic plant. **b** The height and fresh weight of each transgenic line and WT are given as mean ± SD ($n = 5$ plants for each line). The means of each line with the same *small letter* do not differ at $P < 0.05$ by Tukey's HSD test

Table 1 Sugar contents (mg g^{-1} fresh weight) of transgenic and wild-type plants under normal temperature conditions

Line	Fructose	Glucose	Sucrose	Oligosaccharide	Total water-soluble carbohydrate
PFT-10	0.7 (±0.3)a	1.2 (±0.5)a	8.5 (±1.7)a	0.4 (±0.1)a	13.5 (±4.5)a
PFT-31	0.7 (±0.2)a	1.2 (±0.3)a	8.7 (±1.1)ab	0.4 (±0.1)ab	14.5 (±2.2)a
WFT-2	0.8 (±0.1)a	1.3 (±0.1)a	10.0 (±1.1)ab	0.8 (±0.1)c	14.9 (±1.7)a
WFT-14	0.8 (±0.1)a	1.3 (±0.1)a	11.0 (±1.0)b	0.8 (±0.1)c	14.8 (±1.5)a
WFT-20	0.7 (±0.1)a	1.0 (±0.2)a	10.4 (±1.5)ab	0.6 (±0.1)b	14.1 (±1.7)a
WT	0.9 (±0.1)a	1.2 (±0.1)a	10.2 (±0.9)ab	0.5 (±0.1)ab	13.5 (±2.6)a
PFT average	0.7 (±0.2)a	1.2 (±0.4)a	8.6 (±1.4)a	0.4 (±0.1)a	14.0 (±3.4)a
WFT average	0.8 (±0.1)a	1.2 (±0.2)a	10.5 (±1.2)b	0.7 (±0.1)b	14.6 (±1.6)a
WT	0.9 (±0.1)a	1.2 (±0.1)a	10.2 (±0.9)ab	0.5 (±0.1)a	13.5 (±2.6)a

Fructans were included in the oligosaccharide category. *WT* wild type, *WFT* transgenic lines expressing *wft1*, *PFT* transgenic lines expressing *PpFT1*. The results for each line ($n = 5$ plants) and for the combined data of each transgenic type (WFT, $n = 15$; PFT, $n = 10$) are given as mean ± SD. Means with the same small letter do not differ at $P < 0.05$ by Tukey's HSD test among transgenic lines and types, respectively

for 5 weeks after grown at room temperature for 8 weeks were analyzed. In the qualitative HPAEC-PAD analyses, WT had sequential small peaks around the region corresponding to fructans with DP >5; however, the retention times of these peaks did not coincide with those of β(2,6)-linked fructans in the products of the PpFT1 recombinant enzyme with sucrose or β(2,1)-branched β(2,6)-linked fructans in the products of the Wft1 recombinant enzyme (Fig. 3). These retention times also did not accord with those of β(2,1)-linked or linear fructans (inulins) (data not shown). Furthermore, mild acid treatment (data not shown) or incubation with a recombinant Pp6-FEH1 that preferentially hydrolyze β(2,6)-linked fructans but also could hydrolyze β(2,1)-linked fructans (Tamura et al. 2011, Fig. S1) did not alter the pattern of these unidentified peaks. In transgenic plants expressing *wft1*, peaks corresponding to linear β(2,6)-linked fructans with DP = 3–ca. 40 were detected. By comparison, the *PpFT1* transgenic plants had similar peaks to those in WT and also sequential peaks at retention times corresponding to those of fructans with DP >20 to the separation limit (Fig. 3). These peaks were not observed in extracts of WT leaves and they were selectively removed by a treatment with Pp6-FEH1 (Fig. S1). There was no clear peak corresponding to 1-kestotriose in both WT and transgenic lines (Fig. S2). In the quantitative HPLC analysis, broad peaks corresponding to polysaccharide were detected in cold-treated *wft1* and *PpFT1* transgenic plants, and they were at negligible level in extracts of WT (Fig. S3). The polysaccharide peak in the *PpFT1* transgenic plant positioned at higher molecular weight than that in the *wft1* transgenic plant (Fig. S3). These broad peaks were drastically decreased by the recombinant Pp6-FEH1 treatment (data not shown). The mean amounts of oligo- and polysaccharides in the transgenic lines were similar and were significantly higher than in WT (Table 2). In *PpFT1* transgenic lines, the amounts of fructose, glucose and sucrose were significantly higher than those in extracts of WT and *wft1* overexpression plants, resulting in a higher total water-soluble carbohydrate content in *PpFT1* transgenic lines (Table 2). In particular, the glucose content of transgenic lines expressing *PpFT1* was more than 10 times greater than in WT and in those expressing *wft1* (Table 2).

To confirm that the transgenic plants had fructosyltransferase activity, crude enzyme extracts from shoots grown under normal temperature conditions and their enzymatic products with sucrose (a substrate of fructosyltransferase) were analyzed by HPAEC-PAD. Significant 6-SF(S)T and 1-SST activities were confirmed by the generation of fructans with DP = 3, namely, 6-kestotriose and 1-kestotriose, in *PpFT1* and *wft1* transgenic lines (Fig. 4). In the reaction mixture of the crude enzyme extract from the *PpFT1* transgenic line, production of 6,6-kestotetraose (DP = 4) and 6,6,6-kestopentaose (DP = 5) by 6-SFT activity was also observed under this reaction condition (Fig. 4). In the reaction solution from WT plants, signals corresponding to 1-kestotriose and 6-kestotriose were detected at a very low level that was much weaker than in the reaction products of the enzyme extracts from the fructosyltransferase expressing lines.

To compare the freezing tolerance of the transgenic and WT plants, the degree of electrolyte leakage after freezing (termed the index of freezing tolerance) at the cellular level was measured. In leaf blades from plants grown under normal temperature conditions, the degree of electrolyte leakage after freezing at −5 °C did not differ significantly among transgenic plants expressing *wft1* or *PpFT1* and the WT plants (58.9, 59.7 and 54.2 %, respectively). After cold acclimation, electrolyte leakage in two transgenic lines expressing *PpFT1* was significantly lower than in WT and in those expressing *wft1* at −8 and −10 °C of freezing temperatures, whereas difference in freezing tolerance was not clearly confirmed in WT and the *wft1* overexpressing lines (Fig. 5).

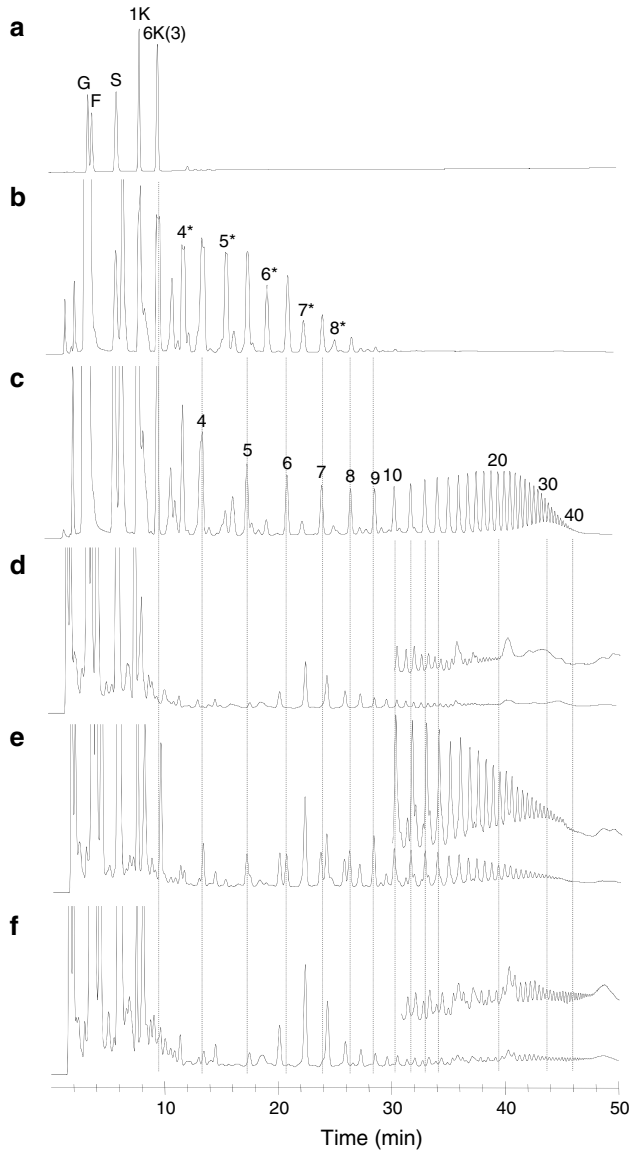

Fig. 3 Anion exchange HPLC (HPAEC-PAD) analysis of water-soluble carbohydrates from the leaf blades of cold acclimated transgenic and wild type *B. distachyon*. Extracted solutions from fully expanded leaves of cold-acclimated wild-type plants (**d**), plants from a transgenic line expressing *wft1,* WFT1-20 (**e**) and plants from a transgenic line expressing *PpFT1*, PFT1-31 (**f**) were analyzed. Standard (**a**), products of the wft1 and PpFT1 recombinant enzyme solution with sucrose (**b** and **c**) were also analyzed. Chromatograms at the latter retention time are redrawn using an extended *y* axis scale (**d**–**f**). Abbreviations for each sugar peak are *G* glucose, *F* fructose, *S* sucrose, *1K* 1-kestotriose, *6K* 6-kestotriose; The *numbers* indicate the putative DPs of β(2,6)-linked and linear fructan oligomers and the *numbers* with *asterisks* indicate the putative DPs of β(2,6)-linked branched fructan oligomers produced by polymerization of fructose units linked to 1-kestotriose

Discussion

Brachypodium distachyon was the first Pooideae species to have its genome fully sequenced (Vogel et al. 2010). It has proved a valuable model plant species for cereals, temperate grasses and dedicated biofuel crops due to its short lifespan, small genome size, and ease of transformation (Opanowicz et al. 2008). Li et al. (2012) confirmed that cold responsive genes, such as those for ice recrystallization inhibition proteins and C-repeat binding factor genes, were conserved among *B. distachyon* and core Pooideae species. They proposed the use of *B. distachyon* as a model for the specific molecular mechanisms involved in low-temperature responses in Pooideae species. The fact that the semi-lethal temperature decreases in *B. distachyon* following a low-temperature conditioning treatment (ca −5 to −10 °C) indicates that this species can respond to cold and increase its freezing tolerance. In the present study, we confirmed that only weak fructosyltransferase activity was present in crude enzyme extracts of WT plants; however, in the extracted water-soluble carbohydrate solution, our HPAEC-PAD analysis did not confirm the presence of β(2,6) and β(2,1)-linked fructans even after cold acclimation. Mild acid and FEH treatments revealed that the unidentified peaks that appeared after cold acclimation did not correspond to fructans, since fructans should have been hydrolyzed by these treatments. It can be concluded that *B. distachyon* does not accumulate fructans. Small fructosyltransferase activity in *B. distachyon* can be regarded as a side activity of invertases because synthesis of the DP3 fructans at high sucrose is a general property of invertases (De Coninck et al. 2005; Ritsema et al. 2006). These suggest that the metabolism of water-soluble carbohydrates, especially for storage, shows evolutionary diversity between *B. distachyon* and core Pooideae species such as wheat and timothy grass.

The significant increase in fructosyltransferase activity and the elevation of oligo- and polysaccharide contents following overexpression of the fructosyltransferase cDNAs, *PpFT1* and *wft1*, indicated the initiation of a heterologous novel fructan synthetic pathway in *B. distachyon*. When testing a crude extract of *PpFT1* transgenic plants with sucrose as the only substrate, higher amounts of 1-kestotriose were generated as compared to 6-kestotriose and more polymerized β(2,6)-linked fructans. This is in accordance with the fact the 6-SST activity of recombinant PpFT1 was lower compared to its 1-SST activity (Tamura et al. 2009). Under cold acclimation conditions, affecting growth more than photosynthesis, increasing sucrose supplies sustain fructan synthesis in PpFT1 transgenic plants. This indicates that PpFT1 acts as a 6-SFT *in planta*, and that endogenous invertases are not able to prevent the accumulation of the synthesized levan-type fructans. Generally, plant fructosyltransferases show low affinity for sucrose as a substrate (high apparent Km; >280 mM); therefore, sucrose availability is a limiting factor in fructan production (Cairns 2003). Under cold acclimation conditions, a significantly higher rate of accumulation of fructans was observed in transgenic

Table 2 Sugar contents (mg g^{-1} fresh weight) of transgenic and wild type plants under cold acclimation conditions

Line	Fructose	Glucose	Sucrose	Oligo- and polysaccharide	Total water-soluble carbohydrate
PFT-10	3.2 (±1.3)ab	28.5 (±16.8)a	28.2 (±6.5)a	28.8 (±12.4)a	88.8 (±26.4)a
PFT-31	4.5 (±2.1)a	18.1 (±3.0)a	35.2 (±5.4)a	34.8 (±13.8)a	92.6 (±19.7)a
WFT-2	0.5 (±0.4)c	1.4 (±0.4)b	11.1 (±3.6)b	19.0 (±10.1)ab	32.1 (±13.6)b
WFT-14	2.2 (±1.5)abc	2.6 (±1.8)b	16.6 (±4.6)b	23.1 (±12.7)a	50.0 (±22.9)b
WFT-20	1.8 (±0.5)bc	2.7 (±1.4)b	14.8 (±4.0)b	30.6 (±7.4)a	49.8 (±12.7)b
WT	1.2 (±0.8)bc	2.0 (±0.6)b	10.1 (±0.8)b	4.3 (±0.5)b	17.5 (±1.4)b
PFT average	3.9 (±1.8)a	23.3 (±12.6)a	31.7 (±6.7)a	31.8 (±12.8)a	90.7 (±22.1)a
WFT average	1.6 (±1.1)b	2.3 (±1.4)b	14.4 (±4.4)b	26.6 (±12.4)a	44.8 (±18.0)b
WT	1.2 (±0.8)b	2.0 (±0.6)b	10.1 (±0.8)b	4.3 (±0.5)b	17.5 (±1.4)c

Fructans were included in the oligo- and polysaccharide category. *WT* wild type, *WFT* transgenic lines expressing *wft1*, *PFT* transgenic lines expressing *PpFT1*. The results for each transgenic line ($n = 5$ plants) and for the combined data of each transgenic type (WFT, $n = 15$; PFT, $n = 10$) are given as mean ± SD. Means with the same small letter do not differ at $P < 0.05$ by Tukey's HSD test among transgenic lines and types, respectively

plants expressing fructosyltransferases compared to under normal temperature conditions; this may reflect higher substrate availability in source leaves, due to the altered source/sink balance under cold. The increase in fructan content in transgenic lines expressing *wft1*, but not in those expressing *PpFT1*, under normal temperature conditions might be due to the higher affinity of Wft1 for sucrose compared to PpFT1 (Tamura et al. 2009). The difference in the DPs of the accumulated fructans, i.e., short fructans (DP = 3–40) in *wft1* transgenic plants and long fructans (DP > 20) in *PpFT1* plants, is consistent with the different affinity of the two fructosyltransferases for fructans of different DPs as acceptors; this difference in affinity was first identified by analysis of yeast recombinant enzymes (Tamura et al. 2009). Little accumulation of low-DP fructans in the *PpFT1* transgenic plants seemed to be caused by the higher 6-SFT activity of PpFT1 to high-DP fructans than to low-DP ones as acceptors, which was previously revealed in the recombinant enzyme analysis (Tamura et al. 2009). As a result, generated low-DP fructans seemed to be gradually converted to high-DP fructans, resulting in the little accumulation of low-DP fructans. In transgenic plants expressing *PpFT1*, the linkage forms of the accumulated fructans could not be determined as the DPs were too high to be separated in this HPAEC-PAD analysis. However, with respect to the known enzymatic properties of PpFT1, it is likely that they had linear β(2,6) linkages similar to those fructans that accumulated in *wft1* expressing plants; further analysis is needed to confirm this supposition. In addition to fructan accumulation, higher levels of hexose and sucrose per unit fresh weight were present in transgenic lines expressing *PpFT1* compared to WT and *wft1* expressing lines. In plants grown under more intense light conditions, a higher accumulation of glucose was also observed in *wft1* expressing lines (data not shown). A significant increase in glucose and/or fructose concentration has been reported in several studies on plant-derived 6-SFT expressing lines (Hisano et al. 2004; Kawakami et al. 2008). Accumulation of these hexoses might result from the release of glucose from sucrose by a fructosyltransferase reaction, hydrolysis of fructans by native invertases, and/or hydrolysis of sucrose by invertase activity of introduced fructosyltransferases. However, it is possible that the reduced growth phenotypes of the transgenic lines, particularly those expressing *PpFT1*, might also affect carbohydrate metabolism, and cause a relatively low consumption of sugars.

The freezing tolerance at the cellular level in leaf blades of *wft1* lines did not differ from that of WT plants despite the former having approximately six times higher accumulation of oligo- and polysaccharides. This suggests that accumulation of fructans might not lead directly to an increase in freezing tolerance. The *PpFT1* expressing lines showed accumulation of oligo- and polysaccharides at a similar level to *wft1* lines and also showed a significant increase in freezing tolerance. The major differences in water-soluble carbohydrate compositions between *wft1* and *PpFT1* plants were (i) the structure of the fructans, especially their DP, and (ii) the amount of mono- and disaccharides, i.e., higher accumulation in *PpFT1* expressing lines and similar levels in WT and *wft1* lines under cold acclimation conditions. Several studies have reported that mono- and disaccharides are involved in freezing tolerance in plant cells (reviewed by Valluru and van den Ende 2008; Livingston et al. 2009). Soluble sugars are also believed to play crucial roles in overall cellular ROS homeostasis (Couée et al. 2006; Keunen et al. 2013; Van den Ende 2013 and references therein). Therefore, the accumulation of high levels of mono- and disaccharides might be involved in the enhanced freezing tolerance of the leaf blades of *PpFT1* expressing plants. Hisano et al. (2004) reported that

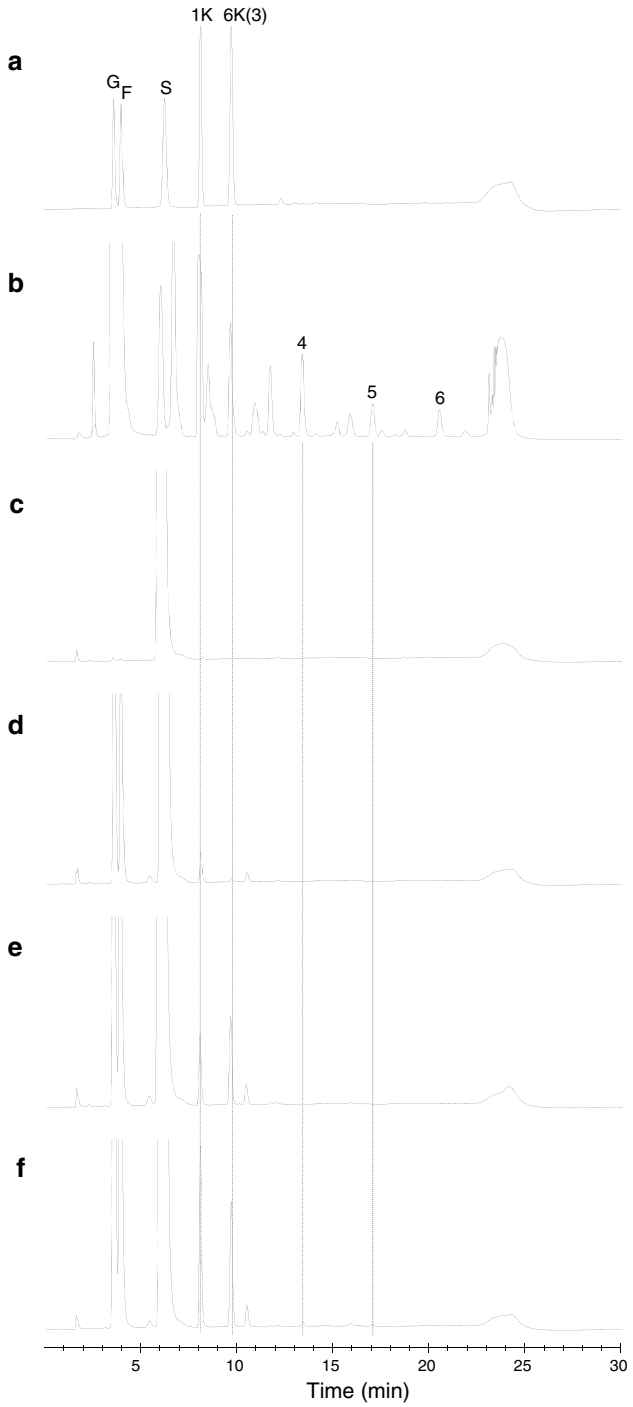

Fig. 4 Anion exchange HPLC analysis (HPAEC-PAD) of the enzymatic products of crude enzyme extracts with sucrose. Crude enzymatic extracts from wild-type plants (**d**), a transgenic line expressing *wft1*, WFT1-20 (**e**) and a transgenic line expressing *PpFT1*, PFT1-31 (**f**) grown under normal temperature conditions were incubated with 1 M sucrose. Standard (**a**), products of the PpFT1 recombinant enzyme solution with sucrose (**b**) and sucrose incubated with buffer not including enzyme extracts (**c**) were also analyzed. Abbreviations for each sugar peak are *G* glucose, *F* fructose, *S* sucrose, *1K* 1-kestotriose, *6K* 6-kestotriose; The *numbers* indicate the putative DPs of β(2,6)-linked and linear fructan oligomers

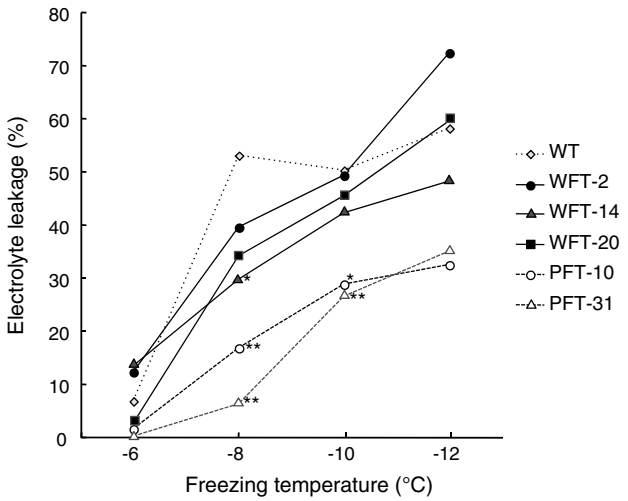

Fig. 5 Freezing tolerance of leaf blades of transgenic *B. distachyon* expressing *wft1* or *PpFT1*. The leaf blades of cold acclimated plants were used for the freezing test. Electrolyte leakage was calculated from the value of electrical conductivity after freezing ($n = 5$). The *asterisks* indicate significant differences compared to wild type by Dunnett's test ($^*P < 0.05$, $^{**}P < 0.01$)

in perennial ryegrass, the overexpression of *wft1* improves freezing tolerance, which was not seen in the *B. distachyon* overexpressing lines in this study. Different response to freezing in the *wft1* overexpressing lines of perennial ryegrass and *B. distachyon* compared to WT might be due to the difference in mono- and disaccharide content; mono- and disaccharide content increased in perennial ryegrass (Hisano et al. 2004) but not in *B. distachyon*. The evidence described above suggests that the enhancement of freezing tolerance caused by 6-SFT overexpression might be related to the increased accumulation of mono- and di-saccharides in addition to fructans. It has also been reported that tobacco plants with heterologous expression of 6-SFT show accumulation of fructans and enhancement of freezing tolerance (del Viso et al. 2011; Bie et al. 2012); however, determination of mono- and disaccharide contents was not performed in these studies. Yoshida et al. (1998) reported that highly freezing-tolerant winter wheat varieties grown under autumn field conditions accumulate high amounts of simple sugars (mono- and disaccharides) and fructans, whereas other varieties (snow mold-resistant cultivars) with higher amounts of fructans and lower amounts of mono-and disaccharides do not have high freezing tolerance compared to varieties with high freezing tolerance. This also suggests the higher contribution of simple sugars than fructans for freezing tolerance.

The effect of the DP of the accumulated fructans on freezing tolerance was more difficult to ascertain in this study because the contents of other forms of carbohydrate

(especially mono- and disaccharides) were very different between *wft1* and *PpFT1* expressing plants. In a study of liposome membrane stability, it was found that a combination of the high glass transition (T_g) effect of hydroxyethyl starch and the depression of gel phase transition temperature (T_m) by glucose could preserve dry liposomes (Crowe et al. 1997). Hincha et al. (2007) also reported "synergistic effects" of low- and high-molecular weight carbohydrates on membrane stability. Thus, the enhanced freezing tolerance of *PpFT1* expressing lines might be a synergistic effect of simple sugars and high-DP fructans.

Transgenic plants expressing fructosyltransferases, particularly those expressing *PpFT1,* showed reduced growth phenotypes. Aberrant phenotypes have been reported for transgenic plants with bacterial levansucrase genes (Cairns 2003). Overexpression of *wft1* in perennial ryegrass and rice did not cause aberrant phenotypes (Hisano et al. 2004; Kawakami et al. 2008) as observed in *B. distachyon* (this manuscript) and *Paspalum notatum* (dwarf phenotype, Muguerza et al. 2013), suggesting that growth aberrations are species-specific. As growth retardation occurred here under normal temperature conditions in which there is little, if any, accumulation of fructans, then the accumulation of fructans is not likely to be a major cause of the phenotypic effect. Further studies will be required to clarify the reason of growth aberrations by the overexpression of plant fructosyltransferases.

In this study, relationship between the structures of fructans and freezing tolerance could not be clarified. However, our analyses here indicate that the increased freezing tolerance after overexpression of 6-SFT genes is caused by the increase of mono- and disaccharides and of fructans with high DPs. Findings obtained in a monocot grass model species *B. distachyon* in this study would contribute to the improvement of the freezing tolerance of plants, especially the most economically important Poaceae family including cereals, temperate forage grasses and dedicated biofuel crops.

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