DOI: 10.1007/s10535-016-0696-1 BIOLOGIA PLANTARUM 61 (2): 235-245, 2017

An intronless sucrose:fructan-6-fructosyltransferase (*6-SFT*) gene from *Dasypyrum villosum* enhances abiotic tolerance in tobacco

X.L. HE[1,2,3], J.W. WANG[3], W.X. LI[4], Z.Z. CHEN[1], J. WU[1], J.X. ZHAO[1], J.N. SU[1], Z.H. WANG[1], and X.H. CHEN[1,2]*

College of Agronomy, Northwest A&F University, Yangling 712100, Shaanxi, P.R. China[1]
Shaanxi Key Laboratory of Genetic Engineering for Plant Breeding, Yangling 712100, Shaanxi, P.R. China[2]
College of Environment and Life Science, Kaili University, Kaili 556011, GuiZhou, P.R. China[3]
Institute for Wheat Research, Henan Academy of Agricultural Sciences, Zhengzhou 450002, Henan, P.R.China[4]

Abstract

Fructans play vital roles in enhancing plant abiotic stress tolerance by reducing oxidative damage, stabilizing cell membranes, improving the osmotic adjustment capacity, and lowering the freezing point. In this study, a sucrose: fructan-6-fructosyltransferase (*6-SFT*) gene involved in the synthesis of fructans was isolated from *Dasypyrum villosum*, *Dv-6-SFT*, using genomic walking and reverse transcription (RT)-PCR. Alignment of the cDNA sequence with its genomic counterpart showed that no introns were present in the *Dv-6-SFT* gene, and thus it differs from all other plant *6-SFTs* that have been cloned previously. Sequence analysis showed that the cDNA of the *Dv-6-SFT* sequence comprised 2 175 bp with a 1 863 bp open reading frame, and its deduced protein comprised 620 amino acids with a predicted molecular mass of 68.47 kDa. The *Dv-6-SFT* gene was transferred into tobacco (*Nicotiana tabacum* L.) cv. W38 *via Agrobacterium*-mediated transformation. The screened plants were tested by PCR and semi-quantitative RT-PCR, and the transgenic plants were evaluated under drought, cold, and salt stresses. The *Dv-6-SFT* transgenic tobacco plants had higher resistance to drought, cold, and salt stress than the non-transgenic plants. Further analysis showed that the transgenic plant expressing *Dv-6-SFT* had increased content of saccharides and proline, but reduced content of malondialdehyde in leaves. The results of this study demonstrate that the *Dv-6-SFT* gene is a potential candidate for conferring abiotic stress tolerance in plants and it could be used in crop improvement breeding programs.

key words: *Agrobacterium tumefaciens*, drought, freezing, *Nicotiana tabacum*, salinity, transgenic plants.

Introduction

Abiotic stresses, such as low water availability, low/high temperatures, and soil salinity are the major constraints that affect plant growth and crop productivity. Biomass and productivity reductions due to abiotic stresses are common in both cereals and legumes, although genotypic variation is also present (Irshad Ahmed *et al.* 2011). Abiotic stresses can also make crops more susceptible to biotic stresses (Shahbaz and Ashraf 2013). Therefore, understanding the mechanisms of plants tolerance to abiotic stresses and improving abiotic stress tolerance in crops are extremely important. Previous research indicates that the accumulation of fructans in plants enhances their performance under abiotic stress conditions.

Fructans are water-soluble polysaccharides, which occur in about 15 % of flowering plants (Hendry 1993) and most cool-season grasses (Chatterton *et al.* 1989). Fructan accumulating species predominate in cold and dry environments whereas they are almost absent in tropical or aquatic conditions (Hendry 1993). Fructans accumulate in the cell vacuoles and they have important functions in assimilate partitioning and storage, in stabilizing cellular membranes (Hincha *et al.* 2007) as well as in cellular

---

Submitted 29 February 2016, *last revision* 13 June 2016, *accepted* 7 July 2016.

Abbreviations: 1-FFT - fructan:fructan 1-fructosyl transferase; cDNA - complementary DNA; 6G-FFT - fructan:fructan 6G-fructosyltransferase; FT - fructosyltransferase; GH32 - glycoside hydrolase family 32; gDNA - genomic DNA; MDA - malondialdehyde; MS - Murashige and Skoog; NT - non-transgenic; ORF - open reading frame; pI - isoelectric point; ROS - reactive oxygen species; RT-PCR - reverse transcription PCR; 6-SFT - sucrose:fructan-6-fructosyltransferase; 1-SST - sucrose:sucrose 1-fructosyltransferase; .

Acknowledgments: The authors thank Dr. D.E. Jackson for useful advice and editing the English language content of the manuscript. Much appreciated financial support was provided by the National Natural Science Foundation of China (31571650) and the Tang Zhong-Ying Breeding Funding Project of the Northwest A & F University.

* Corresponding author; fax: (+86) 029 87082845, e-mail: cxh2089@126.com

osmoregulation (Wei *et al.* 2001). In plants, particularly grasses, graminan- and levan-type fructans have important physiological roles as short-term reserves (Maleux and Van den Ende 2007), long-term storage compounds (Del Viso *et al.* 2009), and in stress protection (Le Roy *et al.* 2007). It is considered that fructans may confer tolerance to cold (Hisano *et al.* 2008), excessive salinity (Kerepesi and Galiba 2000), drought (De Roover *et al.* 2000), and/or oxidative stress (Peshev *et al.* 2013), possibly by protecting the cell membranes in these stressful conditions (Hincha *et al.* 2007). Fructans also contribute to cellular homeostasis during stress (Abdelgawad *et al.* 2014). Increased stress tolerance has been achieved by triggering fructan synthesis using transgenic approaches in tobacco (Pilon-Smits *et al.* 1995, Li *et al.* 2007), rice (Kawakami *et al.* 2008), sugar beet (Pilon-Smits *et al.* 1999), potato (Knipp and Honermeier 2006), triticale (Diedhiou *et al.* 2012), and wheat (Bie *et al.* 2012).

Four types of fructosyltransferase (FT) enzymes are considered to participate in the synthesis of fructan (Ritsema *et al.* 2003). Based on the donor substrate employed, it has been suggested that FTs can be classified into two groups: S-type FTs (1-SST and 6-SFT, using sucrose as a donor substrate) and F-type FTs (1-FFT and 6G-FFT, using fructan as a donor substrate) (Lasseur *et al.* 2009, Schroeven *et al.* 2009). As a key enzyme in monocot fructan biosynthesis (Lammens *et al.* 2012), the *6-SFT* gene was first identified in barley (Sprenger *et al.* 1995) and subsequently cloned in wheatgrass (Wei and Chatterton 2001), bluegrass (Wei *et al.* 2002), perennial ryegrass (Chalmers *et al.* 2003), wheat (Kawakami and Yoshida 2002), timothy grass (Tamura *et al.* 2009), *Bromus pictus* (Del Viso *et al.* 2009) and *Psathyrostachys huashanica* (He *et al.* 2015). In particular, the genes in wheat, timothy grass, and *Bromus pictus* have been functionally analyzed. The 6-SFTs possess three highly conserved motifs, *i.e.*, the (N/S)DPNG box, FRDP box, and WECIDF block (Altenbach *et al.* 2005), whereas the 6-SFT in wheatgrass contains the SDPDG box instead of the usual NDPNG amino acid sequence (Wei and Chatterton 2001). In wheat, the full-length genomic DNAs (gDNAs) of the *6-SFT* genes contain four exons and three introns (Huynh *et al.* 2012), but the size of the *6-SFT* gDNA is variable (Gao *et al.* 2010). The *6-SFT* gene from *Triticum aestivum* was introduced into ryegrass, which increased the fructan content and improved the tolerance to drought and cold (Hisano *et al.* 2004, Kawakami and Yoshida 2002). Bie *et al.* (2012) has found that the fructan content in tobacco transgenic plants (TPs) is positively correlated with drought, cold, and salt stress tolerance. He *et al.* (2015) reported that the introduction of the *6-SFT* gene from *Psathyrostachys huashanica* into tobacco increases the fructan content and improves tolerance to drought, cold, and salt. Knipp and Honermeier (2006) showed that fructan accumulation might have pleiotropic effects on the proline content. Tamura *et al.* (2014a) has found that *6-SFT* lines with resistance to freezing tolerance synthesize higher amounts of soluble sugar in addition to fructans compared with the wild-type plants.

The quantity (Chatterton *et al.* 1989) and structure (Chatterton *et al.* 1993) of fructan oligosaccharides vary greatly among cool-season grass species. Thus, enzyme and gene sequence information from additional plant species will allow us to analyze their molecular relationships and to clarify their evolution (Wei and Chatterton 2001). *Dasypyrum villosum* is a wild relative of common wheat, which possesses many unique and beneficial genes that can be used for wheat improvement (Chalmers *et al.* 2003), and it is particularly well adapted to drought, as reported by Cao *et al.* (2009). The expression of genes that encode enzymes in the fructan biosynthetic pathways of non-host systems is a strategy that has been employed since the mid-1990s, particularly for characterizing the enzymatic properties of FTs, at least for plant (Sprenger *et al.* 1995) and fungal enzymes (Heyer and Wendenburg 2001). However, *D. villosum* FT cDNAs or genomic clones have not been isolated in previous studies. Furthermore, there is relatively little information on the precise physiological effects of *6-SFT* expression and the changes in specific physiological parameters in TPs. Thus, it is still necessary to identify the key functional gene in response to abiotic stresses *via* heterologous expression in tobacco.

In this study, we isolated the *6-SFT* gDNA and cDNA sequences from *D. villosum*, which was designated as *Dv-6-SFT*. After sequencing the gDNA and cDNA, we investigated the exon-intron structure and motif distribution. The *Dv-6-SFT* gene was transformed into non-fructan-accumulating tobacco plants and we determined their tolerance to drought stress, low temperature, and high salinity. We also evaluated the effects of *Dv-6-SFT* expression as well as the physiological relevance of osmoprotectants or the accumulation of compatible solutes in tobacco transgenic plants in various conditions. The results of this study can help to identify potential candidate *6-SFT* genes, thereby facilitating future studies of abiotic stress tolerance in transgenic crops.

Materials and methods

Plants: *Dasypyrum villosum* (L.) Candargy (*Dv*) (syn. *Haynaldia villosa*) ($2n = 2x = 14$, VV) was used for gene cloning. Tobacco (*Nicotiana tabacum* L.) cv. W38 was used as the transformed host. Samples of all the seeds were deposited in the College of Agronomy, Northwest A & F University, China. All plants were cultured in pots in a greenhouse at a temperature of 25 °C and a 12-h photoperiod.

Isolation of the *6-SFT* gene from *D. villosum*: To investigate the intron-exon structure of *Dv-6-SFT*, gDNA was extracted from the roots of *D. villosum* using a plant genomic DNA kit (*Tiangen*, Beijing, China). Based on the expressed sequence tags (EST) sequence of the *D. villosum 6-SFT* fragment (accession No. AF222943) in NCBI, gene-specific primers GSP1 - GSP4 (Table 1 Suppl.) were designed to obtain the full sequence of *D. villosum 6-SFT*, which contained the putative start and stop codons. Following the manufacturer's instructions (genome walking kit, *TaKaRa*, Dalian, China), four fragments were amplified from the *D. villosum* genome by PCR. After purification with a universal DNA purification kit (*Tiangen*), the fragments were inserted into the pMD19-T vector (*TaKaRa*) and transformed into the competent cells of *Escherichia coli* strain DH-5α. Next, the positive clones were submitted for sequencing by *Sangong Company* (Shanghai, China). Sequence analysis shows that the fragments and *D. villosum* reference EST sequence overlapped with each other at the 5′ and/or 3′ ends, and thus a consensus sequence was generated by reassembling the fragments and *D. villosum* reference EST sequence.

Total RNA was extracted from the roots of *D. villosum* using RNA prep pure plant kit (*Tiangen*), and first-strand cDNA synthesis and reverse transcription PCR (RT-PCR) were performed using a *SuperScript II* first-strand synthesis system for RT-PCR kit (*TaKaRa*). The cDNA was used for further PCR in a reaction mixture containing 5 mm^3 of the RT reaction product. Thus, a pair of primers, *i.e.*, Dv-6-SFT-F and Dv-6-SFT-R (Table 1 Suppl.) was designed for amplifying and cloning the putative *6-SFT* gene. A 2 175 bp PCR fragment was obtained, which was purified with a universal DNA purification kit (*Tiangen*). The amplification program was as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. The samples were stored at 4 °C. The amplification products were gel-purified, cloned into the pMD19-T vector (*TaKaRa*), and sequenced. Sequence alignment of the cDNA with its genomic counterpart indicated that this gDNA segment (GenBank acc. No. LN884177) was directly colinear with the cDNA (acc. No. LN884178) of *Dv-6-SFT*.

Sequence and phylogenetic analysis: The nucleotide sequence and deduced amino acid sequence were analyzed using *DNAMAN*. Sequence features such as the signal peptide (predicted using signal peptide prediction software: http://www.cbs.dtu.dk/services/SignalP/), isoelectric point (pI) and molecular mass were evaluated using protein analysis tools (http://expasy.org/tools). The conserved domains of the deduced protein were analyzed with the *ScanProsite* tool. 6-SFT sequences were selected from NCBI and aligned with *CLUSTALX v. 2.0*, and gaps were removed from the alignment. The phylogenetic tree based on the alignment was obtained using the neighbor-joining method with *Mega 5.0* and the bootstrap values were obtained from 1 000 replicates.

Vector construction and plant transformation: To construct a heterologous expression vector for *Dv-6-SFT*, the full open reading frame (ORF) sequence of the *Dv-6-SFT* gene was inserted via *Hin*dIII-*Bam*HI sites into the p1300-35SN vector to obtain the transformation vector p1300-35SN-*Dv-6-SFT* (Fig. 1). The orientation and the ORF integrity of *Dv-6-SFT* in the construct were confirmed by sequence analysis. The p1300-35SN plasmid was transferred into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method (Chen *et al.* 1994). Tobacco cv. W38 was used as the host for *Dv-6-SFT* transformation and transgenic plants (TPs) were selected on Murashige and Skoog (MS) medium with hygromycin (20 mg dm^{-3}) according to Schuler and Zielinski (1989). The rooted shoots were transplanted to soil and the plants were grown in a greenhouse. All of the experiments described in the following were performed using T_0 generation plants.

Molecular characterization of TPs: The gDNA was extracted from the putative tobacco TPs using the cetyltrimethylammonium bromide method (Sambrook *et al.* 1989). The presence of the *Dv-6-SFT* gene in the primary transformants was confirmed by genomic PCR with primers specific to *Dv-6-SFT* (Dv-6-SFT-F and Dv-6-SFT-R) (Table 1 Suppl.). The annealing temperature was 60 °C and the approximate product length was 2 175 bp. The presence of the gene in TPs was further verified by RT-PCR using a gene-specific primer set, *i.e.*, Rp6-SFT-F and Rp6-SFT-R (Table 1 Suppl.), to amplify the *Dv-6-SFT* gene, and Actin-F and Actin-R (Table 1 Suppl.) to amplify an actin fragment as an internal control, where the approximate product lengths were 1 000 bp for *Dv-6-SFT* and 500 bp for *Actin*. RNA was isolated using an RNeasy plant mini kit (*Qiagen*, Hilden, Germany) according to the manufacturer's instructions. One microgram of RNA was used to synthesize cDNA with *SuperScript III* reverse transcriptase (*Invitrogen*, Carlsbad, USA). The RT-PCR amplification program comprised: 94 °C for 5 min; 35 cycles at 94 °C for 45 s, at 55 °C for 1 min, and at 72 °C for 30 s; with a final extension at 72 °C for 10 min. All PCR and RT-PCR reactions were repeated three times.

Abiotic tolerance in tobacco TPs: Drought, cold, and salt stress tolerance were determined according to Bie *et al.* (2012) with slight modifications. At the 5 - 6 leaf stage, the TP and non-transgenic (NT) plants were exposed to drought and freezing stresses. The drought stress treatment involved withholding water for 40 d. The growth status of the plants was monitored. The freezing stress treatment involved exposure of the plants to a temperature of 10°C for 10 d under a 14-h photoperiod (irradiance of 25 μmol m^{-2} s^{-1}), followed by -20°C for 30 min, and returning to room temperature for 9 d. For the salt stress treatment, the leaves of T_0 TPs were removed and cut into 1-cm segments with shoot buds, and these explants were then cultured in MS medium containing 150 or 200 mM NaCl. A visual analysis was used to assess the growth of plants compared with control plants after 2 - 5 weeks.

The physiological parameters measured comprised the fructan, soluble sugars, proline, and malondialdehyde (MDA) content. The samples from Tp and NT T_0 plants were taken before and during drought treatment, where watering was stopped for 20 d, and at the end of the cold treatment (10 °C for 10 d and -20 °C for 5 min). The fructan content was determined colorometrically as described previously (Bie *et al.* 2012). The remaining steps were performed according to the instructions provided with the plant fructan colorimetric assay kit (*GenMed Scientifics*, Wilmington, USA). The soluble sugar content was determined using the anthracene ketone colorimetric method, as described by Fales (1951), with sucrose as the standard. The free proline content was measured according to Bates *et al.* (1973) based on the acid ninhydrin method. The MDA content was evaluated using the thiobarbituric acid reaction method developed by Heath and Packer (1968).

Statistical analysis: The mean fructan, soluble sugar, MDA, and proline content and the standard error of the mean were calculated from four replicates. Significant differences were determined using Tukey's HSD test, where $P < 0.05$ was considered significant. All data were analyzed using the *SAS* (*v. 8.1*) software package.

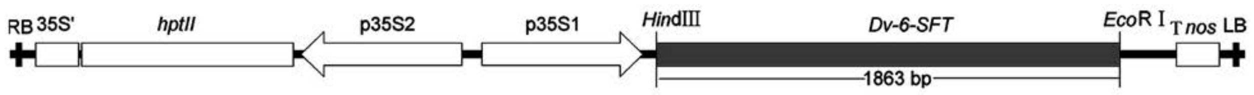

Fig. 1. Structures of the transformation vector. RB and LB - the right and left border of T-DNA, respectively; 35S′ - CaMV 35S poly A; *hptII* - *hygromycin phosphotransferase* II gene; p35S2 - CaMV 35S promoter with a double enhancer sequence; p35S1 - 35S promoter from CaMV; *Dv-6-SFT* - *Dasypyrum villosum* sucrose:fructan-6-fructosyltransferase gene; T *nos* - nopaline synthase terminator.

Results

A comparison of the full-length cDNA sequence with the corresponding gDNA sequence showed that the full length sequence of *Dv-6-SFT* comprised 2.175 bp without any introns. These characteristics indicated that the gene structure of *Dv-6-SFT* differed from that of other *Triticeae* species. The full length cDNA sequence of *Dv-6-SFT* was 2.175 bp, including a 44-bp 5′-untranslated region, a 1.863-bp ORF (Fig. 2), and a 312-bp 3′-untranslated region. The ORF was predicted to encode a protein of 620 amino acids, a calculated pI of 5.00, and an estimated molecular mass of 68.47 kDa for the mature protein. Multiple sequence alignments showed that *Dv-6-SFT* shared a high degree of sequence similarity with the *6-SFTs* or putative *6-SFTs* from other *Poaceae* plants. The predicted signal peptide showed that the cleavage site was located between amino acid positions 52 and 53 (Fig. 2 Suppl.). *ScanProsite* analysis showed that 6-SFT contained three conserved amino acid motifs (S/NDPNG, RDP, and EC), which are the main functional domains of the fructan biosynthesis enzymes and invertases, thereby indicating that the Dv-6-SFT protein belongs to glycoside hydrolase family 32. Phylogenetic analysis was performed using the amino acid sequences of Dv-6-SFT and other *Poaceae* 6-SFTs. The phylogenetic tree showed that all of the 6-FST sequences could be divided into two main groups: *1*) *Triticinae* 6-SFTs and *2*) others. Dv-6-SFT clustered with the *Triticinae* but it was clearly separated from their homologs (Fig. 3). Dv-6-SFT shared high identity with the 6-SFT amino acid sequences from *Agropyron*, wheat, barley, and *Aegilops*.

After selection on MS medium with hygromycin B, the presence of *Dv-6-SFT* in TPs was examined by PCR and semiquantitative RT-PCR analysis. In total, 43 putative transgenic tobacco plantlets were obtained and tested, where 38 plants were confirmed to be positive. The expected band was amplified from the positive transformants, whereas no band was obtained from the NT plants (Fig. 4). The transformed plants did not exhibit any morphological or growth rate differences compared with the NT plants.

The drought, freezing, and salt stress tolerances of the T_0 TPs were compared with those of the NT plants. Before the treatments, there were no visible phenotypic differences between the TPs and NT plants. Wilt symptoms appeared in the NT plants as early as 10 d after water withdrawal, whereas they were not observed in the TPs. The NT plants died after 40 d of drought stress, whereas the top leaves of the TPs remained green (Fig. 3 Suppl.). Similarly, after 10 °C for 10 d and -20 °C for 30 min in the freezing treatment, the TPs exhibited less damage than the NT plants. The leaves of the NT plants were wilted and water-soaked, whereas only the lower leaves of the TPs wilted and the top leaves retained normal growth (Fig. 3 Suppl.). To investigate whether *Dv-6-SFT* expression was correlated with salt stress tolerance in tobacco TPs, tobacco stem segments with shoot buds were cultured on 1/2 MS medium supplemented with NaCl (150 or 200 mM) to impose salt stress. After treatment for 14 d, all of the TPs grew normally and exhibited strong root growth, whereas the NT plants became yellow and their roots stopped growing (Fig. 3 Suppl.). In the salt stress conditions, the TPs exhibited significantly decreased growth inhibition compared with the NT plants in terms of root length.

Based on the results of the PCR and RT-PCR, the TPs and NT plants were selected to quantify the differences in their physiological parameters before and during the drought and cold stress treatments (Fig. 5). In normal growth conditions, the fructan content of the TPs was 6.57 to 10.13 mg g^{-1}(f.m.) (Fig. 5*A*), whereas no fructans was detected in the NT plants. The free proline content in the leaves of the TPs was significantly higher than that in the NT plants (Fig. 5*B*). The MDA content of the TPs was slightly lower than that of the NT plants (Fig. 5*C*). The soluble sugar content in the TPs were significantly (33 %) higher than in the NT plants (Fig. 5*D*). After the plants had been subjected to drought stress for 20 d, the fructan content in the TPs was 15.98 to 18.14 mg g^{-1}(f.m.) (Fig. 5*A*), but no fructan was detected in the NT plants. The proline content in the TPs was significantly higher than in the NT plants (Fig. 5*B*). The MDA content was significantly lower in the TPs compared with NT plants (Fig. 5*C*). The soluble sugar content was 0.2 to 0.24 % of fresh mass in the TPs, but only 0.13 % in the NT plants (Fig. 5*D*), where the leaves of the TPs contained significantly higher levels of soluble sugars than those of the NT plants. After the plants had been subjected to cold stress, the fructan content of the TPs was increased to 16.98 - 18.76 mg g^{-1}(f.m.) (Fig. 5*A*) whereas fructan could not be detected in the NT plants. Thus, the leaves of the TPs contained significantly higher content of fructan, proline, and soluble sugars but significantly lower content of MDA compared with the NT plants under drought or cold stress.

```
1     atggggtcacacggcaagccactgctaccgtacgcgtacaagccgctgccgtccggcgccgccgccgccgacgatggtgagcggaccggc
       M  G  S  H  G  K  P  L  L  P  Y  A  Y  K  P  L  P  S  G  A  A  A  A  D  D  G  E  R  T  G
91    tgtacgaggtggcgcgtgtgtgccaccgtgctgacggcctcggccatggtggtggtggttgtcgccgccacgcccctggcaggagtcagg
       C  T  R  W  R  V  C  A  T  V  L  T  A  S  A  M  V  V  V  V  V  A  A  T  P  L  A  G  V  R
181   ttcgaccaggcggccgacgaggaggcggcgggtgggttcccgtggagcaacgagatgctgcagtggcagcgcagtggttaccatttccag
       F  D  Q  A  A  D  E  E  A  A  G  G  F  P  W  S  N  E  M  L  Q  W  Q  R  S  G  Y  H  F  Q
271   ccggcgaagaactacatgagcgatcccaacggtctgatgtactaccgtggatggtaccacatgtgcttccagtacaaccccgtgggcacc
       P  A  K  N  Y  M  S  D  P  N  G  L  M  Y  Y  R  G  W  Y  H  M  C  F  Q  Y  N  P  V  G  T
361   gattgggatgacggcatggagtggggccacgccgtgtcccggaaccttgtccaatggcgcaccctccctattgccatggtggccgaccag
       D  W  D  D  G  M  E  W  G  H  A  V  S  R  N  L  V  Q  W  R  T  L  P  I  A  M  V  A  D  Q
451   tggtacgacatccagggagtcctgtcgggctctatgacggtgctacccaatggcacagtcatcatgctgtacacggggccaccaatgcc
       W  Y  D  I  Q  G  V  L  S  G  S  M  T  V  L  P  N  G  T  V  I  M  L  Y  T  G  A  T  N  A
541   tccgctgttgaggtccagtgcatcgccacccccgccgaccccaatgacccctcctccgccgatggaccaagcaccccgccaatcccgtc
       S  A  V  E  V  Q  C  I  A  T  P  A  D  P  N  D  P  L  L  R  R  W  T  K  H  P  A  N  P  V
631   atctggtcaccggcgggggtcggcaccaaggatttcagagacccgatgaccgcttggtatgatgaatctgaccacacatggcgcaccctc
       I  W  S  P  A  G  V  G  T  K  D  F  R  D  P  M  T  A  W  Y  D  E  S  D  H  T  W  R  T  L
721   ctcgggtccaaggatgaccactacggccaccacgatggcatcgccatgatgtacaagaccaaggacttccttaactatgagctcatcccg
       L  G  S  K  D  D  H  Y  G  H  H  D  G  I  A  M  M  Y  K  T  K  D  F  L  N  Y  E  L  I  P
811   ggcatcttgcatcgagtcgagaacaccggcgagtgggagtgcatcgacttctaccctgtcggccgcagcggcaacgacaactcatcggag
       G  I  L  H  R  V  E  N  T  G  E  W  E  C  I  D  F  Y  P  V  G  R  S  G  N  D  N  S  S  E
901   atgttgcacgtgttgaaggcgagcatggacgacgaacggcacgactactactcgctaggcaggtacgactcggcggcaaacacgtggacg
       M  L  H  V  L  K  A  S  M  D  D  E  R  H  D  Y  Y  S  L  G  R  Y  D  S  A  A  N  T  W  T
991   ccgattgacccggagctcgacttggggatcgggctgagatacgactggggtaagttttatgcgtccaccacattctatgatccggcgaag
       P  I  D  P  E  L  D  L  G  I  G  L  R  Y  D  W  G  K  F  Y  A  S  T  T  F  Y  D  P  A  K
1081  aagcggcgcgtgctgatggggtacgtcggcgaggtcgactccaagcgggctgacgtcgtgaagggatgggcctcgattcagtcagttcct
       K  R  R  V  L  M  G  Y  V  G  E  V  D  S  K  R  A  D  V  V  K  G  W  A  S  I  Q  S  V  P
1171  aggacggtgactctagatgagaagacccggacgaacctcctcctatggcctgtggaggagattgagaccctccgccttaatgccaccgaa
       R  T  V  T  L  D  E  K  T  R  T  N  L  L  L  W  P  V  E  E  I  E  T  L  R  L  N  A  T  E
1261  cttagcgacgtcaccattaacactggctctgtcttccatgtccccctccgtcaaggcactcagctcgacatcgaggcctctttccgcctt
       L  S  D  V  T  I  N  T  G  S  V  F  H  V  P  L  R  Q  G  T  Q  L  D  I  E  A  S  F  R  L
1351  gatgcttctgccgtcgctaccctcaatgaggctgatgtgggctataactgtagtagcagcggcggtgctgctacccgcggtgcgctaggc
       D  A  S  A  V  A  T  L  N  E  A  D  V  G  Y  N  C  S  S  S  G  G  A  A  T  R  G  A  L  G
1441  cccttcggcctcctcgtcctcgctgctggtaaccgccttggcgagcaaacggcggtgtacttctacgtgtctaggggcctcgacggaggc
       P  F  G  L  L  V  L  A  A  G  N  R  L  G  E  Q  T  A  V  Y  F  Y  V  S  R  G  L  D  G  G
1531  ctccagaccagcttctgccaagacgagttgagatcgtcatgggccaaggatgtgacgaagcgggtgattgggagcacggtaccggtgctc
       L  Q  T  S  F  C  Q  D  E  L  R  S  S  W  A  K  D  V  T  K  R  V  I  G  S  T  V  P  V  L
1621  gatggtgaggcttttcaatgagggtactcgtggaccactccatcgtgcagggcttcgcgatgggcgggaggaccacgatgacatctcgg
       D  G  E  A  F  S  M  R  V  L  V  D  H  S  I  V  Q  G  F  A  M  G  G  R  T  T  M  T  S  R
1711  gtgtacccgatggaggcctatcaggaggcaggagtgtacttgttcaacaatgccaccggtgccggcgtgacagcggaaaggcttgtcgtg
       V  Y  P  M  E  A  Y  Q  E  A  G  V  Y  L  F  N  N  A  T  G  A  G  V  T  A  E  R  L  V  V
1801  catgagatggactcagcacacaaccaactctccaatgaagacgatggcttgtatcttcaatga
       H  E  M  D  S  A  H  N  Q  L  S  N  E  D  D  G  L  Y  L  Q  *
```

Fig. 2. Complementary DNA sequence and predicted amino acid sequence of the *Dv-6-SFT* gene. The stop codon is indicated by an *asterisk*. Three highly conserved active regions are: Suc-binding box (SDPNG) , RDP motif, and EC motif.

Discussion

In the present study, we isolated a putative *6-SFT* gene from *D. villosum*. Amino acid sequence analysis and protein structure prediction indicated that Dv-6-SFT belongs to the plant glycosyl hydrolase family 32. *Dv-6-SFT* contained a 1 863 bp ORF that encoded a protein of 620 amino acids with a predicted molecular mass of 68.47 kDa and a pI of 5.00. Its molecular mass and theoretical pI was slightly lower than that of *Triticeae 6-SFTs* (Gao *et al*. 2010). The predicted Dv-6-SFT protein shared high sequence identity with other plant 6-SFTs and it contained the conserved structural features found in all 6-SFTs. Schroeven *et al*. (2009) suggested that the Arg in an Asp/Arg pair is essential for maintaining Asp in the correct orientation to bind sucrose as the donor substrate in S-type enzymes. Therefore, the presence of an Asp/Arg pair indicates that Dv-6-SFT belongs to the group of enzymes that can use sucrose as a donor substrate: S-type FTs. The phylogenetic analysis indicated that Dv-6-SFT clustered with *Triticinae* and it was clearly separated from their homologs, but it grouped with the 6-SFTs from *Agropyron*, wheat, barley, and *Aegilops* (Fig. 3). These results demonstrate that *D. villosum* is a close relative of the *Triticinae*. Interestingly, the determination of the intron and exon boundaries showed that the *Dv-6-SFT* genomic sequence lacked introns (Fig. 1 Suppl). Previous studies have shown that *OsVIN2* lacks intron 1 and that the mini-exon is combined with the preceding exon as the last nine bases (Ji *et al*. 2007). Ji *et al*. (2007) suggested that intron 1 may have been lost because the mini-exon lies between introns 1 and 2. In addition, the structures of the genomic *1-SST* genes of *Festuca arundinacea* and *Lolium perenne* have been reported to contain three exons, two introns, and no mini-exon (Yoshida *et al*. 2004). Aberrant splicing of the 9-bp mini-exon also occurred in potato invertase when plants were exposed to low temperature (Bournay *et al*. 1996). Its molecular mass and theoretical pI was slightly lower than that of *Triticeae 6-SFTs* (Gao *et al*. 2010). The predicted Dv-6-SFT protein shared high sequence identity with other plant 6-SFTs and it contained the conserved structural features found in all 6-SFTs. Simpson *et al*. (2000) showed that mutations in the intron 1 and 3 splice sites flanking the mini-exon resulted in the mini-exon being skipped, and thus they deduced that both splice sites are required. Bournay *et al*. (1996) suggested that the deletion of the mini-exon could be affected by modifications to the secondary structure of the intron sequences at low temperature. The aberrant splicing of this mini-exon in *Festuca arundinacea* and *Lolium perenne* may play a role in plant metabolism and development in certain conditions. In specific physiological conditions,

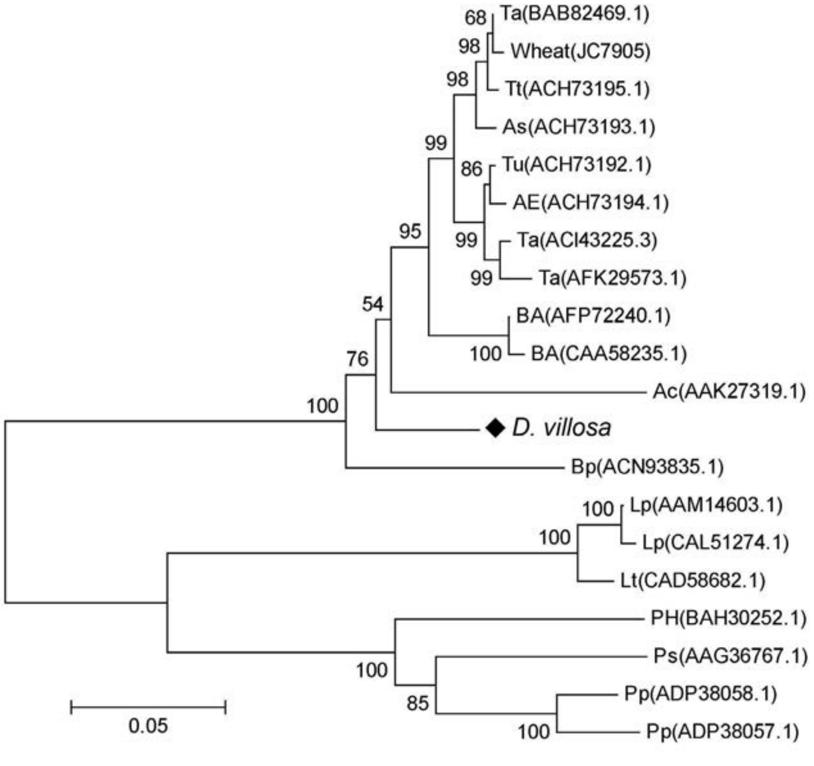

Fig. 3. Phylogenetic tree of 6-SFTs from various species based on amino acid sequences. The bootstrap percentages (> 50 %) are indicated next to the branches (based on 1 000 replicates). *Dasypyrum villosum 6-SFT* sequence is indicated by the *rhombus*. The other sequences were derived from *Triticum aestivum* (Ta), *Triticum turgidum* subsp. *durum* (Tt), *Aegilops searsii* (As), *Triticum urartu* (Tu), *Aegilops tauschii* (AE), *Hordeum vulgare* subsp. *vulgare* (BA), *Agropyron cristatum* (Ac), *Bromus pictus* (Bp), *Phleum pratense* (PH), *Poa secunda* (Ps), *Poa pratensis* (Pp), *Lolium temulentum* (Lt), and *Lolium perenne* (Lp).

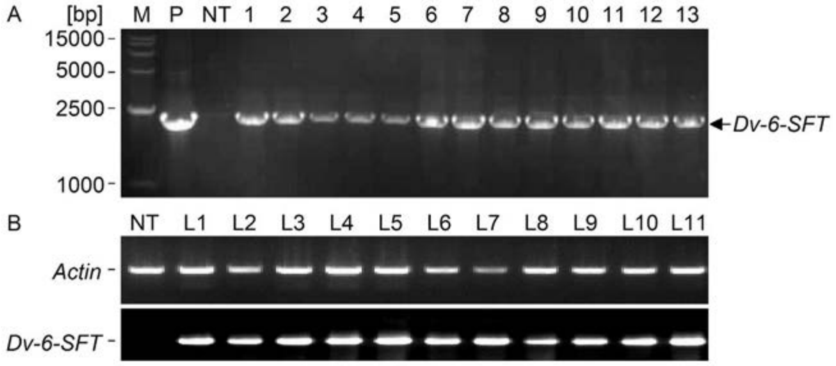

Fig. 4. Detection of transgenic tobacco plants transformed with the *Dasypyrum villosum Dv-6-SFT* gene. *A* - PCR amplification of the *6-SFT* gene in 13 individual tobacco plants. *Lanes*: M - DL15 000 DNA marker; P - positive control (plasmid); NT - negative control (non-transgenic plants); 1 to 13 - putative transgenic plants. *B* - RT-PCR analysis of primary T_0 transgenic plants using *Actin* primers as the internal controls. *Lanes*: NT - negative control (non-transgenic plants); L1 to L11 - LTPs lines L25, L30, L46, L51, L55, L73, L84, L88, L99, L102, and L140.

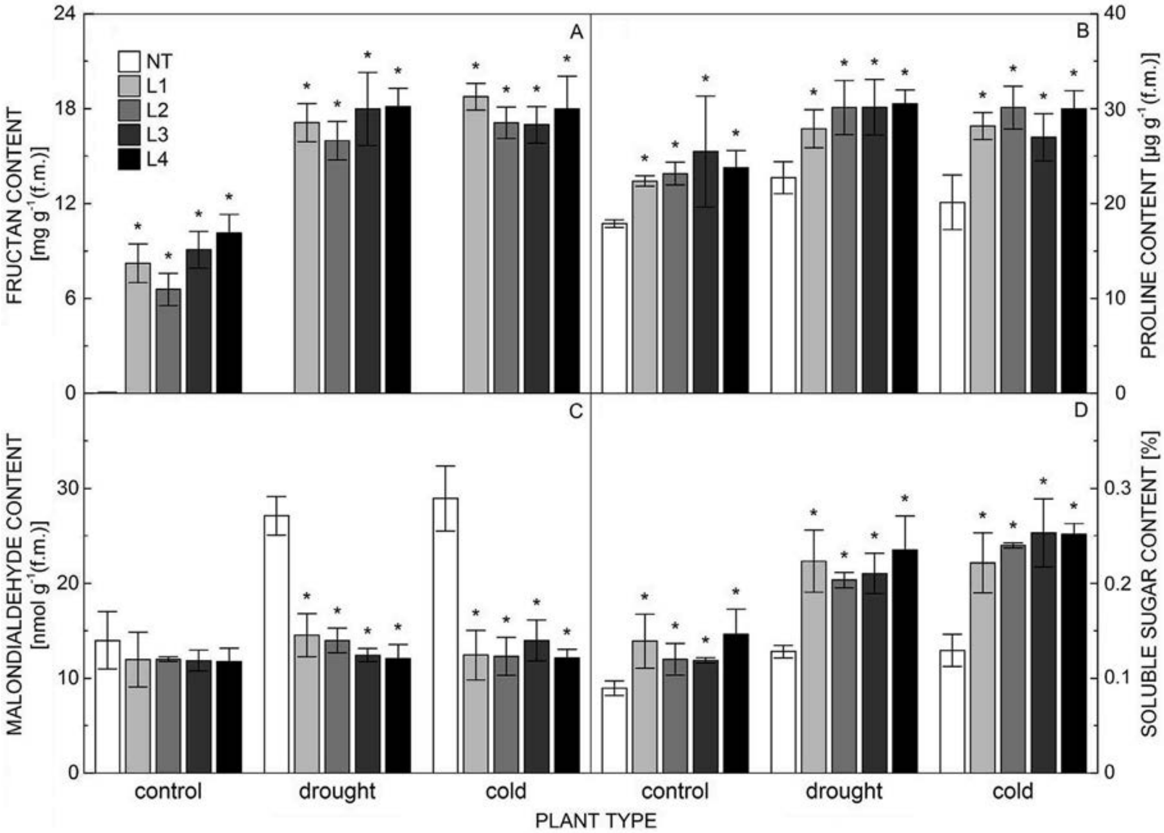

Fig. 5 Physiological analysis of non-transgenic (NT) and *Dv-6-SFT*-transformed tobacco plants (L1 -L4) under drought and cold stresses. *A* - Fructan content, *B* - proline content, *C* - MDA content; and *D* - soluble sugar content. No detectable fructan content in NT plants. Means ± SD, $n = 4$ individual experiments. Significant differences ($P < 0.05$) between NT and *Dv-6-SFT*-transformants are labeled with asterisks (Tukey's HSD test).

splicing might be an element that confers new functions on old genes during the process of evolution. In general, it can be assumed that intron loss and gain are associated with the evolution of primordial genes, although no conclusive link has been demonstrated between the intron-exon structure of a gene and its evolutionary history. Three main hypotheses have been proposed for the evolutionary history of spliceosomal introns: the "introns-early," "introns-late," and "introns-middle" hypotheses (Coulombe-Huntington and Majewski 2007). However, there is insufficient evidence at present to support only one model. The underlying mechanisms or evolutionary origins of intronless genes and the putative advantages obtained by harboring them are not yet clear and further intensive study is required. Previous research indicated that intronless genes are probably in flux between gain and loss, and not static representatives of sequential intron gains or losses (Hartung *et al.* 2002). In evolutionary terms, it is reasonable to assume that the intron-exon structure of a gene may be related to its evolutionary history (Hartung *et al.* 2002) and that intronless genes may be expressed rapidly (Jeffares *et al.* 2008). Kawaguchi *et al.* (2010) proposed that high-expression genes for hatching enzymes frequently lost their introns during the evolution of teleosts, whereas the low-expression genes maintained the exon-intron structure of the ancestral gene. However, this might be an arbitrary conjecture given by the specific physiological and evolutionary features of *Dv-6-SFT*. More research is required to test whether the novel gene has special functions from physiological and evolutionary perspectives. In particular, it is essential to determine whether aberrant splicing also occurs in *D. villosum* when it is exposed to environmental stresses, as well as the effects of the aberrant splicing of *FT* genes on the physiology of the whole plants.

To test the effect of *Dv-6-SFT* on abiotic tolerance, we analyzed the physiological responses of the TPs and NT plants under normal and abiotic stress conditions. The results indicated that *Dv-6-SFT* was expressed in tobacco and the average fructan content in the leaves of the TPs ranged between 6.57 - 10.13 mg g^{-1}(f.m.). In addition, abiotic stress assays indicated that all TPs exhibited significantly enhanced drought, cold, and salt stress tolerance as well as increased fructan content. Fructan functions as a stress protectant (Pontis 1989, Vijn and Smeekens 1999, Crafts Brandner 2005, Livingston *et al.* 2005) and osmoprotectant (Pilon-Smits *et al.* 1995) in a wide variety of organisms, which may explain the enhanced tolerance found in this study. These results agree with the findings of Bie *et al.* (2012), who reported an increased tolerance to drought, cold, and salt in TPs that expressed wheat *Ta6-SFT*, *Ta1-SST*, or *Ta1-FFT* enzymes (range 6.1 - 10.2 mg g^{-1}). In normal growth conditions, the fructan content of the TPs with the *Ph-6-SFT* gene were 6.32 to 11.12 mg g^{-1}(f.m.) which was similar to that in the *Dv-6-SFT* TPs. After drought stress for 20 d, the fructan content of the *Ph-6-SFT* TPs ranged from 15.92 to 20.15 mg g^{-1}(f.m.), whereas those of the *Dv-6-SFT* TPs ranged from 15.98 to 18.14 mg g^{-1}(f.m.). After cold stress, the fructan content of the TPs with the *Ph-6-SFT* gene was 16.13 to 19.47 mg g^{-1}(f.m.), whereas the fructan content of those with the *Dv-6-SFT* gene ranged from 16.98 to 18.76 mg g^{-1}(f.m.) (He *et al.* 2015). Similar results are also obtained in a study where wheat gene *Ta6-SFT* and *Ta1-SST* are expressed in rice (Kawakami *et al.* 2008). Li *et al.* (2007) has also found that expression of the *1-SST* gene from *Lactuca sativa* results in enhanced fructan accumulation and presumably freezing tolerance. The accumulation of fructan and the results of the drought, cold, and salt stress assays in TPs suggest that *Dv-6-SFT* is involved in fructan synthesis in tobacco, which may improve of drought, cold, and salinity stress tolerance of TPs. There was no significant difference in the fructan content of the *Ph-6-SFT* and *Dv-6-SFT* TPs, but their gene structure (in terms of the exon number and exon length) showed that the *Ph-6-SFT* genomic DNA had a complex organization with four exons and three introns, whereas the *Dv-6-SFT* genomic DNA lacked introns and it comprised one large fused exon.

Proline is considered to play an important role in the response to environmental stresses, including the stabilization of cellular proteins and membranes, osmotic adjustment, and the scavenging of reactive oxygen species (ROS) (Ashraf and Foolad 2007). Previous research has shown that hexose sugars from hydrolyzed fructan can lower the intracellular water potential (Darbyshire and Henry 1978, Pollock 1984, Spollen and Nelson 1994). In addition, proline accumulation may be affected by the water potential (Knipp and Honermeier 2006) because proline accumulates at a low water potential (Smirnoff 1994, Voetberg and Sharp 1991). In this study, the proline content of the TPs were obviously higher than those of the NT plants in all conditions, which suggest that *Dv-SFT* may be important for increasing proline accumulation. However, Knipp and Honermeier (2006) found that the proline level was low in most inulin-producing potato plants under drought stress, thereby indicating that the modification of soluble carbohydrate metabolism could affect water stress-induced proline accumulation, although the connections between fructan and proline metabolism are complex.

As a biomarker for oxidative damage (Ren *et al.* 2010), we determined the MDA content, which was lower in TPs than in the NT plants under stress conditions. Only a slight increase in MDA was observed in the TPs after drought and cold stresses, whereas a significant increase was found in the NT plants. The reduced MDA content indicates that less ROS-mediated membrane damage occurred in the TPs (Zhu *et al.* 2012), which may explain why the overall damage caused by drought, cold, and salt stresses in tobacco TPs was much lower than that sustained by the NT plants (Fig. 3 Suppl.). These results agree with those reported by Li *et al.* (2007), who found that the content of MDA in fructan-producing tobacco TPs is significantly lower than that in wild-type tobacco plants and in anti-sense TPs after freezing stress.

Soluble sugars such as sucrose, glucose, and fructose are often accumulated in adverse environments where they are probably associated with abiotic stress responses in plants (Couee *et al.* 2006). The positive effects of soluble sugars on stress responses in plants include decreased photo-oxidative damage, osmoregulation, and as a molecular chaperones for stabilizing protein structures (Shen *et al.* 1997, Livingston *et al.* 2009, Ruelland *et al.* 2009). In this study, the expression of *Dv-6-SFT* in TPs increased the soluble sugar content compared with the NT plants under stressful and non-stress conditions. Similar results have been reported in previous studies (Kawakami *et al*. 2008, Tamura *et al*. 2014a,b). A connection between the fructan content and the starch dynamics is a possible explanation of the increased soluble sugars content in *Dv-6-SFT* TPs (Tarkowski and Van den Ende 2015), although no conclusive evidence was obtained to support this hypothesis. In the current study, *Dv-6-SFT* expression increased the accumulation of fructans, soluble sugars, and proline under the control and stress conditions. As typical compatible solutes they played important roles in drought, cold, and salt stress tolerance in tobacco TPs due to their effects as osmotic adjustment mediators, signaling molecules, cryoprotectants, membrane stabilizers, and antioxidants (Ruelland *et al*. 2009, Szabados and Savouré 2010). However, the exact explanation for the many biochemical changes that generated fructans in TP plants is still unclear.

In conclusion, a *6-SFT* gene in *D. villosum* was successfully isolated using genome walking and RT-PCR. The *Dv-6-SFT* gene was transformed into tobacco to analyze its functional roles. We found that unlike most previously described *6-SFTs*, the *Dv-6-SFT* gene lacked introns. *Dv-6-SFT* expression resulted in fructan accumulation in TPs, which was accompanied by increased content of soluble sugars and proline. The TPs also exhibited much higher tolerance to drought, cold, and high salinity compared with the NT plants. The results of this study provide new insights into the structural diversity of *6-SFT* genes in the plant kingdom, which may have practical applications in crop breeding. Further studies are needed to elucidate the molecular and physiological mechanisms that lead to improved abiotic stress tolerance in *Dv-6-SFT* TPs.

References

Abdelgawad, H., Peshev, D., Zinta, G., Van den Ende, W., Janssens, I.A., Asard, H.: Climate extreme effects on the chemical composition of temperate grassland species under ambient and elevated CO_2: a comparison of fructan and non-fructan accumulators. - PLoS ONE **9**: e92044, 2014.

Altenbach, D., Nüesch, E., Ritsema, T., Boller, T., Wiemken, A.: Mutational analysis of the active center of plant fructosyltransferases: *Festuca* 1-SST and barley 6-SFT. - FEBS Lett. **579**: 4647-4653, 2005.

Ashraf, M., Foolad, M.R.: Roles of glycine betaine and proline in improving plant abiotic stress resistance. - Environ. exp. Bot. **59**: 206-216, 2007.

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Bie, X.M., Wang, K., She, M.Y., Du, L., Zhang, S.X., Li, J.R., Gao, X., Lin, Z.S., Ye, X.G.: Combinational transformation of three wheat genes encoding fructan biosynthesis enzymes confers increased fructan content and tolerance to abiotic stresses in tobacco. - Plant Cell Rep. **31**: 2229-2238, 2012.

Bournay, A.S., Hedley, P.E., Maddison, A., Waugh, R., Machray, G.C.: Exon skipping induced by cold stress in a potato invertase gene transcript. - Nucl. Acids Res. **24**: 2347-2351, 1996.

Cao, Y., Bie, T., Wang, X., Chen, P.: Induction and transmission of wheat-*Haynaldia villosa* chromosomal translocations. - J. Genet. Genomics **36**: 313-320, 2009.

Chalmers, J., Johnson, X., Lidgett, A., Spangenberg, G.: Isolation and characterisation of a sucrose: sucrose 1-fructosyltransferase gene from perennial ryegrass (*Lolium perenne*). - J. Plant Physiol. **160**: 1385-1391, 2003.

Chatterton, N.J., Harrison, P.A., Bennett, J.H., Asay, K.H.: Carbohydrate partitioning in 185 accessions of *Gramineae* grown under warm and cool temperatures. - J. Plant Physiol. **134**: 169-179, 1989.

Chatterton, N.J., Harrison, P.A., Thornley, W.R., Bennett, J.H.: Structure of fructan oligomers in cheatgrass (*Bromus tectorum* L.). - New Phytol. **124**: 389-396, 1993.

Chen, H., Nelson, R.S., Sherwood, J.L.: Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. - Biotechniques **16**: 664-668, 670, 1994.

Couee, I., Sulmon, C., Gouesbet, G., El Amrani, A.: Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. - J. exp. Bot. **57**: 449-459, 2006.

Coulombe-Huntington, J., Majewski, J.: Intron loss and gain in *Drosophila*. - Mol. Biol. Evol. **24**: 2842-2850, 2007.

Crafts Brandner, S.J.: Fructans and freezing tolerance. - New Phytol. **166**: 708-709, 2005.

Darbyshire, B., Henry, R.J.: The distribution of fructans in onions. - New Phytol. **81**: 29-34, 1978.

De Roover, J., Van den Branden, K., Van Laere, A., Van den Ende, W.: Drought induces fructan synthesis and 1-SST (sucrose: sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.). - Planta **210**: 808-814, 2000.

Del Viso, F., Puebla, A.F., Fusari, C.M., Casabuono, A.C., Couto, A.S., Pontis, H.G., Hopp, H.E., Heinz, R.A.: Molecular characterization of a putative sucrose:fructan 6-fructosyltransferase (*6-SFT*) of the cold-resistant Patagonian grass *Bromus pictus* associated with fructan accumulation under low temperatures. - Plant Cell Physiol. **50**: 489-503, 2009.

Diedhiou, C., Gaudet, D., Liang, Y., Sun, J., Lu, Z.X., Eudes, F., Laroche, A.: Carbohydrate profiling in seeds and seedlings of transgenic triticale modified in the expression of *sucrose:sucrose-1-fructosyltransferase* (*1-SST*) and *sucrose:fructan-6-fructosyltransferase* (*6-SFT*). - J. Biosci. Bioeng. **114**: 371-378, 2012.

Fales, F.W.: The assimilation and degradation of carbohydrates by yeast cells. - J. biol. Chem. **193**: 113-124, 1951.

Gao, X., She, M.Y., Yin, G.X., Yu, Y., Qiao, W.H., Du, L.P., Ye, X.G.: Cloning and characterization of genes coding for fructan biosynthesis enzymes (FBEs) in *Triticeae* plants. - Agr. Sci. China **9**: 313-324, 2010.

Hartung, F., Blattner, F.R., Puchta, H.: Intron gain and loss in the evolution of the conserved eukaryotic recombination machinery. - Nucl. Acids Res. **30**: 5175-5181, 2002.

He, X.L., Chen, Z.Z., Wang, J.W., Li, W.X., Zhao, J.X., Wu, J., Wang, Z.H., Chen, X.H.: A sucrose: fructan-6-fructosyltransferase (*6-SFT*) gene from *Psathyrostachys huashanica* confers abiotic stress tolerance in tobacco. - Gene **570**: 239-247, 2015.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hendry, G.A.: Evolutionary origins and natural functions of fructans – a climatological, biogeographic and mechanistic appraisal. - New Phytol. **123**: 3-14, 1993.

Heyer, A.G., Wendenburg, R.: Gene cloning and functional characterization by heterologous expression of the fructosyltransferase of *Aspergillus sydowi* IAM 2544. - Appl. environ. Microbiol. **67**: 363-370, 2001.

Hincha, D.K., Livingston, D.R., Premakumar, R., Zuther, E., Obel, N., Cacela, C., Heyer, A.G.: Fructans from oat and rye: composition and effects on membrane stability during drying. - Biochim. biophys. Acta - Biomembranes **1768**: 1611-1619, 2007.

Hisano, H., Kanazawa, A., Kawakami, A., Yoshida, M., Shimamoto, Y., Yamada, T.: Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing. - Plant Sci. **167**: 861-868, 2004.

Hisano, H., Kanazawa, A., Yoshida, M., Humphreys, M.O., Iizuka, M., Kitamura, K., Yamada, T.: Coordinated expression of functionally diverse fructosyltransferase genes is associated with fructan accumulation in response to low temperature in perennial ryegrass. - New Phytol. **178**: 766-780, 2008.

Huynh, B., Mather, D.E., Schreiber, A.W., Toubia, J., Baumann, U., Shoaei, Z., Stein, N., Ariyadasa, R., Stangoulis, J.C., Edwards, J., Shirley, N., Langridge, P., Fleury, D.: Clusters of genes encoding fructan biosynthesizing enzymes in wheat and barley. - Plant mol. Biol. **80**: 299-314, 2012.

Irshad Ahmed, M., Krishnamurthy, L., Purushothaman, R., Vadez, V., Zaman-Allah, M.: Plant biomass productivity under abiotic stresses in SAT agriculture. - In: Matovic, D. (ed.): Biomass Detection, Production and Usage. Pp 247-264. InTech, Rijeka 2011.

Jeffares, D.C., Penkett, C.J., Bähler, J.: Rapidly regulated genes are intron poor. - Trends Genet. **24**: 375-378, 2008.

Ji, X., Van den Ende, W., Schroeven, L., Clerens, S., Geuten, K., Cheng, S., Bennett, J.: The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the *Pooideae*. - New Phytol. **173**: 50-62, 2007.

Kawaguchi, M., Hiroi, J., Miya, M., Nishida, M., Iuchi, I., Yasumasu, S.: Intron-loss evolution of hatching enzyme genes in Teleostei. - BMC Evol. Biol. **10**: 260, 2010.

Kawakami, A., Sato, Y., Yoshida, M.: Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. - J. exp. Bot. **59**: 793-802, 2008.

Kawakami, A., Yoshida, M.: Molecular characterization of sucrose:sucrose 1-fructosyltransferase and sucrose:fructan 6-fructosyltransferase associated with fructan accumulation in winter wheat during cold hardening. - Biosci. Biotech. Biochem. **66**: 2297-2305, 2002.

Kerepesi, I., Galiba, G.: Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. - Crop Sci. **40**: 482-487, 2000.

Knipp, G., Honermeier, B.: Effect of water stress on proline accumulation of genetically modified potatoes (*Solanum tuberosum* L.) generating fructans. - J. Plant Physiol. **163**: 392-397, 2006.

Lammens, W., Le Roy, K., Yuan, S.G., Vergauwen, R., Rabijns, A., Van Laere, A., Strelkov, S.V., Van den Ende, W.: Crystal structure of 6-SST/6-SFT from *Pachysandra terminalis*, a plant fructan biosynthesizing enzyme in complex with its acceptor substrate 6-kestose. - Plant J. **70**: 205-219, 2012.

Lasseur, B., Schroeven, L., Lammens, W., Le Roy, K., Spangenberg, G., Manduzio, H., Vergauwen, R., Lothier, J., Prud'Homme, M.P., Van den Ende, W.: Transforming a fructan:fructan 6G-fructosyltransferase from perennial ryegrass into a sucrose:sucrose 1-fructosyltransferase. - Plant Physiol. **149**: 327-339, 2009.

Le Roy, K., Lammens, W., Verhaest, M., De Coninck, B., Rabijns, A., Van Laere, A., Van den Ende, W.: Unraveling the difference between invertases and fructan exohydrolases: a single amino acid (Asp-239) substitution transforms *Arabidopsis* cell wall invertase1 into a fructan 1-exohydrolase. - Plant Physiol. **145**: 616-625, 2007.

Li, H.J., Yang, A.F., Zhang, X.C., Gao, F., Zhang, J.R.: Improving freezing tolerance of transgenic tobacco expressing sucrose: sucrose 1-fructosyltransferase gene from *Lactuca sativa*. - Plant Cell Tissue Organ Cult. **89**: 37-48, 2007.

Livingston III, D.P., Hincha, D.K., Heyer, A.G.: Fructan and its relationship to abiotic stress tolerance in plants. - Cell. mol. Life Sci. **66**: 2007-2023, 2009.

Livingston, D., Premakumar, R., Tallury, S.P.: Carbohydrate concentrations in crown fractions from winter oat during hardening at sub-zero temperatures. - Ann. Bot. **96**: 331-335, 2005.

Maleux, K., Van den Ende, W.: Levans in excised leaves of *Dactylis glomerata*: effects of light, sugars, temperature and senescence. - J. Plant Biol. **50**: 671-680, 2007.

Peshev, D., Vergauwen, R., Moglia, A., Hideg, E.V., Van den Ende, W.: Towards understanding vacuolar antioxidant mechanisms: a role for fructans? - J. exp. Bot. **64**: 1025-1038, 2013.

Pilon-Smits, E., Ebskamp, M., Paul, M.J., Jeuken, M., Weisbeek, P.J., Smeekens, S.: Improved performance of transgenic fructan-accumulating tobacco under drought stress. - Plant Physiol. **107**: 125-130, 1995.

Pilon-Smits, E.A., Terry, N., Sears, T., Van Dun, K.: Enhanced drought resistance in fructan-producing sugar beet. - Plant Physiol. Biochem. **37**: 313-317, 1999.

Pollock, C.J.: Physiology and metabolism of sucrosyl-fructans. - Seminar Series Soc. exp. Biol. **19**: 97-113, 1984.

Pontis, H.G.: Fructans and cold stress. - J. Plant Physiol. **134**: 148-150, 1989.

Ren, X., Chen, Z., Liu, Y., Zhang, H., Zhang, M., Liu, Q., Hong, X., Zhu, J., Gong, Z.: ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in *Arabidopsis*. - Plant J. **63**: 417-429, 2010.

Ritsema, T., Joling, J., Smeekens, S.: Patterns of fructan synthesized by onion fructan: fructan 6G-fructosyltransferase expressed in tobacco BY2 cells-is fructan: fructan 1-fructosyltransferase needed in onion? - New Phytol. **160**: 61-67, 2003.

Ruelland, E., Vaultier, M., Zachowski, A., Hurry, V.: Cold signalling and cold acclimation in plants. - Adv. Bot. Res. **49**: 35-150, 2009.

Sambrook, J., Fritsch, E.F., Maniatis, T. (ed): Molecular Cloning., 3rd Ed. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor - New York 1989.

Schroeven, L., Lammens, W., Kawakami, A., Yoshida, M., Van Laere, A., Van den Ende, W.: Creating S-type characteristics in the F-type enzyme fructan:fructan 1-fructosyltransferase of *Triticum aestivum* L. - J. exp. Bot. **60**: 3687-3696, 2009.

Schuler, M.A., Zielinski, R.E.: Transformation of leaf discs with *Agrobacterium*. - In: Schuler, M.A., Zielinski, R.E. (ed.): Methods in Plant Molecular Biology. Pp 145-156. Academic

Press, San Diego 1989.

Shahbaz, M., Ashraf, M.: Improving salinity tolerance in cereals. - Crit. Rev. Plant Sci. **32**: 237-249, 2013.

Shen, B.O., Jensen, R.G., Bohnert, H.J.: Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. - Plant Physiol. **113**: 1177-1183, 1997.

Simpson, C.G., Hedley, P.E., Watters, J.A., Clark, G.P., Mcquade, C., Machray, G.C., Brown, J.W.S.: Requirements for mini-exon inclusion in potato invertase mRNAs provides evidence for exon-scanning interactions in plants. - RNA **6**: 422-433, 2000.

Smirnoff, N.R.M.: The origin of the proline accumulated in primary roots of maize seedlings at low water potential. - J. exp. Bot. **45**: 273-278, 1994.

Spollen, W.G., Nelson, C.J.: Response of fructan to water deficit in growing leaves of tall fescue. - Plant Physiol. **106**: 329-336, 1994.

Sprenger, N., Bortlik, K., Brandt, A., Boller, T., Wiemken, A.: Purification, cloning, and functional expression of sucrose: fructan 6-fructosyltransferase, a key enzyme of fructan synthesis in barley. - Proc. nat. Acad. Sci. USA **92**: 11652-11656, 1995.

Szabados, L., Savouré, A.: Proline: a multifunctional amino acid. - Trends Plant Sci. **15**: 89-97, 2010.

Tamura, K., Sanada, Y., Tase, K., Kawakami, A., Yoshida, M., Yamada, T.: Comparative study of transgenic *Brachypodium distachyon* expressing sucrose:fructan 6-fructosyltransferases from wheat and timothy grass with different enzymatic properties. - Planta **239**: 783-792, 2014a.

Tamura, K., Sanada, Y., Tase, K., Yoshida, M.: Fructan metabolism and expression of genes coding fructan metabolic enzymes during cold acclimation and overwintering in timothy (*Phleum pratense*). - J. Plant Physiol. **171**: 951-958, 2014b.

Tamura, K.I., Kawakami, A., Sanada, Y., Tase, K., Komatsu, T., Yoshida, M.: Cloning and functional analysis of a fructosyltransferase cDNA for synthesis of highly polymerized levans in timothy (*Phleum pratense* L.). - J. exp. Bot. **60**: 893-905, 2009.

Tarkowski, Ł.P., Van den Ende, W.: Cold tolerance triggered by soluble sugars: a multifaceted countermeasure. - Front. Plant Sci. **6**: 203-210, 2015.

Voetberg, G.S., Sharp, R.E.: Growth of the maize primary root at low water potentials III. Role of increased proline deposition in osmotic adjustment. - Plant Physiol. **96**: 1125-1130, 1991.

Wei, J.Z., Chatterton, N.J.: Fructan biosynthesis and fructosyltransferase evolution: expression of the *6-SFT* (sucrose: fructan 6-fructosyltransferase) gene in crested wheatgrass (*Agropyron cristatum*). - J. Plant Physiol. **158**: 1203-1213, 2001.

Wei, J.Z., Chatterton, N.J., Harrison, P.A., Wang, R., Larson, S.R.: Characterization of fructan biosynthesis in big bluegrass (*Poa secunda*). - J. Plant Physiol. **159**: 705-715, 2002.

Wei, J.Z., Chatterton, J.N., Larson, S.R.: Expression of sucrose: fructan 6-fructosyltransferase (*6-SFT*) and myo -inositol 1-phosphate synthase (MIPS) genes in barley (*Hordeum vulgare*) leaves. - J. Plant Physiol. **158**: 635-643, 2001.

Yoshida, M., Lin, D., Kawakami, A.: A mini exon in the *sucrose: sucrose 1-fructosyltransferase* gene of wheat. - J. Plant Physiol. **161**: 1277-1279, 2004.

Zhu, B., Peng, R., Xiong, A., Xu, J., Fu, X., Zhao, W., Jin, X., Meng, X., Gao, J., Cai, R.: Transformation with a gene for myo-inositol O-methyltransferase enhances the cold tolerance of *Arabidopsis thaliana*. - Biol. Plant. **56**: 135-139, 2012.