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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*)

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Constructs with sucrose-sucrose 1-fructosyltransferase (1-SST) from rye and or sucrose-fructan 6-fructosyltransferase (6-SFT) from wheat were placed under the control of wheat aleurone-specific promoter and expressed in triticale using biolistic and microspore transformation. Transgenic lines expressing one or both the 1-SST and the 6-SFT accumulated 50% less starch and 10–20 times more fructan, particularly 6-kestose, in the dry seed compared to the untransformed wild-type (WT) triticale; other fructans ranged in size from DP 4 to DP 15. During germination from 1 to 4 days after imbibition (dai), fructans were rapidly metabolized and only in transgenic lines expressing both 1-SST and 6-SFT were fructan contents significantly higher than in the untransformed controls after 4 days. *In situ* hybridization confirmed expression of 6-SFT in the aleurone layer in imbibed seeds of transformed plants. When transgenic lines were subjected to a cold stress of 4°C for 2 days, synthesis of fructan increased compared to untransformed controls during low-temperature germination. The increase of fructan in dry seed and germinating seedling was generally associated with transcript expression levels in transformed plants but total gene expression was not necessarily correlated with the time course accumulation of fructan during germination. This is the first report of transgenic modification of cereals to achieve production of fructans in cereal seeds and during seed germination.

[**Key words:** Sucrose:sucrose-1-fructosyltransferase (1-SST); Sucrose:fructan-6-fructosyltransferase (6-SFT); Oligofructans; Transcripts; Stable transformation; Triticale]

Fructans are linear or branched polymers of fructose, present in approximately 15% of the angiosperm flora, and are widespread among members of the Poaceae such as wheat (*Triticum aestivum* L.) and grasses (1). Fructans are soluble carbohydrate reserves that increase in content in the leaves and crown during cold hardening in the autumn which are readily mobilized during the winter, and have been implicated in resistance to abiotic winter stresses such as cold and drought (2) and to biotic stresses such as resistance to snow moulds (3). The substrate for fructan synthesis is sucrose (4). Although sucrose is present in the cytoplasm, fructans are synthesized and stored in the vacuole by the action of fructosyltransferases that transfer fructose from sucrose to the growing fructan chain (5). During fructan biosynthesis, sucrose-sucrose 1-fructosyltransferase (1-SST) converts sucrose to 1-kestose that becomes a substrate for inulin-type or levan-type fructans whereas sucrose-fructan 6-fructosyltransferase (6-SFT) converts sucrose to 6-kestose that proceeds to synthesis of levan-type fructans (6,7). Inulin-type fructans consist of linear β(2-1)-linked fructofuranosyl units and occur mainly in dicotyledonous species whereas the more complex and branched levan consists primarily of linear β(2-6)-linked fructofuranosyl units are common in monocots (8,9). Fructans do not normally occur in significant quantities in mature cereal seeds since the vast majority of all carbohydrate stored in the seed is converted to starch (10) although high fructan levels have been observed in mature onions seeds (11). Fructans are the subject of intense research because of their role as prebiotics in human and animal health has been associated with the reduction of disease, namely constipation, infectious diarrhoea, cancer, osteoporosis, atherosclerotic cardiovascular disease, obesity, and non-insulin-dependent diabetes (12).

The cereal seed consists of the embryo and scutellum, the starchy endosperm surrounded by the aleurone layer, and the enveloping seed coat (13). The aleurone layer, with its dense cuboidal cells, serves as the epidermal layer of the endosperm, synthesizing significant quantities of enzymes necessary for the mobilization of endosperm carbohydrates during seed development (14). During germination, nutrients required for the growth of the embryo are initially obtained from the stored nutrients in the embryo itself, but a subsequent mobilization of the storage materials in the endosperm occurs (15,16). The aleurone cells provide

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the required nutrients for development of the embryo by mobilization of numerous enzymes that degrade the starch in the endosperm (15,16).

Genetically modifying plants to augment native quantities of fructans in the roots, leaves or stems have been reported (17). Expression of 1-SST from *Helianthus tuberosus* in leaves of sugar beet resulted in alteration of the carbohydrate profile in the taproot with no visible effect on the morphology and growth rate of the taproot (18). Perennial ryegrass over-expressing the onion 1-SST gene showed increased fructan levels up to 3-fold higher in leaf and stem tissues compared to untransformed lines (17). Rice plants over-expressing *T. aestivum* 6-SFT (*wft1*) and 1-SST (*wft2*) accumulated higher fructans in the leaves and in stems (19). To date, there have been no reports on genetic modification of fructans in cereal seeds. The objective of the present study was to direct the expression of the fructan biosynthesis in triticale (*x Triticosecale* Witt.) seed using aleurone-specific promoters and examine its accumulation in the seed and metabolism during early seed germination. Triticale, which has excellent grain and biomass yields that generally exceed those of current cereal cultivars by 30%, has been adopted as a platform crop for production of biofuels, bioproducts, and designer starches and carbohydrates in Canada (Canadian Triticale Biorefinery Initiative (CTBI), http://www.ctbi.ca/, accessed on March 12, 2012).

MATERIALS AND METHODS

Plant materials and growth conditions A blue aleurone triticale (AC Alta * 6/Porendo 38) designated as wild type (WT), generated in our laboratory, was employed in all studies. For isolation, cloning, and sequencing of the 6-SFT and the 1-SST genes, the wheat cultivar CI14106 which accumulates high levels of fructan during hardening (3), and the rye cultivar Prima were employed. Seeds were planted in 15 cm pots containing a soilless potting mix. Seedlings were reared in a growth cabinet (Conviron, Winnipeg, Canada) set at 20°C (12 h day)/20°C (12 h night) with a light intensity of 350 μmol^{-2} s^{-1} or in a greenhouse set at 22°C with supplemental lighting for 18 h a day. For germination in treatments receiving cold hardening, seedlings were grown 2 weeks at 2°C (16 h day/8 h night) with a light intensity of 250 μmol^{-2} s^{-1} in a growth cabinet. For molecular studies, leaf tissues were harvested, immediately flash frozen in liquid nitrogen and stored at −80°C until used. Seed germination was carried in the dark at either room temperature or at 4°C in a low temperature incubator.

Cloning of *1-SST* and *6-SFT* Reagents and enzymes for recombinant DNA techniques were purchased from Promega (Fisher Scientific Ltd., Canada) and Takara (Clontech Laboratories Inc., USA), and used according to instructions provided by the manufacturer unless otherwise indicated. For the isolation, cloning and sequence analysis of wheat *6-SFT* cDNA gene, total RNA was isolated from wheat leaves by using TRIzol reagent (Invitrogen, CA, USA). Qiagen RNeasy Mini Kit (Qiagen, Canada) was used to clean up total RNA. The Superscript III First-Strand Synthesis System (Invitrogen) was used for the reverse transcription of total RNA. Five μg of total RNA from wheat leaves reared in a growth cabinet was used for the synthesis of the first strand cDNA. Forward primer 5′-atggggtcacacggcaag-3′ and reverse primer 5′-ttcattgaacatacgagtgatcg-3′ were designed for amplifying wheat *6-SFT* cDNA gene. The following PCR protocol was used: 94°C for 2 min, 94°C for 1 min, 70, 68, 66, 64, 62, 60, 58, 56°C each for 1 min, 72°C for 2.5 min for 2 cycles, 94°C for 1 min; 55°C for 1 min, 72°C for 2.5 min for 25 cycles; and, 72°C for 10 min.

The isolation, cloning and sequence analysis of the rye *1-SST* cDNA gene, and reverse transcription of rye cDNA were the same as above. The following degenerate primers ScSST-F: ACCACYTCTTCTACCAGYACAACCC and ScSST-R: YGTSCGGGTCTT-CYYGTCAAGC were designed according to the known *1-SST* genes (*T. aestivum*, GenBank accession no.AB029888; *Hordeum vulgare*, GenBank accession no.AJ567377; *Lolium perenne*, GenBank accession no.AY245431; and *Festuca arundinacea*, GenBank accession no.AJ297369) to amplify the 936 bp-length middle region of rye *1-SST* cDNA gene using the following PCR protocol: 94°C for 2 min, 94°C for 30 s, 60°C for 30 s, 72°C for 1 min for 35 cycles, and 72°C for 10 min. The full-length rye *1-SST* cDNA was obtained by the method of rapid amplification of cDNA ends (RACE) carried out according to protocols supplied by Invitrogen. The primers designed for RACE were: ScSST-5′GSP (ACGATCCCAACGGTCCGGTGTATTATG), ScSST-GSP2 (GCGAGTACGAGTG-CATCGACCTCTACG), ScSST-NGSP2 (GGACGTGCTGTACGTGCTCAAGGAGA), ScSST-3′GSP (GGGTCGTAGAAGGACTTGGACGCGTAG), ScSST-GSP1 (CTTGGACGCGTAG-TACTTGCCCCAGTC) and ScSST-NGSP1 (GTTGCCCGTGTAGAGCAGGATGACCTT).

Description of promoters and construction of expression vectors of rye *1-SST* and wheat *6-SFT* for expression in triticale The 475 bp ns-LTP, seed aleurone layer-specific promoter from wheat (Accession number: AF551849) was used in conjunction with the 1-SST and 6-SFT genes. Four pairs of constructs used for transforming triticale were made using MultiSite Gateway® Three-Fragment Vector Expression System (Invitrogen), according to instructions provided by the manufacturer. The glufosinate-resistant gene (PAT) under the control of the constitutive rice actin promoter was included in all constructs downstream of fructan biosynthesis genes. To facilitate the screening of potential transgenic plants, rye *1-SST* and wheat *6-SFT* gene sequences were fused to yellow fluorescent protein (YFP) or green fluorescent protein (GFP) respectively. The organization of the constructs was as follows: 11, Wheat LTP Promoter:1-SST:GFP:Nos-Actin Promoter-Intron-Pat-Tml; and, 15, Wheat LTP Promoter:6-SFT:GFP:Nos-Actin Promoter-Intron-Pat-Tml. Accuracy of the sequences cloned into expression vectors was confirmed by sequencing.

Transformation of triticale and characterization of putative transformants Plants were transformed by microspore transfection in accordance to Eudes and Amundsen (20). The T_1 and T_2 generations were generated from T_0 seed in a growth cabinet. Putative transformed lines were verified by Southern hybridization (21) using the MultiSite Gateway insert as a probe. Lines identified positive using Southern hybridization were employed to verify the specificity of primers designed for PCR. Specific PCR primers were designed for different regions of GFP or YFP and *1-SST*-YFP and *6-SFT*-GFP. PCR reaction conditions were as follows: 94°C for 3 min, 30 cycles of 94°C for 1 min, 54—60°C for 1 min, 72°C for 1 min, and an additional extension at 72°C for 10 min. Primer pairs for qRT-PCR analyses were designed using Primer 3 software (22). Sequences and general guidelines for primer design were based on the recommendations provided by Qiagen. Lt1 and Lt2 are designations given to two lines that were singly transformed with 1-SST (construct 11) or 6-SFT (construct 15), respectively, and Lt3 corresponds to a line co-transformed with both 1-SST and 6-SFT (constructs 11 and 15).

Seeds from WT and transformed plants were treated with 10% bleach for 10 min, rinsed three times in sterile distilled water, and germinated in the dark on moistened Whatman no. 1 filter paper in Petri dishes at 21°C or 4°C. Quantitative real-time PCR (qPCR) was carried out to analyse the expression of *1-SST* and *6-SFT* under the control of the aleurone-specific wheat promoters at 0, 1, 2, and 4 days following hydration, henceforth referred to as days after imbibition (dai). Samples were frozen in liquid nitrogen and stored at −80°C until used. Seeds were ground to a fine powder in liquid nitrogen, and aliquots were used for RNA extraction. Aliquots were also vacuum-dried and the fructans were extracted and analysed using HPLC according to Huynh et al. (23).

RNA extraction and qRT-PCR Total RNA was extracted from about 200 mg of germinated seed samples using RNeasy Plant Mini Kit (Qiagen, CA, USA) and residual DNA was removed using RNase-free DNase I (Qiagen) according to manufacturer's instructions. Concentration of each RNA sample was measured using a DU 800 Spectrophotometer (Beckman Coulter, USA). Only RNA samples with 260/280 ratio between 1.8 and 2.0 and 260/230 ratio greater than 2.0 were used for the analysis. The integrity of RNA samples was further assessed by agarose gel electrophoresis. First strand cDNA was synthesized using SuperScript™ III M-MLV reverse transcriptase kit (Invitrogen). cDNA was performed according to manufacturers' instructions. Total RNA (4 μg) was incubated in the presence of 50 pmol oligo $(dT)_{20}$ and sterile ddH_2O in 12 μl total volume at 65°C for 5 min and cooled in ice. The synthesis was performed in 1× first strand buffer, 0.1 μmol DTT, 40 U RNaseOut (Invitrogen), 10 nmol of each dNTP and 200 U SuperScript™ III Reverse Transcriptase in 20 μl total volume, incubated for 50 min at 45°C. Synthesis reactions were terminated by 15 min incubation at 70°C. The gene-specific primers employed, their melting temperature (T_m), and the expected size of amplified fragments in base pairs (bp), are listed in Table 1.

qPCR analyses were conducted in an iCycler iQ (BIO-RAD, USA) using Quanti-Tect SYBR Green PCR Kit (Qiagen) as the fluorescence marker for quantitative detection of individual genes according to manufacturer's instructions. qPCR reaction mixtures contained 10 μl SYBR Green mix, 0.5 μl fluorescein (0.5 μM), 6 pM of each primer, 5 μl template (2× diluted cDNA from leaf samples) and sterile distilled water to a total reaction volume of 20 μl. To standardize the data, the amount of target gene was normalized over the abundance of a constitutive wheat translation elongation factor 1 α-subunit (*TEF1*) (GenBank Accession M90077) and contig 5 (GenBank Accession CK155621), a carbohydrate transmembrane transporter. Thermal conditions were 95°C for 13 min followed by 45 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s, then held at 4°C. To detect primer dimerization and other artefacts of amplification, a melting curve analysis was performed immediately after completion of the qRT-PCR by increasing sample temperatures from 40°C to 95°C at a +0.5°C per 10 s period with continuous measurement of fluorescence. Results were analysed with the REST software (24). Based on the data of two reference genes *TEF1* and contig 5, REST was used to analyse the fold differences in target gene expression between the control and the treated samples. Triple biological replicates for each sample were used for real-time PCR analysis and triple technical replicates were analysed for each biological replicate. The experiment was repeated twice using newly extracted RNA and synthesized cDNA samples each time, and representative results are presented. Threshold (Ct) values for real-time PCR results were determined for three individual T_2 seeds and between one and three technical replicates for each treatment.

***In situ* mRNA hybridization** Primers specific to 1-SST and to 6-SFT (Table 1) were combined with plasmid DNA from gene constructs 11 and 15, to amplify PCR fragments which were cloned into pBlueScriptKS+ (Stratagene), sequenced, and used as a template for *in vitro* transcription. The DIG-labelled antisense and sense