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Full Length Research Paper

Fructan metabolism in tall fescue calli under different environmental condition

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Several grasses usually accumulate high levels of fructans at low temperature or in the presence of high sugar supply. Fructans enhance plant resistance in adverse environmental conditions thanks to their vacuolar localization which allow osmotic adjustments. Tall fescue calli do not show mature vacuoles due to its anatomic characteristics, and these *in vitro* cultures can provide more knowledge about the fructan rule in unspecialized tissues especially during stress conditions. The aim of this study was to evaluate the sugar metabolism in embryogenic calli of tall fescue fed with different sucrose concentrations (0, 50 or 100 mM) during growth culture, kept at "control" or "cold" temperatures (23 or 4°C, respectively). In the presence of sucrose (50 or 100 mM), embryogenic calli accumulated fructans under control conditions. On the other hand, the accumulation is inhibited by the simultaneously sugar supply and cold treatment while invertase activity increased. From our data, cold temperature and high sucrose concentration seemed to be negatively correlated in the fructans biosynthetic pathway in tall fescue *in vitro* cultures.

Key words: Cold, *Festuca arundinacea*, invertase, sucrose:sucrose fructosyltransferase (SST), sugars.

INTRODUCTION

The most prominent storage-carbohydrate form in plants is represented by starch, but 15% of flowering plant species prefer fructans as reserve carbohydrates (Vijn and Smeekens, 1999). Fructans are polymers of fructose and form the major component of non-structural carbohydrates; unlike starch, fructans are water-soluble and are localized in the vacuole (Chatterton et al., 1989). Fructans occur particularly in many important crops as asparagus, onion, chicory, wheat, rye, oats and in many temperate C_3 grasses (Wiemken et al., 1986; Michiels at al., 2004).The physiological role of fructans in plants is not fully understood; fructans act as storage compounds, but their presence in specialized organs as bulbs or taproots also indicate a possible role in the plant development, since their utilization occur during regrowth, either after defoliation or during spring sprouting (Ritsema and Smeekens, 2003). Fructans are synthesized from sucrose; therefore, their synthesis promotes a reduction of sucrose levels in the vacuole, preventing the photosynthesis retro inhibition (Pollock, 1986).

Moreover, fructans are important for the protection of plants since they are accumulated during abiotic stresses, as drought, cold or salt stress (Vjin and Smeekens, 1999). The presence of high concentration of these water-soluble carbohydrates in stress tolerant plants supports the hypothesis that fructans do not represent simply storage compounds to maintain plant growth during periods of limited water availability, but they would also play a role as osmoregulators (Hendry and Wallace, 1993) during drought or low temperatures periods (Pilon-Smits et al., 1995). In fact, fructans maintain the stability of the lipid cellular membranes against drought by replacing water around the head groups of membrane phospholipids (Hincha et al., 2000; Vereyken et al., 2001).

During cold stress, fructans reduce the freezing point due to its high concentration in the vacuole, contributing to the change of the osmotic potential, and enhancing

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plant resistance (Van den Ende et al., 2002). Fructans seem to be correlated to mechanism of freezing tolerance, as demonstrated by the presence of these sugars in the apoplastic fluid of second phase cold hardened crown tissue of oat (Livingston and Henson, 1998) and the high expression of 6-kestose exohydrolase (6-KEH) in wheat sink tissues (Van den Ende et al., 2005). 6-KEH degrades apoplastic 6-kestose to sucrose and fructose that seem to protect membrane from freezing.

Fructan synthesis occurs directly from the vacuolar-stored sucrose and it is modulated by light, which led to changes of the availability of sucrose in the cell (Vijn and Smeekens, 1999). According to the classic model for inulin synthesis proposed by Edelman and Jefford (1968) for tubers of *Helianthus tuberosus,* two enzymes are mainly responsible for the synthesis of fructans, the sucrose:sucrose 1-fructosyltransferase (1-SST), and the fructan:fructan 1-fructosyltransferase (1-FFT). Moreover, in grasses, ß(2,6)-linked fructans are mainly accumulated by 6-SFTs in addition to ß(2,1)-linked fructans (Chalmers et al., 2005). In tall fescue, 1-SST and 1-FFT play a crucial role in assimilate partitioning and allocation, for instance in the leaf growth zone; for this reason tall fescue represents a good model to study fructan metabolism and several fructan synthesising enzymes have been previously studied and a 1-SST gene was cloned and characterised (Lüscher and Nelson, 1995; Lüscher et al., 2000).

The biosynthetic enzymes for fructans are evolutionary related to invertases (Vijn and Smeekens, 1999), rather than sucrose synthase (SuSy). These enzymes differently hydrolyse sucrose for further utilization as a source of carbon and energy. Plants contain several forms of invertase; the specific functions of the different invertase isoforms are not clear, but they appear to regulate the entry of sucrose into the different utilization pathways (Sturm, 1999). Because sugars in plants are not only nutrients but also important regulators of gene expression, invertases may be indirectly involved in the control of cell differentiation and plant development (Koch, 1996).

In vitro cultures represent a good model for studying and enhancing different metabolites production, by the modulation of growth medium composition and environmental conditions, as light and temperature. Undifferentiated cell cultures (callus and cell suspensions) are often used for their plasticity and heterotrophic nature (Mulabagal and Tsay, 2004).

The aim of this study was to evaluate the influence of cold and sucrose level in fructan synthesis in tall fescue callus culture without the interference of endogenous sucrose produced by photosynthesis. For this purpose, *in vitro* tall fescue embryogenic calli were produced and subjected to different exogenous sucrose treatments at different temperature (4 or 23°C). Fructans and free sugars levels were measured, the activities of sucrose hydrolysing enzymes were analyzed and the transcriptional expression of 1-SST was determined.

MATERIALS AND METHODS

Plant material

Tall fescue caryopses (*Festuca arundinacea* Schereb., cv Houndog) were used for the initiation of embryogenic callus. After surface sterilization in undiluted commercial sodium hypochlorite (7% active Cl) for 60 min at room temperature, caryopses were rinsed (3 times) in sterile distilled water and sowed in 10 ml sterile distilled water in Petri dishes in the dark at 4°C for 7 days. The caryopses were then sterilized in 10% commercial sodium hypochlorite for 10 min and rinsed (3 times) in sterile distilled water as reported by Dalton (1988). The seeds were then placed on MS medium (Murashige and Skoog, 1962) supplemented with 500 mg/L casein hydrolysed, 5 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 3% (w/v) sucrose, solidified with 1% (w/v) agar, and were kept in the dark at 25°C as reported by Spangenberg et al. (1995). After 4 months, 500 mg of friable, yellowish, embryogenic callus derived from single seeds was individually transferred to 6-well culture dishes (Falcon 3046, Becton Dickinson & Co., New Jersey, USA) with 3 ml of sterile distilled water containing 0, 50 or 100 mM sucrose at 23 or 4°C in the dark on a gyratory shaker at 60 rpm and collected after 3, 7 and 14 days. Samples were immediately used or stored at -80°C before use. All the experiments were conducted in triplicate.

Total RNA extraction and northern blot analysis

Up to 100 mg of callus were ground to a fine powder in liquid nitrogen using a ceramic mortar and pestle and total RNA was extracted according to Skadsen (1993) with minor modifications (Perata et al., 1997).

Ten nanogram (10 ng) RNA was electrophoresed on 1% (w/v) agarose glyoxal gels, and blotted on nylon membrane (BrightStar-Plus, MAbion, Texas, USA) using the procedure suggested by the manufacturers. Membranes were prehybridized and hybridized using the NorthernMax-Gly kit (Ambion) overnight at 55°C. The DNA probe fragment utilized was derived from sequenced tall fescue 1-SST (EMBL accession n^{o}. AJ297369). Radiolabeled probes were prepared from gel-purified cDNAs by using 1-SST primers (5'ATGGCTTCCTCTACCACC3' and 5'CGTTGCCAGTGTAGAGCAT3') labeled with [α^{32}P]dCTP (Takara Chemicals). Equal loading was checked by hybridizing with an rRNA cDNA probe (data not shown). RNA blots were scanned using a Cyclone Phosphoimager (Packard Bioscience, Perkin Elmer). mRNA level was quantified using the Optiquant software (Packard Bioscience, Perkin Elmer).

Soluble sugar extraction and determination

Hundred milligram (100 mg) fresh weight of calli were ground to a powder and extracted as described by Tobias et al. (1992). Amounts of glucose, fructose and sucrose were then determined using a coupled enzymatic assay method (Guglielminetti et al., 1995) measuring the increase in A_{340} due to nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) production. The efficiency of the methods was tested using known amounts of carbohydrates (glucose) as standards. Internal standards were used to evaluate losses taking place during the extraction procedures. Two tests were done for each metabolite by adding known amount of authentic standards to the samples prior to the extraction. The concentrations of the standards added were similar to those estimated to be present in the tissues in preliminary experiments.

The percentage of recovery ranged between 92 and 110%.

Analysis of fructans

Callus cultures (100 mg fresh weight) were ground and extracted in 100 µl distilled water. The extracts were incubated three minutes at 100°C to avoid further enzymatic activities. After cooling, the samples were centrifuged for 10 min at 12,000 g and the supernatants were immediately used or stored at -80°C. Five microliter (5 µl) of supernatants were spotted onto a silica-gel thin layer chromatography (TLC)-ready plates (Sigma-Aldrich, St. Louis, MO, USA) and developed three times with 1-butanol: glacial acetic acid: water in the ratio 50:25:25 according to Spollen and Nelson (1988).

For identification of fructans, dried TLC plates were sprayed with a urea-solution composed of 30 ml 85% phosphoric acid, 176 ml 80% butanol, 44 ml water, 12.5 ml ethanol and 7.5 g urea. After drying and incubating the TLC plates at 100°C for 40 min, the bands appeared and their intensities were measured by Quantity One 4.1.0 Program (Bio-Rad, Hercules CA, USA). Fructan quantification was determined by using fructose band intensity (known amount) as control.

Enzyme extraction and determination of total protein content

All steps for enzyme extraction and desalting were performed at 4°C. Embryogenic calli were homogenized to a fine powder in liquid nitrogen. Proteins were extracted in 50 mM citrate buffer, pH 5.2, 1 mM PMSF, 10 mM $NaHSO_3$, 0,07% (v/v) β-mercaptoethanol, 0.4% (v/v) protease cocktail inhibitor (Sigma-Aldrich, St. Louis, MO, USA), and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged for 15 min at 15,000 g and the supernatants were recovered and desalted by Micro Bio-Spin® Chromatography Columns (Bio-Rad, Hercules CA, USA) pre-equilibrated with 50 mM citrate buffer, pH 5.2, containing 0.1 M sodium citrate tribasic dihydrate and 0.1 M citric acid dihydrate. Total protein contents were determined according to the method of Bradford (1976) by using bovine serum albumin (BSA) as standard.

Enzyme assay

SuSy and alkaline invertase activities were spectrophotometrically measured by detecting the production of NADH (A_{340nm}). Reaction mixtures contained 50 mM HEPES-KOH buffer, 2 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 15 mM KCl, 2 mM adenosine-5'-triphosphate (ATP), 0.75 mM NAD, 2 units of Glc-6-P dehydrogenase, 2 units of hexokinase, 3 units of glucoisomerase and 10 µg of the dialyzed extract. The alkaline invertase reaction started by the addition of 1 M sucrose; otherwise the SuSy activity was measured by supplementary addition of 20 mM UDP.

For acid invertase assay, an aliquot of dialyzed extracts were added with 100 mM sucrose in 100 mM sodium acetate buffer (pH 5.2). Reactions were allowed to progress for 4 h at 37°C and then stopped by boiling the samples for 2 min. Control reactions contained boiled extracts. Five microliter (5 µl) of samples were spotted onto a silica-gel TLC-ready (following the methods previously described). Bands intensities were measured by Quantity One 4.1.0 Program (Bio-Rad, Hercules CA, USA). Acid invertase activity was quantified comparing fructose control band intensity (known amount of this hexose) with the fructose bands intensity derived by sucrose hydrolysis.

1-SST was assayed at 37°C. Aliquots of dialyzed extracts (50 µl) were mixed with an equal volume of 500 mM sucrose in 200 mM citrate/phosphate buffer, pH 5.0. After 3 h of incubation, the reaction was stopped by boiling the incubation mixture in a water bath for 5 min. The assay mixtures were analyzed by TLC as previously reported. GF_2 synthesis was taken as a measure of 1-SST activity.

RESULTS

Northern blotting analysis

Total RNA was isolated from embryogenic tall fescue calli treated at different temperatures and with different sucrose concentrations for 14 days. The transcript level of 1-SST was estimated by northern blotting analysis. The transcript level of 1-SST was very low or undetectable in calli treated at 23 or 4°C without sugar supply (Figure 1). On the other hand, the presence of 50 mM sucrose induced the 1-SST-expression when calli were incubated for at least 7 days at both temperatures, even if gene was strongly expressed at 23°C. When calli were incubated in the presence of 100 mM sucrose at 23°C, the expression of 1-SST increased during all the experimental period. On the contrary, its expression is under the detection limit for all the samples cold treated in the presence of 100 mM.

Free sugars content

Levels of soluble sugars (glucose, fructose and sucrose) during the analyzed period are shown in Figure 2. The glucose and fructose amounts were very low in calli cultured in medium in the absence of sucrose, but its supply promoted a marked increase of these hexoses, independently of temperature treatment (Figures 2A and B). The sucrose content presented a different trend when cultured in the absence of sucrose: a great difference was verified at 4°C, when the sucrose level of 3 days cold treated sample resulted relatively high and then decreased suddenly throughout period analyzed. The highest cellular sucrose level was detected in the presence of 50 mM sucrose in the medium at 4°C after 7 days.

Fructans content

Fructans are analyzed by thin layer chromatography (TLC) in embryogenic calli maintained at 4 and 23°C with different sucrose medium concentrations. The amount of fructans divided into fructans with low (LDP) and high (HDP) degree of polymerisation (HDP), was markedly increased after 7 days of culture, but it was extremely dependent on the adopted culture temperature and on the presence of the sucrose medium (Figure 3B). When embryogenic callus were cultivated in the absence of sucrose and at low temperature (4°C), fructan levels increased at 7 and 14 days. Indeed, at normal temperature (23°C), fructans were synthesized, principally after 1 week of culture, when 50 or 100 mM

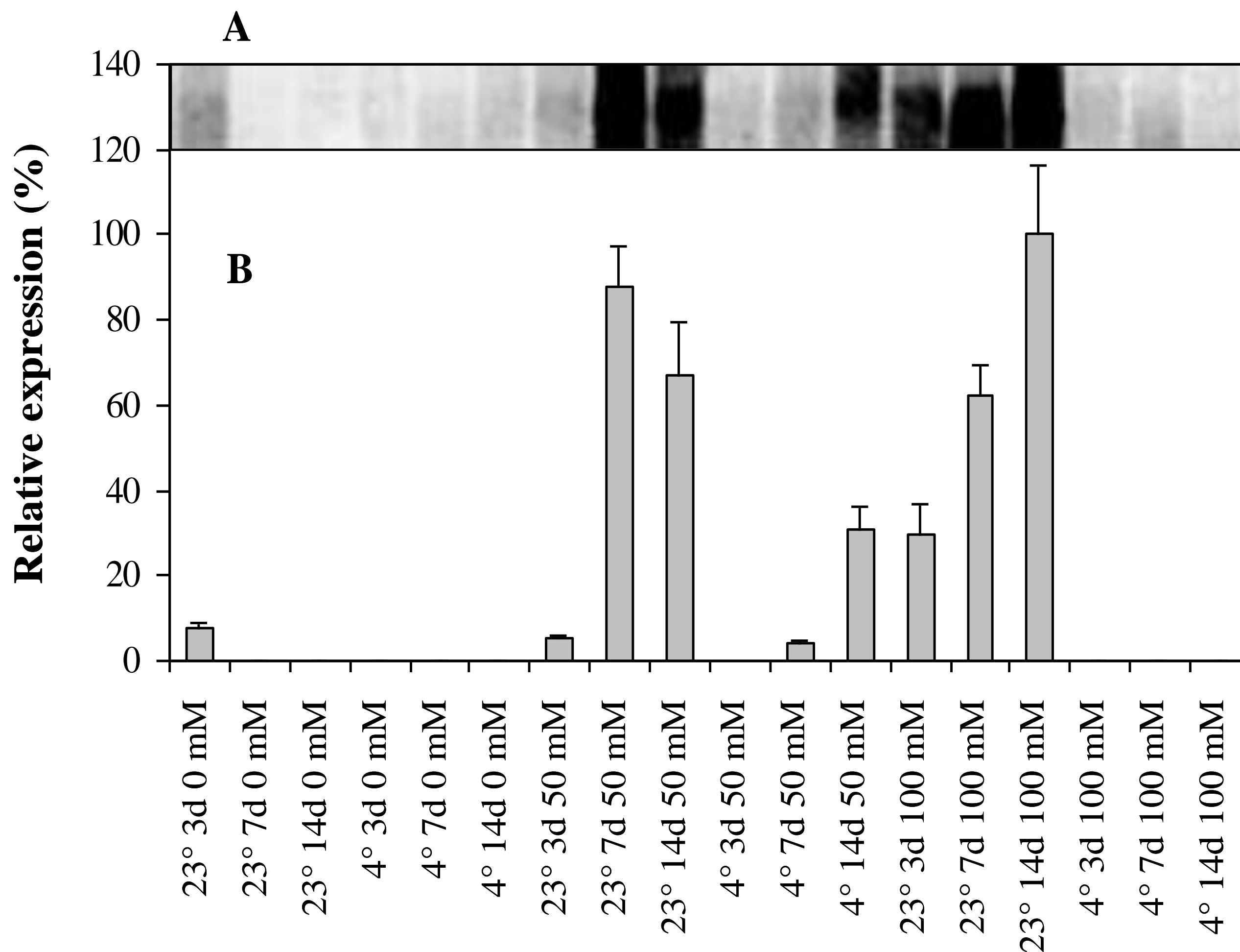

Figure 1. Representative northern analysis of 1-SST expression on cultured embryogenic callus from tall fescue treated in 0, 50 and 100 mM sucrose liquid medium at 4 and 23°C, determined after 3, 7 and 14 days of culture (A). Relative expression level of 1-SST after densitometric analyses (B; the highest expression value was considered 100% relative activity). Data are mean of three independent experiments ± SD.

concentrations of sucrose was added. An opposite behavior is observed when calli were exposed at 4°C in the presence of both 50 and 100 mM sucrose in the medium, since fructan levels remained very low.

Enzymatic activities

Enzymatic activities related to sucrose metabolism as SuSy, 1-SST, acid invertase and cytosolic invertase were assayed on embryogenic callus treated in different culture conditions. The SuSy and 1-SST activities were under detection limit during all analyzed period (data not shown).

The activity of cytosolic invertase was detected in the most samples examined and was always higher than that of acid invertase (Figure 4). High sucrose concentration (100 mM) induced acidic invertase activity at both temperatures while the cytosolic one is strongly induced under cold condition.

Total protein content

The total protein content (Figure 5) in embryogenic callus cultured without sucrose at 23°C strongly decreased after 14 days of treatment while the level remained constant in samples treated at 4°C. When sugar was supplied in the medium, the protein content increased in all the samples, and it was especially marked at higher sucrose concentration (100 mM) independently of temperature condition.

DISCUSSION

In plants it is a known relationship between tolerance to cold stress and high sugar content (Yuanyuan et al.,

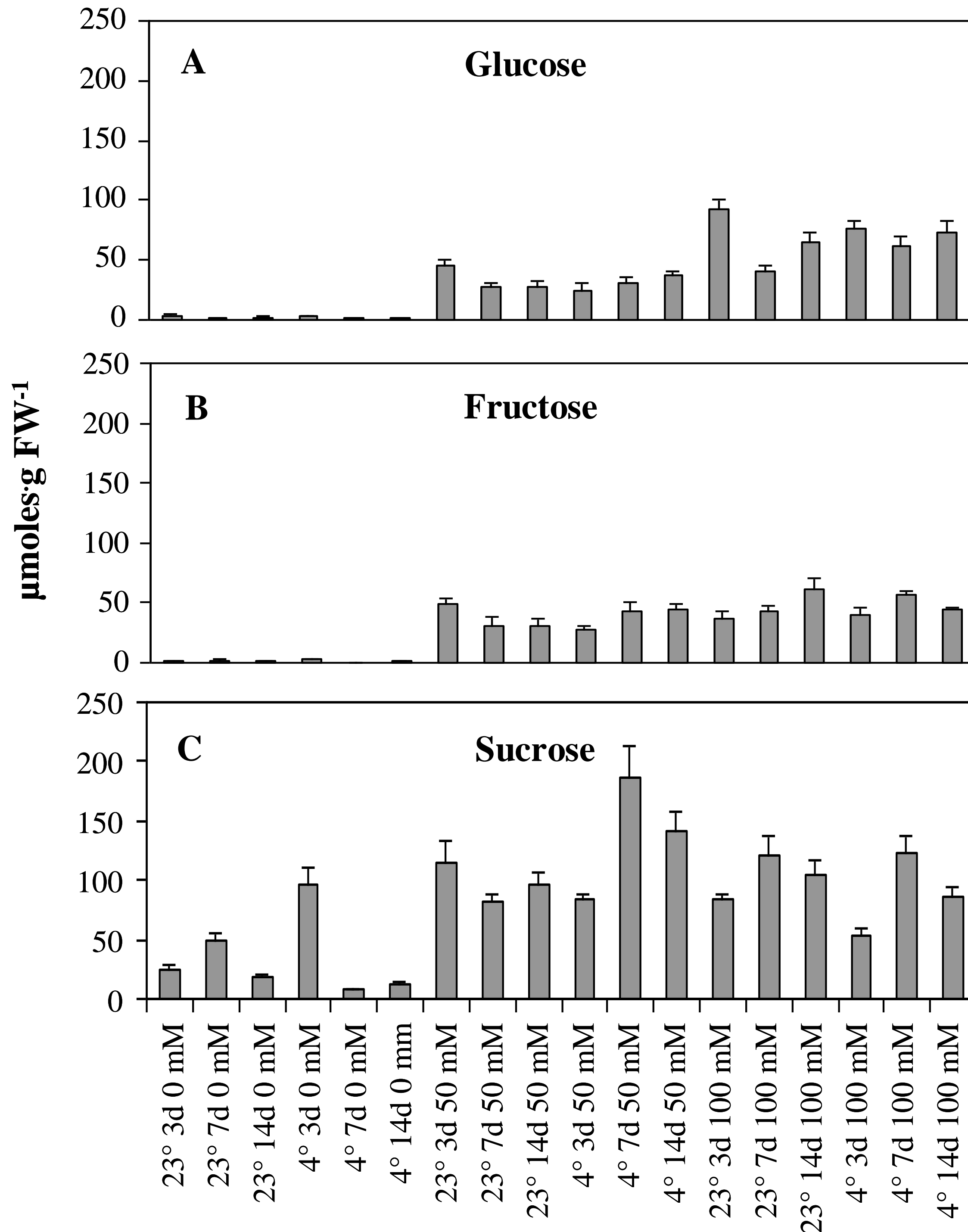

Figure 2. Free sugars content in embryogenic callus from tall fescue treated in 0, 50 and 100 mM sucrose liquid medium at 4 and 23°C, determined after 3, 7 and 14 days of culture. Data are mean of three independent experiments ± SD. (A, Glucose; B, fructose; C, sucrose).

2009). Molecular and physiological studies showed that cold acclimation promote fructans synthesis, particularly in grasses (Hisano et al., 2004; Livingston III et al., 2009). The genes responsible for the fructan synthesis are modulated by various factors (Van den Ende et al., 2002); among these are nitrogen deficiency (Wang et al., 2000), low temperature, defoliation (Wei et al., 2001), drought stress, as well as high sugars concentration

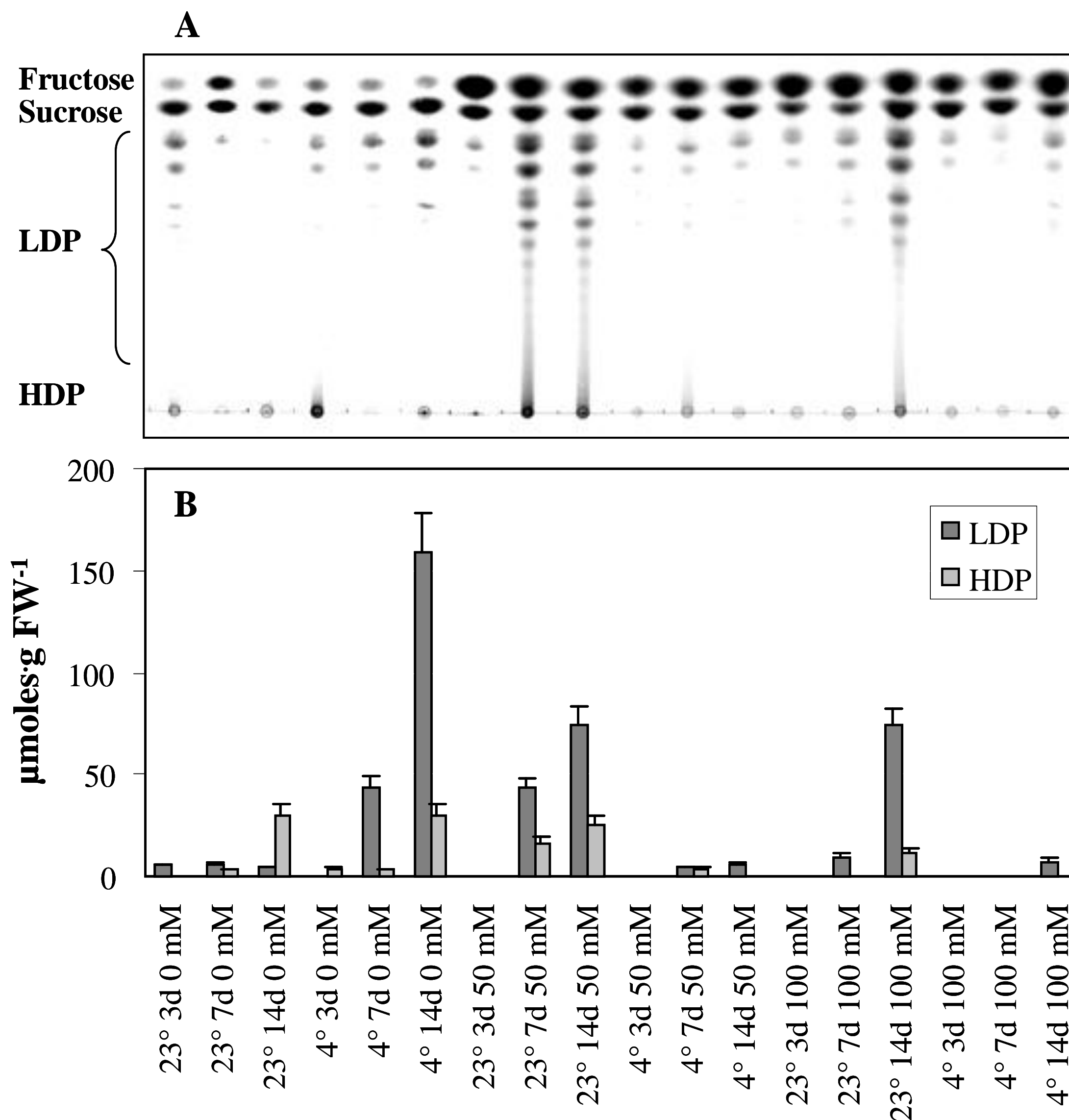

Figure 3. Representative fructans analysis determined by TLC in embryogenic callus from tall fescue treated in 0, 50 and 100 mM sucrose liquid medium at 4 and 23°C, determined after 3, 7 and 14 days of culture (A). B, fructans content estimated by Quantity One 4.1.0 Program (Bio-Rad, USA), in basis of bands intensity (LDP, low degree of polymerisation; HDP, high degree of polymerisation). Data are mean of three independent experiments ± SD.

induce the gene expression for fructans biosynthesis (Muller et al., 2000; Wei et al., 2001). In relation to cold, high sugar supply also induced fructan accumulation (Winter et al., 1994). In this study, embryogenic callus from tall fescue was utilized to study the effect of two different temperatures (4 and 23°C) and two sucrose concentrations on fructan metabolism. Previous *in vitro* experiments showed clearly that the enzymes involved in the fructan synthesis (1-SST, 6G-FFT and 1-FFT) are sequentially induced by sucrose, particularly in onion and chicory shoots placed in sucrose solution (Vijn et al., 1997). Our data supports the role of sucrose in the induction of 1-SST expression under control temperature while cold condition exhibited an opposite behaviour. This is in contrast with previous data concerning 1-SST cold induction in wheat (Kawakami and Yoshida, 2002). However, there are significant differences between mature plants and *in vitro* culture in which plant cells are mainly heterotrophic and therefore sink structures.

Effects of sucrose feeding and exposures to cold stress has been studied in leaves from *Arabidopsis thaliana* at transcriptional level for UDP-glucose pyrophosphorylase (UGPase), an important enzyme in sugar metabolism, producing UDP-glucose for sucrose and cellulose syntheses (Ciereszko et al., 2001). The authors observed an increase in transcript level for UGPase mRNA and

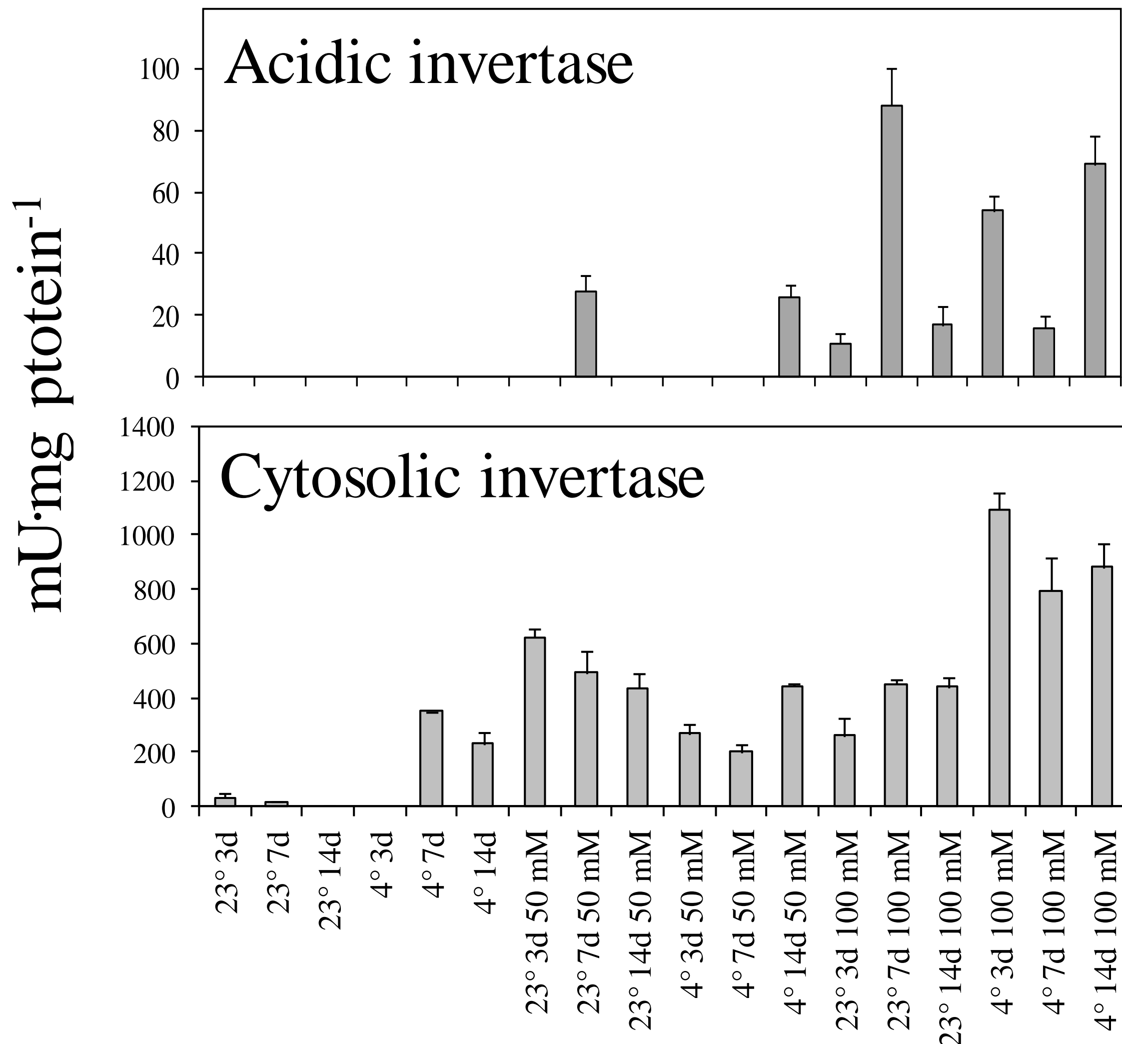

Figure 4. Acid (A) and cytosolic (B) invertase activities in embryogenic callus from tall fescue treated in 0, 50 and 100 mM sucrose liquid medium at 4 and 23°C, determined after 3, 7 and 14 days of culture. Activity is expressed as mU mg protein-1. Data are mean of three independent experiments ± SD.

subsequently a higher of UGPase activity and protein content. Also, tall fescue embryogenic calli cultured on 100 mM sucrose showed high protein content independently of temperature conditions.

In spruce somatic embryogenesis, the accumulation of proteins during the growth period is characterized by a high glucose and fructose demand, produced by sucrose cleavage *via* invertase (Iraqi and Tremblay, 2001). In our experiment, total proteins are accumulated mainly during sugar supply and the activity of both invertases exhibited a similar trend.

Accumulation of water-soluble carbohydrates in plant tissues during cold hardening is associated with freezing tolerance and is essential to winter survival (Kawakami and Yoshida, 2002). Sucrose-containing medium promoted a marked increase in soluble carbohydrates at both temperatures. Changes on free sugars content, principally sucrose, are related with fructans synthesis. Moreover, previous studies reported a role of sucrose in freezing-tolerance (Tabaei-Aghdaei et al., 2003) and its presence promoted a regulation of the metabolic process through intracellular sugar sensing (Loreti et al., 2001). In this work, embryogenic calli exhibited a peculiar behaviour due probably to their heterotrophic sink nature: fructans are synthesized during cold stress without sugar supply. On the other hand, sucrose is necessary for fructan synthesis at control temperature.

The utilization of sucrose as a source of carbon and energy depends on its cleavage into hexoses. In plants either SuSy or invertase catalyses this reaction. Enzymatic analyses of these enzymes showed that in tall fescue embryogenic callus, the preferential way for sucrose hydrolysis occurred by the action of invertases. In this study, the activity of cytosolic invertase was higher

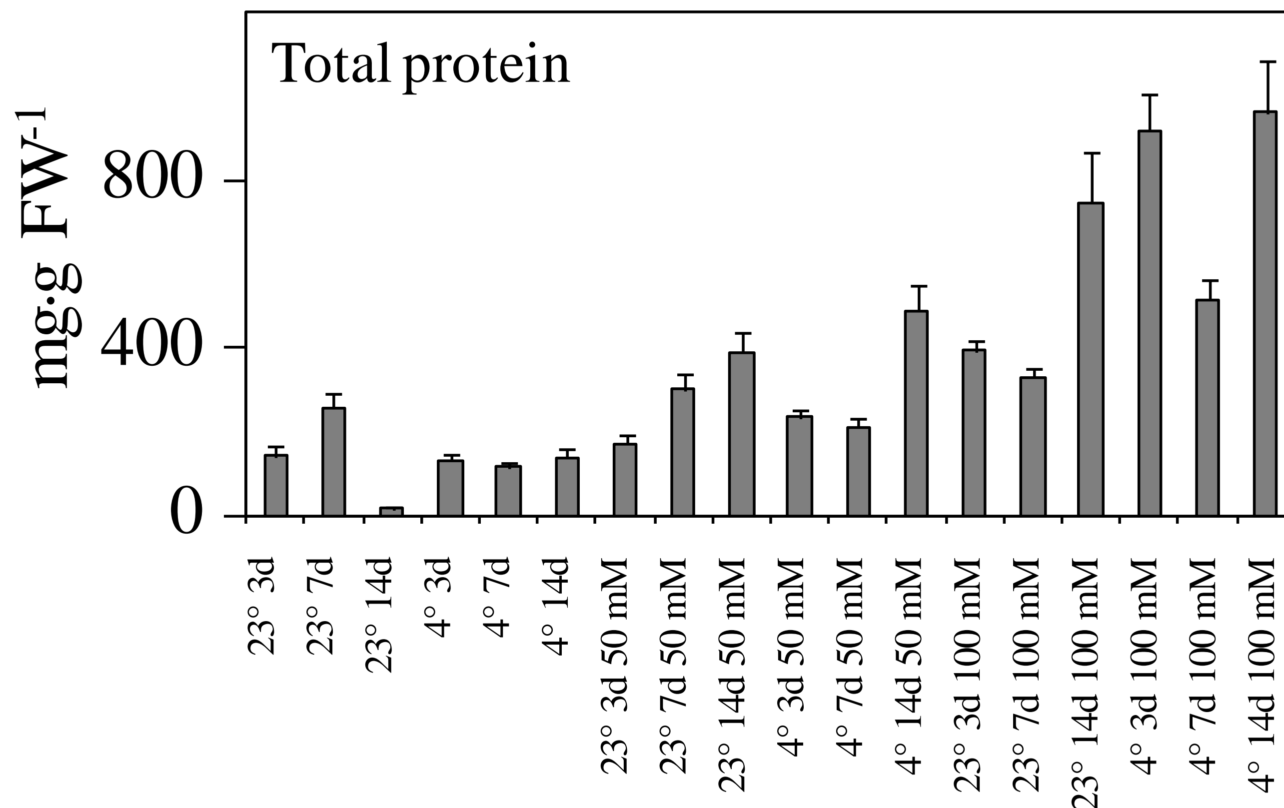

Figure 5. Total protein content in embryogenic callus from tall fescue treated in 0, 50 and 100 mM sucrose liquid medium at 4 and 23°C, determined after 3, 7 and 14 days of culture. Data are mean of three independent experiments ± SD.

than the acidic form in all tested treatments (Figure 4). Unexpected results on the activity of the cytosolic invertase were obtained in samples in the absence of sucrose at low temperature, when the endogenous level of sucrose was relatively low and fructan amount was high.

The catalytic activities of invertase are mainly due to sucrose hydrolysis, but other substrates can be utilized as raffinose (Pollock, 1986) and glucose (Nadkarni et al., 1992). The cytosolic invertase activity and the possible use of fructans as substrate, remains unclear. One possibility for this behaviour should be the presence of fructan in the cytosol. Fructans accumulation in compartments other than vacuoles has been questioned. The presence of fructan specific biosynthetic enzymes has been reported in the apoplastic fluid (Livingston and Henson, 1998), phloem sap (Wang and Nobel, 1998), xylem vessels, xylem and phloem parenchyma cells (Ernest, 1991; Vieira and Figueiredo-Ribeiro, 1993; Van den Ende et al., 2000).

In conclusion, embryogenic calli cultures, differing from mature plants particularly for the absence of vacuoles, offer good advantages for studying the modulation of fructan metabolism. However, more detailed investigations are needed for better understanding of the fructan metabolism in this system.

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