Sucrose Metabolism of Perennial Ryegrass in Relation to Cold Acclimation

Pankaj K. Bhowmik[a,b,*], Ken-ichi Tamura[c], Yasuhara Sanada[c], Kazuhiro Tase[c], and Toshihiko Yamada[a]

[a] Field Science Center for Northern Biosphere, Hokkaido University, Sapporo 060-0811, Japan

[b] Present address: Department of Agriculture, Food and Nutritional Science, Faculty of Agriculture, Forestry and Home Economics, University of Alberta, Edmonton, Alberta, Canada T6G 2P5. E-mail: pbhowmik@hotmail.com

[c] National Agricultural Research Center for Hokkaido Region, Sapporo 062-8555, Japan

* Author for correspondence and reprint requests

Z. Naturforsch. **61c**, 99–104 (2006); received August 2/23, 2005

Sugar metabolism is one of the important factors involved in winter hardiness and since the discovery of sucrose biosynthesis, considerable advances have been made in understanding its regulation and crucial role. This investigation examined the changes in activities of sucrose metabolizing enzymes and sugar content during cold hardening of perennial ryegrass (*Lolium perenne* L.). Changes in acid invertase (AI), sucrose synthase (SS) and sucrose phosphate synthase (SPS) along with all the three soluble sugars glucose, fructose and sucrose were measured in leaves and stem base tissue during cold acclimation. Although fructans were the predominant carbohydrate the changes in glucose, fructose and sucrose were significant. All the three soluble sugars in both leaf and stem tissues started to decrease from the first day and continued up to day 7 and thereafter started to increase until day 28. AI in the soluble fraction showed a higher activity than that in the cell wall bound fraction. In both the leaf and stem bases soluble AI activity increased during the first week and after that it started to decrease gradually. On the other hand both the SS and SPS increased gradually throughout the acclimation period. Sucrose content was negatively correlated with AI and positively correlated with SS and SPS accounting well for the relation between the substrate and enzyme activity. These results suggest that AI, SS and SPS in ryegrass are regulated by cold acclimation and play an important role in sugar accumulation and acquisition of freezing tolerance.

Key words: Lolium perenne L., Cold Acclimation, Sugars

Introduction

Hokkaido is a cold region and contains more than 80% of the grassland in Japan. Recently, perennial ryegrass (*Lolium perenne*) has received more attention as a grass species suitable for sustainable grazing systems in the Hokkaido region because of its particularly high digestibility combined with good tolerance to grazing. However, in general, ryegrass has less winter hardiness compared to other grasses such as timothy (*Phleum pratense*) and orchard grass (*Dactylis glomerata*) because of greater susceptibility to snow mold diseases and lower freezing resistance. Therefore, winter hardiness is one of the important breeding objects for perennial ryegrass in Hokkaido region.

The degree of freezing tolerance of plants varies among species and depends upon developmental stages and environmental conditions. Exposure of grasses from temperate and cool climatic zones to chilling temperatures leads to an accumulation of fructan (Pontis and Campillo, 1985), which is preceeded by an increase in the level of sucrose in the plant cell (Kawakami and Yoshida, 2002). This increment may be caused by the low demand for photosynthates at low temperatures. During growth under these conditions, the metabolism undergoes an adaptation process, and the plants acquire hardiness (Pollock *et al.*, 1983). Recent research has started to define the functional importance of sugar metabolism during cold acclimation and hardening. It is well known that sugar metabolism is affected by temperature stress (Perras and Sarham, 1984), and sugars accumulate in response to low temperature stress (Yoshida *et al.*, 1998). Studies have therefore been done to establish the relationship between the freezing tolerance and

0939–5075/2006/0100–0099 $ 06.00

sugar accumulation in different species (Perras and Sarham, 1984; Guy *et al.*, 1992; Sasaki *et al.*, 1996) and sucrose was found as the most commonly accumulated soluble sugar in response to low temperature. In plant tissues, cleavage of the glycosidic bond in sucrose is by either sucrose synthase (SS) or acud ubvertase (AI). SS requires UDP as co-substrate and produces fructose and UDP-glucose, whereas invertase simply splits sucrose into glucose and fructose (Copeland, 1990). There are two types of acid invertases in plants: AI, which has optimum activity at about pH 5, is present in vacuoles (soluble fraction) and in the apoplast bound to cell walls (cell wall bound fraction). SS is a cytoplasmic enzyme that catalyzes a reversible reaction and under normal conditions acts only in the breakdown of sucrose. On the other hand sucrose phosphate synthase (SPS) catalyses the synthesis of sucrose-6-phosphate, which is then hydrolysed to sucrose by sucrose phosphatase.

Previous reports have shown that when plants are exposed to a chilling shock there is an increase in the level of activity of some enzymes. When wheat seedlings were exposed to chilling temperatures, SS started to increase its activity within 1 h after beginning of the stress (Calderon and Pontis, 1985). In spinach (Guy *et al.*, 1992) and cabbage (Sasaki *et al.*, 2001), the alterations of all the three enzymes invertase, SS and SPS have been reported. However, changes in activities of these enzymes at low temperature varied between the plant species and there is very little information available on the enzymes involved in sucrose metabolism in perennial ryegrass species during cold acclimation.

Consequently the present experimental work was conducted to study the sucrose metabolism in perennial ryegrass during cold hardening.

Materials and Methods

Plant material

Plants (*Lolium perenne* L. cv S23) were grown from seeds in the growth chamber at 15/20 °C day night temperature regime with a photoperiod of 15 h and light intensity of 3,000 lx. Seeds were sown in 7.5 cm i.d. × 28 cm black plastic containers and about 8-week-old plants were used for the experiment. Plants were cold hardened at 2 °C with a 10 h photoperiod and 10,000 lx light intensity. They were cold acclimated for 4 weeks. For sugar and enzyme analysis leaves and stems were collected every week during the hardening period.

Extraction buffers

A 0.2 M citrate-phosphate (C-P) buffer at pH 5.0 for soluble AI and 0.2 M NaCl C-P buffer at the same pH for cell wall bound AI were used. On the other hand, 0.2 M potassium phosphate (K-P) buffer at pH 7.8 containing 10 mM ascorbate, 15 mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol (DTT) was used for the extraction of SS and SPS.

Enzyme extraction for acid invertase

Approx. 1 g of sample from both leaf and stem bases was mixed with 1% polyvinylpolypyrrolidone (PVPP) and 1 g sea sand. The sample was homogenized using a cooled mortar and pestle with 10 ml of 0.2 M C-P buffer (pH 5.0). The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11,000 × *g* for 10 min. The total supernatant was dialyzed with 40 times diluted 0.2 M C-P buffer (pH 5.0) for 12 h and the inner solution was designated as the soluble fraction. The residual tissues were re-extracted in 10 ml 0.2 M NaCl C-P buffer for about 24 h with occasional stirring. The supernatant was dialyzed as described above and the dialyzed solution was designated as cell wall bound fraction. All extraction procedures were carried out at 0–4 °C followed immediately by the enzyme assays.

Enzyme assays for AI

The standard assay medium for acid invertase consisted of 0.2 ml of 0.2 M C-P buffer (pH 5.0), 0.1 ml of 0.5 M sucrose, 0.1 ml of water and 0.1 ml of crude enzyme solution. The blank experiment contained distilled water instead of sucrose. The assay mixture was incubated at 45 °C for 15 min. After the reaction, the assay mixture was neutralized with 0.1 M NaOH or 0.1 M HCl, a coloring Somogyi's copper reagent was added and the mixture was heated for 10 min in boiling water. The amount of reducing sugars was estimated by the method of Somogyi (1952). The soluble protein content was determined by the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin as the standard. The enzyme activity was expressed as the amount (in μmol) of glucose produced per min per mg of protein.

Enzyme extraction for SS and SPS

Approx. 1 g of sample was mixed with 1% PVPP and 1 g sea sand. The sample was homogenized with 10 ml of 0.2 M K-P buffer (pH 7.8) containing 10 mM ascorbate, 15 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT using a cooled mortar and pestle. The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11,000 × *g* for 20 min. The total supernatant was dialyzed with 40 times diluted 0.2 M K-P buffer (pH 7.8) for 16 h and the inner solution was used as the crude enzyme. All extraction procedures were carried out at 0–4 °C.

Enzyme assays for SS and SPS

SS and SPS activities were assayed at 37 °C with the method described by Hubbard *et al.* (1989) with slight modifications. Reaction mixtures (70 μl) contained 50 mM HEPS-NaOH buffer (pH 7.5), 15 mM $MgCl_2$, 25 mM fructose-6-phosphate, 25 mM glucose-6-phosphate and 25 mM UDP-glucose. The mixtures were incubated for 30 min at 37 °C and the reaction was terminated by the addition of 70 μl of 30% KOH. Enzyme blanks were terminated with KOH at 0 min. Tubes were kept at 100 °C for 10 min to destroy any unreacted fructose or fructose-6-phosphate. After cooling, 2 ml of anthrone reagent (150 mg anthrone with 100 ml of 15% H_2SO_4) were added and incubated in a 40 °C water bath for 15 min. After cooling, color development was measured at 620 nm. SS was assayed as above but with 25 mM fructose instead of fructose-6-phosphate, and in the absence of glucose-6-phosphate. The soluble protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. The enzyme activity was measured as μmol of sucrose or sucrose phosphate produced per min per mg protein.

Determination of sucrose, glucose and fructose contents by HPLC

Soluble sugar was extracted from both leaf and stem tissue by boiling in water for 1 h and measured by HPLC (HITACHI L-2000).

Statistics

A randomized complete block design was adopted with three replications. The level of significance was calculated from the *F* value of ANOVA. The relationship between sugars and enzyme activities was described with linear correlation analysis using mean values.

Results

Acid invertase activities

AI activities (soluble and cell wall bound fractions) increased during the first week of cold acclimation and thereafter gradually decreased throughout the experimental period reaching about 45% of its initial activity (Fig. 1). In the soluble fraction a significantly higher AI activity was found in the leaf portion than in the stem bases. But, in the cell wall bound fraction a significantly higher AI activity was found in the stem bases. A highly significant negative correlation (Table I) was found between AI activity and sucrose content.

Sucrose synthase activity

Activities of SS in both leaf and stem bases showed a similar trend during cold acclimation (Fig. 2). SS activity increased during the 4 weeks of cold acclimation and reached a level 2.5 times higher than the activity before cold acclimation. Higher (almost double) SS activity than in the leaf portion was found in the stem bases. A highly significant positive correlation was found between SS activity and sucrose content (Table I).

Sucrose phosphate synthase activity

Activity of SPS also increased during cold acclimation and reached a level about 2 times higher than the activity before cold acclimation (Fig. 2). SPS showed higher activity in the stem bases than in the leaf portion. A highly significant positive correlation (Table I) was also found between SPS activity and sucrose content.

Soluble sugars

All the three soluble sugars sucrose, glucose and fructose in both leaf and stem tissues started to decrease from the first day and continued up to day 7 and thereafter started to increase until day 28 (Fig. 3). The sucrose content remained higher in the leaf portion than that in the stem bases. Among the three sugars, the fructose content remained higher than that of glucose and sucrose. The fructan content also increased throughout the acclimation period (data not shown).

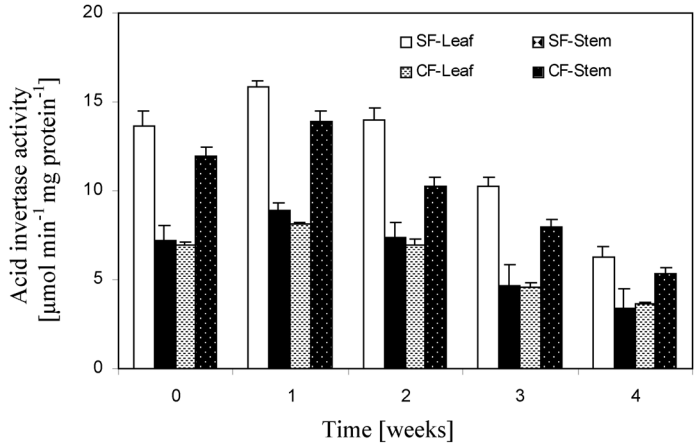

Fig. 1. Changes in acid invertase activities in leaf and stem bases of perennial ryegrass during cold hardening. Each point represents the mean of three replications. Vertical bars indicate S.E. SF, soluble fraction; CF, cell wall bound fraction.

Table I. Correlation coefficients (r) among enzyme activities and sugar contents in leaf and stem bases of perennial ryegrass during cold hardening (n = 24).

Sugar	Portion	Enzyme activity			
		Invertase		Sucrose	Sucrose
		SF[a]	CWBF[b]	synthase	phosphate synthase
Sucrose	Leaf	– 0.712**	– 0.536**	0.609**	0.532
	Stem bases	– 0.612**	– 0.632**	0.574**	0.654
Glucose	Leaf	0.345	0.501*	0.287	0.126
	Stem bases	0.564**	0.756**	0.337	0.023
Fructose	Leaf	0.429*	0.352	0.478*	– 0.145
	Stem bases	0.675**	0.597**	0.438*	– 0.028

[a] Soluble fraction.
[b] Cell wall bound fraction.
* Significant at $p < 0.05$. ** Significant at $p < 0.01$.

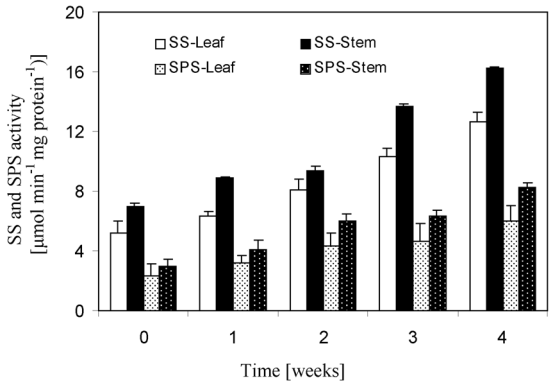

Fig. 2. Changes in sucrose synthase (SS) and sucrose phosphate synthase (SPS) activities in leaf and stem bases of perennial ryegrass during cold hardening. Each point represents the mean of three replications. Vertical bars indicate S.E.

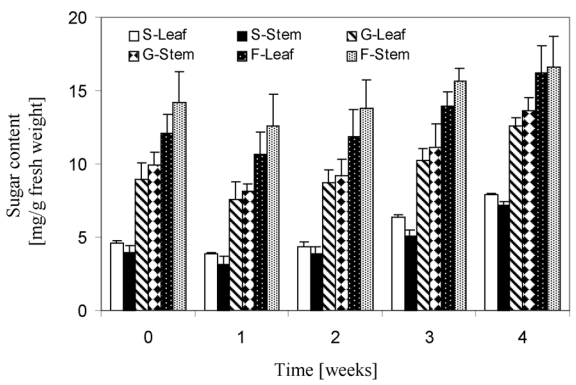

Fig. 3. Changes in soluble sugar content in leaf and stem bases of perennial ryegrass during cold hardening. Each point represents the mean of three replications. Vertical bars indicate S.E. S, sucrose; G, glucose; F, fructose.

Discussion

Changes in soluble sugars and enzyme activities during cold acclimation of perennial ryegrass show close relationship between sugar metabolism and cold acclimation in agreement with results reported in compositae (Pontis, 1966; Edelman and Jefford, 1968; Frehner *et al.*, 1984) and in other Gramineae species (Pollock, 1979, 1984; Wagner *et al.*, 1983; Volence and Nelson, 1984). Particularly the two sucrose metabolizing enzymes, SS and SPS, appear to have a key role during the cold acclimation as both of them were positively correlated with sucrose accumulation throughout the experimental period. The increase in SS activity after a chilling shock has led Calderon and Pontis (1985) to suggest that this enzyme may be associated with the sucrose transport into the vacuole. In general, SS *in vivo* plays the role of sucrose cleavage rather than sucrose synthesis. It would supply UDP-glucose and ADP-glucose (from sucrose) for the synthesis of starch and cell wall polysaccharides by degrading sucrose. Starch accumulated in cabbage seedlings exposed to low temperature (Sasaki *et al.*, 2001). Moreover, hexoses accumulated in cabbage leaves during cold acclimation. These results suggest that SS activity in cabbage leaves enhanced by cold acclimation contributes to the biochemical and physiological changes associated with the freezing tolerance by supplying UDP-glucose and hexose via degradation of sucrose. On the other hand, SPS plays a key role in sucrose synthesis (Huber and Huber, 1996) and sucrose accumulation in strawberry (Hubbard *et al.*, 1991) and tomato (Yelle *et al.*, 1991). The observation that SPS activity was stimulated during cold acclimation but dropped to the same level of activity as before cold acclimation means that SPS activity is also enhanced by cold acclimation (Sasaki *et al.*, 2001).

However, SS activities of spinach (Guy *et al.*, 1992) and algae cultures (Salerno and Pontis, 1989) were not affected by low temperature and SS activity in alfalfa decreased during fall acclimation (Castonguay and Nadeau, 1998). Sasaki *et al.* (2001) reported that SS and SPS, but not acid invertase, are regulated by cold acclimation. Thus, the changes in enzyme activities by cold treatment vary among plant materials. But our results regarding acid invertase, which was coincidental with many reports, are also a characteristic of invertase activity that has also been found in other tissues. The soluble and cell wall bound fractions of acid invertase represent two isoforms and their activities vary with leaf and stem bases due to the difference in maturity levels of the tissues, stem bases comprise actively dividing cells including emerging tillers whereas the leaf bases comprise more mature tissues. A highly significant negative correlation was found between enzyme invertase activities and sucrose content. Other soluble sugars (glucose and fructose) were positively correlated with the enzymes. The correlation results indicate that invertase hydrolyzes sucrose. In contrast, Moriguchi *et al.* (1990) and Suzuki *et al.* (1996) ascertained this role to SS. Further work is necessary to ascertain the roles of each synthase regarding the possibility of either sucrose breakdown or synthesis.

From the above discussion it is apparent that the activity of the three sugar metabolizing enzymes was influenced by cold acclimation changing the amount of soluble sugars present. These findings regarding invertase, SS and SPS activities along with the sugar content might be useful to improve cold hardiness in temperate grasses. Further research is also necessary to know the functional genomics of sugar metabolism in perennial ryegrass.

Acknowledgements

The financial support from the Japan Society for the Promotion of Sciences (JSPS) is gratefully acknowledged.

Calderon P. and Pontis H. G. (1985), Increase of sucrose synthase activity in wheat plants after a chilling shock. Plant Sci. **42**, 173–176.

Castonguay Y. and Nadeau P. (1998), Enzymatic control of soluble carbohydrate accumulation in cold-acclimated crowns of alfalfa. Crop Sci. **38**, 1183–1189.

Copeland L. (1990), Enzymes of sucrose metabolism. In: Methods in Plant Biochemistry. Vol. 3, Enzymes of Primary Metabolism (Lea P. J., ed.). Academic Press, London, pp. 73–86.

Edelman J. and Jefford T. G. (1968), The mechanism of fructosan metabolism in higher plants as exemplified in *Helianthus tuberosus* L. New Phytol. **67**, 517–531.

Frehner M., Keller F., and Wiemken A. (1984), Localization of fructan metabolism in the vacuoles isolated from protoplast of Jerusalem artichoke tubers (*Helianthus tuberosus* L.). J. Plant Physiol. **116**, 197–208.

Guy C. L., Huber J. L. A., and Huber S. C. (1992), Sucrose phosphate synthase and sucrose accumulation at low temperature. Plant. Physiol. **100**, 502–508.

Hubbard N. L., Huber S. C., and Pharr D. M. (1989), Sucrose phosphate synthase and acid invertase as determinants of sucrose concentration in developing muskmelon (*Cucumis melo* L.) fruits. Plant Physiol. **91**, 1527–1534.

Hubbard N. L., Pharr D. M., and Huber S. C. (1991), Sucrose phosphate synthase and other sucrose metabolizing enzymes in fruits of various species. Physiol. Plant. **82**, 191–196.

Huber S. C. and Huber J. L. (1996), Role and regulation of sucrose phosphate synthase in higher plants. Annu. Rev. Plant. Physiol. Mol. Biol. **47**, 431–444.

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

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. (1951), Protein measurement with folin-phenol reagent. J. Biol. Chem. **193**, 265–275.

Moriguchi T., Sanada T., and Yamaki S. (1990), Seasonal fluctuations of some enzymes relating to sucrose and sorbitol metabolism in peach fruit. J. Am. Soc. Hort. Sci. **115**, 278–281.

Perras M. and Sarham F. (1984), Energy state of spring and winter wheat during cold hardening. Soluble sugars and adenine nucleotides. Physiol. Plant. **60**, 129–132.

Pollock C. J. (1979), Pathway of fructosan synthesis in leaf bases of *Dactylis glomerata*. Phytochemistry **18**, 777–779.

Pollock C. J. (1984), Sucrose accumulation and the initiation of fructan biosynthesis in *Lolium temulentum* L. New Phytol. **96**, 527–534.

Pollock C. J., Lioyd E. J., Stoddart J. L., and Thomas H. (1983), Growth, photosynthesis and assimilate partitioning in *Lolium temulentum* exposed to chilling temperatures. Physiol. Plant. **59**, 257–262.

Pontis H. G. (1966), Observations on the *de novo* synthesis of fructosans *in vivo*. Arch. Biochem. Biophys. **116**, 416–424.

Pontis H. G. and Campillo D. E. (1985), Fructans. In: Biochemistry of Storage Carbohydrates in Green Plants (Dey P. M. and Dixon R. A., eds.). Academic Press, New York, London, pp. 205–227.

Salerno G. L. and Pontis H. G. (1989), Raffinose synthesis in *Chlorella vulgaris* cultures after a cold shock. Plant. Physiol. **89**, 648–651.

Sasaki H., Ichimura K., and Oda M. (1996), Changes in sugar content during cold acclimation and deacclimation of cabbage seedlings. An. Bot. **78**, 365–369.

Sasaki H., Ichimura K., Imada S., and Yamaki S. (2001), Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. J. Plant Physiol. **158**, 847–852.

Somogyi M. (1952), Notes on sugar determination. J. Biol. Chem. **195**, 19–23.

Suzuki A., Kanayama Y., and Yamaki Y. (1996), Occurrence of two sucrose synthase isozymes during maturation of Japanese pear fruit. J. Am. Soc. Hort. Sci. **121**, 943–947.

Volence J. J. and Nelson C. J. (1984), Carbohydrate metabolism in leaf meristems of tall fescue. I. Relationship to genetically altered leaf elongation rates. Plant. Physiol. **74**, 590–594.

Wagner W., Keller F., and Wiemken A. (1983), Fructan metabolism in cereals: induction in leaves and compartmentation in protoplasts and vacuoles. Z. Pflanzenphysiol. **112**, 359–372.

Yelle S., Chetelat R. T., Dorais M., Deverna J. W., and Benett A. B. (1991), Sink metabolism in tomato fruit. IV. Genetic and biochemical analysis of sucrose accumulation. Plant Physiol. **95**, 1026–1035.

Yoshida M., Abe J., Moriyama M., and Kuwabara T. (1998), Carbohydrate levels among wheat cultivars varying in freezing tolerance and snow mold resistance during autumn and winter. Physiol. Plant. **103**, 8–16.