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Effects of chilling temperature on the activity of enzymes of sucrose synthesis and the accumulation of saccharides in leaves of three sugarcane cultivars differing in cold sensitivity

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Abstract

The effects of short-term exposure to chilling temperature (10 °C) on sucrose synthesis in leaves of the cold-tolerant sugarcane cultivars *Saccharum sinense* R. cv. Yomitanzan and *Saccharum* sp. cv. NiF4, and the cold-sensitive cultivar *S. officinarum* L. cv. Badila were studied. Plants were grown at day/night temperatures of 30/25 °C, and then shifted to a constant day/night temperature of 10 °C. After 52-h exposure to the chilling temperature, sucrose content in the leaves of NiF4 and Yomitanzan showed a 2.5- to 3.5-fold increase relative to that of the control plants that had been left on day/night temperatures of 30/25 °C. No such increase was observed in Badila leaves. Similarly, starch content in the leaves of NiF4 and Yomitanzan was maintained high, but starch was depleted in Badila leaves after the 52-h exposure. During the chilling temperature, sucrose phosphate synthase (SPS; E.C.2.4.1.14) activity was relatively stable in the leaves of NiF4 and Yomitanzan, whereas in Badila leaves SPS activity significantly decreased. There was no significant change in cytosolic fructose-1,6-bisphosphatase activity for the three cultivars at the chilling temperature. This supports the hypothesis that: (*1*) on exposure to chilling temperature, sucrose content in sugarcane leaves is determined by the photosynthetic rate in the leaves, and is not related to SPS activity; (*2*) SPS activity in sugarcane leaves at chilling temperature is to be determined by sugar concentration in the leaves.

Additional key words: C_4 plants; cold temperature; cytosolic fructose-1,6-bisphosphatase; photosynthesis; saccharides; sucrose phosphate synthase; sucrose metabolism; *Saccharum*.

Introduction

Exposure of plants to chilling temperatures results in accumulation of soluble sugars in a wide variety of plant species or cultivars (Salerno and Pontis 1989, Tognetti *et al.* 1990, Guy *et al.* 1992, Hurry *et al.* 1995b, Strand *et al.* 1997, 1999). The most abundant soluble sugar accumulated usually is sucrose (Guy *et al.* 1992). Concomitant with the accumulation of sucrose in plants, sucrose synthesis capacity increased at chilling temperatures in spinach (*Spinacia oleracea* L.), winter wheat (*Triticum aestivum* L.), winter rye (*Secale cereale* L.), and winter rape (*Brassica napus* L.), as evidenced by increased activities of sucrose phosphate synthase (SPS) and cytosolic FBPase (Holaday *et al.* 1992, Hurry *et al.* 1995a,b, Strand *et al.* 1997, 1999, Sundar and Ramachandra Reddy 2000). The increase in SPS activity was attributed to the increased content of SPS enzyme protein (Guy *et al.* 1992) or to the appearance of new forms of SPS (Reimholz *et al.* 1997). However, not all plants were able to increase sucrose synthesis capacity during exposure to chilling temperatures. Hardy species or cultivars of spinach, winter wheat, winter rye, and winter rape apparently have a much greater ability to increase enzyme activities and accumulate more soluble sugars than the less hardy species or cultivars such as bean (*Phaseolus vulgaris* L.), spring wheat, and spring rape, which usually accumulate much less soluble sugars and do not show increased enzyme activities at chilling temperatures (Holaday *et al.* 1992, Hurry *et al.* 1994, 1995a,b). Although the direct causal relationship between the accumulation of compatible solutes and increased stress tolerance of plants have

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Abbreviations: F6P, fructose-6-phosphate; FBPase, fructose-1,6-bisphosphatase; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; NADP, nicotinamide-adenine dinucleotide phosphate; P_i, orthophosphate; SPS, sucrose phosphate synthase.
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proved difficult to establish, stress-tolerant species or cultivars usually accumulate more solutes than less tolerant counterparts (Hare *et al.* 1998, Lunn and Furbank 1999).

Unlike C_3 plants, C_4 plants are mainly distributed in tropical and subtropical areas. The control of sucrose synthesis in C_4 plants is complicated by the presence of the C_4 pathway and two different photosynthetic cell types. Now much more is known about the regulation of sucrose metabolism in C_3 plants, much less is known for C_4 plants (Lunn and Furbank 1999). Sugarcane is a C_4 plant of NADP-malic enzyme subtype, and a major sugar-production crop. In a previous study (Du *et al.* 1999) we found that sugarcane cultivars differing in original growth habitat showed sharp differences in cold sensitivity of photosynthesis. The subtropical cultivar *Saccharum sinense* R. cv. Yomitanzan and the hybrid cultivar *Saccharum* sp. (*S. officinarum* × *S. spontaneum* × S. *barberi*) cv. NiF4 were more cold-tolerant than the tropical cultivar *S. officinarum* L. cv. Badila with respect to photosynthesis. Because sucrose metabolism in C_3 plants exhibits substantial differences between cold-tolerant and cold-sensitive species or cultivars (Tognetti *et al.* 1990, Holaday *et al.* 1992, Hurry *et al.* 1995a,b), we wanted to know whether the sucrose metabolism in sugarcane is related to the cold sensitivity. If it is, how is the sucrose metabolism regulated when plants are subjected to chilling temperature? In order to answer these questions, we measured the activities of key enzymes associated with sucrose synthesis, SPS, and cytosolic FBPase, and the contents of saccharides and hexose phosphates in leaves of the three sugarcane cultivars on exposure to chilling temperature.

Materials and methods

Plants and chilling treatments: Sugarcane cultivars *Saccharum sinense* R. cv. Yomitanzan, *Saccharum* sp. (*S. officinarum* × *S. spontaneum* × *S. barberi*) cv. NiF4, and S. *officinarum* L. cv. Badila were germinated in vermiculite at day/night temperatures of 30/25 °C for 2 weeks. Then the seedlings were transplanted to 4 500 cm^3 plastic pots containing 2.5 kg soil in a growth chamber (*KG-50HLA, Koito*, Japan) at day/night temperatures of 30/25 °C and photoperiod of 14 h. Relative humidity in the growth chamber was 65 %, photon flux density at leaf height was 700 μmol m^{-2} s^{-1} (400-700 nm), and CO_2 concentration was ambient. The plants were then grown as described in Du *et al.* (1998). After the growth in the growth chamber at day/night temperatures of 30/25 °C for 2 months, plants were transferred to another growth chamber with the same photoperiod and photon flux density but with a constant day/night temperature of 10 °C. The transfer was done at the start of the photoperiod. The control plants were kept in the original growth chamber with the day/night temperatures of 30/25 °C. After exposure of plants to the chilling temperature for 4 h (at 10:30 in the first day of chilling treatment), 28 h (at 10:30 in the second day), and 52 h (at 10:30 in the third day), the upper most fully expanded mature leaves were used for the measurements of gas exchange in the previous experiment (Du *et al.* 1999). Then samples were taken immediately on the same leaf that was used for gas exchange for the measurements of enzyme activities and saccharide and hexose phosphate contents.

Extraction and assays of enzymes: Leaf samples were immediately immersed in liquid nitrogen and then stored at −80 °C until analysed. Leaf pieces (10 cm^2) were ground in a chilled mortar and pestle with 4 cm^3 of extraction medium, 0.5 g sea sand, and 40 mg of polyvinylpolypyrrolidone. The grinding medium contained 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.0, and 2.5 mM dithiothreitol. The homogenate was filtered through one layer of *Miracloth* (*Calbiochem-Novabiochem*, La Jolla, USA), an aliquot of the filtrate was centrifuged at 200 rps for 10 s (*Diskboy Kurabo Fb4000*), and then the supernatant was used immediately for assays of cytosolic FBPase. The remaining filtrate was centrifuged at 38 000×*g* at 4 °C for 10 min, and 1.5 cm^3 of the supernatant was desalted by passing it through a 5 cm^3 *Sephadex G-25* column (*Hitrap Desalting, Pharmacia Biotech,* Sweden) which was previously equilibrated with the extraction buffer. The desalted extract was used immediately for SPS assays.

SPS was assayed using the limiting assay (under limiting substrate with inhibitor P_i) and V_{max} assay (under saturating substrate without P_i) as described by Guy *et al.* (1992). For the limiting assay, the assay mixture (70 mm^3) contained 50 mM Hepes, pH 7.5, 15 mM $MgCl_2$, 1 mM dithiothreitol, 12 mM G6P, 3 mM F6P, 10 mM KH_2PO_4, 10 mM UDP glucose, and an aliquot of leaf extract. The assay mixture for V_{max} assay was the same except that KH_2PO_4 was omitted and the concentrations of F6P and G6P were 10 and 40 mM, respectively. The mixtures were incubated at 30 °C for 10 min, and then adding 70 mm^3 1 M NaOH terminated the reactions. The rest of the reaction procedures were the same as described by Du *et al.* (1998). SPS activity was measured against a blank obtained by the same procedure as above except that 70 mm^3 of 1 M NaOH was added to the reaction mixture before the addition of leaf extract. Cytosolic FBPase was assayed as described by Holaday *et al.* (1992). The assay mixture (1 cm^3) contained 50 mM Hepes, pH 7.0, 2 mM $MgCl_2$, 0.1 mM fructose-1,6-bisphosphate, 0.5 mM NADP, 4 units each of G6P dehydrogenase and G6P isomerase, and an aliquot of leaf extract. The activity was measured at 30 °C.

Determinations of hexose phosphate and saccharide contents: Samples were taken and stored in the same way as for enzyme assays. Hexose phosphates were extracted and determined as described by Du *et al.* (1998) except that 5 % $HClO_4$ was used to extract metabolites instead of

3 % in the previous study. Sucrose, glucose, fructose, and starch were extracted as described by Du *et al.* (1998), and determined photometrically by a modified enzymatic method (Jones *et al.* 1977) with G6P dehydrogenase/ hexokinase/G6P isomerase.

Results and discussion

Phenotypes and photosynthesis: Three sugarcane cultivars used in this study differ in original habitat and cold sensitivity. Field studies showed that the subtropical cultivar Yomitanzan and the hybrid cultivar NiF4 grow well and accumulate sucrose in the stalk at low temperatures, but the tropical cultivar Badila does not. In accordance with these phenotypes, we found previously (Du *et al.* 1999) that NiF4 and Yomitanzan retained more photosynthetic activity than Badila when warm-grown plants were subjected to chilling temperature. For example, after exposure of plants grown at 30/25 °C day/night temperatures to 10 °C for 52 h, the photosynthetic rates measured at 10 °C in the leaves of NiF4 and Yomitanzan increased by 20 to 30 % relative to the unstressed control plants,

Table 1. Changes in saccharide contents in leaves of three sugarcane cultivars on exposure to chilling temperature. Starch contents are expressed as mmol(glucose equivalent) m^{-2}. Calculated total values: the sum of sucrose, glucose, and fructose. Values in parentheses show the percentage of saccharide content in chilling-stressed plants in relation to that in unstressed control plants. Means ± SE of four separate sample preparations from two plants.

Cultivar	Saccharide	Content [mmol m^{-2}] Control	4 h (%)	28 h (%)	52 h (%)
NiF4	Sucrose	4.43 ± 0.35	4.83 ± 0.14 (109)	11.81 ± 4.01 (267)	11.87 ± 0.60 (268)
	Glucose	1.15 ± 0.06	0.53 ± 0.02 (46)	0.86 ± 0.05 (75)	1.43 ± 0.10 (124)
	Fructose	0.50 ± 0.19	0.12 ± 0.04 (24)	0.70 ± 0.10 (140)	1.03 ± 0.06 (206)
	Total	6.08	5.48 (90)	13.37 (220)	14.33 (236)
	Starch	2.35 ± 0.70	1.80 ± 0.24 (77)	3.83 ± 0.63 (163)	3.88 ± 0.28 (165)
Yomitanzan	Sucrose	5.02 ± 0.32	3.97 ± 0.04 (79)	8.11 ± 0.31 (162)	17.31 ± 0.08 (345)
	Glucose	0.49 ± 0.07	0.37 ± 0.04 (76)	0.37 ± 0.04 (76)	1.15 ± 0.21 (235)
	Fructose	0.37 ± 0.17	0.12 ± 0.04 (32)	0.17 ± 0.07 (46)	1.07 ± 0.31 (289)
	Total	5.88	4.46 (76)	8.65 (147)	19.53 (332)
	Starch	1.49 ± 0.42	1.03 ± 0.24 (69)	1.08 ± 0.52 (72)	1.75 ± 0.14 (117)
Badila	Sucrose	2.38 ± 0.05	1.05 ± 0.05 (44)	1.17 ± 0.08 (49)	2.22 ± 0.17 (93)
	Glucose	0.81 ± 0.08	0.18 ± 0.06 (22)	0.12 ± 0.02 (15)	0.01 ± 0.09 (1)
	Fructose	0.72 ± 0.17	0.27 ± 0.09 (38)	0.21 ± 0.07 (29)	0.37 ± 0.10 (51)
	Total	3.91	1.50 (38)	1.50 (38)	2.60 (67)
	Starch	3.43 ± 0.16	1.17 ± 0.34 (34)	0.01 ± 0.10 (0.3)	0.00 ± 0.06 (0)

Table 2. Changes in hexose phosphate (hexose-P) contents in leaves of three sugarcane cultivars on exposure to chilling temperature. Values in parentheses show the percentage of the hexose-P content in chilling-stressed plants in relation to that in unstressed control plants. Total values are calculated as sum of G6P, F6P, and G1P. Means ± SE of four separate sample preparations from two plants.

Cultivar	Hexose-P	Content [µmol m^{-2}] Control	4 h (%)	28 h (%)	52 h (%)
NiF4	G6P	55.8 ± 8.8	72.5 ± 5.0 (130)	161.4 ± 13.1 (289)	138.3 ± 5.1 (248)
	G1P	7.5 ± 1.2	9.4 ± 1.6 (125)	7.5 ± 1.6 (100)	14.1 ± 3.4 (188)
	F6P	17.8 ± 3.4	27.0 ± 4.8 (152)	41.1 ± 2.7 (231)	30.3 ± 4.8 (170)
	Total	81.1	108.9 (134)	210.0 (259)	182.7 (225)
	G6P/F6P	3.1	2.7	3.9	4.6
Yomitanzan	G6P	27.8 ± 3.2	96.2 ± 15.7 (346)	197.7 ± 5.4 (711)	188.4 ± 3.9 (678)
	G1P	4.2 ± 2.4	12.7 ± 2.7 (302)	12.7 ± 2.7 (302)	16.8 ± 4.8 (400)
	F6P	9.9 ± 2.7	27.0 ± 4.8 (273)	48.3 ± 1.7 (488)	37.8 ± 6.0 (382)
	Total	41.9	135.9 (324)	258.7 (617)	243.0 (580)
	G6P/F6P	2.8	3.6	4.1	5.0
Badila	G6P	26.0 ± 4.3	118.3 ± 9.1 (455)	158.6 ± 1.7 (610)	110.0 ± 10.7 (423)
	G1P	9.4 ± 1.6	12.7 ± 4.2 (135)	2.8 ± 1.6 (30)	7.0 ± 2.7 (74)
	F6P	7.1 ± 1.4	32.7 ± 5.3 (461)	36.9 ± 3.7 (520)	35.5 ± 4.2 (500)
	Total	42.5	163.7 (385)	198.3 (467)	152.5 (359)
	G6P/F6P	3.7	3.6	4.3	3.1

whereas the rate in the leaves of Badila decreased by more than 50 % (Du *et al.* 1999).

Saccharide contents: After 52-h exposure to 10 °C, sucrose content in the leaves of the cold-tolerant cultivars NiF4 and Yomitanzan showed a 2.5- to 3.5-fold increase, but there was no such increase in the leaves of the cold-sensitive cultivar Badila (Table 1). Glucose and fructose contents in NiF4 and Yomitanzan leaves declined after a short exposure (4 and or 28 h) but increased after the extended chilling treatment (52 h), whereas in Badila leaves both the glucose and fructose contents decreased rapidly at the chilling temperature. Starch content in the leaves of NiF4 and Yomitanzan was maintained high at the chilling temperature, whereas in Badila leaves starch content decreased rapidly at the chilling temperature, and was depleted after 28 h of exposure. Total soluble sugar (the sum of sucrose, glucose, and fructose) content in NiF4 and Yomitanzan leaves showed a 2.3- to 3.3-fold increase, but decreased by 33 % in Badila leaves after 52 h exposure (Table 1). The saccharide accumulation in leaves of NiF4 and Yomitanzan at chilling temperature resembles the responses to cold temperature of some cold-tolerant C_3 species or cultivars, such as spinach (Holaday *et al.* 1992), winter wheat, rape, and rye (Hurry *et al.* 1994, 1995b). Similarly, the accumulation of saccharides in Badila leaves at chilling temperature was similar to that of the cold-sensitive C_3 plants, such as spring rape (Hurry *et al.* 1995b).

Hexose phosphate contents: Contents of G6P and F6P increased markedly in leaves of all three cultivars after exposure to 10 °C. G1P content increased in the leaves of NiF4 and Yomitanzan, but was maintained low in Badila after a 52-h exposure to the chilling temperature (Table 2). The total content of hexose phosphates (the sum of G6P, G1P, and F6P) in the leaves of the three cultivars exhibited a 2- to 6-fold increase after the exposure for 52 h. The accumulation of hexose phosphates in plant leaves at cold temperatures has been reported for a variety of plants. For example, when warm-grown winter wheat and winter rape were subjected to chilling temperature of 10 °C, the hexose phosphate contents increased 2- to 3-fold (Hurry *et al.* 1995b). However, some species do not seem to accumulate hexose phosphates in leaves at cold temperatures, such as spring rape (Hurry *et al.* 1995b).

The G6P/F6P ratios are related to the proportion of hexose phosphates present in the cytosol and the stroma. When the ratios are high (around 4), hexose phosphates are predominantly located in the cytosol; when the ratios are low (around 1-2), hexose phosphates are predominantly located in the stroma (Gerhardt *et al.* 1987). After exposure to the chilling temperature, the G6P/F6P ratios were well above 2 for all the three cultivars (Table 2), suggesting that hexose phosphates in the three cultivars were predominantly located in the cytosol at the chilling temperature. Cytosol is the compartment of the plant cell where sucrose synthesis occurs (Usuda and Edwards 1980, Furbank *et al.* 1985, Stitt and Heldt 1985).

Enzyme activities: After exposure to 10 °C, SPS activities, both the limiting activity (measured with limiting substrate and inhibitor P_i) and V_{max} (measured with saturating substrate without P_i), in the leaves of NiF4 and Yomitanzan were similar to or higher than in the unstressed controls. For example, the SPS limiting activity in NiF4 leaves significantly increased after 52-h exposure to the chilling temperature (Table 3). However, in Badila leaves both the SPS limiting and V_{max} activities decreased significantly at the chilling temperature (Table 3). SPS activation state, which is defined as the limiting activity expressed as a percentage of the V_{max} activity (Stitt *et al.* 1988, Huber *et al.* 1989), in NiF4 and Yomitanzan leaves increased from 48.2 and 33.4 % in unstressed control plants to 57.8 and 45.7 % in 52 h-chilling-stressed plants, respectively. In Badila leaves the SPS activation state did not apparently change after 52-h exposure to the chilling temperature. Unlike the response of SPS activity to chilling temperature, cytosolic FBPase activity showed no significant changes after the chilling treatment for all the three cultivars (Table 4).

SPS activity can be regulated by the concentrations of effector molecules, through reversible phosphorylation of the enzyme protein, and by a change in the enzyme protein contents (Stitt and Quick 1989, Huber and Huber 1996, Winter and Huber 2000). In spinach leaves, SPS protein content was increased by exposure of plants to 10 °C for 10 d, and the increase in SPS activity resulted from the increased SPS protein content, rather than enzyme activation (Guy *et al.* 1992). In the present study, SPS V_{max} in NiF4 and Yomitanzan leaves did not change at the chilling temperature, suggesting that the SPS protein content was not affected by this relative short-term chilling temperature (Table 3). In Badila leaves, both the limiting and V_{max} activities decreased significantly at the chilling temperature. Since SPS activation state did not show apparent reduction (Table 3), it is likely that the decreased SPS activity in Badila leaves resulted mainly from the reduction of SPS protein.

The mechanism of changes in the concentration of saccharides in leaves: During plant photosynthesis, CO_2 is first assimilated as saccharides in source leaves, and then the fixed carbon is exported from source leaves to storage organs through phloem. Under the natural sunlight in the field, 82 % of the total carbon fixed by photosynthesis in sugarcane source leaves during the day is exported out of the leaves in the day, and 17 % is accumulated as starch in the day, which is exported during the following night (Du *et al.* 2000). Thus, the content of saccharides in source leaves is largely determined by the balance of photosynthesis and sugar transport (Guy *et al.* 1992, Du *et al.* 2000). Photosynthesis is probably the first process affected by adverse temperatures (Berry and

Björkman 1980). However, in sugarcane the phloem sugar transport is very sensitive to chilling temperature (Hartt 1965), probably more sensitive to chilling temperature than photosynthesis (Ebrahim *et al.* 1998a,b). Therefore, the basic reason for the accumulation of saccharides in sugarcane leaves at chilling temperature can be considered as the result of saccharides produced in excess of those being transported. Previously (Du *et al.* 1999) we found that when exposed to 10 °C, the photosynthetic capacity in the leaves of NiF4 and Yomitanzan was maintained, whereas the photosynthetic capacity was seriously damaged in Badila leaves. Thus, a likely interpretation for the differences in saccharide accumulation in the three cultivars observed in this study is that sugar accumulated in the cold-tolerant cultivars NiF4 and Yomitanzan is due to the maintenance of photosynthesis and the inhibition of phloem transport. On the other hand, sugar failing to accumulate in the cold-sensitive cultivar Badila could be due to inhibition of both photosynthesis and phloem transport (Du *et al.* 1999). In other words, the larger sucrose contents in the leaves of the cold-tolerant cultivars NiF4 and Yomitanzan, and the smaller sucrose contents in the leaves of the cold-sensitive cultivar Badila were caused by the higher or lower photosynthetic rate in the respective cultivar at the chilling temperature.

Table 3. Changes in SPS activity in leaves of three sugarcane cultivars on exposure to chilling temperature. SPS limiting activity was measured at limiting substrate with inhibitor P_i, and V_{max} activity was measured at saturating substrate without P_i. SPS activation-state is defined as the limiting activity expressed as a percentage of the V_{max} activity. The statistical difference in SPS activity between chilling-stressed plants and unstressed control plants was tested for the limiting and V_{max} activities, respectively, within the same cultivar. * indicates that the value is significantly different from that of its corresponding unstressed control at the 5 % level. Means ± SE of four separate enzyme preparations from two plants.

Treatment		SPS activity [μmol m^{-2} s^{-1}] NiF4	Yomitanzan	Badila
Control	Limiting	8.04 ± 0.29	4.00 ± 0.32	6.07 ± 0.14
	V_{max}	16.68 ± 0.55	11.98 ± 0.39	15.03 ± 0.24
	Activation (%)	48.2	33.4	40.4
4 h	Limiting	9.00 ± 0.24	3.83 ± 0.62	3.68 ± 0.22*
	V_{max}	17.62 ± 0.17	10.49 ± 0.79	12.60 ± 0.26*
	Activation (%)	51.1	36.5	29.2
28 h	Limiting	9.00 ± 0.74	3.75 ± 0.31	4.55 ± 0.26*
	V_{max}	15.81 ± 0.30	10.44 ± 0.61	12.65 ± 0.74
	Activation (%)	56.9	35.9	36.0
52 h	Limiting	9.95 ± 0.43*	5.06 ± 1.05	4.08 ± 0.60*
	V_{max}	17.20 ± 1.62	11.08 ± 0.49	11.06 ± 1.10*
	Activation (%)	57.8	45.7	36.9

Table 4. Cytosolic FBPase activities in leaves of three sugarcane cultivars on exposure to chilling temperature. Means ± SE of four separate enzyme preparations from two plants.

Treatment	Cytosolic FBPase activity [μmol m^{-2} s^{-1}] NiF4	Yomitanzan	Badila
Control	9.2 ± 0.6	5.3 ± 0.4	20.2 ± 0.8
4 h	10.3 ± 0.8	5.4 ± 0.3	21.7 ± 1.4
28 h	9.5 ± 0.9	4.9 ± 0.3	20.4 ± 1.2
52 h	9.2 ± 0.8	6.0 ± 0.3	19.8 ± 0.9

The mechanism of changes in the activity of enzymes of sucrose synthesis in leaves: In sucrose metabolism, cytosolic FBPase and SPS are two key enzymes that control the sucrose synthesis and the partitioning of photosynthates between sucrose and starch (Stitt and Quick 1989, Winter and Huber 2000). When warm-grown plants are subjected to cold temperatures, cytosolic FBPase and SPS activities usually change, and the altered enzyme activities are generally associated with the higher or lower sucrose content (Holaday *et al.* 1992, Hurry *et al.* 1995a,b, Strand *et al.* 1997, 1999). However, in this study we found that the cytosolic FBPase activity showed no significant changes in the leaves of all the three sugarcane cultivars after exposure of plants grown at 30/25 °C day/night temperatures to 10 °C for 52 h (Table 4), even though the sucrose content substantially changed at the chilling temperature (Table 1). The failure to see a change in cytosolic FBPase activity in the three sugarcane cultivars at the chilling temperature may be attributed to several reasons. First, the response of cytosolic FBPase to chilling temperature is species specific, *i.e.* it changes in some species such as spinach (Holaday *et al.* 1992) but not in others such as sugarcane. Second, because cytosolic FBPase activity is regulated by its potent effector, fructose-2,6-bisphosphate (Stitt and Heldt 1985, Stitt and Quick 1989), it is possible that there was a change in cytosolic FBPase activity *in vivo* in response to the chilling treatment, but the *in-vivo* changes were unable to be identified by *in-vitro* assays, because the fructose-2,6-

bisphosphate had been diluted during enzyme extraction. Third, the changes in cytosolic FBPase activity in spinach and other species at cold temperatures (Holaday *et al.* 1992, Hurry *et al.* 1995a,b, Strand *et al.* 1997, 1999) might have resulted from the increased amount of cytosolic FBPase protein. This might require longer cold treatment, such as 10 d at 10 °C for spinach (cf. Holaday *et al.* 1992). In the present study, the sugarcane was treated at 10 °C for only 52 h (less than 3 d). If the cold treatment was extended to a longer period, cytosolic FBPase may also exhibit responses to the chilling temperature. In contrast to cytosolic FBPase, SPS activity showed substantial differences between the cold-tolerant cultivars NiF4 and Yomitanzan, and the cold-sensitive cultivar Badila (Table 3). After exposure to the chilling temperature, both the SPS limiting and V_{max} activities were maintained high in the leaves of NiF4 and Yomitanzan, but decreased significantly in Badila leaves (Table 3).

When normally-grown plants are subjected to environmental stress, such as water or chilling stress, associated changes between leaf sucrose concentration and SPS activity are observed in various species or cultivars (*e.g.* Tognetti *et al.* 1990, Zrenner and Stitt 1991, Holaday *et al.* 1992, Quick *et al.* 1992, Hurry *et al.* 1994, 1995a,b). Although the mechanisms behind those changes remain unknown, the change in SPS activity is an important part of acclimation response to these stresses (Lunn and Furbank 1999). However, under some circumstances, the change in SPS activity may be a secondary effect of environmental stress, and thus may not be essential for sucrose metabolism in plant leaves. For example, when *Phaseolus vulgaris* was subjected to a mild water stress, SPS activity in mature leaves decreased significantly (Vassey and Sharkey 1989, Vassey *et al.* 1991). However, the decrease in SPS activity was ascribed to the decreased photosynthetic rate, which was caused by the water-stress-induced stomatal closure, and not by the water stress *per se* (Vassey *et al.* 1991). In consistence with this discovery, Kaiser and Förster (1989) also reported that the capacity of nitrate reduction was tightly coupled to net CO_2 assimilation in leaves, and that under mild water stress nitrate reduction decreased as a result of stomata closure. Most recently, studies with transgenic tobacco have also shown that sugar supply is dominating in the regulation of nitrate reductase expression, and low sugar supply strongly inhibits the expression of nitrate reductase in tobacco (Klein *et al.* 2000). These results suggest that a number of plant enzymes, including SPS, are affected by the sugar content in leaves, which in turn is controlled by photosynthetic rate.

Previously (Du *et al.* 1999) we found that on exposure to the chilling temperature of 10 °C the photosynthetic capacity in the leaves of Badila was seriously damaged, leading to marked reduction in photosynthetic rate. Furthermore, at the chilling temperature the photosynthetic carbon intermediates in Badila leaves were largely accumulated as aspartate and alanine, due to the sharp reductions in the activities of NADP malate dehydrogenase and pyruvate,P_i dikinase in the C_4 pathway. Hence the Calvin cycle and sucrose synthesis pathway in Badila leaves might actually be subjected to low carbon supply at the chilling temperature, similar to plants under mild water stress or low CO_2 supply (Kaiser and Förster 1989, Vassey *et al.* 1991). At the chilling temperature, starch content in Badila leaves decreased rapidly, and was depleted after a 28-h exposure (Table 1), further indicating that the carbon flux in Badila leaves was sharply reduced by the chilling temperature. Thus the significant reduction in SPS activity in Badila leaves at the chilling temperature was likely to be caused by the low sugar content in the leaves, and not by the chilling temperature *per se*. Perhaps, the low sugar content inhibited the SPS gene expression in Badila leaves, like the inhibition of nitrate reductase in tobacco (Klein *et al.* 2000). Therefore we conclude that when sugarcane is subjected to chilling stress, the changes in SPS activity in leaves is the secondary effect of chilling stress, the activity in leaves is largely determined by sugar concentrations in the leaves.

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