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BRIEF COMMUNICATION

Sucrose accumulation and enzyme activities in callus culture of sugarcane

F.A. GUTIÉRREZ-MICELI, M.A. RODRÍGUEZ-MENDIOLA, N. OCHOA-ALEJO, R. MÉNDEZ-SALAS, C. ARIAS-CASTRO and L. DENDOOVEN*

Departamento de Biotecnología y Bioingeniería, CINVESTAV-IPN, Av. IPN 2508, CP 07000, AP 14740, México DF, México

Abstract

The activities of sucrose phosphate synthase (SPS), sucrose synthase (SUSY), neutral invertase (NI) and soluble acid invertase (SAI) were measured in callus cultures of four Mexican sugarcane cultivars (*Saccharum* spp.) with a different capacity to accumulate sucrose in stem parenchyma cells. The results indicated that sucrose accumulation in callus was positively correlated to the activity of SPS and SUSY and negatively to the activity of SAI and NI while SPS explained most of the variation found for sucrose accumulation and NI least.

Additional key words: neutral invertase, *Saccharum* spp., soluble acid invertase, sucrose phosphate synthase, sucrose synthase.

Sugarcane is a commercially important crop that accounts for approximately 65 % of world sugar production (Carson and Botha 2002). Parenchyma cells of sugarcane (*Saccharum* spp.) stems accumulate sucrose up to 20 % of their fresh mass or 60 % of dry mass in mature internodes (Moore and Maretzki 1996). Stem sucrose can be catabolized by sucrose synthase (SUSY) or one of three invertases: soluble acid invertase (SAI) found in the apoplast and vacuoles of young internodes, but virtually absent from the mature tissues; acid invertase bound to the cell wall in tissues of all ages; neutral invertase (NI) found in low concentrations in the cytoplasm of young tissues and in larger concentrations in mature tissues (Glasziou 1962). After entering parenchyma cells, hexoses may be metabolized or resynthesized into sucrose by sucrose-phosphate synthase (SPS, EC 2.3.1.14) and sucrose phosphatase (Moore and Maretzki 1996). SUSY can also be involved in sucrose synthesis, but the equilibrium is usually in the direction of degradation.

Suspension cultures of sugarcane have been developed from internode parenchyma cells of a high-yielding strain and used as a model system to investigate sugaruptake (Maretzki and Thom 1972) and metabolism during sucrose storage (Wendler *et al.* 1990). In this system, sucrose storage is regulated by a simultaneous cycle of synthesis and degradation (Wendler *et al.* 1990) and SPS is the key enzyme regulating sucrose accumulation. However, the use of callus cultures would be preferred over cell suspensions or experimentation under field conditions as they are easier to cultivate than suspension cultures, they can be studied in a controlled environment, different cultivars can be used simultaneously and the time of sucrose accumulation is rapid (Gosal *et al.* 1998). Additionally, a callus regeneration system could be used to micropropagate sugarcane and select sugarcane cultivars with the capacity to accumulate large amounts of sucrose.

In this study we investigated sucrose accumulation through the activities of SPS, SUSY, SAI and NI and their relationship with accumulated sucrose in callus culture of four cultivars of sugarcane widely used in Mexico with a different capacity to accumulate sucrose in stems parenchyma cells.

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid, MS medium - Murashige and Skoog medium, NI - neutral invertase, SAI - soluble acid invertase, SPS - sucrose-phosphate synthase, SUSY - sucrose synthase.

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* Author for correspondence; fax: (+52) 55 5061 3313, e-mail: dendoove@mail.cinvestav.mx

Four sugarcane (*Saccharum* spp.) cultivars, *i.e.* Rb 765418 and Mex 57-473 characterised by a large sugar production, Mex 69-290 by a medium production and Zmex 55-32 by a low production, were obtained from the experimental field of the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), México. Callus cultures were developed from stem apices of field-grown plants. Shoot tips (15 cm long) were disinfected in 95 % (v/v) ethanol for 30 s, followed by immersion in 3 % (m/v) aqueous calcium hypochlorite solution (30 min) and three washes with sterile distilled water. The outer leaf sheaths were removed to about the tenth node and the other tender sheaths until only the innermost leaf sheaths remained. Transverse sections (2 mm) were cut aseptically from the apex and placed in 150 cm^3 bottles containing 30 cm^3 of Murashige and Skoog (1962) medium supplemented with 30 g dm^{-3} sucrose, 3 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg dm^{-3} kinetin, and solidified with 1.8 g dm^{-3} *Gelrite.* Bottles with four explants each were incubated under cool white fluorescent light (irradiance of 45.8 μmol m^{-2} s^{-1}) and a 16-photoperiod at a temperature of 26 - 28 ºC for one month.

Calli were transferred to similar vessels containing 30 cm^3 of MS medium with 30 g dm^{-3} sucrose, 10 g dm^{-3} activated charcoal, 1 g dm^{-3} arginine, 0.3 g dm^{-3} yeast extract, 3 mg dm^{-3} 2,4-D, and solidified with 1.8 g dm^{-3} *Gelrite*. Enzyme activities and sucrose concentration were determined using 1 g of fresh callus at time zero and after 8, 17, 21, 27, 34 and 40 d. Time zero was considered the moment that the medium was inoculated with the calli.

Sucrose was extracted from 0.1 g oven-dried callus (vacuum at 70 ºC for 24 h) with 100 cm^3 98 % ethanol for 12 h. Preliminary test confirmed a complete extraction of sucrose after 12 h (no data shown). The volume of extract was reduced in a *461 Rotavapor* (*Buchi*, USA) at 80 ºC under vacuum. Extracts were dried in a *Vortex* evaporator , at 40 rpm and 70 ºC, and redisolved in 1 cm^3 acetonitrile:water (80:20) solution. A 0.02 cm^3 aliquot was analyzed for sucrose on an amino-silica column with a *HPLC* (*Varian*, Palo Alto, USA) fitted with a *R1-4* refraction index detector and a *4400* integrator. Acetonitrile:water (80:20) was used as the mobile phase with a flow rate of 1.0 cm^3 min^{-1}.

Dried callus was weighed and ground to a fine powder in liquid nitrogen in a chilled mortar. The extraction and enzyme assays were done as described by Gutiérrez-Miceli *et al.* (2002). Briefly, SPS and SUSY were extracted, dialyzed, partially purified on a micro-scale ω-amino-hexyl-agarose column as described by Dancer *et al.* (1990) and recovered by washing with 500 mM NaCl solution. Activity of SPS was measured using 0.1 cm^3 extract added with 0.1 cm^3 reaction mixture under V_{max} conditions containing 100 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 4 mM Fru6P, 20 mM Glu6P, and 3 mM UDPglu (Doehlert and Huber 1983). The mixture was incubated at 30 ºC for 30 min and the reaction inhibited by adding 70 μl 30 % KOH. Sucrose formation was estimated as described by Van Handel (1968). Activity of SUSY was measured in the direction of sucrose synthesis and in a similar way as SPS activity but the reaction mixture contained 100 mM Hepes-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 4 mM fructose, and 3 mM UDPglu.

SAI was extracted with phosphate buffer (pH 7.2) and NI with citrate-phosphate buffer (pH 5.2). All extracts were desalted by dialysis using *Spectrapore* membranes (*Thomas Scientific*, Swedensboro, USA). Activity of NI and SAI was measured by adding 0.3 cm^3 dialyzed extract and 0.5 cm^3 0.2 M sucrose to 0.2 cm^3 0.5 mM phosphate buffer (pH 7.2) or 0.2 cm^3 0.5 mM citrate-phosphate buffer (5.2), respectively. The mixture was incubated at 30 ºC and formation of reducing sugars measured by DNSA after 1 h (Hatch *et al*. 1963). Extracts without the reaction mixture acted as controls.

Protein concentration was determined as described by Bradford (1976) in dialysed extract using bovine serum albumin as standard. The data presented are the mean values from at least four replicates. Four sugarcane cultivars were used in the experiment and dynamics of sucrose concentration, SPS, SUSY, IN and SAI were studied after 0, 8, 17, 21, 27, 34 and 40 h using four replicates for each measurement. All statistical analysis were done with *SAS* (*Version 6.04*, 1989).

Sucrose concentration in callus varied among genotypes and reached a maximum by day 8 and 17 and decreased thereafter (Fig. 1). The mean concentration of sucrose in callus was largest in cv. Mex 57-473 and lowest in cv. Mex 69-290. The activity of SUSY increased at the onset of the experiment and then decreased in all cultivars except in cv. Mex 57473 were the activity remained constant (Fig. 2*A*). Changes in SPS activity over time were similar in all cultivars with the largest activity found in cv. Mex 57-473 and lowest in cv. Mex 69-290 (Fig. 2*B*). SAI activity decreased in all cultivars after the onset of the experiment, increased after day 8 and then decreased again except in cv. Zmex 55-32 where no initial decrease was found (Fig. 2*C*). Mean NI activity was lower compared to the activity of the other enzymes and showed less variation over time (Fig. 2*D*). The activity of SPS minus the activity of SAI [SPS-SAI] gave a comparable dynamic for the four cultivars. It increased from day 0 to a maximum at day 8 and then decreased sharply until day 27 or day 34 for cv. Rb765418 (no data shown). Changes in activity of [SPS-SAI] were much smaller thereafter. SAI activity was higher than that SPS, resulting in a negative value at day 17 for cv. Zmex 55-32 and at day 27 for cv. Mex 57-473 while at day 21 for the cv. Mex 69-290 and cv. Rb 765418. The pattern for the activity of

[SPS+SUSY-SAI-NI] was similar as for [SPS-SAI]: an initial increase to a maximum at day 8 followed by a sharp decrease with smaller decrease at the end of the experiment (no data shown). It increased again after day 27 for cv. Zmex 55-32.

In callus cultures, sucrose is hydrolyzed to glucose and fructose by SAI found in the cell wall, incorporated in the cell and resynthesized to sucrose by SPS and SUSY (Wendler *et al.* 1990). The activity of SPS and SUSY was greatest when the largest accumulation of sucrose took place, *i.e.* day 8 or 14. When the cells grew and the concentration of sucrose in the medium decreased, sucrose in the cells was used as an energy source. The SAI activity increased and also NI activity,

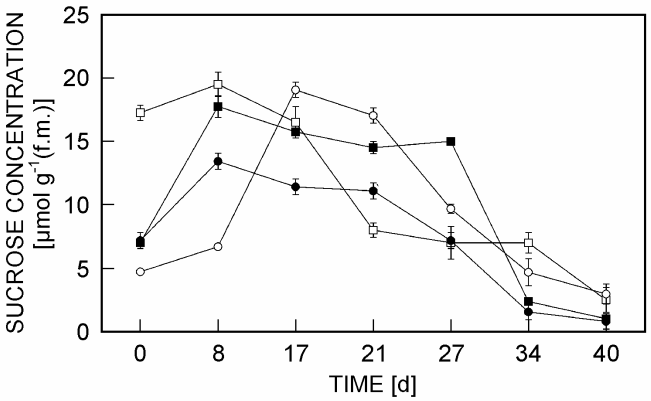

Fig. 1. Sucrose concentration [µmol g^{-1}(f.m.)] in cultivars Zmex 55-32 (*open squares*), Mex 69-290 (*closed squares*), Mex 57-473 (*open circles*), and Rb 765418 (*closed circles*) of sugarcane incubated for 40 d. *Bars* indicate SE of the mean.

but to a lesser extend. The activity of SPS and SUSY decreased, but did not stop completely, so sucrose was still being formed. Wendler *et al.* (1990) described this also in cell suspensions of sugarcane. Sucrose accumulation in callus, however, was much lower than that found in the parenchyma cells of sugarcane stem. *Saccharum* spp. can accumulate sucrose up to 20 % of their fresh mass in mature internodes (Bowen 1972), but only about 1 % accumulated in callus. Accumulation of sucrose in calluses was low when SAI activity was similar to that found in the stem. Zhu *et al.* (1997) reported that in 9-month-old stems SAI played a critical role in limiting sucrose accumulation in sugarcane. No sucrose was accumulated when SAI activity was higher than 7 µmol g^{-1}(protein) min^{-1}, but in calli sucrose was detected even when SAI activity was higher than 15 µmol g^{-1}(protein) min^{-1}. Zhu *et al.* (1997) also reported that the accumulation of sucrose was low when the activity of NI was high as found in callus cultures.

SPS explained most of the variation found for sucrose accumulation in this study. SPS is considered the key enzyme that regulates sucrose synthesis (Stitt *et al.* 1988). Increased activity leads to accumulation of sucrose in stem cells (Zhu *et al.* 1997). Botha and Black (2000) observed a positive correlation between SPS activity and sucrose content in internodes 3 to 11 of sugarcane cv. N19. Pulse labelling experiments with sugarcane cell suspension cultures indicated that sucrose synthesis is catalysed entirely *via* SPS (Wendler *et al.* 1990). Due to its importance, leaf SPS has been exhaustively studied, and some of its inhibitors and activators identified (Doehlert and Huber 1983).

Callus cells also showed high activity of SUSY especially during the phase of sucrose accumulation. SUSY catalyses a readily reversible reaction and a secondary role in sucrose synthesis might be envisaged. Activity of SUSY was positively correlated to the concentration of sucrose in calli. Botha and Black (2000), however, found no significant correlation between SUSY and sucrose content in the internodes 3 to 11 of sugarcane cv. N19, while Wendler *et al.* (1990) reported that the fructosyl moiety of sucrose is derived from the hexose-phosphate pool, ruling out a major contribution from SUSY in the synthesis of sucrose.

SAI activity in stem cells is closely related to the concentration of sucrose: an increased activity reduced sucrose concentration (Zhu *et al.* 1997). The same was found in callus cultures. NI activity did not show a clear relationship with sucrose accumulation in callus cultures nor in the stem (Zhu *et al.* 1997). However, Rose and Botha (2000) reported a significant correlation between sucrose content and NI and this was largely due to a tighter association between the two components in the bottom of the internodes.

Our results indicate three main points: *1*) SPS is a key enzyme in the sucrose synthesis pathway in callus cultures, *2*) SAI is a key enzyme in sucrose degradation and *3*) the difference in activity of SPS and SAI regulates

sucrose storage. However, the relationship between activity of SPS, SUSY, NI and SAI in callus was independent of sugarcane cultivar and the concentration of sucrose in callus was far less than found in parenchyma cells of sugarcane stem limiting its use to select the cultivars that would accumulate most sucrose as a crop.

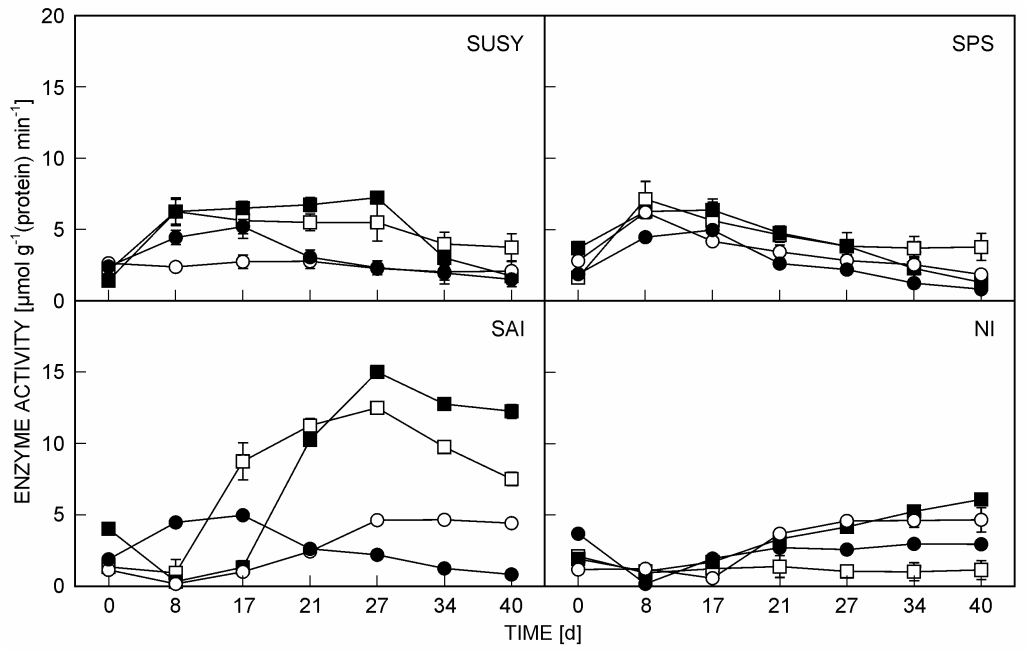

Fig. 2. The activity of SUSY (*A*), SPS (*B*), SAI (*C*), and NI (*D*) [µmol g^{-1}(protein) min^{-1}] in cultivars Zmex 55-32 (*open squares*), Mex 69-290 (*closed squares*), Mex 57-473 (*open circles*), and Rb 765418 (*closed circles*) of sugarcane incubated for 40 d. *Bars* indicate SE of the mean.

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