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REVIEW

Sucrose accumulation in sugarcane: a potential target for crop improvement

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Abstract Sugarcane is a highly productive crop plant with the capacity of storing large amounts of sucrose. Sucrose accumulation in the stem of sugarcane has been studied extensively. The initial recognition and characterization of the enzymes involved in sucrose synthesis and cleavage led to the widely accepted models of how sucrose accumulation occurs in the storage tissue. New insights were gained into the physiological role of individual enzyme activities in the process of sucrose accumulation in sugarcane. Studies on cell cultures and on isolated cell fragments initially supported and strengthened these models, but more recent research has revealed their weaknesses. A dynamic model of rapid cycling of sucrose and turnover of sucrose between vacuole, metabolic and apoplastic compartments explains much of the data, but the details of how the cycling is regulated needs to be explored. Genomic research into sucrose metabolism has been based on the premise that cataloging genes expressed in association with the stalk development would ultimately lead to the identification of genes controlling the accumulation of sucrose. Considerable progress has been made in understanding and manipulating the sugarcane genome using biotechnological and cell biology approaches. Thus, the greater understanding of physiology of sucrose accumulation and the sugarcane genome will play a significant role in the future sugarcane improvement programs and will offer new opportunities to develop it as a new-generation industrial crop.

Keywords *Saccharum* · Sucrose accumulation · Sucrose metabolizing enzymes · Gene expression · ESTs · Genomics

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Introduction

Sugarcane (*Saccharum* species hybrids) is a perennial agricultural crop belonging to the family Graminae and grown primarily for the sucrose-containing juices that are expressed from the stalk. Throughout the world, it is best planted commercially in tropical and subtropical areas that are characterized by warm temperature, humid climates and deep fertile soils (Barnes 1974). The primary use of sugarcane is to produce sucrose, which can be utilized in a range of products.

The biomass yield of sugarcane is the highest for any crop. Approximately 65–70% of the world's sugar is derived from sugarcane (Carson and Botha 2002; Lakshmanan et al. 2005). Improvement in sugar content of sugarcane increases sugar yield with only a small marginal increase in cost of production. This makes gains in sugar content economically more beneficial than corresponding increase in cane yield and means that increased sugar content is an important objective of sugarcane breeding programs. However, the sugarcane breeding programs have delivered increased sugar yield via improvements in cane yield, with much smaller contributions from sugar content. The possible reasons for slow rates of genetic gains in sugar content are as follows: insufficient weighting has been applied to sugar content in comparison with cane

yield in selection of parents, more favorable alleles for sugar content are fixed in current cultivars and that gene effects contributing to level of sugar content above current cultivars are negatively correlated with cane yield (Jackson 2005).

The economic yield of sugarcane is determined by accumulation of sucrose in the stalk. Under conditions favoring sucrose accumulation, the storage parenchyma tissue of sugarcane can store sucrose up to the theoretical maximum value of 62% dry weight or 27% fresh weight (Bull and Glasziou 1963). However, wild relatives of sugarcane accumulate less than 2% fresh weight as sucrose. The striking differences in sucrose storage appear to be regulated within the stem or within the translocation system regulating the partitioning of photoassimilates among alternative sinks. Some source activity also limits sucrose accumulation. Thus, there are multiple pathways in which there might be reaction rates limiting to sucrose accumulation (Inman-Bamber et al. 2010; McCormick et al. 2009; Moore et al. 1997).

1. *Biochemical reactions in leaf* photosynthetic rate, sucrose metabolism and carbon partitioning in different pools.
2. *Biochemical reactions in phloem* phloem reactions including loading in leaf, translocation to and unloading in stalk.
3. *Biochemical reactions in stalk* membrane transport, sucrose metabolism, carbon partitioning into different pools and remobilization of stored sucrose.
4. *Genetic and varietal developmental constraints* including duration and timing of maturation.

Sucrose accumulation has been studied more in sugarcane than any other plant (Hawker 1985) because very high concentration of sucrose is attained and this system was considered simple (Glasziou and Gayler 1972). The characteristics that make sucrose storage in sugarcane simple are that sucrose is the sugar which is produced in the leaf, translocated in the phloem and stored in the stem. Thus, it is not necessary either to consider the synthesis and metabolism of alternative storage carbohydrates or the storage in the specialized reproductive structures of fruit and seed.

The landmark in enzymology of sucrose storage in sugarcane was set by Australian research workers in the 1960s and early 1970s. The data were condensed to a scheme of sucrose metabolism in storage parenchyma by Glasziou and Gayler (1972) which is valid in many aspects even now. Since then, much progress has been made in understanding the mechanisms and regulation of transport processes, of solute compartmentation, of phloem loading and long-distance transport, of apoplastic barriers and symplastic cell continuity and of water relations in plants in general and in sugarcane in particular (Moore 1995). In addition, some more information about metabolic control of sucrose storage is available. Enzymes involved in various key steps have been identified, characterized and evaluated for their role as key regulatory steps. Genes encoding these enzymes are being cloned to transform plants to modify enzyme activity and increase sucrose accumulation. The results suggest that control in major metabolic pathways is shared between enzymes, and therefore, it is more difficult to alter than had been previously appreciated (Moore et al. 1997).

The purpose of this review is to incorporate this data in the previously existing framework of physiological and biochemical aspects of sucrose accumulation in sugarcane and to evaluate the potential for biotechnology and molecular biology study to overcome physiological, biochemical and genetic constraints in order to achieve greater production of sucrose by sugarcane plants.

Sucrose accumulation pathways

The pathway of sucrose accumulation begins with the translocation of sucrose through the phloem sieve elements to the stem internodes. Feeding leaf midribs asymmetrically C^{14}-labeled sucrose confirmed that sucrose is translocated from leaves and that it arrives at sink tissues without breakdown and re-synthesis (Moore 1995). However, 12% of the label was randomized during radial transfer from phloem into storage parenchyma indicating that the part of sucrose translocated was hydrolysed and resynthesised in the storage sink during sucrose accumulation (Hatch and Glasziou 1964).

When labeled sugars were fed to mature internodal tissue slices, sucrose was stored more rapidly from glucose or fructose than from sucrose (Batta and Singh 1986; Hawker and Hatch 1965) indicating preferential uptake of hexoses. When either C^{14}-glucose or fructose was fed to tissue slices, sucrose equally labeled in both the hexoses was recovered. This result showed that adequate intercellular isomerase activity was present to fully randomize the label prior to sucrose resynthesis (Hawker and Hatch 1965). When sucrose was stored from C^{14}-fructosyl sucrose, the ratio of C^{14} in glucosyl to fructosyl moieties in stored sucrose was 0.76 (compared with <0.001 for the C^{14}-fructosyl sucrose supplied to the tissue slices). This result indicates that at least 86% of the stored sucrose was hydrolysed prior to being stored. Uniformly labeled sucrose supplied with unlabeled fructose gave a ratio of 1.76 which was calculated to show that 100% of the sucrose was hydrolysed prior to being stored. This study parallels earlier studies on immature internodal tissue slices (Sacher et al. 1963) which indicated that a minimum of 70% of the labeled sucrose entering the tissue was hydrolysed during movement into the storage

compartment. These results were interpreted to mean that sucrose hydrolysis by invertase in the apoplastic space was obligatory.

The positive correlation between whole-plant dry matter accumulation and apoplastic acid invertase activity also suggested the critical importance of sucrose hydrolysis as photoassimilate moved radially from phloem to the storage compartment of the storage parenchyma cells (Glasziou 1960; Sacher et al. 1963). Then it was found that sugarcane cell cultures expressed high levels of acid invertase and took up reducing sugars but not sucrose (Komor et al. 1981; Maretzki and Thom 1972). This was taken as confirmation of the hypothesis that sucrose was unloaded from the phloem translocating elements into the apoplast where it was cleaved by invertase to produce hexoses which were taken by hexose-specific carriers in the plasmalemma (Bowen and Hunter 1972). These conclusions have been faulted because the experiments were carried out over a period of several hours to a week and could have given false results as a consequence of microbial contamination. Moreover, the model of sucrose accumulation of Glasziou and Gayler (1972) ignored anatomical (Jacobsen et al. 1992) and physiological (Welbaum et al. 1992) evidence for a barrier to post-phloem apoplastic transport.

Reinvestigation of experiments with tissue slice uptake of C^{14}-fructosyl sucrose in 249 mM mannitol medium showed approximately 25% of the label in the apoplast as C^{14}-fructose, indicating some sucrose cleavage (Lingle 1989). However, less than 2.5% of the label was randomized in the sucrose accumulated in the storing internodes and only 1.1% appeared in the glucose moiety of sucrose in the meristematic internodes. This was insufficient to support cleavage and re-synthesis as the sole sucrose transport mechanism. However, in contrast to earlier experiments (Hatch and Glasziou 1964; Sacher et al. 1963), most of the sucrose was accumulated without the randomization of the label. So the conclusion that sucrose could be taken intact was supported by additional research on the uptake of C^{14}-fructosyl sucrose by meristematic internodes (Thom and Maretzki 1992). In one experiment, none of the sucrose was cleaved in the apoplast and there was only 1% randomization of label in the accumulated sucrose. In the repeat of this experiment, 21% of the sugar was cleaved and 22% of the label was randomized. Possible reasons for these differences were not explained. Nevertheless, the researchers interpreted these results as indicating that the cleavage of sucrose was not necessary for its uptake. The authors emphasized that the two alternative pathways for sucrose uptake, either as intact sucrose or as its hexoses after its cleavage, are not mutually exclusive. There could be two uptake systems on the plasma membrane, one for hexoses and another for sucrose. This conclusion was supported by the fact that fluorosucrose, an analog of sucrose that cannot be cleaved by invertase, was taken up by the tissue with a 40% higher efficiency than was sucrose (Thom and Maretzki 1992).

It appears that a primary difference between the later results showing the uptake of sucrose uncleaved (Lingle 1989; Thom and Maretzki 1992) and earlier results showing the requirement for sucrose hydrolysis prior to hexose uptake (Batta and Singh 1986; Hatch and Glasziou 1964; Sacher et al. 1963) involves the treatments used for washing the tissue prior to the uptake studies. Early experiments were conducted on tissues that had been washed for 1 h in water, but later experiments were conducted on the tissues that had been washed for 1 h in 250 (Lingle 1989) or 200 mM mannitol (Thom and Maretzki 1992). The water wash could increase the turgor above that in vivo by about 0.5 MPa while mannitol wash could be nearly isotonic and thus not significantly alter turgor. It is possible that turgor causes conformational changes in the membrane structure such that at high turgor the sucrose transporter is not active so that only hexose can be taken up into the cell while at low turgor the sucrose can also be transported across the membrane.

Sucrose cycling in storage parenchyma cells

Sucrose accumulation is accompanied by continuous cleavage and synthesis of sucrose during accumulation of sucrose in the storage tissue (Batta and Singh 1986; Whittaker and Botha 1997). In storage cells, the sucrose metabolism is achieved by several different enzymes that perform the same or similar reactions and which operate in parallel. The major enzymes directly involved are in the direction of sucrose synthesis: sucrose phosfate synthase (SPS), sucrose synthase (SS) and in the direction of breakdown: soluble acid invertase (SAI), neutral invertase (NI) and sucrose synthase (SS) (Komor 2000). The reaction products glucose, fructose and UDP-glucose can be recycled to precursors of sucrose synthesis again by hexokinase (phosphorylation of glucose and fructose), phosphohexoseisomerase (interconversion of glucose-6-phosphate and fructose-6-phosphate), UDP-glucose:D-fructose2-α-D-glucosyltransferase and UDP-glucose:D-fructose-6-phosphate-2-α-D-glucosyltransferase (sucrose and sucrose phosphate synthesis) and sucrose phosphatases (SPase). The presence of these enzyme activities at the same time in the metabolic compartment of storage tissue had already been found (Batta and Singh 1986; Glasziou and Gayler 1972) and enzymatic cycle of sucrose synthesis and degradation had been postulated (Hatch 1964; Sacher et al. 1963).

The actual rate of sucrose cycling in cell suspension cultures could be determined and was found to be up to fourfold higher than the net rate of sucrose storage (Wendler et al. 1990). In tissue slices of internodes of different

developmental stages, the cycling of sucrose could be quantified too (Komor et al. 1996). The rate of sucrose cycling strongly depends upon the age of internodes. It was highest in young internodes (immature tissue) and decreased successively with the ripening of the internodes. The participation of sucrose synthase and SPS in sucrose synthesis could be calculated after simultaneous feeding of labeled glucose and non-labeled fructose and determination of label partitioning between glucose and fructose moiety in sucrose. Sucrose from sucrose synthase will be labeled in glucose only and sucrose from SPS will be equally labeled in glucose and fructose (Botha and Black 2000). Sucrose synthesis in immature tissue is equally achieved by both the enzymes, degradation mainly by acid invertase. During ripening, where a clear net production of sucrose is achieved in the tissue, i.e. where the rate of synthesis is twice the rate of hydrolysis, synthesis is dominated by sucrose-phosfate synthase and hydrolysis by acid invertase in contrast is strongly decreased. In mature tissue all enzymatic rates become very small and similar. The net import of carbon from phloem into the internodes is equal to growth and energy demand of tissue in immature stage, and during ripening phase approximately half of the imported carbon is diverted to stored sucrose (Komor 2000).

Correlation between enzymes involved in sucrose cycling and sucrose storage

Upon arrival in the stem, sucrose can be catabolised by sucrose synthase or one of the three invertases: SAI (located in vacuole), cell wall bound acid invertase (apoplastic space) or neutral invertase (cytoplasm) prior to being processed for cell growth, respiration, metabolism or storage depending upon the physiological demand of the plant (Hawker 1985; Hawker et al. 1991). Sucrose synthase activity is low in storage parenchyma cells but relatively high in vascular strands from both immature and mature storage tissue (Hawker and Hatch 1965). It was suggested that sucrose synthase does not catabolise sucrose in the metabolic compartment of storage cells because of a highly specific uridinediphosphatase which rapidly hydrolyses UDP rendering it unavailable for transfer to glucose by sucrose synthase (Moore 1995). Indeed, sucrose hydrolysis in storage parenchyma is believed to be accomplished by one of the invertases. Neutral invertase occurring in cytoplasm is active at a low level in very young tissue and at greater levels in older tissue (Batta and Singh 1986; Hatch et al. 1963). SAI activity is high in apoplast and vacuoles of young actively growing internodes but activity is virtually absent from mature internodes. Cell-wall bound invertase is active in both young and mature internodes (Gayler and Glasziou 1972; Hatch and Glasziou 1963; Hawker 1965). The cell wall acid invertase functioned by cleaving sucrose in apoplastic space to control photosynthate import (Hawker and Hatch 1965).

The various physiological studies correlated the invertases and their level with rapidly changing growth rate to find the possible role of invertases in regulating sucrose accumulation in sugarcane. The growth rates were reduced by low temperature, growth inhibitor glyphosate (Su et al. 1992) and drought stress (Hatch and Glasziou 1963) or growth rates were increased with the application of gibberellic acid during cold treatments (Gayler and Glasziou 1972). The amount of invertase in immature internodal tissue was linearly related to the rate of internodal elongation and could be varied more than tenfold during 10-day treatment at different temperatures (Hatch and Glasziou 1963). In all these studies, the SAI, occurring in the vacuole and apoplastic space of elongating internodes, disappeared when internode growth ceased and reappeared when growth resumed. The vacuolar form appeared to be involved with the regulation of turgor and internal sugar pools; the apoplastic form appeared to be the major controller for dry matter import accompanying cell extension growth. The neutral invertase increased during maturation and appeared to be involved in controlling sugar flux in the mature storage tissue. In mature tissue, lacking a measurable SAI, there was a cell wall bound acid invertase which functions in cleaving sucrose in the apoplastic space to control dry matter import for sucrose storage (Hawker and Hatch 1965).

The regulation of acid invertase activity was studied in tissue slices in an attempt to understand how these enzymes are regulated in the whole plant. Acid invertase activity appeared to be regulated in part by an auxin-sugar control system in which auxin stimulated and glucose suppressed the synthesis of invertase (Glasziou and Waldron 1964; Sacher et al. 1963). Subsequent studies reported that these controls were at the level of mRNA synthesis and destruction. In this model it has been suggested that as various factors contribute to the reduction in growth during internode maturation, auxin supply to the stem is reduced, invertase decreases, turnover of sucrose is slowed and more sucrose remains in the storage compartment (Hatch and Glasziou 1963; Hawker and Hatch 1965; Su et al. 1992).

Sugarcane cultivars vary in their potential of sucrose accumulation. The rapid decline in the activity of SAI with the maturity of cane correlated well with the high level of sucrose in early maturing varieties of sugarcane (Batta et al. 1995, 2007). The reduction of SAI activity was dramatic in early-maturing and high sucrose-storing cultivars, whereas the decline in activity was lower in late-maturing and low sucrose-storing cultivars (Sachdeva et al. 2003b; Terauchi et al. 1999). However, low activity of SAI is not sufficient to account for high sucrose accumulation in

high sucrose-storing genotypes. The major differences in sucrose accumulation were attributed to the differences between activity of SAI and SPS, provided the SAI activity is below the critical threshold concentration (Zhu et al. 1997). The similar correlation between SPS–SAI and sucrose concentration was obtained when internodes of various commercial strains were compared during development (Lingle 1999) or when internodes of sugarcane plants were grown at different temperatures (Ebrahim et al. 1998). The differences in SAI activities between low and high sucrose sugarcane lines might be due to difference in expression of essentially identical SAI genes (Zhu et al. 2000).

A similar relationship between growth rate, acid invertase and rate of sucrose accumulation has been reported in batch cultures of sugarcane cell suspensions (Goldner et al. 1991). The switch from mobilization to sucrose storage is accompanied by the small changes in the activities of enzymes and metabolites. The activity of SAI was low while cell wall acid invertase was high in sugarcane cells. The activity of neutral invertase increased with the sucrose storage and decreased in cells with higher sucrose content (Wendler et al. 1990).

For sucrose accumulation, the hexoses in the metabolic compartment of parenchyma cells are resynthesised into sucrose by SPS and SPase (Huber and Huber 1996; Stitt et al. 1987). Potentially, sucrose synthase could also be involved in sucrose synthesis even though equilibrium constant for sucrose formation by sucrose synthase is only 5 as compared with 3,250 for SPS (Moore 1995). The pulse labeling experiments with sugarcane cell suspension cultures indicate that sucrose synthesis is catalyzed entirely via SPS (Wendler et al. 1990). It seems probable that in young internodes both sucrose synthase and SPS contribute to sucrose synthesis. However, SPS activity becomes gradually more important in sucrose synthesis as the internodes get older, exceeding sucrose synthase more than threefold (Botha and Black 2000). The data from Schafer et al. (2004) are in accordance with the labeling data from Botha and Black (2000) which clearly demonstrated the sucrose synthase involvement in sucrose synthesis in young internodes.

Sucrose synthase activity is low in storage parenchyma cells but relatively high in vascular strands from both immature (Hatch et al. 1963) and mature storage tissue (Hawker and Hatch 1965). Hawker and Hatch (1965) recognized that sucrose synthase might be involved in sucrose breakdown but did not speculate the possible role of sucrose synthase in sucrose accumulation. Alternatively, sucrose synthase is thought to be concerned with the generation of UDP-glucose from sucrose for the formation of cell wall material and starch (Chourey et al. 1998). Also sucrose synthase activity in sugarcane has been associated with maturing internodes (Lingle and Smith 1991) and with more mature fully elongated internodes (Buczynski et al. 1993). Some studies showed negative correlation between sucrose synthase and sucrose accumulation rate (Lingle and Smith 1991) and other showed no relation (Botha and Black 2000). The pattern of sucrose synthase activity in relation to sucrose content varied considerably in different internodal tissues. In one study sucrose synthase activity was more than twice as high in internode six than in internode three (Buczynski et al. 1993), while in another the relative activities between these internodes showed reverse distribution (Lingle and Smith 1991). These differences were based on varying experimental protocols, growing conditions and cane varieties. In developing roots of sugarbeet, sucrose synthase was reported to contribute to increasing sink capacity while SPS provided steady increase in sucrose content (Fieuw and Willenbrink 1987). A similar temporal difference in functioning of the two sucrose synthesis pathways, first sucrose synthase and then SPS, might exist in sugarcane but this has not been demonstrated.

As pointed out earlier, sucrose undergoes a cycle of synthesis and breakdown. In theory, sucrose concentration can be increased by shifting up sucrose synthesis activity or by switching down the sucrose hydrolysis activity, whereby each of these metabolic reactions is controlled by different enzymes in parallel. Zhu et al. (1997) analyzed the progeny of a defined cross between *S. officinarum* and *S. robustum* and followed sucrose concentration and enzyme activities during internode ripening of low and high sucrose clones. The best correlation found, comprising all the internodes and all selected clones of progeny, was between sucrose concentration and the difference between SPS and SAI activity. Sucrose accumulation in sugarcane is regulated by the activities of SAI and SPS, where SAI activity alone gave a nearly as good fit. The other enzymes, sucrose synthase, neutral invertase or SPS alone did not correlate with sucrose. The correlation was very robust because it remained valid even if sucrose concentration and SAI activity of the whole stalk (i.e. not separated into individuals internodes) was used in the calculation. The same correlation between SPS–SAI and sucrose concentration was obtained when internodes of various commercial strains were compared during development (Lingle 1999) or when internodes of sugarcane plants grown at three different temperatures (i.e. 15, 27 and 45°C) were analyzed (Ebrahim et al. 1998). No correlation to any enzyme was found when only ripe internodes were considered (Lingle 1999; Zhu et al. 1997) or when seasonal development was compared (Lingle 1997) or in sugarcane suspension cells (Ebrahim et al. 1999). The key sucrose metabolizing enzymes were studied in progeny of an introgression program to identify biochemical markers associated with high sucrose content measured as commercial cane sugar (CCS). The four high and four low CCS clones were derived from an initial cross between *S. officinarum* and commercial

cultivar Q165. The SPS and two soluble invertase isoforms were measured in the clones derived from a backcross of one of the progeny to another commercial cultivar Mida. The SPS activity was significantly higher in upper internodes of high CCS clones as compared with low CCS clones in both populations thus suggesting the role of SPS in establishing metabolic and developmental processes necessary for high sucrose accumulation during stem growth and maturation (Grof et al. 2007).

In sugarcane, sucrose and hexoses are taken up by storage parenchyma cells. During sucrose accumulation, there is significant cycling of carbon between the hexoses and sucrose. As a result an intracellular pool consists of a mixture of imported hexoses and products of sucrose breakdown. Despite this low sucrose concentration is maintained in the mature tissue (Lingle and Smith 1991) and this suggests high fructose phosphorylating activity. Hoepfner and Botha (2003) have recently detected the fructokinase activity from sugarcane culm tissue at different stages of development and activity was reported to decline during maturation. In addition to fructose phophorylating activity, phosphofructokinase and pyrophosphate:fructose-6-phosphate-1-phosphotransferase (PPi:PFP) convert fructose-6-phosphate generated by oxidation of glucose-6-phosphate obtained from sucrose cleavage via pentose phosphate shunt into fructose 1, 6-P2. With the existence of several parallel/alternative steps for interconversion of F-6-phosphate and F1, 6-P2, PFP enzyme activity is found to be correlated with sucrose and fiber accumulation. There is negative correlation between total PFP activity and sucrose content across a range of sugarcane varieties with different level of sucrose. In addition, PFP activity coincided with the increased rate of respiration and decreased ability to accumulate sucrose (Whittaker and Botha 1999). van der Merwe (2005) has revealed that reduced PFP expression led to increased sucrose accumulation by six- and threefold in immature and mature internodes of transgenic sugarcane clones, respectively. However, Groenewald and Botha (2008) have reported that down-regulation of PFP activity resulted in significant increase in sucrose concentration in immature internodes of transgenic plants, but not in the mature internodes. Although these studies do not provide evidence of the role of PFP in sugarcane sink metabolism, it might be related to long-term requirement for sucrose utilization rather than storage, particularly in low sucrose-storing varieties (Whittaker and Botha 1999).

Purification and characterization of enzymes of sucrose accumulation

Metabolism of sucrose, a mobile source of energy, is an absolute requirement for the survival of heterotrophic plant organs (Sturm 1999). In these organs, different isoforms of invertase with discrete subcellular locations hydrolyze the disaccharide into hexoses and thereby feed sucrose in various biochemical pathways. In contrast to acidic pH optima and vacuolar and extracellular location, knowledge about the molecular nature of the cytoplasmic invertases is still scarce (Sturm 1999). Rosario and Santisopasri (1977) characterized soluble invertases from sugarcane juice using ammonium sulfate fractionation followed by SephadexG-200 column chromatography and showed an acid invertase with MW 380 kDa and 23.5% carbohydrate and neutral invertase with MW 66 kDa and 22% carbohydrates. For acid invertase K_m and V_{max} values were 2.8 mM and 2.7 µmol sucrose hydrolysed h^{-1} mg^{-1} protein, respectively. However, for neutral invertase K_m and V_{max} values were 0.32 mM and 2.8 µmol sucrose hydrolysed h^{-1} mg^{-1} protein, respectively. Batta et al. (1991) have investigated the kinetic properties of SAI from sugarcane juice and reported that the enzyme was strongly inhibited by manganese chloride. From the effect of temperature on K_m and V_{max}, the energy of activation (E_a), enthalpy change (ΔH) and entropy change (ΔS) of the enzyme were calculated to be 29.09 kJ mol^{-1}, −13.46 kJ mol^{-1} and −18.56 J K^{-1} mol^{-1}, respectively. The effect of pH on K_m and V_{max} suggested that carboxyl and imidazole groups are involved in catalytic activity of the enzyme. Sachdeva et al. (2003a) purified soluble invertases from cane juice of an early-maturing and high sucrose-storing sugarcane cultivar. Three different acid invertase isoforms A-I, A-I, A-III and one neutral invertase isoform were identified. A-I isoform showed the highest V_{max}/K_m values indicating the maximum kinetic perfection of this enzyme and this may be the major vacuolar enzyme involved in sucrose hydrolysis.

Sugarcane neutral invertase has been partially purified from mature sugarcane stem tissue using sequential ammonium sulfate precipitation and anion-exchange chromatography (Vorster and Botha 1998). The enzyme was not bound by concanavalin A, suggesting that it is not glycosylated. Most of the activity elutes as monomer of active molecular weight 60 kDa. The enzyme displayed typical hyperbolic saturation kinetics for sucrose hydrolysis. It has a K_m of 9.8 mM for sucrose and pH optima of 7.2. An Arrhenius plot shows that energy of activation of the enzyme for sucrose hydrolysis to be 62.5 kJ mol^{-1} below 30°C and −11.6 kJ mol^{-1} above 30°C. Soluble neutral invertase is inhibited by its products, fructose being more effective inhibitor than glucose. Soluble neutral invertase is significantly inhibited by $HgCl_2$, $ZnCl_2$, $CuSO_4$ and $CoCl_2$. Batta et al. (1997) have showed that carboxyl and imidazole groups are also involved in the catalytic activity of soluble neutral invertase from sugarcane storage tissue.

The isozymic forms of sucrose synthase (SS1 and SS2) in sugarcane have been investigated to determine their respective tissue distribution in the plant and differences in

their biochemical characteristics (Buczynski et al. 1993). In most of the plants investigated, sucrose synthase appears to be encoded by at least two genes which are differentially expressed among different genotypes, organs, tissues or stages of development. The total sucrose synthase activity was lowest in mature leaf and generally increased in stalk from a low value at the apex to a maximum value at the 14th internode, the oldest one measured. Western Blots indicated that all of the SS activity in the older internodes was due to the sugarcane isozyme homologous to the maize SS1. The sugarcane isozyme homologous to maize SS2 appeared to be down regulated in the older internodes, the one having highest level of endogenous sucrose. Schafer et al. (2004) partially purified sucrose synthase activity from sugarcane leaf tissue. The product inhibition studies revealed that UDP-glucose is a competitive inhibitor with respect to UDP and a mixed inhibitor with respect to sucrose. Fructose is a mixed inhibitor with regard to both sucrose and UDP. K_m and K_i values for sucrose, UDP, UDP-glucose and fructose were calculated. Replacing the estimated kinetic parameters of sucrose synthase in a kinetic model of sucrose accumulation with the experimentally determined parameters of the partially purified isoform had significant effects on model outputs, with a 41% increase in sucrose concentration and 7.5-fold reduction in fructose. The doubling and halving of sucrose synthase activity reduced and increased steady-state increase in fructose concentration by 42 and 140%, respectively. So the different isoforms of sucrose synthase have significant different effects on metabolite concentrations in vivo, therefore impacting on metabolic regulation. The detailed mechanism of regulation of transcription and translation of the sucrose synthase gene are not yet known but may be activated through carbohydrate or sugar status of the plant (Koch 1996).

During sucrose accumulation, the hexoses in metabolic compartment (cytoplasm) of storage parenchyma cells are resynthesised into sucrose by SPS and SPase (Hatch 1964; Hatch et al. 1963; Hawker et al. 1991). Relatively little work has been done on the purification and characterization of SPS from sugarcane. The studies have revealed the existence of multiple forms of SPS in leaves of different species and that the forms differ in their regulatory properties (Kerr et al. 1987). In general, SPS activity of leaf and stem of sugarcane is regulated by allosteric effectors such as glucose-6-phosphate (activator) and Pi inhibitor (Stitt et al. 1987; Tanouye et al. 1992). The SPS activity is regulated by phosphorylation of specific serine residue in response to light/dark changes (Huber and Huber 1996). Grof et al. (2006) have described five families of SPS genes in *Saccharum* spp. which were differentially expressed in leaves and stem as determined by quantitative real-time PCR. The type B and C families of SPS genes were predominantly expressed in both immature and mature leaves, whereas the two subfamilies making up the type D family (SPS D1 and SPS D2) were expressed at similar levels in all tissues examined. In the type A family, expression was lowest in leaves and increased from the meristem region down to internode 7 of the stem. SPase has been purified from sugarcane storage tissue. The activity of enzyme is increased fourfold by the addition of Mg^{2+} ions and inhibited by EDTA, fluoride, Pi, PPi, Ca^{2+} and Mn^{2+} (Hawker and Hatch 1966). Sucrose, maltose, melezitose and 6-kestose also inhibited SPase activity (Hawker 1967). SPase might be part of a group translocator that operates in the tonoplast of sugarcane (Hawker et al. 1987).

Sugarcane biotechnology

Interspecific hybridization in sugarcane, which has been used for many years, has reduced the disease problem and increased biomass and sugar yield with improved adaptability to grow under various stress conditions (Roach 1972; Srivastava et al. 1994). However, progress in traditional breeding of sugarcane, a highly polyploidy and frequently aneuploid plant, is impeded by its narrow gene pool, complex genome, poor fertility and long breeding/selection cycle. These constraints, however, make sugarcane a good candidate for molecular breeding. The greater understanding of the bioregulation of sucrose accumulation in sugarcane stem and its genome will accelerate the implementation of commercially significant biotechnology outcomes. The improvement in sucrose content is desirable because there are great benefits derived from producing the same amount of sucrose in less biomass, a result of reduced costs of harvesting, transport and processing while the production costs remain the same. Therefore, more strategic approaches have been initiated to increase stem sucrose by identifying the genetic factors controlling sucrose content (Bonnett et al. 2004; Jackson 2005).

The genetic regions associated with the sucrose content have been identified through quantitative trait locus (QTL) analysis of DNA markers scored in the progeny of two segregating populations derived from interspecific crosses between *S. officinarum* × *S. spontaneum*. These two populations were analyzed for a total of 735 DNA marker loci to identify QTLs for sugar yield, POL (sucrose content), stalk weight, stalk number, fiber content and ash content. Among 102 significant associations between these six traits and DNA markers, 61 were located on sugarcane linkage maps, while the other 41 were associated with unlinked markers. Fifty of 61 mapped QTLs were clustered in 12 genomic regions of seven sugarcane homologous groups. The plants with high sugar yield possessed a large number of positive QTLs for sugar yield components (Ming et al. 2002).

Hoarau et al. (2002) using self-progeny of the sugarcane modern cultivar "R570" identified 73 putative QTLs, consistent across both years of evaluation, i.e. with significant association between observed phenotypic variation for a given trait and a particular allele of a locus. Aitken et al. (2006) identified 37 markers-QTL associations for brix and POL in a progeny derived from the cross between clone *S. officinarum* and a cultivar. Each QTL explained 3 to 9% of the observed phenotypic variation, showing both positive and negative effects. The identification of QTLs, however, does not reveal how a region of a particular linkage group contributes to a trait or which particular DNA sequence is involved.

Another approach, involving the transcriptome studies, is used to assess the transcripts associated with sucrose accumulation in sugarcane. This approach involves the sequencing of randomly chosen clones from cDNA libraries representing the genes expressed either in immature stem internodes or internodes that are rapidly accumulating sucrose. These cDNA sequences are termed as expressed sequence tags (ESTs). The initial studies on ESTs were undertaken by Carson and her colleagues at South African Sugar Association Experiment Station (SASEX), now called South African Sugar Research Institute (SASRI), using leaf roll and stem (Carson and Botha 2000, 2002; Carson et al. 2002). The studies led to the understanding of several physiological activities in sugarcane and their genetic control. The genes associated with high sucrose-accumulating internodes were determined by the relative abundance of particular ESTs in cDNA libraries.

The largest collection of ESTs was generated by SUCEST, a consortium of Brazilian researchers who sequenced approximately 238,000 ESTs from 26 diverse cDNA libraries (Vettore et al. 2001, 2003). The Gen Bank database for sugarcane currently lists more than 250,000 EST sequences with 4,850 UniGenes. Interest in the possibility of discovering agronomically important sugarcane genes led an international consortium of sugarcane research institutions (the International Consortium of Sugarcane Biotechnology, ICSB), Australia and South Africa to establish smaller independent sugarcane EST programs. The ICSB program to date is that of a single laboratory analyzing three cDNA libraries—apex, mature leaf and mature internode to develop 9216 ESTs that were clustered into 3,400 non-redundant tags (Ma et al. 2004). About 57% of these ESTs were assigned a putative function based on statistically significant similarity to previously characterized proteins or sequences. Another 28% corresponded to previously identified, but uncharacterized, sequences. Some of the remaining sequences were predicted to be genes that may be new or unique to sugarcane. Comparisons of the sugarcane ESTs with a large sorghum EST database revealed similar compositions of expressed genes between different tissues, suggesting applicability of the more abundant sorghum data (Moore 2005).

Further, the differential expression of particular genes on microarrays were studied while comparing the RNA extracted from immature and maturing stem (Casu et al. 2003, 2004). The most up-regulated genes associated with the carbohydrate metabolism were sugar transporters, the most abundant being the putative hexose transporter (Casu et al. 2003). When the expression of putative hexose transporter, PST2 was examined in the RNA samples from stems of different sugarcane cultivars, a striking difference in the level of expression was found. By correlating the relative expression level with sucrose concentration it may be possible to identify the genes associated with high sucrose accumulation. However, most of the up-regulated genes were apparently unrelated to sucrose accumulation, rather taking part in secondary thickening and lignification as internodes mature (Casu et al. 2004). It is interesting to speculate whether down regulation of some of these genes might redirect photoassimilate from fiber to increased sucrose accumulation. There was also a group of up-regulated genes that were putatively involved in stress tolerance (Casu et al. 2004). The genes in stress tolerance are being investigated further to help understand the processes at a tissue level that are necessary for accumulation of high sucrose in the stem storage cells. The ability to conduct microarray experiments with greater coverage of genome or even more targeted micro-and macro-array experiments (Watt et al. 2005) is now feasible due to large collection of ESTs from sugarcane recently lodged in public databases. Sucrose-accumulating internodes from field-grown plants were assayed using cDNA microarrays containing 1,545 elements (Papini-Terzi et al. 2007, 2009). Transcriptome comparisons aimed at identifying differentially expressed genes were made by comparing high-sugar and low-sugar plants directly, and also by comparing high-sugar and low-sugar internodes. A total of 125 genes were found to have expression patterns correlated with sugar content. Genes encoding SNF-related kinases and involved in auxin signaling were found, providing insights into the regulatory network that might control sucrose accumulation. Intriguingly, several proteins related to stress responses, such as cytochrome P450 monooxygenases, were also found. Approximately half of the sucrose-content-associated genes were found to be developmentally regulated during culm maturation, and many were related to stress responses. A comparison of this differential expression data set with the results obtained when the plants were submitted to drought (Rocha et al. 2007) revealed that approximately half of the genes identified as associated with the sucrose content were responsive to drought. They belonged to several functional categories including calcium signaling,

stress responses, and protein phosphorylation. The data indicated that the sucrose-accumulating tissues activate pathways during culm development, which overlap with drought and other stress responses such as cold and injury. This is corroborated by the observation that several SnRKs associated with the sucrose content and with drought belonged to the SnRK2 and SnRK3 family of kinases involved in osmotic stress responses (Boudsocq and Lauriere 2005). The GeneChip Sugarcane Genome Array produced by Affymetrix was used by Casu et al. (2007) to study culm maturation, leading to the identification of the developmentally regulated genes involved in cellulose synthesis, cell wall metabolism and lignification.

Gene expression profiles, obtained from DNA array analyses, were compared between high-sucrose progeny and low-sucrose progeny of a segregating population during maturation and sucrose accumulation to develop a "genetical genomics" strategy for identifying candidate genes that may control sugar accumulation (Casu et al. 2005). Further, this genetical genomics approach performed using the segregating populations are considered more powerful, as variation in gene expression in non-relevant traits is minimized.

The development of the field of metabolomics has opened the possibility of looking at the end products of both gene expression and protein catalysis (Lakshmanan et al. 2005). Particular metabolites were correlated with the level of sucrose accumulation, which were expected to increase down the stem. Tricarboxylic acid cycle intermediates and amino acids were more abundant in the stem that was actively growing and decreased in more mature internodes down the stem (Glassop et al. 2007). However, other metabolites such as trehalose and raffinose showed positive correlations with sucrose concentration. There are two ways of using the data from metabolic studies in sugarcane which have an immediate appeal in the pursuit of improving sugar content. The first would be analogous to the anonymous DNA marker and QTL approach. Profiles of metabolites would be generated for different groups of genotypes that differ in their level of sucrose accumulation. The data can be used diagnostically without peak identification to determine the pattern of metabolites that discriminate between genotypes (Fiehn et al. 2000). Like the DNA-based analysis, individual metabolite peaks responsible for the difference can be identified and genes encoding the enzymes acting on the metabolites or its precursors are studied further. This is the second way of utilizing the metabolomic data. A more targeted approach is the identification of metabolites more closely related to carbohydrate metabolism, which will rely on an adaptation of standard pre-fractionation and treatment of samples. However, as microarray expression studies have shown, not all of the changes may be directly related to carbohydrate metabolism, so an initial broad sweep may be fruitful (Lakshmanan et al. 2005).

The spatial distribution of a particular transcript and its gene products are being explored using in situ hybridization of RNA and immuno-localization of proteins. The methods were used to identify the cells in which particular transcripts are expressed. Using in situ hybridization, Casu et al. (2003) showed that PST2 was highly expressed in sugarcane phloem companion cells, while Rae et al. (2004) using immuno-localization, showed that sucrose transporter (ShSUT1) could be localized to the layer of cells surrounding the vascular bundles. Through these experiments, it was possible to localize many of the putative sugar transporters and other enzymes of sugar metabolism found in the EST collection. The results of these experiments will give a better understanding of the spatial arrangement of the sugar transport system and as to how this changes developmentally down the stem. The results to date are also providing evidence for a highly regulated movement of different sugars through different cells to the site of storage in the stem parenchyma. The ultimate test of a gene's function is its expression in a heterologous system. The sugarcane sucrose transporter ShSUT1 was expressed in yeast that does not take up sucrose. The complemented yeast was shown to take up sucrose at a faster rate than untransformed yeast (Rae et al. 2004), confirming its function as a sucrose transporter.

Sugarcane sucrose metabolism has been thoroughly studied and much progress has been made to elucidate the physiological and biochemical barriers in efforts to increase the amount of photoassimilate directed towards sucrose in sugarcane stem. The three main targets for molecular manipulation, aimed at increasing sucrose content in the stem have been identified (Grof and Campbell 2001): manipulation of sucrose synthesis process in leaf and stem, improvement of sucrose transport mechanism, and altering the expression, or the activity of enzymes responsible for hydrolysis of sucrose in the cytosol or/and in the vacuole of stem parenchyma. Genetic transformation is thus necessary to clarify the role of genes and define the targets useful for sugarcane improvement programs. The most commonly used method for transformation of sugarcane is particle bombardment of embryogenic callus combined with herbicide resistance gene as a selectable marker (Bower and Birch 1992) or by intact cell electroporation (Arencibia et al. 1995). The first successful report of recovery of morphologically normal transgenic sugarcane plant from co-cultivation of callus with *Agrobacterium tumefaciens* was given by Arencibia et al. (1998). The patterns generated by southern hybridization confirmed that T-DNA was randomly integrated into the sugarcane genome without the persistence of *A. tumefaciens* in the transgenic plant. Genetic engineering, thus, has been used

as a precise tool for identifying the key enzymes involved in metabolic pathways and to successfully manipulate the metabolic processes involved in sucrose accumulation. The direct changes by genetic transformation require a transformation system, a suitable gene, suitable promotor sequences for cell and tissue-specific expression and effective targeting signals to direct the protein products to their final destination within the cell. The design to metabolically alter sugarcane to increase sucrose accumulation can involve either inhibition or over expression of the native plant genes or expression of foreign genes.

To effectively manipulate a metabolic process, the genes must be expressed in a predictable and suitable manner. This may require promoters for organ- and/or cell-specific expression, expression under appropriate environmental conditions and at correct developmental stage. Organ-specific promoters have been reported from storage sink tissue such as seeds, potato tubers and fruits (Edwards and Coruzzi 1990). In sugarcane small subunit Rubisco (rbcs) genes have been isolated, sequenced and promotor elements isolated and fused with a reporter gene to determine the tissue specificity of expression (Tang et al. 1996). The promotor elicited the expression specifically in leaves and preferentially in bundle sheath cells. A similar expression pattern has been observed in transgenic sugarcane using the rice rbcs promotor (Grof et al. 1996). Transgenes may need to be expressed as a fusion protein containing a suitable sequence to target heterologous proteins to the appropriate cellular site. By bioinformative analysis of gene sequences from putative sugarcane vacuolar proteins, a motif has been identified that displays high conservation across plant legumain homologues that are known to function within vacuolar compartments. This five-amino-acid motif represented by the sequence IRLPS in sugarcane is shown to direct an otherwise secreted GFP fusion protein into a large acidic and proteolytic vacuole in sugarcane callus cells as well as in diverse plant species. This targeting motif will be a valuable tool for engineering sugarcane plants for the production of novel products (Jackson et al. 2007). Since sucrose is unloaded into apoplasm, passes through cytoplasm and stored in vacuole, it may be desirable to direct the expression of specific genes to specific sites using targeting constructs. An appropriate sucrose isomerase (SI) expression pattern in sugarcane provides a valuable source of beneficial sugars and overcomes the sugar content ceiling in sugarcane plants. The introduction of an SI gene (Wu and Birch 2007) tailored for vacuolar compartmentation resulted in sugarcane lines with increase in total stored sugar level. The high-value sugar isomaltulose was accumulated in storage tissues without any decrease in stored sugar concentration resulting in up to doubled total sugar concentrations in harvested juice. The transgenic sugarcane lines also showed increased photosynthesis, sucrose transport and higher cell wall acid invertase (CWAI) activity of stalk storage parenchyma which has multiple roles in sink strength.

Metabolism can be altered by decreasing or increasing enzyme activity through genetic transformation. Antisense is used to alter the expression of endogenous genes by transforming the plants to produce transcript in its reverse or antisense orientation to reduce the amount of enzyme. Consequently, attempts have been made to increase sucrose accumulation in sugarcane through transgenesis focusing on the down-regulation of the activity of single genes encoding sucrolytic enzymes (Botha et al. 2001). Ma et al. (2000) studied sucrose accumulation in isolated sink organ by genetic engineering of different levels of invertase activity in three cellular compartments. The CW-INV transformed lines showed that although increased CWI activity supplied higher amount of hexose to the cells, it did not result in more sugar removal from the medium. This implied that hexose uptake rather than hexose availability may be the factor for increased sucrose accumulation. The Cy-INV transformed lines showed that higher cytosolic invertase activity inhibited sucrose accumulation. The antisense SAI-transformed lines showed that partial inhibition of SAI activity could lead to increased sucrose accumulation. Coordinated expression of CWI activity and hexose transporters plus decreased SAI activity may be the primary biochemical factors for maximizing sucrose storage. The pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) activity (Groenewald and Botha 2008) was successfully down-regulated in sugarcane using constitutively expressed antisense and untranslatable forms of the sugarcane PFP-β gene. In young internodal tissue, activity was reduced by up to 70% while no residual activity could be detected in mature tissues. Sucrose concentrations were significantly increased in the immature internodes of the transgenic plants but not in the mature internodes. Both the immature and mature internodes of the transgenic plants had significantly higher fiber contents. These findings suggest that PFP influences the ability of young, biosynthetically active sugarcane culm tissue to accumulate sucrose. However, the equilibrium of the glycolytic intermediates, including the stored sucrose, is restored when ATP-dependent phosphofructokinase and the residual PFP activity are sufficient to sustain the required glycolytic flux as the tissue matures. Moreover, it suggests a role for PFP in glycolytic carbon flow, which could be rate limiting under conditions of high metabolic activity.

Future prospects

Sugarcane is a highly productive crop used for centuries as the main source of sugar and recently to produce ethanol, a

renewable bio-fuel energy source. There is increased interest in this crop due to the impending need to decrease fossil fuel usage. Sucrose accumulation has been studied extensively in sugarcane—an example of a highly productive crop plant with the capacity for storing large quantities of sugar. Sucrose storage in sugarcane is a highly regulated process where anatomical features, metabolic reactions and transport through membranes are closely interacting to achieve the final goal, namely high sucrose concentrations. Molecular studies are beginning to reveal details at the gene and transcriptional levels of the enzymes involved in sucrose transport and metabolism. A dynamic model of rapid cycling and turnover of sucrose between the vacuole, metabolic and apoplastic compartments have been thoroughly discussed in this review. Further, detailed biochemical and genetic information will be needed to develop technologies necessary for long-term success of the sugarcane industry. Genome-based technology such as cDNA microarray data indicates genes associated with sugar content that may be used to develop new varieties improved for sucrose content or for traits that restrict the expansion of the cultivated land. More recently, genes encoding the sucrose-metabolizing enzymes have been isolated, cloned and used in experiments to transform sugarcane to increase or decrease expression of the enzymes with the goal of altering sucrose accumulation. From agricultural prospective, potential definitely exists to increase the yield of sucrose, although careful consideration should be made of growth penalty incurred in transgenic plants. However, results of this reductionist approach towards understanding sucrose accumulation have fallen short of expectations, apparently because of the complex interactions among the multitude of simultaneous processes. Recent rapid expansion of sugarcane molecular datasets and the beginning of a systems approach to metabolic modeling of sucrose accumulation point the way for future research efforts to integrate processes from gene to crop improvement.

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