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Correlation analysis between the key enzymes activities and sugar content in sweet sorghum (*Sorghum bicolor* L. Moench) stems at physiological maturity stage

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Abstract

The contents of Fructose (Fru), Glucose (Glu) and Sucrose (Suc), activities of sugar metabolism enzymes (NI, SAI, SPS and SS) were investigated to identify key enzymes involved in sugar accumulation process in sweet sorghum (*Sorghum bicolor* L. Moench) stems. We also evaluated relationships among these key factors. Ten internodes from top to base of ten sweet sorghum cultivars were used for the experiment at the physiological maturity stage (three-month-old stems). The results showed that Suc was the main sugar in all ten different cultivars, accounting for 85.2% (on average) of the total sugar content (Glu +Fru +Suc). Sweet sorghum stems of different cultivars revealed different patterns of sugar accumulation with the internodal number, increasing from top to base. There was a significant correlation between Glu and Fru contents (r^2=0.96, P<0.001) in individual internodes and the correlation between Suc and hexose (Glu + Fru) content was negative (r^2=0.29, P<0.001), although the quantity was not high. No relationship was detected between Suc content and activities of NI and SS. SAI activity was positively correlated with hexose contents (R^2=0.54, P<0.025) and negatively correlated with Suc content (r^2=0.59, P<0.001). Although the correlation between SPS activity and Suc content was not significant (r^2=0.33, P<0.1), its difference with SAI (i.e. SPS-SAI, net enzyme activity) was more related to Suc content (r^2=0.68, P<0.005). These facts revealed that SAI takes a decisive role in regulating Suc accumulation in sweet sorghum stem, and SPS takes a synergy role.

Keywords: Correlationship, Enzyme activity, Soluble acid invertase, Sucrose accumulation, Sucrose phosphate sybthase, Sugar content, Sweet sorghum.
Abbreviations: BSA-albumin from bovine serum, DNS-3,5-dinitrosalicylic acid, DTT-dithiothreitol, EDTA-ethylenediaminetetraacetic acid, Fru-6-P-fructose-6-phosphate, Fru-fructose, fw-fresh weight, Glu-glucose, Glu-6-P-glucose-6-phosphate, INV-invertase, NI-neutral invertase, SAI-soluble acid invertase, SPS-sucrose phosphate synthase, SS-sucrose synthase, Suc-sucrose, UDP-Glu- uridine diphosphate glucose.

Introduction

Sweet sorghum (*Sorghum bicolor* L. Moench) is a multi-purpose crop with great yield potential. It is used as an alternative feedstock for ethanol production due to its considerable amounts of accumulated sugars, mainly sucrose, in its stem (Ali et al., 2007; Rooney et al., 2007). Calviño and Messing (2011) proposed sweet sorghum as a model among bioenergy crops.

Sugar content is one of the most important traits of sweet sorghum. There are large variations in sugar contents of stem among sweet sorghum varieties. For example, the Brix in two hundred and six cultivars ranged from 8.0% to 19.1% (Zhao et al., 2008). Sucrose (Suc) is the predominant sugar, and the total Suc content is lowest at the boot stage and highest at the soft dough stage (Lingle, 1987). The Suc contents at different internodal region of sorghum stem showed that the sugar content had an up-down tendency with the internodal number increasing (from top to base) (Subramanian et al., 1987). Hoffmann-Thoma et al. (1996) also reported that the uppermost internodes represent strong 'utilization sinks' until final development of the peduncle during anthesis. However, at the physiological maturity stage, how sugar is accumulated in different internodes is not clear.

Suc in the stem can be catabolized by either SS or INV, which is located in apoplast and vacuoles of young internodes. SAI is bound to the cell wall and vacuole in tissues of all ages, and NI exists low in the cytoplasm of young tissues and high in mature tissues (GutiOrrez-Miceli et al., 2002). After entering the parenchyma cells, the hexoses may be metabolized into Callose for plugging or resynthesized into Suc by SPS (Koch, 2004). Sucrose synthase (SS) may also be involved in Suc synthesis, but the equilibrium is usually in the direction of degradation (Goldner et al., 1991).

Invertase (INV), sucrose phosphate synthase (SPS) and sucrose synthase (SS) have been suggested as important regulators of Suc accumulation in storage parenchyma in many plants (Zhu et al., 1997; Miron and Schaffer, 1991; Klann et al., 1993; Davies and Robinson, 1996; Hubbard et al., 1989). In sweet sorghum, Lingle (1987) used only one cultivar 'Rio', where low activities of SAI and SS were associated with a decline in internode elongation and an increase in Suc accumulation in sweet sorghum stems. Onset of Suc accumulation coincided with the extension of the panicle into the sheath of the flag leaf, and was accompanied by a decline in both SAI and SS in stem tissue. The extent of Suc accumulation in stem varies among cultivars, and has been previously related to decline in activities of soluble, Suc-degrading enzymes in sweet sorghum stems. A decline to low levels of invertase and SS might be a prerequisite for Suc accumulation at physiological maturity of the plant (Tarpley et al., 1994). Hoffmann-Thoma et al. (1996) reported a strong positive correlation (r>0.94) between hexose content and the

Table 1. The sweet sorghum accessions used in this study.

No.	Accessions	Origin	Growth period	No.	Accessions	Origin	Growth period
1	Cowley	USA	161	6	Theis	USA	160
2	Smith	USA	149	7	MN-2794	USA	131
3	TianXuan 20	China	136	8	Honey	Australia	142
4	BJK236	China	142	9	M-81E	USA	162
5	Ramada	USA	142	10	MN-2747	USA	109

activity of SS, but invertase was not present. Recently, Qazi et al. (2012) pointed out that contribution of varieties, stage, and internode position was significant for the variation in sugar content.

Obviously none of studies on enzymatic basis of sweet sorghum Suc accumulation can be considered conclusive, since data were obtained from different plant organs, among plants of different ages exposed to divergent environments, and among different plant genotypes. In the present study, we used stems of the physiological maturity of ten sweet sorghum cultivars as experimental materials, and investigated Fru, Glu and Suc contents and sugar metabolism enzymes activities, NI, SAI, SPS and SS. The objective of our study was also to identify key enzymes that regulate sugar accumulation in sweet sorghum stems.

Results

Sugar content of stems at the physiological maturity

At the physiological maturity, the contents of Fru, Glu and Suc all varied in different internodes and genotypes. All cultivars, except MN-2794, showed a bottom-up tendency for Fru and Glu contents from internode no.1 through 11 (Fig 1.A, B). Suc had a different accumulation tendency for different cultivars (Fig 1.C). Other cultivars showed an up-down tendency except Cowley, Tianxuan20, MN-2794 and MN-2747. The highest Suc content and the lowest Fru and Glu contents were found in internode no.3 through 7 for most cultivars. There was a significant correlation between Glu and Fru contents (r^2=0.96, P<0.001) in individual internodes (Fig 2.). There was a significant negative correlation between Suc and hexose (Fru+Glu) contents (r^2=0.29, p<0.001).

Sugar ratio of stems at the physiological maturity

The contents of Fru, Glu, Suc and total sugar of whole-stem were illustrated in Fig 3. Suc was predominant in all cultivars at the physiological maturity. The mean ratio of Suc to total sugar content was 85.2%, ranging from 67.9% in Theis to 96.3% in Smith-1. The mean contents of Fru, Glu, Suc and total sugar were very different among ten cultivars. The Fru content ranged from 1.77 mg/g fw in Smith-1 to 12.43 mg/g fw in Theis. The Glu content ranged from 1.44 mg/g fw in Smith-1 to 14.63 mg/g fw in TheisGlu. For most cultivars, Glu content was slightly higher than Fru. The Suc content ranged from 35.9 mg/g fw in MN-2747 to 95.9 mg/g fw in Cowley.

Enzyme activities of stems at the physiological maturity

SAI activities varied by individual internodes and genotypes, with highest activities in the internode no. 1 of Ramada, lowest in the internode no. 11 of Cowley, and intermediate in other cultivars (Fig 4.B). The high SAI activity of young internodes of the Suc-accumulated cultivars declined rapidly, reaching low levels in internode no. 3 or 5, as the internodes matured and Suc was accumulated. The activities of NI (Fig 4.A), SPS (Fig 4.C), and SS (Fig 4.D) were not significantly correlated with internode age, but showed a decreased tendency during internode maturation in some cultivars. For the whole-stem enzyme activity, SPS and SS were higher than NI and SAI, and activities of NI and SAI were very approximate (Fig 5.). NI ranged from 3.87 μmol h/g fw in Ramada to 7.99 μmol/h/g fw in BJK236. SAI ranged from 2.86 μmol/h/g fw in Cowley to 6.57 μmol/h/g fw in M-81E. The SPS ranged from 8.81 μmol/h/g fw for MN-2794 to 13.67 μmol/h/g fw in BJK236. SS ranged from 8.90 μmol/h/g fw for Cowley to 19.67 μmol/h/g fw in MN-2794.

The correlation between enzyme activities and sugar content

The activities of SAI, NI, SPS, and SS measured on individual internodes were averaged to produce a mean value for the whole stem of each genotype due to the time lag between a shift of enzyme activities and its effect on sugar accumulation, and because the whole-stem analysis is a common agronomic practice. This value was compared with the mean Fru+Glu and Suc contents of the whole stem of each genotype. For the mean internode Fru+Glu content and mean enzyme activity, there was no significant correlation between Fru+Glu content and activities of NI, SPS, or SS. However, the mean Fru+Glu content was positively correlated (r^2 = 0.54, P<0.003) with the mean SAI activity (Fig 6.B). Although there was a significant correlation between mean SAI activity and Fru+Glu contents in individual internodes, the correlation between enzyme activity and Fru+Glu content was not significant (r^2 = 0.23), when activity was expressed as the difference between activities of SPS and SAI (Fig 6.E).

For the mean internode Suc content and mean enzyme activity, there was no significant correlation between Suc content and activities of NI, SPS, or SS. However, the mean Suc content was positively correlated (r^2 = 0.59, P<0.001) with the mean SAI activity (Fig 7.B). Although there was no significant correlation between mean SPS activity and Suc content in individual internodes, the correlation between enzyme activity and Suc content was greater and positive (r^2 = 0.68, P<0.005), when activity was expressed as the difference between activities of SPS and SAI (Fig 7.E).

Discussion

Sugar content and accumulation

In the present study, ten cultivars of sweet sorghum have been investigated with respect to sugar accumulation pattern and Suc metabolism in stem parenchyma. For the whole stem of all ten cultivars, Suc was the dominant nonstructural carbohydrates at the physiological maturity. The cultivars differed in their Suc accumulation pattern, with respect to both its temporal sequence and the Suc amount in the stem. Hoffmann-Thoma et al. (1996) reported that Suc content of the sweet sorghum cultivar Keller could reach 650 mg/g dw at anthesis stage, but the hexoses (Glu and Fru) contents were only about 30 mg/g dw. For the Rio and SSV74 cultivars,

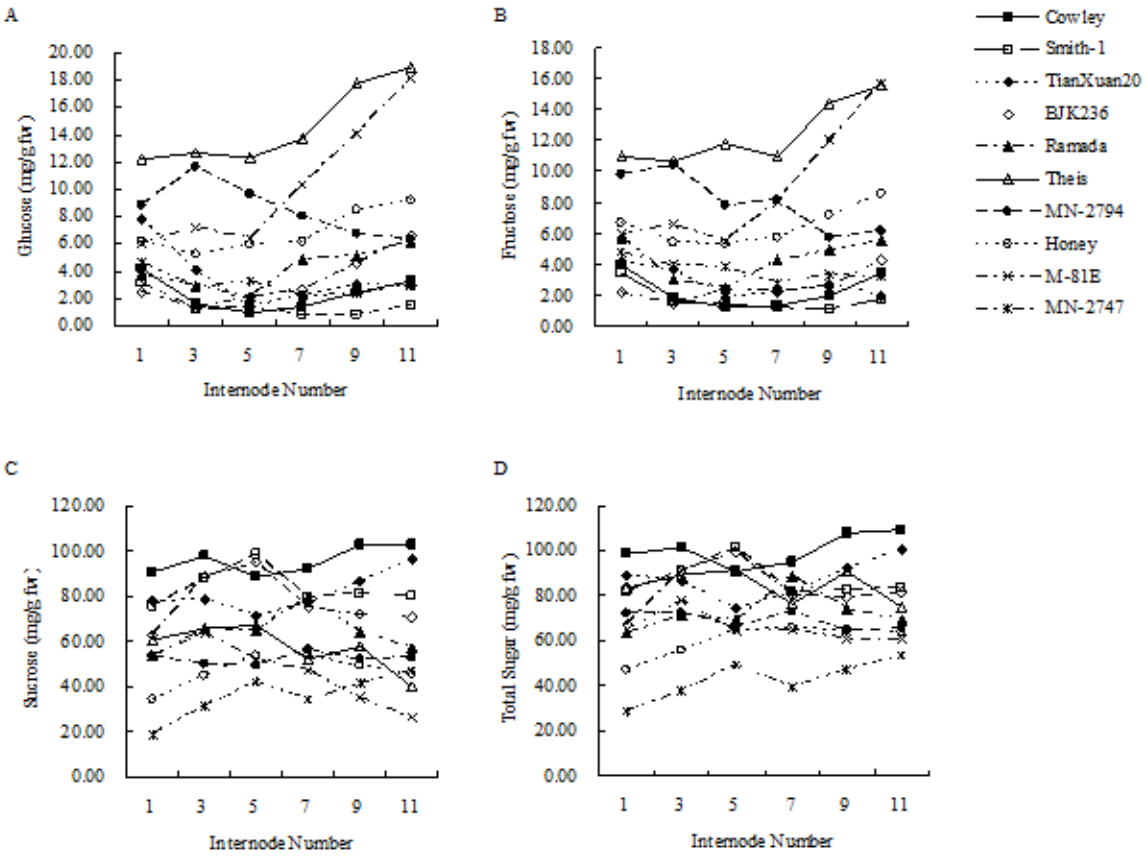

Fig 1. The mean Fru (A), Glu (B) and Suc (C) content in individual internodes of ten sweet sorghum cultivars at maturity. Internode numbers are designated down the stem as no.1 until 11. fw, Fresh weight.

there was a high ratio of Suc/hexoses at the physiological maturity stage (Lingle, 1987; Qazi et al., 2012). In general, hexoses favor cell division and expansion, whereas Suc favors differentiation and maturation (Wobus and Weber, 1999; Weschke et al., 2003; Borisjuk et al., 2003). In the maturing storage of most plants, division and expansion are very little. Therefore, in matured sweet sorghum stems, the high ratio of Suc/hexoses is used for differentiation and maturation.

For SSV74 and SPV1616 cultivars, significant contribution of variety, stage, and internode position was observed for the variation found in sugar content (Qazi et al., 2012). In this study, Cowley, Smith, TianXuan 20, BJK236 and Ramada Cultivars having a high Suc contents (mean 80.5 mg/g fw) had a low hexoses (Glu +Fru) contents (only means 5.7 mg/g fw). On the contrary, Theis, MN-2794, Honey, M-81E and MN-2747 cultivars had a low Suc contents (means 47.8 mg/g fw) with a high hexoses (Glu and Fru) contents (means 16.6 mg/g fw). In addition, the variation in sugar content is very significant in different internode numbers. Therefore, contribution of genotype and internode position is significant for the sugar content. However, for the sugar content variation in different internodes of one cultivar, the maturity stage is lower than the anthesis or heading stage. In maturity stage, the physical activity decreases, metabolism slows down with less demand for hexose. Suc in the upper internode is used to store more rather than broken down into hexose. Therefore, at the maturity stage, sugar content in different internodes tends to be more consistent.

The only known enzymatic paths of Suc cleavage in plants are catalyzed by INV (Suc + H_2O → Glu + Fru) and SS (Suc + UDP ←→ Fru + UDP-Glu) (Koch, 2004). There was a significant positive correlation between Glu and Fru contents (r^2=0.96, P<0.001) in individual internodes. This result showed that Glu (including UDPG) and Fru are produced on the same proportion then Suc might be the only resource of hexoses. During the radial transfer of Suc in ripening internodes of intact sorghum plant, much of the Suc is transferred intact (without hydrolysis and resynthesis) and primarily through a path that include an apoplasmic step (Tarpley and Vietor, 2007). If there is a re-synthesis pathway, the stem should be able to detect a considerable number of hexose, but in the stem, hexose content is very little. It reveals that the re-synthesis path may not exist in the sweet sorghum. Sorghum is closely related to sugarcane (Guimaraes et al., 1997), but the difference is the sugar metabolism.

Correlation between sugar contents and enzyme activities

The correlation coefficients of the relationship between sugar content and metabolism enzymes were much lower when calculated on the basis of individual internodes than that on a whole-stem basis (data not shown). This was consistent with the results for sugarcane (Zhu et al., 1997), where it was attributed to the time lag between a shift of enzyme activities and its effect on Suc accumulation. The whole-stem analysis is a common agronomic practice. The possible reason could be that the sugar content or enzyme activity was significantly correlated between nearby individuals or even different internodes existing far from each other not nearby (data not shown). It implies that a single internode is not an independent organ. Only with the mean value for each

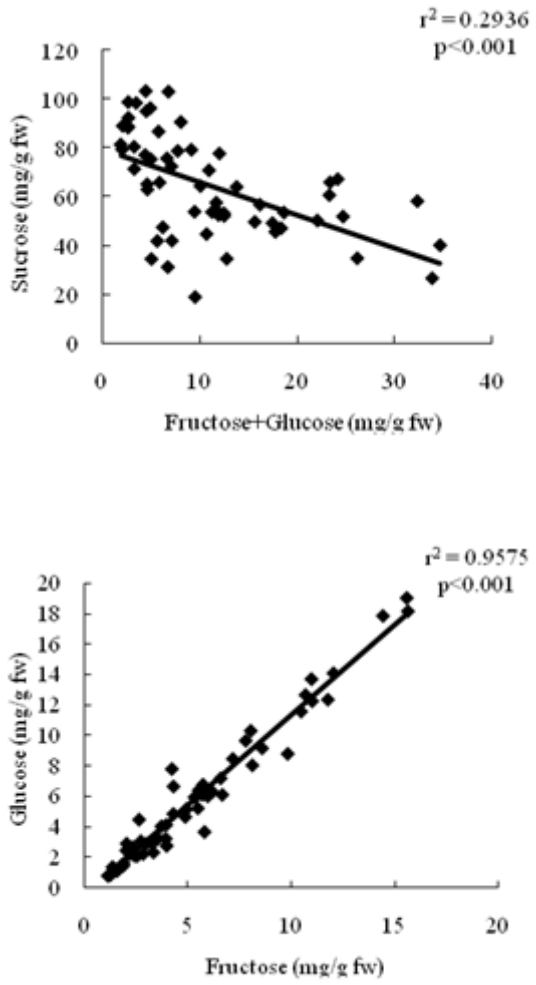

Fig 2. Relationship between Fru and Glu content in individual internodes of ten sweet sorghum cultivars stems. fw, fresh weight.

cultivar could be the real relationship between sugar content and related enzyme be revealed.

In matured sugarcane stem, activity of NI was very low and was not significantly correlated with Suc content (Zhu et al., 1997). For a sweet sorghum variety, Rio, activity of NI in stem was higher during the elongation period, but whether whole stem or different internodes, they were not significantly correlated with Suc content (Lingle, 1987). In the present study, NI and Suc contents also had no apparent correlation, indicating that the NI may not play a key role in the Suc accumulation. SAI is a very important regulatory enzyme regulating Suc accumulation directly in many plants (Zhu et al., 1997; Zrenner et al., 1996; Gao et al., 1999; Giapuinta, 1979; Yelle et al., 1998; Vizzotto et al., 1996; Darnel et al., 1994; Hubbard et al., 1991). In sugarcane, SAI activity is significantly correlated with Suc content ($r = -0.84$, $P < 0.002$) (Zhu et al., 1997). For sweet sorghum, only the lower SAI activity (< 33 μmoL/kg/S fw) can induce Suc accumulation. However, the correlation between SAI activity and Suc content was not significant, (r= -0.58 for the Rio variety (Lingle, 1987). The results of this study indicate that the correlation coefficient of the relation between SAI activity and Suc content can reach -0.71, higher than NI, SPS and SS. SAI is mainly located in the phloem vacuole, and Suc is also stored in the vacuole. In addition, most of the Suc is transported directly to the stem in matured sorghum, without steps of decomposition and re-synthesis (Tarpley and Vietor, 2007). It is also shown that Suc is likely to be transported into the vacuole firstly and then is decomposed mainly into hexose under the effect of SAI. In addition, at the physiological maturity, Suc was decomposed for synthesis of grain starch. Suc decomposition by SAI was likely to be the first step in this process, and results of our study also confirmed this.

The Suc content of sugarcane stem is regulated by SPS in many plants (McCollum et al., 1988; Fieuw and Willenbrind, 1987; MacRae et al., 1992; Komatsu et al., 1999; Dali et al., 1992). Although the correlation between SPS activity and Suc content is not significant in the stem of sugarcane, the SPS-SAI net activity was significantly correlated with Suc content. The correlation coefficient of this relationship could be 0.93 above (for SAI, only r = -0.72) (Zhu et al., 1997). It is also shown that Suc accumulation is collectively regulated by SPS synthesis and SAI decomposition. However, previous studies (Lingle, 1987; Hoffmann-Thoma et al., 1996) and this study all indicate that only SPS does not play a key role in Suc accumulation process in sweet sorghum stem. Tarpley and Vietor (2007) pointed out that Suc accumulation of sweet sorghum was different with sugarcane, without decomposition and re-synthesis steps. Therefore, Suc in the stem is likely to be transported directly, but not re-synthetized by the SPS. In addition, at the physiological maturity, rate of Suc synthesis by SPS slows down, and the role of the SPS will be smaller. So the Suc content in the stem mainly depends on the SAI activity, the SPS only play a supporting role.

Previous research showed that there was a very close relationship between SS activity and sugar accumulation in sweet Sorghum. For Rio (Lingle, 1987), Keller, Tracy and NK405 (Hoffmann-Thoma et al., 1996) varieties, the correlation coefficient of SS activity and hexose is very high. However, Chengappa et al. (1999) decreased SS activity of tomato young fruit by using antisense interference technology, while not affecting the accumulation of starch and sugar in the fruit. This study found that there was no significant correlation between SS activity and Suc content. SS can catalyze both synthesis and degradation of Suc (Elling, 1995). It was very difficult to determine whether the SS activity observed is directed towards the accumulation or the utilization of Suc.

Future work and outlook

The results of our study proved that SAI took a decisive role in regulating Suc accumulation, and SPS took a synergy role at the physiological maturity. This means that lower SAI and higher SPS enzyme activities can promote Suc accumulation. Recent study also indicates that transcriptional expression of SAI (INV3) gene was lower in sweet sorghum as compared to grain sorghum (Qazi et al., 2012). INV3 might be a key gene, regulating the Suc accumulation. However, transcriptional expression of two SPS (SPS2 and SPS3) was lower yet (Qazi et al., 2012). Regulation of SPS was more complicated. Therefore, further research is required to elucidate how these enzymes regulate the sugar metabolism during the sugar accumulation process, which will help eventually to understand the physiological mechanism of sugar accumulation in sweet sorghum stems. In addition, SAI and SPS genes of sweet sorghum have been cloned nearly, and an allelic variation associated with sugar content has been found in our lab (Liu, 2009). More allelic variation of SAI and SPS will be found in order to establish a high efficient double functional marker for sugar content.

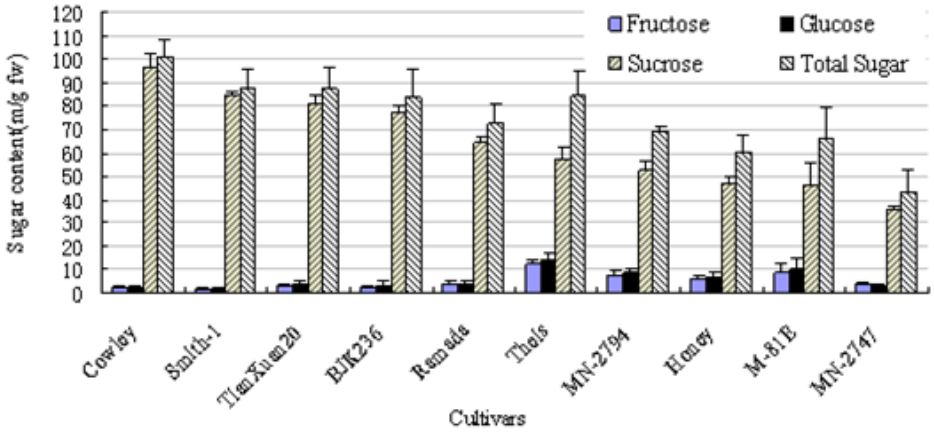

Fig 3. Mean sugar contents in stem fresh weight of the ten cultivars at maturity.

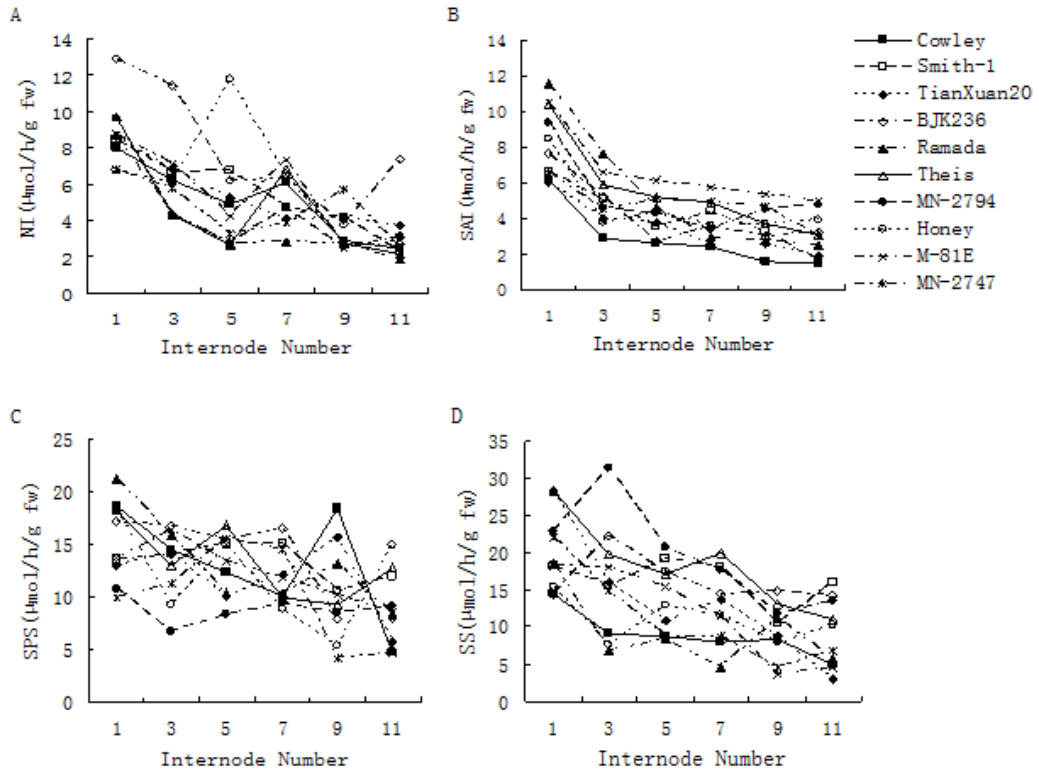

Fig 4. Activities of NI (A), SAI (B), SPS (C) and SS (D) in individual internodes of ten sweet sorghum cultivars. Internode numbers are designated down the stem as no.1 until 11. Fresh weight.

Materials and methods

Plant materials

Ten sweet sorghum cultivars (Table 1.) were grown in research field plots under standard production practices for water and fertilizer application at Changping Experimental Station of Institute of Crop Science, Chinese Academy of Agricultural Sciences in Beijing, China. Stems of the physiological maturity stage (Lingle, 1987) were harvested during September 2007 and stems were harvested in the morning to avoid effects of diurnal fluctuation. The inflorescence was removed and the leaf sheaths were peeled off. The internodes were numbered sequentially down the stem. The top internode was designated internode no.1, with lower internodes given sequentially increasing numbers. The stems were cut into individual internodes and weighed. The internode rind, consisting of a thick epidermis was removed and the remaining internode was cut into quarters lengthwise. Each quarter was chopped into small pieces and frozen in liquid nitrogen before stored in a -80°C freezer until used for sugar and enzyme assays.

Sugar analyses

Sugar assays were done on sub-samples from the same internode that was used for enzyme activity assays. Frozen tissues from each internode were divided into two portions. One portion was weighted and ground to a fine powder in liquid nitrogen in a chilled mortar. This powder was thawed and homogenized in 80% ethanol, then the tissue and extract solution were incubated at 80℃ for 2h. The supernatant was

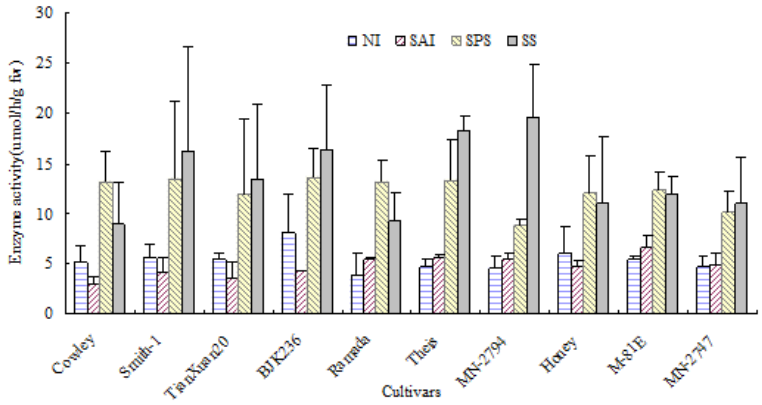

Fig 5. Mean enzyme activity based on stem fresh weight in ten cultivars at maturity.

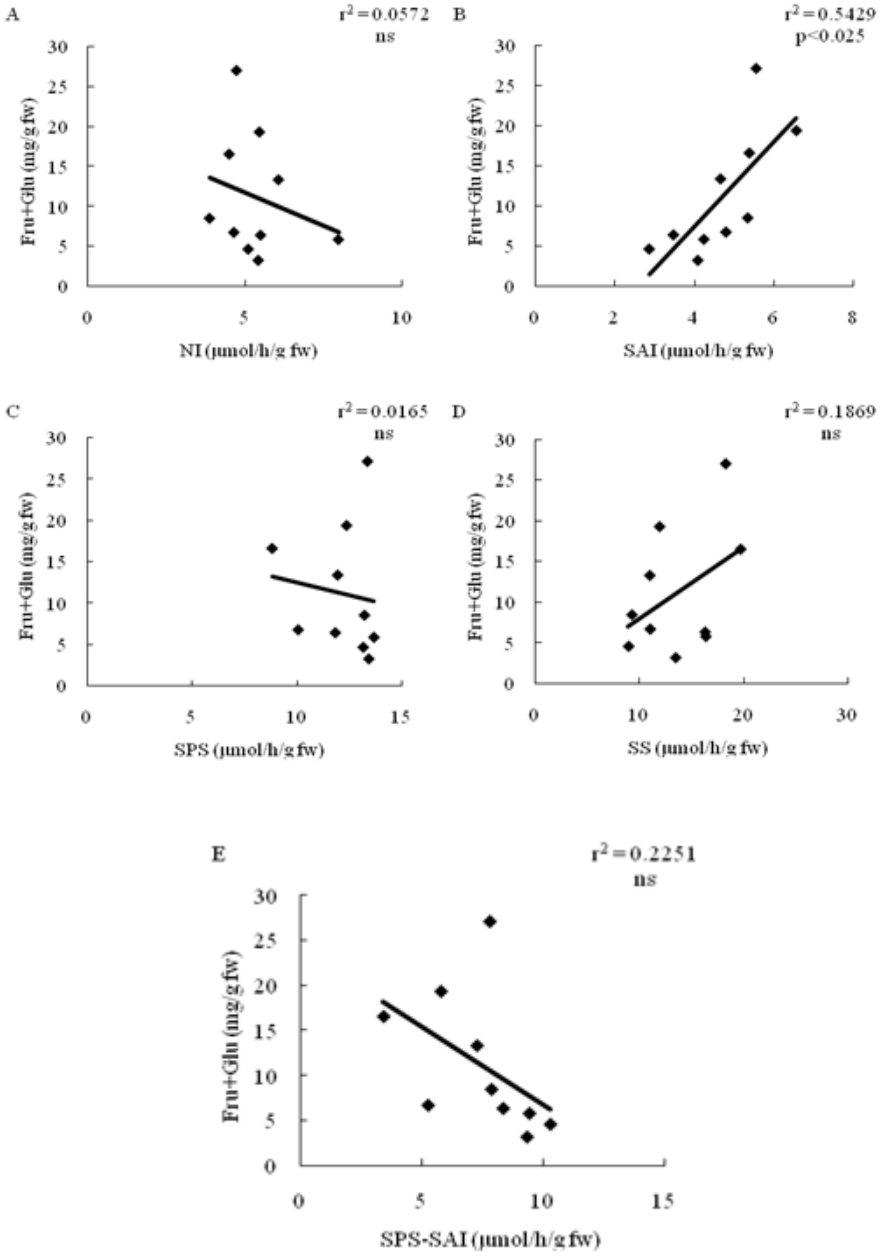

Fig 6. Relationship between mean internode Fru and Glu content and mean enzyme specific activities of NI (A), SAI (B), SPS (C), SS (D) and SPS-SAI (E) in whole stem of ten sweet sorghum cultivars. (fw, fresh weight).

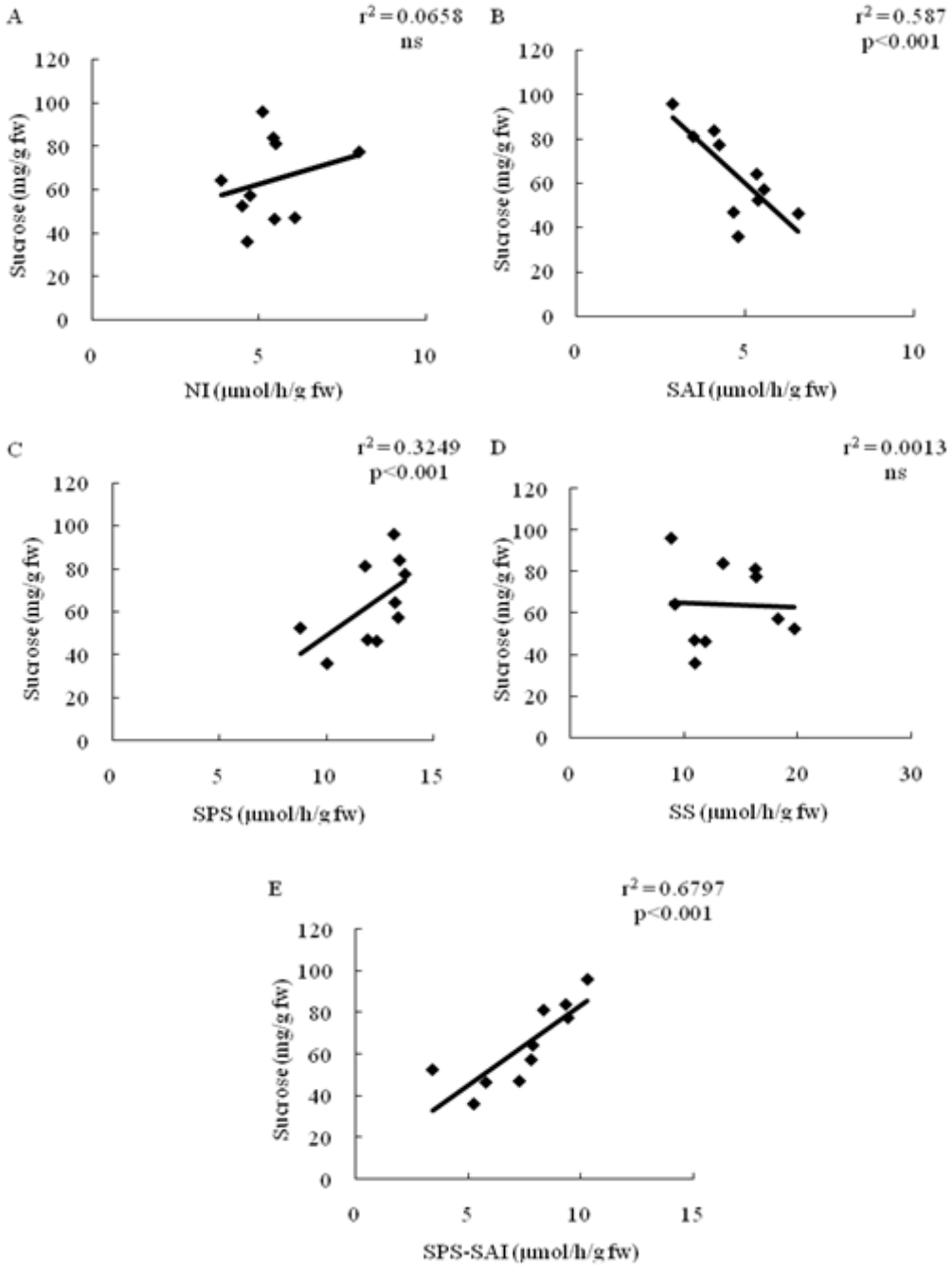

Fig 7. Relationship between mean internode Suc content and mean enzyme specific activities of NI (A), SAI (B), SPS (C), SS (D) and SPS-SAI (E) in whole stem of ten sweet sorghum cultivars. (fw, fresh weight).

removed and concentrated, and the residue was cleaned by 1 mL double-distilled water. The filtered, centrifuged extract was analyzed for sugar content by high performance liquid chromatography (HPLC, Shimadzu) using a CLC-NH2 column produced by Shimadzu company, temperature: 40℃，speed: 1.0mL/min，detector: RID-10A, mobile phase: Acetonitrile: water =70: 30, sample volume: 10μl，Data Processing System: Class-vp. The Suc, Glu, and Fru were identified and quantified by comparison with retention times and peak areas of external standards. The contents of sugars were calculated on each internode and expressed as micromoles per gram fresh weight. The sugar contents of the whole-stem were averaged from individual internode no. 1 to 11.

Enzyme extraction

Enzyme extraction was performed according to Zhu et al. (1997) with minor modification. Frozen tissue of an internode was weighed and ground to a fine powder in liquid nitrogen in a chilled mortar, then transferred to a 2 mL centrifuge tube. Extraction buffer was added into it before mixing thoroughly. The ratio of fresh weight and extraction buffer was 0.2 to 0.3 g/mL fw. The extraction buffer contained 50 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 0.05% (v/v) triton X-100, and 0.5mg/mL BSA. The mixture was filtered

with two layers of gauze and homogenate was recovered. The homogenate was centrifuged at 12,000g for 10 min at 4 °C. The supernatant was recovered and used for assay of SAI and NI. For SPS and SS, the supernatant was added $(NH4)_2 SO_4$ to 80% saturation, deposited for 30 min before centrifuged at 18,000g for 30 min at 4 °C. Supernatant was then removed, the desalted buffer (50mM Hepes, pH=7.5, 5mM Mgcl2, 2.5mM DTT, 0.5mg/mL BAS) was added to the tube, and the deposit was dissolved. The supernatant was desalted with D27 mm dialysis bag. Assays were conducted as soon as the extract was desalted.

Enzyme assays

SAI and NI activities were determined according to the procedure of Lowell et al. (1989) with minor modification. SAI activity at 37°C was started by adding 210 μL of desalted extract to 280 μL assay solution of 140 mM sodium acetate (pH 4.5) and 175 mM Suc. Reactions were incubated at 37°C for 30min and stopped at 30 min by boiling the reaction solution for 3 min. A 490μL DNS reagent was added to terminate the reaction, a boiling water bath for 5 min and A 520 value was measured after cooling. The assay solution without Suc was the control. Reaction in NI activity assay was similar to that for SAI, except that the reaction was conducted at pH 7.5. SPS and SS activity assays were conducted at 37°C and at the pH 7.5 level (Hubbard et al., 1989). Desalted enzyme extract of 35 μL was added to a 35 μL assay solution. The SPS assay solution contained 100 mM Hepes (pH 7.5), 20 mM Glu-6-P, 4 mM Fru-6-P, 3 Mm UDP-Glu, 5 mM $MgCI_2$, and 1mM EDTA. The SS activity assay solution contained 50 mM Hepes (pH 7.5), 15 mM $MgCl_2$, 25 mM Fru, and 25 mM UDP-Glu. Reactions were incubated at 37°C for 60 min and then stopped by boiling it for 3 min. Suc produced in these reactions was determined using the anthrone method (Handel, 1968). The assay solution without UDP-Glu was treated as control. The enzyme activities of the whole-stem were averaged from individual internode numbers 1 to 11. The data presented are the mean values from three independent extractions.

Conclusion

Sweet sorghum stems of different cultivars revealed different patterns of sugar accumulation and Suc was predominant at the physiological maturity. The Glu and Fru are mainly produced by decomposition of Suc. SAI takes a decisive role and SPS takes a synergy role in regulating Suc accumulation at the physiological maturity.

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