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Metabolic Changes Associated with the Sink-Source Transition During Sprouting of the Axillary Buds on the Sugarcane Culm

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Abstract Sucrose, glucose and fructose concentrations, and sucrolytic enzyme activities were measured in the developing shoots and internodes of sprouting sugarcane setts (*Saccharum* spp, variety N19). The most striking change during the sink-source transition of the internode and germination of the axillary bud is a more than five-fold induction of cell wall invertase in the germinating bud. In contrast, soluble acid invertase is the main sucrose hydrolytic activity induced in the internodal tissue. A cycle of breakdown and synthesis of sucrose was evident in both the internodes and the shoots. During shoot establishment, the sucrose content decreased and the hexose content increased in the internodal tissues while both sucrose and hexoses continuously accumulated in the shoots. Over the sprouting period internode, dry mass was reduced by 25 and 30 % in plants incubated in a dark/light cycle or total darkness, respectively. Sucrose accounted for 90 % of the dry mass loss. The most significant changes in SuSy activity are in the synthesis direction in the shoots resulting in a decrease in the breakdown/synthesis ratio. In contrast the SuSy activity in the internodal tissue decrease and more so in the synthesis activity resulting in an increase in the breakdown to synthesis ratio.

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Abbreviations

CWI	Cell wall invertase (EC. 3.2.1.26)
NI	Neutral invertase (EC 3.2.1.26)
SAI	Soluble acid invertase (EC. 3.2.1.26)
SuSy	Sucrose synthase (EC 2.4.1.13)
Bq	Becquerel
V_{max}	Maximum catalytic activity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
EDTA	Ethylenediaminetetraacetic acid
NAD	β-nicotinamide adenine dinucleotide
HK	Hexokinase
G6PDH	Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
ATP	Adenosine 5′-triphosphate
PGI	Phosphoglucose isomerase (EC 5.3.1.9)
UDP	Uridine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
PVPP	Polyvinyl polypyrrolidone

Introduction

The regulatory mechanisms which determine the pattern of carbon allocation between the different plant organs and regulate source-sink transitions are of prime importance in determining crop establishment and performance (Roitsch 1999). Sugars play an important role in the regulation of carbon partitioning between source and sink (Herbers and Sonnewald 1998; Roitsch 1999).

It is widely recognised that sugars not only function as substrate to sustain the heterotrophic growth of sink tissues, but are also important signalling molecules that regulate both source and sink metabolism (Herbers and Sonnewald 1998; Koch 1996).

The sugarcane culm is a strong sink and accumulate more than 55 % of its dry weight in the form of sucrose (for review Moore 1995). Sucrose transport from the leaves to the sinks (culm, roots, etc.) is driven by mass flow and directly dependent on the sucrose gradient within the phloem (for review Grof et al. 2013). Obviously, this gradient depends on conversion or offloading of sucrose at the sink and production or loading at the source. In maturing sugarcane culms, sucrose transport follows a predominantly symplastic pathway when moving from the phloem into the storage parenchyma cells (Lingle 1989; Patrick et al. 2013; Rae et al. 2005).

However, when the crop is propagated through cuttings from the culm the internodal tissue undergoes a transition from sink to source providing the carbon and other nutrients to the sprouting axillary bud, shoot roots and sett roots (Glasziou 1958 and reference therein; van Dillewijn 1952). The germination (axillary bud growth) of sugarcane sets and the subsequent growth of the new shoot is of great economic importance.

Despite several previous studies the biochemical changes and regulation of this switch from sink to source in the internodes and development of a new sink in the shoot, is still not well understood. Meristematic sinks are usually source-limited and storage sinks are sink-limited (Smith and Stitt 2007). Both invertase and sucrose synthase (SuSy) activity are associated with sink strength (Bihmidine et al. 2013 and references therein). Cell wall invertases (CWIs) catalyse the only irreversible step in the apoplastic unloading pathway, which is crucially important in generating the required sucrose gradient to export sucrose through the phloem. CWIs are regulated by a variety of different stimuli, including glucose, phytohormones, and stress, and thus may play a central role in coordinating sink capacity with different signal responses (Roitsch et al. 2003). The gene expression levels of sugar transporters and CWIs appear to be closely coordinated (Bihmidine et al. 2013; Gupta and Kaur 2005; Koch 2004; Wang et al. 2013). The so-called sink/source transition is accompanied by the expression of sucrose symporters in the phloem of all vascular bundle types when they commence their newly acquired functions of phloem loading and axial transport.

Germination and heterotrophic growth of axillary buds and side shoots has been associated with decreasing sucrose levels in sugarcane one-eye setts (suckers) (Bull and Glasziou 1963; Echeverria 1998; Glasziou 1958; O'Neill et al. 2012). When the axillary bud is removed no sucrose mobilisation is evident in the internodes (O'Neill et al. 2012).

According to Glasziou (1958) a first-order reaction (possibly invertase) rather than diffusion is the rate-limiting step in sucrose mobilisation in germinating setts. However, later work suggests that invertase hydrolysis of sucrose in the setts is not required for the mobilisation of sucrose to support growth of the young seedling (Echeverria 1998; O'Neill et al. 2013). O'Neill and co-workers (2013) suggested that it is rather the invertase activity in the developing shoot that plays a crucial role in controlling the rate of sucrose mobilisation in the internode. The levels of sucrose and the main reducing sugars, glucose and fructose, decrease in the internodal tissue (O'Neill et al. 2012). This either implies that sucrose is not hydrolysed, or that the resulting reducing sugars are rapidly phosphorylated and used in metabolism, or exported from the internodal tissue to be utilised by the growing shoot. In a subsequent paper it was shown that invertase activities remain high in the internodal tissue during the time where the reducing sugar levels decrease in the internode (O'Neill et al. 2013). These authors proposed that the movement of sucrose from the internodal parenchyma cells into the phloem and to the sprouting bud occurs down a sucrose gradient and that this coincide with the down regulation of sucrose synthesis and respiratory metabolism in the internodes (O'Neill et al. 2013).

There still remain two neglected aspects in all previous reports on carbon partitioning and regulation of metabolism during sprouting of axillary buds and the transition of the internode form a sink to a source tissue. Firstly, despite the suggestion that the main point of regulation for carbon mobilisation might reside in the invertases in the bud (Glasziou 1958; O'Neill et al. 2013) there are no reports on the metabolic changes in the sprouting bud. Secondly, there is no data on the respiratory carbon loss during the sink/source transition. Based on changes in the activity of some of the glycolytic and sucrose synthesis enzymes it was proposed (O'Neill et al. 2013) that respiration and the cycle of synthesis and breakdown of sucrose (Bindon and Botha 2002) decreases in the internodal tissue as the internode assume a source function.

Here we report that there is a strong induction of invertase activity in both the internodal tissue and germinating bud. In the internodal tissue only SAI and CWI are induced and also represent the great majority of invertase activity, which at least in part can explain the observed changes in glucose and fructose levels in the internode as it transition from a sink to source tissue. In contrast, all three invertases are induced in the germinating bud where CWI and NI activity dominate. In addition we show that as much as 40 % of the mobilised carbon from the internodes is lost to respiration during the development of the roots and shoot.

Results

Redistribution of Carbon from the Internode During Germination

Changes in the dry mass of the internodes, shoots and roots were determined during the 21-day incubation period. Two different light regimes were used to alter the sink strength of the growing shoot. In constant darkness all the carbon form growth must be proved from the internodal tissue (source). In the dark/light regime the young leaves start to green after 14 days and thus becomes at least in part less dependent on reduced carbon from the internodes.

The loss in total dry mass was ascribed to respiratory carbon loss. Shoots are a stronger sink than roots, both in germinating setts incubated in total darkness and a dark/light cycle (Table 1). In both treatments, approximately 43 % (42 mg of 97 mg) of the carbon lost from the internode can be accounted for in biomass yield in the shoots, while only 31 % (31 mg of 97 mg) is allocated to the roots over the first 7 days. This disparate distribution of carbon between the different sinks becomes even more pronounced after 21 days and in addition the allocation pattern between the dark and dark/light cycle treatments also changes (Table 1). During the first seven days of incubation, 25 % (24 mg of 97 mg) of the mobilised carbon is lost to respiration and these increases to approximately 48 % (292 mg of 603 mg) in the dark and 35 % (197 mg of 346 mg) in the dark/light cycle treatments after 21 days (Table 1). The respiratory loss of mobilised carbon in the setts incubated in a light/dark cycle is significantly reduced after 14 days (44 to 35 %) and is lower than the respiratory losses in the dark incubated setts after 21 days (35 vs. 48 %). These rates of biomass accumulation allow for the calculation of carbon flux between the internode and the new shoot or root tissues. The maximum rate of biomass accumulation varies between 14 and 35 μg min^{-1} between days 7 and 14 in the tissue that was incubated in continuous darkness.

Sugar Content in the Shoots and Internodes

The soluble sugar concentrations changed throughout the sprouting period in distinctly different patterns in the internodal and shoots tissue (Fig. 1). The sucrose content of the internodes on the day of planting was 700 mg g^{-1} dry weight and this decreased by approximately 40 % (700 to 436 mg g^{-1}) in total darkness and 30 % (700 to 503 mg g^{-1}) in a dark/light cycle after 21 days (Fig. 1a). Internodal hexose content remained constant during the first 7 days and increased significantly thereafter in both treatments. After 21 days, the hexose content of the internodal tissue incubated in total darkness was significantly higher than that of the dark/light cycle (Fig. 1b & c).

In the shoots, the sucrose content remained constant for the first 7 days and then increased. The sucrose content in the plants incubated in dark/light cycle was significantly higher than that of the plants kept in continuous darkness (Fig. 1d). The hexose content started very low at the day of planting (<20 mg g^{-1} dry weight) and increased sharply (threefold) during the first 7 days and remained constant thereafter (Fig. 1e & f).

Sucrose accounted for 64 % (first 7 days), 85 % (days 7 to 14) and 91 % (days 14 to 21) of the carbon loss from the internode. The decrease in the internode dry mass strongly correlates with changes in sucrose content in both the plants incubated in total darkness ($r^2 = 0.81$, $P < 0.05$) and in the dark/light cycle ($r^2 = 0.83$, $P < 0.05$) throughout the 21 days of shoot establishment.

Invertase Activities

The activity of invertases during the shooting period was determined in both the shoots and the internodal tissues. Distinctly different patterns of changes in the invertase activities are evident between the internodal and shoot tissues (Fig. 2a and b).

In the internodes, the specific activity of SAI and CWI increased throughout the 21 days of the sprouting period regardless of the light treatment (Fig. 2a). There was almost a

Table 1 Allocation of mobilised carbon (mg) from the internode to the developing shoot, roots and respiration. Values are the mean of three replicates ± SE. Values in brackets represent the percentage of carbon allocated to the specific sink

Time	Dark			Dark/Light		
(days)	Shoot mg	Roots	Respiration[a]	Shoot mg	Roots	Respiration[a]
7	42.1 (43.2) ± 1.4	31.2 (32.0) ± 3.2	24.2 (24.8) ± 2.3	42.1 (43.2) ± 1.4	31.2 (32.0) ± 3.2	24.2 (24.8) ± 2.3
14	350.3 (44.1) ± 11.6	95.1 (12.1) ± 19.3	343.3 (43.5) ± 21.6	280.4 (41.6) ± 20.3	99.8 (14.8) ± 18.8	294.6 (43.7) ± 39.0
21	231.2 (38.3) ± 7.8	80.3 (13.3) ± 12.7	292.2 (48.4) ± 32.0	273.2 (47.8) ± 3.1	101.2 (17.7) ± 12.0	197.2 (34.5) ± 18.8

[a] Calculated as the loss in mass from the internode that cannot be accounted for in the increase in shoot and root mass

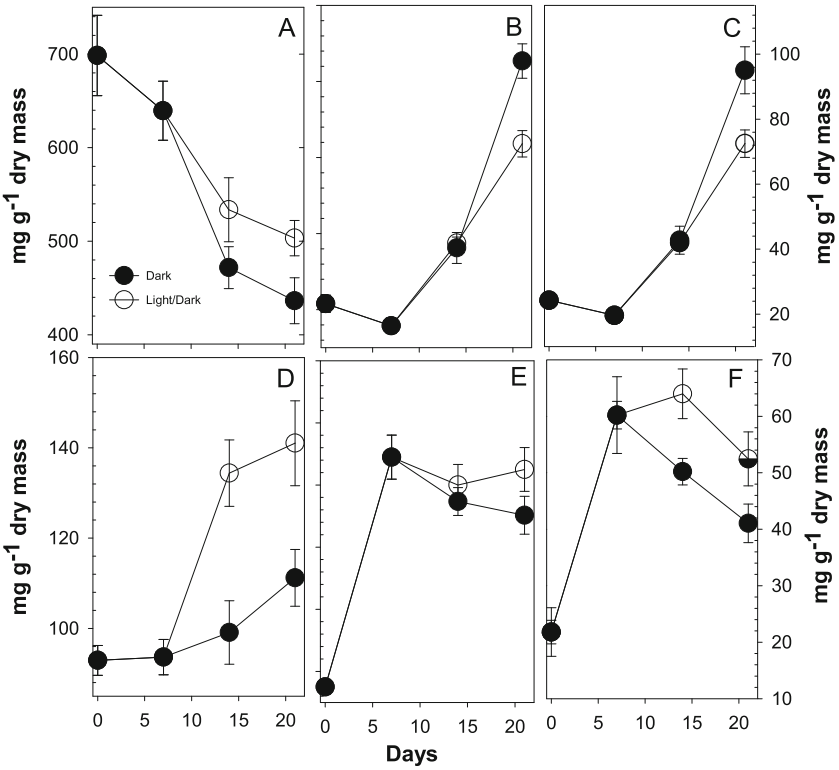

Fig. 1 Sugar content in the internodes (**a**, **b**, **c**) and shoots (**d**, **e**, **f**). Sucrose content (**a**, **d**), glucose (**b**, **e**) and fructose content (**c**, **f**). *Open symbols* are for plants incubated in a dark/light cycle and the *filled symbols* represent plants kept in total darkness. Each data point represents the mean values of three replicates ± standard deviation. *Lower case letters* denote statistically homogenous groups at each time point while *symbol* (*) denote statistically homogenous groups in a component over time ($p < 0.05$)

fivefold increase in SAI activity during the 21-day incubation period. In contrast, NI's specific activity increased slightly during the first 7 days and remained constant thereafter in internodes incubated in total darkness, while in those kept in the dark/light cycle, the activity decreased back to the initial level at the start of incubation (Fig. 2a).

In the shoots, all three invertase activities increased at least fourfold during the incubation period (Fig. 2b). The most striking increase was in CWI activity, which increased sixfold during the first 7 days of incubation and continued to increase to a level tenfold higher than at the start time. While SAI accounts for the dominant invertase activity in the internodes, it is the lowest of the invertase activities in the shoots (Fig. 2a & b).

Activity of Sucrose Synthase During Shoot Establishment

SuSy activity was measured in both the breakdown and synthesis directions in both the internodes and shoots (Fig. 3). In the internodes, SuSy activity, especially in the breakdown

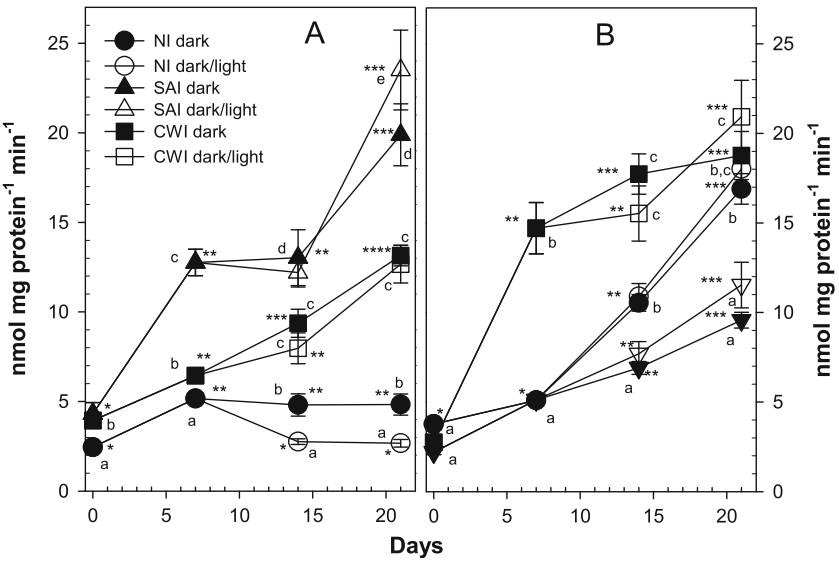

Fig. 2 Soluble acid invertase (SAI), neutral invertase (NI) and cell wall invertase (CWI) in the internodes (**a**) and shoots (**b**). *Open symbols* are for plants incubated in dark/light cycle and the *filled symbols* represent plants kept in total darkness. Each data point represents the mean values of three replicates ± standard deviation. *Lower case letters* denote statistically homogenous groups at each time point while *symbol* (*) denote statistically homogenous groups in a component over time ($p < 0.05$)

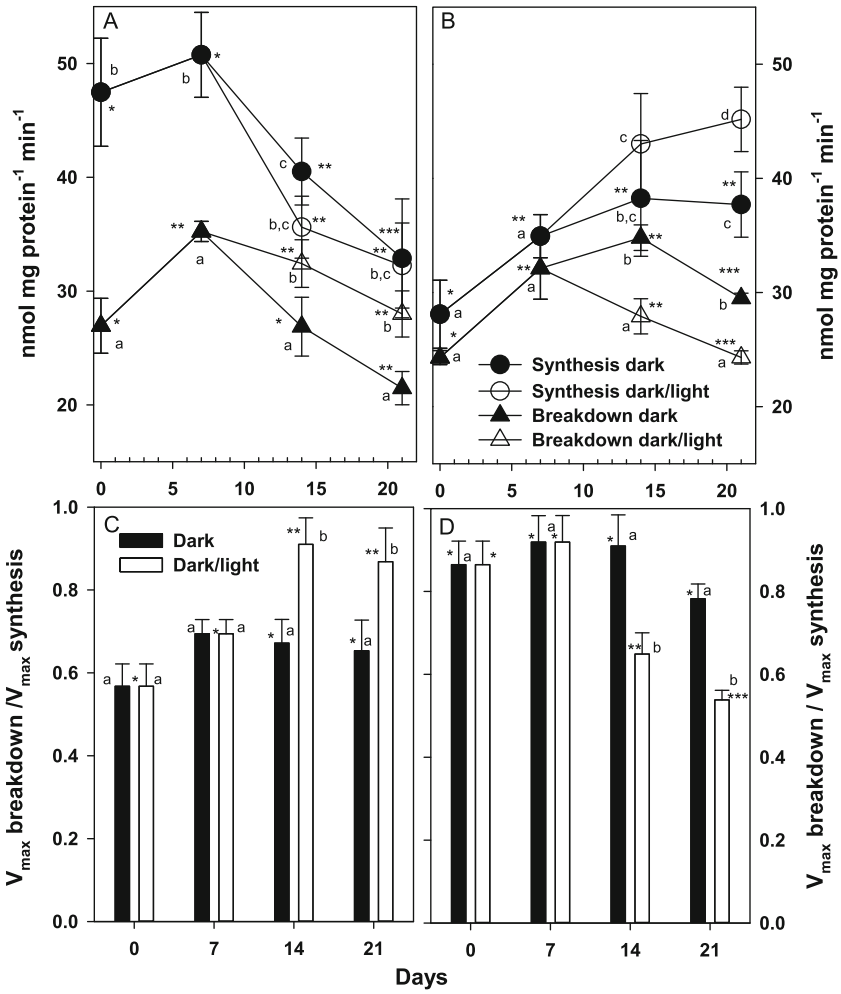

Fig. 3 Sucrose synthase activity in internodes (**a**) and shoots (**b**). Activity was measured in the synthesis (*circles*) and breakdown (*triangles*) direction. *Open symbols* are for plants incubated in dark/light cycle and the *filled symbols* represent plants kept in total darkness. Each data point represents the mean values of three replicates ± standard deviation. *Lower case letters* denote statistically homogenous groups at each time point while *symbol* (*) denote statistically homogenous groups in a component over time ($p < 0.05$)

direction, increased only during the first 7 days, and decreased thereafter to a level lower than activity at the start of incubation (Fig. 3a). There was a significant difference in SuSy breakdown activity in both tissue types and for both light treatments after 14 days, while this difference for the synthesis activity was only noticeable in the shoots (Fig. 3a & b). The ratio between the maximum SuSy activity (V_{max}) in the breakdown vs. synthesis directions remained stable for the dark treatment, but increased and decreased respectively in the internodal and shoot tissues of the dark/light cycle over the 21-day incubation period (Fig. 3c & d. This ratio is indicative of the expression of different isoforms of SuSy (Schafer et al. 2005).

Radiolabeling of Sugars

To characterise the movement of sugars between the internode and shoot and the metabolic fate of sucrose and glucose, radiolabelled sucrose or glucose were injected into the internodal tissue. Most of the radiolabel remained in the internode during the 3 h incubation period following the injection of 14C-glucose or sucrose. The labelling period was not extended beyond 3 h as the results demonstrated that there was significant redistribution of radiolabel between the different cellular constituents over this period. The percentage label distribution was calculated using the total count of the H_2O-soluble components. The other H_2O-soluble components represented the difference between the total H_2O-soluble components and the label in sucrose and the hexoses (Table 2). Within the internode, less than 68 % of the label remained in sucrose, 11.24 % was recovered in the hexose pool and 4.45 % in other H_2O-soluble components after 3 h. With labelled glucose 74.1 % of the recovered label was still in the hexose pool; 15 % was incorporated into the sucrose pool and 2.14 % in other H_2O-soluble components after 3 h. The most striking feature of the labelling work in the internodal tissues was that label readily appear in the other sugars after supplying either ^{14}C-sucrose or ^{14}C-glucose (Table 2).

Irrespective of the fact that the label was supplied to the internodes in the form of sucrose or glucose, most of the

Table 2 Percentage label allocated to the H_2O-soluble component 1 and 3 h after injection of [U-^{14}C]-sucrose and [U-^{14}C]-glucose. Tissue samples were from the internode (Internode) and from 21 day old shoots (Top and Bottom). Values are the mean of three replicates ± SE. Lower case letters denote statistically homogenous groups within tissue types, cellular component and over time with each of the label substrates. The critical values for comparison ($P < 0.05$) are 3.4451 for sucrose labelling and 7.288 for glucose labelling, respectively

^{14}C Source	Time	Component	Radiolabel distribution (%)		
			Tissue		
			Internode	Shoot	
				Bottom	Top
Sucrose	1 h	Sucrose	86.32 a ± 7.70	2.70 efg ± 0.43	1.20 fg ± 0.22
	3 h		67.49 b ± 4.45	7.48 d ± 0.47	4.95 de ± 0.26
	1 h	Hexose	7.16 c ± 1.12	0.23 g ± 0.03	0.10 g ± 0.01
	3 h		11.24 d ± 1.51	0.77 g ± 0.05	0.27 g ± 0.07
	1 h	Other	1.46 g ± 0.34	0.76 fg ± 0.03	0.06 fg ± 0.01
	3 h		4.45 def ± 0.72	3.37 g ± 0.76	1.14 efg ± 0.08
Glucose	1 h	Sucrose	6.06 d ± 0.46	1.20 d ± 0.09	0.40 d ± 0.03
	3 h		15.00 c ± 1.01	3.17 d ± 0.18	0.75 d ± 0.17
	1 h	Hexose	88.23 a ± 9.53	0.96 d ± 0.09	0.27 d ± 0.06
	3 h		74.10 b ± 9.06	2.28 d ± 0.50	0.23 d ± 0.08
	1 h	Other	1.70 d ± 0.22	1.06 d ± 0.40	0.12 d ± 0.02
	3 h		2.14 d ± 0.33	0.91 d ± 0.19	1.20 d ± 0.27

radiolabel in the shoots was recovered in sucrose (Fig. 4). When ^{14}C-sucrose was supplied to the internode, the labelled sucrose in the top half of the shoot was eight fold higher than labelled glucose after 1 h and 18 times higher after 3 h (Fig. 4a & b).

When ^{14}C-glucose was fed to the internode, the ratio of labelled sucrose to hexose increased from 1.5 after 1 h to 3.9 after 3 h (Fig. 4c, d). A similar increase in the ratio of labelled sucrose to glucose was observed in the top half of the shoot.

Sugar Specific Activity

The specific activities of the endogenous sucrose and hexose pools varied between the shoots and the internodes and were significantly lower than those of the original labelling solutions. Both the sucrose and hexose pools' specific activities increased over time in the shoots (Table 3). The most important point to note, however, is that the ratios between the specific activities of the sucrose and hexose pools are similar, regardless of the source of label. This clearly indicates that both the sucrose and hexose pool in the shoots are derived from the same source in both treatments. The reason why the specific activities of the sucrose and hexose pools are different is based on the dilution caused by the endogenous sugar levels in the developing shoots and the cellular compartmentation of the sugars. In the internodal tissues, when applying labelled sucrose, the hexose specific activity increased while the sucrose specific activity decreased (Table 3). A similar pattern was observed when applying labelled glucose (Table 3).

Flux into Sugars in the Internode

The flux into sugars was determined using the calculated sucrose or hexose specific activities in the internode of the injected labelled sugar types (Bindon and Botha 2002) except that we used the internal specific activity of the source sugar. The net flux is derived from the following;

$$\text{Net Flux} = \text{TL1} - \text{TL2}/S_{avg}$$

where L1 and L2 are total label in sucrose (synthesis) or glucose (breakdown) respectively at time T1 and T2, and S_{avg} is the average specific activity in glucose (synthesis) or sucrose (breakdown) over the labelling period T1 (1 h) to T2 (3 h).

This provided an estimated net flux into sucrose from glucose of 0.82 nmol min^{-1} and a flux into glucose from sucrose of 7.35 nmol min^{-1} (Table 4). Sucrose synthesis would be the combined value of synthesis and degradation, i.e. 8.17 nmol min^{-1}. Obviously, these values are at best an approximation, as it does not account for cellular compartmentation and different turnover rates.

Sugar Import to the Shoots

The import rates to the shoots were determined using the internal sucrose specific activity assuming that all the ^{14}C in the shoots were primarily derived from sucrose import. No attempt was made to correct this value for the respiratory loss, which can be as high as 40 % (Table 1).

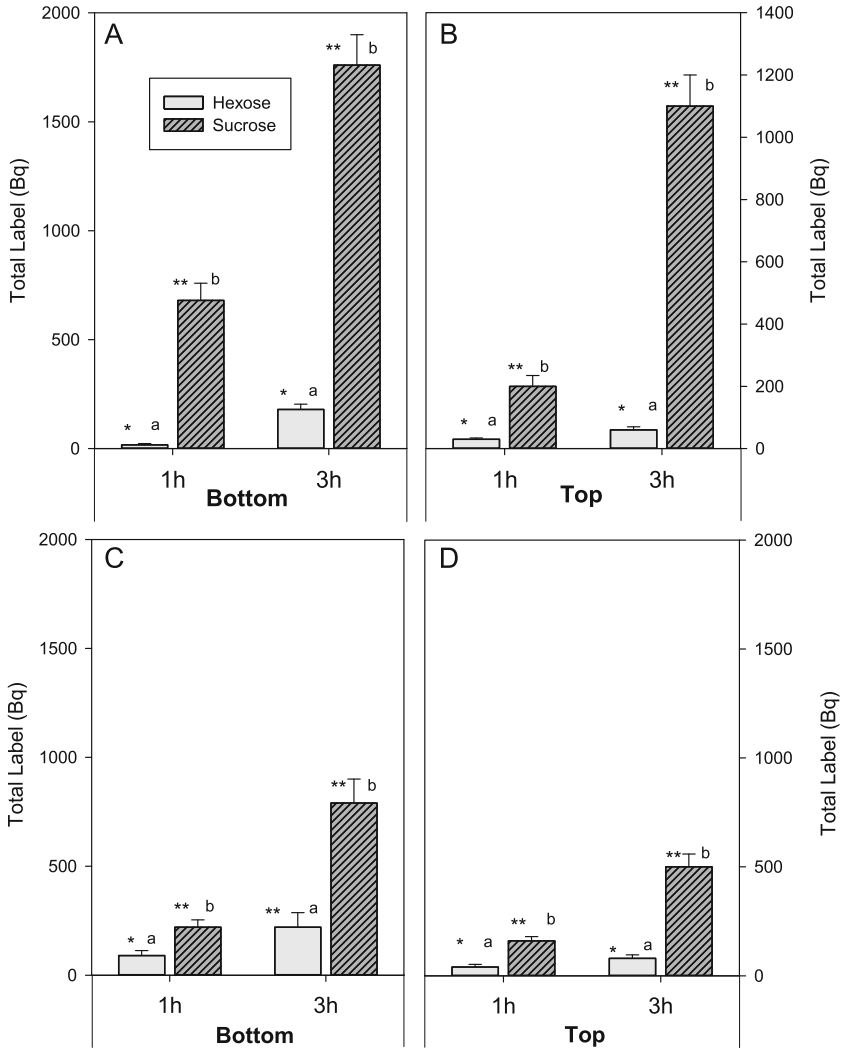

Fig. 4 Radiolabel present in hexoses and sucrose in the bottom of shoot (**a**, **c**) and top of the shoot (**b**, **d**) after feeding ^{14}C-glucose (**a**, **b**) or ^{14}C-sucrose (**c**, **d**) to the internodes (see text for details). Each data point represents the mean values of three replicates ± standard deviation. *Lower case letters* denote statistically homogenous groups at each time point while *symbol* (*) denote statistically homogenous groups in a component over time ($p < 0.05$)

The rate of sucrose transport to the shoot, based on these assumptions was around 110 nmol min^{-1}. Based on the rate of biomass accumulation, and assuming that all the carbon for this increase in mass in the dark incubated tissues were derived from sucrose, the transport of sucrose would vary between 40 (day 7) and 100 nmol min^{-1} (day 14). The similarity in the rates calculated using these two different techniques strongly suggest that sucrose is the only major organic transport molecule between the internodes and developing shoot.

Discussion

The developing shoot is the major carbon sink during the sink to source transition of the internodal tissue, accounting for more than 40 % of the carbon loss from the internode during the first 7 days of bud germination. A significant portion of the carbon in the internode is lost to respiration during bud germination.

The data presented here strongly suggest that one of the earliest changes that occur in the germinating bud, is a more than fivefold increase in CWI over the first 7 days of growth, which coincides with a rapid increase in glucose and fructose levels.

CWI catalyses the only irreversible step in the apoplastic unloading pathway, is crucially important in generating the required sucrose gradient to export sucrose through the phloem, and may play a central role in coordinating sink capacity with different signal responses (Bihmidine et al. 2013; Roitsch et al. 2003). The gene expression levels of sugar transporters and CWIs appear to be closely coordinated, and possibly by the hexose and potentially sucrose concentrations (Bihmidine et al. 2013; Gupta and Kaur 2005; Koch 2004; Wang et al. 2013).

In addition to CWI the other two types of invertases, NI and SAI, are also upregulated in the developing shoot. Sucrose transport into, and metabolism in, the sink are central to sink strength (Bihmidine et al. 2013). A range of genetic, transgenic and environmental stresses have demonstrated that SAI

Table 3 Specific activity (Bq μmol^{-1}) of the endogenous sugars in the shoots (Top and Bottom) and the site of injection (internode) after 1 h and after 3 h injection of [U-^{14}C]-sucrose and [U-^{14}C]-glucose. Values are the mean of three replicates ± SE. Lower case letters denote statistically homogenous groups within tissue types, cellular component and over time with each of the label substrates. The critical values for comparison ($P < 0.05$) are 1.193 for sucrose labelling and 1.611 for glucose labelling, respectively

Tissue	Sampling time	Specific activity of the endogenous sugars in the shoots and internode after injection of:			
		[U-14C]-sucrose		[U-14C]-glucose	
		Hexose	Sucrose	Hexose	Sucrose
	h	Bq μmol-1			
Internode	1	1.19 def ± 0.34	3.12 c ± 0.47	12.27 a ± 1.45	0.34 e ± 0.02
	3	2.34 cd ± 0.49	2.13 cde ± 0.35	10.72 b ± 0.24	0.43 e ± 0.07
Shoots					
Bottom	1	0.42 f ± 0.05	0.68 f ± 0.24	0.12 e ± 0.23	0.45 e ± 0.11
	3	3.16 c ± 0.02	7.53 a ± 0.98	0.87 de ± 0.31	4.56 c ± 0.22
Top	1	0.17 f ± 0.03	0.35 f ± 0.03	0.11 e ± 0.21	0.33 e ± 0.02
	3	1.08 ef ± 0.06	4.41 b ± 0.54	0.59 de ± 0.02	2.12 d ± 0.26

(Andersen et al. 2002) and CWI (Vilhar et al. 2002) are crucial for a strongly defined sink strength. In addition to the invertases, SuSy activity is also very important as an additional sucrose cleavage activity and important for sink strength (Aoki et al. 2012; Koch 2004). During germination of the bud and development of the shoot SuSy activity is strongly up-regulation (Fig. 3). Interestingly the pattern of regulation is different between the dark and dark/light cycle. In the shoot that is greening as a result of the light exposure there is a preference for a high sucrose synthesis to breakdown ratio, which suggest a preferential induction of a SuSy type A activity (Schafer et al. 2005) under these conditions. In the internodal tissue and shoot kept in darkness Susy activity in the breakdown direction dominates. This is taken to indicate that the induction of the type A activity is associated with a transition to autotrophic/photosynthetic like metabolism.

The increases in the sucrose-cleaving enzymes is not only pivotal for the use and distribution of carbon within plant tissues, but also important in establishing the balance between the different sugar signals generated by imported sucrose (Bihmidine et al. 2013; Koch 2004; Koch 1996; Koch et al. 1996; Wang et al. 2013). The rapid increase in glucose and fructose concentrations during the first 7 days in the germinating bud will result in the up-regulation of many genes involved in sucrose import and use (Bihmidine et al. 2013 and references therein), through translation, mRNA stability and protein turnover (Koch 2004; Koch et al. 1996; Tiessen and Padilla-Chacon 2013; Wu et al. 1998). SAI plays an important role in sucrose cleavage in expanding tissues and contribute to considerable hexose flux across the tonoplast and to the entry of hexoses into cytoplasmic metabolism (Koch 2004).

Activation of sucrose hydrolysis by CWI, the probable simultaneous increase in sucrose transport activity, and the further 'sink pull' created by the up-regulation of SAI, NI and SuSy in the germinating bud, lowers the sucrose content in the phloem and creates the necessary gradient to facilitate the movement of sucrose from the internodal parenchyma tissue to the bud. Such a gradient is evident in the phloem between the internodal tissue and the developing shoot (O'Neill et al. 2013). As the sucrose transport in the culm is primarily a symplastic pathway (Lingle 1989; Patrick et al. 2013; Rae et al. 2005), the drawing down of sucrose in the germinating bud will create a pull on the sucrose in the cytosol of the internodal parenchyma cells. It was suggested previously that mobilisation of sucrose from the vacuole in the storage parenchyma might be energy neutral (O'Neill et al. 2013).

In contrast to the germinating bud, the most significant change in the internodal tissue is the up-regulation of SAI. CWI activity also increases over the 21-day incubation period but at a much lower rate. The presence of SAI will result in sucrose hydrolysis in the vacuole and probably explains much of the increased glucose and fructose levels in the internodal tissue. Such an increase in the hexose levels in the internode was not observed previously and in the latter study, no

Table 4 Total radioactivity (Bq) in the endogenous sugars in the internodal tissue at the site of injection after 1 h and after 3 h injection of [U-^{14}C]-sucrose. Values are the mean of three replicates ± SE

Component	Total label (Bq)		Increase in ^{14}C	Flux
	1 h	3 h	Bg min^{-1} g^{-1}FW	nmol min^{-1} g^{-1} FW
Sucrose	1589 ± 2700	2847 ± 2100	10.49 ± 0.56	0.82 ± 0.23
Glucose	1742 ± 213	3957 ± 490	18.46 ± 2.12	7.35 ± 1.23

induction of SAI was evident. This probably indicate genotypic differences between experimental materials used for the two studies.

The distribution of ^{14}C after feeding labelled sucrose and glucose to the internodal tissue indicates that a cycle of sucrose synthesis and breakdown (Glasziou 1961; Whittaker and Botha 1997) still exists in the internodal tissue after transition from a sink to source tissue. However, in contrast to what is evident in the internodes during sucrose accumulation (Bindon and Botha 2002; Whittaker and Botha 1997) this cycle is strongly reduced. At the stage when the internodes are a strong sinks less than 5 % of the label is present in the hexose pool after feeding while more than 70 % remains in the glucose pool when labelled glucose is fed to the internodes when it acts as a source tissue.

Conclusions

The work presented here clearly demonstrates that the proposed first order mechanism that is important for bud germination and shoot development (Glasziou 1961; O'Neill et al. 2013) is CWI. The initial induction of CWI is followed by increases in sugar concentration and these are probably important in the cascade of up-regulation of the other invertases and SuSy activity in the germinating bud. Stored sucrose is the main substrate used during shoot establishment. The decrease in sucrose content was accompanied by a release of hexoses in the internodal tissues as a result of the activity of sucrose hydrolysis/cleavage enzymes.

Although there is evidently still a cycle of sucrose synthesis and breakdown in the internodal tissue when they become a source tissue, this occurs at a much lower rate than in internodes with a sink function.

The continuous sucrose accumulation in the shoots during the latest stage of shoot establishment in plants incubated in dark/light is a result of a source of carbon other than the internodes, probably photosynthesis.

Methods

Shoot Establishment Procedure

Mature non-flowering stalks from field grown sugarcane of the commercial variety N19 were used in all experiments. Setts were cut from nodes 14 to 22 with approximately two centimetres of internode on each side and sealed with candle wax on both ends. The setts were incubated for 21 days in containers (750 cm^2, 10 cm depth) filled with sand. The growth conditions in the controlled environment chamber (Controlled Environments, LTD, Winnipeg, Canada), had a relative humidity of 60 % and temperatures of 22, 27, 32 and 37 °C. Two light regimes were used, i.e. a dark treatment (21 days in complete darkness) and a dark/light treatment (12 h/12 h) starting on the 7th day after planting and continued until day 21. Light was provided by a combination of fluorescent and halogen incandescent lamps, which delivered an intensity of 450 μmol photons.m^{-2}.s^{-1} (measured with a quantum sensor, at approximately 15 cm away from the lamps). After preliminary tests at, the temperature of 32 °C was identified as optimal for growth and used in all further experiments.

Tissue Sampling

Tissue types, the shoots and the corresponding internodal tissues were harvested every 7 days starting from the day of planting (day 0). Samples were collected in triplicate, ground into powder using liquid nitrogen, and stored frozen at −80 °C until use.

Enzyme Extraction

Total soluble protein extractions were carried out in HEPES buffer (100 mM, pH 7.5), containing 10 mM $MgCl_2$, 1 mM ethylene ediamine tetra acetic (EDTA), Roche Complete® protease inhibitor at the recommended concentration, 14 mM β-mercaptoethanol, 0.1 % (*v*/*v*) Triton X-100 and 2 % (m/v) polyvinylpolypyrolidone (PVPP) (insoluble), modified from Albertson et al. (2001); with the following exceptions: soluble proteins were extracted in a 1:2 ratio of tissue to buffer and centrifuged at 10,000 xG for 15 min at 4 °C. The supernatant was immediately desalted on a Sephadex G-25 (Pharmacia PD-10) column equilibrated with desalting buffer (100 mM HEPES buffer pH 7.0), 10 mM $MgCl_2$, and 2 mM EDTA, and 10 % glycerol. Aliquots of eluted protein were rapidly frozen in liquid nitrogen and stored at −80 °C for soluble invertase assays and protein determinations.

For CWI, the pellet from the crude extract was re-suspended in 10 ml of extraction buffer (without PVPP), kept on ice for 10 min with occasional agitation, then centrifuged at 12,000×*g* for 15 min at 4 °C and the supernatant removed. This was followed by a second wash (Albertson et al. 2001). The pellet was kept on ice prior to activity assay.

Invertase Assays

Soluble invertases were assayed in a final volume of 320 μl consisting of 0.5 M sucrose 50 mM HEPES (pH 7.5) for neutral invertase and 50 mM Citrate-phosphate (pH 4.5) for acid invertase, modified from Kingston-Smith et al. (1998). For cell wall bound invertase, the volume of the reaction medium was 2 fold that of the soluble acid (pH 3.6). Aliquots (80 μl) were removed at 30, 60 and 90 min for all invertases and the reaction terminated by a stop solution (2 mM Tris and

22 mM $ZnSO_4$) and a solution of Amidazole (4 M, pH 7.6) was added for soluble and cell wall invertases, modified from Albertson et al. (2001). All aliquots were then used for the determination of reducing sugars as described above. The assay was scaled down for use in microtiter plate format in a final volume of 250 μl. Reactions were carried out in a 96-well microtitre plate and NAD^+ formation was monitored at 340 nm in a Bio-Tek Instruments Power Wave X spectrophotometer (Bio-Tek, Winooski, VT).

Sucrose Synthase Assays

Sucrose synthase was assayed in both synthesis and breakdown directions. In the synthesis direction, a 20 μl aliquot of desalted extract was added to 50 μl reaction cocktail [100 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 10 mM Fructose and 10 mM UDP-Glucose] (Wu et al. 1998). The reaction was incubated at 30 °C for 1 h and subsequently stopped by adding 70 μl of 30 % (*m*/*v*) KOH at 30 min and 1 h. The tubes were then immersed in boiling water for 10 min to remove free hexoses (Wu et al. 1998). Sucrose content was measured using 0.14 % (*m*/*v*) Anthrone reagent in 80 % H_2SO_4 and incubated at 40 °C for 20 min. The concentration of the reaction product (sucrose) was determined spectrophotometrically at 620 nm.

In the breakdown direction, a 10 μl aliquot of the desalted extract was added to 220 μl of reaction cocktail [100 mM Tris-HCl (pH 7.5), 0.32 M sucrose, 2 mM NAD, 2 mM MgCl2, 4 U HK/G6PDH, 1 mM ATP and 4 U PGI]. To initiate the reaction, 20 μl UDP was added, and the amount of free fructose was determined enzymatically (Bergmeyer and Bernt 1974).

Sugar Determination

Sugars were extracted from aliquots of frozen tissues in a 1:10 ratio of mass to extraction buffer [50 mM Tris-HCl (pH 7.0) and 70 % (*v*/*v*) ethanol] and incubated overnight at 65 °C. The samples were then centrifuged at 13,000×*g* for 10 min at 4 °C. The supernatant was immediately used for sugar determination. Sucrose, glucose and fructose concentrations were determined using the standard enzymatic method as described by Bergmeyer and Bernt (1974).

Radiolabelling Experiments

To investigate sugar transport, 10-day-old germinating setts were kept in containers and transferred to a fume hood at room temperature (20–25 °C) for 2 h prior to the labelling experiment. A small cylinder of tissue (diameter 1–2.5 mm) parallel to the developing shoot and 1 cm from the node was removed from both sides of the node with a cork borer. 200 μl (100 μl for each side) of the carrier free buffer containing 250 mM mannitol (pH 5.7), [U ^{14}C]-sucrose or [U ^{14}C]-glucose at final specific activity of 25 Bq/nmol (Bindon and Botha 2002) was injected into the hole with a microsyringe, and the hole sealed with petroleum jelly.

Tissue Sampling and Sugars Extraction

After 1 h and then again after 3 h incubation at room temperature, the young shoot was separated from the internode. The shoots were further divided into a top and bottom half. The internodal tissues were sampled from the site of isotope injection. Three replicates were sampled at each time point during the incubation period. The tissues were finely ground in liquid nitrogen and the resulting powder suspended in a 2:1 ratio (buffer to tissue). The buffer contained 50 mM Tris (pH 7) and 70 % (*v*/*v*) ethanol. The suspension was kept at 70 °C for 3 h and then centrifuged at 12,000×*g* for 10 min and the supernatant transferred to a new tube. The pellet was re-suspended in 500 μl extraction buffer and incubated at 70 °C for a further 30 min and then centrifuged at 12,000×*g* for 10 min and the supernatant added to the original 1 mL. The final supernatant was then reduced to approximately 25 % through evaporation in a vacuum centrifuge.

TLC Sugars Separation

Sugars were separated by loading 2 μl of sample, onto a thin-layer chromatography (TLC) plate (Silica gel 60, Merck); each sample was loaded in triplicate. The TLC plates were developed twice for 3.5 h with a 30 min drying period between each development in a mobile phase containing ethyl acetate: 2-propanol: water (6:3:1). After development, the TLC plate was dried at room temperature for 15 min and then sprayed with a fructose specific stain containing 0.5 M urea, 10 % (*v*/*v*) phosphoric acid, 70 % (*v*/*v*) 1-butanol, and 5 % (*v*/*v*) ethanol and incubated at 80 °C for 15 min. The separated sucrose and fructose from each sample were scraped for radioactivity counting.

Scintillation Counting Procedure

The radioactivity in the separated sucrose and fructose, as well the total soluble extract were counted in 1 ml scintillation cocktail (Ultima Flo™ M) for 20 min in a Beckman LS 1801 scintillation counter. Counts were corrected for quenching using the internal standard of the instrument.

Protein Determination

Aliquots of the desalted extracts were analysed for protein content using a commercial Kit based on the Bradford method (Biorad, Hercule. CA, USA). Bovine albumin (fraction V) was used to generate a standard curve.

Data Analyses

Data presented for sugars content, sucrose cleaving enzyme activities and growth rate are the mean values of three replicates with a replicate containing at least three independent samples.

The Statistix 10.1 software package (Aanalytical Software, Tallahassee, FL, YSA) was used to analyse carbohydrate Analysis of Variance (ANOVA-completely randomised design) was used for determining statistically significant differences. To identify which groups are significantly different a Tukey's HSD All-Pairwise Comparisons Test was then applied to create confidence intervals for all pairwise comparisons. Signifiant differences were deemed to exist at $p < 0.05$. A statistical check for normality was also performed using the Shapiro-Wilk test and where necessary the data was transformed (normalised) prior to performing the Tukey's HSD test.

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