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Changes in Photosynthetic Rates and Gene Expression of Leaves during a Source–Sink Perturbation in Sugarcane

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• *Background and Aims* In crops other than sugarcane there is good evidence that the size and activity of carbon sinks influence source activity via sugar-related regulation of the enzymes of photosynthesis, an effect that is partly mediated through coarse regulation of gene expression.

• *Methods* In the current study, leaf shading treatments were used to perturb the source–sink balance in 12-month-old *Saccharum* spp. hybrid 'N19' (N19) by restricting source activity to a single mature leaf. Changes in leaf photosynthetic gas exchange variables and leaf and culm sugar concentrations were subsequently measured over a 14 d period. In addition, the changes in leaf gene response to the source–sink perturbation were measured by reverse northern hybridization analysis of an array of 128 expressed sequence tags (ESTs) related to photosynthetic and carbohydrate metabolism.

• *Key Results* Sucrose concentrations in immature culm tissue declined significantly over the duration of the shading treatment, while a 57 and 88% increase in the assimilation rate (A) and electron transport rate (ETR), respectively, was observed in the source leaf. Several genes (27) in the leaf displayed a >2-fold change in expression level, including the upregulation of several genes associated with C_4 photosynthesis, mitochondrial metabolism and sugar transport. Changes in gene expression levels of several genes, including Rubisco (EC 4·1·1·39) and hexokinase (HXK; EC 2·7·1·1), correlated with changes in photosynthesis and tissue sugar concentrations that occurred subsequent to the source–sink perturbation.

• *Conclusions* These results are consistent with the notion that sink demand may limit source activity through a kinase-mediated sugar signalling mechanism that correlates to a decrease in source hexose concentrations, which, in turn, correlate with increased expression of genes involved in photosynthesis and metabolite transport. The signal feedback system reporting sink sufficiency and regulating source activity may be a potentially valuable target for future genetic manipulation to increase sugarcane sucrose yield.

Key words: C_4 metabolism, array, hexose, photosynthesis, sucrose, *Saccharum* spp., source–sink, gene expression.

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is the most important source of sucrose worldwide and accounts for >70 % of global sucrose production (Lunn and Furbank, 1999). It has been suggested that the accumulation of high concentrations of sucrose in sugarcane is regulated principally at the level of the sink, where the balance between simultaneous synthesis and degradation of sucrose, often referred to as futile cycling; is believed to be responsible for overall sucrose accumulation (Sacher *et al.*, 1963; Batta and Singh, 1986; Whittaker and Botha, 1997). The high sucrose level (up to 650 mM) in storage tissues of some *Saccharum* spp. hybrids (Welbaum and Meinzer, 1990) makes it an important model genus in the study of the interactions between source (leaf) and sink (culm) tissues. However, sugarcane presents a major challenge for such studies as sucrose is stored in the culm parenchyma tissue and not in specialized storage organs. As such, identifying and interpreting events regulating sucrose partitioning is hampered by the fact that the young culm is the primary growth sink while the mature culm is the primary storage sink.

In addition to improving biomass yield, increasing the concentration of sucrose in the culm is a key objective of most sugarcane breeding programmes. However, recent improvements to sugarcane varieties have been achieved almost entirely through increased cane yield rather than increased culm sucrose content (Jackson, 2005). A more thorough understanding of sucrose metabolism, transport and source–sink interactions that regulate sucrose accumulation may offer greater insight into the processes governing overall culm sucrose concentrations. Despite extensive research, the dynamics and interactions amongst these processes are not well characterized and are only beginning to be explored (Carson and Botha, 2002; Watt *et al.*, 2005; Casu *et al.*, 2007).

Transgenic strategies to increase sucrose concentrations in sugarcane have focused on the manipulation of single enzymes involved in culm sucrose metabolism, primarily those catalysing sucrolytic reactions (Lakshmanan *et al.*, 2005). These genes include those encoding the various isoforms of invertases (EC 2·7·1·90) (Ma *et al.*, 2000; Botha

et al., 2001) and pyrophosphate-dependent phosphofructo-kinase (PFP; EC 2·7·1·90) (Groenewald and Botha, 2007). The mixed success of these attempts may be due to the ability of plants to compensate physiologically for small changes in their genetic environment (Halpin *et al.*, 2001; Luguang and Birch, 2007). A kinetic model of sucrose metabolism in sugarcane culm tissue, developed by Rohwer and Botha (2001), predicts only a limited control on sucrose metabolism for individual genes widely regarded as having a crucial regulatory role. Generally, control over a metabolic flux is shared by several enzymes of a pathway, and large increases in flux cannot be expected from the manipulation of single enzymes, but rather several sites on the pathway (Fell and Thomas, 1995). Without a better understanding of the underlying mechanisms that govern feedback regulation, both within metabolic pathways and between source and sink activity, it may prove difficult to identify potential targets for the effective manipulation of stalk sucrose content.

A key step in understanding the control of sucrose accumulation in sugarcane will be to unravel the complex metabolic and signalling networks that mediate the source–sink relationship. The physiological nature of this relationship has previously been examined in C_3 (Fellows *et al.*, 1979; Wright *et al.*, 1995; Basu *et al.*, 1999; Borrás and Otegui, 2001; Minchin *et al.*, 2002; Franck *et al.*, 2006) and C_4 species (McCormick *et al.*, 2006), and good evidence now exists to support a sink-dependent relationship, whereby carbon levels in storage organs influence the net photosynthetic activity and carbon assimilation of source leaf tissues (Paul and Foyer, 2001; Paul and Pellny, 2003). There is also increasing evidence that the activity of photosynthesis-related enzymes and expression of associated gene transcripts in the leaf, among others, are modified by the local status of the primary transport sugar, sucrose, and/or its constituent hexoses (Pego *et al.*, 2000; Rolland *et al.*, 2002; Franck *et al.*, 2006). However, the mechanisms whereby sugars act to regulate source gene expression are just beginning to be discovered (Rolland *et al.*, 2002; Gibson, 2005), and for C_4 plants, including sugarcane, these remain relatively unexamined (Lunn and Furbank, 1999). Recently, serial analysis of gene expression, which allows for quantitative and qualitative profiling of genome-wide gene expression patterns, has been used to characterize the leaf transcriptome as a potential rate-limiting step for culm sucrose accumulation (Calsa and Figueira, 2007). Although it is possible that C_3 and C_4 plants possess similar regulatory systems, research into source–sink relationships in C_4 plants should be directed towards the possible identification of regulatory elements unique to C_4 plants and the examination of metabolism across hierarchical scales, from the molecular (transcript, enzyme and metabolites) to the crop level (Edmeades *et al.*, 2004).

Study of sugarcane physiology has revealed that the demand for carbon from source tissues is related to the sucrose concentration, age and condition of sugarcane culms (Hartt and Burr, 1967; Marcelis, 1996). Gross photosynthesis is higher in 8-month-old sugarcane plants compared with 4-month old plants, regardless of light intensity (Allison *et al.*, 1997). Furthermore, 3-month-old sugarcane leaves have photosynthetic rates of 45 μmol m^{-2} s^{-1} under intense illumination, while young leaves of 10-month-old plants have a maximum rate of 25 μmol m^{-2} s^{-1} (Amaya *et al.*, 1995). The observed reduction in leaf photosynthetic rates between older and younger crops has been attributed to increased leaf area and reduced leaf nitrogen levels in older leaves (Allison *et al.*, 1997). However, investigations using partial defoliation and shading techniques revealed the existence of a bias in carbon allocation during growth towards culm sucrose accumulation, at the expense of structural growth (Pammenter and Allison, 2002). Gutiérrez-Miceli *et al.* (2004) have additionally demonstrated that partial defoliation of sugarcane plants produces no significant change in culm sucrose concentration compared with control plants, indicating that the remaining intact leaves were capable of maintaining a nominal supply of carbon based on the demand from sink tissues. In a recent study in which source activity was limited to a single unshaded leaf by a shading treatment, a significant increase in photosynthetic rates was observed in the sole source leaf, which was, in turn, negatively correlated with sucrose concentrations in the immature culm tissue (McCormick *et al.*, 2006). Although providing good evidence for sink regulation of photosynthesis in sugarcane, these studies did not address the molecular mechanisms that mediate communication between the source and sink in sugarcane.

Good progress has been made recently in the use of expressed sequence tags (ESTs) as a tool to examine gene expression in sugarcane. EST analysis has been used to examine gene expression behaviour during culm development, with associated increases in sucrose accumulation (Carson and Botha, 2002; Grivet and Arruda, 2002; Casu *et al.*, 2004; Watt *et al.*, 2005). However, while such approaches provide valuable information, they may be ineffective as a sole means to identify factors regulating sucrose storage in the sugarcane culm (Watt *et al.*, 2005). Focus on gene regulation during culm maturation does not provide insights into feedback mechanisms that may operate between the culm and leaf. Hence, experimental systems that permit the study of the expression of genes associated with carbohydrate metabolism in parallel to that of overall plant physiological responses may provide a means to detect mechanisms that mediate the relationship between source and sink tissues (Edmeades *et al.*, 2004).

In the current study on mature sugarcane plants, shading treatments were used to restrict source activity to a single leaf, thereby perturbing the source–sink balance. Gas exchange variables and tissue sugar concentrations were measured in parallel to reverse northern macroarray analysis, which was used to determine relative changes in mRNA expression levels in the sole source leaf over a 14 d period following the source–sink perturbation. A set of ESTs representing 128 genes of photosynthesis and carbohydrate metabolism was used in the gene expression analyses, which were subsequently correlated to changes in photosynthesis and tissue sugar concentrations. This study represents an attempt to determine the changes in the expression of carbohydrate metabolism-related genes that

are associated with a source–sink perturbation in a C_4 crop species.

MATERIALS AND METHODS

Plant material

Twelve-month-old field-grown *Saccharum* spp. hybrid 'N19' (N19), cultivated at Mount Edgecombe, KwaZulu-Natal, South Africa on a 5×15 m plot, was used in this study, which was conducted during December 2004. The plot was located on a north-east-facing slope with a slope of approx. $10°$. Tissue from the third fully expanded leaf (leaf 6) and culm tissue (internodes 4–6) were sampled as described previously (McCormick *et al.*, 2006). The harvested material was immediately frozen in liquid nitrogen (-196 °C). The frozen tissue was then reduced to powder using an A11 Basic Analysis Mill (IKA®) and stored in 50 mL polypropylene centrifuge tubes (Corning®) at -80 °C.

Plant treatment

To modify plant source–sink balance, all leaves except leaf 6 of seven plants per treatment were enclosed in a black sleeve made of 90 % shade cloth. Shade cloth was used so as not to impede gas flow to the plant totally or to cause photomorphogenic effects. Treatments were carried out for 1, 3, 6 and 14 d, effectively rendering leaf 6 the sole full light-receiving source for photosynthetic carbon assimilation over these periods. Treated plants were selected based on similar height and stalk width, and were separated by at least two unshaded plants to negate potential shading effects of the shade cloth on neighbouring plants. Control plants were completely unshaded. Light conditions were checked daily throughout the experiment using a LI-6400 portable photosystem unit (LI-COR Biosciences Inc., Lincoln, NE, USA) to ensure that leaf 6 from control and treated plants received similar levels of light exposure. The start of the shading treatments was staggered so that leaves from all treatments were harvested on the same day.

Sugar determination

Approximately 100 mg of powdered tissue was incubated overnight at 70 °C in 10 vols of sugar extraction buffer consisting of 30 mM HEPES (pH 7. 8), 6 mM MgCl$_2$ and 70 % ethanol (v/v). Extracts were centrifuged for 10 min at 23 200 *g* and sucrose, fructose and glucose concentrations in the supernatant measured by means of a spectrophotometric enzymatic coupled assay modified from Jones *et al.* (1977). The phosphorylation of glucose by hexokinase/glucose-6-phosphate dehydrogenase (EC 1·1·1·49) (Roche, Mannheim, Germany) and fructose by phosphoglucose isomerase (EC 5·3·1·9) (Roche) was quantified by following the reduction of $NADP^+$ to NADPH at 340 nm (A_{340}). Absorbance measurements and data analysis were conducted on a Synergy HT Multi-Detection Microplate Reader using KC4 software (Biotek Instrument, Inc., Winooski, VT, USA).

Gas exchange and fluorescence determinations

A LI-6400 portable photosystem unit was used to measure photosynthetic assimilation (A), transpiration rate (E), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and leaf temperature of the source leaf. Gas exchange measurements were made on 2 cm^2 portions of leaf tissue. Light was provided by a red/blue LED light source (LI-COR Biosciences Inc.) at a photon irradiance of 2000 μmol m^{-2} s^{-1}. All leaf measurements were performed at a constant leaf temperature of 28 °C.

Measurements were performed on the day of harvest for plants that were unshaded or had previously been partially shaded for 1–14 d ($n = 4$). The response of A to C_i ($A : C_i$) was measured by varying the external CO_2 concentration from 0 to 1000 μmol mol^{-1} under a constant photosynthetically active radiation (PAR) of 2000 μmol m^{-2} s^{-1}. An equation

$$A = a\left(1 - e^{(bC_i)} - c\right)$$

was fitted to the A:C_i data using least squares. The portion of the curve where the slope approached zero due to limitation in the supply of substrate (ribulose-1,5-bisphosphate), which is equivalent to the CO_2- and light-saturated photosynthetic rate (J_{max}) (Lawlor, 1987), was calculated from this equation [a, J_{max}; b, curvature parameter; c, dark respiration (R_d)]. Linear regression was performed on the data between a C_i of 0 and 200 μmol mol^{-1} to determine the efficiency of carboxylation (CE) (Lawlor, 1987). The assimilation rate in the absence of stomatal limitations (A_a) was calculated as A, interpolated from the response curve at $C_i = 380$ μmol mol^{-1}.

Chlorophyll fluorescence was determined concurrently with gas exchange measurements using the LI-6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences Inc.). A saturating pulse of red light (0·8 s, 6000 μmol m^{-2} s^{-1}) was applied to determine the maximal fluorescence yield (Fm') at varying external CO_2 concentrations (0–1000 μmol mol^{-1}). The electron transport rate (ETR), defined as the actual flux of photons driving photosystem II (PSII) was calculated from

$$ETR = \left(\frac{Fm' - Fs}{Fm'}\right)fI\alpha_{leaf}$$

where Fs is 'steady-state' fluorescence (at 2000 μmol m^{-2} s^{-1}), Fm' is the maximal fluorescence during a saturating light flash, f is the fraction of absorbed quanta used by PSII, typically assumed to be 0·4 for C_4 plant species (Edwards and Baker, 1993), I is incident photon flux density and α_{leaf} is leaf absorptance (0·85, LI-COR manual). The component fluorescence variables were derived as described by Maxwell and Johnson (2000).

Array target preparation

Target cDNA populations were prepared using mRNA isolated from leaf 6 of unshaded plants (control) and

partially shaded plants where leaf 6 was the sole source leaf for 1, 3, 6 and 14 d. Total RNA was extracted using a modified extraction protocol from Bugos *et al.* (1995). RNA concentration and quality were calculated from ultraviolet (UV) spectrophotometric absorbance measurements using standard photometric equations (Beckman DU-7500 spectrophotometer, Fullerton CA, USA) and confirmed via agarose gel electrophoresis (Ingelbrecht *et al.*, 1998). RNA samples from each time treatment ($n = 6$) were then pooled. To permit accurate and quantifiable comparisons between the array signal intensities produced from each sample, RNA samples (100 μg) were then spiked with two internal mRNA standards, *Arabidopsis thaliana* Rubisco activase (RCA; GenBank accession no. X14212) and *A. thaliana* Rubisco large subunit (rbcL; GenBank accession no. U91966), each at 0·5 ng as per the SpotReport®-3 Array Validation System (Stratagene, La Jolla, CA, USA). Poly(A)$^+$ RNA (mRNA) was isolated from each RNA sample using a Dynabeads Oligo (dT)$_{25}$ mRNA Purification kit (Dynal, Oslo, Norway). Single-stranded cDNA fragments, radiolabelled with [α-^{33}P]dCTP (Amersham BioSciences, UK), were then generated from each mRNA population (1 μg) using a LabelStar™ Array Kit (QIAGEN, Hilden, Germany). Unincorporated dNTPs were removed with a LabelStar™ Array Cleanup Module (QIAGEN).

Array probe preparation and printing

A suite of 128 carbohydrate metabolism and photosynthesis-related ESTs was collected to probe cDNA populations synthesized from total leaf mRNA (see Supplementary Information available online). The suite consisted of ESTs derived from sugarcane (22) and species closely related to sugarcane, including *Oryza sativa* (Kitaake) (55) (MAFF DNA Bank, Tsukuba, Japan) and *Sorghum bicolor* (L.) (51) (PRATT Laboratory, University of Georgia, Athens, GA, USA). EST sizes were verified by gel analysis and homologies confirmed by random partial sequence analysis. Sequences were cross-referenced to the accession numbers of the putative identities contained within the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). Arrays were printed onto a positively charged nylon Hybond-N+ membrane® (Amersham, Germany) by means of a 96-pin manual gridding device (V&P Scientific Inc., San Diego, CA, USA), which facilitated the transfer of aliquots (2 mL) of each sample at a final concentration of 10 ng per spot. For replication, aliquots of each probe were delivered to two adjacent addresses on the array at two randomly selected locations, permitting a total of four query events per probe. External standards were integrated into each array (10 ng), including three heterologous bacterial genes encoding a δ-endotoxin crystal protein [*cry1A(b)*], phosphothricin acetyltransferase (*bar*) and β-glucoronidase (*gus*), and human Cot-1 DNA®, with the latter standard being from the SpotReport®-3 Array Validation System. Furthermore, two internal standards (RCA and rbcL, SpotReport®-3 Array Validation System) were included at final concentrations of 1, 10, 50 or 100 ng. Membranes were air-dried and the probe DNA cross-linked by means of short-wavelength UV radiation (120 kJ cm^{-1}) (Hoefer UV-Crosslinker). The arrays were then wrapped in filter paper, sealed in polyethylene film and stored at room temperature until required.

Array querying and analysis

Array membranes were incubated for approx. 18 h in 20 mL of Church and Gilbert buffer [0·5 M sodium phosphate (pH 7·2); 7% (w/v) SDS; 0·94 mM EDTA] (Church and Gilbert, 1984) containing 10 mg mL^{-1} denatured fragmented salmon or herring sperm DNA (Sigma-Aldrich Inc., St Louis, MO, USA). Incubation was performed at 65 °C in 300 mL volume hybridization bottles within a Hybaid Micro-4 rotary hybridization oven (Hybaid Ltd, Basingstoke, UK). After pre-hybridization, the original solution was discarded and an aliquot of fresh, pre-warmed (65 °C) hybridization buffer containing the cDNA target population was added in the absence of denatured salmon or herring sperm DNA. Following overnight hybridization at 65 °C, the membranes were washed in several aliquots of 1 × SSC (155 mM tri-sodium citrate; 150 mM NaCl), 0·1% (w/v) SDS solution for 10–20 min until unbound labelled target cDNA was removed.

Arrays were exposed to high-resolution Cyclone phosphor screens (Packard Instruments Company, Meriden, CT, USA) and relative abundance captured by means of a Cyclone™ Storage Phosphor Screen imaging system (Packard Instruments Company). Array images were analysed using QuantArray® MicroArray Analysis Software (version 3·0, Packard Bioscience). This software was used to quantify the spot hybridization intensity and corresponding background intensity for each of the probes contained on the array membranes in response to each querying event. Images were visually inspected to identify spots with poor morphology or high local background. These spots were flagged and omitted from further analysis. Array data sets, representing spot and background intensity values, were then imported into Excel spreadsheets for evaluation and comparative analysis.

Overall background consistency was validated by confirming that the coefficient of variation (CV) for the mean background intensity between all query events was <10 % for each array data set. Based on the average ratio of background to spot intensity of external standard query events, a lower intensity-specific threshold was established (Yang *et al.*, 2002), below which probe query events were excluded from further analysis. Following individual background subtraction from query events, replicate query events were compared and excluded from inter-array comparison if their CV exceeded 5 %. Standard curves were then generated for each treatment group from signal intensity data derived from the internal standard query events (see Supplementary Figure available online). To normalize between data from differing arrays, the equation

$$C = e^{\left(\frac{\ln I - b}{a}\right)}$$

was used, where *I* is average query event intensity and *C* is expression level relative to the internal control (*a* and *b* are

curvature parameters). FiRe software was used to detect differential expression between treatments (FiRe Version 2·2, Fribourg, Switzerland) (Garcion *et al.*, 2006). To reduce the possibility of generating false-positive results, only query events with a >2-fold change in expression between treatments were considered for further correlation analysis.

Statistical analysis

Results were subjected to analysis of variance (ANOVA) or Student's *t*-tests to determine the significance of difference between responses to treatments. When ANOVA was performed, Tukey's honest significant difference (HSD) *post hoc* tests were conducted to determine the differences between the individual treatments (SPSS Version 11·5, SPSS Inc., Chicago, IL, USA). SPSS was also used to calculate the Pearson's correlation coefficients for correlation analyses.

RESULTS

Effect of source–sink variations on sugars and photosynthesis

Sugars levels, photosynthetic gas exchange characteristics and leaf chlorophyll fluorescence activities were determined on the sole source leaf of the partially shaded plants and the corresponding leaf of control plants at days 1, 3, 6 and 14. Hexose concentrations in leaf 6 of partially shaded plants declined over the duration of the shading treatment (Fig. 1). Apart from an increase in sucrose detected at 6 d, there were no significant changes in sucrose concentration in leaf 6. In immature internodal tissue, a decline in sucrose over time was observed, while there were no significant changes in hexose concentrations.

Significant increases in J_{max}, *CE* and ETR measured at 380 μmol m^2 s^{-1} CO_2 were observed over the duration of the source–sink perturbation (Table 1). Of note is that plants shaded for 6 d exhibited a 37 % higher J_{max} compared with day 3, which was associated with a significant increase in leaf sucrose levels over the same period (Fig. 1).

Hybridization analysis of leaf transcript abundance

Changes in transcript abundance of selected genes in the source leaf were monitored following the source–sink perturbation induced by the partial shading treatment. Macroarrays were prepared bearing 128 cDNA probes (ESTs), derived primarily from graminaceous species, with known involvement in carbohydrate and photosynthetic metabolism (Table 2; see also Supplementary Information available online). The genes represented on the array were specifically selected to target metabolic activities most likely to be involved in the source–sink relationship. The expression of this set of genes was monitored in leaf 6 of the control plants and in the plants in which all other leaves were shaded at day 1, 3, 6 and 14.

Hybridization between total cDNA populations from leaf 6 and the gene probes on the array was highly consistent between replicate query events. Comparison of the average intensities generated from replicate query events produced R^2 values >0·99 for each treatment, indicating consistent target–probe hybridization (Fig. 2). Following the implementation of a low threshold cut-off, background subtraction and CV analysis, a total of 116 valid gene query events (89·9%) were produced. The majority of query events did not exhibit any substantial difference in hybridization signal intensity between array data sets. However, 27 ESTs showed a 2- to 9-fold change in intensity over
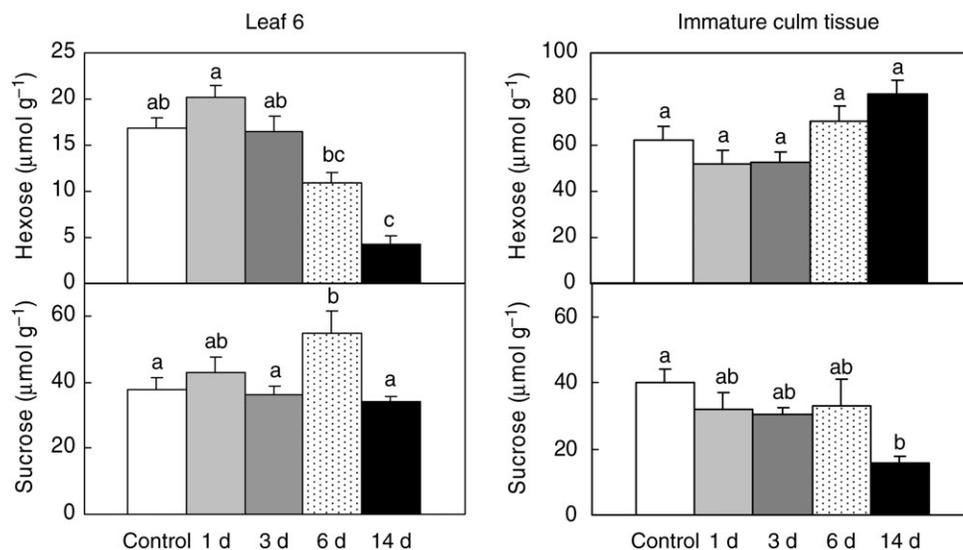
FIG. 1. Comparison of sugar levels in the leaves and immature culm of plants subjected to a source–sink perturbation. Hexose and sucrose (μmol g^{-1} f. wt) measurements for field-grown plants that were completely unshaded (control) and those in which all but leaf 6 (sole source leaf) were shaded for 1, 3, 6 and 14 d prior to sampling ($n = 7$). All plants were harvested and processed concurrently. Sugar levels are shown for leaf 6 and immature culm tissue (internodes 4–6). Letters above the s.e. bars indicate whether the treatment had a significant influence within each tissue type ($P < 0.05$) as determined by ANOVA followed by Tukey's honestly significant difference (HSD) tests.

TABLE 1. *Variables from* A:Ci *curves based on photosynthetic gas exchange and chlorophyll fluorescence variables following a source–sink perturbation*

	Control	1 d	3 d	6 d	14 d
J_{max} (µmol m^2 s^{-1})	$25 \cdot 2 \pm 2 \cdot 6^a$	$22 \cdot 3 \pm 4 \cdot 5^a$	$28 \cdot 7 \pm 3 \cdot 2^a$	$38 \cdot 4 \pm 3 \cdot 4^b$	$39 \cdot 6 \pm 0 \cdot 1^b$
R_d (µmol m^2 s^{-1})	$3 \cdot 8 \pm 0 \cdot 9^a$	$2 \cdot 3 \pm 0 \cdot 3^a$	$2 \cdot 5 \pm 0 \cdot 8^a$	$3 \cdot 7 \pm 0 \cdot 4^a$	$3 \cdot 1 \pm 1^a$
CE (µmol m^2 s^{-1})	159 ± 47^a	120 ± 36^a	231 ± 34^a	343 ± 72^b	$326 \pm 21 \cdot 1^b$
A_a (µmol m^2 s^{-1})	$19 \cdot 8 \pm 2 \cdot 2^a$	$17 \cdot 5 \pm 1 \cdot 3^a$	$26 \pm 2 \cdot 5a$	$32 \cdot 9 \pm 3 \cdot 2^b$	36 ± 1^b
A_i (µmol m^2 s^{-1})	$13 \cdot 2 \pm 1 \cdot 2^a$	$11 \cdot 7 \pm 1 \cdot 9^a$	$20 \cdot 3 \pm 0 \cdot 6^b$	$20 \cdot 9 \pm 0 \cdot 8^b$	$21 \cdot 3 \pm 1 \cdot 7^b$
G_s (mmol m^2 s^{-1})	154 ± 10^a	132 ± 12^a	177 ± 32^a	194 ± 19^a	147 ± 11^a
C_i at $C_a = 380$ (µmol m^2 s^{-1})	$125 \cdot 8 \pm 43 \cdot 2^a$	$183 \pm 30 \cdot 1^a$	$131 \cdot 7 \pm 27 \cdot 9^a$	$153 \cdot 4 \pm 37 \cdot 8^a$	$119 \cdot 8 \pm 25 \cdot 8^a$
ETR at $C_a = 380$ (µmol m^2 s^{-1})	$41 \cdot 4 \pm 7 \cdot 6^a$	$46 \cdot 4 \pm 3 \cdot 3^a$	$73 \cdot 7 \pm 2 \cdot 6^b$	$87 \cdot 7 \pm 9 \cdot 6^b$	$78 \pm 6 \cdot 2^b$

Measurements were taken on leaf 6 of plants in which all the other leaves had been shaded for 1, 3, 6 and 14 d. The control represents measurements taken on leaf 6 of the plants not subjected to shading treatments. All measurements were taken on the same day. Abbreviations are as follows: substrate supply limited assimilation (J_{max}), dark respiration (R_d), carboxylation efficiency (CE), photosynthetic rate in the presence (A_i) and absence of stomatal limitation (A_a), stomatal conductance (G_s), intercellular CO_2 concentration at ambient CO_2 (C_i at $C_a = 380$) and electron transport rate (ETR) at $C_a = 380$. Measurements were performed at an ambient relative humidity of $44 \cdot 6 \pm 3 \cdot 6$ % (mean $\pm$ s.e.) and an irradiance of 2000 µmol m^{-2} s^{-1}. Values represent means $\pm$ s.e. ($n = 4$) and are followed by letters indicating whether treatment time had a significant influence ($P < 0 \cdot 05$), as determined by Student's *t*-tests.

time (Table 3). Within this group, the majority (22) displayed increased hybridization intensity, indicating an overall upregulation of expression in leaf tissue over time.

Within the gene categories represented on the macroarray, a number of trends were evident. During the source–sink perturbation there was a general trend towards the upregulation of genes encoding products involved in photosynthesis, mitochondrial metabolism and sugar transport (Fig. 3). Expression of genes within other EST categories showed little change over time.

Many of the ESTs that depicted an increase in expression over time were homologous to genes associated with the C_4 photosynthetic pathway (Table 3). Overall, the EST group representing photosynthesis showed a 5-fold increase in mRNA abundance (Fig. 3), while, within this group, NADP-dependent malic enzyme (NADPME; EC 1·1·1·40) and pyruvate orthophosphate dikinase (PPdK; EC 2·7·9·1) showed the greatest increase in expression of genes associated with C_4 photosynthesis during the source–sink perturbation (Table 3). Two ESTs related to mitochondrial metabolism, i.e. malate dehydrogenase (MDH; EC 1·1·1·82) and citrate synthase (CS; EC 2·3·3·1), exhibited increased expression, as did triose metabolism-related

ESTs for fructose bisphosphate aldolase (ALD; EC 4·1·2·13) and glyceraldehyde phosphate dehydrogenase (GPDH; EC 1·2·1·12). Increased expression levels were also observed for six ESTs homologues to putative transporter proteins, including three putative monosaccharide transporters, an ADP/ATP plastidic transporter and two triose phosphate transporters.

Included in the small group of five ESTs that were down-regulated were those representing fructokinase (FK; EC 2·7·1·4) and hexokinase (HXK; EC 2·7·1·1) (Table 3). In addition, ESTs homologous to a mitogen-activated protein kinase (MAPK; EC 2·7·1·37), UTP-glucose dehydrogenase (UTP-GD; EC 1·1·1·22) and alcohol dehydrogenase (AD; EC 1·1·1·1) showed reduced expression levels during the perturbation.

In some instances, not all ESTs assigned the same identity displayed consistent changes in expression. For example, only one of the six sucrose synthase (SuSy; EC 2·1·4·13) ESTs (AU173014) showed a >2-fold increase in expression, while only one of the three MAPK ESTs showed reduced expression. This could be due to significant sequence divergence between the ESTs isolated from different species, or, alternatively, these ESTs may represent distinct isogenes or gene family members particular to specific tissues.

TABLE 2. *Functional classification of ESTs used in gene expression analysis*

General classification of gene product function	No. of genes under analysis
Cell wall biosynthesis	9
Sugar sensing and signalling	12
Carbon (starch) metabolism	5
Sucrose metabolism	23
Glycolysis	20
Triose phosphate metabolism	13
Photosynthesis	16
Mitochondrial metabolism	13
Sugar transport	17

The number of genes cited reflects both different genes and variants of single genes.

Correlation analysis

To uncover possible regulatory events induced in leaf 6 by the source–sink perturbation, the observed changes in photosynthesis, sugar concentration and gene expression over time were subjected to Pearson's correlation analyses. With regard to photosynthesis and sugar concentrations, a strong negative correlation between hexose concentrations in source leaf tissue, and J_{max} and CE was revealed (Table 4). This relationship was not evident for sucrose. Furthermore, significant correlations between sucrose and hexose concentrations in immature culm tissue and leaf 6 photosynthesis levels were observed. Immature sink tissues were characterized by decreased sucrose levels
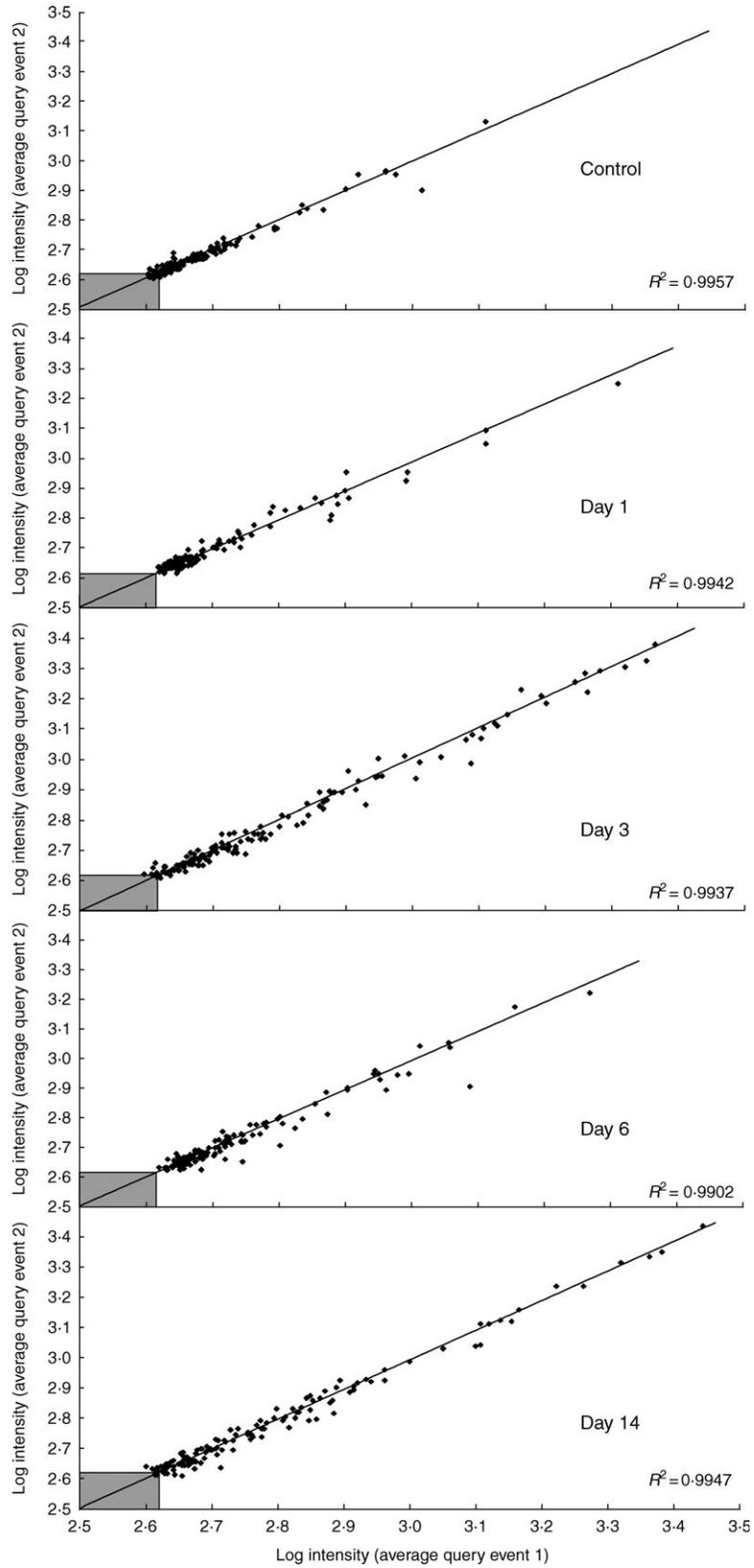
FIG. 2. Comparison of probe–target hybridization intensities of five replicate macroarray query events. Log data represent signal intensities of leaf 6 total cDNA populations hybridized to 128 probes on the macroarray. The data represent the average probe hybridization of two adjacent probes compared with a second set of probe pairs placed at another location on the array. Intensity values below the lower threshold value (shown in grey) were excluded from data analysis.

TABLE 3. *Fold changes in gene expression between leaf 6 mRNA of an unshaded plant (control) and leaf 6 (sole source leaf) at days 1, 3, 6 and 14 following a source–sink perturbation induced by partial shading*

Putative ID		Fold change			
		Day 1	Day 3	Day 6	Day 14
Upregulated	Accession no.				
ATP/ADP transporter	CD423751	0·6	2·4	1·9	9·1
Citrate synthase	BE363510	1·6	3·9	1·6	2·3
Fructose bisphosphate aldolase	AW745533	1·0	1·9	1·8	2·9
Glyceraldehyde-P-dehydrogenase	BG947834	1·7	4·1	2·0	3·4
	PQ0178	0·9	2·1	1·5	1·9
Malate dehydrogenase	AU093830	1·2	1·4	1·5	2·5
NADP-dependent malic enzyme (C$_4$)	CN136258	0·9	2·3	1·3	3·6
	CN146318	0·7	3·0	2·1	8·1
Phenolenolpyruvate carboxylase (C$_4$)	AU088696	0·8	1·3	0·8	2·2
	BG158755	0·9	2·7	2·4	4·3
psbA chloroplast protein	CD212978	0·9	1·7	1·5	3·2
Pyruvate orthophosphate dikinase (C$_4$)	CF071996	0·6	2·6	2·5	8·5
Rubisco (large subunit)	AW678375	0·3	1·1	1·2	3·0
Rubisco (small subunit)	CN150664	0·9	2·7	1·5	2·4
Rubisco activase	BM318446	1·1	1·2	1·4	2·2
Rubisco transition peptide	CN142383	1·1	4·4	3·4	6·9
Sucrose synthase	AU173014	1·6	1·8	1·2	2·6
Sugar transporter (monosaccharide)	CD231617	1·1	3·5	4·9	6·7
	AU163471	0·9	1·9	1·1	3·0
	AU094600	0·9	1·3	1·3	2·0
Triose phosphate transporter	CN149774	1·0	3·6	2·4	4·3
	CN149403	1·1	3·0	3·1	3·0
Down-regulated					
Alcohol dehydrogenase	AU091741	1·0	1·1	1·4	2·3
Fructokinase	CN140006	1·1	1·0	1·2	2·0
Hexokinase	AU057562	1·0	1·1	1·4	2·2
Mitogen-activated protein kinase	CN132740	1·1	0·9	1·2	2·0
UDP-glucose dehydrogenase	AA525658	1·1	1·2	1·4	2·0

Putative ID indicates the homology match of the particular EST probe referenced to accession number records of the National Centre of Biotechnological Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov). See Supplementary Information for further details.

which were correlated with an increase in J_{max} over the 14 d period.

Of the 27 genes that exhibited marked changes in expression over time in leaf 6, 20 showed strong correlations to leaf 6 photosynthetic variables (J_{max} and *CE*) and leaf 6 hexose concentrations (Table 4). Within this group, no correspondence was evident with leaf sucrose concentrations; however, nine genes showed significant correlation with the decreasing sucrose levels observed in immature culm tissue although a secondary correlation through leaf hexose may be indicated. Notably, these included ESTs homologous to the C$_4$ photosynthetic enzymes phospho*enol*pyruvate carboxylase (PEPC; EC 4·1·1·31), NADPME and PPdK, and two putative transporter proteins.

DISCUSSION

Disturbance of the source–sink balance by partial shading of all source leaves, bar one, produced significant changes in the sugar levels of the leaf and subtending internodes (Fig. 1), as well as in photosynthetic activity of the single unshaded leaf (Table 1), in which significant increases in photosynthetic rate, carboxylation efficiency and PSII efficiency were observed. Conversely, shading treatments resulted in decreased sucrose levels in the young immature internodal

tissue. A significant negative linear relationship was observed between maximum photosynthetic assimilation rates (J_{max}) of the source leaf and sucrose concentrations in these immature internodes (Fig. 1; Table 4). These observations support reports that reduced carbon supply to sink tissue is a physiological signal to the source of increased assimilate demand (van Bel, 2003). Partial defoliation has been shown to produce a similar effect in sugarcane, which results in preferential partitioning of available carbon to sucrose culm storage (Pammenter and Allison, 2002). Partial defoliation also has no effect on overall plant sucrose yields (Gutiérrez-Miceli *et al.*, 2004), suggesting that the assimilation capacity of sugarcane leaves is robust and flexible and can readily adjust carbon supply relative to sink demand. In contrast to defoliation studies, the marked increases in photosynthesis resulting from the source–sink perturbation achieved in this study may have been exacerbated by the continued presence of other leaves. Previous work has shown a significant increase in partitioning of a ^{14}C label to shaded leaves, indicating that these leaves were converted to additional sinks during shading treatments (McCormick *et al.*, 2006).

A strong negative correlation was observed between hexose concentrations (Fig. 1) and J_{max} and *CE* (Table 1) in unshaded source leaves (Table 4). In contrast, no
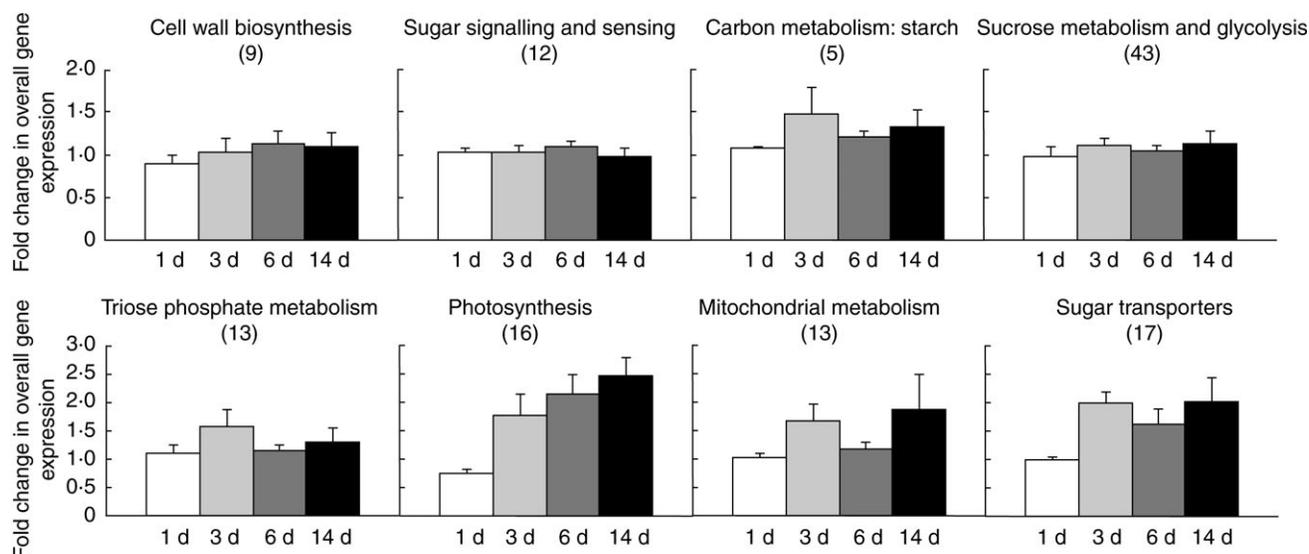
FIG. 3. Average fold changes in gene expression per functional category over time (open symbols, 1 d; light grey symbols 3 d; dark grey symbols 6 d; black symbols 14 d) in the sole source leaf of partially shaded sugarcane plants compared with an unshaded control. The quantity of ESTs per category is indicated in parentheses. See Supplementary Information for EST details.

relationship was observed between source leaf sucrose levels and photosynthesis. This suggests that hexoses, rather than sucrose, may participate in a feedback system for photosynthetic regulation. This contention is supported by observations from maize (*Zea mays* L) in which sucrose concentrations were shown to have no significant short-term feedback inhibitory effects on the synthesis of sucrose itself in leaf tissue (Lunn and Furbank, 1999). Furthermore, hexoses have been shown to inhibit photosynthesis in numerous C_3 species and consequently are believed to play a significant role in regulating carbon accumulation and leaf development (Goldschmidt and Huber, 1992; Kilb *et al.*, 1995; Ehness *et al.*, 1997; Paul and Pellny, 2003). A decreased leaf hexose pool may serve as a signal for increased sink demand, and also reduce negative feedback regulation of photosynthesis (Foyer, 1987), an effect which has been observed previously in sugarcane (McCormick *et al.*, 2006). Due to the compartmentation of enzymes between mesophyll and bundle sheath cells in C_4 species (Edward *et al.*, 2001), the regulation of sucrose accumulation and signalling mechanism may be more complex than for C_3 species. Nevertheless, sugar-mediated regulation of gene expression may be as important in C_4 as in C_3 species for maintaining the balance between the source and sink activity (Lunn and Furbank, 1999).

In the current study, physiological and metabolic effects of a source–sink perturbation have been examined in parallel to changes in the expression of genes associated with photosynthesis and carbohydrate metabolism. To permit comparison of replicate array query events within each array, the hybridization signal intensity values for each EST probe were normalized amongst replicates. To compare array data generated by replicate array hybridizations, a method for the normalization of hybridization signal intensity data was used. An internal standard was introduced into the leaf RNA samples prior to cDNA synthesis and labelling, and then used to normalize hybridization signal intensity data amongst replicate query events. This is an improved means of normalization compared with standard, comparative normalization techniques, such as generation of relative expression values (Cui and Churchill, 2003) in which all measured values are divided by the sum of the values and then compared between arrays. This latter method is not ideal as it is based on the assumption that the amount of mRNAs per sample is constant (Velculescu *et al.*, 1999). A further drawback is that large changes in relative gene expression may impact on the expression of unchanged genes, leading to the generation of false-positive results (Yang *et al.*, 2002). In contrast to such analytical approaches, the method used in this study provides a more stringent approach to the detection of specific changes in gene expression.

During photosynthesis in NADPME-type C_4 species, such as sugarcane, malate is translocated to bundle sheath cells where NADPME catalyses its decarboxylation (Lunn and Hatch, 1995; Edwards *et al.*, 2001). The three key enzymes of C_4 photosynthesis, i.e. PPdK, PEPC and NADPME, are strongly regulated by light (Hatch, 1992; Furbank and Taylor, 1995). However, only PEPC has previously been shown to respond to changing sugars levels (Chollet *et al.*, 1996; Sima and Desjardins, 2001). This study has revealed an increase in gene expression of all three of these enzymes, as well as an increase in expression of Rubisco (both RbcL and RbcS) and Rubisco-related proteins (Table 3). Increased abundance of these transcripts correlated with an increase in photosynthetic activity and decreasing leaf hexose concentrations (Table 4). These observations indicate that hexoses may play a key role in regulating the expression of these enzymes. Sheen (1990) demonstrated that supplying maize protoplasts with glucose or sucrose led to the repression of genes encoding

TABLE 4. *Bivariate Pearson's correlation coefficients between leaf 6 photosynthetic variables* J_{max} *and* CE *(see Table 1 for variable declarations), leaf 6 and immature culm sugar concentrations (hexose and sucrose) and gene expression data of leaf 6 from either unshaded plants or partially shaded plants (sole source leaf 6) between 1 and 14 d*

	Accession no.	Leaf 6		Immature culm	
		J_{max}	CE	Hexose	Sucrose
CE		0·979 (0·00)			
Leaf 6 – hexose		−0·694 (0·00)	−0·655 (0·00)		
Leaf 6 – sucrose					
Immature culm – hexose		0·336 (0·04)			
Immature culm – sucrose		−0·372 (0·03)			
Upregulated					
ATP/ADP transporter	CD423751	0·708 (0·00)	0·621 (0·00)	0·631 (0·00)	−0·487 (0·03)
Citrate synthase	BE363510				
Fructose bisphosphate aldolase	AW745533	0·827 (0·00)	0·799 (0·00)	−0·675 (0·00)	
Glyceraldehyde-P-dehydrogenase	BG947834				
	PQ0178				
Malate dehydrogenase	AU093830	0·772 (0·00)	0·699 (0·00)	−0·632 (0·01)	−0·478 (0·03)
NADP-dependent malic enzyme	CN136258	0·618 (0·01)	0·587 (0·01)	−0·565 (0·01)	−0·515 (0·02)
	CN146318	0·730 (0·00)	0·662 (0·00)	−0·651 (0·00)	−0·493 (0·03)
Phenolenolpyruvate carboxylase	AU088696	0·514 (0·02)	0·581 (0·01)	−0·542 (0·01)	−0·485 (0·03)
	BG158755	0·826 (0·00)	0·815 (0·00)	−0·630 (0·00)	
psbA chloroplast protein	CD212978	0·775 (0·00)	0·719 (0·00)	−0·649 (0·00)	−0·465 (0·04)
Pyruvate orthophosphate dikinase	CF071996	0·775 (0·00)	0·699 (0·00)	−0·647 (0·00)	−0·452 (0·05)
Rubisco (large subunit)	AW678375	0·785 (0·00)	0·706 (0·01)	−0·664 (0·00)	
Rubisco (small subunit)	CN150664	0·826 (0·00)	0·815 (0·01)	−0·489 (0·00)	
Rubisco activase	BM318446	0·830 (0·00)	0·744 (0·01)	−0·648 (0·00)	
Rubisco transition peptide	CN142383	0·789 (0·00)	0·783 (0·00)	−0·603 (0·01)	
Sucrose synthase	AU173014				
Sugar transporter (monosaccharide)	CD231617	0·946 (0·00)	0·934 (0·00)	−0·668 (0·00)	
	AU163471	0·591 (0·01)	0·555 (0·01)	−0·563 (0·01)	−0·532 (0·02)
	AU094600	0·819 (0·00)	0·774 (0·00)	−0·685 (0·00)	
Triose phosphate transporter	CN149774	0·683 (0·00)	0·716 (0·00)	−0·501 (0·00)	
	CN149403	0·683 (0·00)	0·716 (0·00)	−0·501 (0·00)	
Downregulated					
Alcohol dehydrogenase	AU091741				
Fructokinase	CN140006				
Hexokinase	AU057562	−0·730 (0·00)	−0·605 (0·01)	0·576 (0·01)	
Mitogen-activated protein kinase	CN132740				
UDP-glucose dehydrogenase	AA525658	−0·561 (0·01)	−0·449 (0·04)	0·511 (0·02)	0·5 (0·03)

Significance levels (*P*) are reported for the Pearson's correlation coefficients (in parentheses). Missing values indicate genes that were up- or downregulated but not significantly correlated.

products involved in photosynthesis. The depletion or accumulation of sugars has further been shown to activate or repress, respectively, the expression of genes for photosynthetic components of a variety of C_3 species and ultimately influence photosynthesis itself (Krapp *et al.*, 1993; Krapp and Stitt, 1995; Van Oosten and Besford, 1994, 1995; Basu *et al.*, 1999). However, there are few reports describing the effects of sugar levels on the expression of genes encoding components of photosynthesis specific to C_4 plants, and results from C_3 studies may not always be pertinent to C_4 species. For example, in *Spinacia oleracea* (L.), the expression of rbcS, and consequently Rubisco protein activity, has been shown to be regulated by leaf sugar concentrations (Krapp *et al.*, 1991). These results are not easily comparable with C_4 species, where Rubisco levels are typically only 50 % of those of C_3 plants on a chlorophyll basis (Lunn and Furbank, 1999). Furthermore, in C_4 species, sucrolytic and photosynthetic activities are localized in mesophyll and bundle sheath cells, respectively (Lunn and Furbank, 1997). Maize, in particular, shows a

strong asymmetric distribution of activities, with cytosolic sucrose phosphate synthase (SPS; EC 2·4·1·14), sucrose phosphate phosphatase (SPP; EC 3·1·3·24) and fructose 1,6 bisphosphatase (FBPase; EC 3·1·3·11) predominantly localized in the mesophyll (Downton and Hawker, 1973; Furbank *et al.*, 1985), indicating that sucrose is synthesized almost exclusively in the mesophyll of maize source leaves.

In the current study, decreased levels of hexose were correlated with increased expression of several photosynthesis-related genes (Table 4). Drawing on information obtained from studies of maize it also appears likely that in sugarcane, it is a hexose-regulated signal originating primarily in mesophyll cells which serves to regulate PEPC expression levels (Fig. 4). As PEPC has previously been shown to respond to sugars (Chollet *et al.*, 1996; Sima and Desjardins, 2001), PEPC may influence signalling cascades that ultimately result in the upregulation of C_4 photosynthesis under conditions of decreased cytosolic hexose. Of note is that hexoses have been implicated in the regulation of source metabolism via signal transduction
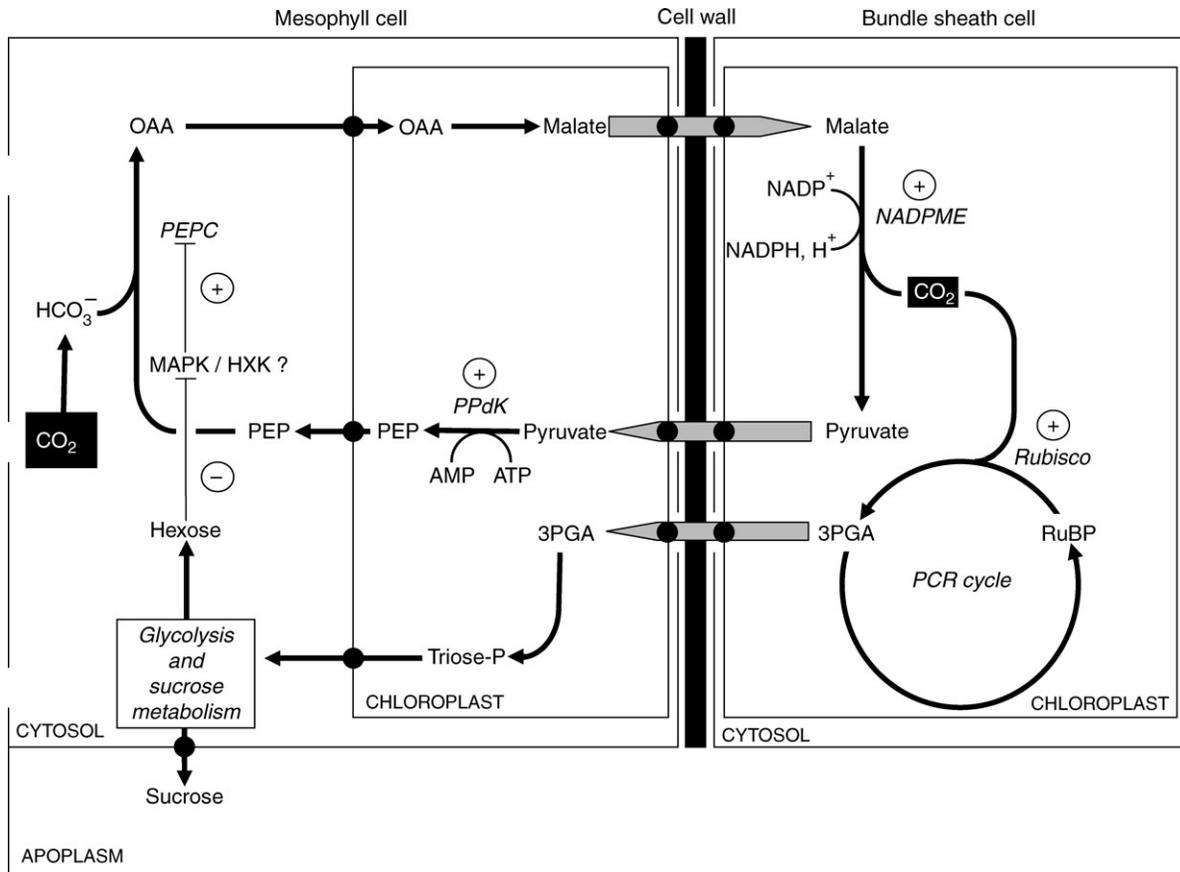
FIG. 4. The C_4 NADP-ME pathway of photosynthesis in sugarcane. Arrows within circles indicate the changes in metabolite and gene expression levels during a source–sink perturbation. The negative correlation observed between hexose and PEPC is indicated. 3PGA, 3-phosphoglycerate; HXK, hexo-kinase (EC 2·7·1·1); MAPK, mitogen-activated protein kinase (EC 2·7·1·37); NADP-ME, NADP-malic enzyme (EC 1·1·1·40); OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase (EC 4·1·1·31); PPdK, pyruvate orthophosphate dikinase (EC 2·7·9·1); RuBP, ribulose bisphosphate; triose-P, triose phosphate.

pathways involving protein phosphorylation via MAPK activities (Ehness *et al.*, 1997). In the present study, MAPK expression was downregulated; however, due to the wide variety of signalling pathways that are associated with MAPKs (Jonak *et al.*, 1996; Lee *et al.*, 2001; Zhang and Klessig, 2002), it is difficult to pinpoint the specific role of this enzyme during the source–sink perturbation (Fig. 4). Regulation of C_4 leaf photosynthesis has been suggested to involve phosphorylation of the PEPC enzyme (Duff and Chollet; 1995; Vidal and Chollet, 1997). Further metabolic and gene expression analyses in sugarcane will aim to examine the post-transcriptional regulation of PEPC by PEPC kinase (Jeanneau *et al.*, 2002) and possible co-mediation by MAPKs and hexoses.

Increased photosynthetic activity was correlated with an increase in several transporter proteins, including a putative ATP/ADP transporter and two triose phosphate transporters (Table 3). Attempts to increase sucrose metabolism in transgenic C_3 *O. sativa* by overexpression of maize PEPC have previously been shown to result in no change in leaf sucrose, but rather a decreased availability of Pi and increased consumption of cytosolic triose phosphate into malate (Agarie *et al.*, 2002). In C_4 plants, this phenomenon may be alleviated by a co-ordinated increase in the supply of cytosolic Pi and triose phosphate when photosynthetic activity increases. Sucrose produced in the mesophyll must, however, additionally pass through the bundle sheath cells to be loaded into the phloem through either a symplastic or an apoplastic pathway, or both (Lunn and Furbank, 1999; Walsh *et al.*, 2005). In sugarcane, the conducting cells of the phloem have been shown not to be connected to other cells of the leaf by plasmodesmata (Robinson-Beers and Evert, 1991). This suggests that phloem loading occurs from the apoplast in sugarcane leaves (Rae *et al.*, 2005). Under conditions of increased photosynthesis and sucrose export, the observed increase in expression of sugar transporter proteins (Table 3) is not unexpected, and may be required to meet increased sink demand, due to the reduction in source supply capacity, efficiently.

Hexokinase and FK were downregulated during the shading treatments. It has been proposed that both enzymes participate in sugar sensing and signalling in plants (Pego and Smeekens, 2000; Rolland *et al.*, 2006), particularly HXK, which showed positive correlation with decreasing hexose concentrations (Table 4). The role of

HXK as a putative sensor of hexose signalling was examined by Jang *et al.* (1997) using sense and antisense constructs of the *A. thaliana* HXK isoforms *Hxk1* and *Hxk2*. Those authors reported that plants overexpressing HXK genes exhibited glucose hypersensitive characteristics, whereas antisense plants were hyposensitive. The results of this study together with those of Jang *et al.* (1997) support the hypothesis that HXK is a putative sensor for hexose signalling. More recently, Moore *et al.* (2003) demonstrated that point mutations in the catalytic domains of HXK resulted in an engineered protein that exhibited no phosphorylation activity, while still being capable of glucose signalling activity. This indicates that HXK may play two functionally distinct roles, at least in C_3 plants (Harrington and Bush, 2003). Two FK isoforms have been isolated and characterized in sugarcane (Hoepfner and Botha, 2004), but little is known about HXK. Further research will be required to clarify the nature of the relationship observed between HXK and hexose concentration (Table 4; Fig. 4).

The observed increases in photosynthetic rates (Table 1) were statistically correlated with a reduction in hexose content and changes in the expression of several genes (Table 4). Although changes in gene expression are indicative of coarse regulation, further study will be required to confirm whether expression patterns correlate with enzyme activity, which may change substantially as a result of post-translational control. Recent studies in *A. thaliana* have indicated that changes in carbohydrates may initiate a significant gene signalling response that does not necessarily lead to long-term changes in plant behaviour (Stitt *et al.*, 2006). Furthermore, analysis of whole tissue sugar levels does not necessarily relate to the precise sugar concentration in the cells actually responding to the signalling. However, the strong correspondence between photosynthesis, hexose and gene expression demonstrated in the present study and the similarity of these responses to those reported in C_3 plants (Krapp *et al.*, 1991, 1993; Franck *et al.*, 2006) provides evidence for hexose as an important signalling molecule in C_4 sugarcane.

Conclusions

This is the first report for sugarcane in which physiological and metabolic changes during a source–sink perturbation have been examined in parallel to changes in leaf gene expression patterns. The work has revealed a strong relationship between source and sink tissues, where demand for carbon from sinks affects source leaf photosynthetic activity, metabolite levels and gene expression. Future research will include a closer examination of the expression patterns of several of the genes highlighted in the current study. This will include comparative expression analysis among different sugarcane cultivars to gauge the extent to which the changes in gene expression observed in this study pertain to other varieties. Clarification of how the sink acts to regulate source activity in sugarcane will provide researchers with additional potential targets for manipulation towards improving sucrose yield. The observation that sink demand limits source activity in sugarcane indicates that the signal feedback system reporting sink sufficiency and regulating source activity may be a potentially valuable target for genetic manipulation in other C_4 species. This study has demonstrated that increased carbon demand from the sink results in increased photosynthetic rates at the source. The communication of this relationship appears to correlate with a decrease in source hexose concentrations, and increased expression of genes involved in C_4 photosynthesis and metabolite transport.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at http://aob. oxfordjournals.org/ and consists of tables of photosynthesis- and carbohydrate metabolism-related ESTs selected for expression analysis, and standard curves generated from the average log intensity values of two mRNA standards that were spiked into the total cDNA population during the array query events.

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