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Identification of proteins and metabolic networks associated with sucrose accumulation in sugarcane (*Saccharum* spp. interspecific hybrids)

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ABSTRACT

The rate of improvement of sugar content in sugarcane remains low for decades worldwide. Our previous transcriptome studies provided an atlas of sucrose accumulation-related gene expression, but little is known about the proteins involved. Here, we conducted a proteomic analysis of experimentally altered sucrose accumulation in sugarcane. Analysis of stem proteomes of sugarcane ripener ethephon treated high- and low-sugar genotypes had identified 2983 proteins of which 139 were significantly differentially expressed (DEPs). These DEPs were found to be associated with sugar metabolism-related processes with 25 of them may have a regulatory role in sucrose accumulation. The key proteins identified include UDP-glucose 6-dehydrogenase associated with amino sugar and nucleotide sugar metabolism; those involved in carbon fixation; and fructokinase, β-D-glucosidase, and α-glucan phosphorylase involved in starch and sucrose metabolism. Distinct genotype- and ethephon-dependent DEP expression was evident providing new insights into one of the most intractable sugarcane traits to breeding.

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Sugarcane; proteomics; differential expression; proteins; sucrose accumulation; sugar metabolism

1. Introduction

Sugarcane is the most important sugar crop grown in tropical and subtropical regions in the world (Cursi et al. 2021). It stores exceptionally high levels of sucrose, as much as 0.7 M, in its stem (Moore 1995), and contributes to ~80% of sucrose production globally. Increasing sucrose content is a major objective of sugarcane breeding worldwide (Ostengo et al. 2021). However, breeding for sucrose content is proving to be much harder than other commercially important traits such as cane yield, fiber content, and disease tolerance (Waclawovsky et al. 2010). For example, the average sugar content of sugarcane variety F134 originally bred and released in Taiwan in 1936 and grown widely in mainland China, and a popular Chinese variety released recently, GT42, is about 14.5% (Guangxi Sugarcane Research Institute 1991; Guangxi Sugar Association 2019), demonstrating the difficulty in improving sugar content in sugarcane through conventional breeding. Modern sugarcane is an aneuploid inter-specific hybrid with a complex genetic background (Hoarau et al. 2001). This genomic complexity, long breeding cycle up to 12–14 years to produce a variety, and non-additive trait genetics are thought to be the bottlenecks for increasing sugar content by conventional breeding. Sugar content in sugarcane germplasm can reach up to 27% of fresh weight (Bull and Glasziou 1963), much higher than the average sugar content of commercial varieties, which is about 15%. This large clonal variation for sugar content, normally seen in sugarcane breeding populations, suggests a big scope for increasing sugar content by conventional breeding. However, despite extensive breeding and considerable molecular and biotechnological research, major breakthroughs on improving sugar accumulation have yet to be reported (Chen et al. 2019; Cursi et al. 2021; Terajima et al. 2021). A better understanding of molecular and biochemical processes, involved in sugar accumulation in sugarcane, is thus needed for developing practically useful approaches to produce high-sugar varieties by conventional and/or molecular breeding.

In the past two decades, based on physiological, biochemical and molecular evidence, activities of a number of genes associated with sugar transport and metabolism were transgenically altered to increase sugar content, but it did not yield the desired outcomes (Lakshmanan et al. 2005; Moore 2005; Singh and Chandra 2021). Recognizing the complexity of sugar accumulation in sugarcane, considerable efforts were directed to genomic research, particularly transcriptomics, to unravel the genetic elements and possible genetic regulatory networks of sucrose accumulation in sugarcane (Huang et al. 2016; Thirugnanasambandam et al. 2017; Chen et al. 2019). These studies provided more clarity on molecular processes and genes likely to be involved in sugar metabolism and related biological processes (Reinders

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et al. 2006; Chen et al. 2016; Huang et al. 2017; Thirugnanasambandam et al. 2019; Miao et al. 2020). This expanded knowledge, while strengthening the rationale for experimental manipulation of growth and sugar content in sugarcane, also highlights the degree of regulatory complexity inherent in sugarcane stem sucrose accumulation process (Garcia Tavares et al. 2018; Chen et al. 2019).

Proteins are macromolecules that perform a vast variety of biological functions including cell metabolism, DNA synthesis and replication, signal transduction, and responses to external stimuli, such as abiotic stresses and pathogen attack. Proteomics is widely used to understand the biochemical regulation of metabolic processes. With the advances in quantitative proteomics, we can accurately identify and quantify proteins expressed in complex biological systems. It can be used to screen and search for differentially expressed proteins (DEPs) in a given condition, and in combination with bioinformatics it could greatly expand our understanding of cell physiological functions such as sugar accumulation in sugarcane stem. Proteomics has been applied in sugarcane to identify proteins that respond to biotic (Singh et al. 2019; Meng et al. 2020) and abiotic stresses (Salvato et al. 2019), and those involved in growth and development (Fonseca et al. 2018). However, its application in studying sugar accumulation in sugarcane has not been reported.

Ethephon is an effective growth regulator and commercially used sugarcane ripener for improving sugar content and sugar yield in sugarcane crops worldwide (Li and Solomon 2003). In our previous studies, we found that ethylene is particularly effective in increasing sugar content in low-sugar sugarcane varieties and thus it can be used to improve sugar content in varieties of genetic background (Chen et al. 2019). A detailed transcriptome analysis of ethylene-induced sugar accumulation process, in sugarcane varieties with inherently low- and high-sugar content, has identified a number of transcripts and genes associated with this phenomenon (Chen et al. 2019). To further advance our understanding of molecular and biochemical processes regulating sucrose accumulation, we studied proteome of sugarcane following ethephon application. In this study, we applied ethephon to field-grown inherently high- and low-sugar genotypes to induce sucrose accumulation (sugarcane ripening). This investigation on proteins at a global scale along with the transcriptomic knowledge-base we already established in the same experimental system (Thirugnanasambandam et al. 2017; Chen et al. 2019), is expected to provide more leads on biochemical aspects of sugar accumulation in sugarcane stem and help identify potential genetic targets involved in sucrose accumulation in sugarcane.

2. Materials and methods

2.1. Plant materials and sample collection

Two sugarcane (*Saccharum* spp. interspecific hybrids) genotypes, ROC22 – a high-sugar (on average 15% sucrose content, ROC5 × ROC69-46) commercial variety grown in China, and GT86-877 – a low-sugar (on average 6% sucrose content, GT82-10 × GT73-11) genotype obtained from a local breeding population, were used for this study (Chen et al. 2021). Each genotype was treated with deionized water or 400 mg/L Ethephon solution (prepared from a commercial product of 40% 2-chloroethyl phosphonic acid, an ethylene-producing compound-trade name Ethephon) as foliar spray till run-off from the lamina in mid-October 2016. The experimental unit (replicate) for both clones was a 5 × 7 m rows plot with 1.2 m interrow spacing, and three replicates were maintained in each treatment. The detailed methodology of plant cultivation and Ethephon treatment (called 'ethylene treatment' hereafter) is given by Chen et al (Chen et al. 2019).

Developing stalk tissues, 20 cm above the node attached to the second youngest fully expanded leaf, which is highly photosynthetically active, were sampled on day 7 following ethylene treatment. The samples were labeled as RCK or R400 for those from ROC22 plants treated with water or 400 mg/L Ethephon, respectively; MCK or M400 for samples from GT86-877 plants treated with water or 400 mg/L Ethephon, respectively. Pooled sample tissues collected from six individual plants from each replicate plot constitute one biological replicate. So, there were three biological replicates for each clone from each treatment and they were flash-frozen in liquid nitrogen for proteome analysis.

2.2. Sample preparation

Sampled tissues (0.5 g per sample) were ground in liquid nitrogen to a fine powder and mixed with 1.5 ml of precooled 90% acetone solution (containing 10% trichloroacetic acid and 0.07% dithiothreitol; DTT) and kept the mixture at −20°C for 2 h for protein precipitation. They were then centrifuged at 10,000 ×*g* for 30 min at 4°C and the precipitate collected was resuspended in the precooled 90% acetone solution (the same composition as above). The suspension was incubated at −20°C for 1 h before centrifuging at 10,000 ×*g* for 30 min at 4°C. The resulting precipitate was washed 3 times by following the same procedure described above, i.e. resuspending the precipitate in precooled 90% acetone solution (the same composition as above) and recovering the precipitate by centrifugation. The washed precipitate was re-dissolved in 300 µL lysis buffer (500 mM triethylammonium bicarbonate, TEAB) and centrifuged at 10,000 ×*g* for 5 min at 4°C, to obtain a clear protein solution. Protein content of the final preparation was determined by Bradford BCA Protein Assay Kit.

2.3. Protein digestion and iTRAQ labeling

Aliquots of the above protein solution (100 µg protein per sample) were transferred to a new 2 mL Eppendorf tube and the volume was adjusted to 100 µL with 100 mM TEAB. To this solution 11 µL 1 M DTT was added and the mixture was incubated at 37°C for 1 h. After incubation samples were transferred to 10 KDa ultrafiltration tube (Millipore, MA, USA) and centrifuged at 14,000 ×*g* for 10 min. Samples were then mixed with 120 µL of 55 mM iodoacetamide and incubated for 20 min under dark at room temperature.

For each sample, proteins were precipitated with ice-cold acetone, and then the precipitated proteins were re-dissolved in 100 µL TEAB, digested with (1:50 mass ratio) sequencing-grade modified trypsin (Promega, Madison, WI), and the resulting peptide mixture was labeled with iTRAQ.

2.4. High pH reverse phase separation

The peptide mixture was re-dissolved in the buffer A (buffer A: 20 mM ammonium formate in water, pH 10.0, adjusted with ammonium hydroxide), and then fractionated by high pH separation using Ultimate 3000 system (ThermoFisher Scientific, MA, USA) connected to a reverse-phase column (XBridge C18 column, 4.6 mm × 250 mm, 5 μm) (Waters Corporation, MA, USA). High pH separation was performed using a linear gradient. Starting from 5% buffer B to 45% buffer B in 40 min (buffer B: 20 mM ammonium formate in 80% acetonitrile, pH 10.0, adjusted with ammonium hydroxide), the column was re-equilibrated at 5% buffer B conditions for 15 min. The column flow rate was maintained at 1 mL/min with its temperature set at 30°C. Twelve fractions were collected and each fraction was dried in a vacuum concentrator.

2.5. Low pH nano-HPLC-MS/MS analysis

The fractions were resuspended in 30 μL buffer C (buffer C: 0.1% formic acid), separated by nanoLC and analyzed by online electrospray tandem mass spectrometry. The experiments were performed on a Nano Aquity UPLC system (Waters Corporation, Milford, MA) connected to a Quadrupole-Orbitrap mass spectrometer (Q-Exactives Plus) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. Five microliter peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm × 2 cm) with a flow of 10 μL/min for 3 min and subsequently separated in the analytical column (Acclaim PepMap C18, 75 μm × 15 cm) with a linear gradient from 2% buffer D to 40% buffer D in 100 min (buffer D: acetonitrile with 0.1% formic acid). The column was re-equilibrated at initial conditions (2% buffer D) for 15 min. The column flow rate was maintained at 300 nL/min at 40°C. The electrospray voltage of 1.9 kV versus the inlet of the mass spectrometer was used.

2.6. Database searching

Tandem mass spectra were extracted, charge state de-convoluted, and de-isotoped by Mascot Distiller version 2.6. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.5.1). Mascot was set up to search the customized RNA-Seq database (86,944 entries) produced in our previous work (Chen et al. 2019). Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine and iTRAQ8plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the n-terminus, and iTRAQ 8-plex of tyrosine were specified in Mascot as variable modifications.

2.7. Criteria for protein identification

Scaffold (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could achieve an FDR below 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.8. Quantitative data analysis and normalization

Scaffold Q+ (version Scaffold_4.7.5, Proteome Software Inc., Portland, OR) was used to quantitate peptide and protein identifications. Normalization was performed iteratively (across samples and spectra) on intensities, as described previously (Oberg et al. 2008). Median values were used for averaging. Spectra data were log-transformed, pruned of those matched to multiple proteins and those missing a reference value, and weighted by an adaptive intensity weighting algorithm. Peptide identifications were accepted for those with less than 1% FDR, as specified by the Peptide Prophet algorithm, or those with at least two identified peptides. Of 194,253 spectra in the experiment at the given thresholds, 171,173 (88%) were quantified. Differentially expressed proteins were determined using Mann–Whitney Test with a significance level $p < 0.05$ adjusted by Benjamini-Hochberg Correction and fold change over 1.2.

2.9. GO analysis

Blast2GO version 4 was used for functional annotation. Whole protein sequence database was analyzed by BlastP using whole database and mapped, annotated with gene ontology database. Statistically altered functions of DEPs were calculated by Fisher's exact test in BLAST2GO (Conesa et al. 2005).

2.10. KEGG analysis

Pathway analysis was processed by KOBAS (http://kobas.cbi.pku.edu.cn/) against Zea mays (Xie et al. 2011). Pathways with P-value < 0.05 were considered as significantly different.

2.11. Protein validation by western blot

To verify the differentially expressed proteins, detected by statistical methods, are indeed biologically relevant to sugar accumulation, the results were further validated using western blot (WB) experiment based on the protocol as follows: SDS-PAGE was used to separate proteins (20 μg) from each sample. They were then transferred to polyvinylidene fluoride membranes which were incubated with appropriate primary antibodies generated by Abmart and the HRP-conjugated anti-mouse IgG secondary antibody (Product No: Abmart, M21001). An enhanced chemiluminescence system (Biouniquer, China) was used to visualize Immune-reactive bands, which were exposed to X-ray film (Kodak). Following this, ImageJ program was used to quantify signal intensities which were normalized to the b-actin signal.

3. Results

3.1. Nearly 140 proteins were differentially expressed between ethylene-treated and untreated high- and low-sugar sugarcane genotypes

A total of 2983 proteins were identified under a peptide threshold of 1.0% FDR and the criterion of 2 unique peptides

by the iTRAQ method (Table S1). The proteins with statistically significant change-fold of more than 1.2 from each pairwise comparison were classified as DEPs. Among them, 14 proteins were down-regulated and 5 of them were up-regulated in the comparison between ethylene-treated and untreated high-sugar genotype (RCK vs. R400). And, 43 proteins were down-regulated and 5 were up-regulated when low-sugar genotypes with or without ethylene treatment (MCK vs. M400) were compared. While 15 proteins were down-regulated and 30 were up-regulated between high- and low-sugar genotype controls (RCK vs. MCK), 40 proteins were down-regulated and 41 were up-regulated (Figure 1(A), Table S2) when ethephon-treated high-and low-sugar plants (R400 vs. M400) were compared. Finally, a total of 139 DEPs were obtained from all pairwise comparisons after eliminating the duplicate proteins (Figure 1(B), Table S3).

3.2. The DEPs identified are mainly involved in the cellular processes related to sugar metabolism

In order to predict the biological functions of DEPs, GO enrichment analysis was conducted. In the GO category of biological processes, DEPs participate in some processes related to sugar metabolism such as carbohydrate synthesis, carbohydrate catabolic processes, photosynthesis, chitin metabolism, and amino sugar metabolism. In the category of molecular function, DEPs are also involved in the sugar metabolism, for example, chitinase activity, chlorophyll binding, 1,4-alpha-glucan branching enzyme activity, and phosphoenolpyruvate carboxykinase activity. While in the category of cellular component, DEPs also take part in sugar metabolism, photosynthetic membrane functions, photosystem function and so on (Figure 2, Table S4).

The KEGG enrichment analysis of DEPs was conducted for a more accurate understanding of the specific metabolic pathways related to sugar metabolism where DEPs are involved. The results showed that DEPs are mainly involved in carbon fixation in photosynthetic tissues, amino sugar and nucleotide sugar metabolism, photosynthesis – antenna proteins, and starch and sucrose metabolism pathway etc. (Figure 3, Table S5), all of which are part of sugar metabolism.

3.3. Identification of proteins involved in sugar metabolism in sugarcane

In order to identify the proteins involved in sucrose metabolism in sugarcane, we further analysed the expression of the DEPs in the metabolic pathways closely related to sugar metabolism.

3.3.1. Carbon fixation pathway

Carbon fixation in plants determines the efficiency of photosynthesis, which may be related to sucrose content in sugarcane. In this study, seven DEPs were found to be involved in this pathway. Among them, m.120013 (fructose bisphosphonate aldolase) and m.2183 (malate dehydrogenase isoform 1) were up-regulated in the comparison group between high- and low-sugar genotypes without ethylene treatment (RCK vs. MCK), i.e. their expression in high-sugar genotype is significantly higher than that in low-sugar genotype (Figure 4; Table S2). But their expression showed no difference in response to

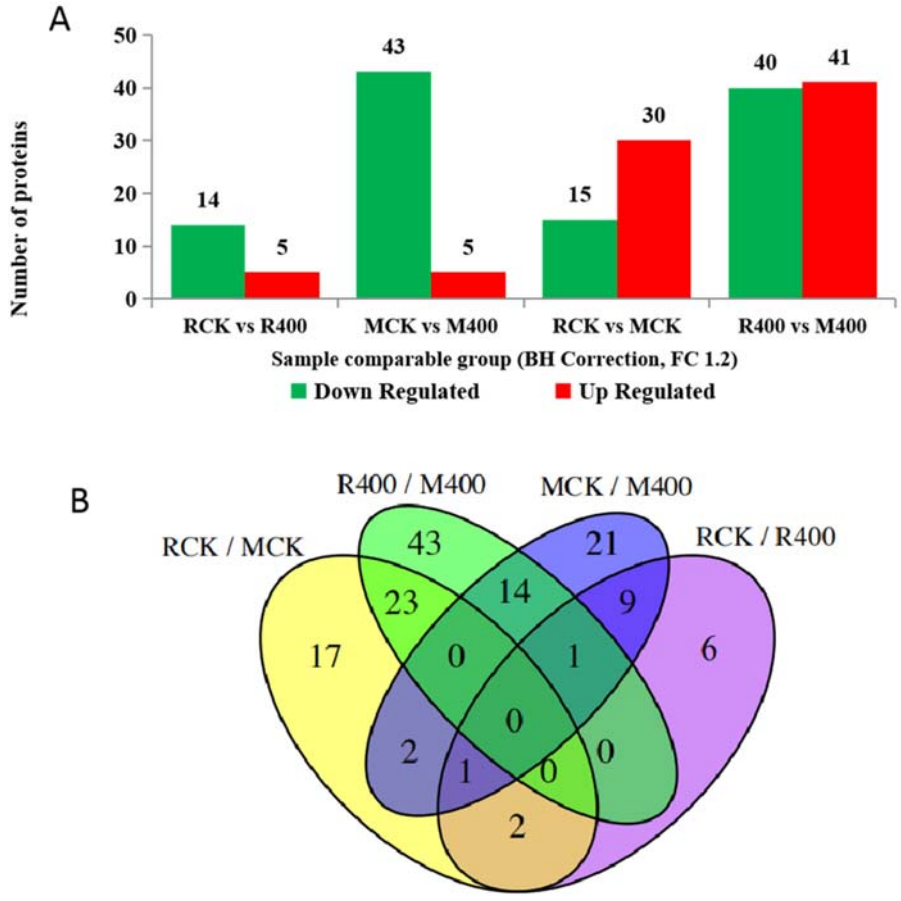

Figure 1. Statistics of differentially expressed proteins by pairwise comparisons. (A) The number of up-regulated and down-regulated proteins in each comparison group; (B) The number of overlapping proteins among the comparison groups.

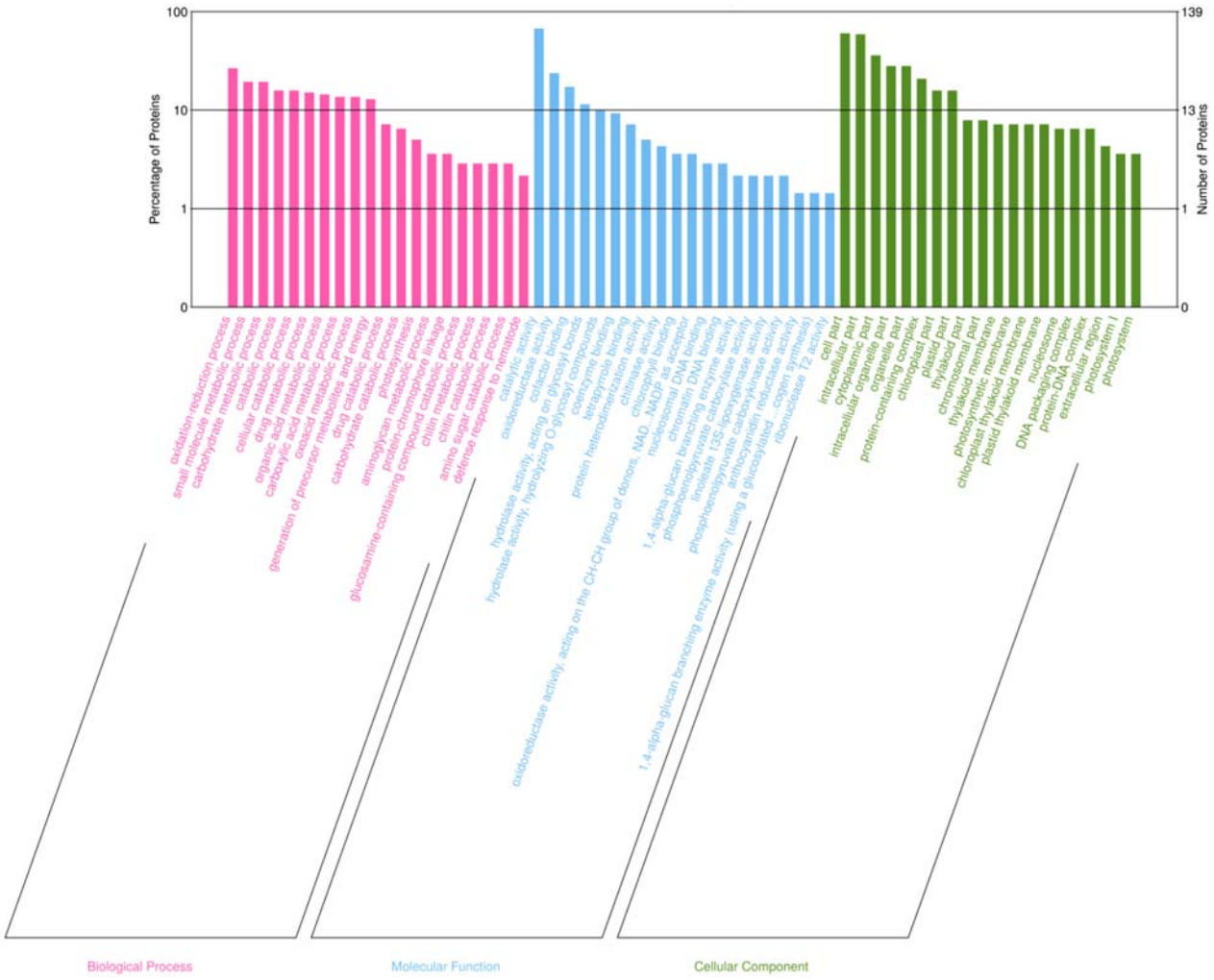

Figure 2. GO enrichment analysis of all differentially expressed proteins identified. The three GO categories are biological processes, molecular function, and cellular components.

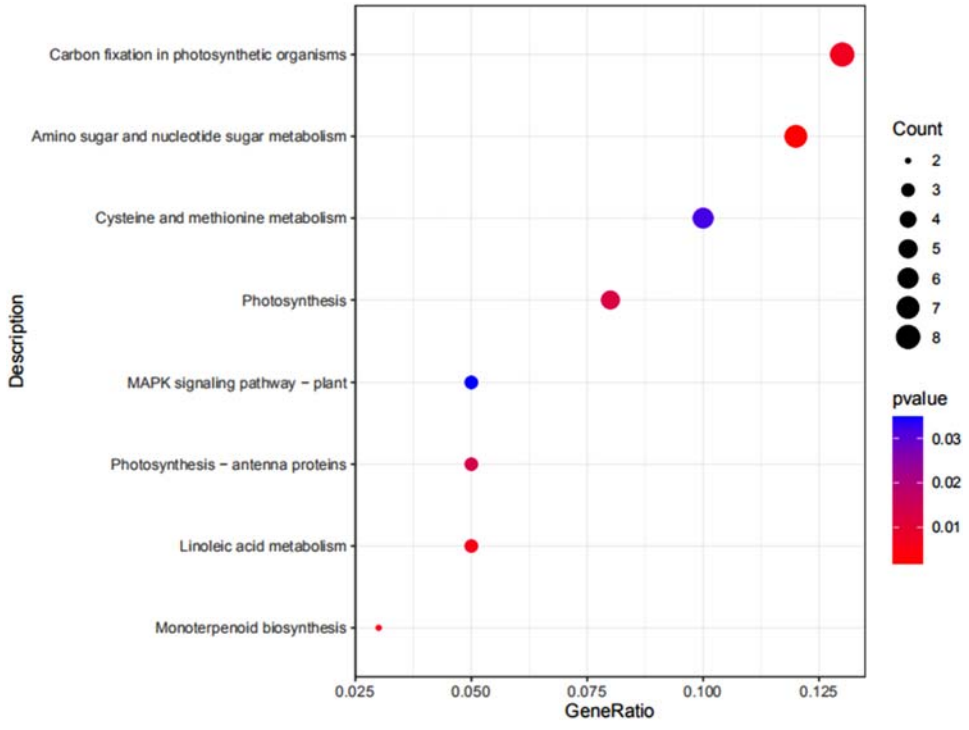

Figure 3. KEGG enrichment analysis of all differentially expressed proteins identified. The size of the dots corresponds to the number of DEPs in each category. The color displays the significance of enrichment.

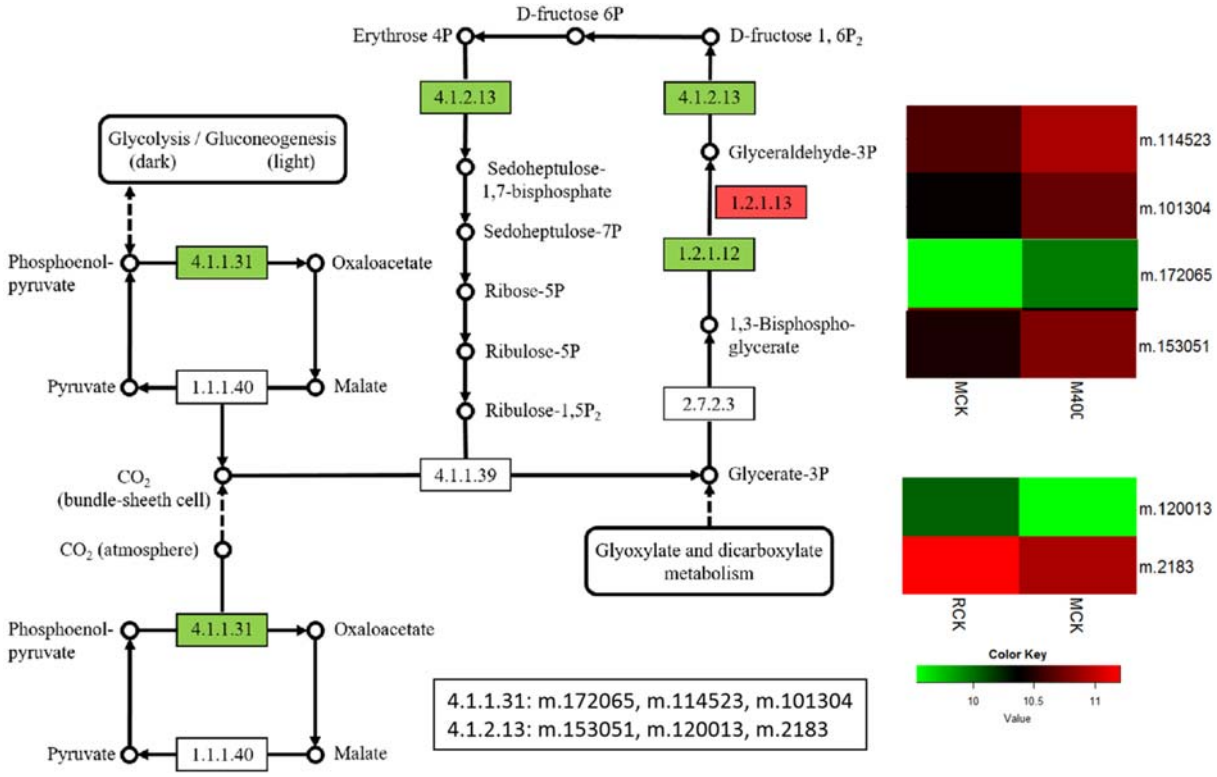

Figure 4. Differentially expressed proteins involved in carbon fixation in photosynthetic organisms pathway by pairwise comparisons. MCK: low-sugar genotype treated with water; M400: low-sugar genotype treated with ethephon: RCK: high-sugar genotype treated with water. Red (high) and green (low) colors represent the relatively abundance of proteins in the color key.

ethylene treatment (Table S2), indicating that the difference in their expression was genotype-dependent. The expressions of m.172065/m., 114523/m.101304 (pep7, phosphoenolpyruvate carboxylase), and m.153051 (fructose bisphosphate aldolase) were induced by ethylene both in high- and low-sugar genotypes, while their abundances did not show any significant variation between high- and low-sugar genotypes, indicating that their expressions were genotype-independent (Figure 4; Table S2). Therefore, the induction of these proteins participating in sugar production may be regulated by ethylene or other genotype-dependent factors.

3.3.2. Amino sugar and nucleotide sugar metabolism pathways

In this category, 6 proteins were identified. The proteins m.91936 (UDP-glucose 6-dehydrogenase), m.48500 (basic endochitinase), m.172302 (chitinase), and m.144144 (hevamine-A precursor) were induced by ethylene in high- and low-sugar genotypes, while m.71264 (chitinase) and m.44939 (chn1, acidic endochitinase-like) were induced by ethylene only in low-sugar genotype. But their expressions showed similar level in high- and low-sugar genotypes. These results indicate that the proteins involved in amino sugar and nucleotide sugar metabolism pathway may be regulated by ethephon, and their expression is genotype independent (Figure S1, Table S2), implicating they may have a role in ethylene-induced sucrose accumulation in sugarcane, the commercial cane ripening process.

3.3.3. Photosynthesis pathway

Plants use photosynthetic pigments such as chlorophyll to capture solar energy to convert CO_2 and H_2O into organic compounds and release O_2 through a series of light and dark reactions. In this study, PsaA (m.120318) and PsaB (m.15233) – components of photosystem I complex, PetA (m.167082) – a component of Cytochrome b6/f complex, and PetE (m.93972, plastocyanin, PC) which is involved in photosynthetic electron transport system, were highly expressed in high-sugar genotype than in low-sugar genotype. But, notably, their expression was not affected by ethylene (Figure S2; Table S2), indicating that the proteins involved in photosynthesis pathway observed in the experimental clones were genotype-dependent, and the differential expression may be, at least in part, contributing to high-sugar phenotype.

3.3.4. Photosynthesis – antenna proteins pathway

The antenna protein complex harvests light energy from sunlight, which is used for driving carbon fixation in plants. In this pathway, m.46259 (chlorophyll a-b binding of LHCII type 1-like), m.46249 (chlorophyll a-b binding chloroplastic-like), and m.69727 (chlorophyll a-b binding chloroplastic), which are the components of light-harvesting chlorophyII protein complex, were highly expressed in high-sugar genotype than in low-sugar genotype (Figure S3; Table S2), indicating that the level of expression of these proteins may be associated with sucrose accumulation, as observed with the photosystem proteins, and it also appears to be a genotype-dependent phenotype.

3.3.5. Starch and sucrose metabolism

The starch and sucrose metabolism pathways determine the distribution of photosynthetic products and ultimately the sucrose content in sugarcane stem. In this study, 6 proteins

participating in these pathways were found differentially expressed between genotypes or treatments. The expression of m.1376/m.1380 (starch-branching enzyme 4) was higher in high-sugar genotype than that in low-sugar genotype. Whereas, the expressions of m.98290 (frk1, fructokinase-1) and m.97819 (beta-glucosidase 30-like) were just the opposite, and the expressions of all these proteins were genotype dependent and not regulated by ethylene. The expression of another protein in this pathway, m.163446 (Alpha-1,4 glucan phosphorylase L-1 isozyme) was higher in high-sugar genotype and was induced by ethephon (Figure S4; Table S2), suggesting that ethylene- and genotype-dependent factors regulate the expression of this enzyme.

3.4. Validation of DEPs relevant to sucrose accumulation

To validate the reliability of proteomics data, the expressions of 4 DEPs involved in sugar metabolism pathways were further verified by western blot. For the protein m.120013 (FBA, fructose-bisphosphate aldolase), we observed a thicker band in the high-sugar content genotype (RCK) than in the low-sugar one (MCK), and it showed no difference in band intensity between low-sugar genotype treated with ethephon (M400) and water control (MCK). This result confirmed the proteomic data on expression of m.120013 in high- and low-sugar genotypes in this study. This result is also consistent with iTRAQ analysis. The western blot results on other three proteins, m.91936 (UGDH, UDP-glucose 6-dehydrogenase), m.97819 (beta-glucosidase 30-like), and m.172302 (chitinase), were also consistent with the results of proteomic analysis, confirming that our experimental results are true and reliable (Figure 5).

RCK: high-sugar genotype with water control; R400: high-sugar genotype with ethylene treatment; MCK: low-sugar genotype with water control. M400: low-sugar genotype with ethylene treatment. Number after sample code (−1, −2, −3) represents the replicate number.

3.5. A few overlaps between DEPs and DEGs

In our previous work, about 25,000 differentially expressed genes (DEGs) were identified from the transcriptome analysis of the same experimental materials (Chen et al. 2019). In this work, 139 DEPs were identified by proteomics analysis with the same samples. The comparisons between the DEPs in this work with DEGs in our previous transcriptome analysis (Chen et al. 2019) were further conducted to identify the genes differentially expressed at both transcriptional and translational levels. To our surprise, only five matching DEGs and DEPs were found, showing the complexity of regulation of biological processes at different molecular hierarchies i.e. at transcriptional and translational levels with genetic and epigenetic operational controls. Three of the matched DEGs and DEPs are related to sugar metabolism. They are m.91936 (UDP-glucose 6-dehydrogenase) in amino sugar and nucleotide sugar metabolism pathway (Figure S1), m.93972 (PetE, plastocyanin) in photosynthesis pathway (Figure S2), and m.97819 (beta-glucosidase 30-like) in starch and sucrose metabolism pathway (Figure S4). The other two are m.72877 (splicing arginine serine-rich 2) and m.80926 (irl1, isoflavone reductase), which may be involved in spliceosome synthesis and catalytic activity pathway (Table S1, Table S5), and may not be related to sucrose accumulation in sugarcane.

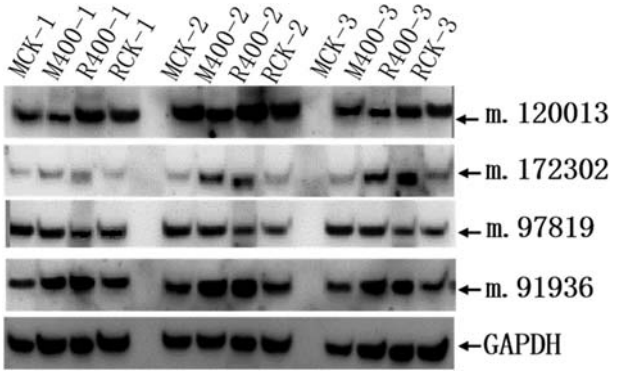

Figure 5. Results of western blot experiment of four differentially expressed proteins.

4. Discussion

4.1. Identification of DEPs associated with sugar metabolism in sugarcane

Proteomics analysis is a powerful tool to study various biological processes and has been applied to understand the molecular and biochemical bases of biotic and abiotic stresses in sugarcane (Barnabas et al. 2015; Fonseca et al. 2018; Salvato et al. 2019; Singh et al. 2019; Meng et al. 2020). For example, proteome analysis helped identify several proteins implicated in resistance to smut disease and leaf scald, and drought tolerance in sugarcane (Salvato et al. 2019; Singh et al. 2019; Meng et al. 2020). These proteins were related to DNA binding, various metabolic processes, defense, stress response, photorespiration, protein renaturation, chloroplast, and nucleus and plasma membrane. However, there is no report on proteomic studies on sugar accumulation process in sugarcane or other crops to date. In this study, we used an advanced proteomics system, iTRAQ technology, to investigate the response of sugarcane proteome to experimental manipulation of stem sugar content in high- and low-sugar genotypes of sugarcane for gaining a better understanding of an unusual biological phenomenon, the accumulation of remarkably high levels of sucrose in stem, a phenomenon not seen in other plants. Analysis of sugarcane developing stem proteome showed that the DEPs identified were involved in pathways associated with sugar metabolism such as carbon fixation, amino and nucleotide sugar metabolism, photosynthesis – antenna proteins, and starch and sucrose metabolism pathways.

4.2. Identification of candidate proteins regulating sucrose accumulation in sugarcane

Sucrose accumulation in sugarcane involves a complex network of multiple metabolic pathways regulated at different levels of organization. Identification of key candidate genes that regulate sucrose accumulation in sugarcane has great practical significance for varietal improvement. Therefore, we further analyzed the expression of DEPs involved in five metabolic pathways closely related to sugar metabolism in sugarcane.

4.2.1. Carbon fixation pathway

Carbon fixation via photosynthesis is the source of sugar metabolism, involving many enzymes and proteins. Fructose-bisphosphate aldolase (FBA, EC4.1.2.13) is one of the key enzymes involved in carbon metabolism and exists widely in prokaryotes and eukaryotes. It catalyzes the cleavage of fructose-1,6-diphosphate (FBP) into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and this reversible reaction is a key step of glycolysis (Lebherz et al. 1984; Tsutsumi et al. 1994). Fructose-bisphosphate aldolase plays important roles in diverse biological processes, such as photosynthesis, sugar and starch biosynthesis (Sonnewald et al. 1994; Haake et al. 1998), CO_2 fixation and plant growth (Uematsu et al. 2012; Cai et al. 2018), as well as biotic and abiotic stresses (Fan et al. 2009; Mutuku and Nose 2012; Khanna et al. 2014). In this study, two FBAs were identified. The expression of one, m.120013 (fructose-bisphosphate aldolase) was higher in high-sugar genotype than in low-sugar genotype, but was not regulated by ethephon. While the other one, m.153051 (fructose-bisphosphate aldolase) was both induced by ethephon in high- and low- sugar genotypes, and its expression was genotype-independent, indicating that fructose-bisphosphate aldolase may have an important role in sucrose accumulation, possibly through multiple ways in sugarcane.

Plant phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a cytoplasmic enzyme, which catalyzes the production of oxaloacetic acid (OAA) from phosphoenolpyruvate (PEP) and HCO^{3-}, which can be converted into various tricarboxylic acid cycle intermediates. PEPC participates in important metabolic processes such as photosynthetic carbon assimilation in plant cells (Shi et al. 2015; Giuliani et al. 2019) and abiotic stress responses (Liu et al. 2017; Waseem and Ahmad 2019). In this work, three PEPC, m.172065/m.114523/m.101304 (pep7, phosphoenolpyruvate carboxylase) were induced by ethephon in high- and low-sugar genotypes, suggesting that ethylene-induced sucrose accumulation, at least in-part, involves upregulation of PEPC, which, in turn, increases photosynthetic carbon fixation.

Malate dehydrogenase (MDH) is a ubiquitous enzyme and reversibly catalyzes the oxidation of malate to oxaloacetate. It participates in many biological processes such as photosynthesis (Tomaz et al. 2010; Lindén et al. 2016), plant growth (Wang et al. 2015), and stress responses (Yao et al. 2011; Huang et al. 2018). A MDH, m.2183 (malate dehydrogenase isoform 1) was found differentially expressed in this study. The expression of m.2183 (malate dehydrogenase isoform 1) in high-sugar genotype was significantly higher than that in low-sugar genotype. Considering its critical role in Kreb's cycle, it was speculated that it might also promote sucrose accumulation indirectly by enhancing sink strength through stalk growth and thereby carbon fixation.

4.2.2. Amino sugar and nucleotide sugar metabolism pathways

There are many enzyme proteins involved in amino sugar and nucleotide sugar metabolism pathways in plants. UDP-glucose 6-dehydrogenase (UGDH) is one of the key enzymes in this pathway, which is involved in the biosynthesis of UDP-glucuronic acid (UDP-GlcA), providing nucleotide sugars for cell-wall polymers (Oka and Jigami 2006). In plant, UGDH is associated with biomass yield (Assanga et al. 2017), polysaccharide synthesis (Xue et al. 2008; Wang et al. 2017), and abiotic stress responses (Yin et al. 2014). In our work, the expressions of m.91936 (UDP-glucose 6-dehydrogenase) were induced in both high- and low-sugar genotypes, indicating that UGDH is responsive to ethylene and might be involved in sucrose accumulation.

Plant chitinases, glycosidase enzymes that hydrolyze β-1,4-glycosidic linkages of chitin to N-acetylglucosamine, are widely found in plant cells (Kasprzewska 2003). And, they are mainly involved in plant development (Kragh et al. 1996), symbiotic nitrogen fixation (Malolepszy et al. 2018), and plant defense against fungal pathogens (Cletus et al. 2013; Durechova et al. 2019). Ethylene, jasmonic acid, and salicylic acid were found to induce chitinase expression (Kasprzewska 2003). In this work, four chitinases were identified. Among them, m.172302 (chitinase) and m.144144 (hevamine-A precursor), two basic vacuolar chitinases with lysozyme activity (Subroto et al. 1996), were induced by ethephon both in high- and low-sugar genotypes. While m.71264 (chitinase) and m.44939 (chn1, acidic endochitinase-like), which also play an important role on disease defense reaction (Kurilla et al. 2019), were induced by ethylene only in low-sugar genotype. The results suggest that different chitinases elicit diverse functions in sugarcane. In our experimental system, whether they are involved in sucrose synthesis or only as a response to ethylene which is also produced during pathogen attack remains unclear.

4.2.3. Photosynthesis pathway

Numerous important protein complexes are involved in photosynthesis, including photosystem II (PSII), cytochrome b6f (Cytb6f), photosystem I (PSI), photosynthetic electron transport system, and ATP synthase (ATPase) (Caffarri et al. 2014; Kouřil et al. 2018). The expression of these proteins determines the level of photosynthesis in most plant growth conditions (Monde et al. 2000; Tozawa et al. 2007; Pesaresi et al. 2009; Millaleo et al. 2013; Ivanov et al. 2015). In this work, the expression of PsaA (m.120318) and PsaB (m.15233) in the photosystem I complex, PetA (m.167082) in cytochrome b6/f complex, and PetE (m.93972) in photosynthetic electron transport complex were not induced by ethylene, but were higher in high-sugar genotype than that in low-sugar genotype (Figure S2; Table S2). This finding indicates that the photosynthetic activity in high-sugar genotype was stronger than that in low-sugar genotype, resulting greater sucrose accumulation in high-sugar genotype. This may be one of the key reasons causing the high sugar content in high-sugar genotype, and it also suggests that sucrose accumulation is positively correlated to photosynthesis at least in some genotypes.

4.2.4. Photosynthesis – antenna protein pathway

The capture of light energy in photosynthetic organs is the beginning of photosynthesis and photosynthetic antenna proteins are an integral part of that process. The photosynthetic antenna is a protein complex carrying pigments, which is organized in a specific way around the reaction center to ensure efficient transmission of photons to the light-active pigments. PS I and PS II have their own antennas (Ruban 2015; Liu and Blankenship 2019). In this work, the

proteins involved in light-harvesting chlorophyII protein complex in the antenna of PS II, m.46259 (chlorophyll a-b binding of LHCII type 1-like), m.46249 (chlorophyll a-b binding chloroplastic-like), and m.69727 (chlorophyll a-b binding chloroplastic) showed higher expression in high-sugar genotype than in low-sugar genotype, but were not induced by ethylene (Figure S3; Table S2). The level of activity of photosynthetic antenna protein complex in sugarcane is thus genotype-dependent. The high-sugar genotype owing to higher expression of photosynthetic antenna protein could capture more energy for driving higher rate of photosynthesis with the potential for increased carbon fixation high sugar accumulation.

4.2.5. Starch and sucrose metabolism

Plants convert sucrose into glucose and fructose using invertase (INV, EC 3.2.1.26) and sucrose synthase (SUSY, EC 2.4.1.13), these sugars are phosphorylated for further metabolic processes. Fructokinases (FRK, EC 2.7.1.4) play an important role in the phosphorylation of fructose (Pego and Smeekens 2000), and the expression of FRK is negatively correlated with sucrose accumulation (Yang et al. 2018). One FRK, m.98290 (frk1, fructokinase-1) was identified in this work. The expression of m.98290 was higher in low-sugar genotype than that in high-sugar genotype (Figure S4; Table S2), but not induced by ethylene, suggesting a relatively higher sucrose degradation and a possible reduction in sucrose accumulation in low-sugar genotype. The FRK is a key enzyme in sucrose metabolism and should be a priority molecule for further functional characterization.

Starch-branching enzyme (SBE) catalyzes the branching point of α – 1, 6-glycosidic bond to form amylopectin. It is a key enzyme involved in starch biosynthesis (Martin and Smith 1995). However, in this study, it was found that the expression of m.1376/m.1380 (starch branching enzyme 4) was higher in high-sugar genotype than that in low-sugar genotype and also not induced by ethylene, implicating that stronger ability of starch synthesis, in addition to sucrose accumulation, exits in the high-sugar genotype. This is an interesting observation and warrants further analysis from a global carbon acquisition and storage perspective in high-sugar clones.

β – D-glucosidase (EC3.2.1.21) is an important member of cellulase system. It hydrolyzes the terminal non-reducing β – D-glucose bond and releases β – D-glucose and the corresponding ligands (Hrmova et al. 1998; Maugard et al. 2002). Plant β – D-glucosidase was reported to be involved in stress responses (Mahajan et al. 2015). In the current study, the expression of two beta-glucosidases, m.27280 (beta-glucosidase 22-like) and m.97819 (beta-glucosidase 30-like), were higher in low-sugar genotype than that in high-sugar genotype, but not induced by ethylene, indicating that the beta-glucosidase may have a negative influence on sucrose accumulation in sugarcane.

Plant α-glucan phosphorylase, also known as starch phosphorylase (EC 2.4.1.1), decomposes starch by phosphorylation (Buchner et al. 1996; Goren et al. 2018; Hwang et al. 2020). In this work, the expression of m.163446 (alpha-1,4 glucan phosphorylase L-1 isozyme) in high-sugar genotype was significantly higher than that in low-sugar genotype, and was further induced by ethylene in high-sugar genotype, providing strong pointers that it might be involved in sucrose accumulation through degradation of starch.

4.3. Comparision of DEPs and DEGs

In our previous work, we identified 24,938 DEGs from the transcriptome analysis with the same samples as those used in this study, and the DEGs were involved in photosynthesis, plant hormone signal transduction, plant-pathogen interaction, starch and sugar metabolism, stress responses, lipid metabolism, apoptosis, and amino sugar and nucleotide sugar metabolism. Further pairwise comparisons of DEGs showed that the expression of unigenes involved in starch and sucrose metabolic pathways, such as genes encoding invertase (INV), polygalacturonase, 6-phosphofructokinase, pectinesterase, phloem sucrose loading enzymes among others, were higher in high-sugar genotype than that in low-sugar genotype. In addition, the genes participating in Photosystem I and Photosystem II protein complexes were also differentially expressed in low- and high-sugar clone comparisons. Moreover, genes involved in sucrose synthesis and transportation, such as the cytosolic acid invertase, cell wall invertases, starch phosphorylase (SPase), sucrose phosphate synthase (SPS), and sucrose synthase (SuSy), also showed genotype-dependent or ethephon-induced responses (Chen et al. 2019). In this work, 139 DEPs were identified by proteomics analysis from the samples. The DEPs mainly participated in the pathway related to sugar metabolism, such as carbon fixation, amino sugar and nucleotide sugar metabolism, photosynthesis, photosynthesis – antenna proteins and starch and sucrose metabolism pathway, which is similar to the pathways the DEGs involved in (Chen et al. 2019).

In our previous work, using the same experimental samples about 25,000 DEGs were identified following transcriptome analysis. However, in this work, only 139 DEPs were identified by proteomics analysis. By comparing the DEPs with the DEGs from previous work (Chen et al. 2019), we found that only five proteins were differentially expressed at both transcriptional and translational levels. Many DEGs identified in our previous transcriptomic study were not represented in DEPs detected in the proteome analysis. The main reason may be the detection accuracy caused by different analysis methods. It is very difficult to detect proteins that are present in very low amounts and more importantly, both transcriptome and proteome are highly dynamic temporarily and spatially (Renaut et al. 2006). Furthermore, post-transcriptional and post-translational modifications may also affect DEGS and DEP expression and presence, and consequently their overlap. Similar result was also found in the proteomics of sugarcane infected with smut, which is thought to be due to both translational and post-translational modifications (Su et al. 2016).

Despite the low colinearity of DEPs and DEGs, DEPs identified in this study provided new knowledge on genotypic variability of sucrose accumulation phenotype which helps formulate specific research questions to further our understanding of sucrose accumulation in sugarcane. The results of proteins involved in carbon fixation and related metabolic processes maybe, at least in part, accounts for sucrose accumulation in high- and low-sugar genotypes (Figure 6, Table 1).

Ethephon (ethylene)-enhanced carbon fixation by accelerated expression of antenna proteins, PsaA, PsaB, PetA, and PetE proteins and key sugar metabolism enzymes, such as fructose-bisphosphate aldolase (FBA), phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase isoform

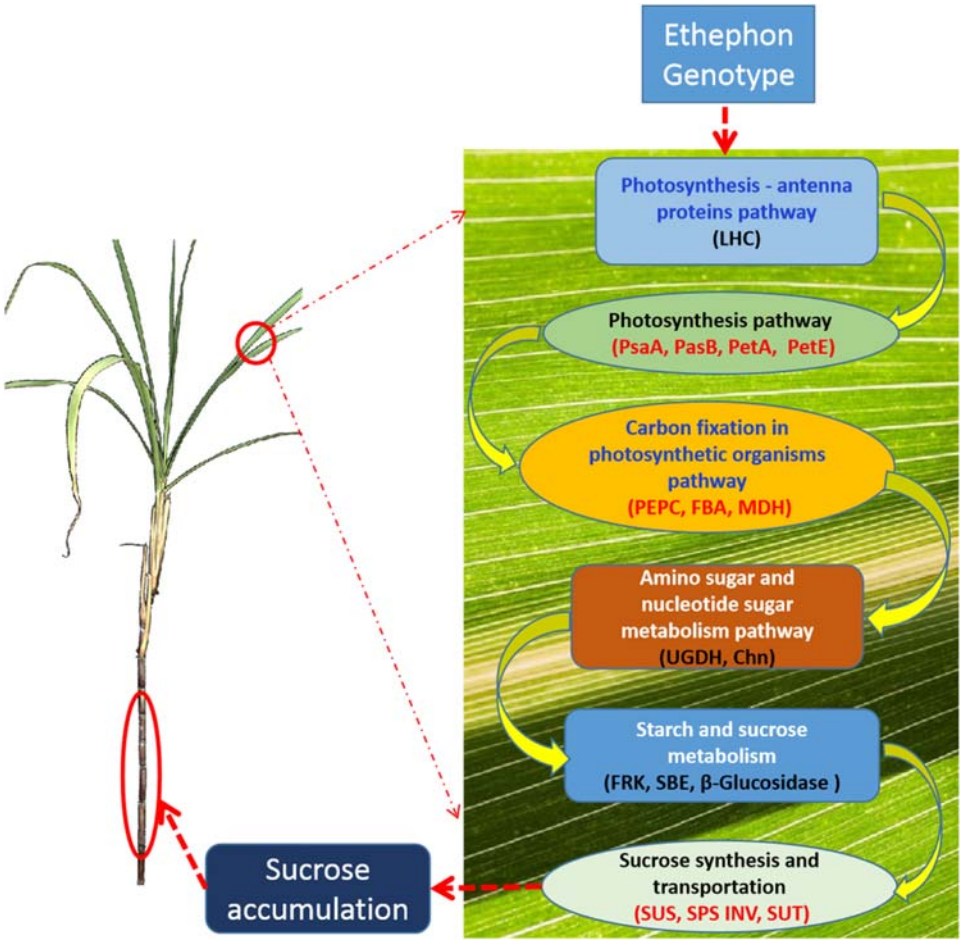

Figure 6. Schematic representation of a model of sucrose accumulation in sugarcane.

1 (MDH) and UDP-glucose 6-dehydrogenase (UGDH) in carbon fixation, and amino sugar and nucleoside sugar metabolism pathways. In addition, ethylene also regulates the expression of key enzymes in starch and sucrose metabolism pathways such as fructokinase (FRK), starch branching enzyme (SBE), α – glucan phosphorylase, and those involved in sucrose synthesis and transport such as sucrose synthase (SUS), invertases (INV), sucrose phosphate

Table 1. Summary of candidate proteins related to sucrose accumulation in sugarcane.

			Change fold between pairwise comparison		
Pathway	Protein ID	Annotation	R400 vs. RCK	M400 vs. MCK	RCK vs. MCK
Carbon fixation in photosynthetic organisms pathway	m.120013	FBA, fructose-bisphosphate aldolase	/	/	+
	m.2183	MDH, malate dehydrogenase isoform 1	/	/	+
	m.172065/m.114523/m.101304	PEPC, phosphoenolpyruvate carboxylase	+	+	/
	m.153051	probable fructose-bisphosphate aldolase	+	+	/
Amino sugar and nucleotide sugar metabolism pathway	**m.91936**	**UGDH, UDP-glucose 6-dehydrogenase**	+	+	/
	m.48500	basic endochitinase	+	+	/
	m.172302	chitinase	+	+	/
	m.144144	hevamine-A precursor	+	+	/
	m.71264	chitinase	/	+	/
	m.44939	chn1, acidic endochitinase-like	/	+	/
Photosynthesis pathway	m.120318	PsaA, photosystem I P700 chlorophyll A apoprotein A1	/	/	+
	m.15233	PsaB, photosystem I P700 apoprotein A2	/	/	+
	m.167082	PetA, cytochrome f	/	/	+
	m.93972	**PetE, plastocyanin**	**/**	**/**	**+**
Photosynthesis – antenna proteins pathway	m.46259	chlorophyll a-b binding of LHCII type 1-like	/	/	+
	m.46249	chlorophyll a-b binding chloroplastic-like	/	/	+
	m.69727	chlorophyll a-b binding chloroplastic	/	/	+
Starch and sucrose metabolism	m.98290	frk1, fructokinase-1	/	/	–
	m.1376/m.1380	starch branching enzyme 4	/	/	+
	m.27280	beta-glucosidase	/	/	–
	m.97819	**beta-glucosidase 30-like**	**/**	**/**	**–**
	m.163446	Alpha-1,4 glucan phosphorylase L-1 isozyme	+	/	+

Note: +: change folds positively above 1.2. –: change folds negatively above 1.2. /: change folds below 1.2. Gray background fonts indicate proteins with differential expression both at transcriptome and proteomics levels in the same comparsion group.

synthase (SPS), and sucrose transporters (SUT). Note that genotype plays a major role in sucrose accumulation in sugarcane and the differential expression of many of the above-mentioned genes seen in this study may underpin the genotype effect.

5. Conclusion

In this study, we identified 25 proteins possibly involved in sucrose accumulation in sugarcane. These proteins include fructose-bisphosphate aldolase (FBA), phosphoenolpyruvate carboxylase (PEPC), and malate dehydrogenase (MDH) involved in carbon fixation; UDP-glucose 6-dehydrogenase (UGDH) associated with amino sugar and nucleotide sugar metabolism; PsaA, PsaB, PetA and PetE in photosynthesis pathway; photosynthesis – antenna proteins; fructokinase (FRK), β – D-glucosidase and α – glucan phosphorylase involved in starch and sucrose metabolism, respectively. Some of these proteins are the candidate targets for further functional analysis by reverse and forward genetics.

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DLH, CXQ, ZLC, MHL, and PL designed the study. CXQ, ZLC, MW, FL, and MQW performed the field and laboratory experiments. AML and MQW conducted data analysis, DLH wrote the manuscript. PL and YRL revised the manuscript. All authors reviewed the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Availability of data and material

All the data supporting the findings of this study are available within the article and its supplementary materials. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al. 2019) partner repository with the dataset identifier PXD024569.

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