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Biological factors driving YCS : Final report submitted to Sugar Research Australia

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Sugar Research Australia Limited

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Biological Factors Driving YCS

Final report submitted to Sugar Research Australia

By:
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Research Organisations:
Sugar Research Australia Limited

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PART A

Section 1: Executive Summary

Yellow Canopy Syndrome (YCS) was first observed in 2012 in the Central and Northern cane-growing regions in Australia and has since spread much further south. The 2015 season is particularly bad in the Mackay and Proserpine regions. YCS is still an undiagnosed condition and is not correlated to mineral nutrient deficiency or any known sugarcane pathogens. When the severity of YCS expression is high and appears several times during the season, more than 50% crop loss can occur.

In this project, we have established that expression of YCS is associated with an increase in soluble sugars, especially sucrose, glucose, fructose, and trehalose in the leaves. There is also a significant suppression of leaf photosynthesis, reduced stomatal conductance, increased variable fluorescence, and accumulation of abscisic acid. This disturbance in leaf metabolism is evident throughout the canopy even in the absence of visual yellowing. It is a common comment from growers and productivity services staff that YCS is associated with a slowdown in growth and sometimes the crop does not respond at all to rainfall and other favourable growth conditions. In many ways, this is typical of the reduced growth phenomenon (RGP). There cannot be any doubt of yield loss occurring when YCS symptoms appear.

We have identified more than 200 metabolites from an extraction cohort of more than 1500. The most striking differences relate to changes in sugars. However, there are additional changes that could be very important. Firstly, there are significant levels of mannitol, kestose, and lactose in the samples prepared from YCS expressing tissues. These are indicative of microorganisms (Leuconostoc) that normally associate with injured tissue, especially where there are significant available carbohydrates. Secondly, there are increases in various organic and amino acids, some of which are indicative of abiotic stress. Thirdly, there are significant increases in several stress related metabolites, such as caffeoyl/chlorogenic type compounds, which are indicative of wounding and activation of plant defence systems.

We have sequenced the transcriptome of control and YCS symptomatic leaves. The results indicate YCS has a wide impact on primary, secondary, and regulatory metabolism. Close to 4000 genes are upregulated greater than 5 fold in the YCS symptomatic leave tissue. We have ascribed a putative identity to 3191 of these sequences. More than 500 of the sequences are upregulated greater than 100 fold. These genes include those involved in protease regulation, sugar metabolism and transport, responses to auxin and abscisic acid (ABA) levels, chloroplast and heat shock protein production, regulation of ubiquitin conjugating enzymes and several cytochrome P450 genes.

More than 2000 genes are down regulated in the YCS symptomatic leaves. A putative identity has been ascribed to 1263 of these. More than 150 of these are down regulated more than 100 fold. Functions associated include those of chloroplast structural and function genes, senescence related genes, transcription factors, phosphofructokinases, glycolytic enzymes, pathogenesis related genes, trehalose metabolism genes, and primary carbon fixation genes.

The metabolome and transcriptome databases that were developed in this project will form the core of continued work on YCS. In addition, this will be an invaluable database for any further research on sugarcane responses to stress and sugar level control.

Based on the results obtained in this study we propose that the development of YCS symptoms in sugarcane is driven by an accumulation of sucrose, glucose and fructose. The increase in sugars is already evident in leaf 3 and older while symptom expression mostly occurs much lower in the canopy. The pattern of yellowing shows remarkable resemblance with those in maize mutants that are defective in sucrose loading of the phloem and phloem translocation of sugars in the minor veins.
The accumulation of sucrose at the site of phloem loading in the minor vein is likely to create feedback inhibition on photosynthesis, as well as partially disrupt electron transport, which then leads to enhanced sucrose levels at the stomata. This would result in partial stomata closure, which further compromises photosynthetic efficiency and evaporative cooling. This combination of reduced stomatal conductance together with electron transport inefficiency will make the crop particularly vulnerable to environmental stresses such as water and heat. In older leaves where photosynthesis and metabolism is already reduced to natural leaf senescence, these stress events are enough to ‘push’ the metabolism over a threshold where photo-oxidation and the disruption of metabolism triggers yellowing.

Section 2: Background

Yellow Canopy Syndrome (YCS) was first observed in 2012 and is still an undiagnosed condition affecting the Central and Northern cane-growing regions in Australia. Initial analyses failed to find any correlation between the development of YCS and nitrogen, iron and all other mineral nutrients as well as any known sugarcane pathogens. The development of YCS is also distinctly different from that of Sugarcane Yellow Leaf Virus (SCYLV) which was detected initially in Hawaii and Brazil (Comstock et al. 1994; Vega et al. 1997) in the 1990s and is now recognised worldwide. The pathogen responsible, the sugarcane yellow leaf virus (ScYLV) was identified as a luteovirus (Moonan et al. 2000; Smith et al. 2000) and YCS affected sugarcane plants consistently test negative for the presence of ScYLV.

It is well known that, under conditions of high carbohydrate accumulation, photosynthetic gene expression can be down regulated and chlorosis can be induced. Studies have shown that soluble sugars and starch rise to a level higher in barley yellow dwarf virus (BYDV) (Esau 1957; Jensen 1996) and ScYLV infected than non-infected leaves (Yan et al. 2010). Indeed, higher hexose concentrations are reported in the leaves and stem juice of ScYLV-infected plants expressing disease symptoms (symptomatic) than in healthy plants (Fontaniella et al. 2003b; Izaguirre-Mayoral et al. 2002) which correlates with ultra-structural changes in the bundle sheath cells of symptomatic leaves (Fontaniella et al. 2003a).

Similarly, yellowing of leaves can be induced by allowing for carbohydrate synthesis while blocking their translocation (Braun et al. 2006; Graham and Martin 2000; Krapp and Stitt 1995; Russin et al. 1996). In maize and other members of the Poaceae family, starch and anthocyanin can accumulate in the leaves when sugar concentrations are abnormally high (Allison and Weinmann, 1970; Thiagarajah et al., 1981; Tollenaar and Daynard, 1982).

The purpose of this study is twofold. Firstly, to determine to what extent photosynthesis, transpiration and fluorescence are impacted in the yellow leaves. Secondly, to determine whether there are changes in the soluble sugars associated with the development of YCS.

References


Section 3: Outputs and Achievement of Project Objectives

Project objectives, methodology, results and discussion

1. Introduction

The project had three distinct phases. Initially we sampled material from the Herbert and Burdekin regions and analysed all leaves on the stool. The variety sampled in the Herbert was Q200^A and in the Burdekin was KQ228^A. Photosynthesis, stomatal conductance, and fluorescence were measured, and leaf disks were collected for biochemical and gene expression studies.

Table 1: Details of the four sampling field visits in this study.

In all the work reported here, leaves on each stalk (Fig 4 A & B) were numbered wherein the leaf with the first visible dewlap was deemed leaf 1 (Bonnett 2014).

<table>
<thead>
<tr>
<th>Field Visit</th>
<th>Date of sampling</th>
<th>Location</th>
<th>Genotype</th>
<th>FVD = Leaf # 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Feb 24-27, 2014</td>
<td>Herbert</td>
<td>Q200^A</td>
<td>All leaves</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burdekin</td>
<td>KQ228^A</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>May 12-15, 2014</td>
<td>Herbert</td>
<td>Q200^A</td>
<td>Leaf 2 &amp; 4</td>
</tr>
<tr>
<td>4</td>
<td>July 1-2, 2014</td>
<td>Herbert</td>
<td>Q200^A</td>
<td>Leaf 2 &amp; 4</td>
</tr>
<tr>
<td>6</td>
<td>Jan 19-22, 2015</td>
<td>Herbert</td>
<td>Q200^A</td>
<td>Leaf 4 &amp; 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Burdekin</td>
<td>KQ228^A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mackay</td>
<td>Q208^A</td>
<td></td>
</tr>
</tbody>
</table>

In phase two we concentrated our efforts on leaf 2 and 4 and the internodes attached to these leaves. In addition to other measurements, xylem sap from the whole stalk was also extracted. This work was primarily restricted to Q200^A in the Herbert.

In phase three we sampled Q200^A from the Herbert, KQ228^A from the Burdekin and Q208^A from Mackay.

In the last round of sampling, we concentrated on chlorophyll fluorescence, biochemical and gene expression analysis, and we collected mid-leaf segments rather than leaf disks. A summary of the four different sample collections are indicated in Table 1.

2. Material

At all collection sites, sugarcane stalks showed a reasonable number of leaves with typical symptoms of YCS, particularly in leaf number 5 and beneath (leaf tissue from YCS expressing stalks was only taken from green lamina tissue).

2.1 Method of sampling

Five stalks were harvested from each YCS-symptomatic or non-symptomatic (control) stool and leaf samples combined to become one replicate. Four replicates were collected for each condition (i.e. 20 stalks per condition). Stalks were selected, tagged and cut in the field. Four leaf disks were punched from the mid-leaf region of each leaf 2 and 4 from each stalk using a standard single hole punch (6mm Ø). This was repeated for leaf sheaths. The leaf and sheath disks were snap frozen in liquid nitrogen before being stored at -80°C for processing later (Fig 2.1 & 2.2).
**Figure 2.1:** General sampling overview for Field visits 2, 3 & 4.

**Figure 2.2** Mid-leaf and disk sampling schematic.
The nodes of each sampled stalk were cut out with secateurs and the upper end (based on plant orientation growing in the field) was cut at a 45 degree angle. Xylem sap was then collected from each of the 5 stalks in 2mL screw cap tubes using an air compressor in conjunction with a pressure release gun attached to a rubber nozzle. Sap was pushed from the internodes in the direction of xylem flow (bottom to top). The sap was snap frozen in liquid nitrogen before being transported and stored, all at -80°C or below.

In the final field collection (FV6), region specific genotypes were sampled. YCS-symptomatic and control leaf 4 and 6 were sampled for mid-leaf section, internode core and sap from three Queensland sites (Herbert: Lawrence DiBella, Ingham; Burdekin: Ian Shepherdson, Home Hill; Mackay: Paul Schembri, Farleigh) (Fig 2.3). In all three sites, leaf 6 displayed typical YCS phenotype while leaf 4 appeared asymptomatic. All control stalks appeared healthy.

Herbert & Burdekin: 10 stools sampled; three YCS and three control stalks from the same stool (total 60 samples/site). Mackay: three YCS and three control stalks not always from the same stool but from the same row in close proximity to each other where possible (total 60 samples).

One senescent leaf was collected from the lower region of a stalk expressing YCS symptoms and one from a control stalk, this was for replicate 1 only; 10 stools were sampled per condition (total 20 samples/site).

For FV2, 3 and 4, five stalks were harvested from each YCS-symptomatic or control stool and leaf samples combined to become one replicate. Four replicates were collected for each condition (i.e. 20 stalks per condition). Stalks were selected, tagged and cut in the field. Four leaf disks were punched from the mid-leaf region of each leaf 2 and 4 from each stalk using a standard single hole punch (6mm Ø). The leaf disks were snap frozen in liquid nitrogen before being stored at -80°C for processing later.
For FV6 sampling was done in the early morning (first light). A 300 mm piece was cut from the middle section of leaves 4 and 6. A 50 mm piece was cut from the bottom end of each 300 mm leaf piece and three pieces of leaf 4 and 6 (i.e. reps 1-3) for each treatment were snap frozen in liquid nitrogen and then stored at -80 °C. The remaining 250 mm leaf samples for each treatment were also snap frozen in liquid nitrogen and then lyophilised in a CHRIST ALPHA 1-4 LSC plus freeze dryer. Scissors were used for all leaf cutting and sprayed with 70% ethanol and wiped between samples.

**Figure 2.4:** A) Schematic diagram of sugarcane leaf numbering system used during sampling; first visible dewlap is leaf 1. B) Photo of sugarcane top with leaf numbering system used during sampling; first visible dewlap is leaf 1. C) Sap extracting apparatus where internode piece is inserted into rubber tubing and regulated compressed air released to push sap out of the xylem into collection tube. D) Diamond Drill Bit Core Blue Ceram (8mm Ø) used to sample internode core section, forceps used to remove sample before placing in collection tube.

Senescent leaf samples were taken as a 250 mm piece cut from the middle of each senescent leaf of the sample replicate 1 stalk for both YCS-symptomatic and control. Samples were collected and snap frozen in liquid nitrogen and then lyophilised as above.

In FV6, xylem sap and internodes were sampled directly beneath leaf sheath 4 and 6 while maintaining correct orientation. Cut ends of the internodes were then blotted with tissue paper for approximately 5 seconds to absorb remnants of ruptured cells. A pressure regulated sap extractor kit was used to push sap (min 70 kPa - max 140 kPa) from the internode under...
constant and controlled pressure (in the direction from bottom to top) into a plastic funnel above a labelled 2 mL screw cap tube (Fig. 2.4C). Sap was snap frozen in liquid nitrogen and stored at -80 °C.

A section from the bottom of the same internode approximately 30mm long was cut and an 8mm Ø cylindrical core was bored off-centre (avoiding the pith) and vertically down using a 12mm cordless drill and Sutton Diamond Drill Bit Core Blue Ceram 8mm Ø (Model Number 370400080) (Fig. 2.4D). Cylindrical samples were placed in a labelled 2mL screw cap tube and snap frozen in liquid nitrogen and stored at -80 °C. The drill bit borer was sprayed with 70% ethanol and wiped between samples.

2.2 Photosynthesis

Measurements on all available green leaves starting from one above the youngest fully expanded leaf were taken throughout the day to encompass a range of vapour pressure deficit (VPD), radiation, light and other environmental stress conditions. Following standard settings recommended for C4 plants, leaf gas exchange measurements were made twice a day on control and YCS-symptomatic plants in group d using two LiCOR 6400 instruments (Long et al., 1996). The stomatal conductance, leaf level photosynthesis, internal CO$_2$ (Ci) and intrinsic transpiration efficiency were also measured during the day.

During gas exchange measurements, the sample CO$_2$ concentration and airflow rate was maintained at 400 µmolm$^{-2}$s$^{-1}$ and 500 mol m$^{-2}$s$^{-1}$, respectively. The photosynthetically active radiation (PAR) was maintained at 1500 mmol m$^{-2}$s$^{-1}$ with the internal red, blue and green light sources. The intensity of blue light in the light source was 10%, while red was 80%. The standard CO$_2$ matching option was used after each set of measurements for greater accuracy.

2.3 Chlorophyll A fluorescence

Chlorophyll a O–J–I–P fluorescence transients (Strasser and Govindjee 1992) were recorded from leaves 1, 3 and 5. Measurements were performed on the broadest midsection of the leaves, of a minimum of 10 plants for each group (with or without visual expression of YCS). Measurements were conducted with a PEA fluorescence meter (Hansatech Instruments Ltd., King’s Lynn, Norfolk, PE 30 4NE, UK). The transients were induced by a red light (peak at 650 nm) of 3,200 µmol m$^{-2}$s$^{-1}$ provided by the PEA instrument through an array of six light-emitting diodes (van Heerden 2014). The JIP-test (Strasser and R.J. 1995) was subsequently employed to analyse each recorded transient. The following data from the original measurements were used: maximal fluorescence intensity (FM); fluorescence intensity at 50 µs (considered as F0); fluorescence intensity at 300 µs (F300 µs) required for calculation of the initial slope (M0) of the relative variable fluorescence (V) kinetics; the fluorescence intensity at 2 ms (the J step) denoted as FJ. VJ was calculated as (FJ - F0)/(FM - F0). The JIP-test (Strasser and R.J. 1995) was used to translate the original recorded data to biophysical parameters that quantify the stepwise energy flow through Photosystem II. A multi-parametric expression performance index ($P_{ABS}$), was also calculated (Strasser et al. 2000). The $P_{ABS}$ considers the three main steps that regulate photosynthetic activity by a Photosystem II reaction center (RC) complex, namely absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET). The formulae used to calculate each of these biophysical parameters from the original fluorescence measurements are as previously detailed (van Heerden et al. 2007b).
2.4 Sample Processing

2.4.1 Lyophilised leaf material

After lyophilisation of mid leaf samples, the midrib was removed and leaves cut into approximately 3 mm² pieces with scissors and then transferred to reinforced 2ml tubes containing two stainless steel grade 316, 5mm Ø balls. The sample was ground to a fine powder using a retch MM300 bead mill (frequency 30 hz, 4 cycles of 2.5 minutes with 2 minutes pause between each cycle to allow any heat to dissipate).

Powder from replicates were then equally combined and thoroughly mixed to make one stock sample. Dry powder portions of 100 mg were sent to the labs outlined below for metabolome and hormone analysis.

- Metabolomics Australia
  - School of Botany
  - The University of Melbourne
  - Parkville
  - Victoria 3010

- National Research Council of Canada
  - Plant Biotechnology Institute
  - 110 Gymnasium Place
  - Saskatoon
  - Saskatchewan S7N 0W9
  - Canada

2.4.2 Xylem sap

A 225 µL combined total of xylem sap from replicates was snap frozen in liquid nitrogen and despatched on dry ice to Metabolomics Australia for analysis.

2.4.3 Sugar and starch extraction from lyophilised mid leaf powder

Sampled lyophilised leaf powder was weighed and 40mg of each sample was used for carbohydrate extraction. Chlorophyll was extracted in 1mL cold acetone (100% v/v), left overnight at -20°C before centrifuging at 6,000 RCF for 5 minutes. Acetone containing chlorophyll was removed and chlorophyll in the pellet re-extracted as above.

The pellet was then left to air-dry before 1mL deionised water was added and incubated at 70°C for 30 minutes followed by centrifugation at 16,000 RCF for 10 minutes. Supernatant was removed and the pellet re-extracted with water as before. The combined supernatant was filtered through a 0.45µm PVDF filter and used for sugar determination. Potassium hydroxide (400µL of 0.2M) was added to the residual pellet and heated at 90°C for 2 h, then cooled, neutralised with acetic acid (70µL of 1M) and centrifuged at 16,000 RCF for 10 minutes. Supernatant was removed and used for starch determination.

2.4.4 Fresh mid-leaf material

Snap-frozen sugarcane mid-leaf tissue had the midrib removed and three sample replicates were combined. Combined samples were ground with a mortar and pestle in liquid nitrogen, with a small amount of sterile sand to aid cell rupture. This material was then used for RNA extraction.
2.5.1 RNA extraction from fresh mid leaf powder

From powder prepared for RNA extraction, 100mg was weighed and used with the Qiagen RNeasy Plant Mini Kit following the protocol supplied by the manufacturer. In brief, 600μL of RLT buffer was added to tissue powder and centrifuged at 15,000 rpm for 5 minutes before passing through the QIAshredder column, and eluting with 60μL RNAse-free water passed through membrane twice. RNA quantity was checked with the NanoDrop for yields > 100ng/μL and A260/A280 > 1.0. RNA quality was checked with 1.5% TBE agarose gel containing 1 % SybrSafe to visualize 18s and 28s ribosomal RNA bands. A final amount of 20μg of RNA was prepared and despatched on dry ice to LC sciences for transcriptome sequencing.

LC Sciences
Sample Receiving
2575 West Bellfort St
Suite 270
Houston Texas 77054, USA

2.4.6 Protein extraction from lyophilised leaf material

Freeze dried and crushed plant material to the weight of 125mg was denatured using 5% SDS, 25mM Tris PH8.0 and 0.07% β-mercaptoethanol (1.2 mL) at 90°C for 10 minutes. Samples were centrifuged at 16,000g for 10 minutes and supernatant transferred to a 15mL Falcon tube. Approximately 6 times the supernatant volume of cold (kept at -20°C) 10% TCA, 0.07% β-mercaptoethanol in acetone (approximately 5.4mL) was added to the supernatant and left at -20°C overnight to precipitate. Samples were centrifuged at 5,000g for 5 minutes and the supernatant discarded. The pellet was washed three times with cold acetone (kept at -20°C) and then dried at room temperature. The pellet was then dissolved in 100 μL of 25mM Tris PH8.0, 0.07% β-mercaptoethanol. The protein concentration was measured and precipitated in 6 volumes of cold acetone. The air dried protein (125 µg per sample) was dispatched on dry ice to Proteomics International for proteome analysis.

Proteomics International Pty Ltd
Harry Perkins Institute of Medical Research
QQ Block, QEII Medical Centre
6 Verdun Street, Nedlands WA 6009

2.5 Sample Analysis

2.5.1 Extraction of metabolites for GC-MS (Untargeted) and LC-MS (Amino Acids and Untargeted Profiling)

Approximately 30 mg of homogenized leaf was added to a cryomill tube. Methanol (100%) (500 μL), and a quantitative internal standard containing 4% [(13C6)-Sorbitol (0.5 mg/mL), 13C5-15N-Valine (0.5 mg/mL); 2-aminoanthracene (0.25 mg/mL) and pentfluorobenzoic acid (0.25 mg/mL)] was added. The sample was vortexed for 30 sec and was subsequently homogenized using a cryomill (Bertin Technologies) using program #2(6100-3 x 45 - 045) at -10°C. The sample mixture was then incubated at 30°C, and agitated at 850 rpm for 15 min and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant containing methanol was then transferred into a new Eppendorf tube. Milli-Q Water (containing formic acid, 2%) (500 μL) was added to the remaining pellet in the cryo-mill tube. The sample was vortexed for 30 sec and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant was then combined with the previous methanolic supernatant. A (50 μL) aliquot and a (5 μL) aliquot were transferred into glass inserts and dried in vacuo for subsequent TMS polar metabolite derivatisation. Extracted leaf tissue samples were placed in a snaplock bag with silica gel prior to
derivatisation for GC-MS analysis. A 10 µL aliquot of the extract was transferred into an Eppendorf tube for subsequent amino acid metabolite derivatisation (LC-QQQ-MS) and a 50 µL aliquot was used for LC-QTOF-MS Profiling. Aliquots for LC-MS (Amino acid quantitation and untargeted profiling) were stored at -20°C prior to analysis.

2.5.2 Derivatisation of Polar metabolites

The dried samples were re-dissolved in 10 µL of 30 mg mL⁻¹ methoxyamine hydrochloride in pyridine and derivatised at 37°C for 120 min with mixing at 500 rpm. The samples were then treated for 30 min with 20 µL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 2.0 µL methanol. Retention time standard mixture [0.029% (w/v) n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-dotriacontane, n-hexatriacontane dissolved in pyridine] with mixing at 500 rpm at 37°C. Each derivatised sample was allowed to rest for 60 min prior to injection.

Two different stock solutions were used, 1) Amino acids, containing a standard mix of 25 amino acids in water 0.1% formic acid and 2) Sulphur containing compounds: a 2.5 mM stock solution containing glutathione and s-adenosyl-homocysteine in water with 10 mM TCEP and 1 mM ascorbic acid. The solutions were mixed and diluted using volumetric glassware with water containing 10 mM TCEP and 1 mM ascorbic acid, 0.1% formic acid to produce the following series of combined standards: 0.1, 0.5, 1, 5, 10, 20, 50, 100 and 150 µM.

2.5.3 Amino acid Quantitation

Derivatisation was done by using 10 µL aliquots of each standard or sample was added to 70 µL of borate buffer (200 mM, pH 8.8 at 25°C) containing 10 mM TCEP, 1 mM ascorbic acid and 50 µM 2-amino-2-methyl-1-propanol. The resulting solution was vortexed, then 20 µL of AQC reagent (200 mM dissolved in 100% ACN) was added and immediately vortexed. The samples were heated with shaking at 55°C for 10 minutes then centrifuged and transferred to HPLC vials containing inserts.

2.6 GC-MS analysis

Samples (1 µL) were injected in split less (lower and higher aliquots) into a GC-MS system comprised of a Gerstel 252 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA). The MS was adjusted according to the manufacturer’s recommendations using tris-(perfluorobutyl)-amine (CF3R). The GC was performed on a 30 m VF-5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150°C. Helium was used as the carrier gas at a flow rate of 1.0 mL min⁻¹. For the polar TMS metabolite analysis, the following temperature program was used; start at injection 70°C, a hold for 1 min, followed by a 7°C min⁻¹ oven temperature, ramp to 325°C and a final 6 min heating at 325°C. For the polar metabolite analysis, the following temperature program was used; start at injection 70°C, a hold for 1 min, followed by a 7°C min⁻¹ oven temperature, ramp to 325°C and a final 6 min heating at 325°C. Both chromatograms and mass spectra were evaluated using either the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B05.00/Build 5.0.291.0 for GC-MS. Mass spectra of eluting compounds were identified using the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp- golm.mpg.de/csbdb/dbma/msri.html) and the in-house Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios (area of analyte divided by area of internal standard, 13C6-sorbitol) per sample FW (mg) for each analysed metabolite as previously described (Roessner et al. 2001). The data was also normalized in order to compare fold differences between groups. If a
specific metabolite had multiple TMS derivatives, the metabolite with the greater detector response and improved peak shape within the dynamic range of the instrument was selected.

2.7 LC-QQQ-MS

LC-QQQ-MS - an Agilent 1200 LC-system coupled to an Agilent 6410 Electrospray Ionisation-Triple Quadrupole MS was used for quantification experiments. Injection volumes of 1 μL of samples or standards were used. Ions were monitored in the positive mode using a Dynamic Multiple Reaction Monitoring (DMRM) method optimized for each analyte. The source, collision energies and fragmentor voltages were optimized for each analyte by infusing a derivatised standard with LC eluent. The following source conditions were used: gas flow 10 L.min⁻¹, nebulizer pressure 45 psi and capillary voltage 3800 V.

An Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 x 50 mm, 1.8 μm column was used with a flow rate of 300 μL min⁻¹, maintained at 30°C, resulting in operating pressures below 400 bar with a 19 minute run time as previously described (Boughton et al. 2011). A gradient LC method (Table 1) was used with mobile phases comprised of (A) 0.1% formic acid in deionized water and (B) 0.1% formic acid in acetonitrile. These conditions provided suitable chromatographic separation of modified amino acids. Co-elution was observed for some of the species, but this could be accounted for by using the mass-selective capabilities of the mass spectrometer using MRM (multiple reaction monitoring).

<table>
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<th>Time (min)</th>
<th>% B</th>
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</table>

2.8 Hormone analyses

An aliquot (100 μL) containing all the internal standards, each at a concentration of 0.2 ng μL⁻¹, was added to homogenized sample (approx. 50 mg). 3 mL of isopropanol:water:glacial acetic acid (80:19:1, v/v/v) were further added, and then samples were shaken in the dark for 14-16 h at 4°C. Samples were then centrifuged and the supernatant was isolated and dried on a Büchi Syncore Polyvap (Büchi, Switzerland). Further, they were reconstituted in 100 μL acidified methanol, adjusted to 1 mL with acidified water, and then partitioned against 2 mL hexane. After 30 min, the aqueous layer was isolated and dried as above. Dry samples were reconstituted in 800 μL acidified methanol and adjusted to 1 mL with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak C18 cartridges (Waters, Mississauga, ON, Canada), and then the final eluate was split in 2 equal portions. One portion (#1) was dried completely (and stored) while the second portion (#2) was dried down to the aqueous phase on a LABCONCO centrifivap concentrator (LabConco Corporation, Kansas City, MO, USA) and partitioned against ethyl acetate (2 mL) and further purified using an Oasis WAX cartridge (Waters, Mississauga, ON, Canada). This GA enriched fraction (#2) was eluted with 2 mL acetonitrile: water (80:20, v/v) and then dried on a centrifivap as described above. An internal standard blank was prepared with 100 μL of the deuterated internal standards mixture. A quality control standard (QC) was prepared by adding 100 μL of a mixture containing all the
analytes of interest, each at a concentration of 0.2 ng μL⁻¹, to 100 μL of the internal standard mix. Finally, fractions #1 and #2, blanks, and QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 ng μL⁻¹ of each of the recovery standards.

2.9 HPLC-ESI-MS/MS

Analysis was performed on a UPLC/ESI-MS/MS utilizing a Waters ACQUITY UPLC system, equipped with a binary solvent delivery manager and a sample manager coupled to a Waters Micromass Quattro Premier XE quadrupole tandem mass spectrometer via a Z-spray interface. MassLynx™ and QuanLynx™ (Micromass, Manchester, UK) were used for data acquisition and data analysis.

The procedure for quantification of ABA and ABA catabolites, cytokinin, auxin, and gibberellins in plant tissue was performed as described (Lulsdorf et al. 2013). Samples were injected onto an ACQUITY UPLC® HSS C18 SB column (2.1x100 mm, 1.8 μm) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (50:50, v/v).

Briefly, the analysis utilized the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces were quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace was integrated and the resulting ratio of signals (non-deuterated/internal standard) compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described (Ross et al. 2004). The QC samples, internal standard blanks, and solvent blanks were also prepared and analysed along each batch of tissue samples.

2.10 Mid-leaf sugar and starch determination

Sucrose content was determined using the standard enzymatic method (Bergmeyer and Bernt, 1974) with a spectrophotometer (BMG-Labtech, FLUOstar Omega) and 96-well UV-clear plate (Thermo Fisher, UV Microtiter).

Glucose composition was determined using Amplex® Red/glucose oxidase enzyme assay (Life Technologies) in a 96-well plate (Thermo Fisher, Microtiter) with a spectrophotometer (BMG-Labtech, FLUOstar Omega).

Fructose content was determined using a BioVision Fructose Flurometric assay kit in a 96-well plate (Thermo Fisher, Microtiter) with a spectrophotometer (BMG-Labtech, FLUOstar Omega). A 1/10 dilution of the OxiRed probe and a running temperature of 37 °C was optimal for this assay.

Starch was digested in a sodium acetate buffer (100mM, pH 5.5) containing 10 U amyloglucosidase per reaction for 2h at 60°C. After cooling down to room temperature, glucose was measured in the resulting solution as described above (Bergmeyer and Bernt 1974; Beutler 1984).

2.11 Transcript Sequencing

Approximately 20μg of RNA per sample was sent to LC Sciences, LLC in Houston, Texas, where the quality was verified using the Agilent 2100 Bioanalyzer before being sequenced. The samples were prepared for sequencing by depleting the ribosomal RNA (rRNA), which allowed the less abundant long-non-coding RNA (IncRNA) and messenger RNA (mRNA) transcripts to be sequenced.
For the library preparation, each of the IncRNA and mRNA transcripts were converted into double-stranded complementary DNA (cDNA) through reverse transcription. Adapter sites were added to each end to allow the sequencing primers to bind to the cDNA, multiplex the samples and bind the samples to the flow cell. Samples were multiplexed 4 per lane and sequenced using the Illumina HiSeq 2000/2500.

The read length was 100 nucleotides, read from both ends of each cDNA fragment to give 2 x 100bp paired-end reads. These paired reads were matched together with the appropriate gaps to generate a paired-read dataset, with the average length of 260bp. The number of reads per sample ranged from 46-106 million, and the raw sequences were put through the Tuxedo suite of software programs (Tophat v2.0.12 and Cufflinks v2.2.1) to align them to the reference sequence and estimate transcript abundance. The reads were aligned against the sorghum reference genome (RefSeq Sorghum bicolor, accession number GCF_000003195.2_Sorbi1) and a limited sugarcane sequence database (Sequence Read Archive, accession number SRP042605).

2.12 Transcriptome Analysis

Transcriptome analysis proceeded in two ways. The first method involved the differential expression analysis of all the transcripts identified in each sample. The second method looked for expression changes in a particular set of enzymes involved in sugar metabolism and photosynthesis. Both methods were carried out using CLC Genomics Workbench v8.01 software.

The differential expression analysis compared each YCS sample with its control, and looked at the changes in gene expression due to YCS. The results were pooled for FV3 and FV4. This analysis showed that around 10,000 genes had changed expression patterns.

From this, the transcripts were analysed to identify those that were up-regulated or down-regulated in the YCS samples. The sequences for those two groups were sent separately through the Blast2GO v3.0.10 software to identify the key metabolic pathways affected by YCS.

Our results also showed large changes in the metabolism of sugars and in the disruption of photosynthesis. In order to understand these changes, and possibly pin-point the cause, we identified key enzymes to examine in detail.

Listed below are enzymes possibly involved in metabolic pathways affected by YCS:

<table>
<thead>
<tr>
<th>Enzyme</th>
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<tbody>
<tr>
<td>ADP glucose pyrophosphorylase</td>
</tr>
<tr>
<td>Alkaline neutral invertase</td>
</tr>
<tr>
<td>Alpha amylase</td>
</tr>
<tr>
<td>Alpha amylase inhibitor</td>
</tr>
<tr>
<td>Alpha glucan phosphorylase</td>
</tr>
<tr>
<td>Alpha glucan water dikinase</td>
</tr>
<tr>
<td>Alpha glucosidase</td>
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<tr>
<td>Alpha phosphofructokinase</td>
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<tr>
<td>Beta amylase</td>
</tr>
<tr>
<td>Beta phosphofructokinase</td>
</tr>
<tr>
<td>Branching enzyme</td>
</tr>
<tr>
<td>Cell wall invertase</td>
</tr>
<tr>
<td>Fructokinase</td>
</tr>
<tr>
<td>Fructose 1,6 bisphosphatase</td>
</tr>
<tr>
<td>Glucose transport</td>
</tr>
<tr>
<td>Hexokinase</td>
</tr>
<tr>
<td>Hexose transporter</td>
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<tr>
<td>Limit dextrinase</td>
</tr>
<tr>
<td>Maltase</td>
</tr>
<tr>
<td>Neutral alkaline invertase</td>
</tr>
<tr>
<td>PEP carboxylase</td>
</tr>
<tr>
<td>Phosphoglucon water dikinase</td>
</tr>
<tr>
<td>Phosphoglucose isomerase</td>
</tr>
<tr>
<td>Phosphoglucose mutase</td>
</tr>
<tr>
<td>Phosphoribulokinase</td>
</tr>
<tr>
<td>Pyrophosphate fructose 6P 1</td>
</tr>
<tr>
<td>phosphotransferase (alpha)</td>
</tr>
<tr>
<td>Pyrophosphate fructose 6P 1</td>
</tr>
<tr>
<td>phosphotransferase (beta)</td>
</tr>
<tr>
<td>Rubisco large subunit</td>
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<tr>
<td>Rubisco small subunit</td>
</tr>
<tr>
<td>Sedoheptulose 1,7 bisphosphatase</td>
</tr>
<tr>
<td>SNRK1 beta subunit</td>
</tr>
<tr>
<td>Soluable acid invertase</td>
</tr>
<tr>
<td>SNRK1</td>
</tr>
<tr>
<td>Starch synthase</td>
</tr>
</tbody>
</table>
The nucleotide sequences for these key enzymes were found on the NCBI website, taken from sugarcane or sorghum whenever possible, and maize, rice, and Arabidopsis otherwise. For the non-sugarcane sequences, a series of sugarcane contigs generated previously in SRA’s laboratory were used to generate a sugarcane consensus version of the enzyme. The sugarcane and sugarcane homolog enzyme sequences were used as the reference sequences for mapping the raw reads, to determine transcript abundance and expression.

The paired-read datasets were mapped against each of the enzyme sequences simultaneously, and the raw mapping result normalised (FPKM) for the size of the gene and the number of sequences available to map to it.

2.13 Statistical Analysis of FV6 Carbohydrates using Statistics 10

Statistical software package Statistix 10 was used to analyse carbohydrate data for FV6. An Analysis of Variance (ANOVA-completely randomised design) was used to compare starch, sucrose, glucose and fructose means from mid-leaf extracts to identify if there are differences between sample groups. To identify which groups are significantly different a Tukey’s HSD All-Pairwise Comparisons Test was then applied to create confidence intervals for all pairwise differences (these are displayed as homogeneous groups A, B, AB, C etc). Different groups indicate that their means are significantly different from each another. A statistical check for normality was also performed using the Shapiro-Wilk test and where necessary the data was transformed (normalised) prior to performing the Tukey’s HSD test.

3. Results

The projects started with work in the Burdekin on KQ228\(^A\) and the Herbert on Q200\(^A\). This pilot study was done to assess the canopy response to onset and expression of YCS. The purpose of this was twofold. Firstly, to determine to what extent photosynthesis and transpiration are impacted in the yellow leaves. Secondly, to determine whether the rest of the canopy compensates for the ‘sick’ leaves, as this would be indicative of a stress response and recovery.

For this purpose, we measured light harvesting, coupling of electron transport, gas exchange, carbon fixation, stomatal conductance, and internal CO\(_2\) levels of leaves. In addition, we measured starch, sucrose and reducing sugar levels, and determined the behaviour of the photosynthetic electron transport system.

3.1 YCS - more than yellowing of the older leaves

3.1.1 Photosynthesis

There was a distinct variation in photosynthesis, conductance, transpiration and internal CO\(_2\) (C\(i\)) between YCS symptomatic and healthy control plants in both varieties and two geographical locations.

CO\(_2\) fixation increased in the younger leaves (-1 to 3) and decreased in the older leaves (Fig 3.1.1). This trend was consistent in both morning and afternoon measurements (results not shown). There was a significant difference in CO\(_2\) fixation in older leaves with YCS. The rate of photosynthesis was lower in older leaves (Fig 3.1.1). This became more evident when readings were taken in the afternoon (data not shown).
The stomatal conductance followed a similar pattern of variation among leaves as observed for photosynthesis (Fig 3.1.2). Under high radiation (PAR) in the morning, stomatal activity in leaves of YCS affected canopies was nearly 50% lower than that of green healthy canopies. The difference in stomatal function and subsequent impact on gas exchange were evident with the variation in photosynthesis.

In healthy leaf tissues, stomatal conductance and photosynthesis are usually linearly related, giving rise to relatively constant internal CO$_2$ (Long et al., 1996). However, this relationship can be disrupted under low conductance and high temperature (Feller, 2006). The lower stomatal conductance in YCS symptomatic plants suggests that these plants are experiencing stress, which results in partial closure of the stomata. Not only will this impact on CO$_2$ diffusion into the leaf, but it will also make the tissue particularly vulnerable to elevated temperatures. The ratio of variable to maximum chlorophyll A fluorescence also suggests that the canopy of the YCS expressing plants are under stress and that electron transport is more uncoupled from photosynthesis, even in the leaves without any noticeable expression of YCS.

Variation in internal CO$_2$ (C) at different leaf levels of the canopies was examined in the morning and the afternoon (data not shown). There were considerable differences between YCS symptomatic and control leaves (Fig 3.1.3). Elevated CO$_2$ levels observed in YCS leaves in the afternoon could be a result of low mid-day stomatal conductance and its carry over effects during the afternoon. This observation needs to be further investigated in YCS affected cane at different stages of growth.
3.1.2 Sugars and starch

Sugar levels in sugarcane follow a diurnal pattern of change, and these levels also vary in leaves of different ages (Lehrer et al. 2009; McCormick et al. 2008b; McCormick et al. 2008a; McCormick et al. 2007; Du et al. 1999). To compensate for this, we normalised the data against the levels in leaf 1 of asymptomatic plants. In this report, only the data for KQ228 is presented (Fig 3.1.4) but similar patterns of change were observed for Q200 (data not shown).

Similar sugar levels are present in the younger leaves of the control and YCS symptomatic plants. However, in the older leaves there are significantly higher levels of sucrose, glucose and fructose.

Figure 3.1.2: Changes in stomatal conductance of asymptomatic (control) and symptomatic (YCS) plants. KQ228 in the Burdekin (A) and Q200 in the Herbert (B). Values ± standard deviation.

Figure 3.1.3: Changes in internal CO$_2$ concentrations stomatal conductance of a symptomatic (control) and symptomatic (YCS) plants. KQ228 in the Burdekin (A) and Q200 in the Herbert (B). Values ± standard deviation.
(Fig 3.1.4 B, C and D). The sucrose and glucose levels in leaf 7 were more than three times higher in the YCS plants than in the controls. In contrast, the starch levels between the YCS symptomatic and control plants were similar (Fig 3.1.4 A).

3.1.3. Electron transport

There is a significant reduction in the Fm/FV ratios between control and YCS plants (data not shown). For this reason, we analysed the chlorophyll A fluorescence characteristics in more detail. The OJIP fluorescence transients clearly indicated an increase in fluorescence intensity at 2ms and beyond (i.e. J–I–P phase) (Fig. 3.1.5). This is normally believed to be the result of accumulation of the reduced QA and PQ pools, mainly because of a blockage of electron flow further downstream (Schreiber and Neubauer 1987; Van Heerden et al. 2007a; Strasser et al. 2000). As reported previously, chlorophyll A fluorescence holds promise as an early and accurate screening methodology. We continue to work with this method to determine its applicability for YCS screening.

![Graphs showing changes in starch, sucrose, glucose, and fructose levels in asymptomatic (control) and symptomatic (YCS) KQ228 plants.](image)

Figure 3.1.4: Changes in starch (A), sucrose (B), glucose (C) and fructose (D) levels in asymptomatic (control) and symptomatic (YCS) KQ228 plants. Values ± standard deviation. Values ± standard
The appearance of a clearly defined I peak in the YCS samples is caused by PSI-associated limitations (Van Heerden et al. 2007a) and inactivation of ferredoxin-NADP+ oxidoreductase (Schansker et al. 2003). It is noteworthy that there is also a clearly defined K peak in the OJIP transient at around 300 µs, which indicates inhibition of the oxygen-evolving complex (OEC) (Jiang et al. 2006). The appearance of a K peak is typically a symptom observed in leaves exposed to elevated temperatures. This could suggest increased leaf temperatures in the YCS expressing plants and a role in the down-regulation of Photosystem II function. As YCS expression leads to lower stomatal conductance and transpiration rates, increases in leaf temperature may easily occur.

Figure 3.1.5: Chlorophyll A fluorescence transients (A) recorded in leaves 1, 3 and 5 of asymptomatic (control) and symptomatic (YCS) KQ228 plants. The different stages in the fluorescence transient (OJIP) are indicated. Difference in variable fluorescence curves (B) constructed by subtraction of normalised (O-P) fluorescence values for the asymptomatic leaves from that recorded for the same age symptomatic leaves.

The involvement of PSI-dependent control of Photosystem II activity during environmental stress has indeed been demonstrated previously (Katona et al. 1992; Van Heerden et al. 2007a).

3.1.4. Conclusions

The data shows that leaf photosynthesis is reduced in the canopy of YCS-symptomatic plants. Given the reduced carbon fixation in such a large portion of the canopy it would be reasonable to predict that there will be significant reductions in cane yield in the YCS-symptomatic plants. The extent of yield loss will be a function of the severity of YCS expression and the frequency it happens during the growth cycle.

Sucrose levels are already elevated in the YCS symptomatic plants in leaf 3. The high levels of carbohydrates will lead to the induction of leaf yellowing. Previous work on other plant species have shown that expressing disease symptoms can often be related to increased sugar levels (Thiagarajah et al. 1981; Izaguirre-Mayoral et al. 2002; Fontaniella et al. 2003).
It is also well established that the yellowing of leaves can be induced by allowing for carbohydrate synthesis while blocking the translocation of carbohydrates (Krapp and Stitt 1995; Graham and Martin 2000; Russin et al. 1996; Braun et al. 2006; Allison and Weinmann 1970; Rajcan and Tollenaar 1999; Tollenaar and Daynard 1982) and in some cases in the Poaceae this coincides with anthocyanin accumulation. Notably, typical YCS symptom expression has a characteristic golden colour, consistent with both chlorophyll disappearance and induction of pigments such as anthocyanins.

However, this could be indirect as water stress can also play a role in the down-regulation of Photosystem II function. Water stress will lead to lower stomatal conductance and transpiration rates and increases in leaf temperature may consequently occur. Wand et al. (2001) specifically demonstrated the effects of elevated growth temperatures on photosynthesis.

In many of the maize yellowing mutants, sucrose transport through the plasmodesmata between the companion and sieve elements in the phloem, or just phloem loading, is restricted (Braun and Slewinski 2009; Slewinski et al. 2012; Slewinski and Braun 2010a; Slewinski and Braun 2010b; Slewinski et al. 2009). In these mutants there is a hyper accumulation of carbohydrates and this is associated with the development of chlorotic patches and early senescence. In most cases the long distance transport of sucrose is not compromised (Slewinski et al. 2012) and hence yellowing and development of chlorotic regions occurs around the smaller veins and inner branches between the veins. This is remarkably similar to the development of YCS-like symptoms, where yellowing occurs between the smaller veins and rarely near the midrib.

In maize, expression of yellowing and chlorosis is dependent on high light intensity (Braun et al. 2006). These authors suggest that yellowing occurs as a result of a threshold which is exceeded under this high light intensity. The mechanism through which high sugar concentration leads to yellowing is not clear, but is probably a combination of feedback inhibition of photosynthetic gene expression and chlorophyll synthesis, which in turn leads to reduced chlorophyll accumulation, and ultimately in yellow coloration of the tissue.

Some of the sucrose in the apoplasm will be carried towards the stomata in the transpiration stream (Kelly et al. 2013 and references therein) and this plays an important role in decreasing stomatal apertures. Initially it was thought that this occurs through an osmotic effect, but recently it was found that the sugar control of stomata opening is mediated through an interaction of hexokinase activity and ABA levels (Kelly et al. 2013).

Increased sucrose in the cell will result in significantly elevated trehalose-6-phosphate (T6P) concentrations (Lunn et al. 2006) and leads to accelerated senescence (Schluepmann et al. 2004; Wind et al. 2010). The effect of elevated T6P may be the inhibition of the sucrose non-fermenting-related kinase-1 (SnRK1), which is involved in plant energy signalling and in turn regulation of plant metabolism (Baena-Gonzalez et al. 2007).

Based on these observations we decided to concentrate all efforts in the remainder of the project on leaf 2 and 4 late in the season and leaf 4 and 6 early in the season. This decision was based on the observation that leaf 4 rarely showed severe yellowing and our results indicated that once visual yellowing is evident many secondary affects appear. This approach in sampling should allow maximum resolution of the impact of YCS on the crop, and assist in identifying the metabolic factors that lead to the expression of YCS.

In addition, we extended the analyses to include another genotype (Q208 A) from another agro-climatic zone (Mackay). This was done to identify those factors that are associated with YCS expression across environments and genotypes.
3.2 **Major changes in the metabolome well before visible yellowing**

We used a GC-MS and LC-MS approach to separate and analyse the metabolome of sugarcane. In the analyses conducted to date we have looked at more than 3000 different metabolite signals. A very large proportion of these have not been conclusively identified.

3.2.1. **Leaves**

For the analyses, data is normalised against the control leaf 2 samples using the algorithms in the software package MetaboAnalyst (Figure 3.2.1). The huge variation in the relative concentrations of the different metabolites makes such normalisation important. Note, for most of these analyses we set the youngest leaf from the control as the reference sample.

Generally the control and YCS samples are well separated by PCA analyses (Figure 3.2.1B). The data shows that morning and afternoon metabolism are distinctly different in the control but not the YCS samples. The control and YCS samples are clearly separated and the soluble sugars are among the main elements in driving this separation into the four clusters, and all are elevated in the YCS expressing samples.

Those metabolites that shows a consistent pattern over the three regions and genotypes are discussed in the rest of this report.

![Figure 3.2.1: Normalisation of log10 transformed GC-MS data from control and YCS expressing Q200 samples (A) followed by a PCA analyses (B).](image)
Nine amino acids are significant (Fig 3.2.2). For six of these amino acids (alanine, valine, proline, serine, GABA and pyroglutamate) the trend is for a decrease between leaf 4 and 6 of asymptomatic leaves. In contrast, these amino acids increase between leaf 4 and 6 of the YCS symptomatic leaves. Noteworthy is the large increase of asparagine and tryptophan in the YCS-symptomatic tissues. Both proline and GABA levels also increase in the YCS-symptomatic tissues.

It is well established in the literature that asparagine and tryptophan levels increase in tissues that develop chlorosis (Kenyon and Turner 1990). Increased proline and GABA levels often strongly correlate with abiotic stress (Rodziewicz et al. 2014; Widodo et al. 2009; Witt et al. 2012).

Although several organic acids are higher in YCS-symptomatic leaves in some of the genotypes in specific environments, only quinate, ribonate and gluconate held up across the three different environments (Fig 3.2.3). Quinate is produced from a branch point of the shikimate pathway (Guo et al. 2014 and references therein). The shikimate pathway is important for both protein synthesis and secondary metabolism. This pathway delivers the precursors for the plant hormones salicylic acid and auxins, and secondary metabolites such as alkaloids, benzenoids, and phenylpropanoids.

Figure 3.2.2: Changes in the levels of amino acids in YCS symptomatic sugarcane plants. Data is normalised against the control leaf two. All these values have a t-test value below $P < 0.05$ (Bonferroni-corrected $P$ value).
When a region or a specific variety was viewed in isolation, more than fifteen sugar and sugar phosphates had significantly higher concentrations in the YCS expressing tissues. However, only seven of these hold true for all the regions sampled and the three varieties (Fig 3.2.4). One of these, gentibiose, is not considered a sugarcane product but rather indicative of micro-organisms. Some of the other sugars that are not consistently present like kestose, raffinose, lactose and melezitose, are associated with secondary infection/contamination with organisms like *Leuconostoc spp.*

![Figure 3.2.3](image)

**Figure 3.2.3:** Changes in the levels of organic acids in YCS symptomatic sugarcane plants. Data is normalised against the control leaf two. All these values have a t-test value below $P < 0.05$ (Bonferroni-corrected $P$ value).

High sucrose levels seem to be strongly linked to the development and expression of YCS. Because of this link between sucrose levels, the data for its 2 reducing sugars, glucose and fructose, is presented in more detail (Fig 3.2.5). Evidently, there is no significant differences between the asymptomatic leaf 2 and 4 samples. However, in all cases these three sugars are at significantly higher levels in leaf 4 of symptomatic plants than in symptomatic leaf 2 as well as asymptomatic leaves. The changes in glucose and fructose are not tightly coordinated with that of sucrose. If this is the case it could suggest that more than sucrose hydrolyses is at play in the YCS samples.

The increase in trehalose levels is interesting. Trehalose is strongly linked to trehalose-6-phosphate (T6P) levels, which is one of the major regulators of plant metabolism. Trehalose 6 phosphate accumulation leads to a reduction in carbohydrate content and an increase in starch levels. T6P content increases when carbon availability is high, and in young growing tissue, T6P inhibits the activity of Snf1-related protein kinase (SnRK1) (Zhang et al. 2009). There is a strong accumulation of T6P in senescing leaves of Arabidopsis (*Arabidopsis thaliana*), in parallel with a
rise in sugar contents (Wingler et al. 2012). There is also a strong correlation between trehalose metabolism, sugar levels, and anthocyanin levels in plants.

3.2.2. Xylem

We have analysed the xylem sap from all three varieties at the three locations. At each location, there were significant differences between the control tissues and YCS symptomatic material. However, we could not identify a single compound across the regions and genotypes that changes in association with YCS expression. Although there are several unknown compounds, we could also not identify any ‘unknown’ compound that increases significantly in the xylem sap across the three regions.

3.3 Quantifying sugar and starch levels

The MS analyses provide valuable insight on the metabolome and specifically in relation to the relative levels of the different components. However, unless standards are used to calibrate for each component the values represent the only information in regards of the relative level of each chemical. For this reason, all the data are normalised against asymptomatic leaf 2 samples.

As sucrose represents the only major form in which carbon is exported from the leaves to the rest of the plant, more information regarding the cellular concentration of sucrose in each leaf is required. In addition, we know that sucrose and its hydrolytic products play a crucial role in signalling and control of cellular metabolism. Starch levels usually reflect the status of leaf metabolism and any excess sucrose is usually converted to starch and stored.

In only one instance, leaf 6 of Q208A in Mackay, were starch levels significantly higher in the YCS-symptomatic tissue than in control plants (Fig. 3.3.1).

![Figure 3.2.4](image-url): Changes in the levels of sugars in YCS symptomatic sugarcane plants. Data is normalised against the control leaf two. All these values have a t-test value below $P < 0.05$ (Bonferroni-corrected $P$ value).
Figure 3.2.5: Changes in the levels of sucrose, glucose, fructose, and trehalose in YCS symptomatic KQ228A, Q200A and Q208A sugarcane plants in three different agroclimatic zones.
In contrast, sucrose levels in YCS symptomatic plants are higher than in the controls. The levels of sucrose in leaf 4 and 6 of the asymptomatic plants are similar to previously reported values (Lehrer et al. 2010; McCormick et al. 2008b). The sucrose levels in leaf 6 of YCS-symptomatic plants significantly exceeds that reported for sucrose accumulation in SCYLV (Lehrer et al. 2010).

Similar to sucrose, the glucose levels in leaf 6 of the YCS expressing plants are always higher than leaf 4 levels, and also higher than that of leaf 4 and 6 of the control plants. In contrast to the GC-MS analyses, fructose levels are not always significantly higher in the YCS-symptomatic leaves.

3.4 Complex Changes in Gene Expression

There are close to 4000 genes that are upregulated more than 5 fold in the YCS-symptomatic leaf tissue. Of these we have ascribed a putative identity to 3191, and 500 of these are upregulated more than 100 fold. In order to understand the molecular mechanisms underlying the development of YCS symptoms we classified the genes according to their putative ascribed cellular function, using the Blast2GO software (Fig. 3.2.6).

The largest group of genes with known function has homology to genes involved in plastid, nucleus, and mitochondrial processes (Fig.3.2.6).

The same upregulated genes can also be classified according to their specific biological function (Fig. 3.2.7). However, care should be taken in deriving specific conclusions as many of these genes can occur in more than one category and, at best, this should be seen as a first high-level putative allocation of function. The upregulated sequences are classified into 117 functions (Fig 3.2.7) and it is noteworthy that aromatic compound, nitrogen, regulation of cellular processes and reactions, carbohydrate metabolism, transporters, organelle organisation, oxidation-reduction processes, and cell communication are among the top 20% of these. A large proportion of these are associated with proteolysis, stress metabolism, sugar metabolism and
transport, auxin and ABA responsive genes, chloroplast and other heat shock proteins, ubiquitin conjugating enzymes and several cytochrome P450 genes.

More than 2000 genes are down regulated in the YCS-symptomatic leaves. We have ascribed a putative identity to 1263 of these. More than 150 of these are down regulated more than 100 fold (>1% of the control level expression). In this group of genes are several chloroplast structural and function genes, senescence related genes, transcription factors, phosphofructokinases, glycolytic enzymes, pathogenesis related genes, trehalose metabolism genes and primary carbon fixation genes.

Most noteworthy in this group are the genes for Photosystem II subunits, chloroplast ATPases, several light harvesting complex components, pyrophosphatases, proteases, PEP carboxylase, isomerasers in respiration and pyruvate orthophosphate dikinase.

These datasets will be analysed in much greater detail in follow-on research.

The accumulation of sugars repress photosynthetic gene expression and chlorophyll abundance in maize and other plants (Sheen 1990, 1994; Goldschmidt and Huber 1992; Krapp and Stitt 1995; Jeannette et al. 2000; Braun et al. 2006; Baker and Braun 2008; Braun and Slewnski 2009; Slewnski and Braun 2010a, b; Koch 1996). Changes observed in sugarcan include the regulation of several genes associated with C4 photosynthesis, mitochondrial metabolism, and sugar transport.

Figure 3.2.7: Biological function classification of the upregulated sugarcane genes in YCS symptomatic plants. This classification was done by using the Blast2GO software.
Figure 3.3.1: Starch, sucrose, glucose and fructose levels in leaf 4 and 6 of asymptomatic and YCS symptomatic leaves from Q200\(^a\) (Herbert), KQ228\(^a\) (Burdekin) and Q208\(^a\) (Mackay). A Tukey HSD All-Pairwise Comparisons Test were done on all the results.
3.5 Can we learn more from analysing the proteome?

Measuring mRNA dynamics through transcriptome sequencing is only a very early step in the assessment of biological systems (Weckwerth 2009). There is an increasing number of publications showing that the correlation between mRNA and protein abundance in the cell in a wide range of organisms can vary greatly (Ponnala et al. 2014). Steady-state mRNA–protein abundances often show a squared Pearson correlation coefficient of approximately 0.40, which implies that approximately 40% of the variation in protein concentration can be explained by the mRNA abundance (Vogel and Marcotte 2012; Friso et al. 2010).

We initiated a pilot study to look at the presence of specific proteins in the leaves of YCS-symptomatic plants to reduce the false discovery rate. More than 6931 proteins were separated and compared against the UniProt *Saccharum Officinarum* database using ProteinPilot™ 4.5 Software [AB Sciex]. The database contains 4,306 sugarcane protein sequences.

Only 73 proteins were identified with a confidence score of more than 95%. Of these, 20 proteins had significant changes in their expression patterns between the YCS symptomatic leaves and control leaves. Obviously much more work is required to build a more comprehensive sugarcane protein database, which would allow more proteins to be identified.

Noteworthy are the following proteins Photosystem I and Photosystem II proteins, PEP carboxylase, heat shock protein 90, Pyruvate orthophosphate dikinase, chloroplast ATP synthase, UDP glucose pyrophosphorylase, glyceraldehyde-3-phosphate dehydrogenase, disease resistance response proteins and a 14-3-3 protein. These findings are consistent with the data that we have obtained in the transcriptome analyses.

3.6 What drives the observed metabolic and gene expression changes

3.6.1 Hormones

When analysing the data on a single genotype by one geographical region basis, many of the plant hormone changes (increases or decreased) correlate with the expression of YCS. However, very few of these changes are consistent over the three agro-climatic zones and three genotypes.

ABA and most of its catabolites are present in all samples. It appears that the main ABA catabolism pathway is through 8'-hydroxylation (which results in PA which is further reduced to dihydrophaseic acid (DPA), as well as conjugation resulting in abscisic acid glucose ester (ABAGE)). Trans-ABA is a product of isomerisation of natural ABA under UV light. The presence of large amounts of DPA suggests that bioactive ABA was produced and rapidly catabolized (Fig 3.6.1).

It is familiar to see very high levels of DPA in plant tissues and it is likely to reflect high turnover of t-ABA. Noteworthy is that the total pool of ABA and its catabolites are higher in the YCS-symptomatic tissues in all three varieties from the three different areas. The higher ABA levels in the YCS symptomatic leaves are consistent with the stomatal conductance results (Fig. 3.1.2).
Environmental signal

Several factors indicate that the YCS symptomatic leaves are experiencing an environmental stress. In most cases, the OJIP chlorophyll A transient fluorescence curves show a typical K peak. The presence of a K peak is typical of heat stress. We also see elevated ABA, proline, and GABA levels, increased expression of heat shock proteins and several cytochrome P450 genes, as well as a down regulation of photosynthetic and glycolytic genes in the YCS symptomatic tissues.

Eleven different cytochrome P450 genes were identified in response to heat stress in switchgrass (Li et al. 2013). In addition, several heat shock proteins, and heat shock transcription factors were upregulated. All of the genes involved in glycolysis were strongly repressed by heat stress in switchgrass.
To gain a better understanding of the changes that occur along the leaf blade during the development of YCS symptoms, we measured changes in chlorophyll a fluorescence kinetics at fixed intervals on the leaf. Obviously, the pattern and position of symptom development varies from leaf to leaf and hence the OJIP kinetics is unique to a particular leaf. However, indicative patterns are starting to emerge (Fig. 3.6.3). The pattern of disruption of the electron transport system is similar on both sides of the leaf, with the ‘green side’ just trailing behind in intensity. This demonstrates that symptom development starts from the younger tissue and expands towards the older tip of the leaf. A similar pattern is observed in the overall photosynthetic driving force PIABS (Fig 3.6.4). Important to note is that these patterns of change in chlorophyll a fluorescence are not evident in asymptomatic leaves (data not shown).

Figure 3.6.3: Difference in variable fluorescence kinetics on different positions of the same leaf. OJIP fluorescence transients were normalised (O.P) and subtracted for the first clip on the greenside of the leaf.

Figure 3.6.4: The overall performance index (PIABS) measured at the same positions on the leaf as in Figure 3.6.2.
3.6.3  **Specific genes in photosynthesis, sugar metabolism and transport**

In addition to analysing the overall changes in gene expression, we targeted a group of 45 enzymes that are involved in CO₂ fixation, sucrose synthesis and breakdown, sugar transport and the regulation of carbohydrate metabolism. The changes in expression for these enzymes are complex to interpret due to variation over season and tissue age. Most of these targeted genes have altered expression during the development of YCS.

A comparison of the expression of a YCS symptomatic leaf of Q200 from the Herbert, normalised against the asymptomatic leaf, indicates that hexokinase, trehalose phosphate synthase and cell wall invertases are most upregulated while alpha amylase, SNRK1, neutral invertase and trehalase most down regulated (Fig. 3.6.5).

However, this represents an over-simplified version as reflected in a comparison spanning different collections of material and leaf stages (Fig. 3.6.6). We will continue to analyse this data and will use this to reconstitute the major metabolic pathways. Only such an analyses will enable conclusion of the changes in metabolism associated with YCS.

![Figure 3.6.5](image1)

**Figure 3.6.5**: Relative changes in the expression of a selected group of genes in YCS symptomatic leaf 6 of Q200 that are involved in primary carbon fixation and the regulation of carbohydrate metabolism. Expression data was normalised against gene size and number of read sequences in the experiment.

![Figure 3.6.6](image2)

**Figure 3.6.6**: Changes in the expression of a selected group of genes in YCS symptomatic leaves of Q200 that are involved in primary carbon fixation and the regulation of carbohydrate metabolism. Expression data was normalised against gene size and number of reads in the experiment.
4. References


Academic Publishers, The Netherlands, pp 977-980


Section 4: Outputs and Outcomes

We have participated in three YCS workshops in Lucinda (2014), Townsville (2014) and Brisbane (2015). The results from this project were presented in a paper at the Annual Plant and Animal Genome meeting in San Diego, USA during January 2015.

We have developed a comprehensive database of all metabolites and changes in gene expression in the sugarcane leaf, and how these change in association with development of YCS. Prior to this project, none of this was available. These databases will be a very valuable asset for any future sugarcane research.

The project has identified several very important leads that can be followed to identify the physiological changes associated with YCS. This work will enable us to eventually identify the causes of YCS and facilitate the development of management strategies.

Section 5: Intellectual Property (IP) and Confidentiality

None

Section 6: Industry Communication and Adoption of Outputs

The results that flowed from this project was widely communicated to industry.

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Section 7: Environmental Impact

None.

Section 8: Recommendations and Future Industry Needs

Despite the good progress, the identification of what causes YCS and potential management strategies will be difficult to achieve. However, we have developed a fundamental background that will be an invaluable platform for future work on this topic. In addition, insights into the link between sugar metabolism and YCS development will not only contribute to finding management solutions for YCS, but might indeed provide an opportunity to break out of the current yield plateau that has haunted sugarcane breeders for the past three decades.

A continued investment in this work could lead to specific genetic targets for crop improvement and management practices to reduce the incidence and impact of YCS.

Section 9: Publications

A first manuscript is in preparation and will be submitted to Tropical Crop Biology by the end of July 2015. We are planning to publish at least two more publications from this work.

A paper was delivered at the Annual Plant and Animal Genome meeting in San Diego, USA during January 2015.