THERE'S YELLOW, AND THEN THERE'S YELLOW—WHICH ONE IS YCS?

By

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Abstract

YELLOW CANOPY SYNDROME (YCS) symptoms include leaf yellowing of the mid canopy, a reduction in photosynthesis and yield decreases of up to 30%. The cause of YCS remains unknown. It has been speculated leaf yellowing from YCS is produced by triggering the early onset of senescence, or through implications of water-stress. Inability to differentiate accurately between YCS and other conditions leading to leaf yellowing is a serious limitation for research and the development of strategies to manage YCS. The aim of this work is to determine if there are distinct metabolic or gene expression changes in YCS which would allow discrimination between YCS, leaf senescence and water-stress induced yellowing. For this purpose, the metabolome and transcriptome of YCS affected leaves, water-stressed leaves and leaves undergoing senescence were compared at the early stages of still-green lamina and after visible yellowing of the lamina. Differential gene expression data uncovered a uniqueness among YCS affected yellow leaves. The separation of YCS yellowing involved genes related to plant diurnal cycle and carbon metabolism. We suggest that YCS yellowing symptoms are produced by an alternate trigger to that determining the developmental (age-related) senescence process, and may be related to excess sugar accumulation in the leaf. Based on the new information, gene expression patterns unique to YCS could be used as a potential diagnostic, and open up an area for further investigation into understanding YCS.

Introduction

Yellow Canopy Syndrome (YCS) is an undiagnosed condition affecting all sugarcane varieties in the north and central cane growing regions of Queensland, Australia. The peak months of symptom expression are December to March. Symptoms begin with yellowing of the leaf lamina midway along the leaf blade, generally on one side of the midrib. Eventually the yellowing spreads and the leaf senesces. Growers with crops affected by YCS can incur significant yield losses.

Early diagnosis of YCS is important not only for industry support but also for research sample collection. If the onset of YCS could be predicted before symptom development, it may facilitate management intervention to prevent the onset of YCS. Accurate identification of YCS versus other forms of leaf yellowing would also be a valuable research tool.

Physiological impacts of YCS include chlorophyll loss, a reduction in photosynthesis of up to 36% and stomatal conductance decline, and is not limited to those leaves displaying yellowing symptoms (Marquardt *et al.*, 2016). Markedly, sugar and starch levels are elevated in YCS-symptomatic leaves and do not show regular diurnal patterns of oscillation.

It is widely recognised that carbohydrate accumulation leads to a downregulation of photosynthetic gene expression (for review see Pinheiro and Chaves, 2011). Sucrose and reducing sugar accumulation is often linked to stress. Water-stress triggers sugars, as well as other osmolytes, amino acids and antioxidants, to build up in leaves (for review see Singh *et al.*, 2015).

Sugar accumulation is known to induce leaf senescence, which causes a reduction in leaf photosynthetic pigments and remobilisation of nutrients (for review see Lim *et al.*, 2007). This differs from natural senescence at the gene expression level (Pourtau *et al.*, 2006). It is not known if sugar accumulation found in YCS leaves triggers the early-onset of senescence.

The aim of this study was to determine if YCS yellowing differs from senescence and/or water-stress-induced leaf yellowing, and whether a potential diagnostic tool for YCS can be developed. We report that metabolite and gene expression in YCS leaf symptomatic leaves differ substantially from leaves that are yellowing as a result of water-stress. In addition, gene expression during developmental (age-induced) senescence differs significantly from that of YCS.

Methods

Plant material and sample collection

Sugarcane plant samples were collected from first ratoon, field grown *Saccharum* spp. hybrids var. Q240(ф) under conventional management practices of the region, except for water-stress samples, which were not irrigated except at the time of planting. Non-stressed (control and senescing) samples and YCS-symptomatic samples were collected from Home Hill in the Burdekin region of Queensland, Australia. Water-stressed plant samples were collected from Brandon in the Burdekin region of Queensland, Australia. Chlorophyll content readings were taken with a SPAD-502 meter (Minolta CO, LTD, Japan) prior to sampling, in both the visually green and yellow portions of the sampled leaves to confirm sample category. All samples were collected in February 2016.

YCS samples

Leaf 6 displaying YCS yellowing symptoms from 21 different culms from separate stools were sampled between 8–10 AM (later to be separated into green- and yellow-lamina YCS leaf tissue samples). Control leaf 6 samples were collected from asymptomatic culms (completely free of YCS symptoms) of plants separated by more than 40 m from symptomatic plants.

Senescence samples

Sixteen senescent leaves were collected. The leaf below the sampled leaf had more than 90% yellow or brown along the leaf lamina, and the next leaf above the sampled leaf was largely asymptomatic.

The senescence phenotype was defined as the leaf tip showing browning with a leading yellow edge around the tip and leaf margins, the lamina around the midrib remaining green. The green and yellow lamina sections of each leaf were later separated to form two samples.

Water-stress samples

Sixteen plants were selected for water-stress affected leaf sampling, identified by the presence of yellowing symptoms on leaf 4 and lack of visual yellowing in leaf 3. All leaves displayed wilting symptoms. Leaf 4 was taken as the water-stress yellowing sample leaf, and leaf 3 as the water-stress green sample leaf.

Leaf sample processing

Leaves were cut from the culm and snap-frozen in liquid nitrogen and kept at –80 °C during processing. Yellow and green leaf lamina were validated by chlorophyll content readings and collected from the middle third of the leaf. Samples were ground to a fine powder in cryovials (Spex SamplePrep 50mL PC, Metuchen, NJ, USA) with a small amount of sterile sand, and stainless steel ball bearings with a homogeniser (Spex SamplePrep 2010 Geno/Grinder, Metuchen, NJ, USA) at 1450 RPM.

Sample powder from four leaves was combined to create one replicate. For each replicate, 100 mg powder was used for RNA extraction for RNA-sequencing. The remaining powder was lyophilised using a freeze dryer (CHRIST ALPHA 1-4 LSC plus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). From this, 30 mg lyophilised powder was used for metabolome analysis.

GC-MS analyses of metabolites

The protocol followed was as described by Botha *et al.* (2016).

RNA isolation, sequencing & data processing

Total RNA was isolated from 100 mg of each sample using Spectrum™ Total Plant RNA kit (Sigma-Aldrich) according to the manufacturer's instructions but with the addition of 2.5% (w/v) PVP-40 (polyvinylpyrrolidone (mw 40 000; Sigma-Aldrich)) in the lysis solution. RNA yield and quality was assessed using a spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE, USA) and by agarose gel electrophoresis.

Samples were sequenced using an Illumina HiSeq2500 platform with TruSeq strand-specific chemistry to create 125bp sequence read length paired end reads. Sequencing yielded 60–100 million reads per sample. Reads were trimmed using Trimmomatic v0.36 (Bolger *et al.*, 2014). Quality was checked before and after trimming with FastQC v0.11.5 (Babraham Institute, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Paired-end reads were imported into CLC Genomics Workbench version 9.5.1 (QIAGEN, Aarhus, Denmark) and mapped to a PacBio sugarcane transcriptome library with length fraction of 0.9 and similarity fraction of 0.8. Between 55–64% of reads mapped to the reference for each sample.

Statistical analyses

Statistical analysis of the metabolite data was performed using MetaboAnalyst 3.0, http://Metabolanalyst.ca, (Xia *et al.*, 2015). Significantly different metabolites between control and different yellow lamina were determined at $P<0.01$ with generalised logarithm transformed values, using One-Way Analysis of Variance (ANOVA) and Fisher's LSD post-hoc analysis. Only these metabolites were used for hierarchical clustering.

Differential expression was performed using total counts of reads mapped to contigs with CLC Genomics Workbench proportion-based statistical analysis, using Baggerley's test comparing proportions between all sample pairs. Significant differential expression of contigs between samples was defined as false discovery rate (FDR)-corrected $P\leq0.01$.

Partial Least Squares-Discriminant Analysis (PLS-DA) and hierarchical clustering (heatmap) were generated in RStudio v0.99.902 (RStudio, Inc, Boston, MA, USA). PLS-DA was performed using mixOmics R package v6.1.1 (LeCao *et al.*, 2016) on RPKM values of transcriptome and metabolite levels of metabolome.

For hierarchical clustering, differentially abundant metabolites and differentially expressed contig RPKM values were natural log-transformed, centered and scaled. Hierarchical clustering was performed using complete linkage clustering method and trees were cut to create five categories of metabolites/contigs. Pheatmap v1.0.8 R package (Kolde, 2015) was used for hierarchical clustering visualisation.

Results

Metabolome

Across all samples, 107 metabolites were detected of which 82 could be identified. Of the 107 metabolites, 82 were significantly different in the yellow leaf lamina and control ($P< 0.01$).

Metabolome PLS-DA

The PLS-DA analyses showed a clear separation of metabolites in the leaf material from all three yellowing conditions (Figure 1). It is interesting to note that the metabolic patterns differentiated the three phenotypes well before visual symptoms were observed (Figure 1A).

Before onset of visual symptoms in the leaves, Component1 accounted for 50% and Component2 for 17% of the total variation. Component2, but not 1, clearly separated control samples from the three stresses, well before the onset of symptom development (Figure 1A).

There was no clear separation between the controls and three stresses in Component1 despite the fact that it accounted for 50% of the overall variation.

After the onset of visual symptoms in the leaves, Component1 accounted for 46% and Component2 for 24% of the total variation (Figure 1B).

Component2 separated the three clusters, control, water stress, and a combination of senescence and YCS (Figure 1B) samples, well before the onset of symptom development (Figure 1A). Component1 separated water stress and control samples.

This suggests that there is more similarity between senescence and YCS than between YCS and water stress.

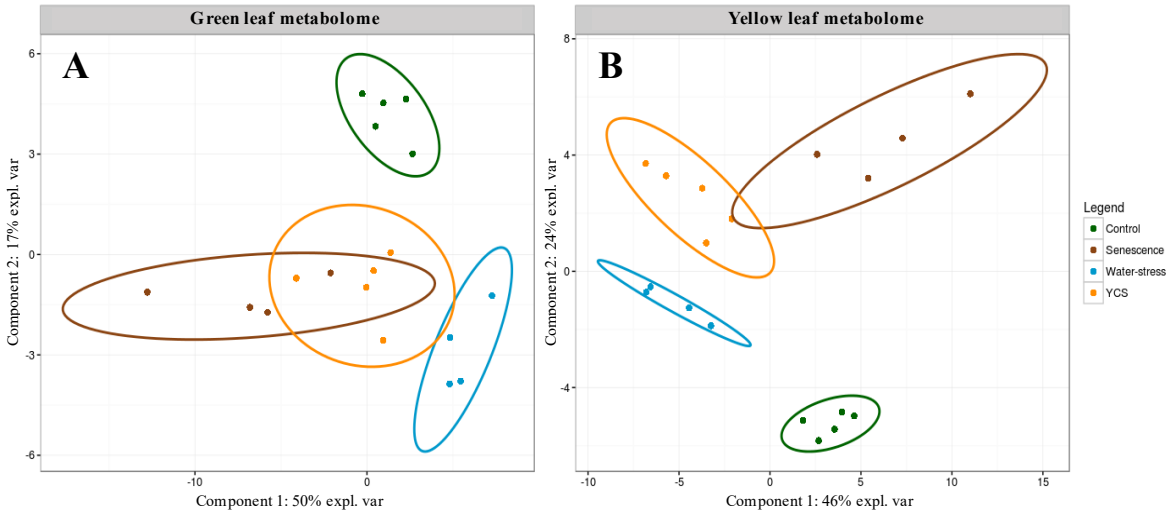

Fig. 1—Partial Least Squares-Discriminant Analysis (PLS-DA) scores plots of metabolome (Component1 vs Component2) using 107 metabolites of control and green leaf lamina (A) and yellow leaf lamina (B) from each YCS, senescence and water-stress samples. Ellipsoids represent 95% confidence interval.

Hierarchical clustering of metabolome differential abundance

Hierarchical clustering of yellow lamina was performed to further determine which metabolites were linked to differentiation of the yellowing types.

The heatmap of the 82 differentially abundant metabolites revealed dissimilarities between yellow lamina samples and control samples (Figure 2).

As could be expected, metabolite levels were up or down in the three stress conditions. However, interestingly some metabolites increased in two stress conditions but decreased in the third.

Cluster1 (seven metabolites) described a group with lower abundance in water-stress samples; this contained largely organic acids.

Cluster2 (27 metabolites) showed increased abundance in water-stress and YCS yellow lamina and was enriched for sugars and amino acids.

Cluster3 (16 metabolites) included metabolites highest in the control, comprising largely other organic acids.

Cluster4 (nine metabolites) increased in water-stress and senescence conditions and contained predominantly sugar alcohols. Cluster5, distinctly higher in water-stress, included a combination of some sugars, sugar alcohols and organic acids.

Transcriptome

For RNA-seq data, across all tissue types between 67 and 83 million reads were generated with a mapping percentage between 55 and 64% to the PacBio generated sugarcane transcriptome assembly built from Australian sugarcane varieties using leaf, culm and root tissues (Table 1). Total gene counts and RPKM values of transcripts mapping to contigs were used for statistical analyses.

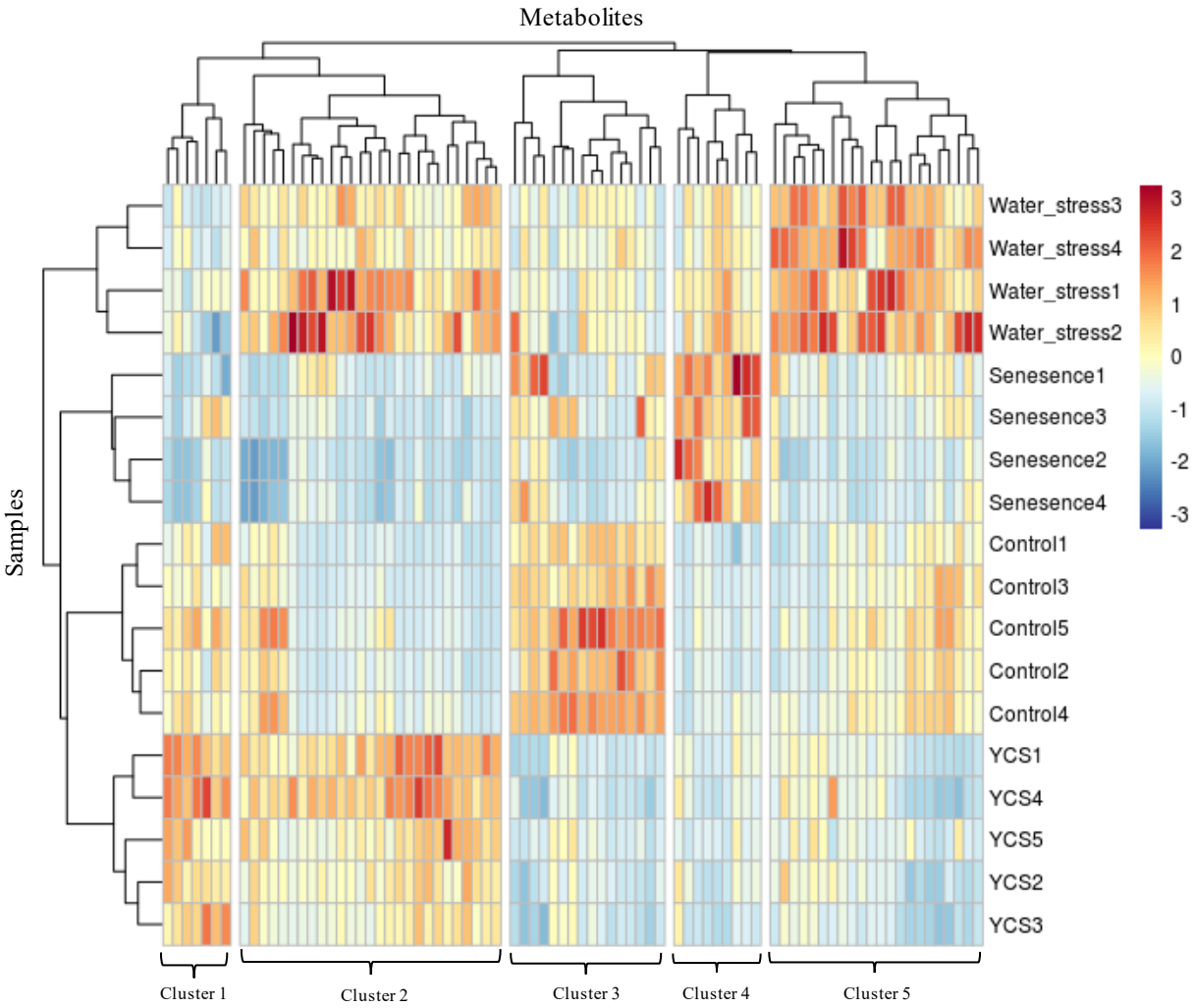

Fig. 2—Hierarchical clustering of differentially abundant (P<0.01) metabolites between control and YCS, senescence and water-stress yellow leaf lamina. Rows represent sample replicates. Columns represent individual metabolites (82 in total, cut tree at five categories to reveal clusters 1–5). Red indicates comparatively higher abundance, blue indicates comparatively lower abundance. Control sample replicates constitute normal metabolite levels.

Table 1—*Sample RNA-seq read and mapping percentage overview.*

Samples			Transcriptome	
Type	Condition	Colour	Average number million reads per sample	Percentage paired reads mapped to PacBio reference
Leaf	Control	–	83.70	63.6%
	YCS	Green	79.41	61.2%
		Yellow	72.51	55.6%
	Senescent	Green	68.80	61.6%
		Yellow	67.12	61.3%
	Water-stressed	Green	73.42	64.5%
		Yellow	72.22	63.8%

Control and green lamina samples had 1 292 contigs with significantly different (P<0.01) expression. In the control and yellow lamina samples, 3 517 contigs were differentially expressed.

PLS-DA was performed using RPKM expression values for all transcriptome contigs. Compared with the metabolome PLS-DA, gene expression PLS-DA contained more variables and displayed greater separation among sample types.

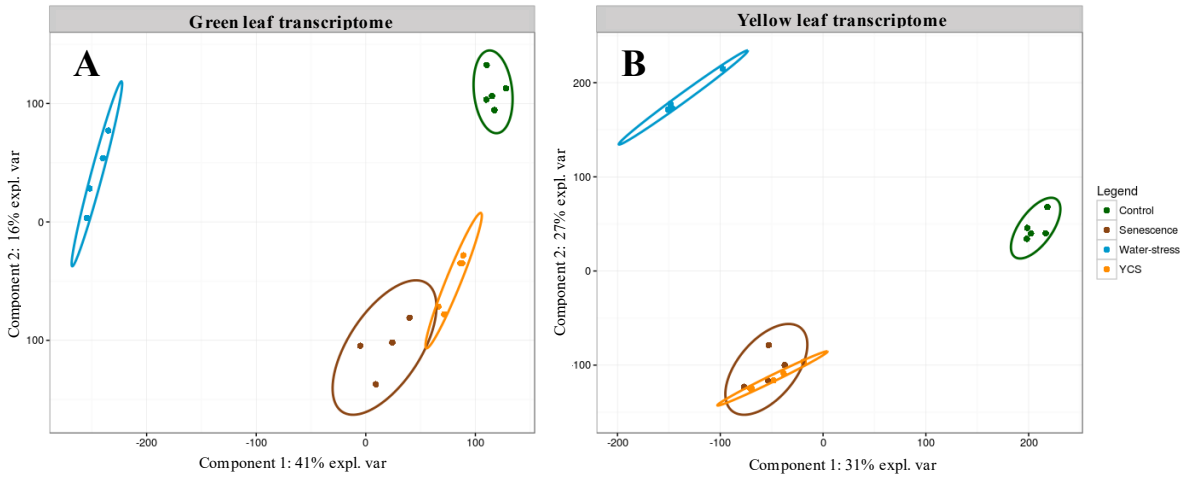

Fig. 3—Partial Least Squares-Discriminant Analysis (PLS-DA) scores plots of transcriptome (Component1 vs Component2) using mapping results against 107,598 contigs of PacBio sugarcane reference transcriptome of control and green leaf lamina (A) and yellow leaf lamina (B) from each YCS, senescence and water-stress samples. Ellipsoids represent 95% confidence interval.

Transcriptome PLS-DA

Transcriptome PLS-DA analyses showed gene expression partitioning for YCS and senescence leaf material, away from both control and water-stress leaf material (Figure 3), both before and after visual yellowing of the symptomatic leaves. In the green lamina for each of the sample types (Figure 3A), Component1 explained 41% of the total variation, and Component2 explained 16%.

Senescence and YCS were only separated slightly through both Component1 and 2. Water-stress samples were separated very clearly by Component1, whereas the control only separated from YCS/senescence by mostly Component2, despite it only accounting for 16% of the total variation.

The leaf material displaying the different yellow symptoms (Figure 3B) showed a separation additional to that of the green material. But the Component1 accounted for 31% of the total variation, and 27% was accounted for by Component2. YCS and senescence did not separate on either component, whereas the control and water-stress samples were separated distinctly by both. This implies YCS and senescence were closely related and, potentially, had common gene expression after the leaf material had become yellow.

Hierarchical clustering of DE transcriptome data

Hierarchical clustering was used to visualise differentially expressed genes in yellow lamina (Figure 4). The differential expression data were separated into five clusters.

Cluster1 (125 contigs) contained those with the highest expression in the control, indicating downregulation during senescence, water-stress and, to a lesser extent, YCS.

This cluster was enriched for photosynthetic electron transport, chlorophyll biosynthesis and chloroplastic ATP synthesis genes. Cluster2 contained contigs most abundant in control and water-stressed yellow lamina. This was the smallest cluster with only 33 contigs, and contained largely photosynthetic electron transport-related gene expression.

Contigs with the highest expression in the water-stressed yellow lamina were in cluster3. The 726 contigs were enriched for translation, protein turnover, protein-folding and signaling genes. Cluster4 (1 208 contigs) showed the highest expression in YCS yellow leaf material.

This cluster was largely made up of stress, signaling, carbohydrate metabolism and mitochondrion-related genes. Cluster5 (1 425 contigs) contained those with the highest expression in yellow senescing leaf material. This covered transcription-regulation, transport/export, chlorophyll catabolism and signaling.

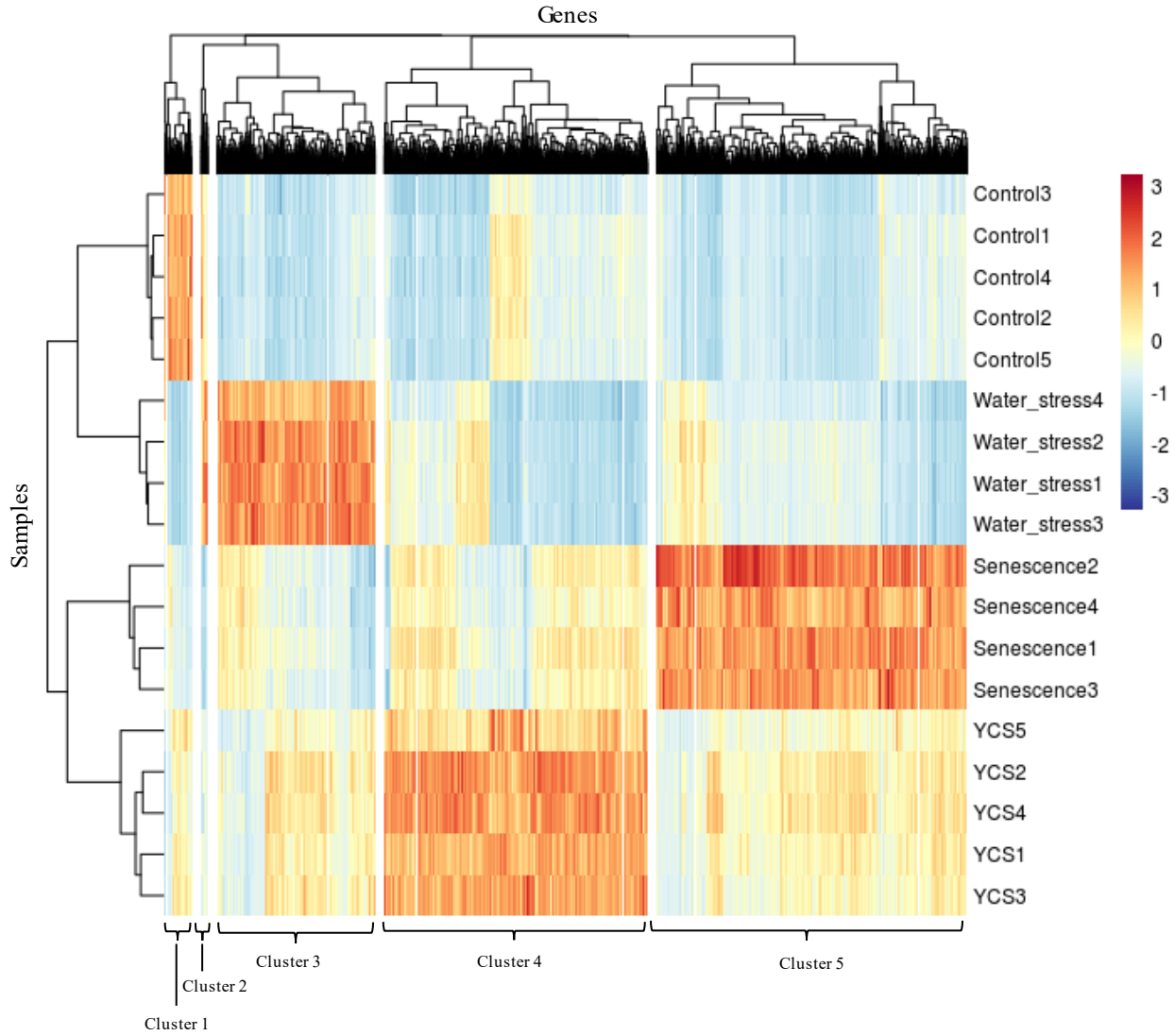

Fig. 4—Hierarchical clustering of differentially abundant (P<0.01) contigs between control and YCS, senescence and water-stress yellow leaf lamina. Rows represent sample replicates. Columns represent individual contigs (3,517 in total, cut tree at five categories to reveal clusters 1–5). Red indicates comparatively higher expression, blue indicates comparatively lower expression. Control sample replicates constitute normal (green, healthy condition) expression levels.

The heatmap showed a predominantly lower expression in the control across the 3 516 contigs used in the analysis. This suggests that different types of yellowing are unique in the upregulation in gene expression, rather than downregulation.

Despite the overlap of YCS and senescence in the PLS-DA of differentially expressed genes, all yellowing leaf material showed distinct differential gene expression profiles.

YCS diagnostic

In order to determine if yellowing, as a result of YCS, can be predicted in green leaf lamina for use as a diagnostic, we further analysed the differential gene expression of both YCS green and yellow leaf material.

Before the onset of yellowing, YCS leaf material had 5 167 contigs with differential expression from the control, and 15 757 after onset of yellowing. Of these differentially expressed contigs, 2 248 were the same in both green and yellow lamina. Of these 2 248, those with inconsistency in up- or downregulation between green and yellow lamina were discarded; similarly, those with less than 2-fold change in expression were also omitted, to leave 538 contigs.

In the 538 contigs, a total of 78 were part of the hierarchical clustering data (had unique expression between control and the different yellow leaf material) (Figure 4). Twenty-five of these were in Cluster4, which contains those most highly expressed in YCS yellow lamina. These would be the most ideal YCS diagnostic candidates. Among these 25 are genes related to transcription factor/DNA binding activity, and plant circadian clock (*REVEILLE 6* and *LHY*).

Discussion

Uniqueness of YCS yellowing

The data presented here show YCS could be differentiated from that of water-stress and senescence yellowing, on the metabolite and differential gene expression level (Figure 2 and Figure 4, respectively). However, transcriptome-wide analysis (PLS-DA) suggested YCS and senescence leaf material (late-phase) contain large similarities (Figure 3B), especially after yellowing of the lamina has occurred (Figure 3B). This is consistent with YCS causing the onset of senescence.

YCS yellowing and sugar

The leaf yellowing in YCS leaves could have been initiated through sugar accumulation, which is an alternative yellowing trigger to plant developmental (age-related) senescence (Lim *et al.*, 2007), also in *Saccharum* spp. (Martins *et al.* 2016). This is supported by significant metabolite differences found between yellow YCS and senescence leaf material (Figure 2), where YCS had accumulation of sugars, and senescence did not.

However, age-related senescence can be associated with, while not necessarily being triggered by, carbohydrate/sugar accumulation (Wingler and Roitsch, 2008). It is unlikely the sugar accumulation in YCS yellow leaves was a result of senescence in this study, because YCS leaves accumulated upwards of 100–400 nmol mg^{-1} DW^{-1} sucrose, whereas senescing leaves rarely exceeded 50 nmol mg^{-1} DW^{-1} (unpublished data, Sugar Research Australia). Control leaves had on average 35 nmol mg^{-1} DW^{-1} sucrose. YCS leaves had much higher levels of carbohydrate accumulation than that of senescing leaves, implying sugar accumulation in YCS could be the cause, rather than the effect, of an early-onset of senescence.

YCS and water-stress

Water-stress is often accompanied by osmolyte accumulation, including sugars, as a mechanism of plant adaptation to insufficient water (for review see Bhargava and Sawant, 2013). Both water-stressed and YCS yellow leaf material showed a higher abundance of sugar-related metabolites. However, water-stressed leaves had an otherwise distinct profile in both metabolites and gene expression data compared to YCS. It is unlikely YCS sugar accumulation in this study was caused by reduced water availability in the leaf.

This is consistent with YCS symptomatic leaves lacking typical water-stress symptoms of wilting and leaf edge curling (data not shown).

Further, distinct differential gene expression of water-stress and YCS leaf material might suggest a different cellular location of sugar accumulation in each. Water-stress causes osmotic adjustment through osmolyte accumulation in primarily the symplast. It is possible YCS sugar accumulation occurs elsewhere (e.g. apoplast). This may cause variation in gene expression, depending on the location of sugar accumulation. Investigating the cellular location of sugar accumulation in YCS leaves is required to clarify this.

Yellowing is not just one-track progression towards senescence

The gene expression results of this study imply leaf yellowing is not a one-directional process (e.g. senescence) in which the different samples of yellow material were at different time points. This is based on two lines of evidence: firstly, the variation seen in metabolite levels and gene expression for each water-stress, senescence and YCS showed increases or decreases from controls; not always a consistent direction of change from the control; secondly, metabolites and genes expression showed perturbances occurred in different metabolic pathways in the different types of leaf yellowing.

It could be argued the metabolic and gene expression changes are due to leaf developmental age. The water-stressed yellow leaf material sampled was younger (leaf 4) than the senescent (leaf 8+) and YCS and control (leaf 6) leaves. However, all leaves sampled were considered mature due to transition from sink to source (ie. leaf number >2). Additionally, enrichment in the differentially expressed gene clusters did not relate to developmental stage nor were typical of a time point in leaf development progression, but rather included plant responses to different conditions. When also considering green and yellow lamina samples were sourced from the same individual leaf, leaf age is considered unlikely to drive the separation seen in the data.

A similar argument could be made for the difference in sample collection site of water-stressed leaf samples and the remaining samples. In order to verify the results were due to water-stress and not site-specific differences, same-site sample collection is required.

We are currently working on establishing the link between the transcriptome and metabolome through the addition of the proteome in these different forms of leaf yellowing. Post-transcriptional and post-translational regulation play large roles in cellular activity, of which the proteome will provide more information. This would allow a better understanding of the similarity/differences of YCS, particularly in the onset of senescence.

The diagnosis of YCS yellow

A reliable method is required for diagnosing the onset of YCS. Sucrose has been implicated as a possible diagnostic due to high levels of accumulation in YCS-symptomatic leaves. However, sucrose can be present at normal levels (~35 nmol mg^{-1} DW^{-1}) in green lamina of YCS leaves, despite surrounding yellow lamina with typical YCS symptoms (unpublished data, Sugar Research Australia).

Gene expression unique to YCS is ideal as a diagnostic and can be performed in a laboratory with high-throughput. Genes involved in plant diurnal cycle regulation were found to be uniquely upregulated in both YCS green and yellow lamina. Of these, *REVEILLE 6* (*RVE6*) is presumed to be an afternoon-activated circadian clock gene due to its homology to *RVE8* (Hsu *et al.*, 2013).

Because samples analysed here were collected in the morning (between 8 and 10AM), this could suggest a unique disruption to the day-night cycling of YCS-symptomatic leaves. This is consistent with implications in sugar and starch metabolism in the leaf that occur during YCS in which diurnal cycling has a strong role.

Conclusions

YCS can be separated from water-stress and senescence leaf yellowing primarily in metabolism surrounding sugars and organic acids, and gene expression changes related to diurnal cycle signalling and carbohydrate metabolism. Twenty-five genes were identified to be predictive of YCS yellowing specifically.

Further work will be conducted to test these as potential diagnostic tools. This work provides a new focus area for further investigation into the effect YCS has on sugarcane leaves on a molecular level, in order to elucidate the cause.

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