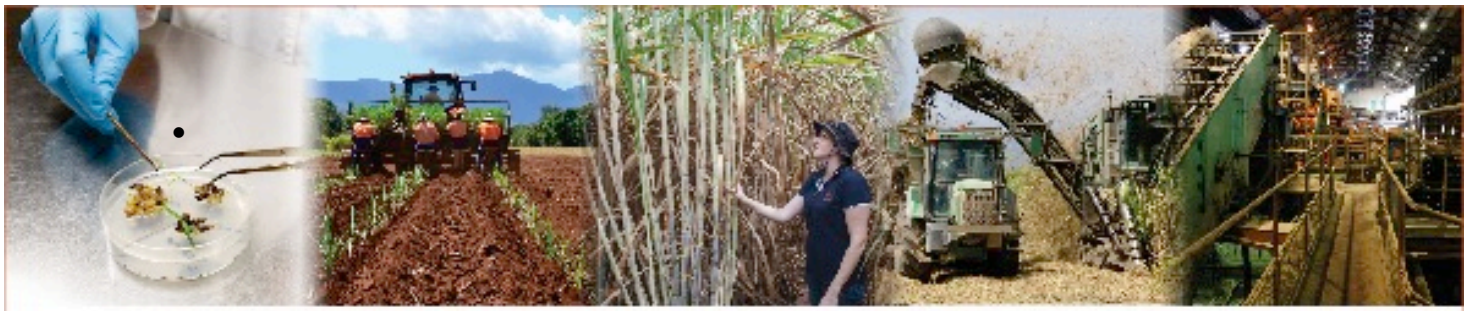

FINAL REPORT 2017/401

SUMO wrestling: understanding the molecular basis of drought tolerance in sugarcane

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Sugar Research Australia

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ABSTRACT

We report here that neither the plant growth hormone gibberellin (GA) signaling, nor SUMOylation post-translational mechanism, a well-known phenomenon triggered by several abiotic stresses, are involved in water stress-induced growth inhibition of sugarcane leaves. The Q208^{A} commercial sugarcane variety was subjected to water stress condition and conducted targeted experiments to understand the molecular aspects of leaf growth regulation under water deficit. Plants were water stressed gradually by withdrawing irrigation. Growth-related parameters, but not photosynthesis, were significantly affected by water deficit. By day 25 following stress imposition, the youngest leaves ceased growing. Analysis of cell division and elongation regions of youngest developing leaves revealed that neither DELLA protein, a negative regulator of GA, nor conjugation of SUMO1 protein increased in response to water stress, suggesting a complex molecular regulation of stress-induced leaf growth inhibition in sugarcane. Analysis of sugar and energy sensing and signaling genes indicate a possible molecular reprogramming occurring in the basal region of developing leaves during water deficit. Understanding the nature of this molecular reprogramming may lead to important insights on sugarcane leaf growth regulation under water stressed and non-stressed conditions.

EXECUTIVE SUMMARY

Drought tolerance is a complex trait which involves thousands of genes with different functions in the plants. Over the past years, the traditional focus has been to use transcriptome analysis to find drought stress marker genes (DSMG) to understand and improve drought tolerance in sugarcane. Unfortunately, this strategy has not led any useful outcomes through molecular or classical breeding. Interest in genetically engineered (GE) sugarcane for commercial sugarcane production is not gaining much momentum in Australia and elsewhere. The innovations proposed in this project are **(1)** to understand the molecular signaling of growth arrest in sugarcane, the first and most visible response upon water deprivation in sugarcane; **(2)** the discovery and application of new molecular regulatory mechanism(s) for improving crop growth and productivity; **(3)** to test an agronomic product to inhibit SUMOylation, allowing sugarcane plants to cope with growth arrest in the period of water deprivation. To achieve these aims, the commercial sugarcane variety Q208[A] was subjected to water stress condition and conducted targeted experiments to understand the molecular aspects of leaf growth regulation under water deficit. Plants were water stressed gradually by withdrawing irrigation. Growth-related parameters, but not photosynthesis, were significantly affected by water deficit. By day 25 following stress imposition, the youngest leaves ceased growing. Analysis of cell division and elongation regions of youngest developing leaves revealed that neither DELLA protein, a negative regulator of GA, nor conjugation of SUMO1 protein increased in response to water stress, suggesting a complex molecular regulation of stress-induced leaf growth inhibition in sugarcane. Analysis of sugar and energy sensing and signaling genes indicate a possible molecular reprogramming occurring in the basal region of developing leaves during water deficit. Understanding the nature of this molecular reprogramming may lead to important insights on sugarcane leaf growth regulation under water stressed and non-stressed conditions.

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1. BACKGROUND

1.1. Gibberellin (GA)-DELLA pathway under drought conditions

Cane yield and commercial cane sugar (CCS) are the two primary traits that determine sugar yield. Cane stalk (culm) growth is a major determinant of cane and sugar yield. Culm growth is highly sensitive to water deficit (Ferreira *et al.*, 2017) and heat stress, and they are important productivity constraints of sugarcane crop in all the major sugarcane growing countries. The true cost of productivity loss due to water is often unrecognised. For instance, it is estimated that the Australian sugar industry loses on average $260 million per annum due to water stress (Inman-bamber, 2007). The economic impact of recurring drought in sugar industries of Brazil, India and China is well documented.

From a plant developmental perspective, stem and leaf elongation are the most affected growth processes by water stress in sugarcane (Ferreira *et al.*, 2017). We have identified a higher order transcriptional regulator, ScGAI, that controls sugarcane growth and development (Garcia Tavares *et al.*, 2018). ScGAI is an inhibitor of both cell division and cell elongation, which are adversely affected by water deficit (Achard *et al.*, 2006). Molecular characterisation of ScGAI showed that it is a DELLA protein, and its action is regulated by gibberellin (GA), a plant hormone, -mediated destruction. Presence of GA reduces DELLA activity and consequently growth inhibition (Nelson and Steber, 2016). In contrast, once GA level decreases in the cell, DELLA proteins become stable and inhibit GA-induced growth (Nelson and Steber, 2016).

Previous studies showed that upregulation of GA levels could not rescue growth in transgenic maize under drought (Nelissen *et al.*, 2017). Interestingly, in sugarcane grown under non-stressed condition, ScGAI was found SUMOylated in the leaf, but not in the culm, in a developmentally regulated manner (Garcia Tavares *et al.*, 2018). ScGAI was SUMOylated maximally in the mature leaf tissue and least in the elongation zone. This finding suggests that SUMOylation of ScGAI may be playing a critical role in effecting organ-specific growth regulation in sugarcane. Over the past years, SUMO (Small Ubiquitin-like Modifier) protein conjugation onto target proteins has emerged as an important class of post-translational modification (PTM) system with a critical role in regulating plant responses to water and salt stress (Castro *et al.*, 2012). HyperSUMOylation of key regulatory proteins has been reported in plants exposed to environmental stress. This is considered as a rapid mechanism to reduce growth to survive stress periods (Conti *et al.*, 2008). Recent studies have demonstrated a key role for SUMOylation in stabilizing DELLA proteins under stress conditions in model plants (Conti *et al.*, 2014). Considering the expanding knowledge of molecular regulation of growth and in model plants and some broad-acre crops, little is known about the molecular basis of growth regulation in sugarcane. In this project, the role of SUMOylation of DELLA in sugarcane growth and development and stress responses have been studied and the results are presented below.

2. PROJECT OBJECTIVES

2.1. Aim

2.1.1. Hypothesis

The key question aimed in this study is whether SUMOylation of ScGAI is acting as a rapid growth retardation mechanism in sugarcane leaf under water deficit. **If so**, that knowledge may pave the way for exploiting the regulation SUMOylation for conferring sugarcane drought tolerance, including the application of natural or synthetic products that have been discovered and produced to inhibit SUMOylation for practical outcomes.

3. OUTPUTS, OUTCOMES AND IMPLICATIONS

3.1. Outputs

A better understanding of sugarcane leaf processes under water deficit.

3.2. Outcomes and Implications

N/A

4. INDUSTRY COMMUNICATION AND ENGAGEMENT

4.1. Industry engagement during course of project

N/A

4.2. Industry communication messages

The key message from this project is that neither DELLA, a growth repressor, nor SUMOylation are involved in water stress-induced growth arrest in young developing sugarcane. The results of this project have not yet been communicated to anyone outside the project.

5. METHODOLOGY

5.1. Plant material, growth condition and stress treatment

A commercial sugarcane variety Q208A was used as the experimental material. The experiments were performed in a shade-house in Sugar Research Australia, Indooroopilly, Brisbane. All plants (19 days old plants produced from stem nodal cuttings) were planted individually in polyethylene pots of 10 dm^3 capacity (54 cm x 15.5 cm diameter) containing a soil mix (Brunnings Garden potting mix/sand, 50:50 ratio, w/w), and fertilized monthly (15 g per pot) with slow-release Osmocote granules (Scotts, Australia). Plants were grown under full irrigation (soil mix moisture level maintained greater than 60 % (v/v) by daily watering) until the initiation of water stress treatment. Stress treatment was imposed by withdrawing irrigation for the rest of the experimental period. Moisture content of top 20 cm layer of soil in the pot was measured daily in both control and stress treatments with the HydroSense II system moisture probe (Campbell Scientific, USA). The meteorological data during the experiment were obtained from an automatic weather station located in the shade house (Campbell Scientific, Inc). The experimental layout followed a randomized complete block design with four replications (block), with 5 plants per treatment in each replicate. Leaves were numbered according to Kuijper (Kuijper, 1915). Shoots generally terminate with three visible growing leaves at different developmental stages just above the youngest fully-

expanded leaf (YFL). The YFL was numbered as L+1 and the successive expanding younger leaves were numbered as L0, L-2 and L-1, respectively, with L-2 being the youngest leaf (Fig.2).

5.2. Measurement of growth and other phenotypic parameters

No visible stalk was formed at the time of this measurement. Length of all elongating leaves was measured with a ruler from the soil surface to the tip of the leaf. The leaf elongation rate (LER) was computed based on leaf length difference over a 24 hour period ($mm.h^{-1}$) (Nelissen *et al.*, 2013). Shoot elongation growth, the distance from soil surface to the dewlap of YFL was determined with a ruler. The measurements were taken between 5 to 56 days after planting (DAP). To determine biomass, shoots were harvested between 54 to 56 DAP and the fresh weight (FW) was determined. The plant material was then dried at 65°C for 7 days, and the weight was recorded.

5.3. Photosynthesis measurements

Photosynthetic parameters were measured using intact L+1 with LI-COR infrared gas analyser LI-6400 (LI-COR Bioscience, USA) in well-watered control and water stressed plants. The chamber light (PAR) level was set to 2000 µmol photons m^{-2} s^{-1} and reference CO_2 concentration to 400 µmol mol^{-1} with a flow rate adjusted to 500 µmol s^{-1}.

5.4. Western blotting

Basal portion of leaf tissue was divided into two parts for analysis; the proximal and distal segments. They were snap frozen and stored at -80°C for analysis. Frozen leaf tissues were ground in liquid nitrogen to a fine powder, and solubilized in a lysis buffer (Hepes 50 mM, NaCl 75mM, KCl 75 mM, 0.05% Tween-20, 1% PEG (MW 3350), 10% ethylene glycol, pH 7.65). After vigorous vortexing, samples were centrifuged at 12 000 g for 10 min at 4°C. The supernatant was collected and its, total protein content was measured by Bradford assay (BioRad, USA). Samples from all extacts, normalised for total protein content, were separated in NuPAGE Novex 4%-12% gradient Bis-Tris gel, transferred onto PVDF membranes and probed with either polyclonal antibody raised (1: 1.000 dilution) against the N-terminal of sugarcane DELLA (Anti-ScGAI) or anti-SUMO1 (Abcam; ab5316) antibodies. Secondary HRP-conjugated anti-rabbit IgG was used at a dilution of 1:8000. Immunoblotted bands were visualized by the SuperSignal West Pico Chemiluminescent substrate (PIERCE). PVDF membranes were stained with Coomassie Blue (CB).

5.5. Droplet digital PCR (ddPCR)

Leaf tissue samples were snap frozen and ground using pre-cooled mortar and pestle with liquid nitrogen to a fine powder, and high-quality total RNAs were isolated and purified according to the Spectrum Plant total RNA kit protocol (Sigma-Aldrich, USA). For cDNA synthesis, 1 µg of total RNA was treated with RQ1 RNase-Free DNase (Promega, USA) at 37°C for 30 minutes to remove genomic DNA contamination, which was confirmed by PCR. Full-length cDNAs were synthesized with Improm-II reverse transcriptase enzyme (Promega, USA). The optimized ddPCR reaction mix (20 µL) contained 10 µL 2x QX200 ddPCR EvaGreen supermix (Bio-Rad), 100 nM of each forward and reverse primers and 1 µL of cDNA (50 ng). For each set of primers, one reaction mix without cDNA was performed as negative template control (NTC). Droplets were generated by the AutoDG (Bio-Rad) and PCR was carried out on C1000 TouchTM thermal cycler (Bio-Rad). PCR cycling conditions were as recommended by the manufacturer: 5 min enzyme activation at 95 °C followed by 40 cycles of 30 s at 95 °C and extension for 1 min at 60 °C with a ramp speed of 2 °C/sec; one cycle of stabilization of 5 min at 4 °C and 5 min at 90 °C. Samples showing good separation between positive and negative droplets were selected for analysis.

5.6. Data and statistical analysis

Data were processed using computer code written in Python shell (Seaborn, Matplotlib and Pandas libraries), and analysed using Scientific Python functions for linear regression, Shapiro-Wilk tests for normality and two-tailed Students's t-tests for significance testing.

6. RESULTS AND DISCUSSION

6.1. Water deficit adversely affected growth-related parameters during the early plant developmental stage (EDS).

The negative effects of water stress on early growth and development of sugarcane is well established (Ferreira *et al.*, 2017). Consistent with the previous reports, similar growth penalties were observed in this experiment (Figure 1). Q208^{A} plants exposed to water stress showed a significant decrease in above-ground biomass accumulation and reduced leaf and tiller numbers (Figure 1). The average shoot elongation rate was reduced by 18.6% in water-stressed plants. (Figure 1).

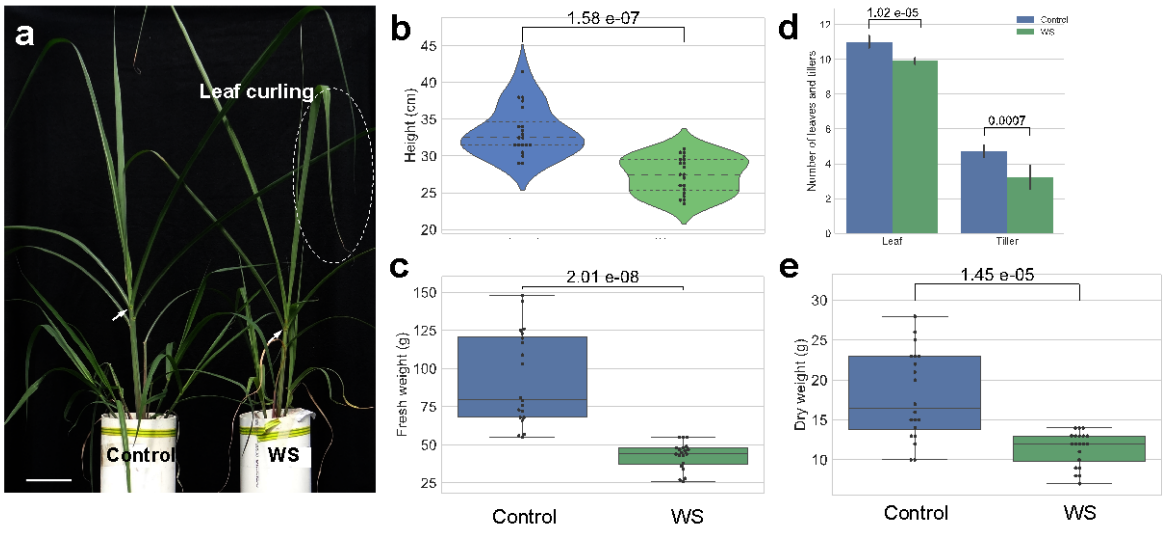

Figure 1. Water stress (WS) reduced different growth attributes of Q208^{A} plants at the early stages of development. (a) Growth inhibition and curled leaves (dashed circle) induced by water stress in Q208^{A} sugarcane plants at 54 days after planting (DAP). Arrows indicate the dewlap of the youngest fully-expanded leaf. Bar= 10 cm. (b) Violin plot showing the distribution of the height data of plants in both water regimes. Individual data points (black dots) are superimposed. Medians and interquartile ranges are exhibited as dashed lines. *P*-value is for equality of means (Student`s *t*-test). (c) Box plot showing the fresh weight of plants immediately after harvest. Individual data points (black dots) are superimposed. *P*-value is for equality of means (Student`s *t*-test). (d) Bar plot showing the number of leaves and tillers for the plants in both water regimes. *P*-values are for equality of means (Student`s *t*-test). (e) Box plot showing the dry weight of plants. The results presented here are based on the data from twenty plants for each treatment.

6.2. The elongation rate (ER) of the youngest leaves (L-2and L-1) was severely affected by water stress.

In order to test the hypothesis, i.e. SUMOylation of DELLA protein restrains sugarcane leaf growth under water deficit, we focused on the elongation rate of the youngest expanding leaves (L-2 and L-1). The ER of the youngest leaves was gradually reduced in response to declining soil moisture until

their growth stopped completely at 54 DAP (Figure 2). It is worth noting that the ER of both leaves followed a similar trend in well-watered and water stress conditions, indicating a potentially similar mechanism controlling cell division and expansion in both treatments.

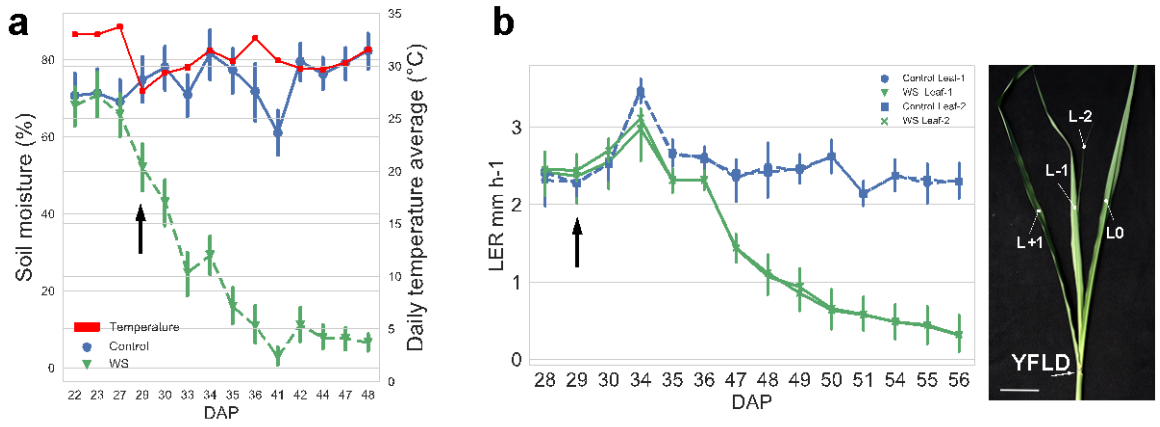

Figure 2. The elongation rate (ER) of the youngest leaves (L-2 and L-1) gradually reduced in response to declining soil moisture. (a) The average daily soil moisture (top 20 cm) in well-watered and water stress conditions (average of 3 measurements per pot per time point). The average daily temperature (between 8-9 am) is also presented. The black arrow (at 29 DAP) indicates the first day of irrigation withdrawal in stress treatment. (b) The ER of the youngest leaves (L-2 and L-1) under well-watered and water stress conditions (20 plants for each treatment). The black arrow (at 29 DAP) indicates the first day of stress imposition (withdrawal of irrigation). On right in more detail, the leaves were numbered according to Kuijper (Kuijper, 1915)**. YFLD means youngest fully-expanded leaf dewlap. Bar = 10 cm; DAP = days after planting.**

6.3. The molecular mechanism(s) triggering water stress-induced reduction in leaf elongation may not be associated with photosynthesis.

It has been known that photosynthesis is less sensitive to water stress than stem growth in sugarcane (Basnayake *et al.*, 2012). But there is no detailed characterization of the relationship between sugarcane stem growth and leaf photosynthesis under water deficit. Hence, we examined whether the initial reduction in leaf elongation (around 40 DAP; Figure 2) was triggered by a decrease in the photosynthesis rate or due to some other reasons. Our data shows that photosynthesis rate is not significantly different between treatments on day 13 after imposing stress by withdrawing irrigation, the time when leaf growth inhibition was evident (Figure 3a and b). It is important to note that the relatively long lag in the onset of leaf elongation despite precipitous drop in moisture content in the top 20 cm soil may be due to roots accessing the residual moisture present in the deeper soil in the pot.

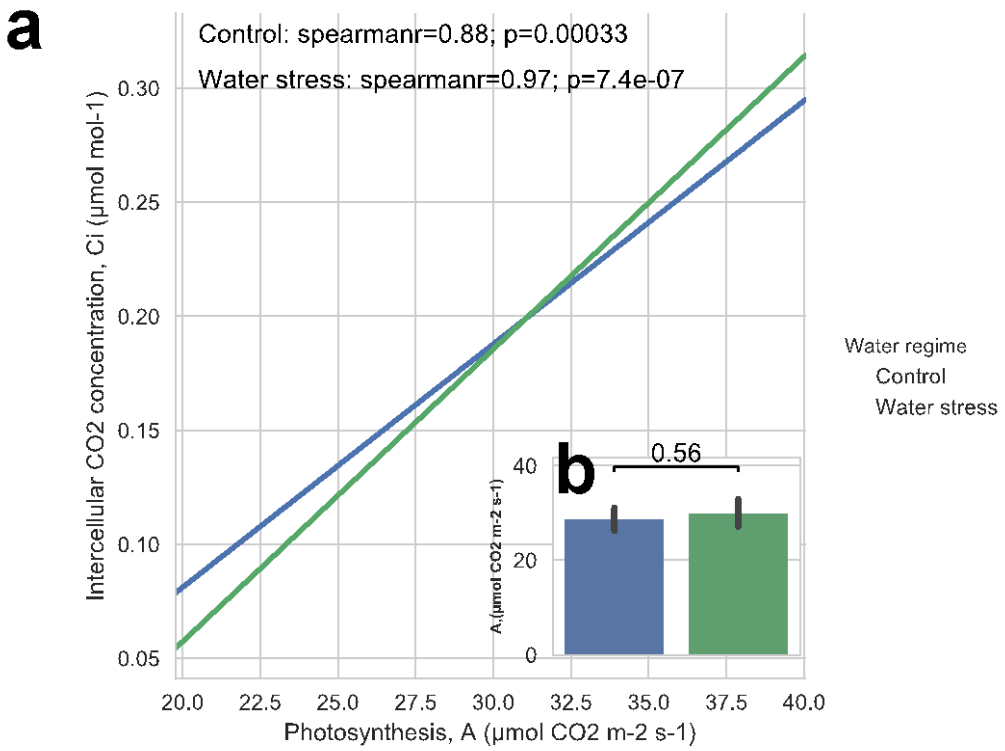

Figure 3. Photosynthesis was not affected under water stress condition. (a) Scatter plot of the intercellular CO_2 concentration plotted against the photosynthesis data (at 41 and 42 DAP; middle region of the leaf+1 from 11 plants for each condition). Spearman's correlation coefficient is showed for each treatment. (b) Bar plot displaying the photosynthesis data between the treatments. *P*-value is for equality of means (Student`s *t*-test).

6.4. DELLA protein is not involved in water stress-induced growth inhibition in the youngest leaves.

Complete growth inhibition of leaf growth was evident on day 54 after planting. Plants at that age were harvested and the old leaves were separated (Figure 2b; detail of leaf numbers). The pre-ligular region (proximal part of the leaf), a narrow and differencing region (Figure 4a; L – arrow), was identified within the youngest leaves (L-2 and L-1), and the leaf lamina was divided into two portions for DELLA protein analysis; the 2-cm actively elongating region above the ligule, termed proximal segment (PS) and the 6 cm-region above PS, termed distal segment (DS) (Figure 4a). Immunoblots probed with anti-DELLA antibodies revealed no expression of DELLA proteins in these tissues, indicating that DELLA is not involved in the water stress-induced growth inhibition of sugarcane leaves.

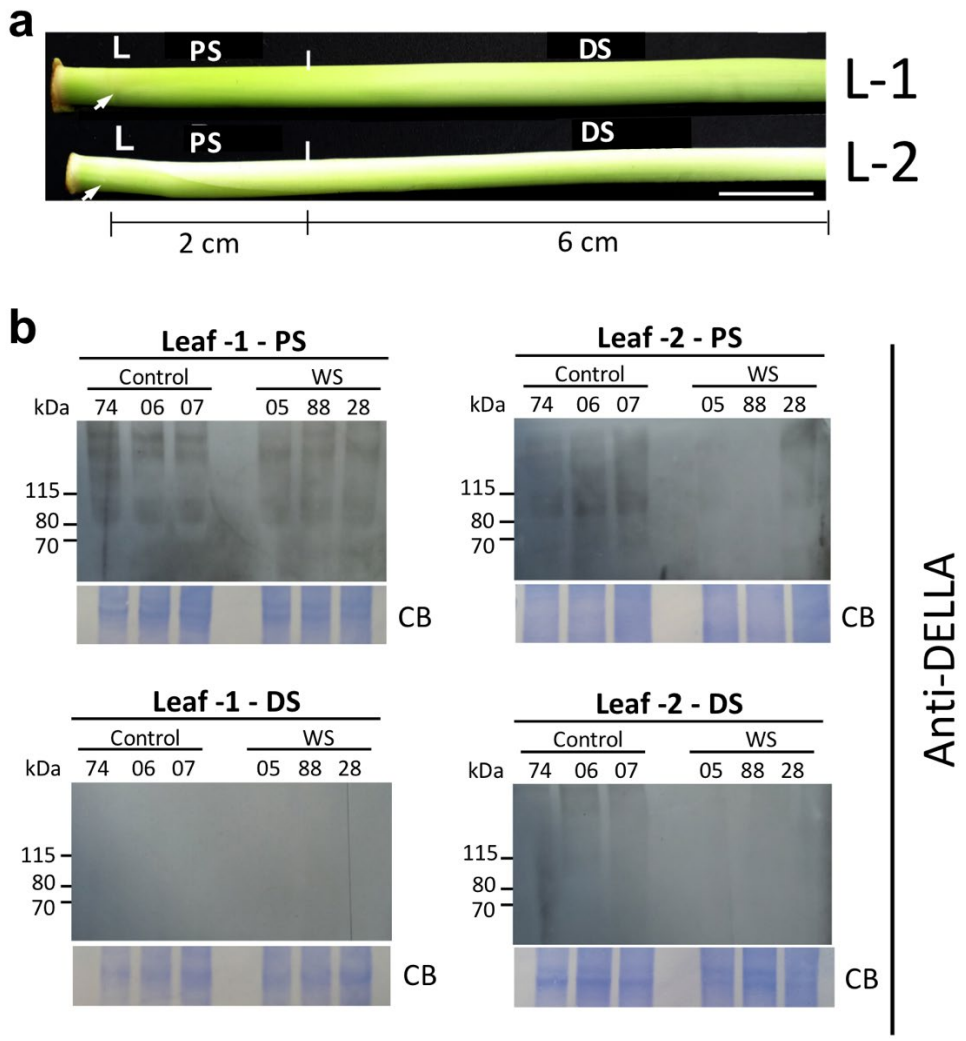

Figure 4. DELLA protein is not involved in the water stress-induced growth inhibition of sugarcane leaves. (a) Representative pictures of the youngest leaves (L-2 and L-1) displaying the proximal segment (PS) and the distal segment (DS) in the laminar portion of leaves (L – pre-ligule; arrow). Bar = 1 cm. (b) Immunoblotting of DELLA protein in PS and DS tissues from well-watered (Control) and water stress (WS) conditions. The randomly chosen numbers above sample lanes correspond to plant identification number; plants for this analysis was selected randomly. CB means coomassie blue-stained membrane as loading control.

6.5. Water stress-induced ScSUMO1 conjugates were not significantly increased in the base of the youngest leaves.

It has been reported that SUMO conjugation level can be induced by several stresses, modifying different sets of target proteins (Castro *et al.*, 2012). As demonstrated in Figure 5, SUMO1 conjugation did not significantly increase in the basal region of leaves under water stress condition compared to control. However, the occurrence of a few target proteins (asterisks) was visualized in the PS of L-1 under stress condition (Figure 5).

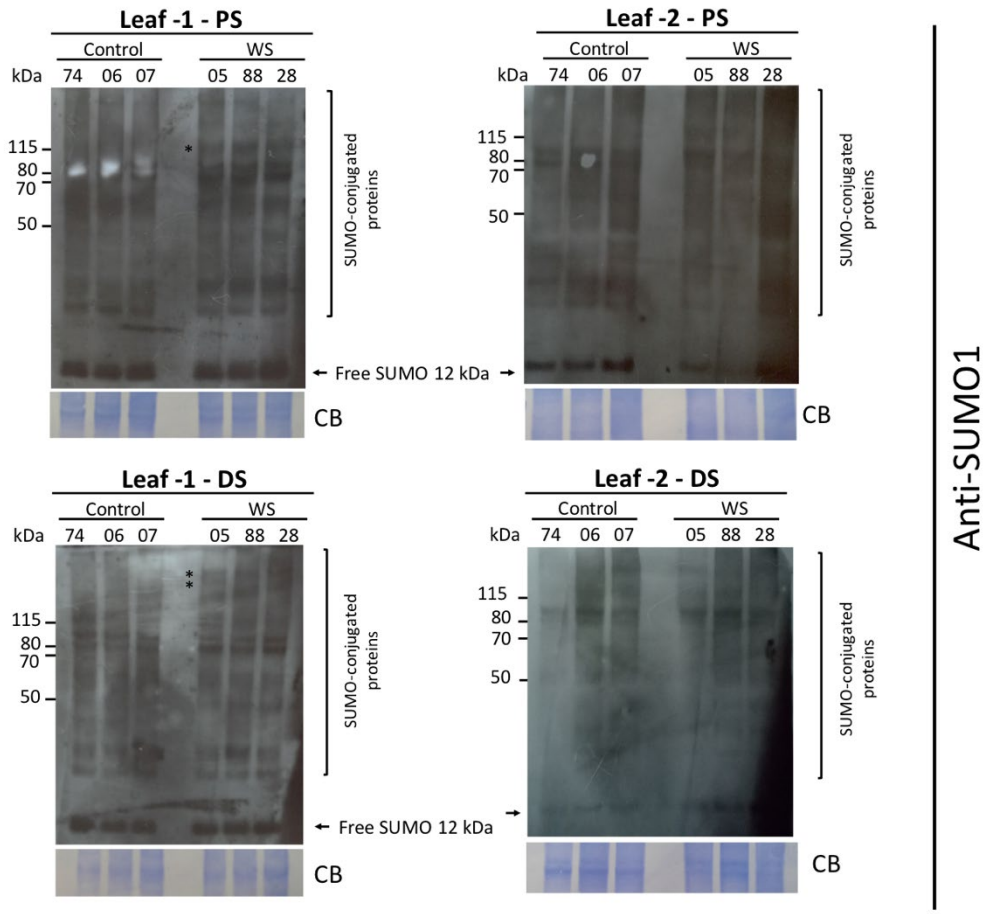

Figure 5. SUMO1 conjugates are not increased by water stress in the basal region of the L-2 and L-1. Immunoblotting of SUMO1 protein in PS and DS tissues from well-watered (Control) and water stress (WS) conditions. The randomly chosen numbers above sample lanes correspond to plant identification number. CB means Coomassie-brilliant blue-stained membrane as loading control. The asterisks depict bands differently expressed in water stress condition.

6.6. *Dehydrin* is severely inhibited in the basal region of the youngest leaves under drought stress.

We continued our analyses evaluating the expression profiles of two well-known drought marker genes, *Dehydrin* and *DREB2* (Figure 6a and 6b). Their expression provides clues as to whether the water stress-induced leaf growth inhibition is linked to ABA-dependent (*Dehydrin*) or ABA-independent (*DREB2*) signaling pathways or by both (Yoshida *et al.*, 2014). While the *DREB2* gene expression remained similar in both treatments, very surprisingly, *Dehydrin* gene expression was strongly repressed in the samples from plants under stress (Figure 6a). To the best of our knowledge, only sucrose and glucose have been demonstrated to repress the *Dehydrin* expression gene (Papini-Terzi *et al.*, 2009). It is likely that plants under water stress may be accumulating sucrose at the base of the leaves due to growth inhibition (Figure 1b, Figure 2b and Figure 3).

We then analysed the activity of *KRP2* gene, a direct inhibitor of cyclin dependent kinases (CDKAs) largely responsible for cell cycle control. The transcriptional analysis of *KRP2* gene can be highly informative, as it is a critical regulatory point for plant hormones such as gibberellins and auxin in cell cycle control (Kalve *et al.*, 2014). In our analysis *KRP2* expression was not significantly different

between the control and water stressed plants (Figure 6c), suggesting the complexity of leaf growth regulation under water stress in sugarcane.

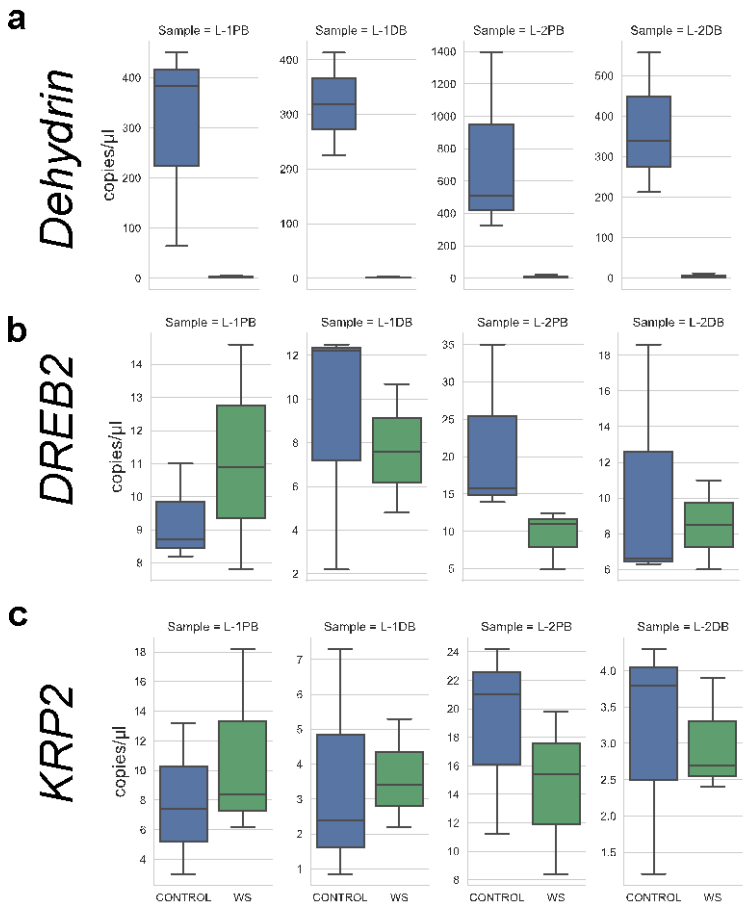

Figure 6. Gene expression analysis of *Dehydrin*, *DREB2* and *KRP2* genes. Box plot showing the distribution of gene expression in three biological replicates. ddPCR results are expressed as the number of target copies per microliter (copies/µl). The samples indicate leaves -1 and -2 (L-1 and L-2) and proximal and distal segments (PB and DB) of the basal part of elongating leaves.

6.7. *KIN10* and *bZIP11* genes are activated in response to water deficit in the basal region of the youngest leaves.

Stress conditions do not necessarily lead to carbon depletion, instead it can increase internal carbon availability in plants. Thus, in order to better understand the molecular mechanisms related to carbon metabolism in the basal region of the youngest leaves under drought stress, we analysed the expression level of *KIN10*, *bZIP11* and *TPS1* genes. The catalytic alpha-subunit, i.e. KIN10, of sucrose-non-fermenting-1-related protein kinase-1 (SnRK1) is a key metabolic sensor that decode energy deficiency signals. It is widely known that KIN10 acts as a regulator of gene expression upon starvation and stress conditions (Hummel *et al.*, 2009). Among the KIN10-activated genes, the members of the S1 class of the basic leucine zipper (bZIP), i.e. *bZIP11* and *bZIP53*, mediate some of the transcriptional changes in many biochemical pathways that reprogram amino acid metabolism (Smeekens *et al.*, 2010). However, SnRK1 activity is suppressed by trehalose-6-P (Tre6P), and Tre6P level reflects the cellular concentration of sucrose in response to high sugar availability. Tre6P is synthesized from UDP-glucose and glucose-6-P by the enzyme trehalose-6-phosphate synthase1

(TPS1) whose regulation is positively related to sucrose concentration (Rolland *et al.*, 2006). According to our data, we could observe an increased expression of *KIN10* and *bZIP11* in the basal region of leaf -1 under drought condition (Figure 7). However, their expressions did not show any difference in both conditions for the segments of the leaf -2, with the exception of *bZIP11* that also increased in response to drought in the proximal segment. Interestingly, *TPS1* showed the opposite trend, with the highest levels of expression occurring in control condition (Figure 7). Therefore, these abovementioned data indicate that there is likely a low sucrose levels under drought conditions in the basal region of the youngest leaves, mainly leaf -1, which results in the reprogramming of amino acid metabolism through the activation of *KIN10* and *bZIP11* genes.

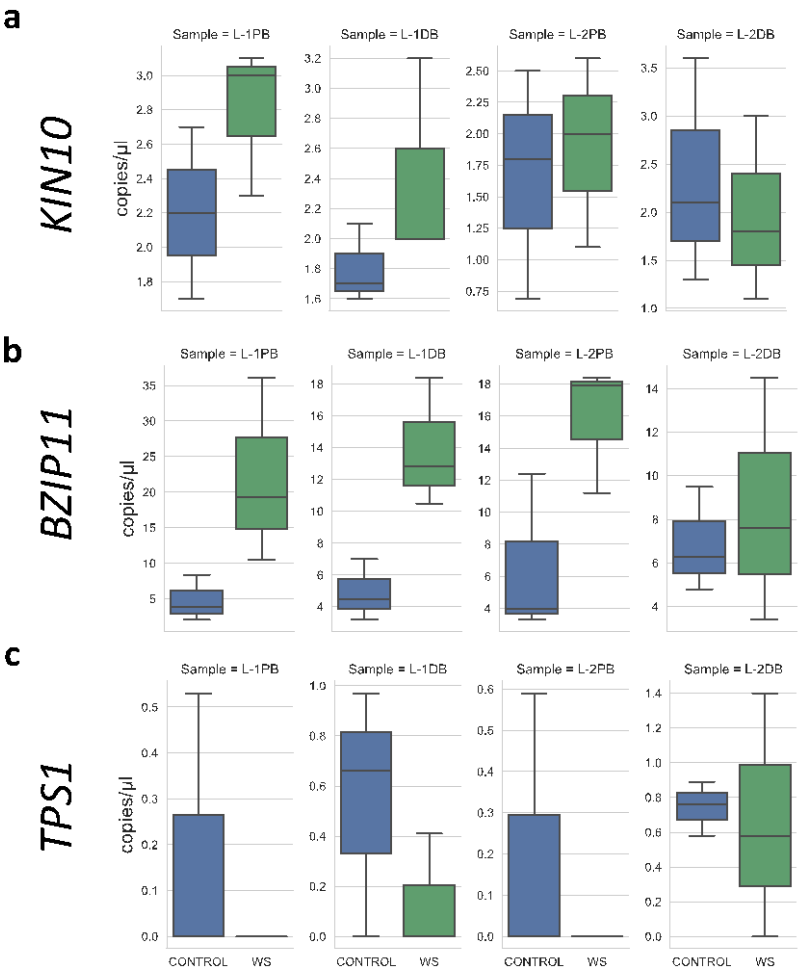

Figure 7. Gene expression analysis of *KIN10*, *BZIP11* and *TPS1* genes. Box plot showing the distribution of gene expression of three biological replicates. ddPCR results are expressed as the number of target copies per microliter (copies/µl). The samples indicate leaves -1 and -2 (L-1 and L-2) and proximal and distal segments (PB and DB).

7. CONCLUSIONS

As sessile organisms, plants must be able to sense and rapidly react to unfavourable environmental conditions. It is now becoming increasingly evident that hormones play an important regulatory role as internal mediators of this complex communication between plants and the environment. Several studies have demonstrated that drought stress effects on elongation growth are at least partly mediated by altered gibberellin metabolism. Therefore, GA-DELLA signaling appears to be a major

point of regulation to restrain growth under water stress. Sugarcane DELLA has been identified recently, and, strikingly, a link between SUMOylation, a post-translational modification process, and DELLA-mediate growth regulation in non-stressed leaves was observed. Apart from many developmental functions, SUMOylation increases rapidly in response to stress conditions. Therefore, the hypothesis of this project was to test whether SUMOylation of ScGAI (sugarcane DELLA) is acting as a rapid growth reduction mechanism in sugarcane under water deficit. Our data revealed that neither DELLA, a growth repressor, nor SUMOylation are involved in the water stress-induced growth arrest in the youngest leaves in sugarcane.

Our next approach was to verify the transcriptional level of well-recognised markers of water stress in plants. While the *DREB2 and KRP2* gene expressions remained similar in both treatments, very surprisingly, *Dehydrin* expression was strongly repressed in the samples from plants under stress. This result provides *Dehydrin* as a novel candidate of drought marker for sugarcane leaves.

Drought condition can also change a set of processes relating to sensing and signaling stress-associated energy deprivation to enable the plant to survive under unfavourable conditions. Thus, we evaluated the expression of genes involved in energy signaling. Our data showed the activation of *KIN10* and *bZIP11* genes indicating a low energy level under drought conditions in the basal region of the youngest leaves. Collectively, these results demonstrate that water stress restrains leaf elongation by a GA-independent mechanism, what appears to be linked to important regulatory genes associated with energy metabolism.

8. RECOMMENDATIONS FOR FURTHER RD&A

Getting a good resolution on the link between energy metabolism, cell cycle and cell growth regulation and the master regulators of these processes in sugarcane under water deficit would be useful.

9. PUBLICATIONS

N/A

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