University of São Paulo
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Comprehensive analysis of sugarcane (*Saccharum* spp) gene expression changes in response to drought and re-watering conditions

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Thesis presented to obtain the degree of Doctor in Science.
Program: International Plant Cell and Molecular Biology

Piracicaba
2017

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Bachelor and Licentiate in Biological Sciences

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RESUMO

Análise global das mudanças na expressão gênica em cana-de-açúcar (*Saccharum spp*) em resposta às condições de seca e reidratação

A exaustão dos combustíveis fósseis juntamente com os efeitos não desejáveis de seu uso, tonaram a cana-de-açúcar uma cultura atrativa para o mercado de biocombustíveis, aumentando a sua importância econômica e ambiental. A posição do Brasil como o principal produtor de cana-de-açúcar e a necessidade de expandir a área plantada para regiões com condições menos favoráveis, tornam o estudo da seca, um dos principais estresses abióticos que afetam a produtividade da cultura, essencial para o futuro do Brasil como o principal exportador dessa comoditie. Este trabalho tem o objetivo de fornecer uma análise global das respostas da cana-de-açúcar à seca, tanto em nível fisiológico quanto molecular. Para isso, foram seguidas quatro estratégias. Primeiro foi realizada uma análise da fisiologia e do transcriptoma (microarranjo) de plantas de cana-de-açúcar cultivadas em casa de vegetação e estressadas por três períodos diferentes (4 dias de estresse, 6 dias de estresse e reidratação). Os tecidos analisados foram folha e raiz. Segundo, com o objetivo de identificar diferentes genes e novos padrões de expressão, foi realizada a análise de RNA-Seq em tecidos de folha e raiz utilizando a condição mais discrepante identificada pelo microarray; terceiro, foi feita a análise de um experimento de progressão da seca por meio da fisiologia e qRT-PCR usando genes candidatos selecionados. A quarta estratégia foi a construção de redes de co-expressão objetivando detectar módulos de genes relacionados à resposta a seca. As análises de fisiologia mostraram que as plantas estavam sob estresse moderado a severo com diminuição de até 97% na fotossíntese. Os dados de microarray levaram à identificação de 7.867 SAS únicos com diferença de razão de expressão maior que 2 ou menor que 0,5, e 575 SAS únicos diferencialmente expressos. A análise das sequencias identificadas permitiu a observação de que em folhas, depois de 4 dias de estresse, há basicamente a transdução dos sinais obtidos a partir do ambiente, enquanto depois de 6 dias e após a reidratação há uma resposta mais funcional da planta, com a última conduzindo o metabolismo de volta à homeostase. No caso das raízes foi observado uma resposta similar, porém, as raízes demoram mais tempo para voltar à condição inicial, de forma diversos genes continuam reprimidos mesmo após a reidratação. Há ainda rotas metabólicas, como o Biosíntese de Fenilpropanoides, que apresentam perfis opostos nos tecidos analisados, sendo ativada em um e reprimida no outro. Além disso, enquanto em folhas há uma restrição na fotossíntese, em raízes parece existir uma restrição no crescimento. A análise *de novo* do RNA-Seq mostrou 28.240 "features" diferencialmente expressos em folhas e 7.435 em raízes, enquanto a utilização do genoma de referência (dados não publicados) identificou 38.317 genes diferencialmente expressos em folha e 7.649 em raiz, sendo que a análise das rotas do KEGG indicam que o ABA tem um papel principal nas respostas à seca em ambos os tecidos, no entanto em folhas existe uma interação entre fitohormônios. O experimento de progressão da seca confirma os resultados obtidos a partir do microarranjo e mostram que quando o estresse é severo, a expressão gênica começa à diminuir, sugerindo que a planta pode estar entrando em senescência. As análises de coexpressão permitiram a determinação de três módulos correlacionados com parâmetros de fisiologia alterados durante o estresse hídrico, e conduziram à identificação de alguns genes centrais que podem ser importantes para as respostas da cana à seca. Além disso, foi possível identificar genes que tanto pela análise de co-expressão quanto pelo qRT-PCR apresentam padrões similar de expressão. Juntos, esses resultados forneceram uma visão global das alterações que ocorrem na cana-de-açúcar em resposta ao estresse hídrico e ajudaram a obter conhecimento para seleção de genes candidatos adequados para o melhoramento genético de plantas.

Palavras-chave: Cana-de-Açúcar; Estresse Hídrico; Expressão Gênica; Transcritoma; Redes de Coexpressão

ABSTRACT

Comprehensive analysis of sugarcane (*Saccharum* spp) gene expression changes in response to drought and re-watering conditions

The exhaustion of oil fields together with the undesirable effects of its use has turned sugarcane into an attractive crop for the biofuel market, increasing its economic and environmental importance. The position of Brazil as the world's major sugarcane producer and the need to expand the planted area to soil with less favorable conditions makes the study of drought, one of the abiotic stresses affecting the most of this crop yield, essential for the future of Brazil as the main exporter of this commodity. This work has the aim of providing a comprehensive analysis of sugarcane drought responses in the physiological and molecular levels. In order to do that we followed four strategies. First, we performed the analysis of physiology and transcriptome (microarray) of drought stressed sugarcane plants in three time points (4 days of stress, 6 days of stress and re-watering) of a greenhouse experiment. The plant material analyzed was leaves and roots. Second, aiming to identify different genes and new patterns of expression it was done the analysis of RNA-Seq from the most discrepant condition, from both leaves and roots, found by the microarray, third, we performed the analysis of a drought progression experiment through physiology and qRT-PCR of selected candidate genes and forth we built co-expression networks to detect interesting patterns. Physiology analysis showed that plants were under moderate to severe water stress with decreases of up to 97% in photosynthesis. Microarray data indentified 7,867 unique SAS with a fold change of more than 2 or less that 0.5, and 575 unique SAS differentially expressed. The analysis of the identified sequences allowed the observation that in leaves after 4 days of stress, the plant is mostly transducing the signal from the environment, while after 6 days and after rehydration there is a more functional response of the plant, with re-watering leading the metabolism back to homeostase. In the case of roots, it was observed a similar response, however roots take longer to go back to the initial condition, since several genes are still being down-regulated even after re-watering. There are also pathways presenting an opposite pattern in the analyzed tissues, being activated in one tissue but repressed in the other, such as Phenylpropanoid Biosynthesis pathway. Furthermore, while in leaves there is a restriction on photosynthesis, on roots it seems to be a restriction on growth. RNA-Seq *de novo* assembly showed 28,240 differentially expressed features in leaves and 7,435 in roots, while using the reference genome (unpublished data) it was possible to identify 38,317 differentially expressed genes in leaves and 7,649 in roots, and the analysis of KEGG pathways indicate that ABA has a major role in both leaves and roots responses to drought, but in leaves there is an interplay of phytohormones. Drought progression experiment confirms the results obtained from microarray and shows that when stress is extreme, gene expression starts to decrease, suggesting the plant might be entering in senescence. Co-expression analysis allowed the determination of three modules correlated with physiological parameters altered during water stress, and lead to the identification of some possible hub genes that may be important for sugarcane responses to drought. Furthermore, it was possible to identify genes that through both co-expression and qRT-PCR analysis had similar patterns of expression. Altogether, these results give us a comprehensive view of the alterations in sugarcane responses to water stress and helped us gain insight for defining better suited candidate genes for plant breeding.

Keywords: Sugarcane; Water Stress; Gene Expression; Transcriptome; Co-Expression Network

1. INTRODUCTION

Sugarcane (*Saccharum* spp.) is a perennial, tropical or subtropical grass that belongs to the Poaceae family and has been cultivated and bred for thousands of years (MING et al., 2006). It is a C4 grass whose economic and environmental importance has been growing (AMALRAJ et al., 2010; LEMBKE et al., 2012; ROCHA et al., 2007; ZHOU et al., 2012), especially because it is a highly competitive source of sucrose and ethanol in tropical and subtropical countries (CALSA; FIGUEIRA, 2007). This crop contributes to about 60% of the white sugar produced in the whole world, being its biomass still used to produce bioethanol and generate electricity (AMALRAJ et al., 2010).

The importance of the sugarcane agroindustry has been growing due to the decreasing oil reserves and high prices of petroleum allied to the increase of the population conscience regarding the environment and the undesirable effects of using fossil fuels in the balance of atmosphere carbon and in the increase of greenhouse gases emission (National Company of Supply – Conab, 2016). In this aspect, sugarcane is considered a great option for the biofuel market, in which its economic and environmental value can be exemplified by the decrease in greenhouse gases emission, lower cost for production and higher ethanol productivity, when compared with other crops, such as corn and sugar-beet (GOLDEMBERG; GUARDABASSI, 2010).

Brazil is the main world producer of sugarcane, with a planted area of about 8.7 million hectares. It is responsible for more than half of the sugar commercialized in the world, and the average increase rate of production is estimated to reach up to 3.25% by 2018/19 (Ministry of Agriculture, Livestock and Food Supply – MAPA, 2012). Therefore, the rise in the world demand for ethanol derived from renewable sources associated with the amount of arable land and the edafoclimatic conditions, has turned Brazil into a promising country for the exportation of this commodity (National Company of Supply – Conab, 2016).

In Brazil, sugarcane production is centered in the Northeast and South-central regions (Sugarcane Industry Union – ÚNICA, 2012), with the major expansion of planted area being localized in the Southeast region, especially in São Paulo state (National Company of Supply – Conab, 2012). This region is responsible for about 60.7% of sugar production and more than 60% of ethanol production in the country (National Company of Supply – Conab, 2016). However, the increasing world demand for biofuels is requiring the occupation of new tillage areas, including less favorable lands (ENDRES, 2010).

Sugarcane modern cultivars are polyploid and aneuploid clones, derived from interspecific hybridization between S. officinarum and S. spontaneum (HOARAU et al., 2001). Commercial varieties have 8 to 12 sets of the haploid genome with 100 – 130 chromosomes, and different levels of aneuploidy (MING et al., 2006). This aspect makes sugarcane a particularly challenging crop for plant breeding. Furthermore, crossing of large genomes, which suffered recent duplications in order to allow the chromosomal pairing and recombination turns each genotype of each progeny into a unique genome.

Abiotic stresses are the primary causes of crop loss worldwide, reducing average yields for most major crops by more than 50% (RODRIGUEZ; CANALES; BORRAS-HIDALGO, 2005). These environmental constraints have also been shown to impair sugarcane productivity (ROCHA et al., 2007; SEKI et al., 2003), whose cultivation faces considerable losses due to inappropriate or unfavorable soil and climatic conditions (KIDO et al., 2012).

Water deficit constitutes one of the major stresses that affect the development of sugarcane (PRABU; THEERTHA PRASAD, 2012; SUGIHARTO et al., 2002), limiting its productivity (ROCHA et al., 2007; SEKI et al., 2003) and leading to considerable yield losses (KIDO et al., 2012). During 2014, the decrease in the rain in the southeast region, especially in the states of São Paulo and Minas Gerais, led to a harvest reduction of 8.5% compared to the

previous season (Brazilian Sugarcane Industry Association – UNICA, 2014). Even a non-constant dry climate for a small period is capable of modifying the formation of internodes that become shorter despite not affecting the overall productivity (National Company of Supply – Conab, 2016).

Taking into account these aspects, breeding new varieties that have better water use efficiency or that are able to tolerate/resist drought has become of major importance (LI et al., 2016a) and a necessity for the future of agriculture (PRABU et al., 2011). Nevertheless, owing to its genetic complexity and polyploid nature with significant levels of chromosomal mosaicism (MENOSSI et al., 2008), breeding through conventional techniques is challenging and not sufficient anymore to develop new varieties, being necessary to build up new tools that will allow the obtainment of more tolerant or resistant varieties.

Characterization of genes underlying drought responses and elucidation of regulatory mechanisms related to sugarcane drought tolerance can be of great help to direct strategies that will ultimately lead to an increase in productivity and an expansion of the cultivated area to regions such as the northeast of Brazil, which has a dry climate due to the lack of rain (LEMBKE et al., 2012; SILVA et al., 2013a). Gene expression profiling associated to rehydration and drought responses is a way to comprehend the regulatory mechanisms that govern the tolerance and adaptation to drought (GENTILE et al., 2013; LEMBKE et al., 2012; NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014; PERRONE et al., 2012).

The knowledge of the mechanisms underlying drought responses and its relation with carbon partition and other characteristics of interest (LEMBKE et al., 2012), such as the lignocellulose content (VEGA-SÁNCHEZ; RONALD, 2010) can also be of great help to the increase of productivity. Furthermore, improved drought tolerance is important to the development of biofuel-focused canes (energy canes – high sucrose levels, increased biomass yield and cell wall altered for enhanced saccharification) that would not compete for tillage areas with other food crops (WACLAWOVSKY et al., 2010). Altogether, this knowledge can be used to develop better breeding strategies for drought tolerance, which can sustainably reduce the impact of drought stress (MWENYE et al., 2016).

The results of our work provide a comprehensive view of the changes on sugarcane transcriptome in response to one of the major stresses affecting its yield. In order to do that, it was analyzed (a) leaf and roots tissues following not only the traditional pipeline to identify differentially expressed genes, but also the identification of unknown sugarcane genes by RNA-Seq; (b) the physiology analysis in both, specific time points and during drought progression; (c) the evaluation of the expression level of candidate genes during drought evolution in a time-course experiment and (d) a co-expression analysis using data from this work and previous works of the group. These results provide targets for plant breeding for drought tolerance, especially since rehydration had not yet been contemplated in the previous works of our group (LEMBKE et al., 2012; ROCHA et al., 2007) and physiological data was not as detailed as done in this study. Furthermore, there is an absence of studies analyzing the changes in sugarcane roots transcriptome in response to drought (KIDO et al., 2012).

Hence, this work aimed to analyze sugarcane gene regulation mechanisms involved in drought responses and to start to understand the architecture of sugarcane drought-related networks. It was intended to direct future studies about water stress sugarcane responses, providing a comprehensive view of the alterations in gene expression due to this stress imposition.

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3. CHAPTER I – CHANGES IN THE SUGARCANE TRANSCRIPTOME IN RESPONSE OF DROUGTH AND RE-WATERING CONDITIONS

Abstract

In Brazil, sugarcane has an increasing economical importance represented by its role in the sugar and biofuel markets, and, therefore, there is an increasing need to use land with unfavorable conditions to expand the plantations. In this context, understanding the changes in sugarcane transcriptome that are interconnected with alterations in plant physiology provide usefull information to direct plant breeding works. This chapter aims to provide such integrated view by using the correlations between a variety of physiological parameters and the alteration of the expression of genes involved with the plant's response to water stress. In order to do that it was performed a greenhouse experiment using sugarcane plants from the variety SP80-3280, stressed for 4 days and 6 days, and also after two days of recovery. Physiological parameters evaluated included phostosynthesis, transpiration, stomatal conductance, water use efficiency, effective quantum yield, among others. Transcriptome analysis was done using microarray for all three conditions and RNA-Seq for the most discrepant condition (6 days without water). Results show that after 6 days without water there was a huge drop on the values for physiological parameters. Photosynthesis, for instance, decreased 98%. Those alterations were reflected on the gene expression, once genes involved with photosynthetical processes were enriched in the down-regulated group of differentially expressed transcripts for stressed leaves. In the case of roots, there was also an enrichmed of genes involved with Cell Wall and Cell Wall Organization and Biogenesis in down-regulated transcripts and might be a reflection of the repression of root growth in greenhouse plants submitted to drought stress. The down-regulation of genes involved with Cell Wall Organization and Biogenesis was also observed in the RNA-Seq results. In general RNA-Seq allowed the identification of more than 28,000 differentially expressed features in leaves and more than 7,000 in roots, with classes only up-regulated and others only down-regulated. The orthologous group classification of identified sequences indicated the importance of Signal Transduction Mechanisms and Posttranslational modification, protein turnover, chaperones in leaves and Carbohydrate Transport and Metabolism and Signal Transduction Mechanisms in roots. The precursors of several micro RNAs involved with drought responses were also identified among the differentially expressed features from RNA-Seq. The study of KEGG pathways shed light on some pathways differential expression in leaves and roots, such as Phenylpropanoid Biosynthesis, Galactose Metabolism, Lipid Metabolism and Plant Hormone Signal Transduction. The last one indicated a major role for ABA independtly of the plant tissue and an interplay of several hormones in the leaves responses to water stress. The results of this chapter helped in the understanding of differences on the response to a major abiotic stress in distinct parts of sugarcane plants and also how these changes in the molecular level along with the transduction of the signals between the different parts of sugarcane organism have consequences to the whole plant physiological response.

Keywords: Sugarcane; Drought; Re-watering; Physiology; Transcriptome

3.1. Introduction

Brazil is considered the world's largest producer of sugarcane, whose economical importance goes beyond the production of sugar and biofuels. Lately the perception that sugarcane can be used for a variety of processes, like bioprocessing for the production of chemicals such as polymers and new molecules has made the interest on study this amazing plant even bigger (SOUZA; FILHO, 2016). In this context, the promising role of sugarcane as a bioenergy crop makes the expansion of plantations to areas of severe drought common (GENTILE et al., 2015).

All the way through a plant's life cycle, they are submitted to different kinds of environmental stresses, that include water deficit, temperature extremes, salinity (HAYAT et al., 2012), among the abiotic, and pathogen infections among the biotic stresses. Drought is considered one of the major constraints for plant productivity worldwide (HAYANO-KANASHIRO et al., 2009)). In the countryside of São Paulo, for instance, the lack of rain caused a 15% loss in sugarcane fields (PALHARES, 2014), which was reflected on the producer profit, causing finantial losses.

Drought is a complex trait and in order to understand its underlying mechanisms it is necessary to have a global view (WANG et al., 2016a), once the changes in expression of transcription factors, enzymes and other proteins must be translated to the alterations in plant morphology and physiology aiming the survival of the organism.

Root development, photosynthetic mechanisms, transpiration, stomatal control of CO_2 diffusion, phostsynthetic efficiency (SAIBO; LOURENÇO; OLIVEIRA, 2009) are among the physiological parameters altered after water privation.

The investigation of changes in plant transcriptomes in response to environmental stresses is of fundamental importance for the selection of candidate genes for plant breeding programs. The use of large scale transcriptomic techniques to understand and characterize molecular changes due to water stress has been widespread in different plant species, stress conditions (from severe stress to re-watering) and using plants with different characteristics of drought tolerance or susceptibility (HAYANO-KANASHIRO et al., 2009; LU et al., 2017; ZHAO et al., 2016; ZHOU et al., 2014). The study of genes associated with drought avoidance or tolerance traits, for example, was performed in C4 hybrid bermudagrass plants (ZHOU et al., 2014) and found genes associated to dehydration-protective proteins, stress signaling, oxidant stress defense. The study of rehydration is also interesting once tolerant genotypes seems to activate mechanisms that will lead to a more efficient recovery process (HAYANO-KANASHIRO et al., 2009).

The analysis of the whole organism's reactions to an environment stimulus is essential for the comprehension of the role that differentially expressed genes have in the plant's response to the environmental stresses. Most studies focus either on physiological and morphological changes caused due to drought or on the molecular aspects represented by data obtained from transcriptomic, proteomic, metabolomics works. Only a few, such as Hayano-Kanashiro et al., (2009) presents a correlation between both aspects.

Therefore, this part of the work aimed at analyzing sugarcane's response to drought stress not only in the molecular, but also in the physiological level, once the last one represents the phenotypical changes that parallel the molecular ones. An integrated and interconnected view of alterations in sugarcane due to water stress is provided in this chapter.

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4. CHAPTER II – CO-EXPRESSION ANALYSIS OF SUGARCANE DROUGHT EXPERIMENTS

ABSTRACT

Gene networks are considered the basis of biological complexity and have become the core area of research in systems biology. Their analysis help in the identification of patterns of responses related to specific conditions as well as hypothesize about the function of unknown genes, once transcriptionally co-expressed genes tend to be functionally related. Moreover, the possibility of relating the co-expressed genes with physiological responses or to hormone crosstalk may help us getting insight into the genes coordinated action that culminate in a specific response. This chapter aimed to obtain a co-expression network of genes involved in drought by relating them with (a) physiological parameters considered important for water stress responses; (b) analyzing the co-expression modules in which players of the signal transduction pathway of ABA, auxin and ethylene belong to. These hormone related genes were also used for the study of the changes in gene expression during the progression of drought, with the goal of observing if their pattern of expression and their co-expression are similar. Co-expression analysis used data from two greenhouse and one field drought related experiments, in which materials from different sugarcane varieties and plant parts (leaves, roots and internodes) were used. The analysis of gene expression changes during drought was performed using leaves of water stressed sugarcane SP80-3280 plants, and the genes studied were chosen accordingly to data obtained from previous experiments. Co-expression results showed the existence of 43 modules with genes expressed in a similar way, from these, 3 were correlated with physiological parameters of interest, such as photosynthesis and transpiration, and 15 contained genes chosen for the evaluation of gene expression changes in the daily basis in response to drought. Dark Orange2, Dark Red and Maroon were the modules correlated with physiological parameters, and interestingly showed genes from the top nodes involved with the avoidance of damage in photosynthetical membranes, protection of macromolecules, antioxidant response, protein ubiquitination and transcription factor activity. In the case of the modules from genes studied in the drought progression experiments, most of the nodes belong to Function Unknown orthologous group category, Posttransational Modification, protein turnover, chaperones, Transcription and Signal Transduction Mechanisms, and highlighted the importance of some group of genes in sugarcane`s response to water stress. Among those are calcium binding proteins, transcription factors and cryptochromes. The further analysis of the results are helping in the selection of interesting genes to be identified in the sugarcane genome and used in the future for biotechnological approaches. The study of drought progression showed that the expression of several genes start to decrease after the sixth day and may indicate the plant might be entering the senescence process, and also that the peak of expression for several genes known to have roles in drought responses happens between the forth and the sixth day.

Keywords: Sugarcane; Co-Expression; Greenhouse; Field; Drought

4.1. Introduction

The molecular response of plants to abiotic stresses has been often considered as a complex process mainly based on the activation of some signaling pathways and the modulation of transcriptional activity of stress-related genes (COSTA et al., 2008; MAZZUCOTELLI et al., 2008). Some genes respond to water stress very rapidly, whereas others are induced slowly after the accumulation of ABA (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997). Such genes are thought to function not only in protecting cells from water deficit by producing important metabolic proteins, but also in the regulation of genes for signal transduction in the water-stress response (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997).

The identification of signaling and gene regulatory pathways in cells affected by different stresses, as well as the interaction between the pathways constitutes one of the major interests in the signal transduction pathway research area (COSTA et al., 2008; SEKI et al., 2003). These interactions define the temporal and spatial pattern of expression of a specific group of genes (MEJIA-GUERRA et al., 2012). The importance of these studies relies on the fact that gene expression is frequently the prime mover, in other words, the first step of a response (BLAIS, 2005). Therefore, the identification and comprehension of regulatory networks, as well as the way they are built and interact, are fundamental for the understanding of tolerance mechanisms and provide targets for genetic manipulation (MARCOLINO-GOMES et al., 2013; VAN DRIEL, 2003).

The modulation of expression of several genes help plants to cope with water stress and optimize their growth and development (SINGH; LAXMI, 2015). Abscisic Acid (ABA) is considered a master player on such responses. Plants responses to drought can be divided in two major pathways: ABA-dependent and ABA-independent pathways (SINGH; LAXMI, 2015). Other well known phytohoromes players involved in plants drought responses are ethylene, involved in senescence, and auxin, involved in stomatal movements.

Moreover, stress responses are not linear pathways, and involve changes at molecular, cellular and physiological levels, constituting integrated circuits that involve a plethora of pathways, cell compartments, tissues and the interaction of additional cofactors and signaling molecules (RODRIGUEZ; CANALES; BORRAS-HIDALGO, 2005).

Drought stress changes plant morphology leading to alterations on several physiological parameters that will ultimately promote photoinhibition and oxidative damage on the cells (PINHEIRO; CHAVES, 2011). In this aspect, understanding only the molecular responses characterized by sensing of the stress, transducing the signal and activating stress-responsive genes, along with the cellular mechanisms that will involve genes related to protection of macromolecules and membranes, detoxification enzymes, proteases and osmoprotectants (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997), is not enough.

Such an intricate response demonstrates that studies focusing on only a specific aspect tend to be incomplete and to neglect important factors. Nowadays it is necessary not only to understand one or another characteristic of the response, but instead to integrate the physiology, gene regulation and cellular mechanisms and, therefore, obtain a comprehensive view of the plant responses.

In this part of the work it was aimed to obtain a drought co-expression network using the data from the drought experiment previously analyzed in this work, as well as using data formerly generated by the group, also related to the same environmental stress. Furthermore, it was aimed to analyze the relationship between genes related to the main hormones involved in water stress responses, ABA, auxin and ethylene, on the co-expressed networks as well as

to evaluate the relative expression of genes involved in ABA-dependent and ABA-independent pathways during the progression of the stress.

4.2. Specific Objectives

- Analyze the physiology and changes in gene expression of selected genes during the progression of drought in sugarcane.
- Compare microarray data obtained from this experiment with data obtained from previous experiments of the group and generate a sugarcane drought co-expression network;
- Identify genes that have a similar pattern of expression during drought progression and also belong to the same co-expression module;
- Identify interesting genes that may be targets for future studies.

4.3. Material and Methods

4.3.1. Drought Progression Physiology and Gene Expression Analysis

4.3.1.1. Plant Material

Sugarcane plants (*Saccharum* spp) cultivar SP80-3280 were grown in greenhouse under irrigated and drought conditions. Plants were seven-month-old and cultivated in 20 liters pots. The tissues collected were leaves L+3 for RNA extraction during the progression of the drought. One eye sett of sugarcane was planted in 20 liter pots containing a mixture of 2 parts soil: 2 parts substrate: 1 part vermiculite. Until the beginning of the drought treatments, the soil was kept near the field capacity (FC) in the same manner as the experiment in Alagoas Federal University. The soil water content was monitored by three measurements of soil humidity per pot, in a depth of 10 cm each day after stress treatments, using the soil moisture meter MO750 (EXTECH Instruments, Nashua – New Hampshire).

4.3.1.2. Analysis of physiology parameters

The physiology parameters evaluated were photosynthesis, stomatal conductance and transpiration. Measures were done with a LICOR LI-6400 Portable Photosynthesis System (LI-COR, Lincoln – Nebraska). All measures were performed between 9h to 11h am on leaves L+1. A recovery time period of 24 hours (between day 7 and day 9) was given to the plants after they were watered again. Therefore, there are no physiology measures on day 8.

For physiological analysis, the t-test was used to determine statistical significant difference between irrigated, drought and rehydrated samples. Excel was used for the t-test and graphs were built using GraphPad Prism 5.

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5. FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

A comprehensive understanding of the organization, function and evolution of plant genes is essential to disentangle important biological processes and to advance crop engineering and breeding strategies, therefore, the ultimate goal in deciphering complex biological processes is the discovery of causal genes and regulatory mechanisms controlling the processes of interest (SERIN et al., 2016).

Several studies of plant responses to abiotic stresses have been done in the past years, but only a few try to integrate physiological and molecular changes caused by these stresses, especially drought. Most of all, it is difficult to find a work that integrates different strategies to interpret and obtain a deeper knowledge of the transcriptional changes happening in a plant during the stress. To our knowledge there are no works integrating both parameters for the study of the stress progression in a daily basis. Both physiology and transcriptomic data suggested that water privation for 4 and 6 days caused a moderated to severe stress, which was reversed by the rehydration period, characterizing the capacity of the plant to respond and recover from such stresses. After 6 days of water privation it was able to detect photochemical damage which was reflected on the transcripts detected.

Microarray allowed the identification of 7,867 SAS with FC $\geq$ 2 and FC $\leq$ 0.5 and 585 considered differentially expressed by the HTSelf method. The update in the annotation from SUCEST database allowed the decrease in the number of unknown or hypothetical proteins, and helped in the standardization of the annotation. Therefore, analysis such as Fisher's Enrichment test could be performed along with the identification of orthologus groups. The annotation of DEGs determined by the HTSelf allowed the determination that classes related to photosynthesis were enriched only after 6 days of stress on the down-regulated genes and also that classes related to root growth were down-regulated during the stress.

This work also allowed the identification of LEA proteins, ABA related factors and transcription factors among other genes involved with classical drought responses, along with the observation of some genes altered in different drought experiments, such as Dehydrin and SAPK1 which can be interesting targets for transgenic approachs. Furthermore, pathview analysis showed the emphasis of plant metabolism on sensing and transducing the signal in the beginning of the stress and functionally reacting to it once the stress is severe. In addition, the crosstalk between stresses and between drought and lignin biosynthesis could be identified. Pathview analysis using microarray data shed light on some important pathways with different responses on leaves and roots, such as galactose and phenylpropanoid metabolism.

Interestingly in the case of phenylpropanoids biosynthesis, transcriptome analysis indicated the difference in expression of genes related to lignin metabolism, but quantification of the metabolite did not indicate significant changes, probably because the stress was not severe enough or because the quantification allowed the determination of only total lignin and not specific variants. Sugar metabolism appeared to be essential for roots responses to osmotic stress, as well as modification of lipids metabolism in the leaves. After re-watering, protein synthesis and turnover seems to have a fundamental role for the plants recovery.

Moreover, although it seems to be a systemic response of the plant to the stress, there are some pathways, such as the antioxidant and DNA repair related ones, that are preferentially expressed either in leaves or in roots. This could mean that leaves tend to avoid damage, while roots tend repair it. The up-regulation of differentially expressed genes in leaves might mean that in this tissue there is a tendency to stimulate gene expression, due maybe to the activation of new routes to respond and tolerate drought. In roots, the repression of most of the genes seems to show

an opposite tendency, which could be that roots are shutting down some processes, as, exemplified by the down-regulation of cell division and expansion related genes.

Furthermore, the down-regulation of aquaporins may be a response of the plant to increase roots hydraulic resistance and avoid loss of water to the soil. The expression of classical drought response pathways, such as proline, ABA and lipid metabolism, as well as the stimulation of less known routes, that are also related to important functions during drought responses, like lipoic acid pathway, show the agreement between the results found and the hypothesis of an attempt by the plant to tolerate the stress and recover from it after rehydration. The combination of data obtained from differentially expressed and significantly expressed genes allowed a global view of what seems to be happening in the plant during water stress and rehydration, such as the up-regulation of ABA-dependent and independent pathways, ethylene, lipid metabolism, DNA repair, osmolytes and terpenoids pathways, along with the down-regulation of aquaporins and gibberellin. Opposing to that, rehydration seem to repress ABA and ethylene biosynthesis.

RNA-Seq allowed the assembly of a huge amount of transcripts, both through *de novo* RNA-Seq assembly and using a reference genome. RNA-Seq *de novo* analysis corroborates with some conclusions obtained from microarray and allowed the observation that some terms from classification are exclusive for up or down-regulated transcritps, while terms such as "Transcription" and "Signal transduction Mechanisms" are important in both groups. In the case of using the reference genome, this work shed light on the possible role for a variety of hormones in leaves responses to drought, such as jasmonic acid and brassinosteroids. Also ABA seems to play a central function in water stress responses no matter the plant tissue.

The search of miRNA sequences on the differentially expressed genes lead to the identification of interesting classes of miRNA and shows the importance of studying sugarcane miRNAs differentially expressed on diverse sugarcane varieties and plant tissue during stresses.

The analysis of drought progression experiment, show a daily decrease in the parameters of photosynthesis, stomatal conductance and transpiration. These values were close to 0 on the seventh day of stress, but 24 hours after re-watering plants already had these parameters back to normal values. qRT-PCR using characteristic drought related genes, show that their expression starts to increase after 3 or 4 days of stress and then, begin to decrease after the 7th day without water, indicating that maybe in this point, when physiology parameters indicate a severe stress, the plant is starting the senescence process. Furthermore, a couple of genes with similarity on their expression during drought progression also appeared in the same co-expression modules.

The analysis of co-expression networks and their relationship with physiological parameters of interest allowed the determination of three interesting modules, which contained Top nodes related to both protection of photosynthetical membranes and antioxidant responses, and shows that the group in successfully stablishing a pipeline for the analysis of Co-Expression networks. Despite that, since the experiments used in this analysis were very diverse, several genes were excluded on the analysis because did not reach the cut off value. Therefore, for future experiments it will be interesting to use the same pattern of experiment in different varieties.

Overall, the results of our project could provide a complete and comprehensive view of sugarcane response to one of the most affecting abiotic stress for this crop. For the next steps, the analysis of qRT-PCR using primers for genes from auxin and ethylene biosynthesis and signaling pathways will be performed in order to understand their pattern of expression and correlate the results with their co-expression modules. Furthermore, the interpretation of RNA-Seq data will be improved and the analysis will focus on the groups in common between leaves and roots samples as well as on the search for transcription factors sequences differentially expressed during water stress, aiming to select targets on the new assembly of the sugarcane genome for future studies.

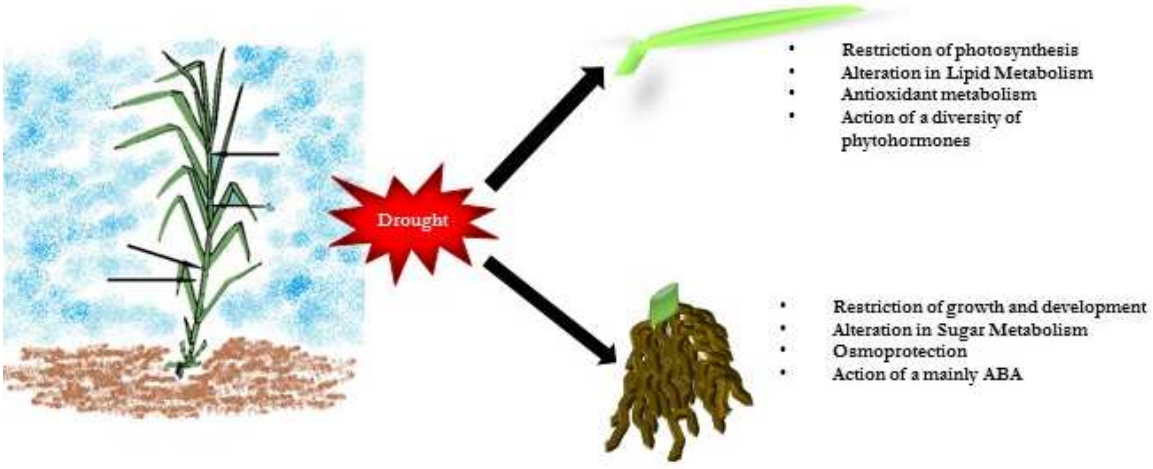

Figure 60. Summary of sugarcane leaves and roots responses to water stress as detected in this work