University of São Paulo
"Luiz de Queiroz" College of Agriculture

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

Danielle Izilda Rodrigues da Silva

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

Piracicaba
2018

Danielle Izilda Rodrigues da Silva
Bachelor and Licentiate in Biological Sciences

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

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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1. Cana-de-Açúcar 2. Estresse Hídrico 3. Expressão Gênica 4. Redes de Coexpressão I. Título

DEDICATION

To my family that has been by my side, supporting me during every and each moment, and in special, to my parents, the strongest and kindest people I know and who have turned me into the person I am now.

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EPIGRAPH

“If we knew what it was we were doing,
it would not be called research, would it?.”.

Albert Einstein

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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 RT-qPCR 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, estas demoram mais tempo para voltar à condição inicial, de forma que 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 RT-qPCR 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 RT-qPCR 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 RT-qPCR 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

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LIST OF ABBREVIATIONS AND ACRONYMS

ABA	Abscisic Acid
ABRE	ABA-responsive element
AcBr	Acetyl Bromide
AOX	Alternative Oxidase
AP2/EREBP	APETALA2/Ethylene Responsive Element Binding Protein
AP2/ERF	APETALA2/Ethylene Responsive Factor
APX	Ascorbate Peroxidase
AREB/ABF	ABRE-binding protein/ABRE-binding factors
ATP	Adenosine triphosphate
BR	Brassinosteroid
CAT	Catalase
cDNA	complementary DNA
CDPK	Calcium-dependent protein kinase
CIA	24 parts Chloroform:1 part Isoamyl alcohol
CK	Cytokinin
COG	Cluster of Orthologous Groups
CPIK	Calcineurin B-like protein-interacting protein kinase
cRNA	complementary RNA
CTAB	Cetyltrimethylammonium bromide
CWR	Cell Wall Residue
Cys	Cysteine
DEPC	Dietilpirocarbonate
DNA	Deoxyribonucleic acid
DOD	Days of Stress
DRE/CRT	Dehydration-responsive element/C-repeat
DREB2	DRE-/CRT-binding protein 2
DRIP	DREB2A-INTERACTING PROTEIN
EDTA	Ethylenediaminetetraacetic acid
ERF	Ethylene Response Factor
FC	Field capacity
FDR	False Discovery Rate
GA	Gibberellins
GO	Gene Ontology
GPX	Glutathione peroxidase
GRF7	Growth-Regulating Factor 7
GSH	Glutathione
GSSG	Oxidized glutathione
GST	Glutathione *S*-transferase

MAPK	Mitogen-Activated Protein Kinase
ME	Module Eigengene
MG	Methylglyoxal
mRNA	messenger RNA
MT	Metallothioneins
NACR	NAC recognition sequence
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced Nicotinamide Adenine Dinucleotide
nt	Nucleotide
P5C	Δ'-pyrroline-5-carboxylate
PP2C	Protein Phosphatase 2C
PrxR	Peroxiredoxin
PVP	Poly(vinylpyrrolidinone)
PYR/PYL/RCAR	Pyrabactin/Pyrabactin-like/Regulatory Components of ABA Receptor
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RBOH	Respiratory Burst Oxidase Homolog
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RO	Alkoxy Radical
ROS	Reactive Oxygen Scavenging
Rpm	Revolutions per minute
RSEM	RNA-Seq by Expectation Maximization
RuBisCO	Ribulose-1,5-biphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-biphosphate
RWC	Relative Water Content
SAS	Sugarcane Assembled Sequence
SOD	Superoxide Dismutase
TFs	Transcription Factors
WT	Wild Type
WUE	Water Use Efficiency

LIST OF SYMBOLS

A	Photosynthesis rate
cm	Centimeter
CO2	Carbon dioxide
E	Transpiration
g	grams
gS	Stomatal Conductance
H2O2	Hydrogen Peroxide
HCl	Hydrochloric Acid
K+	Potassium
LiCl	Lithium chloride
M	Molar
m	meters
mg	miligrams
mL	mililiter
mM	milimolar
mm	millimeter
NaCl	Sodium chloride
ng	nanogram
nm	nanometer
nM	nanoMolar
O2	Molecular Oxygen
O2 -	Superoxide Radical
O2 1	Singlet Oxygen
OH-	Hydroxyl Radical
µg	microgram
µL	microliter
µM	microMolar

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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2. LITERATURE REVIEW

2.1. Sugarcane, its biology and importance

Sugarcane (*Saccharum* spp.) is a unique crop that belongs to the Poaceae family, Panicoideae subfamily, Andropogoneae tribe and has the ability to accumulate high levels of sugar, being also a commercially viable source of biomass for bioelectricity and second-generation bioethanol (FERREIRA et al., 2017). In Brazil, sugarcane was introduced in the colonial period and has become one of the most important crop for the country's economy. Nowadays, Brazil is responsible for more than half of the sugar commercialized in the world, being the average increase in production until 2018/19 estimated at 3.25% in relation to the previous year (Ministry of Agriculture, Livestock and Food Supply – MAPA, 2012).

Sugarcane is usually vegetatively propagated from axillary buds on stems (or stalks) cuttings. The first, "plant" crop is generally harvested from 12 to 24 months after planting; thereafter, "ratoon" crops may be harvested at shorter to equal time periods (MING et al., 2006). The crop tillers at the base, producing unbranched stems from around 2 to 4 meters and about 5 cm in diameter. Its structure can be basically divided in stems, leaves and roots.

The stems are differentiated into joints consisting of nodes and internodes, with the first ones containing a lateral bud in the axil of the leaf, a band with root primordia, and a growth ring. Furthermore, the stalks have a hard, wax-covered rind (epidermis), that surrounds a mass of softer tissue, named parenchyma, which is interspersed with fibers, the vascular bundles (JAMES, 2004). The leaves are attached to the base of the nodes, alternating on opposite sides of the stalk, and consists of two parts: the sheath and the blade or lamina. The first one is tubular, broader at the base, tightly involves the stem and is separated from the blade by a ligule and the dewlaps. The blade broadens from the ligule to up to 10 cm in width and then, narrows towards the pointed tip. Sugarcane leaves also have a strong midrib (JAMES, 2004). As new leaves unfold, old ones are shed, so that the number remains approximately constant throughout the life of a stalk (ARTSCHWAGER; BRANDES, 1958). Leaves can be numbered according to the "Kuijper System" that helps in the standardization of leaf growing, nutrition and diagnosis studies (SCARPARI; BEAUCLAIR, 2010). In this system, the leaf that shows the first visible dewlap (Top Visible Dewlap, TVD) in the top of the plant where new leaves are localized, receives the denomination of Leave +1 (SCARPARI; BEAUCLAIR, 2010). The older leaves get crescent numbers (+2, +3, etc) and the youngest, in the direction of the apical gem, gets the numeration of 0, -1, -2, etc. The same pattern of numbering is used for the nodes and internodes (SCARPARI; BEAUCLAIR, 2010).

Roots are divided in two types, the sett roots, which are the first ones to develop from the initials on the root band and are thin and branched, and the shoot roots, that develop from the root primordia on the new developing tillers and are thicker, fleshier, less branched (JAMES, 2004). The root system has two main functions, first it enables the intake of water and nutrients from the soil; and second, it anchors the plant to the soil (MILLER; GILBERT, 2009). Soon after a seedpiece or "sett" (stem cuttings or sections of the stalks) is planted, two kinds of roots develop, the sett and the shoot roots, previously mentioned. The root system as a whole is renewed as each new shoot produces its own roots (JAMES, 2004). The rate of the process of rejuvenation is governed by the periodicity of tillering (emission of new culms by the same plant – tillers) and the continuous production of new roots is important since it allows the plant to adjust itself to changing environmental conditions (JAMES, 2004; MILLER; GILBERT, 2009) (figure 1).

Sugarcane is a C4 crop with four phenological stages: germination, tillering, grand growth and maturation. The first one is characterized by the leaves appearing on the stems and the establishment of the first roots. It occurs 20 to 30 days after planting. The second one is represented by the production of stems by the plant, a process regulated by hormones and that occurs 20 to 30 days after the emergence of the primary stem. In the grand growth phase, the stems become higher and start to accumulate sugar; root growth is also more intense. The maturation phase start with intense growth of the stalks, then the tillers get 2 m or more in height and leaves start to turn yellow and dry and finally there is intense storage of sugar (MARIN, 2012).

There has been some controversy regarding the origins of sugarcane, although, there is some agreement that selection, movement and introgression resulted in two centers of diversity, one in India for *S. spontaneum* and another in New Guinea for *S. robustum* (DANIELS; ROACH, 1987). The species *S. officinarum*, with high sucrose content, is believed to have derived from *S. robustum* in New Guinea (MING et al., 2006). Nowadays, the varieties of sugarcane cultivated are mainly complex interspecific hybrids between *S. officinarum* (noble cane) and *Saccharum spontaneum*, with contributions of *S. robustum*, *S. sinense*, *S. barberi*, in addition to other related grasses, such as *Miscanthus* (DANIELS; ROACH, 1987). Commercial sugarcane varieties are highly polyploidy (8 to 12 sets of haploid genome) with 100-130 chromosomes, with different levels of aneuploidy (MING et al., 2006).

SP80-3280 is a sugarcane variety with good ratoon and demanding in watered and fertile soils, conditions in which the yield is high. Harvesting time is between July and October (UDOP – Union of Bioenergy Producers, 2014). It has been extensively cultivated in Brazil in around 300 thousand ha in different regions (UDOP – Union of Bioenergy Producers, 2014). Such superior agronomic performance in a vast area implies that breeders have selected adaptability traits responsible for yield stability (FIGUEIRA et al., 2012) and turned this variety into an interesting model of study. Furthermore, most of the cDNA libraries used for EST sequencing carried by the Sugarcane EST Project – SUCEST (VETTORE et al., 2001) were from SP80-3280, and the sugarcane genome sequencing project is also using this variety.

To obtain high productivity and other characteristics of interest to develop an energy cane, research must unravel the complexities of sugarcane genome, develop statistical tools to be used in highly polyploidy genomes and identify genes associated with the content of sucrose, drought resistance, biomass and cell wall recalcitrance (WACLAWOVSKY et al., 2010).

The study of the sugarcane genome started with the generation of 237,954 high quality ESTs, assembled in 43,141 possible transcripts (SUCEST project) (VETTORE, 2003). The next step was the development of the database SUCEST-FUN, which stores data obtained by SUCEST project and by the catalogues SUCAST (Sugarcane Signal Transduction Catalogue) and SUCAMET (Sugarcane Metabolism Catalogue) (NISHIYAMA JR et al., 2012). These catalogues led to the identification of ESTs involved in signal transduction and metabolic pathways (PAPINI-TERZI et al., 2005, 2009; ROCHA et al., 2007; SOUZA et al., 2001).

The initiative was of great importance, and the group is now dedicated to the sequencing and analysis of this plant complete genome. To accomplish genetic breeding, besides sequencing, it is necessary to characterize functionally the genes already identified, as well as sugarcane regulatory networks (NISHIYAMA JR et al., 2012). In this aspect, ally the information already existent in the genome project with data obtained by other studies becomes important to the progress of the knowledge of mechanisms that control the development of this plant.

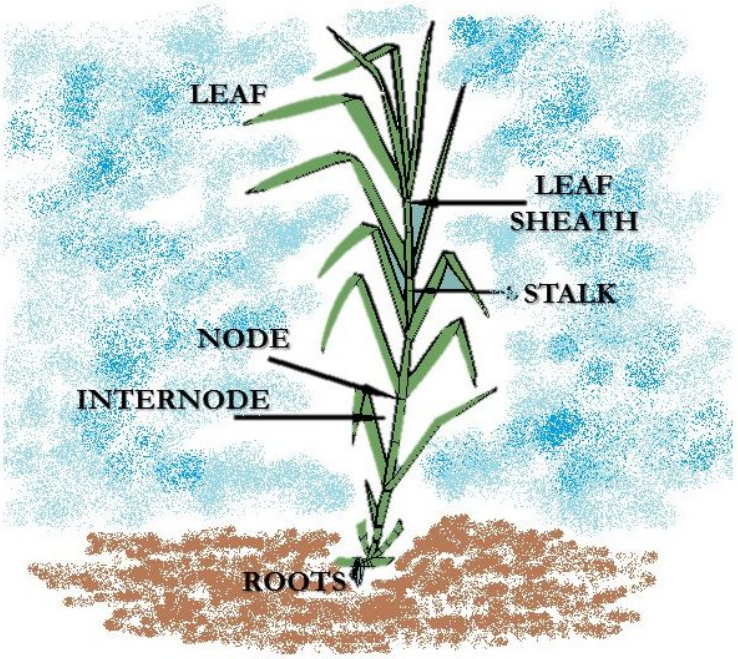

Figure 1. Sugarcane morphology showing the different parts of the plant. Adapted from: University of Florida <http://edis.ifas.ufl.edu/LyraEDISServlet?command=getImageDetail&image_soid=IMAGE%20SC:SC034F01&document_soid=SC034&document_version=95982>

2.2. Drought as an abiotic stress

Plants are sessile organisms that usually face adverse growth conditions caused by various environmental challenges such as freezing, salinity, drought and high temperatures, and, therefore, need to develop adaptive robustness at molecular, cellular and physiological levels (MATTOS; MORETTI, 2016; YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014). According to Mattos and Moretti, (2016) and Ferreira et al., (2017), these adverse but not always lethal conditions that plants confront during their life cycles restrict normal functions. They delay growth and development, reduce productivity, lead to starvation, depletion of energy and fiber production and in extreme cases, cause plant death, and are usually known as Stress.

Drought, salinity and extreme temperatures are considered the most important environmental stresses that have evolved with plants (ROSA et al., 2009). Drought is defined as a complex phenomenon characterized by an intricate interplay of limiting water availability, lower rainfall, reduced ground water level and increase in the temperature (SINGH et al., 2015). It is considered an inevitable and recurring feature of world climate (MIRZAEI et al., 2012), which reduces agricultural production mainly by disrupting the osmotic equilibrium and membrane structure of the cell (TUTEJA, 2010).

The unpredictability along with variations in severity and duration and the expectancy of an increase of the effects thanks to climate change, growing water scarcity and long-term effects of global warming (FAROOQ et al., 2012; HARB et al., 2010; JOSHI et al., 2016), indicate the urgent need to develop adaptive agricultural strategies for a changing environment (TUTEJA, 2010). Despite our enhanced skills to forecast its onset and modify the impacts, drought remains as the single most important factor affecting world plant production, as well as the condition and stability of the land resources from which plants are derived (MIRZAEI et al., 2012).

Drought impairs normal growth, disturbs water relations and reduces water use efficiency in plants (FAROOQ et al., 2012). It is characterized by a reduction of water content, decreased leaf water potential and turgor loss, closure of stomata, reduced cell expansion and growth and alteration in enzyme activity among others (MATTOS; MORETTI, 2016). Photosynthesis rate, in particular, is reduced by stomatal closures, membrane damage and disturbed activity of various enzymes, such as the ones involved in ATP synthesis (FAROOQ et al., 2012).

The intricate nature of plant characters involved in water acquisition and the heterogeneity of water in the environment turns drought into a complex process (MIRZAEI et al., 2012). The plant's responses will depend not only on the species inherent genetics, but also on the duration and severity of the drought period (CRUZ DE CARVALHO, 2008; MATTOS; MORETTI, 2016), which will causes several symptoms such as drying of leaf tips, leaf wilting and curling, yellowing, chlorosis and necrosis (PRABU et al., 2011).

Plants adaptations to drought stress can be classified as belonging to four organizational levels. The highest level, named "Developmental Traits", encompasses the most complex mechanisms and requires the interplay of many gene products (e.g., time of flowering); the following one, named "Structural Traits", includes characteristics such as root patterns or leaf waxiness. The next lower level comprises "Physiological Traits", like water use efficiency; and the lowest level, known as "Metabolic or Biochemical Resistance Traits" involves enzymes, regulatory mechanisms and other gene products that can be considered possible targets for biotechnology (MCCUE; HANSON, 1990).

Besides these organizational levels, plants can exhibit two distinct mechanisms to cope with water deficit: drought escape and drought resistance, the last one further classified into drought avoidance (maintenance of tissue water potential) and drought tolerance (PRICE et al., 2002).

Drought escape can be defined as the plant ability to complete its life cycle before the onset of drought and to undergo dormancy earlier than the onset of the dry season (FAROOQ et al., 2012). It can be exemplified by the accelerated flowering at the beginning of drought (BHASKARA; YANG; VERSLUES, 2015). Such mechanism is advantageous where chances of terminal drought is more recurrent and it is helpful to match crop phenology with climate conditions (FAROOQ et al., 2012). In the case of sugarcane, this strategy does not apply due to the perennial nature of this crop (FERREIRA et al., 2017).

Drought avoidance is described as the plant capability to maintain high tissue water potential despite a soil water deficit, using mechanisms such as improved water uptake reached, for example, through the development of deep-rooted system, and the capacity of plant cells to hold acquired water and further reduce water loss (PRICE et al., 2002; ROSA et al., 2009). These mechanisms are achieved by roots plasticity, reduced leaf size, reduced transpirational loss (FAROOQ et al., 2012). It is important in areas with severe or terminal drought, and in sugarcane is characterized by lower stomatal conductance, leaf rolling and senescence and impaired growth (FERREIRA et al., 2017).

Drought tolerance is the ability to withstand water deficit and maintain metabolic activities with low tissue water potential (BHASKARA; YANG; VERSLUES, 2015; FAROOQ et al., 2012; INGRAM; BARTELS, 1996). It involves metabolic adjustments, mediated by alteration in gene expression, to help improve the plant's functionality (ROSA et al., 2009). Therefore, drought tolerance can be achieved by several mechanisms, such as maintaining cell turgor and reducing evaporative water loss by accumulating compatible solutes (osmotic adjustment) (YANCEY et al., 1982), antioxidant defense system and changed dynamics of phytormones (FAROOQ et al., 2012). Tolerance mechanisms in sugarcane are characterized by higher leaf chlorophyll content, higher stomatal conductance, photosynthesis and growth maintenance and osmotic adjustment. They are favorable in areas with mild and moderate stress (FERREIRA et al., 2017).

Osmotic adjustment or osmoregulation can be defined as the accumulation of organic and inorganic solutes under drought and/or salinity, contributing to lower the water potential without decreasing actual water contents (SERRAJ; SINCLAIR, 2002). It helps plants under drought in two ways: in the maintenance of leaf turgor to improve stomatal conductance for efficient intake of CO_2 (KIANI et al., 2007) and in the promotion of the root's ability to uptake more water (CHIMENTI; MARCANTONIO; HALL, 2006). Some examples of compounds involved in this process are proline (accumulated particularly in young leaves) (PÉREZ-PÉREZ et al., 2009), soluble sugars, free amino acids and glycinebetaine.

Phytormones such as abscisic acid (ABA), auxins, gibberellins (GA), cytokinins (CKs) and ethylene are substances involved in the control of plant growth and development (FAROOQ et al., 2012). Drought stress alters the endogenous synthesis of these substances, therefore, normally growth promoters (GA and CKs) decrease in concentration while growth retardants (ABA and ethylene) increase to regulate plant water budget (FAROOQ et al., 2009; TAIZ, 2010).

Antioxidants act as defense system to drought tolerance since limited water supply under drought promotes oxidative stress with overproduction of ROS (FAROOQ et al., 2012). ROS (O_2^- – superoxide radical; O_2^1 – singlet oxygen; H_2O_2 – hydrogen peroxide; RO – alkoxy radical; OH^- – hydroxyl radical) are highly reactive and deteriorate normal plant metabolism through oxidative damage to lipids, DNA, protein and other macromolecules (ROUT; SHAW, 2001). The antioxidant defense system in the plant cell includes both enzymatic and non-enzymatic components. The first group is compounded mainly by peroxidases, glutathione reductase, catalase, superoxide dismutase and ascorbate peroxidase, while the second group is formed by constituents such as ascorbic acid, proline, amino acids, γ-amino butyric acid (GABA), reduced glutathione (FAROOQ et al., 2012).

Drought tolerance mechanism has been investigated using three main approaches in plants: (a) examining tolerant systems, such as seeds and resurrection plants, (b) analyzing mutants from genetic model species and (c) analyzing the effects of stress on agriculturally relevant plants (INGRAM; BARTELS, 1996). Tolerance to abiotic stress is a complex trait that is influenced by coordinate and differential expression of a network of genes (GARG et al., 2002).

Water deficit is considered the single largest abiotic stress affecting sugarcane productivity, therefore, the development of water use efficient drought tolerant cultivars is an imperative for all major sugarcane producing countries (FERREIRA et al., 2017).

2.2.1. Leaves and roots suffer morphological and physiological changes due to drought

Abiotic stresses can directly or indirectly affect the physiological status of an organism by altering its metabolism, growth and development (GARG et al., 2002).

During drought, reductions in leaf area (size and number) and stomatal closure, impaired activities of carboxylation enzymes and ATP synthesis, and destruction of photosynthetic apparatus are among the key factors lowering carbon fixation (YAMANE et al., 2003). Other leaf adaptation to drought are a low ratio of external leaf surface to its volume, leaf rolling (common in grasses), changes in color (such as yellowing), wilting and also senescence (DE MICCO; ARONNE, 2012).

Stomata closure may be hydropassive or hydroactive. In the first case, it results from direct evaporation of water from the guard cells with no metabolic involvement, while in the second one it is metabolically dependent and involve processes that result in reversal of the ion fluxes that cause stomatal opening (TUTEJA, 2010). Furthermore, plant phytohormone ABA promotes the efflux of K^+ ions from the guard cells, which results in the loss of turgor pressure leading to stomata closure (TUTEJA, 2010).

The decrease in stomatal aperture limits CO_2 influx resulting in a decline in the rate of photosynthesis (FAROOQ et al., 2012) which is also related with the impaired activities of essential photosynthetic enzymes such as ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase, pyruvate phosphate dikinase, NADP-malate dehydrogenase and NADP-malic enzyme (FAROOQ et al., 2008, 2009; RAMACHANDRA REDDY; CHAITANYA; VIVEKANANDAN, 2004). Interestingly, the closure of stomata does not always depend upon the perception of water-deficit signals arising from the leaves. In fact, stomata closure also responds directly to soil desiccation even before there is any significant reduction in leaf mesophyll turgor pressure (TUTEJA, 2010).

Photosynthetic pigments such as chlorophyll (chl) contents and carotenoids are also important in leaves carbon fixation due to their involvement in capturing solar radiation to drive photosynthetic mechanism (FAROOQ et al., 2012) and seems to be decreased during drought stress (ASRAR; ELHINDI, 2011). This also diminish photosynthetic rate, declining photochemical efficiency (Fv/Fm) of chlorophyll fluorescence in leaves (MIYASHITA et al., 2005). Impaired photosynthesis along with low CO_2 influx downregulates carbon fixation which consequently lowers the oxidation of reduced nicotinamide adenine dinucleotide (NADPH) in the Calvin Cycle (FAROOQ et al., 2012). This process happens because a decrease in the intracellular CO_2 levels lead to the over-reduction of the components within the electron transport chain, which causes the transference of the electrons to the oxygen at photosystem I (TUTEJA, 2010), thus, the primary electron acceptor $NADP^+$ is not sufficiently available, causing the generation of ROS including O_2^-, H_2O_2 and OH^-, consequently leading to photoinhibition and photo-oxidation as a result of reactive oxygen species overproduction when plants are exposed to high irradiance (TAUSZ et al., 2001; TUTEJA, 2010).

Longer drought situations can also cause plant cells to undergo shrinkage, leading to mechanical constraints on cellular membranes, impairing the functioning of ions and transporters as well as membrane-associated enzymes (TUTEJA, 2010). Leaf senescence in drought stressed plants also contributes to nutrient remobilization, allowing the rest of the plant to benefit from the nutrients accumulated during the lifespan of the leaf (FAROOQ et al., 2012).

The plant's first organ to detect a limitation on the water supply is the root system (CRUZ DE CARVALHO, 2008), but its role in drought tolerance is less investigated when compared to knowledge already known for aerial parts (AROCA; PORCEL; RUIZ-LOZANO, 2012). The increase of root density over leaf area is one well-stablished response of roots to drought (DE MICCO; ARONNE, 2012). However, the capacity of absorbing water varies along a given root. This variation happens due to apoplastic barriers such as suberin layers, lignin deposition and the development of casparian bands. Moreover, the exact role of aquaporins in the regulation of root hydraulic properties is not yet understood (AROCA; PORCEL; RUIZ-LOZANO, 2012).

Root traits associated with drought stress tolerance can be grouped based on their key strategy to match plant physiological functions to water supply. These are: (a) adjusting spatial distribution to enhance soil water exploration, access and uptake; (b) optimizing resource partitioning (between shoot and roots) to enhance soil water acquisition and (c) special sensitivity for soil water uptake under drought stress (MWENYE et al., 2016). In the first case, it includes total root length (cm), taproot length (cm), root length density (mm mm^{-3} or m mm^{-3}), center of root length density with depth, root dry matter (g), root fresh weight (g) center of root dry matter with depth, root weight

density (g cm^{-3} or kg m^{-3}), total root surface area (m^2), root branching density and root system architecture. In the case of resource partitioning, drought-tolerant genotypes tend to adjust the partitioning of resources (dry matter) towards the roots (allometry) under water-limited stress conditions and/or reduce lateral root branching (MWENYE et al., 2016).

Other putative metabolic root traits with potential to confer tolerance include osmotic adjustment in root tips, which assists in maintenance of root growth under drought, modification of cell wall extension properties, the growth sustenance effect of ABA, protection of oxidative damage (YAMAGUCHI; SHARP, 2010) and decrease in root hydraulic conductance and aquaporin activity (MWENYE et al., 2016). Furthermore, plants produce compounds in the roots that are transported to the shoots via the xylem sap and are vital to signaling and adaptation for drought stress (TUTEJA, 2010).

2.2.2. In the cellular level drought leads to alteration of different proteins and metabolites

The need to deal with abiotic stresses made plants evolve multiple and interconnected signaling pathways that allows the regulation of different groups of stress-responsive genes for producing various classes of proteins, e.g. protein kinases, transcriptional factors, enzymes, molecular chaperones, among others, resulting in diverse physiological and metabolic responses that might confer tolerance to environmental stresses (YOU; CHAN, 2015).

Acclimation of plants to changes in their environment requires a new state of cellular homeostasis achieved by a delicate balance between multiple pathways that reside in different cellular compartments (MILLER et al., 2010). The response is initiated when a plant recognizes a stress at the cellular level. This will activate signal transduction pathways that transmit information within individual cells and throughout the plant. The changes in gene expression, which occur at the cellular level are, then, integrated into a response by the whole plant that may modify growth and development and even influence reproductive capabilities (BRAY; BAILEY-SERRES; WERETILNYK, 2000).

The machinery leading to the expression of drought related genes is similar to the general model mentioned and can be divided in three basic steps: (a) perception of the stimulus; (b) processing, which includes amplification and integration of the signal, and (c) response reaction in the form of *de novo* gene expression (INGRAM; BARTELS, 1996). Thus, in summary, plant cells perceive abiotic stress signals and transduce them through various signaling pathways, including secondary signaling molecules, plant hormones and transcriptional regulators (YOU; CHAN, 2015), that will lead to a response represented by the synthesis of new proteins and metabolites.

In the cellular level, water stress results in both displacement of membrane proteins in the lipid bilayer, which contributes to loss of membrane integrity and selectivity, along with disruption of cellular compartmentalization, and loss of membrane-based enzyme activity (TUTEJA, 2010). Cellular metabolism is disrupted thanks to the high concentration of cellular electrolytes caused by the protoplasm's dehydration (TUTEJA, 2010). Furthermore, cell shrinkage is followed by a marked decline in cellular volume and the greater accumulation of solutes causes toxicity and negatively affects functioning of some enzymes, often leading to reduced photosynthesis and water use efficiency (WUE) (JOSHI et al., 2016).

The expression patterns of dehydration-inducible genes are complex. Some genes respond to water stress very rapidly, whereas others are induced slowly after the accumulation of ABA (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997).

Several genes have been reported to respond to water stress in a variety of plant species, and are thought to function not only in protecting cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the water stress response (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997). These gene products can be separated into two groups. The first group probably functions in stress tolerance and includes water channel proteins, enzymes required for the biosynthesis of osmoportectants (sugar, proline, glycinebetaine), proteins that may protect macromolecules and membranes (LEA protein, osmotin, antifreeze protein, chaperon, and mRNA binding proteins), proteases for protein turnover (thiol proteases, Clp protease and ubiquitin) and detoxification enzymes (glutathione *S*-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase and ascorbate peroxidase) (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997).

An interesting trait related to roots responses to drought are aquaporin expression. Aquaporins enhance membrane permeability to water in both directions depending on osmotic pressure differences across a membrane (BOHNERT; SHEN, 1998). During drought stress, the importance of aquaporins is exemplified by the roots need to decrease their hydraulic conductivity and their osmotic potential in order to avoid dehydration in the root cells and the flow of water outside of the roots (AROCA, 2012; AROCA; PORCEL; RUIZ-LOZANO, 2012; NORTH; NOBEL, 1992). When transpiration is restricted at night or under stressful conditions, water flows predominantly through the cell-to-cell pathway (symplastic plus transcellular pathways) due osmotic forces (AROCA; PORCEL; RUIZ-LOZANO, 2012). Furthermore, results that show a descending hydraulic conductivity and an increase in aquaporin protein amount could be due to the localization of these proteins in internal membranes where they cannot contribute to water transport from external medium (AROCA; PORCEL; RUIZ-LOZANO, 2012).

Among the signal transduction pathways observed in plant roots submitted to drought stress, one could cite an early-warning response mechanism in which the hydrogen pump ATPase protein (H+-ATPase) is activated on plasma membrane of root hairs before a substantial decline in plant RWC (relative water content). This activation triggers the amplified biosynthesis of key osmolytes such as leaf proline to maintain the water budget of plants (FAROOQ et al., 2012).

LEA proteins and chaperones seem to be involved in protecting macromolecules like enzymes, lipids and mRNAs from degradation due to cell dehydration (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). Dehydrins (DHNs) belong to the group of late embryogenesis abundant II proteins (group 2 LEA) and represent important dehydration-inducible proteins whose accumulation is induced by developmental processes (in maturing seeds) and by several abiotic stresses (in vegetative tissues) (HANIN et al., 2011; KOSOVÁ; VITÁMVÁS; PRÁŠIL, 2014).

To cope with drought stress plants need to perform osmotic adjustments through decreasing the cellular osmotic potential by the synthesis/accumulation of compatible solutes (MOHAMMADKHANI; HEIDARI; MOHAMMADKHANI, 2008; TUTEJA, 2010). These molecules are low molecular weight, highly soluble organic compounds that are not toxic at high cellular concentration (HAYAT et al., 2012; MCCUE; HANSON, 1990). Enzymes of sugar metabolism are probably critical in desiccation tolerance, since certain sugars may be central for the protection of a wide range of organisms against drought (INGRAM; BARTELS, 1996). Detoxification enzymes such as glutathione *S*-transferase, soluble epoxide hydrolase and glutathione peroxidases are involved in the protection of cells from active oxygens (INGRAM; BARTELS, 1996; RODRIGUEZ MILLA et al., 2003; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000). It is possible that the sum of different compatible solutes accumulated is more protective of cellular structure than any one of them alone which leads to a significant osmotic adjustment (VERSLUES; SHARMA, 2010).

Proteases such as Clp protease and ubiquitin are thought to be required for protein turnover and recycle of amino acids (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000), since they function removing denatured and damaged proteins. Their importance in stress metabolism may reside in the release of amino acids for the massive synthesis of new proteins (INGRAM; BARTELS, 1996). HVA22 proteins constitute highly conserved stress-inducible protein which may play an important role in protecting cells from damage under stress conditions in many eukaryotic organisms (SHEN et al., 2001). Heat-shock proteins and chaperonin may be involved in protein repair, by helping other proteins to recover their native conformation after denaturation or misfolding during water-stress (INGRAM; BARTELS, 1996).

The other group of genes code for protein factors involved in further regulation of signal transduction and gene expression that probably function in stress responses, such as protein kinases, transcription factors and 14-3-3 proteins (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997). The water stress signaling pathways are also divided into ABA-dependent and ABA-independent pathways (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997, 2006) and will be discussed in the next sections.

Some of the above-mentioned compounds will be further discussed.

2.2.2.1. Proline

Biotic and abiotic stresses lead to the accumulation of a plethora of metabolites, in special amino acids, from which a particular attention, in the context of water stress, is given to proline (HAYAT et al., 2012). The observation of proline accumulation in many plant species induced by drought led to the hypothesis that proline accumulation would promote drought tolerance (VERSLUES; SHARMA, 2010). Proline metabolism is highly regulated and therefore, its accumulation in response to drought cannot be considered a symptom of the abiotic stress, nor a passive accumulation caused by reduced growth (BHASKARA; YANG; VERSLUES, 2015).

Proline and its metabolism can be distinguished from other amino acids in different ways, from which the most fundamental is the fact that this metabolite is the only one of the proteogenic amino acids where the *a*-amino group is present as a secondary amine (VERSLUES; SHARMA, 2010), this unique characteristic may contribute for proline distinguished roles. In plants, proline is synthetized by two major pathways: glutamate pathway, mainly responsible for proline accumulation during osmotic stress (VERBRUGGEN; HERMANS, 2008), and ornithine pathway. The synthesis from glutamatic acid occurs through an intermediate Δ'-pyrroline-5-carboxylate (P5C) in reactions catalyzed by Δ'-pyrroline-5-carboxylate synthetase (P5CS) and Δ'-pyrroline-5-carboxylate reductase (P5CR) (SEKHAR et al., 2007), while the synthesis from ornithine happens after its transamination to P5C by orinithine-δ-aminotransferase (VERBRUGGEN; HERMANS, 2008). Proline degradation is the reverse process of biosynthesis; therefore, it is catalyzed by Proline dehydrogenase (PDH) and P5C dehydrogenase (P5CDH) (VERBRUGGEN; HERMANS, 2008). The interconversion of proline and glutamate is often referred as "proline cycle" (VERSLUES; SHARMA, 2010). Proline synthesis occurs in the chloroplast and cytoplasm while its catabolism happens in the mitochondria (VERSLUES; SHARMA, 2010).

Among the possible functions of proline in stress resistance, it can be highlighted its function in osmotic adjustment, protection of cellular structure during dehydration (acting as molecular chaperons stabilizing the structure of proteins), redox and pH buffering, storage and transfer of reductant (and nitrogen), signaling molecule and reactive oxygen scavenging (VERSLUES; SHARMA, 2010). Proline accumulation varies from plant to plant during osmotic stress, but it can be 100 times greater than in control situation, and is mainly due to increased synthesis and reduced

degradation (VERBRUGGEN; HERMANS, 2008). Furthermore, the internal proline content is not only determined by biosynthesis and catabolism, but also by the transport between cells and different cellular compartments (HAYAT et al., 2012).

Osmotic adjustment is related to the fact that accumulation of proline helps in the decreasing of cellular osmotic potential while allowing turgor and water content to be maintained. The protection of cellular structure during dehydration relates to the fact that proline, other solutes and some intrinsically unstructured proteins can act as "water substitutes" to stabilize cellular structure through hydrophilic interactions and hydrogen bonding. Redox buffering occurs because adjusting the balance of proline synthesis and degradation can alleviate imbalances in cellular redox, since proline synthesis uses NADPH and catabolism releases reductants. Storage and transfer of reductant (and nitrogen) refers to the importance of proline movement within the plant and separation of synthesis and catabolism between different tissues, which relates to the signaling molecule function as well (VERSLUES; SHARMA, 2010). The scavenging of ROS refers to the fact proline itself can reacts with and detoxifies those types of molecules (SMIRNOFF; CUMBES, 1989). Proline functions cannot be considered mutually exclusive and probably, plant accumulate this metabolite in order to fulfil several of these roles at once (VERSLUES; SHARMA, 2010).

Nevertheless, proline accumulation is not only a physiological response to several stresses, but also part of the developmental program in generative tissues (e.g. pollen), and it is imperative for the osmoprotective and developmental functions of proline that a balance between biosynthesis and degradation occurs (VERBRUGGEN; HERMANS, 2008). As stated by Bhaskara; Yang; Verslues, (2015), it is proline metabolism and turnover that functions to maintain growth during water limitation. More proline is not a guarantee of better drought tolerance. Thus, the amount of proline that accumulates is dependent on metabolic and signaling pathways (BHASKARA; YANG; VERSLUES, 2015). Furthermore, proline seems to accumulate to higher concentrations in tolerant than in in sensitive plant genotypes (MWENYE et al., 2016).

According to Hayat et al., (2012) low concentration of exogenous proline applied to plants exposed to stress results in enhanced growth and other physiological characteristics of plants. In special, it affects plant water relations by maintaining turgidity of cells under stress and increases the photosynthetic rate.

2.2.2.2. Soluble sugars

Soluble carbohydrates (sucrose, glucose and fructose) function primarily as metabolic resources and structural components of the cell (ROSA et al., 2009). They are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source to sink organs (ROSA et al., 2009). They may either act directly as negative signals or as modulators of plant sensitivity and thus, can also play important roles in cells responses to stress-induced remote signals (ROSA et al., 2009). Ecological and agronomic studies have stablished soluble sugars as important metabolites in plants under drought stress after uncovering a strong correlation between their concentration and stress tolerance (ARABZADEH, 2012; ROSA et al., 2009).

Soluble sugars can act as primary messengers and regulate the growth and metabolism by modulating gene expression and enzymes activities in both sugar exporting (source) and importing (sink) tissues (ROSA et al., 2009). Therefore, they have a dual role in plants – they are involved in various metabolic events and act as molecule signals regulating different genes, especially those involved in photosynthesis, sucrose metabolism and osmolyte synthesis (ROSA et al., 2009). Their role in reducing water potential prevents from oxidative destruction and helps in keeping the structure of proteins and membranes under average dehydration (ARABZADEH, 2012).

Sucrose and hexoses have the dual-function of up-regulating growth-related genes and down-regulating stress-related ones (ROSA et al., 2009). Trehalose is a non-reducing disaccharide of glucose found in some resurrection plants and has been shown to be involved in stabilizing dehydrated enzymes, proteins, and lipid membranes efficiently, as well as protecting biological structures from damage during desiccation (GARG et al., 2002) (CHAMOLI; VERMA, 2014; TUTEJA, 2010). It is the reversible property of water-absorption of trehalose that protects biomolecules from desiccation induced damage (CHAMOLI; VERMA, 2014).

Fructans are highly polymerized polyfructose molecules stored in vacuoles in soluble form mainly in species experiencing water stress condition (CHAMOLI; VERMA, 2014). Fructans synthesizing species show better survival rate under unfavorable environment conditions, furthermore, tolerance and better growth in plants might be due to pronounced development of rooting system (CHAMOLI; VERMA, 2014).

Glucose and ABA signaling act in coordination for regulating plant growth and development. A high concentration of ABA and sugars can inhibit growth under severe drought stress, while low concentrations can promote growth. Oligosaccharides such as raffinose and galactinol are among the sugars synthesized in response to drought. These compounds seem to function as osmoprotectants rather than providing osmotic adjustment (TUTEJA, 2010).

Increasing the level of compatible solutes through genetic engineering does not provide a straightforward solution, probably due to the highly integrated nature of sugar metabolic pathways; therefore, it seems that source-sink relationships at the whole-plant level must be considered in attempts to enhance stresses tolerance through conventional breeding programs and/or other techniques (ROSA et al., 2009).

2.2.2.3. Reactive Oxygen Species (ROS)

The excessive production of ROS, also called active oxygen species (AOS) or reactive oxygen intermediates (ROI) such as hydroxy radical (OH^-), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^1) as a result of the partial reduction of atmospheric O_2 is one of the most know and inevitable stress response in photosynthetic organisms, such as plants and cause large cellular damage (CHAMOLI; VERMA, 2014; CRUZ DE CARVALHO, 2008; HAYAT et al., 2012). They are produced in different tissues and at different sub-cellular compartments generating numerous divergent signals, which calls for a high degree of control and coordination in the plant cells (MILLER et al., 2010). Accumulation of toxic products from ROS with lipids and proteins definitely contributes to the damage of crop plants under biotic and abiotic stresses (YOU; CHAN, 2015).

The osmotic stresses induced by adverse conditions in plant cells lead to decreasing water availability, causing loss of cell turgor and the accumulation of the detrimental ROS (NXELE; KLEIN; NDIMBA, 2017). ROS cause considerable damage through peroxidation of membrane lipid components and also through direct interaction with different kinds of macromolecules, leading to a process named “oxidative stress” that can culminate in extensive cellular injury or damage and ultimately cell death (CRUZ DE CARVALHO, 2008; HAYAT et al., 2012; MATTOS; MORETTI, 2016; YOU; CHAN, 2015). Oxidative stress is one of the major causes of plant injury under drought conditions, therefore, limited water availability favors a shift in the balance between ROS production and elimination (MATTOS; MORETTI, 2016; NOCTOR; MHAMDI; FOYER, 2016). Furthermore, decreased water availability may involve a progressive, rather than an acute, oxidative stress (NOCTOR; MHAMDI; FOYER, 2016).

Under optimal growth conditions, ROS are naturally produced at low level in organelle such as chloroplasts, mitochondria and peroxisomes in special by the electron-transport chains of the first two cellular compartments

(CHAMOLI; VERMA, 2014; CRUZ DE CARVALHO, 2008). However, during stress, their rate of production is dramatically elevated (MILLER et al., 2010). Mitochondria has a lowering antioxidant buffering capability, and therefore, is most vunerable to oxidative damage and play a crucial role in setting the cellular redox state and initiating the signal transduction cascades under drought stress (CRUZ DE CARVALHO, 2008).

In general, the drought stress related ROS response has three successive phases. In the first one, the normal ROS steady-state level is disturbed by drought stress, the second is characterized by enhancement on ROS production since stomatal closure shifts the equilibrium upwards, which will trigger signal transduction defense pathways. In the third level, prolonged drought stress will result in exacerbated ROS production that will no longer be counterbalanced by the antioxidant system, causing oxidative damage, resulting in cell death (CRUZ DE CARVALHO, 2008).

The main cellular components susceptible to damage by free radicas are lipids, proteins, carbohydrates and nucleic acids. Peroxidation of unsaturated fatty acids in membrane and denaturation of functional and structural macromolecules (e.g. proteins) is the well-known result of ROS production in cells. However DNA nicking, amino acids, protein and photosynthetic pigments oxidation are also reported effects (MATTOS; MORETTI, 2016).

In the chloroplasts, during photosynthesis, energy from the sunlight is captured and transferred to two light-harvesting complexes (photosystems II and I) present in the thylakoidal membranes. Subsequently, a succession of redox reactions occurs within the electron transport chain in the photochemical phase, until electrons finally reach the CO_2 in the chemical phase of photosynthesis (CRUZ DE CARVALHO, 2008). During drought, there is a limitation on CO_2 fixation, which will reduce $NADP^+$ regeneration in the Calvin Cycle, and will not keep pace with NADPH production, ultimately provoking an over reduction of the photosynthetic electron transport chain and, therefore, increasing electron leakage to O_2. Furthermore, there is also the production of the hydroxyl radical in the thylakoids through "iron-catalyzed" reduction of H_2O_2 by superoxide dismutase (SOD) and ascorbate (CRUZ DE CARVALHO, 2008; MILLER et al., 2010; NOCTOR; MHAMDI; FOYER, 2014). Chloroplast production of ROS has long been proposed as a major driver of redox signaling or damage during drought (NOCTOR; MHAMDI; FOYER, 2014).

Peroxisomes also produce H_2O_2 and O_2^- at high rates through several metabolic processes with the amount produced regulated by a delicate balance between production and scavenging (MILLER et al., 2010). The decrease in CO_2 influx leads to ROS generation through photorespiration, a process in which RuBisCO use O_2 to oxygenate ribulose-1,5-biphosphate (RuBP) yielding glycolate that is transported to peroxisomes and oxidized by glycolate oxidase, generating H_2O_2 (CRUZ DE CARVALHO, 2008).

In the mitochondria, the effects of ROS production are alleviated by the alternative oxidase (AOX) system that acts diverting electrons flowing through electron-transport chain and decreasing the possibility of electron leaking to O_2 to generate O_2^- (YOU; CHAN, 2015).

In order to cope with the deleterious effects caused by ROS, plants have developed mechanisms to keep ROS levels in a beneficial state (HAYAT et al., 2012). The tight control of ROS production is kept due to a versatile and cooperative antioxidant system that modulates their intracellular concentration and sets the redox-status of the cell (CRUZ DE CARVALHO, 2008). ROS can be scavenged by low molecular weight antioxidative metabolites, such as ascorbic acid and glutathione or metabolized by antioxidative enzymes, like SOD, catalase, ascorbate peroxidase (APX) (HAYAT et al., 2012). By definition, the primary function, of antioxidant defenses is to restrict the accumulation of ROS (NOCTOR; MHAMDI; FOYER, 2014).

Basically, in plants, ROS detoxification system comprises two main components, the enzymatic, characterized by the action of enzymes such as SOD and APX; and the glutathione cycle (glutathione *S*-

transferase/glutathione peroxidase - GST/GPX); and the non-enzymatic composed by metabolites like flavones, anthocyanins, carotenoids, ascorbic acid, among others (CHAMOLI; VERMA, 2014) that act synergistically in the protection against oxidative damage and in the fine modulation of low levels of ROS for signal transduction ((YOU; CHAN, 2015).

The enzymatic component is characterized by the Halliwell-Asada pathway (water-water cycle) and the GST/GPX cycle, responsible for the elimination of O_2 ^{1} and OH^- and H_2O_2 (CHAMOLI; VERMA, 2014; MILLER et al., 2010). In the first case, the superoxide radicals are eliminated by the SOD in a reaction that yields H_2O_2, which is consumed through its conversion to O_2 and water by the action of catalase or to water alone through the oxidation of ascorbate (CHAMOLI; VERMA, 2014). The generation of ascorbate occurs by two mechanisms, the enzymatic reduction of monodehydroascorbate in the plastids, followed by the spontaneous conversion of monodehydroascorbte to dehydroascorbate, that reacts with glutathione (GSH) to produce ascorbate and oxidized glutathione (GSSG) in a reaction catalyzed by dehydroascorbate reductase (CHAMOLI; VERMA, 2014). Ultimately, GSSG is reduced by glutathione reductase in a reaction that requires NADPH (CHAMOLI; VERMA, 2014).

AOX reduces the production rate of O_2^- in thylakoids (MILLER et al., 2010). ROS that escape this cycle and/or are produced in the stroma undergo detoxification by SOD and stromal ascorbate-glutathione cycle (MILLER et al., 2010).

Peroxiredoxin (PrxR) and GPX are also involved in the H_2O_2 removal in the stroma (MILLER et al., 2010). Excited chlorophyll (Chl) in its triplet state at the light-harvesting complex (LHC) can generate O_2 ^{1} when the electron transport chain is over reduced (MILLER et al., 2010). ROS produced in peroxisomes during fatty acid oxidation, photorespiration or other reactions are decomposed by SOD, catalase (CAT) and APX (MILLER et al., 2010). SOD and other components of the ascorbate-glutathione cycle are also present in the mitochondria (MILLER et al., 2010). In addition, AOX prevents oxidative damage in mitochondria (MILLER et al., 2010). In principle, the cytosol contains the same set of enzymes as the stroma (MILLER et al., 2010). NADPH oxidases are the major producers of ROS-associated signals required in a wide range of biological activities (MILLER et al., 2010). The enzymatic components responsible for ROS detoxification in the apoplast and cell wall are only partially known, and the ROS-scavenging pathways at the vacuole and nucleus are unknown (MILLER et al., 2010).

CAT is also an important enzyme involved in ROS detoxification that seems to be enhanced under severe drought stress while under moderate drought stress H_2O_2 scavenging is preferably made through the ascorbate/glutathione cycle. CAT's affinity to H_2O_2 is lower than APX, which suggests its role in counteracting excessive H_2O_2, despite that, excess H_2O_2 may attack and inhibit APX, highlighting CAT's importance during extreme stress (CRUZ DE CARVALHO, 2008).

Metallothioneins (MT) are a group of low molecular weight proteins with the characteristics of high cysteine (Cys) residue content and metal-binding ability, and the presence of several Cys residues in MTs suggests their involvement in the detoxification of ROS or in the maintenance of REDOX levels (YOU; CHAN, 2015).

ROS signaling is an integral part of the acclimation response of plants to drought or salinity stresses (MILLER et al., 2010). It is used to sense the stress due to enhanced ROS production caused by metabolic imbalances, as well as to actively send different signals via enhanced production of ROS at the apoplast by different RBOH (respiratory burst oxidase homolog) proteins (MILLER et al., 2010).

ROS enhancement during stress responses can also function as an alarm signal that triggers acclimation/defense responses by specific signal transduction pathways (CRUZ DE CARVALHO, 2008; MILLER et al., 2010). During the initial phase of abiotic stresses, elevated ROS levels might act as a vital acclimation signal (YOU;

CHAN, 2015). Their involvement in signal transduction implies that there must be a coordinated function of regulation networks to maintain ROS at non-toxic levels in a delicate balancing act between ROS production, involving ROS generating enzymes and the unavoidable production of ROS during basic cellular metabolism, and also the ROS scavenging pathways (MATTOS; MORETTI, 2016; YOU; CHAN, 2015).

H_2O_2 is the most stable ROS and has the capacity to easily diffuse from one cellular compartment to the other and can also be readily metabolized by an efficient cellular antioxidant system, which allows a fast switch "on" and "off" of the signal, a condition essential for an effective second messenger (CRUZ DE CARVALHO, 2008). The stress response signaling pathways related to this metabolite (H_2O_2) promote the accumulation of several cellular protectants that may act directly or indirectly in the regulation of the cellular redox-status, and consequently control the extent of the signal itself (CRUZ DE CARVALHO, 2008). Among the H_2O_2-induced genes are the ones involved in cellular repair; protection mechanisms (like DNA damage repair protein and LEA protein) or in signal transduction pathways (e.g. serine/threonine protein kinase) (CRUZ DE CARVALHO, 2008). It also has an active role in modulating Ca^{2+} signaling and MAPK (mitogen-activated protein kinase) cascades (CRUZ DE CARVALHO, 2008).

ABA-induced antioxidant defense has been well documented in plants (YOU; CHAN, 2015). Both, ABA biosynthesis and catabolism are involved in antioxidant defense and abiotic stresses (YOU; CHAN, 2015). In addition to the hormone, ROS signaling mechanism is also linked Ca^{2+} fluxes and sugar sensing and probably is both, upstream and downstream of the ABA-dependent signaling pathways involved in drought stress (CRUZ DE CARVALHO, 2008).

ROS scavenging and antioxidative metabolism is a result of the gene expression regulation by different transcription regulators, that ultimately lead to the expression of stress-responsive genes, such as calcium-dependent protein kinase (CDPK), calcineurin B-like protein-interacting protein kinase (CPIK), MAPK, protein phosphatases, similar to RCD (radical induced cell death) one (SRO) (YOU; CHAN, 2015).

Elucidation of the mechanisms that control ROS signaling in cells during drought and salt stresses could provide a powerful strategy to enhance the tolerance of crops to these environmental stress conditions (MILLER et al., 2010).

2.2.2.4. Regulatory Mechanisms involves the action of Transcription Factors and Signal Transduction Cascades

Abiotic stress responses are regulated by several genes, from which special attention should be given to the ones belonging to the class of transcription factors (TFs) (YOU; CHAN, 2015). TFs act on enabling plants to withstand unfavorable conditions and represent key molecular switches orchestrating the regulation of plant's developmental processes in response to a variety of stresses (JOSHI et al., 2016). They control transcription of their downstream genes by interacting with other proteins and binding to a consensus sequence in promoters, therefore, identification of these downstream genes, among which are proteins involved is osmolyte production, ROS scavenging and detoxification, macromolecule protection and ubiquitination, is also imperative to elucidate molecular machineries of gene activation or repression (JOSHI et al., 2016).

Typically, plant cells perceive the stress stimulus through sensors or receptors mostly localized in the cell membrane surface, such signal is then transduced by second messengers such as inositol phosphate, calcium ions, ROS, sugars and ultimately lead to the activation of TFs that will directly regulate the expression of associated genes

via serving as molecular switches that will interact with determined cis-elements located in the promoter region of the genes they regulate (JOSHI et al., 2016).

The global transcriptional network activated in response to osmotic stress is cooperatively but not exclusively regulated by ABA-dependent and ABA-independent pathways (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014). Transcriptional regulation of gene expression by drought is mainly governed by AREB/ABFs and DREB2A operating respectively in ABA-dependent and ABA-independent signaling pathways (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014).

The cis-acting element, ABA-responsive element (ABRE) (PyACGTGG/TC), and a group of transcription factors, ABRE-binding protein/ABRE-binding factors (AREB/ABFs) have pivotal functions in ABA-dependent gene expression. AREB/ABF TFs family members, AREB1/ABF2, AREB2/ABF4, ABF3 and ABF1 are known to regulate most downstream genes of the three subclass III SnRK2s in ABA-dependent manner (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014). AREB1/ABF2 has a particular role in ABA-signaling during drought stress at the vegetative stage (JOSHI et al., 2016). The cis-element dehydration-responsive element/C-repeat (DRE/CRT) and DRE-/CRT-binding protein 2 (DREB2) TFs play key roles in ABA-independent gene expression in response to osmotic stress (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014).

GRF proteins are putative TFs from which, the GRF7 (growth-regulating factor 7) interacts with a short DREB2A promoter region that suppresses its expression under unstressed conditions and may act as a repressor regulating a wide range of osmotic-stress responsive genes during normal conditions (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014). DREB2A and DRIPs (DREB2A-INTERACTING PROTEIN) are well-characterized transcription factor/ubiquitin E3 ligase combination that regulates osmotic stress-responsive genes (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014).

Transcription factors from the AP2/EREBP (APETALA2/Ethylene Responsive Element Binding Protein) superfamily, also known as AP2/ERF (APETALA2/Ethylene Responsive Factor), along with DRE/CRT regulation of gene expression are some of the most studied genes (MARCOLINO-GOMES et al., 2013; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2006). The AP2/EREBP superfamily is composed of the AP2, ERF and RAV families (MARCOLINO-GOMES et al., 2013). AP2-type transcription factors DREB1 and DREB2 are important in dehydration and high salinity stress-responsive gene expression (AGARWAL; ZHU, 2005; NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2006). They bind to the DRE element and trans-activate stress-responsive genes (AGARWAL; ZHU, 2005; JOSHI et al., 2016). DRE cis-element is involved in the regulation of genes not only by drought and salt, but also by cold stress (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2006).

NAC TFs have a highly conserved DNA binding NAC domain in the N-terminal region and a variable C-terminal transcriptional regulatory region (JOSHI et al., 2016). NAC genes have differential expression patterns such as tissue-specific, developmental stage-specific or stress-specific expression, thereby suggesting their active involvement in the complex signaling networks during plant stress responses (JOSHI et al., 2016).

Some NAC and HD-ZIP transcription factors are also involved in ABA-independent pathways by the expression of genes such as ERD1 (EARLY RESPONSIVE TO DEHYDRATION 1), which encodes a Clp protease regulatory subunit, ClpD (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2006).

Moreover, abiotic stress-responsive NAC-type transcription factors (SNAC) are involved in abiotic stress tolerance through the regulation of expression of genes related to soluble sugar biosynthesis, free proline levels,

antioxidant system and Na^+ accumulation (NAKASHIMA et al., 2012). Genes of the SNAC group bind to the NAC recognition sequence (NACR) and regulate the expression of such NAC responsive genes (NAKASHIMA et al., 2012).

NACs can also be regulated by miRNAs and alternative splicing at post-transcriptional level, be activated by phosphorylation or glycosylation at translational level or even undergo protease or ubiquitin proteasomal-mediated degradation in response to environmental cues (PURANIK et al., 2012).

The interaction between multiple TFs from both ABA-dependent and independent pathways in drought responses has already been reported (JOSHI et al., 2016). The DRE/CRT motif in the promoters of drought responsive genes is a binding region for the ABA-independent DREB/CBF TF and function as a CE for ABRE in ABA-dependent gene expression (NARUSAKA et al., 2003). DREB1A/CBF3, DREB2A and DREB2C proteins can interact physically with AREB/ABF (LEE et al., 2010). Furthermore, according to Puranik et al., (2012), NAC TFs regulatory processes involve upstream TFs like DREBs (ABA-independent pathways) or ABREs (ABA-dependent pathways) that may bind to stress-related cis-regulatory elements in the promoter of regulated NAC genes and influence transcription. Activated NACs can then bind to promoters of their target genes, controlling their expression.

The crosstalk between pathways can also be exemplified by the interaction between DREB/CBFs and other kinds of AP2/ERFs. ERF1, an upstream TF in both ethylene and jasmonate signaling, seems to regulate gene expression by binding to two kinds of cis-elements, the GCC box in response to biotic stress and DRE/CRT under abiotic stress (CHENG et al., 2013). Thus ERF1 may have a role in the integration of ABA, ethylene and jasmonate signaling pathways (NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014). Furthermore, kinases from the subclass III SnRK2s might work as a convergence point in the crosstalk between ABA-dependent and ABA-independent gene expression and DREB2A is induced by AREB/ABFs (YOSHIDA; MOGAMI; YAMAGUCHI-SHINOZAKI, 2014).

ABA-, stress- and ripening-induced (ASR) proteins are also plant-specific TFs and are considered to be important regulators of plant responses to various stresses (YOU; CHAN, 2015).

2.2.3. Hormones

Plant hormones or phytohormones are defined as a "group of naturally occurring, organic substances which influence physiological processes at low concentrations" (DAVIES, 1987). They can regulate plant growth and development, as well as responses to changing environmental conditions. Therefore, by modifying the production, distribution or signal transduction of these hormones, plants are able to regulate and coordinate both growth and/or stress tolerance to promote survival or escape from environmental stresses (COLEBROOK et al., 2014). Abscisic acid (ABA), gibberellins (GA), cytokinins (CK), ethylene, brassinosteroids, auxins, jasmonic acid, salicylic acid are some of the known phytohormones. The ways in which crop plants generate, transport, and regulate both local and long-distance hormone-based chemical signals are important research targets (WILKINSON et al., 2012). It is almost impossible to find a single process in plant life that is not affected by both stress and hormones directly or indirectly (POSPÍŠILOVÁ; SYNKOVÁ; RULCOVÁ, 2000).

Phytohormones have been recognized as a strong tool for sustainably alleviating adverse effects of abiotic stresses in crop plants (KHAN et al., 2015). In the case of drought and other stresses, the net outcome is regulated by a balance between the hormones that promote and those that inhibit the traits, rather than individual ones (BASU et al., 2016). They play a central role in the integration of diverse upstream signals into different adaptive outputs such as

changes in the activity of ion-channels, protein modifications, protein degradation, and gene expression (RIEMANN et al., 2015).

Drought stress induces many physiological and biochemical changes that alter the metabolic status of the plant which could influence the cellular susceptibility mainly to ABA accumulation (CRUZ DE CARVALHO, 2008). The phytohormone ABA is a tiny molecule classified as a sesquiterpene, which has a non-planar configuration and multiple functional moieties (VISHWAKARMA et al., 2017). It is a key endogenous messenger that plays pivotal roles in adaptive stress responses to environmental stimuli in plants (FUJITA et al., 2011; WILKINSON et al., 2012). It is also required to fine-tune growth and development under non-stress conditions, since it controls process such as growth, stomatal aperture, hydraulic conductivity, seed maturation and dormancy (FINKELSTEIN; GAMPALA; ROCK, 2002; LEUNG; GIRAUDAT, 1998; PARENT et al., 2009; RAGHAVENDRA et al., 2010; UMEZAWA et al., 2010).

The core regulatory network involved in ABA responses is composed by three major components: PYR/PYL/RCAR (pyrabactin/pyrabactin-like/regulatory components of ABA receptor), an ABA receptor; PP2C (type 2C protein phosphatase), a negative regulator; and SnRK2 (SNF1-related protein kinase 2), a positive regulator (UMEZAWA et al., 2010).

The phosphorylated ABA binds to the PYR/PYL/RCAR receptors, which in turn binds to PP2C, forming a RCAR-ABA-PP2C trimeric complex. PP2C is inhibited by the formation of the complex and its inhibition allows SnRK2 to be activated and to phosphorylate AREB/ABFs. The last ones bind to ABRE motifs and, therefore, can regulate ABA-dependent gene expression (FUJITA et al., 2011; MA et al., 2009; NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014; RAGHAVENDRA et al., 2010; UMEZAWA et al., 2010).

There also seems to be an intricate relation between the hormone ABA and ROS (CARVALHO, 2008). One of the best characterized ABA-induced physiological responses under drought stress is leaf stomatal closure, a response that operates precociously on the onset of the drought period, depending on the plants' inherent strategy (and acclimation) towards drought stress (CRUZ DE CARVALHO, 2008).

The discovery of genes that are induced by drought, salt and cold in ABA-deficient or ABA-insensitive Arabidopsis mutants suggested that these genes do not depend on ABA for their expression (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997). Such molecular studies have revealed, thus, that ABA-independent gene expression is also important in stress tolerance in plants (NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014) and, therefore, other hormones should also have roles in plant abiotic stress responses.

Salicylic acid (SA) is not only involved in plant defense, but also mediates plant abiotic stress tolerance through modulation of osmolyte synthesis, activation of antioxidant system, production of secondary metabolites and optimization of mineral nutrients status (HARA et al., 2012; KHAN et al., 2015). SA plays a major role in the resistance to pathogens by inducing the production of "pathogenesis related proteins", enhance flower longevity, inhibit ethylene biosynthesis and seed germination, block the wound response and reverse the effects of ABA (DAVIES, 1987). Brassinosteroids (BRs) form a large group of steroidal plant hormones that have emerged in the past years as a new paradigm in this category of substances and influence various processes, such as senescence, abscission and maturation, but the specific mechanisms of BR-induced drought tolerance have yet to be determined (KAHLAOUI et al., 2016).

Auxins and cytokinins in low physiological concentrations under water stress promote stomatal opening, while high concentrations lead to stomatal closure (KAUR; ASTHIR, 2017). CKs promote cell division, control development and senescence of the whole plant, leaf expansion, accumulation of chlorophyll and conversion of etioplasts into chloroplasts, and delay leaf senescence, promoting stomatal opening and transpiration rate (DAVIES,

1987; POSPÍŠILOVÁ; SYNKOVÁ; RULCOVÁ, 2000). CKs under drought stress are known mainly to delay premature leaf senescence and death (BASU et al., 2016). They antagonize many physiological processes induced by water stress, mainly those mediated by ABA, such as ABA-induced stomatal closure (POSPÍŠILOVÁ; SYNKOVÁ; RULCOVÁ, 2000). The amount of endogenous CK mostly decreases under water stress which amplifies the response of shoot to increasing concentration of ABA, furthermore, exogenous application of CKs might partially ameliorate negative effects of water stress, by, for example, stimulation of osmotic adjustment, delay of stress induced senescence, and reversion of leaf and fruit abscission (POSPÍŠILOVÁ; SYNKOVÁ; RULCOVÁ, 2000).

Auxins act on several processes, such as cell enlargement and division, leaf and fruit abscission, delays leaf senescence, promotes flowering, among other effects (DAVIES, 1987). Auxin has been shown to negatively regulate drought adaptation in plants (BASU et al., 2016), therefore, drought is expected to reduce auxins levels and this reduction along with the interaction with ABA is supposed to help in survival and adaptive growth under stress (DU; LIU; XIONG, 2013).

Jasmonates [Jasmonic acid (JA) and methyl jasmonates (MeJAs)] are known to take part in various physiological processes, such as senescence (AHMAD et al., 2016), furthermore, its exogenous application has proved effective in improving plant stress tolerance and contributing to regulation of stomatal closure during drought stress (RIEMANN et al., 2015).

GAs are suggested to positively regulate plant adaptation to drought stress (BASU et al., 2016). It has been shown that a reduction of GA levels and signaling contribute to plant growth restriction on exposure to several stresses (e.g. osmotic stress), while increased GA biosynthesis and signaling promote growth in plant scape responses to shading and submergence (COLEBROOK et al., 2014).

Ethylene stimulates fruit ripening, flower and leaf senescence, leaf and fruit abscission, adventitious root formation, among others (DAVIES, 1987). Ethylene is a key mediator of biotic and abiotic stress responses in plants and considered a negative regulator of drought stress (BASU et al., 2016). It is essential for many developmental processes (MÜLLER; MUNNÉ-BOSCH, 2015). Ethylene signaling and response pathways includes Ethylene Response Factors (ERFs), considered regulatory hubs, integrating ethylene, abscisic acid, jasmonate and redox signaling of the plants' abiotic stress responses (MÜLLER; MUNNÉ-BOSCH, 2015). The presence of ethylene leads to the inactivation of ethylene receptors and CTR1 kinase activity, which will result in the dephosphorylation and cleavage of the EIN2 (ethylene insensitive protein) C-terminus and its translocation to the nucleus, where they regulate EIN3/EIL1 activation directly or indirectly, these will consequently target the TF genes ERFs, that will activate biotic or abiotic stress response genes by binding to the specific cis-acting GCC-box and DRE elements (MÜLLER; MUNNÉ-BOSCH, 2015).

Figure 2 summarizes plant responses to drought in all levels presented above.

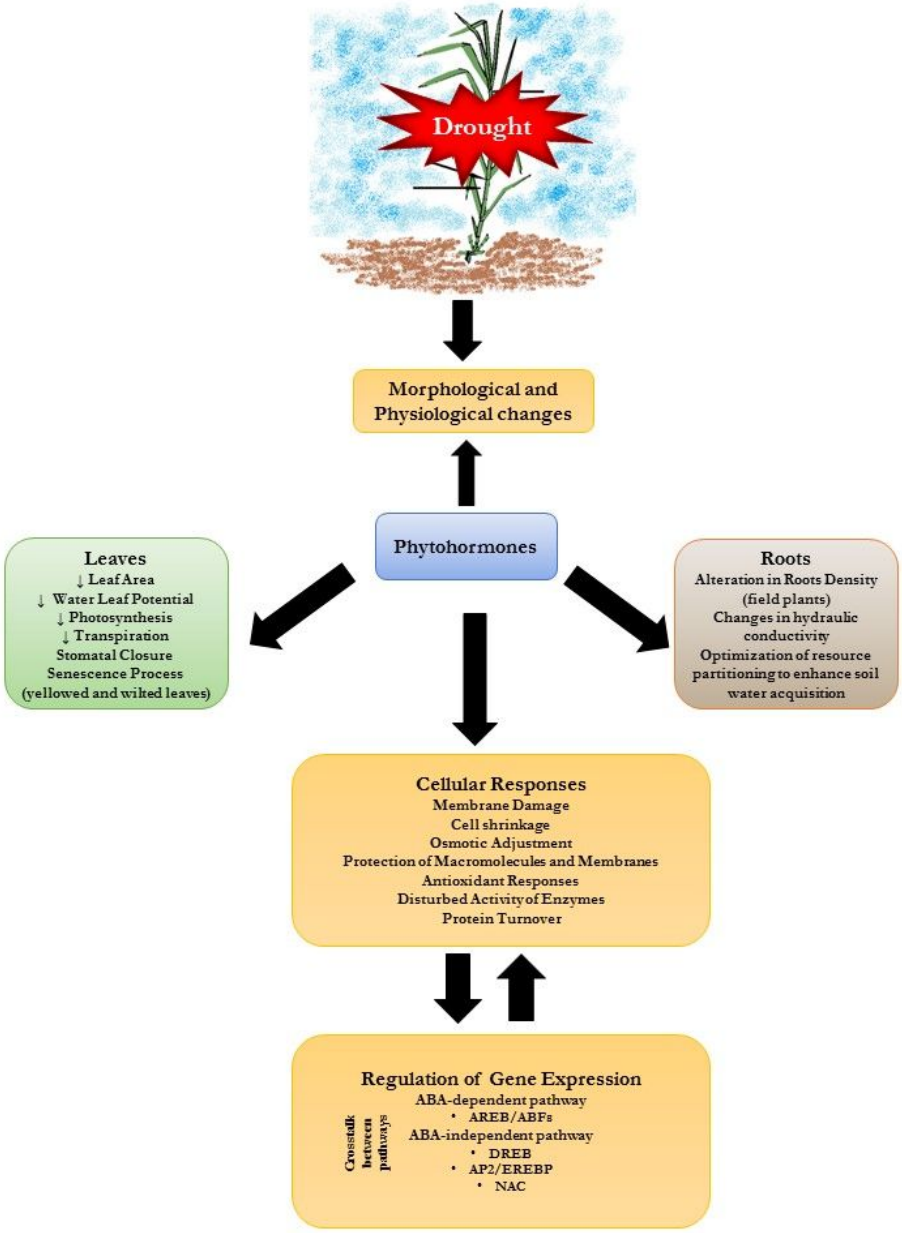

Figure 2. Schematic representation of a plant's responses to drought from morphological to molecular aspects

2.3. Transcriptome Studies provide a strategy to detect drought related biological functions and processes

Works related to the study of the changes in the transcriptome in response to drought are found in several species, such as Arabidopsis, maize, wheat, rice, sugarcane (HARB et al., 2010; LENKA et al., 2011; LI et al., 2012; MOUMENI et al., 2011; PAYTON et al., 2011; ZHENG et al., 2010).

The comparison between gene expression profiles of roots from susceptible and tolerant genotypes of wheat demonstrated the enrichment of classes related to abiotic stresses, biosynthesis of organic acids and metabolism of lipids among others and covered a wide range of cellular activities, indicating that wheat roots respond to drought

treatment in a global fashion (LI et al., 2012). Transcripts of Dehydrin, Late Embryogenesis Abundant Proteins (LEA), cold responsive proteins, heat shock proteins, ABA-responsive proteins and Germin-like proteins were found.

Moumeni et al., (2011) evaluated the root transcriptome of drought-tolerant rice lines and found that the main up-regulated classes were related to specialized metabolism, amino acid metabolism, response to stimulus, defense response, transcription and signal transduction, and down-regulated genes were involved in photosynthesis and cell wall growth.

Payton et al., (2011) analyzed the changes in the cotton transcriptome in response to drought stress in both leaves and roots and found that almost twice as many stress-responsive genes were in leaf compared to root. The three major functional groups showing stress induction in leaves were cell signaling, stress response and regulation of transcription; for roots, the major responsive categories were stress response, carbon metabolism and regulation of transcription.

In maize, a study also comparing tolerant and susceptible inbred lines found an enrichment of the classes of metabolism, use of energy, cell transportation, signal transduction and biogenesis of cellular components and of ABA signaling and antioxidant pathways (ZHENG et al., 2010). A similar study analyzing contrasting drought tolerant genotypes was done in rice, but using whole seedling (combined root and leaf samples). It showed the up-regulation in the expression of genes from classes related with carbon fixation, glycolysis, and flavonoids biosynthesis and downregulation of genes related to sucrose and starch metabolism in rice (LENKA et al., 2011).

Studies related to rehydration can be found in *Sinapis alba* (DONG et al., 2012) and show differential expression of genes related specially with cell division and metabolic and catalytic processes, and in grape (PERRONE et al., 2012), which shows the enrichment of specialized metabolism, sugar metabolism and transport classes, as well as aquaporin genes. Harb et al., (2010)studying the expression of genes after drought treatment of *Arabidopsis* plants observed the upregulation of genes involved in the processes of stress response, desiccation and the up-regulation of genes regulated by ABA. A RNA-Seq work in maize (KAKUMANU et al., 2012) showed that, in the leaf meristem, there is an increase in the expression of genes related to carbohydrate metabolism, specialized metabolism and cell cycle, and, in the ovaries, an increased response in the ABA related processes.

In sugarcane, the study of the transcriptome in response to drought has been the target of several works in the last few years (GENTILE et al., 2013; ISKANDAR et al., 2011; KIDO et al., 2012; LEMBKE et al., 2012; ROCHA et al., 2007; RODRIGUES et al., 2011), which shows its increasing importance. Gentile et al., 2012 analyzed the effects of drought in the micro-transcriptome of field-grown sugarcane plants and found 13 mature miRNAs differentially expressed in drought-stressed plants, being the target genes of such miRNAs mostly related to transcription factors, transporters, proteins associated with senescence and proteins involved with flower development.

Rodrigues; De Laia; Zingaretti, (2009) found an induction of genes related to transcription factors, signal transduction, transport and stress response. Genes regulated by plant hormones, such as ABA showed an induction during the whole period of drought treatment. Changes in the sugarcane root transcriptome in response to drought stress were evaluated by Kido et al., (2012) by SuperSAGE technique and allowed the identification of 213 up-regulated unitags related basically to abiotic stresses. Transcripts such as Delta-1-Pyrroline-5-Carboxylate Synthetase, Serine-Threonine Kinase SAPK1; Glutathione Transferase, AP2/EREBP were among the represented ones.

Iskandar et al., (2011) investigated the expression of genes implicated in abiotic stress to determine their expression in the context of sucrose accumulation by studying mature and immature culm internodes of a high sucrose accumulating sugarcane cultivar (cultivar Q117). Correlation analysis identified genes encoding enzymes involved in amino acid metabolism, a sugar transporter and a transcription factor. Proline seems to be negatively correlated with

sucrose concentration and transcripts from LEA proteins showed no correlation. Rocha et al. (2007) analyzed the changes in the transcriptome in response to several stresses like drought, phosphate starvation, herbivory and N2-fixing endophytic bacteria. This study showed that drought elicited higher changes in the late experimental data points (72 hours and 120 hours after drought treatment), and transcripts belonging to the group of transcription factors, signal transduction, hormone responses and antioxidant were found.

The transcriptome analysis of sugarcane subjected to 24, 72 and 120 hours of water stress using a customized Agilent oligoarray (the same platform used in the present work), showed the alteration in the expression of 987 transcripts (using a statistical significance of p=0.9), being 928 in the sense and 59 in the antisense orientation. This work used plantlets of SP90-1638, a drought sensitive cultivar, cultivated for 15 days prior to stress treatment. The main categories found after 72 h of stress reflects a modification in the plant metabolism and include the categories of redox, cell wall, carbohydrate metabolism and photosynthesis. After 120 h of water stress, the signal transduction category continued to be enriched, along with categories related to other stress response processes, such as carbohydrate and lipid metabolism, oxidative phosphorylation and light harvesting (LEMBKE et al., 2012).

In most of the studies, several transcripts had not yet been annotated and belonged to the Unknown functional class. These transcripts represent a source of new and potentially interesting targets for further works.

2.4. Co-Expression Networks as a tool to unveil biologically important genes

Gene networks are the basis of biological complexity and have become the core area of research in systems biology, becoming a new strategy to analyze large, high-dimensional data sets. These networks are modeled as graphs where nodes represent the functional units, such as genes, proteins, metabolites, etc, and edges are dependencies or interaction between the nodes (SMITA et al., 2013). Weighted Gene Co-Expression Network Analysis (WGCNA) is a systems biology method for describing the correlation patterns among the expression of genes across microarray samples, and can be used for finding clusters (modules) of highly co-expressed genes (LANGFELDER; HORVATH, 2008; SERIN et al., 2016).

This interaction may be the expression correlation between the paired genes that is generally measured in terms of Pearson Correlation Coefficient (PCC), a parameter used by the WGCNA (Weighted Gene Co-Expression Network Analysis) tool to assemble both signed and unsigned co-expression networks (SMITA et al., 2013). The construction of a co-expression network usually consists of three steps, the first is to calculate a measure of similarity or relatedness for each of the possible gene pairs, then the resulting list of gene pairs is filtered using a threshold value for the similarity scores that will result in a network following a power-law distribution, and, finally, the remaining gene pairs will form a list of edges from which the network is constructed (SERIN et al., 2016).

Genome-wide high-throughput techniques that made it possible to analyze thousands of genes in one shot (SMITA et al., 2013) along with the fact that co-expression networks construction is generally a straightforward process, led to the conclusion that complex network can be very tricky to interpret (SERIN et al., 2016). Therefore, it is necessary to stablish strategies to extract biologically important information. One way of doing that is through measures of node centrality that allows the detection of genes with critical functional roles, once highly connected genes are also candidate hubs and deserve further investigation (AZUAJE, 2014).

Thus, after the co-expression network is obtained, relevant biological information can be mined by gene prioritization, a process that consists in integrating diverse data sources to allow the ranking of the nodes in the network and the identification of groups of functionally related genes (SERIN et al., 2016). This process function by selecting

highly connected nodes as hub nodes and is a facile approach for better understanding and interpretation of the network and overall biological complexity (SMITA et al., 2013). Generally, hubs are identified by analyzing parameters such as the connectivity, defined as the total number of links in the network; the node degree, that corresponds to the number of connection of a node with other nodes in the network; and the betweenness, which is the sum of the shortest paths connecting all pair of nodes in the network, passing through that specific node (SERIN et al., 2016).

From the premise that genes with many, strong network connections are probably co-regulated with their co-expressed genes and have influence in phenotype-specific processes, the centrality scores calculated from the networks may provide complementary approaches in the interpretation of biological information, once significant connectivity patterns may represent biologically meaningful interactions (AZUAJE, 2014).

Co-expression networks can be used to functionally annotating unknown genes by the guilty-by-association principle (SERIN et al., 2016), since transcriptionally co-expressed genes tend to be functionally related and may interact with each other at physiological or molecular level (SMITA et al., 2013).

Signed networks are defined as the ones in which positively and negatively correlated nodes are clustered in separate modules (SMITA et al., 2013). Differently, the unsigned networks find the node's correlation by their absolute value (SMITA et al., 2013). Furthermore, correlation networks have undirected edges, therefore, no causality can be inferred from two connected genes (SERIN et al., 2016).

Co-expression relationships derived from condition-dependent gene expression data is also an efficient way to identify functional association between genes that are part of the same biological process and may be under similar transcriptional control (SIRCAR; PAREKH, 2015). This type of analysis has an important role, since genes that are co-expressed across various tissues and environmental stresses are biologically interesting and most probably play coordinated function in similar biological processes (SMITA et al., 2013). Therefore, co-expression network analysis appears as an important tool to decipher the underlying mechanisms of plant's adaptations to stresses, avoiding the bias that evaluation of only a few datasets would promote and allowing the analysis of different aspects of plant's stress tolerance that might be operating simultaneously (SHEN; HOUR; LIU, 2017).

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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 correlation 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 differential expression os some pathways 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 studying 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.

3.2. Specific Objectives

- Analyze physiological parameters obtained from a greenhouse drought and re-watering experiment;
- Evaluate by microarray the transcriptome of plants subjected to drought stress and rehydration conditions in a greenhouse experiment;
- Validate gene expression data obtained from microarray by RT-qPCR (reverse transcription quantitative PCR);
- Perform RNA-Seq on the most contrasting condition selected from the microarray results.

3.3. Material and Methods

3.3.1. Plant Material

Sugarcane plants (*Saccharum* spp) cultivar SP80-3280 were grown in greenhouse conditions with and without irrigation. The experiment was conducted in collaboration with Professor Lauricio Endres (Alagoas Federal University – UFAL). Two one eye sett of sugarcane were planted in 20 liters plant pots containing 18 kg of soil and cultivated for 5 months before submission of stress. Samples were collected after 4 and 6 days of water privation (a stress period longer than previously done by the group) and after 6 days of drought followed by 2 recovering days (Table 1). The tissues collected were the leaf L+1 and the roots. Analysis of the soil are shown on table 2 and figure 3. Until the beginning of the drought treatments, the soil was kept near the field capacity (FC). In order to do that, the soil from the pots was irrigated until the point in which the water was flowing through the bottom. After that, the pots were placed at rest for 24 hours and then, the soil volumetric humidity was measured with the help of a probe. After five months, half of the pots were keep under FC and in the other half the irrigation was ceased. The soil water content was monitored by three measurements of soil humidity per pot, in a depth of 5 cm each day after stress treatments, using the humidity sensor model SM200 (DELTA-T Devices, Cambridge - England) (figure 4a). It was used a completely randomized design. Leaves and roots samples were collected, immediately frozen in liquid nitrogen and kept in dry ice until storage in -80ºC.

Table 1.SP80-3280 drought/rehydration greenhouse experiment.

Tissue	Irrigated	Water Privation
Leaves	4 days	4 days
	6 days	6 days
	8 days	6 days + 2 rehydration days
Roots	4 days	4 days
	6 days	6 days
	8 days	6 days + 2 rehydration days

Table 2. Chemistry Analysis of the soil.

	Parameter	Value
pH	CaCl2	4,5
	Water	5,7
	SMP	5,8
	MO (g/dm$^{3)}$	27
	P (g/dm$^{3)}$	17
	K (mmolc/dm^3 TFSA)	1,3
	Ca (mmolc/dm^3 TFSA)	11
	Mg (mmolc/dm^3 TFSA)	5
	H + Al (mmolc/dm^3 TFSA)	52
	SB (mmolc/dm^3 TFSA)	17,3
	CTC (mmolc/dm^3 TFSA)	69,3
%	V%	24,9
Relations	Ca/Mg	2,2
	Mg/K	3,84

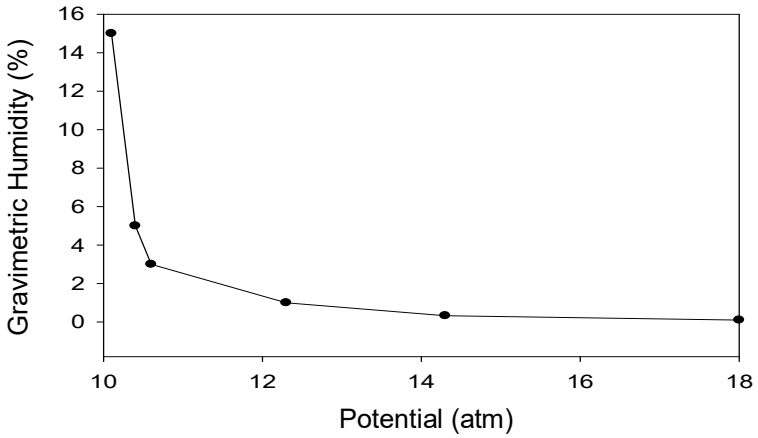


Figure 3. Soil water retention curve.

3.3.2. Physiology Analysis

3.3.2.1. Leaf Water Potential

Leaf water potential was measured with the help of a Scholander Pressure Chamber (Soil Moisture, Equipament Corporation, Santa Barbara, USA) at 4:30 (predawn), using the leaf L+2. Leaves were sectioned on the ligule, packed in an insulated box with ice (without direct contact with ice) with the aim of minimizing humidity loss. Immediately after harvesting, samples were taken to the laboratory to make the measures. All graphs from physiology analysis data were build using GraphPad Prism 6.

3.3.2.2. Gas Exchange Measurements

The gas exchange measurements were performed between 8:00 and 11:00 am with a portable infrared gas analyzer (IRGA, ADC Bioscientific Ltd., Hoddesdon, UK), with a light source of 1.123 μmol $m^{-2}s^{-1}$ on leaf +1. The following variables were evaluated: photosynthesis rate (A), stomatal conductance (gs), and transpiration (E). Carboxylation efficiency (A/ci) and intrinsic water use efficiency (A/gs) were calculated using the measured values of A, Ci and gs.

3.3.2.3. Chlorophyll *a* fluorescence analysis

The photochemical efficiency of photosynthesis was obtained through the evaluation of the chlorophyll a fluorescence on the same leaves for which the gas exchange was evaluated.

The measurements of the maximum quantum efficiency of PSII (Fv/Fm) were determined at predawn and at midday after the leaves were adapted to the dark for 20 minutes, using a modulated fluorometer 051-FL (OPTI-SCIENCES, Hudson, NH, USA). The measurements were taken after the exposure to saturating light pulses of 1 s.

The effective quantum yield (φPSII) was measured between 10:00 am and 12:00 pm (midday) on the same leaf that was used to quantify Fv/Fm as described in Maxwell; Johnson, (2000). This parameter measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry (MAXWELL; JOHNSON, 2000).

3.3.3. Lignin Analysis

3.3.3.1. Cell Wall Preparation

In order to prepare the cell wall residue, an amount of around 20 mg of dry plant material (leaves and roots grinded in liquid nitrogen) was weighted on an empty 2 mL microtube (Safe Lock). Then, 1 mL of water was added and samples were incubated at 98 °C while shaking (750 rpm) for exactly 15 minutes. Samples were centrifuged for 5 minutes at 14,000g and supernatant was removed. Then, 1 mL ethanol was added and samples were incubated at 76 °C while shaking for exactly 15 minutes. The material was centrifuged for 5 minutes at 14,000g and supernatant was removed. A volume of 1 mL chloroform : ethanol (1:1) was added and samples were incubated at 59ºC, 750 rpm for

15 minutes. Samples were centrifuged one more time for 5 minutes at 14,000g and supernatant was removed. The volume of 1 mL acetone was added, and incubation was at 54 °C, 750 rpm for 15 minutes. Samples were centrifuged for 5 minutes at 14,000g and supernatant was removed. The pellet was left drying overnight in at oven at 80 °C. Samples were weighted to obtain the cell wall residue ("CWR").

3.3.3.2. Acetylbromide

The volume of 250 µL of freshly made acetyl bromide (AcBr) solution (25% AcBr in glacial acetic acid) was gently added down to the tube walls to prevent splashing. Samples were heated at 50 °C for 2 hours, without shaking, and then heated to an additional hour vortexing every 15 minutes. The material was cooled down on ice and centrifuged for 15 minutes at 10,000 rpm.

In a new 2 mL microtube 400 µL of 2 M sodium hydroxide, 75 µL of freshly prepared 0.5 M hydroxylamine hydrochloride and 50 µL of AcBr reaction supernatant were added. Samples were mixed by vortexing. After that, 1.475 mL of glacial acetic acid was added, tubes were capped and inverted several times to mix. The blank was a 50 µL of 25% AcBr solution made in glacial acetic acid. Measures were done in a Nandrop at 280 nm wave length. The amount of lignin was calculated using the average absorbance value from three technical replicates of each biological replicate divided by 23.0772 (universal coefficient, Fukushima; Kerley, (2011)) multiplied by 0.1 and by 10 (dilution factor). This value was divided by the "CWR" and multiplied to 100 to obtain the percentage of lignin.

3.3.4. Transcriptome Analysis

3.3.4.1. RNA Extraction

Total RNA was extracted from leaves and roots (3 biological replicates, from which 2 were used for microarray) of irrigated and water deprived samples in all conditions evaluated, according to Zeng and Yang (2002) with modifications. Briefly, the extraction buffer (2% CTAB, 4% PVP, 10 mM Tris-HCl (pH 8), 25 mM EDTA, 2 M NaCl, 4 mL DEPC (dietilpirocarbonate) Milli-Q Water, 2% β-mercaptoethanol (added just before use)) was pre-warmed to 65°C in a water bath. Then 100 mg – 300 mg of tissue was added to a 2 ml tube. After that, 0.9 mL of the prewarmed extraction buffer was quickly added. The plant material and the buffer were mixed completely by inverting the tube. Samples were incubated at 65ºC for 15 minutes with vigorous shaking every 5 minutes. Then, an equal volume of CIA (1 isoamyl alcohol : 24 chloroform) was added and shaken vigorously. Samples were centrifuged at 20,000g for 10 minutes.

The supernatant was transferred to a new tube and extracted with an equal volume of CIA four times. Supernatant was again removed and 0.25 volumes of 10 M LiCl was added, well mixed and stored at 4ºC overnight. RNA was recovered by centrifugation at 16,000 g during 40 minutes at 4ºC. The viscous supernatant was completely discarded and the pellet was washed twice with 75% ethanol and air dried for 10 minutes. RNA was re-suspended in 50 µL of DEPC treated water, and its concentration was determined with a NanoDrop (Thermo Scientific). In order to eliminate genomic DNA contamination, RNA samples were treated with DNase I, amplification grade (Invitrogen) according to the manufacturer's instructions with some modifications. Briefly, it was used 10 µg of total RNA per sample, 10 µL 10X DNase I Reaction Buffer, 10 µL DNase I, Amp Grade, 1 U/µL and DEPC-treated water up to

100 µL. Tubes were incubated for 10 minutes at room temperature, then the reaction was stopped by the addition of 10 µL of 25 mM EDTA solution. The next step was to clean the treated RNA with RNeasy® Mini Kit (Qiagen, Catalogue number 74104) following the RNA cleanup steps from the manufacturer's instructions.

Finally, RNA integrity was assayed by using 2100 Bioanalyzer (Agilent Technologies) and evaluated with the RIN (RNA Integrity Number) parameter (above 7) provided by the equipment. Samples were prepared and analyzed using the Agilent RNA 6000 Pico Kit (Agilent, Catalogue number 5067-1513), according to the manufacturer's instructions.

3.3.4.2. Microarray

Microarray analysis was done according to Lembke et al., (2012), using a customized sugarcane oligoarray in the Agilent platform and validated by qRT-PCR of the sense and antisense transcripts. The oligos are distributed in a 4x44K arrangement and are composed by a total of 45,220 elements, being 21,901 represented in duplicate in the arrangement, from which 14,522 hybridize with sense transcripts and 7,380 with anti-sense transcripts.

For microarray sample preparation, cRNA was marked with Cy3 and Cy5 for the hybridizations in the customized oligos chip (LEMBKE et al., 2012) using the kits Low Input Quick Amp Labeling Kit, Two-Color (Agilent, catalog number 5190-2306), according to the manufacturer's indications, with an input total RNA of 200ng. For cRNA amplification and marking, mRNA control synthetized in vitro (Agilent RNA Spike-In kit, two-Color, catalogue number 5188-5279) was used as a spike. Gene Expression Hybridization Kit (Agilent, Catalogue Number 5188-5242), Gene Expression Wash Buffer Kit (Agilent, catalogue number 5188-5327) and RNeasy Mini Kit (Qiagen, catalogue number 74104) were also used according to the manufacturer's instructions.

After the hybridizations and washes, microarrays were scanned in GenePix 4000B scanner (Molecular Devices) and image data was extracted with the aid of the Feature Extraction 9.5.3 (Agilent Technologies), using the two-color microarray referential in the Agilent platform (KERR, 2007). Data intensity from Cy3 and Cy5 of the same sample were corrected and normalized using the Lowess function (YANG et al., 2002) of the R software tool available at our server and taking into consideration the signals from the spike controls. Differentially expressed genes were defined by HT-self method (VENCIO; KOIDE, 2005), modified from Rocha et al., (2007) and adapted to the Agilent oligonucleotide microarray platform and to the Feature Extraction.

3.3.4.3. cDNA synthesis

For cDNA synthesis SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, catalogue number 18080-400) with oligo(dT) was used according to the manufacturer's instruction, using 500 ng of total RNA for each sample. In the case of the separate analysis for transcripts in the sense and anti-sense orientation, cDNA synthesis was done in a strand-specific manner, using the kit above mentioned as described by Lembke et al., (2012). For synthesis of the sense transcripts strand, 2 µM of the primer with an anti-sense sequence was used, whereas, 2 µM of the sense primer was used for the anti-sense transcript.

3.3.4.4. RT-qPCR

RT-qPCR was performed for the three biological replicates with the aim of validating the expression level of the genes identified as differentially expressed. In each reaction 5 μL of 1.5 μM primer mix, 1 μL of cDNA in the dilution 1:10 and 6 μL of Fast SYBR® Green Master Mix (Applied Biosystems, catalogue number 4385612) were used. The final reaction volume was12 μL and the final concentration of the primer was 625 nM. The equipment applied was 7500 Fast Real-Time PCR System (Applied Biosystems). In the case of the primer SCSGLR1045D05.g we decreased the primer concentration to 312.5 nm.

The primers were designed using the Primer Express 2.0 (Applied Biosystems) program and analyzed for amplification efficiency. Only primers with an efficiency between 90 e 110% were used. Reaction specificity was verified by the melting curve of the amplification products generated by the equipment.

The relative expression was calculated as described by Vandesompele et al., (2002) using as normalization factor the geometric mean of, at least, two constitutive genes. The ratio of expression was analyzed with the help of the REST 2009 (Relative Expression Software Tool, Quiagen) according to Pfaffl; Horgan; Dempfle, (2002), also using at least two more stable endogenous genes for the calculation of the normalization factor. Actin (ACT, SCCCLR1069D05.g), ATP Synthase subunit delta (SCCCLR1072A03.g), 60S ribosomal subunit (60S, SCJFRZ2009G01.g), glyceraldehyde-3-phosphate dehydrogenase (GAPDH,(ISKANDAR et al., 2004)), tubulin (TUB, SCCCRZ1002H03.g), E2 ubiquitination enzyme (UBE2, SCBGLR1002D06.g) and ubiquitin (UB, SCCCLR1048F12.g) were tested for homogeneous expression among the samples. The most appropriated genes for each tissue were identified by the geNorm program (VANDESOMPELE et al., 2002) (table 3), therefore, it was obtained a different normalization factor specific for each tissue. Primers sequences are found in appendix A.

Table 3. Reference Genes chosen for qRT-PCR assays for both leaves and roots tissues

SAS (sugarcane assembled sequence)	Annotation	Tissue
SCCCLR1072A03.g	Mitochondrial ATP Synthase subunit delta	Leaves / Roots
SCCCLR1048F12.g	ubiquitin	Leaves / Roots
SCJFRZ2009G01.g	60S ribosomal subunit	Roots
SCBGLR1002D06.g	E2 ubiquitination enzyme	Roots
Sequence obtained from Iskandar et al., (2004)	glyceraldehyde-3-phosphate dehydrogenase	Roots

3.3.4.5. RNA-Seq

Three biological replicates (BRs) from root and leave samples were used for RNA-Seq, for both, control (watered) and stressed conditions (6 days without watering), totaling 12 samples (3 BRs of control leaves, 3 BRs of stressed leaves, 3 BRs of control roots and 3 BRs of stressed roots). The stressed condition chosen was the one that showed a high number of differentially expressed genes after the microarray analysis. RNA was extracted according to Zeng and Yang (2002) with modifications. RIN was assayed through the use of 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 Pico Kit (Agilent, Catalogue number 5067-1513) according to the

manufactures' instructions. Only samples with the RIN above or equal to 6.5 and a 25S:18S ratio above or equal to 1 were selected for sequencing. Library preparation and sequencing was performed at BGI (Beijing Genomics Institute) using the TruSeq Low Input Library Prep Kit (catalogue number FC-134-2002) according to the manufacturer`s instructions and starting with 1 μg of RNA material. Sequencing was performed with the Illumina Sequencing Machine HiSeq4000, PE150. A total of 6G of data was generated for each sample.

3.3.4.6. Microarray and RNA-Seq Data Analysis

In the case of the microarray, the analyses were done using the tools from the database and web environment SUCEST-FUN. Therefore, differentially expressed genes (DEGs) were defined by two strategies, which were genes with a fold change (FC) ≥ 2 and ≤ 0.5 (LI et al., 2016a) and the modified HTSelf method (LEMBKE et al., 2012). The first one allowed us to identify a high number of sugarcane assembled sequences (SAS) differentially expressed and the results found were used to identify enriched pathways on KEGG database (http://www.kegg.jp/). The KEGG metabolic pathways and the expression of the SAS found were plotted together with the help of the tool Pathview, from Bioconductor (https://www.bioconductor.org/) using the R software. The DEGs identified by the HTSelf method, which is a more stringent methodology, were used to build biological networks with the help of Pathway Studio Software (plant.pathwaystudio.com - Elselvier), to perform functional classifications and enrichment analysis.

The modified HTSelf strategy use tools based on three methodologies for gene expression analysis. Two for the identification of differentially expressed genes, one by ratio of expression (by the HTSelf adapted methodology (LEMBKE et al., 2012; VÊNCIO; KOIDE, 2005), another by Pearson correlation, and the third method by identification of significantly expressed genes in each experimental condition / individual channel (LEMBKE et al., 2012).

The SUCEST-FUN web environment also allows the analysis integrating metabolic pathways, with levels of expression in different conditions and treatments. The pathway activity analysis was accomplished using Pathway Activity Scores calculated through an algorithm which aimed to integrate transcript expression profiles and metabolic pathways, therefore, estimating the level of activity of each pathway found and the level of correlation between pathways (NISHIYAMA et al., 2014). The results are shown as a heatmap that allows the identification of pathway activity patterns and the grouping of pathways and conditions with high correlation.

For the *de novo* assembly of the RNA-Seq transcripts (performed in collaboration with doctorate student Mauro Medeiros), sequence quality was assessed by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adaptor and low quality sequences were removed by Trimmomatic software (BOLGER; LOHSE; USADEL, 2014), using the sliding window (20 nt) option to crop sequences with quality Phred score < 20. Filtered reads were assembled using Trinity *de novo* assembler (GRABHERR et al., 2011).

Therefore, all filtered reads, from all samples and biological replicates were combined into a single RNA-Seq assembly in order to generate a single reference transcriptome. Then, original RNA-Seq reads were aligned back to the reference transcriptome assembled using Bowtie (http://bowtie-bio.sourceforge.net) and transcript abundance was evaluated using RSEM (RNA-Seq by Expectation Maximization) (LI; DEWEY, 2011).

The pipeline is described at Haas et al., (2013) and was followed according to: https://github.com/trinityrnaseq/RNASeq_Trinity_Tuxedo_Workshop/wiki/Trinity-Denovo-Transcriptome-

Assembly-Workshop. The assembled transcripts were used in Blast2GO software to the determination of differentially expressed features according to the software pipeline and default parameters, using an FDR=0.05 and log Fold Change of 2/-2.

For both microarray and RNA-Seq, the transcripts were annotated using Blast2GO software and characterized by gene ontology terms (biological process, molecular function and cellular component) and orthologous group annotation. In the case of Blast, I used blastx, nr database, an e-value of 1.0e-5 and a number of blast hits of 10. In parallel to blast, InterProScan was run. After both parameters finished, mapping, annotation and GoSlim were done. Furthermore, in order to improve the analysis, GO-Enzyme Code mapping, merge InterProScan GOs to annotation and ANNEX tools were also used. In general, sequences were identified based on the concept of homology which is related to sharing a common ancestor. Sequences considered to be homologous are identified normally by BLAST searches in a way that the highly similar ones can also have common ancestry and possibly a similar role in different organisms (PEARSON, 2013). In the case of sugarcane genome there is yet no published complete annotation and several sequences are unidentified, therefore BLAST strategy is widely used. The sequences identified in the present work had an e-value of at least 1e-5 and were, therefore, considered homologous.

The orthologous group annotation from the software allows to assign cluster of orthologous groups (COG) to sequences using the EggNOG database. COGs are group of proteins that share a high level of sequence similarity, which can be usually associated to evolutionary convergence and can be used to infer properties that may improve the characterization of the sequences (https://www.blast2go.com/). In the case of microarray, all SAS from SUCEST-Fun database were annotated in order to update annotation and allow all the annotations to be in the same format for the enrichment analysis using Fisher's Exact Test tool from the software. The reference file used was the one containing all the SAS from the CaneRegNet database, therefore, the SAS found in the customized sugarcane Agilent oligoarray, and the test files were the ones from DEGs found in each condition in leaves and roots. The filter value was FDR 0.05 and the terms used were GO IDs from biological process, cellular component and molecular function. The pipeline and default parameters from Blast2GO were followed.

In the case of using a reference genome, in order to perform differential expression analysis, reads from all libraries were mapped to the most recent genome assembly of Sugarcane commercial hybrid SP80-3280 (unpublished data) using software Bowtie 2 (LANGMEAD; SALZBERG, 2012) with standard parameters (table 4). After the alignment, gene coverage was calculated using HTSeq (ANDERS; PYL; HUBER, 2015) and used as input to identify differential expressed genes with R package DESeq2 (LOVE; HUBER; ANDERS, 2014) combining technical replicates and applying a cut-off of 0.01 for p-value adjusted by FDR procedure (BENJAMINI; YEKUTIELI, 2001).

Differential expressed genes were searched against KEGG Orthology (KANEHISA et al., 2016) database using KAAS web service (MORIYA et al., 2007) and most relevant pathways were analyzed using Pathview Web (LUO et al., 2017). The RNA-Seq assembly using sugarcane reference genome was done in collaboration with Dr. Felipe Ten Caten.

Table 4. Mapped reads for each RNA-Seq library using the most recent genome assembly of Sugarcane commercial hybrid SP80-3280.

Sample	Condition	Tissue	Library	# reads	# mapped reads	% mapped reads
L5	control	leaf	201_L6	18,231,525	15,430,967	84.64
L5	control	leaf	201_L7	18,234,706	15,393,813	84.42
L6	control	leaf	202_L6	18,250,248	14,836,748	81.30
L6	control	leaf	202_L7	18,257,761	14,812,849	81.13
L7	control	leaf	203_L6	18,220,505	15,643,832	85.86
L7	control	leaf	203_L7	19,439,533	16,649,347	85.65
L15	treated	leaf	204_L6	18,120,194	14,669,562	80.96
L15	treated	leaf	204_L7	19,339,147	15,613,713	80.74
L16	treated	leaf	205_L6	18,241,125	14,570,655	79.88
L16	treated	leaf	205_L7	18,246,826	14,544,346	79.71
L17	treated	leaf	206_L6	18,252,119	14,976,478	82.05
L17	treated	leaf	206_L7	18,258,553	14,948,909	81.87
R5	control	root	207_L6	18,233,580	7,401,199	40.59
R5	control	root	207_L7	18,237,873	7,409,449	40.63
R6	control	root	208_L6	18,223,492	4,967,848	27.26
R6	control	root	208_L7	18,229,208	4,978,133	27.31
R7	control	root	209_L6	18,205,533	4,147,210	22.78
R7	control	root	209_L7	19,424,212	4,433,921	22.83
R15	treated	root	210_L6	18,233,791	2,798,195	15.35
R15	treated	root	210_L7	18,240,684	2,811,250	15.40
R16	treated	root	211_L6	18,208,268	5,613,941	30.83
R16	treated	root	211_L7	18,219,240	5,616,509	30.83
R17	treated	root	212_L6	18,243,971	3,298,690	18.08
R17	treated	root	212_L7	18,249,687	3,315,561	18.17

3.4. Results

3.4.1. Physiology

Drought treatment led to some morphological alterations in the leaves which turned curly and yellowish. According to Zhang et al. (2015) the most direct effects of drought on sugarcane plants are yellowed, curled and wilted leaves, and a reduction in the number of green leaves/plants. The same author states that after rehydration, the leaves expanded gradually and the number of green leaves are related to the rate of photosynthesis and to the maintenance of life activities under stress.

Four days after irrigation suppression there was an approximate decrease of 81% in soil humidity (figure 4b) and the leaf water potential (LWP) increased 0.92 times in absolute value (though negative) (figure 4a). The decrease percentage of soil humidity after 6 days of irrigation suppression was 90% (figure 4b) and LWP increased 14.5 times in absolute, but negative, value (figure 4a). After rehydration, there was an increase in soil humidity, and the difference between the values found for watered (control) and rehydrated samples was 25% (figure 4b). After re-watering there was not anymore a statistically significant difference in leaf water potential between control and re-watered plants (figure 4a).

There was a progressive decrease in transpiration (figure 4d), stomatal conductance (figure 4e) and photosynthesis (figure 4a) (59%, 90%; 70%, 98%; 61%, 98% - approximated values - respectively), with no statistically

significant difference after rehydration. Carboxylation efficiency (figure 4f) showed a decrease of 99% after 6 days of water privation and no significant difference in the other conditions.

Instantaneous water use efficiency (figure 4g) showed a significant decrease of 78% after 6 days of drought, with no statistically significant difference on the other conditions. The same pattern was followed by intrinsic water use efficiency (figure 4h) (74% decrease after 6 days of water privation). The intense photoinhibition was observed only after 6 days of drought with a difference of 14% in maximum quantum yield at 5 AM (figure 4i), 31% in maximum quantum yield at midday (figure 4j), and 55% in effective quantum yield (figure 4k).

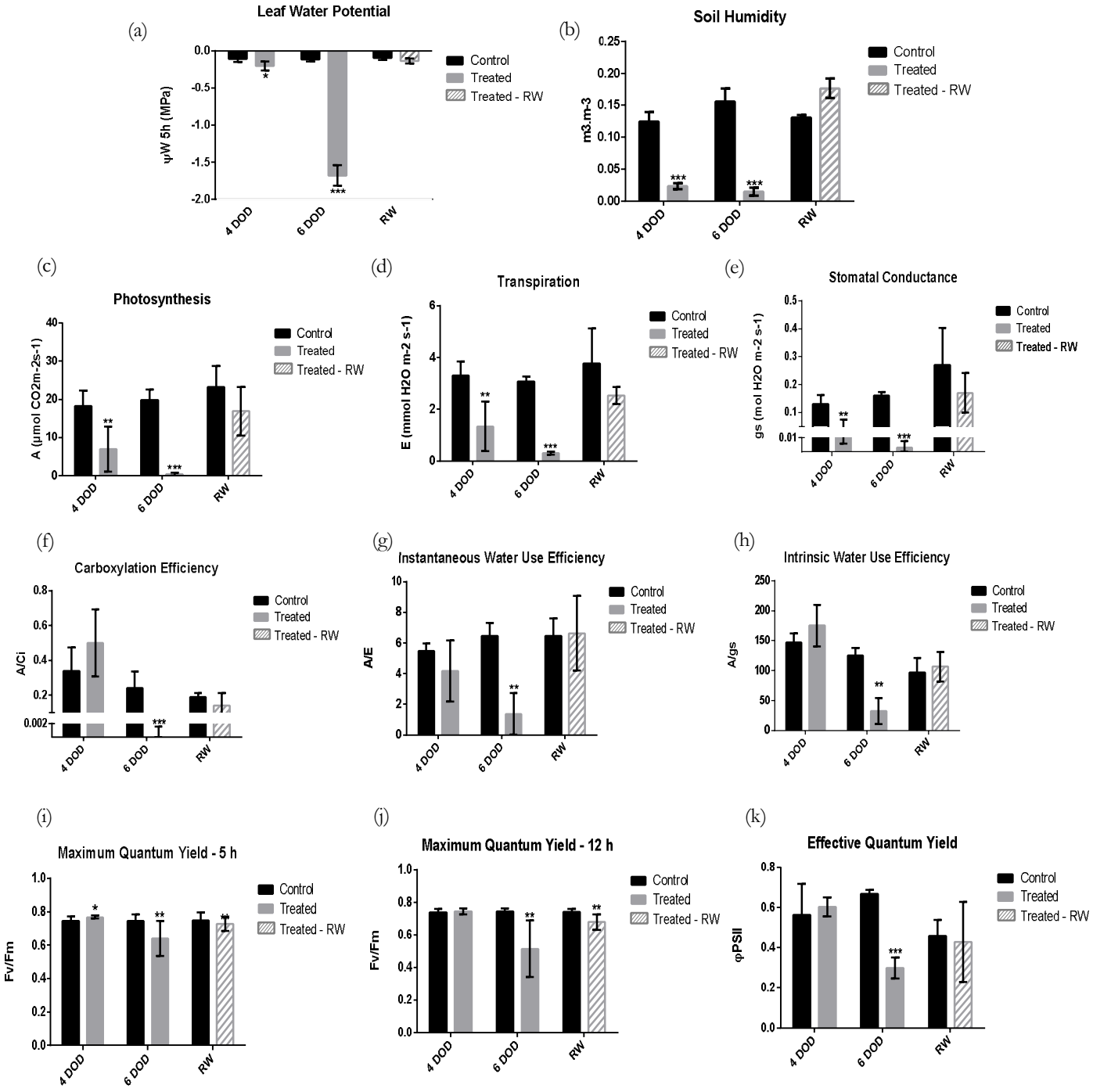


Figure 4. Physiology data from drought stresses samples shows that stress was moderate after 4 days but severe after 6 days. Asterisks indicate a significant statistical difference, according to t test, therefore, *; **; *** for $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively.

3.4.2. Microarray Data Analysis

The results in figure 5 demonstrated the similarity between leaves samples in all three conditions and root samples also in all three conditions, with leaves stressed for 6 days and leaves re-watered more correlated. The same pattern occurring with roots samples.

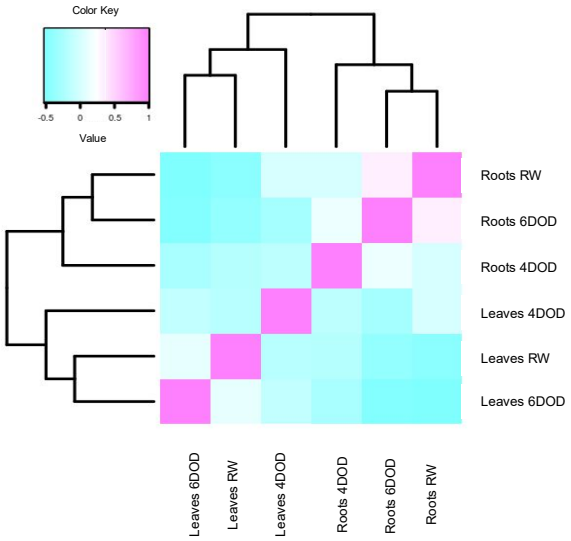


Figure 5. Correlation Matrix showing more similarity among the samples from the same plant tissue

Microarray results showed a total of 57,438 significantly expressed SAS (with a signal above the background), considering all samples together. From these, 11,844 were unique and appeared only in a specific condition (figure 6). Considering a FC $\geq$ 2 and FC $\leq$ 0.5 (LI et al., 2016a), the number of differentially expressed probes are 13,446, corresponding to 7,867 unique SAS (figure 6). When we apply the HTSelf analysis, which is a very stringent methodology, we obtain 713 differentially expressed probes, corresponding to 585 unique SAS (figure 6).

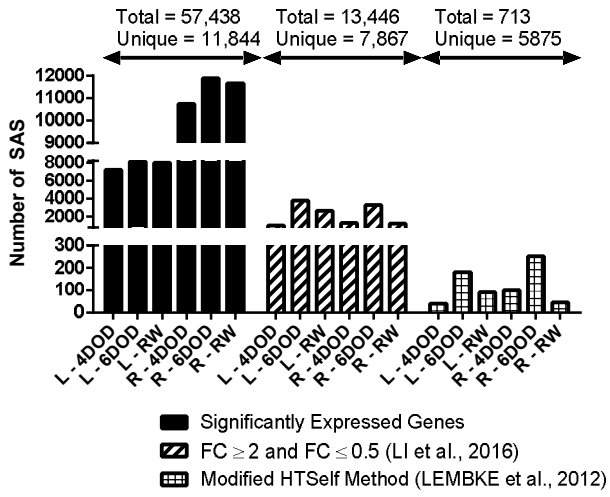


Figure 6. Graph showing that most differentially expressed SAS are found after 6 days of drought, in both root and leaves and using different analysis strategies

The differentially expressed SAS identified by the HTSelf and by the FC methods were annotated using the Blast2GO software (https://www.blast2go.com/) and gene ontology analysis was done for the SAS identified by the HTSelf method (figures 7, 8, 9, 10, 11 and 12).

The description of the HTSelf annotated SAS is on appendix C. In the case of leaves, the classes of Organic Substance Biosynthetic Process, Oxoacid Metabolic Process, Cellular Nitrogen Compound Metabolic Process, Cellular Biosynthetic Process, Protein Dephosphorylation and Single-Organism Carbohydrate Metabolic Process were found exclusively after 4 days of stress (figure 7). Cellular Nitrogen Compound Biosynthetic Process, Transport, Biological Regulation, Nucleic Acid Metabolic Process, Generation of Precursor Metabolites and Energy, Photosynthesis, Small Molecule Metabolic Process and Single-Organism Cellular Process were exclusive after 6 days without water when stress is extreme. Cellular Amino Acid Metabolic Process, Translation, Protein Folding, Anatomical Structure Development and ncRNA Metabolic Process were represented only after re-watering (figure 7). Interestingly, Response to Stress, Oxidation-Reduction Process and Catabolic Process were found in the three conditions, with Catabolic Process more represented in the second and third conditions (figure 7). Cellular Component Organization and Cellular Protein Modification Process were found only after 6 days of stress and after re-watering, and Lipid Metabolic Process and Regulation of Cellular Processes were found at 4 days of stress and after re-watering. The only term found exclusively in conditions 1 and 2 was Single-Organism Biosynthetic Process (figure 7).

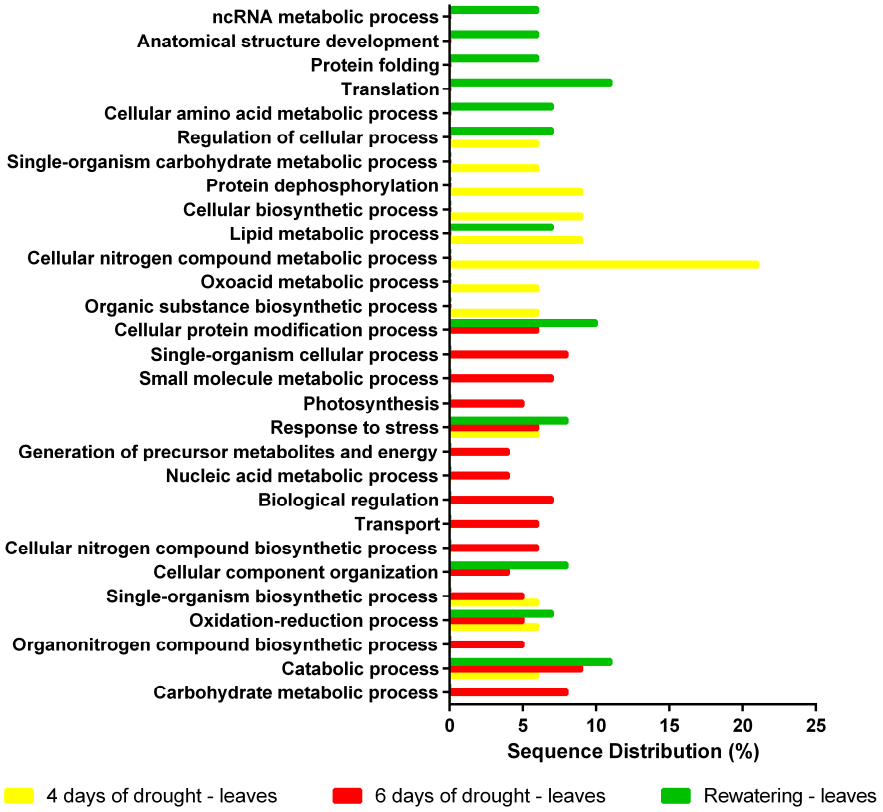


Figure 7. Biological Process gene ontology analysis on leaves show terms that were exclusive for each time point while classes as Response to Stress, Oxidation-Reduction Process and Catabolic Process appeared in all time-points analyzed

In the case of cellular component, sequences related to the term Plastid, Protein Serine/Threonine Phosphatase Complex, Integral Component of Membrane and Endomembrane System appeared only after 4 days of stress (figure 8), while Plasma Membrane and Thylakoid were represented after 6 days of stress and Cytosol and

Intracellular Non-Membrane-Bounded Organelle after re-watering (figure 8). The terms of Nucleus, Intracellular Organelle Part and Mitochondrion appeared in all three conditions, with Nucleus more represented in the first condition (4 days without water) (figure 8).

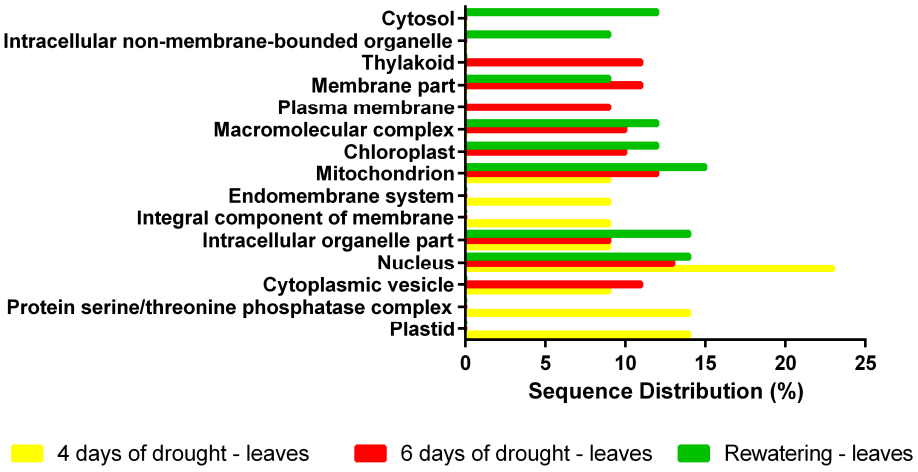


Figure 8. Analysis of Cellular Component gene ontology terms on leaves points the attention to the representation of Thylakoid term only after 6 days of drought which may be a reflection of chloroplast membrane damage due to the stress

The figure 9 shows the sequence distribution of the terms related to the Molecular Function, and once more we can notice some terms represented only in determined conditions. For example, the terms of Metal Ion Binding, Protein Serine/Threonine Phosphatase Activity, Isomerase Activity and Nucleic Acid Binding Transcription Factor Activity were observed after 4 days of stress, while the terms Ion Binding, Transferase Activity and Nucleic Acid Binding after 6 days of stress. Transition Metal Ion Binding, Transferase Activity, Transfering Phosphorus-Containing Groups, ATP binding and RNA binding appeared only after re-watering. The single term that appeared in all three conditions was Oxidoreductase Activity.

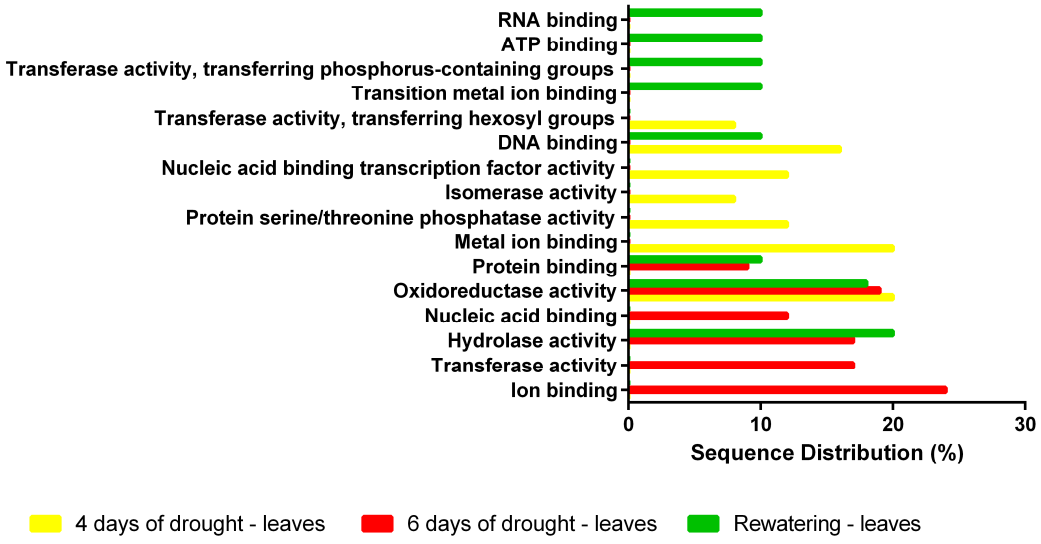


Figure 9. Molecular Function gene ontology term analysis shows that in leaves Nucleic Acid Binding Transcription Factor Activity was represented exclusively after 4 days of stress highlighting the possibility of this time point be mostly related to changes in the control of gene expression

In the case of roots, the sequence count of Biological Process Gene Ontology terms shows that Carboxylic Acid Metabolic Process, Transmembrane Transport, Phosphate-Containing Compound Metabolic Process, Lipid Metabolic Process, Cellular Protein Modification Process and Ion transport were represented after 4 days without water (figure 10). The condition of more extreme stress, in which plants were without water for 6 days showed the representation of, for example, Organic Cyclic Compound Metabolic Process, Cellular Macromolecule Metabolic Process, Regulation of Cellular Process, Organic Substance Biosynthetic Process (figure 10). After re-watering, classes such as Cytoskeleton Organization, Cellular Homeostasis, Biosynthetic Process and Nitrogen Compound Metabolic Process were represented (figure 10). Catabolic Process and Response to stress appeared in all three conditions, while interestingly Carbohydrate Metabolic Process was observed on conditions 1 and 2 (4 and 6 days without watering) (figure 10).

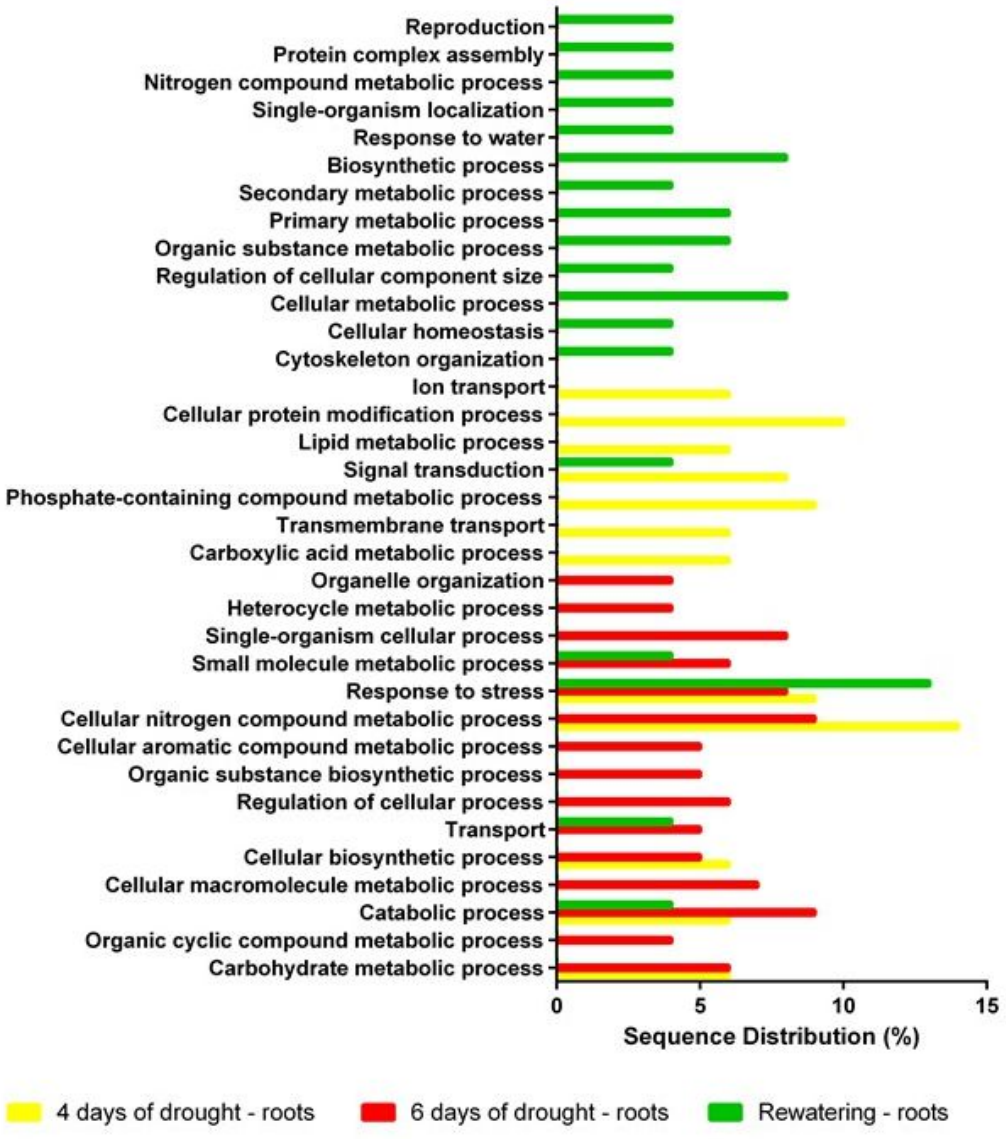


Figure 10. In roots, the analysis of Biological Process GO terms shows transmembrane transport and ion transport after 4 days of stress while Carbohydrate Metabolic Process appeared after 4 and 6 days of drought and may demonstrate the major role of osmoregulation and osmoprotection in roots of sugarcane stressed plants

The analysis of sequence count from the Cellular Component terms observed in roots shows that Cytoplasmic Vesicle, Plasma Membrane, Nucleus and Integral Component of Membrane are represented in all conditions, while Plastid appears only after 4 days without water, Extracellular Region only after 6 days and Cytosol only after re-watering (figure 11).

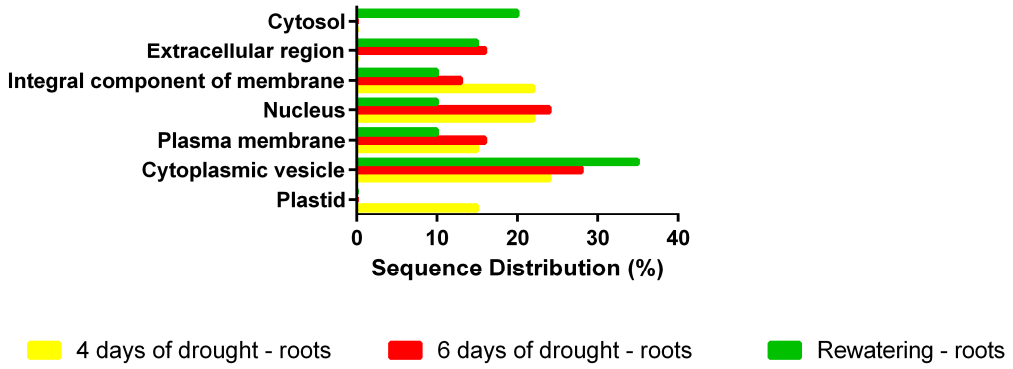


Figure 11. Analysis of Cellular Component terms in roots highlights the importance of transcripts found in nucleus in all three conditions

In the case of Molecular Function, the only terms represented after 6 days of stress were Transporter Activity and Transferase Activity, while Nucleic Acid Binding Transcription Factor Activity and Nucleotide Binding appeared after 4 days of stress and DNA Binding after 4 and 6 days of stress. The classes of Water Channel Activity, Glycerol Channel Activity, Asparaginase Activity among others were observed only after re-watering.

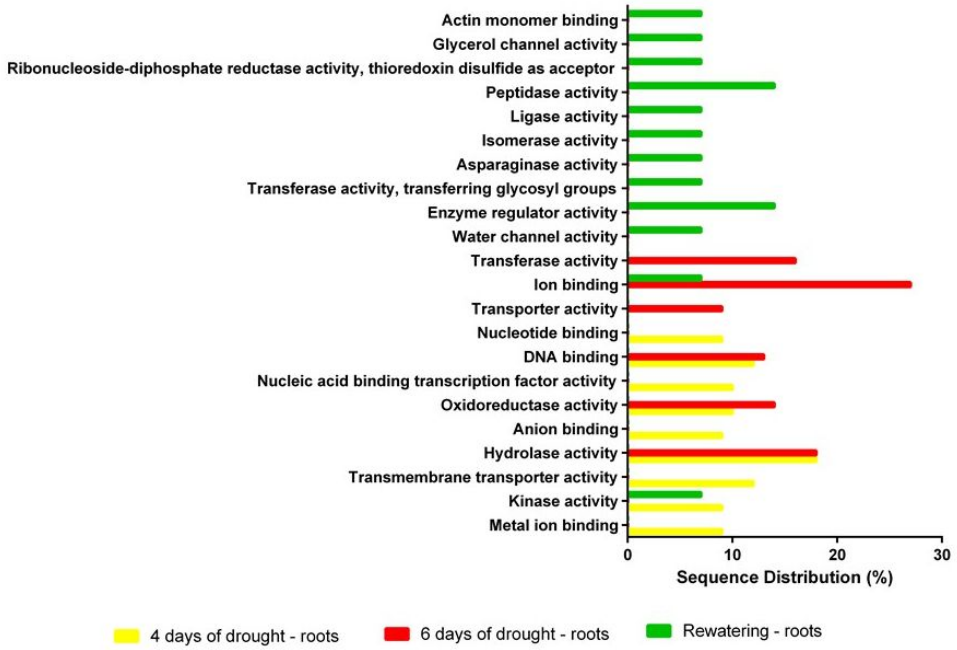


Figure 12. The analysis of Molecular Function shows the presence of Water Channel Activity term representing the role of aquaporins in root's responses to water stress

In order to perform an enrichment analysis, all SAS belonging to SUCEST database were re-annotated using Blast2GO (figure 13). This annotation was performed aiming to update the SAS description and also to standardize the annotation in a way Fisher's Enrichment Test could be performed using Blas2GO tool.

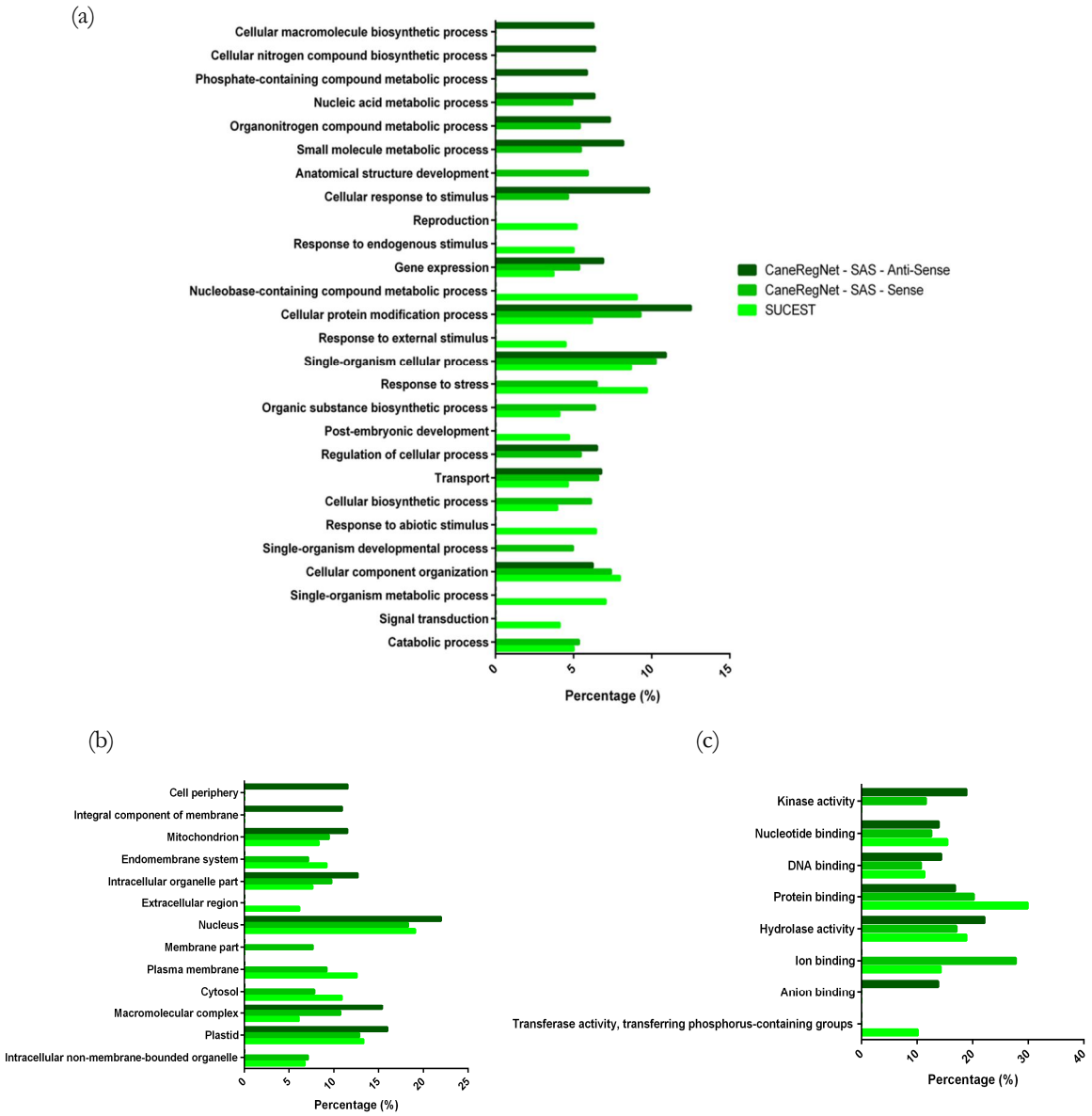


Figure 13. Updated annotation of SAS found in SUCEST database shows a diversity of terms belonging to the three Gene Ontology categories: (a) Biological Process, (b) Cellular Component and (c) Molecular Function

Data analysis show that there are some terms that appear in all three catalogues, such as Cellular Component Organization, Transport, Single-Organism Cellular Process, Gene Expression (Biological Process – figure 13a). In addition, Plastid, Macromolecular Complex, Nucleus, Intracellular Organelle Part, Mitochondrion (Cellular Component – figure 13b), Hydrolase Activity, Protein Binding, DNA Binding, Nucleotide Binding (Molecular Function – figure 1c), and others like Anatomical Structure Development appeared in just one catalogue (CaneRegNet-SAS-Sense).

According to Fisher's Enrichment Test (table 5), there were enriched categories in roots stressed for 4 days, leaves and roots stressed for 6 days and re-watered samples from both tissues. Most of the categories enriched in leaves

after 6 days of stress are related to Chloroplast and Photosynthesis and are down-regulated, reflecting the impaired photosynthesis observed on the physiology data. In re-watering samples, there was an enrichment in up-regulated genes related to Plastid and to the molecular function of Ribosomal Large Subunit Binding, which may show a recovery in photosynthesis and in the protein synthesis. In the case of roots, there was an enrichment of terms only in the down-regulated genes. The down-regulation of terms related to Cell Wall and Cell Wall Organization and Biogenesis may reveal the constriction in root growth of drought stressed plants cultivated in pots.

Table 5. Fisher's Enrichment Test showing over-represented categories on differentially expressed genes found by HTSelf method. FDR=0.05

	GO ID	GO Name	GO Category	FDR	P-Value
	GO:0009536	Plastid	Cellular Component	3.15E-10	5.19E-14
Leaves	GO:0009579	Thylakoid	Cellular Component	1.07E-08	3.52E-12
6 days of stress	GO:0015979	Photosynthesis	Biological Process	2.08E-06	1.03E-09
DOWN	GO:0009507	Chloroplast	Cellular Component	0.004006017	2.65E-06
	GO:0044444	Cytoplasmic Part	Cellular Component	0.01533011	1.27E-05
Leaves	GO:0009536	Plastid	Cellular Component	3.25E-06	5.37E-10
Re-watering UP	GO:0043023	Ribosomal Large Subunit Binding	Molecular Function	0.042654128	1.41E-05
Roots	GO:0016781	Phosphotransferase Activity, Paired Acceptors	Molecular Function	0.021779979	7.19E-06
4 days of stress DOWN	GO:0050242	Pyruvate, Phosphate Dikinase Activity	Molecular Function	0.021779979	7.19E-06
	GO:0031410	Cytoplasmic Vesicle	Cellular Component	3.13E-10	1.42E-13
	GO:0097708	Intracellular Vesicle	Cellular Component	3.13E-10	1.42E-13
	GO:0031982	Vesicle	Cellular Component	3.13E-10	1.55E-13
	GO:0005576	Extracellular Region	Cellular Component	1.93E-05	1.27E-08
Roots	GO:0071554	Cell Wall Organization or Biogenesis	Biological Process	2.77E-04	2.29E-07
6 days of stress DOWN	GO:0009056	Catabolic Process	Biological Process	0.007826126	7.75E-06
	GO:0005618	Cell Wall	Cellular Component	0.012975162	1.50E-05
	GO:0030312	External Encapsulating Structure	Cellular Component	0.013662973	1.80E-05
	GO:0016798	Hydrolase Activity, Acting on Glycosyl Bonds	Molecular Function	0.033058673	4.91E-05
Roots	GO:0031410	Cytoplasmic Vesicle	Cellular Component	0.026889605	1.30E-05
Re-watering	GO:0097708	Intracellular Vesicle	Cellular Component	0.026889605	1.30E-05
DOWN	GO:0031982	Vesicle	Cellular Component	0.026889605	1.33E-05

The analysis of the Top Orthologous Groups found show that in both, leaves and roots the "Function Unknown" category is the most represented (figures 14 and 15). The categories of "Signal Transduction Mechanisms", "Transcription" and "Energy Production and Conversion" have a higher representativeness after 4 days of stress, while "Posttranslational modification, protein turnover, chaperones" and "Translation, Ribosomal Structure and Biogenesis"

are more represented on re-watered plants. "Lipid Transport and Metabolism" category has a higher percentage of sequences on the re-watered and stressed for 4 days leaves samples (figure 14).

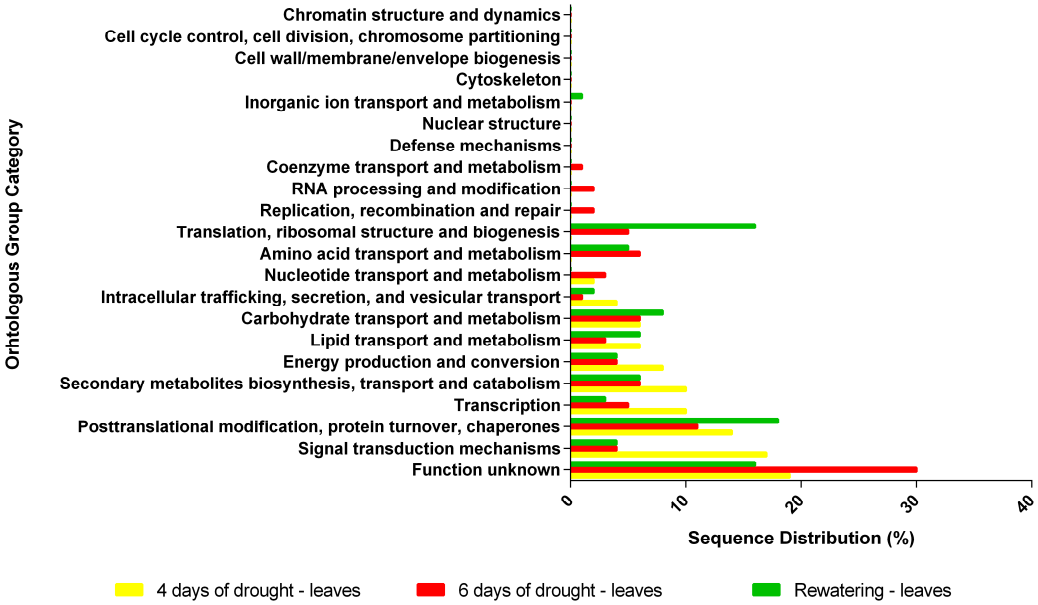


Figure 14. Top Ortholgous Groups found in leaves shows a predominance of "Signal Transduction Mechanisms" and "Transcription" after 4 days of stress while categories such as "Translation, Ribosomal Structure and Biogenesis" after re-watering which corroborates with the hypothesis that on moderate stress plants are detecting the condition and changing the expression of genes while after re-watering there is a change on the synthesis of proteins

In the case of roots, the categories of "Transcription", "Amino Acid Transport and Metabolism", "Intracellular Trafficking, Secretion, and Vesicular Transport", "Inorganic Ion Transport and Metabolism", "Translation, Ribosomal Structure and Biogenesis" and "Energy Production and Conversion" are observed with a higher representativeness after 4 days of stress. "Signal Transduction Mechanisms" are found with a higher percentage of sequences after 4 and 6 days of stress, "Carbohydrate Transport and Metabolism", "Cell Cycle Control, Cell Division, Chromosome Partitioning", "Nucleotide Transport and Metabolism" and "Cytoskeleton" after 6 days of stress and after re-watering, and "Chromatin Structure and Dynamics" exclusively found after condition 2 (6 days of stress) (figure 15).

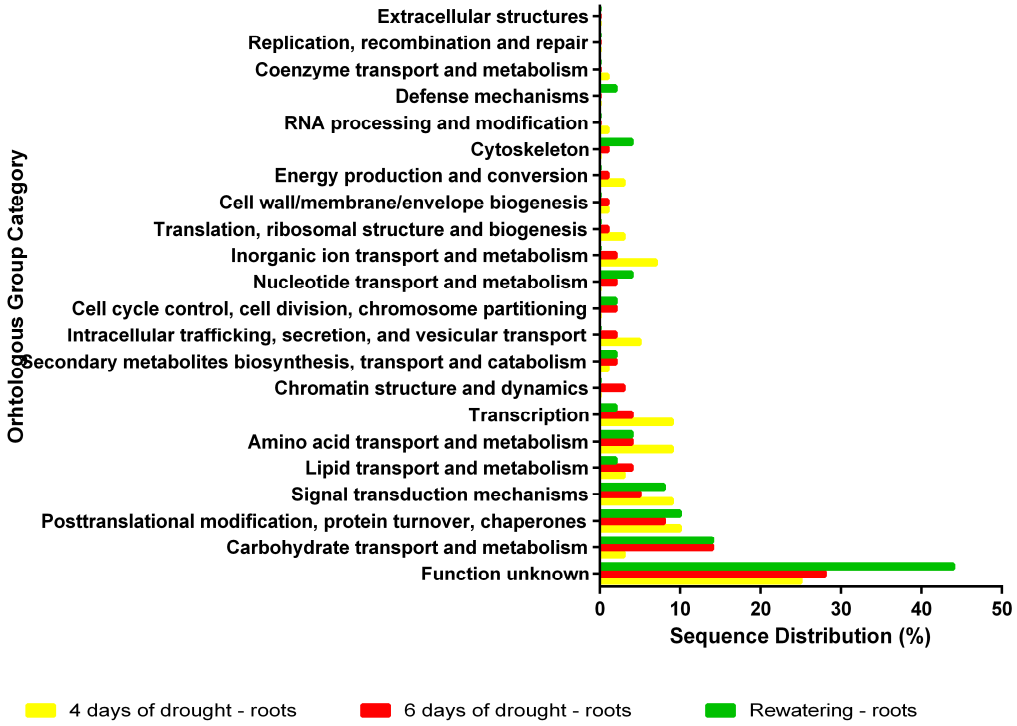


Figure 15. Top Orthologous Group showing "Transport" related functions are more frequent after 4 days of stress, maybe leading to osmoregulation due to ion transportation, for instance, in roots stressed samples. "Cell Cycle Control, Cell Division, Chromosome Partitioning" may be reflecting the limitation of root growth on stressed material

Using the differentially expressed genes detected through the HTSelf method we were able to generate, through Pathway Studio, biological networks for each tissue (roots and leaves) and each condition (Figure 16 and appendix D). The biological network generated using the differentially expressed genes from leaves after 4 days of stress showed a predominance of transcripts involved on the transcriptional control from both ABA-dependent and ABA-independent pathways (appendix D).

Most of the identified transcripts were associated with signal transduction and gene regulation. Among the transcripts that showed the higher number of connections, we found the lysine-specific histone demethylase 1-1, a transcript whose down-regulation will stop the inhibition of ABA and ROS related genes. This is also associated with chromatin remodeling. We can notice the crosstalk between stresses and the presence of the biological process of oxidative stress, stomatal conductance, response to dehydration and cell death (appendix D).

Furthermore, after 6 days of stress (figure 16), there is a down-regulation of transcripts related to photosynthesis and carbon fixation and the up-regulation of genes involved with lignin and ABA biosynthesis, sugar and lipid metabolism. Several processes, such as leaf senescence, heat shock response, drought tolerance, ROS generation, leaf chlorosis, cell death, response to osmotic stresses, among others were up-regulated. After re-watering, we can still see some genes involved in ABA-responses, but photosynthesis related genes are no longer down-regulated, and despite of the residual drought response mechanisms, the cells are going back to their normal state, even stomatal conductance is going back to normal values (appendix D).

On roots, after 4 days of stress we can see the presence of transcripts related to signal transduction and gene regulation, such as a couple of transcription factors involved in ABA-dependent and ABA-independent pathways, as well as transcripts involved in sugar metabolism and amino acid metabolism. There is also the downregulation of nitrogen transporters and the inhibition of cell division. Furthermore, we can observe that genes expressed in roots

may be involved with the response of the aerial part of the plant, such as stomatal movements. Processes like senescence, cell death and the crosstalk between stresses are observed (appendix D).

After 6 days of stress, the response in roots involve a wide variety of processes, such as down-regulation of chromatin remodeling factors, the inhibition of cell expansion, cell cycle and roots growth and development, and the down-regulation of aquaporins. An up-regulation of transcripts involved in sugar metabolism, lignin biosynthesis, response to osmotic stress, turgor, heat tolerance, ROS generation, among others was found (appendix D).

After re-watering, the transcripts involved in processes such as cell division and elongation, cell development and differentiation, aquaporins are still down-regulated, and transcripts involved in processes related to drought tolerance, water loss, defense response, cuticle development are still up-regulated, showing that despite re-watering, the root tissue is not yet back to homeostasis (appendix D).

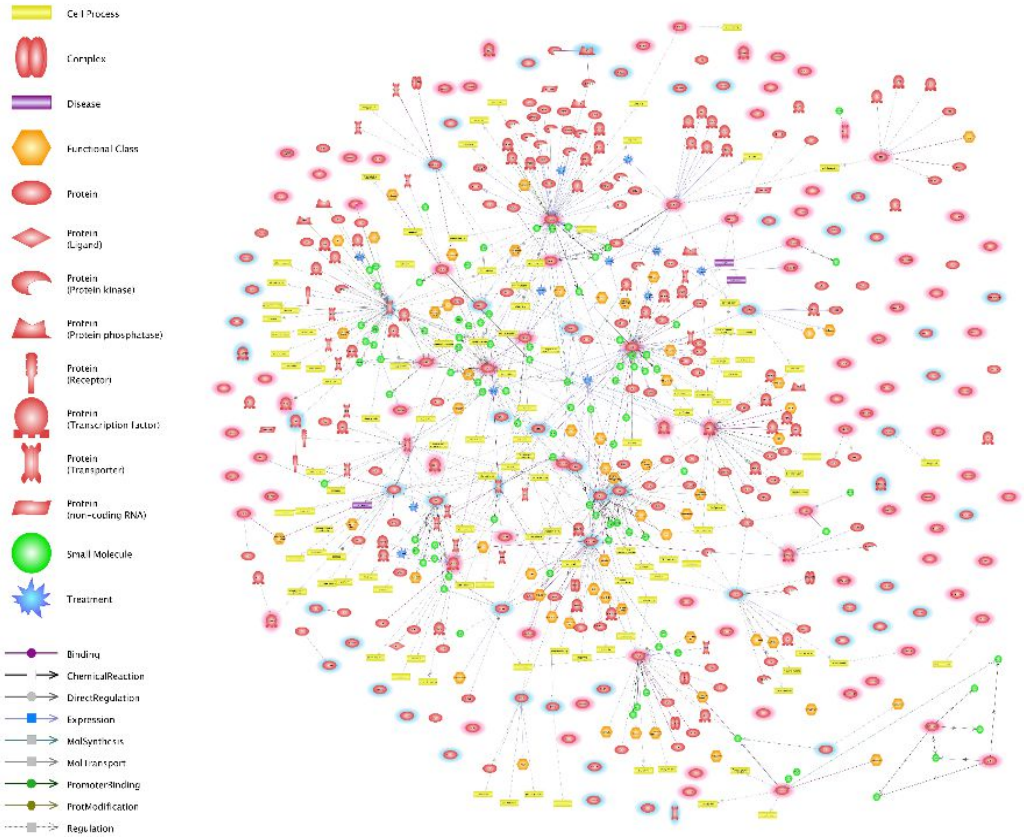


Figure 16. The Biological Network from leaves stressed for 6 days (generated by Pathway Studio (Elselvier) highlights the down-regulation of Photosynthesis and Carbon Fixation in parallel to the up-regulation of Leaf Senescence and Chlorosis, Heat Shock Response and Cell Death. Legend in a bigger size in appendix D

The analysis using Pathview of the differentially expressed genes with FC ≥ 2 and FC ≤ 0.5 (figures 17 and 18) allowed the expression of the differentially expressed genes to be plotted on KEGG pathways, and some interesting patterns could be distinguished. Among these, one that catches our attention relates to the galactose metabolism, which in roots presents up-regulation of several genes from the pathway since the first data point (4 days without water), while in leaves the up-regulation of most of the genes from the pathway found in our study starts only after 6 days of stress (figure 17). The other pathway that can be highlighted is the phenylpropanoid biosynthesis pathway that appears to be up-regulated in leaves and down-regulated in roots (figure 18).

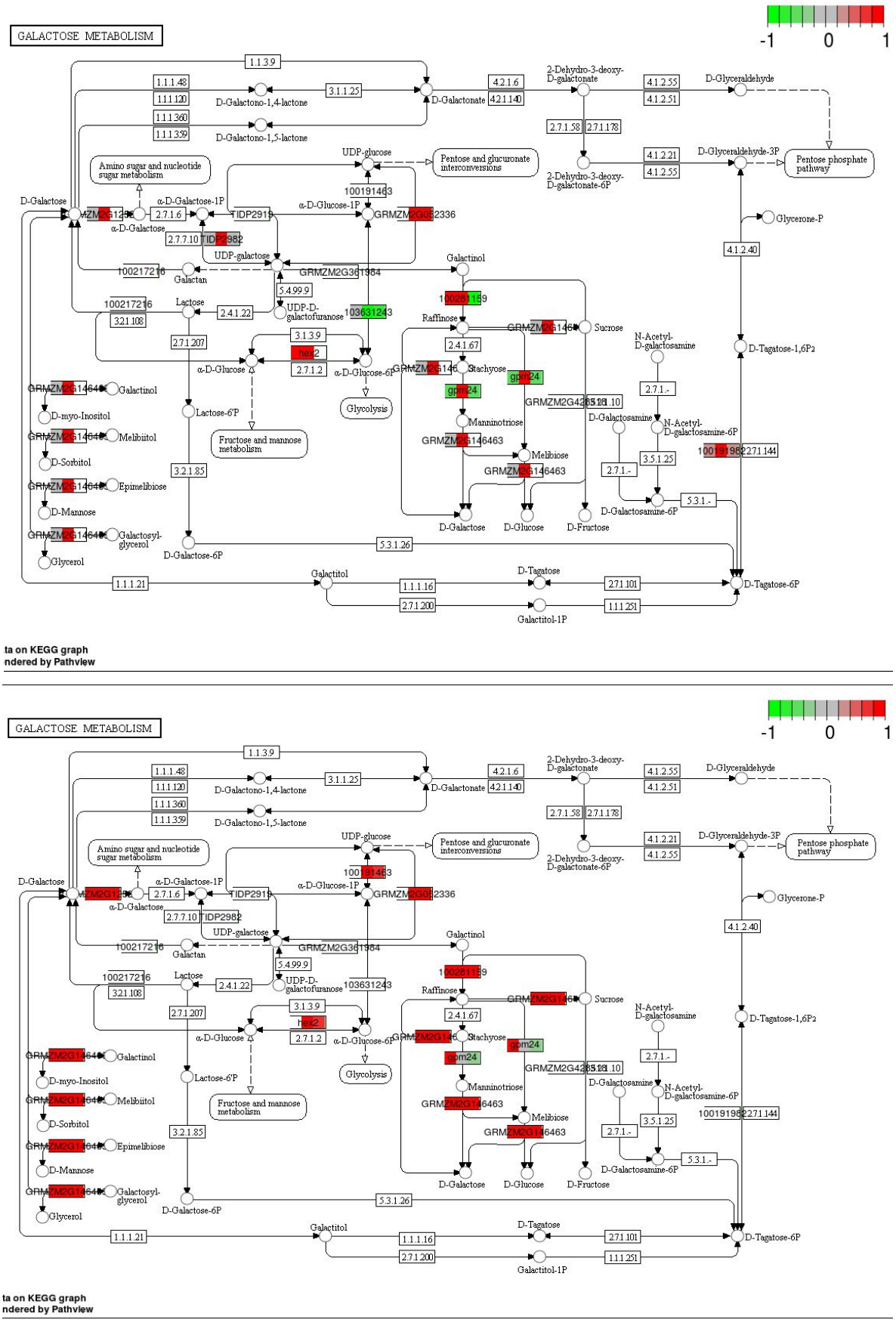


Figure 17. Pathview analysis showing Galactose Metabolism pathway from KEGG highlights the up-regulation of enzymes involved in the synthesis of RFOs sugars during the three time points on roots.

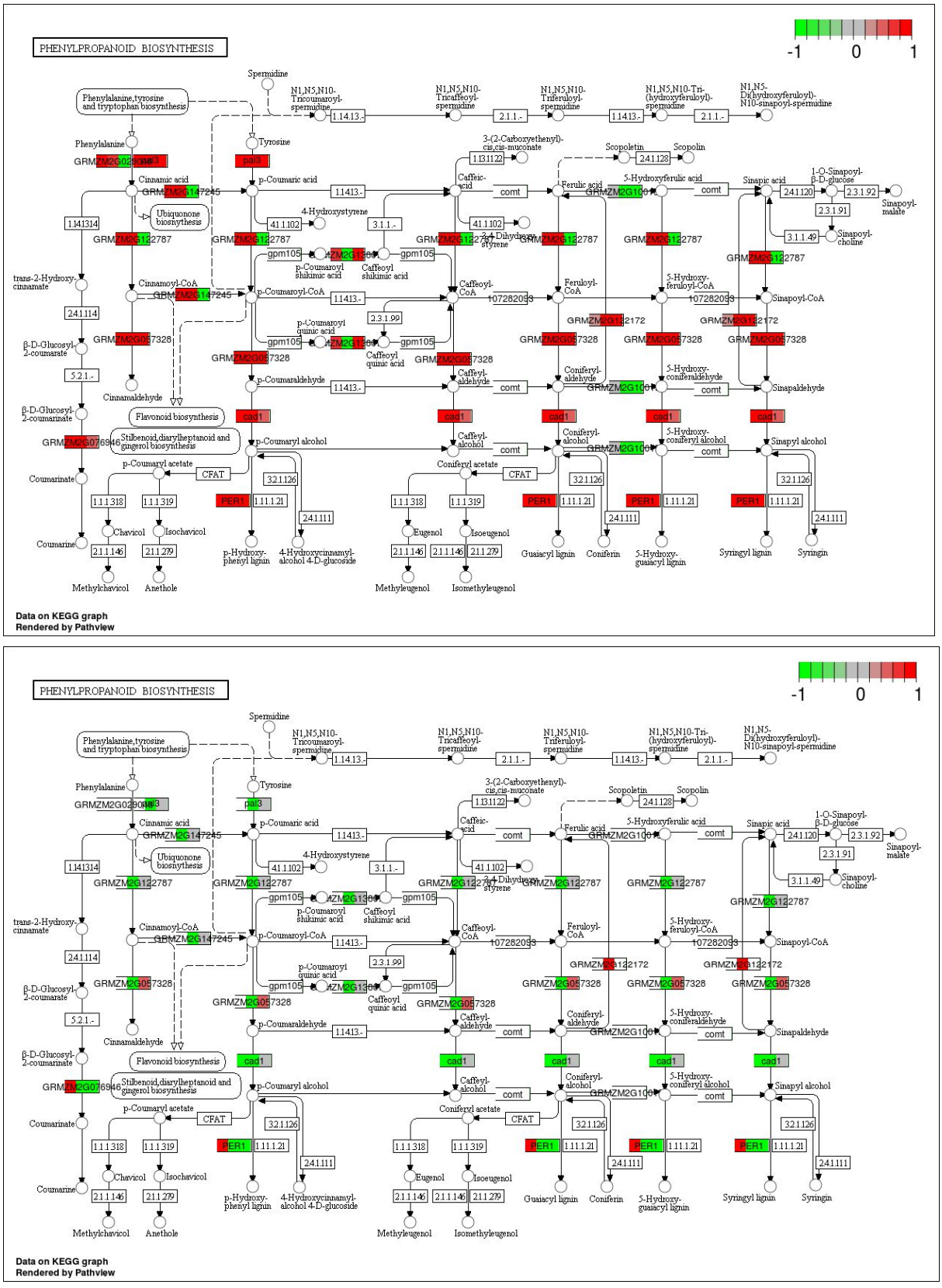


Figure 18. Pathview analysis showing Phenylpropanoid Biosynthesis pathway on leaves samples as opposed to its down-regulation or lack in expression difference on roots.

3.4.3. RT-qPCR Data

For RT-qPCR validation of microarray data a total of 46 data points were used. The percentage of validation using the ratio of expression from REST 2009 Software for positives and false positives was 86.95% (figures 19 and 20). To calculate the relative expression we used the method described by Vandesompele et al. (2002), the percentage of validation was 89.13% (appendix B). The SAS not validated were SCEQLB1063D10.g and SCCCCL4005E12.g (sense transcript) for leaves; and SCCCLB1023H08.g and SCQGST1032H03.g (anti-sense transcript) for roots (figures 19 and 20, and appendix B). All the remaining 43 differential gene expression profile confirmed data obtained using the microarray procedures. The expression profiles using REST 2009 software are represented on figures 19 and 20.

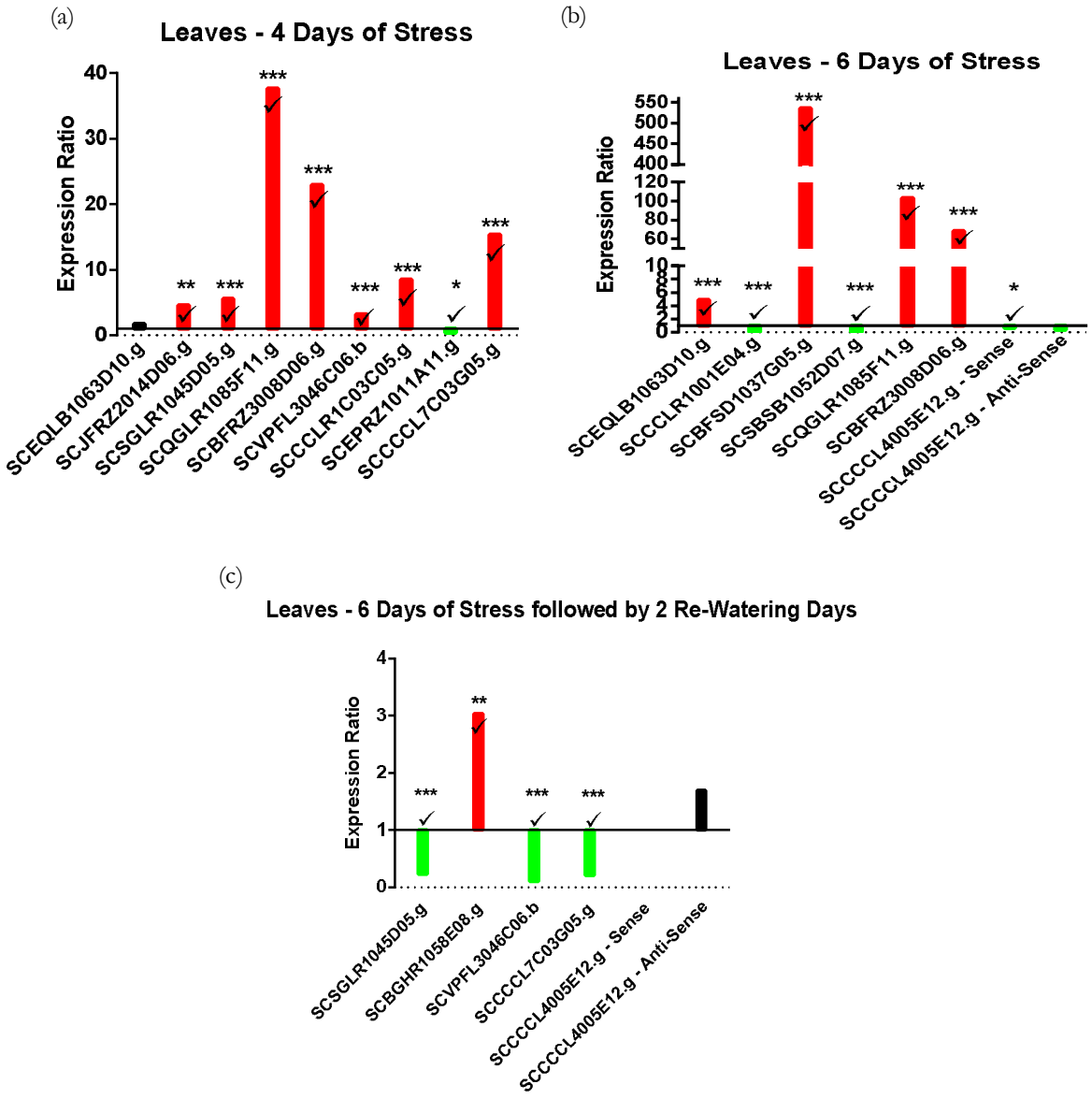


Figure 19. Microarray qPCR validation of leaves samples showing that most of the analyzed genes were validated. (a) 4 days without water, (b) 6 days without water and (c) 6 days without water followed by 2 days of re-watering. $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$

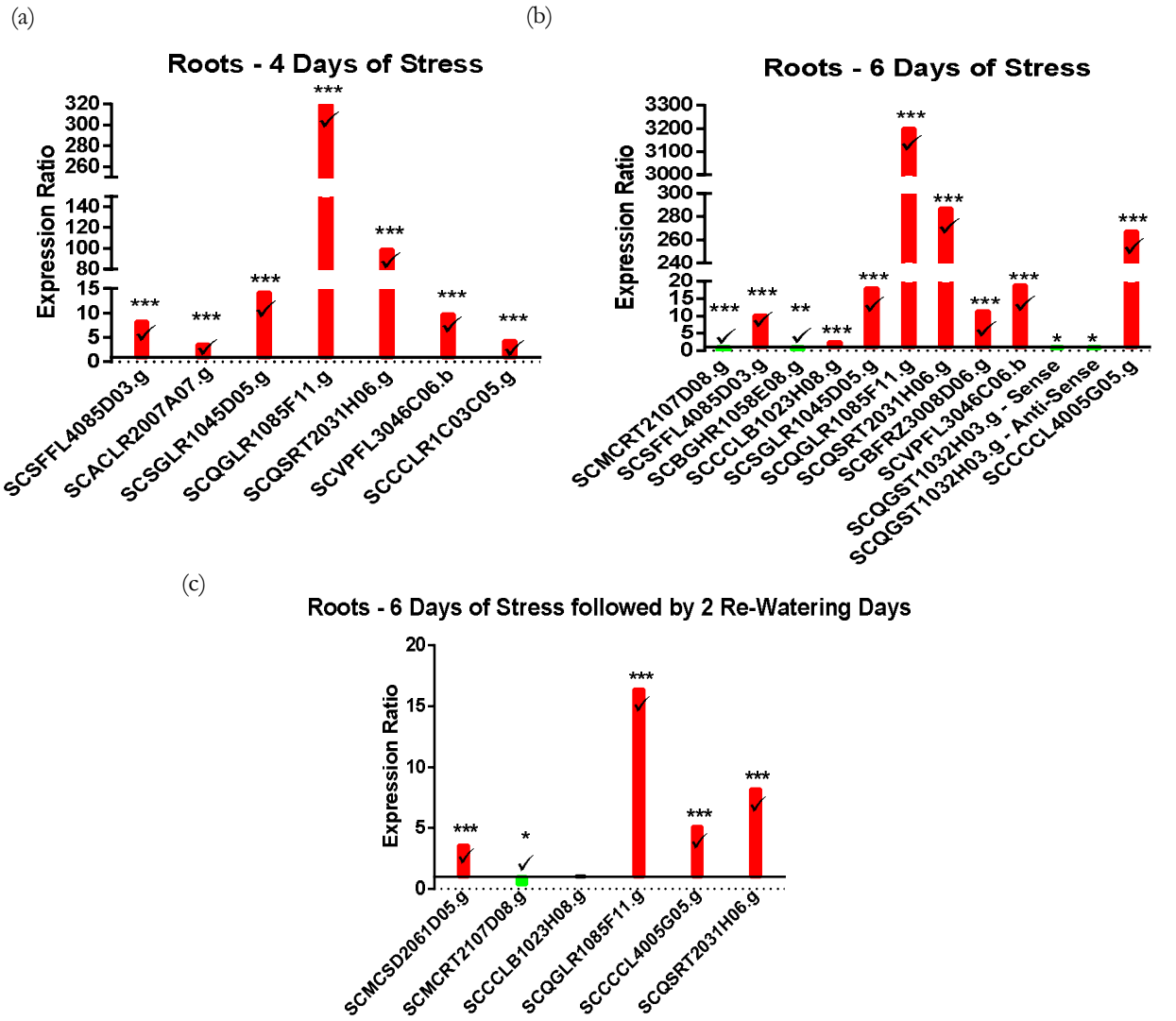


Figure 20. Microarray qPCR validation of roots samples also showing that most of the analyzed genes were validated. (a) 4 days without water, (b) 6 days without water and (c) 6 days without water followed by 2 days of re-watering. $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$

3.4.4. Expressed pathways analysis based on the Activity Score

The level of correlation between samples allows the clustering of leaves and roots in separate groups (figures 5 and 21), which indicates that these two tissues show different activity scores in their expression profiles. The pathway correlation in leaves and roots shows that some of the pathways activated/deactivated in condition 1 (C1 – 4 days without irrigation) appears to continue with the same pattern in condition 2 (C2 – 6 days without irrigation), when other pathways become activated/deactivated in condition 3 (C3 – re-watering) (figure 21).

The Brassinosteroid Biosynthesis Pathway was up-regulated after 4 days of drought and rehydration period and down-regulated after 6 days of drought in leaves. In roots, this pathway was down-regulated in all conditions. Valine, leucine and isoleucine degradation pathways were down-regulated after 4 days of drought and then became up-regulated in the 6th day of drought. Both ether lipid and glycerophospholipid metabolism were down-regulated after

4 days of drought in leaves and roots; down-regulated after rehydration period in roots and up-regulated after 6 days of drought in leaves. Fatty acid biosynthesis was down-regulated in roots and up-regulated in leaves after 6 days of drought and after rehydration period. Diterpenoid biosynthesis was down-regulated after 4 days of drought and after the rehydration in roots and up-regulated after 6 days of drought and after rehydration in leaves and after 6 days of drought in roots (figure 21).

Lipoic acid metabolism pathway was up-regulated in leaves and down-regulated in roots. Arginine and proline metabolism were up-regulated in leaves after 6 days of drought and after rehydration, being the pathway expression higher after the 6th day without water. The same pattern was observed in roots. Interestingly, synthesis and degradation of ketone bodies was up-regulated in leaves after 6 days of drought and after rehydration and down-regulated in leaves after 4 days of drought and in roots after 4 and 6 days of drought (figure 21).

Inositol phosphate metabolism and phosphatidylinositol signaling system pathways were up-regulated after 6 days of drought and after rehydration in leaves, and after 4 days of drought in roots. The metabolism of several amino acids, such as cysteine, methionine, glycine, serine and threonine also showed the same pattern. Base excision repair, nucleotide excision repair and mismatch repair pathways were up-regulated after 6 days of drought in roots. Non-homologous end-joining was up-regulated after 6 days of drought in leaves. Homologous recombination was down-regulated after 4 days of drought and up-regulated after 6 days of drought in roots (figure 21).

Sesquiterpenoid and triterpenoid biosynthesis was up-regulated in leaves after 6 days of drought and down-regulated in all the other conditions, but 4 days after drought in roots. Folate biosynthesis was up-regulated in roots after 6 days of drought. Sphingolipid metabolism was up-regulated after 6 days of drought. In leaves glycerolipid metabolism was down-regulated after 4 days of drought, but their expression increased after 6 days of drought and after rehydration period. Glycosphingolipid biosynthesis (ganglio and globo series) and glycosaminoglycan degradation pathways were up-regulated after 6 days of drought and after rehydration period (figure 21).

Limonene and pinene degradation and caffeine metabolism were up-regulated in roots after 4 and 6 days of water deprivation, and down-regulated in leaves after 4 days of drought and after rehydration (figure 21).

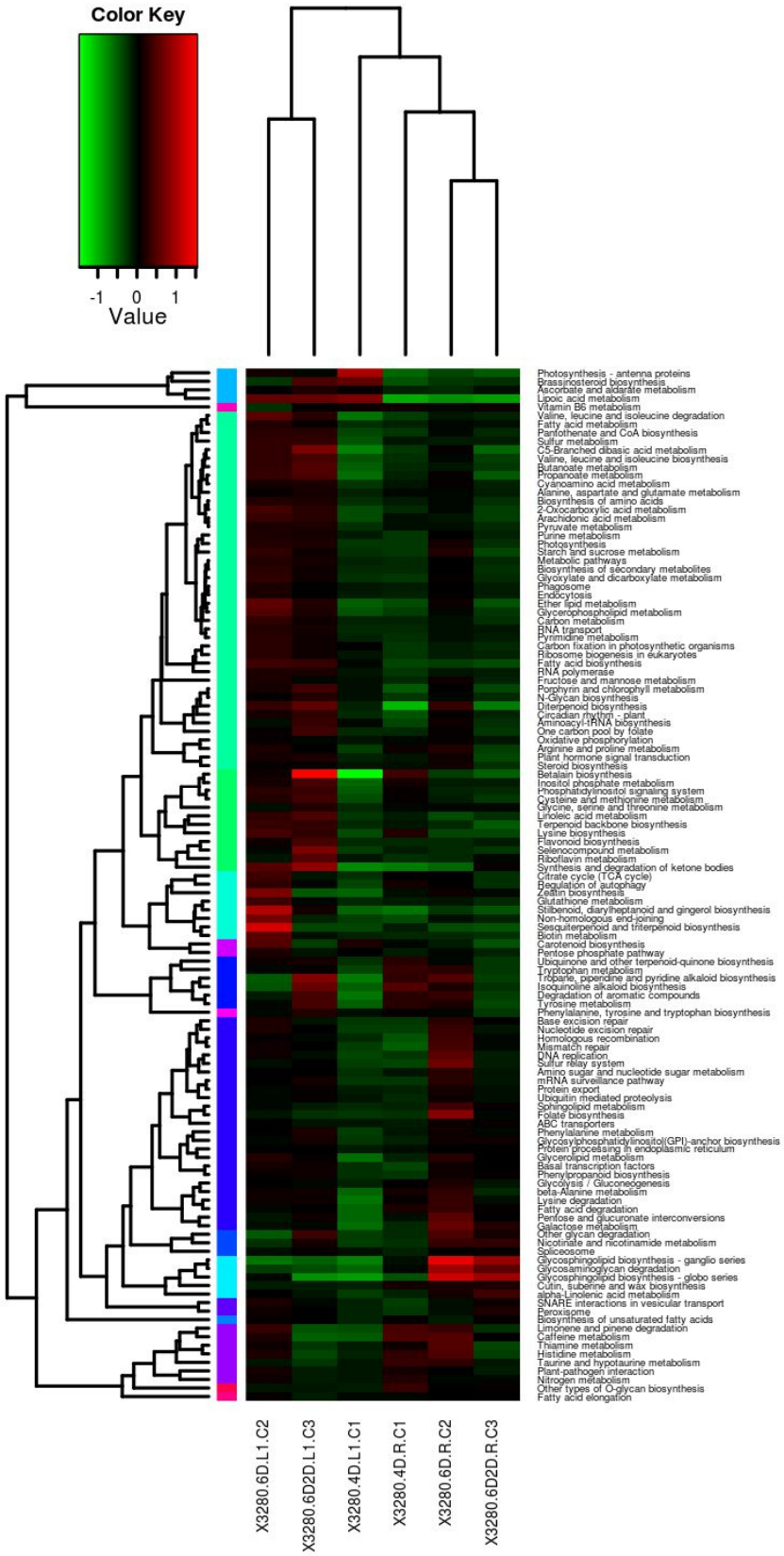


Figure 21. Activity level of expressed pathways shows Brassinosteroid Biosynthesis higher expression after 4 days of drought and rehydration period in leaves. In roots, pathways such as Base excision repair, nucleotide excision repair and mismatch repair had a higher expression on severe stress. L=leaves; R=roots; C1=4 days of drought, C2=6 days of drought, C3= 6 days of drought followed by 2 days of rehydration

3.4.5. RNA-Seq Data Analysis

The RNA-Seq data analysis was done using the same samples from the most contrasting condition identified by microarray data. This analysis aimed to complement the information obtained from microarray as well as to provide a data set of transcripts to be used in the future for characterization of promoters from genes involved in drought responses, transcription factors and other regulators having as a base the new assembly of sugarcane genome (unpublished data).

The analysis allowed the identification of 437,214 total Trinity genes and 623,447 total Trinity transcripts, with %GC 49.04. The average contig N10 is 3,116 nt and the contig N50 is 905 nt. The smallest contig found had 224 nt, while the longest contig had 17,329 nt. Library size had an average value of 63,368,676.5 read counts, with the smallest size being 60,946,485, and the highest being 65,388,462. The correlation matrix between all the biological replicates showed a high correlation between the biological replicates and also between the plant tissues (Figure 22).

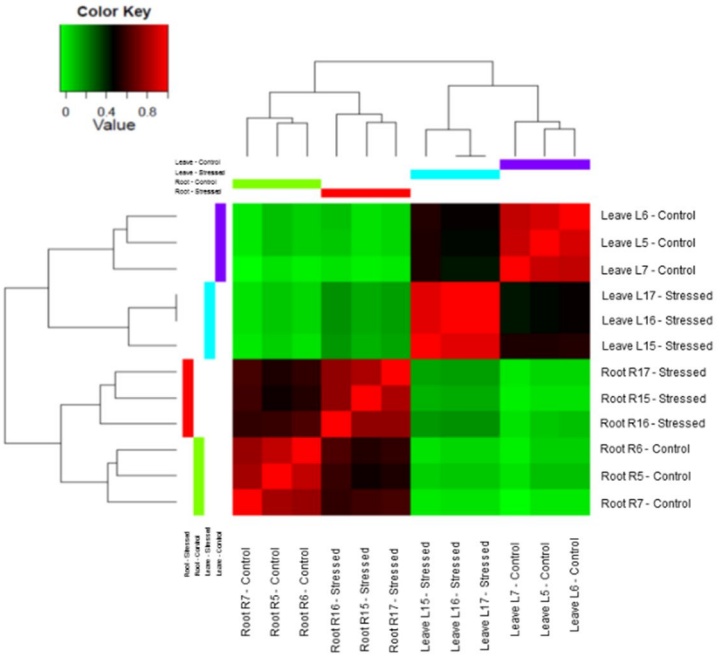


Figure 22. Correlation matrix between the biological replicates demonstrates a high correlation between roots and leaves, stressed and control samples

Using Blast2GO tool for pairwise differential expression analysis and an FDR < 0.05, a CPM value of 0.5 and the TMM (Trimmed mean of M values) for normalization, it was observed 28,240 differentially expressed (DE) features for leaves and 7,435 DE features for roots. From these, in leaves 13,928 had logFC > 2.0 and, therefore, were up-regulated, while 14,312 had logFC < -2.0 and were considered down-regulated. For roots, those numbers were respectively 3,827 and 3,608 DE features. MA plot, which demonstrates the LogFC vs average of Log CPM (counts per million) for each gene, shows the statistically significant genes (FDR<0.05) in red (figures 23 and 24). The Volcano plot, that presents the negative Log FDR vs Log FC for each gene, displays up-regulated genes in green and down-regulated genes in red (figures 25 and 26). Both analysis demonstrate a higher number of DEGs in leaves than in roots.

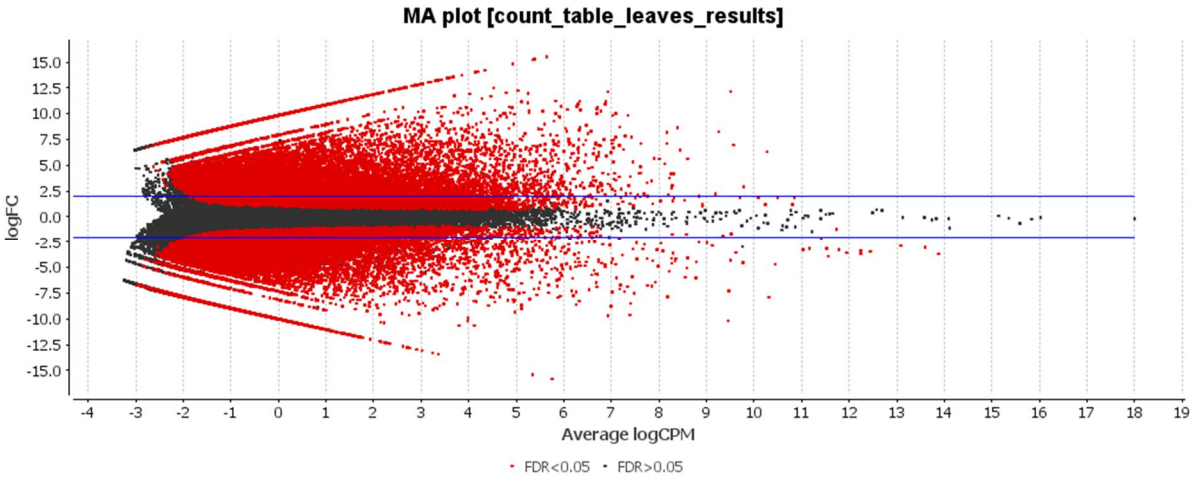


Figure 23. MA plot shows the detection of a high number of statistically significant genes in the *de novo* assembly of the RNA-Seq from leaves samples water stressed for 6 days.

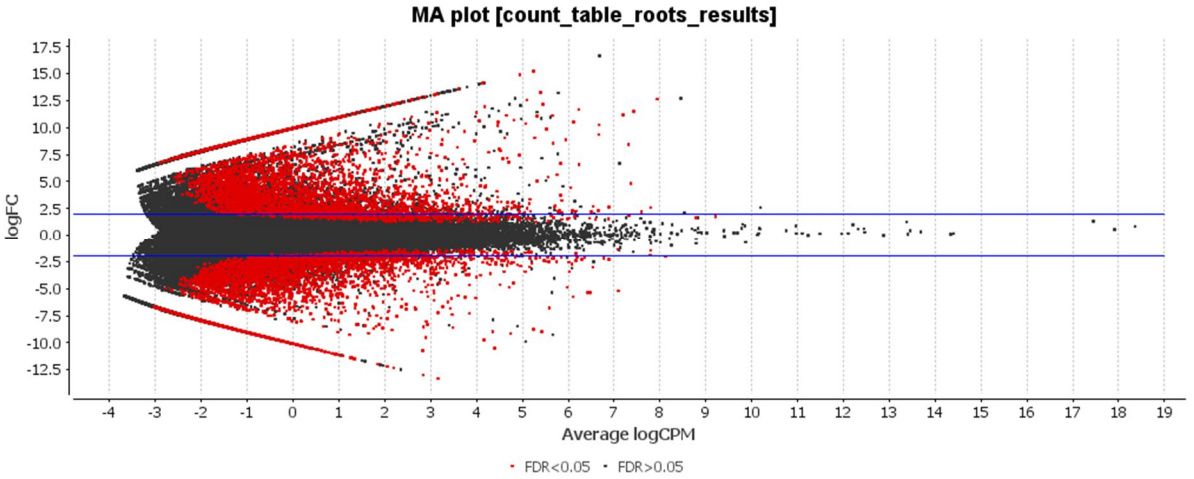


Figure 24. MA plot shows the detection of a interesting number of statistically significant genes in the *de novo* assembly of the RNA-Seq from roots samples water stressed for 6 days.

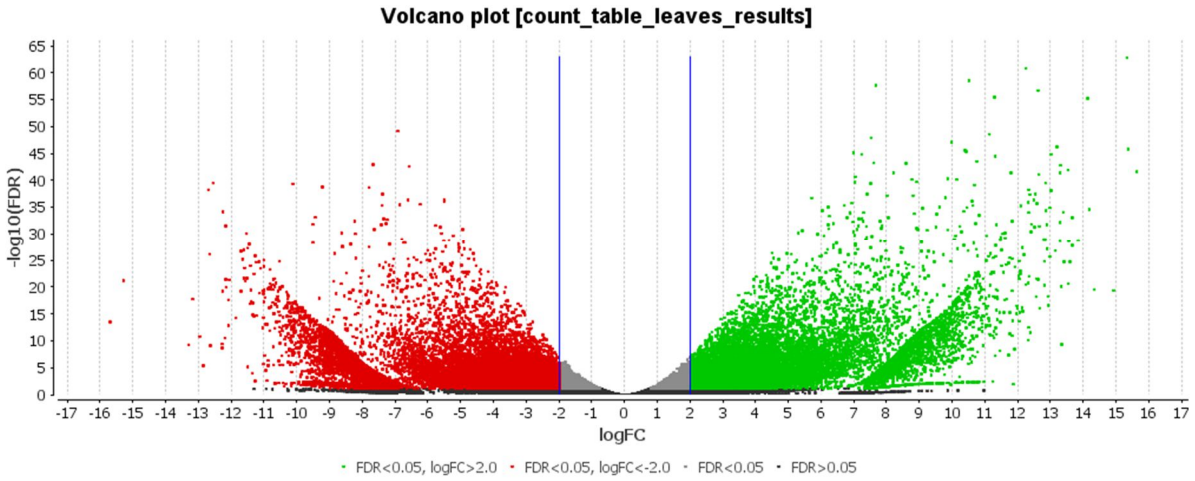


Figure 25. Volcano plot showing a high number of up and down-regulated genes detected in the *de novo* assembly of the RNA-Seq from leaves samples water stressed for 6 days.

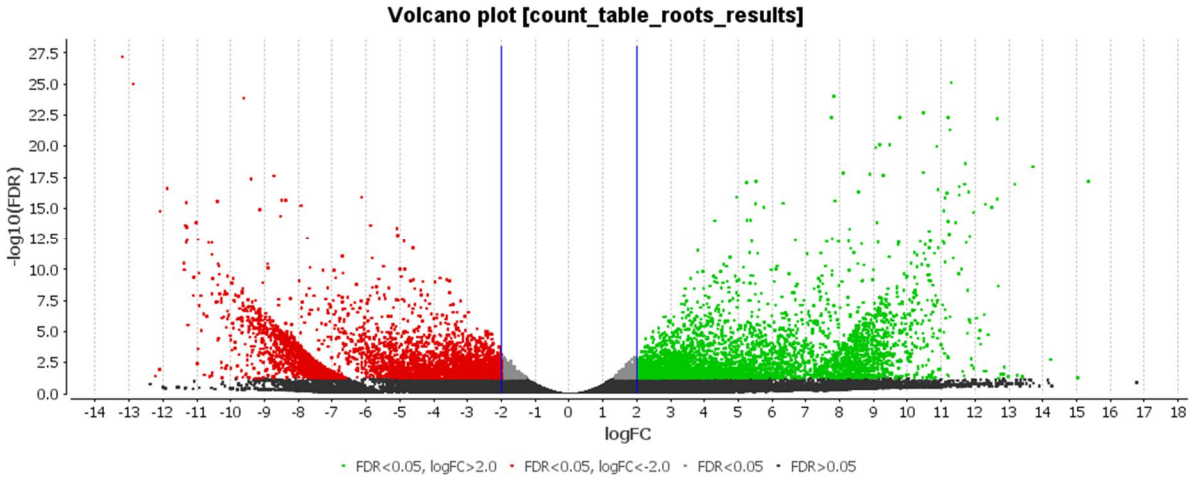


Figure 26. Volcano plot showing the number of up and down-regulated genes detected in the *de novo* assembly of the RNA-Seq from root samples water stressed for 6 days.

The results observed in figures 27 and 28 demonstrates a similar number of up and down regulated genes in both leaves and roots. Despite that, there is a slightly higher number of down-regulated genes in leaves and up-regulated genes in roots.

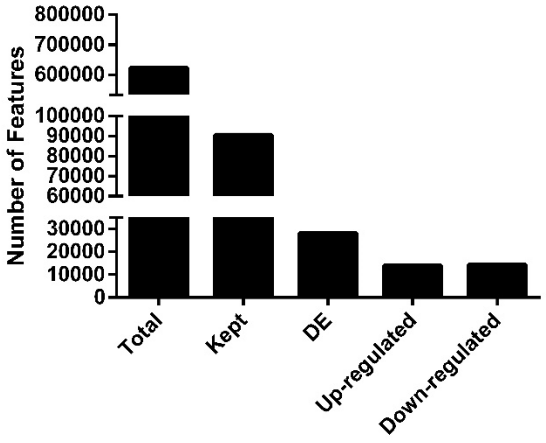


Figure 27. Graph shows that among more than 600,000 assembled transcripts, 28,240 were considered differentially expressed features in leaves, considering *de novo* RNA-Seq assembly and a FDR value of 0.05

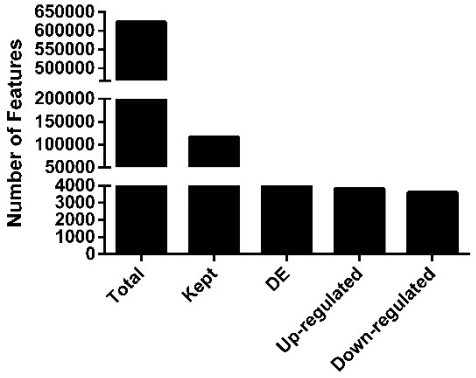


Figure 28. Graph also showing that among more than 600,000 assembled transcripts, 7,435 were considered differentially expressed features in roots, considering *de novo* RNA-Seq assembly and a FDR value of 0.05

3.4.6. Functional Classification – RNA-Seq *de novo* assembly

The differentially expressed transcripts were annotated using Blast2GO software (https://www.blast2go.com/). Differentially expressed transcripts were characterized using both the gene ontology analysis and the orthologous group annotation.

In the case of leaves, several classes such as Biological Regulation, Transport, Cellular Protein Modification Process were represented in both induced and repressed transcripts, while Response to Stimulus, Cellular Nitrogen Compound Metabolic Process, Catabolic Processes and Carbohydrate Metabolic Processes were induced and Cellular Component Organization or Biogenesis was repressed (figure 29).

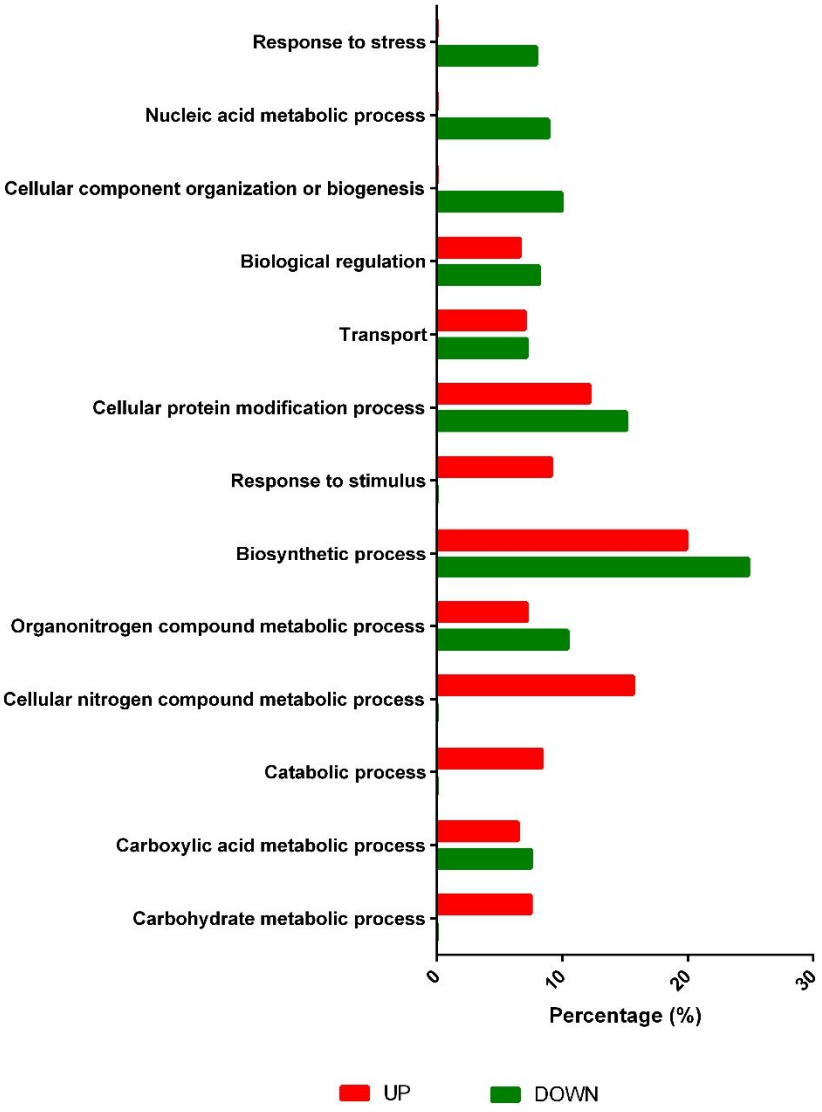


Figure 29. Gene Ontology classification of Biological Process in leaves demonstrates the repression after 6 days of stress of terms related to biogenesis of cellular components and up-regulation of terms involved in osmoprotection and response to stimulus

Interestingly in the case of roots, sequence distribution show only four terms represented in both up and down-regulated transcripts (figure 30). Those terms were Cellular Protein Modification Process, Transport, Oxidation-Reduction Process and Carbohydrate Metabolic Process and in all the cases more up-regulated sequences were found. Among the up-regulated classes were Transcription - DNA-templated, Response to Stress, Regulation of Primary Metabolic Process and Regulation of Gene Expression (figure 30). Cell Wall Organization or Biogenesis, Phosphorylation and Biosynthetic Process related to Cellular Macromolecule, Organic Cyclic Compound, Cellular Nitrogen Compound, Aromatic Compound and Single-Organism were among the classes found exclusively in the down-regulated DE features (figure 30).

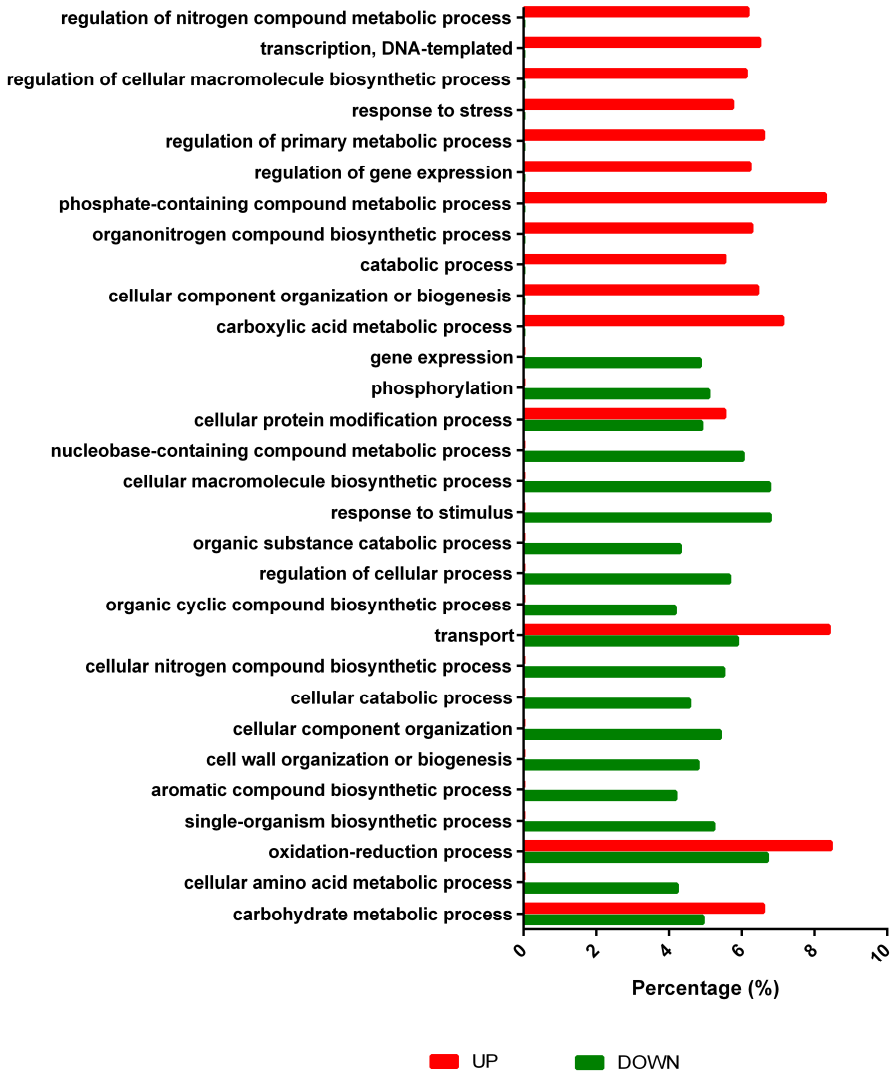


Figure 30. Biological Processes gene ontology terms shows "Cell Wall Organization or Biogenesis" exclusively on down-regulated DE features, corroborating with the repression of root growth due to stress while "Transcription - DNA-templated", "Response to Stress", "Regulation of Gene Expression" were found on induced DE features demonstrating the importance of gene expression control even during severe stress

The Cellular Component term from leaves RNA-Seq show a higher representation of Nucleus and Mitochondrion on the up-regulated transcripts and an opposed pattern on Plastid term (figure 31).

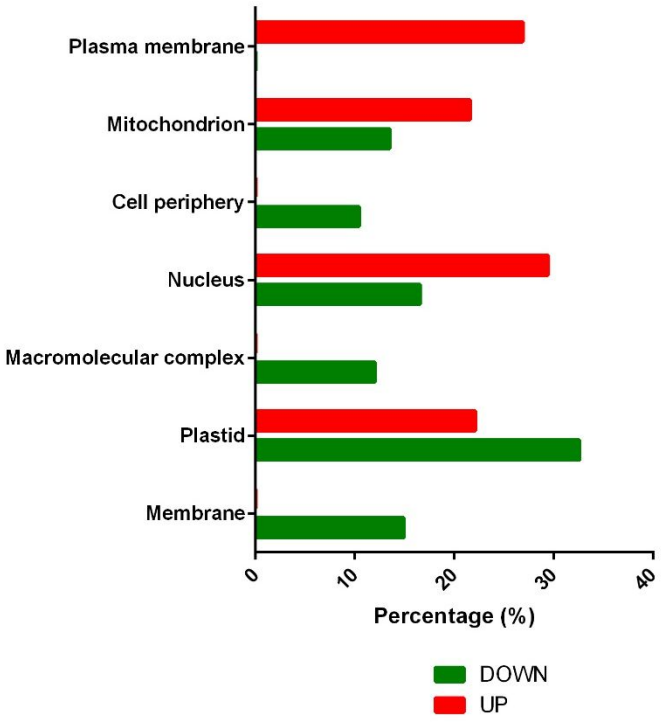


Figure 31. In leaves, the analysis of gene ontology terms related to Cellular Component corroborates with the results from microarray analysis and also shows more features related to "Plastid" on the repressed transcripts

The cellular component gene ontology terms also reveal classes that were exclusively found in the up or in the down-regulated root transcripts (figure 32). Among those, are Cell Periphery and Protein Complex in the up-regulated and Integral Component of Membrane in the down-regulated (figure 32). The compartments of Intracellular Organelle Part, Nucleus and Cytoplasmic Part are represented in up- and down-regulated transcripts, but similar to the Biological Process terms, more up-regulated sequences associated to those organelles were found (figure 32).

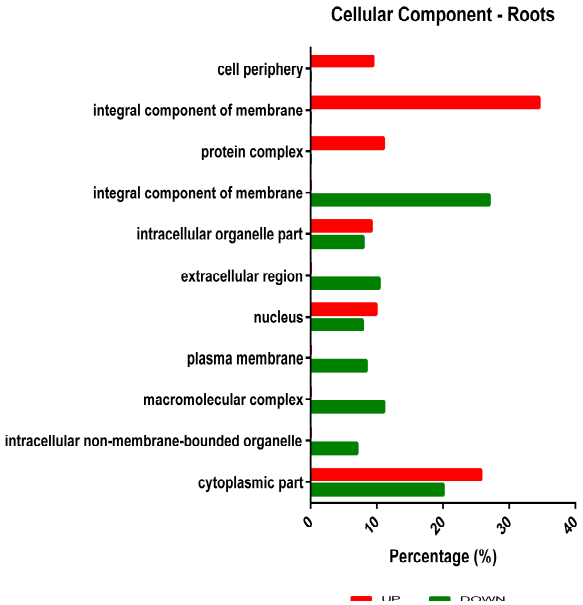


Figure 32. Cellular Component analysis in roots shows that the term "Integral Component of Membrane" is exclusive for the down-regulated features and may represent down-regulation of aquaporins, which would corroborate with microarray results

In leaves, all the terms from Molecular Function were represented in a similar way (figure 33).

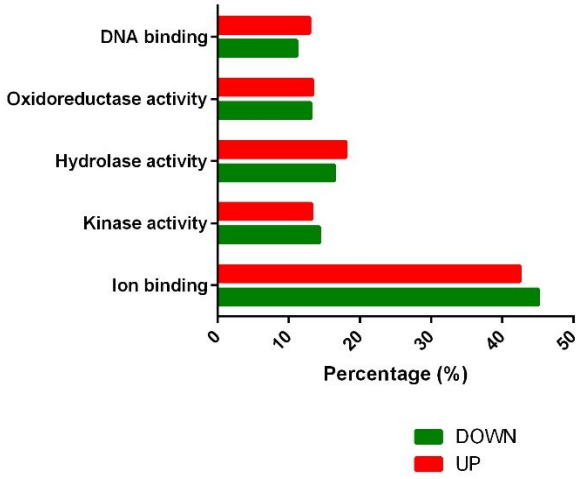


Figure 33. The Molecular Function gene ontology terms analysis showed that all terms found were represented in both, induced and repressed differentially expressed features

In the case of Molecular Function, the term associated with DNA binding was found in the sequences from up-regulated features, while the Kinase Activity in the down-regulated ones, which corroborates with the fact that the Phosphorylation Biological Process was found only in the down-regulated features (figure 34).

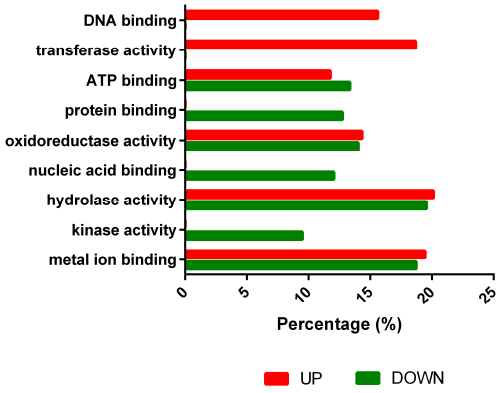


Figure 34. The analysis of Molecular Function GO terms shows "DNA binding" only on induced features, while kinase activity is on down-regulated features

The Orthologous Group annotation from leaves reveals that most of the transcripts belonged to the "Function Unknown" orthologous group, but the "Signal Transduction Mechanisms", "Posttranslational modification, protein turnover, chaperones", "Transcription" and "Carbohydrate Transport and Metabolism" were among the most represented (figure 35). In the case of up-regulated features, the most represented categories after the "Function Unknown" were in order: "Signal Transduction Mechanisms", "Posttranslational modification, protein turnover, chaperones", "Carbohydrate Transport and Metabolism" and "Transcription" (appendix F); while for down-regulated they were (also in order): "Signal Transduction Mechanisms", "Posttranslational modification, protein turnover, chaperones", "Translation, ribosomal structure and biogenesis" and "Transcription" (appendix F).

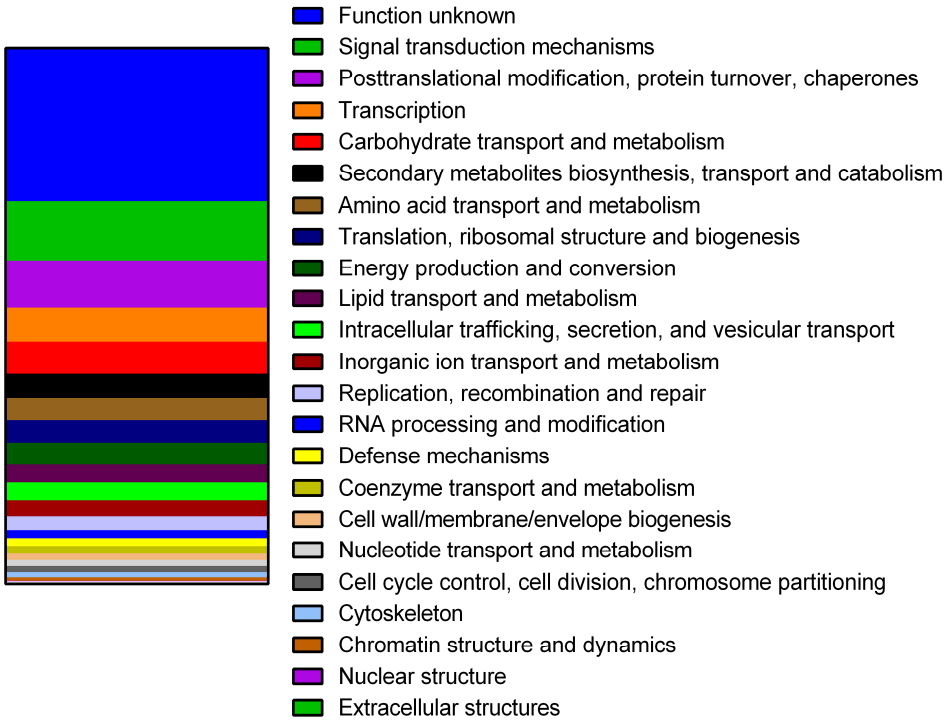


Figure 35. Top Orthologous Group analysis shows the importance of "Signal Transduction Mechanisms" in leaves responses to severe stress

The Orthologous Group annotation from roots also reveals that most of the transcripts belonged to the "Function Unknown" orthologous group, but the "Carbohydrate Transport and Metabolism", "Signal Transduction Mechanisms", "Posttranslational modification, protein turnover, chaperones" and "Transcription" were among the most represented (figure 36). In the case of up-regulated features, the most represented categories after the "Function Unknown" were in order: "Posttranslational modification, protein turnover, chaperones", "Signal Transduction Mechanisms", "Transcription" and "Carbohydrate Transport and Metabolism" (appendix F); while for down-regulated they were (also in order): "Carbohydrate Transport and Metabolism", "Signal Transduction Mechanisms", "Posttranslational modification, protein turnover, chaperones" and "Secondary Metabolites Biosynthesis, Transport and Catabolism".

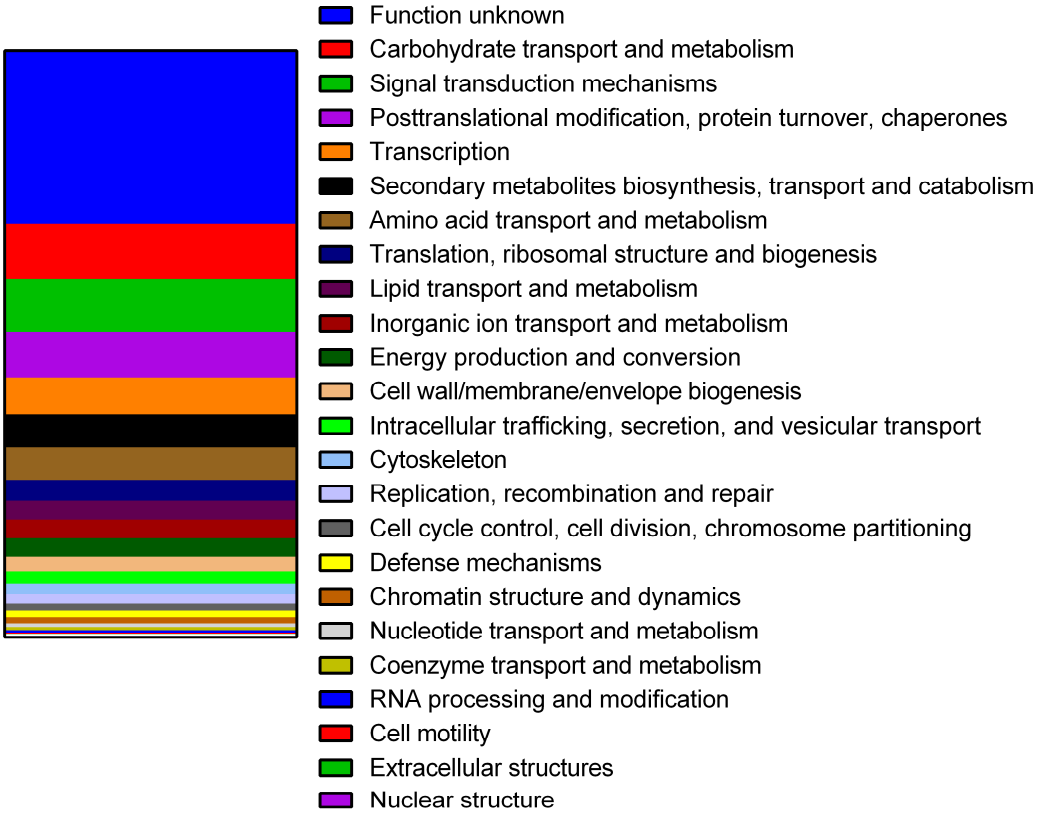


Figure 36. Top Orthologous Group analysis highlight the role of "Carbohydrate Transport and Metabolism" in roots responses to severe stress corroborating with previous microarray results

3.4.7. Analysis of miRNAs precursor sequences found in the *de novo* RNA-Seq assembly

The analysis of features identified by the *de novo* RNA-Seq assembly using RFAM tool from Blast2GO showed that in leaves, 0.6% sequences had RFAM hits, while in roots the number was 3.86%. From those, most of the sequences presented similarity with the ones from the database that belonged to the "Gene; rRNA" in both leaves and roots (figures 37 and 38).

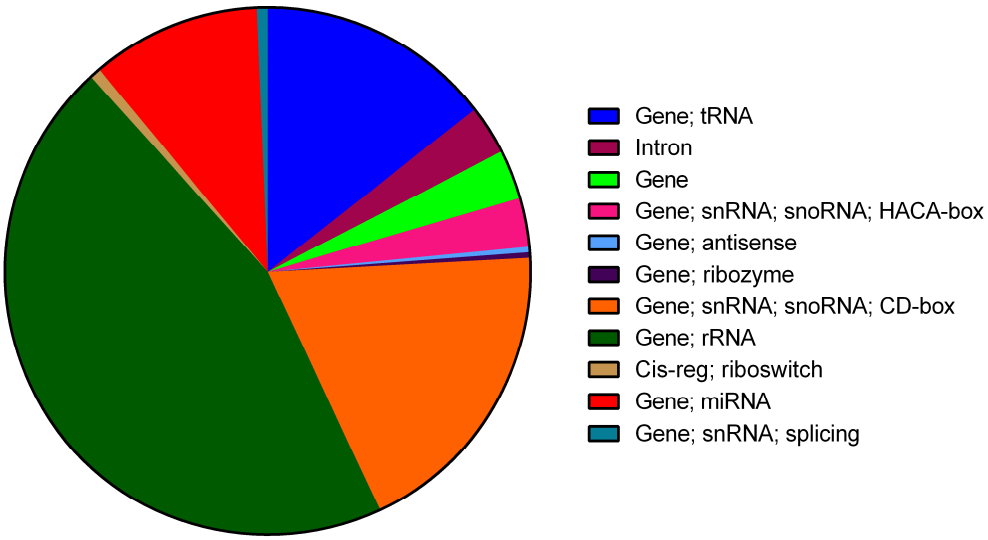


Figure 37. RFAM biotype distribution obtained from leaves sequences shows the identification of features with homology to miRNAs precursors

Furthermore, in the case of leaves, several sequences were found to belong to the classes of "Gene; snRNA; snoRNA; CD-box", "Gene; tRNA" and "Gene; miRNA" (figure 37). For the roots, the classes of "Gene" and "Gene; ribozyme" were very represented, following the "Gene; rRNA" (figure 38). The RFAM class of "Gene; miRNA" was also found.

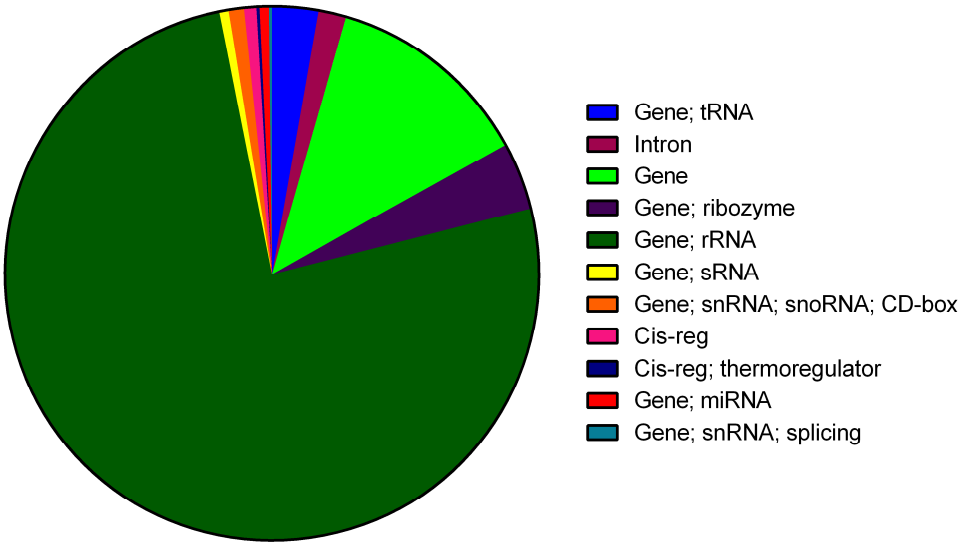


Figure 38. In roots, the amount of sequences with homology to miRNAs was smaller but sill detected

The analysis of the micro-RNAs precursors found reveals the identification of only two down-regulated in roots (table 7) and twenty-five sequences in leaves, from these eight were up-regulated and seventeen were down-regulated (table 6).

Table 6. Micro-RNAs precursors differentially expressed identified on leaves RNA-Seq samples

Sequence	Score	e-Value	Family ACC	Family ID	LogFC	PValue	FDR	UP/DOWN
TR116626|c0_g1_i1	65.4	3.4E-16	RF00445	mir-399	7.212429	1.7E-49	9.07E-46	UP
TR131470|c1_g1_i11	68.1	1.2E-15	RF00677	mir-168	-9.50902	0.000822	0.003351	DOWN
TR80934|c0_g1_i2	47.4	1.4E-7	RF02000	mir-1846	3.795358	2.11E-11	4.82E-10	UP
TR131470|c1_g1_i5	68.1	1.2E-15	RF00677	mir-168	-10.3229	1.82E-19	1.69E-17	DOWN
TR85389|c1_g1_i1	66.4	1.3E-14	RF00247	mir-160	-8.28404	3.53E-09	5.21E-08	DOWN
TR85389|c1_g1_i2	66.4	1.3E-14	RF00247	mir-160	-8.28416	2.2E-09	3.4E-08	DOWN
TR86488|c0_g1_i1	62.5	1.1E-12	RF00742	mir-162_2	-5.43848	7.82E-08	8.67E-07	DOWN
TR114307|c0_g2_i10	59.7	8.0E-13	RF00695	mir-398	-8.13167	8.2E-08	9.04E-07	DOWN
TR148431|c0_g1_i2	74.9	6.7E-15	RF00865	mir-169_5	-6.32831	2.1E-08	2.64E-07	DOWN
TR148431|c0_g1_i3	62.6	7.3E-12	RF00865	mir-169_5	-7.93303	0.000181	0.000893	DOWN
TR148431|c0_g1_i4	62.6	3.8E-12	RF00865	mir-169_5	-6.35025	2.83E-09	4.26E-08	DOWN
TR148431|c0_g2_i1	79.2	1.3E-16	RF00865	mir-169_5	-8.27347	6.26E-08	7.09E-07	DOWN
TR153173|c0_g1_i1	73.4	1.9E-16	RF02516	mir-393	-8.48906	3.7E-05	0.000219	DOWN
TR78616|c1_g1_i1	60.7	3.2E-15	RF00445	mir-399	8.449218	4.88E-10	8.55E-09	UP
TR78616|c1_g1_i2	60.7	3.5E-15	RF00445	mir-399	8.905397	5.14E-10	8.96E-09	UP
TR80998|c0_g1_i1	73.7	4.7E-14	RF00643	mir-171_1	10.69138	7.38E-36	6.49E-33	UP
TR119994|c1_g1_i1	46.7	2.8E-9	RF00843	mir-228	3.158001	1.38E-05	9.05E-05	UP
TR143357|c1_g1_i1	74.3	5.4E-18	RF00640	mir-167_1	3.135274	5.21E-14	1.93E-12	UP
TR70982|c1_g1_i4	64.9	1.8E-13	RF00073	mir-156	-2.77083	5.94E-05	0.000333	DOWN
TR113245|c0_g1_i2	66.4	1.7E-15	RF00640	mir-167_1	-7.08889	0.003547	0.011806	DOWN
TR114307|c0_g2_i7	59.7	5.5E-13	RF00695	mir-398	-4.38935	0.007604	0.022434	DOWN
TR124218|c0_g1_i3	61.5	6.6E-12	RF00704	mir-397	-4.06296	3.99E-05	0.000233	DOWN
TR124218|c0_g1_i4	70.0	4.4E-14	RF00704	mir-397	-3.33348	6.68E-06	4.75E-05	DOWN
TR136472|c2_g1_i6	123.6	1.2E-27	RF00638	mir-159	2.237127	1.88E-07	1.91E-06	UP
TR146295|c0_g1_i3	65.9	4.9E-18	RF00645	mir-169_2	-2.42076	6.49E-05	0.00036	DOWN

Table 7. Micro-RNAs precursors differentially expressed identified on roots RNA-Seq samples

Sequence	Score	e-Value	Family ACC	Family ID	LogFC	PValue	FDR	UP/DOWN
TR78600|c0_g1_i4	80.5	4.0E-21	RF00647	mir-164	-7.72444	1.48E-05	0.000722	DOWN
TR105549|c0_g1_i2	80.8	1.7E-18	RF02516	mir-393	-7.17386	0.002135	0.03489	DOWN

3.4.8. Analysis of hormone related pathway using RNA-Seq assembly based on sugarcane reference genome

RNA-seq libraries from leaf and roots samples showed a very distinct alignment rate, with a mean of 82.3 % mapped reads for leaves libraries and 25.8 % for root libraries (table 4). Lower alignment rates at roots may be associated with a presence of contaminant reads from microorganisms from soil community and it would be interesting to perform a meta-genome analysis aiming to identify these sequences. Despite the low alignment rate for roots samples, the high amount of initial sequenced reads input yielded a good coverage for subsequent analysis. Considering a cut-off p-value of 0.01 it was possible to identify 19,889 up-regulated and 18,428 down-regulated genes at leaf samples and 3,556 up-regulated and 4,093 down-regulated genes at root samples. 2,228 genes exhibit a similar expression pattern in both tissues, with 432 genes down-regulated and 1,856 genes up-regulated at leaves and roots, in the other hand 458 genes have an antagonist expression at each tissue, with 89 genes down-regulated at leaves and up-regulated at roots and 369 genes up-regulated at leaves and down-regulated at roots (Figure 39).

Considering all 43,220 differentially expressed genes it was possible to associate 22,209 of them to an ortholog group present in KEGG database, enabling the identification of interesting pathways where these genes are present such as Photosynthesis, Ribosome and MAPK signaling pathway for leaves and Galactose Metabolism for roots. From the pathways identified, a fascinating pattern was observed in the "Plant Hormone Signal Transduction" (figures 40 and 41).

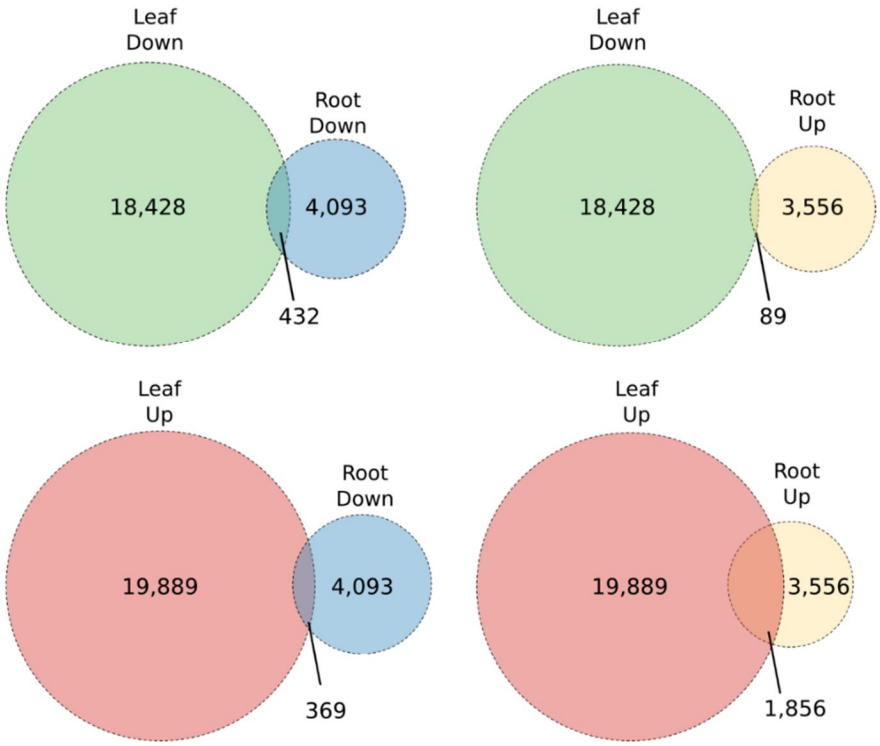


Figure 39. The Venn-Diagram shows the intersection between induced and repressed transcripts found in both leaves and roots samples and highpoints the intersection between leaves up-regulated and roots down-regulated as well as leaves repressed and roots induced. Those transcripts may be representing interesting pathways that characterize responses to drought exclusive for each plant part.

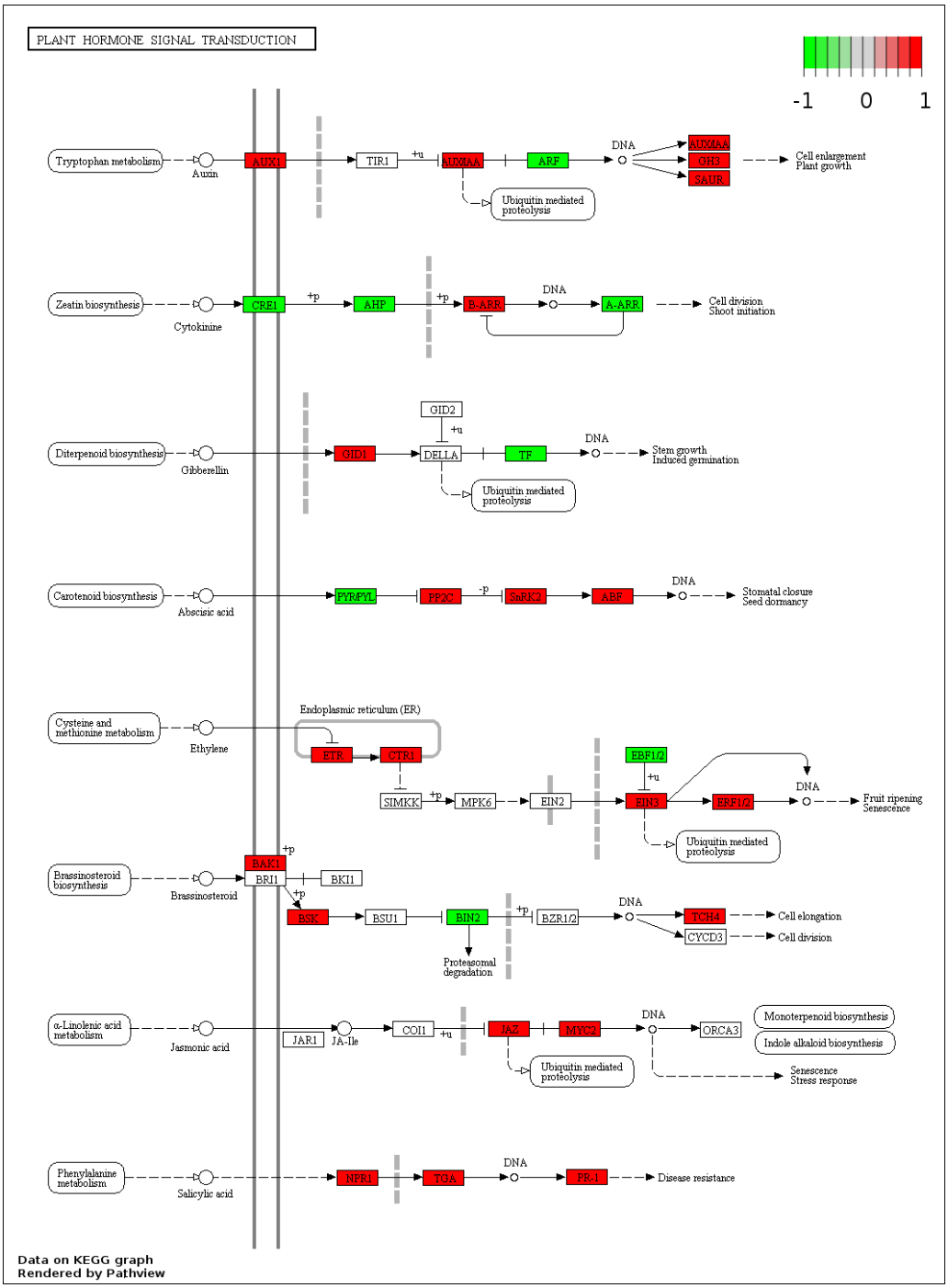


Figure 40. KEGG analysis shows that ABA has a fundamental role in sugarcane drought responses, but in the case of leaves, other hormones such as brassinosteroids and salicylic acid also plays important roles

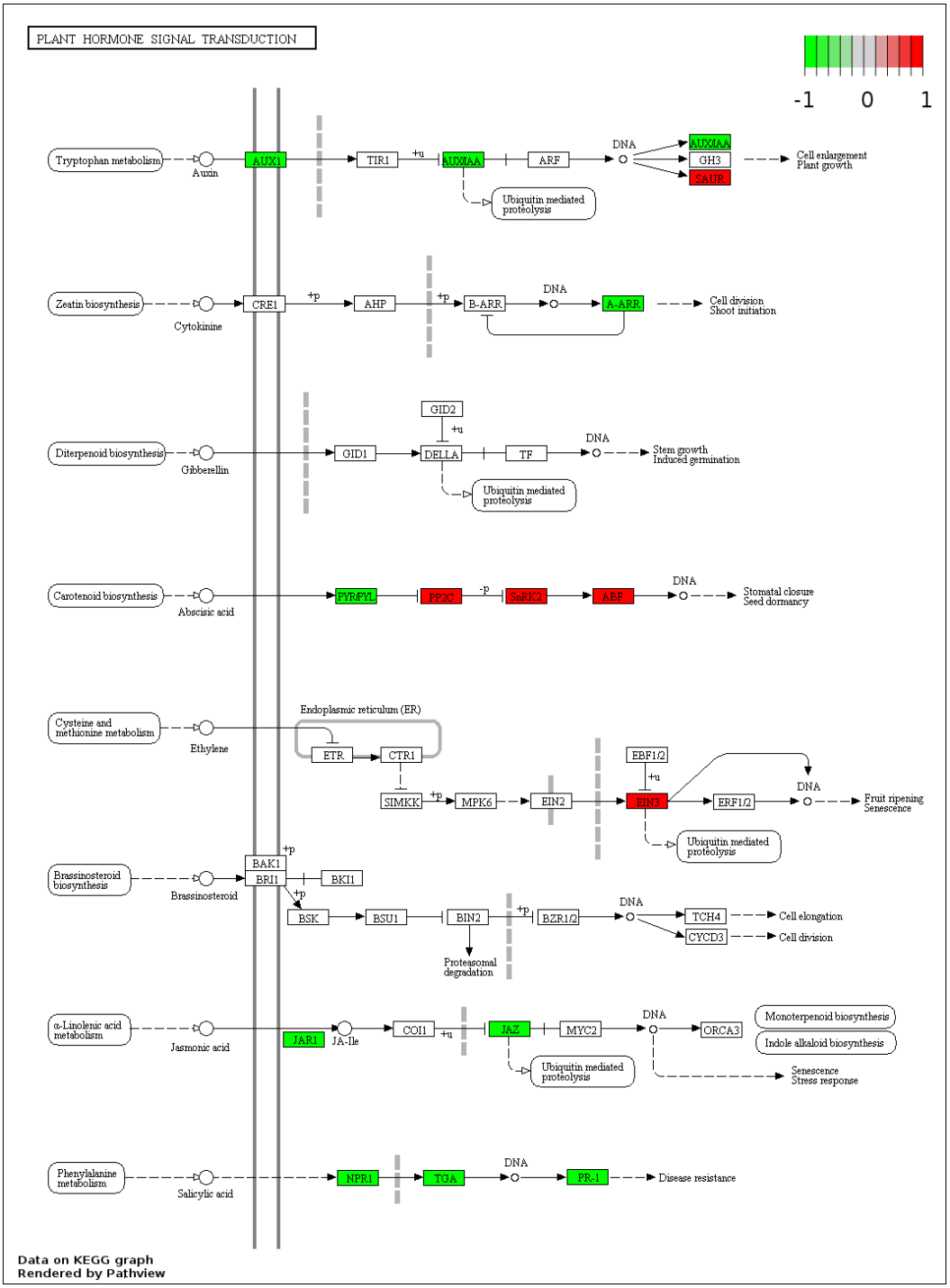


Figure 41. KEGG analysis shows that in roots, the main hormone acting on the response to drought is ABA

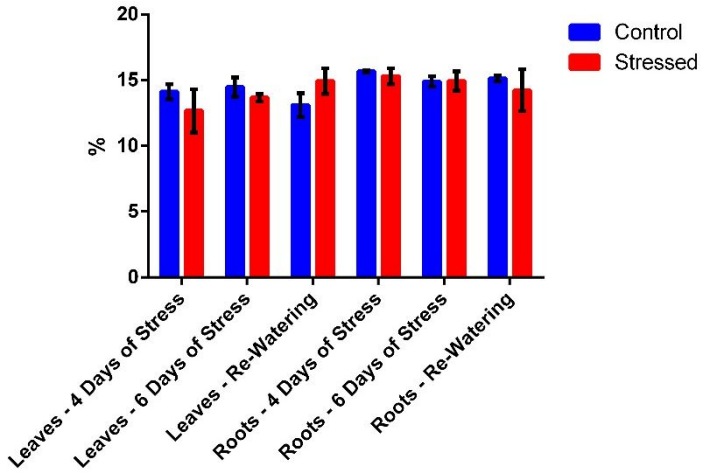
The results suggest that ABA signal transduction is involved on sugarcane drought responses in both leaves and roots (figures 40 and 41), but in the case of leaves, auxin, ethylene, brassinosteroids, jasmonic acid and salicylic acid may also have an important role (figure 40).

Interestingly, the signal transduction pathways from auxin, jasmonic acid and salicylic acid seems to be down-regulated on roots (figure 41).

3.4.9. Lignin Analysis

The analysis of DEGs by microarray showed a differential pattern of expression in the genes related to phenylpropanoid biosynthesis in both leaves and roots (figure 18) and, therefore, led to the hypothesis that the amount of lignin in both plant parts could be altered due to drought treatment. Thus, cell wall and lignin content were quantified (figure 42 and table 8).

(a)



(b)

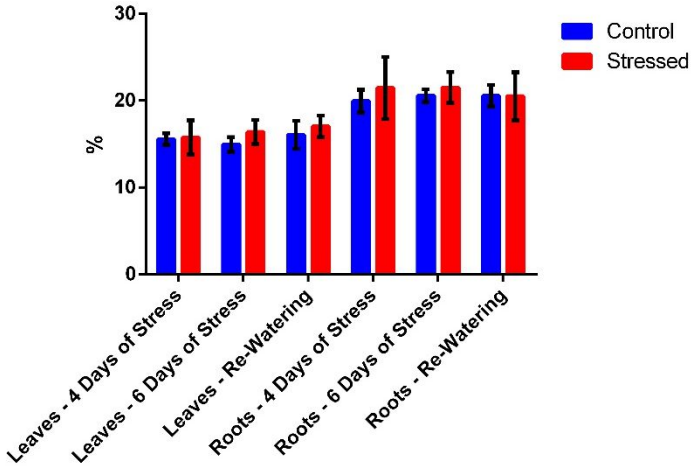


Figure 42. Lignin and Cell Wall analysis shows a general tendency of the increase in their contents in response to water stress, despite that, there was no significant statistical difference (a) Cell Wall Residue from sugarcane leaves and roots; (b) Lignin per percentage of cell wall

The results obtained demonstrate a general tendency of the increase in the amounts of lignin in response to drought, in oppose to the decrease in the CWR (cell wall residue). Despite that tendency, the difference between control and stressed samples is not statistically significant, which lead to the conclusion that even after a stress of 6 days, the alteration in gene expression is not yet reflected in the phenotypic level.

Table 8. Difference between the amounts of lignin per percentage of cell wall on stressed and control sugarcane leaves and roots

Experimental Condition	Difference between stressed and control lignin (% cell wall)
Leaves - 4 Days of Stress	0.19567318
Leaves - 6 Days of Stress	1.42194764
Leaves - Re-Watering	0.9752388
Roots - 4 Days of Stress	1.52280273
Roots - 6 Days of Stress	0.94775677
Roots - Re-Watering	-0.0637244

3.5. Discussion

3.5.1. Physiology data indicates moderate to severe stress and full recovery after re-watering

Abiotic stresses, such as heat, cold, salinity and drought, are among the major causes limiting crop yields worldwide (GARG; VARSHNEY; JAIN, 2014; RODRIGUEZ; CANALES; BORRAS-HIDALGO, 2005; YOU; CHAN, 2015). Sessile organisms, such as plants, need to adapt to different environmental conditions and stresses, and in order to do that, an intricate machinery at both, physiological, molecular and biochemical levels was developed (CRUZ DE CARVALHO, 2008; GARG; VARSHNEY; JAIN, 2014; JOSHI et al., 2016).

Drought is considered the major yield-limiting factor of crop plants, that actively and continuously determines the natural distribution of plant species (CRUZ DE CARVALHO, 2008). It exacerbates the effect of the other stresses to which plants are submitted, both biotic and abiotic, and several different abiotic stresses (such as cold and salt) result in water stress (CRUZ DE CARVALHO, 2008). In the case of sugarcane, as previously mentioned, the 8.5% reduction in harvesting caused by the 2014 decrease in rain (Brazilian Sugarcane Industry Association – UNICA, 2014) demonstrates the importance of understanding the mechanisms underlying *Saccharum* responses to drought and selecting candidate genes for breeding programs.

Quantifying the severity of water stress through a physiological analysis is important and can improve plant breeding studies (MEDEIROS et al., 2013), plus several physiological characteristics are very sensitive to water stress and may become reliable tools for this purpose (ENDRES, 2010; SILVA et al., 2007). Sugarcane growth can be divided in four phenological stages, where the tillering and grand growth phase (between 120 and 270 days) are known as critical stages of drought-sensitivity due to the high need of water for growth (RAMESH, 2000) being important from a crop production perspective (FERREIRA et al., 2017). Environmental stresses during this period can drastically reduce the yield of this crop as well as affect its potential for re-growth and longevity (CASTRO et al., 2015).

We evaluated sugarcane plants (variety SP80-3280) submitted to drought stress by water privation for 4 and 6 days, and 6 days plus 2 days of rehydration in greenhouse conditions. Despite of a slight decrease of soil humidity between 4 and 6 days (figure 4b), the predawn leaf water potential showed a large difference between these two conditions (figure 4a). When soil water content decreases to a dryer condition a small difference in soil water content reflects in a great decrease in soil water potential (STOOF; WESSELING; RITSEMA, 2010) and predawn leaf water potential tends to equilibrate with soil water potential (WILLIAMS; ARAUJO, 2002).

Significant reductions in leaf water potential (LWP) during stress treatments were also observed by Medeiros et al., (2013) with values 11-fold smaller than those found in the control treatment, with recovery after re-watering. In our case, we found values 14.5-fold smaller than the ones in the control treatment (figure 4a). Recovery after rehydration was also observed (figure 4b). Such decreases in LWP might represent a low level of cell turgor on stressed plants when compared to control and re-watering (MEDEIROS et al., 2013).

During drought, reductions in leaf area (size and number) and stomatal closure, impaired activities of carboxylating enzymes and ATP synthesis, membrane damage and destruction of photosynthetic apparatus are among the key factors reducing carbon fixation (FAROOQ et al., 2012; YAMANE et al., 2003). The decrease in stomatal aperture limits CO2 influx resulting in a decline in the rate of photosynthesis (FAROOQ et al., 2012) which is also related to the impaired activity of essential photosynthetic enzymes such as ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCo) (FAROOQ et al., 2008, 2009; RAMACHANDRA REDDY; CHAITANYA; VIVEKANANDAN, 2004).

Impaired photosynthesis along with low CO_2 influx down-regulates carbon fixation which in turn lowers the oxidation of reduced nicotinamide adenine dinucleotide (NADPH) in the Calvin Cycle (FAROOQ et al., 2012). Thus, the primary electron acceptor $NADP^+$ is not sufficiently available, leading to photoinhibition as a result of reactive oxygen species (ROS) overproduction when plants are exposed to high irradiance (TAUSZ et al., 2001).

In our work, gas exchange and chlorophyll a fluorescence parameters suggest that at 4 days after stress stomatal conductance (figure 4e) limited the photosynthesis (figure 4c) and transpiration (figure 4d) but there was limited damage to the photochemical machinery and Calvin Cycle. After 6 days of water stress, plants experienced a much more severe damage of the whole photosynthesis apparatus with a reduction of carboxylation and intrinsic water use efficiency and a high photoinhibition, even at predawn (figures 4f, 4h 4i and 4j). Similar results were found by Gonçalves et al., (2010), who observed only photochemical damage when sugarcane was under severe drought stress. Medeiros et al., (2013) found that stomatal conductance (gS) in the variety RB867515 was reduced 100% in the water suppression treatment compared to the control after 4 days of stress and that recovery happened 4 days after re-watering. In the case of RB962962 a reduction of 96% in gS occurred only after 8 days of stress, while recovery occurred after 3 days of re-watering. In the case of SP80-3280 a decrease of 98% was observed only after 6 days of stress, with recovery observed 2 days after rehydration (figure 4e).

It has also been shown reductions of 98.8% in transpiration (E) and 99.6% in photosynthesis rate (A) after 4 days of drought in RB867515 variety (MEDEIROS et al., 2013). RB962962 underwent reductions of 93.3% in transpiration and 97.7% in photosynthesis after 8 days of stress (MEDEIROS et al., 2013). In the case of transpiration, recovery happened after 2 days of re-watering for both varieties, but in the case of photosynthesis, recovery happened at the forth day of re-watering for RB867515 and after 2 days of re-watering for RB962962 (MEDEIROS et al., 2013). The partial or total recovery of net photosynthetic rate (A), stomatal conductance (gS) and transpiration (E) after re-watering of sugarcane plants from the variety RB867515 was also observed by Ribeiro et al., (2014). In the present study, the variety SP80-3280 showed a decrease of 90% and 98% in photosynthesis and transpiration (figures 4c and 4d), respectively, after 6 days of stress. Recovery was observed after 2 days of re-watering. Together, these parameters might indicate an intermediary position (between RB867515 and RB962962) of SP80-3280 regarding drought tolerance, since huge drops in the physiological parameters happen only after 6 days of drought and recovery demands about 2 days of re-watering. The fast recovery is an important feature of drought tolerance, and might mean an increase in the survival and cell damage repair after the stress period (MEDEIROS et al., 2013). These observations though need confirmation by doing experiments that compare these varieties side-by-side.

The plants fully recovered leaf photosynthesis apparatus after 2 days of re-watering, similarly to what has been previously found (KIM; MALLADI; VAN IERSEL, 2012; SILVA et al., 2013b). These results are in agreement with transcriptomic data, since most of the differentially expressed genes were found after 6 days of drought (figure 6). In addition, photosynthesis related genes were down-regulated after drought, but showed no difference in expression after rehydration (table 5). Moreover, the metabolic damage over the Calvin Cycle observed by the decrease in the carboxylation efficiency (figure 4f) and photosynthesis (figure 4c) was reflected in the instantaneous and intrinsic water use efficiency (WUE) (figures 4g and 4h). Interestingly, after 4 days of drought there was an increase in intrinsic water use efficiency (iWUE) (figure 4h). This might be due to the fact that in the beginning of the water stress, the partial reduction in the stomata conductance (gS) causes a greater reduction of transpiration than of CO_2 internal concentration, characterizing a non-linear relationship between carbon assimilation and stomata conductance (gS) (MORISON et al., 2008; REZA SEPASKHAH; AHMADI, 2010). This relationship could result in an increase in the intrinsic water use efficiency (iWUE) (MORISON et al., 2008; REZA SEPASKHAH; AHMADI, 2010).

The maximum quantum yield reflects the damage in the photosystem II (MAXWELL; JOHNSON, 2000). Water privation caused an intense photoinhibition in plants under 6 days of stress (figure 4j). Furthermore, photosystem II did not recover during night period (figure 4i) in plants under severe stress. After rehydration, the value of Fv/Fm increased back to values similar to the ones found for plants kept under irrigation. Water suspension for 6 days also caused a decrease in the effective quantum efficiency (figure 4k), probably due to the decrease of the Calvin Cycle activity and damages to the photosystem II (figures 4i and 4j). The intense photoinhibition observed after 6 days of drought was accompanied by the downregulation of genes such as Photosystem II PsbP, which did not show difference in expression after rehydration.

Quantifying the severity of water stress through physiological analysis is important since it may help in the identification of more tolerant varieties, therefore, improving plant breeding studies (MEDEIROS et al., 2013). The terms "moderate" and "severe" drought stresses are also quite subjective and vary from one group to another (CRUZ DE CARVALHO, 2008). Our results show that plants were under moderate to severe stress caused by stomatal restriction after 4 days of stress and metabolic damage after 6 days of stress.

3.5.2. Transcriptome analysis by microarray and RNA-Seq allowed the identification of different transcripts and pathways

The changes in sugarcane transcriptome were evaluated by microarray in three different conditions (4 days of stress, 6 days of stress and 6 days of stress plus 2 re-watering days) and two different plant tissues (roots and leaves). Microarray data was evaluated by 2 different methodologies, the HTSelf method, that allowed the identification of a smaller number of differentially expressed genes (DEGs) (very stringent statistical analysis) and FC ≥ 2 and FC ≤ 0.5 that lead to the identification of a higher amount of DEGs, and therefore, allowed a broader characterization of the differences in expression on KEGG pathways (figure 6).

The annotation of Sugarcane Assembled Sequences (SAS) found on SUCEST database was updated, therefore, the sequences found in the whole SUCEST as well as the sequences found in CaneRegNet sense and anti-sense catalogues were described according to their Gene Ontology terms (figure 13), and the results were used for Fisher's Enrichment Test (table 5). The category of gene expression was found in all three catalogues and was reflected

by nucleus cellular component term and DNA binding molecular function term also present in all three catalogues (figure 13). Response to stress is mainly found in SUCEST and CaneRegNet sense sequences (figure 13).

In the case of microarray, the updated annotation of the transcripts using Blast2GO resulted in a decrease on the number of unknown and/or hypothetical proteins. This annotation also leaded to the description of a higher number of transcripts involved with stress responses, such as late embryogenesis abundant protein class, different transcription factors, ABA-related transcripts and differentially expressed histones and histone methyltransferase (appendix C), the last ones shed a light on the role of chromatin remodeling in response to stresses. The relation between stresses and histones and/or histone modifications has been described and may have a role in providing a flexible, global and stable means for the transcriptional reprogramming of plant cells, leading to plant stress adaptation (SOKOL et al., 2007; YUAN et al., 2013; ZHU; DONG; SHEN, 2012). Down-regulation of several histones (appendix C), similarly to our work, has also been observed by Zhu; Dong; Shen, (2012).

Microarray data analysis shows that there is a high correlation between samples from the same plant tissue, and also that correlation between severe stress represented by the 6 days without irrigation in both leaves and roots samples is higher with re-watering samples than with moderate stress (figure 5). This may be an indicative of a shift in the expressed genes between moderate to severe stress, and that even with physiological parameters being back to normal values after re-watering, gene expression is not yet completely back to the status found in control samples (figure 5).

There were 11,844 unique SAS on the microarray slide with a signal above the background, from which 7,867 had a FC ≥ 2 and FC ≤ 0.5 and 585 were considered differentially expressed by the HTSelf methodology, which belonged to several orthologous groups, KEGG pathways and biological functions (figures 6, 7, 8, 9, 10, 11, 12, 14 and 15).

Results demonstrate that as the stress becomes more severe, we can observe the differential expression of genes related to ROS responses, like detoxification enzymes and with the protection of macromolecules, such as heat shock proteins and dehydrins among others (appendix C). There is also the repression of processes such as photosynthesis in the leaves and cell division and expansion in the roots (figure 7, 15 and table 5). Therefore, despite the fact that after 6 days of stress we can still notice a strong presence of transcripts related to signal transduction and gene regulation, the emphasis seems to switch for the functional response of the cell. Interestingly we can notice, as previously described, a crosstalk between several stresses, such as cold and salt stresses (RODRIGUEZ; CANALES; BORRAS-HIDALGO, 2005; SINGH; LAXMI, 2015) and between drought and lignin biosynthesis (KUMAR et al., 2016; MOURA et al., 2010) (appendix D).

Several of the classical drought responsive genes were found in our work. Some examples include dehydrins (HANIN et al., 2011; KOSOVÁ; VITÁMVÁS; PRÁŠIL, 2014), chaperones (INGRAM; BARTELS, 1996), phospholipid (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000); LEA proteins (INGRAM; BARTELS, 1996; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000), detoxification enzymes (INGRAM; BARTELS, 1996; RODRIGUEZ MILLA et al., 2003; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000), sugar metabolism (INGRAM; BARTELS, 1996; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000), proline rich proteins (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000) (appendix C). Regulation of transcription functional category was represented by DRE related motifs, transcription factors the from AP2, NAC, AUX/IAA, bZIP, MYB, MYC, EREBP/AP2 and zinc fingers families (LEMBKE et al., 2012; ROCHA et al., 2007; SHINOZAKI; YAMAGUCHI-SHINOZAKI, 2000) (appendix C).

The SAS of SCBGLR1023D05.g (LSD1 isoform X2) and SCVPLR2005H03.g (Auxin-responsive IAA13) were down-regulated in this work and also in Rocha et al., (2007) and Papini-Terzi et al., (2009) after drought, SCSBAM1085B06.g (fatty acid partial) was also down in Rocha et al., (2007) as well as SCCCLR1001E04.g (chloroplast ribulose-1,5-bisphosphate carboxylase oxygenase small subunit). The IAA12 - auxin-responsive Aux IAA family member (SCEQRT2093D08.g) was down also in Rocha et al., (2007) (appendix C).

Dehydrin 9 (SCQGLR1085F11.g) was induced in this work and also Lembke et al., (2012); Papini-Terzi et al., (2009) and Rocha et al., (2007), the SCSGHR1069F04.b (hypothetical protein SORBIDRAFT_07g008860) was found induced also by Rocha et al., (2007). The Ribonuclease 1 (SCJLRT1016G06.g) induced in roots after stress was found induced by Papini-Terzi et al., (2009) and Rocha et al., (2007) on drought stressed plants (appendix C).

Microarray and RNA-Seq results have been shown to complement each other (KOGENARU et al., 2012), therefore we chose the time point of 6 days of stress to perform the RNA-Seq on samples from leaves and roots, control and stressed. RNA-Seq results show a high number of total transcripts (623,447) and 'genes' (437,214). Huang et al., (2016) were able to obtain 101,255 unigenes, while Que et al., (2014) found 148,605 unigenes. Li et al., (2016b) found 275,018 transcripts and 164,803 'genes', while Nishiyama et al., (2014) found 195,765 transcripts and Cardoso-Silva et al., (2014) found a total of 119,768 transcripts and 72,269 unigenes. Therefore, in our work we obtained a huge amount of transcripts, by both *de novo* Assembly and reference genome assembly, for further analysis.

Despite that, we have found a contig N50 of 905 nt, which is smaller than 1,177; 1,385; 1,300 and 1,367 bp obtained by Li et al., (2016b), Vicentini et al., (2015), Que et al., (2014) and Cardoso-Silva et al., (2014) respectively. This number is similar to previously obtained by the group (963 bp) (NISHIYAMA et al., 2014) and higher than obtained by Huang et al., (2016) (640 bp). The correlation matrix shows that biological replicates from the same tissue and condition have a strong correlation between them, indicating that they behave similarly in each condition and, therefore, they are good biological replicates, which is an important feature for statistical analysis (figure 22).

The *de novo* assembly and annotation of RNA-Seq allowed the identification of 28,240 differentially expressed (DE) features for leaves and 7,435 DE features for roots after 6 days of stress (figures 27 and 28), as well as some up and down-regulated microRNAs (figures 37, 38 and tables 6 and 7). Furthermore, a high number of transcripts without blast results or considered unknown or hypothetical proteins were observed. This result is interesting and may indicate that among exclusive transcripts, several may be either long non-coding RNAs (lncRNAs), undescribed genes or sugarcane-specific transcripts. According to Li et al., (2016a), the characterization of such undescribed genes is important in the study of drought since they may represent new sources of variability and contribute to the comprehension of the interaction between molecular and physiological responses involved with this stress. Therefore, further analysis of RNA-Seq data is still necessary in order to obtain more complete and applied conclusions regarding the data.

In general, the results show differential expression of peroxidases, sugar metabolism transcripts, photosynthesis related genes, ABA-related transcripts, among others, such as previously described (LI et al., 2016a; PINHEIRO; CHAVES, 2011; RODRIGUEZ; CANALES; BORRAS-HIDALGO, 2005; SINGH; LAXMI, 2015). Despite a huge agreement between results from several works found in the literature, the clear view of event chronology triggered by drought will be only understood once there is an homogenization of treatments amongst researchers, since it is difficult to compare a plant's responses to different types of drought stress, such as PEG treatment, progressive drought, water privation (CRUZ DE CARVALHO, 2008).

3.5.2.1. Transcription Factors and signal transduction

Several genes and pathways may act synergistically to help the plant to detect and adapt to the stress condition by different ways. Mitogen-activated protein kinase (MAPK) cascades are involved in diverse processes from plant growth and development to stress response (YOU; CHAN, 2015), while SAPKs are MAPKs that sense and transduce stress signals (FLEMING et al., 2000). These kinases are involved in carrying osmotic signals from sensory to target elements of the cell (KÜLTZ, 2001) and Calcineurin B-like proteins (CBLs) and their target proteins, CBL-interacting protein kinases (CIPKs), which are typical Ser/Thr protein kinases. CIPKs have emerged as a key Ca^{2+}-mediated signaling network in response to stresses in plants (CHEN et al., 2012, 2011; CHEONG et al., 2003; PIAO et al., 2010). The influx of Ca^{2+} into the cytosol is countered by pumping Ca^{2+} out from the cytosol to restore the basal cytosolic level, and this may be achieved either by P-type Ca^{2+} ATPases or antiporters (YOU; CHAN, 2015). The SAS related to this class of proteins can be exemplified by SCBFRZ2048D04.g - CBL-interacting kinase family, SCEZAM2034C10.g - probable calcium-binding CML10, up-regulated in leaves both at 4 and 6 days of stress, SCCCST1004A07.g - serine threonine kinase SAPK4, SCCCCL3001E05.b - SNF1-related kinase regulatory subunit beta-1, SCMCST1053F09.g - calcium-transporting ATPase plasma membrane-type-like, SCMCSD2061D05.g - CBL-interacting kinase 2 the last ones induced in roots (appendix C).

The SAS SCCCRZ3001D06.g (LOV domain-containing) found in Rocha et al., (2007) kinome catalogue and down-regulated in response to MeJa was up-regulated by 6 days of drought in this work. The SCRFLR2034A09.g - serine threonine-kinase SAPK1, also classified as part of sugarcane kinome by the same work was up-regulated in roots after 6 days of stress (appendix C).

We could find several genes involved in the control of gene expression in an ABA-dependent and ABA-independent manner. Examples include Type 2C protein phosphatases (PP2C) and SnRK2 kinases. In the case of PP2C, the SAS from probable phosphatase 2C 50 (SCEPRZ1010E06.g) was also found induced by ABA and drought by Papini-Terzi et al., (2009) and by Rocha et al., (2007) 72 and 120 h of stress. Other PP2Cs were SCVPFL3046C06.b, SCCCCL3005D01.b (appendix C).

ABRE-binding protein, ABRE-binding factor TFs (COHEN et al., 2010; LEONHARDT, 2004; MA et al., 2009; NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014; ROCHA et al., 2007; SAEZ et al., 2006), were represented by SCCCLR1C03C05.g - ABA responsive element binding factor 1, SCVPLB1018G11.g - ABSCISIC ACID-INSENSITIVE 5 5, SCSFFL4082E10.g - ABI five biding 3. The AUX/IAA, DREB, EREBP/AP2 and NAC TFs (NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014; ROCHA et al., 2007) by DRE binding factor (SCVPRZ3026C06.g), auxin response factor (SCCCCL3120A10.b), ethylene-responsive factor 1 (SCCCCL4002B07.g), auxin-responsive IAA31-like (SCEPLR1008A10.g), ethylene-responsive transcription factor ERF073-like (SCCCCL7002A04.g), IAA12 - auxin-responsive Aux IAA family member (SCEQRT2093D08.g) (appendix C). Among the TFs found, SCJFRZ2014D06.g - NAC transcription factor, up-regulated after 4 days was also up-regulated after 72 and 120 hours after stress by Lembke et al., (2012) (appendix C).

Other TFs found inlcude Transcription factor MYB108 (SCSGAD1007F02.g), WRKY transcription factor 19 (SCUTRZ3069A01.g), WRKY transcription factor 21(SCQGLR2025A07.g), MYB DNA-binding domain superfamily (SCEQRT1024G12.g, SCBFRZ2018G02.g, SCVPRT2084A03.g), MYB DNA-binding domain superfamily, transcription factor SCREAM2 (SCRURT3061D05.g), Transcription factor bHLH96 (SCEZAM2035A03.g). The whole list of transcripts with their respective differential expression can be found at appendix C.

3.5.2.2. LEAs and Chaperones

Among the differentially expressed genes found, cell protection was represented by transcripts such as HVA22 e (SCSGLR1045D05.g), which has a role in protecting cells from damage under stress conditions (SHEN et al., 2001) and negatively regulates GA mediated processes (GUO; DAVID HO, 2008). We also found the desiccation-related protein PCC13-62 (SCBFSD1037G05.g), which is expected to be detected only after severe drought. Involved with chaperone function are peptidyl-prolyl cis-trans isomerase FKBP20- chloroplastic (SCSBAD1052A06.g), FKBP-type peptidyl-prolyl cis-trans isomerase family (SCCCLB1C06D11.g), BAG family molecular chaperone regulator 6 (SCBFAD1088H04.g). The last two up-regulated. Furthermore, several transcripts related to heat shock proteins, such as Heat shock 70 kDa 8 (SCRLLR1038B09.g), class III heat shock (SCCCLR2001C04.g), Small heat shock (SCCCCL4005G05.g), heat shock (SCSGLR1025D03.g) (appendix C) were found. Plants submitted to drought stress are also subjected to heat stress because of the reduced transpiration flux due to stomatal closure (CRUZ DE CARVALHO, 2008).

Dehydrin 9 (SCQGLR1085F11.g) a SAS also found induced by drought at Lembke et al., (2012); Papini-Terzi et al., (2009) and Rocha et al., (2007), and LEA 3 (SCQSRT2031H06.g) represented the LEAs (appendix C).

3.5.2.3. ROS

Superoxide dismutase (SOD) acts as the first line of defense converting O_2^- into H_2O_2, while catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) then detoxify H_2O_2 (YOU; CHAN, 2015).

Catalase is an enzyme predominantly located in peroxisomes and its activity may be particularly important to metabolize photorespiratory H_2O_2, which can be produced at higher rates when water becomes limiting (NOCTOR; MHAMDI; FOYER, 2014).

Related to hydrogen peroxide, enzymes involved in its removal are divided into catalases, which dismutate H_2O_2 to water and O_2 through heme-dependent dismutation, and peroxidades, which require reducing co-factors (NOCTOR; MHAMDI; FOYER, 2014). Peroxidases are diverse enzymes, but include two major groups – heme-based and thiol-based peroxidases (NOCTOR; MHAMDI; FOYER, 2014). In plants, the first group includes the best characterized antioxidative heme-based peroxidades, APX (a class I heme peroxidades), and class II heme peroxidades (important in both ROS generation and consumption) (NOCTOR; MHAMDI; FOYER, 2014). APX reduces H_2O_2 at the expense of ascorbate, which is then regenerated by monodehydroascorbate reductases and dehydroascorbate reductases using NAD(P)H and GSH, respectively (NOCTOR; MHAMDI; FOYER, 2014). Thiol-based peroxidades include enzymes that notably use reducing equivalents from TRX- and/or GSH (NOCTOR; MHAMDI; FOYER, 2014).

Several SAS identified as peroxidases were found, such as the SCCCCL2001D02.b, SCVPRZ2035F03.g, SCSBSD1033E10.g, SCCCCL3002F08.b, SCJFRZ2032H11.g among others. Also SCJLAM1062A05.g (probable phospholipid hydroperoxide glutathione peroxidase), SCCCCL4007F05.g (glutathione *S*-transferase GSTU6) were found.

Oxidative signaling is now considered to be a key element of complex gene networks, underpinning cross tolerance responses to stress and leading not only to defense but also to regulation of growth (NOCTOR; MHAMDI; FOYER, 2014). Therefore, identification of differentially expressed transcripts involved on these processes sheds lights on its importance also for sugarcane drought responses.

3.5.2.4. Sugars

The Galactose Metabolism pathway, shown in figure 17, is involved with the galactinol, raffinose and stachyose family of oligosaccharides (RFOs) which are known to have roles in osmoprotection and in the protection of plant cells from oxidative damage against abiotic stresses (NISHIZAWA; YABUTA; SHIGEOKA, 2008; SANTOS et al., 2015), demonstrating that in roots, this stress response may be activated earlier than on leaves. The SAS from stachyose synthase precursor (SCJFLR1017E09.g) induced after 4 days of stress in leaves and 6 days of stress in roots (appendix C) was also found up-regulated after 72 h and 120 h of stress by Lembke et al., (2012).

Other SAS involved with sugar metabolism found were SCSGSB1009B08.b (soluble starch synthase II-2) down-regulated in leaves after 6 days of stress, SCQGLR1019G02.g (UDP-glucose 6-dehydrogenase), SCEQRT1025C10.g (Glucan endo-1,3-beta-glucosidase 13), SCEPCL6023F02.g (sucrose synthase 4).

3.5.2.5. Proline and other amino acids

Proline (Pro) accumulation is a common physiological response in many plants to a wide range of biotic and abiotic stresses (VERBRUGGEN; HERMANS, 2008).

In this work, we found the Δ-1-pyrroline-5-carboxylate synthetase 1 (P5CS1) (SCJFLR1073H12.g) (appendix C). During osmotic stress, availability of atmospheric CO_2 is reduced because of increased stomatal closure and consumption of NADPH by the Calvin Cycle is decreased. Activity of P5CS in the chloroplast can recycle $NADP^+$, the last acceptor of the photosynthetic electron transfer chain, which may reduce ROS production in the photosystem I. Also glucose-6-phosphate dehydrogenase, the first and rate limiting enzyme of the pentose phosphate pathway (PPP) requires $NADP^+$ and is inhibited by NADPH (VERBRUGGEN; HERMANS, 2008). P5CS (EC 2.7.2.11/1.2.1.41) is a bifunctional enzyme that converts glutamate into the intermediate glutamic semialdehyde, which spontaneously cyclizes into Δ-1-pyrroline-5-carboxylate (P5C) (VERSLUES; SHARMA, 2010).

Several amino acids, in particular the branched chain ones (leucine, isoleucine and valine) and threonine are present at higher level than other amino acids and have synthesis pathways that consume NADPH in the plastids and catabolism pathways that release reductant in the mitochondria. Thus, they could participate in similar redox buffering and energy transfer mechanisms as proline metabolism (VERSLUES; SHARMA, 2010). Alteration in the metabolism of amino acids was found in this work as well (figures 7 and 15, appendix).

Proline overproduction helps in the maintenance of cell turgor or osmotic balance, stabilize membranes, prevents electrolyte leakage and brings the concentrations of reactive oxygen species (ROS) within normal ranges, which prevents oxidative burst in plants (HAYAT et al., 2012). Furthermore, Pro metabolism has roles in redox buffering and energy transfer and is involved in plant pathogen interaction and apoptosis (VERSLUES; SHARMA, 2010).

Recently, quantitative comparison of P5CS1 (proline biosynthesis gene) expression to known ABA-induced genes found largely ABA-independent regulation of P5CS1, implying that the underlying mechanisms controlling P5CS1 expression are distinct from that of many commonly studied stress marker genes (VERSLUES; SHARMA, 2010). Mechanisms independent of ABA or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery.

3.5.2.6. Aquaporins

The capacity of roots to take up water is determined in part by the resistance of the living tissues to radial water flow (JAVOT; MAUREL, 2002). Aquaporin-rich membranes may be needed to facilitate intense water flow across epidermal, endodermal and surrounding xylems vessels cells and may represent critical points where an efficient and spatially restricted control of water uptake can be exerted (JAVOT; MAUREL, 2002). Roots can alter their water permeability over a short term period in response to many stimuli such as drought stress. These fast changes can be mostly accounted for by changes in cell membrane permeability and are mediated by aquaporins (JAVOT; MAUREL, 2002).

Drought decrease hydraulic conductivity (Lp) and aquaporins are thought to mediate these adjustments (JAVOT; MAUREL, 2002). The hydraulic resistance will be high at low rates of transpiration, i.e. during the night or during periods of water shortage. Under these conditions, roots may be protected from excessive water loss to the soil by their high hydraulic resistance (STEUDLE; PETERSON, 1998). The down-regulation of aquaporin genes could be a response or adaptation of the plant to increase the hydraulic resistance in the cell-to-cell path, being related to the decrease in transpiration and water use rates after six days of drought. The continued down-regulation after rehydration may be a reflection of the previous attempt of the plant to deal with the stress, since transpiration rates were not yet completely back to the values found in the irrigated plants. Aquaporins were also found as down-regulated in rice leaves after re-watering (MIRZAEI et al., 2012).

The Water Channel Activity molecular function in roots is found only after re-watering (figures 12 and 32). Aquaporins found were: aquaporin TIP2-3 (SCBGRT1052E01.g), Aquaporin TIP2-1(SCJFRT1010C08.g), aquaporin PIP2-6 (SCEQRT1024B11.g) all down-regulated in roots (appendix C).

3.5.2.7. Cell Cycle and Cell Wall Related Genes

Ribeiro et al., (2014) in an experiment of partial root drying (PRD) found that although total root dry mass was not affected in plants under the PRD treatment, the root volume in the dry side was reduced (a 48.8% reduction in volume compared to the irrigated portion). Therefore, the weight and volume of roots in the irrigated side compensated for the other side. In the same experiment, the weight and volume of roots in the non-irrigated treatment were lower than the same parameter in the full-irrigation treatment, representing a reduction in root growth. Medeiros et al., (2013) also observed a significant reduction in root dry mass after water suppression in the same variety (RB867515). Thus, the reduction in root growth in response to drought found by these works are in agreement with the down-regulation of expansin genes (appendix C) and a high percentage of sequences belonging to the "Cell Cycle Control, Cell Division, Chromosome Partitioning" on the down-regulated orthologous groups as compared with the up-regulated one from RNA-Seq. Furthermore, Cell Wall and Cell Wall Organization or Biogenesis gene ontology terms were enriched in the down-regulated root transcripts found by microarray analysis on 6 days stressed samples (table 5).

Expansins SAS found were SCCCCL4006H09.g, SCEQRT2027A10.g, SCMCRT2107D08.g, SCEPRT2043B01.g and others, repressed in roots, and cyclin by SCVPLR1049E05.g, SCUTLR1058G02.g also down-regulated. Cell wall represented by glycine-rich cell wall structural 1 (SCACHR1035G08.g), cell wall integrity and stress response component 1-like (SCJFRT1007H04.g), galactoside 2-alpha-L-fucosyltransferase-like (SCEQRT2100C04.g),

alpha-galactosidase precursor (SCUTLR1037C07.g), beta-D-xylosidase 4-like (SCCCCL4009F05.g) all repressed in roots after 6 days of stress (appendix C).

In roots, the anti-sense transcript for SCQGST1032H03.g - Oligosaccharyl transferase STT3 subunit was found up-regulated after 6 days of drought by microarray and down-regulated by RT-qPCR. Oligosaccharyl transferase STT3 is a salt tolerance induced gene (KOTTAPALLI; SARLA; KIKUCHI, 2006) (appendix C), characterizing a hyperosmotic/saline adaptation determinant of *Arabidopsis* (KOIWA et al., 2003). Since osmotic and drought stress responses appears to be similar and SP80-3280 is a sugarcane variety that demands fertile and watered soils to achieve a good yield, it does not seems to have a good adaptation to drought stress. Therefore, I expected a down-regulation of oligosaccharyl transferase STT3. *Arabidopsis* stt3a-1 and stt3a-2 mutations cause NaCl/osmotic sensitivity that is characterized by reduced cell division in the root meristem (KOIWA et al., 2003). In our work we also found the down-regulation of the transcripts that belongs to Cell Cycle Control (figure 15) which would better agree with a down-regulation of oligosaccharyl transferase STT3 transcripts and a reduced cell division.

Furthermore, after re-watering, genes related to aquaporins and to cell division and expansion continue to be down-regulated in roots (appendix C), while photosynthesis is not repressed anymore in leaves, indicating that roots take more time to go back to normal conditions. Plant cell expansion is dependent on cell turgor (CHAUMONT; TYERMAN, 2014; JOHANSSON et al., 2000), and the down-regulation of these genes in roots is a direct effect of the stress. These results might be showing that root growth by both cell division and cell expansion is inhibited during drought responses.

3.5.2.8. Plant Hormones

Drought stress also alters synthesis of several phytohormones. Usually, growth promoters (GA and CK) decrease in concentration while growth retardants (ABA and ethylene) increase to regulate the plant water budget (FAROOQ et al., 2009; TAIZ, 2010). In this context, the phytohormone ABA is a key endogenous messenger that plays pivotal roles in adaptive stress responses to environmental stimuli in plants (FUJITA et al., 2011). For this reason, water stress signaling pathways are classified into ABA-dependent and ABA-independent pathways (SHINOZAKI; YAMAGUCHI-SHINOZAKI, 1997, 2006), but the crosstalk between these pathways have also been described (CHENG et al., 2013; NAKASHIMA; YAMAGUCHI-SHINOZAKI; SHINOZAKI, 2014).

The results from the RNA-Seq assembly using the reference genome demonstrates the difference on plant hormone signal transduction pathways between leaves and roots after 6 days without water (figures 40 and 41). In the case of leaves, there is an up-regulation of several genes from the growth retardants ABA and ethylene, as well as auxin, brassinosteroids, jasmonic acid and salicylic acid. It is interesting that the analysis of activity score from pathways using microarray results demonstrates that after 4 days without irrigation, brassinosteroids biosynthesis pathway is up-regulated in leaves (figure 21) and RNA-Seq shows the up-regulation of its signaling pathway after 6 days of stress consistent with the chronological order of biosynthesis and signaling.

Roots show an up-regulation of transcripts belonging to the signaling pathway of ABA only, while there is a down-regulation of transcripts from auxin, jasmonic acid and salicylic acid signal transduction pathways and no change in the brassinosteroid signal transduction, showing the fundamental role of ABA in the whole plant level and more specifically in roots.

3.5.2.9. Phenylpropanoids and lignin biosynthesis

The up-regulation of phenylpropanoid biosynthesis in leaves opposed to its down-regulation in roots was another interesting pattern observed (figure 18). This result is curious, since according to Moura et al. (2010) growth reduction and tolerance to desiccation were associated with more lignin in the roots, while lower levels of lignin on the leaves suggests an adaptation to drought. Despite that, the activity of the enzymes from the pathway varies depending on the period that the plant was exposed to drought (MOURA et al., 2010). As a hydrophobic polymer, increased lignin deposition may be important to reduce loss of water in stressed plants. Furthermore, it was shown that in maize, leaf lignin content is a useful index for evaluation of drought tolerance (HU et al., 2009). Phenylpropanoid biosynthesis was also shown altered in sugarcane submitted to drought by Li et al., (2016a).

Although pathview analysis show a difference in the gene expression, in the present work there is only a tendency of increase in the lignin content after drought treatment (figure 42) and, therefore, possibly plants were not stressed long enough to show a phenotypical change in the content of lignin.

3.5.3. Functional Categorization suggests different responses on moderate and severe stresses

The analysis of the leaves gene ontology terms, from the data obtained using the HTSelf method, for cellular component shows that after 4 days without water the plant is still activating the expression of response genes, since we can observe a high number of sequences associated with Nuclear Localization (figure 8). The representation of protein serine/threonine phosphatase activity, nucleic acid binding transcription factor activity and DNA binding molecular function after 4 days of stress corroborates with the affirmative (figure 9). The molecular functions of Nucleic Acid Binding Transcription Factor Activity and Nucleotide Binding are also found only in the beginning of the stress in roots, while water channel activity is found after re-watering in the same tissue (figure 12).

In contrast, the number of terms associated with the chloroplast cellular localization in the other two conditions (6 days without water and 6 days without water plus two re-watering days), reflect the down-regulation of photosynthesis related genes after 6 days of stress and the attempt of the plant to recover photosynthesis after re-watering (figure 7 and table 5). Furthermore, thylakoid GO is observed only after 6 days of stress which might reflect the metabolic damage on the photochemical apparatus (figure 8). Corroborating with that is the high representation of the Photosynthesis biological process in these experimental points. Fisher's Enrichment Test also demonstrates the over-representation of plastid, thylakoid, photosynthesis and chloroplast GO terms on the down-regulated transcripts after 6 days of stress and the over-representation of Plastid on the up-regulated transcripts after re-watering, which further corroborates with the impaired photosynthesis after 6 days of stress and its recovery after re-watering demonstrated by physiological data (table 5).

The Response to Stress Class is represented in all three conditions and both plant tissues, which indicates that even after the stress condition is removed the genes involved continue being transcribed (figures 7 and 10). The same pattern happening for catabolic process, which might be involved with the mobilization of nutrients, such as nitrogen, for different processes during stress response (figures 7 and 10). The biological processes related to cellular nitrogen metabolic compound process indicates a possible role for nitrogen mobilization after stress in the beginning of the stress on leaves. In roots, this biological process is found both after 4 and 6 days of stress (figure 10).

Carbohydrate metabolic process on roots is on these two conditions as well and after 6 days without irrigation on leaves (figure 10).

In leaves the lipid metabolic process is represented after 4 days of stress and after re-watering (figure 7), indicating a possible role for membrane lipid reorganization in these two conditions. The same BP class in roots is altered after 4 days of stress only (figure 10).

Signal Transduction GO class is also observed after 4 days without irrigation and after re-watering in roots and might mean that this tissue is sensing the differences on the soil humidity and transducing the signal to alter gene expression (figure 10). Observation that in leaves translation and protein folding are biological classes found after re-watering shed some light on protein turnover and biosynthesis of new proteins after re-watering (figure 7).

Analyzing the Top Orthologous Groups for leaves, the categories of "Signal Transduction Mechanisms" and "Transcription" have a higher percentage of representation after 4 days of stress, while "Lipid Transport and Metabolism" is observed after 4 days without water and after re-watering and "Translation, Ribosomal Structure and Biogenesis" after re-watering (figure 14). These results agree with the one found by Gene Ontology classification. "Energy Production and Conversion" is more observed after 4 days without irrigation (figure 14).

In the case of roots Top Orthologous Groups, the categories that catches my attention are the classes of "Carbohydrate Transport and Metabolism" and "Cell Cycle Control, Cell Division, Chromosome Partitioning" after 6 days of stress and re-watering showing the importance of sugars possibly in osmoregulation in this tissue, and the restriction of root growth (figure 15). "Signal Transduction Mechanisms" after 4 days without water and after re-watering, "Transcription" and "Amino Acid Transport and Metabolism" in the beginning of stress, and "Chromatin Structure and Dynamics" after 6 days of stress are also interesting (figure 15). The first one agrees with GO terms indication, the second demonstrates the alteration in gene expression on the beginning of stress, the third sheds light on protein metabolic process, amino acid related osmoprotection and nitrogen remobilization; and the last one on histone modification and chromatin remodeling in response to stresses.

The results from RNA-Seq *de novo* analysis shows biological processes up or down-regulated on roots. It is particularly interesting how Catabolic Process, Regulation of Gene Expression, Response to Stress, Transcription-DNA-templated and Regulation of Nitrogen Compound Metabolic Process are found only up-regulated (figure 30) and show the roles of mobilization of compounds, such as amino acids, and the importance of signaling and control of gene expression even during severe stress. The significance of these processes are also observed by the analysis of Top Orthologous Groups with the categories of "Posttranslational modification, protein turnover, chaperones", "Signal Transduction" and "Transcription" among the mainly up-regulated (appendix F). According to Wang et al (2016) protein synthesis and turnover is considered one of the fundamental metabolic processes for plants to cope with drought stress.

Li et al., (2012) found an enrichment in the metabolism of lipids, abiotic stresses and biosynthesis of organic acids classes in roots from susceptible and tolerant genotypes of wheat submitted to drought treatment. Also evaluating the root transcriptome, Moumeni et al., (2011) studying rice drought-tolerant lines, found a high representation of classes related to amino acid metabolism, transcription and signal transduction and cell wall growth, similarly to our results.

Payton et al., (2011) analyzing the changes in the cotton transcriptome in response to drought stress on leaves and roots, found more DEGs in leaves, differently from our results, that show 313 DEGs in leaves and 400 DEGs in roots (considering the total number of SAS). Furthermore, in leaves, the major functional groups detected were cell signaling, stress response and regulation of transcription, while in roots they were stress response, carbon

metabolism and regulation of transcription. In this work, as previously mentioned, the stress response class was also among the most represented ones in both tissues studied.

The enrichment of classes related to metabolism, energy use, cell transport, signal transduction, biogenesis of cellular components and antioxidant pathways were observed in maize (ZHENG et al., 2010), while in rice, a study using whole seedlings found classes related to carbon fixation, glycolysis, flavonoids biosynthesis and sucrose and starch metabolism (LENKA et al., 2011). Carbohydrate, lipid and protein metabolism, as well as energy, redox homeostasis, cell wall biogenesis and photosynthesis were found affected by drought and/or salinity stresses on chickpea roots, demonstrating also the crosslink between stresses (GARG et al., 2016). Some of these classes and related processes, such as glycolysis and sucrose and starch metabolism, were found in our work. The representation of cell division class after re-watering in roots was also observed in a study done in leaves of Sinapis alba (DONG et al., 2012).

The study of sugarcane transcriptome in response to drought has been addressed in a couple of works. Rodrigues et al., (2011) analyzed sugarcane leaves submitted to mild, moderate and severe stress and also found a high number of DEGs related to protein and amino acid metabolism, stress response and photosynthesis, but in our work, photosynthesis related genes were repressed, while in Rodrigues et al., (2011), they were induced. In the present work, signal transduction Gene Ontology Biological Process was represented on roots DEGs, but not on leaves, but the "Signal Transduction Mechanisms" orthologous group appeared in both leaves and roots.

Similar to previous works of the group (LEMBKE et al., 2012; ROCHA et al., 2007), the number of DEGs increased in the late experimental data point, and transcripts related to stress response, hormone responses, such as ABA, carbohydrate and lipid metabolism, photosynthesis and cell wall metabolism were also found. Prabu et al., (2011) noticed a high representation of response to stress, carbohydrate and protein metabolism, and Rodrigues; De Laia; Zingaretti, (2009) found protein metabolism, stress response, signal transduction and transport classes highly represented. These results together with ours suggest a key role of carbohydrate and protein metabolism on sugarcane responses to drought.

The stress response category was also found by Kido et al., (2012) on sugarcane roots submitted to stress, along with several transcripts with description similar to the ones found in this work, such as viviparious 14 (leaves), ABA responsive element binding factors (leaves and roots), serine/threonine protein kinases (leaves and roots) among others. Despite a smaller number of DEGs after 4 days of stress, Pathway Studio analysis (appendix D) indicates the importance of signal transduction and gene regulation in the beginning of the stress, as stated in the literature (LI et al., 2016a), being both ABA-dependent and ABA-independent pathways represented. We could find several transcription factors, such as ABRE, NAC, WRKY and DREB, among others (appendix D).

3.5.4. KEGG enriched pathways

The analysis of the genes with FC $\geq$ 2 and FC $\leq$ 0.5 allowed us to notice interesting patterns, such as up-regulation of galactose metabolism from the beginning of the stress in roots (appendix E).

Furthermore, the up-regulation of fatty-acid biosynthesis and degradation (appendix E) on leaves may demonstrate that in this tissue there is a higher need for the stress adaptive reorganization of membranes and maintenance of the cellular energy supply under water stress conditions (GOLLDACK et al., 2014).

Ribosome and ribosome biogenesis were up-regulated in leaves (appendix E). The change in sugarcane ribosomal protein gene expression in stressed plants was also shown by Li et al., (2016a) and Rodrigues; De Laia;

Zingaretti, (2009), and may suggest a function in restoring the protein synthesis processes and complement the results from Gene Ontology and Orthologous Group functional annotation.

KEGG enriched pathways (appendix E) also demonstrated the importance of signal transduction and cellular nitrogen compound metabolic process in the beginning of the stress in both tissues, the last one possibly related to nitrogen mobilization, since nitrogen status of plants under stress is a critical factor in stress tolerance (KOHLI et al., 2012). The importance of sugar metabolism in roots and photosynthesis and carbon fixation in leaves are also evidenced, confirming to the above mentioned results about RFOs in roots and photosynthesis related genes in leaves.

Li et al., (2016a) analyzing sugarcane differential profile on leaves in response to drought, found a high representation of some of the same enriched pathways that we found in this work (appendix E), such as plant hormone signal transduction, ribosome and purine metabolism. Furthermore, other pathways less represented on the data were also in common. Such as starch and sucrose metabolism, oxidative phosphorylation, ubiquitin mediated proteolysis, carbon fixation in photosynthetic organisms, phenylpropanoid biosynthesis, spliceosome, fatty acid degradation, among others.

The activity level of the pathways expressed in the different conditions and samples analyzed show a distinct separation between the expressed pathways found in leaves from the ones found in roots (figure 21). The up-regulation of pathways involved in the metabolism of several types of lipids after 6 days of drought in both roots and leaves was expected. Lipids are the major constituents of biological membranes that can sense extracellular conditions, functioning as signaling lipids in response to various environmental stresses and as stress mitigation, to reduce the intensity of the stressors, such as drought (OKAZAKI; SAITO, 2014).

In leaves, the up-regulation of terpenoid pathways may be related to the fact that drought significantly increase the terpene concentration in some species such as Pinus halepensis, Quercus ilex, Pinus sylvestris L. and Picea abies (L.) Karst. (BLANCH et al., 2008; TURTOLA et al., 2003). Further conclusions about the relationship between terpenoids and drought are difficult since there is not much information about terpenoid metabolism and its regulation in plants (MANSOURI; ASRAR; MEHRABANI, 2009).

Cells accumulate reactive oxygen species (ROS) during biotic and/or abiotic stresses and damage cellular structure and function (PRABU et al., 2011). The expression of antioxidant compounds is, therefore, highly desirable for the protection of the cell during such stresses. The avoidance of leaf damage is essential for the recovery of sugarcane plants from diverse biotic and abiotic stresses. This is the tissue responsible for the photosynthesis and the fixation of atmospheric carbon, thus, its well-functioning is indispensable for the response of the whole plant in face of different types of stresses.

Sugarcane is a C4 plant, therefore, photorespiration is nearly absent, decreasing the H2O2 generated by this process. In this context, most of the damage generated by ROS comes from the impairment of the processes that occurs in the chloroplast. The over-reduction of the components from the electron transfer chain along with the reduction in CO2 influx lead to a decrease in the oxidation of the NADPH in the Calvin Cycle, making NADP+ not sufficiently available and causing the generation of ROS, and consequently, photoinhibition and damage on cellular membranes.

In agreement with that is the observation that drought effects are more pronounced under higher PAR (Photosynthetically Active Radiation) (ZHAO; GLAZ; COMSTOCK, 2013), which can lead to chlorophyll degradation as well as the higher excitation of the chlorophyll molecules which could cause leakage of electrons once the acceptor is not available in enough quantities. This process can culminate in the generation of ROS. Furthermore,

drought leads to an energetic pressure at the PSII and at chloroplasts, making damage avoidance mechanisms, such as the activities of SOD and APX in the consumption of excess electrons necessary to avoid ROS accumulation and help sugarcane plants to keep its metabolism even in severe stress conditions, as demonstrated by Sales et al (2013). The ROS scavenging helps in the protection of its direct effects and also in relaxing the photon (electron) excess stress (ASADA, 2006).

In conjunction with that, my results demonstrate in leaves the up-regulation of Beta-1,3-glucanase precursor (SCQSRT2031D12.g) after 6 days of stress. This enzyme may constitute a link between biotic and abiotic stresses in sugarcane plants, once they act in fungal cell walls inducing the accumulation of phytoalexins, that has both antibiotic and antioxidant properties, highlighting the role of antioxidant protection in leaves (SOUZA, DIAS, SILVA-FILHO, 2017). This interplay between biotic and abiotic stresses as well as the necessity of avoidance of leaf damage is also demonstrated by the down-regulation of genes involved in photosynthesis in response to a variety of biotic stresses such as arthropods, fungi, bacteria and viral pathogens in Arabidopsis data sets (BILGIN et al., 2010) and the need to, despite that, keep photosynthesis working, allowing the recovery of the plant.

Regarding the KEGG pathways related to antioxidant responses, Lipoic acid metabolism was up-regulated in all the conditions in leaves opposing its down-regulation in all the conditions in roots which might mean that this oxidative stress response pathway has a tissue specific pattern of expression (figure 21).

The potential of free lipoic acid as a powerful metabolic antioxidant that is also able to scavenge the reactive oxygen species, to protect membranes by interacting with vitamin C and glutathione has been pointed out by some studies (GUEGUEN et al., 2000; PACKER; WITT; TRITSCHLER, 1995; SEN; ROY; PACKER, 1996). In roots, most of the pathways were down-regulated. Among the up-regulated pathways (figure 21), are: DNA repair and folate biosynthesis, and sulfur relay system. Folate has a role in DNA repair and turnover and might be required for adaptation to stress situations (RÉBEILLÉ et al., 2006). Sulfur relay system seems to be required for survival at extremely high temperatures in *E. coli* (MURATA et al., 2011) and, thus, has possible roles against stresses. These pathways are related to each other and might mean that in this tissue there is a tendency to repair the damage in the DNA. Therefore, our data may suggest that in leaves there is a tendency to avoid damage due to the stress, since ROS related pathways are up-regulated. In contrast, in roots there is a tendency to repair the damage, once several pathways related to DNA repair are activated.

3.5.5. miRNAs precursors identified

The analysis of RNA-Seq *de novo* assembly provided data for searching against RFAM database in an attempt to identify miRNAs precursors differentially expressed on sugarcane is response to severe drought. The search allowed the identification of 25 possible miRNA sequences in leaves, from which 8 were induced and 17 were repressed.

According to Ferdous, Hussain and Shi (2015), microRNAs are small noncoding RNAs with about 22 nucleotides in length and act as important regulators of genes at post-transcriptional level in a variety of organisms, being functionally conserved across plant species. They may be involved in various aspects of plant development, like leaf pattering, meristem function and abiotic stresses such as drought, freezing, salinity, high temperatures (HAMZA et al., 2016) and regulate their target mRNAs by transcript cleavage and/or inhibition of protein translation (SORIN et al., 2014).

The up-regulated miRNAs precursors found were: mir-399, mir-1846, mir-171_1, mir-228, mir-167_1 and mir-159. The down-regulated ones were: mir-168, mir-160, mir-162_2, mir-398, mir-169_5, mir 393, mir-156, mir397, mir-169_2, mir-167_1 (table 6). In roots, mir-164 and mir-393 were also found repressed (table 6).

Among the induced ones, mir-171 has a role in response to abiotic stresses and floral development and targets GRAS transcription factors and, NaCl stress response, floral asymmetry and leaf development. The mir-399 has a role in response to phosphate starvation (FUJII et al., 2005). The mir-1846 may target proteasome subunit alpha type 1 or other hypothetical/expressed proteins (XUE; ZHANG; XUE, 2009) and opposing to this work was found down-regulated by drought in rice (ZHANG et al., 2017) and mir-228 appears to be a sequence from *C. elegans* (LIM, 2003).

In the case of repressed miRNA sequences, it was found a mir-398 which targets copper superoxide dismutases. The mir-162 seems to target DCL1 and has a role in miRNA biogenesis; the mir-156 targets SQUAMOSA promoter binding protein-like (SPL) genes (GAO et al., 2016) and SBP family of transcription factors to promote phase transitions, flowering time and the mir-393 targets AFB2 and AFB3 and act on susceptibility to virulent bacteria.

Interestingly mir-160 that targets ARF 10, 16 and 17 and has a role in seed germination and post-germination was also down-regulated in rice (ZHANG et al., 2017). Studying drought-responsive miRNAs in *Sorghum bicolor* (L.) Moench, Katiyar et al (2015) found the up-regulation of members from mir-160 family in M35-1 drought-tolerant genotype and down-regulation of mir-169 family members in both drought-tolerant and susceptible genotypes. The last one agrees with the analysis from the present work.

Gao et al., (2016) found mir-162, mir-398 and mir-156 families of miRNAs in *Ammopiptanthus mongolicus*, a desert plant. One member of the mir-156 family was up-regulated, and from the mir-398 family there were members both induced and repressed by drought stress.

The mir-167, mir-168, mir-393, mir-397 were also found deregulated in a study performed on 11 different Sudanese *Sorghum* plants exposed to drought stress (HAMZA et al., 2016). The isoforms of mir-169 seems to target subunits A of NF-Y (nuclear factor Y) TFs. These transcription factors are ubiquitous and may have a role in drought responses. Therefore down-regulation of miRNA 169 is expected during drought stress as found by this work and also by Li et al., (2008) who observed that overexpression of miR169a in *Arabidopsis* caused enhanced leaf water loss and sensitiveness to drought stress and its down-regulation was through an ABA-dependent pathway.

According to Liu et al., (2008), miR-168 and miR-171 are responsive to drought, salinity and cold stresses and miR-393 was found responsive to drought stress induced by treatment with 200 mM mannitol. This work proposed roles for some miRNAs, such as mir-159 targeting MYB and TCP transcription factors and having a role in ABA response. The mir-167 targets ARF6 and ARF8 and has a role in gynoecium and stamen development; mir-168 that target Argonaute1 also has a role in MAPK-miRNA biogenesis, mRNA degradation and plant development and mir-397 appears to target laccases and act on lignin biosynthesis, ion absorption and stress response (GAO et al., 2016).

Gao et al., (2016) also found mir-164 and mir-393 families as responsive to drought, despite that, the member from mir-164 was down-regulated as in this work, but the member from mir-393 was induced. Liu et al., (2017) found members of the mir-168 family down-regulated in tomato sensitive genotype, but up-regulated in the tolerant line, the same thing for members of the mir-171, while mir-399 was up-regulated in both. In the case of the present work, mir-168 was down-regulated (similar to tomato drought sensitive genotype), but mir-171 was up-regulated (similar to tolerant genotype) and mir-399 was also up-regulated.

Similar to the study of Zhang et al., (2017) in *Oriza rufipogon*, putative miRNAs sequences found in this study in sugarcane seems to be responsive to drought stress, and could play different roles in the regulation of Auxin and ABA pathway, salt stress, abiotic stress and even flowering pathway. Furthermore, in the present work, we found a higher number of down-regulated miRNAs which agrees with the stated by Hamza et al., (2016) who states that in general the expression levels of miRNAs are deregulated by the environmental stresses.

Gentile et al., (2015), also found, in two sugarcane differing in their tolerance to drought, members of the micro RNAs families whose precursors were found here. Among those members were mir-160, mir-164, mir-169, mir-171, mir-393, mir-397, mir-399. From these, mir-164 was found repressed in RB855536 and its precursor was also repressed in roots in the present work. Furthermore, mir-397 was repressed in RB867515 after 4 days of stress in greenhouse, mir-393 after 2 days in RB867515 and after 4 days in RB855536; and mir-169 was repressed in 7 months RB867515 in field. Their precursors sequences were also found down-regulated after 6 days of stress in the present work.

These results indicate it would be interesting to make small RNA libraries of leaves and roots samples aiming to analyze drought related miRNAs found in different sugarcane genotypes, such as more commercial or more drought tolerant and susceptible varieties.

3.6. Conclusion

The analysis of differentially expressed genes by different strategies allowed the uncover of several layers of information that being strict to a unique methology would not allow. Data showed differential expression of hormone related genes, phenylpropanoid biosynthesis, osmoprotection, antioxidant response in leaves and roots, and demonstrated the correlation between physiological responses and changes in gene expression. For the next steps it would be interesting to analyze metabolites, such as galactose and also phytohormones to draw further conclusions.

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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 13 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 15 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 comprise 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.

4.3.1.3. RNA Extraction and cDNA synthesis

Total RNA from leaf samples of drought progression experiment was extracted according to Zeng; Yang, (2002) with modifications, as described in Chapter 1. RNA was re-suspended in 30 μL of nuclease free water, and its concentration was determined with a NanoDrop (Thermo Scientific).

In order to eliminate genomic DNA contamination, RNA samples were treated with DNase I, amplification grade (Invitrogen) according to the manufacturer's instructions with some modifications and subsequently submitted to cDNA synthesis using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

4.3.1.4. RT-qPCR

The reactions were performed using Fast SYBR® Green Master Mix (Applied Biosystems, catalogue number 4385612) and the 7500 Fast Real-Time PCR System (Applied Biosystems). In each reaction 5 μL of 1.5 μM primer mix, 1 μL of cDNA in the proportion of 1:10 and 6 μL of Fast SYBR® Green Master Mix was used. The whole reaction was composed of 12 μL and the final concentration of the primer was 625 nM. Primers of genes of interest were chosen according to previous results obtained through microarray analysis (chapter 1), and reference genes were selected using geNORM excel applet. Gene expression analysis was performed using REST software.

4.3.2. Co-Expression Network

The Weighted Gene Correlation Network Analysis (WGCNA) was performed in collaboration with Dr. Augusto Lima Diniz. The analysis considered two experiments previously done by the group in addition to the microarray expression data generated in this study. The first one was carried out under greenhouse conditions with the variety SP90-1638 and samples from leaves at 24, 72 and 120 hours of dehydration (and controls) were collected for gene expression analysis by microarray (LEMBKE et al., 2012). The second experiment included leaves and internode I from 6-month-old plants belonging to the varieties RB86-7515, RB85-5536 and RB92-579 grown under field conditions and also collected for expression analysis (COSTA, 2011). In all cases, two biological replicates and dye swaps were used for oligoarray hybridization and data processing, normalization and analysis, as described in section 2.5.3 and in Lembke et al., (2012). The output was a data set including 52 samples and 17.097 significant expressed genes.

The WGCNA R package (LANGFELDER; HORVATH, 2008) was used for cleaning and pre-processing the expression data, as well as for co-expression analysis. Genes and samples with excessive missing value (> 10%) were filtered and outliers were identified by cluster analysis and manually removed from the final dataset, which consisted of 48 samples and 11.985 genes.

The adjacency matrix was calculated by estimating the Pearson's correlation coefficient between all pair of genes, and raised to a soft thresholding power (β) of 13. The power β was chosen based on the scale-free topology criterion (ZHANG; HORVATH, 2005), resulting in a scale-free topology index (R2) of 0.79 (figure 43). To minimize effects of noise and spurious associations, the Topological Overlap Measure (TOM), representing the overlap in shared neighbors, was calculated using the adjacency matrix. The dissimilarity TOM was used as input for the hierarchical clustering analysis, and modules (clusters of highly co-expressed genes) were detected. The module eigengene (ME)

was used to represent each module, which was calculated by the first principal component, thereby capturing the maximal amount of variation of the module. The MEs clustering dendrogram was evaluated, and similar modules (distance ≤ 0.1) were manually merged. In addition, Module-Trait relationships were estimated by calculating the Pearson's correlations coefficient between the ME and the following traits of interest: Fv/Fm, leaf water potential, photosynthesis, transpiration and stomatal conductance. Those Module-Trait relationships were used to select potential biologically interesting modules for downstream analysis. Furthermore, modules of interest were also chosen having in consideration the presence of genes belonging to the ABA-dependent and independent pathways selected from the microarray experiment described on chapter 1.

Node and edge files were generated for use with the Cytoscape 3.5.1 network visualization program (SHANNON, 2003). In Cytoscape 3.5.1, it was chosen the undirected type of interaction for network generation. The networks created were analyzed by estimating the following parameters: degree, betweenness and closeness. Subnetworks of genes related with signal transduction and transcription were built in addition to the complete network in which the nodes with highest degree, betweenness and closeness centralities were highlighted (appendix G).

4.4. Results

4.4.1. Drought Progression

4.4.1.1. Physiology

The results from the experiment done in the CAPS greenhouse show that plants were visually stressed after 7 days without watering (figure 43b). The leaves showed the classical symptoms of drought stress, such as wilting and yellowing of the plant tissue (figures 44b and 47a).

Figure 43. Pictures shows an extreme difference between treatment and control plants ((b) treatment and (d) control) after drought imposition, with plants before the treatment (a) and (c) showing no signal of stress

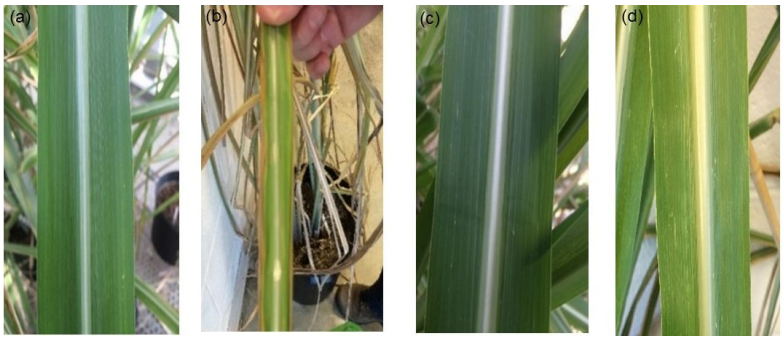

Figure 44. Leaves of plants before the treatment (a) and (c) show no morphological alteration, while after the treatment, control leaves (d) continues showing no symptoms and stressed leaves (b) shows the classical symptoms of drought such as wilting

The parameters of photosynthesis (figure 45a), stomatal conductance (figure 45b), transpiration (figure 45c) and soil humidity (figure 45d) show a progressive decrease after the treatment started when compared with control samples. In the case of photosynthesis, transpiration and soil humidity a significant statistical difference was observed after day 2, for the stomatal conductance, such difference was observed from day 4 and on. In day 7 there was a decrease of about 63% in soil humidity, which reflected in a decrease of 98% in photosynthesis, 83% in stomatal conductance and 82% in transpiration. All parameters show a full recovery of the plants 24 hours after re-watering.

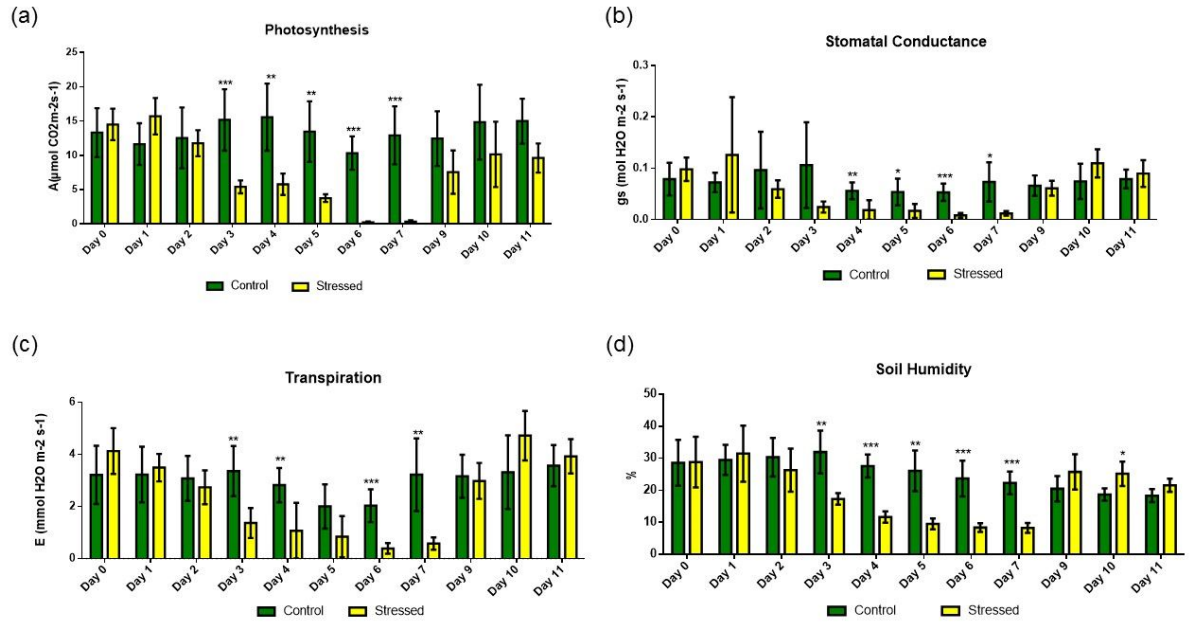

Figure 45. Graphs of (a) Photosynthesis; (b) Stomatal conductance; (c) Transpiration and (d) Soil Humidity during the seven days of drought experiment and after re-hydration (day 9) shows that stress was extreme in the seventh day and plants fully recovered 24 hours after re-watering. Bars show standard deviation and asterisks indicate a significant statistical difference, according to t test, therefore, *; **; *** for $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively. Arrows indicates the first measures after re-watering the plants.

The correlation coefficient between stomatal conductance (figure 46a), photosynthesis (figure 46b) and transpiration (figure 46c) and soil humidity indicates that the progressive drying of the soil was reflected in the physiology status of the plants.

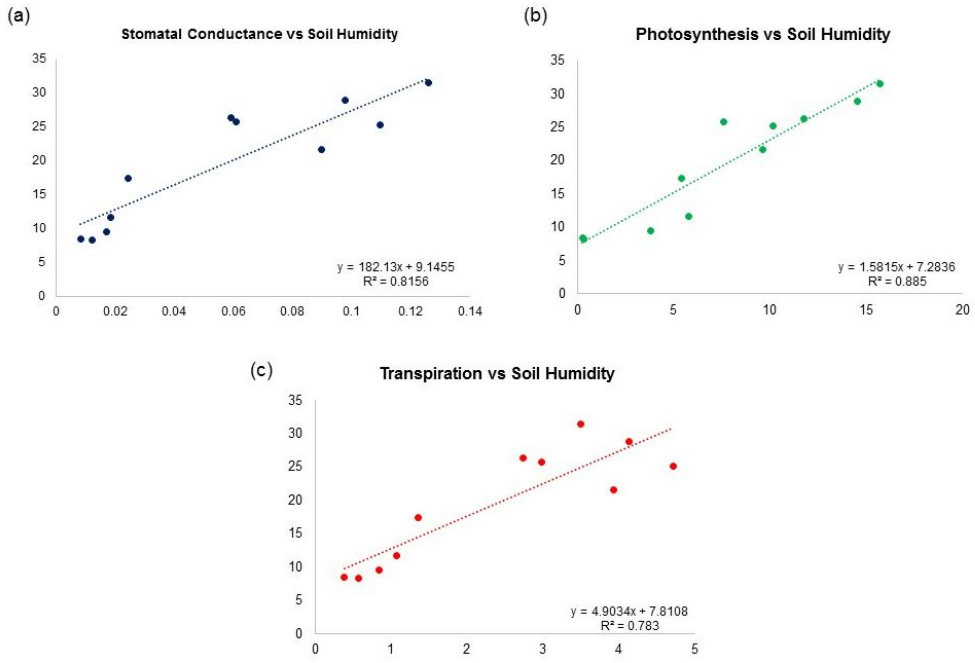

Figure 46. Correlation analysis showing that progressive drying of the soil was reflected on the plant's physiology (a) stomatal conductance vs soil humidity; (b) photosynthesis vs soil humidity; (c) transpiration vs soil humidity.

4.4.1.2. RT-qPCR

Leaves samples from the drought progression experiment performed at The Ohio State University greenhouse (35º C) were submitted to RNA extraction and cDNA synthesis which was then, used for RT-qPCR reactions. GeNorm software was used for the selection of the most suitable reference genes, therefore, three reference genes were chosen: SCCCLR1072A03.g – Mitochondrial ATP Synthase subunit delta, SCJFRZ2009G01.g – 60S Ribosomal Subunit and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (ISKANDAR et al., 2011) (primer sequences on appendix A).

The genes for the RT-qPCR reactions were selected according to the differentially expressed transcripts found from the microarray experiments described on chapter 1. A total of 24 genes involved in ABA-dependent, ABA-independent and hormone crosstalk reactions were selected. Furthermore, a datamining on SUCEST database was also performed with the aim of selecting genes involved with the biosynthesis and signaling pathways of other hormones, such as ethylene and auxin for future analysis. Interestingly, most of the selected genes were also present in different modules obtained from gene co-expression analysis and had correspondence with genes already described in the literature that have some involvement in drought responses. The reason of choice for each gene and their presence on microarray data and co-expression modules are shown in table 9.

(continue)

Table 9. Genes selected for qRT-PCR reactions for drought progression experiment

SAS	Description	Reason for Choice	Microarray HTSelf	Microarray FC	SAS data mining	Co-Expression Module	Literature Validation	Reference
SCBFRZ3008D06.g	9-cis-epoxycarotenoid dioxygenase chloroplastic-like	ABA Biosynthesis	✓	✓		Light Yellow	✓	Endo et al., (2008)
SCCCLR1048H03.g	Viviparous 14	ABA Biosynthesis	✓	✓		Dark Green	✓	Lu et al., (2017)
SCVPRZ3030G09.g	Probable esterase PIR7A	ABA Receptor	✓			Green Yellow		
SCVPFL3046C06.b	Phosphatase 2C family	ABA Signaling	✓	✓		Bisque 4	✓	Zhang et al., (2013)
SCEPRZ1010E06.g	Probable phosphatase 2C 50	ABA Signaling	✓	✓		Bisque 4	✓	Zhang et al., (2013)
SCCCLR1C03C05.g	ABA responsive element binding factor 1	ABA TF	✓	✓		Pale Violet Red	✓	Amir Hossain et al., (2010)
SCQGLR1085F11.g	Dehydrin 9	ABA Induced	✓	✓		Sienna 3	✓	Hassan et al., (2015)
SCQSRT2031H06.g	Late embryogenesis abundant 3	ABA Induced	✓			Sienna 3	✓	Chen et al., (2015)
SCSGLR1045D05.g	HVA22 e	ABA Induced	✓	✓		Black (AS) / Light Cyan (SS)	✓	Hayano-Kanashiro et al., (2009)
SCBFRZ2046E11.g	Aspartic protease in guard cell 1	ABA Induced	✓			Light Yellow (AS/SS)	✓	Yao et al., (2012)
SCBFSD1037G05.g	desiccation-related PCC13-62-like	ABA Induced	✓	✓		Plum 1	✓	Zhae et al. (2015)
SCVPRZ2041G12.g	indole-3-acetic acid amido synthetase	Auxin Biosynthesis	✓			Green Yellow	✓	Zhang et al., (2009)

(continuation)

SAS	Description	Reason for Choice	Microarray HTSelf	Microarray FC	SAS data mining	Co-Expression Module	Literature Validation	Reference
SCMCRT2085F03.g	probable indole-3-pyruvate monooxygenase YUCCA10	Auxin Biosynthesis (rate limiting step)			✓		✓	Lee et al., (2012) (YUCCA7)
SCCCRT2001C12.g	Auxin-binding 5	Auxin Receptor			✓			members of the family identified
SCBGFL5079F06.g	Auxin Signaling F-BOX 3	Auxin Signaling			✓	Floral White (AS)		
SCCCCL3120A10.b	Auxin response factor	Auxin TF	✓					members of the family identified
SCCCRT1C01F08.g	SAUR11 - auxin-responsive SAUR family member	Auxin Induced		✓	✓	Light Yellow		
SCCCRZ2003H05.g	auxin-responsive IAA17	Auxin Induced		✓	✓	Black		members of the family identified
SCJLRT1006C03.g	1-aminocyclopropane-1-carboxylate synthase (ACC synthase)	Ethylene Biosyntheis			✓			reference to ethylene in general
SCEZLR1009E06.g	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	Ethylene Biosyntheis		✓	✓	Light Green		reference to ethylene in general
SCSBAD1086C02.g	Ethylene response sensor 2	Ethylene Receptor			✓		✓	Hopper et al., (2016)
SCSBHR1056H08.g	Ethylene insensitive 2	Ethylene Signaling			✓			reference to ethylene in general
SCCCCL4002B07.g	Ethylene-responsive factor 1	Ethylene TF	✓	✓		Green Yellow	✓	Cheng et al., (2013)

(continuation)

SAS	Description	Reason for Choice	Microarray HTSelf	Microarray FC	SAS data mining	Co-Expression Module	Literature Validation	Reference
SCJFLR1073H12.g	Delta 1-pyrroline-5-carboxylate synthetase 1	Ethylene Induced (Proline Biosynthesis)	✓	✓		Green	✓	Kishor et al., (1995)
SCJLAM1062A05.g	probable phospholipid hydroperoxide glutathione peroxidase	Ethylene Induced	✓	✓		Dark Green	✓	Miao et al., (2006)
SCCCCL4007F05.g	glutathione *S*-transferase GSTU6	Ethylene Induced	✓	✓		Light Cyan	✓	Rezaei et al., (2013)
SCBFRZ2048D04.g	CBL-interacting kinase family	Hormone Crosstalk - Signaling	✓			Dark Olive Green	✓	Wang et al., (2016a)
SCEZAM2034C10.g	Probable calcium-binding CML10	Hormone Crosstalk - Signaling	✓				✓	Zeng et al., (2015) - family
SCJFRZ2014D06.g	NAC transcription factor	Hormone Crosstalk - TF	✓	✓		Light Cyan	✓	Hong et al., (2016) - family
SCVPRZ3026C06.g	DRE binding factor	Hormone Crosstalk - TF	✓			Dark Grey	✓	Kizis; Pagès, (2002) - family
SCCCCL6024F07.g	Cinnamoyl-reductase 1-like	lignin biosynthesis	✓	✓		Plum 1	✓	Fracasso; Trindade; Amaducci, (2016)
SCSGLV1008B03.g	Flowering-promoting factor 1 1	involved in flowering	✓	✓				
SCEPLB1043F06.g	Aspartic proteinase nepenthesin-2	catalytic activity similar to pepsin	✓	✓		Green Yellow		
SCJFLR1017E09.g	Stachyose synthase precursor	involved in the biosynthesis of RFOs sugars	✓	✓		Sienna 3	✓	Zhou et al., (2014)

(conclusion)

SAS	Description	Reason for Choice	Microarray HTSelf	Microarray FC	SAS data mining	Co-Expression Module	Literature Validation	Reference
SCCCCL3003E07.b	Chitinase partial	cleavage of chitin	✓	✓		Salmon (AS)	✓ (down)	Fracasso; Trindade; Amaducci, (2016)
SCCCRT3001A02.g	Endochitinase A-like	cleavage of chitin	✓	✓		Light Cyan	✓	Kwon et al., (2007) - family
SCSGAD1007F02.g	Transcription factor MYB108	negative regulator of abscisic acid-induced cell death	✓			Light Green	✓	Baldoni; Genga; Cominelli, 2015)
SCEPSD1007C11.g	Pathogenesis-related class partial	induced in response to infection	✓	✓			✓	Gregorová et al., (2015)
SCJLLR1107D06.g	hypothetical protein SORBI_002G141800	Signal Peptide - SignalP-TM	✓	✓		Sienna 3		
SCBGLR1117C01.g	---NA---	Signal Peptide - Non cytoplasmic	✓	✓		Plum 1		
SCEZAD1C01D10.g	---NA---	Signal Peptide - Non cytoplasmic	✓	✓		Light Yellow		
SCRLLR1038F07.g	hypothetical protein SORBIDRAFT_06g026710 (interessei nela)!	ABA related Interpro domain	✓	✓			✓	Hou et al., (2013)

The analysis of gene expression during the progression of drought indicate that SCBFRZ3008D06.g – 9-cis-epoxycarotenoid dioxygenase chloroplastic-like and SCSGAD1007F02.g – MYB108 transcription factor have a similar pattern of expression with the expression starting to increase in the forth day, having a peak in the fifth and sixth days and then starting to decrease (figure 47). The SCCCCL6024F07.g – cinnamoyl-reductase 1-like is down-regulated in the first day, but starts to become induced on the forth day, while SCBFSD1037G05.g – Desiccation-related PCC13-62-like is highly up-regulated from day four and on (figure 47).

The putative esterase SCVPRZ3030G09.g – PIR7A, SCCCCL3003E07.b – chitinase partial, SCCCRT3001A02.g – endochitinase A-like, SCSGLV1008B03.g – flowering promoting factor 11, SCEPSD1007C11.g – pathogenesis-related class were also up-regulated from day 4 and on, but the first and the second ones are down-regulated on the second day (figure 47).

The SCJFLR1017E09.g – Stachyose synthase precursor and SCQGLR1085F11.g – dehydrin 9 are slightly down-regulated in the first day, but became up-regulated from the third day and on (figure 47). The up-regulation starting on the third day is also true for SCQSRT2031H06.g – LEA3, SCCCLR1C03C05.g – ABA responsive element binding factor 1, SCSGLR1045D05.g – HVA22 e, SCJFRZ2014D06.g – NAC transcription factor and SCEPRZ1010E06.g – PP2C 50 (figure 47).

The SCCCLR1048H03.g – Viviparous 14 is down-regulated in the first day, up-regulated from the third to the sixth day, and not differentially expressed anymore in the seventh day (figure 47). A gene from the PP2C family (SCVPFL3046C06.b) is also down in the first day and then become up-regulated from the forth to the seventh say, the same pattern happening for the SCBFRZ2046E11.g - aspartic protease in guard cell 1 (figure 47). The SCEZAM2034C10.g – calcium-binding CML10 is up-regulated from day five to seven and SCVPRZ3026C06.g – DRE binding factor is up-regulated only on days five and six (figure 47).

The genes of Auxin Response Factor (SCCCCL3120A10.b), Ethylene-responsive factor 1 (SCCCCL4002B07.g) and CBL-interacting kinase family (SCBFRZ2048D04.g) that showed a difference in the expression on the microarray showed no significant difference in expression on the drought progression experiment. Interestingly, the expression of most of the genes start to decease on the seventh day (figure 47).

Moreover, from the chosen genes for evaluation by RT-qPCR (both the ones evaluated in the present work as well as the ones selected for future evaluation) 32 belonged to 15 modules found on the co-expression analysis (item 3.4.3) (table 9). Green yellow, sienna3 and light green modules harbored 4 genes; plum1 and light cyan 3 genes; bisque4, black, dark green and light green had 2 of the selected genes; and dark grey, dark olive green , floral white, green, pale violet red and salmon hosted only one of the genes of interest (table 9).

Interestingly, from the genes with a smaller distance observed at the heatmap (figure 47), only Dehydrin 9, Late Embryogenesis Abundant 3 and Stachyose Synthase Precursor belong to the same co-expression module (table 9).

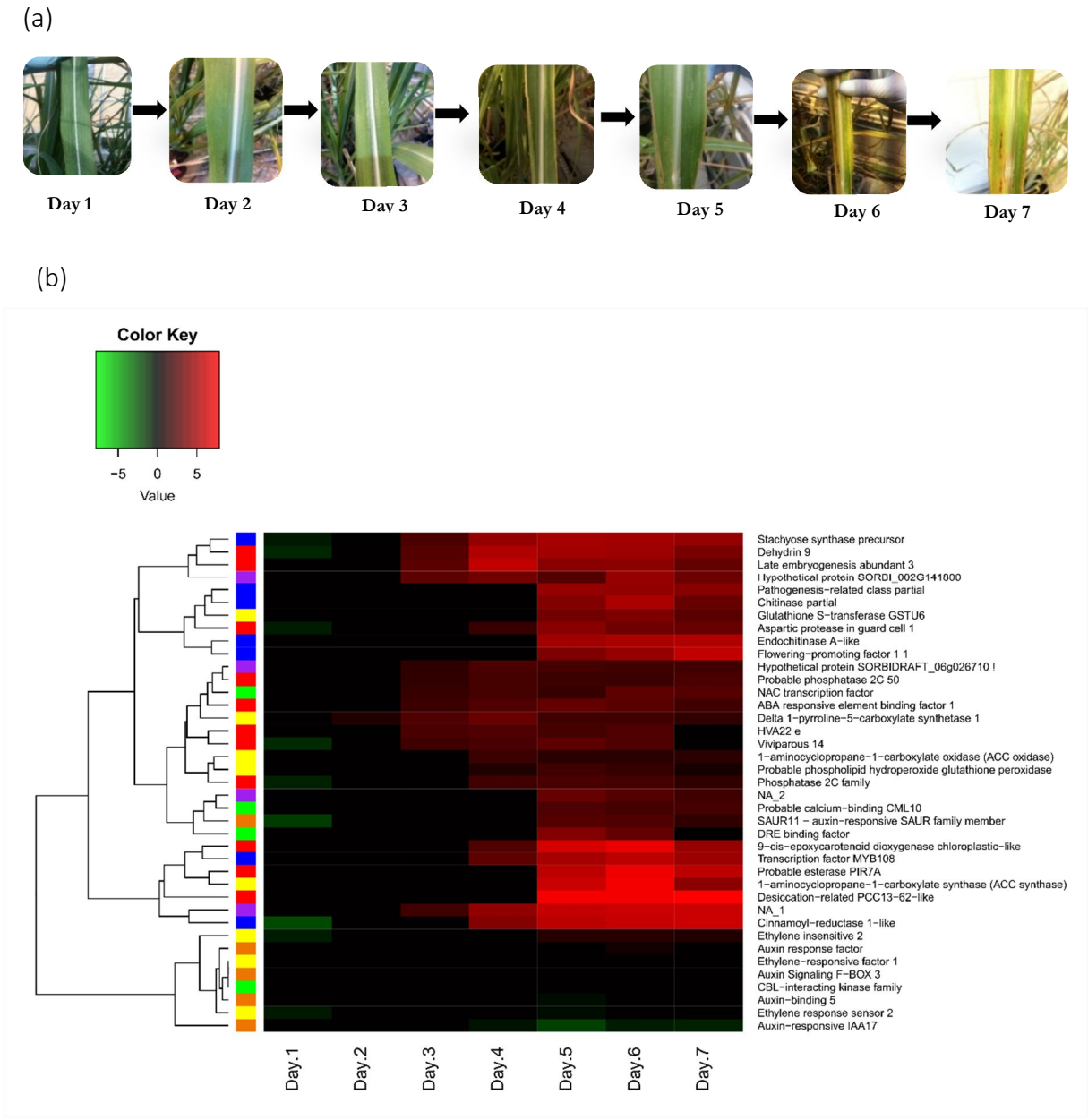

Figure 47. The Heat map shows the pattern of expression of chosen genes during the progression of drought and pinpoint the similarity in the expression of Dehydrin 9, Late Embryogenesis Abundant 3 and Stachyose Synthase Precursor, all of them belonging to Sienna3 co-expression module. (a) morphological changes on sugarcane leaves during the progression of the stress; (b) heatmap; *NA_1-SCBGLR1117C01.g, NA_2-SCEZAD1C01D10.g.

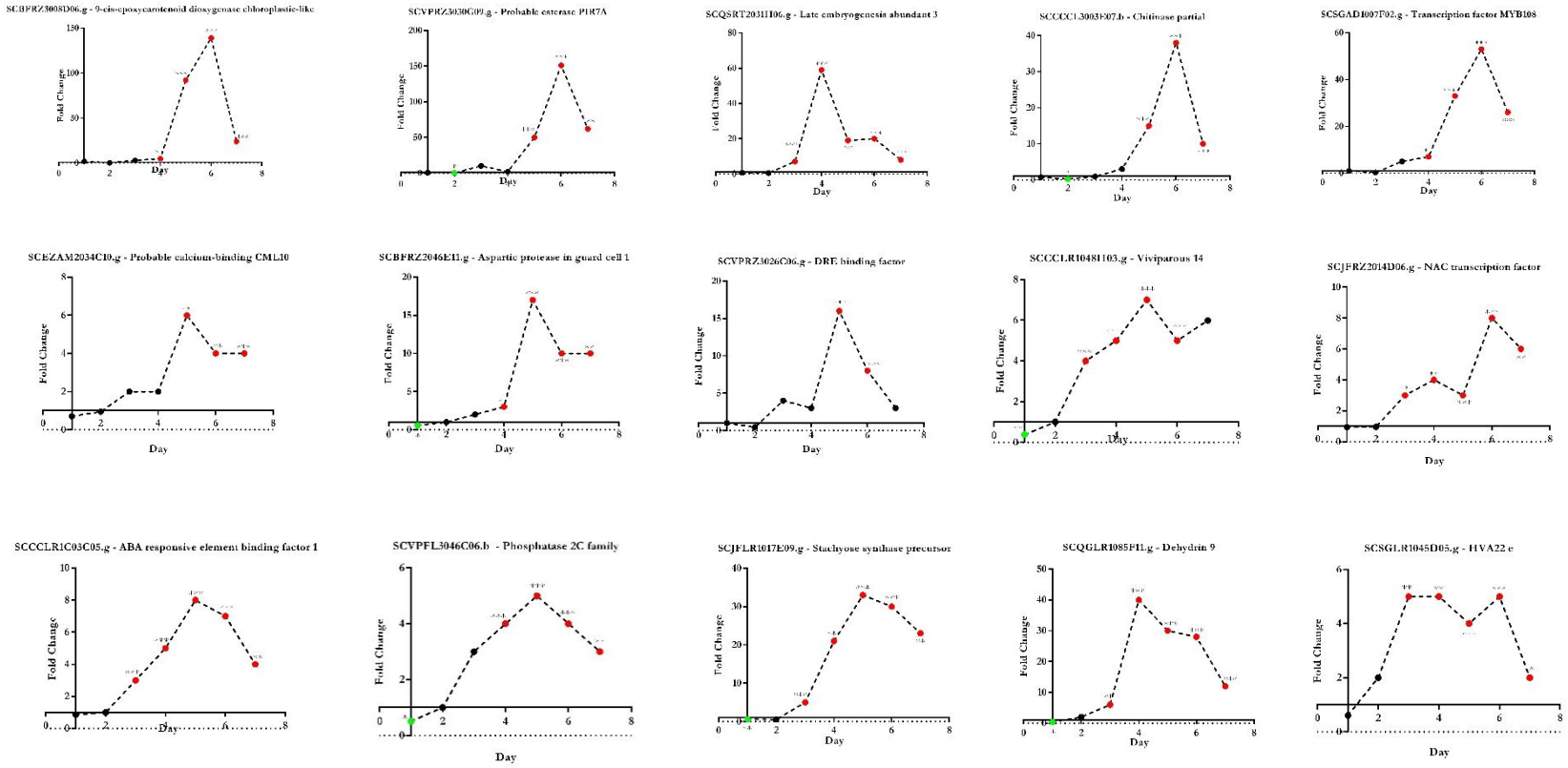

Figure 48. RT-qPCR results from drought progression experiment shows that most genes become induced after the third day (part 1) (graphs shows a subset of 24 from the 39 chosen genes). Asterisks indicate statistical significance, therefore, *; **; *** for $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively

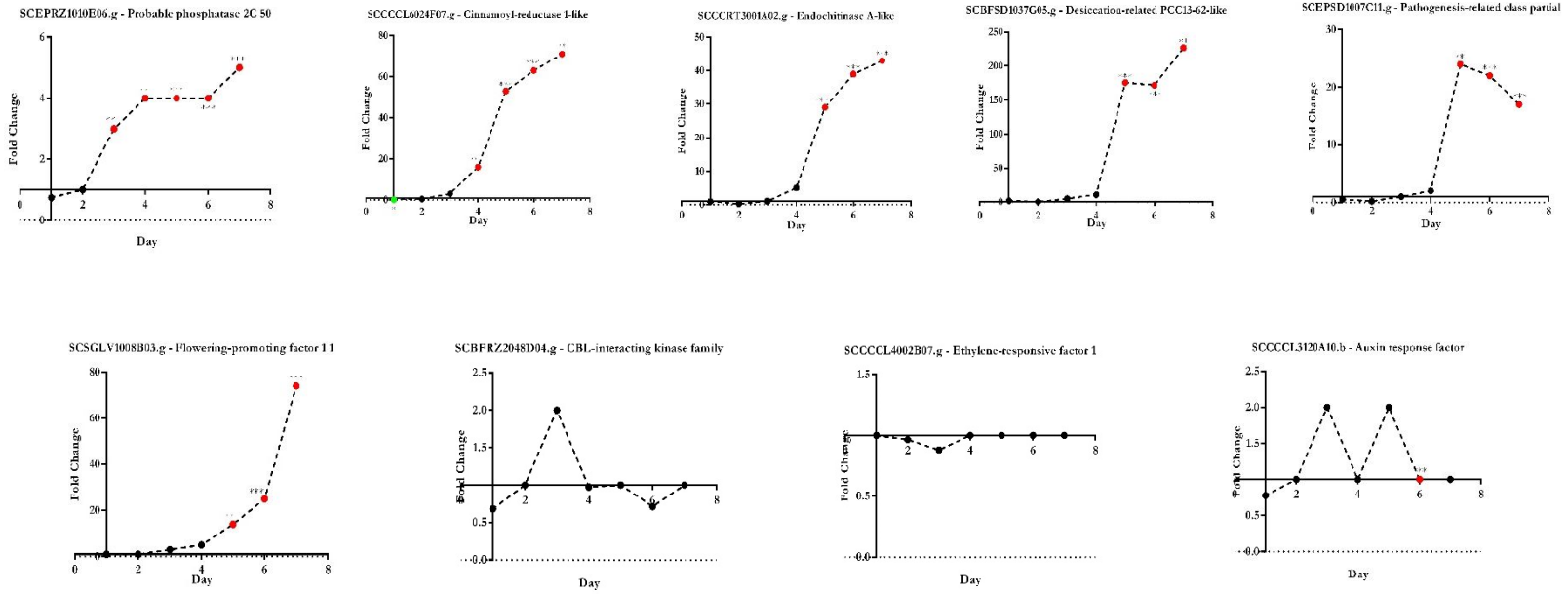

Figure 49. RT-qPCR results from drought progression experiment shows that most genes become induced after the third day (part 2) (graphs shows a subset of 24 from the 39 chosen genes). Asterisks indicate statistical significance, therefore, *; **; *** for $p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$, respectively

4.4.2. Co-expression Network

4.4.2.1. Co-Expression Network Construction

Co-Expression Networks were built using a data set of 52 samples containing 17,097 significantly expressed genes, from which the ones with and excessive missing value (>10%) along with the outliers identified by cluster analysis were filtered and removed from the final data set, that resulted of 48 samples and 11,985 genes (figure 50).

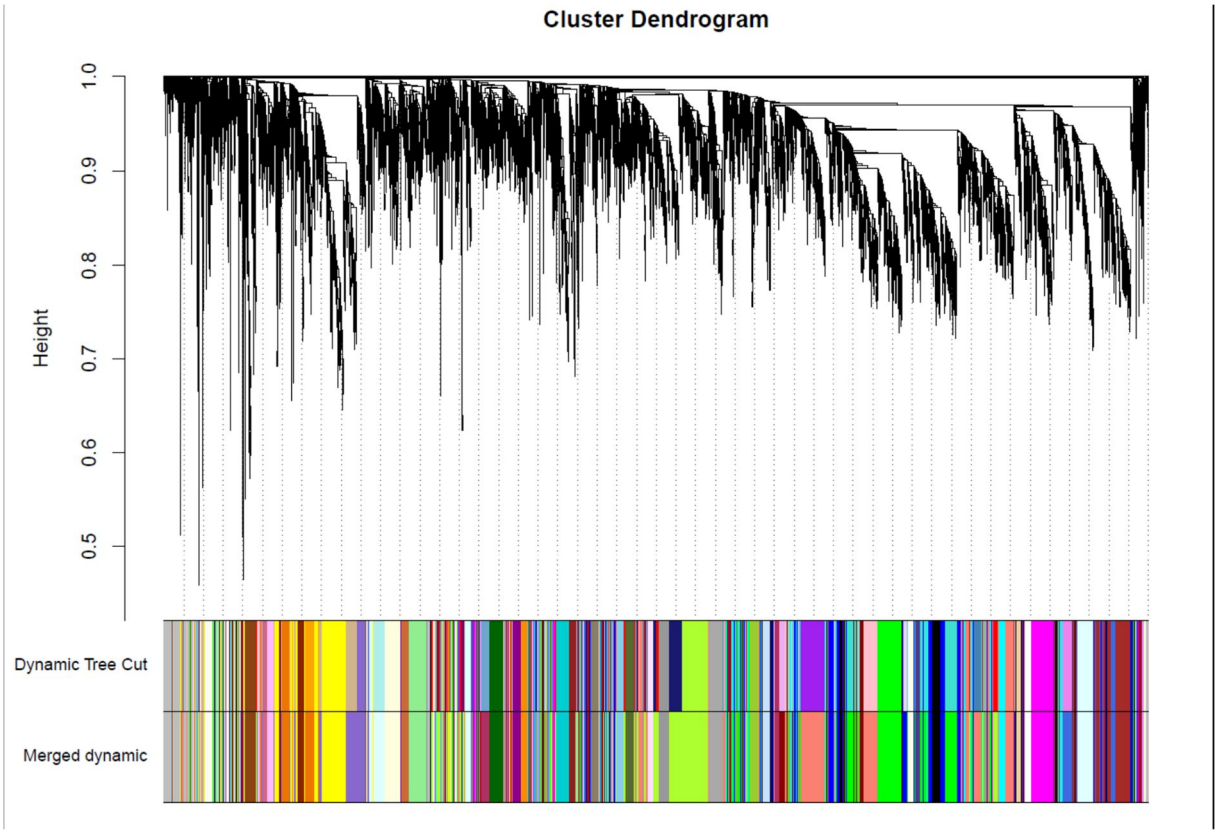

Figure 50. The filtered 11,985 significantly expressed genes from 48 samples were grouped into 56 modules which were lately merged into 43 final modules. The "Height" value refers to the topological overlap (TO) distance between the genes. The different colors represent the different modules, which contain at least 30 genes.

The filtered genes were analyzed and grouped into 55 modules of highly co-expressed genes and one module, denominated "grey module" that contained the genes that were not co-expressed with any of the other genes. The modules were further merged and generated a set of 43 more similar modules (figures 50 and 51).

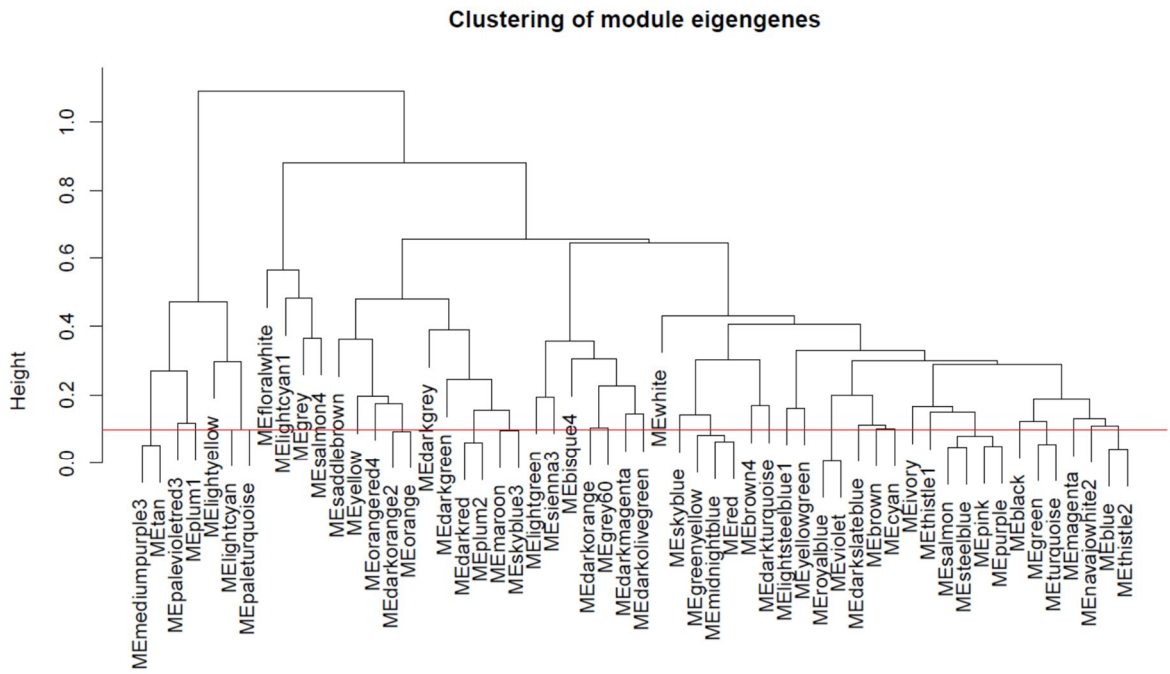

Figure 51. Clustering of module eigengenes (distance ≤ 0.1) reduces the number of modules from 56 to 43 more similar ones.

Two strategies were adopted to relate modules to a priori information and find the biologically interesting ones. The first was to perform a Module-Trait relationship analysis, which allowed the selection of three modules that had a high positive and significant ($p<0.01$) correlation to the following physiological parameters of intrest: Fv/Fm, leaf water potential, photosynthesis, transpiration and stomatal conductance. These parameters are highly changed by drought stress (figure 52). The modules selected using this strategy were "Dark Orange 2", "Dark Red" and "Maroon", which counted with 236 SAS in the sense strand (SS) and 11 in the anti-sense strand (AS); 275 SS and 28 AS SAS; and 165 SS and 9 AS SAS, respectively (figure 52).

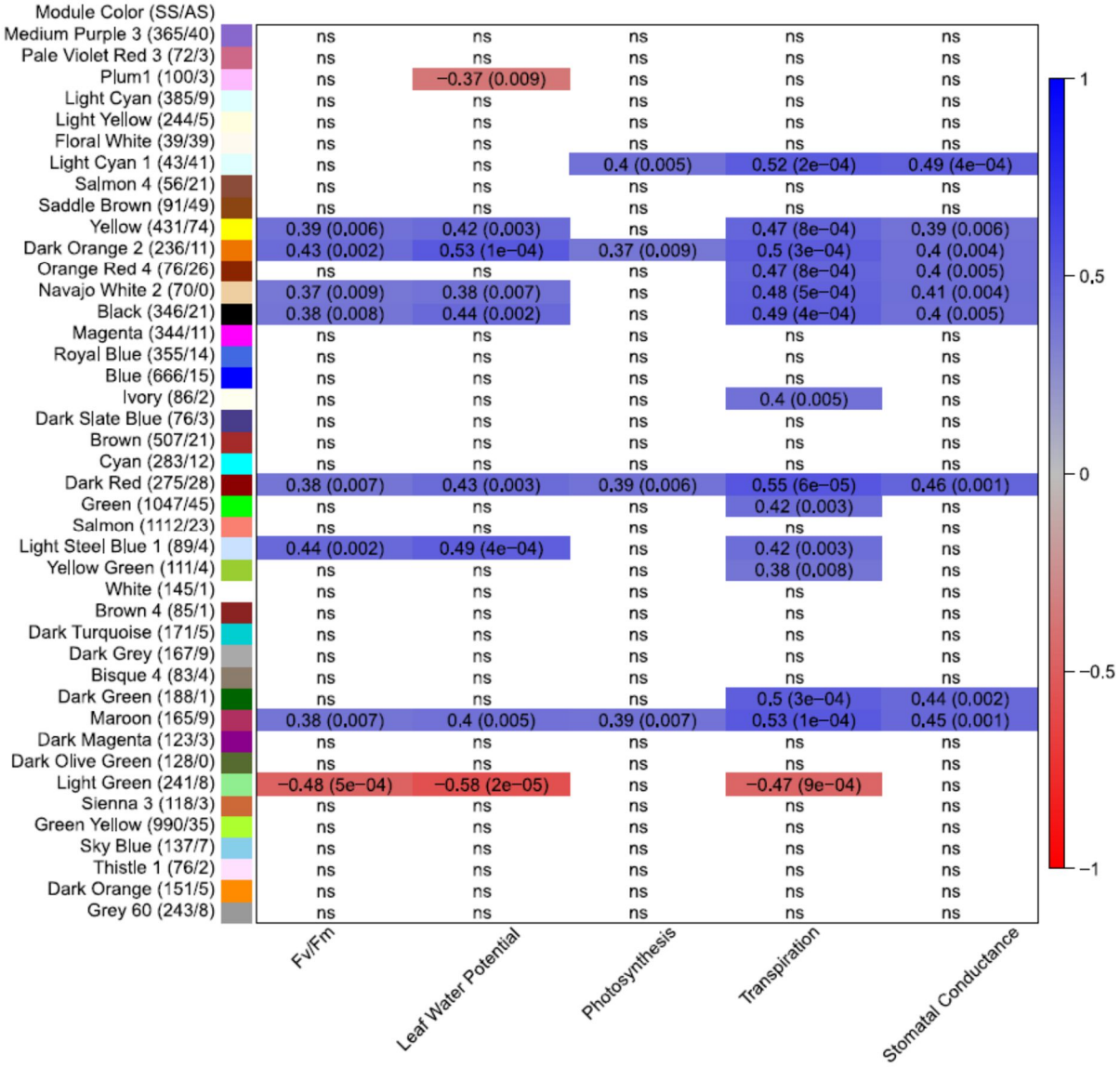

Figure 52. The Module-Trait relationship analysis helped in the identification of the modules positively correlated with the physiology parameters of interest. $p < 0.01$

The second strategy was to analyze the modules in which the genes chosen for the drought progression experiment appeared. That group counted with genes that belonged to ABA-dependent as well as to ABA-independent and hormone crosstalk pathways and involved 15 of the co-expressed modules found, already mentioned on the previous section (table 9).

4.4.2.2. Analysis of selected Co-Expression Networks

4.4.2.2.1. Networks Selected from Module-Trait Relationship

The analysis of the selected modules was done initially trying to find an Orthologous Group Annotation that would summarize the role of the modules of interest. Unfortunately, no annotation could summarize the modules

individually and all three modules had "Function Unknown", followed by "Posttranslational modification, protein turnover, chaperones" on Dark Orange 2 module and "Signal Transduction Mechanisms" on Dark Red and Maroon modules as the most represented terms (table 10).

Table 10. Top Orthologous Group sequence distribution (percentage) of nodes found in the modules selected from Module-Trait Relationship analysis

Orthologous Group	Dark Orange 2	Dark Red	Maroon
Posttranslational modification, protein turnover, chaperones	12	9	10
Function unknown	21	30	28
Intracellular trafficking, secretion, and vesicular transport	4	4	4
Transcription	5	7	9
Signal transduction mechanisms	3	10	13
Carbohydrate transport and metabolism	5	2	6
RNA processing and modification	3	3	2
Energy production and conversion	5	3	3
Amino acid transport and metabolism	7	2	4
Replication, recombination and repair	3	4	3
Inorganic ion transport and metabolism	3	3	2
Cell wall/membrane/envelope biogenesis	2	1	0
Translation, ribosomal structure and biogenesis	9	7	2
Defense mechanisms	2	1	1
Cell cycle control, cell division, chromosome partitioning	1	2	1
Lipid transport and metabolism	4	4	4
Chromatin structure and dynamics	1	0	1
Cytoskeleton	0	1	1
Secondary metabolites biosynthesis, transport and catabolism	2	1	2
Nucleotide transport and metabolism	2	2	1
Nuclear structure	0	0	0
Coenzyme transport and metabolism	4	2	2
Extracellular structures	0	0	0
Cell motility	0	0	0

Therefore, it was selected the top 10 genes with a highest betweenness and from these, the ones which also had the highest closeness and degree. Furthermore, the sub-networks of genes related to the orthologous groups of "Transcription" and "Signal Transduction" were also studied.

In the case of Dark Orange 2 module it was found five genes with the highest betweeness, closeness and degree. Those genes were: SCEZRZ3101E10.g_SS - Hypothetical protein SORBIDRAFT_01g000446, partial, SCVPAM1056E09.g_SS - Sulfoquinovosyl transferase SQD2; SCEZHR1047B03.g_SS - tRNA (cytidine(34)-2 -O)-methyltransferase; SCRUHR1076B12.g_SS - Probable lactoylglutathione chloroplastic - EC:2.7.11; EC:4.4.1.5 and SCJLRZ1021F06.g_SS - Hemolysin A (figure 53).

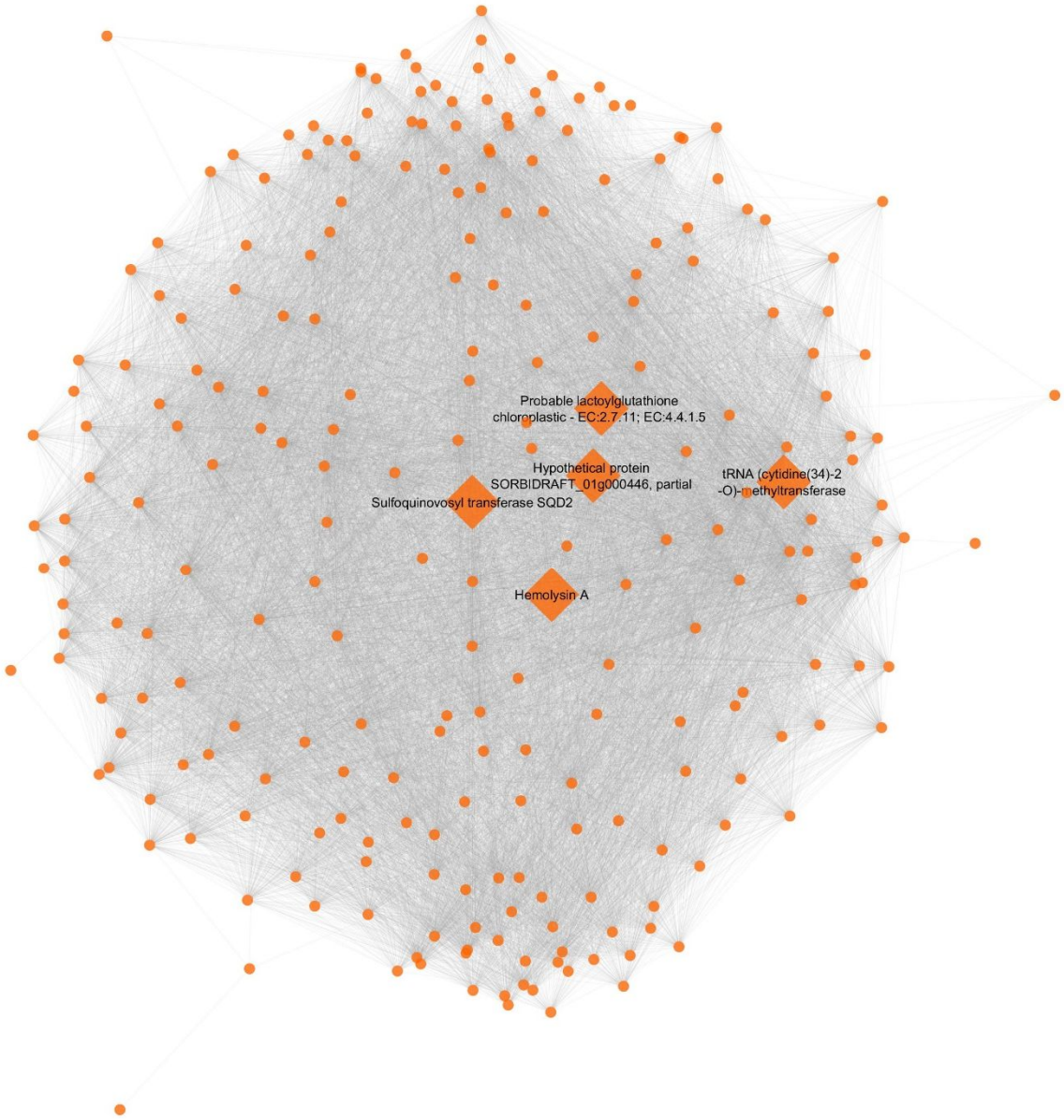

Figure 53. The co-expression network of dark orange 2 module highlights the five TOP nodes of the network

The subnetwork of signal transduction is composed of 7 genes, from which we can recognize kinases, phosphatases, some receptors and a calmodulin (figure 54b). In the case of the subnetwork related to transcription, it was found 15 genes, including a transcription factor ICE1-like, a bHLH107, a MADS-box transcription factor 18 and a heat shock factor 2 (figure 54a).

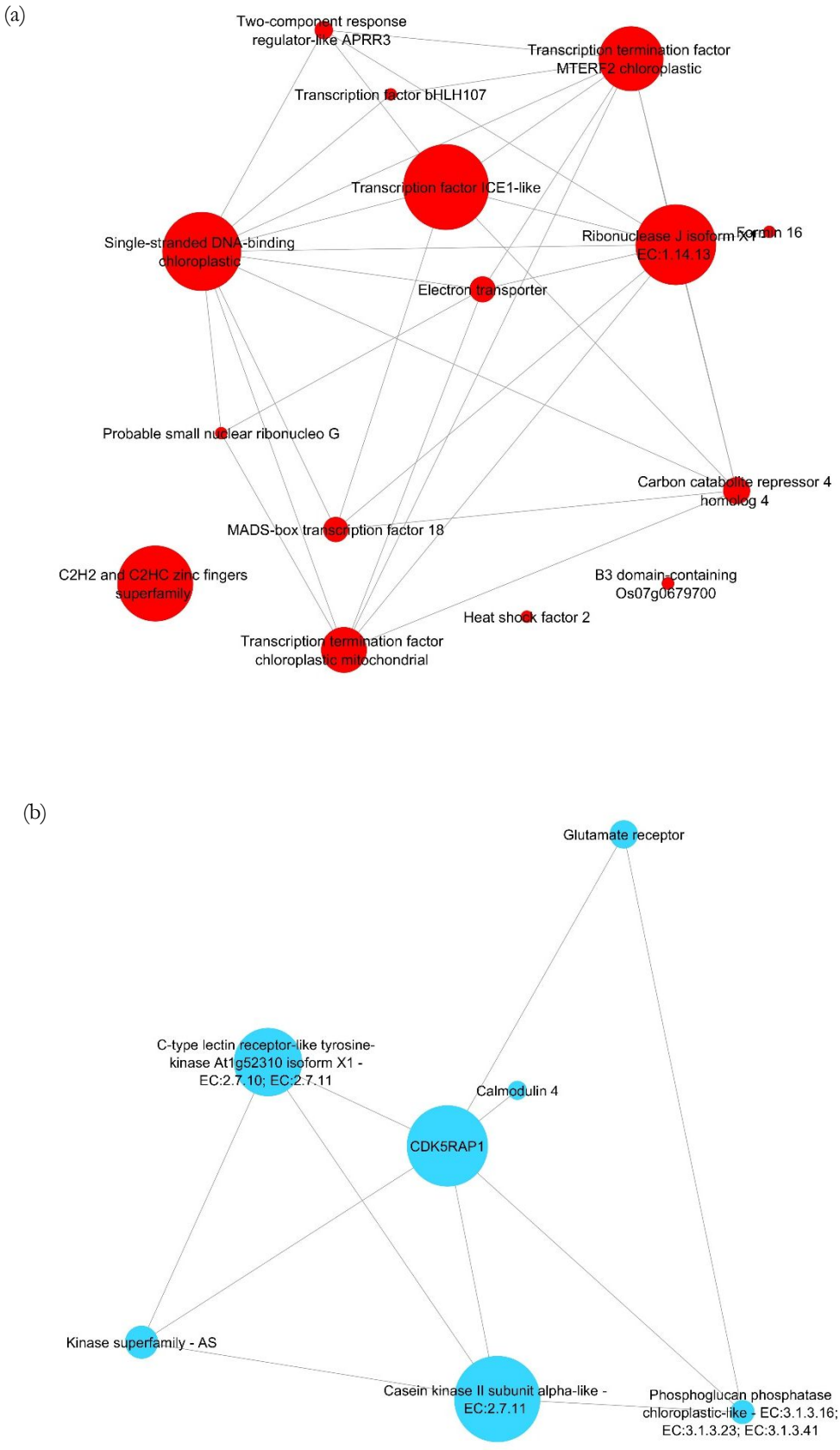

Figure 54. The Dark Orange2 subnetworks of Transcription (a) and Signal Transduction Mechanisms (b) show nodes representing interesting transcription factors as well as kinases. Nodes with a higher betweeness in the complete network are shown as bigger nodes in size

In the case of the Dark Red co-expression network (figure 55), there was only four nodes concomitantly higher in betweenness, closeness and degree. Those nodes were: SCJLLR1033C06.g_SS - MYB DNA-binding domain superfamily; SCRUFL1120E08.g_SS – NA (Unknown); SCQGLR1062F07.g_SS - Serine arginine repetitive matrix 1-like isoform X1 and SCVPLB1018A01.g_SS - E3 ubiquitin- ligase SPL2.

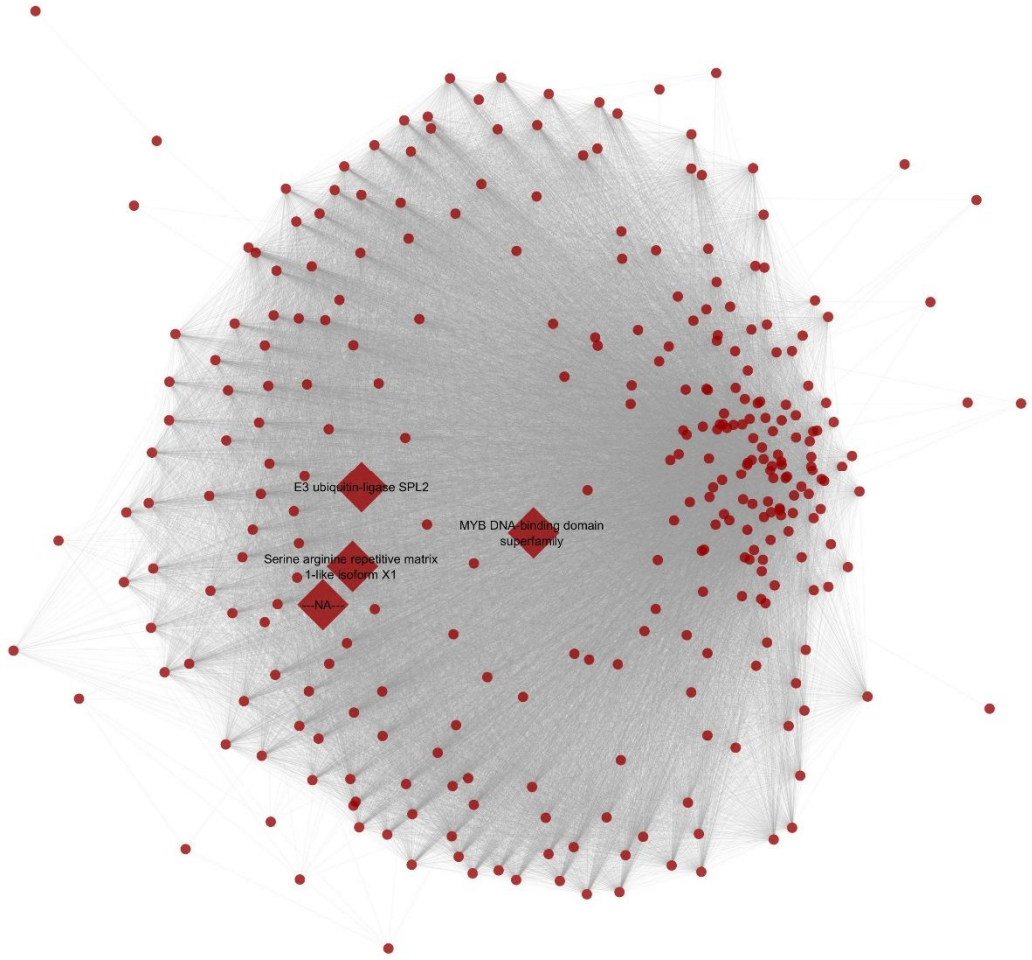

Figure 55. Top nodes from Dark Red module reveals transcripts of MYB family and ubiquitin ligase

The "Signal Transduction" subnetwork from Dark Red module has 27 nodes and reveals an interesting set of genes that includes several kinases, counting with the ones involved with calcium signaling, some phosphatases and GTPases, and also CRY1 (cryptocrome 1) and a cyclin-dependent kinase (figure 56b).

The "Transcription" related subnetwork from the same module, by its turn, has 19 nodes and includes genes described as MADS-box transcription factors, Auxin response factor 26, basic leucine zipper 6, MYB DNA-binding domain superfamily, among others (figure 56a).

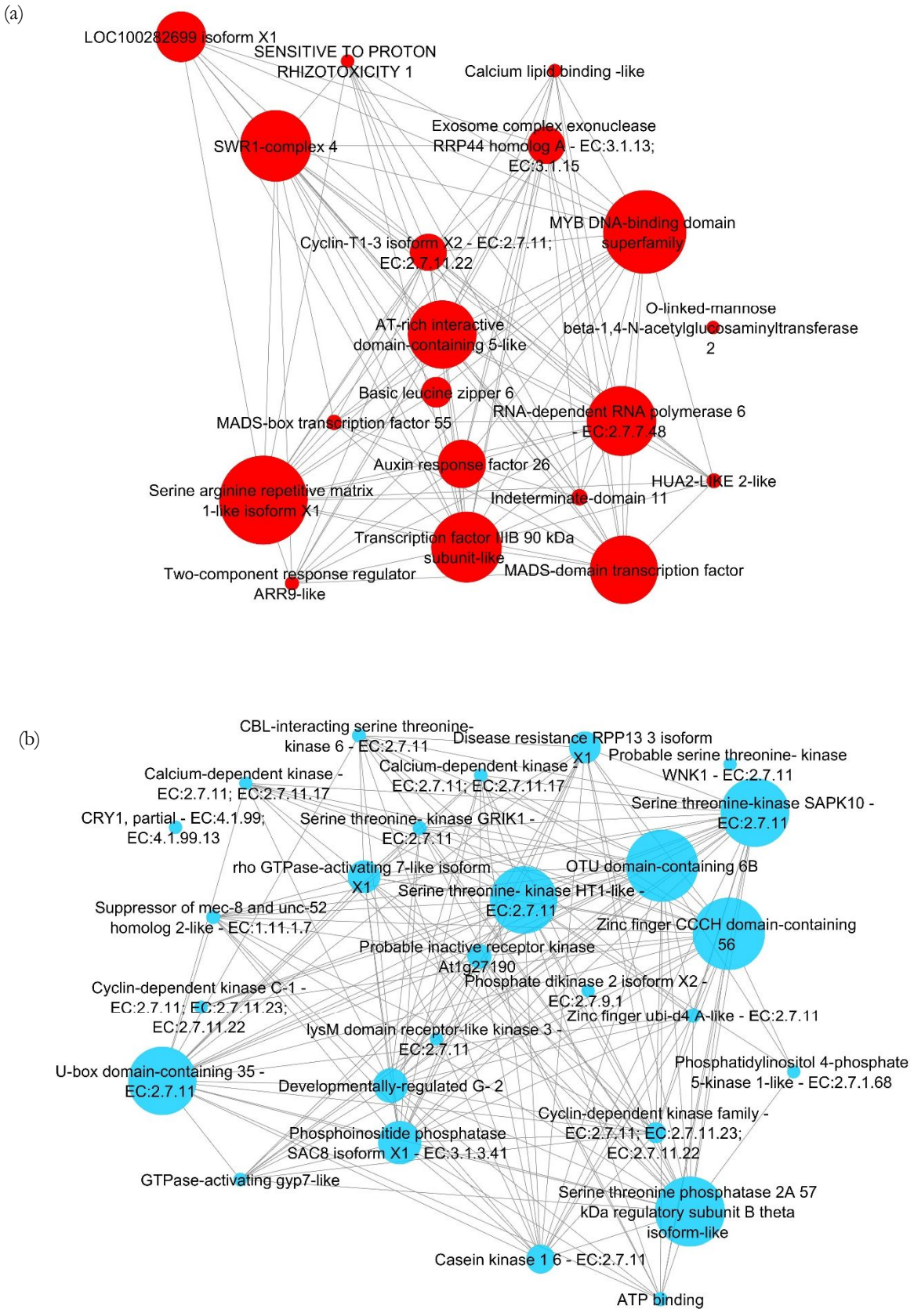

Figure 56. Dark Red subnetwork of Transcription (a) and Signal Transduction (b) co-expressed transcripts reveals the presence of nodes described as cryptochrome, cyclin, MADS-box TF, ARF among others. Nodes with a higher betweeness in the complete network are shown as bigger nodes in size

The third module selected from the correlation with physiological parameters is the Maroon, and has 5 nodes with high betweeness, closeness and degree. Those nodes are: SCAGLB1070G02.g_SS - seed specific Bn15D14A; SCCCRT2004B05.g_SS - G-patch domain containing; SCJFFL3C04B03.g_SS - DNA binding; SCQGAM2029C04.g_SS - Uncharacterized protein LOC100194005; SCQGAM2109F09.g_SS – NA (Uncharacterized) (figure 57).

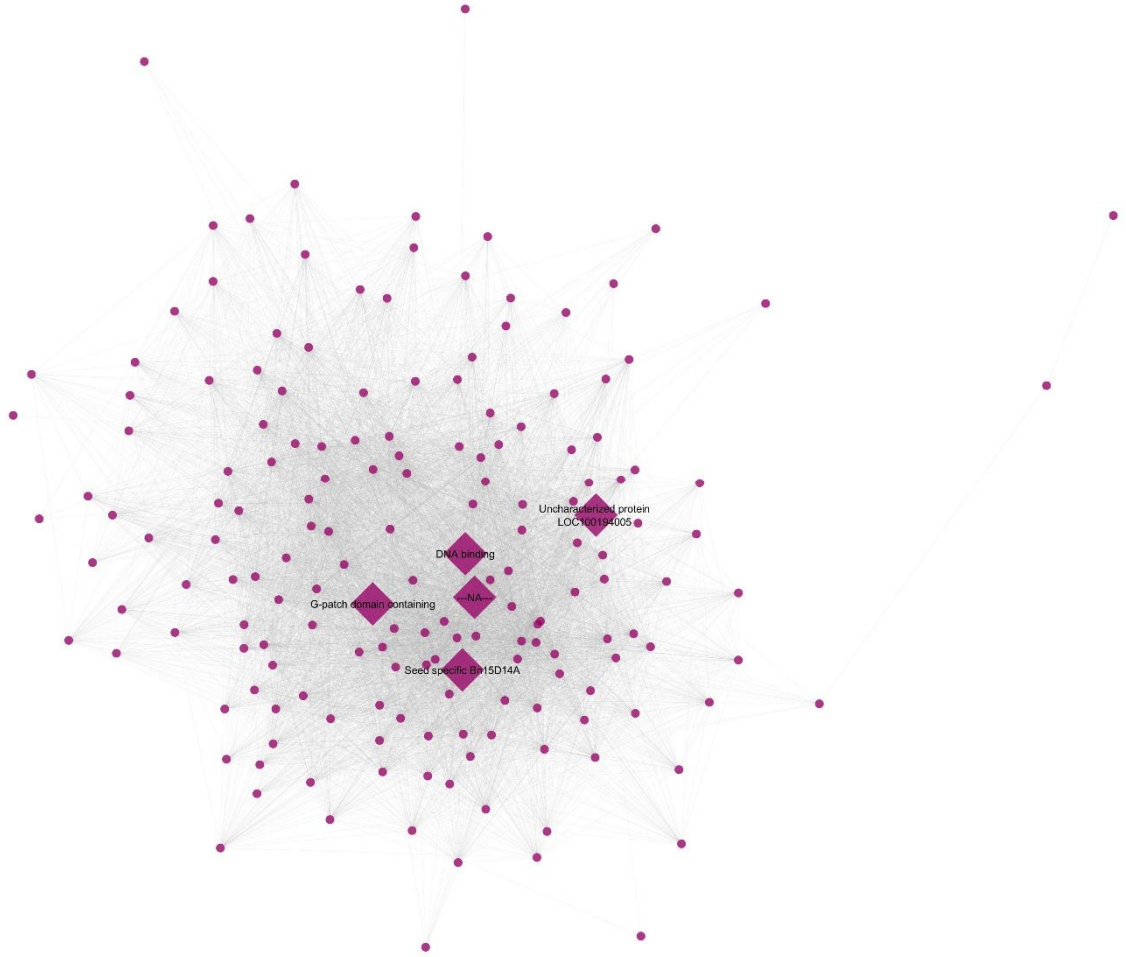

Figure 57. Maroon co-expression module network shows most top nodes are unknown or uncharacterized

The Maroon "Signal Transduction" subnetwork has 19 node genes, among those, one could point attention to the MAP kinase 6 - EC:2.7.11; EC:2.7.11.24, Cryptochrome 2, Catalytic phosphatase type 2C - EC:3.1.3.16; EC:3.1.3.41, Probable phosphatase 2C 64 - EC:3.1.3.16; EC:3.1.3.41; Serine threonine- kinase SAPK5 - EC:2.7.11 and Serine threonine phosphatase 2A 57 kDa regulatory subunit B (figure 58 b).

In the case of "Transcription" Maroon subnetwork, there was 16 node genes among which a Heat stress transcription factor A-2e, CCR4-NOT transcription complex subunit 7, Scarecrow 21 and transcription factors from the bZIP family (figure 58 a).

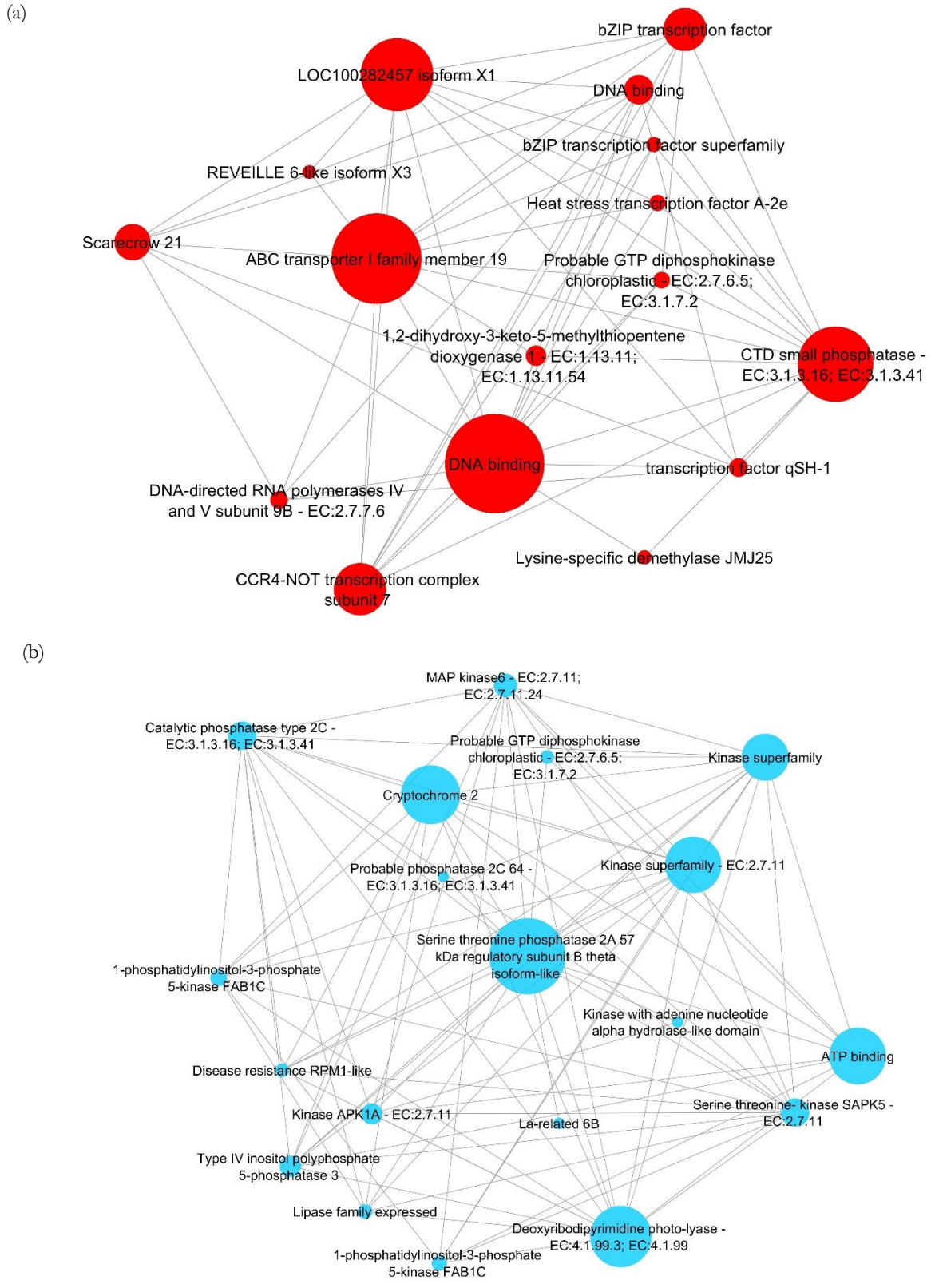

Figure 58. The analysis of Maroon subnetworks related to Transcription (a) and Signal Transduction (b) points the attention to MAPKinases, PP2C and SAPK as well as Heat Stress TF and bZIP TFs. Nodes with a higher betweeness in the complete network are shown as bigger nodes in size

4.4.2.2.2. Networks Selected from Drought Progression Analysis of Genes of Interest

The analysis of the networks in which the genes from drought progression analysis belong reveals that in all the modules analyzed, the most common Orthologous group category is "Function Unknown". This category is followed by "Posttransational Modification, protein turnover, chaperones" in bisque4, green yellow, light green, plum1 and salmon; "Transcription" in pale violet red; and "Signal Transduction Mechanisms" in black, dark green, dark grey, dark olive green, green, light cyan and light yellow (table 11).

Therefore, similar to the results from the previous networks analyzed, there was not an Orthologous Group that could characterize a specific network, and the analysis was focused on signal transduction and transcription subnetworks from each module and on the function of interesting top nodes.

The Bisque4 module counts with an ADP-glucose phosphorylase - EC:2.7.7.12 among the modules with highest betweenness, closeness and degree and with three PP2Cs in the "Signal Transduction" subnetwork (appendix G). In the Black module, SCUTAM2088B01.g_SS - Switch sucrose nonfermenting 3C is among the four with highest betweenness, closeness and degree (appendix G). In the "Signal Transduction" subnetwork there are 30 nodes most of them consisting on protein kinases, while in the "Transcription" subnetwork it was found Auxin-responsive IAA17, Growth-regulating factor, Heat stress transcription factor A-2 isoform X2 and ARF transcription partial (appendix G).

In the Dark Green module, there were 8 top nodes, among which SCEZRZ1012A01.g_SS - 2-oxoglutarate dehydrogenase E1 component - EC:1.2.4.2 (appendix G). The "Signal Transduction" subnetwork had five PP2C (PP2C 55; PP2C 2; PP2C 48; PP2C 47 and PP2C 38) and one PP2A (PP2Ac-5 - Phosphatase 2A isoform 5 belonging to family 2 - EC:3.1.3.16; EC:3.1.3.41), a Blue-light photoreceptor PHR2 and a Calcium Ion binding protein (appendix G). The "Transcription" subnetwork counted with Ethylene-responsive transcription factor 3 and ETHYLENE INSENSITIVE 3-like 1 (appendix G).

Dark Grey module has also 4 top nodes, SCCCCL3140H06.g_SS - PHD and RING finger domain-containing 1, SCCCRT2C01C11.g_SS - Hypothetical protein, SCCCRZ2002C11.g_SS - Patellin family and SCCCLR1078G01.g_SS - Hypothetical protein SORBIDRAFT_09g019360 (appendix G). The "Transcritption" subnetwork shows the co-expression of Ethylene-responsive element binding 2, ETHYLENE INSENSITIVE 3-like 3 and DRE binding factor, whereas "Signal Transduction" has a variety of kinases and phosphatases, including PP2C 26 and PP2C 7, Mitogen-activated kinase kinase kinase 1 - EC:2.7.11 and Calcium-binding CML16-like (appendix G).

The Dark Olive-Green module has 5 top nodes: SCCCLR1065G12.g_SS - Uncharacterized protein LOC100272356; SCCCLR1065G09.g_SS - la-related 6B-like; SCAGAM2125G10.g_SS - PRA1 family F3; SCCCRZ2002F06.g_SS - Casein kinase I isoform delta-like - EC:2.7.11 and SCCCLR1024G02.g_SS - Oxysterol-binding-related 3B (appendix G). In the "Signal Transduction" subnetwork there is the co-expression of CBL-interacting kinase family - EC:2.7.11 and CDPK-related kinase 3-like - EC:2.7.11; EC:2.7.11.17 (appendix G).

(continue)

Table 11. Top Orthologous Group sequence distribution (percentage) of nodes found in the modules selected from genes chosen for drought progression experiment

Orthologous Group	Bisque4	Black	Dark Green	Dark Grey	Dark Olive Green	Green	Green Yellow	Light Cyan	Light Green	Light Yellow	Pale Violet Red	Plum1	Salmon
Posttranslational modification, protein turnover, chaperones	36	8	12	8	13	9	12	9	14	9	5	12	12
Function unknown	22	24	21	31	23	26	22	27	26	29	28	18	26
Intracellular trafficking, secretion, and vesicular transport	8	3	5	2	8	6	8	3	9	3	1	4	7
Transcription	6	6	5	5	3	8	5	6	5	7	18	5	7
Signal transduction mechanisms	6	11	15	14	13	10	9	12	5	20	12	11	10
Carbohydrate transport and metabolism	4	4	5	5	4	4	7	9	6	4	4	1	3
RNA processing and modification	3	4	1	1	1	6	1	1	1	1	0	0	4
Energy production and conversion	2	1	3	3	4	2	4	3	5	1	3	2	3
Amino acid transport and metabolism	2	3	5	3	6	1	4	6	6	2	10	5	2
Replication, recombination and repair	1	7	1	1	1	4	1	0	1	1	1	0	2
Inorganic ion transport and metabolism	1	1	4	1	6	2	1	1	3	3	1	6	2
Cell wall/membrane/envelope biogenesis	1	2	0	2	1	1	2	0	1	1	0	2	1
Translation, ribosomal structure and biogenesis	1	4	3	1	1	4	3	1	2	1	0	1	3

(conclusion)

Orthologous Group	Bisque4	Black	Dark Green	Dark Grey	Dark Olive Green	Green	Green Yellow	Light Cyan	Light Green	Light Yellow	Pale Violet Red	Plum1	Salmon
Defense mechanisms	1	1	1	1	1	1	1	1	0	1	2	3	1
Cell cycle control, cell division, chromosome partitioning	1	2	1	2	0	2	1	1	0	1	0	1	1
Lipid transport and metabolism	0	3	6	7	5	2	3	2	6	1	3	9	3
Chromatin structure and dynamics	0	2	0	1	0	2	1	0	0	1	1	2	2
Cytoskeleton	0	2	2	3	0	1	1	2	1	1	0	1	1
Secondary metabolites biosynthesis, transport and catabolism	0	1	3	1	2	1	2	6	1	4	0	5	1
Nucleotide transport and metabolism	0	1	0	0	0	0	1	1	1	0	0	0	1
Nuclear structure	0	1	0	0	0	0	0	0	0	0	0	0	0
Coenzyme transport and metabolism	0	1	1	1	2	0	1	0	0	1	1	5	1
Extracellular structures	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell motility	0	0	0	0	0	0	0	0	0	0	0	0	0

Green module has 1092 nodes, but no top node. In the case of "Transcription" subnetwork there are several typical drought related elements, such as Dehydration responsive element binding 2 isoform a, Auxin response factor 7, NAC domain-containing 43-like, MADS-box transcription factor 50 isoform X2, Auxin response factor 2, Ethylene-responsive transcription factor 3 – AS, Myb family transcription factor APL-like, Heat stress transcription factor A-5 Growth-regulating factor 5 – AS, AP2-like ethylene-responsive transcription factor AIL5 – AS, Nuclear transcription factor Y subunit A-4-like, ABI3VP1 transcription partial along with CCR4-associated factor 1 homolog 11 - EC:3.1.13; EC:3.1.15; EC:3.1.13.4 and GATA transcription factor 25 - EC:2.6.1.85 (appendix G). The "Signal Transduction" subnetwork shows Phytochrome C - EC:2.7.13.3, Mitogen-activated kinase kinase kinase 1-like - EC:2.7.11, CBL-interacting serine threonine- kinase 18 - EC:2.7.11, Calcium-dependent kinase 3-like - EC:2.7.11; EC:2.7.11.17, Cryptochrome partial and Phytochrome B - EC:2.7.13.3 among others (appendix G).

In the Green-Yellow module there are 5 nodes with highest betweenness, closeness and degree which are SCEQRT2097F03.g_SS - L-type lectin-domain containing receptor kinase, SCEZRZ1016A05.g_SS - CASP C, SCCCLR1070D04.g_SS - Pyruvate kinase cytosolic - EC:2.7.1.40, SCEPLR1030H09.g_SS - Dynamin-related 3A - EC:3.6.1.15, SCAGLR1064D05.g_SS - Ubiquitin interaction motif-containing (appendix G). Curiously, in the "Transcription" subnetwork there are also nodes described as Nuclear transcription factor Y subunit B-4, Auxin response factor, Auxin response factor 15-like, Ethylene response element binding, Ethylene-responsive transcription factor 1-like, CCR4-NOT transcription complex subunit 1 - EC:3.1.13; EC:3.1.15; EC:3.1.13.4 (appendix G). In the "Signal Transduction" subnetwork there are nodes described as Probable calcium-binding CML7, Calcineurin subunit B, MAP kinase family - EC:2.7.11; EC:2.7.11.24, CBL-interacting kinase 24 - EC:2.7.1, Ethylene receptor 3 - EC:2.7.13.3, Ca^{2+} calmodulin-dependent kinase phosphatase - EC:3.1.3.16; EC:3.1.3.41, Ca^{2+} calmodulin-dependent kinase phosphatase - EC:3.1.3.16; EC:3.1.3.41, Ethylene receptor 1-like - EC:2.7.13.3 (appendix G).

Light Cyan has three top nodes, SCJFRZ2009A12.g_SS - Glycosyltransferase family 61, SCJLRT2051G07.g_SS - Alpha-amylase trypsin inhibitor-like, SCJFRT1009C10.g_SS - Serine carboxypeptidase 2-like - EC:3.4.21; EC:3.4.16 (appendix G). "Transcription" subnetwork reveals nodes related to Transcription factor bHLH128-like, NAC transcription factor, AP2 EREBP transcription factor superfamily, Transcription factor MYB1R1-like - EC:2.3.1.48 Ethylene-responsive transcription factor RAP2-3-like, GATA transcription factor 16 and others (appendix G). The "Signal Transduction" subnetwork counts with Probable calcium-binding CML32, CBL-interacting kinase 31-like - EC:2.7.11 along with other kinases and phosphatases (appendix G).

The Light Green module has 8 top nodes SCRLLR1059C02.g_SS - 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily, SCVPAM1056C05.g_SS - Acyl-coenzyme A oxidase 2 - EC:1.3.99.3; EC:1.3.3.6, SCVPCL6044F07.g_SS - Transmembrane adipocyte-associated 1 homolog, SCMCRT2089A05.g_SS - Hypothetical protein SORBI_009G141200, SCQGRT1039G03.g_SS - Transmembrane BAX inhibitor motif-containing 4, SCQGLR2025G04.g_SS - Cytochrome b5, SCUTRZ2024B07.g_SS - Hypothetical protein SORBIDRAFT_02g007530, SCEZRT2015F08.g_SS - Long chain acyl- synthetase 4-like isoform X2 (appendix G). "Signal Transduction" subnetwork shows Calcium-dependent kinase isoform 11 - EC:2.7.11; EC:2.7.11.17, Calmodulin-7 - EC:1.3.1.74 among other kinases, and "Transcription" subnetwork counts with SCARECROW 1-like, NAC domain-containing 68, Transcription factor MYB108, AP2-like ethylene-responsive transcription factor At1g16060, Transcription factor bHLH77 isoform X2 – AS and others (appendix G).

In the Light-Yellow module, there are 9 nodes with concomitantly high degree, closeness and betweenness. These nodes are: SCJFRT1007A11.g_SS - Probable galacturonosyltransferase-like 9, SCACLR1036G02.g_SS - Syntaxin 121, SCVPRZ3029A08.g_SS - Mitogen-activated kinase kinase kinase YODA-like - EC:2.7.11,

SCEPAM2054B05.g_SS - Phosphatidylinositol:ceramide inositolphosphotransferase-like isoform X2, SCEPRT2048G02.g_SS - G-type lectin S-receptor-like serine threonine-kinase SD2-5 - EC:2.7.11, SCJFRT1008B03.g_SS - Probable bifunctional riboflavin biosynthesis RIBA chloroplastic - EC:3.5.4.25; EC:4.1.99.12, SCVPLB1019E10.g_SS – NA (Unknown), SCCCRT2001D11.g_SS - Probable carboxylesterase 17, SCRFLR1034D08.g_SS - E3 ubiquitin- ligase RING1-like (appendix G).

"Transcription" and "Signal Transduction" subnetworks have nodes related to Probable CCR4-associated factor 1 homolog 11 - EC:3.1.13; EC:3.1.15; EC:3.1.13.4, Probable WRKY transcription factor 50, Ethylene-responsive transcription factor 4, NAC domain-containing 68, Ethylene-responsive transcription factor 7, Dehydration-responsive element-binding 1E in the first case (appendix G). In the other there are E3 ubiquitin- ligase RGLG2-like isoform X1, Calmodulin-related touch-induced, PP2C 44, PP2C 34, PP2C 65, PP2C 32, calcium-dependent kinase family - EC:2.7.11 among other kinases (appendix G).

Pale Violet Red module also has 9 top nodes, SCCCST3C01A07.g_SS - dnaJ domain containing, SCEZLR1031D07.g_SS - Sucrose-phosphatase 1 - EC:3.1.3.23; EC:3.1.3.24; EC:3.1.3.41, SCSBRZ3118B10.g_SS - Transmembrane amino acid transporter family, SCJFRZ2013H10.g_SS - CTD-phosphatase isoform X1 - EC:3.1.3.16; EC:3.1.3.41, SCCCLR1C10C04.g_SS - F-box kelch-repeat At1g22040, SCCCLB1C02H10.g_SS - UDP-glycosyltransferase 87A1-like, SCMCCL6050E02.g_SS - Hypothetical protein SORBIDRAFT_08g004650, SCAGLR1021F02.g_SS - Phosphoinositide phosphatase SAC2 and SCMCLB2084G10.g_SS - ABC transporter I family member 20 - EC:3.6.1.3; EC:3.6.1.15 (appendix G). "Signal Transduction" module counts with CIPK expressed - EC:2.7.11, CBL-interacting kinase - EC:2.7.11, PP2C 34, Probable phosphatase 2C BIPP2C1 , and "Transcription" subnetwork with ABA responsive element binding factor 1, ABC transporter I family member 20 - EC:3.6.1.3; EC:3.6.1.15 (appendix G).

In the Sienna3 module, the 5 top nodes were SCQGLR1086A04.g_SS - Uncharacterized protein LOC101756814, SCQGAM2108C06.g_SS - Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase mitochondrial, SCMCCL6055D10.g_SS - Autophagy 9; SCEPLR1051D11.g_SS - Probable acyl-activating enzyme peroxisomal isoform X2; SCCCLR1C05G02.g_SS - 3-ketoacyl- thiolase peroxisomal; SCCCCL3001B09.b_SS - Aconitate cytoplasmic - EC:4.2.1.3, SCAGLB1069A07.g_SS - Peroxisomal fatty acid beta-oxidation multifunctional - EC:1.1.1.35; EC:4.2.1.17 (appendix G). In the "Transcription" subnetwork, the nodes of WRKY DNA-binding domain superfamily and Heat stress transcription factor A-1a catches our attention (appendix G).

The Salmon module has 1135 nodes and interestingly, there is only one node with high betweenness, closeness and degree. The node is SCQGLR1085D04.g_SS – Ethylene-responsive transcription factor ERF118-like and may be a hub in the network (appendix G). Among the interesting nodes in the "Signal Transduction" subnetwork, one could cite Mitogen-activated kinase kinase kinase 5 - EC:2.7.11, CBL-interacting kinase 8 - EC:2.7.11, Cytochrome b561 and DOMON domain-containing, Calcium-binding CML50 - EC:3.4.22, Phosphatase 2C ABI2 - EC:3.1.3.16; EC:3.1.3.41, SNF1-related kinase catalytic subunit alpha KIN10-like - EC:2.7.11, PP2A regulatory subunit TAP46, Phytochrome-associated serine threonine- phosphatase - EC:3.1.3.16; EC:3.1.3.41 (appendix G). Among the interesting nodes found in the "Transcription" subnetwork, there are Probable WRKY transcription factor 32, Auxin response factor 15-like, Growth-regulating factor 11, Scarecrow 9, Nuclear transcription factor Y subunit B, ABSCISIC ACID-INSENSITIVE 5 2, Auxin response factor 4, ABSCISIC ACID-INSENSITIVE 5 5, Ethylene-induced calmodulin-binding 4, Auxin response factor 22 and MADS-box transcription factor 50 isoform X2 (appendix G).

Plum1 module has 9 top nodes, SCSFRT2072D01.g_SS - Vacuolar-sorting receptor 1, SCEPAM2015D12.g_SS - Probable phosphatase 2C 44 - EC:3.1.3.16; EC:3.1.3.41, SCRLRZ3041C03.g_SS - Myb family transcription factor APL-like, SCCCLR1C05E03.g_SS - ATG8-interacting 1, SCCCRZ2C04B08.g_SS - Serine threonine-kinase 19 isoform X2, SCQSLR1040A10.g_SS - Hypothetical protein SHCRBa_144_K17_F_300, SCVPAM1057E02.g_SS - NEP1-interacting-like 2, SCCCRZ2004B10.g_SS - Isocitrate dehydrogenase [NAD] catalytic subunit 5 mitochondrial - EC:1.1.1.41, SCMCCL6049E02.g_SS - Pyrrolidone-carboxylate peptidase - EC:3.4.19 (appendix G). Among the ones related to "Signal Transduction" subnetwork there are CBL-interacting kinase 2 - EC:2.7.11, Calcineurin B 4, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor - EC:2.7.11 and "Transcription" subnetwork counts with MADS-box transcription factor 47-like isoform X1, Myb family transcription factor APL-like, Basic leucine zipper 9 (appendix G).

The Floral White module is curious because it counts with basically anti-sense nodes. It has 6 nodes with high betweenness, closeness and degree: SCSGAD1008H08.g_SS – NA (Unknown), SCAGHR1019F01.g_AS - Vacuolar import degradation Vid27-related – AS, SCMCLB2083G12.g_AS – NA (Unknown), SCSGLR1025G01.g_AS - Hypothetical protein SORBIDRAFT_03g003300 – AS, SCCCST1001B03.g_AS - ruvB-like 2 - EC:3.6.1.3; EC:3.6.1.15 – AS, SCCCSD2091D04.g_SS – NA (Unknown) (appendix G). In the case of "Transcription" and "Signal Transduction" there are nodes described as Glycosyltransferase family 61 – AS, Myb-related 2, ruvB-like 2 - EC:3.6.1.3; EC:3.6.1.15 – AS, WRI1 transcription factor1 – AS in the first (appendix G). And Serine threonine- kinase CCR4 - EC:2.7.11 – AS, AMSH-like ubiquitin thioesterase 2 – AS, LRR receptor-like serine threonine-kinase - EC:2.7.11, U-box domain-containing 35 - EC:2.7.11 – AS in the last (appendix G).

The transcription factor MYB108 (SCSGAD1007F02.g_SS) is highly induced during drought, as shown in figure 49, but the analysis of the co-expression networks reveals that this transcript does not have a high betweenness in the module it belongs. Despite that, the subnetwork built having as a base the first-neighbors shows its connection to interesting transcripts, such as Acyl-coenzyme A oxidase 2 - EC:1.3.99.3; EC:1.3.3.6, 26S proteasome non-ATPase regulatory subunit 6, Ubiquitin-conjugating enzyme E2 4 (figure 60). Also, Glutamate decarboxylase - EC:4.1.1.15, Cytochrome b5, Fructokinase 1 - EC:2.7.1.15; EC:2.7.1.4; EC:2.7.1.1, NAC domain-containing 78, Glutamate dehydrogenase 2-like - EC:1.4.1.3; EC:1.4.1.2, Glutathione cytosolic - EC:1.8.1.7, among others (figure 59)

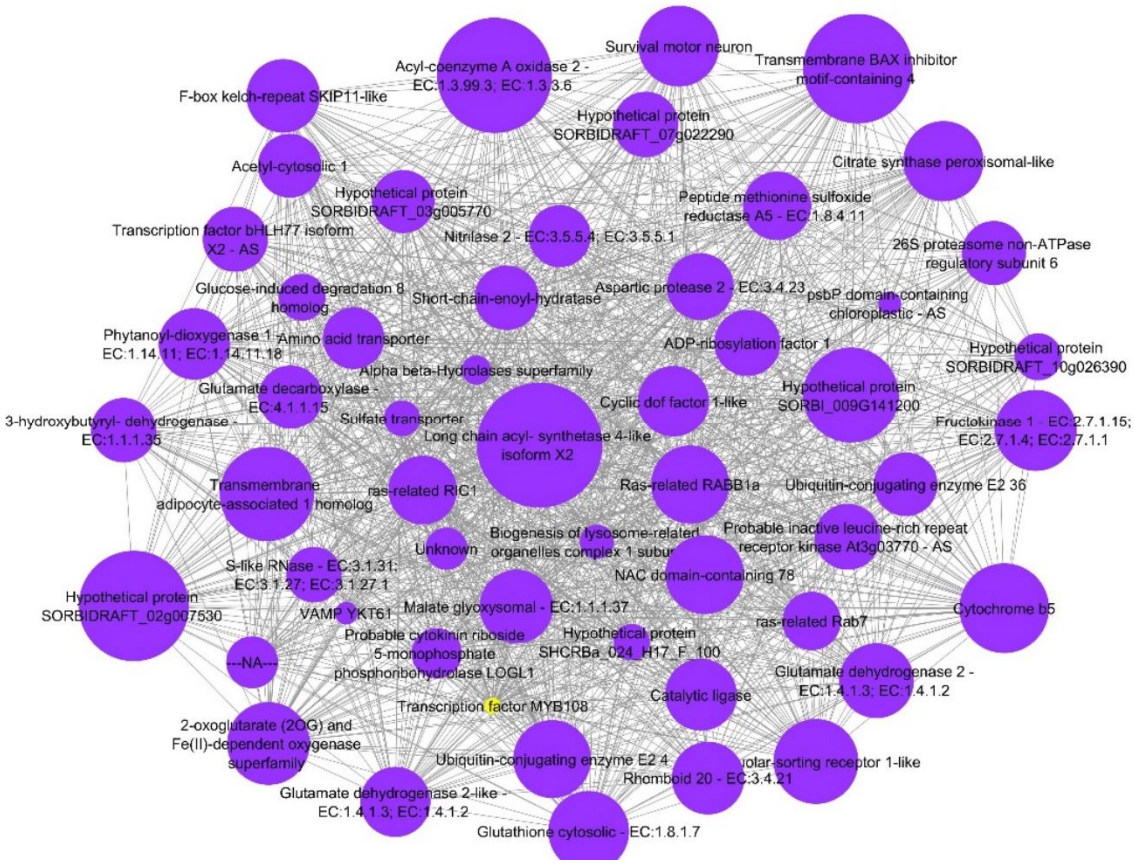

Figure 59. Subnetwork of first-neighbors from transcription factor MYB108 shows several transcripts related to ROS responses, such as Glutamate dehydrogenase

4.5. Discussion

4.5.1. Co-expression reveals 3 modules correlated with physiological parameters

The analysis of networks has been increasingly used by molecular biologists and bioinformatics with the aim of finding meaningful biological patterns among genes, transcripts, protein and metabolites and extract information to direct further studies. The co-expression network analysis is an attempt to describe correlation patterns among expressed transcripts across a big input data set compounded of microarray or RNA-Seq samples (JIANG et al., 2016). WGCNA can be used to find modules or clusters of highly correlated genes that can be summarized using module eingene aiming to relate modules with each other and to external sample traits (JIANG et al., 2016).

In the present work, it was used WGCNA algorithm and co-expression networks were obtained using 52 samples from three different drought related experiments. These experiments encompassed greenhouse and field conditions, different sugarcane varieties as well as leaves, roots and internodes plant tissues, in a way that input data set was diverse, with drought and the Agilent microarray platform being the single aspects in common between all of them. After the exclusion of outliers, data set was reduced to 48 samples and 11,985 genes (figure 50).

The nature of the condition analyzed as well as the variety of the input data set made the analysis of the modules very difficult, since no specific characteristic could be found as determinant for a specific module. Besides

that, in the present data, filtering lead to the removal of more than 5,000 genes. A similar trend was found by Jiang et al., (2016), who states that after merging data sets from all biochips used in the study, the number of genes was so reduced that did not cover the entire *Mycobacterium tuberculosis* (the organism studied) genome.

The present work found 55 modules with possible association to sugarcane drought responses. These modules were grouped into 43 final modules, from which grey color was assigned for genes that did not fell into other modules (figures 50 and 51). According to Langfelder; Horvath, (2008), the hierarchical clustering of module eigengenes summarize the modules found in the clustering analysis and branches of the dendrogram group puts together eigengenes that are positively correlated, such as shown in figure 51.

Smita et al., (2013) trying to identify conserved drought stress responsive gene-networks across tissues and developmental stages in rice found 11 modules, being the grey color module also reserved to genes that were not part of any other module. Sircar; Parekh, (2015), also using the network approach to study drought-responsive module in *Oryza sativa*, identified 16 co-expressed modules with a minimum size of 200. A microarray meta-analysis done with the aim of exploring abiotic stress-specific gene expression patterns in *Arabidopsis* found 19 gene modules (of differentially expressed genes) with a minimum size of 60 genes (SHEN; HOUR; LIU, 2017).

According to Jiang et al., (2016), the use of a single dataset with various experimental conditions caused the differences within dataset to have more influence on the gene modules than the differences within the chips. Therefore, some important modules ended up being missed, so, if the experimental conditions were simplified instead of diversified, modules associated with specific conditions would have been identified. This aspect was also observed in our data, since most modules analyzed were associated with the same Top Orthologous Group which were "Posttranslational modification, protein turnover, chaperones", "Signal Transduction Mechanisms" and "Transcription" (tables 10 and 11).

Furthermore, since the "Function Unknown" was by far the most represented one in all the modules analyzed due to the fact that sugarcane genome is still not fully annotated, module characterization by function and the hypothesizing of function of unknown transcripts by the guilty-by-association principle faced an increased level of difficulty.

One strategy used to select modules for further evaluation was to perform a Module-Trait Relationship analysis to identify modules that positively correlates with physiology parameter associated with drought responses, such as photosynthesis, transpiration and stomatal conductance (figure 52). This analysis allowed the selection of 3 modules (discussed in the next sections): Dark-Orange2, Dark-Red and Maroon, that led to the determination of transcripts that, despite of being not genotype-dependent, may positively influence in the plant adaptation process under drought stress (KIDO et al., 2012).

Despite that, further optimization of the co-expression analysis pipeline is still necessary, since some of the barriers faced may be due to limitations of the methods implemented in the WGCNA package. Such limitations involve the assumption of proper pre-processing and normalization of microarray data, the bias or invalid analysis thanks to technical artefact, tissue contamination or poor experimental design, use of inadequate method for module detection on your specific dataset, and the limitation of the package for undirected networks (LANGFELDER; HORVATH, 2008).

4.5.1.1. Dark orange 2 module reveals highly connected genes involved with detoxification and protection of photosynthetic membranes

The SQD2 found in Dark Orange 2 module catalyzes the transfer of the sulfoquinovose moiety from UDP-sulfoquinovose to diacylglycerol during sulfolipid biosynthesis. These compounds may be important during drought responses helping to avoid damage of photosynthetic membranes maintaining photosynthesis working, since they contribute to keep a negatively charged lipid-water interface, a requirement for proper function of photosynthetic membranes (YU; XU; BENNING, 2002). Furthermore, the overexpression of SQD2.2 in rice lead to reduced starch content, enhanced flavonoids and increased soluble sugars relative to WT plants (ZHAN et al., 2017).

The tRNA (cytidine (34)-2 -O)-methyltransferase might be related to the protection of macromolecules such as N2-dimethylguanosine tRNA methyltransferase and tRNA stability (HU et al., 2006). The lactoylglutathione (EC:4.4.1.5) is also called glyoxalase I and is involved in the glyoxalase pathway, a detoxification system found in plants (HOSSAIN et al., 2012). This system acts to detoxify the methylglyoxal (MG), an oxygenated short aldehyde produced in plants as a by-product of a number of metabolic reactions and that can accumulate to high levels when plants are exposed to abiotic stresses, such as drought (HOQUE et al., 2016). Similar to ROS, stress-induced MG are toxic molecules, that inhibit developmental processes such as germination, photosynthesis and root growth while at low levels are important signaling molecules, involved in regulating diverse events, like cell proliferation and survival, control of the redox status of cells and cellular homeostasis (HOQUE et al., 2016). Furthermore, there is a crosstalk between ROS and MG detoxifying systems (HOSSAIN et al., 2012).

The "Transcription" related subnetwork (figure 54a) showed TFs such as Heat shock factor 2, MADS-box transcription factor 18, Transcription factor bHLH107 (discussed in the next section) and also Transcription factor ICE1-like. The ICE1 is a MYC-type bHLH transcription factor that regulates the expression of CBF3/DREB1A (SAIBO; LOURENÇO; OLIVEIRA, 2009), therefore it is interesting to find it in a co-expression module chosen by its correlation with physiological parameters altered by drought.

In the case of "Signal Transduction" subnetwork (figure 54b) it was observed a calmodulin connected with a CDK5RAP1 (CDK5 Regulatory Subunit Associated Protein 1) both involved in calcium signaling (discussed in the next section), a Casein kinase II subunit alpha-like - EC:2.7.11, which was shown to regulate negatively ABA activated SnRK2s (VILELA et al., 2015), among others.

4.5.1.2. Dark Red high betweenness genes reveals a MYB and an E3 ubiquitin ligase as possible hub in water stress responses co-expression networks

The E3 ubiquitin ligase SPL2 is involved in the pathway of protein ubiquitination and, therefore, protein modification (uniprot website). The process of ubiquitination refers to a protein degradation system that efficiently degrade detrimental cellular proteins and control the pool of regulatory components, therefore, plants adaptation to a diversity of abiotic stresses occurs via ubiquitination (LEE; KIM, 2011). This process is also important for protein turnover. According to Gao et al., (2015) in several cases, the change in expression of the E3 ubiquitin ligase gene also caused an alteration in drought resistance and plant hormone action by regulation of downstream target genes.

The family of MYB transcription factors is large, diverse, represented in all eukaryotes and function as TFs with varying numbers of MYB domain repeats conferring their ability to bind DNA (AMBAWAT et al., 2013). They

are distributed across plant kingdom and control processes like biotic and abiotic stresses, development, differentiation, metabolism, defense, being also implicated in ABA-responses (AMBAWAT et al., 2013).

In the subnetwork related to "Transcription" (figure 56a) there were MYB DNA-binding domain superfamily, MADS-domain transcription factor, Auxin response factor 26 (discussed in the next section) and SWR1-complex 4. The last one is part of the SWR1 complex and the NuA4 histone acetyltransferase, mediating ATP-dependent exchange of histone H2A for the variant HZT1 in the first case and gene activation by acetylation of H4 and H2A in second one, being therefore, involved in chromatin remodeling.

The "Signal Transduction" subnetwork (figure 56b) found counts with Calcium-dependent kinase - EC:2.7.11; EC:2.7.11.17, CBL-interacting serine threonine-kinase 6 - EC:2.7.11, Calcium-dependent kinase - EC:2.7.11; EC:2.7.11.17 (discussed in section 4.5.2.1), Casein kinase 1 6 - EC:2.7.11 (discussed in section), CRY1, partial - EC:4.1.99; EC:4.1.99.13 (discussed in section 4.5.2.1). Also, Phosphate dikinase 2 isoform X2 - EC:2.7.9.1, phosphoinositide phosphatase SAC8 isoform X1 - EC:3.1.3.41, Phosphatidylinositol 4-phosphate 5-kinase 1-like - EC:2.7.1.68. The last two are involved in phospholipid signaling that may occur in cells in response to stresses, such as osmotic and temperature (XUE; CHEN; MEI, 2009; ZHU, 2002), since phosphoinositols mediate ABA and stress signal transduction in plants and their turnover is critical for attenuating ABA and stress signaling. The phosphatidylinositol 4-phosphate 5-kinase is involved in the synthesis of PIP2, a precursor of IP3, whose levels increase upon treatment with exogenous ABA and caused guard cell shrinking and stomatal closure (LEE et al., 1996), furthermore, superinduction of ABA- and stress-responsive gene transcription correlated with elevated IP3 accumulation (XIONG, 2001).

The first one, phosphate dikinase 2, has been shown to be involved in the acclimation of *Nicotiana tabacum* L. to drought stress, since its activity increased 11 days after stress imposition (DOUBNEROVÁ HÝSKOVÁ et al., 2014).

4.5.1.3. Maroon high betweenness genes

The G-patch domain containing (named after its glycine-rich sequence signature) refers to a protein conserved among eukaryotes, such as plants, animals and fungi and seems to have a function in RNA processing (ARAVIND; KOONIN, 1999). The seed specific Bn15D14A has a domain that belongs to the TPX2 family and targets a kinesin-like protein.

Maroon's "Signal Transduction" subnetwork (figure 58a) reveals nodes related to MAP kinase6 - EC:2.7.11; EC:2.7.11.24 (discussed in section 4.5.2.1), Cryptochrome 2 (discussed in section 4.5.2.1), Catalytic phosphatase type 2C - EC:3.1.3.16; EC:3.1.3.41, Probable phosphatase 2C 64 - EC:3.1.3.16; EC:3.1.3.41 (discussed in section 4.5.2), Serine threonine- kinase SAPK5 - EC:2.7.11 (discussed in chapter 1). Also present were: Type IV inositol polyphosphate 5-phosphatase 3, 1-phosphatidylinositol-3-phosphate 5-kinase FAB1C which is part of the Phosphatidylinositol signaling system (explained in better details in the previous section), Deoxyribodipyrimidine photo-lyase - EC:4.1.99.3; EC:4.1.99, involved in repair of UV radiation-induced DNA damage and required for plant survival in the presence of UV-B light.

In the case of "Transcription" subnetwork (figure 58b) it was found Heat stress transcription factor A-2e, CCR4-NOT transcription complex subunit 7, Scarecrow 21, bZIP transcription factor (discussed in section 4.5.2.2). In addition, it was detected Lysine-specific demethylase JMJ25, which is a histone demethylase involved in the control of several developmental processes by protecting genes from silencing and protecting a number of transcribed genes

from non-CpG methylation. It may have a role in silencing of gene expression through small RNA-directed DNA methylation (RdDM)-directed repression.

4.5.2. Analysis of drought progression gene expression patterns reveals transcripts found in the same drought co-expression network modules

In this stage of the work, SP80-3280 cuttings were cultivated in order to perform a time-course drought experiment and focus gene expression analysis to some genes highlighted by the previous microarray analysis (chapter I). These genes are involved in ABA-dependent and ABA-independent signal transduction pathways (table 9) and it was aimed to observe some pattern of expression on a daily basis and associate the RT-qPCR expression results with the presence of the genes in the modules obtained on the co-expression network analysis.

Plants were grown for 7 months before the stress was imposed for 7 days. They were re-watered and the physiology parameters continued to be analyzed to confirm the recovery of the plants. Previous results (chapter I) might indicate an intermediary position of SP80-3280 regarding drought tolerance, being its fast recovery an important feature in the survival and cell damage repair after the stress period (MEDEIROS et al., 2013). Using that information as a base, differently from the previously published studies, in which the physiology measures were taken for just some evaluation points (ANDRADE et al., 2015; DA SILVA et al., 2012; GRAÇA et al., 2010; JAIPHONG et al., 2016; RIBEIRO et al., 2014; RODRIGUES et al., 2011; RODRIGUES; DE LAIA; ZINGARETTI, 2009; SALES et al., 2015), these results show the daily evolution of the stress. Photosynthesis, stomatal conductance, transpiration and soil humidity were measured every day during the whole experiment, until the severe stress, when photosynthesis was close to zero.

Our results demonstrate that plants were stressed, affected by drought (figure 43b) and presented the classical symptoms of drought stress, like drying of leaf tips, leaf wilting and curling, yellowing, chlorosis and necrosis (PRABU et al., 2011) (figure 44b) and reductions in the leaf area (FAROOQ et al., 2012; YAMANE et al., 2003) (figure 45b). After 7 days of stress, soil humidity showed a decrease of about 63% while photosynthesis, stomatal conductance and transpiration had values close to zero, representing a decrease of 98%, 83% and 82% respectively (figures 45a, b and c). Photosynthesis was the most affected parameter and the correlation between physiology parameters and soil moisture was high. Similarly, to the previous experiment, it was observed a fast recovery of the plants after re-watering reflected by the values of photosynthesis, stomatal conductance and transpiration 24 hours after the treatment stopped (figures 45a, b and c).

These results demonstrate that morphological changes reflected the alterations on photosynthesis, transpiration and stomatal conductance, and will lead to impaired carbon assimilation and fixation and ultimately cause photoinhibition and oxidative damage on the cells (PINHEIRO; CHAVES, 2011). Samples from this experiment were harvested on every day of the treatment for transcriptomic and possibly metabolomics analysis of drought progression, complementing the results already obtained during the whole project. Therefore, RNA was extracted, cDNA synthetized and RT-qPCR already performed for most of the selected genes.

From the sequences selected, one can mention PP2C, Dehydrin, LEA3, 9-cis-Epoxycarotenoid dioxygenase and Viviparous 14. PP2C is a known player in the signaling cascade involved in ABA-dependent responses, and similarly to our results, has been found both up and down-regulated (LI et al., 2016a). The PP2C SAS chosen for the

RT-qPCR was up-regulated after 4 and 6 days of stress corroborating to our microarray results (figure 48 and 49). PP2Cs appeared co-expressed on Bisque4, Dark Green, Pale Violet Red, Salmon and Light Yellow.

Dehydrin, LEA3, 9-cis-Epoxycarotenoid dioxygenase and Viviparous 14 are also very important and characteristic players on a plant response to drought. The first one has its expression induced after several stresses, such as drought, salinity or cold, and is thought to play a protective role during cellular dehydration (HANIN et al., 2011). The second one is involved in the biosynthesis of ABA.

Dehydrin and 9-cis-Epoxycarotenoid dioxygenase SAS chosen were also up-regulated in our microarray experiment of samples after 4 and 6 days without water (figure 48 and 49). It is interesting to note that although 9-cis-Epoxycarotenoid dioxygenase and Viviparous 14 are involved with the biosynthesis of ABA, changes in expression during drought progression shows that Viviparous 14 is induced earlier and is not differentially expressed in the 7th day, while 9-cis-Epoxycarotenoid dioxygenase starts to be induced on the 4th day and continues induced in the seventh (figures 47b and 48). Therefore, it seems that during the increase in the stress severity, both are active to lead to a higher Abscisic Acid biosynthesis.

The results for gene expression analysis shown in both chapters (I and II) demonstrates that despite the fact that microarray and drought progression experiments were done in different greenhouses, using different plants from the same variety, the outcomes agree largely, with gene up-regulation starting around the third day and having a peak between 5 and 6 days of stress (figures 47b, 48 and 49). Furthermore, expression starts to decrease on the seventh day for several genes, possibly indicating that plants may be entering on senescence stage (figures 48 and 49).

Besides that, from the 42 genes chosen from data mining of microarray results and SUCEST database, 32 appeared in the co-expression modules, and directed the second strategy to analyze the co-expression network (table 9). According to Serin et al., (2016), network based strategies should be largely determined by the biological question addressed as well as the prior knowledge available since they can be powerful to accelerate the elucidation of molecular mechanisms underlying important biological processes.

Among the modules from which more than one of the selected genes belong, the Bisque4 module covers the two PP2Cs, Dark Green module has an ABA biosynthesis and an ethylene induced protein, Green Yellow has an ABA receptor, an auxin biosynthesis, an ethylene transcription factor and a proteinase (table 9). The Light Cyan module has an ethylene induced, a transcription factor involved in hormone crosstalk and an endochitinase, while Light Green has an ethylene biosynthesis gene and a transcription factor that is a negative regulator of ABA induced cell death (table 9). Light yellow has an ABA biosynthesis, an ABA induced, an auxin induced and an unknown genes, Plum1 has an ABA induced, a lignin biosynthesis and an unknown genes, whereas Sienna3 has two ABA induced, one involved with RFOs sugars and a hypothetical protein (table 9).

Interestingly, the analysis of gene expression observed on the heatmap (figure 48b) shows that Dehydrin 9, LEA3 and Stachyose Synthase precursor that are found in the Sienna3 module are closely clustered, being the first two induced by ABA. Cinnamoyl-reductase 1-like and Desiccation-related PCC13-62-like from Plum1 module also, but the up-regulation of the last one starts at day 5 (figure 47b).

Aspartic protease in guard cell 1 and 9-cis-epoxycarotenoid dioxygenase chloroplastic-like from Light Yellow module are not clustered closely on the heatmap (figure 48b), possibly because, despite of both starting the up-regulation in day four, the aspartic protease is repressed on day one. This may be related to ABA-dependent signal transduction pathway, since 9-cis-epoxycarotenoid dioxygenase chloroplastic-like is involved in ABA biosynthesis and is up-regulated in the fourth day, which may increase ABA levels leading to the induction of aspartic protease in guard cell 1, which is an ABA induced gene.

Furthermore, a couple of interesting genes with high betweenneess, closeness and degree were observed from the modules selected using this section strategy. Among those are: ADP-glucose phosphorylase - EC:2.7.7.12 (Bisque4), Switch sucrose nonfermenting 3C (Black), 2-oxoglutarate dehydrogenase E1 component (Dark Green), Pyruvate kinase cytosolic - EC:2.7.1.40 and Ubiquitin interaction motif-containing (Green Yellow), Glycosyltransferase family 61 (Light Cyan), Probable galacturonosyltransferase-like 9, Mitogen-activated kinase kinase kinase YODA-like - EC:2.7.11, E3 ubiquitin- ligase RING1-like (Light Yellow). Also: 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily, Acyl-coenzyme A oxidase 2, Cytochrome b5, Long chain acyl-synthetase 4-like isoform X2 (Light Green), Sucrose-phosphatase 1 - EC:3.1.3.23; EC:3.1.3.24; EC:3.1.3.41, ABC transporter I family member 20 - EC:3.6.1.3; EC:3.6.1.15 (Pale Violet Red), Ethylene-responsive transcription factor ERF118-like (Salmon), Isocitrate dehydrogenase [NAD] catalytic subunit 5 mitochondrial - EC:1.1.1.41 and Pyrrolidone-carboxylate peptidase - EC:3.4.19 (Plum1). Most of them are involved with processes already discussed.

Overall, it was observed that networks with a higher number of nodes presents less nodes that have high betweeness, closenesss and degree at the same time. Interestingly, it seems that genes involved directly in the signal transduction pathway in response to ABA have a different pattern of expression than other drought related genes. Furthermore, a couple of classes were recurrent on the networks and will be further discussed (sections 4.5.2.1, 4.5.2.2, 4.5.2.3).

4.5.2.1. Calcium-signaling and MAPK

Several Calcium signaling and MAPK signal transduction pathways were found in the selected modules (appendix G). From the calcium signaling pathway we can highlight: Calcium Ion binding protein (Dark Green), Calcium-binding CML16-like (Dark Grey), CBL-interacting kinase family - EC:2.7.11 and CDPK-related kinase 3-like - EC:2.7.11; EC:2.7.11.17 (Dark Olive Green), CBL-interacting serine threonine-kinase 18 - EC:2.7.11, Calcium-dependent kinase 3-like - EC:2.7.11; EC:2.7.11.17 (Green), Probable CML32, CBL-interacting kinase 31-like - EC:2.7.11 (Light Cyan). Others found were: Calcium-dependent kinase isoform 11 - EC:2.7.11; EC:2.7.11.17, Calmodulin-7 - EC:1.3.1.74 (Light Green), Probable CML7, Calcineurin subunit B, CBL-interacting kinase 24 - EC:2.7.1, Ca2+ calmodulin-dependent kinase phosphatase - EC:3.1.3.16; EC:3.1.3.41, Ca2+ calmodulin-dependent kinase phosphatase - EC:3.1.3.16; EC:3.1.3.41 (Green Yellow), Calmodulin-related touch-induced, calcium-dependent kinase family - EC:2.7.11 (Light Yellow). The other ones are: CIPK expressed, CBL-interacting kinase - EC:2.7.11 (Pale Violet Red), CBL-interacting kinase 8 - EC:2.7.11, CML50 - EC:3.4.22, SNF1-related kinase catalytic subunit alpha KIN10-like - EC:2.7.11 (Salmon), CBL-interacting kinase 2 - EC:2.7.11, Calcineurin B 4 (Plum1).

Calcium (Ca^{2+}) regulates numerous signaling pathways involved in growth, development and stress tolerance (YOU; CHAN, 2015), and its cytosolic free concentration has been shown to change in response to mannitol (drought) in *Arabidopsis* seedlings (KNIGHT; TREWAVAS; KNIGHT, 1997). During stresses, such as drought, according to Huang et al., (2012), the perception of signals is followed by the generation of second messengers, as calcium, that can itself further regulate its intracellular level, which is then sensed by calcium binding proteins (Ca^{2+} sensors). These sensors change their conformation in a calcium dependent manner and interact with their respective interacting partners, often initiating a phosphorylation cascade that will end targeting stress responsive genes or the transcription factors controlling their expression. These products will lead to plant's adaptation and survival not only in the individual cell level, but also synergistically as an organism and may participate in plant hormones responses, which corroborates with their presence in the studied modules.

The CDPKs, for instance, are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs that can directly bind Ca^{2+}, and CBL-CIPK signaling pathways, has been shown to have key roles in the response to abiotic stresses. The CBL-Interacting Protein Kinase TaCIPK2 regulates stomatal movement and confers drought tolerance in tobacco transgenic plants, playing a positive regulatory role in drought stress responses (WANG et al., 2016b).

In the MAPK pathway, there were: Mitogen-activated kinase kinase kinase 1 - EC:2.7.11 (Dark Grey), Mitogen-activated kinase kinase kinase 1-like - EC:2.7.11(Green Module), MAP kinase family - EC:2.7.11; EC:2.7.11.24 (Green Yellow) and Mitogen-activated kinase kinase kinase 5 - EC:2.7.11 (Salmon), BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor - EC:2.7.11 (Plum1).

The signal transduction pathway through MAP kinases (mitogen-activated protein kinases) lead to a diversity of cellular responses, including the ones to environmental stresses (SINHA et al., 2011). They are represented by a conserved multigene family involved in transducing the signals to the nucleus for appropriate cellular adjustment, and their signaling cascade consists basically of 3 components, a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase) and a MAPK interconnected by the event of phosphorylation (SINHA et al., 2011).

The BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 precursor - EC:2.7.11 is also involved in the MAPK signaling pathway and was found differentially expressed in *Linum usitatissimum* (DASH et al., 2014). A maize MAPK kinase has also been shown to positively regulate salt and drought tolerance in transgenic *Arabidopsis* (CAI et al., 2014).

4.5.2.2. Transcription Factors

The analysis of the selected modules allowed the observation of several classes of drought related transcription factors that may have some functions in sugarcane response to water deficit stress (appendix G). The Auxin related transcription genes found were, for example, Auxin-responsive IAA17, ARF transcription partial (Black), Auxin response factor 7, Auxin response factor 2 (Green), Auxin response factor, Auxin response factor 15-like (Green Yellow), Auxin response factor 15-like, Auxin response factor 4, Auxin response factor 22 (Salmon).

The Ethylene related ones may be represented by: Ethylene-responsive TF 3 and ETHYLENE INSENSITIVE 3-like 1 (Dark Green), Ethylene-responsive element binding 2, ETHYLENE INSENSITIVE 3-like 3 (Dark Grey), Ethylene-responsive TF 3 – AS, AP2-like ethylene-responsive TF AIL5 – AS (Green), Ethylene response element binding, Ethylene-responsive TF1-like (Green Yellow), AP2 EREBP TF superfamily, Ethylene-responsive TF RAP2-3-like (Light Cyan). Also Ethylene-responsive transcription factor 4 Ethylene-responsive transcription factor 7 (Light Yellow), Ethylene-induced calmodulin-binding 4 (Salmon), AP2-like ethylene-responsive transcription factor At1g16060 (Light Green).

According to Müller; Munné-Bosch, (2015) ERFs are key regulatory hubs that belongs to the APETALA2/ERF family, regulating pathogen attack by binding to the GCC-box cis element, but can also bind to DRE and integrates ethylene, abscisic acid, jasmonate and redox signaling in abiotic stress responses.

ABA is a major phytohormone regulating plants' responses to drought and some of the transcription genes involved with ABA found were: ABA responsive element binding factor 1 (ABF 1), ABC transporter I family member 20 - EC:3.6.1.3; EC:3.6.1.15 (Pale Violet Red), ABSCISIC ACID-INSENSITIVE 5 2, ABSCISIC ACID-INSENSITIVE 5 5 (Salmon), ABI3VP1 transcription partial (Green). As previously mentioned (section 2.2.2.4) ABA-dependent gene expression is related to the presence of ABRE-motif on the cis-regulatory region of promoters.

Transcripts such as Abscisic Acid-Insensitive 5 2 are induced by drought, salt, ABA and glucose or 2-deoxy-glucose (2DG) and may play a role in senescence, while ABFs are a subfamily of bZIP proteins which binds strongly to ABRE and induce the expression of several ABA/stress responsive genes (CHOI et al., 2000).

Furthermore, there were members of DREB, transcription factors mentioned earlier that recognize DRE/CRT and activate downstream genes responsive to drought (SINGH; LAXMI, 2015). Dehydration responsive element binding 2 isoform a (Green), DRE binding factor (Dark Grey), Dehydration-responsive element-binding 1E (Light Yellow) were among those. NAC family of TFs identified can be represented by NAC domain-containing 43-like (Green), NAC transcription factor (Light Cyan), NAC domain-containing 68 (Light Green), NAC domain-containing 68 (Light Yellow). They are a big family of plant-specific TFs compounded by NAM, ATAF and CUC (NAC), being the ones involved in stress response and tolerance known as SNAC (stress-responsive NAC) (SINGH; LAXMI, 2015).

GRFs are a family of transcription factors identified as strongly expressed in growing and developing tissues (KIM; CHOI; KENDE, 2003). In this work they were represented by Growth-regulating factor (Black), Growth-regulating factor 5 – AS (Green), Growth-regulating factor 11 (Salmon). GRF7 was found to repress DREB2A expression in *Arabidopsis* (KIM et al., 2012) and represents a connection between this group of TFs and drought stress.

Heat Stress TFs characterized by Heat stress transcription factor A-2 isoform X2 (Black), Heat stress transcription factor A-5 (Green), Heat stress transcription factor A-1a (Sienna3). This family of TFs execute its role in plant tolerance against abiotic stresses by binding to HSE cis-acting elements in promoters of stress-inducible genes (GUO et al., 2016).

The MADS-box family were exemplified by MADS-box transcription factor 50 isoform X2 (Green), MADS-box transcription factor 50 isoform X2 (Salmon), MADS-box transcription factor 47-like isoform X1 (Plum1), and MYB family by Myb family transcription factor APL-like (Green), Transcription factor MYB1R1-like - EC:2.3.1.48 (Light Cyan), Transcription factor MYB108 (Light Green), Myb family transcription factor APL-like (Plum1), Myb-related 2 (Floral White). MADS-box were also found to be induced by drought and salt in *Brassica rapa* (SAHA et al., 2015).

MYB TFs have a conserved DNA-binding domain, MYB domain, with up to four imperfect amino acid sequence repeats (R) (DUBOS et al., 2010). They have been implicated in several processes, among those, abiotic stresses, as drought. AtMYB60, for example, was characterized by its involvement in the regulation of stomatal movements, since it is expressed in guard cells and is repressed during drought (COMINELLI et al., 2005). The null mutation leads to reduction of stomatal opening and decrease of wilting during water privation (COMINELLI et al., 2005). According to Baldoni; Genga; Cominelli, (2015), they are good candidates to improve stress tolerance and productivity in plants, since they are implicated not only in drought but also other stress.

MYB108, shown as a negative regulator of ABA induced cell death (CUI et al., 2013), has as first neighbors (figure 60) TFs, such as NAC domain 78, and transcripts as Aspartic protease 2, ubiquitin and proteasome related, Glutathione cytosolic, Cytochrome b5, Acyl-coenzyme A oxidase 2 and may also be related to processes of protein turnover, ROS responses, lipid metabolism.

Other TFs such as NF-Y, CCR4, GATA, bHLH, Scarecrow and WRKY also had representatives. Some were NF-Y subunit A-4-like (Green), NF-Y subunit B-4 (Green Yellow), NF-Y subunit B (Salmon); CCR4-associated factor 1 homolog 11 - EC:3.1.13 (Green), CCR4-NOT transcription complex subunit 1 - EC:3.1.15 (Green Yellow), Probable CCR4-associated factor 1 homolog 11 - EC:3.1.13.4 (Light Yellow); GATA TF 25 - EC:2.6.1.85 (Green), GATA TF 16 (Light Cyan); TF bHLH128-like (Light Cyan), TF bHLH77 isoform X2 – AS (Light Green);

SCARECROW 1-like (Light Green), Scarecrow 9 (Salmon), Probable WRKY TF 50 (Light Yellow), WRKY superfamily (Sienna3), Probable WRKY TF 32 (Salmon).

Nuclear Factor Y (NF-Y), for instance, are ubiquitous transcription factors composed of three distinct subunits (NF-YA, NF-YB, and NF-YC). NFYA5 was found by Li et al., (2008) to be strongly induced by drought stress in an ABA-dependent manner and that nfya5 knockout *Arabidopsis* plants had enhanced leaf water loss and were more sensitive to drought than WT plants, while the overexpression of this TF caused reduction in water loss and more resistance to drought stress. Furthermore, the same work found that NFYA5 is crucial for the expression of a number of drought stress–responsive genes.

4.5.2.3. Phytochromes and Cryptocromes

Interesting nodes found were Phytochrome, represented by Blue-light photoreceptor PHR2, having a Photolyase/cryptochrome alpha/beta domain (Dark Green), Phytochrome C - EC:2.7.13.3 and Phytochrome B - EC:2.7.13.3 (Green), Phytochrome-associated serine threonine- phosphatase - EC:3.1.3.16; EC:3.1.3.41 (Salmon); and Cryptochrome partial (Green) (appendix G).

Phytochromes and Cryptochromes are photoreceptors that seems to have some roles in abiotic stress responses. Phytochrome B has a function in the adjustment of morphological and physiological responses according to the changes in the red : far-red ratio (R:FR), and it was also shown to increase drought tolerance in *Arabidopsis thaliana* through enhancing ABA sensitivity when soil water becomes limiting (GONZÁLEZ et al., 2012).

Cryptochromes are blue light receptors found in plants, microbes and animals that regulates several processes, from circadian rhythms to stomata opening (YU et al., 2010). It has been shown that CRY1 may mediate the expression of blue light induced genes that trigger singlet-oxygen mediated programmed cell death in *Arabidopsis* (DANON; SANCHEZ COLL; APEL, 2006), also, another work made in *Arabidopsis* demonstrated that stomata from cry1 cry2 double mutant showed reduced blue light response and were drought-tolerant (MAO et al., 2005).

4.6. Conclusion

Co-Expression analysis allowed the detection of genes that can be used to direct future studies. Futhermore, the detection of genes with similar expression by RT-qPCR that belong to the same module indicates this strategy might be useful to study genes involved with specific processes. Despite that, its is necessary to further develop strategies to analyze the data obtained, once most of sugarcane genes are still considered unknown.

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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. RT-qPCR 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 is 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 RT-qPCR 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.

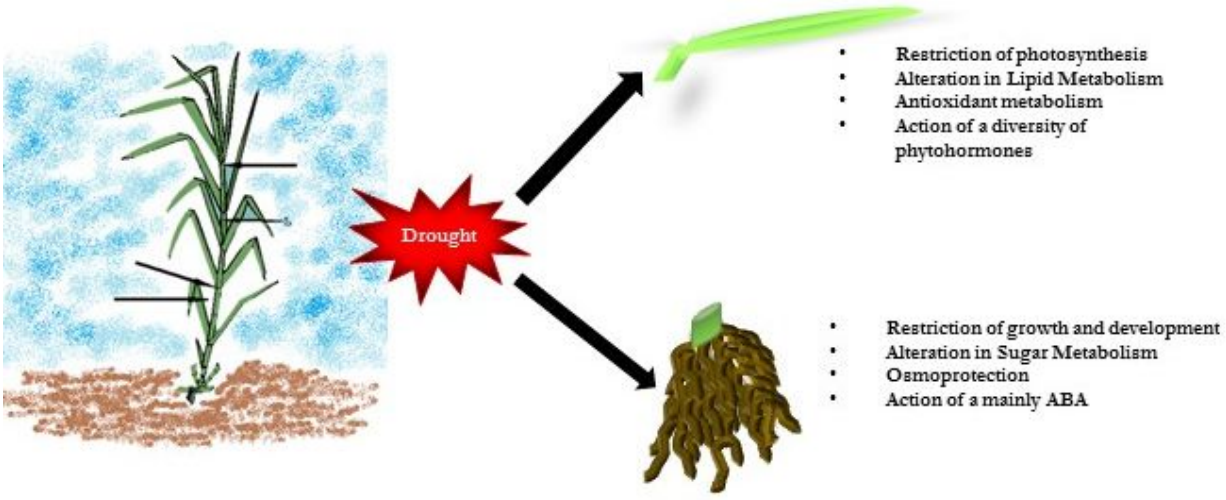


Figure 60. Summary of sugarcane leaves and roots responses to water stress as detected in this work

APPENDIX

APPENDIX A.

(continue)

Table 12. Sequence of primers used for qRT-PCR.

SAS	Description	FORWARD	REVERSE
SCACLR2007A07.g	LIP5	TGCATGCAGGTGGAACAGA	TGCATGAACCCGTGAAACAC
SCBFRZ3008D06.g	9-cis-Epoxycarotenoid dioxygenase	GCAGCGGCAGGCCTAGA	GTTAGGTGGTCGAGTTCGTGATC
SCBFSD1037G05.g	Desiccation-related protein PCC13-62	GCGCCAACCCCATCATC	CAGCAGCCCTGCCAGAAG
SCBGHR1058E08.g	Class III peroxidase 135 precursor	CAATCGGGCTCGCCTAGA	CCAGAGGCGCCAAATTGT
SCCCCL4005E12.g	RuBisCO large subunit-binding protein, chloroplast precursor	CGTGGCCTACAATTGCATGT	CCAACGAAACGGATCTGCAT
SCCCCL4005G05.g	Heat shock protein 26	AACGCACACTACCGCTACACAA	CGTTTATCGCCCCTTTTTCA
SCCCCL7C03G05.g	Gibberellin 2-oxidase	TGCTCTAATGGCCTGTTTTGG	GCATGCCGAAATAGGCAAGT
SCCCLB1023H08.g	Beta-expansin 1a	TCTGCCCAGGGTTAATTGGA	CGCCGGCATCATCGA
SCCCLR1001E04.g	Ribulose bisphosphate carboxylase small chain	CGACAGCGTCTCGAACTTCTT	AAGGATCCGGTGCATGCA
SCCCLR1C03C05.g	ABA responsive element binding factor 1	CACCGGTGCCGTACGTTT	TCCATAGCTGGTGGCTTCCTT
SCEPRZ1011A11.g	PP2C	TCCCAATCAGGTGCCACTTC	GAGGGAGTAGCCACCAATCG
SCEQLB1063D10.g	Senescence-associated protein-like	ATGGATCCGAAGATGACACAGA	GCGCACACCCACGAGAAG
SCJFRZ2014D06.g	NAC domain transcription factor	GGTGGTGCCGTCGTTGAT	GAGCAGGAGGGCTCAACTGT
SCMCRT2107D08.g	Beta-expansin 3	CAATCCAAGTACCTACGGATCG	GCATAGACTGCTGGAAATGGAA
SCMCSD2061D05.g	CBL-interacting protein kinase 19	AACCTTCAGTCCTAAACTCTTT	ACATCTACCAACACAACTTCAA
SCQGLR1085F11.g	Dehydrin	TGCGGCTTGGTGGTTTCT	GCACGCAGAGCTCTAGCTTAGC
SCQGST1032H03.g	Oligosaccharyl transferase STT3 subunit	GGATTTGCACCAGACGTTACAC	TCCGTCCTTGCTCGTGAAA
SCQSRT2031H06.g	Late embryogenesis abundant protein 3	GTTCCTGGCCGGTTCAATTA	AGGGTCTCTCAAGCTCGTGAAG
SCSBSB1052D07.g	Glyceraldehyde-3-phosphate dehydrogenase A,chloroplastic	CCAGGCTCTTCCCTGATGAA	GGCCTCGATTTCGCATTATTAC

(continuation)

SAS	Description	FORWARD	REVERSE
SCSFFL4085D03.g	ABRE-binding factor BZ-1	TCGGCTCGTGTCGCTAAA	TCTCGACGGGCCTTCGA
SCSGLR1045D05.g	HVA22 e	GAGCAGTGGCTCGCCTACTG	ACTCGAGGACCATCTCCATGA
SCVPFL3046C06.b	Phosphatase 2C family	CGCGTCCTGGGAGTCTTG	CACGTACGGCTTCAGGTAGTAGTC
SCEPRZ1010E06.g	Probable phosphatase 2C 50	CGGAGAACTTCATTTCCTAATC	GGATTTGACTGTCTGGATATTG
SCCCRZ2003H05.g	auxin-responsive IAA17	CCATGAGGTTTTACTGGCGG	TGTAGGGCAGGTCTTGGAAC
SCCCLR1048H03.g	Viviparous 14	CCATGTCATCCAATCCAAAG	CTAGAGATCGAGCAACTAGAG
SCJLAM1062A05.g	probable phospholipid hydroperoxide glutathione peroxidase	AGCGGTACGCACCAACAAC	TCCCTAGTAGTTTCTTGATGTCCTTCT
SCVPRZ3026C06.g	DRE binding factor	GGCTTCCCTCTTCTGTTTCGT	CTGCCGTGCCAATCTATCGT
SCBFRZ2048D04.g	CBL-interacting kinase family	AGGTTTGTGGCAGCCTTCAT	GCCCCAGTGCTTGCTACAAG
SCBGFL5079F06.g	Auxin Signaling F-BOX 3	GCAAGGATCTACACGAACT	CGGCAGCCTGATGATATT
SCJFLR1073H12.g	Delta 1-pyrroline-5-carboxylate synthetase 1	GATAGTGCCGCTGTGTTTCA	CGCCCTGTACTTATGCCAAC
SCVPRZ3030G09.g	Probable esterase PIR7A	CAGAGGAAGCGTCCATTAC	CGAGTACGAGCGTCTATCTA
SCVPRZ2041G12.g	indole-3-acetic acid amido synthetase	ACATCGTTCTCTGCTCTTC	GGTCATCACCGCCATTTA
SCCCCL4002B07.g	Ethylene-responsive factor 1	GGAACGAACCCTGGAATTA	AGGCTTTGGCTGTAGAAG
SCEPLB1043F06.g	Aspartic proteinase nepenthesin-2	GAAAAACCACTCCCTCTCCA	CGCCCTATGATGATTGATGA
SCCCCL4007F05.g	glutathione *S*-transferase GSTU6	CCTCCTACGTCGAAGACAAGTTGTA	CCCGTCCTGCCTCTGAAA
SCCCRT3001A02.g	Endochitinase A-like	GAAGTGTTCACGTCAATCTG	AGAGTGCGTAGTGGTATTG
SCEZLR1009E06.g	1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase)	CTTCAGGCTTCAATGGTTATG	GACGCTACACACTTTACAATAC
SCSGAD1007F02.g	Transcription factor MYB108	CTAGTGTGACATCTCGATCAT	CTAGCTCGCTAAAGCCTAC
SCCCRT1C01F08.g	SAUR11 - auxin-responsive SAUR family member	CTCACCCCTCAGCCTCAG	ACACAACGAAACGGGACAAG
SCEZAD1C01D10.g	---NA---	GGAAAGGATGGTGAGTGAGC	CCAACAAGAGAGAGGGTCGT
SCBFRZ2046E11.g	Aspartic protease in guard cell 1	GTCCTCGGCTTGATTGAT	ATTCCTAACCTTCTCTCCTTG
SCCCCL6024F07.g	Cinnamoyl-reductase 1-like	CGCTGCTTTTGTTTGATGACA	CTGTACGAGACTGTGGTGTGCTT

(conclusion)

SAS	Description	FORWARD	REVERSE
SCBGLR1117C01.g	---NA---	CATGGTGGTCTTGTAAATAGC	CCGACGACCCTTAAACTT
SCCCCL3003E07.b	Chitinase partial	CCACTTGTTCGACCTGAATA	TCGTAAGTACAAGCGATCTG
SCJFLR1017E09.g	Stachyose synthase precursor	CACGACCATTGCCATTTG	AAAGCATACTCTCTCCATACC
SCJLLR1107D06.g	hypothetical protein SORBI_002G141800	AGGAGGAGGCAAGGCAACA	CTGACGCAACCAATCTAAAATCG
SCMCRT2085F03.g	probable indole-3-pyruvate monooxygenase YUCCA10	CCCTCCTTCTTCTTCTACCT	CGCTGTGTGTGTGTATGT
SCCCRT2001C12.g	Auxin-binding 5	CTCACTGGTGAGAGACATAAG	CACTTCCACCTCCTTCATC
SCCCCL3120A10.b	Auxin response factor	CAGGTTGAGGAAGGGTTT	TCAGCAAGTCGGTAGATATG
SCJLRT1006C03.g	1-aminocyclopropane-1-carboxylate synthase (ACC synthase)	GGTGCTCCATGATCCATTGC	CGAGAACCAACTGTCGCTTG
SCSBAD1086C02.g	Ethylene response sensor 2	AGGTGGCATTTTGGTGGTTG	CAATCGCATCCATCCATCCG
SCSBHR1056H08.g	Ethylene insensitive 2	GCGCACACTTCCCAACTGT	ACACCAAAACTAACAACGAGTGATG
SCEZAM2034C10.g	Probable calcium-binding CML10	GGCTCCGAACCACTGCTTT	CGTGAACCGCTGAGGATATTG
SCSGLV1008B03.g	Flowering-promoting factor 1 1	TCTCGCTGAATTTGATTTCG	ATCATCACTCCCAGACGACA
SCEPSD1007C11.g	Pathogenesis-related class partial	GTCGTCGGACAGAGCCCATA	CATGCGATGCTGAAAGCGTAT
SCRLLR1038F07.g	hypothetical protein SORBIDRAFT_06g026710	CTCTGCTCCCGTTGTTTCTG	CACACACCCTCACCCAAAAG
SCCCLR1072A03.g	Mitochondrial ATP Synthase subunit delta	GGGCAGAAGGTGTTTGATATGTC	ATCCAAAAGGCTGTTAGAATTCAAG
SCCCLR1048F12.g	Ubiquitin	GGTGGCCGGCTTGGA	TTTGTTTCGGTTTCAAGTCGATAA
SCBGLR1002D06.g	E2 ubiquitination enzyme	CAGGTCCTGCTGGTGAGGAT	CACCTCCAGCATATGGACTATCAG
SCJFRZ2009G01.g	60S ribosomal subunit	GCGAGTGCCTCACCTTTGAC	TCTTAGGTCCCCTCAGCAGAAC
Iskandar et al.	GAPDH	CACGGCCACTGGAAGCA	TCCTCAGGGTTCCTGATGCC

APPENDIX B.

(continue)

Table 13. qRT-PCR validation data for leaves and root samples in all conditions evaluated in the present work. 0 = absence of validation; OK = success in validation. Asterisks indicate a significant statistical difference, according to t-test, therefore, *; **; *** for p≤5%; p≤1% and p≤0,1%, respectively.

Experiment	SAS	Description	Microarray	Vandersompele	t Test	Statistical Signficance	Validation
Leaf - 4 DOD	SCEQLB1063D10.g	Senescence-associated protein-like	DOWN	UP	0.048488566	*	0
	SCJFRZ2014D06.g	NAC domain transcription factor	UP	UP	5.25E-05	***	OK
	SCSGLR1045D05.g	HVA22-like protein e	UP	UP	3.99E-08	***	OK
	SCQGLR1085F11.g	Dehydrin	UP	UP	3.68E-07	***	OK
	SCBFRZ3008D06.g	9-cis-Epoxycarotenoid dioxygenase	UP	UP	3.64E-07	***	OK
	SCVPFL3046C06.b	PP2C	UP	UP	1.32E-05	***	OK
	SCCCLR1C03C05.g	ABA responsive element binding factor 1	UP	UP	4.86E-07	***	OK
	SCEPRZ1011A11.g	PP2C	DOWN	DOWN	0.002503023	**	OK
	SCCCCL7C03G05.g	Gibberellin 2-oxidase	UP	UP	6.97E-09	***	OK
Leaf - 6 DOD	SCEQLB1063D10.g	Senescence-associated protein-like	UP	UP	3.71E-05	***	OK
	SCCCLR1001E04.g	Ribulose bisphosphate carboxylase small chain	DOWN	DOWN	7.27E-08	***	OK
	SCBFSD1037G05.g	Desiccation-related protein PCC13-62	UP	UP	1.90E-05	***	OK
	SCSBSB1052D07.g	Glyceraldehyde-3-phosphate dehydrogenase A,chloroplastic	DOWN	DOWN	1.44E-09	***	OK
	SCQGLR1085F11.g	Dehydrin	UP	UP	5.49E-09	***	OK
	SCBFRZ3008D06.g	9-cis-Epoxycarotenoid dioxygenase	UP	UP	5.42E-08	***	OK
	SCCCCL4005E12.g -Sense	RuBisCO large subunit-binding protein, chloroplast precursor	DOWN	DOWN	1.36E-05	***	OK
Leaf – Re-watering	SCSGLR1045D05.g	HVA22-like protein e	DOWN	DOWN	1.31E-07	***	OK
	SCBGHR1058E08.g	Class III peroxidase 135 precursor	UP	UP	9.83E-05	***	OK
	SCVPFL3046C06.b	PP2C	DOWN	DOWN	5.19E-05	***	OK

(continuation)

Experiment	SAS	Description	Microarray	Vandersompele	t Test	Statistical Signficance	Validation
Leaf – Re-watering	SCCCCL7C03G05.g	Gibberellin 2-oxidase	DOWN	DOWN	1.16E-07	***	OK
	SCCCCL4005E12.g-Sense	RuBisCO large subunit-binding protein, chloroplast precursor	UP	DOWN	0.965534183	-	0
	SCCCCL4005E12.g-Anti-Sense	RuBisCO large subunit-binding protein, chloroplast precursor	UP	UP	1.45E-06	***	OK
Root – 4 DOD	SCSFFL4085D03.g	ABRE-binding factor BZ-1	UP	UP	0.001988091	**	OK
	SCACLR2007A07.g	LIP5	UP	UP	5.13E-09	***	OK
	SCSGLR1045D05.g	HVA22-like protein e	UP	UP	4.26E-09	***	OK
	SCQGLR1085F11.g	Dehydrin	UP	UP	3.12E-07	***	OK
	SCQSRT2031H06.g	Late embryogenesis abundant protein 3	UP	UP	1.62E-07	***	OK
	SCVPFL3046C06.b	PP2C	UP	UP	1.32E-05	***	OK
	SCCCLR1C03C05.g	ABA responsive element binding factor 1	UP	UP	2.18E-09	***	OK
Root – 6 DOD	SCMCRT2107D08.g	Beta-expansin 3	DOWN	DOWN	0.005328676	**	OK
	SCSFFL4085D03.g	ABRE-binding factor BZ-1	UP	UP	0.000146353	***	OK
	SCBGHR1058E08.g	Class III peroxidase 135 precursor	DOWN	DOWN	2.28E-06	***	OK
	SCCCLB1023H08.g	Beta-expansin 1a	DOWN	UP	6.05E-06	***	0
	SCSGLR1045D05.g	HVA22-like protein e	UP	UP	1.72E-09	***	OK
	SCQGLR1085F11.g	Dehydrin	UP	UP	2.00E-07	***	OK
	SCCCCL4005G05.g	Heat shock protein 26	UP	UP	2.90E-06	***	OK
	SCQSRT2031H06.g	Late embryogenesis abundant protein 3	UP	UP	7.95E-08	***	OK

(conclusion)

Experiment	SAS	Description	Microarray	Vandersompele	t Test	Statistical Signficance	Validation
Root – 6 DOD	SCBFRZ3008D06.g	9-cis-Epoxycarotenoid dioxygenase	UP	UP	2.49E-09	***	OK
	SCVPFL3046C06.b	PP2C	UP	UP	7.19E-08	***	OK
	SCQGST1032H03.g – Anti-Sense	Oligosaccharyl transferase STT3 subunit	UP	DOWN	7.05E-05	***	0
Root – Re-watering	SCMCSD2061D05.g	CBL-interacting protein kinase 19	UP	UP	1.37E-05	***	OK
	SCMCRT2107D08.g	Beta-expansin 3	DOWN	DOWN	0.00014268	***	OK
	SCCCLB1023H08.g	Beta-expansin 1a	DOWN	UP	0.517445425	-	0
	SCQGLR1085F11.g	Dehydrin	UP	UP	1.65E-05	***	OK
	SCCCCL4005G05.g	Heat shock protein 26	UP	UP	2.58E-07	***	OK
	SCQSRT2031H06.g	Late embryogenesis abundant protein 3	UP	UP	0.001160097	**	OK

APPENDIX C. Annotation of leaves and roots differentially expressed genes determined by HTSelf method (continue)

Table 14. Leaves differentially expressed genes after 4 days of stress determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCAGLR1021A05.g	GS1_SS_03786_09462	Hypothetical protein SORBIDRAFT_04g028585	NA	---NA---	402	3	1.91E-19	71.33%
SCBFRZ3008D06.g	GS1_SS_15333_16913	9-cis-epoxycarotenoid dioxygenase chloroplastic-like	LOC103642321	↑	588	10	3.99E-65	95.40%
SCBGLR1023D05.g	GS1_SS_02103_17384	LSD1 isoform X2	LDL1	↓	950	10	7.26E-65	98.10%
SCCCCL3005D01.b	GS1_SS_01463_17788	Probable phosphatase 2C 30	OsP2C30	↑	1677	10	0.00E+00	84.70%
SCCCCL4001D06.g	GS1_SS_06096_02741	EXORDIUM-like 1	EXL1	↑	1304	10	8.91E-129	81.40%
SCCCCL7C03G05.g	GS1_SS_21236_03669	Gibberellin 2-beta-dioxygenase-like	LOC103636636	↑	697	10	1.40E-28	92.40%
SCCCLR1048H03.g	GS1_SS_20675_19867	Viviparous 14	ZmVP14	↑	1275	10	1.42E-166	96.30%
SCCCLR1075D11.g	GS1_SS_04303_00173	NA	NA	↑	718	NA	NA	NA
SCCCLR1C03C05.g	GS1_SS_01335_10053	ABA responsive element binding factor 1	OsABF1	↑	1149	10	7.00E-111	86.50%
SCCCLR1C03C11.g	GS1_SS_10186_07079	Glycerol-3-phosphate transporter 1	PS3	↓	2038	10	0.00E+00	92.40%
SCCCLR1C06D03.g	GS1_SS_12746_10371	Alpha-L-fucosidase 1	FUC1	↑	1829	10	0.00E+00	91.50%
SCCCRT1002F09.g	GS1_SS_11716_03359	TPA: bZIP transcription factor superfamily	ZmbZIP34	↑	1182	10	4.34E-104	83.40%
SCEPLB1043F06.g	GS1_SS_18547_10976	Aspartic proteinase nepenthesin-2	LOC100283965	↑	547	10	1.53E-91	92.70%
SCEPRT2048A10.g	GS1_SS_22981_14481	NUCLEAR FUSION DEFECTIVE 4	NFD4	↓	999	10	1.91E-109	91.80%
SCEPRZ1010E06.g	GS1_SS_17276_20176	Probable phosphatase 2C 50	OsP2C50	↑	2403	10	4.57E-87	92.70%
SCEPRZ1011A11.g	GS1_SS_14297_03846	Phosphatase 2C containing expressed	AT3G17090	↓	2076	10	0.00E+00	84.40%
SCEPRZ3132A06.g	GS1_SS_20556_07822	Ubiquitin ligase	T8K14.20	↑	435	4	6.26E-09	84.25%
SCEQLB1064H09.g	GS1_SS_09110_10359	Peptidyl-prolyl cis-trans isomerase FKBP16-chloroplastic	LOC103643054	↓	885	10	1.39E-48	91.40%
SCEQLB1066G04.g	GS1_SS_00806_21326	Probable mixed-linked glucan synthase 6	LOC103641257	↑	4081	10	0.00E+00	94.80%
SCEQLB1067F01.g	GS1_SS_12045_19144	Bark storage A	NA	↓	1260	10	0.00E+00	88.20%
SCEZAD1C01D10.g	GS1_SS_00508_12015	NA	NA	↑	634	NA	NA	NA

(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJFLR1017E09.g	GS1_SS_08724_06083	Stachyose synthase precursor	LOC103641257	↑	1336	10	0.00E+00	87.90%
SCJFRZ2014D06.g	GS1_SS_12278_15377	NAC transcription factor	NAC069	↑	1329	10	1.48E-141	88.60%
SCJLLR1107D06.g	GS1_SS_11621_09365	Hypothetical protein SORBI_002G141800	NA	↑	768	5	4.46E-17	83.80%
SCJLLR2013D11.g	GS1_SS_11458_09280	Random slug 5-like	LOC103626942	↓	1072	10	5.60E-170	82.20%
SCJLRZ3077F12.g	GS1_SS_16018_00210	Undecaprenyl pyrophosphate synthetase	OsUPPs	↑	716	10	7.32E-112	89.30%
SCQGLR1085F11.g	GS1_SS_11161_19643	Dehydrin 9	Zmdhn-2	↑	760	10	2.03E-23	71.40%
SCRFLB2060E04.g	GS1_SS_25725_08917	Disulfide isomerase	PDIL2-2	↑	1426	10	0.00E+00	95%
SCRFRZ3056G09.g	GS1_SS_16192_16179	Refined Structure Of The Cytochrome B Reductase Fragment	NA	↓	1126	10	2.33E-180	96.70%
SCRLLR1038F07.g	GS1_SS_12299_12089	Hypothetical protein SORBIDRAFT_06g026710	NA	↑	810	10	2.59E-41	93.70%
SCRLRZ3113A05.g	GS1_SS_25731_08523	NA	NA	↑	478	NA	NA	NA
SCSBAM1085B06.g	GS1_SS_15769_16971	Fatty acid partial	NA	↓	810	10	9.45E-125	94.30%
SCSBSD1056F06.g	GS1_SS_25729_14516	NA	NA	↓	296	NA	NA	NA
SCSFFL4082E10.g	GS1_SS_16146_13026	ABI five biding 3	AFP3	↑	767	10	1.66E-118	92.50%
SCSGLR1045D05.g	GS1_SS_24901_21213	HVA22 e	HVA22E	↑	758	10	8.32E-76	89.70%
SCUTLR2008H10.g	GS1_SS_09610_06203	Hypothetical protein BAE44_0024388	NA	↑	570	2	1.79E-07	99%
SCVPFL3046C06.b	GS1_SS_16946_20384	Phosphatase 2C family	NA	↑	805	10	7.74E-94	95.50%
SCVPRT2078D10.g	GS1_SS_14106_09883	NA	NA	↑	569	NA	NA	NA
SCVPRZ2041G12.g	GS1_SS_06726_11141	Indole-3-acetic acid amido synthetase	ZmGH3	↑	2365	10	0.00E+00	92.40%
SCVPRZ3026C06.g	GS1_SS_19681_18294	DRE binding factor	Zmdbf1	↑	1324	10	9.67E-125	76.10%

(continue)

Table 15. Leaves differentially expressed genes after 6 days of stress determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCACLR1057H07.g	GS1_SS_04477_20876	Oil yellow-1	ZmOY1	↓	1635	10	0.00E+00	94.60%
SCACLR1126G08.g	GS1_SS_09620_01141	NA	NA	↓	159	NA	NA	NA
SCACSD2018E08.g	GS1_SS_25481_14374	Stem 28 kDa glyco - like	umc2754	↓	603	10	1.83E-116	86.90%
SCACST3159D04.g	GS1_SS_18393_15001	Inosine-5'-monophosphate dehydrogenase	T8K14.11	↑	1262	10	0.00E+00	96.80%
SCAGAM2018H09.g	GS1_SS_03687_06940	Rhodanese-like domain-containing chloroplastic	Os02g0596000	↓	977	10	8.43E-149	92.90%
SCAGAM2126G09.g	GS1_SS_20067_01031	NAD(P)H-quinone oxidoreductase subunit chloroplastic	LOC103641886	↓	813	10	3.61E-61	89.90%
SCAGFL3022F05.g	GS1_SS_22556_11250	Hypothetical protein SORBI_005G178000	NA	↑	856	1	2.27E-26	77%
SCAGLR1021F10.g	GS1_SS_07364_07335	Hypothetical protein SORBIDRAFT_10g023690	NA	↓	796	10	3.20E-76	87.80%
SCAGRT2039D11.g	GS1_SS_12834_13883	Hypothetical protein SORBIDRAFT_10g006790	NA	↑	560	4	2.40E-19	84.75%
SCAGRT2041F09.g	GS1_SS_12859_09283	NA	NA	↑	574	NA	NA	NA
SCBFLR1005H06.g	GS1_SS_16572_16695	Cytochrome P450 87A3-like	LOC103627541	↓	1503	10	0.00E+00	83.30%
SCBFRZ2019C12.g	GS1_SS_17262_00357	Translation initiation factor IF-2-like	ZmIF-2	↑	764	10	2.15E-60	68.50%
SCBFRZ2019D02.g	GS1_SS_03259_08864	TPA: bZIP transcription factor superfamily	LOC103644180	↓	826	10	6.77E-56	81.80%
SCBFRZ2046E11.g	GS1_SS_04484_01565	Aspartic protease in guard cell 1	AT3G18490	↑	1644	10	5.20E-169	89.40%
SCBFRZ2048D04.g	GS1_SS_03974_02428	CBL-interacting kinase family	NA	↑	810	10	3.59E-118	88.40%
SCBFRZ2048F01.g	GS1_SS_26284_05683	Transport and Golgi organization 2 homolog	NA	↑	614	10	1.67E-121	97%
SCBFRZ3008D06.g	GS1_SS_15333_16913	9-cis-epoxycarotenoid dioxygenase chloroplastic-like	NCED5	↑	588	10	3.99E-65	95.40%
SCBFSD1034C06.g	GS1_SS_26225_05201	Polyphenol oxidase	NA	↑	883	10	3.60E-102	91.70%
SCBFSD1037G05.g	GS1_SS_25364_10919	desiccation-related PCC13-62-like	LOC103641612	↑	752	10	1.12E-118	88.80%
SCBGLR1002B09.g	GS1_SS_16279_00786	NA	NA	↓	460	NA	NA	NA
SCBGLR1117C01.g	GS1_SS_25660_05461	NA	NA	↑	469	NA	NA	NA

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCBGLR1120B07.g	GS1_SS_10078_07251	High-light-induced chloroplastic	LOC103637819	↓	560	10	5.72E-59	82%
SCCCAD1002F05.g	GS1_SS_00031_10674	Exonuclease chloroplastic mitochondrial isoform X3	NA	↑	1131	10	0.00E+00	91.90%
SCCCCL2001D02.b	GS1_SS_07118_15123	Peroxidase 5-like	LOC103653548	↑	1220	10	0.00E+00	93.90%
SCCCCL3001B09.b	GS1_SS_12984_15678	Aconitate cytoplasmic	Zmaco2	↑	2960	10	0.00E+00	97.40%
SCCCCL3003E07.b	GS1_SS_06601_18767	Chitinase partial	ZmCHN1	↑	1153	10	8.21E-162	96.20%
SCCCCL3080B09.b	GS1_SS_05864_20064	Probable indole-3-acetic acid-amido synthetase	NA	↑	554	10	3.53E-36	97.20%
SCCCCL3120A10.b	GS1_SS_05899_00214	Auxin response factor	OsARFL	↑	1098	10	3.09E-68	99.80%
SCCCCL4001F08.g	GS1_SS_05956_20395	Malate synthase	MLS	↑	940	10	7.36E-121	94.20%
SCCCCL4002B07.g	GS1_SS_06324_18757	Ethylene-responsive factor 1	ZmERF1	↑	1708	10	4.00E-79	81%
SCCCCL4003D06.g	GS1_SS_05985_18582	Anthocyanidin 5,3-O-glucosyltransferase	LOC103626938	↑	1176	10	0.00E+00	85.80%
SCCCCL4005E12.g	GS1_SS_09119_19261	Rubisco large subunit-binding subunit chloroplastic	LOC103629384	↓	1803	10	0.00E+00	95.30%
SCCCCL4007D09.g	GS1_SS_06055_10447	NA	NA	↑	725	NA	NA	NA
SCCCCL6024F07.g	GS1_SS_25287_16690	Cinnamoyl-reductase 1-like	CCR1	↑	1494	10	0.00E+00	93%
SCCCFL1097H04.g	GS1_SS_24385_14343	WD40-like beta propeller repeat family	LOC103625729	↓	540	10	2.26E-61	91.20%
SCCCHR1004C05.g	GS1_SS_07375_12099	Hypothetical protein SORBIDRAFT_09g002380	NA	↓	604	9	9.91E-32	87.78%
SCCCLB1001E04.g	GS1_SS_03025_20848	Acid phosphatase 1-like	LOC100191194	↓	847	10	1.55E-141	84.90%
SCCCLB1021E12.g	GS1_SS_16038_21347	Protoporphyrinogen chloroplastic	TOPP2	↓	919	10	2.82E-138	97.80%
SCCCLR1001E04.g	GS1_SS_09575_15878	Chloroplast ribulose-1,5-bisphosphate carboxylase oxygenase small subunit	ZmSSU1	↓	1062	10	1.15E-113	95.90%
SCCCLR1048C12.g	GS1_SS_10109_00767	Alkaline alpha galactosidase 2	ZmAGA2	↑	1123	10	0.00E+00	93.30%
SCCCLR1065C08.g	GS1_SS_10150_13292	Hypothetical protein SORBIDRAFT_10g005620	NA	↑	1000	10	2.97E-84	79.90%
SCCCLR1072G11.g	GS1_SS_10129_08956	Photosynthetic NDH subunit of subcomplex B chloroplastic	ndhB	↓	823	10	1.92E-32	65.70%
SCCCLR1C04G11.g	GS1_SS_19109_01089	Outer envelope pore 16- chloroplastic	LOC103635971	↑	848	10	1.36E-106	90%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCLR2003C05.g	GS1_SS_10887_09804	Cytochrome P450 87A3-like	LOC103627541	↓	825	10	1.47E-69	88.10%
SCCCLR2C01F06.g	GS1_SS_11286_19433	Hypothetical protein SORBIDRAFT_06g029880	NA	↑	546	10	1.60E-37	88.20%
SCCCRT3001A02.g	GS1_SS_24414_20650	Endochitinase A-like	LOC103646280	↑	481	10	5.26E-47	92.80%
SCCCRZ2C03C09.g	GS1_SS_03914_01548	Probable AMP deaminase	FAC1	↓	1190	10	1.33E-97	95.60%
SCCCRZ2C04C11.g	GS1_SS_01867_08709	F-box domain containing	F1M20.19	↑	749	10	1.54E-75	93.30%
SCCCRZ3001D04.g	GS1_SS_25370_19926	Triose phosphate phosphate chloroplastic isoform X2	LOC103628720	↓	1008	10	9.23E-122	97.10%
SCCCRZ3001D06.g	GS1_SS_17037_20889	LOV domain-containing	LOV1	↓	1901	10	0.00E+00	95.80%
SCCCSD2002A10.g	GS1_SS_25196_11886	O-acyltransferase WSD1	WSD1	↑	748	10	1.73E-110	90.60%
SCCCST2004E05.g	GS1_SS_17839_02986	Probable flavin-containing monooxygenase 1	LOC103631795	↓	743	10	1.59E-146	90.80%
SCEPAM2011H12.g	GS1_SS_16579_19888	Mitochondrial uncoupling 3	LOC103646382	↑	1447	10	0.00E+00	95.40%
SCEPAM2014A10.g	GS1_SS_03976_08701	Initiator-binding 2	NA	↑	1305	10	0.00E+00	91.60%
SCEPLB1043F06.g	GS1_SS_18547_10976	Aspartic proteinase nepenthesin-2	AT3G18490	↑	547	10	1.53E-91	92.70%
SCEPLR1008A10.g	GS1_SS_23522_14785	Auxin-responsive IAA31-like	IAA31	↓	1464	10	8.48E-94	72.40%
SCEPRT2048D07.g	GS1_SS_12960_09973	NAD(P)H-dependent oxidoreductase	IDP2346	↑	1255	10	0.00E+00	92.60%
SCEPRZ3045E10.g	GS1_SS_20157_14564	Probable serine threonine- kinase WNK5	NA	↑	1359	10	0.00E+00	96.60%
SCEPRZ3046F03.g	GS1_SS_18833_01293	Probable glucuronosyltransferase Os04g0650300	LOC103648921	↑	1622	10	0.00E+00	91.80%
SCEPRZ3129G09.g	GS1_SS_19353_07185	Eukaryotic peptide chain release factor subunit 1-2-like	LOC103642704	↑	573	10	1.11E-97	98.10%
SCEPRZ3132A06.g	GS1_SS_20556_07822	Ubiquitin-ligase	NA	↑	435	4	6.26E-09	84.25%
SCEPSB1136F09.g	GS1_SS_21332_12694	Probable glycerol-3-phosphate acyltransferase 3	LOC103648202	↑	1258	10	1.90E-126	80.60%
SCEPSD1007C11.g	GS1_SS_25637_17940	Pathogenesis-related class partial	PR1	↑	700	10	9.34E-74	97%
SCEPSD2005D11.g	GS1_SS_25416_00588	NAD(P)H-quinone oxidoreductase subunit chloroplastic	ndhA	↓	774	10	2.63E-124	82.60%
SCEPSD2008F06.g	GS1_SS_25775_17841	Phosphoribulokinase precursor	OsPRK	↓	953	10	0.00E+00	98.10%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCEQLB1063D10.g	GS1_SS_09088_14427	Seven-transmembrane-domain 1	LOC100273830	↑	1154	10	6.72E-154	89.50%
SCEQLB1063H01.g	GS1_SS_18982_10957	Non-specific lipid transfer GPI-anchored 2-like	NA	↑	822	10	2.79E-46	90.80%
SCEQLB1063H05.g	GS1_SS_09095_14043	Photosynthetic NDH subunit of subcomplex B chloroplastic	ndhI	↓	851	10	2.82E-148	87.90%
SCEQLB1068B05.g	GS1_SS_18596_08565	Hypothetical protein SORBIDRAFT_03g005770	NA	↑	942	1	1.67E-06	81%
SCEQLR1091E07.g	GS1_SS_10378_04719	Hypothetical protein SORBI_004G274100	NA	↓	1223	10	4.03E-171	94.10%
SCEQRT1024H10.g	GS1_SS_13487_20028	Pathogenesis-related 5	ZmPR-5	↑	900	10	1.52E-96	89.50%
SCEQRT1025C04.g	GS1_SS_11924_18787	Alkane hydroxylase MAH1	CYP96A15	↑	792	10	2.10E-103	93.60%
SCEQRT1029F09.g	GS1_SS_11796_19381	Glutamate decarboxylase	LOC100501579	↑	1686	10	0.00E+00	94%
SCEQRT2027C06.g	GS1_SS_24536_12695	Wound induced	LOC100280811	↑	478	10	2.12E-36	78.60%
SCEQRT2027G07.g	GS1_SS_12262_12384	Universal stress A	LOC103634867	↓	954	10	7.13E-92	79.50%
SCEQRT2091H09.g	GS1_SS_13053_10192	Hypothetical protein SORBIDRAFT_01g017040	NA	↑	892	10	6.40E-91	94.50%
SCEQRT2098E08.g	GS1_SS_13124_20300	NA	NA	↑	597	NA	NA	NA
SCEZAD1081D01.g	GS1_SS_19544_12238	Uncharacterized protein LOC100277183	LOC100277183	↓	662	10	3.07E-55	87.70%
SCEZAD1C01D10.g	GS1_SS_00508_12015	NA	NA	↑	634	NA	NA	NA
SCEZAM2034C10.g	GS1_SS_04207_10459	Probable calcium-binding CML10	NA	↑	516	10	1.55E-15	95.50%
SCEZAM2058C09.g	GS1_SS_04238_10821	Diacylglycerol O-acyltransferase 2	AT3G51520	↑	731	10	6.02E-47	74.10%
SCEZFL5089C05.g	GS1_SS_23691_13712	Glycerol-3-phosphate acyltransferase 3	LOC103648202	↑	737	10	2.86E-156	91.70%
SCEZHR1049C07.g	GS1_SS_07647_21820	50S ribosomal chloroplastic	ZmPRPL35-1	↓	539	10	3.93E-94	87.40%
SCEZLB1006C12.g	GS1_SS_08332_12007	Hypothetical protein SORBIDRAFT_10g006240	NA	↑	831	10	4.19E-56	74.90%
SCEZLB1006F11.g	GS1_SS_17085_18093	Phosphoglycerate kinase	PGK	↓	1604	10	0.00E+00	96.90%
SCEZLB1007G07.g	GS1_SS_09918_08397	Wound stress	NA	↑	577	10	3.27E-40	93%
SCEZLB1009D04.g	GS1_SS_13925_19329	Hypothetical protein SETIT_027185mg	NA	↑	744	1	6.96E-06	100%
SCEZLB1013H04.g	GS1_SS_09241_18534	Adenine phosphoribosyltransferase 2	LOC100283282	↑	1019	10	4.68E-135	87.10%
SCEZLR1031G02.g	GS1_SS_10424_20078	Chlorophyll a-b binding 1B- chloroplastic	LOC103642498	↓	1109	10	5.60E-171	95.60%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCEZLR1052F03.g	GS1_SS_02033_04411	Copper transport ATX1	ATX1	↑	1219	10	3.88E-37	90%
SCEZRT2019G08.g	GS1_SS_13220_12766	40 partial	NA	↑	602	10	1.31E-18	81.90%
SCEZRZ1014E09.g	GS1_SS_18010_10601	kDa heat shock-like	OsHSP90	↓	1103	10	7.72E-79	81.30%
SCJFAD1011C01.g	GS1_SS_00551_14059	Acyl-thioesterase 1 homolog 1-like	LOC103635415	↑	1124	10	2.01E-57	85.50%
SCJFLR2036D12.g	GS1_SS_23119_08244	50S ribosomal L35	ZmPRPL35-1	↓	536	10	9.23E-59	94%
SCJFRT1007H07.g	GS1_SS_20498_19027	TPA: Lipoxygenase1	ZmLOX1	↓	2141	10	0.00E+00	91.90%
SCJFRT1008G05.g	GS1_SS_13327_19746	Lichenase-2 precursor	NA	↑	1649	10	0.00E+00	95.90%
SCJFRT1010E08.g	GS1_SS_12205_00613	3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase-like	LOC100283365	↑	1269	10	6.95E-147	93.20%
SCJFRT1060C03.g	GS1_SS_12307_02901	Hypothetical protein SORBIDRAFT_03g040515, partial	NA	↑	1354	10	5.06E-137	63.50%
SCJFRT1060E10.g	GS1_SS_12310_06740	F-box kelch-repeat At1g74510	F1M20.19	↑	1340	10	0.00E+00	97.70%
SCJFRT1061B11.b	GS1_SS_31280_20412	Endo-1,4-beta-xylanase A-like	RXF12	↑	650	10	2.73E-60	82%
SCJFRT2057H02.g	GS1_SS_13381_00259	Short-chain dehydrogenase TIC chloroplastic-like	AT4G23430	↓	790	10	5.89E-99	89.60%
SCJFRZ2007G11.g	GS1_SS_14778_01986	PREDICTED: Glutaredoxin-C10-like	LOC103652731	↓	595	10	8.35E-51	84.70%
SCJFRZ2014H12.g	GS1_SS_24786_13539	Flavo wrbA	NA	↑	840	10	8.78E-122	89.70%
SCJFST1013B05.g	GS1_SS_17632_13040	NA	NA	↑	853	NA	NA	NA
SCJLFL3011A10.g	GS1_SS_22718_14492	TPA: homeodomain-like transcription factor superfamily	NA	↓	831	10	8.60E-41	84.40%
SCJLHR1028E06.g	GS1_SS_08176_19578	Benzoate carboxyl methyltransferase	LOC100281293	↑	951	10	0.00E+00	88.90%
SCJLLR1102C07.g	GS1_SS_10657_10822	Photosynthetic NDH subunit of lumenal location chloroplastic	ndhA	↓	872	10	7.71E-153	92%
SCJLLR1107D06.g	GS1_SS_11621_09365	Hypothetical protein SORBI_002G141800	NA	↑	768	5	4.46E-17	83.80%
SCJLRT1016E05.g	GS1_SS_03488_07857	Hypothetical protein SORBIDRAFT_03g033420	NA	↑	866	10	1.10E-31	59.60%
SCJLRT1019F05.g	GS1_SS_13714_19154	Glucan endo-1,3-beta-glucosidase-like	E13L3	↑	864	10	3.49E-59	79.40%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJLRT2050G12.g	GS1_SS_13486_05014	Urate oxidase	T1D16.13	↑	1336	10	0.00E+00	96.30%
SCJLRT2052C09.g	GS1_SS_13522_14567	Bisdemethoxycurcumin synthase	LOC103626945	↑	1373	10	0.00E+00	81.90%
SCJLST1022G06.g	GS1_SS_17806_20038	Chlorophyll a-b binding chloroplastic-like	LOC103626067	↓	1261	10	0.00E+00	94.20%
SCMCAM2080E02.g	GS1_SS_02481_16398	Transcriptional corepressor LEUNIG-like isoform X1	LOC103634873	↑	2079	10	0.00E+00	93.90%
SCMCLV1033A03.g	GS1_SS_26273_06159	Transcription termination factor chloroplastic mitochondrial	T22I11.17	↓	467	10	1.38E-88	96.10%
SCMCRZ3064F11.g	GS1_SS_20198_12809	Ring-H2 zinc finger	XERICO	↑	1442	10	2.90E-95	86.40%
SCMCRZ3066A03.g	GS1_SS_06624_01251	TPA: Mitochondrial transcription termination factor family	EMB93	↓	1113	10	5.55E-97	87.40%
SCQGAM1048F02.g	GS1_SS_02913_06742	DEAD-box ATP-dependent RNA helicase 25	STRS2	↓	1990	10	0.00E+00	86.60%
SCQGFL4079D05.g	GS1_SS_14500_13207	PAP fibrillin domain containing, expressed	pco102443(516)	↓	1013	10	2.81E-109	93.60%
SCQGLR1085F11.g	GS1_SS_11161_19643	Dehydrin 9	Zmdhn-2	↑	760	10	2.03E-23	71.40%
SCQGLR2025B12.g	GS1_SS_07917_21846	Photosystem I reaction center subunit chloroplastic	ZmPSAJ	↓	1072	10	2.35E-120	96.50%
SCQGRT1040E10.g	GS1_SS_05578_19435	Nematode resistance-like HSPRO1	NA	↑	1902	10	0.00E+00	94.20%
SCQGRT1041D10.g	GS1_SS_31422_20960	ATP-dependent Clp protease	K21G20.5	↑	520	10	8.92E-77	98.70%
SCQGRT1041G07.g	GS1_AS_12676_05999	F-box kelch-repeat SKIP11-like	T17M13.4	↑	1050	10	3.74E-147	91.20%
SCQGRT1041G07.g	GS1_SS_12676_06000	F-box kelch-repeat SKIP11-like	T17M13.4	↑	1050	10	3.74E-147	91.20%
SCQGSD1045G04.g	GS1_SS_25462_11885	Lysine histidine transporter 1	LHT1	↑	713	10	5.24E-30	94.50%
SCQGSD2047F01.g	GS1_SS_25860_10456	Hypothetical protein SORBI_005G062400	NA	↑	529	2	7.97E-07	86.50%
SCQSAD1055E08.b	GS1_SS_25290_14289	Magnesium chelatase H partial	OsChlH	↓	607	10	1.74E-81	99.60%
SCQSRT1034D09.g	GS1_SS_12729_12754	Sodium-coupled neutral amino acid transporter 1-like	LOC103654018	↑	1902	10	0.00E+00	95.30%
SCQSRT1034G09.g	GS1_SS_18176_12363	Probable sarcosine oxidase	LOC103635090	↑	677	10	2.33E-95	90.60%
SCQSRT1035D12.g	GS1_SS_24797_18959	TPA: Thaumatin domain family	ZmPR-5	↑	952	10	2.00E-135	84%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCQSRT1036B03.g	GS1_SS_12504_13385	Lysine-ketoglutarate reductase saccharopine dehydrogenase1	ZmLKRSDH	↑	673	10	2.99E-106	96.10%
SCQSRT1036B10.g	GS1_SS_25808_17599	Acidic endochitinase	CHIA	↑	1185	10	0.00E+00	89.80%
SCQSRT2031H06.g	GS1_SS_13683_17635	Late embryogenesis abundant 3	AT3G53770	↑	905	10	2.88E-27	88.40%
SCQSRT2033C07.g	GS1_SS_13695_06895	HLH DNA-binding domain superfamily	gpm693	↑	724	4	2.65E-33	81.25%
SCQSRT2034D09.g	GS1_SS_06805_06778	Carbonyl reductase 1	LOC100273052	↑	683	10	3.53E-91	86.70%
SCQSST1039E07.g	GS1_SS_06871_19113	Initiator-binding 2	NA	↑	1540	10	2.76E-148	95.10%
SCRFHR1006H10.g	GS1_AS_07908_17435	NA	NA	↓	331	NA	NA	NA
SCRFLB1056D05.g	GS1_AS_24218_01518	LOC100282293 isoform X1	AI665421	↑	1519	10	0.00E+00	95.70%
SCRFLR1012G08.g	GS1_SS_10963_08117	Selenium-binding -like	SBP1	↓	469	10	5.45E-69	97.70%
SCRFRZ3055F03.g	GS1_SS_16181_09065	Basic blue-like	umc1431	↑	700	10	3.74E-60	81.80%
SCRFST1043C02.g	GS1_SS_17125_05715	CMV 1a interacting 1	LOC100284521	↓	560	10	8.87E-96	96.50%
SCRLLR1059C02.g	GS1_SS_14164_13949	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily	AT3G18210	↑	1410	10	0.00E+00	96.90%
SCRLRZ3043D02.g	GS1_SS_23626_18022	Hexose carrier HEX6	ZmHEX6	↓	2164	10	0.00E+00	92.80%
SCRLSB1041A05.g	GS1_SS_22283_05680	Hypothetical protein SORBIDRAFT_04g003670	NA	↑	888	10	1.29E-85	89.30%
SCRUAD1063A04.g	GS1_SS_00564_00688	Hypothetical protein SORBIDRAFT_01g007470	NA	↑	658	10	3.81E-97	97.10%
SCRURT2008D11.g	GS1_SS_13790_10162	TPA: Exhydrolase II	umc1721	↑	424	10	5.97E-40	96.50%
SCRURT2012H06.g	GS1_SS_13826_14124	LOC100281229 isoform X1	NA	↑	493	10	7.89E-90	95.50%
SCSBAD1052A06.g	GS1_SS_01122_02959	Peptidyl-prolyl cis-trans isomerase FKBP20-chloroplastic	AT3G55520	↓	616	10	7.91E-107	90.30%
SCSBFL1040G03.g	GS1_SS_19978_05175	NA	NA	↑	931	NA	NA	NA
SCSBSB1052D07.g	GS1_SS_21449_18655	Glyceraldehyde-3-phosphate dehydrogenase chloroplastic	GAPCP-1	↓	1574	10	0.00E+00	98.10%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCSBSD1033E10.g	GS1_SS_25423_10062	PER1_SORBI ame: Full=Cationic peroxidase SPC4 Flags: Precursor	LOC103655313	↑	654	10	1.85E-65	85.30%
SCSBSD2029E08.g	GS1_SS_25567_05330	Tyrosine N-monooxygenase-like	LOC103645625	↑	750	10	8.25E-55	87.70%
SCSFLR2024H10.g	GS1_SS_11606_14655	Probable polyribonucleotide nucleotidyltransferase chloroplastic	LOC103643360	↓	1322	10	1.47E-142	93.40%
SCSGAD1007F02.g	GS1_SS_01384_10946	Transcription factor MYB108	MYB108	↑	945	5	7.85E-29	63.60%
SCSGAM2078C07.g	GS1_AS_05162_06150	Ferredoxin	ZmFDX2	↓	466	10	1.30E-73	95.50%
SCSGFL4C08B10.g	GS1_SS_22942_15410	NRT1 PTR family-like	NRT1.1	↓	1465	10	0.00E+00	81.50%
SCSGHR1066A02.g	GS1_SS_03000_19495	50S ribosomal chloroplastic	ZmPRPL35-1	↓	982	10	4.29E-125	91.60%
SCSGHR1070G09.g	GS1_SS_08601_04578	BTB POZ domain-containing	F8K7.22	↓	547	3	4.40E-16	81.33%
SCSGLR1045E01.g	GS1_SS_11123_09145	Ribulose-1,5 bisphosphate carboxylase oxygenase large subunit N-chloroplastic isoform X1	F18O22.50	↓	366	10	9.03E-17	98.40%
SCSGLV1008B03.g	GS1_SS_26120_13402	Flowering-promoting factor 1 1	OsFPF1	↑	879	10	6.74E-57	94.20%
SCSGRT2065E10.g	GS1_SS_07126_19446	Endonuclease 2-like	LOC103644365	↑	1208	10	9.95E-148	92.30%
SCSGRZ3061E08.g	GS1_SS_02338_02948	Armadillo beta-catenin-like repeat family expressed	LOC100217267	↑	1005	10	3.27E-153	93.60%
SCSGSB1008G07.g	GS1_SS_23483_11148	NA	NA	↓	1433	NA	NA	NA
SCSGSB1009B08.b	GS1_SS_17356_10984	Soluble starch synthase II-2	OsSSS2	↓	628	10	4.27E-35	97%
SCUTAD1031D04.g	GS1_AS_01483_00725	NA	NA	↑	1038	NA	NA	NA
SCUTLR2008H10.g	GS1_SS_09610_06203	Hypothetical protein BAE44_0024388	NA	↑	570	2	1.79E-07	99%
SCUTRZ3069A01.g	GS1_SS_01829_15967	WRKY transcription factor 19	LOC103642110	↑	1247	10	0.00E+00	87.70%
SCUTRZ3069H08.g	GS1_SS_04737_12358	NA	NA	↑	430	NA	NA	NA
SCUTRZ3071H01.g	GS1_SS_16511_04168	Nucleolar RNA helicase 2	LOC100281387	↓	2101	10	0.00E+00	97.60%
SCUTST3089H07.g	GS1_AS_16325_07363	Hypothetical protein SORBIDRAFT_01g045990	NA	↑	877	10	1.82E-119	94.50%
SCVPCL6046C06.g	GS1_SS_05641_02503	Glutamate dehydrogenase 2-like	GDH2	↑	812	10	1.35E-74	99.20%
SCVPCL6061B12.g	GS1_SS_15679_05046	E3 ubiquitin-ligase MIEL1 isoform X1	NA	↑	1117	10	2.92E-179	90%

(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCVPFL3047B03.g	GS1_SS_22356_16195	N utilization substance B homolog	NA	↓	726	10	1.10E-44	96.30%
SCVPHR1094H06.g	GS1_SS_18536_09986	NA	NA	↓	519	NA	NA	NA
SCVPLR2005H09.g	GS1_SS_11666_17875	3-oxoacyl-[acyl-carrier-] synthase chloroplastic-like	T1O3.5	↓	2190	10	0.00E+00	95.60%
SCVPRT2075A05.g	GS1_SS_05292_13126	Like transcriptional regulator family expressed	AT4G12750	↓	1203	10	1.76E-116	94.90%
SCVPRT2078D10.g	GS1_SS_14106_09883	NA	NA	↑	569	NA	NA	NA
SCVPRT2080G12.g	GS1_SS_25867_08379	Aspartic proteinase 1	AT3G18490	↑	949	10	1.18E-99	88.20%
SCVPRZ2035F03.g	GS1_SS_13355_15631	Peroxidase 2-like	LOC103633480	↑	1377	10	0.00E+00	92.10%
SCVPRZ3026C06.g	GS1_SS_19681_18294	DRE binding factor	Zmdbf1	↑	1324	10	9.67E-125	76.10%
SCVPRZ3030G09.g	GS1_SS_22543_20618	Probable esterase PIR7A	LOC103652304	↑	1027	10	2.14E-171	77%

(continue)

Table 16. Leaves differentially expressed genes after 6 days of stress plus 2 re-watering days determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCACAD1039E02.g	GS1_SS_21707_20459	Methionine gamma-lyase-like	LOC103628064	↓	1785	10	0.00E+00	92%
SCACAM1072F01.g	GS1_SS_00252_08835	PCI domain containing	TIDP3728	↑	641	10	4.37E-63	92.60%
SCACLB1047F10.g	GS1_SS_08706_21371	Ribosome-recycling chloroplastic	LOC103655693	↑	1123	10	2.26E-167	94.10%
SCACLR1126B12.g	GS1_SS_09614_09565	Hypothetical protein SORBIDRAFT_09g022720	NA	↓	412	1	5.11E-06	97%
SCACLR1127B12.g	GS1_SS_03586_07058	Pentatricopeptide repeat-containing At1g19720	F14P1.33	↑	595	10	1.56E-120	85.60%
SCAGAM2016C01.g	GS1_SS_03638_14593	ATP binding	NA	↑	1244	10	1.62E-174	97.30%
SCAGLR2011E12.g	GS1_SS_11370_11622	Mitochondrial glyco	pco079267	↑	1237	10	4.09E-119	87.20%
SCAGLR2033C03.g	GS1_SS_23263_10883	RNA polymerase alpha subunit (chloroplast)	rpoA	↑	938	10	0.00E+00	99.30%
SCBFLR1083F02.g	GS1_SS_09769_21010	40S ribosomal S9-2	LOC100283332	↑	873	10	6.99E-128	98.10%
SCBFRZ2045E10.g	GS1_SS_04724_09988	NA	NA	↑	409	NA	NA	NA
SCBGHR1058E08.g	GS1_SS_07477_19258	Peroxidase 4-like	LOC103640022	↑	852	10	1.48E-102	95.90%
SCBGLR1002E05.g	GS1_SS_10549_10737	TPA: Tify domain CCT motif transcription factor family	LOC100274370	↓	1356	10	4.20E-94	75%
SCBGLR1027B09.g	GS1_AS_04140_00359	Rubisco large subunit-binding subunit chloroplastic isoform X2	LOC103633537	↑	1085	10	5.32E-156	88.50%
SCBGLR1100F03.g	GS1_SS_26117_09177	Acyl carrier	ACP1	↑	671	10	2.67E-43	87.40%
SCBGLR1113F02.g	GS1_SS_26158_07638	PREDICTED: uncharacterized protein LOC102703397 isoform X1	NA	↑	1394	10	1.54E-125	85.60%
SCBGRT1050A08.g	GS1_SS_25394_21770	Chitinase 8	OsCHI8	↑	999	10	1.50E-143	89%
SCCCCL3001E05.b	GS1_SS_05729_16932	SNF1-related kinase regulatory subunit beta-1	CIPK16	↑	1503	10	7.83E-140	93.50%
SCCCCL3001H12.g	GS1_SS_05756_04348	Isoflavone 3-hydroxylase-like	isoflavone 3'-hydroxylase	↑	1973	10	0.00E+00	89.60%
SCCCCL3120G07.g	GS1_SS_16843_15376	Probable mediator of RNA polymerase II transcription subunit 37c	LOC103635762	↓	2299	10	0.00E+00	98.70%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCCL4005E12.g	GS1_AS_09119_19260	Rubisco large subunit-binding subunit chloroplastic	LOC103647277	↑	1803	10	0.00E+00	95.30%
SCCCCL4005E12.g	GS1_SS_09119_19261	Rubisco large subunit-binding subunit chloroplastic	LOC103647277	↑	1803	10	0.00E+00	95.30%
SCCCCL4011D01.g	GS1_SS_06105_17851	Phospho-2-dehydro-3-deoxyheptonate aldolase chloroplastic	LOC103628810	↓	1786	10	0.00E+00	97.80%
SCCCCL4012A05.g	GS1_SS_14996_13074	Hypothetical protein SORBI_010G218100	NA	↑	672	10	5.15E-97	85.10%
SCCCCL5001D04.g	GS1_SS_07223_04063	50S ribosomal chloroplastic	LOC100283234	↑	1171	10	2.10E-143	97%
SCCCCL7C03G05.g	GS1_SS_21236_03669	Gibberellin 2-beta-dioxygenase-like	LOC103636636	↓	697	10	1.40E-28	92.40%
SCCCHR1003H12.g	GS1_SS_07532_01845	Hypothetical protein SORBI_001G460300	NA	↓	1032	10	7.75E-81	90%
SCCCLB1C06D11.g	GS1_SS_04112_13280	TPA: FKBP-type peptidyl-prolyl cis-trans isomerase family	AT3G55520	↑	1031	10	1.75E-116	84.10%
SCCCLR1C11C06.g	GS1_SS_09664_02008	Hypothetical protein SORBIDRAFT_04g023950	NA	↓	528	10	1.58E-43	71.70%
SCCCLR2004G08.g	GS1_SS_09639_06668	20 kDa chloroplastic	NA	↑	1084	10	2.44E-154	92.40%
SCCCRT1002D04.g	GS1_AS_04538_19991	Hydroxymethylglutaryl-lyase	AT2G26800	↑	1877	10	0.00E+00	85.40%
SCCCRT1C01F08.g	GS1_SS_12175_14061	SAUR11 - auxin-responsive SAUR family member	ZmSAUR11	↓	825	10	9.12E-56	81.40%
SCCCRZ1002D11.g	GS1_SS_14185_10664	Subtilisin-like protease	ARA12	↑	1061	10	8.99E-89	88.30%
SCCCRZ1C01E09.g	GS1_SS_10514_16646	Cytosolic sulfotransferase 14-like	NA	↓	1208	10	5.34E-179	81.30%
SCCCRZ1C01F03.g	GS1_SS_14388_20616	Serine threonine-kinase SAPK5	OsSAPK5	↓	415	9	1.63E-14	80%
SCCCRZ2001G05.g	GS1_SS_25349_19582	Hypothetical protein SORBIDRAFT_09g001320	NA	↑	747	1	5.05E-07	97%
SCCCRZ2C02C08.g	GS1_SS_15053_18095	Acyl carrier chloroplastic-like	acyl carrier protein	↑	564	10	2.17E-88	93.80%
SCCCRZ2C03A12.g	GS1_SS_11946_09704	LON peptidase N-terminal domain and RING finger 1	ZmLON1	↑	1378	10	1.12E-160	76.10%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCRZ3003H07.g	GS1_SS_15220_12028	Cytokinin dehydrogenase 11	OsCKX11	↓	496	10	8.55E-18	96.70%
SCCCST3005E08.g	GS1_SS_04718_20859	Cationic peroxidase 1-like	LOC103633557	↑	1268	10	4.71E-169	84.70%
SCEPRZ1008A06.g	GS1_SS_00605_14380	Membrane-like	NA	↓	1730	10	0.00E+00	93.90%
SCEPRZ3046D03.g	GS1_SS_15375_04366	DNA polymerase I	OsPolA	↑	720	10	1.47E-105	94.80%
SCEPSB1131A04.g	GS1_SS_21034_04042	Hypothetical chloroplast RF19 (chloroplast)	NA	↑	656	10	9.64E-18	92.20%
SCEQLB1064H09.g	GS1_SS_09110_10359	Peptidyl-prolyl cis-trans isomerase FKBP16-chloroplastic	FKBP16-2	↑	885	10	1.40E-48	91.40%
SCEQLB1067F01.g	GS1_SS_12045_19144	Bark storage A	NA	↑	1260	10	0.00E+00	88.20%
SCEQRT1024E08.g	GS1_SS_12016_09780	Cytochrome b5	LOC103643306	↓	621	10	4.82E-71	84.30%
SCEQRT1024H10.g	GS1_SS_13487_20028	Pathogenesis-related 5	PR5	↑	900	10	1.52E-96	89.50%
SCEQRT2027G07.g	GS1_SS_12262_12384	Universal stress A	NA	↑	954	10	7.14E-92	79.50%
SCEZAD1081D01.g	GS1_SS_19544_12238	Uncharacterized protein LOC100277183	LOC100277183	↑	662	10	3.07E-55	87.70%
SCEZAM1080E12.g	GS1_SS_22297_14025	Methionine-tRNA chloroplastic mitochondrial	tRNA methionine	↑	1112	10	7.50E-146	93.50%
SCEZAM2059G06.g	GS1_SS_04261_06658	Ribosome-recycling factor	RRF	↑	844	10	7.93E-136	95.70%
SCEZHR1049C07.g	GS1_AS_07647_21819	50S ribosomal chloroplastic	LOC100283234	↑	539	10	3.94E-94	87.40%
SCEZLB1006E12.g	GS1_SS_12023_11525	Alliin lyase-like	OsAlli	↓	712	10	1.21E-55	88.70%
SCEZLR1009E06.g	GS1_SS_24926_20825	ACC oxidase	ACO2	↓	1161	10	3.71E-157	92.50%
SCEZSB1090F08.g	GS1_SS_19911_19392	Cullin 3B	CUL3B	↑	1110	10	2.65E-53	60.70%
SCJFHR1034A11.g	GS1_SS_07804_03675	UPF0307 KPN78578_45570	NA	↑	998	10	9.71E-126	87%
SCJFRZ1007H05.g	GS1_SS_17513_09918	Remorin -like	F4I18.20	↓	2792	10	0.00E+00	89.30%
SCJLAM1061F07.g	GS1_SS_02528_10084	Dihydrodipicolinate reductase	LOC100194039	↑	834	10	0.00E+00	95.70%
SCJLLR1107G06.g	GS1_SS_22777_13393	Hypothetical protein SORBIDRAFT_02g035960	NA	↓	1000	10	1.03E-80	72.30%
SCJLLR2006B06.g	GS1_SS_11419_13966	PREDICTED: Uncharacterized protein LOC103629542	LOC103629542	↓	781	2	9.12E-09	79.50%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJLLR2013F11.g	GS1_SS_24597_12226	Probable inactive heme oxygenase chloroplastic	TED4	↑	663	10	7.02E-107	96%
SCJLRT1014H03.g	GS1_SS_15946_21311	RRP5 homolog	LOC103632100	↑	1358	10	0.00E+00	96.60%
SCJLRZ1020C01.g	GS1_SS_14491_06752	Non-lysosomal glucosylceramidase isoform X1	LOC103652830	↓	2897	10	0.00E+00	98.40%
SCJLRZ1021F11.g	GS1_SS_02406_12748	50S ribosomal L18	RPL18	↑	799	10	2.17E-97	89.40%
SCJLRZ1024D10.g	GS1_SS_16564_08300	Cell division cycle ATPase	NA	↓	640	10	6.32E-31	97.60%
SCJLRZ1027B10.g	GS1_SS_23569_16523	Phytoene synthase 3	OsPSY3	↓	922	10	1.91E-156	93.20%
SCMCLV1033A03.g	GS1_SS_26273_06159	Transcription termination factor chloroplastic mitochondrial	transcription termination factor	↑	467	10	1.38E-88	96.10%
SCMCST1049C08.g	GS1_SS_17869_13453	Monofunctional aspartate kinase 2	ASK2	↑	473	7	1.66E-10	90.86%
SCQGLB2043C04.g	GS1_SS_40544_21586	Leucine--tRNA chloroplastic mitochondrial	NA	↑	800	10	1.22E-144	94.10%
SCQGLR1019G02.g	GS1_SS_04353_15253	UDP-glucose 6-dehydrogenase	AT5G15490	↓	1919	10	0.00E+00	98.70%
SCQGLR2025A07.g	GS1_SS_08675_21761	WRKY transcription factor 21	OsWRKY21	↓	1856	10	0.00E+00	88.90%
SCQSAM2047G01.g	GS1_SS_04643_05813	ATP-dependent Clp protease proteolytic subunit-related chloroplastic	CLPP4	↑	1383	10	0.00E+00	97%
SCQSRT2031D12.g	GS1_SS_25268_17084	Beta-1,3-glucanase precursor	BGL2	↑	1287	10	0.00E+00	89.10%
SCRFLR1055F04.g	GS1_SS_00296_18314	Cycloartenol synthase	OsCAS	↓	2509	10	0.00E+00	94.80%
SCRLFL1010H02.g	GS1_SS_18101_08806	Histone-lysine N- H3 lysine-9 specific SUVH4-like	SUVH4	↑	944	10	5.24E-162	97.80%
SCRLRZ3114C04.g	GS1_SS_16282_12967	Double-strand break repair mus-23-like	MRE11	↓	783	10	1.79E-89	59.90%
SCSBFL5022D04.g	GS1_SS_23080_13675	NA	NA	↑	798	NA	NA	NA
SCSBSB1057D05.g	GS1_SS_16966_14693	Starch partial	NA	↓	861	10	7.63E-128	98.60%
SCSBSD1030E08.g	GS1_SS_25321_14015	AAA-ATPase At3g28580-like	At3g28580	↓	627	10	4.40E-59	91.40%
SCSBSD1056F06.g	GS1_SS_25729_14516	NA	NA	↑	296	NA	NA	NA
SCSFAD1069H10.g	GS1_SS_01211_13210	LHCP translocation defect	LOC100277526	↑	686	10	2.97E-101	85.60%

(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCSFSD1065G12.g	GS1_SS_25767_13854	CK5P1_ORYSJ ame: Full=CDK5RAP1	OsCK5P1	↑	1028	10	1.86E-146	93.40%
SCSFSD1066G06.g	GS1_SS_25776_00124	Ubiquitin-ligase CIP8	CIP8	↓	734	10	2.95E-28	71.20%
SCSGHR1069F04.b	GS1_SS_01095_20796	Hypothetical protein SORBIDRAFT_07g008860	NA	↑	584	10	8.11E-62	93.50%
SCSGLR1045D05.g	GS1_SS_24901_21213	HVA22 e	HVA22E	↓	758	10	8.34E-76	89.70%
SCSGRZ3061G02.g	GS1_SS_24939_12545	Dehydrodolichyl diphosphate synthase 6	umc2219	↓	1351	10	0.00E+00	94.40%
SCUTLR1058C09.g	GS1_SS_06649_10194	RNA polymerase beta subunit (chloroplast)	rpoB	↑	1153	10	0.00E+00	99.30%
SCUTLR2030B11.g	GS1_SS_11653_21205	Heat shock factor HSF30 isoform X1	ZmHSF30	↓	1166	10	2.30E-139	89.70%
SCVPFL3046C06.b	GS1_SS_16946_20384	Phosphatase 2C family	LOC100193064	↓	805	10	7.76E-94	95.50%
SCVPLR1028G03.g	GS1_SS_11203_13165	Cytokinin riboside 5 -monophosphate phosphoribohydrolase LOG	OsLOG	↓	1045	10	9.00E-18	91.30%
SCVPLR2005D10.g	GS1_AS_09886_05400	NA	NA	↑	911	NA	NA	NA
SCVPRZ3030D01.g	GS1_SS_18238_16749	Cytochrome P450 714B3	CYP714B3	↓	759	10	3.81E-123	94.90%

Table 17. Roots differentially expressed genes after 4 days of stress determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓ (continue)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCAD1004H06.g	GS1_SS_00075_12059	Myo-inositol-1-phosphate partial	MIPS2	↑	488	10	4.41E-17	99.70%
SCBFAD1048A12.g	GS1_SS_00293_13720	NA	NA	↑	851	NA	NA	NA
SCEQRT1024G12.g	GS1_SS_00606_15578	MYB DNA-binding domain superfamily	LOC100192961	↓	1142	10	1.05E-137	79.70%
SCCCLR1C03C05.g	GS1_SS_01335_10053	ABA responsive element binding factor 1	OsABF1	↑	1149	10	7.00E-111	86.50%
SCCCCL7002A04.g	GS1_SS_01459_19871	Ethylene-responsive transcription factor ERF073-like	HRE1	↓	1154	10	3.04E-109	61.60%
SCCCCL3005D01.b	GS1_SS_01463_17788	Probable phosphatase 2C 30	OsP2C30	↑	1677	10	0.00E+00	84.70%
SCBFRZ2018G02.g	GS1_SS_01696_17365	MYB transcription partial	ZmMYB67	↓	1225	10	1.03E-156	93.60%
SCBFRZ2017C12.g	GS1_SS_02279_09129	Zeaxanthin epoxidase	LOC100285076	↑	857	10	1.91E-35	96.90%
SCJFRZ3C05F08.b	GS1_SS_02878_10420	Elicitor peptide receptor 1	NA	↑	570	10	6.98E-47	89.20%
SCCCAM2C06A10.g	GS1_SS_03534_00583	External alternative NAD(P)H-ubiquinone oxidoreductase mitochondrial	LOC103629626	↑	1850	10	0.00E+00	93.60%
SCUTRZ2024B01.g	GS1_SS_04272_10998	P-loop NTPase domain-containing LPA1-like	LPA1	↑	1021	10	0.00E+00	95.50%
SCEQAM2038H01.g	GS1_SS_05584_17809	Cold acclimation WCOR413 gamma form	COR413-PM1	↑	1024	10	2.19E-22	89.20%
SCCCCL2001H12.b	GS1_SS_05715_13689	Dermokine-like	LOC103654415	↑	558	10	2.64E-25	90.10%
SCCCCL3001C02.b	GS1_SS_05720_06578	Oleosin 16 kDa	ZEAMMB73_000220	↑	659	10	3.34E-31	79.30%
SCCCCL4005A01.g	GS1_SS_06011_18741	Phosphate dikinase 2	OsPPDK	↓	1315	10	0.00E+00	91.10%
SCEPLR1051C10.g	GS1_SS_06158_03726	EF-Hand containing	Os02g0608400	↑	1057	10	0.00E+00	90.10%
SCVPLB1018G11.g	GS1_SS_06186_17337	ABSCISIC ACID-INSENSITIVE 5 5	LOC103631350	↑	1092	10	1.39E-99	86.40%
SCACCL6007G08.g	GS1_SS_06356_15055	Phosphate dikinase 2 isoform X1	OsPPDK2	↓	1186	10	2.04E-126	88.70%
SCCCCL4009D03.g	GS1_SS_06380_13519	Fatty acyl coA reductase	Os08g0557800	↑	1754	10	0.00E+00	84.70%
SCEZHR1088C09.g	GS1_AS_06761_04380	Nucleolar complex 3 homolog	LOC103642562	↑	1426	10	0.00E+00	93.20%
SCRLCL6031A08.g	GS1_SS_06851_15580	Probable isoaspartyl peptidase L-asparaginase 2	LOC103630215	↓	1300	10	0.00E+00	94.40%
SCUTCL6035F07.g	GS1_SS_06988_06904	Seed maturation	gpm885	↑	448	3	1.26E-11	73.67%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCQSLR1040A09.g	GS1_SS_07214_10254	DUF21 domain-containing At2g14520	T13P21.10	↑		1480	10	0.00E+00	95%
SCMCST1053F09.g	GS1_SS_07355_21364	Calcium-transporting ATPase plasma membrane-type-like	LOC103625922	↑		2243	10	0.00E+00	95.50%
SCJLAM1062A05.g	GS1_SS_07495_18169	Probable phospholipid hydroperoxide glutathione peroxidase	LOC103634626	↑		1026	10	2.76E-132	93.30%
SCCCRZ1003B11.g	GS1_SS_08088_15207	Cyclin-dependent kinase inhibitor 6	KRP6	↑		729	10	6.18E-28	84.20%
SCQGHR1010E03.g	GS1_SS_08213_06176	Xyloglucan galactosyltransferase KATAMARI1 homolog	LOC103631711	↑		617	10	2.41E-63	94.80%
SCSGHR1069A02.g	GS1_SS_08555_02448	Peroxidase 70	LOC103628533	↓		596	10	6.73E-72	89.30%
SCEQRT2092H02.g	GS1_SS_08739_04099	NDX1_ORYSJ ame: Full= NEOXANTHIN-DEFICIENT 1	NA	↑		1062	10	1.37E-143	90.90%
SCVPRT2084A03.g	GS1_SS_08741_13464	MYB DNA-binding domain superfamily	LOC100192961	↓		646	2	3.10E-18	72.50%
SCCCLB1021F07.g	GS1_SS_08968_09368	Lipase	AT3G61680	↑		1193	10	0.00E+00	95.70%
SCAGLR1064H05.g	GS1_SS_09173_13309	Arginine decarboxylase 1	ADC1	↑		531	10	6.13E-14	90.50%
SCEZLB1012C12.g	GS1_SS_09216_00883	Notum homolog	LOC103636409	↑		482	10	3.12E-45	80.60%
SCACLR1127F10.g	GS1_SS_09622_19535	14-3-3 GF14-H	GRF6	↑		1051	10	2.18E-164	92.20%
SCBGLR1023B02.g	GS1_SS_09797_12040	G-box-binding factor 3-like	NA	↑		621	10	3.30E-74	84.80%
SCVPLR2012E12.g	GS1_SS_10380_14287	Translation initiation factor IF-2	FUG1	↑		799	10	6.33E-63	84%
SCEQLR1092C09.g	GS1_SS_10389_20048	NA	NA	↑		646	NA	NA	NA
SCCCLR1078G03.g	GS1_SS_10546_12685	AC074105_12 transposon	NA	↑		1425	10	5.24E-36	70.50%
SCMCLR1010E10.g	GS1_SS_10766_14340	Hypothetical protein SORBIDRAFT_01g002540	NA	↓		646	10	1.99E-42	81.40%
SCQGLR1085F11.g	GS1_SS_11161_19643	Dehydrin 9	ERD14	↑		760	10	2.03E-23	71.40%
SCCCLR2C02G07.g	GS1_SS_11310_09979	UPF0014 membrane STAR2	OsSTAR2	↓		1001	10	2.92E-155	93.40%
SCACLR2007A07.g	GS1_SS_11335_18086	NA	NA	↑		832	NA	NA	NA
SCRFLR2038G09.g	GS1_SS_11573_09279	Senescence-associated DIN1	SEN1	↓		479	10	2.54E-63	88.80%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJLLR1107D06.g	GS1_SS_11621_09365	Hypothetical protein SORBI_002G141800	NA	↑		768	5	4.46E-17	83.80%
SCVPLR2012E08.g	GS1_SS_11675_13721	Seed maturation PM41	ZmPM41	↑		562	10	2.89E-43	91.50%
SCEQRT1024C07.g	GS1_SS_12011_14550	Partial	NA	↓		1080	10	4.41E-150	96.20%
SCCCRT1003E03.g	GS1_SS_12123_03618	Hypothetical protein SORBIDRAFT_06g001170	NA	↓		805	10	1.32E-93	72.30%
SCEQRT2027G07.g	GS1_SS_12262_12384	Universal stress A	NA	↓		954	10	7.12E-92	79.50%
SCJLRT1016G06.g	GS1_SS_12487_19039	Ribonuclease 1	RNS1	↑		939	10	2.11E-143	91.50%
SCEPRT2046C06.g	GS1_SS_12672_13178	von Willebrand factor A domain-containing DDB_G0292028-like	LOC103633419	↑		1523	10	0.00E+00	89.20%
SCEQRT1029E10.g	GS1_SS_12675_11753	O-methyltransferase 2	LOC103646955	↑		1463	10	0.00E+00	85.80%
SCCCRT2001D04.g	GS1_SS_12781_18391	Cortical cell-delineating precursor	LOC103638954	↓		798	10	7.47E-54	94.30%
SCEQRT1025C10.g	GS1_SS_12911_14632	Glucan endo-1,3-beta-glucosidase 13	LOC103633263	↓		2422	10	0.00E+00	78.30%
SCBFLR1046A09.g	GS1_SS_13551_16733	Vacuolar cation proton exchanger 1a-like	LOC103636964	↑		1258	10	1.97E-162	90.60%
SCQSRT2031H06.g	GS1_SS_13683_17635	Late embryogenesis abundant 3	AT3G53770	↑		905	10	2.88E-27	88.40%
SCEZRZ1013F08.g	GS1_SS_14325_11048	Diacylglycerol kinase 1-like	DGK1	↑		1082	10	0.00E+00	93%
SCCCRZ2C02F06.g	GS1_SS_14651_02386	NA	NA	↓		588	NA	NA	NA
SCJFRZ2011B07.g	GS1_SS_14829_07728	Subtilisin-like protease	LOC103632537	↓		841	10	2.03E-167	90.10%
SCCCAM1073C01.g	GS1_SS_15860_00617	Potassium channel	LOC100101523	↑		2790	10	0.00E+00	92.60%
SCCCLR1048A03.g	GS1_SS_15979_14228	Vegetative cell wall gp1 precursor	NA	↑		886	10	7.83E-20	83.80%
SCJLRZ3077F12.g	GS1_SS_16018_00210	Undecaprenyl pyrophosphate synthetase	LOC100285459	↑		716	10	7.32E-112	89.30%
SCMCRZ3068E07.g	GS1_SS_16080_10052	TPA: nuclear	NA	↓		609	10	7.85E-34	69.20%
SCSFFL4082E10.g	GS1_SS_16146_13026	ABI five biding 3	AFP3	↑		767	10	1.66E-118	92.50%
SCRURZ3080C06.g	GS1_SS_16319_11047	MAO HUZI chloroplastic	NA	↑		498	10	8.56E-30	65.40%
SCCCCL4006G06.g	GS1_SS_16357_13298	Cysteine protease 1 precursor	CP1	↑		1670	10	0.00E+00	93.40%
SCJFST1016G10.g	GS1_SS_16389_09885	Pepsin A	LOC100193625	↑		1855	10	0.00E+00	93.10%
SCRLCL6030F07.g	GS1_SS_16870_04945	CLP protease regulatory subunit mitochondrial	CLPX	↓		704	10	9.73E-33	59.90%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCVPFL3046C06.b	GS1_SS_16946_20384	Phosphatase 2C family	ZmABI1	↑		805	10	7.74E-94	95.50%
SCVPLB1015F06.g	GS1_SS_17991_12078	Chloride channel CLC-c-like	LOC100502544	↑		1598	10	0.00E+00	97.60%
SCEQLB1067C03.g	GS1_SS_18192_21167	Probable 1-deoxy-D-xylulose-5-phosphate chloroplastic	NA	↑		1390	10	0.00E+00	93.50%
SCBFST3136G10.g	GS1_SS_18490_06616	Cyclic nucleotide-gated ion channel 2	DND1	↑		1079	10	0.00E+00	96.60%
SCJFLR1073H12.g	GS1_SS_18573_16684	Delta 1-pyrroline-5-carboxylate synthetase 1	P5CS2	↑		2627	10	0.00E+00	96.40%
SCCCST1004A07.g	GS1_SS_18938_20926	Serine threonine kinase SAPK4	OsSPK3	↑		1502	10	0.00E+00	96.20%
SCCCLR1C01B12.g	GS1_SS_19095_07571	Hypothetical protein SORBIDRAFT_09g022700	NA	↑		541	10	4.20E-34	73.50%
SCCCLR1C04G11.g	GS1_SS_19109_01089	Outer envelope pore 16- chloroplastic	LOC103629251	↑		848	10	1.36E-106	90%
SCSBFL1036C06.g	GS1_SS_20246_00899	Catalytic hydrolase	NA	↑		697	10	3.86E-99	90.70%
SCSBFL1039F08.g	GS1_SS_20403_19091	YZ1	OsYZ1	↑		635	10	2.80E-118	92.50%
SCSGFL1078H06.g	GS1_SS_20492_15481	Cyclic nucleotide-gated ion channel 2	DND1	↑		767	10	1.86E-111	97.50%
SCEPRZ3132A06.g	GS1_SS_20556_07822	Ubiquitin-ligase	T8K14.20	↑		435	4	6.26E-09	84.25%
SCCCLB1004F06.g	GS1_SS_20880_05341	Nudix hydrolase chloroplastic	LOC103631375	↑		1294	10	1.23E-144	96.50%
SCBGST3107A01.g	GS1_SS_21249_09170	SPIRAL1-like 4	SP1L4	↑		592	10	1.42E-48	78.30%
SCQSLB1049B08.g	GS1_SS_22106_00067	RNA-dependent RNA polymerase	NA	↓		1164	10	3.04E-150	91%
SCSGFL4C08B10.g	GS1_SS_22942_15410	NRT1 PTR family -like	OsNRT1	↓		1465	10	0.00E+00	81.50%
SCJFRZ2027B02.g	GS1_SS_23174_20218	Cytochrome P450 78A11	OsPLA1	↑		1048	10	2.09E-136	97%
SCQGLR2017A03.g	GS1_SS_23262_04279	kDa proline-rich	NA	↑		1302	10	2.47E-54	96%
SCRFST3142A03.g	GS1_SS_23459_03749	Hypothetical protein SORBIDRAFT_08g022870	NA	↓		861	3	2.34E-12	60.67%
SCJLRZ1027B10.g	GS1_SS_23569_16523	Phytoene synthase 3	OsPSY3	↑		922	10	1.91E-156	93.20%
SCSFFL4085D03.g	GS1_SS_23616_03654	ABRE-binding factor BZ-1	ZmBZIP1	↑		762	10	1.35E-72	82.20%
SCMCSB1112G10.g	GS1_SS_24052_04293	CSC1 RXW8	F1N20.220	↑		1849	10	0.00E+00	92.60%
SCVPRZ3028C03.g	GS1_SS_24211_00167	LOC100282928 isoform X1	AY110191	↑		898	10	0.00E+00	95.30%
SCACAD1036B09.g	GS1_SS_24432_14831	Soluble starch synthase chloroplastic amyloplastic	OsSSIIa	↑		919	10	7.27E-36	97.40%

(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJLRT1016B01.g	GS1_SS_24593_08755	Hypothetical protein SORBI_003G356400	NA	↑		1242	10	3.02E-44	89.60%
SCQSRT1035D12.g	GS1_SS_24797_18959	TPA: thaumatin domain family	ZmSIP1	↑		952	10	2.00E-135	84%
SCSGLR1045D05.g	GS1_SS_24901_21213	HVA22 e	HVA22E	↑		758	10	8.32E-76	89.70%
SCACCL6009B02.g	GS1_SS_24913_15490	Heat shock factor 1	HSF1	↑		1346	10	9.28E-89	81.70%
SCRUHR1075B01.g	GS1_SS_25033_10293	Ultraviolet-B receptor UVR8	UVR8	↑		809	10	1.07E-106	92.80%
SCCCSD2001G01.g	GS1_SS_25685_11811	Peptide transporter PTR2	PTR2	↑		599	10	9.68E-92	88.40%
SCSBSD1054A04.g	GS1_SS_25722_09520	TPA: RING zinc finger domain superfamily	AC212353.4_FG004	↑		550	10	3.16E-82	92.60%
SCRFLB2060E04.g	GS1_SS_25725_08917	Disulfide isomerase	ZmPDI7	↑		1426	10	0.00E+00	95%
SCQGSD2047F01.g	GS1_SS_25860_10456	Hypothetical protein SORBI_005G062400	NA	↑		529	2	7.97E-07	86.50%
SCUTRZ3069C11.g	GS1_SS_33296_17210	Receptor kinase	transmembrane receptor protein kinase	↑		608	10	1.47E-118	92.20%

Table 18. Roots differentially expressed genes after 6 days of stress determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓ (continue)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCACAD1037B06.g	GS1_SS_00142_20039	Aconitate cytoplasmic	Zmaco2	↑	1179	10	0.00E+00	84.90%
SCVPRZ3029F03.g	GS1_SS_00314_13853	Trichome birefringence-like 38	TBL38	↓	903	10	3.00E-155	96.80%
SCCCRZ2C03F05.g	GS1_SS_00324_15487	Phospholipase A2	PLA2-ALPHA	↓	1036	10	3.22E-87	91.10%
SCJFRZ1006B02.g	GS1_SS_00402_07756	High mobility group B6-like	HMGB6	↓	1491	10	2.62E-150	86.60%
SCEPCL6023F02.g	GS1_SS_00431_19531	Sucrose synthase 4	SUS4	↑	2174	10	0.00E+00	98.60%
SCBGRT1047G10.g	GS1_SS_00614_14931	Germin 5-1	OsGL51	↓	921	10	8.60E-116	86.70%
SCACLR2007A10.g	GS1_SS_00783_13825	Formin 15	OsFH15	↑	904	10	1.20E-52	86.20%
SCEZAM2035A03.g	GS1_SS_00835_12289	Transcription factor bHLH96	LOC103645107	↓	1098	10	1.09E-129	78.60%
SCRLLR1038D02.g	GS1_SS_00894_14222	Hypothetical protein SORBIDRAFT_07g025110	NA	↓	1392	10	1.35E-158	85.30%
SCEZAM2031E09.g	GS1_SS_01306_09932	Deoxyuridine-5-triphosphate nucleotidohydrolase	LOC103643771	↓	727	10	4.16E-47	98%
SCQGST1032H03.g	GS1_AS_01498_06863	Dolichyl-diphosphooligosaccharide - glycosyltransferase subunit STT3B	STT3B	↑	1199	10	0.00E+00	98.60%
SCUTAD1032E08.g	GS1_SS_01501_02824	Dirigent pDIR3	ZmpDIR3	↓	385	10	2.71E-38	94.70%
SCCCAM1001G06.g	GS1_AS_01599_02840	Hypothetical protein BAE44_0012701	NA	↓	587	10	2.18E-32	76.40%
SCCCAM1001G06.g	GS1_SS_01599_02839	Hypothetical protein BAE44_0012701	NA	↓	587	10	2.18E-32	76.40%
SCACLR2029E11.g	GS1_SS_01653_10908	Downstream of FLC-like	NA	↓	1015	10	9.72E-88	87.60%
SCUTAM2005D03.g	GS1_SS_01666_12791	Metal ion binding	cl3469_11b	↑	1102	10	2.31E-62	95.20%
SCUTLR1058G02.g	GS1_SS_02358_19984	Cyclin IIIZm	ZmCYC3	↓	1445	10	0.00E+00	91.90%
SCVPLR2005H03.g	GS1_SS_02369_18630	Auxin-responsive IAA13	IAA13	↓	1232	10	3.49E-92	87.50%
SCCCLR2002D04.g	GS1_SS_02378_18759	Histone H4	HIS4	↓	666	10	2.38E-52	97.50%
SCJLAM1063F08.g	GS1_SS_02558_09443	Bifunctional epoxide hydrolase 2-like	LOC103625737	↓	1146	10	0.00E+00	90.30%
SCEZLB1006G08.g	GS1_SS_02651_18123	Cellulase containing	LOC100284407	↓	1029	10	0.00E+00	93.60%
SCJLLR1033H06.g	GS1_SS_02986_16193	Cyclin B2	CYCB2;2	↓	987	10	9.68E-168	95.70%
SCEZLB1005E07.g	GS1_SS_03013_18904	NRT1 PTR family-like	NRT1.5	↓	2751	10	0.00E+00	93%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCCL4007C09.g	GS1_SS_03185_18026	Probable histone H2A variant 1	OsH2A1	↓	778	10	1.40E-69	98.90%
SCBGLR1117C12.g	GS1_AS_03261_03602	TPA: hypothetical protein ZEAMMB73_397316	NA	↑	1301	3	1.56E-09	69%
SCCCST1008B08.g	GS1_SS_03268_18427	RNA-binding chloroplastic-like	NA	↑	1089	10	1.27E-135	92.20%
SCJFLR2036E03.g	GS1_SS_03274_15357	Kinase superfamily	NA	↑	1377	10	0.00E+00	93.40%
SCEPRZ1010A12.g	GS1_SS_03849_07370	PREDICTED: uncharacterized protein LOC103648073	LOC103648073	↑	2255	10	0.00E+00	86.30%
SCJLLR1106B06.g	GS1_SS_03996_21860	Carbohydrate transporter sugar porter transporter	LOC100191783	↓	1909	10	0.00E+00	89.10%
SCEPAM2056G07.g	GS1_SS_04089_16415	Peroxidase 64	MJC20.29	↓	1468	10	0.00E+00	90.40%
SCEZAM2034C10.g	GS1_SS_04207_10459	Probable calcium-binding CML10	LOC103633363	↑	516	10	1.55E-15	95.50%
SCUTLR2023A03.g	GS1_SS_04374_14495	GDSL esterase lipase At5g45910-like	LOC103630572	↓	1460	10	0.00E+00	94.60%
SCJFRT2057H11.g	GS1_SS_04616_10915	Protease inhibitor seed storage LTP family	LOC100281222	↓	778	6	6.05E-30	79.33%
SCEZHR1087B05.g	GS1_SS_04633_07348	Homeobox-leucine zipper HOX25	NA	↑	1585	10	4.27E-85	66.20%
SCRFLB1055H11.g	GS1_SS_04715_01331	WVD2-like 1 isoform X2	WDL1	↓	1680	10	0.00E+00	80.10%
SCCCST3005E08.g	GS1_SS_04718_20859	Cationic peroxidase 1-like	LOC103633557	↓	1268	10	4.70E-169	84.70%
SCEZAM2058D02.g	GS1_SS_05300_21664	Ribonucleoside-diphosphate reductase small chain	RNR2A	↓	1273	10	0.00E+00	96.50%
SCCCRT1C05E01.g	GS1_SS_05544_19640	DNA binding	NA	↓	847	10	7.01E-114	95.10%
SCVPCL6046C06.g	GS1_SS_05641_02503	Glutamate dehydrogenase 2-like	GDH2	↑	812	10	1.35E-74	99.20%
SCCCCL2001H01.b	GS1_SS_05710_02918	Stem-specific TSJT1	ZmTSJT1	↑	1089	10	2.43E-157	86.60%
SCCCCL3001C02.b	GS1_SS_05720_06578	Oleosin 16 kDa	ZEAMMB73_000220	↑	659	10	3.34E-31	79.30%
SCEPLR1051C10.g	GS1_SS_06158_03726	EF-Hand containing	Os02g0608400	↑	1057	10	0.00E+00	90.10%
SCVPLB1018G11.g	GS1_SS_06186_17337	ABSCISIC ACID-INSENSITIVE 5 5	LOC103631350	↑	1092	10	1.39E-99	86.40%
SCACCL6007G08.g	GS1_SS_06356_15055	Phosphate dikinase 2 isoform X1	OsPPDK	↓	1186	10	2.32E-126	88.70%
SCCCCL3003E07.b	GS1_SS_06601_18767	Chitinase partial	CHI	↑	1153	10	8.21E-162	96.20%
SCEZHR1088C09.g	GS1_AS_06761_04380	Nucleolar complex 3 homolog	LOC103642562	↑	1426	10	0.00E+00	93.20%
SCRLCL6031A08.g	GS1_SS_06851_15580	Probable isoaspartyl peptidase L-asparaginase 2	LOC103630215	↓	1300	10	0.00E+00	94.40%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCAGLR2033H02.g	GS1_SS_06970_09580	Oleosin 16 kDa	ZEAMMB73_000220	↑	889	10	6.20E-65	95.40%
SCUTCL6035F07.g	GS1_SS_06988_06904	Seed maturation	ZmPM41	↑	448	3	1.26E-11	73.67%
SCRLLR1038B09.g	GS1_SS_07016_13921	Heat shock 70 kDa 8	LOC100285374	↑	2255	10	0.00E+00	95.50%
SCEQRT2093D08.g	GS1_SS_07042_18864	IAA12 - auxin-responsive Aux/IAA family member	IAA12	↓	935	10	3.43E-83	78.30%
SCCCRT2C03A04.g	GS1_SS_07176_04916	Carotenoid cleavage dioxygenase	CCD8	↓	560	10	3.51E-108	97.20%
SCCCCL7038A01.g	GS1_SS_07217_18359	Mannan endo-1,4-beta-mannosidase 2	OsMAN2	↓	899	10	1.21E-77	83.80%
SCVPRZ2039B05.g	GS1_SS_07297_12565	Leucine-rich repeat (LRR) family	LOC100192830	↓	1159	10	0.00E+00	89.30%
SCMCST1053F09.g	GS1_SS_07355_21364	Calcium-transporting ATPase plasma membrane-type-like	ACA9	↑	2243	10	0.00E+00	95.50%
SCCCCL4006H09.g	GS1_SS_07367_19801	Expansin-B3 precursor	ZmexpB5	↓	1120	10	3.39E-171	93.70%
SCBGHR1058E08.g	GS1_SS_07477_19258	Peroxidase 4-like	LOC103642595	↓	852	10	1.48E-102	95.90%
SCCCST2002A09.g	GS1_SS_07635_18343	2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like	LOC103651775	↑	688	10	1.75E-146	97.40%
SCCCST2002A09.g	GS1_AS_07635_18344	2,3-bisphosphoglycerate-independent phosphoglycerate mutase-like	LOC103634611	↑	688	10	1.75E-146	97.40%
SCQGHR1014E03.g	GS1_SS_07879_15885	WAT1-related At3g30340-like	AT3G30340	↓	1316	10	0.00E+00	91.70%
SCCCHR1001B01.g	GS1_SS_07957_10799	Fibroin heavy chain precursor	LOC101027116	↓	1133	2	2.52E-09	92.50%
SCACHR1035G08.g	GS1_SS_07969_08697	Glycine-rich cell wall structural 1	LOC103642234	↓	899	10	3.67E-54	94.70%
SCJLHR1028E06.g	GS1_SS_08176_19578	Benzoate carboxyl methyltransferase	LOC100281293	↑	951	10	0.00E+00	88.90%
SCSGHR1069A02.g	GS1_SS_08555_02448	Peroxidase 70	LOC103628533	↓	596	10	6.73E-72	89.30%
SCAGLR1064H08.g	GS1_SS_08700_17136	Aspartic proteinase nepenthesin-1 precursor	LOC101027131	↓	2097	10	0.00E+00	93.30%
SCJFLR1017E09.g	GS1_SS_08724_06083	Stachyose synthase precursor	NA	↑	1336	10	0.00E+00	87.90%
SCRFLR2034A09.g	GS1_SS_08753_18806	Serine threonine-kinase SAPK1	OsPKABA1	↑	1395	10	0.00E+00	96.50%
SCCCLB1003H12.g	GS1_SS_08851_04211	Blue copper precursor	LOC100285874	↓	930	10	7.62E-66	93.80%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCACLB1046B10.g	GS1_SS_08871_08840	Hypothetical protein SORBIDRAFT_03g045310	NA	↓	1011	1	7.06E-11	92%
SCVPLR2012E03.g	GS1_SS_08936_21690	Inositolphosphorylceramide-B C-26 hydroxylase	LOC100282761	↓	1041	10	3.25E-171	95%
SCEZLB1005A11.g	GS1_SS_09153_01408	NA	NA	↓	487	NA	NA	NA
SCCCLR2001D01.g	GS1_SS_09157_06293	Histone H4-like	HIS4	↓	709	10	1.08E-43	100%
SCEZLB1012E05.g	GS1_SS_09220_12743	Peroxidase 19	LOC103651385	↓	527	10	1.18E-65	95.10%
SCEQRT2027A10.g	GS1_SS_09271_13480	Expansin Os-EXPA2	EXPA2	↓	863	10	5.33E-53	96%
SCCCST1002H07.g	GS1_SS_09343_09069	NA	NA	↓	300	NA	NA	NA
SCCCLB1024F05.g	GS1_SS_09346_13580	Histone H2A-like	HTA2	↓	781	10	3.09E-48	99.30%
SCEPRZ1009H07.g	GS1_SS_09358_02863	Arabinogalactan peptide 16-like	AGP16	↓	616	10	2.51E-13	99.10%
SCQSLB1052A10.g	GS1_SS_09362_05645	Pentatricopeptide repeat-containing partial	F27F23.8	↓	612	10	1.12E-106	81.50%
SCCCCL3004D04.b	GS1_SS_09393_12844	CRAL TRIO domain containing	LOC103633731	↓	1652	10	0.00E+00	87%
SCQGLB1029D12.g	GS1_SS_09460_14046	Hypothetical protein SORBIDRAFT_02g007160	NA	↓	523	10	1.11E-48	78.60%
SCEZLB1014G11.g	GS1_SS_09548_09048	Pollen-specific C13	LOC100285910	↓	547	10	4.73E-78	91.90%
SCACLR1127F10.g	GS1_SS_09622_19535	14-3-3 GF14-H	GF14 PHI	↑	1051	10	2.18E-164	92.20%
SCACLR1127H10.g	GS1_SS_09625_10814	Plasma membrane ATPase 1-like	OsPMA1	↑	1409	10	0.00E+00	97.60%
SCBGLR1023B02.g	GS1_SS_09797_12040	G-box-binding factor 3-like	NA	↑	621	10	3.30E-74	84.80%
SCBGLR1096A03.g	GS1_SS_09883_20653	Uncharacterized GPI-anchored At4g28100	LOC103633290	↓	700	10	3.51E-56	87.60%
SCCCRZ2C03C04.g	GS1_SS_09916_17814	Histone H2A	OsH2A1	↓	706	10	3.73E-53	98.80%
SCEPLR1008G07.g	GS1_SS_10294_13665	NA	NA	↑	1003	NA	NA	NA
SCVPLR2012E12.g	GS1_SS_10380_14287	Translation initiation factor IF-2	FUG1	↑	799	10	6.33E-63	84%
SCCCLR2001C04.g	GS1_SS_10381_13545	kDa class III heat shock	LOC103634094	↑	833	10	9.00E-55	82.60%
SCEQLR1092C09.g	GS1_SS_10389_20048	NA	NA	↑	646	NA	NA	NA
SCEZLR1052E03.g	GS1_SS_10436_11125	Thioredoxin superfamily	F18O22.30	↑	595	10	8.34E-18	94.90%
SCCCLR1078G03.g	GS1_SS_10546_12685	AC074105_12 transposon	NA	↑	1425	10	5.24E-36	70.50%
SCJLLR1104A12.g	GS1_SS_10677_05236	B3 domain-containing Os03g0184500-like	LOC103640421	↓	1029	10	1.63E-102	92.40%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCMCLR1123E09.g	GS1_SS_10817_07976	Lipase YDR444W isoform X3	NA	↑	549	10	5.39E-83	95.10%
SCCCLR1C11G04.g	GS1_SS_10833_13109	NA	NA	↑	499	NA	NA	NA
SCQGLR1062C09.g	GS1_SS_10874_10877	Pyrimidine-specific ribonucleoside hydrolase rihA	LOC100282256	↓	1451	10	0.00E+00	98.20%
SCQGLR2032B01.g	GS1_SS_10886_01209	Hypothetical protein SORBIDRAFT_03g042590	NA	↓	736	10	1.24E-84	95.80%
SCEZLB1007D12.g	GS1_SS_10899_12734	Rapid alkalinization factor 1 precursor	RALF1	↓	834	10	3.80E-40	93.40%
SCRFLR1055B07.g	GS1_SS_10987_08624	Glucan endo-1,3-beta-glucosidase 4 precursor	LOC100282931	↓	740	10	6.85E-67	93.60%
SCQGLR1085F11.g	GS1_SS_11161_19643	Dehydrin 9	Zmdhn-2	↑	760	10	2.03E-23	71.40%
SCCCLR2C02C04.g	GS1_SS_11301_17591	NA	NA	↓	634	NA	NA	NA
SCACLR2014F01.g	GS1_SS_11342_08704	Farnesylated 2	umc2310	↑	410	10	4.61E-46	95.80%
SCJLLR1107D06.g	GS1_SS_11621_09365	Hypothetical protein SORBI_002G141800	NA	↑	768	5	4.46E-17	83.80%
SCVPLR2012E08.g	GS1_SS_11675_13721	Seed maturation PM41	ZmPM41	↑	562	10	2.89E-43	91.50%
SCCCRZ2002F11.g	GS1_SS_11680_09616	Hypothetical protein SORBIDRAFT_01g040070	NA	↑	791	10	1.82E-112	88%
SCCCRT1002A07.g	GS1_SS_11710_08920	Lysine histidine transporter 2	LHT2	↓	684	10	8.79E-53	90.60%
SCEQRT1028D12.g	GS1_SS_11752_04522	Peroxidase 2-like	AT1G05250	↓	1343	10	0.00E+00	94.40%
SCEQRT1024F02.g	GS1_SS_11853_16153	Peroxidase 1	LOC103637120	↓	1265	10	0.00E+00	92.90%
SCBGRT1052E01.g	GS1_SS_11960_00886	Aquaporin TIP2-3	TIP2;3	↓	1037	10	3.23E-165	97.40%
SCEQRT1024C07.g	GS1_SS_12011_14550	Partial	NA	↓	1080	10	4.42E-150	96.20%
SCEQLB1067F01.g	GS1_SS_12045_19144	Bark storage A	NA	↓	1260	10	0.00E+00	88.20%
SCEQRT1029H04.g	GS1_SS_12121_10055	Cortical cell-delineating precursor	LOC103638956	↓	782	10	1.72E-54	93.40%
SCCCRT1003E03.g	GS1_SS_12123_03618	Hypothetical protein SORBIDRAFT_06g001170	NA	↓	805	10	1.32E-93	72.30%
SCJFRT1007H04.g	GS1_SS_12151_00246	Cell wall integrity and stress response component 1-like	LOC103645916	↓	618	10	4.97E-94	81.50%
SCCCLR2004B09.g	GS1_SS_12164_09995	Transcription factor bHLH96-like	LOC103645107	↓	494	10	2.58E-43	93.90%
SCJFRT1010C08.g	GS1_SS_12200_11806	Aquaporin TIP2-1	DELTA-TIP	↓	884	10	8.59E-156	97.70%
SCBFAD1088H04.g	GS1_SS_12231_10753	BAG family molecular chaperone regulator 6	LOC103626967	↑	1454	10	1.11E-102	63.70%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCCL3002F08.b	GS1_SS_12334_19734	Peroxidase 2	AT1G05250	↓	1403	10	0.00E+00	90.40%
SCJLRT1015G08.g	GS1_SS_12472_19393	Hypothetical protein SETIT_027065mg	NA	↓	663	7	3.09E-10	51.86%
SCJFRT1058B04.g	GS1_SS_12617_11405	65-kDa microtubule-associated 6	ATMAP65-6	↓	1304	10	2.59E-140	82.40%
SCQGRT1040C08.g	GS1_SS_12657_04555	Chemocyanin partial	LOC100280893	↓	570	10	1.02E-57	87.90%
SCCCRZ1C01F02.g	GS1_SS_12696_16186	Boron transporter 1	BOR1	↓	2640	10	0.00E+00	97%
SCMCRT2107D08.g	GS1_SS_12765_10611	AF332176_1 beta-expansin partial	ZmexpB5	↓	1226	10	2.38E-71	96%
SCCCRT2001D04.g	GS1_SS_12781_18391	Cortical cell-delineating precursor	LOC103638956	↓	798	10	7.49E-54	94.30%
SCAGRT2038E09.g	GS1_SS_12822_11192	Thiosulfate sulfurtransferase chloroplastic-like	thiosulfate sulfurtransferase	↓	549	10	7.29E-74	85.40%
SCAGRT2042E10.g	GS1_SS_12869_16367	Tropinone reductase	AY110052	↑	969	10	1.14E-155	81.70%
SCEQRT2092B02.g	GS1_SS_12879_17793	Zinc C2H2 type family	NA	↓	1048	10	6.28E-92	77.80%
SCEPRT2043B01.g	GS1_SS_12904_00991	AF332176_1 beta-expansin partial	ZmexpB5	↓	586	10	2.41E-59	86.30%
SCJLRZ1027E04.g	GS1_SS_12905_02854	Cytosolic sulfotransferase 18-like	AT3G45080	↓	655	10	2.18E-120	84.60%
SCEQRT1025C10.g	GS1_SS_12911_14632	Glucan endo-1,3-beta-glucosidase 13	LOC103633263	↓	2422	10	0.00E+00	78.30%
SCCCCL4007F05.g	GS1_SS_12928_14208	Glutathione *S*-transferase GSTU6	LOC100192043	↑	878	10	1.47E-103	82.20%
SCEPRT2047F02.g	GS1_SS_12953_15288	Phytoene synthase 2	LOC103649117	↓	1086	10	0.00E+00	97.70%
SCJFRZ1007G02.g	GS1_SS_13139_06873	Polyamine oxidase	PAO1	↓	1197	10	0.00E+00	97.30%
SCEQRT2100C04.g	GS1_SS_13145_18270	Galactoside 2-alpha-L-fucosyltransferase-like	LOC103637888	↓	797	10	7.39E-117	80.90%
SCCCLB1001E11.g	GS1_SS_13187_02951	Leucine-rich repeat extensin 3	EXT3	↓	1190	5	5.51E-08	73.80%
SCJLLR1107C08.g	GS1_SS_13249_09692	Probable carbohydrate esterase At4g34215	LOC103632302	↓	521	10	1.15E-64	87.60%
SCEZRT2023A09.g	GS1_SS_13261_11151	NA	NA	↑	853	NA	NA	NA
SCJFRT1007F09.g	GS1_SS_13319_20910	O-methyltransferase ZRP4	LOC100282209	↓	1468	10	0.00E+00	85.80%
SCEQRT1024B11.g	GS1_SS_13442_16670	Aquaporin PIP2-6	ZmPIP2F	↓	1193	10	0.00E+00	97.20%
SCJLRT2051F02.g	GS1_SS_13500_16098	UTP-glucose-1-phosphate uridylyltransferase	UGP2	↓	1073	10	0.00E+00	96.50%
SCJFRZ2032H11.g	GS1_SS_13528_11402	Peroxidase 5-like	LOC103642599	↓	1551	10	0.00E+00	90.90%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCMCRT2089E02.g	GS1_SS_13592_20671	Pyruvate decarboxylase	PDC1	↓	1406	10	0.00E+00	95.70%
SCCCLR1C05G08.g	GS1_SS_13638_17704	Peroxidase 72-like	LOC103635254	↓	1810	10	0.00E+00	95.70%
SCQSRT2031H06.g	GS1_SS_13683_17635	Late embryogenesis abundant 3	AT3G53770	↑	905	10	2.88E-27	88.40%
SCQSRT2033C07.g	GS1_SS_13695_06895	HLH DNA-binding domain superfamily	LOC100191589	↓	724	4	2.65E-33	81.25%
SCVPRT2083B12.g	GS1_SS_13708_12497	Phosphoglycerate cytosolic	PGK	↓	1065	10	8.57E-83	88%
SCRURT2009D02.g	GS1_SS_13800_07255	Hypothetical protein SORBIDRAFT_06g023850	NA	↓	535	9	1.19E-60	86.67%
SCEQRT2027H09.g	GS1_SS_13921_13907	PREDICTED: uncharacterized protein LOC103649874	LOC103649874	↓	1198	10	0.00E+00	91.50%
SCSGRT2063H08.g	GS1_SS_13976_00142	Dirigent 2-like	F12P19.3	↓	631	10	1.92E-46	58.80%
SCEQHR1082B01.g	GS1_SS_14031_15728	Beta-glucosidase 29-like	OsBGL29	↓	1966	10	0.00E+00	89.20%
SCVPRT2075E05.g	GS1_SS_14070_09154	Hypothetical protein SORBIDRAFT_05g018540	NA	↑	931	10	2.80E-63	90%
SCVPRT2080B02.g	GS1_SS_14119_14599	NA	NA	↓	781	NA	NA	NA
SCEZRZ1016E04.g	GS1_SS_14365_12782	NA	NA	↓	490	NA	NA	NA
SCJLRZ1025A05.g	GS1_SS_14535_04361	NA	NA	↑	850	NA	NA	NA
SCRFLR1012G02.g	GS1_SS_14537_01329	GAST1 precursor	GASA1	↓	849	10	1.64E-34	92.80%
SCCCRZ2C02F06.g	GS1_SS_14651_02386	NA	NA	↓	588	NA	NA	NA
SCBFRZ2017F11.g	GS1_SS_14694_03476	Dihydroflavonol-4-reductase	DFR	↑	520	10	9.52E-68	90.10%
SCBFRZ2045A12.g	GS1_SS_14710_13413	S-adenosyl-L-methionine: phosphoethanolamine N-methyltransferase	ZmPEAMT	↓	739	10	3.65E-33	92.70%
SCJFRZ2030D03.g	GS1_SS_14939_21284	Cytochrome P450 71C1	ZmCYP71C1	↑	984	10	0.00E+00	86.50%
SCVPRZ2036F10.g	GS1_SS_14947_00522	Fasciclin-like arabinogalactan 7 precursor	LOC103641751	↓	1118	10	1.61E-113	89.40%
SCBFRZ2019F01.g	GS1_SS_14978_01433	hsp20 alpha crystallin family	OsHsp20	↓	571	9	2.50E-77	60.11%
SCBFRZ3008D06.g	GS1_SS_15333_16913	9-cis-epoxycarotenoid dioxygenase chloroplastic-like	NCED3	↑	588	10	3.99E-65	95.40%
SCQGLR1085E04.g	GS1_SS_15452_11659	Aspartic proteinase nepenthesin-2	LOC100283965	↓	484	10	8.08E-65	94.60%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCLR1065A10.g	GS1_SS_15508_08491	Beta-expansin 1a precursor	ZmEXPB1A	↓	1180	10	0.00E+00	91%
SCUTLR1037C07.g	GS1_SS_15768_19034	Alpha-galactosidase precursor	LOC100856962	↓	1638	10	0.00E+00	89.10%
SCJFST1015A07.g	GS1_SS_15858_05084	Aspartic proteinase	T26N6.7	↓	2006	10	0.00E+00	92.70%
SCSFFL4082E10.g	GS1_SS_16146_13026	ABI five biding 3	AFP3	↑	767	10	1.66E-118	92.50%
SCSBRZ3118B10.g	GS1_SS_16371_09107	Amino acid transporter-like	F16N3.4	↑	886	10	7.62E-123	96.90%
SCSBRZ3125F02.g	GS1_SS_16436_20397	NA	NA	↑	1140	NA	NA	NA
SCSBLB1033B10.g	GS1_SS_16580_15645	Dirigent 1-like	LOC103642957	↓	1073	10	1.12E-72	92.40%
SCBFLR1005H01.g	GS1_SS_16661_12921	Hypothetical protein SORBIDRAFT_01g006350	NA	↓	657	10	2.90E-24	87.50%
SCVPFL3046C06.b	GS1_SS_16946_20384	Phosphatase 2C family	ZmABI1	↑	805	10	7.76E-94	95.50%
SCQGSB1083B11.g	GS1_SS_17026_03848	Aspartic proteinase nepenthesin-1 precursor	LOC101027131	↓	897	10	2.44E-123	95.60%
SCBFRZ2019C12.g	GS1_SS_17262_00357	Translation initiation factor IF-2-like	T22B4.140	↓	764	10	2.16E-60	68.50%
SCJLST1024H09.g	GS1_SS_17586_12600	Cysteine-rich repeat secretory 12-like	LOC103629988	↓	1452	10	3.30E-131	82.50%
SCJFST1014C02.g	GS1_SS_17645_06164	PREDICTED: uncharacterized protein LOC101766165 isoform X2	NA	↑	778	10	2.88E-32	98.80%
SCCCST1C02D05.g	GS1_SS_17710_19657	Beta-hexosaminidase 3-like	HEXO3	↓	588	10	3.97E-132	96.40%
SCVPRZ2038B09.g	GS1_SS_18185_11153	Aspartic proteinase nepenthesin-1 precursor	LOC101027131	↓	851	10	1.39E-94	96.30%
SCEQLB1067C03.g	GS1_SS_18192_21167	Probable 1-deoxy-D-xylulose-5-phosphate chloroplastic	NA	↑	1390	10	0.00E+00	93.40%
SCCCCL4005G05.g	GS1_SS_18344_01636	Small heat shock	HSP17.6A	↑	1006	10	4.14E-102	83%
SCACLR2014H03.g	GS1_SS_18482_16104	VAMP SEC22	SEC22	↓	1167	10	1.40E-131	89.30%
SCBFST3136C07.g	GS1_SS_18486_08015	Profilin A	Zmprofilin A	↓	782	10	1.98E-90	98%
SCJFRZ1006E06.g	GS1_SS_18676_07921	Tubulin beta chain	LOC103645713	↓	1760	10	0.00E+00	98.20%
SCAGRT3047G04.g	GS1_SS_18687_13647	Hypothetical protein SORBIDRAFT_09g004130	NA	↓	360	10	4.73E-25	94.80%
SCVPLB1020A04.g	GS1_SS_18847_01946	Alpha-L-fucosidase 2 precursor	LOC101027129	↓	1229	10	0.00E+00	94.40%
SCJFRZ2005H01.g	GS1_SS_18926_10412	Pectate lyase 8 isoform X1	LOC100281162	↓	1554	10	0.00E+00	98%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCCCLR1C04G11.g	GS1_SS_19109_01089	Outer envelope pore 16- chloroplastic	LOC103635971	↑	848	10	1.36E-106	90%
SCSGLR1025D03.g	GS1_SS_19125_12207	Heat shock	T10M13.11	↑	940	10	1.81E-72	80.80%
SCAGAD1073C08.g	GS1_SS_19747_12612	MYB18 transcription factor	MYB18	↓	716	10	1.65E-62	98.10%
SCAGFL1089A10.g	GS1_SS_19891_09996	Cold shock -1 precursor	CSDP1	↓	969	7	4.76E-08	85.57%
SCEPAM1021B02.g	GS1_SS_20282_12750	High mobility group nucleosome-binding domain-containing 5-like isoform X1	LOC103648259	↓	1713	10	5.14E-180	68.80%
SCSGFL1082F01.g	GS1_SS_20285_01633	Hypothetical protein SORBI_004G344400	NA	↓	1201	10	0.00E+00	86.20%
SCSBFL1038F08.g	GS1_SS_20355_20318	Ent-copalyl diphosphate partial	GA1	↓	654	10	2.28E-153	93.30%
SCEZRZ3019D12.g	GS1_AS_20358_04794	Hypothetical protein SORBI_007G049700	NA	↑	2019	2	1.23E-17	86.50%
SCRLAD1102G08.g	GS1_SS_21122_19815	NA	NA	↓	190	NA	NA	NA
SCQGLR1019F12.g	GS1_SS_21199_20863	Plastidic ATP/ADP-transporter-like	LOC100281461	↑	785	10	3.06E-89	95%
SCVPLR1049E05.g	GS1_SS_21231_17810	Cyclin m	CYCB1;3	↓	1942	10	0.00E+00	91.60%
SCBGST3107A01.g	GS1_SS_21249_09170	SPIRAL1-like 4	SP1L4	↑	592	10	9.97E-48	78.30%
SCEPSB1136F09.g	GS1_SS_21332_12694	Probable glycerol-3-phosphate acyltransferase 3	LOC103650549	↑	1258	10	1.91E-126	80.60%
SCCCLR1079C08.g	GS1_SS_21356_20703	High mobility group B7	NA	↓	1090	10	1.59E-111	80.50%
SCCCCL4009F05.g	GS1_SS_21374_15489	Beta-D-xylosidase 4-like	XYL4	↓	2020	10	0.00E+00	95.90%
SCRLSB1044E01.g	GS1_SS_21526_15823	Meiotic spindle formation mei-1-like	NA	↑	2212	10	0.00E+00	91.10%
SCCCLB1023H08.g	GS1_SS_21848_03892	Beta-expansin 1a precursor	ZmEXPB1A	↓	1179	10	4.23E-167	96.90%
SCRFFL4009G09.b	GS1_SS_22001_11071	BCL-2 binding anthanogene-1	LOC100273657	↓	860	10	4.78E-78	92.90%
SCUTHR1065C09.g	GS1_SS_22291_14920	Syntaxin-related KNOLLE	SYP111	↓	557	10	9.09E-30	96%
SCJLFL3017H03.g	GS1_SS_22497_16253	F-box LRR-repeat 17-like	NA	↓	532	10	1.90E-32	82.40%
SCCCRZ2001C03.g	GS1_SS_22652_11155	Hypothetical protein SORBIDRAFT_01g012230	NA	↓	1864	10	0.00E+00	73.10%
SCCCLR2001D10.g	GS1_SS_23214_16731	Histone H3-like	T24H18.80	↓	642	10	3.28E-72	95.30%
SCRLFL4060B07.g	GS1_SS_23344_01746	Probable RNA-binding ARP1	RP1	↓	1138	10	1.77E-96	77.90%
SCCCLB1002E02.g	GS1_SS_23497_10219	C2 domain-containing expressed	K23L20.10	↓	913	10	2.02E-124	96.50%

(continuation)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCJLRZ1027B10.g	GS1_SS_23569_16523	Phytoene synthase 3	OsPSY3	↑	922	10	1.91E-156	93.20%
SCCCFL5062D05.g	GS1_SS_23589_00797	POLLENLESS 3-LIKE 2	LOC100274072	↓	466	10	3.21E-22	81.50%
SCSFFL4085D03.g	GS1_SS_23616_03654	ABRE-binding factor BZ-1	ZmBZIP1	↑	762	10	1.35E-72	82.20%
SCBGFL5077B07.g	GS1_SS_23640_09836	NA	NA	↓	144	NA	NA	NA
SCCCFL5062H06.g	GS1_SS_23772_11629	Mechanosensitive ion channel 6-like	LOC103653893	↑	529	10	1.73E-75	97.80%
SCVPRZ2036F02.g	GS1_SS_23960_16424	Replication A 70 kDa DNA-binding subunit	NA	↓	1149	10	0.00E+00	95.70%
SCRUFL1119E04.g	GS1_SS_24015_09336	E3 ubiquitin-ligase LIN-1 isoform X1	LOC103630225	↓	612	10	3.38E-133	95.20%
SCUTLR1037G02.g	GS1_SS_24077_16084	DNA (cytosine-5)-methyltransferase 3	ZmET5	↓	1553	10	0.00E+00	95.20%
SCEQAM2039A10.g	GS1_SS_24094_21208	Scarecrow 28	LOC103652157	↓	1415	10	0.00E+00	89.80%
SCSGAM2075E07.b	GS1_SS_24321_03224	Treacle -like	LOC103628465	↓	639	10	2.87E-92	86.90%
SCCCRT3002G10.g	GS1_SS_24423_08264	Jacalin-related lectin 9-like	T2O4.11	↓	1264	10	1.10E-118	69%
SCSGRT2063H01.g	GS1_SS_24431_09386	Cationic peroxidase SPC4-like	LOC103655313	↓	1383	10	6.73E-161	79.90%
SCACAD1036B09.g	GS1_SS_24432_14831	Soluble starch synthase chloroplastic amyloplastic	ZmZSSIIA	↑	919	10	7.28E-36	97.40%
SCCCRT1C05A07.g	GS1_SS_24436_08415	Beta-hexosaminidase subunit B2	LOC100280704	↓	398	10	3.08E-42	96.90%
SCQGRT1038H03.g	GS1_SS_24489_14281	Probable carbohydrate esterase At4g34215	LOC103632302	↓	681	10	4.74E-64	91.10%
SCCCRT3004A10.g	GS1_SS_24498_15150	NA	NA	↓	821	NA	NA	NA
SCCCLB1003E01.g	GS1_SS_24511_01373	Cortical cell-delineating precursor	LOC103638956	↓	732	10	1.41E-51	96.10%
SCEQRT3C03F04.g	GS1_SS_24517_08410	Hypothetical protein SORBI_004G088600	NA	↑	598	10	7.37E-30	87.10%
SCVPRT3088A07.g	GS1_SS_24523_09862	Wall-associated receptor kinase 2-like	LOC103655899	↓	543	10	3.88E-77	80.40%
SCJLRT1016B01.g	GS1_SS_24593_08755	Hypothetical protein SORBI_003G356400	NA	↑	1242	10	3.03E-44	89.60%
SCQSRT1035D12.g	GS1_SS_24797_18959	TPA: thaumatin domain family	LOC100192090	↑	952	10	2.00E-135	84%
SCSGLR1045D05.g	GS1_SS_24901_21213	HVA22 e	HVA22E	↑	758	10	8.34E-76	89.70%
SCACCL6009B02.g	GS1_SS_24913_15490	Heat shock factor 1	HSF1	↑	1346	10	9.30E-89	81.70%
SCJLLB2078D10.g	GS1_SS_24956_01082	Hypothetical protein SORBI_004G278400	NA	↑	772	10	5.07E-35	77%
SCEQRT3C05D04.g	GS1_SS_24982_19345	Phospholipase D alpha 2-like	PLDALPHA2	↓	625	10	1.77E-137	84.50%

(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCRURT3061C01.g	GS1_SS_24990_09009	Endonuclease 2	ENDO2	↓	884	10	7.46E-158	82.10%
SCRURT3061D05.g	GS1_SS_24992_10526	Transcription factor SCREAM2	SCRM2	↓	729	10	1.78E-98	88.40%
SCEQRT3017F02.g	GS1_SS_25015_02194	32 kDa	NA	↓	462	10	2.59E-48	64%
SCRURT3064D10.g	GS1_SS_25017_11727	Dirigent 1	LOC103627194	↓	797	10	2.92E-106	64.80%
SCCCRT2001D02.g	GS1_SS_25042_08456	Elicitor-responsive 3	OsERG3	↓	581	10	8.44E-88	92.50%
SCCCCL6024F07.g	GS1_SS_25287_16690	Cinnamoyl- reductase 1-like	CCR1	↑	1494	10	0.00E+00	93%
SCRLSD2011D08.g	GS1_SS_25447_13585	Low-molecular-weight cysteine-rich LCR70 precursor	ZmLCR70	↑	422	4	1.13E-45	80.75%
SCSBSD2029D04.g	GS1_SS_25485_15384	Calvin cycle CP12 - chloroplastic	CP12-1	↑	439	10	4.99E-58	86.50%
SCACSD2018G06.g	GS1_SS_25493_01997	NA	NA	↑	278	NA	NA	NA
SCVPRT2073C10.g	GS1_SS_25628_13940	WIN1 precursor	WIN1	↑	736	10	1.81E-77	93.30%
SCRFSD1022C11.g	GS1_SS_25632_12776	Branched-chain-amino-acid aminotransferase chloroplastic-like	pco098993(576)	↑	356	10	2.49E-09	95.80%
SCEPSD1007C11.g	GS1_SS_25637_17940	Pathogenesis-related class partial	HCHIB	↑	700	10	9.36E-74	97%
SCRFSD2022A08.g	GS1_SS_25659_13116	NA	NA	↑	248	NA	NA	NA
SCCCSD2001G01.g	GS1_SS_25685_11811	Peptide transporter PTR2	PTR2	↑	599	10	9.70E-92	88.40%
SCRFLB2060E04.g	GS1_SS_25725_08917	Disulfide isomerase	LOC100192725	↑	1426	10	0.00E+00	95%
SCQGSD2047F01.g	GS1_SS_25860_10456	Hypothetical protein SORBI_005G062400	NA	↑	529	2	7.99E-07	86.50%
SCSBSD2055F08.g	GS1_SS_25879_13953	Hypothetical protein SORBIDRAFT_01g043980	NA	↑	332	1	9.23E-08	85%
SCCCSD1093B10.g	GS1_SS_41310_20526	60S ribosomal L15	SAG24	↑	685	10	6.17E-81	77.90%

(continue)

Table 19. Roots differentially expressed genes after 6 days of stress plus 2 re-watering days determined by HTSelf method and annotated using Blast2GO. Up-regulated genes = ↑; Down-regulated genes = ↓

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean
SCRLAM2049E07.g	GS1_SS_00943_09461	Hypothetical protein SORBIDRAFT_05g002650	NA	↑	784	10	1.91E-118	85.90%
SCBGAM1093E09.g	GS1_SS_01813_10587	Uncharacterized protein LOC100277515	LOC100277515	↓	810	10	6.84E-80	90%
SCUTLR2023A03.g	GS1_SS_04374_14495	GDSL esterase lipase At5g45910-like	LOC103630572	↓	1460	10	0.00E+00	94.60%
SCJFRT2057H11.g	GS1_SS_04616_10915	Protease inhibitor seed storage LTP family	NA	↓	778	6	6.05E-30	79.33%
SCEZAM2058D02.g	GS1_SS_05300_21664	Ribonucleoside-diphosphate reductase small chain	RNR2A	↓	1273	10	0.00E+00	96.50%
SCCCCL2001H01.b	GS1_SS_05710_02918	Stem-specific TSJT1	ZmTSJT1	↑	1089	10	2.43E-157	86.60%
SCACLR1036D09.g	GS1_SS_06828_14213	SEC14 cytosolic factor-like	SEC14	↓	1847	10	0.00E+00	90.90%
SCRLCL6031A08.g	GS1_SS_06851_15580	Probable isoaspartyl peptidase L-asparaginase 2	LOC103630215	↓	1300	10	0.00E+00	94.40%
SCACLB1046B10.g	GS1_SS_08871_08840	Hypothetical protein SORBIDRAFT_03g045310	NA	↓	1011	1	7.06E-11	92%
SCQGLB1029D12.g	GS1_SS_09460_14046	Hypothetical protein SORBIDRAFT_02g007160	NA	↓	523	10	1.11E-48	78.60%
SCEQLR1092B01.g	GS1_SS_10388_08647	Hypothetical protein	NA	↓	499	10	7.88E-19	86.20%
SCEQLR1092C09.g	GS1_SS_10389_20048	NA	NA	↑	646	NA	NA	NA
SCQGLR2032B01.g	GS1_SS_10886_01209	Hypothetical protein SORBIDRAFT_03g042590	NA	↓	736	10	1.24E-84	95.80%
SCQGLR1085F11.g	GS1_SS_11161_19643	Dehydrin 9	LEA	↑	760	10	2.03E-23	71.40%
SCCCLR2C02C04.g	GS1_SS_11301_17591	NA	NA	↓	634	NA	NA	NA
SCJLLR1107D06.g	GS1_SS_11621_09365	Hypothetical protein SORBI_002G141800	NA	↑	768	5	4.46E-17	83.80%
SCVPLR2012E08.g	GS1_SS_11675_13721	Seed maturation PM41	ZmPM41	↑	562	10	2.89E-43	91.50%
SCEQRT1024F02.g	GS1_SS_11853_16153	Peroxidase 1	LOC103637120	↓	1265	10	0.00E+00	92.90%
SCBGRT1052E01.g	GS1_SS_11960_00886	Aquaporin TIP2-3	TIP2;3	↓	1037	10	3.23E-165	97.40%
SCCCRT1003E03.g	GS1_SS_12123_03618	Hypothetical protein SORBIDRAFT_06g001170	NA	↓	805	10	1.32E-93	72.30%
SCEQRT2027G07.g	GS1_SS_12262_12384	Universal stress A	NA	↑	954	10	7.12E-92	79.50%
SCAGLR1043F07.g	GS1_SS_12317_14614	Brown planthopper-induced resistance 1	NA	↑	972	10	1.15E-139	86.80%
SCMCRT2107D08.g	GS1_SS_12765_10611	AF332176_1 beta-expansin partial	OsEXPB3	↓	1226	10	2.38E-71	96%
SCCCRT2001D04.g	GS1_SS_12781_18391	Cortical cell-delineating precursor	LOC103638956	↓	798	10	7.47E-54	94.30%

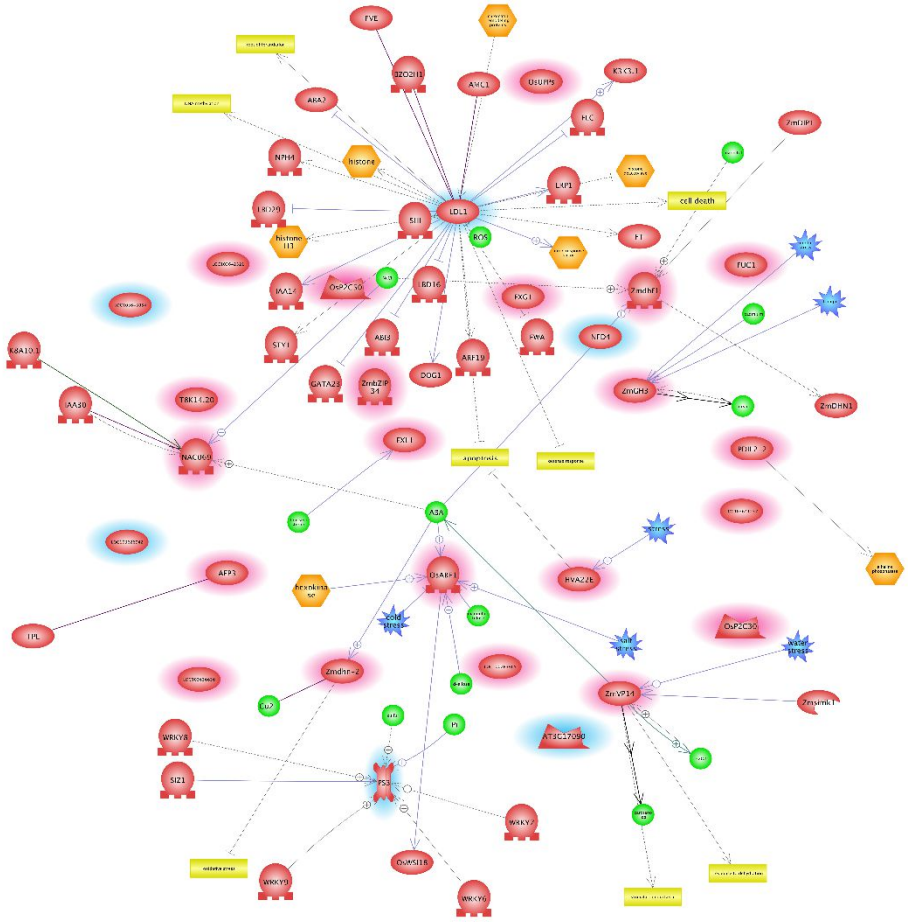
(conclusion)

Transcript Name	Probe Name	Description	Pathview	Expression	Length	#Hits	e-Value	sim mean	
SCQSRT2031A09.g	GS1_SS_13365_10130	Benzothiadiazole-induced -like		OsBIHD1	↓	578	10	1.55E-92	69.80%
SCQSRT2031H06.g	GS1_SS_13683_17635	Late embryogenesis abundant 3		AT3G53770	↑	905	10	2.88E-27	88.40%
SCEQRT2027H09.g	GS1_SS_13921_13907	PREDICTED: uncharacterized protein LOC103649874		LOC103649874	↓	1198	10	0.00E+00	91.50%
SCCCRZ2C02F06.g	GS1_SS_14651_02386	NA		NA	↓	588	NA	NA	NA
SCUTSB1076D12.g	GS1_SS_17469_11499	Photosystem II repair PSB27-chloroplastic		PSB27	↑	674	10	9.82E-68	97.60%
SCJFST1014C02.g	GS1_SS_17645_06164	PREDICTED: uncharacterized protein LOC101766165 isoform X2		NA	↑	778	10	2.88E-32	98.80%
SCJLRT2049D05.g	GS1_SS_17684_13787	Heme-binding 2		LOC100281923	↓	1195	10	1.96E-136	91.90%
SCVPRZ2038B09.g	GS1_SS_18185_11153	Aspartic proteinase nepenthesin-1 precursor		LOC101027131	↓	851	10	1.39E-94	96.30%
SCCCCL4005G05.g	GS1_SS_18344_01636	Small heat shock		LOC100285715	↑	1006	10	4.13E-102	83%
SCBFST3136C07.g	GS1_SS_18486_08015	Profilin A		Zmprofilin A	↓	782	10	1.97E-90	98%
SCAGFL1089A10.g	GS1_SS_19891_09996	Cold shock-1 precursor		CSDP1	↓	969	7	4.75E-08	85.57%
SCRLAD1136F04.g	GS1_SS_21443_14179	Unknown		NA	↓	741	10	1.30E-56	85.50%
SCVPLB1018H01.g	GS1_SS_21633_09324	Microtubule-associated MAP65-1a		ZmMAP65-1a	↑	3000	10	0.00E+00	92.40%
SCCCLB1023H08.g	GS1_SS_21848_03892	Beta-expansin 1a precursor		LOC100282311	↓	1179	10	4.22E-167	96.90%
SCCCRT3002G10.g	GS1_SS_24423_08264	Jacalin-related lectin 9-like		JAL31	↓	1264	10	1.10E-118	69%
SCRLLB2032D09.g	GS1_SS_24497_14077	Flower-specific gamma-thionin precursor		LOC100280572	↓	490	10	1.63E-26	94.80%
SCQSRT1035D12.g	GS1_SS_24797_18959	TPA: thaumatin domain family		LOC100192090	↑	952	10	2.00E-135	84%
SCRURT3061D05.g	GS1_SS_24992_10526	Transcription factor SCREAM2		SCRM2	↓	729	10	1.77E-98	88.40%
SCMCSD2061D05.g	GS1_SS_25565_17459	CBL-interacting kinase 2		LOC100141385	↑	707	10	1.23E-105	77.70%
SCVPRT2073C10.g	GS1_SS_25628_13940	WIN1 precursor		SHN1	↑	736	10	1.80E-77	93.30%
SCRFLB2060E04.g	GS1_SS_25725_08917	Disulfide isomerase		LOC100192725	↑	1426	10	0.00E+00	95%
SCBFRZ2048F01.g	GS1_SS_26284_05683	Transport and Golgi organization 2 homolog		NA	↑	614	10	1.67E-121	97%

APPENDIX D. Biological Networks from Pathway Sutdio (Elselvier)

Figure 61. Pathway Studio Biological Network from leaves up and down-regulated genes after 4 days of stress (a); Biological Network legend (b)

(a)



(b)

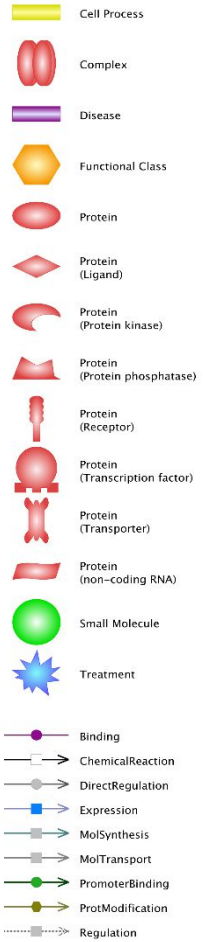


Figure 62. Pathway Studio Biological Network from leaves up and down-regulated genes after 6 days of stress

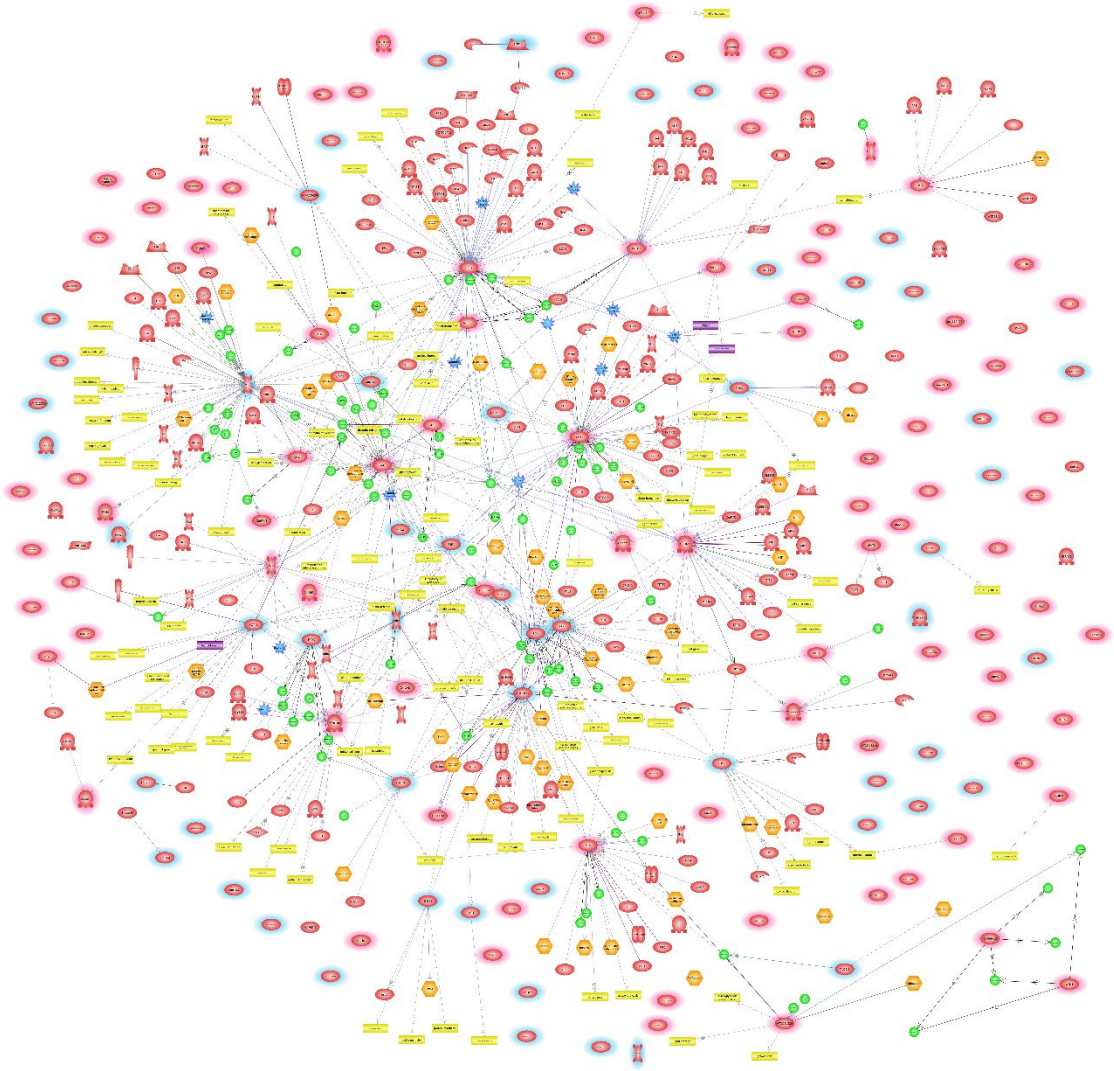


Figure 63. Pathway Studio Biological Network from leaves up and down-regulated genes after re-watering

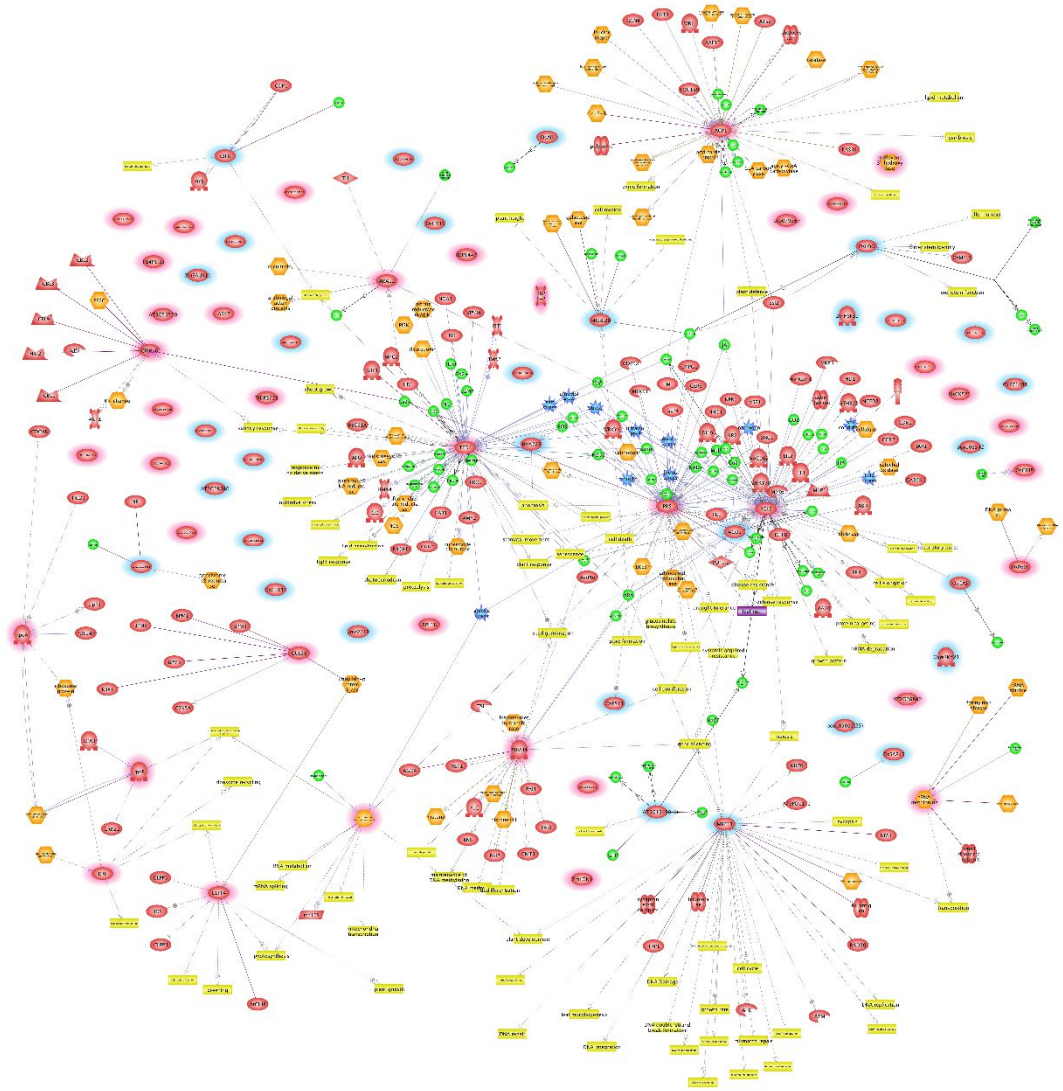


Figure 64. Pathway Studio Biological Network from roots up and down-regulated genes after 4 days of stress

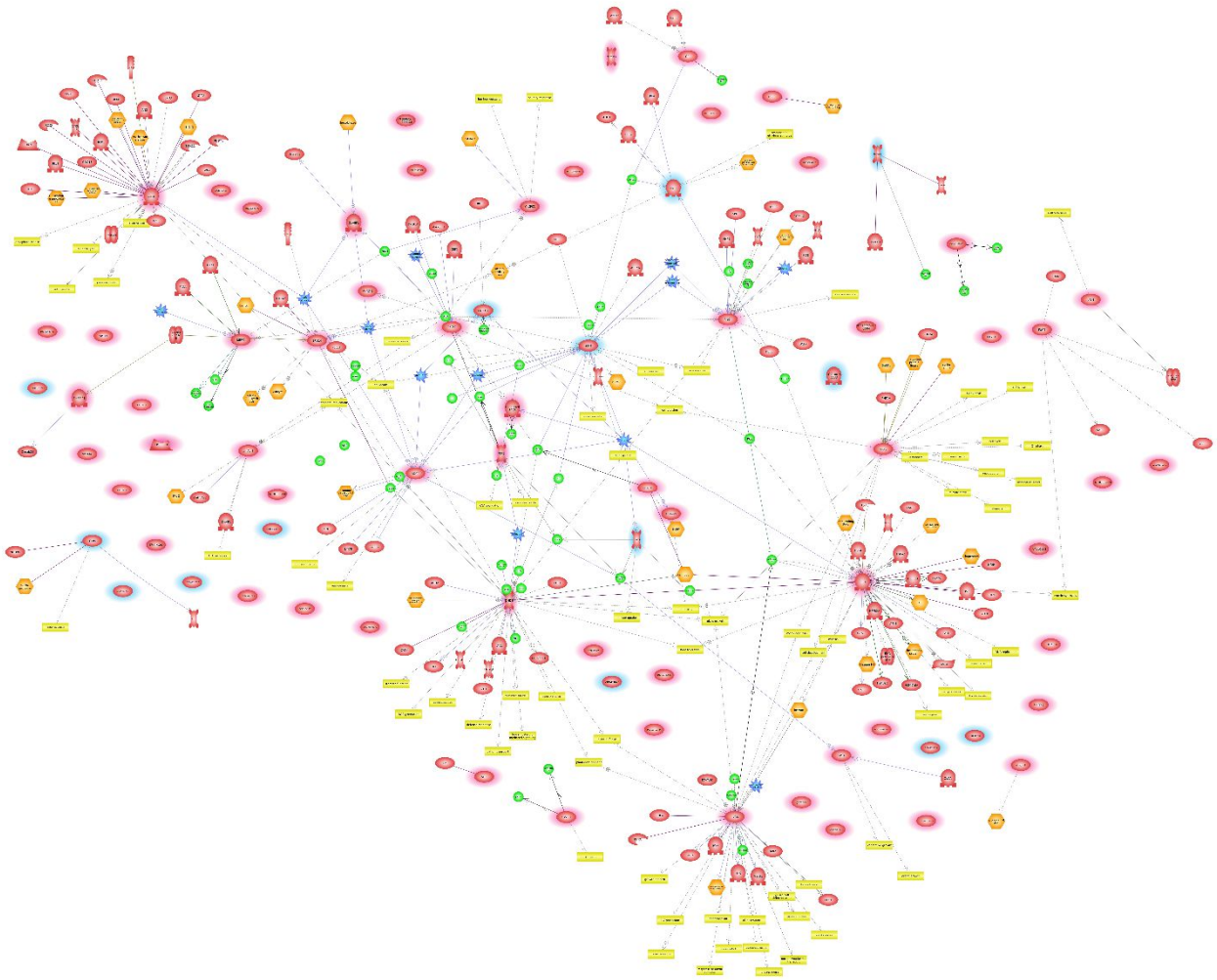


Figure 65. Pathway Studio Biological Network from roots up and down-regulated genes after 6 days of stress

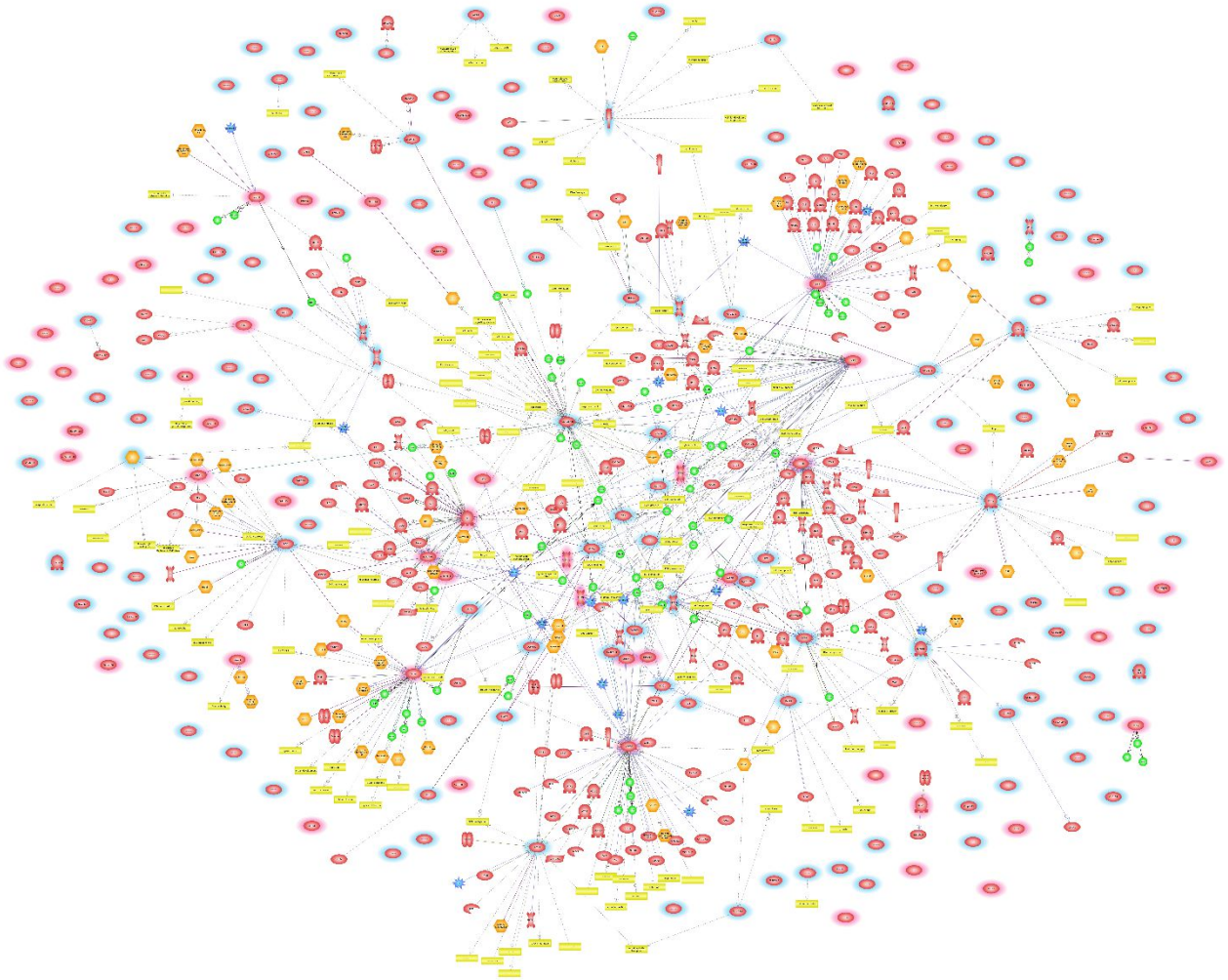
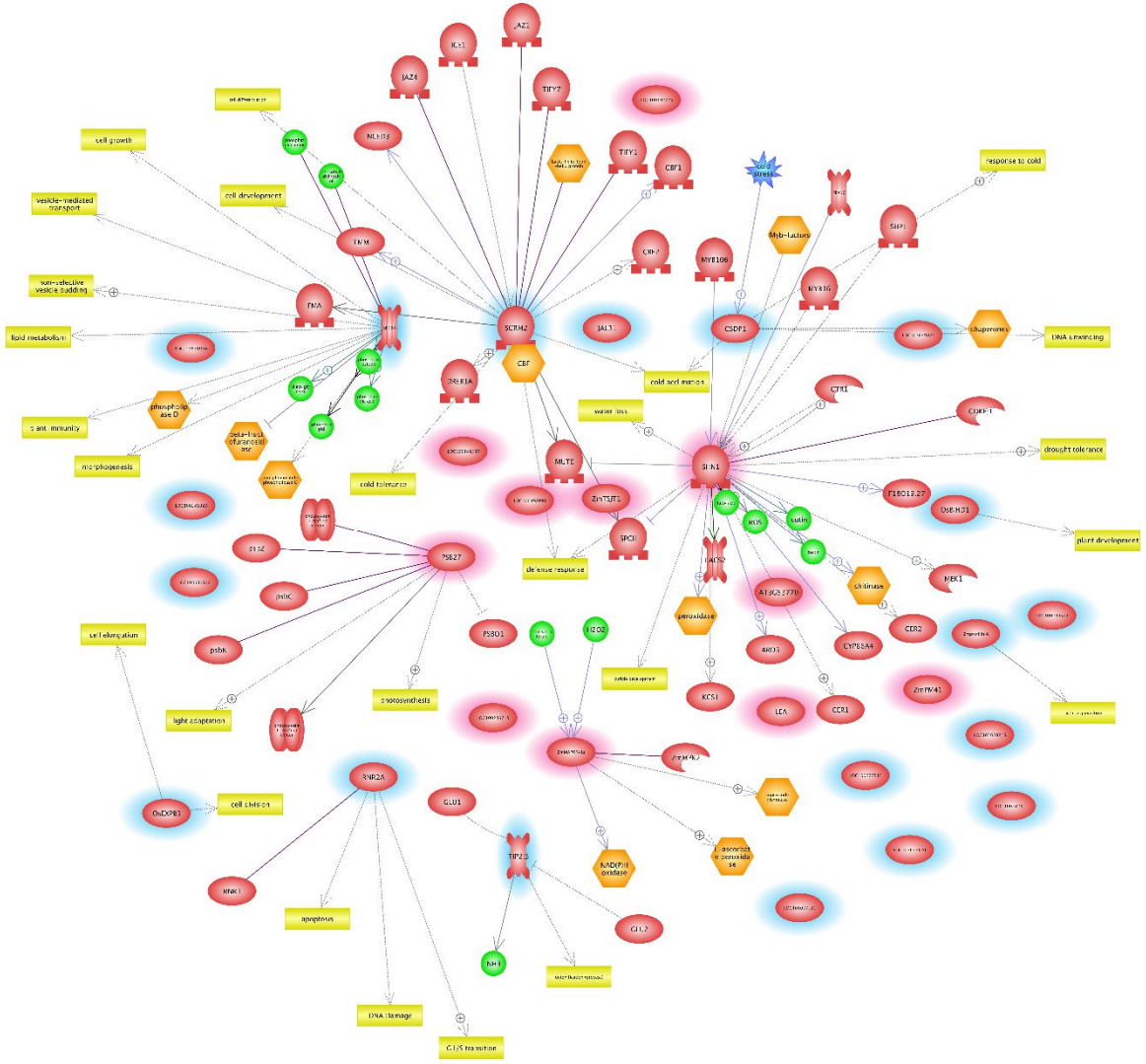


Figure 66. Pathway Studio Biological Network from roots up and down-regulated genes after re-watering



APPENDIX E. Differentially Expressed Pathways

Figure 67. Differentally Expressed Pathways in leaves

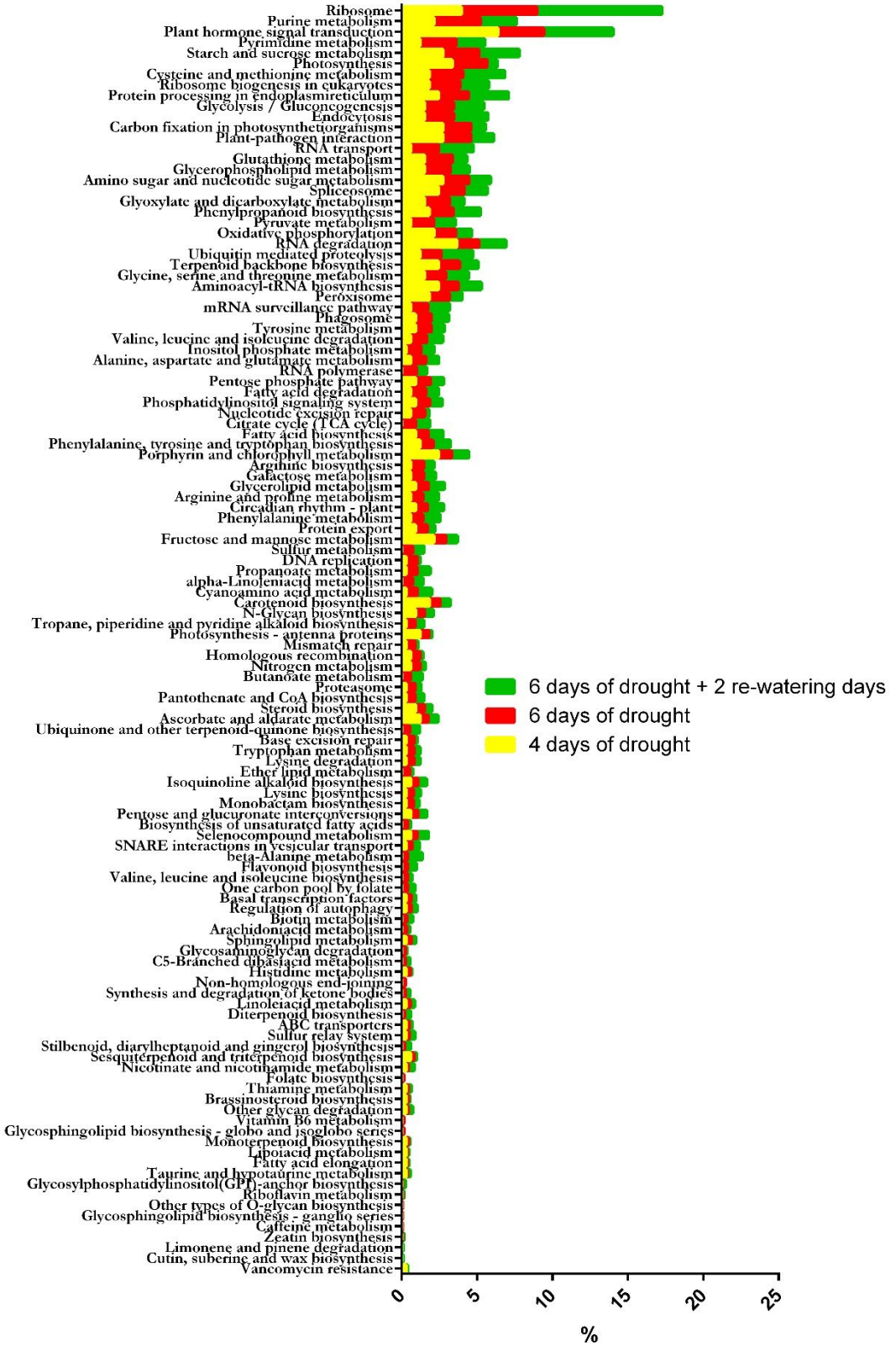
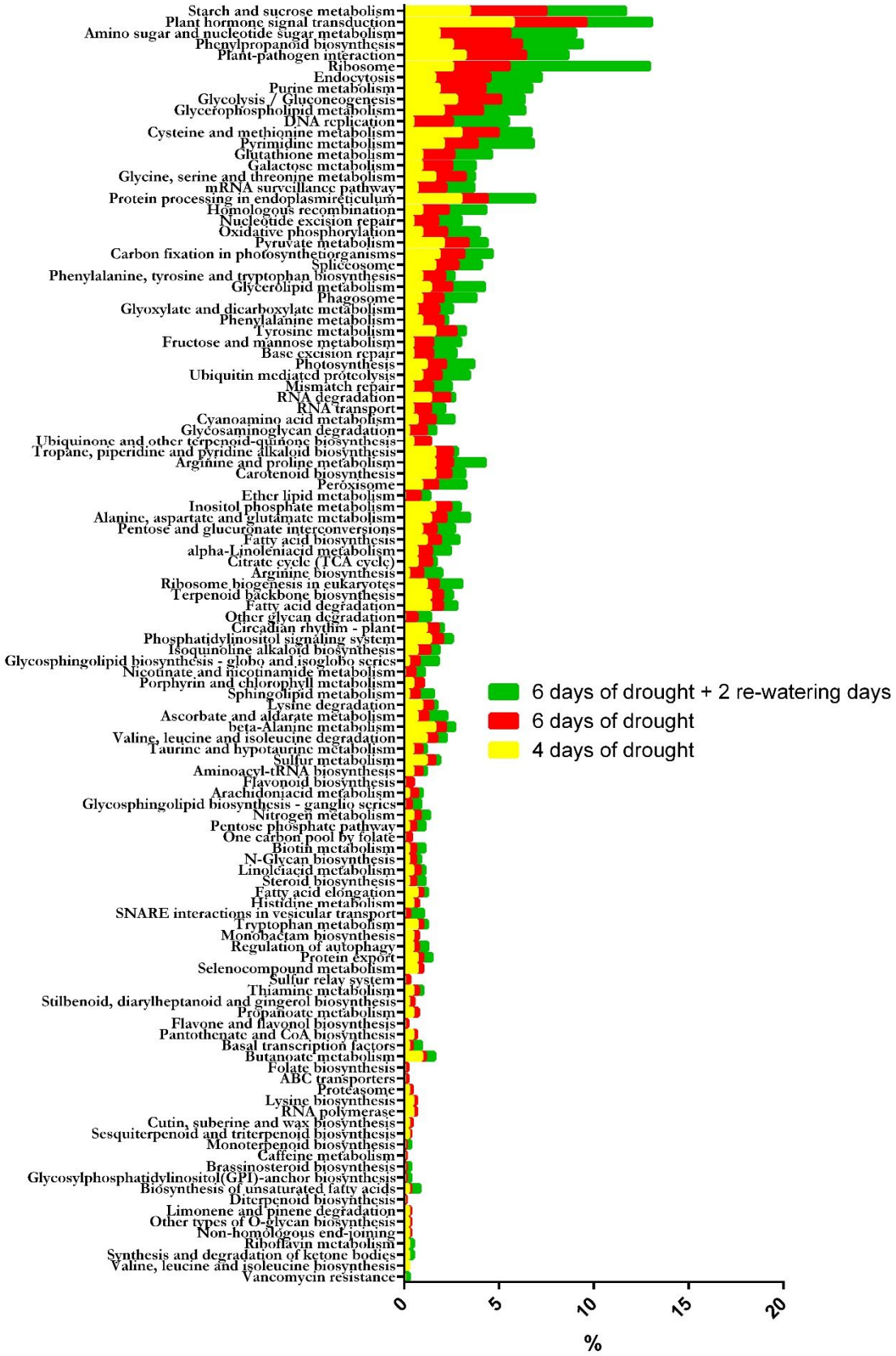


Figure 68. Differentially Expressed Pathways in roots



APPENDIX F. Orthologous Group Annotation for differentially expressed features identified at *de novo* RNA-Seq assembly

Figure 69. Top Orthologous Group from leaves (a) up and (b) down-regulated features detected from *de novo* RNA-Seq assembly

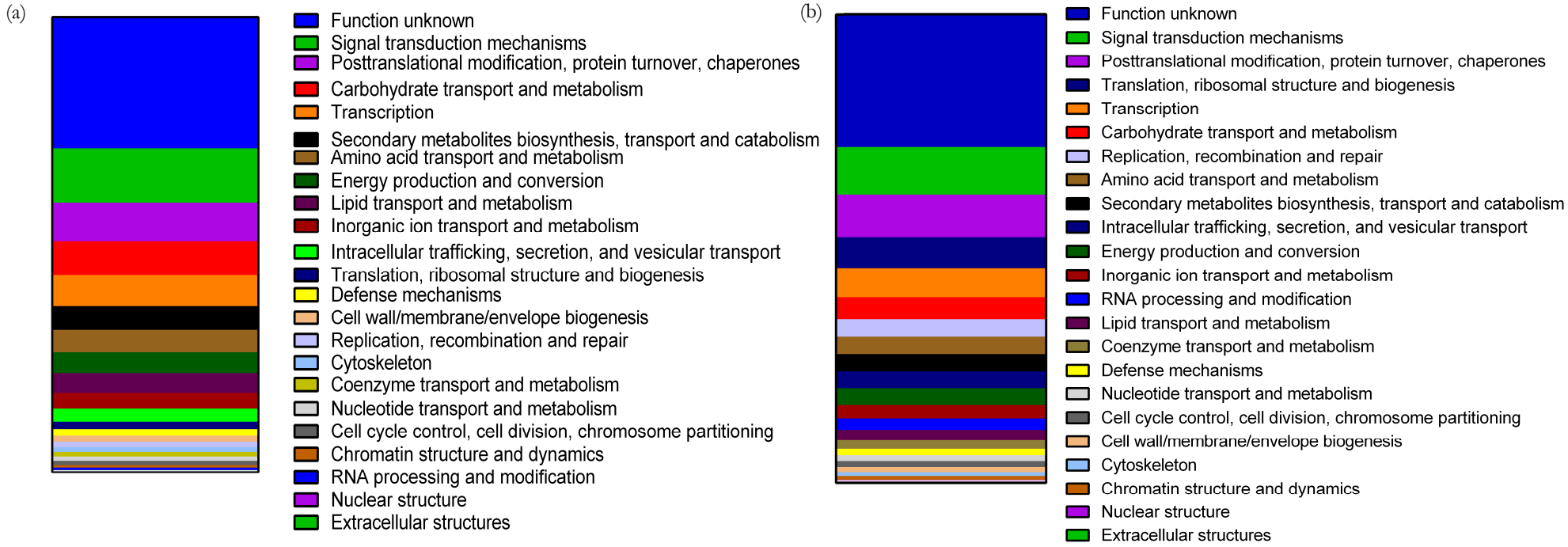
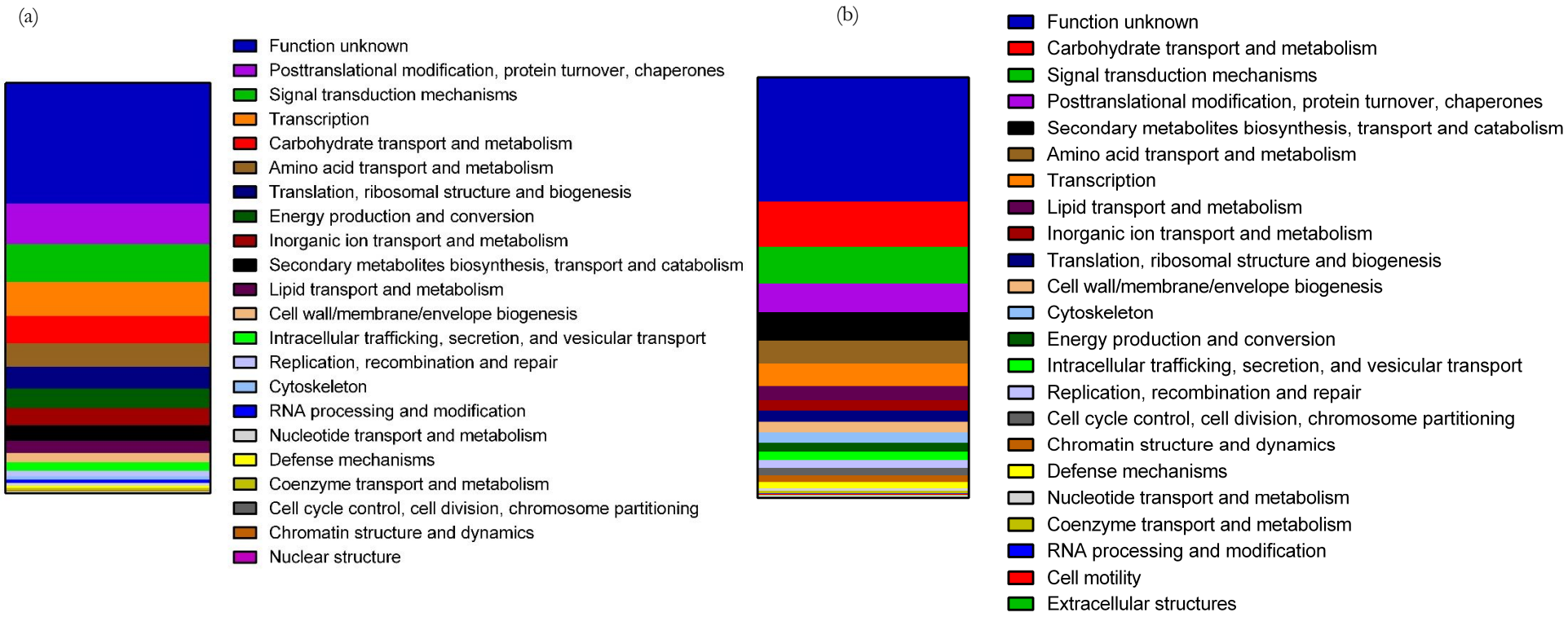


Figure 70. Top Orthologous Group from roots (a) up and (b) down-regulated features detected from *de novo* RNA-Seq assembly



APPENDIX G. Co-Expression Networks identified from the modules where drought progression transcripts selected belonged

Figure 71. Bisque4 Co-expression network. (a) full; (b) signal transduction; (c) transcription

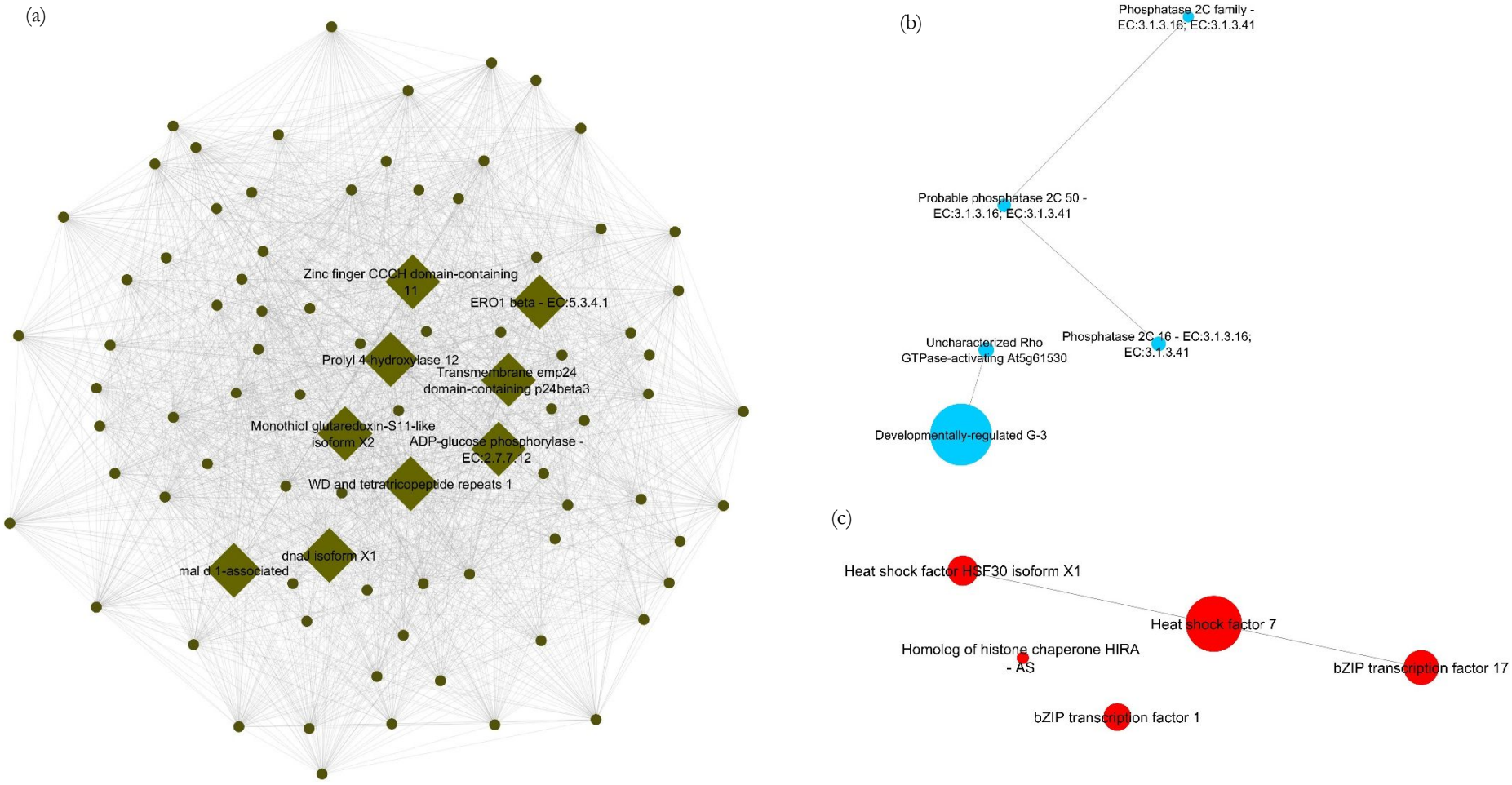


Figure 72. Black Co-expression network. (a) full; (b) signal transduction; (c) transcription

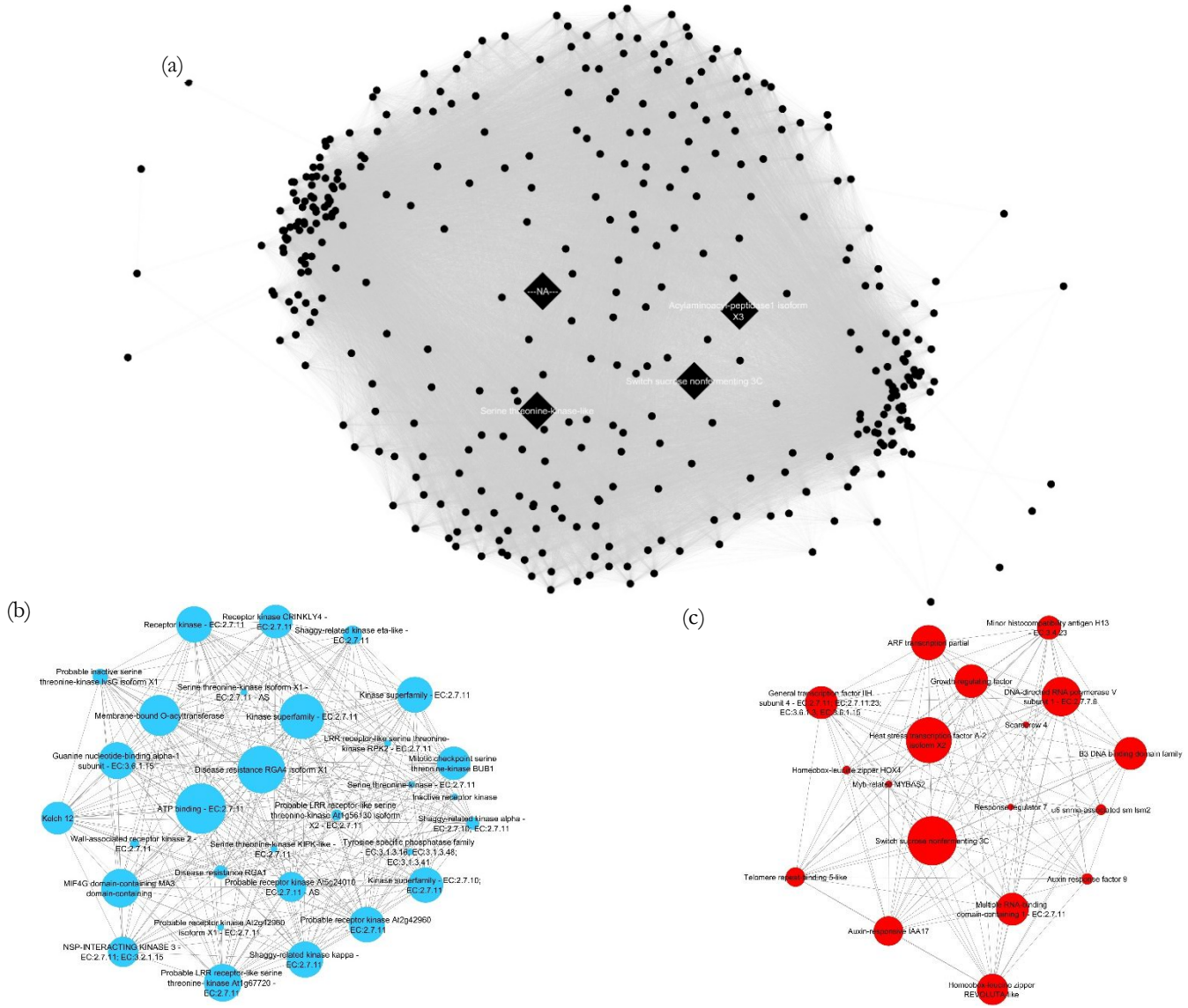


Figure 73. Dark Green Co-expression network. (a) full; (b) signal transduction; (c) transcription

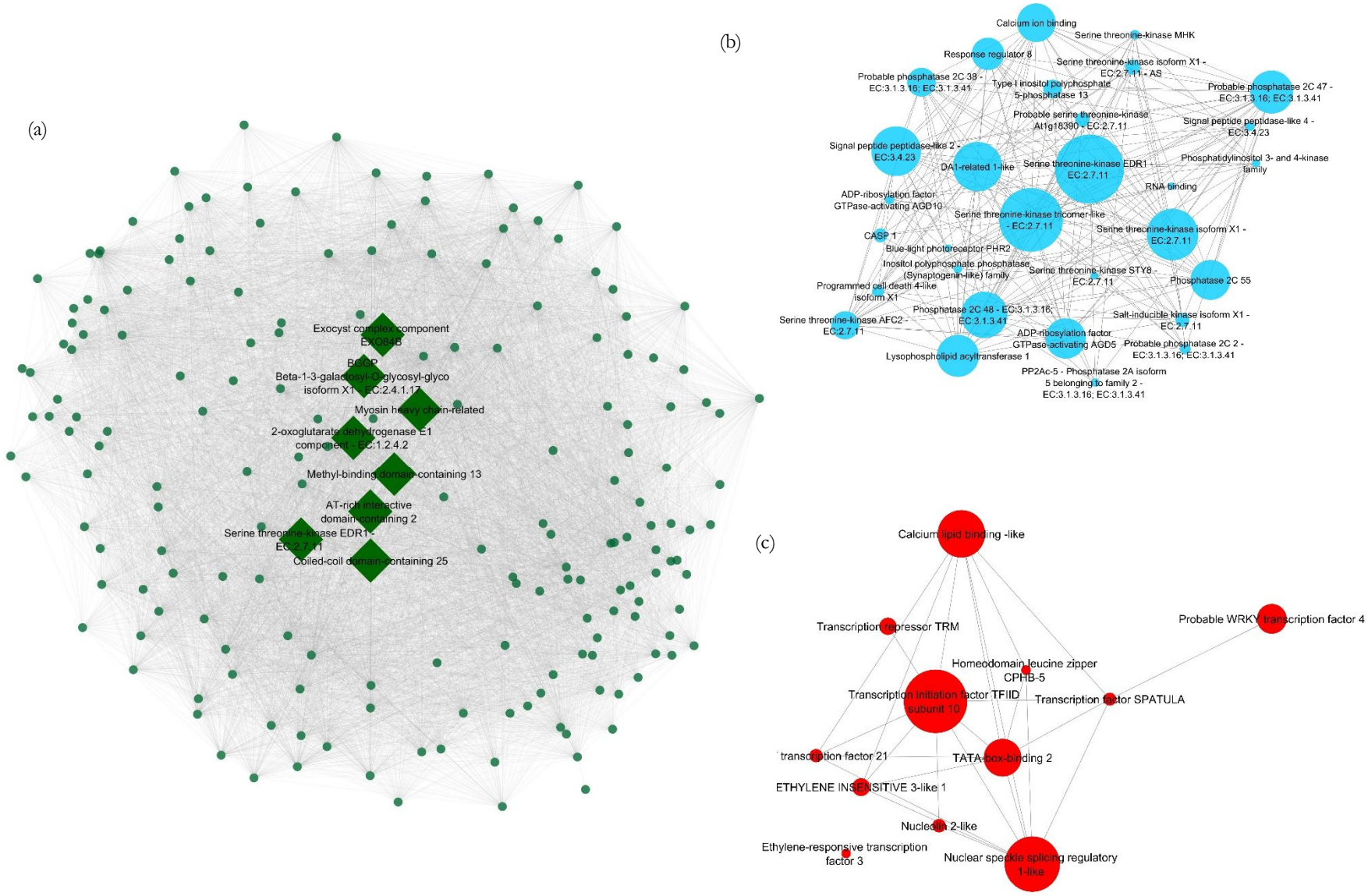


Figure 74. Dark Grey Co-expression network. (a) full; (b) signal transduction; (c) transcription

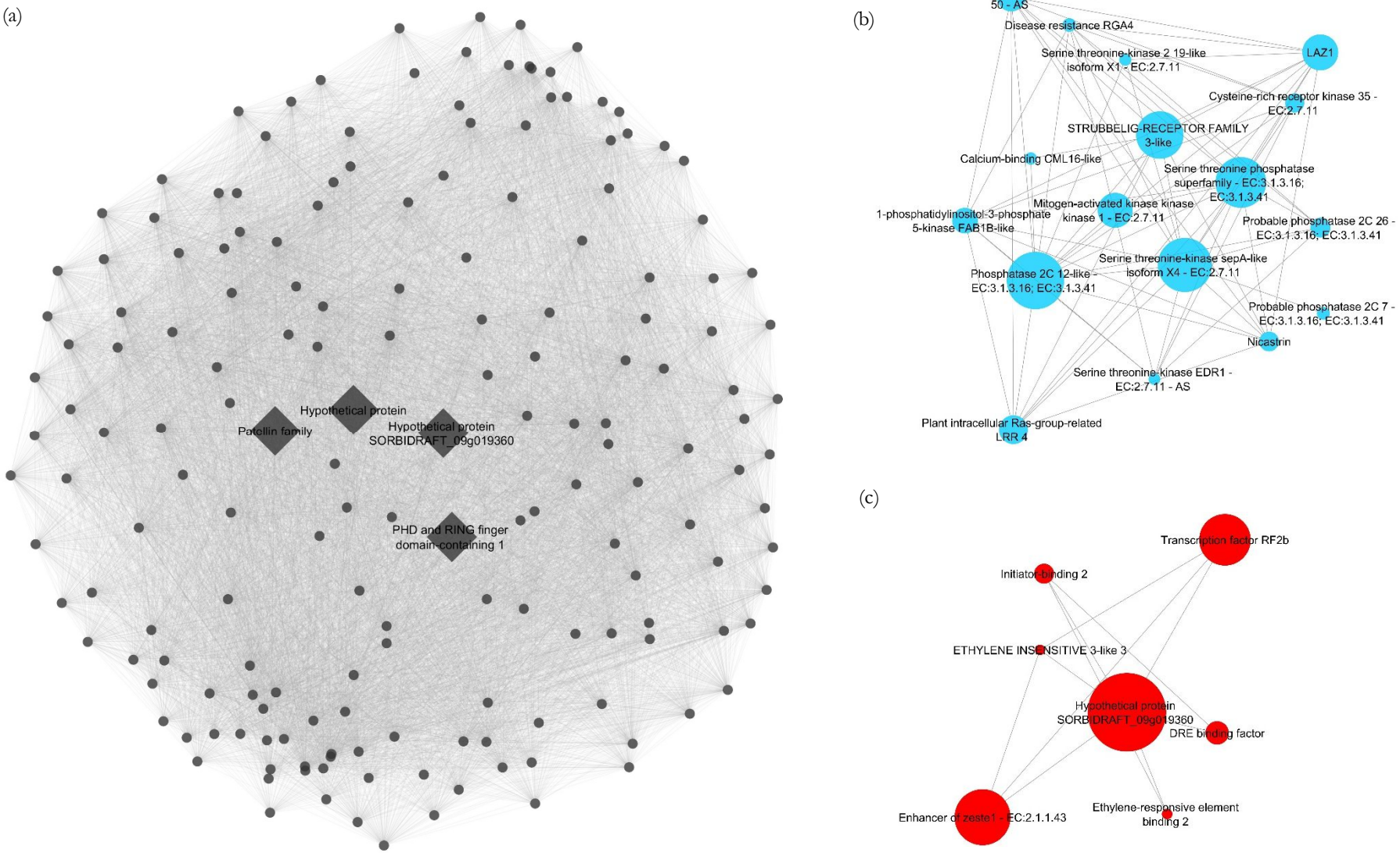


Figure 75. Dark Olive Green Co-expression network. (a) full; (b) signal transduction; (c) transcription

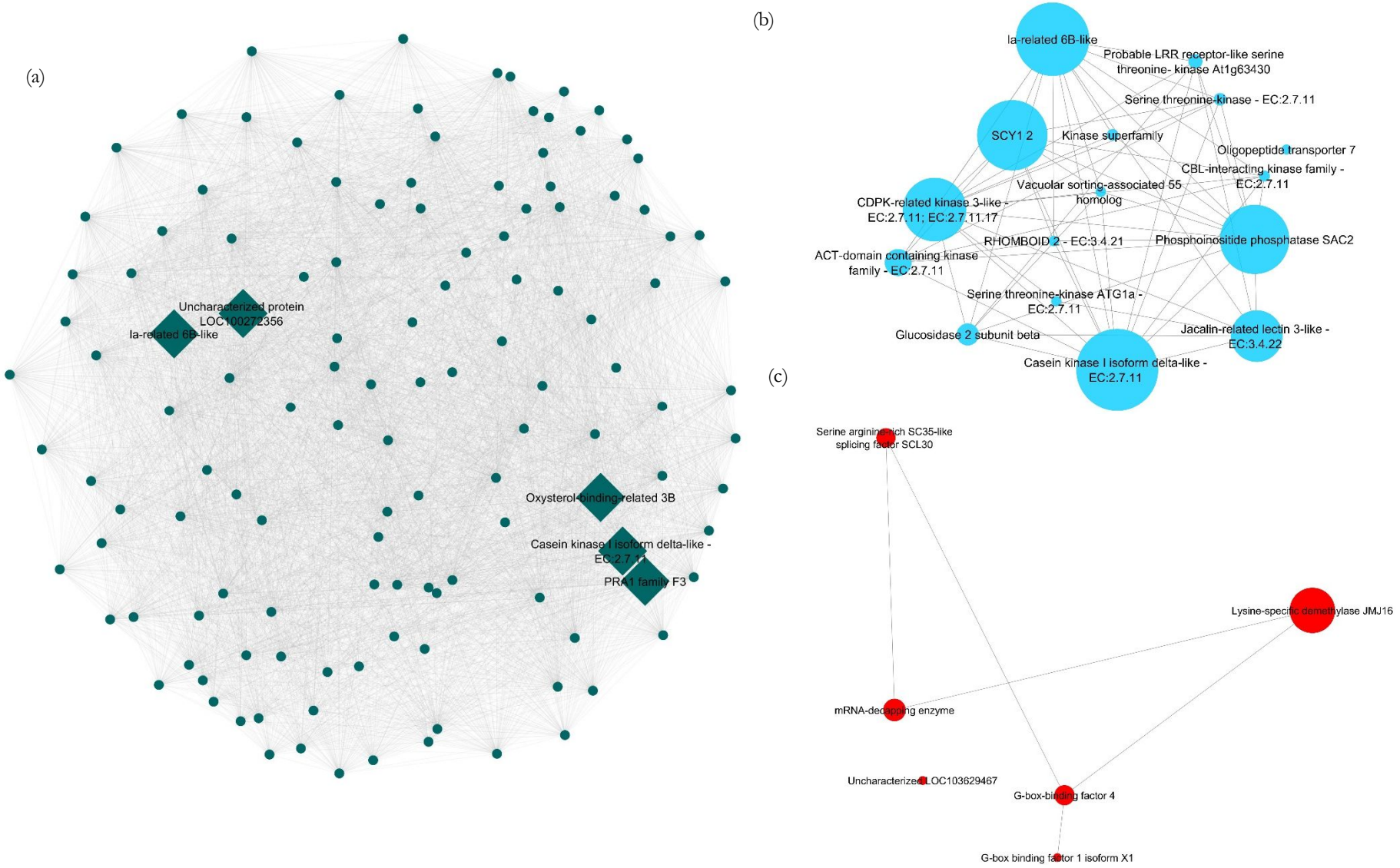


Figure 76. Green Co-expression network. (a) full; (b) signal transduction; (c) transcription

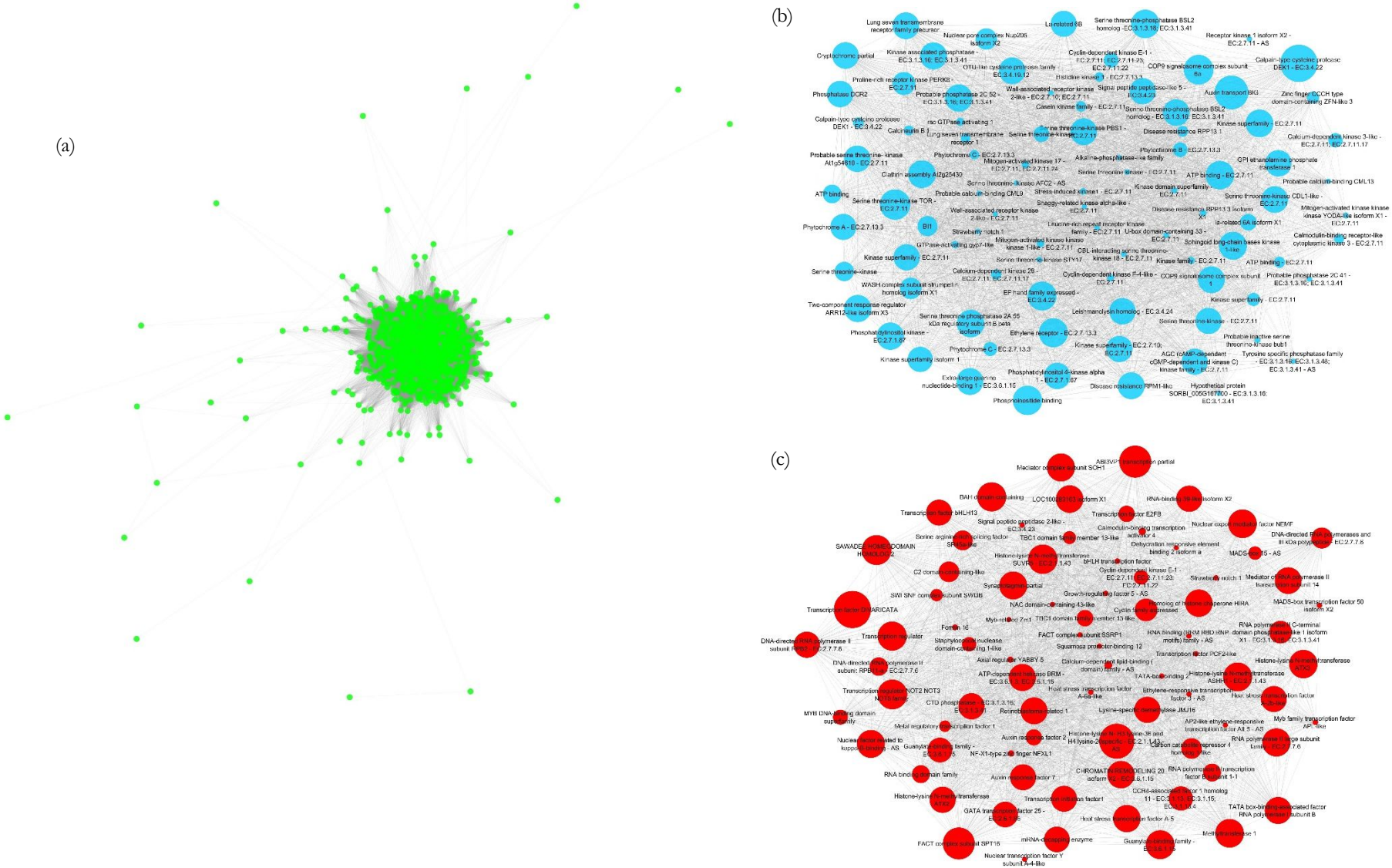


Figure 77. Green Yellow Co-expression network. (a) full; (b) signal transduction; (c) transcription

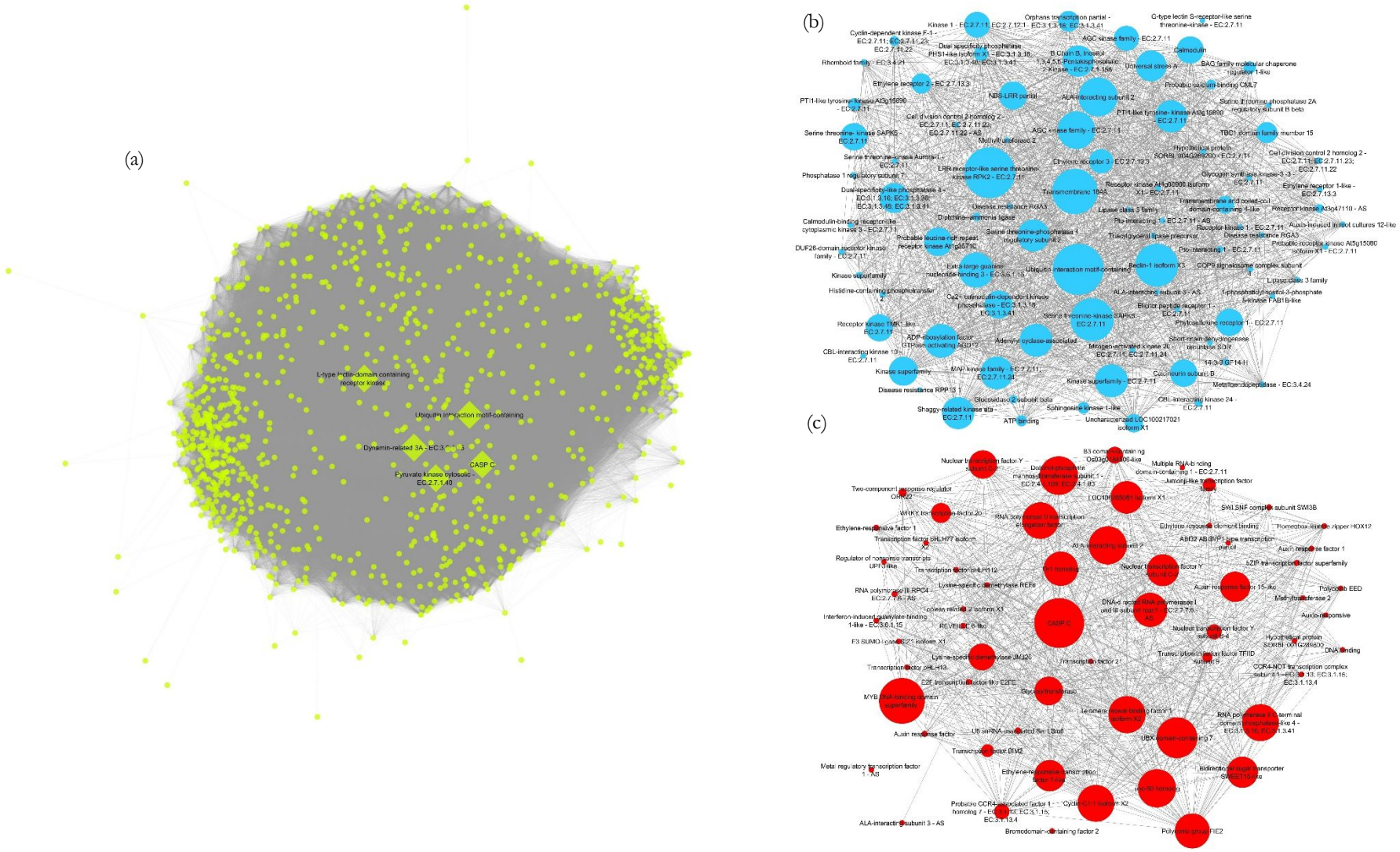


Figure 78. Light Cyan Co-expression network. (a) full; (b) signal transduction; (c) transcription

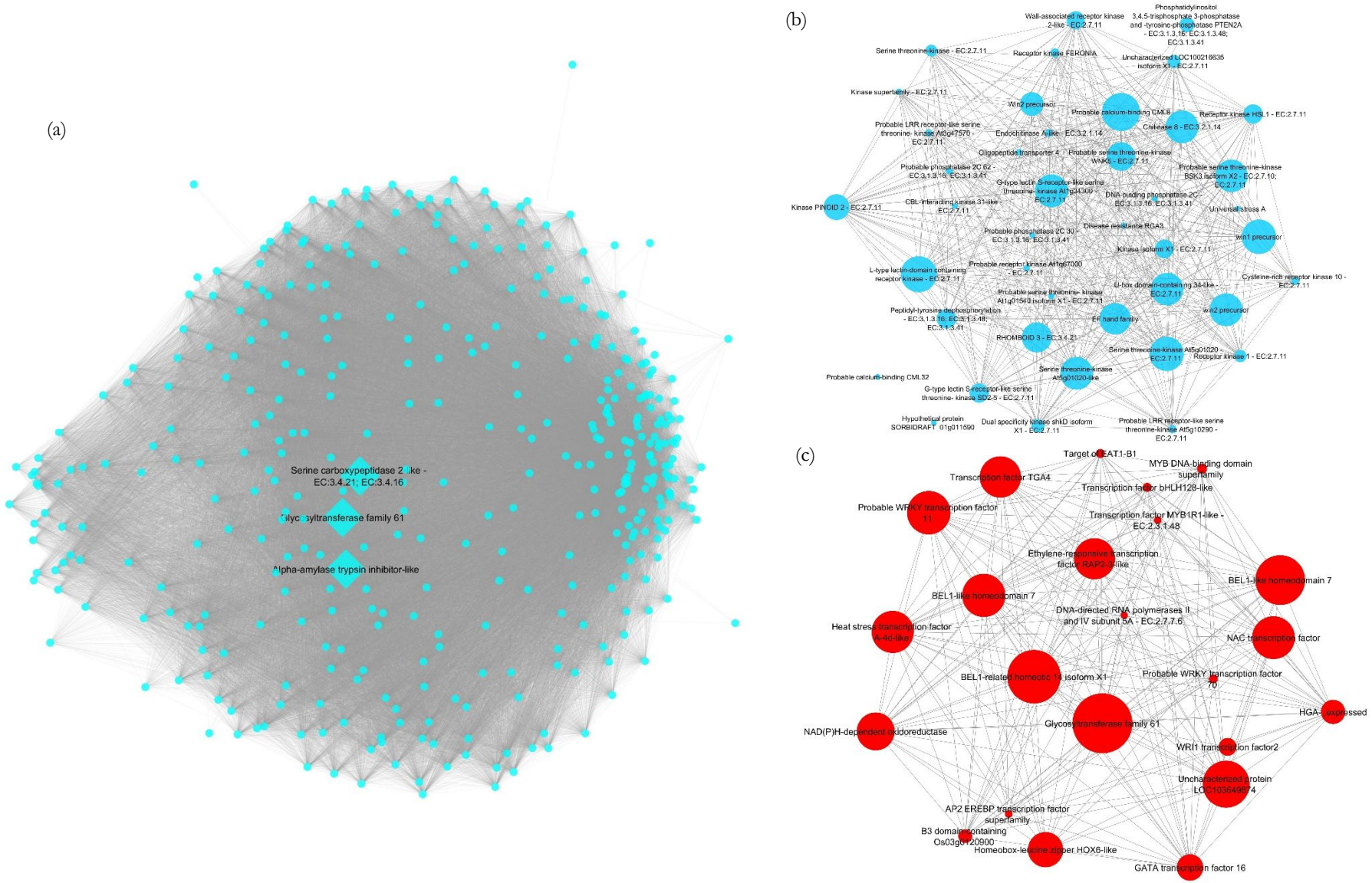


Figure 79. Light Green Co-expression network. (a) full; (b) signal transduction; (c) transcription

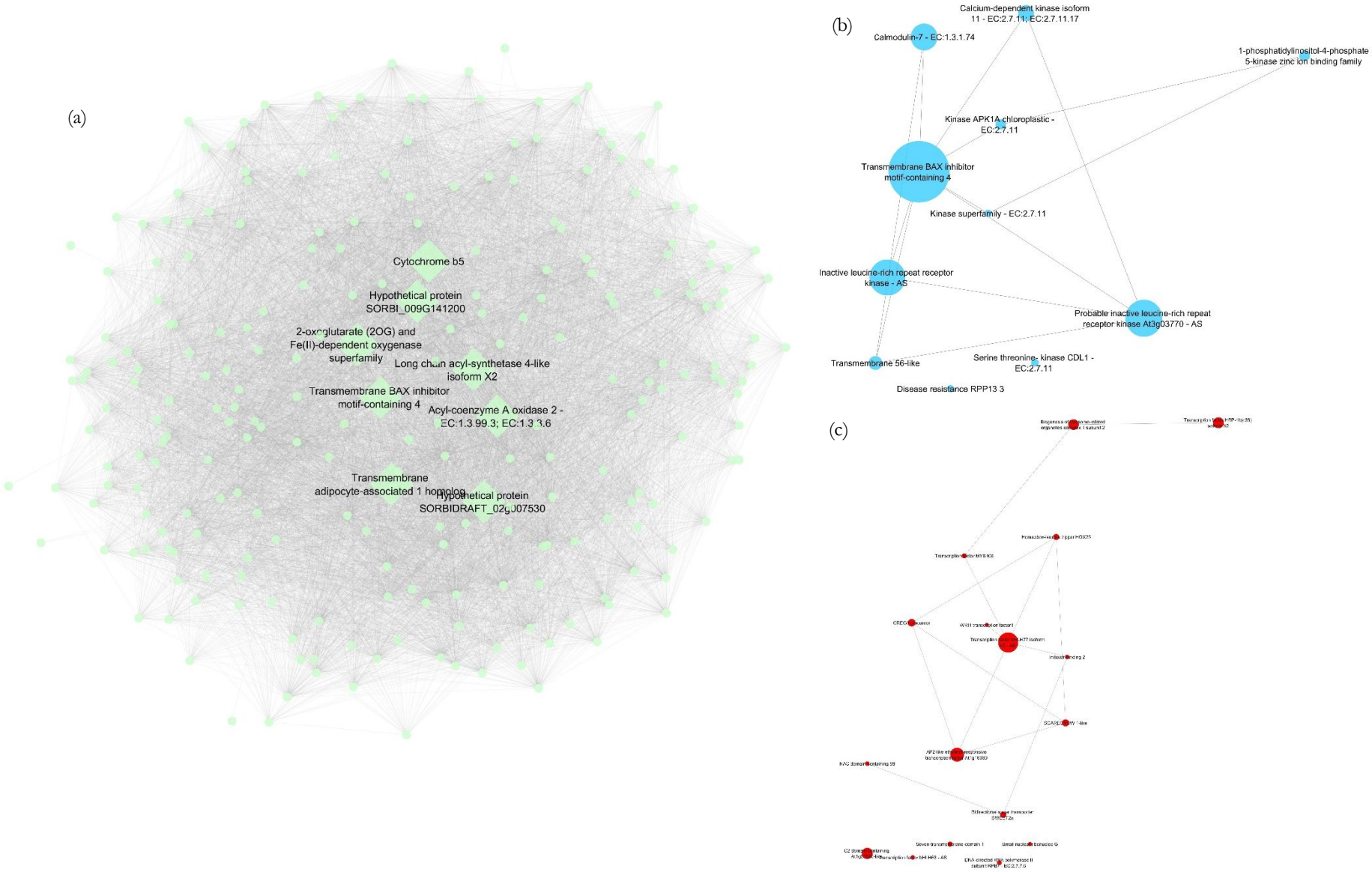


Figure 80. Light Yellow Co-expression network. (a) full; (b) signal transduction; (c) transcription

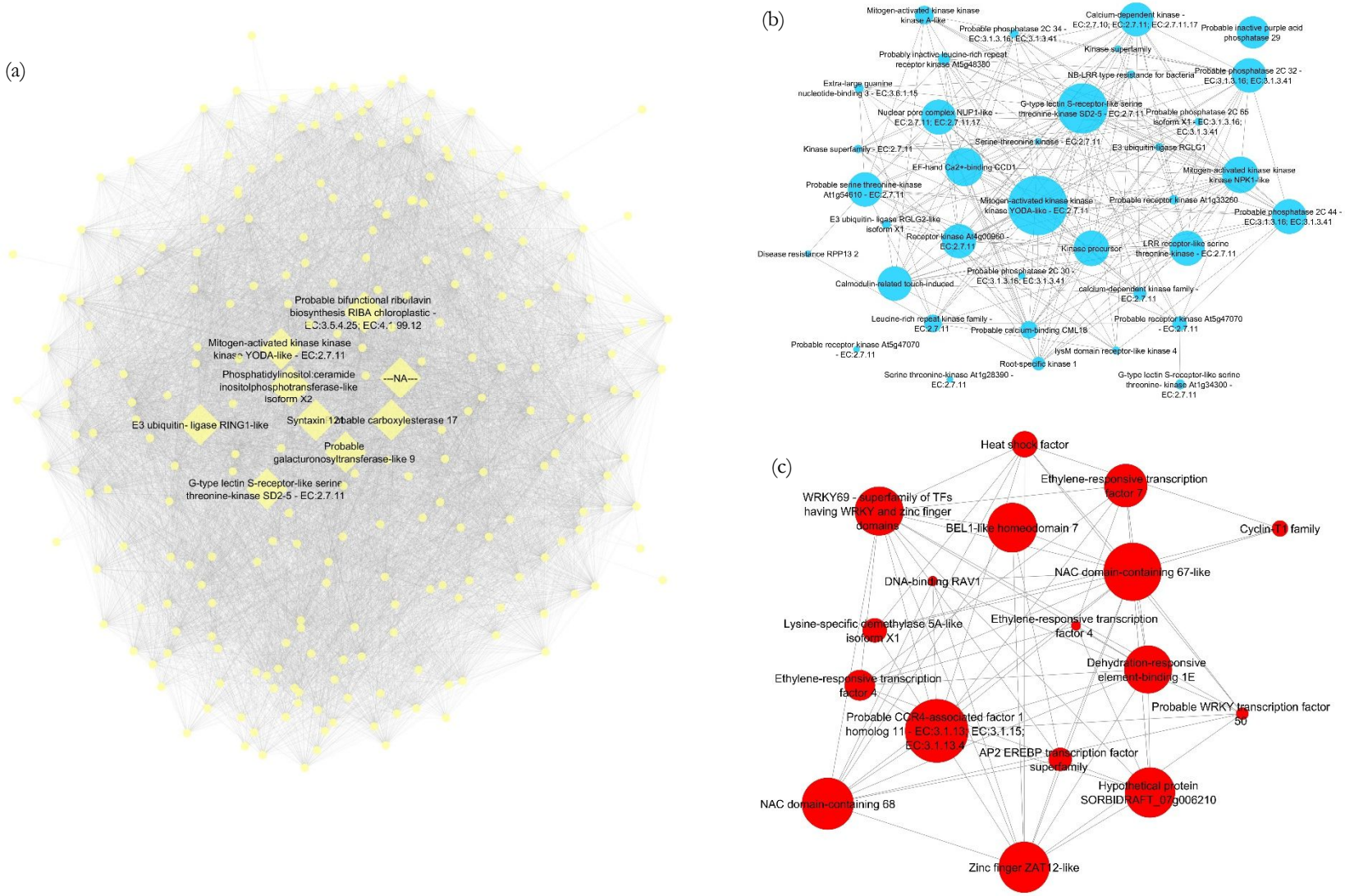


Figure 81. Pale Violet Red Co-expression network. (a) full; (b) signal transduction; (c) transcription

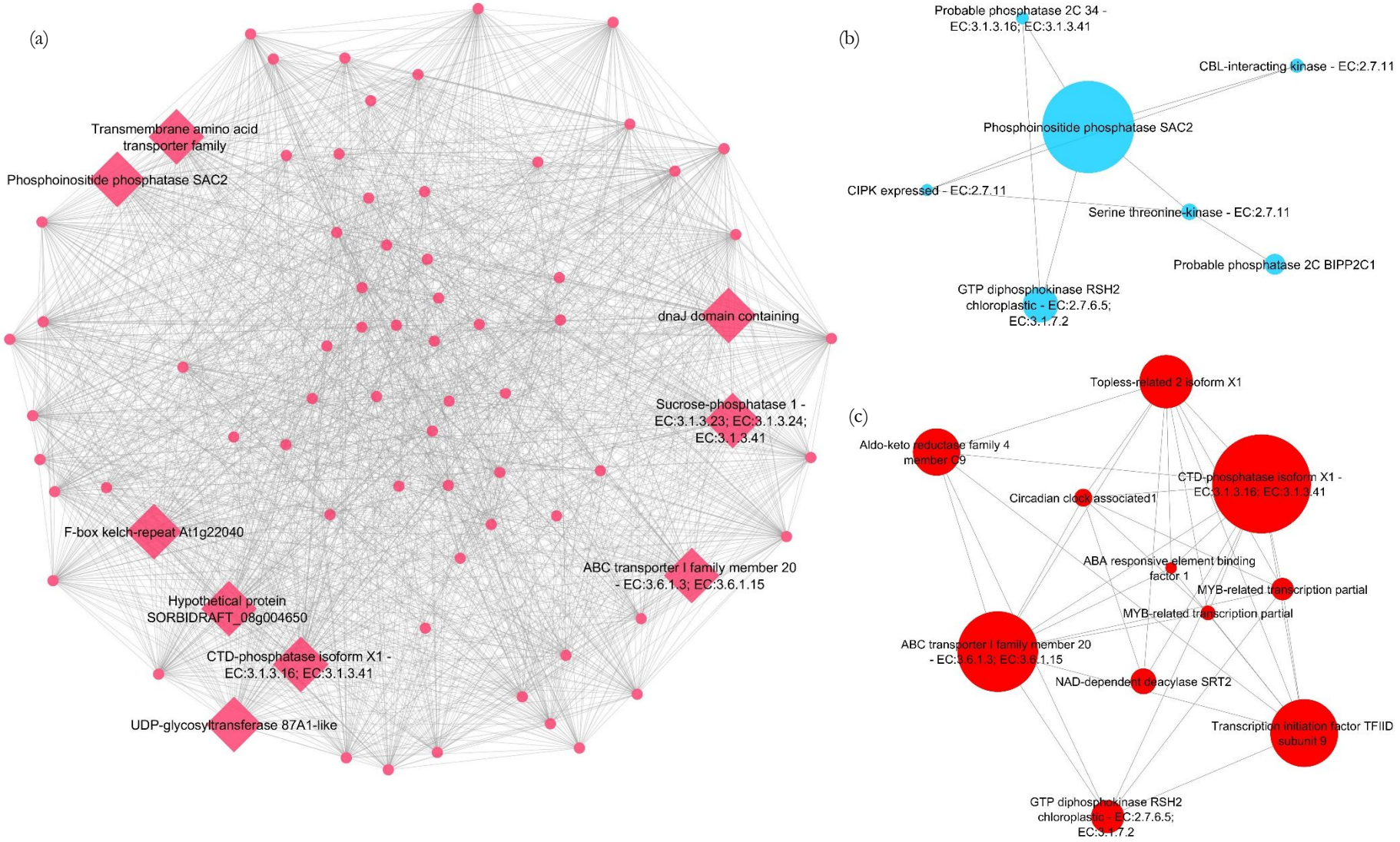


Figure 82. Plum1 Co-expression network. (a) full; (b) signal transduction; (c) transcription

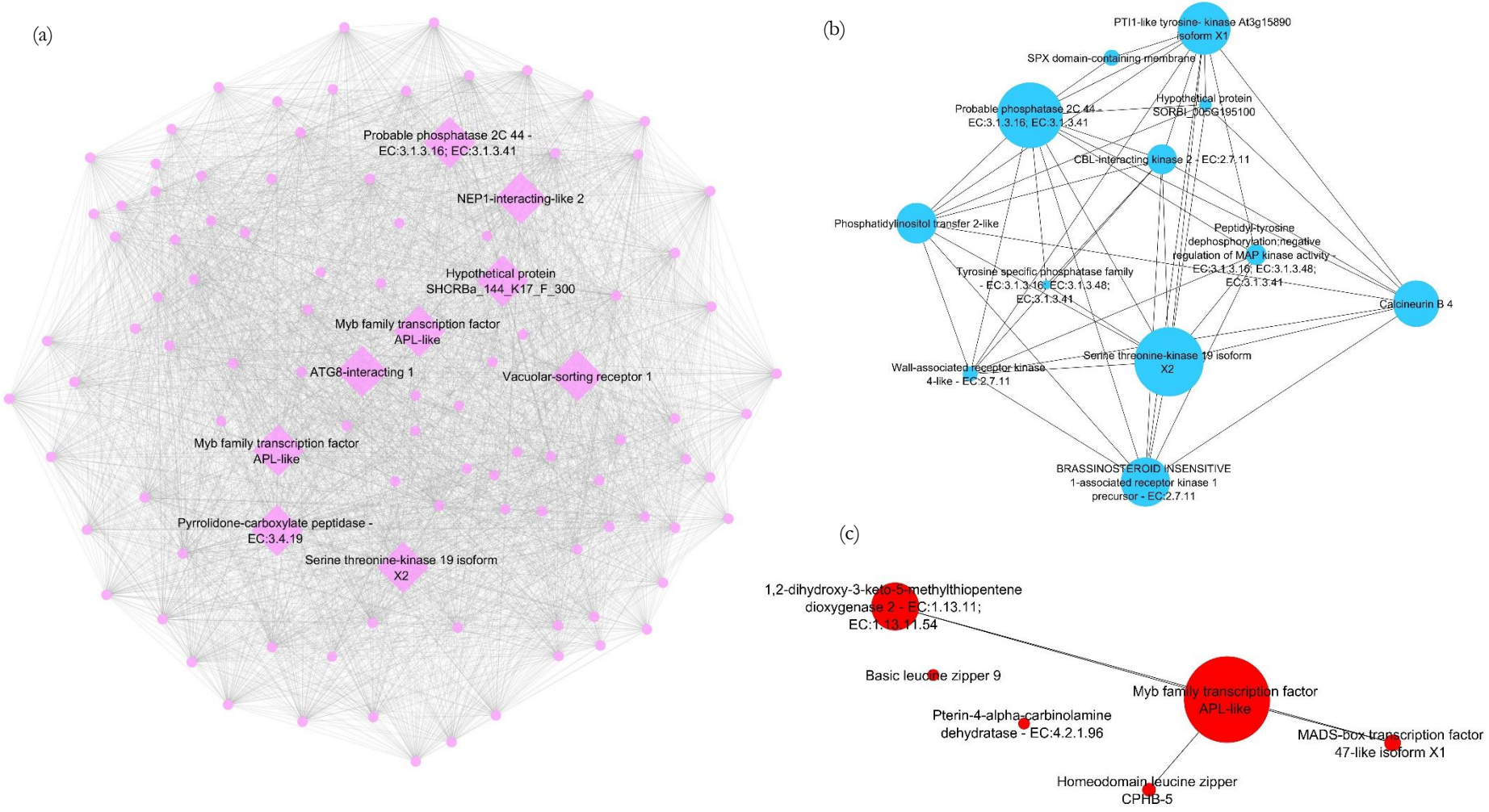


Figure 83. Salmon Co-expression network. (a) full; (b) signal transduction; (c) transcription

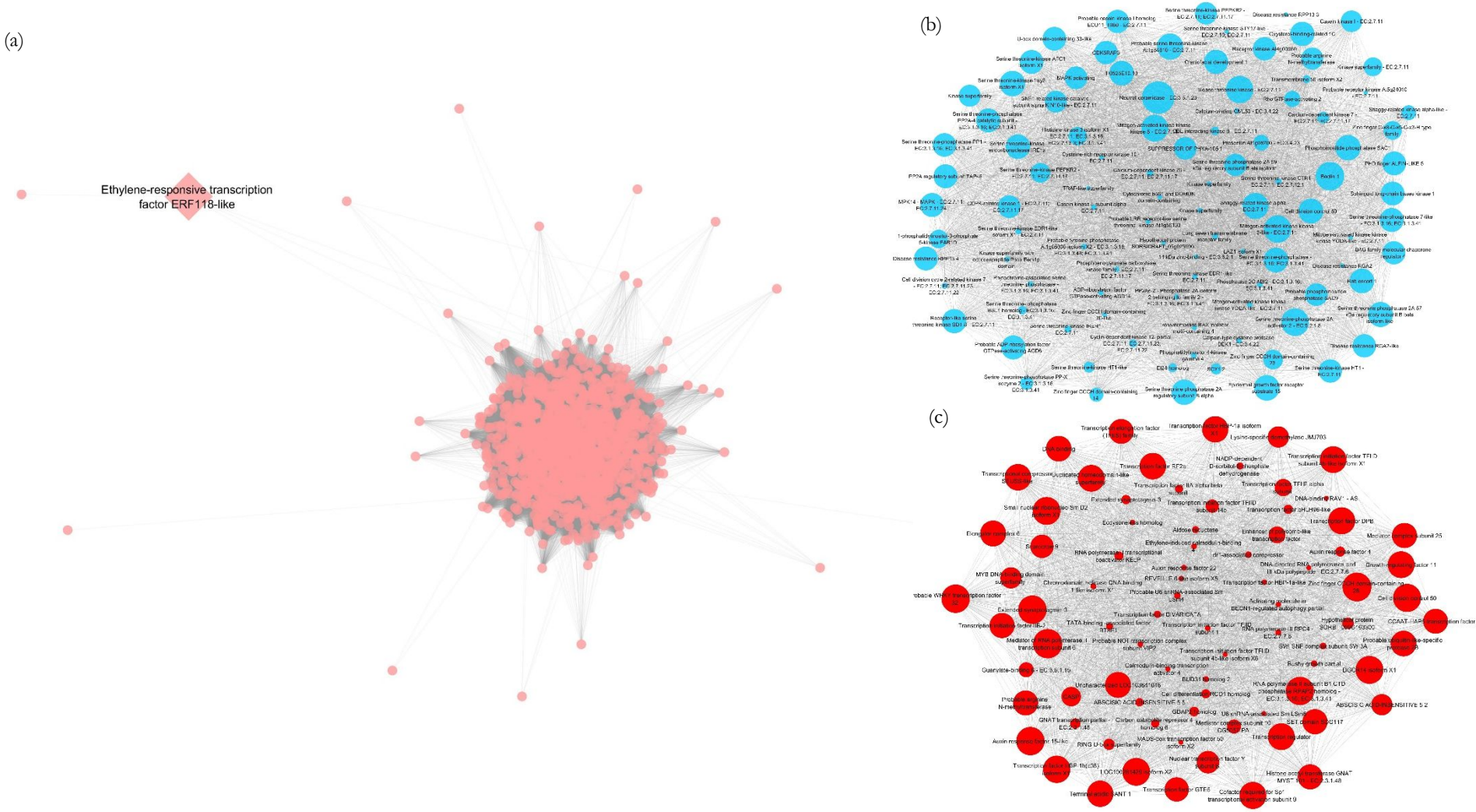


Figure 84. Sienna3 Co-expression network. (a) full; (b) signal transduction; (c) transcription

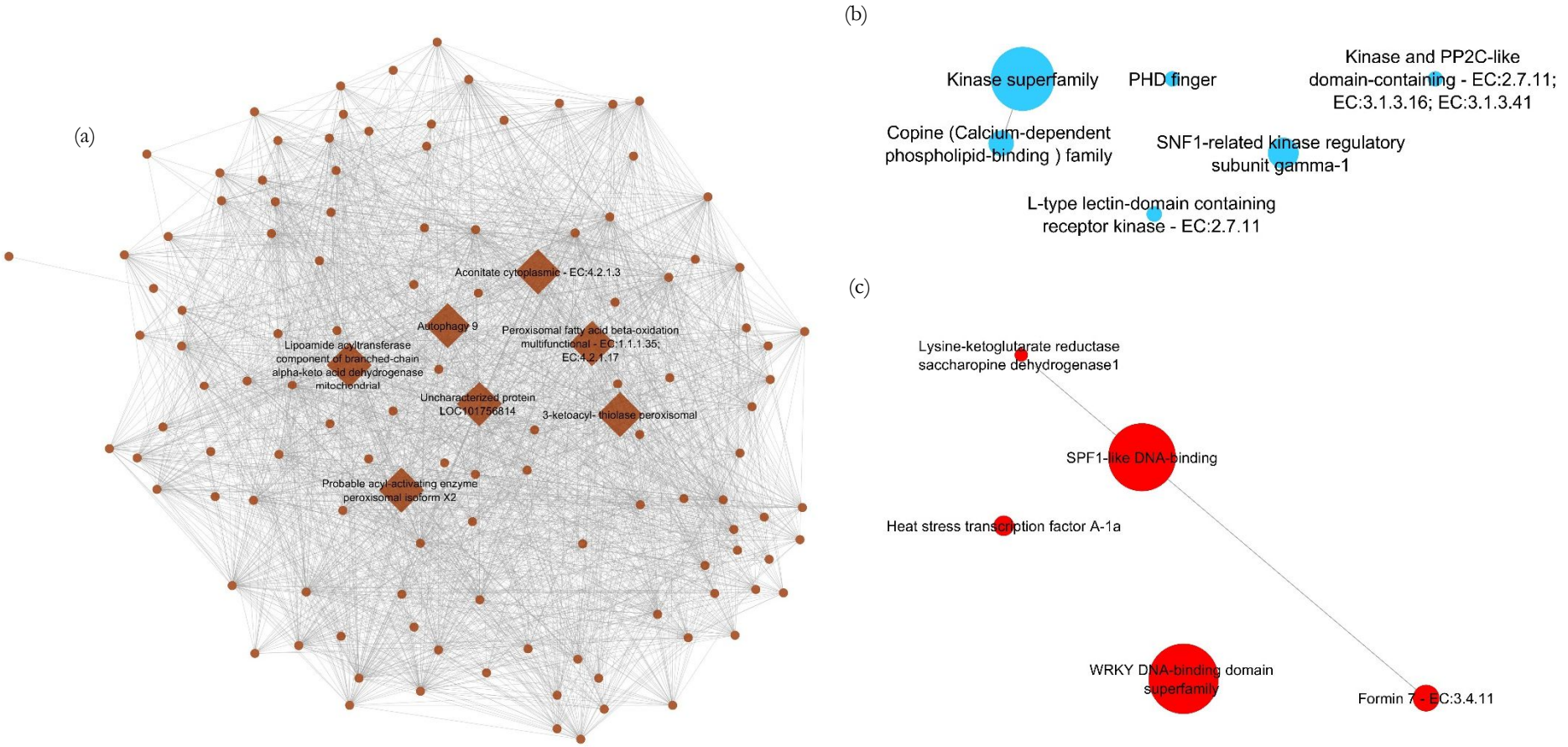
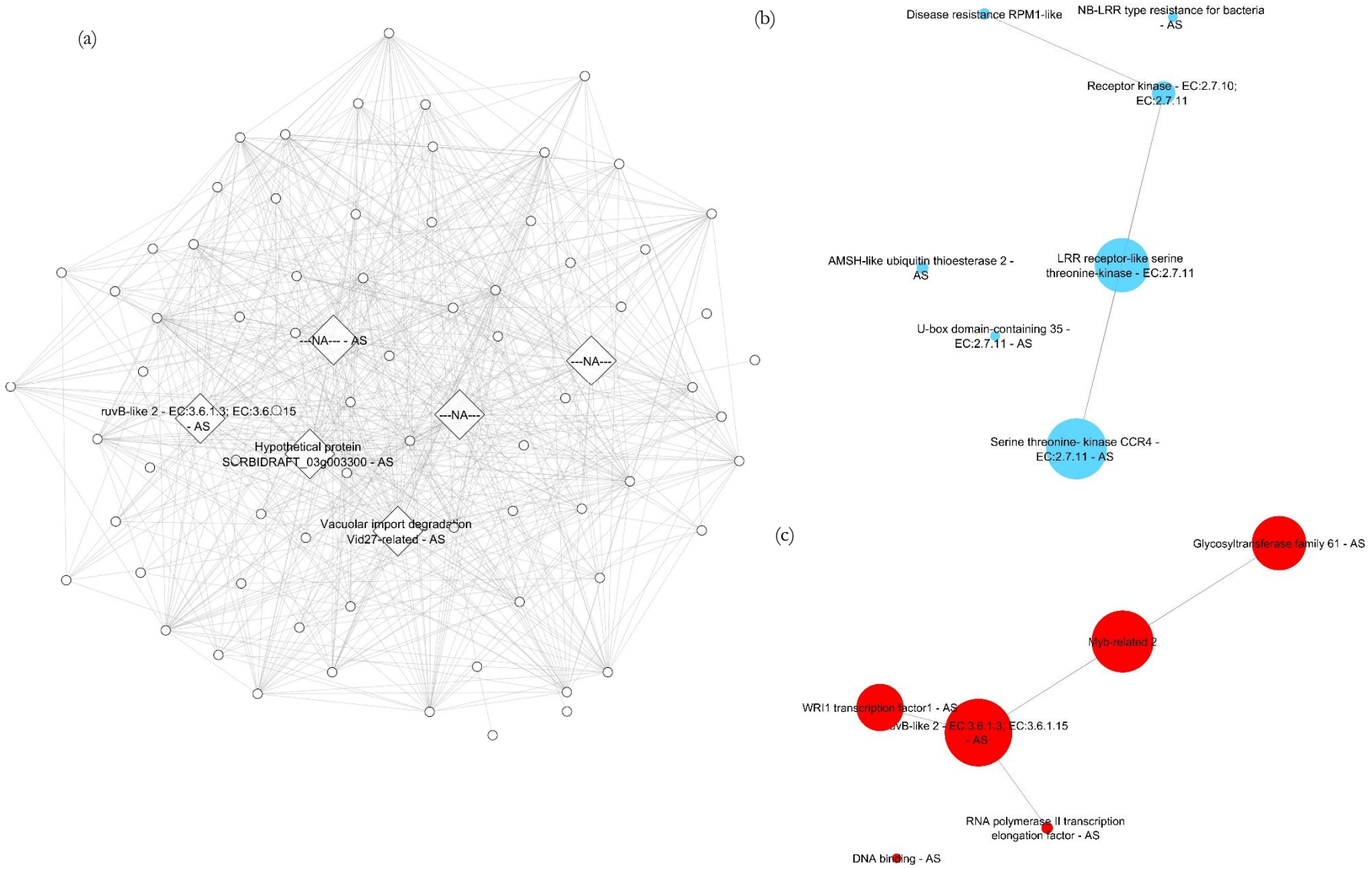


Figure 85. Floral White Co-expression network. (a) full; (b) signal transduction; (c) transcription



APPENDIX H. Curriculum

Danielle Izilda Rodrigues da Silva (http://lattes.cnpq.br/0973693375124593)

I am graduated at Biological Sciences by the São Paulo State University "Júlio de Mesquita Filho", with conclusion at december/2008, and specialist at Mastership, Auditorship and Environmental Management at Oswaldo Cruz University. I performed my master's degree at Genetics and Plant Breeding at the "Luiz de Queiroz" College of Agriculture (ESALQ). Currently I am performing my PhD at International Plant Cell and Molecular Biology program, also from ESALQ. I have experience with genetics and molecular biology with emphasis to Plant Genetics and at the environmental and animal care areas.

Personal Information

Full name - Danielle Izilda Rodrigues da Silva
Parental information - Wilson Florêncio da Silva and Rosana Aparecida Rodrigues da Silva
Birth information - 08/09/1987 - São Paulo/SP - Brazil
Identification document - 437766354 SSP - SP - 20/09/2004
CPF Number - 352.345.898-62
Phone number: +55 11 20529449
Mobile: +55 11 982867886
e-mail: daniiizilda080987@gmail.com
alternative e-mail: wfsdani@terra.com.br

Formal Education

2013 – now – Doctorate in International Plant Cell and Molecular Biology program.
"Luiz de Queiroz" College of Agriculture, ESALQ, Brazil
with Sandwich Doctorate in Ohio State University (Advisor : Erich Grotewold)
Title: Comprehensive analysis of sugarcane (*Saccharum* spp) gene expression changes in response to drought and re-watering conditions
Advisor: Glaucia Mendes Souza
Scholarship from : São Paulo Research Foundation

2010 – 2012 – Master's in Genetics and Plant Breeding.
"Luiz de Queiroz" College of Agriculture, ESALQ, Brazil
Title: Caracterização do proteoma nuclear de folhas de cana-de-açúcar (Saccharum spp) de 1 e 4 meses de idade, Year of degree: 2012
Advisor: Carlos Alberto Labate
Scholarship from : São Paulo Research Foundation

2009 – 2011 - Specialization in Perícia, Auditoria e Gestão Ambiental.
Oswaldo Cruz University, FOC, Sao Paulo, Brazil
Title: Plantas transgênicas - Questões técnicas e legais
Advisor: André Camargo Tozadori

2005 – 2008 – Graduation in Ciencias Biológicas.
São Paulo State University "Júlio de Mesquita Filho", UNESP, Sao Paulo, Brazil
Title: Caracterização cromossômica de acessos de Capsicum
Advisor: Mônica Rosa Bertão

Professional Experience

1. São Paulo State University "Júlio de Mesquita Filho"- UNESP

2006 – 2008 – Undergrad trainee in Cellular Biology and Plant

2. Butantan Institute - IBU

2008 (January – February) – Internship in the Biological Museum in the area of animal care

2008 (July) – Internship in the Genetics Laboratory

3. "Luiz de Queiroz" College of Agriculture - ESALQ

2009 (July) – Internship in Max Feffer Laboratory

4. Suzuki Orchidarium - OS

2006 (July) – Internship in the area of Orchid cultivation

5. Ubatuba Aquarium - AU

2007 (January – February) – Internship in the area of animal care and environmental education

Areas of Expertise

1. Plant Genetics
2. Molecular Biology
3. Conservation of the Animal Species
4. Biochemistry

Languages

English Understanding Fluent, Speaking Fluent, Writing Fluent, Reading Fluent

Español Understanding Fluent, Speaking Fluent, Writing Fluent, Reading Fluent

Français Understanding Functional, Speaking Functional, Writing Functional, Reading Functional

Articles published in annals of events

1. SILVA, D. I. R.; SOUZA, G. M.; ENDRES, L.; COSTA, M. D. L.

Transcriptomic Analysis of Sugarcane Plants Submitted to Drought Stress and Rehydration Conditions In: 2º Brazilian BioEnergy Science and Technology Conference, 2014, Campos do Jordão.

2º Brazilian BioEnergy Science and Technology Conference - Abstracts. , 2014.

2. SILVA, D. I. R.; GUIDETTI-GONZALEZ, S.; LABATE, C. A.

Nuclear Protein Profile From Young Sugarcane Leaves In: European Proteomics Association 2012 Scientific Congress 'New Horizons and Applications for Proteomics', 2012, Glasgow.

EuPA 2012 Scientific Congress - New Horizons and Applications for Proteomics. , 2012. p.169 – 169

3. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Development of an optimized protocol for nuclear protein extraction of young sugarcane (Saccharum spp) leaves In: 1º Brazilian BioEnergy Science and Technology Conference, 2011, Campos do Jordão.

Abstracts. , 2011.

4. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Nuclear Protein Identification of Young Sugarcane (Saccharum spp) Leaves by MudPit and 1-D SDS-PAGE In: XIII Congresso Brasileiro de Fisiologia Vegetal e XIV Reunião Latinoamericana de Fisiologia Vegetal, 2011, Búzios.

Brazilian Journal of Plant Physiology - Suplemento. Londrina: Brazilian Society of Plant Physiology, 2011. v.23. p.217 – 218

5. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Nuclei isolation and nuclear protein identification of young sugarcane (Saccharum spp) leaves with emphasis to the aplication of subcellular localization predictors. In: 57º Congresso Brasileiro de Genética, 2011, Águas de Lindóia.

Resumos do 57º Congresso Brasileiro de Genética. Ribeirão Preto: Sociedade Brasileira de Genética, 2011. p.65 - 65

Book Chapter

BUDZINSKI, I. G. F.; SILVA, D. I. R.; REGIANI, T.; LABATE, M. T. V.; GUIDETTI-GONZALEZ, S.; RODRIGUES, M. J. C.; BORGES, J. S.; MOZOL, I. M.; LABATE, C. A.

Cap 7 - Proteômica; Book Ômicas 360º: Aplicações e Estratégias para o Melhoramento de Plantas. Visconde do Rio Branco : Suprema, 2013, v.1. p.289.

Key words: Proteômica, Subproteômica, Fosfoproteômica

Oral Presentation

1. SILVA, D. I. R.

Analysis of sugarcane physiology and gene expression changes in response to drought, 2017. (Conference or lecture,Presentations in Events)

2. SILVA, D. I. R.; SOUZA, G. M.; GROTEWOLD, E.; ENDRES, L.; COSTA, M. D. L.

Analysis of sugarcane physiology and gene expression changes in response to drought, 2016. (Other,Presentations in Events)

3. SILVA, D. I. R.

Identification of Sugarcane Regulatory Networks Involved in Drought Responses, 2014. (Other,Presentations in Events)

4. SILVA, D. I. R.

Proteômica Subcelular e fosfoproteômica - Olhando menos para ver mais, 2011. (Other,Presentations in Events)

Poster Presentations

1. SILVA, D. I. R.; SOUZA, G. M.; ENDRES, L.; COSTA, M. D. L.

Transcriptomic Analysis of Sugarcane Plants Submitted to Drought Stress and Rehydration Conditions, 2014. (Conference or lecture,Presentations in Events)

2. SILVA, D. I. R.; LABATE, C. A.; GUIDETTI-GONZALEZ, S.

Nuclear Protein Profile From Young Sugarcane Leaves, 2012. (Congress,Presentations in Events)

3. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Development of an optimized protocol for nuclear protein extraction of young sugarcane (Saccharum spp) leaves, 2011. (Conference or lecture,Presentations in Events)

4. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Nuclear Protein Identification of Young Sugarcane (Saccharum spp) Leaves by MudPit and 1-D SDS-PAGE, 2011. (Congress,Presentations in Events)

5. SILVA, D. I. R.; SALVATO, F.; LABATE, C. A.

Nuclei isolation and nuclear protein identification of young sugarcane (Saccharum spp) leaves with emphasis to the aplication of subcellular localization predictors., 2011. (Congress,Presentations in Events)

Participation in Events

1. 9th Tripartite Meeting: Chemistry, Biochemistry and Molecular Biology for Wellness, 2016.
2. 2014 Practical Summer Workshop in Functional Genomics., 2014.
3. 2º Brazilian BioEnergy Science and Technology Conference, 2014.
4. 4th Practical Summer Workshop in Functional Genomics, 2014.
5. Latin American eScience Workshop "Turning Data into Insight", 2013
6. USP Conference on Synthetic Biology for Biomass and Biofuels Production, 2013.
7. European Proteomics Association 2012 Scientific Congress, 2012.
8. 1º Brazilian BioEnergy Science and Technology Conference, 2011.
9. 28º Encontro Sobre Temas de Genética e Melhoramento, 2011.
10. 57º Congresso Brasileiro de Genética, 2011.
11. XIII Congresso Brasileiro de Fisiologia Vegetal e XIV Reunião Latinoamericana de Fisiologia Vegetal, 2011.
12. 26º Encontro sobre temas de Genética e Melhoramento, 2009.
13. I Encontro sobre Animais Selvagens, 2007.
14. II Seminário do Comitê de Ética em Pesquisa, 2007.
15. IX Encontro de Biociências e Biotecnologia: "Terra: Biodiversidade Científica e Tecnológica", 2007.
16. I Encontro Paulista sobre Clonagem Animal, 2006.
17. I Fórum de Biotecnologia do Vale do Paranapanema - Novos rumos para o desenvolvimento, 2006.
18. VIII Encontro de Biociências e Biotecnologia de Assis, 2005.

Paticipation in mini courses

1. Sugarcane Breeding / Sugarcane Agricultural Practices, 2014.
2. Treinamento em ajuste de modelos lineares e mistos no ambiente 'R", 2014.
3. Controle da expressão gênica por RNAs de interferência e microRNAs, 2011.
4. Mutagênese ambiental: biomonitores e biomarcadores, 2011.
5. Proteômica em Plantas, 2011.
6. Treinamento sobre técnicas em microscopia eletrônica de transmissão, 2011.
7. Auditor Ambiental - ISO 14001:2004, 2010.

8. DNA: Espiral da Evolução Humana., 2008.
9. Identificação Humana pelo DNA: Genética Forense e Sistema HLA., 2008.
10. Tópicos em Fisiologia Vegetal, 2008.
11. Ecofisiologia da Germinação e Alelopatia, 2007
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