Functional analysis of sugarcane genes modulated by drought in roots

Análise funcional de genes de cana-de-açúcar modulados por seca em raízes

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Functional analysis of sugarcane genes modulated by drought in roots

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Genetics and Molecular Biology, in the area of Plant Genetics and Breeding.

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RESUMO

A cana-de-açúcar é uma cultura de importância econômica mundial, atuando como matéria-prima principalmente na produção de açúcar e etanol. A cultura de cana é afetada por vários tipos de estresses bióticos e abióticos, sendo a seca um dos principais fatores que reduzem a sua produtividade. A seca atua sobre as variedades vegetais de diversas formas, provocando alterações na parte aérea e no sistema radicular. As raízes desempenham um papel importante na manutenção das funções da planta em condições de estresse, de modo que suas propriedades exercem grande influência na resposta à escassez hídrica. Entretanto, o conhecimento acerca das alterações da expressão gênica em raízes de cana sujeitas à seca ainda é muito restrito, embora seja uma etapa importante para a identificação de genes com potencial para a produção de plantas mais tolerantes ao deficit hídrico. Deste modo, este projeto objetivou a identificação de genes modulados por seca em raízes de cana para estudos funcionais empregando plantas transgênicas de Arabidopsis thaliana e cana-de-açúcar. Utilizando dados de RNA-Seq de dois cultivares de cana (RB92579 e RB72454) foram selecionados 10 genes potencialmente relevantes nas respostas radiculares frente à condições de seca. Testes de estresse severo e moderado utilizando plantas transgênicas de Arabidopsis e cana-de-açúcar superexpressando alguns desses genes apontaram o envolvimento no mecanismo de tolerância à seca.
ABSTRACT

Sugarcane is a crop of global economic importance, acting as raw material especially in the production of sugar and ethanol. Sugarcane is affected by various types of biotic and abiotic stresses, and drought is one of the main factors reducing sugarcane productivity. Drought acts on the plant varieties of several ways, causing alterations in shoots and roots. Roots play an important role in the maintenance of plant functions under stress conditions, and their properties have great influence on responses to water scarcity. However, the knowledge on gene expression changes in plant roots subject to drought is still very restricted, although it is an important step for the identification of genes with potential for the production of plants more tolerant to water deficit. Therefore, this project aimed to identify genes modulated by drought in sugarcane roots for functional studies using Arabidopsis thaliana and sugarcane transgenic plants. Using RNA-Seq data from two sugarcane cultivars (RB92579 and RB72454) we selected 10 potentially relevant genes in root responses in drought conditions. Severe and moderate stress tests using Arabidopsis and sugarcane transgenic plants overexpressing some of these genes indicated the involvement in drought tolerance mechanisms.
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1. GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp.) is a plant of the Poacea family (Gramineae) currently constituted of aneuploidy and polyploidy hybrids, resulting from interspecific crosses between *S. officinarum* and *S. spontaneum* (Irvine, 1999). This crop is of great economic importance worldwide, being responsible for approximately 80% of world sugar production (International Sugar Organization, 2016) and with increasing relevance in the production of renewable energy. Brazil stands out as the main producer of this culture, with a cultivated area estimated at 8,766.5 thousand hectares (harvest 2017/2018), producing 646.4 millions of tons, of which 47.9% is destined to sugar production and 52.1% to ethanol production (Conab, 2017).

The growing concern about polluting gases emission from the use of fossil fuels has raised the demand for alternative sources of energy. The use of ethanol is a sustainable option, increasing sugarcane demand and consequently expanding cultivated areas (Martinelli and Filoso, 2008). Therefore, the development of new cultivars, combining tools from classical plant breeding and biotechnology, emerges as a promising alternative to increase sugarcane productivity, reducing the pressure for new cropping areas.

Sugarcane is cultivated in tropical and subtropical regions (Akintola, 1978), with water supplementation in some places, such as Australia and South Africa, where 60% and 40% of the crop, respectively, depend on irrigation (Inman-Bamber, 2004; Inman-Bamber and Smith, 2005). Irrigated cultivation significantly increases production costs and intensifies the dependence of fresh water in the agriculture. These factors combined with future predictions of water scarcity boost the development of better irrigation practices and cultivars with improved capacity for efficient water use and drought tolerance (Carr and Knox, 2011).

Drought represents one of the main factors that reduce sugarcane productivity (Basnayake *et al*., 2012, Ferreira *et al*., 2017). Water deficit affects the plant as a whole, from the shoot to the roots, causing significant and sometimes irreversible changes that can lead to death. Drought causes several physiological alterations in the aerial part of the sugarcane plants including: inhibition of buds germination, inhibition of new leaves appearance and reduction of leaf area, reduction of the elongation of leaves and stems, tillers loss, senescence and winding of young leaves. In the root portion, the main changes involve root development, increase in length and dry/fresh weight ratio (as a sign of tissue dehydration) and decrease in hydraulic conductance (Franco *et al*., 2011;
Inman-Bamber and Smith, 2005).

Since plants absorb water through the roots, this tissue is the first to detect soil water deficit and to signal to other parts of the plant, playing an essential role in maintaining plant development and nutritional functions under stress. Roots act at the interface between plant and soil, so that their growth and function determine the plant response to underground stress situations. Despite this, stress influence on root development is still a poorly studied subject, probably due to the limited accessibility of roots (Franco et al., 2011).

The sugarcane root system is traditionally divided into three functional types: superficial roots, responsible for water and nutrients uptake from surface soil layers; buttress roots, adapted to support the plant; and rope roots, able to provide access to deeper water reserves in the soil (Evans, 1935). Despite this typical profile, root development exhibits some plasticity in relation to soil characteristics, especially water content.

Root development is strongly influenced by availability and distribution of water in the soil (Smith et al., 2005). Sugarcane plants grown under drought conditions developed deeper roots compared to irrigated plants (van Antwerpen, 1998), presenting higher capacity for water uptake from deeper soil resources (Wood and Wood, 1967). Similarly, sugarcane crops under different irrigation regimes showed more deeply distributed roots when irrigated at lower frequencies, possibly in response to surface drying, reducing plant vulnerability against the lack of water in the soil (Baran et al., 1974). Overall, these data demonstrate that root characteristics exert influence on sugarcane response under water deficit and, therefore, their study is essential to understand the mechanisms associated with plant responses to drought.

From the perspective of molecular biology, the analysis of sugarcane roots subjected to water deficit represents a promising way for a better understanding of phenotypes associated with drought responses. However, there is a scarcity of data regarding the molecular basis of sugarcane roots response to water suspension. This knowledge not only contributes to elucidate adaptive mechanisms underlying drought tolerance, but also provides prospects for biotechnological applications related to the production of transgenic plants.

In the present work, sugarcane genes induced by drought in roots were functionally evaluated. This thesis is organized in four chapters. Chapter 1 presents the gene selection from a RNA-Seq dataset of sugarcane roots under drought conditions.
Chapters 2 and 3 show the effects of sugarcane genes overexpression in transgenic *Arabidopsis* and sugarcane plants, respectively. Chapter 4 corresponds to the article accepted for publication and the patent deposited in the National Institute of Industrial Property (INPI) concerning the development of a new vector (pGVG) for gene overexpression and silencing in sugarcane and other monocots.

2. GENERAL OBJECTIVES

The main goal of this research was to perform a functional evaluation of genes modulated by drought in sugarcane roots to produce transgenic plants more tolerant to water deficit. For that, it was evaluated the changes in the root transcriptome of two sugarcane cultivars submitted to water stress. This allowed selecting genes with potential to increase drought tolerance for further studies. Then, the effects of the overexpression of selected sugarcane genes in the physiology of *A. thaliana* and sugarcane plants submitted to drought were analyzed. This is a key step in determining if the genes are useful for developing new commercial cultivars.
3. **CHAPTER 1 - Transcriptional analysis of sugarcane roots under drought**

### 3.1. Introduction

The development of next-generation sequencing (NGS) technologies has enabled advances in the study of non-model species of economic interest whose genome is not fully sequenced, such as sugarcane. Among these tools, RNA sequencing (RNA-Seq) technique stands out, providing information about transcripts content and expression levels in specific tissues at a given time and condition (O'Rourke et al., 2013). The use of this technique reduces problems associated with genomic complexity (Guimarães et al., 1997; Cuadrado et al., 2004) and unavailability of complete genome maps, because it allows the evaluation of transcripts structure and expression levels without information about their complete sequence (Kawahara et al., 2012). Therefore, this tool enables the identification of genes involved in processes that affect plant response to drought, including non-coding RNAs and unknown coding RNAs, which is estimated to correspond to more than 10,000 sugarcane genes (Vicentini et al., 2012).

### 3.2. Objectives

The main objective of this chapter was to identify the changes in the transcriptome of two sugarcane cultivars in response to water scarcity, in order to select 10 genes for functional analysis.

### 3.3. Materials and Methods

#### 3.3.1. Plant material

In this work we used two commercial sugarcane varieties: RB92579 and RB72454. The RB92579 cultivar (drought tolerant) was selected based on its high productivity under water deficit, good recovery after drought periods, high responsiveness to irrigation and elevate water use efficiency (WUE), being one of the most important cultivars from RIDESA, the Inter Universities Network for the Development of the Sugar/Alcohol Industry (Daros et al., 2010). The RB72454 cultivar was selected based on its drought tolerance characteristics (Silverio et al., 2017), although there are controversies regarding its productivity under water deficit. Nevertheless, due to similar physiological profile of drought response presented by RB92579 and RB72454 cultivars after one and three days of drought stress
(unpublished data), we considered RB72454 with drought tolerance characteristics in this work.

3.3.2. RNA-Seq

For drought stress test, RB92579 and RB72454 plants were grown in 30 L plastic pots containing autoclaved soil, in greenhouse at the Federal University of Pernambuco (UFRPE, Recife), for three months (tillering stage). After this period, plants were subjected to irrigation suspension or normal watering regime for one and three days. To collect the roots, the plants were removed from the pots, washed with running water, selected, washed with DEPC water, dried and then frozen in liquid nitrogen. Transcriptional profile of sugarcane roots were evaluated by RNA-Seq. Root total RNA was extracted from a set of 24 plants: two cultivars (RB72454 and RB92579), two treatments (irrigated and water deficit), two collect times (1 and 3 days) and three biological replicates. The verification of RNA quality, library production, sequencing and previous statistical analysis of gene expression were performed by the Central Laboratory of High Performance Technologies (LaCTAD). Sequencing was conducted using the Illumina HiSeq 2000 platform, with paired-end reads of 100 base pairs (bp) in length. The generated sequences (reads) were edited by removing the adapters and deleting low-quality sequences and used in the transcriptome assembly.

3.3.3. De novo transcriptome assembly

A de novo assembly of the transcripts was performed for all reads obtained from the 24 libraries from both cultivars using the Trinity platform (Grabherr et al., 2011). This reconstruction process was based on the alignment of reads by overlapping corresponding sequences using minimum k-mer coverage of 15 and contigs minimum size of 200 bp.

3.3.4. Transcripts annotation

The resulting transcripts were annotated using BLASTx (cut-off e-value 1e-5) against several protein database, including NR (NCBI non-redundant database), Uniprot (http://www.uniprot.org), TAIR (http://www.arabidopsis.org) and sorghum protein database (Sorghum bicolor) (http://www.phytozome.net/sorghum.php). In addition, BLASTn (cut-off e-value 1e-10) was used for annotation against SUCEST transcripts (Vettore et al., 2003) and BLAST2GO (Conesa et al., 2005) for functional annotation.
3.3.5. Calculation of transcripts expression

The reads of each library were aligned with transcripts resulting from assembly using the RSEM program (RNA-Seq by Expectation Maximization), described by Li and Dewey (2011). Expression calculation of each transcript was performed using the value of RPKM (Reads per Kilobase per Million of Reads mapped) (Mortazavi et al., 2008), defined according to the formula: $\text{RPKM} = (N \times 10^9) / S \times T$; where $N =$ Number of reads (fragments) mapped in a transcript; $S =$ transcript size; and $T =$ Total reads mapped from the library.

3.3.6. Identification of differentially expressed transcripts

DESeq statistical program (Anders and Huber, 2010) was used to identify differentially expressed transcripts (DETs). Transcripts with FDR (False Discovery Rate) $\leq 0.05$ were considered differentially expressed and used in the subsequent analysis.

3.3.7. Phylogenetic analysis

The transcripts were submitted to phylogenetic analysis for search of orthologs, including GO (gene ontology) and Mapman terms (http://mapman.gabipd.org). This step was carried out in collaboration with Prof. Dr. Renato Vicentini. The global annotation of the transcriptome was performed using Blastx, Sim4 (Florea et al., 1998), RepeatMasker (http://repeatmasker.org) and CPAT (Wang et al., 2013). Blastx was used to identify coding sequences, Sim4 to find putative non-annotated genes/ncRNAs, RepeatMasker to identify possible repetitive sequences and CPATs to check for the probabilities of coding and non-coding RNA for some sequences. After this process, contaminations (viruses, fungi and bacteria) derived from transposons and repetitive sequences were removed, and ncRNAs and exclusive sugarcane genes were identified.

3.3.8. Differential expression

A general analysis of activated and repressed DETs in the two cultivars in three days of stress (period with significant differences in plant physiology - unpublished data) was performed. Transcripts with induction level (fold change) $\geq 2$ comparing drought vs irrigated condition, RPKM $\geq 1$ in at least one biological replicate and mean expression of the three replicates in the drought condition $\geq 2$ for non-expressed
transcripts in the irrigated treatment were considered as induced by drought. A similar strategy was adopted for the identification of genes repressed by water deficit.

Gene expression analysis focused on DETs that responded in a similar way between the cultivars, since our central interest is to discover potential candidates involved in drought response conserved between these two genotypes, assuming that both are tolerant to water deficit (Daros et al., 2010). Since this study is based on the use of gene overexpression to develop cultivars with higher tolerance to drought, strict emphasis will be placed on genes induced by stress.

The analysis of DETs activated by the drought in the two cultivars in three days of stress was performed using Venn diagram (http://www.pangloss.com/seidel/Protocols/venn.cgi). The obtained gene set was analyzed in terms of Gene Ontology (GO), using the BLAST2GO program, classified into functional categories (bins) using the Mercator program (http://mapman.gabipd.org) and the resulting mapping was evaluated in the MapMan program using Blastx against Arabidopsis proteins. For the enrichment analysis Wilcoxon test was used, with multiple correction FDR Benjamini-Hochberg and ORA cutoff = 1, via PageMan platform (http://mapman.gabipd.org).

3.3.9. Gene selection for functional analysis

From the list of genes induced by drought in both cultivars, 10 genes were selected for further analyses. We used as main parameters: gene function and relevance of metabolic pathway, which is correlated with the perspective of publication in journals of high impact; absence of gene overexpression articles and patents covering their use in transgenic plants for improved drought tolerance; expression level; and presence of the complete coding sequence (ATG to STOP codon).

3.3.10. Gene expression analysis via qRT-PCR

The confirmation of selected genes expression was performed by qRT-PCR. For this experiment, the same biological triplicates (irrigated and drought treatments) from RNA-Seq were used. Initially, root samples of 12 sugarcane plants (two cultivars, third day of stress, two treatments, and three biological replicates) were macerated in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen, USA), quantified by spectrophotometry in a NanoDrop 2000 (ThermoScientific) and further evaluated in 1% agarose gel electrophoresis.
Samples were treated with DNase I (Fermentas) and reverse transcription (RT) was performed with oligo dT primers using SuperScript® II enzyme (Life Technologies). The cDNA was subjected to qPCR using SYBR Green PCR Master Mix kit (Applied Biosystems) and AmpliTaq Gold® DNA Polymerase. For each reaction, 3 μL of cDNA (1:100 dilution) and 0.3 μL of the respective forward and reverse gene-specific primers (10 μM) were used in a final volume of 15 μL. Reactions were conducted on the 7500 Real Time PCR System (Applied Biosystems) under the follow conditions: holding stage - 50 °C (2 minutes) and 95 °C (10 minutes); cycling stage (40 cycles) - 95 °C (15 seconds) and 60 °C (1 minute).

Gene relative quantification was performed using $2^{\Delta\Delta CT}$ method. The endogenous polyubiquitin gene (SCCCST2001G02; NCBI accession CA179923.1) was used as internal control (Papini-Terzi et al., 2005) for data normalization. Expression level of target genes in the treated sample (drought) was compared with the control group (irrigated), expressed as fold change. The correlation coefficient between gene expression results of RNA-Seq and qRT-PCR was calculated using fold change average from the three biological replicates.

The template sequence used for oligonucleotides design was inferred from the sequencing data (RNA-Seq) and confirmed by alignment with the corresponding in the SUCEST (SAS and genome) and homologues in sorghum (Sorghum bicolor). The primers (Table 1) were preferably designed in non-conserved regions of the protein, using Custom Primers - OligoPerfect™ Designer (Life Technologies) and Beacon Designer™ Real Time PCR Oligo Design (PREMIER Biosoft) tools. The following parameters were used: primers with a length of 18 to 22 nucleotides; average amplicon size between 100 and 200 bp; Tm (melting temperature) ranging from 55 °C to 62 °C; and GC content between 40 and 60%. Primers specificity was confirmed by BLAST against NCBI and SUCEST databases. Primers functionality was tested by evaluating their CT and melting curve, and their efficiency was measured by analyzing the efficiency coefficient of standard curve.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer forward (5’-3’)</th>
<th>Primer reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWPM</td>
<td>GCAGGAATCAAGAAAGAGATCG</td>
<td>TAGTTGGTCTGCCCCGTGTGAT</td>
</tr>
<tr>
<td>PSK</td>
<td>AAGGTGGCGCTGGGCAATG</td>
<td>CACTCCTGGTGCTCTCCTCCT</td>
</tr>
<tr>
<td>NRX</td>
<td>TCTCCATCAACGGAAGAGAG</td>
<td>GCCCTTGAGGATGCTGTAGA</td>
</tr>
<tr>
<td>RTNL</td>
<td>GACCTCCTCACCTTGATATACAT</td>
<td>GGCATCCAGCACCTCATACCT</td>
</tr>
<tr>
<td>GRAM</td>
<td>GACTTCTTCAACACCTGGAGC</td>
<td>GGTAAGCAAGCAGACGTCTTC</td>
</tr>
<tr>
<td>AUX</td>
<td>CTTCTGGTGACGGTTCTGTGT</td>
<td>AATCAGCGACACCTACTCA</td>
</tr>
<tr>
<td>DNAJ</td>
<td>CAGGAGATGCTGGCTGTGCTCAT</td>
<td>ACTCTGCTCTTGTCTGCTC</td>
</tr>
<tr>
<td>HIPP</td>
<td>AGCAGCAGCAGCAGCAGCAT</td>
<td>CACAGATCCGCAAGTGGTGTC</td>
</tr>
<tr>
<td>HP</td>
<td>TGGCTGTCCTCTTGTCGTCAT</td>
<td>CTGTGGTTGTTGGTGTTGAG</td>
</tr>
<tr>
<td>HP2</td>
<td>GGAAGTTTAGACCCACTCGT</td>
<td>CTTCTCCTTACCTCCGACC</td>
</tr>
<tr>
<td>PUB</td>
<td>CCGGTCCCTTTAAACCAACTCAGT</td>
<td>CCGGTCTCTGCACCTCCATT</td>
</tr>
</tbody>
</table>

Table 1. Primers used in the qPCR. AWPM: AWPM-19-like membrane family protein; PSK: phytosulfokine; NRX: nucleoredoxin; RTNL: reticulon-like protein; GRAM: (glucosyltransferases, Rab-like GTPase activators, and myotubulins) GRAM domain family protein; AUX: dormancy/auxin associated family protein; DNAJ: chaperone DNAJ; HIPP: heavy metal-associated isoprenylated plant protein; HP: hypothetical protein 1; HP2: hypothetical protein 2. Nomenclature according to the homologs identified using BLAST against NCBI database.

3.4. Results and Discussion

3.4.1. RNA-Seq and phylogenetic analysis

Sequenced libraries generated a total of 185.1 Gb, with 174.6 Gb (94.3%) having a phred quality value ≥ 20 and 167.8 (90.6%) with a phred value ≥ 30. The obtained reads were filtered for low quality sequences and used for transcriptome assembly, producing a total of 146,361 transcripts, including all isoforms. For the following analyses only the major isoform of each gene was considered, resulting in a total of 97,587 transcripts. Phylogenetic analysis of these data (Figure 1) allowed us to determine the ontology for 44,770 transcripts conserved in grasses (~ 36,000 in sorghum and ~ 8,000 in rice), whereas 45,862 transcripts showed no similarity with plant genes. After removal of contaminations, transposons and repetitive sequences, 17,841 ncRNAs and 2,754 exclusive sugarcane genes were identified.
Figure 1. Phylogenetic analyses of RNASeq data to search orthologs, ncRNAs and specific genes in sugarcane. Blastx was used to find coding sequences in the database of Sorghum and other Viridiplantae. Subsequently, a Blastx was performed against the database of yeast, virus and bacterial proteins for contaminations removal. The resulting transcripts were evaluated in the RepeatMasker for the identification of repetitive sequences. The set of unidentified transcripts was subjected to search for possible non-annotated genes/ncRNAs using the Sim4 program and then analyzed for the presence of coding and non-coding RNA via CPAT.

3.4.2. Differential analysis and functional classification

Evaluating the DETs (Figure 2a), a higher number of drought-modulated transcripts was observed in RB92579 plants (1519 up-regulated and 2342 down-regulated), compared to RB72454 (551 up-regulated and 164 down-regulated). It is worth to note that in RB92579 plants, the number of repressed transcripts was higher than the number of induced transcripts, and the opposite was observed in RB72454.

DETs shared between the two sugarcane cultivars presented the same expression profile, each one with 467 drought-induced and 101 drought-repressed transcripts (Figure 2b). From common up-regulated transcripts, we selected 10 genes to perform in-depth analyses, as explained in the next section.
Figure 2. Number of DETs in 2 sugarcane cultivars in 3 days of stress. (a) Number of DETs in each genotype. (b) Number of DETs shared between the cultivars.

The analysis of DETs functional categories using the Mercator program (Figure 3) revealed that most genes (28.60% for RB92579 and 30.05% for RB72454) do not have assigned functions. This high percentage of unregistered genes may have been caused by the databases used [TAIR (Arabidopsis), SwissProt/UniProt Plant Proteins, TIGR5 rice proteins and clusters of orthologous eucaryotic genes database (KOG)] or by the cutoff considered (equal to 50, suggested by the program). Among the annotated transcripts, the four best represented categories in the two cultivars were: protein, signaling, misc (miscellaneous enzyme families) and RNA. The mapping file generated in the Mercator was used to visualize DETs functions in MapMan.
A general analysis of regulatory components and metabolic pathways (Figure 4) showed that both genotypes present a very distinct profile in relation to DETs number and their expression levels. In RB92579 cultivar, DETs number is much higher than that observed for RB72454, suggesting greater disturbance of gene regulation and metabolism under drought conditions. On the other hand, for RB72454 cultivar, despite the reduced number, most of differentially expressed transcripts are induced.
Figure 4. Overview of DETs involved in different regulatory and metabolic pathways under drought stress, using MapMan program. Alterations in transcripts expression after three days of drought in sugarcane roots of cultivars RB92579 (a, c) and RB72454 (b, d).
The enrichment analysis in the RB92579 cultivar (Figure 5a) indicates an overrepresentation of the class of induced genes related to heat stress and protein synthesis and repressed genes involved with cell wall lipid metabolism, signaling (kinase receptors), misc and cell organization. For RB72454 (Figure 5b), there is enrichment in only one class of repressed genes related to cell wall cellulose synthesis, while the enriched induced genes do not have a function defined by the program.

![Figure 5](image-url) DETs enrichment analysis in cultivars RB92579 (a) and RB72454 (b), using the Pageman program.

After gene selection and initial functional analyses, a contamination of RNA-Seq library was detected. Therefore, a new assembly is being performed using sugarcane sequenced genome (Riaño-Pachón and Mattiello, 2017) as reference and it will be used to obtain a new transcriptome analysis. Once the gene selection was also based on other parameters, we decided move on with the already selected genes.
3.4.3. Gene selection for functional analysis

Ten genes induced by drought in the roots of the two sugarcane cultivars were selected as potential candidates to produce sugarcane plants more tolerant to drought. Eight of these genes are homologs of genes involved in drought tolerance mechanisms in other plant species. The other two genes encode hypothetical proteins with unknown function, representing potential new genes not yet described in the sugarcane database (table 2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWPM</td>
<td>Transport</td>
</tr>
<tr>
<td>PSK</td>
<td>Root cell differentiation and proliferation</td>
</tr>
<tr>
<td>NRX</td>
<td>Regulation of transcriptional activity, detoxification, oxidoreductase activity</td>
</tr>
<tr>
<td>RTNL</td>
<td>Membrane stabilization, intracellular transport, endocytosis, apoptosis</td>
</tr>
<tr>
<td>GRAM</td>
<td>Binding of intracellular proteins and lipids, responses to pathogen infection, apoptosis, hormone metabolism (ABA)</td>
</tr>
<tr>
<td>AUX</td>
<td>Proteins associated with dormancy and auxin</td>
</tr>
<tr>
<td>DNAJ</td>
<td>Protein folding, response to stress (heat), binding to HSP70</td>
</tr>
<tr>
<td>HIPP</td>
<td>Homeostasis of heavy metals and detoxification, transcriptional responses to cold and drought, plant-pathogen interactions</td>
</tr>
<tr>
<td>HP</td>
<td>Not assigned</td>
</tr>
<tr>
<td>HP2</td>
<td>Not assigned</td>
</tr>
</tbody>
</table>

Table 2. Selected genes and their functions.

Genes that promote cellular proliferation and root growth such as phytosulfokines (Kutschmar et al., 2009; Matsubayashi et al., 2006) are potential candidates to increase drought tolerance in plants. In sugarcane, the root system characteristics influence the plant response to water deficit stress. Under restrictive water conditions, sugarcane and other grasses tend to decrease shoot development, prioritizing root growth (Ramesh, 2000; Dias-Filho, 2011). The development of deeper root systems allows capturing deep water, reducing plant vulnerability to drought (Kulkarni et al., 2008).

Plants responsiveness to drought stress is regulated, among other factors, by abscisic acid (ABA) levels. Water deficit induces the biosynthesis of this hormone, which acts as an endogenous messenger in the regulation of plant water status (Swamy et al., 1999), controlling the expression of several genes. One of these genes is AWPM, which encodes a membrane protein associated with cold tolerance (Koike et al., 1997) and whose function is related to cell transport (mapman. gabipd.org/web/guest/mercator). Under drought conditions, genes with carrier function may help to control the flow of substances through the membrane and to maintain osmotic balance.
Besides ABA, other hormones are involved in the signaling cascade triggered by drought as, for example, auxins. This phytohormone plays a central role in the coordination of plant development, regulating many processes, including adaptive responses to biotic and abiotic stresses (Iglesias et al., 2011). The dormancy auxin associated proteins family, besides being related to auxin pathway is also associated with cellular dormancy mechanism and physiological activity reduction in plants, making them less dependent on the environment and thus more tolerant to external stresses, such as drought (Koller, 1969).

Plants under drought are more susceptible to the effects of secondary stresses (Zhuang et al., 2007), such as oxidative stress. The production of reactive oxygen species (ROS) occurs naturally in the cell during electrons transport in photosynthetic reactions (Miller et al., 2010). However, under water deficit condition, there is a reduction in carbon assimilation resulting in insufficient dissipation of electrons and subsequent overproduction of ROS (Edreva, 2005), which causes deleterious cell effects. Cells respond to increased ROS levels by activating antioxidant systems that limit oxidative damage. One of the effects caused by oxidative stress is root growth inhibition due to reduction in ABA content. In this scenario, root antioxidants are able to reduce ROS levels, allowing high production of ABA and enhanced root growth (MacDonald et al., 2009; Karni et al., 2010). Thus, genes with detoxifying functions found in sugarcane roots, as nucleoredoxins and HIPPs, may be important in combating oxidative damage caused by drought, maintaining cellular homeostasis and root development. Metallochaperones, such as HIPPs, are involved in heavy metals homeostasis/detoxification mechanisms and are also induced by cold and drought (de Abreu-Neto et al., 2013). It is interesting to mention that Arabidopsis HIPP26, induced by cold, salt and drought, interacts with the transcription factor ATHB29 (Barth et al., 2009), which activates the expression of several genes modulated drought (Tran et al., 2007). Thus, the HIPP protein from sugarcane can act by modulating the action of transcription factors that would activate several other genes induced by drought.

Additionally, phytotoxic levels of ROS resulting from drought stress may trigger signaling cascades that lead to programmed cell death (apoptosis). This is a cellular suicide process commonly found in organisms which is important for the development and adaptation to environmental stresses (Hückelhoven, 2004). There are evidences that apoptosis-related genes identified in rice (Ubaidillah et al., 2013) are involved with drought stress tolerance. In fact, Ramiro et al. (2016) found that the overexpression of
the *Arabidopsis* Bax Inhibitor-1 can enhance drought tolerance in sugarcane. Thus, proteins associated with this physiological process, such as reticulon-like and GRAM domain proteins, may play an important role in sugarcane plants adaptation to drought (Alam *et al*., 2010a). Reticulon-like proteins are found predominantly associated with the endoplasmic reticulum, probably acting in membranes stabilization, endocytosis, intracellular transport and apoptosis (Nziengui *et al*., 2007). In *Arabidopsis*, GRAM domain proteins are involved in regulation of programmed cell death and defense responses observed in vascular tissues (Lorrain *et al*., 2004). This domain is predicted to function in intracellular protein binding or lipid binding during membrane-associated processes (Doerks *et al*., 2000) and includes ABA-responsive proteins (Jiang *et al*., 2008).

Drought stress usually causes protein dysfunction. The maintenance of functional conformation and prevention of proteins aggregation are particularly important for cell survival under stress conditions. Chaperones have a crucial role in membrane and protein stabilization, reestablishing the normal protein conformation (Wang *et al*., 2004). Additionally, stomatal closure caused by drought may increase leaf temperature. Plants subjected to heat and drought stresses express heat shock proteins (HSPs), such as chaperone DnaJ (Hsp40) involved in the protection of enzymes and structural proteins from denaturation and aggregation (Rizhsky *et al*., 2004), exerting an important role in maintaining of cellular homeostasis.

### 3.4.4. Gene expression analysis via qRT-PCR

The qRT-PCR results corroborate the gene expression pattern observed in the RNA-Seq. Although the expression amplitude between the two techniques is different, the general tendency of gene expression is consistent, presenting average fold change values very similar (Figure 6). As shown in Figure 7, qRT-PCR results demonstrated significant similarity with the sequencing data, presenting a high correlation coefficient ($R^2 = 0.99$, for both cultivars). These data indicate that the contamination of one library in the RNAseq experiment did not induce to errors in the selection of the genes up-regulated by drought in this work.

Induction levels of selected genes were wide, ranging from 2 to 5,000 fold induced. The inclusion of genes with variable expression levels is due to the fact that induction degree is not directly related with gene importance in the protection against drought, that is, not necessarily the most induced genes are more relevant for drought
tolerance. An example are the transcription factors, whose role is extremely important in controlling water deficit response, but changes in their expression levels may have minor magnitudes and even be restricted to small periods of stress.

**Figure 6.** Comparison of gene induction levels under drought obtained in RNA-Seq and qPCR for the selected genes. The fold change values represent the mean of the 3 biological replicates. Bars indicate the standard deviation. (a) Cultivar RB92579. (b) Cultivar RB72454.

**Figure 7.** Correlation coefficient between RNA-Seq (y-axis, log10) and qPCR (x-axis, log10) for the 10 selected genes. (a) Cultivar RB92579. (b) Cultivar RB72454.
4. CHAPTER 2 - Sugarcane genes related with drought tolerance in *Arabidopsis*

4.1. Introduction

4.1.1. The use of model plants for functional analysis

The use of model species, such as *Arabidopsis thaliana*, to study more complex organisms, such as sugarcane, is a common and efficient strategy employed in genetic engineering. *Arabidopsis thaliana* stands out as the most used model plant for genes characterization even those coming from distinct and evolutionarily distant species (Dong, 2001; Murakami *et al.*, 2007), since most of the proteins present conserved functions (Koornneef and Meinke, 2010; Rine, 2014).

*Arabidopsis* plants shows several advances that justifies its use: small size that eliminates the use of large laboratory facilities; short life cycle, allowing analyzing next generations in short time period; self-fertilization; production of large number of seeds; small and sequenced genome, among others (Koornneef and Meinke, 2010). On the other hand, sugarcane plants present features that make manipulation more difficult such as: big size; long life cycle and polyploid genome (Ming *et al.*, 2001), which justifies the use of easy-to-handle plants to study crop genes.

Several crop genes involved in stress response mechanisms were characterized using model plants. A new sugarcane gene was associated with drought, salt and oxidative tolerance in tobacco transgenic plants, by improving physiological and biochemical drought-related parameters (Begcy *et al.*, 2012). A sugarcane ethylene responsive factor (ERF) was also evaluated using tobacco plants, showing increased salt and drought tolerance (Trujillo *et al.*, 2008). Vannini *et al.* (2003) demonstrated that a rice MYB transcription factor is capable of increased chilling and freezing tolerance in *Arabidopsis* transgenic plants, improving photosystem II stability and survival rate. The overexpression of *DBP3* gene from maize, belonging to AP2/ERF superfamily, enhanced drought and cold stress tolerance in *Arabidopsis* plants (Wang and Dong, 2009). The function of a wheat *MYB* gene in stress response was also analyzed using *Arabidopsis* transgenic plants. These plants showed improved drought tolerance by changing the expression of stress-responsive genes and physiological parameters (Zhang *et al.*, 2012).

Therefore, the use of the model plant *Arabidopsis thaliana* to overexpress sugarcane genes is a promising approach to study the role of sugarcane genes in drought stress response.
4.1.2. Severe and mild drought

Plant response to drought varies according to several factors, including stress intensity. Severe stress conditions are often used to evaluate plant responses to dehydration. However, tolerance mechanisms activated in response to severe drought may not have a direct correlation with those active under moderate stress, closer to field conditions. Thus, reproducing moderate conditions of water deficit in tests will increase the chances of identifying genes that may enhance drought tolerance under field conditions (Skirycz et al., 2011; Clauw et al., 2015).

Therefore, we evaluated the overexpression effects of sugarcane genes in the growth of Arabidopsis plants under severe and mild stress conditions. However, the reproduction of moderate water deficit stress requires a well-monitored experimental assay, with a rigorous control of soil water content and precise timing to start the drought, since the plant developmental stage influences stress response (Skirycz et al., 2010; Verelst et al., 2010). Taking this into account, we performed the moderate drought assay in the laboratory of Dr. Dirk Inzé at VIB (Belgium). His research group developed the robotic platform WIWAM (Weighing, Imaging & Watering Machines) which automatically weighs, irrigates and images the plants, ensuring strict control over the applied water regime (Figure 8) (Skirycz et al., 2010; Dubois et al., 2017). WIWAM has capacity to assess 384 plants per experiment, allowing the determination of the leaf area of each plant and inference of stress tolerance level. Additionally, survival tests under severe stress with Arabidopsis plants overexpressing independently the genes were performed and compared with mild stress tests.
Figure 8. The WIWAM robotic system. The automated system adjusts water content in each pot and takes pictures of the rosettes on a daily basis and the images are stored in a database. Source: www.wiwam.be.

4.1.3. Root analysis

Roots are responsible for water absorption by plants, being the first to detect water deficit in soil and to send signals to other parts of the plant, playing an essential role in adjusting plant development under stress. Nevertheless, due to technical difficulties, the effects of drought stress on root development are largely unknown, compared to data from aerial parts. This knowledge will contribute to elucidate the adaptive mechanisms underlying drought tolerance and to produce plants with higher tolerance to water deficit. Therefore, we also performed root analysis under drought conditions in vitro and in soil.

Several strategies are used to study the root system architecture (RSA) traits in Arabidopsis. These plants can be grown in different substrates, but their thin and small roots make it difficult to evaluate the root system in situ. Hydroponic culture is other strategy that can be used, since it facilitates root visualization. However, roots tangle after a short period of time (15 days) making hard to analyze them and the root morphogenesis is affected because there is no mechanical restriction. Additionally, solid culture medium can also be used, but again the analysis time is limited because of the restricted space (Devienne-Barret et al., 2006). Another alternative is the rhizotron-based platform (Ara-rhizotron), developed by Dr. Tom Beeckman’s group (VIB), that was used in this work (Figure 9).

This system allows evaluating the growth of thin and small roots of Arabidopsis plants in soil during the whole period of its vegetative growth, in a non-destructive way
in conditions closer to reality. Additionally, we analyzed the root development of
*Arabidopsis* plants under drought stress *in vitro* and the results were compared.

![Representative scheme of the Ara-rhizotron system.](image)

**Figure 9.** Representative scheme of the Ara-rhizotron system. (a) Visualization of the rhizotron box showing the plates slanted in the system. (b) *Arabidopsis* plants grown in the Ara-rhizotron. Source: www.iuap-mars.be/techniques/rhizotrons.

## 4.2. Objectives

The main objective of this chapter was to perform a functional evaluation of six sugarcane genes that were induced by drought in sugarcane roots, using *Arabidopsis* plants in water deficit conditions. Specifically, the objectives were:

- Isolate and clone the genes sequence in expression vectors;
- Transform *Arabidopsis thaliana* via floral dip using *Agrobacterium*;
- Select single copy and homozygous transgenic events;
- Analyze phenotypic and physiological parameters of the transgenic plants grown using the robotic phenotyping platform WIWAM for controlled mild drought;
- Evaluate the survival rates of the transgenic plants under severe water deficit;
- Perform *in vitro* roots and shoots analysis using mannitol to induce stress;
- Characterize the root development of transgenic plants in soil under water deficit stress, using the Ara-rhizotron platform.

## 4.3. Materials and Methods

### 4.3.1. Gene cloning using Gateway system

The coding region of the selected genes, delimited by the initiator ATG and the stop codon, was amplified by PCR, using gene-specific oligonucleotides. Beacon Designer™ Real Time PCR Oligo Design (PREMIER Biosoft) program was used for primer design, following the guide from pENTR™ Directional TOPO® Cloning kit
(Invitrogen, 2006). To this end, CACC sequence followed by ATG (start codon) was added to the 5’ end of the forward primers, whereas in the reverse primers the STOP codon was not included since target genes will be fused in frame to histidine tag C-terminus (table 3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer forward (5’-3’)</th>
<th>Primer reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWPM</td>
<td>CACCATGGCCGGGGTTGGGAGG</td>
<td>GACTCTGGGGGGCCGCCCTT</td>
</tr>
<tr>
<td>PSK</td>
<td>CACCATGGCGTCGTCCGCGCCACG</td>
<td>CGGCTTGCCCTTTGTGCTGC</td>
</tr>
<tr>
<td>NRX</td>
<td>CACCATGGCTGAGATCCGGGAGG</td>
<td>AGCAGTTCTGGTCGGCTGCC</td>
</tr>
<tr>
<td>RTNL</td>
<td>CACCATGGCCGATCGCGGCCGAGG</td>
<td>GTTCTGCTTCTTGAGATTTGACCC</td>
</tr>
<tr>
<td>GRAM</td>
<td>CACCATGGGAGGGAAAAGGACGGA</td>
<td>CGGCTTGCCGAGCAGTTCG</td>
</tr>
<tr>
<td>AUX</td>
<td>CACCATGGGCTCTGGGACCAG</td>
<td>CCACCAGTCGTACACGATGT</td>
</tr>
<tr>
<td>DNAJ</td>
<td>CACCATGAGCGGGGAGAGGAGATGCA</td>
<td>TCGTGAGAAAGCGGCTGCGG</td>
</tr>
<tr>
<td>HIPP</td>
<td>CACCATGGCGATTCTGGAGACCTTT</td>
<td>CATGACCCTGACCCGTTGTA</td>
</tr>
<tr>
<td>HP</td>
<td>CACCATGAGCATGAGGTGGCGCATG</td>
<td>AGGACCAGGGGTGGCCTGG</td>
</tr>
<tr>
<td>HP2</td>
<td>CACCATGTACCCCTACACTCCGT</td>
<td>GGCCTGGGATCGTCTTTGC</td>
</tr>
</tbody>
</table>

**Table 3.** Primers used to amplify the sequences to clone into pENTR/D-TOPO vector.

The PCR reaction was performed by the enzyme Phusion High-Fidelity DNA polymerase (2 u/μL) (ThermoScientific) in a final volume of 50 μL. For genes with high GC content, DMSO (dimethyl sulfoxide) (5%) was added to the reaction. PCR conditions were: 94 °C (1 minute); 94 °C (30 seconds), annealing temperature of each primer (45 seconds) and 72 °C (3 minutes) - (33 cycles); 72 °C (10 minutes) and 14 °C (∞). As reaction template, cDNA samples (dilution 1:1) of drought group replicates (RNA-Seq) were used, due to higher expression of genes of interest under stress conditions.

The PCR product was subjected to 1% agarose gel electrophoresis stained with ethidium bromide using GeneRuler 1kb Plus (Thermo-Scientific) as molecular weight marker. The gel bands with size corresponding to the target genes were cut and purified with Wizard® SV Gel and PCR Clean-Up System (Promega). Purified PCR products were concentrated in the Speedy Vacuum to the volume of 15 μL and quantified by spectrophotometry in NanoDrop 2000 (ThermoScientific). Subsequently, 3 μL of samples were analyzed on 1% agarose gel in order to confirm genes amplification.

Purified PCR products were cloned in a directional way into pENTR/D-TOPO entry vector (Life Technologies), using the enzyme topoisomerase I. This commercial vector (Figure 10a) presents a TOPO® cloning site, which allows rapid directional cloning; a kanamycin resistance gene for selection; pUC (ori): origin of replication; and
attL1 and attL2 recombination sites to transfer target gene from entry constructions to Gateway destination vector.

Subsequently, *Escherichia coli* competent cells (DH5α) were transformed with entry vectors containing the target genes by heat shock. The obtained colonies were used for plasmid DNA extraction via alkaline lysis. DNA minipreparations were confirmed to the presence of target genes through restriction enzymes digestion and sequencing. All restriction sites analyses and double digestions were performed using Serial Cloner 2.6.1 program and the online tool (http://www.thermoscientificbio.com/webtools/doubledigest).

The plasmid DNA of the entry construct was used in a recombination reaction with pGWB608 destination vector (Nakamura *et al*., 2010). This vector (Figure 10b) has Gateway® system recombination sites (attR1 and 2); a CaMV 35S promoter (P35S) for constitutive gene overexpression; the selection gene for spectinomycin antibiotic (Spcr); selection for glufosinate-ammonium herbicide (BASTA) and the epitope Hexahistidine-tag (6xHis).

The recombination reaction between entry and destination vectors was performed using Gateway LR Clonase II Enzyme (Life Technologies). The cloning reaction product was used to transform chemically competent *E. coli* cells (DH5α), following the same procedure mentioned before. The pGWB608 constructions confirmed for the presence of the taget genes were used in the next step.

4.3.2. *Agrobacterium tumefaciens* transformation

*Agrobacterium*–mediated transformation has some advantages over biolistic direct transformation, such as high transformation efficiency, high proportion of simple
integration events with intact transgenes, stable expression, heritability and lower cost (Singh et al., 2013), becoming a preferred vehicle for genes insertion into plants (Manickavasagam et al., 2004; Santosa et al., 2004). Therefore, this was the transformation method used in this work.

Agrobacteria competent cells (GV3101) were prepared following the protocol adapted from Weigel and Glazebrook (2002). The competent cells were transformed with the construction target genes::pGWB608 via heat shock and plated in solid LB medium containing the antibiotics rifampicin (10 μg/mL) and gentamicin (30 μg/mL) for bacterial selection, as well as spectinomycin (100 μg/mL) for pGWB608 vector selection. After 3 days growing at 28 °C, the colonies obtained were inoculated in liquid LB medium with antibiotics and kept at 28 °C in the dark at 250 rpm overnight. The bacteria were used for plasmid DNA extraction, following the protocol describes by Ebert and Dean (2003). Plasmid minipreparations were quantified, and the genes of interest were amplified by PCR, to eliminate false positive constructs (empty vectors). Positive minipreparations were sequenced and used for Arabidopsis transformation.

4.3.3. Arabidopsis thaliana growth conditions

Arabidopsis seeds from Columbia ecotype (col-0) were germinated directly on soil with substrate and vermiculite (ratio 3:1) and vernalized at 4 °C for three days to break dormancy. After this period, plants were kept under growth conditions of 22 to 24 °C and photoperiod 16 h (~ 120 μE m−2 s−1). Approximately 30 plants for each gene were used for transformation, organized in 15 cm² plastic pots, containing on average eight plants each. After five weeks of germination, the first inflorescence was cut to stimulate the growth of multiple inflorescences. By reaching this stage, wild type plants (WT) were prepared for transformation by removing open flower whorls and silicas, leaving only closed flower buds in order to potentiate transformation efficiency.

4.3.4. Arabidopsis transformation

The transformation of Arabidopsis plants with Agrobacterium was performed following the floral dip method (Clough and Bent, 1998). A single Agrobacterium colony transformed with pGWB608 vector (for each gene) was first grown in LB medium (pre-inoculum) at 28 °C overnight, under 250 rpm in the dark. The culture was transferred to YEB medium (inoculum), containing the appropriate antibiotics (spectinomycin 100 mg/mL, gentamicin 50 mg/mL and rifampicin 30 mg/mL), under
the same pre-inoculum conditions. After inoculum reached OD$_{600}$ from 0.8 to 2.0, a fresh solution of 5% sucrose and 0.03% silwet L-77 (surfactant) was added in a volume three times bigger than agrobacteria volume. WT plants were immersed in this final solution for one minute. After this period, plants were covered with a plastic film for 24 hours and kept in the growth chamber (22 °C) until seed collection (3 to 4 weeks). Plants transformed with the pGWB608 empty vector and untransformed WT plants were used as negative controls in the drought experiments.

4.3.5. Transgenic plants selection

T1 seeds obtained from transformed plants (T0) were seeded directly in soil and selected with glufosinate-ammonium herbicide (0.1% v/v from FINALE commercial solution of 200 mg/L active compound). The herbicide was sprayed every two days during two weeks for the selection of transgenic events, using the resistance gene presents in pGWB608 vector.

In order to confirm the identity of the gene inserted into the plant genome, leaf tissue samples from each T1 resistant plant were collected and its DNA extracted. Subsequently, a PCR of foliar DNA was performed using gene-specific forward primers and a common reverse primer drawn from NOS terminator sequence of pGWB608 vector (Figure 11). About 4 to 8 events of each gene in T1 generation were selected. The confirmed plants were individualized and cultivated until seeds production (generation T2).

![Figure 11. Primers position in the pGWB608 vector. The forward primers (Fw) were the same as that used for cloning into entry vector. The primer reverse (Rv) was drawn in the terminator region (T-NOS). Therefore, genes amplification will result in a band 104 bp bigger than the original size of the respective genes.](image)

To evaluate the number of copies of each gene inserted into the genome as well as its homozygosity, one hundred T2 seeds for each T1 event were plated on Murashige-Skoog (MS) medium containing 0.0044% (v/v) of the herbicide Finale.
Following the precepts of Mendelian segregation, confirmed T1 plants (hemizygotes for the target gene) were expected to produce a proportion of 25% homozygous plants for the target gene, 50% hemizygous plants and 25% of plants without the target gene, in cases where a single copy of the transgene has been inserted. In selective medium, only homozygous and hemizygous transgenic plants will survive, corresponding to 75% of the plants. Thus, if the ratio 3:1 (survivors: dead) is obtained in this step, it indicates that only one copy of the gene has been inserted into the plant. For distinct proportions, more than one copy is present. About 8 to 10 survivors of single copy events for each T1 event were selected. Such plants may be homozygous or hemizygous for the gene of interest. In the next round of selection, the seeds of these plants (T3 generation) were plated again in herbicide medium. For homozygous plants, 100% of the seeds germinated and developed. For hemizygous plants, only 75% of the plants survived. Three independent events single copy in homozygosity overexpressing the transgene on T3 seeds were selected and used in *Arabidopsis* drought tolerance experiments.

### 4.3.6. Gene expression analysis

The expression levels of transgenes in the single copy homozygous plants were evaluated by qRT-PCR. For each gene, three independent events were selected. A pool of 10-15 plants (for each event) was collected and macerated using Mini-BeadBeater. Total RNA was extracted using Trizol reagent. The qRT-PCR was performed following the method described in section 3.3.10 and the same primers were used. The differences are that here we used cDNA (dilution 1:20), $2^{-\Delta CT}$ method for transgenes relative quantification and actin-2 gene (*AT3G18780.2*) as internal control. Expression values will be given in relation to actin-2, since there is no sugarcane transgene amplification in *Arabidopsis* plants of the negative control (empty vector).

### 4.3.7. Phenotyping analysis under mild stress

The phenotyping experiment using the WIWAM platform followed the VIB standard protocol. In total, six sugarcane genes successfully transformed in *Arabidopsis* were evaluated. For that, two screens were performed, each one with three genes (three independent events each) and the controls (WT/empty vector), once the maximum capacity of the platform is 384 plants and 32 plants were used for each event (16 plants per treatment).
Plants were grown in cylindrical polypropylene pots (200 ml, diameter 53 mm, height 88 mm; VWR International, Leuven, Belgium) with 85 g±2 g of Saniflor compost (Van Israe N.V., Geraardsbergen, Belgium), under a long-day regime (16 h light) at 21°C and 110-120 µmol m-2 s-1 light intensity. Plants were kept on the platform under control conditions for 6 DAS (days after sowing), after which the irrigation remained normal for the control plants (water content at 68%) and the water was suspended for the stressed plants until the set stress level (water content at 40%) was reached and subsequently kept constant. During the experiment, the automatic WIWAM system daily captured images, controlled irrigation and weighed the plants, allowing a precise control of the stress conditions. Furthermore, the environment was monitored by the integration of light, temperature and humidity sensors, for a detailed recording of the experimental growth conditions. The test was finished 23 DAS and the images were analyzed to determine shoot growth, using the software PIPPA, developed by VIB.

Additionally, 4-5 plants of each event and treatment (mild drought and well-watered) were collected to calculate biomass and leaf chlorophyll levels (Hiscox and Israelstam, 1979).

4.3.8. Survival assays under severe drought

For the survival tests, each plant was grown in a separate pot (55 mm) filled with jiffy-7 peat pellet (Jiffypot, Netherlands), under a long-day regime (16 h light) at 21°C and 110-120 µmol m-2 s-1 light intensity. Transgenic and control plants were randomized among four trays (5 x 7 pots), assuring that each tray received at least one plant of each event to minimize technical variations. Additionally, the pots were moved (within each tray) three times a week to avoid position effects. Seven plants were used for each transgenic event and 10 plants for the control events. Since the reproducibility of survival assays is a challenge due to environmental variability that affects the soil drying rate, the rehydration moment is a critical stage. Thus, some control plants were previously re-watered in each assay repetition to assure the right moment to rehydrate all the plants. The WT and empty vector transformed plants were used as control in this experiment.

The plants were grown under normal conditions (16 h day regime, at 21 °C) for 14 days. After that, the water content of all pots was normalized to 50 g. Water was withheld for approximately two weeks until the majority of the plants showed clear
symptoms of wilting and then plants were rewatered. One day after, survived plants were counted and the survival rate was scored, comparing the transgenic and control plants.

4.3.9. *In vitro* shoot and root analysis under drought stress

Shoot and root parameters of transgenic plants were evaluate *in vitro* under stress conditions induced by different concentrations of mannitol. For root analysis, 12 plants of each gene event were sowed in MS plates supplemented with 0 and 100 mM of mannitol, held in vertical position. For this experiment it was also used an *Arabidopsis* homozygous mutant (SALK_037546C) from the *AT4G11220.1* gene, homologous to the *RTNL* gene from sugarcane. Parameters as primary root length, root depth and width, and root convex hull (root area measured as the smallest convex polygon containing the roots) were measured 15 days after stratification.

For the shoot analysis, taking into account the results from the survival assays, we focused on the genes *ScNRX* and *ScRTNL*, which presented higher survival in all 3 events compared with controls. Forty plants of each line were grown in MS medium supplemented with 0, 25 and 100 mM of mannitol and the effects of drought on seeds germination and plants rosette growth were examined.

4.3.10. Root analysis using the Ara-rhizotron system

The rhizotron experiment (Figure 12) begins with the assembly of the rhizosheets. The lay back panel (white PVC) and plexiglass panel are separated by two spacers in the left and right side and all the system is kept together by three layers of micropore tape. After that, the sandwich is fixed by applying binders at the sides. Before fill the rhizosheets, the soil is sieved twice to guarantee no crumbs and residue left. The process of filling the rhizotrons follows the pattern:

1. Fill to top, compact to 1/2 the height of the rhizosheet = 80 g of soil
2. Fill to top, compact to 2/3 the height of the rhizosheet = 120 g of soil
3. Fill to top, compact to 3/4 the height of the rhizosheet = 140 g of soil
4. Fill to top, compact to 7/8 the height of the rhizosheet = 160 g of soil
5. Fill to top, compact to 15/16 the height of the rhizosheet = 165 g of soil

In the next step, the rhizosheet is filled to the top (168 g of soil). After that, soil is put onto the top of the rhizosheet and pressed, being this step repeat three times to assure no separation between the top and deep layers of soil. At this point the loaded
soil should be around 170 g. A layer of micropore is applied at the top of the rhizotron to avoid soil drying. Under normal water treatment, the rhizotron is put in the water bath overnight to equalize any humidity difference among rhizosheets and the seeds are sown. For water bath and plants irrigation was used purified water with fertilizer.

To test drought stress using the rhizotrons we performed a pilot experiment using WT plants. For that, four rhizotrons were submitted to four conditions: water bath + 5 mL of water during the experiment (normal treatment); no water bath + 5 mL of water during the experiment (stress treatment); no water bath + 2.5 mL of water during the experiment (stress treatment) and no water bath + no water during the experiment (stress treatment). In each rhizotron five seeds were sowed and after germination just one seedling was kept. The plants were grown for about two weeks. From the results of this pilot experiment we decided to use as drought treatment the rhizotrons submitted to no water bath and no water addition (data not shown).

For the main rhizotron experiment we used seeds from ScRTNL-OE2 line (event 2 overexpressing the gene ScRTNL), based on the results of in vitro root tests, and the WT as control. Due to maximum capacity of the system, it was used six rhizotrons for each treatment (well-watered and drought) and each genotype (ScRTNL-OE2 and WT), totalizing 24 rhizotrons. Following the VIB protocol, the rhizotrons were filled during four days (six a day) and the sowing was performed on the fifth day. The first loaded rhizotrons were put in the water bath on the fourth day and left there overnight, while the last ones were used for the drought treatment. Despite adding 6 mL of solution before sowing the seeds in the drought rhizotrons, after germination and removal of the foil, seedlings started dying and the experiment was not successfully concluded.

Another attempt was made, reducing the filling time for three days (eight rhizotrons a day) and performing the sowing on the third day. These modifications were done because the biggest problem in the previous experiment was that the soil was too dry, making difficult to infiltrate water to allow seed germination. This possibly occurred because the rhizotrons were filled during the week and not in the same day of sowing as we did in the pilot experiment. However, again, after removing the foil, the dry treated seedling started dying, and just two of them were left (one of each genotype).

Taking this into account, a third attempt was performed. For this experiment, the water bath was used for the drought treatment a week up front of the well-watered treatment rhizotrons, leaving them for a week to leak out (covered). Meanwhile, the preparation of the irrigated rhizotrons had begun, following the standard protocol. By
the end of the second week, the seeds were sown in both treatments. The dry condition
did not receive any water afterwards, while the normal condition received 5 mL of
water. Once the water loss surface of the rhizotrons is very small the treatments were
not incubated at the same time in the water bath, because this would require a longer
time to dry and the roots would hit the dimensions limits of the sheets. After 30 days,
the roots were scanned and the data analyzed.

**Figure 12.** Steps of the rhizotron experiment. (A) Assembly of rhizosheets. (B) Rhizotron filled with soil. (C) Rhizotrons in the water bath. (D) Rhizotrons positioned sloping, containing five seeds each and covered with foil to keep high humidity for seeds germination. (E) Top view of two-weeks-old *Arabidopsis* plants grown in the rhizotron system. (F) Detail of the root development in plants grown in the rhizotron.

### 4.3.11. **Statistical analysis**

The results of drought tests in transgenic *Arabidopsis* plants were compared with the control group (WT and empty vector) by statistical analysis using t-Student test, adopting as statistically different results that show a value of \( p \leq 0.05 \).
4.4. Results and Discussion

4.4.1. Gene cloning using Gateway system

All 10 genes were successfully amplified. Most of them were isolated from cDNA, but some of them faced difficulties in amplification via cDNA. The genes sequences ScAWPM, ScGRAM and ScAUX were isolated from complete SUCEST clones (SCVPCL6042G06.g, SCJLRT1015C02.g and SCCCCL3120D11.g, respectively). ScDNAJ was amplified from sugarcane root cDNA using primers designed in the UTR region of this gene. Then, a PCR of this PCR product was performed with specific-gene primers made for cloning into TOPO vector.

Bands obtained by PCR were cut, purified and analyzed in agarose gel. The genes expected size corresponds to bands size observed in the gel (from ATG to the last codon before STOP codon): ScAWPM: 513 pb; ScPSK: 375 pb; ScNRX: 1185 pb; ScRTNL: 768 pb; ScGRAM: 846 pb; ScDNAJ: 573 pb; ScHIPP: 453 pb; ScHP: 384 pb; ScAUX: 409 pb and ScHP2: 516 pb (Figure 13).

In the next step, the purified PCRs were cloned into the entry vector. These constructs were confirmed by enzymatic digestion using SacII and EcoRV and subsequently by sequencing. The observed bands matches the expected bands sizes in the digestion reaction: ScAWPM: 2735, 358 and 301 bp; ScNRX: 2434, 1124 and 207...
bp; ScGRAM: 2434, 881 and 111 bp; ScAUX: 2434, 432 and 123 bp; ScDNAJ: 2434 and 719 bp; ScHP: 2434, 458 and 69 bp; ScHP2: 2434, 508, 111, 27 and 13 bp; ScPSK: 2434 and 524 bp; ScRTNL: 2434, 584 and 333 bp; ScHIPP: 2434 and 602 bp (Figure 14).

**Figure 14.** Entry vectors containing the target genes digested with the enzymes SacII and EcoRV. (a) ScPSK, (b) ScRTNL, (c) ScHP, (d) ScAUX, (e) ScAWPM, (f) ScDNAJ, (g) ScHIPP, (h) ScGRAM, (i) ScHP2 and (j) ScNRX. The numbers represent the colonies analyzed. Digestions indicate that colonies ScPSK-1, 3 and 5; ScRTNL-3; ScHP-3, 4 and 7; ScAUX-3 to 6; ScAWPM-2 to 8; ScDNAJ-1; ScHIPP-2 and 5; ScGRAM-1, 3, 4 and 6; ScHP2-1, 2, 3 and 5; and ScNRX-2 have the target genes. The remaining samples are false positive. M: GeneRuler 1kb plus. ND: undigested vector.

Entry vectors were recombined with destination vector, via Gateway system. The resulting vectors were digested with the enzymes SacI and EcoRV. Observed and expected bands present the same sizes: ScAWPM: 9692 and 749 bp; ScNRX: 9692, 639,
549 and 233 bp; \textit{ScGRAM}: 9692 and 1082 bp; \textit{ScAUX}: 9692 and 645 bp; \textit{ScDNAJ}: 9692 and 809 bp; \textit{ScHP}: 9692 and 617 bp; and \textit{ScHP2}: 9692 and 749 bp (Figure 15). These results were confirmed by sequencing.

\textbf{Figure 15.} Destination vectors digested with the enzymes SacI and EcoRV. (a) \textit{ScAWPM}, (b) \textit{ScDNAJ}, (c) \textit{ScAUX}, (d) \textit{ScGRAM}, (e) \textit{ScPSK}, (f) \textit{ScRTNL}, (g) \textit{ScHIPP}, (h) \textit{ScHP}, (i) \textit{ScNRX} and (j) \textit{ScHP2}. The numbers represent the colonies analyzed. All plasmids present the correspondent target genes. M: GeneRuler 1kb plus. ND: undigested vector.
4.4.2. *Agrobacterium* transformation

Sequenced pGWB608 vectors containing the target genes were used to transform agrobacteria competent cells (GV3101). The 10 selected genes were successfully transformed into GV3101 as we can see by bands size in Figure 16. Once the PCR primers used are the same from TOPO cloning, expected bands present the same size that in Figure 13. For positive control it was used pGWB608 vector miniprep with the target gene e for negative control no plasmid is present.

*Figure 16. PCR of the genes of interest from DNA minipreparation of transformed agrobacteria. (a) ScPSK, (b) ScRTNL, (c) ScHP, (d) ScHP2, (e) ScGRAM, (f) ScAWPM, (g) ScHIPP, (h) ScDNAJ, (i) ScNRX and (j) ScAUX. The numbers represent the colonies analyzed. All colonies contain the respective target genes. M: GeneRuler 1kb plus. C +: miniprep of pGWB608 vector containing the gene of interest. C - negative control of PCR. The second lower band observed for the ScGRAM and ScAWPM genes is*
probably due to the non-specific binding of the primers to another region of the vector, since it was also observed in the C +. The genes sequences were confirmed by sequencing.

4.4.3. Arabidopsis transformation and transgenic selection

Transgenic agrobacteria were used to transform Arabidopsis plants. In the first herbicide selection, resistant transgenic plants stand out due to their size and green color, while untransformed plants have a yellowish color at first and end up dying (Figure 17).

![Figure 17](image-url) Selection of T1 plants transformed with one of the target genes. (A) Seedlings before selection. (B) Seedlings after selection with the herbicide Finale 0.1% (200 mg/L). The red circles show some of the herbicide-resistant plants.

The presence of the target genes in transformed plants selected with herbicide was confirmed by PCR of leaf DNA (Figure 18). Six genes were successfully confirmed in T1 transgenic plants, however, no distinct phenotype visible was observed in these plants. Plants transformed with sugarcane genes AWPM, DNAJ, PSK and HP2 presented problems in obtaining resistant events (T1), with no seed surviving to herbicide selection. This may be due to transformation problems or lethality in plants overexpressing of these genes. Therefore, the analyses continued only with the six remaining genes: ScHIPP, ScRTNL, ScNRX, ScAUX, ScGRAM and ScHP.
Figure 18. PCR of leaf tissue DNA from Arabidopsis plants overexpressing (a) ScHIPP (b) ScRTNL (c) ScAUX (d) ScHP (e) ScGRAM and (f) ScNRX. The numbers represent the plants analyzed. All evaluated plants contain the target genes, except for the ScGRAM-4. M: GeneRuler 1kb plus. C +: pGWB608 miniprep containing the target genes. WT: wild plants. C -: PCR negative control. The bands are 104 bp bigger than gene original size because the reverse primer was drawn in the terminator region (T-NOS).

These genes were selected for single copy (Figure 19) and homozygous events (Figure 20). The selection of single copy events avoids the insertion of transgene multiple copies into the plant genome which may cause gene silencing instead of overexpression (Lechtenberg et al., 2003; Kihara et al., 2006), and the use of homozygous events also helps with the stability of the phenotypes.

Figure 19. Selection of single copy events (T2 generation). (A) T2 plants with the insertion of more than one copy of the transgene (93.75% of herbicide resistant plants). (B) T2 plants with a single copy of the transgene (72.91% of herbicide resistant plants). (C) The blue rectangle illustrates plants resistant to selection, while the red rectangle shows some of the plants sensitive to the selective agent.
4.4.4. Gene expression analysis

Transgenes expression level of single copy homozygous plants was evaluated by qRT-PCR. Figure 21 shows transgenes expression levels relative to the endogenous actin-2 gene, a gene with high expression in *Arabidopsis* (An *et al.*, 1996). For all events it is possible to detect transgenes expression, despite the variable expression levels, which are expected due to several factors, as the transgene insertion site.
Figure 21. Transgenes expression in relation to endogenous actin-2 gene. (a) ScRTNL, (b) ScHP, (c) ScNRX, (d) ScPSK, (e) ScHIPP and (f) ScAUX. Bars represent the standard error (n=3).

4.4.5. Phenotyping analysis under mild stress

Shoot growth analyses of transgenic plants under mild drought using the WIWAM platform was assessed via rosette area and biomass. A transgene was considered relevant when at least two of the three events presented similar phenotypes. In the first WIWAM screen we evaluated the genes ScHIPP, ScHP and ScRTNL, while in the second screen we evaluated the genes ScNRX, ScPSK and ScAUX.
The overexpression of \textit{ScHP} (all events) and \textit{ScRTNL} (events 2 and 3) resulted in bigger plants (higher rosette area) compared with control empty vector (EV) under mild drought. Under well-watered conditions, plants overexpressing these same genes (\textit{ScHP}: events 1 and 3; \textit{ScRTNL}: events 2 and 3) and \textit{ScHIPP} (all the events) presented increased rosette area in relation to control (Figure 22a). Furthermore, higher biomass accumulation (dry weight) was observed for \textit{ScHP-OE} (events 1 and 2) and \textit{ScRTNL-OE} (events 2 and 3) plants compared to control when expose to mild stress, while \textit{ScHIPP-OE} (events 1 and 2) increased shoot biomass under normal irrigation. Accordingly, smaller reductions in biomass were observed in \textit{ScHP-OE} (all events) and \textit{ScRTNL-OE} (events 2 and 3) plants under stress comparing to irrigated conditions in relation to control plants (Figure 22b).

Overall, these results indicate that \textit{ScHP} and \textit{ScRTNL} genes are related with plant shoot growth mechanisms under normal and mild drought conditions, while \textit{ScHIPP} gene just affects the rosette growth under non-stressed conditions. Field drought conditions usually present short periods of moderate stress that do not threaten plant survival. Thus, the lower water deficit-induced reductions of rosette area and biomass observed in \textit{ScRTNL-OE} and \textit{ScHP-OE} plants under mild drought would be a drought tolerance trait in this environment that might help plants to sustain yield (Skirycz et al., 2011), as observed for other plant genes overexpressed in \textit{Arabidopsis}.

In the second WIWAM screen, \textit{ScNRX-OE2} and 3 plants presented the smallest rosettes under normal conditions and smaller reduction in the rosette area under drought, with a surprising increase of 39\% in \textit{ScNRX-OE2} rosette area (although with no statistical significance) (Figure 23a). A possible explanation for these results is that smaller plants are less affected by mild drought because less water is dissipated through their surface. Additionally, biomass analysis showed that \textit{ScPSK-OE} (events 1 and 3) and \textit{ScAUX-OE} (events 1 and 3) plants have higher shoot biomass under well-watered conditions, but similar rosette dry weight under drought compared with control plants (Figure 23b). Increased biomass under well-watered condition with no penalties under mild drought stress can also be considered an interesting trait.

The chlorophyll content of transgenic leaves was also measured for both WIWAM screens, but no significant differences were noticed, indicating that the overexpression of these sugarcane genes does not affect chlorophyll metabolism in \textit{Arabidopsis} plants (Figures 22c and 23c).
Figure 22. Phenotyping analysis of the genes ScHIPP, ScHP and ScRTNL (screen 1) under mild stress, using the WIWAM platform. (a) Rosette area. (b) Rosette biomass (dry weight). (c) Total leaf chlorophyll content. Percentages represent reductions in the parameters under moderate drought compared to well-watered condition for each event. Bars represent the standard error (n=16 for rosette area and biomass; n=5 for total chlorophyll). The asterisk represents significant differences comparing the transgenic event with the control (EV) in the same treatment [Student’s t-test (p ≤ 0.05)].
Figure 23. Phenotyping analysis of the genes *ScNRX*, *ScPSK* and *ScAUX* (screen 2) under mild stress, using the WIWAM platform. (a) Rosette area. (b) Rosette biomass (dry weight). (c) Total leaf chlorophyll content. Percentages represent reductions in the parameters under moderate drought compared to well-watered condition for each event. Bars represent the standard error (n=16 for rosette area and biomass; n=5 for total chlorophyll). The asterisk represents significant differences comparing the transgenic event with the control (EV) in the same treatment [Student’s t-test (p ≤ 0.05)].
4.4.6. Survival assays under severe drought

*Arabidopsis* transgenic plants overexpressing the genes *ScNRX*, *ScRTNL* and *ScHIPP* (events 1 and 3) exhibited higher survival rates than controls under severe stress, which can be useful for drought tolerance in more arid regions (Figure 24). As stated above, we considered as positive result only when at least 2 of the 3 independent events behaved in the same way. One day after rehydration, a maximum of 16% of control plants survived, whereas transgenic lines of *ScRTNL*, *ScNRX* and *ScHIPP* reached 43%, 50% and 43% of survival, respectively.

Comparing these results with the WIWAM data it is possible to note that transgenic lines with better survival under severe drought do not match perfectly with transgenic lines that presented better shoot growth performance under mild stress. The increase in the survival rate is frequently associated with reduction of water loss by stomatal closure, which negatively affects CO₂ uptake, leading to growth reduction. Additionally, the plants response varies according to different levels of stress (Claeys et al., 2014). Therefore, these data are in agreement with the recent findings indicating that genes involved in drought tolerance under severe stress may not enhance tolerance under mild stress (Claeys and Inzé, 2013; Skirycz et al., 2011).
4.4.7 In vitro tests under drought stress

4.4.7.1 Germination

All tested ScRTNL-OE lines and ScNRX-OE1 and 3 lines showed no obvious phenotypes in the germination process compared with WT plants in MS medium with and without mannitol, whereas the germination of ScNRX-OE2 plants was significantly reduced in all conditions: non-stressed (no mannitol) and stressed (25 and 100 mM of mannitol)] (Figure 25a, b). These results show that the overexpression of ScRTNL and
ScNRX does not affect the germination rate in Arabidopsis plants under normal and stressed conditions, while the opposite was observed for the ScNRX-OE2 line. The differences between ScNRX events are possibly caused by position effects regarding the locus of the transgene insertion into the genome which may disrupt endogenous gene affecting plant development (Lambirth et al., 2015).

**Figure 25.** Mannitol effects on germination of transgenic plants. (a) Plants overexpressing ScRTNL. (b) Plants overexpressing ScNRX. The number of seedlings germinated was counted and the mean was determined. Asterisks indicate statistical differences comparing transgenic and control group in the same treatment (Student’s t-test, p ≤ 0.05). Bars indicate the standard error (n = 40).

### 4.4.7.2. Rosette area

In general, ScRTNL-OE plants did not differ from WT plants when exposed to mannitol (Figure 26a). The only exception was the ScRTNL-OE3 line, which presented smaller rosette area compared with WT in MS medium with 0 or 25 mM mannitol and bigger rosette area under 100 mM mannitol. It is worth to mention that events 2 and 3 increased shoot area compared with control plants under mild stress in soil, but did not show similar behavior in the in vitro moderate stress (25 mM mannitol). This might be explained because of the differences between in vitro and in soil drought experiments. Besides the substrate difference, in the in vitro tests, plants were sown on osmotic medium and the seedlings developed continuously in stable stress conditions while in soil experiments the drought treatment is a gradual process (Clauw, 2010).

Mannitol effect on ScNRX-OE rosettes produced mixed results (Figure 26b). While ScNRX-OE2 plants showed smaller rosettes than WT under 0 and 25 mM mannitol, ScNRX-OE3 plants presented bigger rosette area under severe stress (100 mM mannitol).

Comparing these data with WIWAM results, we noticed a consistence in the ScNRX-OE2 line which presents reduced shoot area under normal and mild stress.
condition in both experiments (soil and in vitro), despite of differences in the drought stress conditions. Moreover, in the survival assays, it was possible to visually notice smaller rosettes for this event (data not shown) and thus smaller surface to lose water by transpiration, which probably explain why these plants better survived under severe drought.

For both genes (ScRTNL and ScNRX), the rosette area reduction of transgenic plants was bigger than WT from 0 to 25 mM mannitol, but smaller from 25 to 100 mM mannitol (Figure 26a, b).

**Figure 26.** In vitro rosette area of ScRTNL-OE and ScNRX-OE plants under different concentrations of mannitol. (a) ScRTNL-OE events. (b) ScNRX-OE events. Asterisks indicate statistical differences comparing transgenic and control group in the same treatment (Student’s t-test, p ≤ 0.05). Percentage values represent the rosette area reduction from 0 to 25 mM and 25 to 100 mM of mannitol. Bars indicate the standard error (n = 40).

### 4.4.7.3. Root analysis

The first root parameter evaluated was primary root length. From these results and visual analyses we focused on genes that showed most interesting results to continue the analyses. Arabidopsis plants overexpressing ScRTNL gene presented promising data in the three transgenic events (Figure 27a, b, c). While in the absence of mannitol, WT and ScRTNL-OE transgenic plants did not differ in root characteristics, at 100 mM mannitol, the three ScRTNL-OE lines presented higher root depth and primary root length compared to WT. Additionally, ScRTNL-OE1 and 2 plants showed significant increase in root width and convex hull under severe stress. The ScRTNL-OE3 event also presented higher average in these parameters and lower reduction comparing drought with normal treatment, but these differences were not statistically significant. On the other hand, the Arabidopsis mutant for AT4G11220.1 gene, homologous to ScRTNL, showed significant reduction in root development under mannitol stress.
represented by decrease in all root parameters evaluated, but no alterations were noticed in non-stressed conditions (Figure 27d).

Together, these results show that RTNL genes from sugarcane and Arabidopsis are involved with alterations in the root system architecture that can enhance drought tolerance. The overexpression of ScRTNL improves root growth in plants under severe stress, developing longer primary roots, deeper and wider root system, and larger root convex hull which would improve the uptake of water and nutrients, positively contributing to drought tolerance (Yu et al., 2008). Additionally, the silencing of a RTNL gene from Arabidopsis corroborates the importance of this gene class in the root development under stress conditions.

For the other genes (ScNRX-OE, ScPSK-OE, ScHIPP-OE, ScAUX-OE and ScHP-OE) no significant differences were observed in the primary root length (Figure 28a-e).
Figure 27. Mannitol effects on root growth of transgenic plants overexpressing ScRTNL. Primary root length, root depth, root width and root convex hull parameters were measured for each event: (a) ScRTNL-OE1, (b) ScRTNL-OE2, (c) ScRTNL-OE3 and for (d) Arabidopsis mutant. Percentages represent reductions in the parameters under drought (100 mM mannitol) compared with normal condition (control). Asterisks indicate statistical differences comparing transgenic and control group in the same treatment (Student’s t-test, p ≤ 0.05). Bars indicate the standard error (n=12).
Figure 28. Mannitol effects on root growth of transgenic plants overexpressing (a) ScNRX, (b) ScPSK, (c) ScHIPP, (d) ScAUX and (e) ScHP. Only the primary root length was evaluated for these genes. Data are presented as mean and error bars represent standard error (n=12). Asterisks indicate statistical differences comparing transgenic and control group in the same treatment (Student’s t-test, p ≤ 0.05).
4.4.8. Root analysis using the Ara-rhizotron system

The Ara-rhizotron results did not show significant differences between WT and transgenic line for root system features evaluated under drought and well-watered conditions (Figure 29). However, it is possible that other parameters are affected by drought stress and could be essential for ScRTNL-OE2 line. Taking this into account, other analyses are being performed by Tom Beeckman’s laboratory, evaluating new parameters, such as, root gravity center and lateral root density.
Figure 29. Root analysis of three-weeks-old ScRTNL-OE2 plants under water deficit, using the rhizotron system. (a) Roots development in rhizotron. Col-0: WT control plants. Ret2: ScRTNL-OE2 line. D: drought stress. W: irrigated conditions. The number represents the replicates. (b) Primary root length. (c) Root depth. (d) Root width. (e) Root convex hull. Bars represent the standard error (n=6).
5. CHAPTER 3 - Effects of sugarcane genes overexpression in sugarcane drought tolerance

5.1. Introduction

Most of works regarding transgenic sugarcane plants tolerant to drought uses genes from model species with functions associated to drought stress well consolidated in the literature (Reis et al., 2014; Raza et al., 2016; Ramiro et al., 2016). In spite of the use of model plants to study more complex organisms, it is necessary to evaluate target genes in their species of origin. Our research group presents several patent applications protecting the use of sugarcane genes that confer drought tolerance, based on data from tobacco transgenic plants. However, during the licensing process, biotechnology companies require the proof of concept in the target crop species.

The development of molecular tools has promoted advances in the understanding of sugarcane drought responses, but there are still many challenges to engineer crops with drought tolerance characteristics (Wang et al., 2016; Ferreira et al., 2017). Drought response is a complex, multigenic and quantitative trait which varies according several factors, including species and genotype (Campos et al., 2004), and stress intensity and duration (Lopes et al., 2011; Reis et al., 2014). Besides that, sugarcane presents high heterogeneity, polyploidy and large genome which make more difficult to develop water deficit tolerant varieties (Basnayake et al., 2012; Singh, 2013; Reis et al., 2014).

Taking this into account, physiological and molecular studies involving drought-related sugarcane genes are essential to uncover stress response mechanisms used by this crop, contributing for the development of genetically modified cultivars suitable for commercial purposes (Lam et al., 2009).

5.2. Objectives

The main objective of this chapter was to perform a functional evaluation of four sugarcane genes using transgenic sugarcane under water deficit conditions. Specifically, the objectives were:

- Cloning the genes sequence into pGVG expression vector and transform agrobacteria;
- Transform sugarcane calli via Agrobacterium;
- Select events using transgene expression levels quantified by qRT-PCR;
- Analyze phenotypic and physiological parameters of the transgenic plants grown in greenhouse under drought stress conditions.

5.3. **Materials and Methods**

5.3.1. **Gene selection**

The initial proposal of this study predicted that the experiments with the model plant *Arabidopsis* would be a basis to select the genes that would be transformed into sugarcane. However, due to time constraints to obtain at least three independent homozygous events for each target gene and problems in cultivation of this model plant, we decided to initiate sugarcane transformation with four genes selected according to data from the literature: *NRX, DNAJ, HIPP and HP*. The work with the genes with positive profiles in the transgenic *Arabidopsis* plants will be continued in the near future.

5.3.2. **Sugarcane transformation vector**

A new vector named pGVG was constructed to transform sugarcane calli and is described in detail in chapter 4 as well as the patent application. Briefly, this vector presents a Gateway cassette under control of *ZmUbi1* promoter and CaMV 35S terminator for gene overexpression or silencing. Additionally, a FLAG-tag sequence was inserted upstream the CaMV 35S terminator for C-terminal fusion with the target protein. This vector was validated using GUS staining and qRT-PCR assays and showed to be able to efficient and fast overexpression or silencing of genes in sugarcane plants.

5.3.3. **Sugarcane transformation**

The meristematic region from shoot apex of six-months-old sugarcane plants (SP80-3280) was used to produce embryogenic calli. This material was cultivated in MS maintenance medium [4.33 g/L MS salts (Murashige and Skoog, 1962), 1 mL/L MS vitamins, 0.15 g/L citric acid, 0.5 g/L casein hydrolysate, 25 g/L sucrose, 12 g/L mannitol, 100 mg/L proline, 3 mg/L 2-4 dichlorophenoxyacetic acid (2,4-D) and 2.8 g/L phytage] at 26 °C in the dark, until the generation of embryogenic calli.

The four selected genes already insered into pENTR/D-TOPO vector were transferred to pGVG destination vector, using Gateway recombination. The constructs were inserted into EHA105 *A. tumefaciens* strain by heat shock. Bacterial cultures were incubated with sugarcane calli under vacuum pressure for five minutes and transferred
to co-cultivation medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.15 g/L citric acid, 25 g/L sucrose and 3.5 g/L phytagel) at 22 °C, in the dark for 3 days. After that, the calli were kept in resting medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.15 g/L citric acid, 25 g/L sucrose, 100 mg/L proline, 2.8 g/L phytagel and 200 mg/mL timentin) at 26 °C, in the dark for 6 days. Following the resting phase, the transformed calli were transferred to a selective regeneration medium [4.33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 5 mg/mL CuSO$_4$, 1 mg/mL benzylaminopurine (BAP), 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin] at 26 °C, during 14 days with 16 h photoperiod. The transgenic events were kept in medium without fitohormones (4.33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin) to induce growth and rooting. Plants transformed with pGVG empty vector and wild-type plants were used as negative controls.

5.3.4. Selection of transgenic events

Putative transgenic lines that survived to neomycin phosphotransferase (NPTII) selection were analyzed for transgene overexpression using qRT-PCR. Approximately twenty independent events from each gene were selected for initial screening, using a pool of leaves from three plants. Wild type and empty pGVG transformed plants (5 events) were used as control. The qRT-PCR analysis followed the methods previously described, except here it was used the enzyme iScript (Biorad), cDNA (dilution 1:20) and guanidine reagent for RNA extraction (Logemann, 1987). Gene relative quantification was performed using $2^{\Delta\Delta CT}$ method and the endogenous polyubiquitin gene (PUB) (SCCCST2001G02.g) was used as internal control. The same primers used for RNA-Seq confirmation were used here. After screening of putative transgenic lines, three events with higher gene expression were selected for further analysis. For these events, qRT-PCR was repeated using leaves from three biological replicates (three individual plantlets) in technical triplicate.

5.3.5. Drought tolerance assay in transgenic sugarcane

The selected transgenic events were transferred to 415 mL plastic pots containing Tropstrato HT Hortaliça (VidaVerde, Mogi Mirim, SP) and expanded vermiculite (1:1), kept in culture room at 25 °C and photoperiod 12h for two weeks and moved to the greenhouse for two more weeks, to acclimatization. After that, plants were
transferred to 18 L individual pots equalized with a mixture of latosol, Tropstrato HT Hortaliça (VidaVerde, Mogi Mirim, SP) and expanded vermiculite (65, 30 and 5%, respectively) and kept under normal irrigation for 3 months.

Drought stress assay were performed with four genes (NRX, DNAJ, HIPP and HP) and WT as control, using a randomized complete block design. The experiment was divided in four independent blocks, each one corresponding to one gene. Each block contained 40 plants: five plants to each event (three events per gene and WT) and treatment (irrigated and drought). The irrigated treatment refers to plants kept at pot capacity (PC - water content) 80% and the drought treatment, at PC 30%. Soil water percentage was calculated collecting 10 soil samples. This material was dried in drying oven at 70 °C for five days and then soaked with water until weight stabilization (PC 100%). The average weights were used to determined soil water percentage under PC 100%.

On day one of the assay, sugarcane plants were soaked with water to achieve PC 100%. The pots weights average was measured and the weights for irrigated and drought treatments were inferred. From this point, the watering was controlled in the irrigated group at PC 80% and irrigation was suspended in the drought group until achieve PC 30%, being maintained in this level, until plants show signals of severe stress (photosynthesis close to zero and significant differences between irrigated and drought group). After that, drought plants were rehydrated for 5-9 days.

The effects of transgene overexpression on drought tolerance were evaluated by measuring several parameters: gas exchange, water relative content (RWC), chlorophyll content (SPAD value), biometric factors, shoot and root biomass and root development, comparing the control and transgenic groups under normal and stressed conditions, as described bellow.

5.3.5.1. Gas exchange parameters

Measurements of gas exchange, i.e., photosynthetic rate (A), stomatal conductance (gs) and transpiration rate (E), were taken during the experiment, using a portable photosynthesis system (LCi, ADC BioScientific Ltd.) with ambient CO₂ concentration and light intensity ranging from 800 to 1200 µmol m-2 s-1. The data were taken in the middle portion of leaf +1, from 9:00 am to 01:00 pm.
5.3.5.2. Relative water content and chlorophyll content

The leaf relative water content (RWC) was determined in the last day of drought and rehydration using samples of leaf +2 and +1, respectively. Five 7 mm diameter leaf discs were collected from the middle portion of the leaf without midrib, immediately weighted (fresh weight - FW) and kept in distilled water overnight. Water excess of leaf discs were removed with filter paper and the turgid weight (TW) was recorded. Then, samples were dried at 60 °C for 48 h and the dry weight (DW) was measured. Leaf RWC percentage was calculated using the equation: \[ \text{RWC} = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100 \] (Barrs and Weatherley, 1962).

Chlorophyll content estimation was performed at the end of drought and rehydration in the leaf +2 and +1, respectively, using a chlorophyll meter SPAD-502Plus (Konica Minolta, Japan). Three measurements in the middle of the leaf (without midrib) were taken for each plant and the average was used in the analyses.

5.3.5.3. Biometry and biomass

Biometric agronomic traits considered yield components (stalk number, height and circumference) were taken at the beginning of the assay, end of drought and end of rehydration. Stalk circumference was measured in the base of the plant using a measuring tape and the height was considered from base until leaf +1 insertion. The biometric data were plotted as growth rates during the stress assay.

By the end of rehydration period, roots were carefully removed from the soil, washed and photographed for further root development analysis. The shoot and root of all plants were harvested, weighted (fresh weight) and dried at 60 °C for 15 days to obtain biomass. Samples of fresh root and leaf tissues (leaf +1) were collected for further biochemical and molecular analyses. Leaf +2 was collected at the end of drought stress.

5.3.5.4. Statistical analysis

Drought stress test was performed with five biological replicates. However, two outliers were identified with GraphPad Outlier calculator (Grubb's test) and excluded from statistical analysis. The data were evaluated using ANOVA (p-value < 0.05), followed by a least significant difference test (LSD, p-value < 0.05) to compare means. The analyses were conducted using the package Agricolae in R software.
5.4. Results and Discussion

5.4.1. Selection of transgenic events

Expression level of transgenic events was evaluated using qRT-PCR. For the first screening, most of the lines presented gene expression higher than control (empty vectors) (Figure 30). Three events of each gene with highest induction levels were selected for further analysis. The *ScHIPP-OEI* line was selected despite not having the highest induction, because presented more tillers for drought experiment. For each gene, two graphs are presented, corresponding to individual 96-well qPCR plates, analyzed separately with the respective controls. Five events transformed with the pGVG empty vector were evaluated and the one with the greatest expression was considered as control (fold change = 1) in the $2^{\Delta\Delta CT}$ calculation in order to avoid overestimating transgene expression.
Figure 30. Expression analysis of putative transgenic sugarcane events via qRT-PCR. (A, B) ScHIPP, (C, D) ScDNAJ, (E, F) ScNRX and (G, H) ScHP. Arrows indicate the selected events. pGVGs represent the five events of the empty vector plants.

In the final screening (Figure 31), the three events in biological triplicates were confirmed for overexpression using WT plants as control, the same control used in the drought stress test (the choice of WT control over the empty vector follows the pattern of most publications in this area).
Figure 31. Expression level of three independent transgenic events selected for drought stress assay, using qRT-PCR. (A) ScDNAJ, (B) ScHP, (C) ScNRX and (D) ScHIPP. Data represent the mean of three biological replicates. Bars indicate the standard error (n=3). WT was used as control for relative expression (fold change = 1).

5.4.2. Drought stress assay in transgenic sugarcane

According to Chaves et al. (2003), drought tolerance trait is related with the maintenance of plant functions under water deficit and the ability to fast recover after stress, restoring plant water status and function. Taking this into account, transgenic sugarcane plants overexpressing independently the four genes were evaluated during drought stress and rehydration (Figure 32), using phenotypic, physiological, biometric and biomass analyses in order to find characteristics related with improved drought tolerance.
Figure 32. Representative image of sugarcane transgenic plants (ScHIPP-OE) under drought stress and rehydration. (a, c) Plants kept under well-watered conditions. (b) Plants after 21 days of stress. (d) Plants after 5 days of rewatering.

5.4.2.1. Gas exchange parameters

In general, the results showed a similar behavior of sugarcane plants regarding gas exchanges under drought (Figures 33, 34, 35, and 36). At the beginning of the assay, gas exchange parameters are very close between the irrigated and drought groups, with some differences caused by the events. Increasing time and intensity of water deficit there was a decline in photosynthesis rate (A), stomatal conductance (gs) and transpiration (E). Under water deficit, plants trigger stomatal closure in order to avoid water loss by transpiration, which results in decreased CO₂ availability for
photosynthesis (Cornic et al., 1992; Machado et al., 2009), causing the simultaneous gas exchange reductions observed in our results.

As drought becomes more severe, non-stomatal limitations take place, inhibiting CO₂ assimilation by affecting photosynthetic machinery (Keck and Boyer, 1974; Farquhar and Sharkey, 1982; Irigoyen et al., 1992). During drought and rehydration, plant responses will depend on stress intensity and duration, as well as species and genotype (Campos et al., 2004; Xu et al., 2010; Lopes et al., 2011; Reis et al., 2014). In cases where drought is very intense, irreversible damages may occur making it impossible full recovery after rehydration (Xu et al., 2010) as noticed for WT stressed plants in the $ScHIPP$ group, while for transgenic $ScHIPP$-OE plants $A$, $gs$ and $E$ return to control (irrigated) levels, indicating complete recovery (Figures 32 and 33).

Sugarcane plants kept at PC 80% (irrigated group) maintained their gas exchange parameters relatively constant within a variation range. Since we are focusing on drought response, the differences between the transgenic and control groups under irrigated conditions were not considered. Additionally, as previously mentioned, a transgene was considered relevant when at least two of the three events present similar results.

For the $HIPP$ gene (Figure 33), $ScHIPP$-OE3 plants presented significant higher foliar photosynthetic rate compared to WT plants from day 8 (7 days after drought treatment, DAT) until day 22 (when all plants were irrigated) and during all rehydration time. These periods were also accompanied by significant increase in stomatal conductance and transpiration during almost all the time. Drought-stressed $ScHIPP$-OE1 and $ScHIPP$-OE2 events also recovered better than WT after rewatering, and $ScHIPP$-OE1 plants still presented increase in $A$ and $gs$ at day 20 (19 DAT).

For sugarcane plants overexpressing $NRX$ gene (Figure 34), WT plants showed higher CO₂ assimilation rate ($A$) and stomatal conductance at the beginning of drought stress compared to transgenic lines. However, $ScNRX$-OE1 and $ScNRX$-OE3 events presented better photosynthetic performance as drought stress continued. Significant improve in photosynthesis was observed in all $ScNRX$-OE plants at the final periods of rehydration.

For the $HP$ gene (Figure 35), $ScHP$-OE2 plants demonstrated significant higher levels of $A$, $gs$ and $E$ in relation to WT during most of the time under drought stress, while $ScHP$-OE1 and $ScHP$-OE3 lines showed higher CO₂ assimilation rate than WT plants in specific times during the stress (days 8 and 21; and day 6, respectively). Four
days after rewatering (day 31), all plants overexpressing HP gene presented statistically significant increase in A, gs and E compared with WT.

In plants overexpressing DNAJ (Figure 36), the ScDNAJ-OE2 line showed significant higher levels of A than WT plants from the beginning (day 6) until the end of stress (day 18, exception for day 13), almost always accompanied by improved gs and E. ScDNAJ-OE3 event presented higher CO₂ assimilation at days 6 and 17 (after 5 and 16 days of water deficit, respectively).

These results provide evidence that drought-stressed transgenic sugarcane plants overexpressing HIPP, NRX and HP genes recover better and faster than WT plants during rehydration, after a long period of severe stress. ScHIPP-OE3, ScDNAJ-OE2 and ScHP-OE2 plants were able to maintain CO₂ assimilation rate higher than WT during almost all periods of water deficit. For other events, such as, ScHIPP-OE1, ScNRX-OE1 and 3, ScDNAJ-OE3 and ScHP-OE1 and 3 the photosynthesis level was higher than WT during specific times. Taken all together, these results suggest that the overexpression of these genes can attenuate drought effects and facilitate the recuperation of photosynthetic metabolism after drought stress.
**Figure 33.** Gas exchange analysis in sugarcane plants overexpressing *HIPP* gene submitted to normal irrigation, drought stress and rehydration. (a), (d), (g) Photosynthesis rate - *A*. (b), (e), (h) Stomatal conductance - *gs*. (c), (f), (i) Transpiration rate - *E*. The measures were taken for three transgenic events: (a), (b), (c) *ScHIPP-OE1* (H1); (d), (e), (f) *ScHIPP-OE2* (H2) and (g), (h), (i) *ScHIPP-OE3* (H3) under drought (D) and irrigated (I) treatment. PC 100% represents water capacity of 100%. WT plants were used as control. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in drought-stressed and rehydrated plants. Statistical differences between transgenic and control group under normal irrigation were not considered. Data are presented as mean and error bars represent the standard error (n=3).

**Figure 34.** Gas exchange analysis in sugarcane plants overexpressing *NRX* gene submitted to normal irrigation, drought stress and rehydration. (a), (d), (g) Photosynthesis rate - *A*. (b), (e), (h) Stomatal conductance - *gs*. (c), (f), (i) Transpiration rate - *E*. The measures were taken for three transgenic events: (a), (b), (c) *ScNRX-OE1* (N1); (d), (e), (f) *ScNRX-OE2* (N2) and (g), (h), (i) *ScNRX-OE3* (N3) under drought (D) and irrigated (I) treatment. PC 100% represents water capacity of 100%. WT plants were used as control. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in drought-stressed and rehydrated plants. Statistical differences between transgenic and control group under normal irrigation were not considered. Data are presented as mean and error bars represent the standard error (n=3).
Figure 35. Gas exchange analysis in sugarcane plants overexpressing HP gene submitted to normal irrigation, drought stress and rehydration. (a), (d), (g) Photosynthesis rate - $A$. (b), (e), (h) Stomatal conductance - $gs$. (c), (f), (i) Transpiration rate - $E$. The measures were taken for three transgenic events: (a), (b), (c) $ScHP$-OE1 (P1); (d), (e), (f) $ScHP$-OE2 (P2) and (g), (h), (i) $ScHP$-OE3 (P3) under drought (D) and irrigated (I) treatment. PC 100% represents water capacity of 100%. WT plants were used as control. Asterisks indicate statistical differences [ANOVA, followed by LSD test ($p$-value < 0.05)] between transgenic and control group in drought-stressed and rehydrated plants. Statistical differences between transgenic and control group under normal irrigation were not considered. Data are presented as mean and error bars represent the standard error (n=3).
**Figure 36.** Gas exchange analysis in sugarcane plants overexpressing DNAJ gene submitted to normal irrigation, drought stress and rehydration. (a), (d), (g) Photosynthesis rate - $A$. (b), (e), (h) Stomatal conductance - $g_s$. (c), (f), (i) Transpiration rate - $E$. The measures were taken for three transgenic events: (a), (b), (c) ScDNAJ-OE1 (D1); (d), (e), (f) ScDNAJ-OE2 (D2) and (g), (h), (i) ScDNAJ-OE3 (D3) under drought (D) and irrigated (I) treatment. PC 100% represents water capacity of 100%. WT plants were used as control. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in drought-stressed and rehydrated plants. Statistical differences between transgenic and control group under normal irrigation were not considered. Data are presented as mean and error bars represent the standard error (n=3).

### 5.4.2.2. Relative water content and chlorophyll content

Leaf relative water status and chlorophyll levels are physiological parameters related with drought tolerance (Silva et al., 2007). These traits were evaluated in sugarcane transgenic plants under water deficit conditions. After 21 days of drought, ScHIPP-OE2 and 3 plants showed relative water content (RWC) of 53 and 69%, respectively, significantly higher than WT (~17%). Additionally, WT plants reduced on average 79% of RWC under drought in relation to well-watered treatment, while for ScHIPP-OE2 and 3 events, the reduction was less than half of this value (35 and 11%,
respectively). After nine days of rewatering, only the drought-stressed *ScHIPP-OE3* line kept higher RWC (~85%) compared to WT (~49%), while *ScHIPP-OE2* event presented values lower than WT. Despite *ScHIPP-OE1* plants presented higher RWC (~69%) than WT in rehydrated group, this difference was not statistically significant. For the irrigated treatment, no statistical differences were observed between the transgenic and control groups (Figure 37a, b).

Leaf water status of *ScNRX-OE1* (~89%) and 3 (~68%) plants was maintained at levels significantly elevated compared to control (~15%) after 17 days of drought stress, with lower RWC reduction under drought (0.5 and 29%, respectively) than WT (83%). However, drought-stressed WT plants recovered better with rehydration reaching *ScNRX-OE1* and 2 levels and overcoming RWC of *ScNRX-OE3* (Figure 38a, b).

Sugarcane *ScHP-OE1* plants presented significant higher RWC than WT after 26 days of water deficit and six days of rewatering. *ScHP-OE2* event showed better recuperation after rehydration overcoming WT water content (Figure 39a, b). For the DNAJ gene (Figure 40a, b), under drought conditions and after rewatering, *ScDNAJ-OE3* event kept water content below the WT level, while *ScDNAJ-OE1* plants showed internal water similar to WT under stress, but decreased after rehydration. For *ScDNAJ-OE* no significant differences were noticed.

Drought tolerant sugarcane plants tend to present higher RWC under water limitation. This parameter indicates cell hydration level, which is essential to maintain plant metabolism and growth under water deficit (Silva et al., 2007; Silva et al., 2011). Our results demonstrated that sugarcane *ScNRX-OE* plants have enhanced capacity to retain water under drought, which may contributes for better photosynthesis performance under drought and after rehydration observed in these plants. The overexpression of *ScHP* gene showed to be related with improved water retention under rewatering conditions, which is accompanied by higher CO₂ assimilation rate in the same environment. Sugarcane *ScHIPP-OE* plants showed to be able to maintain higher RWC levels under stress, suffering less during stress, thus enabling better photosynthetic recuperation capacity after rewatering.

The determination of chlorophyll content using SPAD values is an efficient parameter to infer drought tolerance (Silva et al., 2013). The correlation between chlorophyll levels and genotypes classification is evidenced in several works that show higher SPAD value in drought tolerant sugarcane genotypes compared with less tolerant
ones under water deficit conditions (Jangpromma et al., 2010; Silva et al., 2007; Silva et al., 2013).

All sugarcane *ScHIPP-OE* plants showed significant increase in chlorophyll content compared to WT under drought stress and rehydration (Figure 37c, d). Under stress, SPAD values of transgenic events were above 25 units, dropping 12 to 15% from irrigated to drought treatment, while WT plants presented SPAD index below 10 units and reduction of 78% between treatments.

Besides high RWC, *ScNRX-OE1* (~23 units) and 3 (~23 units) plants also presented significantly higher estimated leaf chlorophyll content than WT (~10 units) under water deficit and relative reductions of 19% (event 1) and 39% (event 3), lower compared with WT (73%) (Figure 38c). This was accompanied by elevate photosynthesis rates during stress (Figure 34).

For the other 2 genes (*ScHP* and *ScDNAJ*), there was not promising results regarding transgenic plants enhanced chlorophyll index, showing SPAD values not statistically different or lower than WT under normal irrigation, drought and rehydration (Figures 39c, d and 40c, d).

Drought stress results in chlorophyll degradation which affects plant photosynthetic capacity (Arjenaki et al., 2012). Transgenic sugarcane plants overexpressing *ScHIPP* and *ScNRX* genes presented better chlorophyll retention under drought and *ScHIPP-OE* plants also increased chlorophyll content after rehydration, which helps to keep chloroplast functionality, reducing stress damage and assisting photosynthesis recovery after drought (Augustine et al., 2015). These high SPAD indexes may contribute for improved photosynthesis observed in *ScNRX-OE* plants under prolonged water deficit and in *ScNRX-OE* and *ScHIPP-OE* plants after rewatering.

Very low levels of chlorophyll such as evidenced in stressed WT plants from *HIPP* group (Figure 37 c, d) together with gas exchange decline (Figure 33) show profound and irreversible drought effects in the plants physiology, that were no able to restore their stomatal conductance, photosynthetic activity, transpiration and chlorophyll content after rehydration.
Figure 37. Physiological analysis of sugarcane plants overexpressing the ScHIPP gene. Leaf relative water content (RWC) (a, b) and chlorophyll content (SPAD index) (c, d) in sugarcane plants under drought (a, c) and rehydration conditions (b, d). Three transgenic events were evaluated: ScHIPP-OE1, 2 and 3. WT plants were used as control. Rehydrated treatment represents drought-stressed plants after rewatering. Percentages represent reductions/increases in the parameters under drought/rewatering compared to well-watered condition for each event. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).
Figure 38. Physiological analysis of sugarcane plants overexpressing the ScNRX gene. Leaf relative water content (RWC) (a, b) and chlorophyll content (SPAD index) (c, d) in sugarcane plants under drought (a, c) and rehydration conditions (b, d). Three transgenic events were evaluated: ScNRX-OE1, 2 and 3. WT plants were used as control. Rehydrated treatment represents drought-stressed plants after rewatering. Percentages represent reductions/increases in the parameters under drought/rewatering compared to well-watered condition for each event. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).
Figure 39. Physiological analysis of sugarcane plants overexpressing the ScHP gene. Leaf relative water content (RWC) (a, b) and chlorophyll content (SPAD index) (c, d) in sugarcane plants under drought (a, c) and rehydration conditions (b, d). Three transgenic events were evaluated: ScHP-OE1, 2 and 3. WT plants were used as control. Rehydrated treatment represents drought-stressed plants after rewatering. Percentages represent reductions/increases in the parameters under drought/rewatering compared to well-watered condition for each event. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).
**Figure 40.** Physiological analysis of sugarcane plants overexpressing the *ScDNAJ* gene. Leaf relative water content (RWC) (a, b) and chlorophyll content (SPAD index) (c, d) in sugarcane plants under drought (a, c) and rehydration conditions (b, d). Three transgenic events were evaluated: *ScDNAJ-OE1*, 2 and 3. WT plants were used as control. Rehydrated treatment represents drought-stressed plants after rewatering. Percentages represent reductions/increases in the parameters under drought/rewatering compared to well-watered condition for each event. Asterisks indicate statistical differences [ANOVA, followed by LSD test (p-value < 0.05)] between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).

### 5.4.2.3. Biometry and biomass

Biomass and biometric traits such as stalk height, circumference, number and weight were evaluated during the experiment once they are considered yield indexes (Zhang *et al*., 2006). For stalk height and circumference we considered only the main stalk data.

For all genes evaluated there were clear differences in the growth rate in plant height between irrigated and drought groups under water deficit, showing that stress reduces stalk growth (Figures 41a, 42a, 43a and 44a). Plants subjected to drought inhibit cell division and elongation processes (Machado *et al*., 2009), making stalk height one of the most affected traits (Inman-Bamber *et al*., 2008; Ferreira *et al*., 2017; Silva *et al*., 2008), which is in accordance with our results. When sugarcane plants overexpressing the genes *ScHIPP*, *ScNRX*, *ScHP* and *ScDNAJ* are grown under PC 30%, no statistically significant increase in stalk height growth rate were observed in transgenic plants.
comparing with WT during water deficit stress (Figures 41a, 42a, 43a and 44a). This is also true for rehydration period (Figures 41b, 42b, 43b and 44b).

ScHP-OE1 and 2 sugarcane plants increased growth rate in stalk circumference under drought compared with WT plants, which did not growth at all. ScHP-OE1 line also kept this high growth level during rehydration time (Figure 41b, d). For other genes, just few alterations in individual events were noticed for this biometric trait. For example, in NRX-OE plants, only ScNRX-OE1 exhibited increased stalk circumference growth rate under drought, but decreased rate for stalk height and circumference after rehydration (Figure 42). ScHIPP-OE3 sugarcane plants under drought stress and after rewatering presented higher growth in stalk circumference compared with WT, however, no significant differences were seen for other two events (Figure 41). For DNAJ gene, only the event 2 improved stalk circumference growth rate during rewatering (Figure 44).

![Figure 41](image)

**Figure 41.** Growth rate of biometric traits in sugarcane transgenic plants overexpressing the *ScHIPP* gene. Three independent events were subject to drought stress (a, b) and rehydration conditions (c, d). Growth rate of main stalk height: a, c; Growth rate of main stalk circumference: b, d. WT plants were used as control. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3). GR: growth rate.
Figure 42. Growth rate of biometric traits in sugarcane transgenic plants overexpressing the Sc:NRX gene. Three independent events subjected to drought stress (a, b) and rehydration conditions (c, d). Growth rate of main stalk height: a, c; Growth rate of main stalk circumference: b, d. WT plants were used as control. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3). GR: growth rate.
Figure 43. Growth rate of biometric traits in sugarcane transgenic plants overexpressing the *ScHP* gene. Three independent events were subjected to drought stress (a, b) and rehydration conditions (c, d). Growth rate of main stalk height: a, c; Growth rate of main stalk circumference: b, d. WT plants were used as control. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3). GR: growth rate.
Figure 44. Growth rate of biometric traits in sugarcane transgenic plants overexpressing the ScDNAJ gene. Three independent events were subjected to drought stress (a, b) and rehydration conditions (c, d). Growth rate of main stalk height: a, c; Growth rate of main stalk circumference: b, d. WT plants were used as control. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3). GR: growth rate.

Regarding the number of tillers, there were no relevant results involving at least two transgenic events for the four sugarcane genes evaluated (Figures 45-48). ScHIPP-OE3 plants start the experiment with bigger tiller average number compared with WT in both treatments: well-watered and drought. These values were kept high during throughout the stress, while after rehydration only irrigated ScHIPP-OE3 plants remained average above WT control. The number of new tillers significantly increased for ScHIPP-OE1 during water deficit and for ScHIPP-OE3 plants under normal irrigation (Figure 45d). For HP gene (Figure 47), the ScHP-OE1 line showed enhanced number of tillers before, during and after the stress, for both treatments. The ScHP-OE3 event developed more tillers during rehydration period. No statistical differences were seen in sugarcane plants overexpressing the genes NRX and DNAJ regarding the average number of tillers and new tillers developed during the experiment (Figures 46 and 48, respectively). Since only individual events from each gene showed interesting results for these parameters, no relevant conclusions can be taken from the role of theses genes in the tillers number under stress.
Figure 45. Tillers number of sugarcane plants overexpressing the \textit{ScHIPP} gene under normal irrigation, drought and rehydrated conditions. Tillers number average in: (a) the beginning of the assay, (b) end of water deficit and (c) end of rehydration. (d) Number of new tillers arised from pre-drought until the end of stress. (e) Number of new tillers arised from the end of drought until rehydration. Asterisks indicate statistical differences (ANOVA, followed by LSD test, $p < 0.05$) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error ($n=3$).
Figure 46. Tillers number of sugarcane plants overexpressing the ScNRX gene under normal irrigation, drought and rehydrated conditions. Tillers number average in: (a) the beginning of the assay, (b) end of water deficit and (c) end of rehydration. (d) Number of new tillers arised from pre-drought until the end of stress. No new tillers arised from the end of drought until rehydration. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).
Figure 47. Tillers number of sugarcane plants overexpressing the *ScHP* gene under normal irrigation, drought and rehydrated conditions. Tillers number average in: (a) the beginning of the assay, (b) end of water deficit and (c) end of rehydration. (d) Number of new tillers arised from pre-drought until the end of stress. (e) Number of new tillers arised from the end of drought until rehydration. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).
Figure 48. Tillers number of sugarcane plants overexpressing the ScDNAJ gene under normal irrigation, drought and rehydrated conditions. Tillers number average in: (a) the beginning of the assay, (b) end of water deficit and (c) end of rehydration. (d) Number of new tillers arised from pre-drought until the end of stress. No new tillers arised from the end of drought until rehydration. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are presented as mean and error bars represent the standard error (n=3).

The analysis of biomass and root length parameters indicated a significant increase in total biomass (events 1 and 3) for sugarcane ScHIPP-OE plants under well-watered conditions with no penalties under drought (Figure 49). ScHIPP-OE plants (events 1 and 2) presented higher shoot dry weight under normal irrigation, but lower shoot biomass accumulation (3 events) under rehydrated conditions compared with WT. No statistically significant differences were observed for root biomass after rehydration and for root length under normal irrigation in plants overexpressing ScHIPP. The ScHIPP-OE-3 event showed increased root biomass under well-watered conditions and decreased root length after rewatering.

ScNRX plants (events 1 and 3) had higher root biomass under well-watered condition, and, as observed for ScHIPP-OE plants, no differences in relation to WT were observed under drought stress (Figure 50). Elevate shoot dry weight was seen in ScNRX-OE2 plants compared with WT for both treatments (irrigated and rehydrated). ScNRX-OE1 plants developed statistically larger roots with 12% increase in length from irrigated to rehydrated treatment. Additionally, ScNRX-OE3 line improved root length (5%) between treatments compared with WT plants (decrease in 13%) (Figure 50d).
For *ScDNAJ* (Figure 52), despite no statistical differences in total biomass (events 1 and 3) and root maximum length (3 events) compared with WT, the transgenic plants presented a trend of lower reduction in these parameters comparing both treatments, which was also observed for some plants overexpressing *ScHP* (Figure 51).

Other statistical differences observed in biomass and root length for these genes did not show relevant results, since they involve individual events or are normally related with reduced transgenic performance.

**Figure 49.** Biomass and root length in transgenic plants overexpressing the *ScHIPP* gene. Alterations in shoot (a), root (b) and total (c) biomass and root length (d) in sugarcane plants under normal irrigation and drought followed by rehydration. Percentages represent reductions/increases in the parameters under rehydration compared to well-watered condition for each event. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are mean and error bars represent the standard error (n=3).
Figure 50. Biomass and root length in transgenic plants overexpressing the ScNRX gene. Alterations in shoot (a), root (b) and total (c) biomass and root length (d) in sugarcane plants under normal irrigation and drought followed by rehydration. Percentages represent reductions/increases in the parameters under rehydration compared to well-watered condition for each event. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are mean and error bars represent the standard error (n=3).
Figure 51. Biomass and root length in transgenic plants overexpressing the ScHP gene. Alterations in shoot (a), root (b) and total (c) biomass and root length (d) in sugarcane plants under normal irrigation and drought followed by rehydration. Percentages represent reductions/increases in the parameters under rehydration compared to well-watered condition for each event. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are mean and error bars represent the standard error (n=3).

Figure 52. Biomass and root length in transgenic plants overexpressing the ScDNAJ gene. Alterations in shoot (a), root (b) and total (c) biomass and root length (d) in sugarcane plants under normal irrigation and drought followed by rehydration. Percentages represent reductions/increases in the parameters under rehydration.
compared to well-watered condition for each event. Asterisks indicate statistical differences (ANOVA, followed by LSD test, p < 0.05) between transgenic and control group in the same treatment. Data are mean and error bars represent the standard error (n=3).

Under more severe drought stress, water preservation strategies are more advantageous. These traits involve to reduce water loss by decreasing stomatal conductance that consequently reduces other gas exchanges, such as photosynthesis and transpiration, or to increase water uptake by developing root system to improve soil water capture (Lopes et al., 2011). This way plants can face stress without serious damages, allowing a better recovery after stress. However, these strategies can compromise plant biomass accumulation, since by closing stomata to avoid transpiration the CO$_2$ assimilation is also reduced, dropping the production of photoassimilates (Araus et al., 2008; Blum, 2009; Blum, 2011; Lopes et al., 2011). This mechanism may help to explain the biomass results obtained.

Root characteristics are important to determine drought tolerance. Under water deficit, sugarcane plants tend to develop deeper and larger roots which improve water uptake, favoring the maintenance of adequate plant water status and less injury (Smith et al., 2005; Park et al., 2005; Ferreira et al., 2017). According to Lopes et al. (2011), the ability of root system to capture water is more related with their spatial distribution in the soil and not your biomass, which might explain the absence of differences in root biomass and length, once we did not access root distribution in soil.
6. CHAPTER 4 - pGVG: a new vector for sugarcane transformation

6.1. Scientific article

pGVG: a new Gateway-compatible vector for transformation of sugarcane and other monocot crops

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Abstract

The successful development of genetically engineered monocots using Agrobacterium-mediated transformation has created an increasing demand for compatible vectors. Here, we have developed a new expression vector, pGVG, for efficient transformation and expression of different constructs for gene overexpression and silencing in sugarcane. We modified the pCAMBIA2300 binary vector by adding Gateway recombination sites for fast gene transfer between vectors and the maize polyubiquitin promoter Ubi-1 (ZmUbi1), which is known to drive high gene expression levels in monocots. Transformation efficiency using the pGVG vector reached up to 14 transgenic events per gram of transformed callus. Transgenic plants expressing the β-glucuronidase (GUS) reporter gene in pGVG showed high levels of GUS activity. qRT-PCR evaluations demonstrated success for both overexpression and hairpin-based silencing cassettes. Therefore, pGVG is suitable for plant transformation and subsequent applications for high-throughput production of stable transgenic sugarcane.
The use of an expression cassette based on the ZmUbi1 promoter opens the possibility of using pGVG in other monocot species.

**Keywords:** monocots, sugarcane, vector, Gateway technology, genetic transformation.

Sugarcane (*Saccharum* spp. L.) is one of the most economically important crops due its bioenergetic potential and is recognized as a source of renewable energy (Gianotto *et al.*, 2011). Genetic transformation methods are powerful biotechnological tools to improve yield and *Agrobacterium*-mediated transformation, initially restricted to dicots, has been successfully used in many monocots plants (Gelvin, 2003; Shrawat and Lörz, 2006; Hiei *et al.*, 2014; Slamet-Loedin *et al.*, 2014; Mayavan *et al.*, 2015). This method became one of the main approaches used to produce transgenic plants due to its simplicity, low-cost equipment needs and delivery of one or few copies of larger gene insertions. Furthermore the transfer DNA has greater stability, favoring its heritability in comparison to other transformation methods (Elliott *et al.*, 1998; Hansen and Wright, 1999; Travela *et al.*, 2005). Despite that, the availability of vector systems compatible for monocots is limited and most expression vectors are based on the CaMV 35S promoter, which generates lower expression levels in monocots (Mann *et al.*, 2012). There are other vectors for monocot transformation, however they are limited to gene silencing (Karimi *et al.*, 2002), lack epitope tags for protein detection/isolation (Mann *et al.*, 2012), present ZmUbi1 promoter driving both gene of interest and selection cassettes, which can cause gene silencing (De Wilde *et al.*, 2000; Butaye *et al.*, 2005; Himmelbach *et al.*, 2007; Mann *et al.*, 2012) or show regeneration problems due to the use of hygromycin selection (Joyce *et al.*, 2010).

In this study, we describe the construction and functional validation of a vector (pGVG) for gene functional analysis in sugarcane and other monocots. The pGVG (Figure 1) is based on the backbone from pCAMBIA2300 binary vector (CAMBIA, Canberra, Australia) that possesses the *NPTII* gene as selection marker, one of the most efficient markers for transgenic sugarcane callus selection and certified for use in commercial transgenic species (Zhangsun *et al.*, 2007; Joyce *et al.*, 2010). The pGVG vector presents a Gateway cassette (*att*R1-Cm*-ccdB-*att*R2) under control of ZmUbi1 promoter and CaMV 35S terminator for gene overexpression or silencing. By incorporating the Gateway cloning technology, pGVG allows a fast and easy exchange of DNA fragments between vectors, without using restriction endonucleases and ligases.
from traditional cloning. Target DNA flanked by **attL** recombination sites are easily transferred to **attR** site-compatible destination vectors using the LR clonase enzyme. In this process, the lethal **ccdB** gene is moved from destination plasmid to entry vector, facilitating the selection of recombinant constructions (Katzen, 2007). Entry vectors such as pCR8GW TOPO (Invitrogen, Life Technologies, USA), with resistance to spectinomycin, are suitable for direct recombination with pGVG. In cases where both entry and pGVG destination vectors have the same bacterial selectable marker it is indicate to use the PCR product flanked by the recombination sites to assure high efficiency of recombination. The **ZmUbi1** promoter was cloned in pGVG with the 5’ untranslated region and the first intron of **Ubi-1** gene, which is associated with enhanced transgene expression in monocots (Callis et al., 1988; Bruce and Quail, 1990; McElroy et al., 1990; Vasil et al., 1993; Christensen and Quail, 1996). This promoter allows high levels of gene expression or RNAi-mediated suppression in most tissue types during most stages of plant development (Cornejo et al., 1993; Mann et al., 2012), being used to produce stable transgenic monocots plants (Gallo-Meagher and Irvine 1996; Ma et al., 2000; Kinkema et al., 2014). Additionally, pGVG presents a FLAG-tag sequence (DYKDDDDK) inserted upstream the CaMV 35S terminator for C-terminal fusion with the target protein.

To produce transgenic lines, sugarcane plants (SP80-3280) were cultivated in greenhouse (IAC, Ribeirão Preto, Brazil) for six months and the meristematic region from shoot apex was used to generate explants. This material was cultivated in MS maintenance medium [4.33 g/L MS salts (Murashige and Skoog, 1962), 1 mL/L MS vitamins, 0.15 g/L citric acid, 0.5 g/L casein hydrolysate, 25 g/L sucrose, 12 g/L mannitol, 100 mg/L proline, 3 mg/L 2-4 dichlorophenoxyacetic acid (2,4-D) and 2.8 g/L phytage] at 26 °C in the dark, until the generation of embryogenic calli. Several constructs based on pGVG (see below) were transferred to *Agrobacterium tumefaciens* (EHA105 strain) by heat shock. Bacterial cultures were incubated with sugarcane calli under vacuum pressure for five minutes and transferred to co-cultivation medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.15 g/L citric acid, 25 g/L sucrose and 3.5 g/L phytage) at 22 °C, in the dark for 3 days. After that, the calli were kept in resting medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.15 g/L citric acid, 25 g/L sucrose, 100 mg/L proline, 2.8 g/L phytage and 200 mg/mL timentin) at 26 °C, in the dark for 6 days. Following the resting phase, the transformed calli were transferred to a selective regeneration medium [4.33 g/L MS
salts, 1 mL/L MS vitamins, 25 g/L sucrose, 5 mg/mL CuSO₄, 1 mg/mL benzylaminopurine (BAP), 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin] at 26 °C, during 14 days with 16 h photoperiod. The transgenic events were kept in medium without fitohormones (4.33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin) to induce growth and rooting. Plants transformed with pGVG empty vector and wild-type plants were used as negative controls.

The functionality of pGVG was evaluated using the GUS reporter gene. The coding sequence of GUS gene was amplified from the construction pENTR™-gus (Invitrogen, Life Technologies, USA), using specific primers designed in the attL Gateway recombination sites. The purified PCR product was recombined with pGVG using Gateway® LR Clonase® II enzyme (Invitrogen, Life Technologies, USA). The resulting vector was introduced into Agrobacterium and used for calli transformation. Transgene expression was assessed by GUS histochemical staining (Jefferson, 1987). Strong GUS activity was detected in callus and whole plants (Figure 2), confirming that pGVG is suitable for sugarcane transformation.

To further evaluate the transformation capacity of pGVG, sugarcane genes related with different biological processes were tested using overexpression and RNAi-mediated silencing constructs (Table 1). The vector was able to transform sugarcane plants with genes of different sizes for both construct types. The overall transformation efficiency showed variation, probably reflecting differences in callus quality, culture medium, age and selective subculturing, which affect both transformation and plant regeneration (Păcurar et al., 2008; Basnayake et al., 2011).

Analyses of gene expression were performed through qRT-PCR using gene-specific primers and the polyubiquitin gene (SCCCST2001G02.g) as internal control for normalization (Papini-Terzi et al., 2005) (Figure 3). The results demonstrated that sugarcane genes 1 and 2 were up-regulated in different levels in the transgenic lines when compared with endogenous levels observed in the empty vector control (Figure 3a and b). Additionally, a unique hairpin construction that targets three genes of the same family (genes 15, 16 and 17) silenced each member reaching up to 92% of down-regulation (Figure 3c). These data show that pGVG is able to produce efficient transgene overexpression and suppression of target genes.

Therefore, the combination of adequate plant selectable markers, Gateway technology and stable and strong promoters in the pGVG vector assures effective
transformation and plant regeneration demonstrated by GUS reporter gene expression and qRT-PCR assays. This vector can be used in overexpression and RNAi-mediated silencing of sequences of interest in sugarcane plants that will greatly facilitate the functional characterization of genes. All characteristics incorporated into pGVG certainly will allow it to be used successful in several other monocot species.

ACKNOWLEDGMENTS

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FIGURES

Figure 1. Schematic structure of the pGVG vector. This vector contains the backbone from pCAMBIA2300, modified by the insertion of the ZmUbi1 promoter (including 5’ untranslated exon and first intron) for strong transgene overexpression and the CaMV 35S terminator. The sequences from the Gateway system were inserted between the ZmUbi1 promoter and the CaMV 35S terminator. A FLAG-tag is positioned upstream the terminator to facilitate target protein isolation. The vector also contains the NPTII.
gene as plant selectable marker under control of the enhanced CaMV 35S promoter. Cm\(^r\): chloramphenicol-resistance gene. ccdB: lethal gene. RB: right border. LB: left border.

**Figure 2.** GUS expression on transgenic sugarcane tissues obtained from Agrobacterium-mediated transformation system using the pGVG vector. Transformed (a) and untransformed (b) calli, three weeks after the co-culture period; (c) leaves from a transformed (left) and untransformed (right) plant; (d) transformed (GUS, pGVG empty) and untransformed (Wild Type) plants, 4 months after the co-culture period.
Figure 3. Expression levels of different sugarcane genes induced or repressed in transgenic sugarcane plants using the pGVG vector. Leaves from transgenic plants were used to extract RNA and the transcripts were quantified using RT-qPCR (a) Overexpression of gene 1 (drought stress-related) in three independent lines (E1, E2, and E3) compared with control (pGVG empty). (b) Overexpression of gene 2 (drought stress-related) in three independent lines (E4, E5 and E6) compared with control (pGVG empty). (c) RNAi-mediated suppression of the genes 15, 16 and 17 (development related; triple silencing) in three independent lines (E7, E8 and E9) compared with control (pGVG empty). Data represent the mean of three biological replicates. Bars indicate the standard error. The expression data refer to the transgene and the correspondent endogenous gene levels. The genes named here are the same as described in Table 1.
## TABLES

### Table 1. Transformation efficiency in sugarcane using pGVG.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Type of cassette</th>
<th>Events</th>
<th>Callus (g)</th>
<th>Efficiency</th>
<th>Construct size (bp)</th>
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<tr>
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*OE: overexpression, HS: hairpin silencing; Transformation efficiency expressed as the number of transgenic plants per gram of fresh callus matter.*
6.2. Patent application

**FINAL CONCLUSIONS**

From the results obtained it is possible to conclude that the overexpression of the sugarcane gene *RTNL* increases the survival rate in *Arabidopsis* plants under severe drought, induces alterations in the shoot growth (rosette area and biomass) under mild stress and improves root development (primary root, depth, width and convex hull) in severe stress induced by mannitol, which would help to increase drought tolerance.

The overexpression of *ScNRX* in *Arabidopsis* plants conferred higher survival under severe drought. Additionally, *ScNRX-OE* plants have small rosettes under normal conditions which contributed for a smaller reduction in the rosette area under drought, since smaller plants lose less water. This might also explain why these plants had higher survival levels under severe stress. The *NRX* gene also showed to be related with drought tolerance in transgenic sugarcane plants. Plants overexpressing *ScNRX* are able to maintain increased photosynthesis levels compared with WT under drought stress and after rehydration, as well as higher relative water content and chlorophyll levels under water deficit conditions. Biomass analyses found that *ScNRX* can improve root biomass in sugarcane under irrigated conditions without penalties under water deficit. These agronomic traits showed to contribute for drought tolerance.

The *ScHP* gene is involved with drought tolerance in *Arabidopsis* plants subjected to mild stress, enhancing rosette growth and biomass accumulation which decrease the yield losses in moderate drought environments. Transgenic sugarcane overexpressing this gene also enhanced physiological parameters that contribute for water deficit tolerance. *ScHP-OE* sugarcane plants maintained higher CO₂ assimilation compared with WT during stress and rewatering periods, and recovery water status faster after stress. Biometric analysis revealed that *ScHP* gene is also able to increase stalk circumference under drought stress.

The *ScHIPP* gene showed a role in drought tolerance in *Arabidopsis* and sugarcane plants. In *Arabidopsis*, the overexpression of this gene increases survival rate under severe drought and improved plant shoot growth under normal conditions, producing larger rosette area and increased biomass. In sugarcane, *ScHIPP* accelerated and improved plant recovery after rewatering by increasing photosynthetic capacity. Additionally, *ScHIPP* promoted higher leaf water retention and chlorophyll levels under severe drought, and also improved SPAD values in well-watered conditions. Biomass analysis showed that *ScHIPP* can increase sugarcane total dry weight under normal irrigation without reduction in stress conditions which is also an interesting trait.
The pGVG expression vector was successfully validated in sugarcane by GUS histochemical staining and qRT-PCR. The development of a vector containing the Gateway technology is extremely useful in sugarcane transformation experiments since it allows the insertion of any gene without the need for restriction enzymes and ligases, saving time and reagents. The use of *ZmUbi1* promoter showed to be able to enhance transgene expression in high levels. In addition, the insertion of a flag-tag in the pGVG vector, confirmed by western blot assay, can facilitate further recombinant protein analyses.
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8. ATTACHMENTS
8.1. Attachment 1

INFORMAÇÃO

INFORMAMOS que o projeto CIBio 2007/03 - Genômica Funcional de Plantas, cujo pesquisador responsável é o Prof. Dr. Marcelo Menossi Teixeira, sub-projeto Análise funcional de genes de cana-de-açúcar modulados por seca em raízes, da pós-graduanda Giovanna Vieira Guidelli, encontra-se devidamente aprovado e regularizado junto a CIBio/IB-UNICAMP e a CTNBio, conforme legislação vigente.

Cidade Universitária “Zeferino Vaz”,
14 de dezembro de 2017.

Prof. Dr. JOSÉ LUIZ PROENÇA MÓDENA
Presidente da CIBio
Instituto de Biologia - UNICAMP
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