CÁSSIA ÂNGELA PEDROZO

MORPHOPHYSIOLOGICAL AND MOLECULAR RESPONSES OF SUGARCANE GENOTYPES TO WATER STRESS

Thesis presented to the Federal University of Viçosa - Brazil as part of the demands of the Genetic and Breeding Program, for obtaining of *Doctor Scientiae* title.

VIÇOSA
MINAS GERAIS – BRAZIL
2010

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RESUMO

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, julho, 2010. **Respostas morfo-fisiológicas e moleculares de genótipos de cana-de-açúcar submetidos ao estresse hídrico.** Orientador: Márcio Henrique Pereira Barbosa. Co-orientadores: Luiz Alexandre Peternelli e Jorge Alberto da Silva.

O objetivo deste trabalho foi estudar respostas morfo-fisiológicas e moleculares de dois genótipos de cana-de-açúcar (tolerante à seca: TSP05-4 ou sensível à seca: TCP02-4589) ao estresse hídrico. Características morfo-fisiológicas foram avaliadas em cinco diferentes tempos [dois dias antes do início do estresse hídrico (T0), dois (T1), doze (T2) e vinte dias (T3) após início do estresse hídrico de moderada intensidade, e oito dias após re-irrigação (T4)]. Características de desenvolvimento foram avaliadas no final do experimento (após T4). A técnica cDNA-AFLP foi utilizada para identificar genes diferencialmente expressos em T1, T2 e T4. Tanto sob condições controle (plantas bem irrigadas) quanto sob condições de estresse hídrico o genótipo tolerante mostrou os maiores valores de indíce de esverdeamento da folha (SPAD), eficiência quântica máxima do PSII (F_v/F_m) e relação biomassa da raíz/planta inteira. Além disso, sob condições de estresse hídrico, as taxas de fotossíntese, transpiração e condutância estomática foram significativamente maiores no genótipo tolerante. O genótipo susceptível apresentou colmos mais altos e mais pesados. No entanto, a condição de estresse hídrico causou significativa redução na altura deste genótipo, enquanto que nenhuma alteração foi observada no genótipo tolerante. Um total de 15 fragmentos derivados de transcritos (TDF) diferencialmente expressos foram caracterizados e 8 desses foram validados por RT-PCR em tempo real. Três TDFs, os quais mostraram similaridade a um pentatricopeptideo putativo, à subunidade regulatória CK2β3 da proteina kinase CK2 e ao transportador glicose-6-fosfato/fosfato 2, foram diferencialmente expressos no genótipo susceptível. Um TDF similar ao mRNA de uma proteína induzida pela seca foi também induzido no genótipo tolerante em T2. Tanto as características fisiológicas quanto o padrão de expressão gênica que foram alterados pelo estresse hídrico foram completamente revertidos após o período de re-irrigação, demonstrando a plasticidade dos genótipos de cana-de-açúcar em responder a mudanças nas condições hídricas. Os resultados encontrados neste estudo demonstram a robustez do genótipo tolerante em responder a condições estresse hídrico e enfatiza diferenças fisiológicas e moleculares entre os dois genótipos que podem auxiliar em programas de melhoramento que visam tolerância à seca.

Termos de indexação: estresses abióticos, *Saccharum* spp, mecanismos fisiológicos, cDNA-AFLP.

ABSTRACT

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, July, 2010. **Morphophysiological and molecular responses of sugarcane genotypes to water stress.** Adviser: Márcio Henrique Pereira Barbosa. Co-Advisers: Luiz Alexandre Peternelli and Jorge Alberto da Silva.

The objective of this work was to study morphophysiological and molecular responses of two sugarcane genotypes (drought-tolerant: TSP05-4 or drought-susceptible: TCP02-4589) to water stress. Morphophysiological traits were evaluated at five different times [two days before water stress initiation (T0), two (T1), twelve (T2) and twenty (T3) days after moderate water stress initiation, and at eight days after re-watering (T4)]. Growth traits were evaluated at the end of the experiment (after T4). The cDNA-AFLP technique was used to identify differentially-expressed genes at T1, T2 and T4. Under control (well-watered) and water stress conditions, the tolerant genotype showed the highest values of leaf greenness index (SPAD), maximum quantum yield of PSII (F_v/F_m), and root:shoot ratio. Besides, under water stress conditions, photosynthesis, transpiration and stomatal conductance were significantly higher in that genotype than in the susceptible genotype. The susceptible genotype had taller and heavier stalks. However, water stress caused a significant reduction in stalk length in this genotype, while no differences were observed for the tolerant genotype. A total of 15 differentially-expressed transcript-derived fragments (TDF) were characterized and 8 of them were validated by real-time RT-PCR. Three TDFs showing significant sequence similarities to genes encoding a putative expressed pentatricopeptide, a protein kinase CK2 regulatory subunity CK2β3, and a glucose-6-phosphate/phosphate translocator 2 were differentially expressed in the susceptible genotype. One TDF similar to a drought-inducible protein mRNA was also up-regulated in the tolerant genotype at T2. The physiological traits and gene expression which were altered by water stress were completely reversed after re-watering, demonstrating the plasticity of sugarcane genotypes in being able to respond to changing water conditions. The results found in this study demonstrate the robustness of the tolerant genotype in response to water stress and highlights physiological and molecular differences between the two genotypes that could help in sugarcane improvement programs for stress tolerance.

Indexation terms: abiotic stress, *Saccharum* spp, physiological mechanisms, cDNA-AFLP.

GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp; Poaceae) is an economically important perennial crop of tropical origin that is grown in more than 90 countries (FAO, fttp://apps.fao.org) for sugar production. About 80% of the world's sugar (sucrose) supply is from sugarcane, producing 111.8 million tonnes (ton) of sugar for 2002/2003 (Licht; 2003), while the other 20% is obtained from sugar beet (*Beta vulgaris* L., Chenopodiaceae). Sugarcane is also a leading alternative energy feedstock crop both for ethanol and for biomass production.

Sugarcane is widely adapted within the tropical zone (±30° of the equator) and its tolerance to a range of temperatures has allowed cultivation to expand into sub-tropical regions such as Louisiana, Florida and Texas in the United States of America. For optimum growth and productivity, sugarcane requires temperatures of above 20ºC and a period of 8 to 24 months to reach maturity, depending on location and agronomic practices. The leading three sugarcane-producing-countries, Brazil, India, and China, each produced more than 100 Mton of cane sugar in the year 2002/2003. The other major sugarcane-producing countries include Australia, Mexico, Thailand, Pakistan, USA, South Africa, Colombia, Cuba, and Philippines.

Even though sugarcane and other C_4 perennial grasses have many adaptive qualities (such as high productivity, water use efficiency, vegetative propagation, and wide environmental adaptation), for sugar or bioenergy production, ample water supply and nutrients are required to assure optimum productivity (Wiedenfeld, 2000; Menossi *et al*., 2008; Cha-um and Kirdmanee, 2009). The annual water requirements for optimum yields have been estimated at about 1500 to 2500 mm (Doorenbos and Pruitt, 1976). Since sugarcane production is concentrated in many tropical regions where water supply is either inadequate or irrigation infrastructures are underdeveloped, moisture deficit is a major limitation to optimal productivity (Inman-Bamber and Smith, 2005; Hemaprabha *et al*., 2006; Jangpromma *et al*., 2007). The major determinants of sugarcane yield (stalk yield and sucrose content) are highly sensitive to various biotic and abiotic stresses, of which, drought is the most critical.

A basic understanding of mophophysiological and molecular processes responsible for high performance under water stress conditions and its interactions is necessary in sugarcane breeding programs aimed at increasing productivity by enhancing stress tolerance. Thus, the objective of this study was to characterize and compare morphophysiological and

molecular responses of two sugarcane genotypes during moderate water stress exposure and during a recovery period after re-watering.

SCIENTIFIC PAPER 1

MORPHOPHYSIOLOGICAL RESPONSES OF SUGARCANE GENOTYPES TO WATER STRESS

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RESUMO

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, julho, 2010. **Respostas morfo-fisiológicas de genótipos de cana-de-açúcar submetidos ao estresse hídrico.** Orientador: Márcio Henrique Pereira Barbosa. Co-orientadores: Luiz Alexandre Peternelli e Jorge Alberto da Silva.

O objetivo deste estudo foi melhor entender a natureza de características morfo-fisiológicas e de desenvolvimento de dois genótipos de cana-de-açúcar (tolerante à seca: TSP05-4 ou sensível à seca: TCP02-4589) ao estresse hídrico. Características morfo-fisiológicas foram avaliadas em cinco diferentes tempos (2 dias antes do início do estresse hídrico, dois, doze e vinte dias após início do estresse hídrico de intensidade moderada, e oito dias após re-irrigação). Características de desenvolvimento foram avaliadas no final do experimento (após o período de re-irrigação). Sob condições controle (plantas bem irrigadas) e de estresse hídrico, o genótipo tolerante apresentou os maiores valores de indíce de esverdeamento da folha (SPAD), eficiência quântica máxima do PSII (Fv/Fm) e relação biomassa raíz/planta inteira (50.83, 0.8085 e 0.6182, respectivamente). Adicionalmente, sob condições de estresse hídrico, as taxas de fotossíntese (P_n), condutância estomática (G_s) e transpiração (E) foram significativamente superiores no genótipo tolerante (14.61, 133.8 e 3.49 $\mu mol.m^{-2}.s^{-1}$, respectivamente) que no susceptível (11.23, 110.5 e 2.96, respectivamente). A redução nas taxas de P_n, G_s e E devido ao estresse hídrico foi completemente revertida após o período de re-irrigação, demonstrando a plasticidade dos genótipos de cana-de-açúcar em responder a mudanças nas condições hídricas e suportam a hipótese de que o controle fotossintético durante condições de moderado estresse hídrico foi principalmente estomático. Os colmos do genótipo susceptível apresentaram maior peso e altura que aqueles do genótipo tolerante. No entanto, as condições de estresse causaram significativa redução na altura dos colmos daquele primeiro genótipo (de 1.69 m para 1.32 m) enquanto nenhuma alteração foi observada para o segundo. Os resultados encontrados neste estudo demonstram a robustez do genótipo tolerante em responder a condições de estresse hídrico e enfatiza diferenças, principalmente fisiológicas, entre os dois genótipos que podem auxiliar em programas de melhoramento que visam tolerância à seca.

Termos de indexação: estresse abiótico, *Saccharum* spp, mecanismos fisiológicos, melhoramento genético.

ABSTRACT

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, July, 2010. **Morphophysiological responses of sugarcane genotypes to water stress**. Adviser: Márcio Henrique Pereira Barbosa. Co-adviser: Luiz Alexandre Peternelli and Jorge Alberto da Silva.

The objective of this study was to better understand the nature of morphophysiological traits in two sugarcane genotypes (tolerant: TSP05-4 or susceptible: TCP02-4589) to water stress. Physiological and morphological traits were measured at five different times (two days before water stress initiation, two, twelve and twenty days after moderate water stress initiation, and at eight days after re-watering) and growth traits were measured at the end of the experiment (after re-watering). Under control (well-watered) and simulated water conditions, the tolerant genotype showed the highest values of leaf greenness index (SPAD), maximum quantum yield of PSII (F_v/F_m), and root:shoot ratio (50.83, 0.8085 and 0.6182, respectively). Moreover, under water stress, net photosynthesis (P_n), stomatal conductance (G_s) and transpiration rate (E) were significantly higher in the tolerant genotype (14.61, 133.8 and 3.49 $\mu mol.m^{-2}.s^{-1}$, respectively). The decrease in P_n, G_s and E due to water stress was completely reversed after re-watering, demonstrating the plasticity of these genotypes to respond to changing water conditions and supporting the hypothesis that the control on photosynthesis during moderate water stress was mainly stomatal. Stalks of the susceptible genotype were taller and heavier than those of the tolerant genotype. However, water stress caused significant reduction in stalk length of the susceptible genotype (from 1.69 m to 1.32 m), while no differences were observed for the tolerant genotype. These data demonstrate the robustness of the tolerant genotype in response to water stress and highlights differences, mainly physiological, between the two genotypes that could help in sugarcane improvement programs for stress tolerance.

Indexation terms: abiotic stress, *Saccharum* spp, physiological mechanisms, genetic improvement.

1. INTRODUCTION

Sugarcane (*Saccharum* spp; Poaceae) is an economically important perennial crop of tropical origin that is grown in more than 90 countries (FAO, fttp://apps.fao.org) for sugar, ethanol and biomass production. However, since sugarcane production is concentrated in many tropical regions where water supply is either inadequate or irrigation infrastructures are underdeveloped, moisture deficit is a major limitation to optimal productivity (Inman-Bamber and Smith, 2005; Hemaprabha *et al*., 2006; Jangpromma *et al*., 2007). The major determinants of sugarcane productivity (stalk yield and sucrose content) are highly susceptible to various biotic and abiotic stresses, of which, drought is the most critical.

Drought stress reduces plant physiological performance and ultimately productivity, although the magnitude and direction of the response depends on the genotype, duration and intensity of stress exposure as well as the developmental stage at which stress is applied (Chaves *et al*., 2003). Four distinct developmental phases have been identified in sugarcane: germination, tillering, grand growth and maturity (Van Dillewijn, 1952). The tillering and early grand growth stages are collectively known as the formative phase, which runs between 60 to 150 days of crop age. Although sugarcane can withstand some degree of water stress without affecting biomass and sucrose accumulation, formative phase has been identified as a critical water demand period (Venkataramana *et al*., 1984; Vasantha *et al*., 2005). Water deficit stress during this phase affects growth, morphological, physiological and biochemical traits, and consequently, cane and sugar yields. In many sugarcane growing areas, the formative phase coincides with periods of high temperature and water deficit stress.

Therefore, successful culture of sugarcane in areas characterized by inadequate water availability depends, among other factors, on using appropriate cultural management practices, such as irrigation, and on growing tolerant varieties. In many production regions of the world, the high cost of water for irrigation, coupled with poor irrigation infrastructure and efficiency makes irrigation an expensive and non-sustainable practice. An economic and sustainable alternative approach would be to plant cultivars that are drought tolerant. There are currently very few drought tolerant commercial cultivars, however, even though many sugarcane breeding programs around the world have been selecting for drought tolerance as a suitable trait for yield improvement.

Drought tolerance is a complex trait that depends on many genes and, thus is determined by many interactive processes. The polygenic nature of drought tolerance mechanisms is probably one reason why many conventional selection/breeding approaches

have not been fruitful. Moreover, in sugarcane, the high ploidy and large size of the genome make conventional breeding for this crop more laborious when compared with other crops (Hogarth, 1987). Hence, yield, sugar content, and disease resistance have received major attention in sugarcane breeding programs. Nonetheless, some progress in genetic improvement of sugarcane drought tolerance has been obtained (Ramesh *et al*., 2000, Hemaprabha *et al*., 2004; Vasantha *et al*., 2005; Hemaprabha *et al*., 2006; Silva *et al*., 2008).

Studies have focused on the evaluation of genotypes and families for drought tolerance-related growth traits such as stomatal behavior, osmotic adjustment, relative water content, among others (Srivastava *et al*., 1996; Cha-um and Kirdmanee, 2009; Srivastava *et al*., 1997; Ramesh, 2000; Ramesh and Mahadevaswamy, 2000; Hemaprabha *et al*., 2004). According to Sleper and Poehlman (2006), combination of genes for frost and drought hardiness of the *S. barberi* and *S. sinensi* with genes for high sugar yield from *S. officinarum* should be a major aim in the breeding of sugarcane for marginal environments.

A basic understanding of growth, morphological and physiological processes responsible for high performance under drought conditions and its interactions are still poor in sugarcane. This could play a major role in identifying traits for yield improvement and genetic advance in stress environments. Growth analysis procedures have been utilized to identify drought-tolerant varieties in certain sugarcane breeding programs (Venkataramana *et al*., 1984; Hemaprabha *et al*., 2004). In this crop, the growth or yield traits that are negatively impacted by drought stress during the formative phase usually include: stalk height, number of internodes per stalk, number of millable stalks, stalk diameter, stalk weight and Brix (Wagih *et al*., 2003; Hemaprabha *et al*., 2004; Silva *et al*., 2008). Among these, stalk height and stalk weight are generally the most affected traits.

In theory, it is possible to improve productivity either by increasing the photosynthetic rate, reducing respiration rate, or by increasing allocation of photosynthates to storage sinks (Zhang *et al*., 2000). Other drought-responsive physiological traits, such as stomatal resistance, transpiration, relative water content, chlorophyll content and chlorophyll a fluorescence traits also influence productivity and constitute useful traits to select drought tolerant genotypes (Müller *et al*., 2010; Srivastava *et al*., 1996). One caveat about these traits is that even though some of them may be highly correlated with drought tolerance, they may be of little use during screening because they are too expensive or laborious and time-consuming.

Morphological responses of plants to drought generally include alterations on leaf traits (area, shape size, stomatal density, etc), root systems, and mass-area-volume

relationships, such as specific leaf area and root-to-shoot ratios (Marron *et al*., 2002; Marron *et al*., 2003; Anyia and Herzog, 2004; Liu and Stützel, 2004; Songsri *et al*., 2008; Songsri *et al*., 2009; Painawadee *et al*., 2009).

Studies on how plants respond to, and recover from drought stress can reveal differences in plasticity among genotypes and could be a useful tool for screening for stress tolerance. Plant physiological/phenotypic plasticity or the ability of the plant to alter its physiology, morphology and/or behavior in response to a change in the environmental conditions has not been extensively studied in relation to drought tolerance. Following re-watering, plants could immediately show a high rate of biological activity, including photosynthetic capacity and new organ growth, which can be considered as alternative functional states that may overcompensate for the limitation to plant growth and metabolic activity due to previous drought (Cai *et al*., 2004; Montanaro *et al*., 2007; Efeoglu *et al*., 2009; Flexas *et al*., 2009; Xu *et al*, 2009). Such overcompensation has also been reported in sugarcane (Ashton, 1956; Inman-Bamber, 1995). Even after several weeks of severe stress, it took only few days (3 to 5 days) for leaf extension rates to resume to those rates under normal conditions (Inman-Bamber, 1995). Genetic variation in physiological/phenotypic plasticity with respect to drought has not been explored in sugarcane. The objective of this study was to characterize and compare morphophysiological responses of two sugarcane genotypes during water stress exposure and during a recovery period after re-watering. The two genotypes used had previously been classified as drought tolerant and drought susceptible based on yield performance tests in a field study.

2. MATERIALS AND METHODS

2.1. Planting materials, irrigation treatments, and growing conditions

This study was performed during spring-summer (February-May) 2009 in a ventilated greenhouse at the Agrilife Research and Extension Center, Texas A&M University, Weslaco (latitude 26° 12’ N , longitude 97° 57’ W and 18.90 meters of altitude), TX, USA. The two genotypes of sugarcane selected and evaluated in this study have been previously classified as drought tolerant (TSP05-4) and drought susceptible (TCP02-4589), based on field yield trials (Da Silva, personal communication). Among a group of 80 genotypes, TSP05-4 and TCP02-4589 showed one of the smallest and highest reductions in stalk productivity under drough stress conditions, respectivelly.

Single-node segments containing one lateral bud were germinated in plastics trays filled with a peat-based substrate (MetroMix MM200, Scotts-Sierra Horticultural Products Co, Marysville, OH). After two weeks, plantlets were transplanted into 15-L pots containing MetroMix substrate. Plants were watered at least once per day, and fertilized two times per week with a complete water-soluble fertilizer (10N-4.4P-8.3K, Peter's Corp., St. Louis, Mo.). The average daily photosynthetic photon flux (*PPF*) at the canopy level was 15 ± 3.8mol·m^{-2}. Average day/night temperatures were 28.8 ± 4.4 / 21.7 ± 3.2 °C and average day/night relative humidity were 48 ± 11 / 68 ± 11%.

Water supply treatments were imposed from 70 to 90 days after planting (DAP) and after this period the plants were adequately re-watered for eight days. The stress period coincided with the formative phase, which is the most sensible phase to drought stress in sugarcane (Venkataramana *et al*., 1984; Vasantha *et al*., 2005). Plants were subjected to two water supply regimes (15% volumetric moisture content, designated "moderate water stress" and 30% volumetric moisture content, designated as "control" or "well-watered treatment", respectively). Volumetric soil moisture content was monitored continuously using soil moisture sensors (EC5, Decagon Devices, Inc) connected to dataloggers (Em5b, Decagon Devices, Inc).

2.2. Measurement of morpho-physiological traits

Physiological traits were measured two days before water stress initiation (T_0), and then also at two, twelve and, twenty days after stress initiation (T1, T2 and T3, respectively) and finally at eight days after re-watering (T4). Net photosynthesis rate (P_n), stomatal conductance (G_s), and transpiration (E) were measured using a portable gas exchange system CIRAS-2 (PPSystems), under ambient temperature, light saturation (1,500 µmol m^{-2} s^{-1}), and CO_2 partial pressure of 35 Pa. Leaf chlorophyll a fluorescense measurements were conducted immediately after P_n measurements following the procedures of Maxwell and Johnson (2000), using a pulse amplitude modulation fluorometer (Model OS5-FL, Opti-Sciences, Tyngsboro, MA, USA). The maximum quantum yield of photosystem II (PSII) was measured as the dark-adapted F_v/F_m ratio where F_v is the variable fluorescence (F_m-F_0), Fm is the maximal fluorescence yield following a 1 s saturating pulse at 7500 µmol m^{-2} s^{-1}, and F_0 is the minimal fluorescence yield obtained by modulated light at intensity of 0.2 µmol m^{-2} s^{-1}. Leaves were dark-adapted for 30 minutes with leaf-clips (FL-DC, Opti-Science) prior to F_v/F_m measurements. Two measurements were collected per plant.

Leaf greenness index (SPAD) measurements were made using a Minolta SPAD-502 chlorophyll meter (Minolta Corp., Ramsey, NJ, USA). The SPAD value is an indicator of the leaf chlorophyll content (Fanizza *et al*., 2001; Zaharieva *et al*., 2001; Anand and Byju, 2008). Two SPAD readings made on two different leaves were measured per plant. All the above physiological traits were measured between 10 and 12:00 am, using the 3rd or 4th leaf from the top-most visible dewlap of stalk.

Leaf relative water content (RWC: water content relative to the water content of the tissue at full turgor) was estimated from leaf disks (ten disks per plant). The leaf disks (4,5 mm diameter each) were weighted immediately after collection to determine fresh weight (FW), and then placed in dislilled water and kept in the dark at room temperature for 24 h before turgid weight (TW) was recorded. Finally, leaves were dried at 80 ºC for 48 h to measure the dry weight (DW) (Matin *et al*., 1989, Silva *et al*., 2007). RWC trait was calculated according to the following expression:

$$RWC\ (\%) = ((FW - DW) / (TW - DW)) \times 100$$

Growth and morphological measurements included stalk height (SH), stalk diameter (SD), stalk weight (SW), root:shoot ratio (RS ratio) and specific leaf area (SLA) were collected at the end of the experiment (after T_4). SLA or the ratio between leaf area and leaf mass was obtained using the dry weight of the same discs used to measure the RWC trait, by the following expression:

$$SLA\ (cm^2 g^{-1}) = (discs\ leaf\ area) / DW$$

SLA has been used to draw conclusions about density and thickness of leaves and the response of leaf structure to adverse environmental conditions.

Shoot height (SH) was measured from the base of the top-most visible dewlap to the soil level. Basal stem diameter (SD) was measured with a pair of calipers at 10cm from the soil level. Single stalk fresh weight was obtained in each pot using a precision scale. Root:shoot ratio was determined after final harvest by drying root and whole plant tissues at 70ºC for 72 h in an oven and recording dry weights.

2.3. Experimental design and data analysis

The physiological investigation experiments were set in a completely randomized block design with four replications, arranged in a triple factorial 2 x 2 x 5 (two genotypes, two water treatments and five evaluation times). The growth and morphological investigation experiments were set in a completely randomized block design with four replications, arranged in a double factorial 2 x 2 (two genotypes and two water treatments). Analyses of variance (ANOVA) for each trait were performed to assess the main effects of factors and interaction between different factors. Genotypes, irrigations treatments and evaluation times were considered fixed effects. Standard errors were used to detect differences between treatments means. Statistical tests were considered significant at $P \leq 0.05$. All statistical analyses were performed with SAS statistical package (SAS Institute, Inc.,Cary, NC).

3. RESULTS AND DISCUSSION

Analysis on net photosynthesis (P_n), transpiration (E) and stomatal conductance (G_s) showed significant effects for water supply regime (WR), evaluation time (ET) and the interactions ET x WR and G x WR (Table 1). Besides, analysis on P_n showed significant effects for genotype (G). There were no significant differences in P_n, G_s and E between the genotypes at control (well-watered) treatment, while under water stress treatment tolerant genotype had values significantly higher for these traits compared to the susceptible genotype (Fig.1). Water stress reduced Pn, E and Gs rates of the susceptible genotype in 30, 32.70 and 26.53%, respectively. On the other hand, for the tolerant genotype the reductions in these traits were 6.94, 13.93 and 10%, respectively. Reductions in these physiological traits were initially non-significant (T0) between the water treatments but with increasing duration of water stress exposure, differences became more apparent (Fig. 2).

Table 1. Summary of analysis of variance for net photosynthesis (P_n), transpiration rate (*E*), stomatal conductance (G_s), leaf greenness (SPAD), PSII photochemical efficiency (F_v/F_m) and leaf relative water content (RWC) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stresss) and evalueted at five times (T0: two days before water stress initiation, T1, T2 and T3: two, twelve and twenty days after moderate water stress initiation, and T4: 8 days after re-watering).

SV	DF	P_n ($\mu mol\ m^{-2}\ s^{-1}$)	*E* ($\mu mol\ m^{-2}\ s^{-1}$)	G_s ($\mu mol\ m^{-2}\ s^{-1}$)	SPAD	F_v/F_m	RWC (%)
		Mean square					
Evaluation time (ET)	4	113.7343*	8.9363*	15906.5125*	109.99*	0.001878*	0.000740
Water regime (WR)	1	186.6605*	10.6142*	28388.1125*	42.490	0.001268*	0.006541*
Genotype (G)	1	40.0445*	0.74110	1058.51250	1214.46*	0.005977*	0.007380
ET x WR	4	67.6343*	2.0989*	5716.3625*	27.550	0.000326	0.001221
ET x G	4	3.61700	0.56800	1400.63750	4.410	0.000238	0.000194
WR x G	1	77.2245*	2.2916*	5136.0125*	0.780	0.000043	0.000798
ET x WR x G	4	8.66830	0.34250	1106.51250	9.420	0.000064	0.000313
Block	3	16.39250	0.29590	1918.64580	75.730	0.000458	0.000168
Error	57	7.41190	0.43810	1135.68970	21.170	0.000180	0.000431
Mean		14.45	3.5853	140.99	46.93	0.7998	91.67
CV (%)		18.84	18.46	23.90	9.80	1.68	2.27

SV: Source of variation; DF: degree of freedom; CV: coefficient of variation; * significant at $p < 0.05$.

Studies have shown that stomatal conductance is one of initial short term responses to water limitation (Jones, 1992) compared to other traits such as leaf expansion, root growth, gene expression and proteins (Yordanov *et al*., 2000). A reduction in G_s caused by stomatal closure is considered one of the first lines of defense against dehydration damage, and serves to conserve plant water and maintain high cell and tissue turgor pressure (maintain high leaf water potential) (Teare *et al.,* 1973; Blum 1974). Stomatal closure and reduction in G_s caused by water limitation also limits photosynthetic CO_2 assimilation and transpirational water loss, as was observed in the present study, suggesting that P_n was inhibited by gas phase processes (stomatal limitation). The traits P_n and G_s showed a high positive correlation (0.8795, data not showed).

Differences between plant species and among genotypes of a species in the degree of stomatal closure and reduction in G_s, P_n and E can be indicative of the ability to tolerate mild-to-moderate water deficit stress. The ability to tolerate low water availability means that the plant can continue to carry out metabolic processes even during periods of sub-optimal water

supply. In the present study, the tolerant genotype was able to maintain significantly higher rates of P_n, G_s and E under water stress conditions (Fig.1).

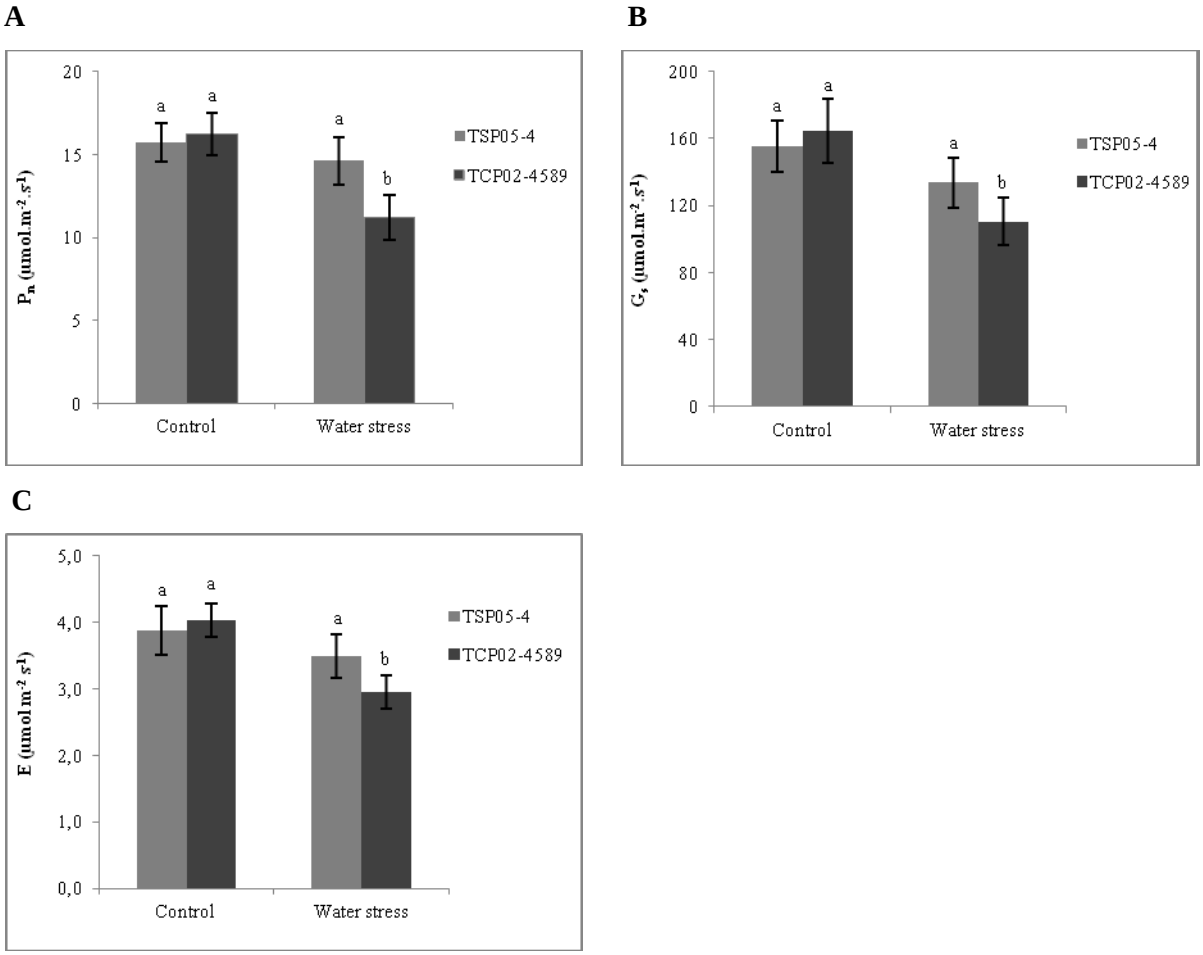

Figure 1. Mean net photosynthesis (P_n), stomatal conductance (G_s) and transpiration rate (E) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress) (mean, ± S.E.). Different letters indicate significant differences between genotypes within each water supply regime.

The reductions in P_n, G_s and E due to water stress were most severe within 2 days after water stress initiation (T1). Thereafter, further changes in P_n and G_s were not expressive (Fig. 2). A leveling off in the rate of decline in these traits probably suggest onset of mechanisms to counteract the effects of water deficit stress, for instance, accumulation of compatible solutes or adjustments in source-sink allometric relationships such that shoot water potential is ameliorated. Similar results have also been reported in kidney bean (Miyashita *et al*., 2005) and sugarcane leaves (Cha-um and Kirdmanee, 2009; Vu and Allen, 2009).

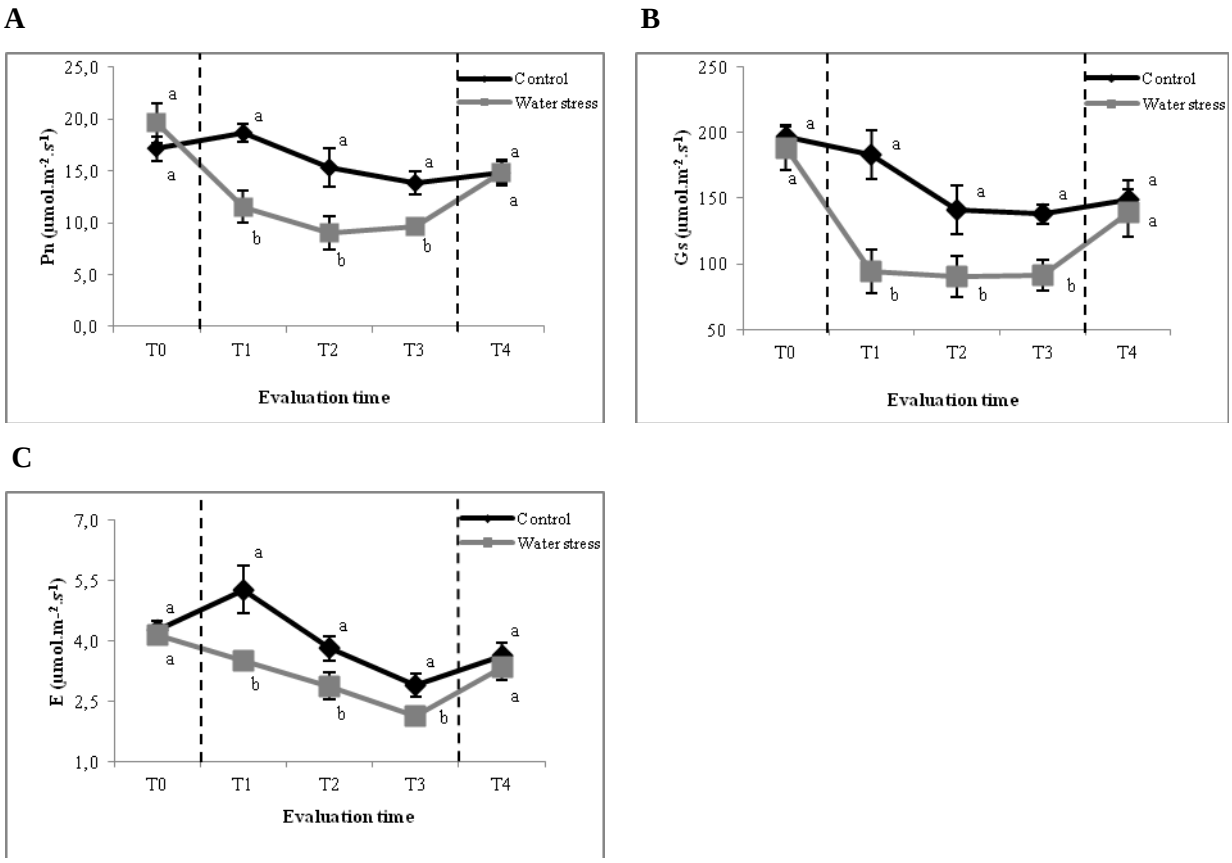

Figure 2. Mean net photosynthesis (A), stomatal conductance (B) and transpiration rate (C) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress) and evaluated at five times (T0: two days before water stress initiation, T1, T2 and T3: two, twelve and twenty days after moderate water stress initiation, and T4: 8 days after re-watering) (mean, ± S.E.). Different letters indicate significant differences between water supply regimes within each evaluation time.

Radiation use efficiency as estimated by PS II chlorophyll a fluorescence (F_v/F_m) was significantly altered by water supply regime, genotype and evaluation time. On average, F_v/F_m was lower in water-stressed plants and in the susceptible genotype (Fig. 3), compared to control and to tolerant genotype. A decline in F_v/F_m ratio under water stress conditions is indicative of photoinhibition associated with over-reduction of PSII or photoinhibitory damage (Maxwell and Johnson, 2000; Colom and Vazzana, 2003). Thus, the ability to maintain high F_v/F_m under water stress indicates a high efficiency of radiation use possibly for photochemistry and carbon assimilation. According to Quiles (2005) the values of F_v/F_m in unstressed dark-adapted plants adapted to dark are in the range of 0.75 - 0.80. Zhang *et al*. (2000) found that in unstressed sugarcane plants the F_v/F_m values were in the range of 0.71 to 0.82.

The leaf greenness index (SPAD) was significantly influenced by genotype and evaluation time but not by water supply regime (Table 1). SPAD values of the susceptible genotype were on average 15% lower than those of the tolerant genotype, which is consistent with their classifications as susceptible and tolerant, respectively (Fig. 3). Similar responses of SPAD to drought have been reported in the literature (Silva *et al*., 2007; Cha-um and Kirdmanee, 2009). SPAD values are indicative of leaf chlorophyll and hence leaf nitrogen contents (Zaharieva *et al*., 2001; Fanizza *et al*., 2001; Anand and Byju, 2008). Photoinhibitory damage to chlorophyll degradation resulting from water stress-induced photoinhibition probably accounted for the reduction in leaf greenness indices of the susceptible genotype. Mechanisms responsible for protection of prevention of chlorophyll degradation and hence high SPAD values are unclear but probably involve antioxidant activity of carotenoid pigments. The rates of SPAD and F_v/F_m have been considered as rapid and easy tools to identify drought tolerant genotypes in several species (Fanizza *et al*., 1991; Rong-hua *et al*., 2006; Silva *et al*., 2007; Arunyanark *et al*. 2009).

Leaf relative water content (RWC) was significantly altered only by water supply regime (Table 1), with the control treatment having higher value compared to that of the water stress treatment (Fig. 3). Even though water supply regime had a significant effect on RWC, differences between the two water regime treatments only amounted to ~2%. Thus, RWC values were still high (~91%) compared to results found in other crops such as potato (Schafleitner *et al*., 2007) and wheat (Tavakol and Pakniyat, 2007), which have shown drastic reduction in RWC under water stress. A possible explanation for the lack of differences found among the genotypes and for the high value of RWC maintained under water stress may be due the time of the day at which this trait was analyzed. Consistent with observations of Sarker *et al*. (1999), RWC measurements tended to be higher in the morning, declined around midday, and showed some recovery in the afternoon. Thus, the best time to analyze the RWC was in the afternoon, when evapotranspiration is highest and real differences among treatments are likely to be revealed. In the present study, RWC samples were collected at 3:00 pm, when the plants likely have already recovered from drought stress that had previously occurred. Besides, Painawadee *et al*. (2009) have found that RWC is an insensitive indicator of water status in peanut when water deficit was mild.

Relative water content is an easily measured indicator of the current water content in sampled leaf tissue relative to the water content of the same tissue at full turgor (Matin *et al*., 1989). RWC has been used to indicate the plant water status under different water conditions, and in a diversity of studies (Sarker *et al*., 1999; Medici *et al*., 2003; Silva *et al*., 2007;

Bayoumi *et al*., 2008; Boussadia *et al*., 2008). While maintenance of high RWC during water stress is indicative of drought tolerance, the mechanisms for maintaining high RWC are varied and often confounding (Rampino *et al*., 2006; Silva *et al*., 2007). In the present study there were no significant differences in RWC between the two genotypes studied. This result indicates that both genotypes may possess the same ability to absorb water from the soil and/or the same ability to control water loss under moderate stress. Similarly, in crops such as maize (Efeoglu *et al*., 2009), common bean (Martínez *et al*., 2007) and wheat (Tavakol and Pakniyat, 2007) no differences among genotypes subjected to different water conditions were detected for RWC.

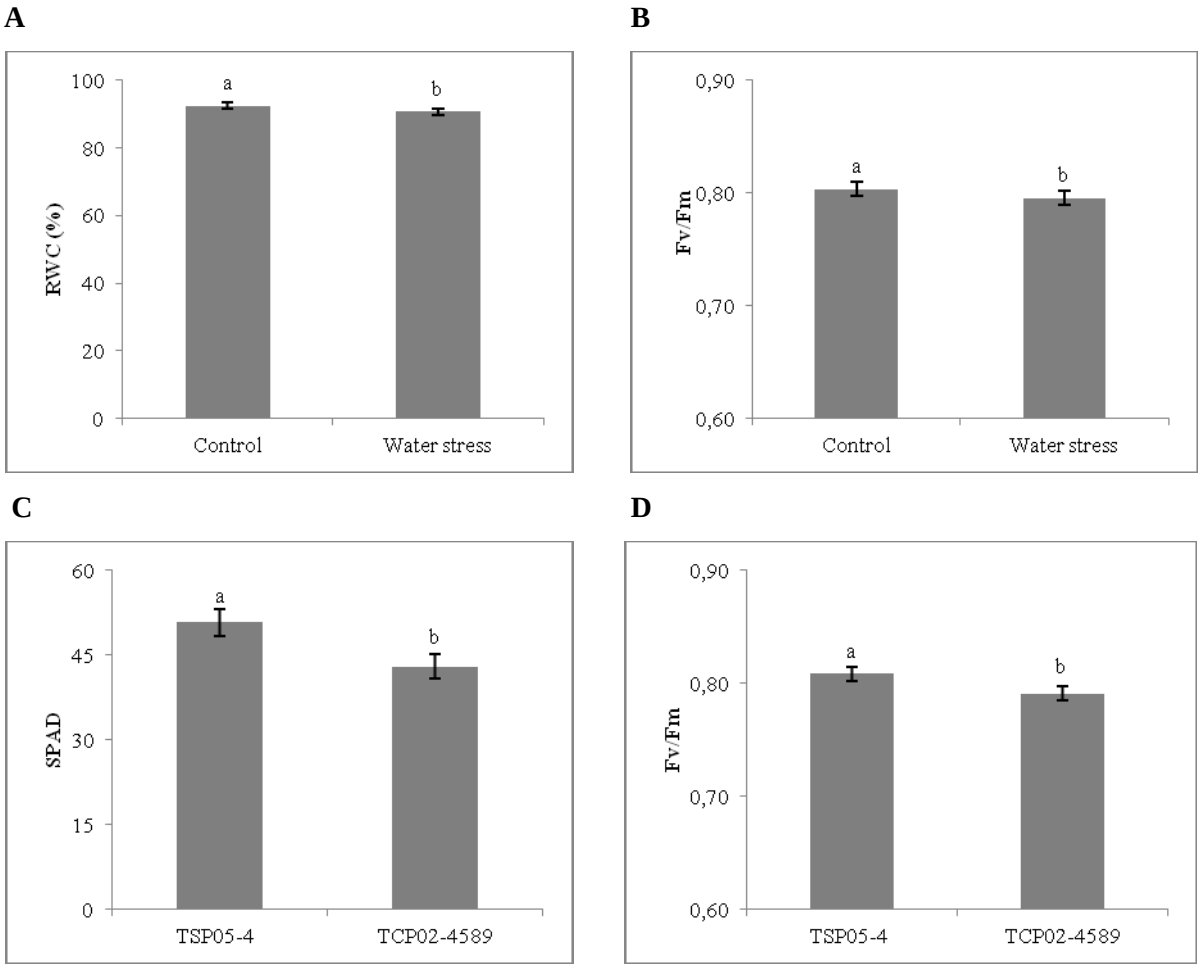

Figure 3. Mean relative water content (RWC), maximum quantum yield of photosystem II (F_v/F_m) and leaf greenness index (SPAD) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under water supply regimes (control and moderate water stress) and evaluated at five times (T0: two days before water stress initiation, T1, T2 and T3: two, twelve and twenty days after moderate water stress initiation, and T4: 8 days after re-watering) (mean, ± S.E.). Different letters indicate significant differences between water supply regimes (A and B) or between genotypes (C and D).

The non-significant interactions for SPAD, F_v/F_m and RWC (Table 1) may indicate that these traits could be described as constitutive traits, once their expression is independent of the environment. A constitutive trait is not expected to show high genotype x environment interaction and could be of advantage as a tool for selection to drought stress (Bayoumi *et al*., 2008). The results suggest that F_v/F_m could be carried out under any evaluation time, genotype and water condition under investigation with similar results. This fact was also observed for SPAD in relation to evaluation time and genotype. This last trait has previously showed to be a stable trait in peanut genotypes (Arunyanark *et al*., 2009). The stable performance of sugarcane genotypes under both water stress and well-watered control for SPAD and F_v/F_m is a flexible tool for breeders to evaluate genotypes for drought tolerance, once the screening can be carried out without drought conditions.

Plant responses and adaptation to drought stress reflect in changes in photosynthetic rates. Research on the effect of drought in photosynthesis has been carried out to a less extent in sugarcane. The inhibition of photosynthesis in water-stressed plants may be attributed to limited CO_2 diffusion to the leaf intercellular spaces as a consequence of the reduced stomatal opening (stomatal control) and/or by direct inhibition of biochemical and photochemical processes (non-stomatal control) imposed by the water deficit (Ashton, 1956; Colom and Vazzana, 2003; Chaves *et al*., 2003). Thus, the water stress tolerance was well correlated with the genotypes capacity to maintain high G_s and P_n under water stress and indicate that these physiological traits were confident to use in early screening for drought tolerance in sugarcane.

The recovery point upon re-watering following a water shortage is an important and useful index for the research of recovery processes and for screening of faster-recovery cultivars. The velocity of recovery after a stress period depends on the species analyzed, intensity and duration of the stress previously reached, leaf age, light intensity and many other factors (Flexas *et al*., 2004; Miyashita *et al*., 2005; Flexas *et al*., 2006). The decrease in P_n, G_s and E due the water stress was completely reversed eight days after re-watering (Fig.2). These results demonstrate the plasticity of the sugarcane genotype to respond to changing water conditions and support the hypothesis that the basic mechanisms of photosynthetic biochemistry and photochemistry (non-stomatal control) were not impaired by water stress and that control on net photosynthesis during water stress was mainly stomatal.

The results on recovery of P_n, G_s and E rates found in this study agree with those reported by Ashton (1956) and Inman-Bamber (1995). In those studies a fast recovery of sugarcane stressed plants was observed, taking only about few days for photosynthesis and

leaf extension rates to return to unstressed conditions. Some 80 to 90 % of normal photosynthetic activity was recovered within two days in response to irrigation after a series of high and low soil water content alternating cycles (Ashton, 1956). A fast recovery of drought effects due the re-watering has also been found in grapevine (Flexas *et al*., 2009), maize (Efeoglu *et al*., 2009), coffee (Cai *et al*., 2004) and kiwifruit (Montanaro *et al*., 2007).

Specific leaf area (SLA) and root:shoot ratio (RS) were significantly affected by evaluation time and genotype, respectively (Table 2). These traits are sensitive to water stress and have also been correlated with the net photosynthetic capacity (McClendon, 1962, Zhang *et al*., 2004). SLA has been used for selecting genotypes for drought tolerance in crops, such as peanut (Anyia and Herzog, 2004; Songsri *et al*., 2008; 2009; Painawadee *et al*., 2009) and amaranth (Liu and Stützel, 2004). Specific leaf area varies considerably between species and is a very plastic trait.

Studies have shown that low SLA is associated with slow growth (Poorter and Remkes, 1990) and this is indicative of low leaf areas available for light interception and hence photosynthetic carbon assimilation. On the other hand, the inverse of SLA, generally referred to as specific leaf weight (SLW or leaf mass per unit area) is positively correlate with leaf thickness and, in some instances, Pn, often through the total number of mesophyll cells per unit leaf depth (Beadle, 1993). In this regard, a genotype with low SLA may be more tolerant to water deficit stress since the pathway for water loss is greater and photosynthesis per unit leaf area is potentially greater. The decrease of SLA under water stress may be associated with the accumulation of soluble compounds and/or thickening of the cell wall (Marron *et al*., 2003). According to Tardieu *et al*. (1999), a decrease in SLA occurs when environmental conditions cause a greater depression in growth rate than on photosynthesis (i.e. the same amount of carbon gain is distributed to a reduced leaf area). However, studies which emphasize the effects of the water stress in SLA have been contradictory.

Some studies have showed that SLA decrease due to drought (Liu and Stützel, 2004; Schumacher *et al*., 2008; Marron *et al*., 2003; Lal *et al*., 2009; Painawadee *et al*., 2009), while in other ones increased SLA rates were found (Anyia and Herzog, 2004; Aspelmeier and Leuschner, 2006; Montanaro *et al*., 2007). Increase in SLA suggests loss in leaf weight in relation to leaf expansion to compensate for reduced assimilation (Anyia and Herzog, 2004). The formation of thinner leaves represent less costly leaves. These contradictory results should be expected in reason of the differences on species, environmental conditions, stress intensity, stress duration and, plant and leaf age used by different researchers for SLA determination.

Table 2. Summary of analysis of variance for specific leaf area (SLA) and rate among root and shoot biomass (RS ratio) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress). SLA was evalueted at five times (T0: two days before water stress initiation, T1, T2 and T3: two, twelve and twenty days after moderate water stress initiation, and T4: 8 days after re-watering) and RS ratio was measured at the end of the experiment (after T4).

SV	**SLA (**$cm^2.g^{-1}$)	**RS ratio**
	Mean square	
Evaluation time (ET)	1437.7424*	-
Water regime (WR)	49.4718	0.0014
Genotype (G)	69.6458	0.2626*
ET x WR	108.8795	-
ET x G	14.9686	-
WR x G	194.0844	0.0031
ET x WR x G	84.6787	-
Block	640.8661	0.0196
Error	80.7958	0.0040
Mean	171.5896	0.4901
CV (%)	5.24	12.94

SV: Source of variation; DF: degree of freedom; CV: coefficient of variation; * significant at $p < 0.05$.

In the present study SLA did not differ among genotypes and water supply regimes (Table 2) showing that the tolerance and the water stress did not affect the leaf mass in sugarcane. Similar results were found in quinoa plants (González *et al*., 2009). The lack of treatment effects on SLA may be due to the early sampling date, which was not enough to discriminate differences between genotypes and water regimes. Similarly, SPAD values did not differ in water stressed and non-stressed treatments until two weeks of stress in *Vitis vinifera* (Fanizza *et al*., 1991). Songsri *et al*. (2009) have also reported that too early or too late evaluation times were not appropriate to discriminate differences between peanut genotypes.

The ratio between root and shoot biomass (RS ratio) seems to be governed by a balance between water absorption by roots and shoot growth. Cell and leaf expansion as well as stem elongation are more sensitive to water deficit stress than to root growth. In general, water limitation often results in an increase in RS ratio (Liu and Stützel, 2004). In this study

RS ratio was unaffected by water stress (Table 2). This result is in agreement with the finding of González *et al*. (2009) who did not observe changes for RS ratio among three treatments in quinoa plants: drought, water logging and control.

A high RS ratio could reflect an increased capacity of water uptake, thereby maintaining the shoot in a well-hydrated condition (Blum, 1996). Despite the lack of water treatments effects, the tolerant genotype had the highest RS ratio (0.62) in both control and water stress conditions (Fig. 4) and this may have contributed to the higher P_n, G_s and E observations under water stress conditions.

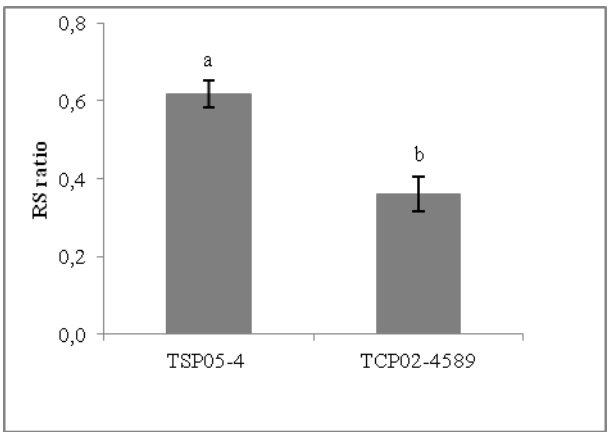

Figure 4. Mean rate among root and shoot biomass (RS ratio) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress) (mean, ± S.E.). Different letters indicate significant differences between genotypes.

Sugarcane yield components such as brix, stalk weight, stalk height, stalk diameter and stalk number are usually affected by drought stress during the formative phase (Srivastava *et al*., 1997; Ramesh and Mahadevaswamy, 2000; Hemaprabha *et al*., 2004; Vasantha *et al*., 2005; Silva *et al*., 2008). Among these, stalk height and stalk weight are generally the most affected traits. In the present study, stalk height (SH) and stalk weight (SW) were significantly affected by genotype and water supply regimes ($P<0.05$). SH was also affected by the interaction between these two factors (Table 3).

Stalk diameter (SD) was a stable trait across genotypes and water supply regimes. This result is consistent with studies conducted by Domaingue (1996), Vasantha *et al*. (2005) and Silva *et al.* (2008), who showed that SD was one of the yield component less affected by

water shortage. On the other hand, reduction in SH and SW under drought has been observed by several authors (Domaingue, 1996; Ramesh and Mahadevaswamy, 2000; Hemaprabha *et al*., 2004; Vasantha *et al*., 2005; Cha-um and Kirdmanee, 2009).

Table 3. Summary of analysis of variance for stalk height (SH), stalk diameter (SD) and stalk weight (SW) of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress).

SV	DF	SH (m)	SD (cm)	SW (kg)
		Mean square		
Genotype (G)	1	0.9409*	0.1444	0.1266*
Water regime (WR)	1	0.2209*	0.0121	0.0990*
G x WR	1	0.0729*	0.064	0.0323
Block	3	0.0052	0.0147	0.0123
Error	9	0.0097	0.0451	0.0164
Mean		1.26	2.63	0.5735
CV (%)		7.81	8.08	22.34

SV: Source of variation; DF: degree of freedom; CV: coefficient of variation; * significant at $p < 0.05$.

Among four yield components (SH, SD, stalk number and brix) studied by Silva *et al*. (2008), SH was the most affect by unirrigated conditions. In this study, water stress caused a significant reduction in SH of the susceptible genotype (from 1.69 to 1.32 m), but no differences were observed for the tolerant genotype (Fig. 5). The reduction in SH in the susceptible genotype may be caused by suppression of cell division and expansion due to lower turgor pressure. The mean SH for the susceptible and tolerant genotypes were 1.50 and 1.02 m, respectively.

Water stress reduced SW by 26% in comparison to control treatment and the tolerant genotype had the smallest single stalk weight (Fig. 5). The diminished weight observed for tolerant genotype (0.48 kg) is due to the lowest stalk length measured for this genotype (Silva *et al*., 2009).

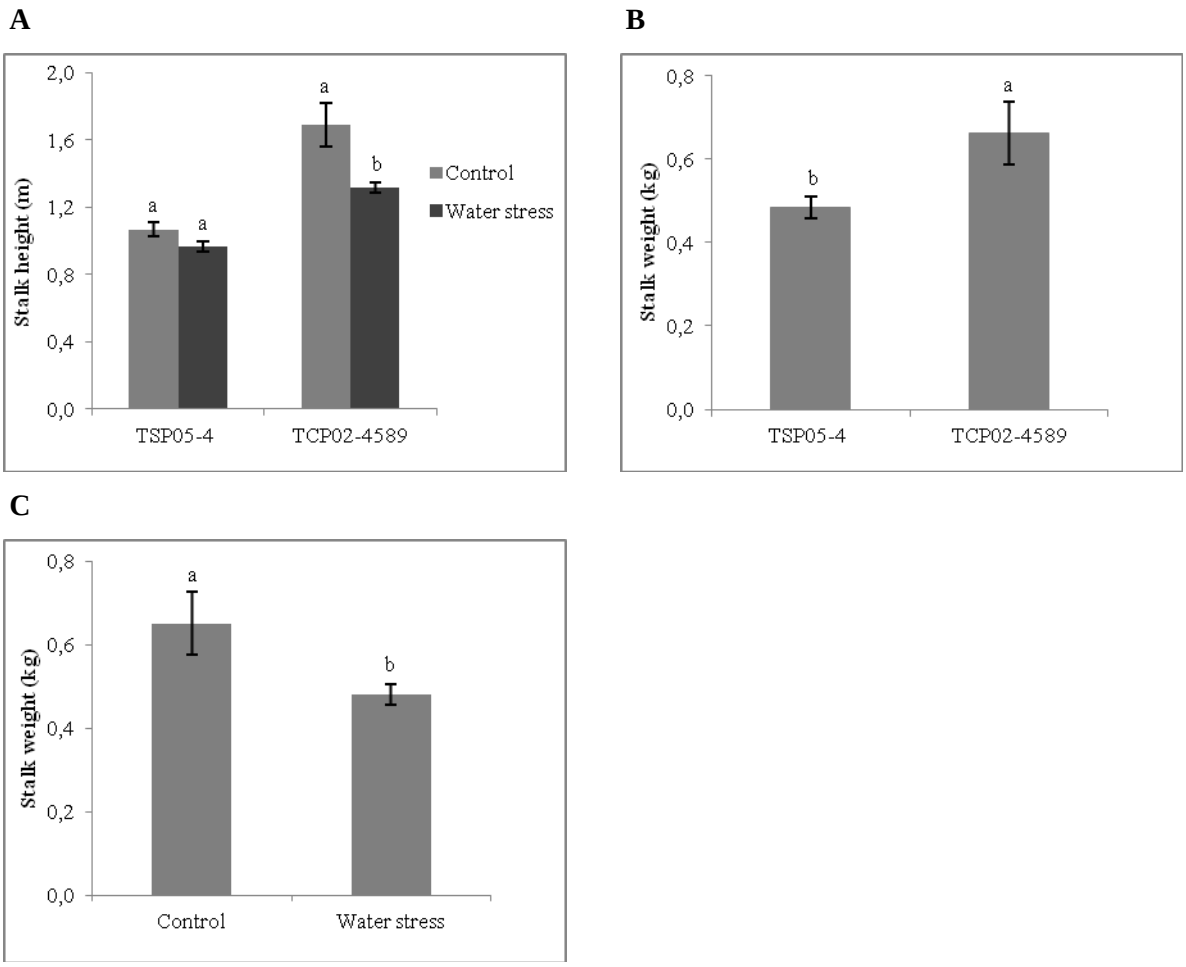

Figure 5. Mean stalk height and mean stalk weight of two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress) (mean, ± S.E.). Different letters indicate significant differences between water supply regimes within genotype (A), between genotypes (B) or between water supply regimes (C).

Genotypes which show proportionally less reduction in yield attributes under drought conditions, could be considered more drought tolerant, but only if the reduction in the expression of an attribute is associated with a high mean, because it is of little value if the mean expression of the attribute is too low to satisfy the minimum required criteria (Domaingue, 1996; Silva *et al*., 2008). The smaller SH and SW found for the tolerant genotype suggest that it could have a slower early growth during water stress when compared to the susceptible genotype. Consequently, the yield superiority cannot be determined solely on the basis of SH and SW. These traits should be evaluated in plants subjected to a longer stress period, during the formative phase (60 to 150 days of crop age) to be applied as drought tolerance indicators. In the present study the plants were submitted to a shorter stress period (70 to 90 days of crop age), which corresponds to the beginning of the tillering phase.

4. CONCLUSIONS

- Physiological traits, especially gas exchange traits, were able to discriminate among water stress tolerant and susceptible genotypes even at early and moderate stress.
- After a re-watering period, the rates of gas exchange traits altered by water stress returned completely to the rates observed in the control treatment, demonstrating the plasticity of sugarcane genotypes in responding to water stress.
- Morphological traits were not influenced by the imposed water stress.
- Growth traits, especially stalk height could be useful to differentiate between tolerant and susceptible genotypes. However these traits should be evaluated in an advanced water stress stage during the formative phase to select to drought tolerance and higher productivity genotypes.

5. ACKNOWLEDGEMENTS

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SCIENTIFIC PAPER 2

DIFFERENTIAL GENE EXPRESSION IN SUGARCANE GENOTYPES IN RESPONSE TO WATER STRESS

VIÇOSA
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RESUMO

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, julho, 2010. **Expressão gênica diferencial em genótipos de cana-de-açúcar em resposta ao estresse hídrico.** Orientador: Márcio Henrique Pereira Barbosa. Co-orientadores: Luiz Alexandre Peternelli e Jorge Alberto da Silva.

A seca é o principal fator limitante à produtividade da cana-de-açúcar em muitas regiões do mundo e é causada por períodos de estresse hídrico prolongado ou por irrigação inadequada. Os mecanismos pelos quais as plantas respondem as condições de seca são variados e ainda pobremente entendidos. A identificação de genes, bem como a caracterização de suas funções e regulação em resposta à seca é necessária em programas de melhoramento que visam ao aumento da produtividade por meio da obtenção de cultivares mais tolerantes. O objetivo deste estudo foi usar a técnica cDNA-AFLP para identificar e caracterizar genes diferencialmente expressos em dois genótipos de cana-de-açúcar (tolerante à seca: TSP05-4 ou susceptível à seca: TCP02-4589) ao estresse hídrico. As plantas foram avaliadas dois (T1) e doze dias após início de estresse hídrico de intensidade moderada (T2), e oito dias após re-irrigação (T3). Um total de 15 fragmentos derivados de transcritos (TDFs) diferencialmente expressos foram clonados e caracterizados e, 8 destes foram validados por RT-PCR em tempo real. Três TDFs mostrando similaridade com genes codificando para um pentatricopeptídeo putativo, um translocador glicose-6-fosfato/fosfato 2 e para a subunidade regulatória CK2β3 da proteína kinase CK2 foram diferencialmente expressos no genótipo susceptível. Os dois primeiros TDFs foram reprimidos enquanto que o último foi induzido. Por outro lado, um TDF similar a um mRNA de uma proteína induzida pela seca foi também induzido no genótipo tolerante em T2. A expressão gênica alterada pelo estresse hídrico foi completamente revertida para todos os fragmentos após o período de re-irrigação demonstrando, assim, a plasticidade dos genótipos de cana-de-açúcar em responder a alterações nas condições hídricas. Os resultados encontrados neste estudo demonstram a robustez do genótipo tolerante em responder a condições de estresse hídrico e enfatiza diferenças moleculares entre os dois genótipos que podem auxiliar em programas de melhoramento que visam tolerância à seca.

Termos de indexação: estresse abiótico, cDNA-AFLP, mecanismos moleculares, melhoramento genético.

ABSTRACT

PEDROZO, Cássia Ângela, D.Sc., Universidade Federal de Viçosa, July, 2010. **Differential gene expression in sugarcane genotypes in response to water stress**. Adviser: Márcio Henrique Pereira Barbosa. Co-adviser: Luiz Alexandre Peternelli and Jorge Alberto da Silva.

Drought is a major factor limiting the productivity of sugarcane in many regions of the world and is caused by prolonged dry conditions or inadequate irrigation. The mechanisms by which plants respond to drought are varied and are still poorly understood. Identifying relevant genes and characterizing their functions and regulation in response to drought is necessary in crop improvement programs aimed at increasing productivity by enhancing stress tolerance. The objective of this study was to use the cDNA-AFLP technique to identify and characterize differentially-expressed genes in two sugarcane genotypes (tolerant: TSP05-4 or susceptible: TCP02-4589) to drought stress. Plants were evaluated at two (T1) and twelve days after moderate water stress initiation (T2), and again at eight days after re-watering (T3). A total of 15 transcript-derived differentially-expressed fragments (TDF) were cloned and characterized and 8 of them were validated by real-time RT-PCR. Three TDFs showing sequence similarities to genes encoding a putative expressed pentatricopeptide, a glucose-6-phosphate/phosphate translocator 2, and a protein kinase CK2 regulatory subunit CK2β3 were differentially expressed in the susceptible genotype. The two formers TDFs were down-regulated and the last one was up-regulated. On the other hand, one TDF similar to a drought-inducible protein mRNA was also up-regulated in the tolerant genotype at T2. The genes that had their expression altered in response to water stress were completely reversed after re-watering period, thus demonstrating the plasticity of sugarcane plants in being able to respond to changing water conditions. The results found in this study demonstrate the robustness of the tolerant genotype in response to water stress and highlights molecular differences between the two genotypes that could help in sugarcane improvement programs for stress tolerance.

Indexation terms: abiotic stress, cDNA-AFLP, molecular mechanisms, genetic improvement.

1. INTRODUCTION

Modern sugarcane varieties are hybrids derived mostly from hybridization between *Saccharum officinarum* L. (2n=80) and *Saccharum spontaneum* L. (2n=40-128), followed by a series of backcrosses with *Saccharum officinarum*, in a process known as 'nobilisation'. However, other species, such as *S.robustum*, *S. barberi* Jesw., and *S. sinensi* Roxb. have also been involved to a lesser extent in the development of modern varieties. These varieties are highly polyploids, heterozygotes, aneuploids, and on average contain 100-130 chromosomes (Irvine, 1999).

Crop cultivation is negatively affected by abiotic stresses such as drought, high salinity, cold, flooding, and heat (Grover *et al*., 2001; Luan, 2002; Wang *et al*., 2003). These stresses elicit a variety of responses in plants from alteration of gene expression to changes in growth and final yield. Among the abiotic stress, drought is the most severe stress limiting the sugarcane productivity in Brazil and other sugarcane producing countries (Ellis and Lankford, 1990; Wiedenfeld, 1995; Wiedenfeld, 2000). The level of tolerance or sensitivity of plants to drought stress is influenced by the time of exposure and severity of stress, previous exposure to stress, genotype, and developmental age of the plants (Bray, 2002; Passioura, 2004). In sugarcane, the formative phase (60 to 150 days of crop age) has been identified as a critical water demand period (Venkataramana *et al*., 1984; Vasantha *et al*., 2005). Water deficit stress during this phase affects growth, morphological and physiological traits, and consequently, cane and sugar yields.

Drought is a condition of special interest, since increasing water scarcity has been observed throughout the world. According to Riera *et al*. (2005), irrigated agriculture currently accounts for approximately 65% of global fresh water use indicating that the development varieties tolerant to drought stress can have a potentially huge impact on productivity in the future. However, drought-tolerance is a polygenic trait and it is difficult to be improved by traditional breeding (Beever, 2000). Thus, the integration of molecular approaches with plant breeding and physiology may be advantageous in increasing sugarcane productivity under water shortage. For molecular breeding, the identification and characterization of genes regulated by drought stress is essential for understanding the drought-tolerance mechanisms and developing tolerant genotypes, either for transgenic plant development or for marker-assisted breeding.

It has been shown that tolerance/sensitivity mechanisms of plants in response to drought are revealed by changes in the expression level of genes regulated by this adverse

condition (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki and Yamagushi-Shinozaki, 2007). A variety of drought stress-inducible genes encodes for proteins which have different functions, such as detoxification, water channels, transporters, protection factors of macromolecules (LEA proteins), osmolyte biosynthesis (proline, sugars), transcription factors, protein kinases, phosphatases, ABA biosynthesis etc (Shinozaki and Yamagushi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Wang *et al*., 2003; Shinozaki and Yamagushi-Shinozaki, 2007). These gene products are included in three major categories: (1) those involved in signaling cascades and in transcriptional control; (2) those that function as protectors of membranes and proteins; (3) those involved in water and ion uptake and transport.

Techniques like suppression subtractive hybridization (SSH), serial analysis of gene expression (SAGE), macroarray, microarray, differential display reverse transcription-polymerase chain reaction (DDRT-PCR) and cDNA-amplified fragment length polymorphism (cDNA-AFLP) are available to monitor gene expression under different biotic and abiotic stresses. Among them, macroarray and microarray technologies are the most important and most powerful tools for studying the whole genome transcription (Decorosi *et al*., 2005). However, these techniques are relatively expensive and require prior sequence knowledge of the genes to be investigated, and therefore unable for some laboratories and for some species.

cDNA-AFLP (Bachem *et al*., 1996) is a powerful and relatively inexpensive tool for genome-wide expression analysis, especially when genome sequence information is limited. Its sensitivity and specificity is compared to those found for the microarray approach (Reijans *et al*., 2003). Moreover, cDNA-AFLP enables the identification of new and/or poorly expressed genes. This technique has been successful to characterize the mechanisms underlying tolerance to several biotic and abiotic stresses, such as pathogens (Cadle-Davidson, 2006; Adhikari *et al*., 2007; LaO *et al*., 2008), salt (Jayaraman *et al*., 2008, Roshandel and Flowers, 2009), cold (Sun *et al*., 2007; Meng *et al*., 2008), and heat (Simões-Araújo *et al*., 2002). For drought stress there are very few reports on gene expression using cDNA-AFLP analysis (Yang *et al*., 2004; Si *et al*., 2009).

In sugarcane, attempts to reveal the gene expression under drought stress have been performed using microarray (Rocha *et al*., 2007) and macroarray (Rodrigues *et al*., 2009) analysis. In both these studies, a variety of genes, such as transcription factors, kinases, phosphatases, transporters, and auxin biosynthesis enzymes were related. Moreover, Rodrigues *et al*. (2009) have showed that the number of expressed genes increased with the

severity (mild to severe stress) of drought. Despite these positive results, little is known about molecular mechanisms of drought stress response in sugarcane. Research in this specific subject may provide new strategies to improve the stress tolerance of sugarcane crop.

The aim of this study was to use the cDNA-AFLP technique to identify and to characterize differentially expressed genes in leaves of two sugarcane genotypes subjected to moderate water stress.

2. MATERIALS AND METHODS

2.1. Planting materials, irrigation treatments, and growing conditions

This study was conducted during spring-summer (February-May) 2009 in a ventilated greenhouse at the Agrilife Research and Extension Center, Texas A&M University, Weslaco (latitude 26° 12' N , longitude 97° 57' W and 18.90 meters of altitude), TX, USA. The two genotypes of sugarcane selected and evaluated in this study had been previously classified as drought tolerant (TSP05-4) and drought susceptible (TCP02-4589), based on field yield trials (Da Silva, personal communication). Among a group of 80 genotypes, TSP05-4 and TCP02-4589 showed one of the smallest and highest reductions in stalk productivity under drough stress conditions, respectivelly.

Single-node segments containing one lateral bud were germinated in plastics trays filled with a peat-based substrate (MetroMix MM200, Scotts-Sierra Horticultural Products Co, Marysville, OH). After two weeks, plantlets were transplanted into 15-L pots containing MetroMix substrate. Plants were watered at least once per day, and fertilized two times per week with a complete water-soluble fertilizer (10N-4.4P-8.3K, Peter's Corp., St. Louis, Mo.). The average daily photosynthetic photon flux (*PPF*) at the canopy level was 15 ± 3.8mol·m^{-2}. Average day/night temperatures were 28.8 ± 4.4 °C / 21.7 ± 3.2 °C and average day/night relative humidity were 48 ± 11% / 68 ± 11%.

Water supply treatments were imposed from 70 to 90 days after planting (DAP) and after this period the plants were adequately re-watered for eight days. The stress period coincided with the formative phase, which is the most sensible phase to drought stress in sugarcane (Venkataramana *et al*., 1984; Vasantha *et al*., 2005). Plants were subjected to two water supply regimes (15% volumetric moisture content, designated "moderate water stress" and 30% volumetric moisture content, designated as "control" or "well-watered treatment", respectively). Volumetric soil moisture content was monitored continuously using soil

moisture sensors (EC5, Decagon Devices, Inc) connected to dataloggers (Em5b, Decagon Devices, Inc).

2.2. Sampling procedures

To study the differential gene expression of two sugarcane genotypes under water stress, samples of the 3rd leaf counted from the top-most visible dewlap of stalk were collected at three evaluation times: two days (T1) and twelve (T2) days after water stress initiation, and eight days after re-watering (T3). Leaf tissues of three plants for each treatment (evaluation date/water regime/genotype) were collected and frozen immediately in liquid nitrogen and latter stored at -80 ºC prior to analysis.

2.3. Total RNA extraction, poli (A)+ RNA isolation and cDNA synthesis

Approximatelly 100 mg of leaf tissues were frozen with liquid nitrogen and ground to a fine powder to extract the total RNA using the QIAGEN Rneasy Plant Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The total RNA concentration was measured with a Nanodrop spectrophotometer (NanoDrop ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies), while the RNA quality were checked using 1 µg of total RNA by electrophoresis in agarose gel 1% (w/v). Poli $(A)^+$ RNA was isolated from 10 µg of total RNA using the MicroPoly(A) Purist™ Kit (Ambion), according to the manufacturer's protocol. Single and double stranded-cDNAs were then synthetised from 10 µl of poli $(A)^+$ RNA, using the SuperScript™ Double-Stranded cDNA Synthesis (Invitrogen).

2.4. cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis

The cDNA-AFLP analysis was performed as described by Bachem *et al*. (1996), using the AFLP® Expression Analysis kit of LI-COR (LI-COR, Lincoln, NE), with minor modifications. Restriction enzymes *Taq*I e *Mse*I were used to digest the resulting cDNA and to generate pre-amplification PCR products. A non-selective pre-amplification was performed using non-selective adaptor primers without additional nucleotides. The pre-amplification products were diluted 10-fold before being used in the final selective amplification. Selective PCRs were performed with a total of 22 primer combinations obtained by the eight *Mse*I+2 primers and the eight *Taq*I+2 primers (+2 represents two

selective nucleotides: +GA, +GT, +TC, +TG, +CT, +CA, +AG and +AC on both adaptor primers) provided in the AFLP Expression Analysis Kit. The sequences of adapter strands and primers used for pre-amplification and selective amplification are summarized in the Table 4. The *Taq*I+2 selective primers were labeled with 700 and 800-nm infrared dye (LI-COR, IRDye 700 and IRDye 800). PCR reactions were performed with *Taq* DNA polymerase from Promega (Promega, Madison, WI). Selective PCR products were separated and visualized by electrophoresis on 6.5% denaturing polyacrylamide in a LI-COR DNA analyser (model 4300 LI-COR®). Eletrophoretic run parameters were: 1500 V, 40 W, 40 mA, 45 ºC, with a 25-min pre-run and 2 h main run.

Table 4. Sequence of adapter strands and primers used for pre-amplification and selective amplification.

Primer or adapter	**Sequence**
*Mse*I adapter	5'-GACGATGAGTCCTGAG-3'
	3'-TACTCAGGACTCAT-5'
*Taq*I adapter	5'-CTCGTAGACTGCGTAC-3'
	3'-CGGTACGCAGTCT-5'
*Mse*I pre-amplification primer	5'-GACGATGAGTCCTGAGTAA-3'
*Taq*I pre-amplification primer	5'-GTAGACTGCGTACCGA-3'
*Mse*I amplification primer	5'-GATGAGTCCTGAGTAANN-3'
*Taq*I amplification primer	5'-GTAGACTGCGTACCGANN-3'

Data images were collected using LI-COR's Saga AFLP Analysis software. Gel images were analyzed visually and the transcript-derived fragments (TDFs) were selected based on its presence/absence (qualitative variants) between the water regimes. Only the fragments with reproducible pattern of expression among the three plants sampled were considered. Use of replicated samples in this study could eliminate false-positive banding patterns and also allows for the identification of fragments that are water stress-related only.

2.5. Isolation and sequencing of transcript-derived fragment (TDF)

Isolation of TDFs of interest was perfomed according to the AFLP® Expression Analysis kit, mentioned previously. The TDFs were excised from gels and incubated at -20 ºC in Eppendorf tubes containing 40 µl 1X TE buffer. Three freeze-thaw cycles were

performed and the solution was centrifuged at 15,000 x g for 20 minutes at 4 ºC. After centrifugation, 5 µl of the resuspended DNA solution was used for PCR re-amplification using the same primers and PCR conditions as those in the selective amplification procedures. To determine the purity and confirm the size of isolated fragments, 10 µl of the PCR products were run on the 1.5% agarose gel (w/v). After gel visualization, the TDFs were excised from gel and purified using the Zymoclean™ Gel DNA recovery Kit (Zymo Research). Subsequentely the purified TDFs were cloned into the pGEM®-T Easy vector (Promega Corp., Madison, WI) according to the manufacturer's protocol, then used to transfom *Escherichia coli* DH5α competent cells.

Recombinant plasmids from six bacterial colonies of each cloned fragment were isolated using Zyppy™ plasmid Miniprep kit (Zymo Research) following the manufacturer's protocol. Before sequencing, the identity of the cloned fragments were verified comparing the size of the cloned fragment with the size of the fragment in the original polyacrylamide gel. Purified plasmids containing the insert were sequenced with an automated DNA sequencer (Applied Biosystem, Inc.) at the DNA Facility, Iowa State University, USA. Sequences not corresponding to the selective amplification were omitted from further analysis.

2.6. Analysis of sequences

After removal of vector sequence database searches were performed. The nucleotide as well as translated sequences were analysed for their homology with nucleotide and protein sequences, respectively, against the data available in the GenBank (http.//www.ncbi.nlm.nih.gov/BLAST) database using the BLASTx and BLASTn search tools. Significance of the similarity was based on E-value. Similarities with E-values $\leq 10^{-10}$ were considered significant.

2.7. Validation of cDNA-AFLP experiments by real-time reverse transcription PCR

Real-time reverse transcription PCR (RT-PCR) was used to confirm the differential expression of 7 TDFs isolated by cDNA-AFLP. The RT-PCR primers for these fragments were designed using the Primer Express Version 3.0 (Applied Biosystems, Inc). The following criteria were used for selecting each primer pair: 18 - 25 pb long; melting temperature of 59 - 61 ºC; 40-60% GC content; amplicon length of 60-150 pb. The primer

sequences were searched against the NCBI database using the Blast tool to verify specificity of the sequences (Table 5).

Single strand cDNA was synthetized from 20 ng of poli $(A)^+$ RNA using the iScript™ cDNA Synthesis Kit (Invitrogen), following the manufacturer's instructions. The cDNA of the three plants sampled in each treatment (genotype/water regime/evaluation time) was pooled in identical quantities to prepare the RT-PCR reactions. These reactions were performed with iQ™ SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA, USA) in a BioRad iCycler iQ5 thermocycler (Bio-Rad laboratories, Hercules, CA, USA).

Table 5. Primer sequences of reference gene (RF) and of target TDFs used in the RT-PCR expression analysis.

	Primer name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
RF	GAPDH*	CACGGCCACTGGAAGCA	TCCTC AGGGTTCCTGATGCC
Target TDF	SugF01	CCCTCAAATGCAGGGAACTA	GCCAGCTGTTTTCTGAGACC
	SugF02	CCTACGATGACGAGGTCCAT	CCTTTGCTGCAACAATTTCA
	SugF05	AGCAACTAACCAACCCATCG	CTTGTTGGAGGGAGATCGAG
	SugF06	ATGAGGAAATGGAGCGTGTC	CCATGTGAACCAATCTGTGC
	SugF10	AACGCCGAAACTTCTTCTGA	GAGTCGAACTCGGGAACTGA
	SugF11	ATCTGGCAGGCGTGAGTTTA	TTCCACTGCTCACTTGCATC
	SugF15	TTCTCCAAGAAGGGGATGAA	ATGGAGAGGCAGGCGTAGTA
	SugF16	GCAGCAACCGGATATCTCTT	CTGCCTTGGCCTATTTCTTG

PCR was performed for each sample in triplicates. To normalize the gene expression glyceraldehide-3-phosphate de-hydrogenase (*GAPDH*) was used as endogenous reference gene (Iskandar *et al*., 2004; Table 5). For each RT-PCR reaction, a total of 25 µl was prepared containing 2 µl of template cDNA, 0.4 µM of each fragment specific primer and reference gene primers, 8.5 µl water, and 12.5 µl of iQ SYBR Green Supermix. For negative controls (NTC), cDNA templates synthetized without iScript reverse transcriptase (Invitrogen) were used. No-template controls were also included to detect any spurious signals arising from amplification of any DNA contamination or primer dimer formed during the reaction. The following amplification program was applied: 50 ºC for 2 min, 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

To ensure optimal PCR efficiencies of TDFs standard curves were generated. Also, melting curve analyses were performed after the final cycle of amplification to exclude the occurrence of primer dimers and unspecific PCR products. The relative expression rates of

each fragment normalized to the reference gene were calculated using Ct values (threshold cycle value). The following expression was used to calculate the Ct value (Livak and Schmittgen, 2001):

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)}$$

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$

$$\Delta(\Delta Ct) = \Delta Ct \text{ (interess sample)} - \Delta Ct \text{ (control sample)}$$

3. RESULTS AND DISCUSSION

3.1. cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis

By using 22 cDNA-AFLP primer combinations, about 1548 TDFs ranging in size from ~ 50 bp to ~ 500 bp were detected. There were observed few fragments larger than 500 bp but visualization and evaluation of them were very difficult to perform without errors. An average of 70 TDFs per primer combination was produced. These results are in agreement with those showed by Yang *et al*. (2004), Gigliotti *et al*. (2004), and Polesani *et al*. (2008). Figure 6 represents an example of a section of a typical cDNA-AFLP gel, containing banding patterns of two sugarcane genotypes analyzed under two water supply regimes and at three evaluation times. The primer combination used in this example was *Taq*I GT x *Mse*I AG.

Among the total number of TDFs visualized in this study, 30 (about 2% of all TDFs) were classified as differentially-expressed. A total of 24 differentially expressed TDFs were down-regulated and 6 were up-regulated. TDFs of interest were classified into three categories: (1) TDFs that were present only in the tolerant genotype, (2) TDFs present only in the susceptible genotype, and (3): TDFs that were simultaneously present in both tolerant and susceptible genotypes. The susceptible genotype had 23 differentially-expressed TDFs, while the tolerant genotype had only 6 TDFs. Only one down-regulated differentially-expressed TDF was simultaneously present in both genotypes. Despite the higher number of TDFs expressed by the susceptible genotype, most of them (19) were down-regulated. Among six TDFs found exclusively in the tolerant genotype, four were down-regulated and two were up-regulated. The expression profile exhibited by the susceptible genotype suggests that stress was detected earlier in the susceptible genotype. These results are in concert with a previous study by Rodrigues *et al*. (2009), who observed that the number of differentially expressed TDFs in sugarcane was increased with water stress severity. In accordance to the results

found by these authors, the TDFs differentially expressed in a drought-tolerant cultivar (SP83-5073) appear as induced or repressed preferentially in severe water stress conditions. Under moderate drought-stress, SP83-5073 had only one down-regulated TDF. On the other hand, under the same moderate drought conditions, 36 TDFs were induced and 136 were repressed in a susceptible cultivar (SP90-1638).

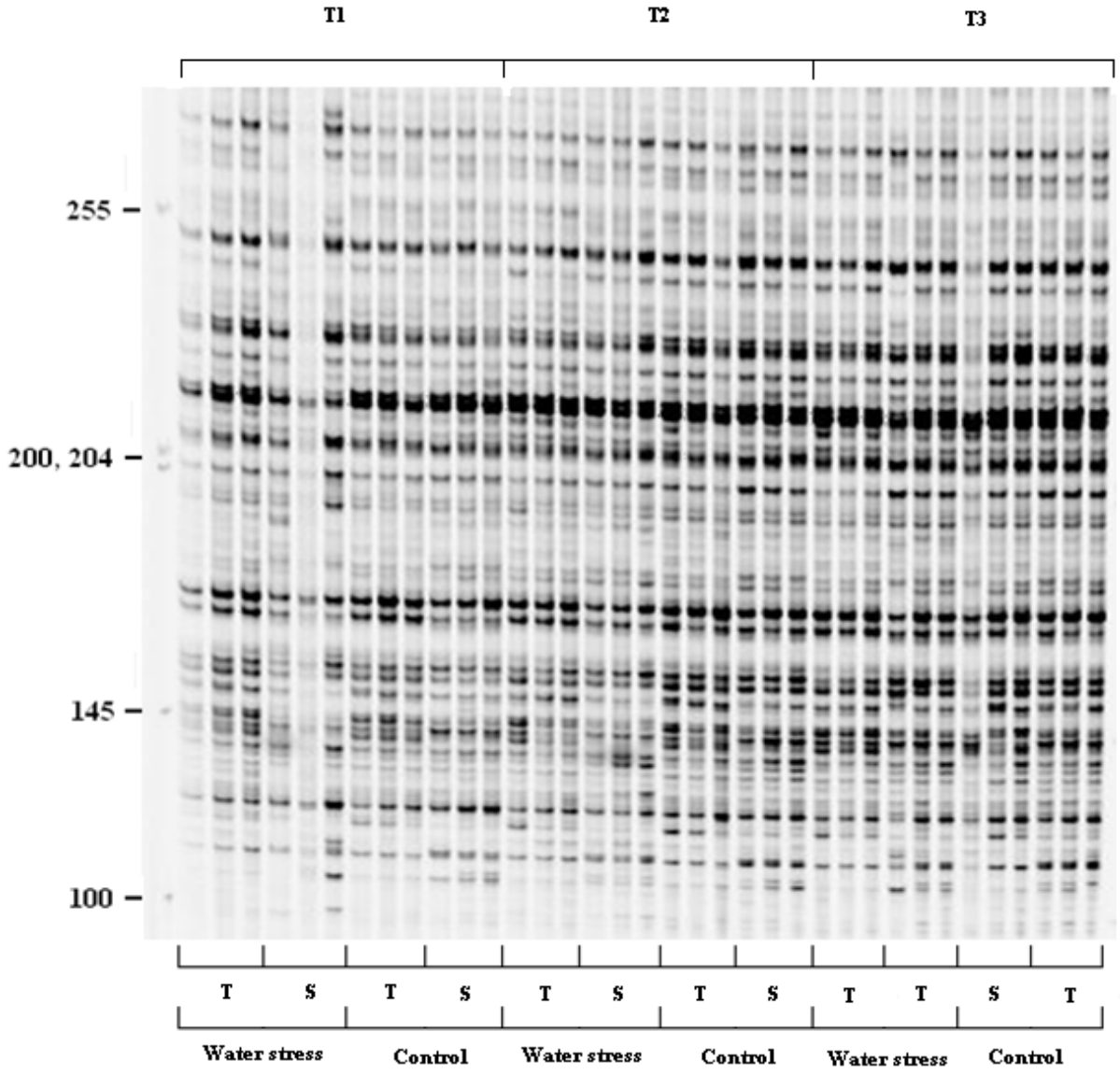


Figure 6. cDNA-AFLP fingerprint generated from two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) under two water supply regimes (control and moderate water stress) evaluated at three times (T1 and T2: two and twelve days after water stress initiation, respectively ,and T3: eight days after re-watering). The primer combination used was *Taq*I GT x *Mse*I AG.

Among other factors, time of exposure and severity of stress is very important for plant survival under drought conditions (Passioura, 2007). A total of 11 TDFs were differentially-expressed at T1. 8 TDFs were differentially-expressed only at T2, while 11

TDFs were responsive at both evaluation times. These results indicate that some TDFs overlapped between the two evaluation times, while others are expressed early on or later on during stress exposure. Only 1 TDF was up-regulated at T1, while 5 TDFs were up-regulated at T2. On the other hand, 10 TDFs were down-regulated at T1, 3 at T2 and 11 at both evaluation times. The higher number of down-regulated TDFs compared to those up-regulated was also observed by Yue *et al*. (2008) in maize plants exposed to one day and seven days of water deficit stress treatment.

Following re-watering, plants could immediately show a high rate of biological activity, including photosynthetic capacity and new organ growth, which can be considered as alternative functional states that may overcompensate for the limitation to plant growth and metabolic activity due to previous drought (Xu *et al*., 2009). In the present study all the TDFs which were differentially expressed in the susceptible or tolerant genotypes had their normal expression pattern recovered after re-watering. These results show the plasticity of sugarcane genotypes in being able to respond rapidly to changing water conditions.

3.2. Isolating and characterization of transcript-derived fragment (TDF)

A total of 15 differentially expressed TDFs was excised from polyacrylamide gels, re-amplified, cloned and sequenced. The size of these TDFs varied from 84 to 343 bp. The sequencing failed for 2 TDFs and these fragments were not characterized further. The remaining sequenced TDFs were renamed as SugDR (sugarcane drought-responsive) and then compared to nucleotide and protein databases using the BLASTn and BLASTx tools, respectively. Table 6 describes TDFs along with the measure of similarity (in case of the TDFs with similarity in the GenBank), regulation (down or up-regulation) under water stress, and evaluation time when the TDFs were regulated.

Table 6. Differentially-expressed transcript derived fragments (TDFs) of two sugarcane genotypes [drought-tolerant: TSP05-4 (T) and drought-susceptible: TCP02-4589 (S)] grown under moderate water stress conditions and evaluated at two times [Two (T1) and twelve days after moderate water stress initiation (T2)]. The TDFs were up-regulated (U) or down-regulated (D).

TDF name	TDF size	Sequence homology	ID	*E*-Value	R.P.	G	E.T.
SugDR01*	201	Putative expressed pentatricopeptide, *Oriza sativa*	ABA99065.2	$1.0e^{-27}$	D	S	T1 and T2
SugDR02*	247	Hypothetical protein OsJ-08616, *Oriza sativa*	EEE57919.1	$2.0\ e^{-40}$	D	S	T1 and T2
SugDR04	112	No significant similarity	-	-	D	S	T1
SugDR05*	84	22 kDA drought-inducible protein mRNA, *Saccharum* hybrid cultivar	AY496271.1	$3.0\ e^{-33}$	U	T	T2
SugDR06*	93	Hypothetical protein OsI-08927, *Oriza sativa*	EEC74003.1	$2.0\ e^{-07}$	D	T	T2
SugDR08	264	Protein kinase CK2 regulatory subunit CK2β3, *Zea mays*	NM001111505.1	$2.0\ e^{-79}$	U	S	T2
SugDR09	217	No significant similarity	-	$1.0\ e^{-19}$	U	S	T2
SugDR10*	111	No significant similarity	-	-	D	T	T1
SugDR11*	143	Hipothetical protein LOC100273728, *Zea mays*	NP001141610.1	$1.0\ e\text{-}^{19}$	D	S	T1 and T2
SugDR14	281	No significant similarity	-	-	D	S	T1 and T2
SugDR15*	282	Glucose-6-phosphate/phosphate translocator 2, *Zea mays*	NP001147439.1	$1.0\ e^{-21}$	D	S	T2
SugDR16*	343	Putative tocopherol polyprenyltransferase, *Oryza sativa*	BAC83059.1	$5.0\ e^{-52}$	U	S	T2
SugDR19	131	No significant similarity	-	-	D	S and T	T1

ID: Identification on GenBank; RP: regulation pattern; G: genotype; ET: evaluation time
* TDFs selected for RT-PCR analysis

Of the total 13 TDFs sequenced, 5 TDFs showed significant similarity to genes with known or putative function, 3 were similar to hypothetical proteins and 5 did not show significant similarity to any sequence or protein in the non-redundant database. The identified and unknown proteins were similar to rice, sugarcane and maize sequences. Approximately 40% of the sequenced TDFs represent yet uncharacterized genes. The characterization of new genes may provide interesting information to the complex plant response network. The unclassified genes also represented the largest category in a study previously performed in sugarcane (Rodrigues *et al*., 2009). Three differentially expressed TDFs are presented in the Figure 7.

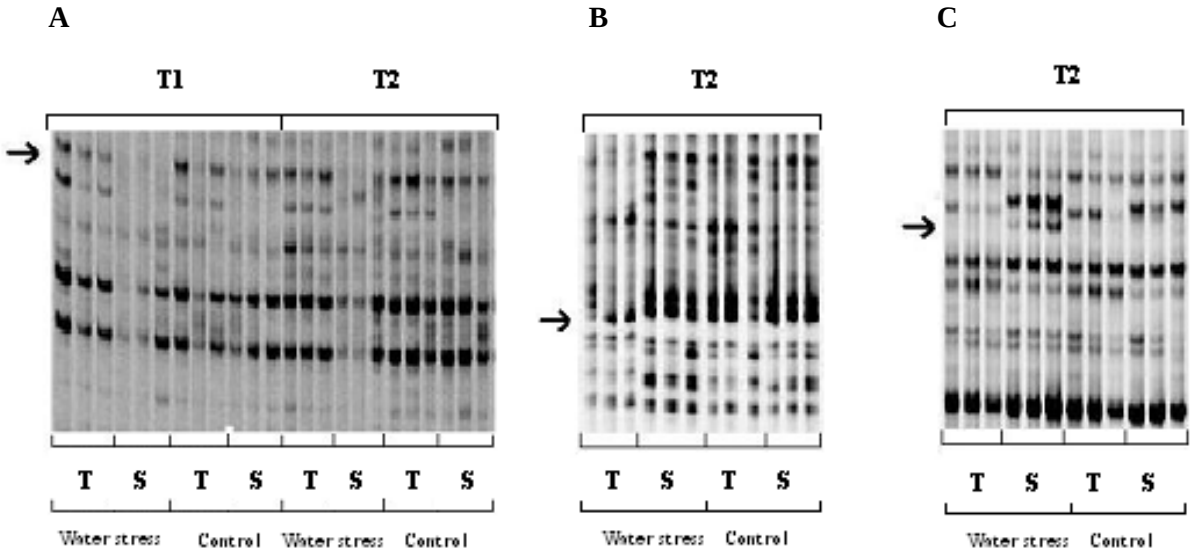


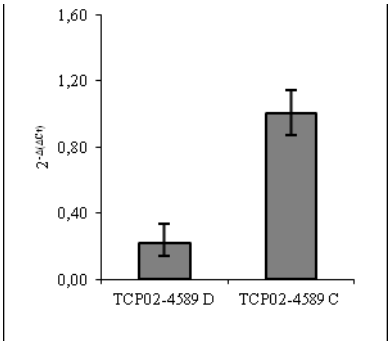
Figure 7. Three differentially-expressed transcript derived fragment (A: putative expressed pentatricopeptide, B: 22 kDA drought-inducible protein mRNA and C: protein kinase CK2 regulatory subunit CK2β3) generated from two sugarcane genotypes [drought-tolerant: TSP05-4 (T) and drought-susceptible: TCP02-4589 (S)] grown under two water supply regimes (control and moderate water stress) and evaluated at two times (T1: two days after moderate water stress initiation and T2: twelve days after moderate water stress initiation).

A databank search revealed the relationship of the TDFS SugDR01, SugDR05, SugDR08, SugDR15, and SugDR16 with other genes involved in environmental stress such as drought, cold, high salinity, and attack of pathogens. Three of these TDFs showing significant sequence similarities to genes encoding a putative expressed pentatricopeptide (SugDR01), a protein kinase CK2 regulatory subunity CK2β3 (SugDR08), and a glucose-6-phosphate/phosphate translocator 2 (SugDR15) were differentially expressed in the susceptible genotype. One TDF (SugDR05) similar to a drought-inducible protein mRNA

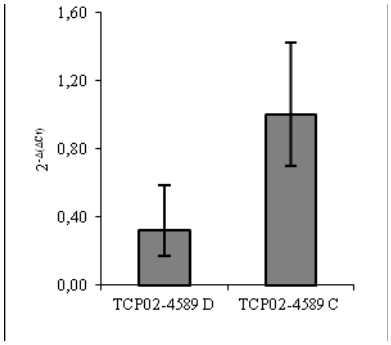
was up-regulated in the tolerant genotype at T2. Finally, one TDF (SugDR16) similar to a tocopherol polyprenyltransferase gene was down-regulated in both genotypes at T2.

3.3. Validation of cDNA-AFLP experiments by real-time reverse transcription PCR (RT-PCR)

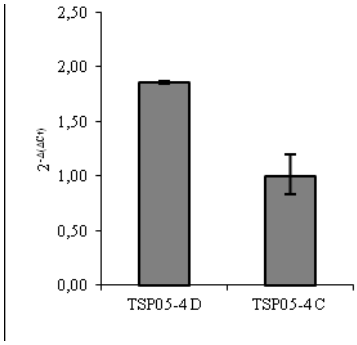
Real-time reverse transcription PCR (RT-PCR) is one of the most reliable method for gene expression analysis. Thus, to validate the changes in mRNA abundance detected by cDNA-AFLP and to quantitatively evaluate the expression level of transcripts in the drought-tolerant and susceptible genotypes, RT-PCR experiment was performed on five down regulated and two up-regulated TDFs. Those TDFs selected for RT-PCR analysis are indicated with an asterisk in the Table 6. The Ct values were normalized using the Ct value of the *GAPDH* gene, which was used as a housekeeping gene. No significant variation in the expression of *GAPDH* was observed among water treatments (data not shown). Five TDFs (SugDR01, SugDR02, SugDR05, SugDR11 and SugDR15) were confirmed as either down or up-regulated in response to water stress. The fragments SugDR10 and SugDR16 did not show changes in gene expression level among control and water stress treatments.



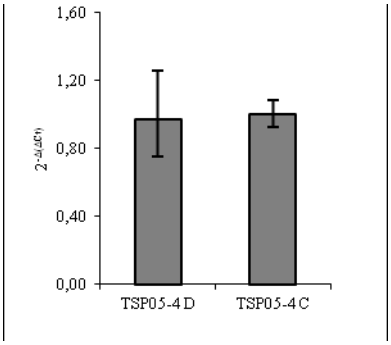
Putative expressed pentatricopeptide -T1



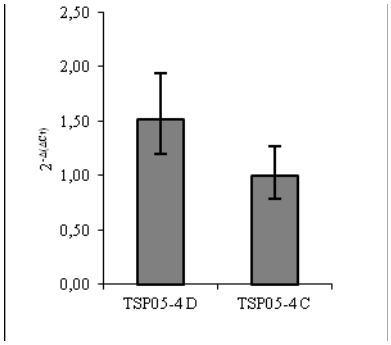
Hypothetical protein *OsJ-08616* - T1



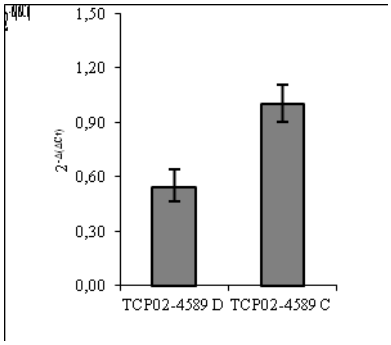
22 kDA drought-inducible protein mRNA - T2



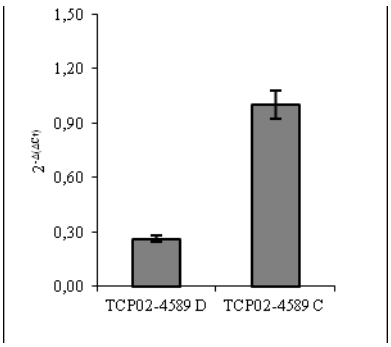
Hypothetical protein *OsI-08927* - T2



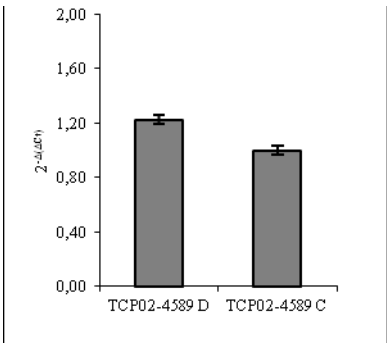
SugDR10 - No significant similarity - T1



Hipothetical protein LOC100273728 - T1



Glucose-6-phosphate/phosphate translocator 2 -T2



Putative tocopherol polyprenyltransferase -T2

Figure 8. Real time RT-PCR analysis of 8 differentially-expressed transcript derived fragment generated from two sugarcane genotypes (drought-tolerant: TSP05-4 and drought-susceptible: TCP02-4589) grown under two water supply regimes (control and moderate water stress) and evaluated at two times (T1: two days after moderate water stress initiation and T2: twelve days after moderate water stress initiation). All data were normalized to the glyceraldehide-3-phosphate de-hydrogenase (*GAPDH*) expression level. Data represent fold change of the gene expression in water-stressed vs. control plants. Bars indicate the standard deviation obtained of three biological replications.

3.4. Functional classification of drought-responsive genes

The pentatricopeptide repeat (PPR) is a protein family capable of specific binding to both protein and RNA molecules (Lurin *et al*., 2004; Schmitz-Linneweber and Small, 2008). Some PPR proteins are involved in plant development (Schmitz-Linneweber and Small, 2008), organelle biogenesis (Lurin *et al*., 2004), restoring of cytoplasmic male sterilities (Bentolila *et al*., 2002; Koizuka *et al*., 2003), RNA processing and editing in mitochondria and chloroplasts (Meierhoff *et al*., 2003; Kotera *et al*., 2005), and responses to environmental stresses (Rodrigues *et al*., 2009). PPR proteins are required for a wide range of different post-transcriptional processes in plant organelles, and the lack of particular PPR proteins often leads to phenotypes owing to lack of expression of a specific organelle gene (Schmitz-Linneweber and Small, 2008). However, there is a little evidence that any of the known PPR proteins meaningfully regulates expression of organelle proteins under physiological conditions.

TDFs with similarity to PPR repeat proteins already been found in sugarcane (Rodrigues *et al*. (2009), mandarin (Gimeno *et al*., 2009), and rice (FengHua *et al*., 2009) exposed to drought stress conditions. In this study, the PPR like-protein (SugDR01) was down-regulated at T1 and T2 days after water stress initiation in the susceptible genotype. Similarly, Rodrigues *et al*. (2009) have found one TDF (CA128234) which was down-regulated under moderate water stress conditions in a susceptible genotype and up-regulated under severe water stress in both tolerant and susceptible genotypes. These authors also found another TDF (CA120224) which was up-regulated only in the tolerant genotype subjected to severe water stress conditions. In the present study PPR protein expression was also suppressed in the susceptible genotype under water stress conditions. An *Arabidopsis* mutant in the PPR gene *At3g09650*, designated as *high-chlorophyll-fluorescence (hcf152-1)*, shows a defect in photosynthetic-electron transport (*petB*) and *psbH* mRNA processing in the chloroplast, resulting in reduced levels of the cytochrome *b6f* complex (Meierhoff *et al*., 2003). The up-regulation of SugDR01 in the susceptible genotype may be indicative of the sensitivity of this genotype to water stress conditions.

SugDR05 which was slightly up-regulated at T2 in the tolerant genotype showed similarity to a drought inducible protein (SoDip22) in *Saccharum officinarum* (Sugiharto *et al*., 2002). Because of the hydrophilic nature of SoDip22, it is plausible that it belongs to the Asr (abscisic acid-ABA, stress, and ripening induced) protein family and it functions to adapt to drought stress in the bundle sheath, and the signaling pathway for the induction is, at least

in a part, mediated by ABA. *Asr* genes represent a gene family that is usually induced by a wide range of stress such as, drought (Maskin *et al*., 2001; Yang *et al.,* 2005; Philippe *et al*., 2010), salt (Yang *et al.,* 2005), ABA (Çakir *et al*., 2003; Carrari *et al*., 2004) and pathogen response (Liu *et al*., 2010).

Kinase CK2 protein, also known as casein kinase II, is a highly conserved serine/threonine kinase and it has been classified as a stable tetrameric complex, consisting of two catalytic subunits (CK2α and CK2α') and two regulatory subunits (CK2β1 and CK2β2) (Pinna, 2002; Litchfield, 2003). However, plant kinase CK2 proteins contain several isoforms for both catalytic and regulatory subunits, creating the potential for a wide variety of CK2 holoenzyme combinations that may have a role in regulating CK2 activity or substrate specificity (Riera *et al*., 2001). The plant CK2 is involved in many different processes such as, DNA transcription, RNA translation, and cell-cycle regulation.

SugDR08 which was up-regulated at T2 in the susceptible genotype showed similarity to the regulatory subunit CK2β3. Although several studies have shown that kinase protein groups are regulated under drought stress conditions (Montalvo-Hernández *et al*., 2007; Rocha *et al*., 2007; Clement *et al*., 2008; Rodrigues *et al*., 2009; Mizogushi *et al*., 2010), few studies have reported on the regulation of CK2 regulatory subunits under environmental stress. In sugarcane, Rocha *et al*. (2007) and Rodrigues *et al*. (2009) reported that several kinases are regulated under drought conditions. Rodrigues *et al*. (2009) have also found two TDFs similar to serine/threonine kinase-like proteins that were up-regulated under severe drought conditions. One protein was induced in both tolerant and susceptible sugarcane cultivars, while two other ones were induced only in the susceptible genotype. They also found other kinases that were simultaneously up-regulated in the tolerant and susceptible genotypes. Rocha *et al.* (2007) have similarly identified ten kinase proteins differentially-expressed in response to drought.

Changes in osmotic response are associated with changes in the location of cellular components via transporters and changes in the synthesis of secondary metabolites (Sahin-Çevic and Moore, 2006; Meng *et al*., 2008). The induction of transporters enables osmoprotectants to move to their functional sites. The glucose-6-phosphate/phosphate translocator (GPT) represents a distinct member of the phosphate translocator protein family and its proposed physiological function is import glucose 6-phosphate into amyloplasts of heterotrophic tissues for use as a precursor for starch and fatty acid biosynthesis and a substrate for the oxidative pentose phosphate pathway (Fischer and Weber, 2002; Hua-wu *et al*., 2003). The expression of transcripts similar to GPT was induced by cold (Sahin-Çevic

and Moore, 2006; Lee *et al*., 2010) and heat conditions (Qin *et al*., 2008) and by combined drought and heat stress treatments (Rizhsky *et al*., 2004). However, a TDF (SugDR15) similar to a glucose-6-phosphate/phosphate translocator was found to be repressed at T2 in the susceptible genotype. Similarly, in a study achieved by Xue *et al*. (2008) a significant reduction in expression was observed for one (TaGPT) of the three chloroplasts GPT genes analyzed in droughted-wheat plants.

4. CONCLUSIONS

- The tolerant and susceptible genotypes had different responses when subjected to water stress conditions.
- Gene response to water stress was quicker in the susceptible genotype, since a higher number of differentially expressed TDFs were detected in this genotype compared to the tolerant genotype.
- The higher number of up-regulated genes in the susceptible genotype may be an indicator of its sensitivity to water stress.
- The normal gene expression pattern were restored in both genotypes within 8 days after re-watering, perhaps demonstrating the plasticity of sugarcane plants in being able to respond rapidly to changing water conditions.

5. ACKNOWLEDGEMENTS

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GENERAL CONCLUSIONS

- Physiological traits, especially gas exchange traits were more able to discriminate among drought tolerant and susceptible genotypes even at early and moderate water stress.
- Growth and morphological traits should be evaluated in an advanced water stress stage during the formative phase to select to drought tolerance and higher productivity genotypes.
- It was possible see that tolerant and susceptible genotypes had different responses when subjected to water stress conditions and that gene response to drought stress was quicker in susceptible compared to the tolerant genotype.
- After a re-watering period the gas exchange traits and gene expression pattern returned completely to the rates observed to the control treatment, demonstrating the plasticity of sugarcane genotypes in responding to changing water conditions.