Chapter 3-3

Development of drought-tolerant sugarcane overexpressing the *AtDREB2A CA* gene

Wagner Rodrigo de Souza [1,2], Nelson Geraldo de Oliveira [1], Felipe Vinecky [1], Ana Paula Ribeiro [1], Marcos Fernando Basso [1], Raphael Augusto das Chagas Noqueli Casari [1], Bárbara Andrade Dias Brito da Cunha [1], Alexandre Lima Nepomuceno [3], Adilson Kenji Kobayashi [1], Kazuo Nakashima [4], Kazuko Yamaguchi-Shinozaki [5, 6], Hugo Bruno Correa Molinari [1*]

[1] Genetics and Biotechnology Laboratory, National Center for Agroenergy Research (CNPAE), Brazilian Agricultural Research Corporation (Embrapa), Brasília, Distrito Federal 70297-400, Brazil

[2] Center for Natural and Human Sciences, Federal University of ABC (UFABC), Santo André, SP, 09210-580, Brazil

[3] Embrapa Soybean, Rod. Carlos João Strass, s/n, Londrina, PR 86001-970, Brazil

[4] Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki 305-8686, Japan

[5] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[6] Research Institute for Agricultural and Life Sciences, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

[*] *Corresponding author; Email: hugo.molinari@embrapa.br*

Abstract

Sugarcane is considered an important economic crop not only for sugar production, but also for ethanol generation, serving as an expandable green alternative to the usage of crude oil. To take advantage of the use of sugarcane as a renewable source for bioethanol production, it is important to increase its productivity without increasing land usage, which includes sugarcane cultivation under hostile conditions, such as water-limited environments. In this study, we demonstrated that stress-inducible overexpression of the transcription factor *AtDREB2A CA* conferred drought tolerance in sugarcane subjected to water deficiency under greenhouse conditions. In addition, transgenic plants expressing *AtDREB2A CA* accumulated higher levels of sucrose than non-transgenic plants. These results indicate that the *AtDREB2A CA* gene under the control of the stress-inducible promoter *ZmRab17* represents a promising strategy for the development of new sugarcane varieties with improved drought tolerance.

Keywords: abiotic stress, *AtDREB2A CA*, *ZmRab17* promoter, *Saccharum* spp. hybrid, transcription factor

Introduction

Sugarcane is an important economic crop not only for production of sugar but also for biofuel production. The importance of sugarcane cultivation has increased in the recent years due to ethanol production, which is considered one of the most viable alternatives to fossil fuels (Savage 2011). The production of biofuels is important not only to reduce oil imports, but also to decrease CO_2 emissions contributing to global warming and mitigate climate change (Reis et al. 2014). Drought is considered the most deleterious abiotic stress affecting crop productivity worldwide, and water availability is the main factor that influences sugarcane productivity as it directly affects tillering, culm height, and sucrose production (Sugiharto 2004).

Currently, the development of drought-tolerant genotypes is one of the main objectives of sugarcane research programs. However, the achievement of this goal is hampered by the high ploidy level of modern sugarcane varieties and by the fact that drought tolerance is a multigenic quantitative trait. In addition, plant responses to drought are influenced by the time, intensity, duration, and frequency of the stress as well as by diverse plant-soil-atmosphere interactions, making breeding selection procedures difficult (Basnayake et al. 2012). Therefore, the use of different genetic engineering strategies to improve drought tolerance in sugarcane is desirable.

Dehydration-responsive element-binding proteins (DREBs) play vital regulatory roles in abiotic stress responses in plants. The transcription factor DREB2A interacts with a *cis*-acting dehydration-responsive element (DRE) sequence to activate the expression of downstream genes that are involved in abiotic stress responses in *Arabidopsis thaliana* (Sakuma et al. 2006). In the present study, we evaluated the effects of stress-inducible overexpression of *AtDREB2A CA* in sugarcane. The results demonstrated that sugarcane transgenic lines presented significantly enhanced drought tolerance without yield penalty under greenhouse conditions. These promising results prompted us to evaluate these sugarcane plants in field trials conducted in Brazil, and the results obtained from the field trials will be briefly discussed here.

Materials and Methods

Plant material and transformation

Sugarcane *ZmRab17::AtDREB2A CA* lines were generated and analyzed at the molecular level as described by Reis et al. (2014). Sugarcane embryogenic calli were generated from immature leaf segments

of 6-8-month-old plants of the RB855156 variety and bombarded with the expression vector pBract 302, containing the *A. thaliana DREB2A CA* (Sakuma et al. 2006), which is driven by a stress-inducible promoter Rab17 from *Zea mays*. pBract 302 also contains the bar cassette used as a selective marker. Regenerated plantlets were transferred to planting trays containing a commercial propagation substrate (Plantmax^{TM}) and grown under controlled greenhouse conditions.

Molecular analysis

The presence of the *AtDREB2A CA* transgene in leaf samples from regenerated sugarcane plantlets was confirmed by standard polymerase chain reaction (PCR) using specific primers. Positive PCR plants and non-transgenic plants were sprayed with 1% (v/v) glufosinate ammonium and evaluated 8 days after spraying, obtaining eight independent events that were further evaluated under drought conditions.

Screening of transgenic sugarcane events under water-deficit conditions

One-month-old plants from the eight events obtained from plant transformation were subjected to 21 days of water-deficit stress to select the best events for further analysis. One out of five events demonstrated outstanding drought tolerance, as suggested by reduced leaf rolling and decreased senescence when compared with non-transformed (NT) plants. Therefore, this independent event was further characterized in detail under drought stress conditions.

Physiological measurements of sugarcane under water-deficit conditions

Eight-month-old plants were grown in 28-L PVC cylinders (25 cm diameter, 150 cm height). Water stress trials were carried out in a randomized block design (RBD) with five replications for both transgenic and non-transgenic plants. The water tension in the xylem was measured daily (8:00 – 11:00), using a Scholander pressure chamber, in the fully expanded photosynthetically active leaf (+4 leaf). These measurements were assumed to represent the leaf water potential (Ψ_L). The relative water content (RWC) was estimated in the +5 leaf (base, middle, top) by measuring the fresh and dry weights, and the RWC was calculated as: RWC (%) = [(FM - DM)/ (TM - DM)] × 100, where FW, DM, and TM are the fresh, dry, and turgid weights, respectively.

The net photosynthetic rate (A), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration (E) were assessed using an open gas exchange system with a 6 cm^2 clamp-on leaf cuvette (LI-6400XT, LICOR, Lincoln, NE, USA). Leaf gas exchange was evaluated in the middle third of the second fully expanded leaf with visible ligule (+2 leaf). These measurements were taken between 8:00 and 11:00 hours, for 4 days after withholding water every day. The photosynthetic photon flux density (PPFD) was fixed at 1.500 μmol m^{-2} s^{-1}, using a red-blue LED light source built into the leaf cuvette, although other

environmental factors, such as air humidity and temperature, were not controlled; in other words, natural variation was permitted. The vapor pressure deficit in the cuvette was maintained below 2.5 kPa to prevent stomatal closure due to the low air humidity effect. The air collected outside the greenhouse was passed through a buffering zone and then pumped into the system, with a mean CO_2 concentration of 380 μmol mol $^{-1}$.

Agronomic characterization of sugarcane plants under water-deficit conditions

The agronomic characterization of the 8-month-old transgenic (T) and non-transgenic (NT) sugarcane plants was performed after four days of withholding water. The agronomic parameters consisted of shoot dry weight, root dry weight, internode length, culm length, sucrose content, and bud sprouting rate. Shoot and root dry weights were determined after drying the leaves or roots in an oven at 80 °C until a constant weight was obtained. The culm length, diameter, and number and length of internodes were measured using a graduated meter ruler and a digital caliper Vonder model (PPV-1506). Sucrose content was measured at the end of the experiment, when aliquots of cane juice from the last, penultimate, and antepenultimate internodes were collected and the sucrose content was determined as prescribed by Reis et al. (2014). Briefly, the juice was separated by high-performance liquid chromatography (HPLC) and subsequently quantified using refractive index detection (RID). HPLC-RID was conducted with an Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, CA) equipped with an Aminex HPX-87H anion-exchange column, 300 × 7.8 mm (Bio-Rad, Hercules, CA). Samples were diluted with 9 volumes of H_2O, injected into the HPLC-RID system (50-μl injection volume), and eluted isocratically with 0.02 N H_2SO_4 at a flow rate of 0.5 mL min^{-1} (RID flow cell, 45°C; column, 50°C). Reference sucrose (Fisher Scientific) was diluted in H_2O and used to generate a standard curve.

Bud sprouting rate was measured after harvesting the middle portion of each stalk from 9-month-old transgenic (T) and non-transgenic (NT) sugarcane plants that were subjected to water deprivation as described above. Each measurement consisted of 24 buds grown in vermiculite substrate on plastic trays under greenhouse conditions. At the end of 30 days, the bud sprouting rate was calculated and expressed as percentage.

Results

Screening of drought-tolerant sugarcane lines under greenhouse conditions

Eight independent events of sugarcane *AtDREB2A CA* plants and non-transgenic plants (NT) were subjected to 6 days of water deprivation, which demonstrated a better performance of transgenic lines than NT plants (**Fig. 1a**). From these lines, event 24.2 presented the best characteristics to be considered an elite

event, that is, a very strong "stay-green" phenotype, compared to NT plants (**Fig. 1b** and **1c**) and high levels of *AtDREB2A CA* expression under water-deficit conditions (**Fig. 1d**). Therefore, this event was chosen for further detailed analysis, and from this point onwards, it will be designated as a transgenic event (T).

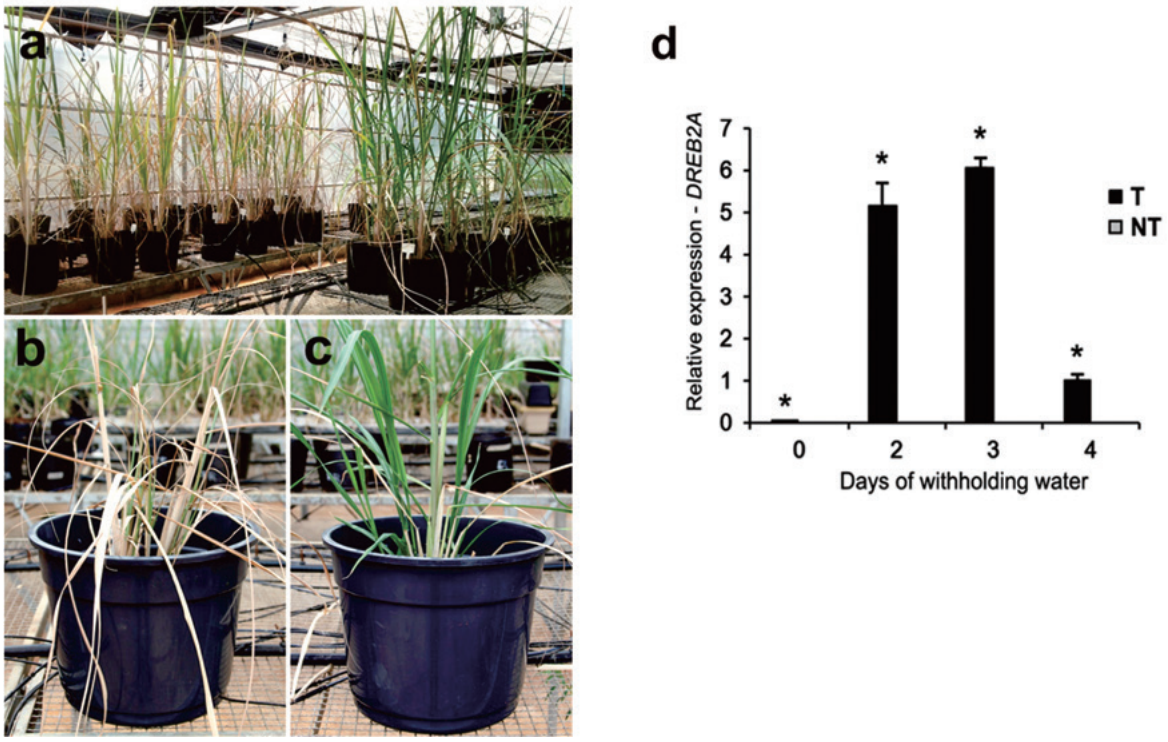

Fig. 1. Sugarcane with drought-inducible expression of *AtDREB2A CA* during a 'survival' drought tolerance test.
a – Three-month-old plants subjected to 6 days of water deprivation. Left side: non-transgenic plants; right side: eight independent transgenic events. **b** and **c**: Independent transgenic event (24.2) and control (non-transgenic plants) sugarcane plants that are 1 month old grown in 8-L plastic pots and subjected to 21 days of water deficit stress period. **d** – Transcript abundance of *AtDREB2A CA* during drought treatment. The geometrical mean of *GAPDH* and *25S rRNA* was used as a reference to measure the relative quantification, which corresponds to the mean of three biological repetitions ± SE. Statistical differences (*$p < 0.05$) were analyzed with ANOVA, followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Plant water status and gas exchange analysis

Under well-watered conditions, it was verified that the difference in leaf water potential (Ψ_L), relative water content (RWC), net photosynthesis rate (A), concentrations of CO_2 in the substomatal chamber (Ci), transpiration (E), and stomatal conductance (g_s) were not statistically significant between transgenic (T) and non-transgenic (NT) plants (**Fig. 2**). However, under drought stress, the Ψ_L and RWC in T and NT plants were similar until day 3 (i.e., after the start of water deficit), with mean values of −1.41 MPa and 74%, respectively. In addition, on day 4 of water deprivation, the Ψ_L and RWC of the NT plants dropped to nearly −2 MPa and 60%, respectively, whereas the Ψ_L and RWC of the T plants increased to −1.42 MPa and 75%, respectively. In general, the gas exchange measurements related to A, g_s, and E were higher for T plants than for NT plants, and the Ci was smaller for T plants than for NT plants. However, statistical significance was

only found for *A*, g_s, and *Ci* at days 2 and 3 of withholding water (**Fig. 2**). By the end of day 4 of water deprivation, the NT plants appeared completely wilted and exhibited leaf rolling, while the leaves of T plants were still turgid and expanded (data not shown). These results suggest that differential responses to the drought stress of T and NT plants were observed only on the 2nd and 3rd days when the plants experienced moderate levels of stress (RWC = 80% and water potential ~ −1.5 MPa for the NT and T plants) or a more severe stress (RWC = 70% and water potential lower than −1.5 MPa for NT plants).

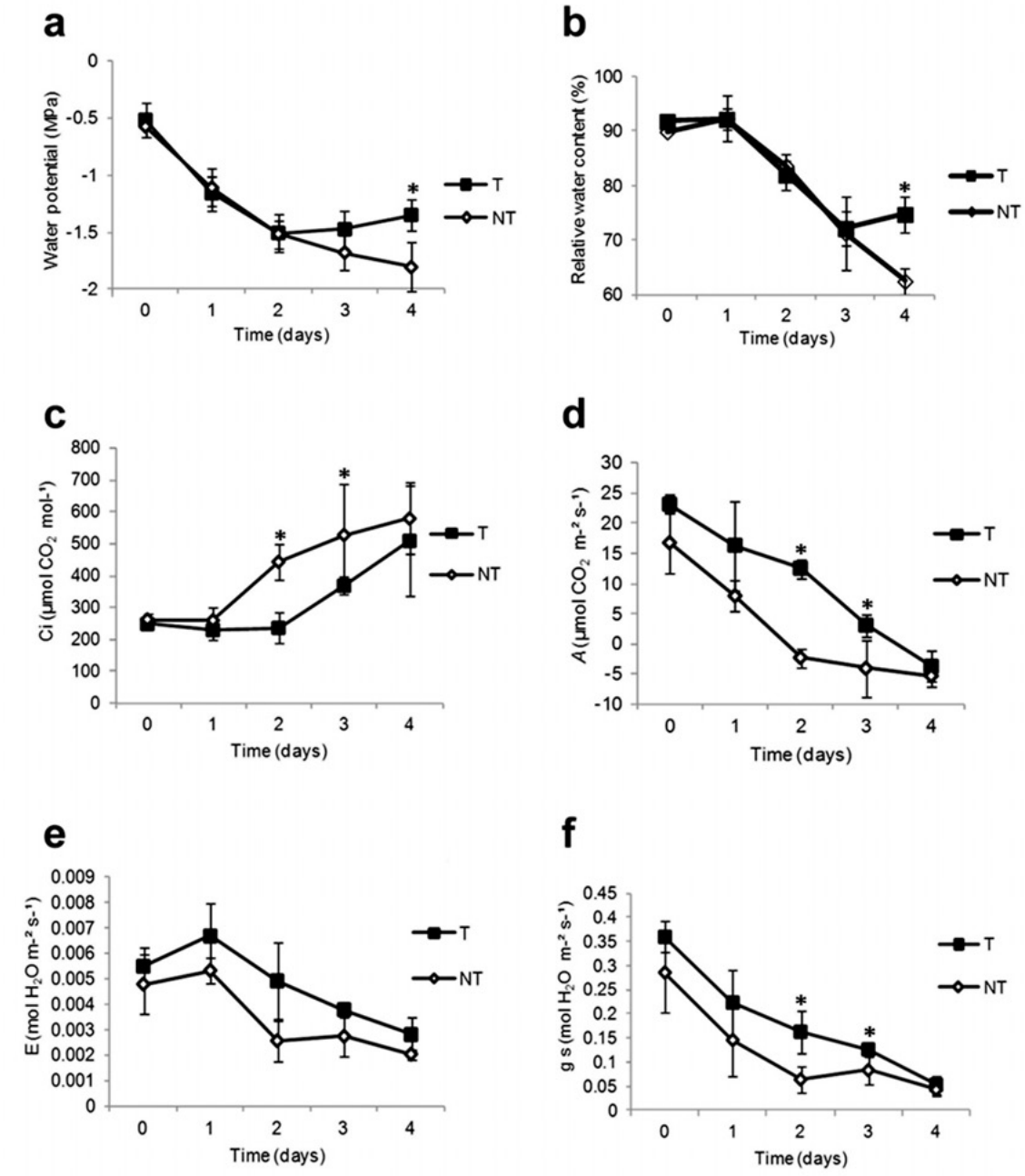

Fig. 2. Physiological characterization of transgenic (T) and non-transgenic (NT) sugarcane plants under water deprivation for four days of withholding water.

a) Water potential; **b**) relative water content (RWC). **c - f** Gas exchange parameters including: **c** - concentrations of CO_2 in the substomatal chamber (*Ci*), **d** is the net photosynthesis rate (*A*), **e** is the transpiration (*E*), and **f** is the stomatal conductance (*gs*). Values are the mean ± S.E. (n = 5). Statistical differences (*$p < 0.05$) were analyzed with ANOVA followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Agronomic characteristics of transgenic sugarcane under greenhouse conditions

As observed in **Fig. 3**, we found no significant differences between T and NT plants in the shoot and root dry weight after a period of water deprivation for 4 days (**Fig. 3a** and **3b**). However, statistical differences were found in the culm length and internode length (**Fig. 3c** and **3d**), which were higher for the T plants. **Fig. 3e** demonstrates that the sucrose content in the culms of T plants was 33.8% higher than that in NT plants, while the bud sprouting rates of T plants that descended from plants subjected to water deprivation were 82% higher than that of NT plants (**Fig. 3f**).

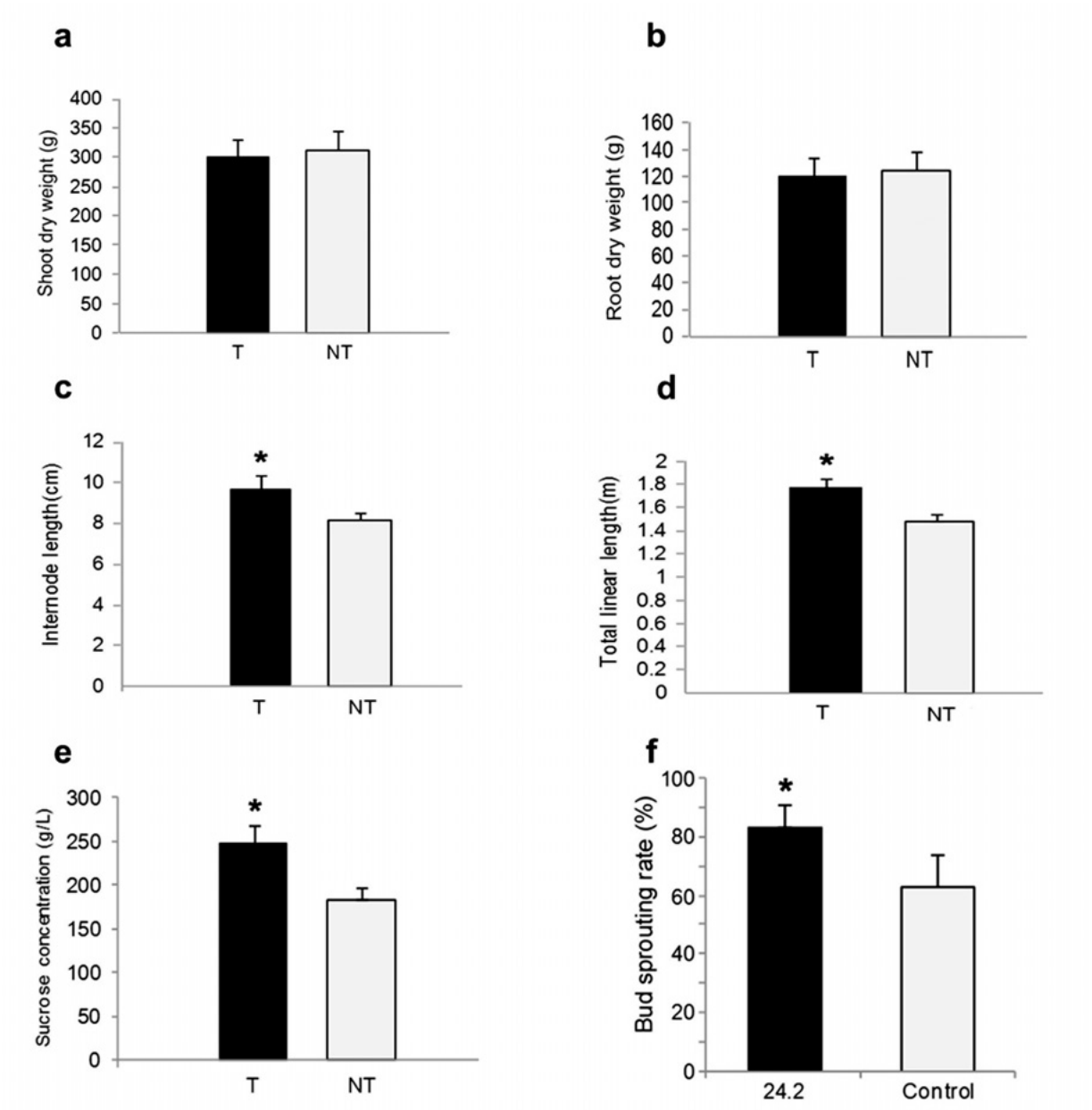

Fig. 3. Agronomic characterization of 8-month-old transgenic (T) and non-transgenic (NT) sugarcane plants after four days of withholding water.

Shoot dry weight **(a)**, root dry weight **(b)**, internode length **(c)**, culm length **(d)**, sucrose content **(e)** and bud sprouting rate **(f)**. Values are the mean ± S.E. (n = 5 for a-e and n = 24 for f). Statistical differences (*$p < 0.05$) were analyzed with ANOVA followed by Tukey's test (Figure modified from Reis et al., 2014 with permission).

Discussion

AtDREB2A is a transcription factor involved in drought stress responses, and its native form is not sufficient for the activation of drought-responsive genes because of the presence of a negative regulatory domain (NRD). In *Arabidopsis thaliana*, the deletion of the central region in the NRD makes DREB2A constitutively active (*DREB2A CA*) (Sakuma et al. 2006). The constitutive expression of *DREB2A* leads to severe growth defects, but the overexpression of the transcription factor under a stress-inducible promoter, such as ZmRab17, allows regular plant development (Sakuma et al. 2006, Engels et al. 2013, Reis et al. 2014). Here, we used the construct *ZmRab17::AtDREB2A CA* to transform the drought-sensitive sugarcane RB855156 variety as background. Under greenhouse conditions, our results demonstrated that *ZmRab17::DREB2A CA* plants subjected to drought stress displayed better performance related to increased sucrose content and bud sprouting rate verified with sugarcane descended from plants subjected to water deprivation (**Fig. 3**). In addition, the physiological measurements showed that plants transformed with the *ZmRab17::DREB2A CA* construct presented higher stomatal conductance and photosynthetic and transpiration rates, even under normal water regimes (**Fig. 2c**, **2d,** and **2f**).

As discussed by Reis et al. (2014), one of the most important characteristics of sugarcane varieties is their capacity for initial sprouting. The RB855156 variety used in the present study has high yields and sucrose levels; however, low initial sprouting is a major concern for this variety, especially after a period of water deprivation (Silva et al. 2007). It is possible that physiological and molecular adaptations obtained by *AtDREB2A CA* plants are not only during water stress trials but also during all survival tests performed previously, and during the whole plant cycle with the plants under control conditions, as shown in **Fig. 1d**, revealing the basal activity of the *ZmRab17* promoter in the leaves of the transgenic plants, could be responsible for higher rates of bud germination and effects observed in transgenic plants. The higher photosynthetic rates of transgenic plants prompted us to speculate that induced overexpression of *AtDREB2A CA* gene was capable of activating the sucrose synthesis pathways, as these plants demonstrated increased accumulation of sucrose. However, at this point, we are not able to rule out a plausible explanation for the increased sucrose accumulation in transgenic plants. Thus, additional studies on gene expression analysis would be required to investigate the role of *DREB2A CA* overexpression in the modulation of sugar biosynthesis pathways.

The promising results obtained from this study prompted us to evaluate these sugarcane transgenic lines under field conditions. Two distinct lines, including line 24.2, were analyzed in two seasonally independent dry regions of Brazil. As presented by de Souza *et al.* (2019), *AtDREB2A CA* sugarcane lines demonstrated higher yield and productivity than non-transformed plants under drought conditions. The

agronomical performance of these lines was measured in terms of the content of soluble solids (°Brix), sugar content in the culm juice (Pol%), tons of cane per hectare (TCH), and tons of Pol% per hectare (TPH). In both the dry locations, the sugarcane lines performed better, demonstrating increased levels of °Brix, %Pol, TCH, and TPH corresponding to 11.6%, 18.0%, 20.3%, and 41.7%, respectively, than NT plants. The performance of *AtDREB2A CA* sugarcane lines in the field under drought conditions was comparable to that of a high-yield and drought-tolerant elite variety grown in Brazil (CTC9001). These results corroborate that overexpression of *AtDREB2A* in sugarcane might be incorporated as a new biotechnological strategy for the development of drought-tolerant varieties.

Conclusions

The results presented here show that transformation of sugarcane with the *AtDREB2A CA* gene under the control of the *ZmRab17* promoter enhanced the tolerance of the sugarcane RB855156 variety to water deficiency. Transgenic sugarcane had improved initial bud sprouting, increased culm and internode lengths, and higher sucrose content (33.8%), than non-transgenic plants under water deficiency in greenhouse conditions. The results described here were presented by Reis et al. (2014) and extracted with permission. As the success of any biotechnological strategy would ultimately be determined by the final yield under field conditions, the analysis of two sugarcane transgenic events overexpressing *AtDREB2A CA* in two different dry regions of Brazil. These results indicated that these lines presented higher productivity and yield than control plants under drought conditions. Therefore, the results obtained from field trials corroborated that the overexpression of *AtDREB2A CA* in sugarcane might be a useful strategy for the development of new drought-tolerant varieties.

Acknowledgment

We would like to thank Editage (www.editage.com) for English language editing.

References

Basnayake J, Jackson PA, Inman-Bamber G, Lakshmanan P (2012). Sugarcane for water-limited environments. Genetic variation in cane yield and sugar content in response to water stress. *J Exp Bot* **63**: 6023–6033.

de Souza WR, de Oliveira NG, Vinecky F, Ribeiro AP, Basso MF, Casari RACN, da Cunha BADB, Duarte KE, Santiago TR, Martins PK, Aucique-Perez CE, Cristofoletti Júnior SC, Nepomuceno AL, de Sousa CAF, Kobayashi AK, Nakashima K, Yamaguchi-Shinozaki K, Molinari HBC (2019). Field evaluation of *AtDREB2A CA* overexpressing sugarcane for drought tolerance. *Journal of Agronomy and Crop Science* **00**:1-9. https://doi.org/10.1111/jac.12341

Engels C, Fuganti-Pagliarini R, Marin SRR, Marcelino-Guimarães FC, Oliveira MCN, Kanamori, N, Mizoi J, Nakashima, K., Yamaguchi-Shinozaki K, Nepomuceno AL (2013) Introduction of the *rd29A:AtDREB2ACA* gene into soybean (*Glycine max* L. Merril) and its molecular characterization in the leaves and roots during dehydration. *Genet Mol Biol* **36:** 556–565. doi: 10.1590/S1415-

47572013000400015
Reis RR, da Cunha BADB, Martins PK, *et al*. (2014). Induced overexpression of AtDREB2A CA improves drought tolerance in sugarcane. *Plant Science* **221–222**: 59–68.
Sakuma Y, Maruyama K, Qin F, Osakabe Y, Sek, M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Dual function of an *Arabidopsis* transcription factor DREB2A in water stress-responsive and heat-stress-responsive gene expression. Proc. Nat. Acad. Sci. USA **103**:18828-18833.
Savage, N (2011) Fuel options: the ideal biofuel. *Nature* **474**: S9–S11.
Silva MA, Jifon JL, Da Silva JAG, Sharma V (2007) Use of physiological parameters as fast tools to screen for drought tolerance in sugar. *Brazilian Journal of Plant Physiology* **19**: 193-201.
Sugiharto, B (2004) Biochemical and molecular studies on sucrose-phosphate synthase and drought inducible-protein in sugarcane (*Saccharum officinarum*). *ILMU Dasar* **5**: 62–67.