Plant Science 221–222 (2014) 59–68

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Induced over-expression of *AtDREB2A CA* improves drought tolerance in sugarcane

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ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form 4 February 2014
Accepted 6 February 2014
Available online 15 February 2014

Keywords:
Abiotic stress
AtDREB2A CA
ZmRab17 promoter
Saccharum spp.
Transcription factor

ABSTRACT

Drought is one of the most challenging agricultural issues limiting sustainable sugarcane production and, in some cases, yield losses caused by drought are nearly 50%. DREB proteins play vital regulatory roles in abiotic stress responses in plants. The transcription factor DREB2A interacts with a *cis*-acting DRE sequence to activate the expression of downstream genes that are involved in drought-, salt- and heat-stress response in *Arabidopsis thaliana*. In the present study, we evaluated the effects of stress-inducible over-expression of *AtDREB2A CA* on gene expression, leaf water potential (Ψ_L), relative water content (RWC), sucrose content and gas exchanges of sugarcane plants submitted to a four-days water deficit treatment in a rhizotron-grown root system. The plants were also phenotyped by scanning the roots and measuring morphological parameters of the shoot. The stress-inducible expression of *AtDREB2A CA* in transgenic sugarcane led to the up-regulation of genes involved in plant response to drought stress. The transgenic plants maintained higher RWC and Ψ_L over 4 days after withholding water and had higher photosynthetic rates until the 3rd day of water-deficit. Induced expression of *AtDREB2A CA* in sugarcane increased sucrose levels and improved bud sprouting of the transgenic plants. Our results indicate that induced expression of *AtDREB2A CA* in sugarcane enhanced its drought tolerance without biomass penalty.

1. Introduction

The importance of sugarcane cultivation has increased in recent years due to ethanol production, which is considered as one of the most viable alternatives to fossil fuels [1]. Production of biofuels are important not only to reduce dependence on oil but also to ameliorate CO_2 emissions generated by burning fossil fuels as gas emissions contributes to global warming and climate changes [2].

Brazil is the world leader in sugarcane production; the crop has an important social role in Brazilian agribusiness, improving opportunities for farmers and rural communities. According to UNICA

Abbreviations: ABA, abscisic acid; DREB, DRE-binding protein; DREB2A, DRE-binding protein 2A; DRE, dehydration-responsive element; AP2/ERF, APETALA2/ethylene responsive-element binding factor.

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http://dx.doi.org/10.1016/j.plantsci.2014.02.003
0168-9452/

(Sugarcane Industry Union – Brazil), the sugar-ethanol sector creates 1.2 million direct rural jobs, which include seventy thousand producers in 2011/2012 milling season [3].

The Brazilian sugarcane agroecological zoning mapped the cerrado degraded pasture land as a promising area for expanding sugarcane production. The cerrado is a savannah-like biome with climate classified as Aw type (Koppën-Geiger), with long period of drought. The soil is predominantly latosoil, acid with low fertility [4]. However, the irregular rainfall in the cerrado usually leads to severe drought stress which affects the crop growth [5]. Drought is the main factor that influences sugarcane productivity as it directly affects tillering and culm height, resulting in decreased sucrose production [6].

Development of genotypes tolerant to drought stress is one of the main objectives of sugarcane research programs in Brazil; however, achievement of such goal is hampered by the high-ploidy level of modern sugarcane varieties and by the fact that drought tolerance is multigenic and is a quantitative trait [7]. Besides, 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 [8].

The use of biotechnology in public and private breeding programs allows for the development of events with improved traits, such as higher sugar content [9], higher resistance to diseases and pests [10–12], better tillering and deeper roots [13], increased tolerance to drought, heat and low-temperature [14,15], and through improved agronomical and industrial qualities [16,17]. In addition, the introduction of some specific genes allowed for better understanding of molecular, cellular and physiological mechanisms of plant responses to environmental stresses [18].

One alternative approach to the development of drought tolerant plants is to genetically engineer plants to introduce stress-tolerant genes, including genes for transcription factors (TFs). TFs recognize specific DNA sequences in the regulatory regions of target genes and lead to activation of downstream genes responsive to abiotic stresses. One relevant class of transcription factors is the DREBs [19].

The DRE, which has a core sequence of A/GCCGAC, is a *cis*-acting element originally isolated from the promoter of the *Rd29a* gene of *Arabidopsis thaliana* and is involved in both cold- and dehydration-inducible gene expression by an ABA-independent pathway [20]. DREB2A is a transcriptional activator that recognizes DRE in *Arabidopsis*. It is a member of the DREB subfamily within the AP2/ERF family of TFs and contains a single conserved DNA-binding domain [21]. Its expression is induced by dehydration, high salinity and heat shock [22]. The transcription of *DREB2A* in response to dehydration and heat shock is independently regulated by different regions in the *DREB2A* promoter [23]. The inducible response to stress conditions is a common feature among DREB2-type transcription factors of various plants, and the promoter structure, including these *cis*-elements, is conserved among many eudicot species [24].

The over-expression of *DREB2A* gene in *Arabidopsis* [22] and several other transgenic plants such as maize [25], rice [26], tobacco [27] and wheat and barley [28] resulted in improved stress tolerance. Because accumulation of *DREB2A* mRNA is not sometimes sufficient for the induction of downstream genes due to posttranslational regulation, Sakuma et al. [22] obtained the constitutively active form of the *AtDREB2A* gene (*AtDREB2A CA*) through the removal of a negative regulatory domain downstream of the DNA-binding domain.

In our study, it was demonstrated that transgenic plants of sugarcane containing the *AtDREB2A CA* gene under the control of the stress-inducible promoter *Rab17* had improved drought tolerance and higher sucrose content with no biomass penalty.

2. Materials and methods

2.1. Tissue culture and plant transformation

Immature leaf segments of 6–8-month-old plants of the sugarcane variety RB855156 were surface sterilized with 2.5% (v/v) sodium hypochlorite solution, and the outer leaves were aseptically removed. Transverse segments 2–3 mm wide were excised above the apical meristem and placed on solid MS medium [29] supplemented with 20 g L^{-1} sucrose and 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 8 g L^{-1} Bacto Agar. Embryogenic calli were selected and cultured at 3-week intervals on the same medium prior to bombardment [30]. The microprojectile suspension was prepared as described by [31].

The expression vector used for transformation of sugarcane (pBract 302) contains the *A. thaliana DREB2A (AtDREB2A) CA* [22] coding sequence driven by a stress-inducible promoter *Rab17* from maize (*Zea mays*) [32]. *Hin*dIII-Rab17 promoter-*Sma*I fragment was inserted through *Hin*dIII and *Eco*RV sites of pBract302. Then *Pst*I-*AtDREB2A CA*:NosT-*Eco*RI fragment was blunted and inserted through *Sma*I site. The pBract 302 also contains the *bar* cassette used as selectable marker.

Following bombardment, calli were transferred to MS medium for 10 days without plant growth regulators and then placed on MSC3 [30] with 3 mg L^{-1} glufosinate ammonium. Resistant calli were placed on 2,4-D and glufosinate ammonium-free MS medium for regeneration under 27 ± 1 °C with a 16-h photoperiod (30 μmol m^{-2} s^{-1}). The rooted plantlets were transferred to planting trays containing a commercial propagation substrate (Plantmax™) and grown in a controlled greenhouse conditions.

2.2. Molecular analyses and selection of events

Standard polymerase chain reaction (PCR) techniques were used to detect the presence of the *AtDREB2A CA* transgene in leaf samples from regenerated putative transgenic sugarcane plantlets. The *Rab17::DREB2A CA*-specific primer sequences 5′-TGTTATGCAGTTCGCTCTGG-3′ and 5′-CTACATCGGCTATTCTCTGG-3′ were used to generate a 674-bp PCR product. Plants that presented amplification of the expected size product and non-transgenic plants were sprayed with 1% (v/v) glufosinate ammonium and evaluated 8 days after spraying.

Southern blot analysis was performed to confirm the integration of the *AtDREB2A CA* gene in transgenic plants. DNA samples (20 μg) were digested overnight with *Bcl*I, eletrophoresed on 1% (w/v) agarose gel, transferred onto nylon membranes (Hybond-N^+, GE Healthcare, Piscataway, NJ) and fixed through incubation of 2 h at 80 °C. A 1.75-kb sequence corresponding to *Rab17::DREB2A CA::TNos* was used as probe. Labeling was performed using [α-^{32}P]dCTP by random primer labeling. Hybridization and washings were performed at 42 °C. Membranes were exposed with BAS-MS 2340 IP support, and the data was acquired using a Fluorescent Image Analyzer FLA-3000 (Fujifilm Life Science).

2.3. Water-deficit assays

The events obtained by transformation of sugarcane with the *Rab17::DREB2A CA* were subjected to water deficit assays in order to select the best event (s) for further analysis. Twenty four plants of the T0 transgenic events and fifteen plants of the non-transgenic (NT) grown in 8L pots were subjected to water deficit for 6 days by suspending irrigation (Fig. 1a). From these, plants of five independent events were selected based on its reduced leaf rolling and senescence when compared with the NT plants. Leaf samples of such events were used for the Southern blot analysis (Fig. 2). Out of the five selected events, the event 24.2 was chosen for further