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AtDREB2A-CA Influences Root Architecture and Increases Drought Tolerance in Transgenic Cotton

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Abstract

Drought is a major environmental factor limiting cotton (*Gossypium hirsutum* L.) productivity worldwide and projected climate changes could increase their negative effects in the future. Thus, targeting the molecular mechanisms correlated with drought tolerance without reducing productivity is a challenge for plant breeding. In this way, we evaluated the effects of water deficit progress on *AtDREB2A-CA* transgenic cotton plant responses, driven by the stress-inducible *rd*29 promoter. Besides shoot and root morphometric traits, gas exchange and osmotic adjustment analyses were also included. Here, we present how altered root traits shown by transgenic plants impacted on physiological acclimation responses when submitted to severe water stress. The integration of *AtDREB2A-CA* into the cotton genome increased total root volume, surface area and total root length, without negatively affecting shoot morphometric growth parameters and nor phenotypic evaluated traits. Additionally, when compared to wild-type plants, transgenic plants (17-T_0 plants and its progeny) highlighted a gradual pattern of phenotypic plasticity to

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some photosynthetic parameters such as photosynthetic rate and stomatal conductance with water deficit progress. Transgene also promoted greater shoot development and root robustness (greater and deeper root mass) allowing roots to grow into deeper soil layers. The same morpho-physiological trend was observed in the subsequent generation (17.6-T_2). Our results suggest that the altered root traits shown by transgenic plants are the major contributors to higher tolerance response, allowing the *AtDRE2A-CA*-cotton plants to maintain elevated stomatal conductance and assimilate rates and, consequently, reducing their metabolic costs involved in the antioxidant responses activation. These results also suggest that these morpho-physiological changes increased the number of reproductive structures retained per plant (26% higher) when compared with its non-transgenic counterpart. This is the first report of cotton plants overexpressing the *AtDRE2A-CA* transcription factor, demonstrating a morpho-physiological and yield advantages under drought stress, without displaying any yield penalty under irrigated conditions. The mechanisms by which the root traits influenced the acclimation of the transgenic plants to severe water deficit conditions are also discussed. These data present an opportunity to use this strategy in cotton breeding programs in order to improve drought adaptation toward better rooting features.

Keywords

Dehydration Responsive Element Binding Factors, Water Deficit Tolerance, *Gossypium hirsutum*, Physiological Phenotyping, Transcription Factor, Stress-Inducible Promoter

1. Introduction

Drought is often the most yield-limiting factor for plants. Water supplies will continue to decrease worldwide as the global population will increase from the current 7 billion to over 9 billion people in 2050 [1]. Along with water scarcity, the rate of land expansion under irrigation is decreasing substantially [2]. Therefore, it is imperative to maximize food production vertically, e.g., to minimize the use of cropland and water use and develop higher-yielding varieties resistant to pests and diseases, as well as more tolerant to future climate scenarios [3] [4]. Facing these global agricultural challenges, there is an urgent need to develop crops with delayed stress-onset to manage risk and increase yield and quality under both optimal and suboptimal conditions.

Cotton is one of the most important fiber crops worldwide and annually generates nearly US$ 12 billion. Its production involves more than 350 million people, from farming to logistics, textile ginning, processing and packaging. Currently, cotton is produced by over 60 countries in five continents and Brazil stands amongst the top five cotton producers globally [5]. Nevertheless, cotton crop has been severely threatened by different biotic and abiotic stresses. Water deficit has been considered one of the main limiting factors to crop harvests

during the critical season [6].

Various traditional and molecular genetics approaches have been applied to improve drought tolerance. Conventional breeding for water deficit tolerance has had and continues to have success, but it is labor-intensive and time-consuming. These constraints primarily arise due to the complex identification of quantitative trait loci that controls yield, as well as the difficulty to control moisture levels in the field [7] [8] [9], which can lead to increase trial error, decreasing its precision and, consequently, making it difficult to quantify the treatment effects. In contrast, rapid progress in plant biotechnology has opened up new possibilities for creating drought tolerant crops by identifying key genes and introducing them through genetic engineering [10].

Numerous drought-inducible genes have been identified and used as candidate genes in genetic engineering including a number of transcription factors that regulate stress-inducible gene expression [11] [12]. Among them, the dehydration-responsive element binding (DREB) transcription factors from *Arabidopsis thaliana* have been reported to enhance drought tolerance in transgenic plants [13]. Both DREB1A and DREB2A regulatory proteins bind to the same *cis*-acting element, *DRE*, present in the promoter of numerous genes activated under water stress. However, these two proteins function in different signal transduction pathways under low-temperature and dehydration stress conditions [14] [15]. The *AtDREB1A* gene improves tolerance to cold acclimation and dehydration [16] [17]. On the other hand, the *AtDREB2A* gene or its constitutively active form (*DREB2A-CA*) is strongly involved in water deficit response but only slightly involved in freezing tolerance [18] [19]. Afterwards, several DREB genes have been identified in a large number of plant species [20] [21] [22] [23].

Early attempts to develop transgenic plants tolerant to water stress focused on the use of constitutive promoters such as *CaMV*35S. However, it has been demonstrated that the use of a stress-inducible promoter is important to minimize deleterious side-effects including reduced vegetative growth and delayed flowering [16] [24]. Although promoters that are constitutively expressed at high levels are still widely used, they are not appropriate for all transgenes [10] [25] [26].

In cotton, concurrent efforts to identify appropriate transgene-promoter combinations in suitable backgrounds have been undertaken by several research groups as an alternative to circumvent unfavourable climate conditions (Additional File 1—**Table S1**) [25]-[39]. These reports demonstrate that these gene products protect cotton under water deficit conditions but none of these genes has been used as commercial traits.

Drought stress lead to a considerable reduction in photosynthetic performance mediated through stress-induced stomatal or non-stomatal limitations [40]. Thus, the evaluation of physiological parameters is very important to discriminate between the effects of these limitations on the plant photosynthetic capacity [41] [42]. Moreover, the assessment of variability in specific traits such

as root architecture and morphology, water use efficiency and yield components is considered critical, in addition to several other physiological aspects [43].

The current study describes the production of genetically modified cotton plants containing the *AtDREB2A-CA* gene driven by the *rd*29A stress-inducible promoter. Our morpho-physiological data demonstrate that the over expression of *AtDREB2A-CA* in transgenic cotton plants improved shoot development, morphometrics roots traits as such total volume, length and surface area, beyond deeper roots with greater biomass, contributing to yield advantage under water deficit without displaying any penalty under normal irrigated conditions.

2. Material and Methods

Molecular Analysis

Cotton transformation

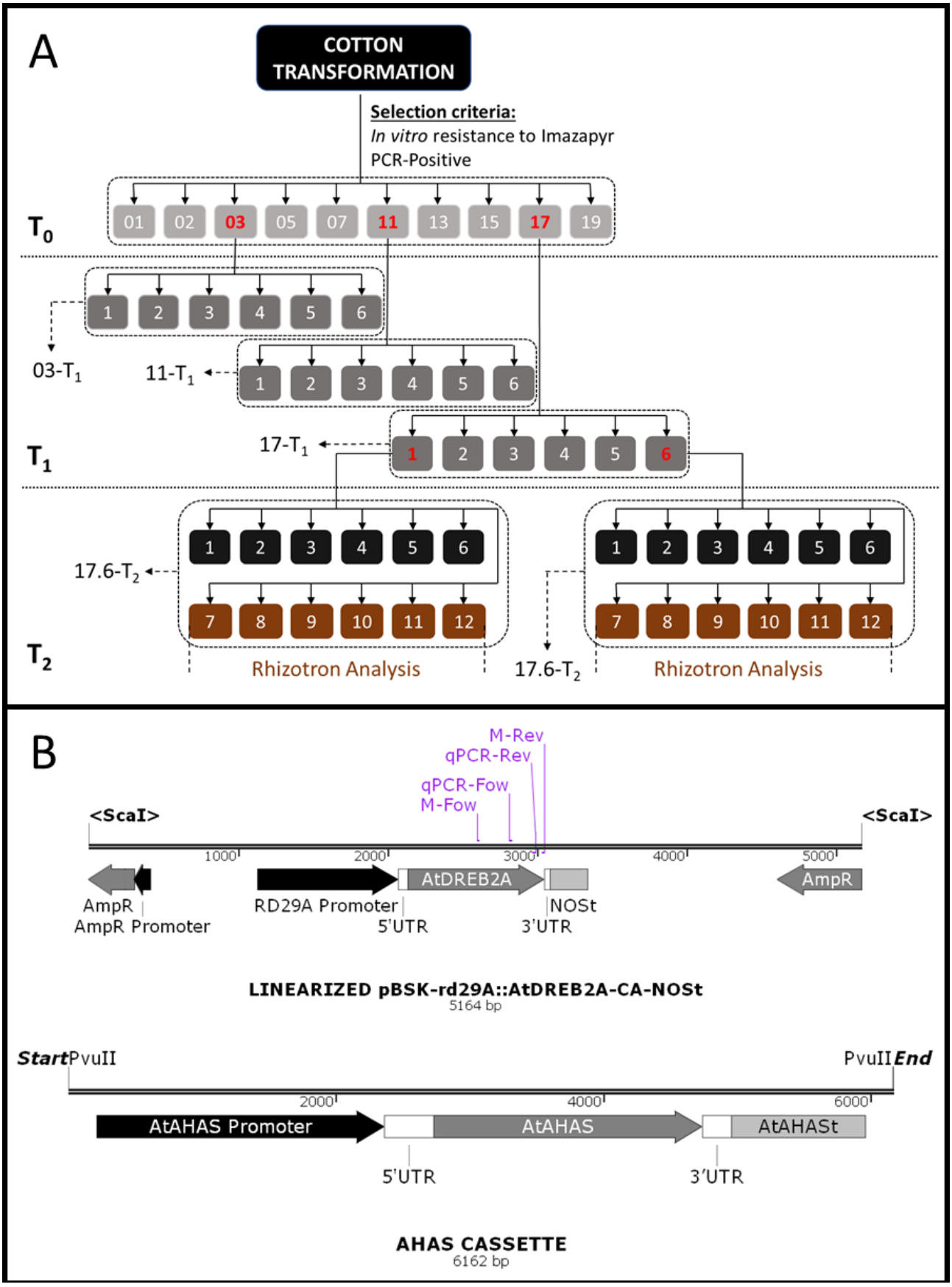
Embryos of Embrapa 113-7MH cotton cultivar were co-transformed with 25 µg of the following DNA fragments: *AHAS* cassette (obtained by cleavage of pAC321 vector with *Pvu*II restriction enzyme) [44] and pBSK-*rd*29A::*AtDREB2A-CA*-NOSt (linearized with *Sca*I restriction enzyme) [45] (**Figure 1(A)**). The methodology employed was microparticle bombardment (biolistic) and the resultant transformed plantlets were selected as previously described [46] [47]. The pAC321 vector (8669 bp) contained the AHAS cassette (*AtAHAS* promoter + *AtAHAS* coding sequence + *AtAHAS* terminator) with open reading frame (ORF) mutated at position 653, which resulted in a substitution of a serine by an asparagine that confers herbicide tolerance against the imidazolinone chemical group such as Imazapyr [46]. The pBSK-*rd*29A::*AtDREB2A-CA*-NOSt vector (5164 bp) containing the *rd*29A:*AtDREB2A-CA* construction (*rd*29A promoter + *AtDREB2A-CA* coding sequence + NOS terminator) was kindly provided by JIRCAS (Japan) in collaboration with Embrapa (Brazil). The restriction enzyme *Sca*I was used to linearize the pBSK-*rd*29A::*AtDREB2A-CA*-NOSt vector in order to interrupt the *β*-lactamase gene (AmpR).

PCR Analysis

In the T_0 generation, the genomic DNA was extracted from young leaves of transformed and wild-type (WT) cotton plants [48]. In the T_1 and T_2 generation, the genomic DNA was extracted from seeds [49]. The primers M-Fow and M-Rev (Additional File 2—**Table S2**) were used to analyze the insertion of *AtDREB2A-CA* gene in the cotton genome. The PCR mixture consisted of: 300 ng DNA, reaction buffer (50 mM KCl; 10 mM Tris-HCl pH 8.4; 0.1% Triton X-100); 1.5 mM $MgCl_2$; 0.4 mM dNTP; 0.25 mM of each primer and 2.5 U *Taq* DNA polymerase (Phoneutra©). PCR amplifications were performed at the following conditions: 94˚C for 5 min; followed by 30 cycles of 94˚C for 1 min; 55˚C for 1 min, 72˚C for 1 min; and a last elongation step of 72˚C for 5 min. The amplicon obtained had 450 bp.

Real Time Quantitative PCR (*RT-qPCR*) *assays*

The *AtDREB2A-CA* transgene expression levels were measured in all plants


A
COTTON TRANSFORMATION
Selection criteria:
In vitro resistance to Imazapyr
PCR-Positive
T0
01 02 03 05 07 11 13 15 17 19
1 2 3 4 5 6
03-T1
11-T1
1 2 3 4 5 6
17-T1
T1
1 2 3 4 5 6
17.6-T2
1 2 3 4 5 6
7 8 9 10 11 12
T2
Rhizotron Analysis
17.6-T2
1 2 3 4 5 6
7 8 9 10 11 12
Rhizotron Analysis
B
M-Rev
qPCR-Rev
qPCR-Fow
M-Fow
<ScaI>
<ScaI>
1000
2000
3000
4000
5000
AtDREB2A
AmpR
AmpR
AmpR Promoter
RD29A Promoter
5'UTR
NOSt
3'UTR
LINEARIZED pBSK-rd29A::AtDREB2A-CA-NOSt
5164 bp
StartPvuII
PvuIIEnd
2000
4000
6000
AtAHAS Promoter
AtAHAS
AtAHASt
5'UTR
3'UTR
AHAS CASSETTE
6162 bp

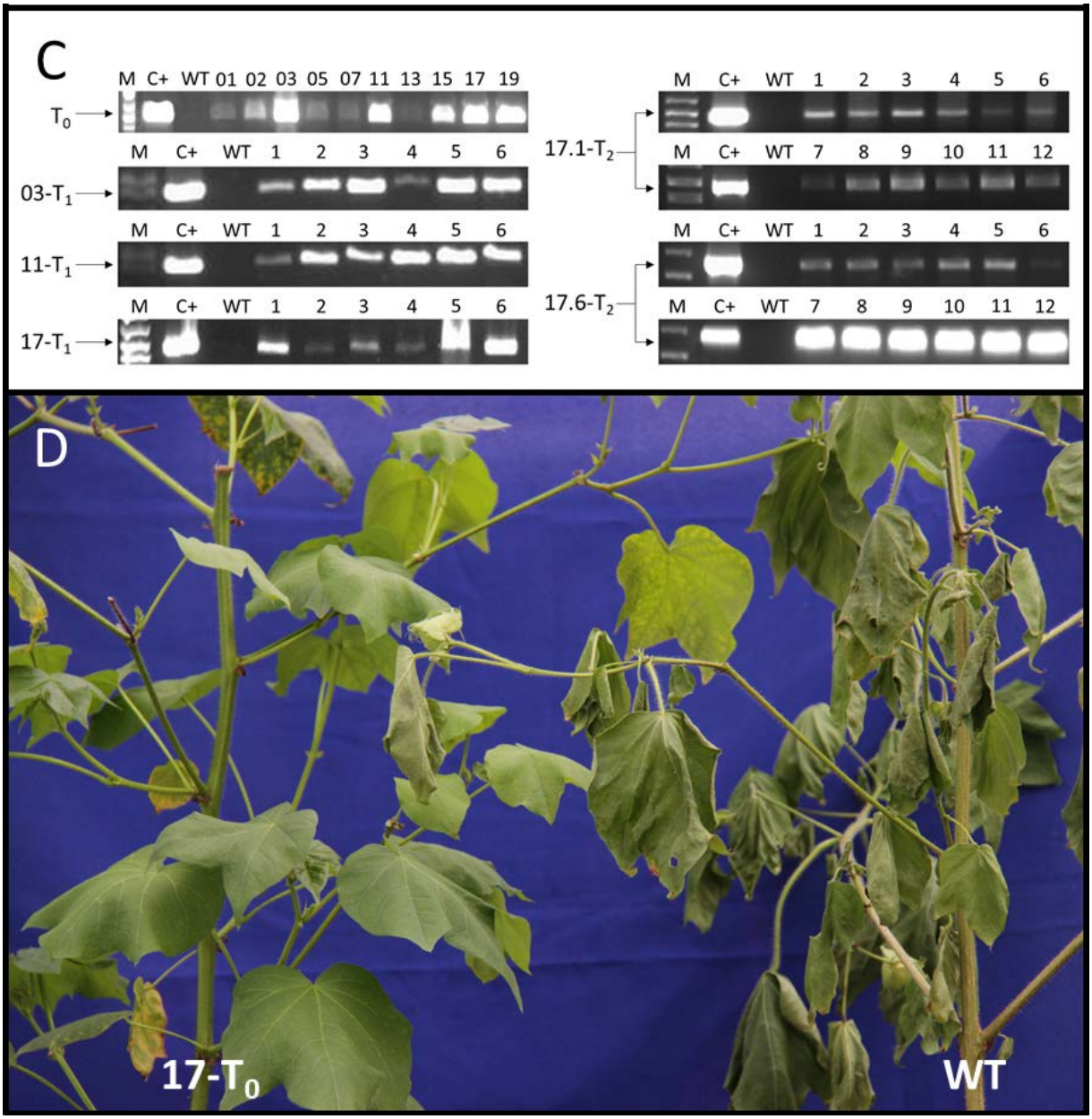


Figure 1. PCR and Phenotypic Selection of $T_0/T_1/T_2$ Cotton Transgenic Plants. The overall experimental design is described in (A). After co-bombard embryos of Embrapa 113-7MH cotton cultivar with linearized pBSK-*rd*29A::*AtDREB2A-CA*-NOSt and *AHAS* cassettes (B), the transformed cotton plants were selected by resistance to Imazapyr conferred by *AHAS* gene Once acclimated in a greenhouse, the seedlings were characterized by PCR amplification of the*AtDREB2A-CA* transgene with the M-Fow and M-Rev primers (amplicon with 450 bp) (C). In this way, ten T_0PCR-positive plants (01, 02, 03, 05, 07, 11, 13, 15, 17 and 19) were selected. These ten T_0 cotton plants were phenotypically characterized with regard to drought tolerance. Three of them (03-T_0, 11-T_0 and 17-T_0) with the healthiest phenotypes when compared to WT after seven days without irrigation were selected, as shown in (D). Six *AtDREB2A-CA* PCR-positive seeds from the three selected T_0 lines were planted and then subjected to physiological analysis. Among the eighteen plants in the T_1 generation, the two with the best physiological results were selected (17.1 and 17.6). Finally, twelve*AtDREB2A-CA* PCR-positive seeds from the T_1 selected plants represented the T_2 generation (17.1-$T_2$and 17.6-T_2). Legends: *Pvu*II and *Sca*I (restriction enzyme sites); AmpR (interrupted β-lactamase open reading frame); AtAHASt (acetohydroxyacid synthase gene terminator); C+ (positive control; PCR template is the original pBSK-*rd*29A::*AtDREB2A-CA*-NOSt used for cotton transformation); M (1.0 kb ladder; Invitrogen® Cat. # 10787018); UTR (untranslated region); NOSt (NOS gene terminator); WT (wildtype non-transgenic plants).

after seven days without irrigation. The total RNA of each leaf replicate (*i.e.* a leaf disc of 1.0 cm in diameter) of WT and transgenic cotton plants was isolated using Concert Reagent (Invitrogen®) following the manufacturer's instructions. All RNA extractions were performed in biological triplicate. Prior to the cDNA synthesis, all of the RNA samples were treated with 1.0 U of Ambion® DNase I RNase-free™ (Invitrogen®). The cDNA was reverse transcribed from 1.0 μg of total RNA using 200 U of Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen®) and oligo-NVdT_{30}, following the manufacturer's instruction. Informations about the primers used in these experiments (qPCR-Fow and qPCR-Rev for*AtDREB2A-CA* amplification, as well as GhUBQ14-Fow, GhUBQ14-Rev, GhPP2A1-Fow and GhPP2A1-Rev for amplification of reference controls) are presented in Additional File 2—**Table S2**. The raw SYBR-fluorescence data from all qPCR amplification runs were imported into the Real-time PCR Miner software [50] to determine the corrected threshold cycle (C_t) value and efficiency of the primers. Gene expression analyses were completed using qBASE Plus software [51]. Statistical analysis was performed using REST software (Qiagen®).

Determination of AtDREB2A-CA transgene expression cassette copy number in the transgenic cotton genome by qPCR-based $2^{-\Delta\Delta Ct}$

The copy number of *AtDRE2A-CA* transgene in T_0, T_1 and T_2 generations of the transgenic cotton plants was determined according to the literature [52] [53] through qPCR-based $2^{-\Delta\Delta Ct}$ method. Genomic DNA from WT and *AtDREB2A-CA* genetically modified (GM) cotton plants was extracted from fresh leaves (100 mg) using the DNeasy Plant Maxi Kit (Qiagen®) according to the manufacturer's instructions. The DNA concentration was determined spectrophotometrically and the DNA integrity was checked by agarose gel electrophoresis. A reference plasmid pBSK-*AtDREB2A-CA-ubc*1 was manufactured containing a fragment of ubiquitin C (*ubc*1) gene from *G. hirsutum*, which is a single-copy gene, as well as a fragment of *AtDREB2A-CA* gene, which was also present in the *AtDREB2A-CA* cassette. The copy number of *AtDREB2A-CA* cassette in the genome of $T_0/T_1/T_2$ GM cotton plants was calculated from the relative qPCR quantification of *AtDREB2A-CA* against the endogenous reference gene *ubc*1 using the primers qPCR-Fow, qPCR-Rev, GhUBC1-Fow and GhUBC1-Rev (Additional File 2—**Table S2**). Firstly, the absolute quantity of the two genes *ubc*1 and *AtDREB2A-CA* was determined by qPCR in reference to standard curves, which were obtained by plotting the C_t values against the log-transformed concentration of serial tenfold dilutions (10^1, 10^2, 10^3, 10^4 and 10^5) from the same reference plasmid, pBSK-*AtDREB2A-CA-ubc*1. Subsequently, the absolute copy number of the plasmid pBSK-*AtDREB2A-CA-ubc*1 was calculated using C_t values based on the standard curves. Finally, the relative copy number of the *AtDREB2A-CA* target gene was obtained using the ratio between *AtDREB2A-CA* and *ubc*1 absolute concentrations in the same sample.

Phenotypic and Physiological Analysis

Water deficit treatment

Transgenic and WT plants were grown in substrate containing turf and vermiculite (1:1 v/v), natural phosphate and macronutrients (Terral, TrueMix). PVC tubes (1.2 m height and 0.35 m diameter) were filled with 43.0 kg of substrate previously dried at 105˚C. Subsequently, the tubes were watered from the top until water drained from the bottoms, covered with plastic bags to minimize evaporation, and allowed to drain for 24 h. Then, the drainage holes were sealed and the tubes were weighed for field capacity determination. The assay was carried out under a climatized greenhouse conditions (28˚C ± 2˚C and 60% ± 10% relative humidity) and monitored via a Hobo Micro Station Data Logger (Onset Computer). After emergence, 0.5 L of half-strength Hoagland solution [54] was applied twice a week. The plants were irrigated regularly with water status maintained at the substrate capacity until the first flower occurrence.

A preliminary test was performed to analyze the behavior of transgenic cotton plants under drought conditions. Ten PCR-positives T_0 transgenic cotton plants (01, 02, 03, 05, 07, 11, 13, 15, 17 and 19) and non-transformed counterparts were grown in 10 L plastic pots for 30 days under greenhouse conditions. The T_0 transgenic plants were visually assessed for tolerance to water deficit stress by withholding irrigation for seven days and compared with WT. Plants were monitored daily for wilting.

Based in result of preliminary test, those plants that maintained greater visual turgescence were chosen for subsequent evaluations. In the next step, the experiment was performed using a completely randomized design using WT plants and six plants from T_1 generation of threeindependent*AtDREB2A-CA* transgenic cotton events (03, 11 and 17). Three seeds of each transgenic lines and WT were sown per PVC tube and, after the 12th day, thinning was performed leaving only one plant per PVC tube. Stress treatments were imposed at first flower occurrence and finished after nine days. The evaluation of T_2 generation was carried out similarly to the procedures described above. In this phase, based in greatest physiological performance shown before, six transgenic plants from 17.1 and 17.6 T_1 transgenic lines were used aiming its morpho-physiological characterization when submitted to water deficit.

Leaf water potential

At the first flower occurrence, the leaf water potential at predawn (LWPpred—between 4:30 and 5:00 a.m.) was monitored every three days to define the most suitable leaf water status for gas exchange analyses in both transgenic and WT plants [55]. These procedures were conducted using an Oregon Corvallis pressure chamber, 97,330 (PMS Instrument Company) and water potential measurements were performed as described in literature [56]. Briefly, fully expanded cotton leaves were excised with a razor blade near the base of the petiole and immediately inserted into the pressure chamber. Pressure from a tank of compressed nitrogen was applied at the rate of 0.2 to 0.3 bar sec^{-1} until

the moment that a drop of exudate appeared on the cut face of the petiole.

Gas exchange measurements

Gas exchange analysis was also carried out for all trangenic plants (T_1 and T_2) when the WT plant showed approximately −0.20 MPa (no stress), −1.20 MPa (moderate stress) and −1.79 MPa (severe stress) at predawn. Measurements were done using LI-6400 Portable Photosynthesis System (LI-COR, USA). The variables (net photosynthetic rate—P_n; stomatal conductance—g_s; transpiration rate—E and intercellular carbon concentration—C_i) were measured simultaneously between 9:00 and 11:00 a.m. under artificial photosynthetic photon flux (PPF) (1100 μmol m^{-2} s^{-1}). The sample chamber was programmed to maintain 400 ppm CO_2 at 25˚C and relative air humidity at 35% - 50% for all measurements. The CO_2 concentration in the cuvette (Ca, μbar) was controlled using a 12 g CO_2 cartridge in the injector system. Leaves were acclimated in the leaf chamber for two to four minutes until steady-state gas exchange was achieved. For all stressed and control treatments measurements were started on the uppermost fully expanded leaf in the middle third of each plant. The leaves were tagged in order to use the same sample in all measurements and the mid- to distal-portion of each leaf blade was inserted into the LI-6400 chamber.

Shoot and root architecture analysis

At the end of the stress period, shoots were cut at the cotyledonary node for leaf area (LA) measurements using a portable LA meter (Model LI-3000A) and subsequent shoot dry mass determination in both T_1 and T_2 generations. For T_1 root architecture and morphology analysis, roots were cut and separated from the stems and carefully washed in order to avoid any disturbance to them. The cleaned individual root systems were floated in 5.0 mm water layer in a 0.5 × 0.4 m Plexiglas tray and untangled with a plastic paintbrush to minimize root overlap.

Additionally, as an alternative for non-destructive root trait measurements of T_2 generation, rhizotrons were constructed to analyze, visualize and preserve the root architecture and morphology of WT and T_2 transgenic lines (17.1-T_2 and 17.6-T_2) in order to compare the root trait attributes. Each rhizotron consisted of two transparent glass sheets of 100.0 cm high, 50.0 cm wide and 4.0 mm thick. The two sheets were separated on three sides (two long sides and one of the short sides) by 0.9 cm aluminum spacers and were held with foldback paper clips. The rhizotrons were filled with 1220 g of black substrateas described in detail above. Rhizotrons were placed with an inclination of 42˚ relatively to vertical position and covered with black plastic sheet to exclude light from the soil and roots, leaving small slits for seedlings to emerge. Before sowing, the rhizotrons were watered to field capacity and the plants were subjected to the same water stress procedures imposing and monitored as previously described.

After stress period, imaging and analysis of root architecture were performed with a camera (Sony Cyber Shot DSC-HX1, Optical Zoom 20X) and the photographed images were analyzed with WinRHIZO PRO to determine total root

length, surface area and volume.

Osmotic adjustment analysis

The youngest fully developed leaves from17.1-T_2 and 17.6-T_2 transgenic cotton lines were rehydrated for 12 h and sampled according to LWP pred thresholds values defined above. Then, each leaf was washed with deionized water, sealed in a 20.0 mL plastic syringe, immediately frozen in liquid nitrogen and stored at −20˚C until required. For osmolality analysis, syringes containing leaf samples were manually pressurized and the sap was transferred to a 1.5 mL plastic microtube for subsequent centrifugation (10,000 g for 10 min at 0˚C - 4˚C). Sap samples were taken for osmolality determinations using a Vapor Pressure Osmometer (Vapro 5600, Wescor Biomedical Systems Inc.). The osmotic potential was determined using the ideal gas equation ($\psi s = -RTcs$), where R is the gas constant (8.32 J mol^{-1} K^{-1}), T is the absolute temperature (in Kelvin degrees) and cs is the solute concentration in the solution, expressed as osmolality. Osmotic adjustment was calculated by the rehydration method [57], which was calculated by the difference between osmotic potential measured in non-stressed and stressed leaves after full rehydration.

Effect of water deficit on yield

The productivity of transgenic cotton progenies was estimated as the mean number of reproductive structures, including squares (flower bud stage before blooming), flowers (perfect flowers) and bolls (fruits)retained in the WT and T_2 transgenic lines (17.1-T_2 and 17.6-T_2) seven days after re-watering establishment.

Statistical analyses

Data were subjected to analysis of variance (ANOVA, $p < 0.05$) and comparison of means was performed with Student's *t-test* ($p < 0.05$) using SPSS Statistics software. The physiological parameters obtained for the transgenic lines were compared with those of WT plants for each water regime.

3. Results

Plant Transformation and Molecular Analysis

The overall experimental design was in accordance with the flowchart in Fig. 1A.Two different vectors, one containing the *AtDREB2A-CA* gene and the other containing the *AHAS* gene (selective marker that confers resistance to Imazapyr) were used to co-bombard apical meristems of mature cotton embryos of Embrapa 113-7MH cultivar (**Figure 1(B)**). From 3000 embryos bombarded, 148 (4.93%) putative *AHAS*-transformed plantlets were selected by resistance to Imazapyr. Genomic DNA was extracted from leaves of the surviving transgenic plants and was used for PCR analysis to verify the presence of *AtDREB2A-CA*. Thus, ten T_0PCR-positive independent transgenic events were identified (**Figure 1(C)**), which indicated a transformation frequency of 0.33%. Three T_0 primary transformed plants (03-T_0, 11-T_0 and 17-T_0) were selected on the basis of a drought tolerance phenotype after seven days without irrigation, as shown in

Figure 1(D). After this period, WT plants became totally withered while the selected T_0 transgenic plants were erect with turgid leaves. Then, the three selected T_0 generation plant were self-pollinated and the seeds were used to obtain the T_1 progeny. Six *AtDREB2A-CA*PCR-positive plants from T_1 generation of each selected primary transformation event were phenotipically and physiologically evaluated. After detailed physiological analysis (results described below), twelve PCR-positive seeds of the two T_1 trangenic plant with the best results (17.1-T_1 and 17.6-T_1) were sown for T_2 generation analysis. Six PCR-positive seeds of each selected T_2 transgenic line (plants 7-12 of both 17.1-T_2 and 17.6-T_2; Figure 1(C)) were used for rhizotron analysis.

The expression profile of the *AtDREB2A-CA* transgene in the selected transgenic lines was evaluated after seven days without irrigation (Figure 2). The 17-T_0 transgenic plant presented the highest level of transgene expression in this condition, followed by its T_1 and T_2 progeny. In addition, 17-T_0 carried in its

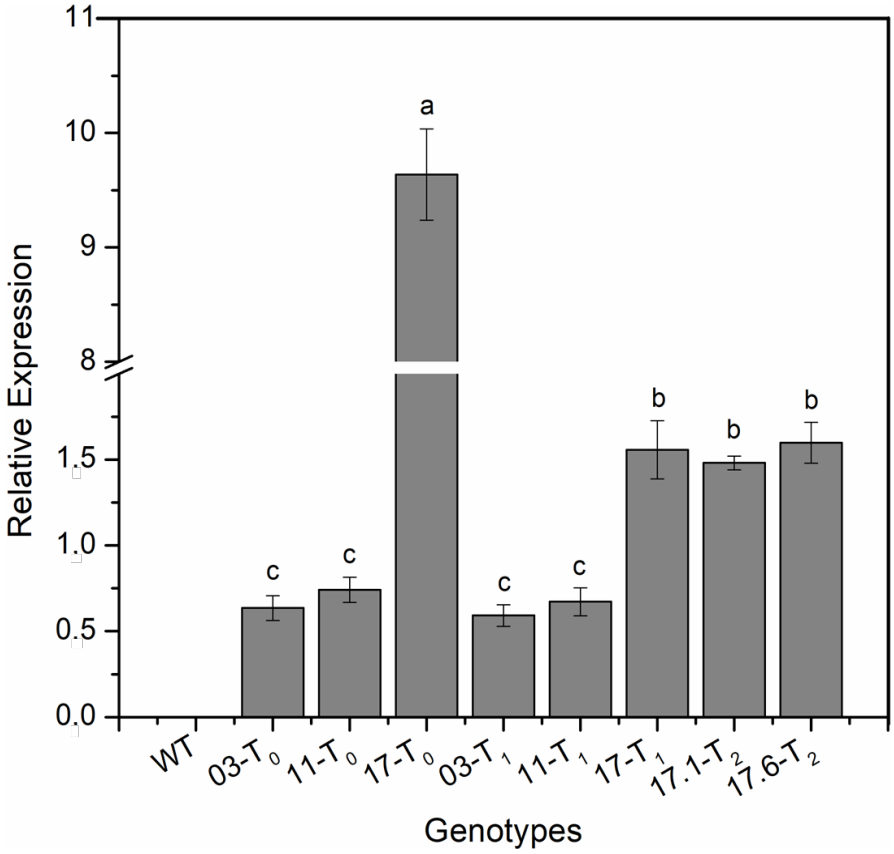


Figure 2. Relative Expression of *AtDREB2A-CA* Transcript. The expression of the *AtDREB2A-CA* transgene was measured in transgenic and WT plants after seven days without irrigation with qPCR-Fow and qPCR-Rev primers. Each bar represents the transcript expression level of three technical and biological replicates of the six T_1 (03-T_1, 11-T_1, and 17-T_1) and twelve T_2 (17.1-T_2 and 17.6-T_2) PCR-positive selected transgenic plants ± standard error.Statistical analysis was performed according to the Student's *t-test* ($p < 0.05$). Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wildtype non-transgenic plants).

genome two copies of the transformation cassette containing the *AtDREB2A-CA* transgene, while all the other selected transgenic plants had only one copy (Table 1). These combined results indicate transgene stabilization in the transformed plants, especially in the T_2 generation.

Physiological Analysis

Leaf water potential and gas exchange analysis

At the beginning of water with drawal, the LWP pred of T_1 and T_2 plants was always kept at −0.5 MPa or greater (less negative) (Figure 3(A) and Figure 3(B),

Table 1. Copy number of the *AtDREB2A-CA* DNA cassette in GM cotton plants.

Plant ID	*AtDREB2A-CA*/*UBC* ratio	Estimated copy number
WT	−0.34	0
03-T_0	1.27	1
11-T_0	1.26	1
17-T_0	2.35	2
03-T_1*	1.22 - 1.39	1
11-T_1*	1.13 - 1.28	1
17-T_1*	1.14 - 1.24	1
17.1-T_2**	1.17 - 1.21	1
17.6-T_2**	1.19 - 1.30	1

*The number of copies was evaluated in all the six T_1 plants of each analyzed transgenic line (03, 11 and 17). The *AtDREB2A-CA*/*UBC* ratio presented is the range of values found in the analysis of the six plants. **The number of copies was evaluated in all twelve T_2 plants of each analyzed transgenic line (17.1 and 17.6). The *AtDREB2A-CA*/*UBC* ratio presented is the range of values found in the analysis of the twelve plants.

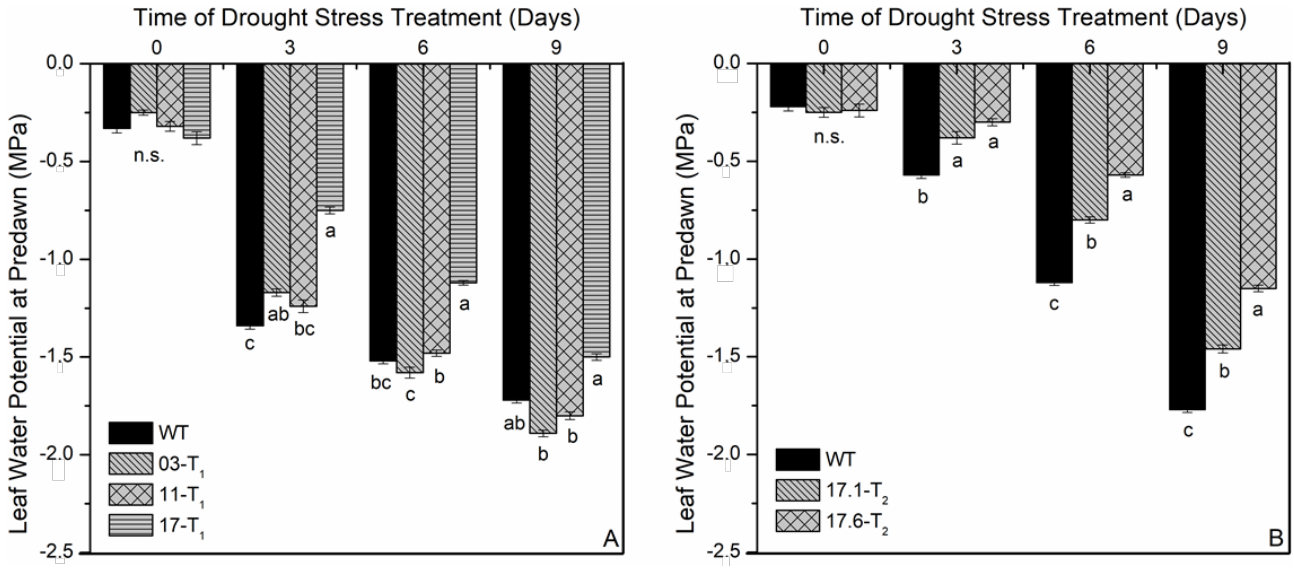


Figure 3. Leaf Water Potential at Predawn in T_1/T_2 Transgenic Cotton Plants at Flowering. (A) T_1 generation (03-T_1, 11-T_1 and 17-T_1); (B) T_2 generation (17.1-T_2 and 17.6-T_2). All measurements were recorded at three, six and nine days after imposing water stress. Each bar represents the mean of six replicates ± standard error. Statistical analysis was performed according to Student's *t-test* ($p < 0.05$). Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wild type non-transgenic plants); *n.s.* (non-significant).

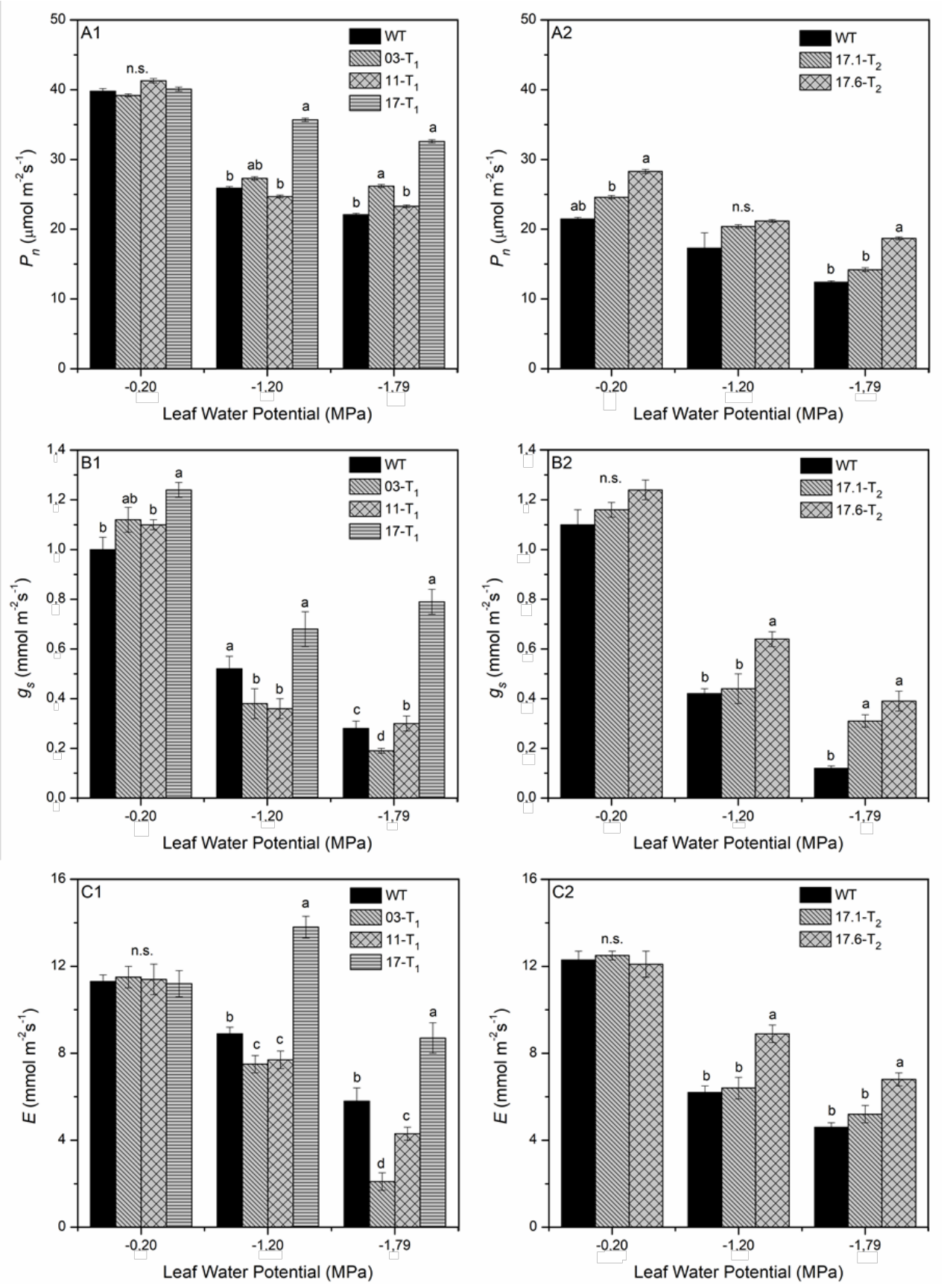
respectively). As shown in the T_1 cotton plants, after nine days without irrigation, WT plants and descendants from the transgenic line 17 reached −1.72 and −1.50 MPa LWPpred, respectively. The 17.1-T_2 and 17.6-T_2 transgenic lines reached on average −1.46 and −1.15 MPa, respectively, at predawn during the same stress period, while the LWPpred of the WT plants was −1.77 MPa.

In respect to LWPpred thresholds for gas exchange analysis, the leaf water status was allowed to progress until approximately −1.20 (moderate stress) and −1.79 MPa (severe stress) in WT plants, considering their more accelerated LWPpred decline compared to the transgenic lines, as observed in preview tests with cotton leaf and whole canopy photosynthesis [55] [58].

Increased water deficit leaded to greater gas exchange performance in transgenic plants when compared with WT plants for both T_1 and T_2 generations (**Figure 4**). P_n values decreased for all genotypes as the stress imposing progressed (**Figure 4(A1)** and **Figure 4(A2)**). However, in general, the results in the T_2 progeny from 17 transgenic line were significantly different from those in WT plants at moderate (−1.20 MPa) and severe (−1.79 MPa) stress level, which indicated that cotton lines over expressing *AtDREB2A-CA* improved their net photosynthetic rate even when maintained under water deficit regime. Stomatal conductance (g_s) values were greatly influenced by stress, but descendants from 17 transgenic line showed the highest values for this variable in comparison with other genotypes under the most stressful condition (**Figure 4(B1)** and **Figure 4(B2)**). The same trend was registered for E (**Figure 4(C1)** and **Figure 4(C2)**) and C_i (**Figure 4(D1)** and **Figure 4(D2)**), highlighting the 17 transgenic line outperformance. As water deficit treatment progressed, the proportional decrease in the P_n was significantly less affected than the decrease in stomatal conductance, demonstrating the different roles of stomatal responses on the gas exchange parameters in the analyzed lines. Data from the T_2 generation demonstrated that the most evidenced differences between the transgenic lines and WT plants were observed at severe stress level for all gas exchange variables analyzed. There was a strong *gs* decrease in WT plants when compared with the transgenic lines. This trend also extended for the *E* and *Ci* parameters. On the other hand, P_n performance was less affected in terms of magnitude across stress levels, with reduced values for WT plants varying from 25.81% (moderate stress) to 56.63% (severe stress) in comparison with well-watered conditions. On the other hand, the 17.1-T_2 transgenic line showed a slow progress and a small final proportional decrease in P_n, varying from 21.64% to 28.47% in both described stress levels.

Shoot morphology and root traits

Analysis of growth variables on WT and transgenic lines under the most severe stress condition is shown in **Figure 5**. Significant differences were observed in plant height (**Figure 5(A)**), dry mass (**Figure 5(B)**) and LA (**Figure 5(C)**) among 17 progeny and WT plants, indicating that this transgenic line showed a similar (sometimes even better) growth pattern under drought stress conditions,


A1
WT
03-T1
11-T1
17-T1
Pn (μmol m-2s-1)
n.s.
b
ab
b
a
b
a
b
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)
A2
WT
17.1-T2
17.6-T2
Pn (μmol m-2s-1)
ab
b
a
n.s.
b
b
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)
B1
WT
03-T1
11-T1
17-T1
gs (mmol m-2s-1)
b
ab
b
a
a
b
b
a
c
d
b
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)
B2
WT
17.1-T2
17.6-T2
gs (mmol m-2s-1)
n.s.
b
b
a
b
a
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)
C1
WT
03-T1
11-T1
17-T1
E (mmol m-2s-1)
n.s.
b
c
c
a
b
d
c
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)
C2
WT
17.1-T2
17.6-T2
E (mmol m-2s-1)
n.s.
b
b
a
b
b
a
-0.20
-1.20
-1.79
Leaf Water Potential (MPa)

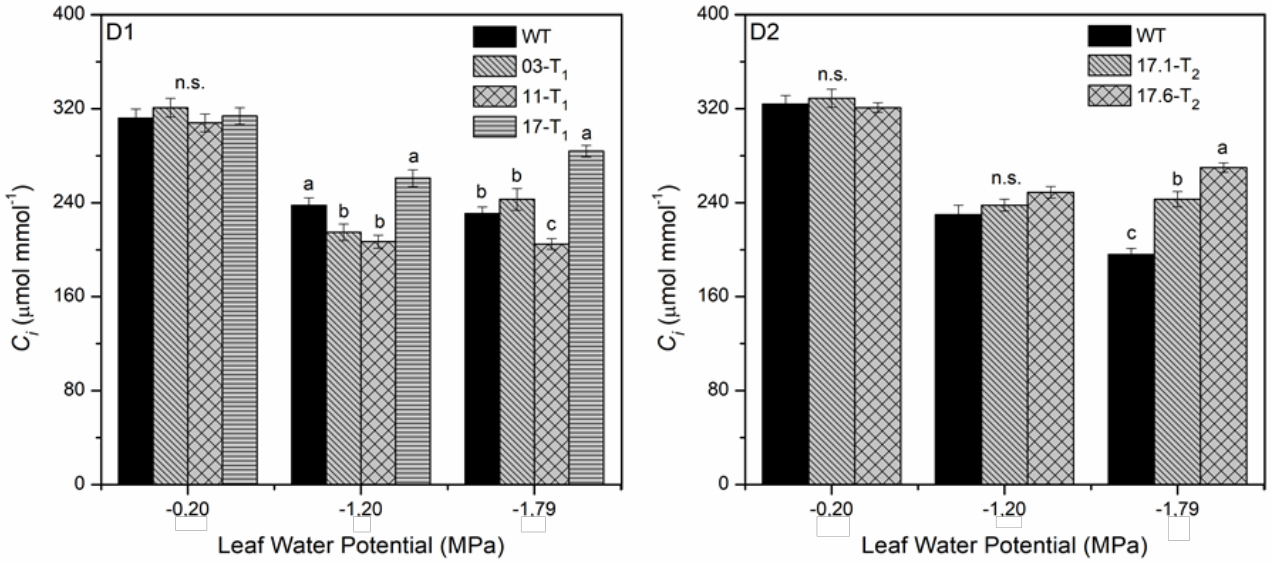


Figure 4. Gas Exchange Measurements in T_1/T_2*rd*29A::*AtDREB2A-CA* Cotton Plants. (A) net photosynthetic rate—P_n; (B) stomatal conductance—g_s; (C) transpiration rate—E and (D) intercellular carbon concentration—C_i monitored in WT plants and transgenic lines exposed to drought stress. Column (1) T_1 generation (03-T_1, 11-T_1 and 17-T_1); Column (2) T_2 generation (17.1-T_2 and 17.6-T_2). Drought stress treatment was applied according to leaf water status: −0.20 MPa (no stress), −1.20 MPa (moderate stress) and −1.79 MPa (severe stress) at predawn. Each bar represents the average of six replicates ± standard error. Statistical analysis was performed according to the Student's *t-test* ($p < 0.05$). Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wildtype non-transgenic plants); *n.s.* (non-significant).

when compared with WT plants. In addition, the imaging analysis of the root system showed that the 17-T_2 progeny rooted into deeper soil layers than the other genotypes (**Figures 5(D)-(F)**). The total root volume, length and surface area of this transgenic line were more than 2-fold higher than WT plants and significantly superior to other transgenic lines. The rhizotron assay confirmed this root pattern, showing that the 17-T_2transgenic roots were longer than WT one (**Figures 5(G1)-(G3)**).

Osmotic adjustment

A markedly contrasting response for osmotic adjustment was observed when comparing the T_2transgenic lines 17.1-T_2 (0.63 MPa) and 17.6-T_2 (0.45 MPa) with WT plants (0.90 MPa) at the most severe drought stress level (**Figure 6**). These results suggested that possibly the favourable morpho-physiological traits shown by transgenic lines (deeper and denser root system) allowed them to reduce the metabolic costs involved in osmotic adjustment response and in other antioxidant pathways activation.

Yield components

For cotton crop grown under real field conditions, the number of reproductive structures retained per plant, specially squares (flower bud stage before blooming), flowers (perfect flowers) and young bolls (fruits less than two centimeter in diameter) is the most important yield component, which can influence cotton productivity. Even though the experiments were carried out under controlled conditions (greenhouse), drought stress consistently led to noticeable

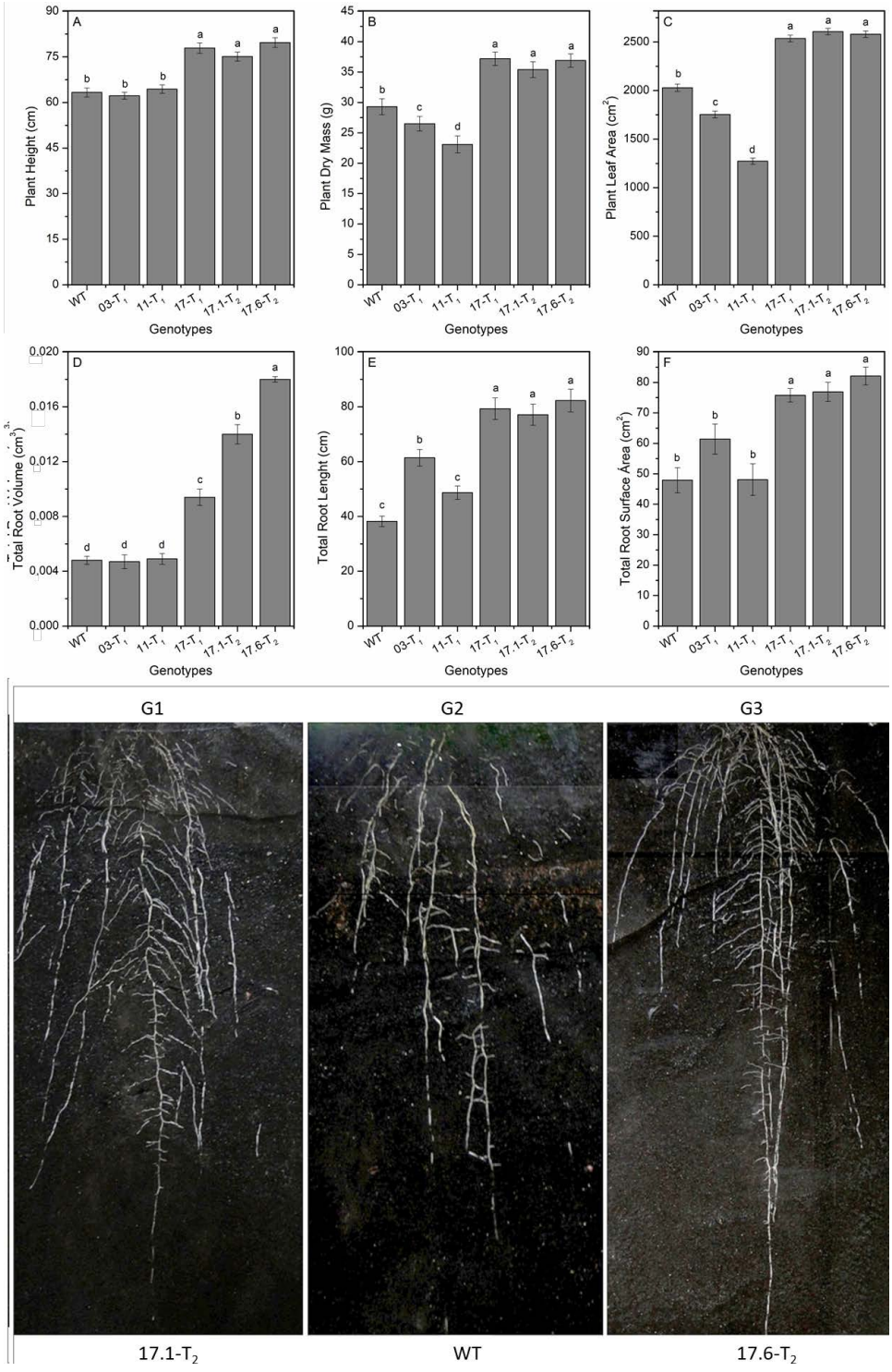


Figure 5. Evaluation of Shoot and Root Morphology of the T_1/T_2*rd*29A::*AtDREB2A-CA* Cotton Plants. Shoot traits ((A)-(C)) and root architecture ((D)-(F)) quantified in WT plants and transgenic lines exposed to severe drought stress (leaf water status of −1.79 MPa at predawn). Main features of the 17-T_2 roots in rhizotron analysis: (G1) 17.1-T_2; (G2) WT; and (G3) 17.6-T_2. *AtDREB2A-CA* cotton plants displayed deeper root when compared with WT plants at LWPpred at −1.79 MPa, approximately. Each bar represents average of six replicates ± standard error. Statistical analysis was performed according to the Student's *t-test* ($p < 0.05$). Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wildtype non-transgenic plants).

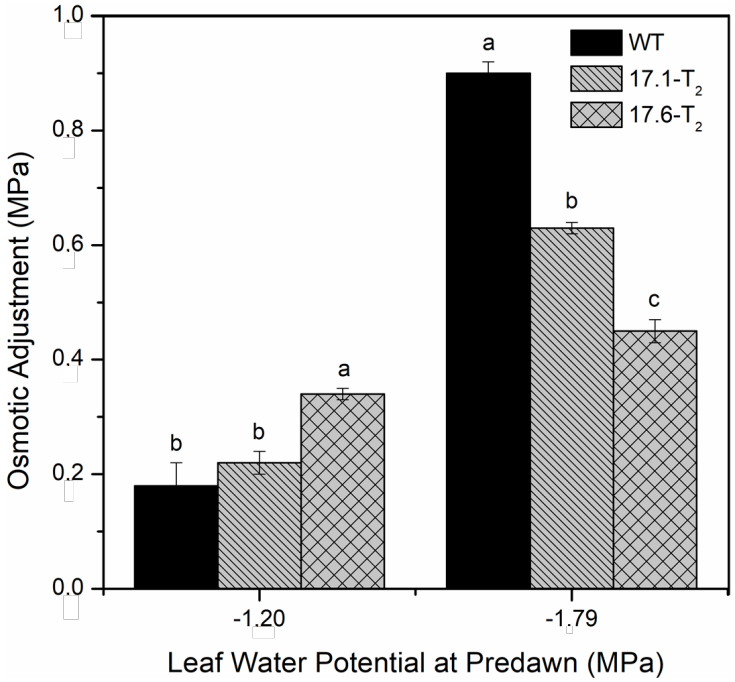


Figure 6. Osmotic Adjustment of T_2*rd*29A::*AtDREB2A-CA* Cotton Plants. Osmoregulation was analyzed in WT plants and T_2 transgenic lines (17.1-T_2 and 17.6-T_2). Drought treatment was applied according to leaf water status: −1.20 MPa (moderate stress) and −1.79 MPa(severe stress) at predawn. Each bar represents the average of six replicates ± standard error. Statistical analysis was performed according to the Student's *t-test* ($p < 0.05$). Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wildtype non-transgenic plants).

differences between the 17.6-T_2 transgenic line and WT plants in the number of retained reproductive structures (**Figure 7**) per plant, which was approximately 26.0% higher in the transgenic line under severe water shortage conditions.

4. Discussion

The present investigation provides the first report of a GM cotton plant tolerant to drought stress due to the presence of the*AtDREB2A-CA* gene driven by the *rd*29A inducible promoter, instead of the *CaMV* 35S constitutive promoter. Employing an optimized biolistic transformation protocol [47], a high transformation frequency was obtained with the cotton cultivar Embrapa 113-7MH.

The particle bombardment process often results in complex transgene integration patterns [59] [60]. In contrast to this statement, the present study showed that just one or two copies of *AtDREB2A-CA* cassette were integrated into the cotton genome. Low number of transgene copies was also observed in a previous study of transgenic cotton plants transformed by a biolistic procedure [53]. Molecular analysis confirmed the successful integration of the transgene in the T_0 plants, as well as in the subsequent generations (T_1 and T_2).

The use of the *rd*29A promoter allowed cotton plants to preserve their physiological functions and did not affect any other relevant feature. Similarly, in

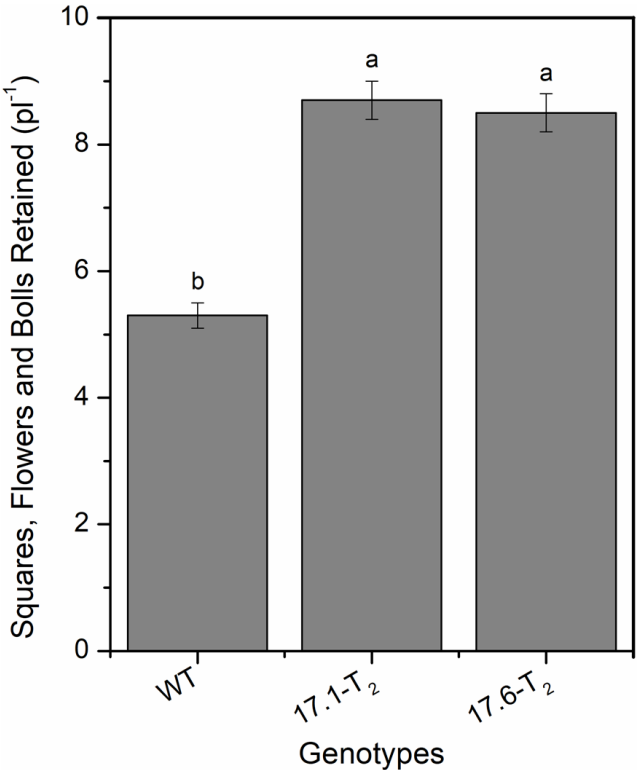


Figure 7. Effect of Drought on Yield Components of T_2*rd*29A::*AtDREB2A-CA* Cotton Plants. The number of reproductive structures (squares, flowers and boll fruits) retained per plant was evaluated in WT plants and T_2 transgenic lines (17.1-T_2 and 17.6-T_2) seven days after re-irrigation establishment. Each bar represents the average of six replicates ± standard error. Statistical analysis was performed according to the Student's *t-test* ($p < 0.05$).Identical letters above the bars indicate that the means were not significantly different. Legends: WT (wildtype non-transgenic plants).

other studies, the over expression of *DREB* genes under the control of the *rd*29A promoter conferred increased drought and freezing tolerance into transgenic Arabidopsis, tobacco, wheat, peanut, groundnut and rice plants without growth retardation [16] [24] [61] [62] [63] [64]. Conversely, the use of *CaMV* 35S promoter has led to growth and yield penalties in a variety of *DREB*-transgenic plants [18] [61] [65]. Only few exceptions showed few or no changes in the phenotypes of transgenic plants the *DREB* constitutively over expressing *DREB* genes [66] [67] [68]. Another important feature of the *rd*29A promoter is that it can be induced in all plant organs including leaves and roots, working as a component of stress perception and signaling in response to water deficits [69]. Qualitative and quantitative assays in soybeans cultivars with the *rd*29A::*GUS* cassette showed significant *rd*29A promoter activity in soybean plants subjected to dry-down conditions. In addition, the reporter gene expression was notably higher in roots than in leaves and the *rd*29A promoter did not result in agronomic penalties through leaky expression when no abiotic stress was imposed [70].

Currently, only a few *DREB*-related genes have been introduced into different

cultivars aiming to improve water deficit tolerance around the world (Additional File 3: **Table S3**) [14] [18] [45] [71] [72] [73] [74]. Several studies have been performed in order to establish parameters to assess GM drought-tolerant cotton plants (Additional File 4: **Table S4**) [27]-[36]. The employment of physiological and agronomic traits has been able to distinguish tolerant and sensitive genotypes to dehydration [58] [62]. Water deficit perception occurs later in GM drought-tolerant plants compared with WT plants, *i.e.*, wilting, reduced stomatal conductance, transpiration and photosynthesis [12]. In the present study, under well-watered conditions, the genotypes displayed similar physiological behaviors for most parameters, which indicate that the insertion of *AtDREB2A-CA* did not negatively affect their performance. The same observation was previously reported for *rd*29A::*DREB*1*A*-peanut plants under normal irrigated conditions [62].

In this report, all the gas exchange variables were higher among the descendants from 17 transgenic cotton line, when compared with WT plants as drought intensity progressed from −1.20 MPa (moderate stress) to −1.79 MPa (severe stress). This observation reflected the greater ability of the transgenic plants to acquire a larger amount of water as a consequence of its deeper and greater-biomass roots. These results suggest that this transgenic line could tolerate a suboptimal water supply, most likely due to the situational activation of the stress-responsive*AtDREB2A-CA* gene driven by the *rd*29A promoter. The stress induced expression of*rd*29A::*DREB*1*A* in transgenic peanut plants demonstrated that it caused no morphological alterations in comparison with WT control plants, which may be attributed to stress-inducible expression of the transgene [62]. In contrast, other studies have shown that both non-transformed and *rd*29A::*SbDREB2*-rice plants subjected to drought conditions showed leaf rolling symptoms, which indicated that the water regulation mechanisms were similar in transgenic and WT plants [63]. Enhanced drought tolerance in transgenic wheat and barley transformed with 2x35S-*TaDREB2* and 2x35S-*TaDREB3* resulted in slower growth, delayed flowering and lower grain yields than control plants under water deficit conditions, most likely due to the pleiotropic promoter effect [61].

Compared with the T_1 and T_2 descendants from 17 line, the other transgenic cotton lines (03-T_1 and 11-T_1) showed restricted water loss, as evidenced by their similar or inferior stomatal conductance and transpiration rate performance compared with WT plants, indicating a conflict between water conservation and net CO_2 assimilation in these plants. Moreover, the same plants showed similar or lower values for plant height, dry mass, LA, root volume, root length and root surface area when compared with WT plants at the end of stress period. Among several possibilities, the transgene stability could be one reason that explains the differences observed in each transformation event. The *position effect*, which reflects the influence of genomic DNA surrounding the transgene introduction site, as well as *locus structure*, which influences the likelihood of physical inte-

ractions and further recombination within the locus, can determine effective transgene stability and plant metabolic rearrangement, culminating in the expected phenotype for GM plants [75] [76]. Studies show that the extent of the differences between the phenotypes of transgenic and control plants may also be explained by variation in the number and type of target genes induced by *DREB* transgene over expression in certain plant species [23].

The maximum cotton rooting depth and density may help plants to maintain their water status at a relatively higher level due to an increased capacity to exploit water in the soil deeper layers [58] [77]. The root data shown in this study clearly indicated that the *AtDREB2A-CA* gene conferred an advantage on the descendants from 17 transgenic line under drought stress conditions, by improving total root length, volume and surface area. This response would be beneficial if these transgenic cotton plants were grown in the field, where they could access water in the deeper soil layers via their root architecture traits and robustness. Under some unstable climatic conditions, better root architecture helps plants to avoid water deficit and consequently reduces the metabolic cost of the osmotic adjustment pathway, decreasing the negative impact on gas exchange performance and its yield components. Thus, the expression of these traits allows plants to maintain greater stomatal conductance and higher CO_2 rate diffusion, increasing the net photosynthesis, growth and yield. Studies have shown that water stress treatment promoted better root growth in *rd*29A::*DREB*1*A* transgenic peanut lines in comparison with WT plants and that this was partially related to the stimulation of root penetration into deeper soil layers [78]. In contrast, sugarcane plants transformed with *AtDREB2A-CA* did not show significant differences in root diameter, volume or length [73]. As reported in other study, roots may explore a larger volume of soil to obtain more water, however, this important parameter is rarely considered during GM analysis [12].

Since the 17-T_1 transgenic plants (manly 17.1 and 17.6) showed better morpho-physiological results, their progenies were selected for further studies in T_2 generation to confirm the effects of transgene expression. Both T_1 and T_2 generations exhibited similar physiological performance during severe stress, which is in agreement with the proposed function of *rd*29A::*AtDREB2A-CA*. During the most severe stress, the T_2 transgenic lines, especially the 17.6-T_2, maintained a higher LWPpre (−1.15 MPa) than WT plants (−1.67 MPa), which was coupled with improved gas exchange performance. Mechanisms of delayed drought perception can result from decreased water loss (stomatal closure, reduced LA and senescence of old leaves) or increased water absorption (deeper root systems) [79] [80] [81]. The enhanced physiological (photosynthetic indexes) and rooting depth traits observed in the 17-T_1 descendants contributed to better morphological and yield performance.

The deeper rooting depth and density shown by the 17.1-T_2 and 17.6-T_2 transgenic lines allowed them to maintain greater cell turgor and consequently lower the demand on activating osmotic adjustment pathways, when compared

with non-transgenic plants. The key role of osmotic adjustment is cell-turgor preservation during water deficit conditions, which is essential for the maintenance of turgor-related processes, especially stomatal regulation [82] [83] [84]. Osmotic adjustment is considered a crucial process for plant adaptation to drought because it sustains tissue metabolic activity and enables regrowth upon re-watering, although it varies greatly among genotypes [85]. Nevertheless, osmotic adjustment is important for root traits, enabling their sustained growth under decreasing water availability in soil [86].

The expression of *AtDREB2A-CA* in cotton plants also significantly improved drought tolerance during the reproductive stage. The transgenic lines had substantially improved retention of reproductive structures (squares, flowers and bolls), approximately 26.0% higher than that of WT plants. Although yield components were not evaluated under non-stressed conditions, we assume that they would not be lower, once *AtDREB2A-CA* insertion did not cause any morpho-physiological penalty to the plants in non-stressed conditions. In this report, it was evident that the modifications in the root architecture of transgenic lines were correlated with physiological attributes and yield components. For cotton breeding, strategies using stomatal conductance regulation, osmotic adjustment capability and promotion of a larger and deeper root are pursued to favour better water status and fiber yield when plants are subjected to water deficiency [58] [77]. The performance of cotton lines over expressing stress responsive *NAC*1 (*SNAC*1) under drought and salt stress conditions was significantly better than WT plants in terms of boll number [36]. Similarly, cotton transgenic lines transformed with *GHSP*26 (Heat Shock Protein Gene), *GUSP*1 (Universal Stress Protein Gene) and *Phyto-B* (Phytochrome-B Gene) showed a significant increase in the number of bolls per plant, single boll weight, and seed cotton yield under drought stress, when compared with WT plants [34]. An improvement of yield components up to 24.0% in drought trials under field conditions was achieved using *DREB*1*A*-peanut plants across a wide range of stress intensities and resulted in higher harvest indices [62]. Thus, our study highlighted that, despite being a proof of concept, the increase in production observed in the T_2 descendants from 17 transgenic lines can be considered a promising result in obtaining transgenic cotton more tolerant to drought.

5. Conclusions

Based on our findings, the 17-*AtDRE2A-CA*-cotton line exhibited enhanced drought tolerance in comparison with control plants via a more robust root system trait. The high and stable physiological features followed by better gas exchange indexes displayed by this line were also maintained among its descendants (T_2 generation). The superior rooting depth and density of this transgenic line resulted in higher ability to acquire water in deeper layers, which likely contributed to its higher yield components in drought conditions, when compared to its WT counterpart. Moreover, this high performance was confirmed by im-

proved photosynthetic data, leading to higher numbers of retained reproductive structures in these plants. To the best of our knowledge, the present work is the first report in cotton demonstrating a physiological and yield advantage attributes under drought stress using the *AtDREB2A-CA* transcription factor. Further evaluation will be performed under field conditions in order to assess whether *AtDREB2A-CA* gene can be used to increase cotton crop production in water-limited real field trials.

Acknowledgements

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Supporting Information

Additional File 1—Table S1. Transformed cotton plants tolerant to water stress.

Gene inserted	Gene product and function	Promoter	Transformation method	Stress tolerance	References
AtGF14λ	14-3-3 protein regulates primary metabolism, ion transport, cellular traffic, enzyme activities and gene expression.	*CAMV* 35S	*Agrobacterium tumefaciens*	Drought	Yan et al., 2004
AtNHX1	Vacuolar sodium/proton antiporter that mediates the exchange of cytosolic Na^+ for vacuolar H^+.	*Supermas*	*Agrobacterium tumefaciens*	Drought, Salinity	He et al., 2005
GaHSP26	Molecular chaperones that assist in protein folding and prevent protein denaturation.	*CAMV* 35S	*Agrobacterium tumefaciens*	Drought	Maqbool et al., 2010
SceSAMDC	SAMDC catalyzes S-adenosyl methionine (SAM) to form decarboxylated SAM.	*CAMV* 35S	Particle bombardment	Drought	Momtaz et al., 2010
GhMPK16	Mitogen-activated protein kinase (MAPK) gene that plays a role in mediating biotic and abiotic stress responses.	*CAMV* 35S	*Agrobacterium tumefaciens*	Drought, Pathogen	Shi et al., 2011
AtAVP1	Vacuolar pyrophosphatase that functions as a proton pump on the vacuolar membrane.	*CAMV* 35S	*Agrobacterium tumefaciens*	Drought, Salinity	Pasapula et al., 2011
AtLOS5	Molybdenum co-factor, essential for activating aldehyde oxidase, which is involved in the hormone abscisic acid (ABA) biosynthesis.	*CAMV* 35S	*Agrobacterium tumefaciens*	Drought	Yue et al., 2012
GHSP26	Molecular chaperones that assist in protein folding and prevent protein denaturation.	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Shamim et al., 2013
GUSP1	Bacteria, archaea, fungi, flies, and plants protein which show specific expression patterns during drought stress.	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Shamim et al., 2013
Phyto-B	Controls the transition from vegetative to reproductive growth, seedling establishment and entrainment of circadian clock.	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Shamim et al., 2013
AtuIPT	Isopentenyltransferase, which has a role in cytokinin biosynthesis	*pSARK*	*Agrobacterium tumefaciens*	Drought	Kuppu et al., 2013
OsSNAC1	NAC transcription factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought, Salinity	Liu et al., 2014
GhAnn1	Putative annexin protein	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought, Salinity	Zhang et al., 2015
GhNAC2	NAC transcription factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Gunapati et al, 2016
GhABF2	bZIP transcription factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought, Salinity	Liang et al., 2016
AtABF3	ABA-responsive element binding factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Kerr et al., 2017
GhABF2D	ABA-responsive element binding factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought	Kerr et al., 2017
OsSIZ1	SUMO E3 Ligase	*pZmUbi*	*Agrobacterium tumefaciens*	Drought, Heat	Mishra et al., 2017
ZmABP9	bZIP transcription factor	*CaMV* 35S	*Agrobacterium tumefaciens*	Drought, Salinity	Wang et al., 2017
AtDREB2A-CA	DRE binding proteins.	*rd*29A	Particle bombardment	Drought	This report

Abbreviations: **At** – *Arabidopsis thaliana*; **Atu** – *Agrobacterium tumefaciens*; **Ga** – *Gossypium arboreum*; **Gh** – *Gossypium hirsutum*; **Os** – *Oriza sativa*; **Sce** – *Saccharomyces cerevisiae*; **Zm** – *Zea mays*.

Additional File 2—Table S2. Summary of the used primers.

Primer name	Sequence (5'-3')	T_M (°C)[1]	Amplicon length (bp)	Assays
M-Fow	AAT GGT GCG GAA GAG ATG AA	55	450	Detection of pBSK-*rd29A*::*AtDREB2A-CA*-NOSt transformation cassette in transgenic cotton plants.
M-Rev	GTT CTC CAG ATC CAA GTA AC			
qPCR-Fow	TGT TTGA TGT CGA TGA GCT TCT	60	173	Determination of transgene transcript levels and cassette copy number in GM cotton plants (RT-qPCR).
qPCR-Rev	CTG AAA CGG AGG TAT TCC GTA G			
GhUBQ14-Fow	CAA CGC TCC ATC TTG TCC TT	60	75	Determination of transgene transcript levels in GM cotton plants (RT-qPCR).
GhUBQ14-Rev	TGA TCG TCT TTC CCG TAA GC			
GhPP2A1-Fow	GAT CCT TGT GGA GGA GTG GA	60	100	Determination of transgene transcript levels in GM cotton plants (RT-qPCR).
GhPP2A1-Rev	GCG AAA CAG TTC GAC GAG AT			
GhUBC1-Fow	TGG CAT TAT ATT GTC ATT GTT ACT ATC C	60	121	Determination of cassette copy number in transgenic cotton genome.
GhUBC1-Rev	ACC ATG TTA TCT TAT TCT AAG ACA AGC TC			

[1]Anneling (or melting) temperature used in experiments.

Additional File 3—Table S3. Transgenic plants transformed with *AtDREB2A*.

Species	Promoter	Tolerance test	References
Arabidopsis thaliana	*CaMV* 35S	Not shown	Liu et al., 1998
Arabidopsis. thaliana	*CaMV* 35S *rd29A*	Drought Freezing	Sakuma et al., 2006a
Arabidopsis thaliana	*CaMV* 35S	Drought, salt H_2O_2 Heat Shock	Sakuma et al., 2006b
Tobacco cells	*CaMV* 35S	No one	Schramm et al., 2008
Soybean	*rd29A*	Drought	Engels et al., 2013
Sugarcane	*rab17*	Drought	Reis et al., 2014
Peanut	*CaMV* 35S	Drought Salinity	Pruthvi et al., 2014

Additional File 4—Table S4. Analyses performed to screen transgenic cotton tolerant to water stress.

INSERTED GENES	ASSAYS																REFERENCES
	MOLECULAR ANALYSIS						PHYSIOLOGICAL ANALYSIS						MORPHOLOGICAL ANALYSIS			OTHER ASSAYS	
	PCR	Southern blot	Northern blot	qPCR	Western blot	ELISA	Photosynthesis	Water use efficiency	Transpiration rate	Chlorophyll content	Stomatal conductance	Intercellular carbon	Biomass	Fiber content	Phenotype differences		
AtGF14λ	Yes	Yes					Yes	Yes		Yes	Yes				Yes	Intercellular to ambient CO_2 concentration ratio	Yan et al., 2004
AtNHX1	Yes		Yes		Yes		Yes							Yes	Yes	Nitrate reductase activity and field tests	He et al., 2005
GaSP26	Yes			Yes											Yes	Immunohistochemical localization	Maqbool et al., 2010
SAMDC	Yes	Yes		Yes											Yes	Transformed protein concentration through HPLC	Momtaz et al., 2010
GhMPK16	Yes	Yes													Yes	Immunohistochemical localization and Rapid H_2O_2 accumulation	Shi et al., 2011
AtAVP1	Yes	Yes	Yes				Yes		Yes		Yes		Yes		Yes	Immunohistochemical localization	Pasapula et al., 2011
AtLOS5	Yes			Yes					Yes							Relative water content (RWC), electrolyte leakage, peroxidation, ABA and proline content, Antioxidative enzyme activities	Yue et al., 2012
GHSP26	Yes			Yes											Yes	Inheritance and Productivity (cotton bolls number, single boll weight, seed cotton yield, Yield/plant	Shamim et al., 2013
GUSP1	Yes			Yes											Yes	Inheritance and Productivity (cotton bolls number, single boll weight, seed cotton yield, Yield/plant	Shamim et al., 2013
Phyto-B	Yes			Yes											Yes	Inheritance and Productivity (cotton bolls number, single boll weight, seed cotton yield, Yield/plant	Shamim et al., 2013
IPT	Yes	Yes	Yes	Yes			Yes		Yes	Yes			Yes	Yes	Yes	Number of bolls per plant and internal carbon	Kuppu et al., 2013
SNAC1	Yes	Yes	Yes				Yes		Yes		Yes		Yes		Yes	Proline and Malondialdehyde (MDA) content and number of bolls per plant	Liu et al., 2014
AtDREB2A-CA	Yes			Yes			Yes		Yes	Yes	Yes	Yes	Yes		Yes	Transgenic copy number by qPCR, osmotic adjustment, photosynthetic pigment content and yield components	This report

Abbreviations: **At** – *Arabidopsis thaliana*; **Ga** – *Gossypium arboreum*; **Gh** – *Gossypium hirsutum*.