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ORIGINAL PAPER

Phenotyping soybean plants transformed with rd29A:AtDREB1A for drought tolerance in the greenhouse and field

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Abstract The development of drought tolerant plants is a high priority because the area suffering from drought is expected to increase in the future due to global warming. One strategy for the development of drought tolerance is to genetically engineer plants with transcription factors (TFs) that regulate the expression of several genes related to abiotic stress defense responses. This work assessed the performance of soybean plants overexpressing the TF DREB1A under drought conditions in the field and in the greenhouse. Drought was simulated in the greenhouse by progressively drying the soil of pot cultures of the P58 and P1142 lines. In the field, the performance of the P58 line and of 09D-0077, a cross between the cultivars BR16 and P58, was evaluated under four different water regimes: irrigation, natural drought (no irrigation) and water stress created using rain-out shelters in the vegetative or reproductive stages. Although the dehydration-responsive element-binding protein (DREB) plants did not outperform the cultivar BR16 in terms of yield, some yield components were increased when drought was introduced during the vegetative stage, such as the number of seeds, the number of pods with seeds and the total number of pods. The greenhouse data suggest that the higher survival rates of DREB plants are because of lower water use due to lower transpiration rates under well watered conditions. Further studies are needed to better characterize the soil and atmospheric conditions under which these plants may outperform the non-transformed parental plants.

Keywords BR 16 · *DREB1A* · Embrapa 48 · Transpiration · Transcription factor · Water deficit

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Introduction

Water deficit is a major abiotic stress factor limiting crop yield. Drought periods in the 2007 season caused

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a US $787.2 million loss in agricultural soybean production in the USA, the world's largest soybean producer. The southern states of Brazil, which account for 40 % of the soybean production by the second leading producer worldwide, lost more than 20 % of their production due to water deficits during the 2003/2004 and 2004/2005 seasons, resulting in US $2.3 billion in economic losses (Embrapa 2004; Conab 2005). Recently, the national production in Brazil reached 75 million tons in the 2010/2011 season, but in 2011/2012 the production decreased to 65.6 million tons due to drought (Conab 2012).

It is difficult to breed for drought tolerance using conventional approaches because tolerance is a multigenic and quantitative trait. Plant responses to drought are also influenced by the time, intensity, duration, and frequency of the stress as well as by diverse plant–soil–atmosphere interactions (Bhatnagar-Mathur et al. 2007).

One alternative approach to the development of drought tolerant plants is to genetically engineer plants to introduce stress-tolerance genes, including genes for transcription factors (TFs). These TFs recognize specific DNA sequences in the regulatory regions of target genes and lead to the activation of downstream genes responsive to abiotic stresses. One relevant class of transcription factors is the dehydration-responsive element-binding proteins (DREBs), which are transcriptionally up-regulated by water deficit or low temperature (Liu et al. 1998).

DREB/CBF (C-repeat (CRT)-binding factor) proteins have a single 60 amino acid-long DNA binding AP2 domain, which permits them to specifically recognize and bind as a single molecule to so-called drought/cold/salt-stress responsive promoter elements with the consensus sequence (A/G)CCGAC. The expression of individual DREB/CBFs is regulated by drought, salt, heat, and/or cold. Most DREBs/CBFs that have been studied exhibit low or very low expression in the absence of stress and moderate expression induced by stress (Lopato and Langridge 2011).

Due to the central role of DREBs/CBFs in abiotic stress responses and their ability to regulate a large number of target 'stress-responsive' genes, they have become popular targets for genetic engineering to improve abiotic stress tolerance in various plant species (Lopato and Langridge 2011).

Improvements in tolerance to drought, salinity and low-temperature stresses have been reported in *Arabidopsis* (Kasuga et al. 1999), potato (Behnam et al. 2006), tobacco (Kasuga et al. 2004), rice (Oh et al. 2005) and wheat (Pellegrineschi et al. 2004). In transgenic wheat, *DREB1A* overexpression delayed death following withdrawal of irrigation (Pellegrineschi et al. 2004). More recently, constitutive over-expression of two wheat DREB factors in barley substantially improved survival under severe drought or cold (Morran et al. 2011).

On the other hand, transgenic plants constitutively over-expressing DREB/CBFs express undesirable developmental traits such as stunted growth and delayed flowering (Kasuga et al. 1999; Kasuga et al. 2004; Morran et al. 2011; Saint Pierre et al. 2012). Stress inducible promoters such as the *rd29A* promoter apparently overcome the difficulties encountered when using constitutive promoters.

In our laboratory, transgenic soybean plants have been generated that overexpress the DREB1A TF

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under the control of the *rd29A* promoter. The drought tolerance of the transformed lines under moderate and severe water stress was reported by Polizel et al. (2011). Senescence was found to occur later for the DREB1A line named P58 than for its isoline BR16. After severe water stress (2.5 % gravimetric humidity), this line maintained higher values of net photosynthesis and photosynthetic efficiency, and therefore it was considered drought tolerant.

According to Passioura (2012), the results obtained under controlled conditions may not be well connected to the way that plants behave over the entire season in the field. Thus, it is important to test DREB plants in the field to accurately gauge whether the technology is successful. Considering that few studies have reported results from genetically modified crops under realistic field conditions and that there is a lack of understanding of the mechanisms of tolerance of DREB transgenic plants, the present work had the following aims:

- To test whether soybeans can be genetically engineered for enhanced abiotic stress tolerance by stress inducible expression of the transcriptional factor DREB1A without any detrimental effects on plant growth and development;
- To investigate the performance of the transgenic plants under field conditions in drought and non-drought environments.
- To provide new insights into the mechanism of tolerance of the DREB1A soybean plants.

Plant material

The drought-sensitive Brazilian soybean cultivar BR16 (Oya et al. 2004) was transformed with the *rd29A:AtDREB1A* (Patent Nos. P3183458 and P3178672) construct by particle-bombardment according to Aragão et al. (2000) and Rech et al. (2008). The transformation method, selection, and regeneration procedures were described in Polizel et al. (2011). The lines obtained in this way (P58, P59, P1142, P1378 and P3069) were submitted for molecular analysis, and based on these studies the P58 and P1142 lines were selected for further experimentation (see northern blot analysis). The P58 line was further characterized through physiological and agronomical studies along several generations under greenhouse conditions (Polizel et al. 2011). From these studies, the P58 (T8) and P1142 (T5) lines were chosen for further characterization and/or performance evaluation in the field. The P58 line was crossed with its isoline, the BR 16 cultivar, producing the genotype 09D-0077. Plants resulting from this cross that were PCR positive for the transgene were selected based on recuperation after water stress in a greenhouse and allowed to produce seeds, which were then used in the field experiment.

Northern blot analysis

Soybean seeds were sown in triplicate and grown in soil:vermiculite (1:1) for 20 days at 28 °C with a 12/12 h light/dark cycle. Hoagland's solution was added to the pots twice a week until the plants reached the V_4 developmental stage. At this stage, the pots were divided into three subsets, leaf samples were harvested from subset one, and irrigation of subsets two and three was suspended for four and 6 days, respectively. At these times leaf samples were harvested, placed in liquid nitrogen and stored at −80 °C for RNA extraction.

Total RNA was extracted from all lines using TRIzol (*Invitrogen—Life Technologies*) reagent according to the manufacturer's instructions. For the northern blot analysis, 10 μg of total RNA was separated by electrophoresis in a 1 % agarose-formaldehyde gel for 2 h and transferred to an Amersham HybondTM-N+ (GE Healthcare) membrane. A probe was prepared using cDNA from the P58 GM line that was amplified using specific primers for the target gene *AtDREB1A*, and labeled with [a^{32}P]d-CTP. Hybridization was performed for 16 h at 42 °C, after which the membranes were washed with a series of buffers (1× SSC/0.1 % SDS; 0.1x SSC/0.1 % SDS) to remove the excess probe and then imaged using Kodak BioMax film.

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Recovery of DREB1A plants after water deficit

Seedlings were cultivated in 1.0 kg pots containing a 1:3:1 soil:sand:manure mixture (26 % water capacity) in well-watered conditions (70 % of capacity) until the V_3 developmental stage (Fehr et al. 1971; supplementary data Fig. S1b). Prior to the initiation of stress, the pots were saturated with water and then irrigation was stopped. The plants were re-irrigated after approximately 6 days, and recovery after water stress was assessed after 3 days by counting the number of plants that survived the stress period.

The phenotype, transpiration, relative growth ratio and percentage reduction in growth of DREB1A plants under water stress

Soybean seeds from generations T8 and T5 of lines P58 and P1142, respectively, were germinated along with the BR16 cultivar on filter paper for 4 days in a growth chamber where the temperature and relative humidity were set to 25 ± 1 °C and 100 %, respectively. Seedlings were then cultivated in 3.0 kg pots (supplementary data Fig. S1a) containing a 2:2:1 soil:manure:sand mixture (39 % water capacity) in well-watered conditions (70 % of capacity) until the V_4 developmental stage (Fehr et al. 1971). Greenhouse temperature and air humidity were monitored every 5 min using a Hobo U14-002 thermo-hygrograph (Onset®). The vapor pressure deficit (VPD) was calculated from the atmospheric temperature and relative humidity (RH) according to the formula VPD = (100 − RH)/100*PVsat (kPa). PVsat was calculated using the psychrometric chart available at http://physics.holsoft.nl/physics/ocmain.htm.

At the V_4 stage, 30 days after sowing (DAS), the pots were divided into three subsets. For subset one, six plants were harvested per genotype and divided into roots and shoots to estimate the initial dry plant biomass (W1). The other two subsets were subjected to control (C) or drought stressed (DS) treatments. The experimental design was a completely randomized block design with a factorial scheme (2 × 3) with three blocks and two plants per block. The treatments included two water regimes (control, C and drought stressed, DS) and three genotypes, the cultivar BR 16 and the DREB1A lines P58 and P1142.

Prior to the initiation of stress at 30 DAS, the pots of both the C and DS treatments were saturated with water and left overnight to drain the excess water. On the following morning, the pots were enclosed in polyethylene bags to prevent any loss of water by evaporation from the soil surface (supplementary data - Fig. S1a). Thereafter, the pots were weighed every morning between 09:00 and 10:00 h Brazilian Standard Time (IST). For the control plants, water lost by transpiration on the previous day was compensated on the following day by adding water to 70 % of the soil capacity. For the DS plants, the water in the pot was progressively adjusted to 40 % (moderate) and thereafter 20 % (severe stress). The transpiration of each plant was calculated as the difference between pot weights on successive days plus the water added on the previous day.

At the end of the experiment, the plants were harvested and divided into roots and shoots to estimate the final dry plant biomass (W2). The total transpiration was calculated as the sum of the daily transpiration from the initial day when the plants were bagged to the day when the plants were harvested. The transpiration efficiency (TE) could then be calculated as the plant biomass gained between the first and final plant samplings divided by the total transpiration during that period.

Growth was analyzed by determining the mean relative growth rate (RGR; g g^{-1} day^{-1}) across one harvest interval (t2−t1) using the equation: RGR = (ln(W2)−ln(W1))/(t2−t1), where W2 and W1 represent the dry matter of the plants at 30 DAS and after final harvest. Reduction in weight (%) was calculated by dividing the whole plant dry weight (g) in the water stressed condition by the whole plant dry weight (g) in the control condition and multiplying by 100. Phenotype was analyzed by measuring the number of leaves, leaf area (length*width), height, and length of the internode.

Transpiration, transpiration efficiency, stomatal conductance and photosynthesis under two different vapor pressure deficits

To determine the plants' responses to changes in the atmospheric vapor pressure deficit (VPD), an experiment was performed in phytotron CE cabinets. Seeds were germinated on filter paper for 4 days in a growth chamber where the temperature and relative humidity were set to 25 ± 1 °C and 100 %, respectively. The plants were transferred to pots containing 1.0 kg of

soil:sand:manure (1:3:1; 26 % water capacity) and kept in an environment with a low vapor pressure deficit (RH = 60 % and day/night temperature = 25/20 °C) under well watered conditions (100–70 % capacity) until the V_3 developmental stage. At this stage, the pots were divided into two subsets, with one set of ten plants harvested to estimate initial dry plant biomass and the other set saturated with water and left overnight to drain the excess water. On the following morning, the pots were enclosed in polyethylene bags to prevent any loss of water by evaporation from the soil surface. Then, the photosynthetic rate (A) and stomatal conductance (g_s) were evaluated using a Model LI-6400 Portable Photosynthesis System (Li-Cor, Inc.) Parameters were measured on the middle leaflet from the second leaf node that was totally expanded under a photon flux density of 1,000 μmol m^{-2} s^{-1}. Thereafter, the pots were weighed every morning between 09:00 and 10:00 h Brazilian Standard Time (IST).

For 7 days after the initiation of stress, the plants were kept under a low vapor pressure deficit (RH = 60 % and day/night temperature = 25/20 °C). After 7 days without irrigation, the relative humidity and temperature of the cabinets were changed to 35 % and 35/30 °C (day/night), respectively, for 3 days. At the end of this period, the plants were harvested and divided into roots and shoots to estimate the final dry plant biomass. Transpiration and transpiration efficiency were calculated as previously described.

The experimental design was completely randomized in a factorial scheme (3 × 2). The treatments were the three genotypes (P58, P1142 and BR16) and the two water regimes (control and drought stressed).

Evaluation of growth and yield under field conditions

To test the plants' performance under field conditions, an experiment was carried out at Embrapa Soybean (Londrina-PR, Brazil) during the 2011/12 season. Soil chemical corrections and cultivations were performed according to recommendations for this crop (Embrapa 2011). Daily precipitation and temperature (maximum, minimum and average) during the season were obtained from a meteorological station at Embrapa Soybean. From these data, a water balance was calculated according to Thornthwaite and Mather (1955) as shown by Crusiol et al. (2012). Evapotranspiration was greater than precipitation in several stages (supplementary data - Fig. S2).

The experimental design was a randomized complete block with treatments arranged in a split plot and with four replicates for each condition. The main plots received four different water regimes consisting of irrigation (I, matric soil–water potential maintained between −0.03 and −0.05 MPa), non-irrigated (NI, natural rainfall) and plants artificially drought stressed at the vegetative (ER) or reproductive (EV) stages. To simulate drought stress, the plants were sheltered from rain using rainout shelters programmed to automatically close upon rainfall and open as soon as the rain stops. Soil humidity was monitored daily by tensiometers placed at a soil depth of 30 cm and weekly by the gravimetric methods. The treatments in the sub-plots were the BR 16 soybean cultivar, regarded as drought sensitive, the GM line DREB1A P58, and the cross 09D-0077, resulting from crosses between DREB1A plants and the cultivar BR 16. Measures of growth were recorded such as height (H), number of nodes (NN), leaf area (LA), leaf area index (LAI) and shoot fresh (SFW) and dry weights (SDW). The leaf area index was calculated as the ratio between leaf area and the area of land occupied by the plant. Apparent harvest index, yield and its components such as the number of seeds (NS), number of pods (NP), and 100-seed weight (100-SW) were also evaluated when all plants had reached the R8 stage. Plot grain yields (at 13 % humidity) were calculated using the equation: Yield (Kg/ha) = (100—grain humidity at harvest, %) × (harvested grain weight, kg × 10,000)/plot harvested area, m^2).

Statistics

Response variables were statistically analyzed by an exploratory diagnostic checking for assumptions of normality, the independence of the residue, the additivity of the model, and the homogeneity of treatment variances, followed by analysis of variance (ANOVA). After these analyses and when the F test indicated statistical significance, the Duncan test for multiple comparisons among treatment means was applied, with $\alpha = 0.05$ as the level of significance.

Results

Northern blot analysis and recovery of DREB1A plants after stress

The *DREB1A* gene expression was induced in the transformed plants under water stress, and also under control (well watered) conditions at a lower intensity (Fig. 1). Expression was higher in the P58 line in all conditions. The survival rates of the DREB and BR 16 plants after water stress were 70 % (P58), 60 % (P1142) and 40 % (BR 16) (supplementary data - Fig. S3).

The phenotype, transpiration, relative growth ratio and percentage of reduction in growth of DREB1A plants under water stress

Figure 2 presents the growth analysis of the plants under well watered (C) and water deficit (DS) conditions. The DREB1A plants exhibited a lower height (C/DS), the same number of nodes (C/DS), a slightly higher number of leaves (DS) and a greater leaf area (C/DS-P58) than BR 16 plants. However, statistical analysis of these data showed that none of these differences were statistically significant, indicating that transformation of soybean plants with the *DREB1A* gene under the control of the *rd29A* promoter did not lead to any retardation of growth of the transformed plants.

The relative growth ratio (Fig. 2) and the percentage of reduction in growth (Fig. 2) in the water stress condition as compared to the plants in the control condition were calculated. When water was available (control condition), the P58 plants had a slightly lower RGR, but both transgenic plants had a slightly higher RGR under water stress. Although these differences were not statistically significant, they are an indication that the DREB lines exhibit a more conservative growth pattern under control conditions and slightly increase their growth rates under water stress conditions.

The stress level was quantified by the stomatal conductance data according to Flexas et al. (2004). The plants were considered to be under control (well watered), moderate and severe water stress conditions when their *gs* values were ≥ 0.2 molH_2O m^{-2} s^{-1}, $0.1 < gs < 0.2$ molH_2O m^{-2} s^{-1} and ≤ 0.1 molH_2O m^{-2} s^{-1}, respectively. The plants designated to the control treatment and maintained under well watered conditions exhibited *gs* values that varied between 0.16 (BR 16 and P58) to 0.25 molH_2O m^{-2} s^{-1} (P1142), and this variation was not statistically significant (supplementary data – Fig. S4). In the same way, plants under water stress exhibited *gs* values below 0.1 molH_2O m^{-2} s^{-1}, within the range expected by Flexas et al. (2004).

These findings indicated that the plants in our experiments were subjected to the same level of water stress after the suspension of irrigation, enabling comparisons between the BR 16 plants and line P58.

Gas exchange (*A* and *gs*) was found to be statistically significantly different between the well watered and water stress conditions, but no differences were observed in gas exchange among the genotypes within each treatment (supplementary data - Fig. S4).

Different from the BR 16 plants, the DREB1A plants did not show any symptoms of drought under moderate water stress (supplementary data - Fig. S5a). Under severe water stress, wilting symptoms were similar between P1142 and BR 16 plants (supplementary data - Fig. S5b).

For some genotypes, declines in transpiration have been observed when the soil is still relatively wet as a mechanism of saving water in the soil for use at later stages. Daily transpiration under the control (Fig. 3a) and water stress (Fig. 3b) conditions was assessed in the DREB plants by evaluating the pots daily.

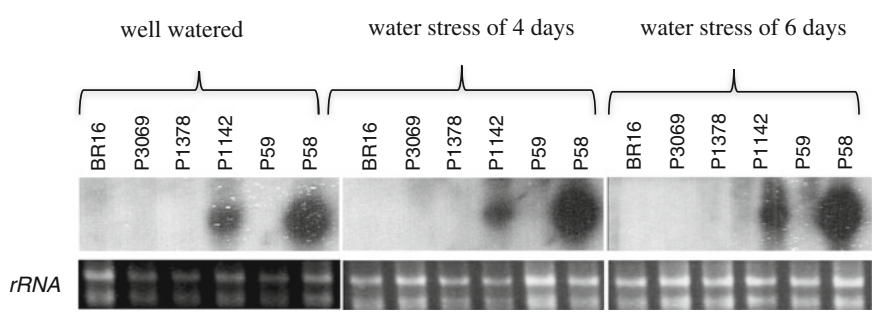

Fig. 1 Northern blot analysis of the DREB1A lines

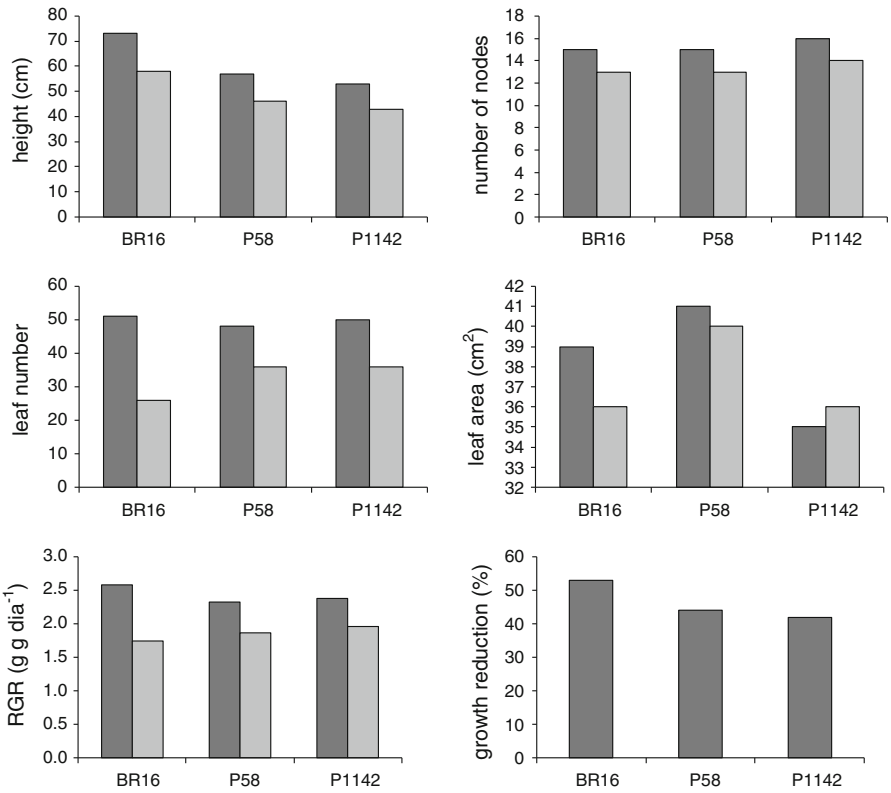

Fig. 2 Growth characteristics of the *AtDREB1A* plants and the cultivar BR16 under control (C-*dark bars*) and moderate water stress (DS-*grey bars*) conditions in the greenhouse. Differences were not statistically significant (Duncan 5 %) (n = 6)

The transpiration of P58 plants was lower than the other two genotypes at some time points under control conditions, when water was fully available to supply the plant′s demand (Fig. 3a). Under water stress conditions no significant differences in transpiration were observed among the genotypes, except at the beginning of the water stress period (Fig. 3b). VPD analysis in the green house verified that major differences in transpiration among the genotypes occurred at high VPD (28th Aug and 5th Sept) (Supplementary data – Fig. S6). The pot weight data verified that on these dates (28–30th Aug), the plants still had high water availability.

To test the effects of the VPD on the plant′s transpiration, an experiment was carried out under controlled conditions (phytotron cabinet) in which the plants were subjected to a change in the atmospheric VPD within the period of suspension of irrigation. This experiment confirmed that when VPD was altered by modifying the temperature and air humidity of the cabinet from 1.2 to 2.3 kPa, the P58 line exhibited a decrease in transpiration compared to the BR 16 plants and the P1142 line. However, at the end of the water stress period, the transpiration of BR 16 plants was lower than that of the DREB plants (Fig. 3c).

The data on transpiration and gain of dry mass over the water stress period were used to calculate transpiration efficiency (TE) (Fig. 3d). The DREBs plants have higher TE values than the BR 16 plants. The negative TE value for the BR 16 plants can be explained by the fact that for some of these plants, the final dry weight minus the initial dry weight (obtained at the beginning of the water stress period) was negative, indicating that these plants were losing weight through respiratory metabolism. Occurrence of respiration instead of photosynthesis was verified by the negative readouts of *A* by the Licor equipment.

Evaluation of growth and yield under field conditions

The supplementary data (Fig. S7a) show the experiment performed under field conditions, including the rain out shelters used to simulate water stress. Fig. S7b shows the same experiment later in the season. Note

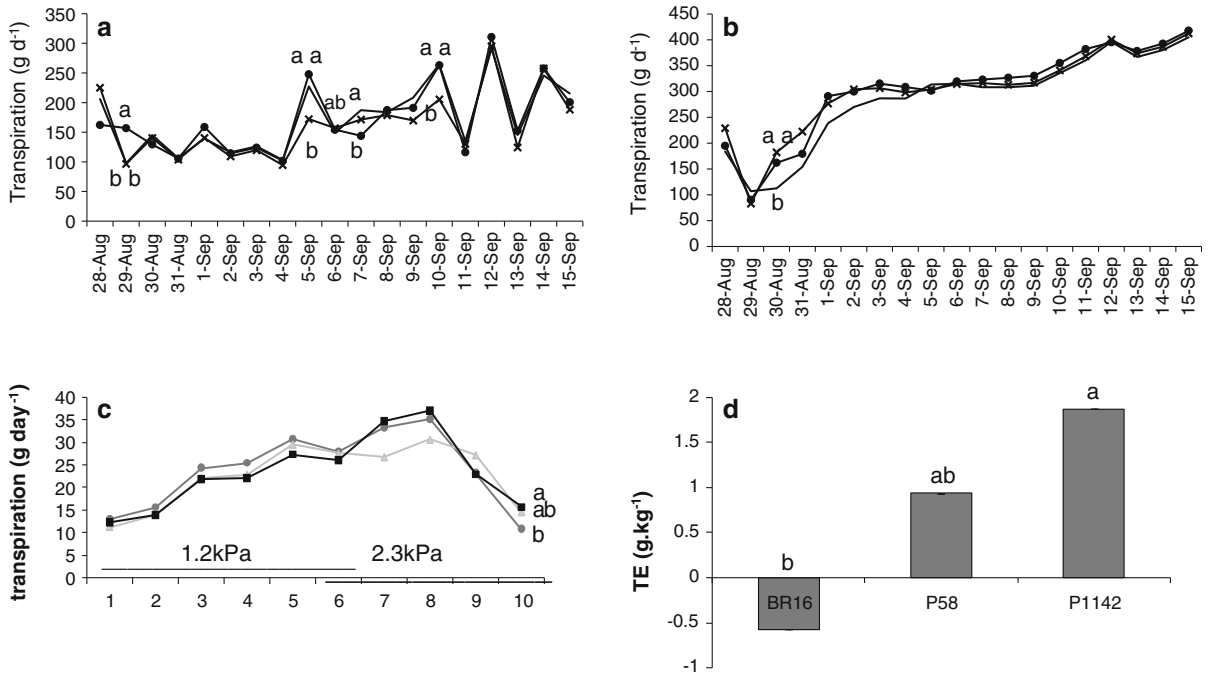

Fig. 3 Transpiration of *AtDREB1A* plants and the cultivar BR16 under control (**a**) and water stress conditions (**b**) in the greenhouse. P58, x; P1142, continuous line; BR16, circle. Days with different letters showed significant differences between groups at 5 % (Duncan) (n = 6). **c** Transpiration of *AtDREB1A* plants and the cultivar BR16 under control (1–6 days) and water stress (7–10 days) conditions in phytotron. Circle, BR 16; square, P1142; triangle, P58. Different letters represent significant differences at 5 % (Duncan) (n = 6). **d** Transpiration efficiency of *AtDREB1A* plants and the cultivar BR16 under water stress conditions in the phytotron. Different letters indicate significant differences at 5 % (Duncan) (n =10)

that the slow wilting phenotype of the plants of the P58 line was also observed under field conditions.

Figure 4 shows that no significant differences were observed in the measures of growth of the P58 line and the crosses between the P58 line and the BR 16 cultivar (09D-0077) under field conditions.

No significant differences were observed among genotypes in yield components (Fig. 5) except for the number of nodes, which was higher for the P58 line in the NI treatment and for the 09D-0077 cross under the water stress condition in the vegetative stage. However, when the water stress was applied in the vegetative stage, there was a clear tendency toward reduced yield components (number of seeds and total number of pods) of the BR16 plants when compared to the plants of the P58 line and the 09D-0077 cross.

Discussion

The development of drought tolerant plants is of high interest, an increase in the area suffering from drought is expected in the future. One strategy for the development of drought tolerance is to genetically engineer plants with transcription factors (TFs) that regulate the expression of several genes related to abiotic stress. As drought-adaptive mechanisms are normally under multigenic control, this seems to be a wise strategy (Blum 2005; Pinto et al. 2010; Saint Pierre et al. 2012).

Previous reports have shown that the TF DREB1A specifically interacts with dehydration response element (DRE) and induces the expression of several genes related to stress tolerance. In our studies, soybean plants overexpressing the *DREB1A* gene under the control of the *rd29A* promoter were obtained and two lines (P58 and P1142) were tested for drought tolerance under greenhouse and/or field conditions.

We found that overexpression of the *DREB1A* gene in the transformed soybeans was induced not only under water stress conditions but also at a lower level under control conditions (Fig. 1). The *Arabidopsis rd29A* promoter is drought-inducible, but induction of *AtRD29A:DREB1A* in transgenic tobacco under

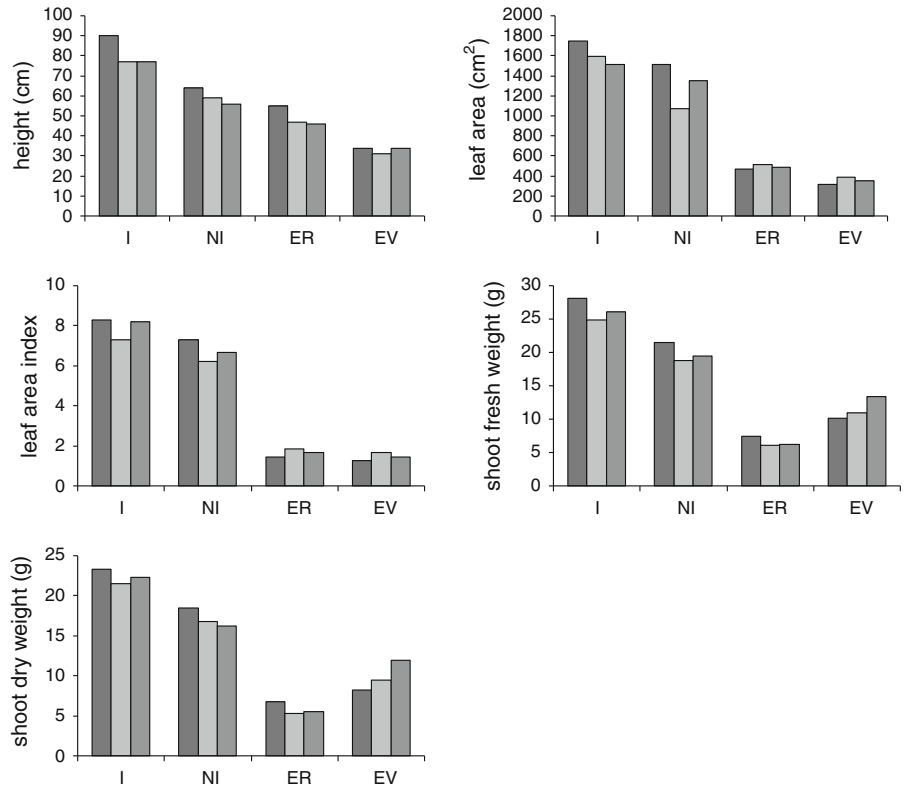

Fig. 4 Growth components of *AtDREB1A* plants and the cultivar BR16. The bars in each treatment represent BR16, P58 and 09D-0077 from left to the right. *I* irrigated, *NI* non irrigated or natural drought, *ER* water stress in the reproductive stage, *EV* water stress in the vegetative stage. Differences were not statistically significant (Duncan 5 %)

normal conditions has already been described (Kasuga et al. 1999). According to Kasuga et al. (1999, 2004), even when there is some activity under control conditions, the *rd29A* promoter is still considered stress induced as it should induce a higher level of expression under stress if compared to the *35S* promoter. The use of stress inducible promoters also overcomes the difficulties encountered when using constitutive promoters such as the *35S* promoter, which is frequently associated with retardation of plant growth (Kasuga et al. 1999; Kasuga et al. 2004; Morran et al. 2011).

Growth analysis of the two DREB1A lines under greenhouse and field conditions revealed that differences in growth were not statistically significant and that they were mainly related to the shortening of internodes in plants of the P58 line, which in turn resulted in shorter plants. For the P1142 line, no differences in growth were observed under either water stress and control conditions (Figs. 2, 4).

We demonstrated here that under well irrigated conditions, DREB soybean plants seemed to exhibit a more conservative growth pattern due to lower RGR when compared to BR16 plants (Fig. 2). Under water stress, however, the growth ratios of the BR16 plants slowed down, resulting in a slightly higher RGR for the transgenic plants. This behavior resulted in a lower percentage reduction in growth (shoot dry mass under stress/shoot dry mass under stress under well watered conditions *100) for the transgenic plants under water stress conditions (Fig. 2). Although not statistically significant, DREB plants seemed to have a higher number of leaves and a greater leaf area than the BR 16 plants, at least in the latter stages of development (Figs. 2, 4).

Previous studies under laboratory and greenhouse conditions (Polizel et al. 2011) showed that the DREB1A P58 line had a slow wilting phenotype and was able to maintain a higher rate of photosynthesis and a higher photosynthetic efficiency under water stress (Polizel et al. 2011).

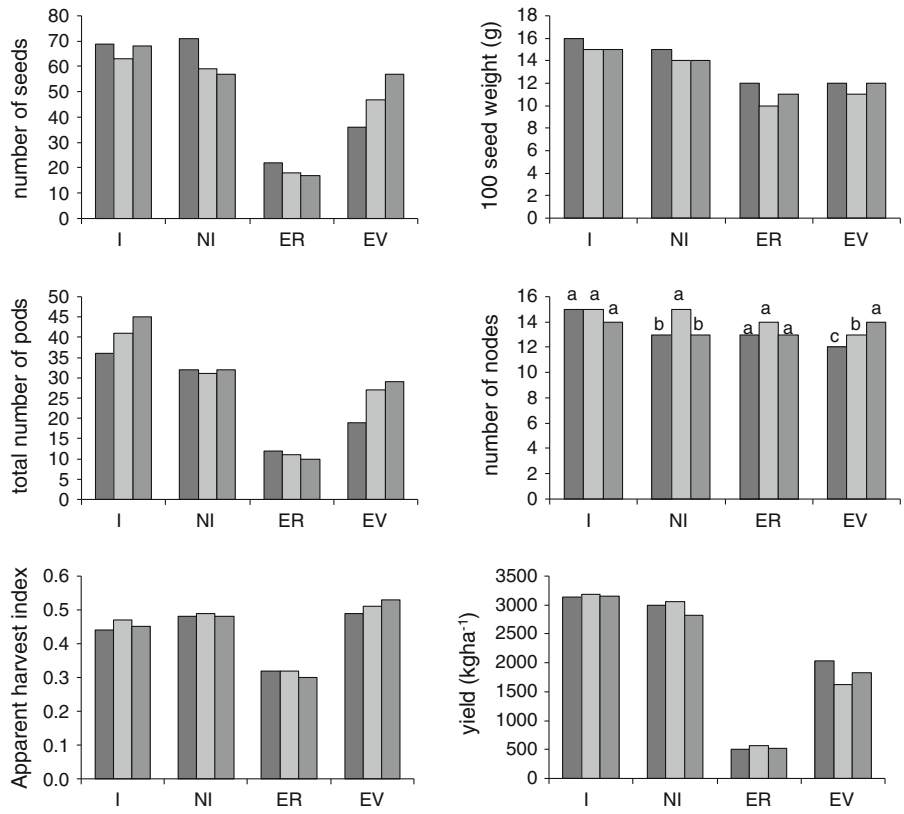

Fig. 5 Yield components of *AtDREB1A* plants and the cultivar BR16. The bars in each treatment represent BR16, P58 and 09D-0077 from left to the right. *I* irrigated, *NI* non irrigated or natural drought, *ER* water stress in the reproductive stage, *EV* water stress in the vegetative stage. Data (bars) without letters are not statistically significantly different at 5 % by the Duncan test

We found that P58 (70 %) and P1142 (60 %) survival rates after severe water stress were higher than the BR 16 survival rate (40 %). Thus, the plants were considered to have improved drought response under greenhouse conditions. The slow wilting phenotype was also observed under field conditions (supplementary data - Fig. S7).

The results from pot experiments for the DREB gene in wheat (Pellegrineschi et al. 2004; Gao et al. 2009) and other crops, such as tobacco (Kasuga et al. 2004), rice (Dubouzet et al. 2003), maize (Qin et al. 2004), groundnut (Bhatnagar-Mathur et al. 2004), and soybean (Li et al. 2005), also showed higher survival and recovery after severe water deficit.

The improved survival of genetically modified plants relative to controls after severe drought has been suggested to be associated with either the activation of genes related to drought resistance or to a reduced consumption of water resulting from smaller plant sizes, i.e., a more conservative growth pattern in the transgenic plants compared with controls (Bhatnagar-Mathur et al. 2004; Morran et al. 2011; Saint Pierre et al. 2012).

Reduction in water consumption under well watered conditions could be one explanation for the higher survival rates and maintained growth capability of the soybean DREB plants. In fact, the P58 plants' transpiration under well watered conditions (Fig. 3a) was lower than that of the BR16 plants despite the absence of differences in leaf area and the number of leaves among these genotypes. No differences in transpiration were observed under water stress (Fig. 3b).

Other authors have implicated a decline in transpiration rate when the soil is relatively humid as a mechanism that results in water conservation, and this mechanism have been verified in maize (Ray and Sinclair 1997), soybean (Vadez and Sinclair 2001 and Hufstetler et al. 2007) and peanuts (Bhatnagar-Mathur et al. 2007).

Using the data of transpiration and the gain of dry mass over the stress period, we calculated the plants' transpiration efficiency (TE, Fig. 3d). TE is related to the water use efficiency through the formula: WUE (biomass) = TE/(1 + E_s/T) proposed by Richards (1991), where E_S is the water lost by evaporation from the soil surface and T is the water lost through transpiration. Because E_S is null in our study as the pots were bagged with plastic bags to prevent evaporation from the soil surface, TE = WUE.

Dehydration-responsive element-binding protein plants had higher TE, at least at the beginning of the water stress period, but not under well watered conditions. These findings differ from that obtained for DREB peanuts, where except for one event, all DREB plants under study achieved higher TE under well irrigated conditions due to lower stomatal conductance. For DREB1A groundnut, improved water use efficiency appeared to be mainly related to a large modification in the root/shoot ratio under water deficit (Vadez et al. 2007). More recently, Vadez et al. (2013) found that under water deficit, some DREB1A transgenic events of *Arachis hypogaea L.* extracted more water from the soil because they rooted into the deep layers of the soil.

In this study, the higher TE of the soybean DREB plants seemed to be related to higher dry mass accumulation over the stress period rather than to decreased transpiration due to changes in the stomatal conductance under water stress. This is supported by the negative values found when the final dry weight of BR 16 plants was subtracted from the initial dry weight during the water stress period. Therefore, it seems that while DREB plants were accumulating organic matter at low rates during the water stress period, BR16 plants were dying or losing weight, most likely through respiratory metabolism.

Polizel et al. (2011) showed that the DREB1A-P58 soybean plants have reduced palisade parenchyma, most likely due to the higher proximity of the cellular layers and a thicker abaxial epidermis. While changes in the anatomy of the mesophyll cells can lead to modifications in transpiration due to evaporation of water from the intercellular spaces and increased mesophyll resistance, it also may represent an adaptation to reduced water availability. Diminished proximity increases the cell surface contact and facilitates the capture of light energy and gaseous elements, which are necessary for the photosynthetic process. On the other hand, a thicker abaxial epidermis may diminish water vapor loss through cuticle transpiration.

Thus far, it has been demonstrated increased drought resistance in DREB transgenic plants under laboratory and greenhouse conditions for several crops (Dubouzet et al. 2003). However, very little is known about the performance of DREB plants under field conditions (Xiao et al. 2009; Yang et al. 2010; Saint Pierre et al. 2012).

For Grain Crops, the success of any selection strategy would ultimately be determined by the reproductive success and thus by the final yield (Saint Pierre et al. 2012). As stated earlier, it is particularly important to test DREB plants in the field, considering that few studies have reported results from genetically modified crops grown in realistic field environmental conditions (Saint Pierre et al. 2012 and Passioura 2012). The field performance of P58 and 09D-0077 plants was evaluated under four different water regimes: irrigated, natural drought and stress simulated by sheltering the plants from rain in the vegetative or reproductive stages (Figs. 4, 5).

The drought treatments affected the plant′s productivity as well as its growth and yield components. Under water deficit in the vegetative or reproductive stages, the main effects of the *DREB1A* gene were changes in the plant height due to a shortening of the internode, at least at the initial stages of crop growth in the field. However, Fig. 4 shows that there were no significant differences in growth components between P58 and 09D-0077 plants under field conditions.

No significant differences were observed in yield components (Fig. 5) among genotypes except for the number of nodes, which was higher for the P58 line in the NI treatment and for the 09D-0077 cross under water stress in the vegetative stage.

Although the DREB plants did not outperform their isoline the cultivar BR16 in terms of yield, there was a clear tendency toward superiority for some yield components such as the number of seeds and the total number of pods when stress was applied in the vegetative stage. Saint Pierre et al. (2012) found that DREB1A wheat plants did not generally outperform the controls in terms of grain yield under water deficit. However, according to his studies, events selected for WUE in the greenhouse were identified as lines that combined an acceptable yield—even higher yield under well irrigated conditions—and stable performance across the different environments generated by

the experimental drought treatment. The positive association between WUE and total biomass suggests that it is possible to increase grain yield by increasing WUE in transgenic plants, assuming that HI (harvest index) is maintained (Wright 1996; Saint Pierre et al. 2012).

In summary, the acquisition a better drought response in the transgenic plants in this study did not occur at the cost of growth and yield. Therefore, further studies are needed to better characterize the conditions (soil and atmosphere) under which these plants may outperform the non-transformed parental line, as plant responses to drought are influenced by the time, intensity, duration and frequency of the stress, and plant developmental stage as well as by diverse plant-soil-atmosphere interactions (Saint Pierre et al. 2012).

The drought response of DREB plants may also involve water conservation mechanisms when water is available to fully supply the plant's demand, and this may be due to DREB1A gene expression under well watered conditions. However, ongoing studies are needed to gain more insight into the mechanisms of stress tolerance under both greenhouse and field conditions and to identify other physiological traits linked to drought tolerance.

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