GENETIC TRANSFORMATION AND HYBRIDIZATION

Stress-inducible expression of *At* DREB1A in transgenic peanut (*Arachis hypogaea* L.) increases transpiration efficiency under water-limiting conditions

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Abstract Water deficit is the major abiotic constraint affecting crop productivity in peanut (*Arachis hypogaea* L.). Water use efficiency under drought conditions is thought to be one of the most promising traits to improve and stabilize crop yields under intermittent water deficit. A transcription factor *DREB1A* from *Arabidopsis thaliana*, driven by the stress inducible promoter from the *rd29A* gene, was introduced in a drought-sensitive peanut cultivar JL 24 through *Agrobacterium tumefaciens*-mediated gene transfer. The stress inducible expression of *DREB1A* in these transgenic plants did not result in growth retardation or visible phenotypic alterations. T3 progeny of fourteen transgenic events were exposed to progressive soil drying in pot culture. The soil moisture threshold where their transpiration rate begins to decline relative to control well-watered (WW) plants and the number of days needed to deplete the soil water was used to rank the genotypes using the average linkage cluster analysis. Five diverse events were selected from the different clusters and further tested. All the selected transgenic events were able to maintain a transpiration rate equivalent to the WW control in soils dry enough to reduce transpiration rate in wild type JL 24. All transgenic events except one achieved higher transpiration efficiency (TE) under WW conditions and this appeared to be explained by a lower stomatal conductance. Under water limiting conditions, one of the selected transgenic events showed 40% higher TE than the untransformed control.

Abbreviations

NTR	Normalized transpiration rate
FTSW	Fraction of transpirable soil water
TE	Transpiration efficiency
HI	Harvest index
DS	Drought stressed
WW	Well-watered
DAS	Days after sowing
SIM	Shoot induction medium
SEM	Shoot elongation medium
RIM	Root induction medium

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Introduction

Water deficit is a major abiotic stress factor limiting crop yields and affecting livelihoods of millions of resource-

poor farmers in arid and semi-arid regions. Yield losses due to drought are highly variable in nature depending on the stress timing, intensity, and duration. This variability coupled with other location-specific environmental stress factors such as high irradiance and temperature makes breeding for drought tolerance difficult through conventional approaches. In peanut, the progress in breeding for drought tolerance has been very slow due to a limited characterization of associated traits and the fact that potential component traits of drought tolerance such as Transpiration (T), Transpiration Efficiency (TE), or Harvest Index (HI) (Passioura 1977) do not have simply additive effects. Molecular markers would probably help the breeding of drought tolerant groundnut but the low level of polymorphism in cultivated groundnut has so far hampered that approach. Hence, the use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance. Indeed, increasing research efforts in engineering for abiotic stress-tolerance in crops are being employed over the last few years (Holmberg and Bulow 1998; Umezawa et al. 2006). Certain genes are expressed at elevated levels when a plant encounters stress (Bray 1993). Specific proteins induced by abiotic stress with known functions include water channel proteins, key enzymes for osmolyte biosynthesis, detoxification enzymes, and transport proteins (Tarczynski et al. 1993; Xu et al. 1996; Kasuga et al. 1999; Sivamani et al. 2000; Vinocur and Altman 2005).

However, tolerance to stress as complex as drought is very unlikely to be under the control of a single gene. A wiser strategy may be to use genetic engineering to switch on a transcription factor regulating the expression of several genes related to abiotic stress (Bartels and Sunkar 2005; Chinnusamy et al. 2005). Transcription of stress inducible genes has been shown to involve various ABA-dependent as well as ABA-independent regulatory mechanisms (Iuchi et al. 2001). A *cis*-acting dehydration responsive element (DRE), identified in *Arabidopsis thaliana*, is involved in ABA-independent gene expression under drought, low temperature, and high salt stress conditions in many dehydration responsive genes (Nordin et al. 1991; Yamaguchi-Shinozaki and Shinozaki 1993; Iwasaki et al. 1997). DRE-binding genes *DREB1* and *DREB2* are transcription factors that bind to the promoter region of dehydration-responsive genes such as *rd29A*, thereby inducing their expression in response to different abiotic stresses (Stockinger et al. 1997; Shen et al. 1997). *DREB1* and *DREB2* are members of the AP2/EREBP plant-specific family of transcription factors (Yamaguchi-Shinozaki and Shinozaki 1993; Shinozaki and Yamaguchi-Shinozaki

2000) where the cDNAs encoding the DRE-binding proteins, *DREB1A* and *DREB2A*, have been isolated from *A. thaliana*, and proteins shown to specifically bind and activate the transcription of genes containing DRE sequences (Liu et al. 1998). The overexpression of *DREB1A* has been shown to delay plant death following withdrawal of irrigation in transgenic wheat (Pellegrineschi et al. 2004), while an improved tolerance to salinity in potato (Behnam et al. 2006) and low-temperature has been reported in tobacco (Kasuga et al. 2004). However, such procedures to impose stress for the phenotypic evaluation of transgenic plants for their response to drought and other stresses have been questioned (Sinclair et al. 2004). The protocols used for the evaluation of transgenic plants for abiotic stresses often involved the use of young plants grown in small pots, disregarding water content in pots, usually maintained under inappropriate light and growth conditions (Tarczynski et al. 1993; Pilon-Smits et al. 1996; Xu et al. 1996; Pellegrineschi et al. 2004). Here, a more realistic physiological response to progressive soil drying has been proposed to include a proper control of soil moisture depletion so as to ensure that plants are exposed to stress levels and kinetics of water-deficits approaching those occurring under field conditions. This approach is very novel in the field of transgenic plant evaluation for tolerance to water deficit. Also, our focus is to quantify traits (here, TE) that are components of the overall tolerance to drought and explore a possible effect of the transgene on that trait.

Peanut or groundnut *(Arachis hypogaea* L.) is one of the important food legume and oilseed crops of the semi-arid tropics of the world where the major constraint for crop productivity in the rain-fed areas is often the erratic and unpredictable rainfall patterns. Annual estimated losses in peanut production due to drought stress have been estimated at over US $520 million (Sharma and Lavanya 2002). In our efforts to improve drought tolerance in this important crop, we have developed transgenic peanut plants expressing *DREB1A* driven by the *rd29A* promoter from *A. thaliana*.

The first objective of this work was to test if the genetic engineering of peanut for abiotic stress tolerance can be achieved by stress inducible expression of transcriptional factor *DREB1A* without any detrimental effects on plant growth and development. The second objective was to investigate and document the genetics of transgenic events of peanuts under abiotic stress. Finally, the third objective was to carry out a phenotypic evaluation of *DREB1A*-containing peanut transformants for TE, an important component trait of drought tolerance, under containment greenhouse conditions.

Materials and methods

Plasmid constructs

The *Agrobacterium* strain C 58 harboring the binary vector pBI29NotAP (Liu et al. 1998) containing the rd29A : DREB1A or a modified EI2 Ω-35S : DREB1A (Mitsuhara et al. 1996) gene construct was used for transformation (Fig. 1a, b). The rd29A : DREB1A construct contained a *Bam*HI fragment of *DREB1A* cloned into the *Bam*HI site of pBI29ApNot. The plasmid pBI29ApNot was constructed by ligating the *Hin*dIII fragment of the *A. thaliana rd29A* promoter into the *Hin*dIII fragment of the pBI101 (Clontech, Palo Alto, CA, USA), as described earlier by Kasuga et al. (1999). The selectable marker *nptII* was driven by a 35S CaMV promoter. The plasmid 35S : DREB1A was as previously reported Liu et al. (1998).

Plant transformation and selection of transgenic events

Mature seeds of peanut (*Arachis hypoagaea* L. var. JL 24) were surface sterilized with 0.1% (w/v) mercuric chloride followed by soaking for 3–4 h in sterile distilled water. The seed coat was then removed and cotyledons were separated with a pointed forceps. The embryonic axis was removed surgically and each cotyledon was cut into vertical halves to obtain the cotyledon explants used for regeneration and transformation system, as described previously (Sharma and Anjaiah 2000). The cotyledon explants were co-cultivated for 72 h with *A. tumefaciens* strain C 58 containing the plasmids 35S : DREB1A or rd29A : DREB1A, and then transferred to the shoot induction medium (SIM) supplemented with cefotaxime (250 μg ml^{-1}) for 2–3 weeks.
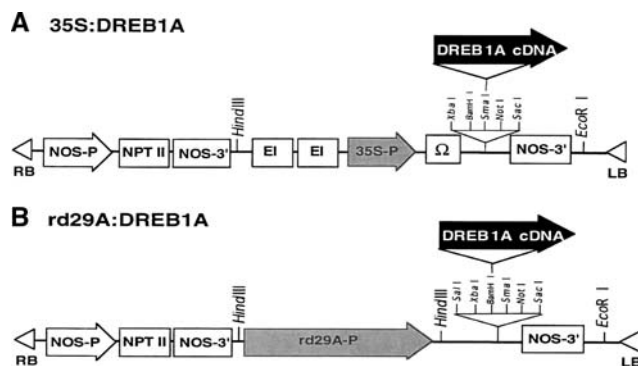
Fig. 1 a and **b** T-DNA regions of the binary plasmids used for *Agrobacterium tumefaciens*-mediated transformation. **a** Construct containing *npt*II and *DREB1A* genes under the control of a modified EI2 Ω-35S promoter with two repeats of the 5′ enhancer sequence (*E*) of CaMV 35S promoter and an Ω sequence from the tobacco mosaic virus (*TMV*). **b** rd29A : DREB1A binary plasmid containing *npt*II and rd29A driven *DREB1A* gene (*LB* left border, *RB* right border)

Following the induction of multiple shoot buds in at least 70% of the explants, they were transferred to SIM containing 250 μg ml^{-1} cefotaxime and 50 μg ml^{-1} kanamycin for selection of the putative transformants. After the shoots attained a size of 2–3 cm, they were subcultured twice on the shoot elongation medium (SEM) containing 100 μg ml^{-1} kanamycin. Elongated shoots (4–5 cm) were then transferred to the root induction medium (RIM) without any selection pressure followed by the transfer of the rooted plants to pots containing a mixture of sand and soil (1:1). Pots containing the transplanted plants were covered with a clear polythene bag and incubated in a growth chamber at 25–28°C under continuous lighting provided by white cool fluorescent lamps of 60 μE m^{-2} s^{-1} light intensity for 1 week before being transferred to a greenhouse for further plant growth to maturity.

Molecular analysis of transgenic plants

Genomic DNA was isolated from young leaves of the putative transformants as described earlier (Sharma et al. 2000). Initial screening of the transformants was done by PCR for presence of the *npt*II and *DREB1A* genes. The PCR reaction was performed with 50 μl of a total reaction mixture containing 150 ng of genomic DNA, 5 μl of 10× PCR buffer (MgCl$_2$), 1.5 μl of 50 μM MgCl$_2$, 1 μl of 10 mM dNTP mix, 1 μl of 10 μM gene specific Primer I, 1 μl of 10 μM gene specific Primer II, and 0.25 μl of 1.25 U of *Taq* DNA *polymerase*. The total volume was made up to 50 μl with sterile distilled water. The control devoid of the template DNA was used in each set of reactions with each primer. The 700 bp region of *npt*II gene was amplified using 22-mer oligonucleotide primers (Hamill et al. 1991). Presence of the introduced *DREB1A* gene was detected using 24-mer primers for the rd29A : DREB1A junction region (forward *rd29A* primer: 5′-GGC CAA TAG ACA TGG ACC GAC TAC-3′and reverse primer: *DREB1A*: 5′-GTT GAT TCC GGG ATT CGG AGT CTC-3′) designed to obtain a 760 bp amplicon with a PCR profile of 30 cycles (94°C for 45 s, 64°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 5 min. Fidelity of the amplicons were verified by resolving the fragments on to a 1.2% agarose gel followed by transfer to Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by Southern blotting (Sambrook et al. 1989) and probing with either the *pst*I fragment of the *npt*II gene or the 648 bp *Bam*HI fragment of the *DREB1A* region using NEB Blot® Phototope® and Phototope-star Detection Kit (New England Biolabs, Beverly, MA, USA).

For Southern hybridization analysis, genomic DNA (25–30 μg) from each of the putative transformants was separately digested with *Eco*RI to ascertain the integration pattern based on the number of copies of the inserted DNA.

The blot was probed with a Biotin-labeled 648 bp *DREB1A* fragment [NEB Blot Phototope and Phototope-star Detection Kit (New England Biolabs)].

RT-PCR analysis was carried out using the Thermoscript® RT-PCR system (Invitrogen, Carlsbad, CA, USA) on total leaf RNA isolated using the TRIzol® reagent (Invitrogen) from plants at various levels of soil moisture following the withdrawal of irrigation. Primer sequences for the *npt*II transcripts were the same as described in PCR analysis and forward and reverse primers used for the *DREB1A* transcripts were 5-CGAGTCTTCGGTTTCCT CAG-3 and 5-CAAACTCGGCATCTCAAACA-3, respectively (Pellegrineschi et al. 2004). Duplex PCR was performed after initial denaturation at 94°C for 5 min, followed by 34 amplification cycles (94°C for 45 s, 60°C for 1 min, and 72°C for 1 min) with a final extension cycle at 72°C for 5 min.

Screening of the transformants under water deficit conditions

Fourteen T2 transgenic events along with the WT JL 24 were evaluated for physiological responses to drought by assessing their transpiration response to water deficit using a dry down technique (Sinclair and Ludlow 1986) as briefly described below. A dendrogram was generated (Fig. 6) based on the threshold value of the Fraction of Transpirable Soil Water (FTSW, i.e., an index of soil moisture available to plant transpiration), when plant transpiration began to decline (see below for the calculation of FTSW) and the number of days to reach FTSW = 0, i.e., a stage equivalent to the permanent wilting point. The purpose of that preliminary assessment was to select a few transgenic lines for further assessment of TE. Five events were selected from different clusters of the dendrogram because there is, a priori, no reason to speculate that a particular position in the dendrogram might be related to higher-lower TE. Therefore, our objective was to select transgenic lines ranging from very different to very similar to JL 24. This approach is very novel in transgenic research, where transgenic lines showing extreme phenotypes compared to the wild types are usually the only ones considered for further study.

Soil dry down experiments

Two experiments were conducted in a P2-level containment greenhouse during June–July and October–November 2004. In both the experiments, five of the 14 T3 transgenic events along with WT JL 24 were used to evaluate their specific responses under dry down conditions. The seeds were inoculated with rhizobium strain NC 92 (IC 7001) and sown in 8 in. pots containing 5.0 kg alfisol : sand : compost mixture (3:2:1; 20% water holding capacity) along with SSP (Single super phosphate; 300 mg kg^{-1}).

Sixteen and eighteen plants per genotype (all positive in the case of transgenics) used in experiments 1 and 2, respectively, were grown in the greenhouse with 28/20°C day/night temperatures under WW conditions until 28 days after sowing (DAS). The pots were divided into three subsets; set one with four and six replicated plants in experiments 1 and 2, respectively, was harvested at 28 DAS while the other two were either used as WW (six replicated plants) or drought stressed (DS; six replicated plants) treatments.

Prior to the stress initiation at 28 DAS, the pots of both WW and DS treatments were saturated with water and left overnight to drain the excess water. On the following morning, the pots were enclosed in polythene bags so as to prevent any water loss by evaporation from the soil surface (Fig. 7a). Thereafter, the pots were weighed every morning between 0900 and 1000 hours Indian Standard Time (IST). The WW control plants were maintained at about 80% field capacity (200 g below the initial pot weight) by daily compensating the water loss by transpiration. In order to expose the DS plants to a progressive water deficit, these plants were allowed to lose a maximum of 70 g of water per day by compensating for transpiration losses exceeding 70 g per day per pot. The transpiration of each plant was then calculated as the difference in pot weights on successive days plus water added on the previous day.

Two normalizations of transpiration data (normalized transpiration rates, NTR) were carried out to ease the comparison of transpiration between DS and WW plants and to minimize the effects of plant-to-plant variation (Ray and Sinclair 1997). First, the daily transpiration data of each individual DS plant was divided by the average daily transpiration of WW plants for each genotype. This allowed comparison of the transpiration of DS plants relative to WW plants for each genotype. The second normalization consisted in dividing each first-normalization data (for each day) by the average of these first-normalization data over the first 3 days (when DS plants were still under WW conditions). The second normalization was done to take care of the plant-to-plant variation in the first-normalization data, in order to ease the comparison of the profile of transpiration response to water stress.

Drought stressed plants were considered to have extracted all the available water for transpiration from the pot when their NTR was <10% of the NTR of WW control plants. The difference between the initial and final pot weight provided an estimate of the total transpirable soil water (TTSW) available in each pot. This information was

used to compute the daily FTSW remaining in the pot (FTSW) as follows:

$$FTSW = \frac{1 - Initial\ pot\ weight - Daily\ pot\ weight}{Initial\ pot\ weight - Final\ pot\ weight}.$$

Individual plants in the dry down treatment were harvested as soon as their normalized transpiration values dropped below 10% of that in control. Plants of an individual genotype under WW conditions were harvested when all DS plants of that genotype had reached NTR <0.1. The daily NTR was then plotted against daily FTSW and by using NTR as a function of FTSW. A plateau regression procedure using SAS (SAS Institute 1996) was used to calculate the FTSW threshold value where NTR begins to decline (Ray and Sinclair 1997). Basically, the plateau regression procedure carries out iterations of the NTR data, starting at FTSW = 1 (wet soil) and fits them to a $y = 1$ equation. From the FTSW level onwards where $y = 1$ is no longer the best fit for NTR, data are fitted to a linear decline equation. The FTSW threshold (with confidence interval) where NTR begins to decline is then taken as the intersection between the plateau ($y = 1$) and linear decline equations.

Harvest and transpiration efficiency measurements

The plants were carefully separated into shoot and root components. The leaf area of the plants was measured by using the Licor® leaf area meter. The dry weight of the leaves, shoot, roots, nodules, and pods was determined by drying in a forced air oven at 80°C for 3–4 days. While the subset of plants harvested at 28 DAS before the imposition of treatments was used to assess the pre-treatment biomass, the post-treatment biomass was that of either DS or WW plants. The TE in DS and WW plants was estimated as the total biomass produced during the experimental period (from pot saturation to harvest) divided by the cumulative water transpired during the same period. The second experiment was an exact repeat of the first experiment where the stomatal conductance of the WW plants was also measured. Data were recorded using a LCA 4-leaf gas analyzer (ADC Bio-Scientific Ltd., Herts, UK) on the fully expanded leaf between 1000 and 1200 hours, under saturating light conditions (PAR > 1,200 μmol m^{-2} s^{-1}).

Statistics

The data were subjected to average linkage cluster analysis and a dendrogram was generated using the Euclidian distance of NTSYS pc (Version 2.10 d) during the preliminary experiment. The physiological data were analyzed using Tukey's test for the comparison of different transgenic events with WT JL 24. A plateau regression procedure using SAS was used to compute the threshold value where NTR begins to decline as explained above.

Results

Transformation

Co-cultivation of the cotyledon explants with the *Agrobacterium tumefaciens* strain C 58 carrying the plasmids of interest for 48–72 h resulted in over 70% transformation frequency. All responding explants produced multiple adventitious shoot buds. The shoot buds differentiated into shoots within 2–3 weeks of culture on SIM. Healthy green shoots were then selected and further transferred for elongation on SEM. After three to four subcultures on SEM, over 85% of the shoots attained a size of 3–4 cm. The shoots exhibiting bleaching on the selective medium containing 125 mg l^{-1} kanamycin sulphate were discarded at each stage and only the green healthy shoots were maintained until rooting. Roots appeared within 2 weeks on RIM and were allowed to develop for another 4 weeks. Over 90% of the rooted shoots transformed with the rd29A : DREB1A construct survived and appeared to be phenotypically normal (Fig. 2a), while the shoots containing 35S : DREB1A showed stunted growth and high rates of mortality (Fig. 2b). The rooted shoots were transplanted to pots containing a mixture of sand and soil (1:1) and were maintained under high humidity conditions before being transferred to the P2 containment greenhouse until harvest. Fifty-three independently transformed plants with rd29A : DREB1A and 18 plants with 35S : DREB1A were successfully transplanted to the greenhouse and their T1 seed collected. Thirty-four transgenic events of rd29A : DREB1A and all eighteen events of 35S : DREB1A were advanced to T2. Under greenhouse conditions, over 40% of the 35S : DREB1A events showed delayed germination and severe growth retardation within 7 days of emergence; where 70% of these resumed suboptimal growth thereafter (Fig. 2c). However, the seed from rd29A : DREB1A plants showed normal germination and growth behavior (Fig. 2d).

Integration, inheritance, and expression of the transgenes

In PCR analysis, the oligonucleotide primers specific to the coding region of the *npt*II and *DREB1A* genes amplified the expected sizes of gene fragments from 75% of the analyzed putative tranformants (Fig. 3a, b). Southern blot analysis of T1 individuals indicated that the

Fig. 2 a–d Phenotypes of the plants transformed with 35S : DREB1A and rd29A : DREB1A constructs growing under controlled in vitro and greenhouse conditions. Growth differences in 45-day-old plants grown in test tube conditions. **a** A rd29A : DREB1A transgenic plant growing in vitro showing root initiation. **b** A 35S : DREB1A transgenic plant growing in vitro on rooting medium showing lack of root induction. **c** and **d** Transgenic plants containing 35S : DREB show highly stunted growth while those containing rd29A : DREB1A show no negative effects on growth and development 4 weeks after germination of T1 seed
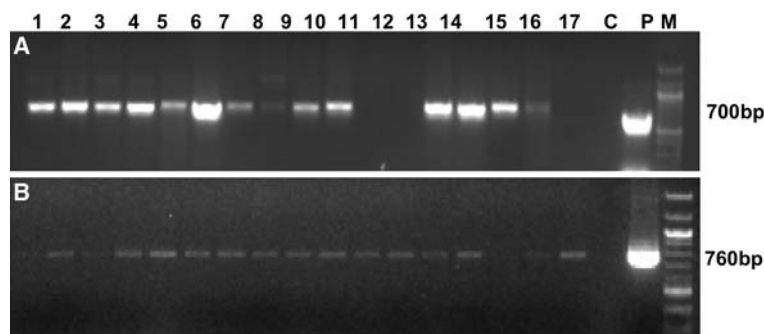
Fig. 3 a and b PCR amplification of the *npt*II and *DREB1A* genes in T_1 progenies of independent transgenic events. **a** Lanes 1–16 carry samples from putative transformants, lanes 17 and 18 are untransformed controls, lane 19 is the DNA from plasmid rd29A : DREB1A, and lane 20 is the 100 bp marker ladder. **b** PCR amplification of genomic DNA from 16 T_1 plants showing amplification of the 769 bp fragment of rd29A : DREB1A junction region, lanes 1–17 carry samples from putative transformants, lane 18 is the untransformed control, lane 19 has the plasmid DNA, and lane 20 is the 100 bp marker ladder

number of copies of the *DREB1A* transgene in the tested plants varied from one to four. The transgenic events having one to two copies of the transgene were selected for subsequent gene expression and phenotyping studies (Fig. 4). Segregation analysis of the T1 and T2 progeny for the *npt*II gene indicated segregation in a 3:1 Mendelian ratio (data not shown). RT-PCR studies of cDNA from the putative transgenic plants (35S : DREB1A and rd29A : DREB1A) showed positive amplification of the 700 bp *npt*II gene fragment in the selected transgenic events under unstressed as well as stressed conditions. The transcripts of *rd29A*-driven *DREB1A* were detected only in the plants subjected to 5 days of water deficit where the FTSW was about 65% following the dry down procedure (Fig. 5); similar pattern was found in both experiments. However, we have no data to confirm whether transcripts might have been detected earlier and this will be the objective for future studies.

Preliminary evaluation of the transgenics

Initial assessment of 14 transgenics showed they differed in their transpiration responses to soil drying, with all transgenic events taking more days to deplete the soil water (Bhatnagar et al. 2004). A dendrogram (Fig. 6) based on similarity in FTSW threshold values and the number of days to deplete the soil water under water deficit conditions revealed that these transgenic events could be broadly classified into four groups (at a similarity index value of 0.6). From the 14 tested transgenic events, five events from across different clusters of the dendrogram, and having transpiration responses ranging from very similar to very
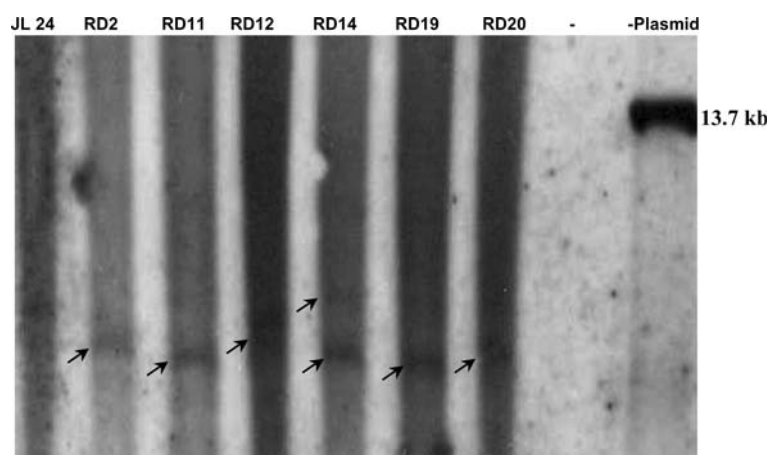
Fig. 4 Southern blot analysis of the *DREB1A* gene in the genomic DNA of T2 transgenic plants transformed with the rd29A : DREB1A construct. The DNA was restricted using *Eco*RI to provide a single cut within the T-DNA region. The blots were probed with Biotin labeled *DREB1A* fragment (Lanes 2–7 carry genomic DNA from events RD2, RD11, RD12, RD14, RD19, and RD20; lanes 1 and 9 carry DNA from the untransformed controls and a plasmid carrying the rd29A : DREB1A construct, respectively)
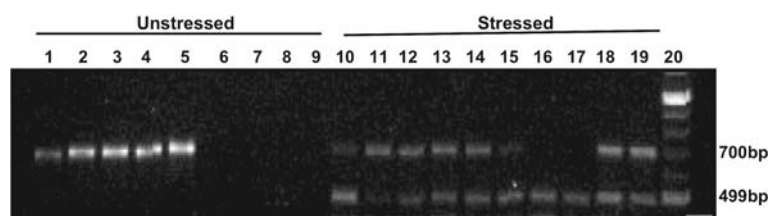
Fig. 5 RT-PCR analysis of *npt*II (700 bp) and *DREB1A* (499 bp) gene expression in control and transgenic plants (rd29A : DREB1A) before and after water stress. Lanes 1–7 carry RNA isolated from plants grown under non-stress conditions with non-transgenic control in lanes 8 and 9; lanes 10–19 have RNA isolated from plants after 5 days of water stress; lane 20 has a 100 bp marker ladder

different from the WT JL 24, were selected for subsequent experiments.

Plant growth and transpiration under well-watered conditions

Overall, the initial shoot weights of the transgenic events were more or less similar to WT JL 24 across the experiments. In contrast, WT JL 24 had somewhat lower final shoot weight under WW conditions than the transgenic events RD19, RD12, and RD20 (Table 1). Similarly, the initial and final root weights of all were similar to that of WT JL 24 across both experiments (Table 1). Moreover, the initial leaf area of event RD20 was the highest across the two experiments. Similarly, the final leaf areas of events RD11, RD12, RD19, and RD20 were higher than that of JL 24 under WW conditions (Table 1).

The cumulated transpiration of WW plants was compared in the initial 12 days after initiating the stress treatment. Overall, across both WW experiments, the cumulative transpiration of event RD20 and WT JL 24 were similar and were significantly higher than in event RD11 (Table 2). The remaining transgenic events showed intermediate WW transpiration values. The stomatal conductance of the events under WW conditions was measured in the second experiment. Transgenic events exhibited significantly lower stomatal conductance, with RD2 and RD11 having less than half of stomatal conductance than that of WT JL 24. Moreover, the TE of WW plants of all the transgenic events except RD20 was higher than that of WT JL 24 across both experiments (Table 2).

Plant response to water stress

The biomass of plants exposed to progressive drying was more than two-thirds of that in WW conditions. Overall, amongst all the transgenic events, RD11 had consistently the lowest shoot biomass under drought stress (Table 1). Across both experiments, the root dry weight of all transgenic events was lower than that in WT JL 24, except for event RD20. The leaf area under drought stress was lower in RD2 and RD11 than in RD12, RD20, and WT JL 24 across both the experiments (Table 1).
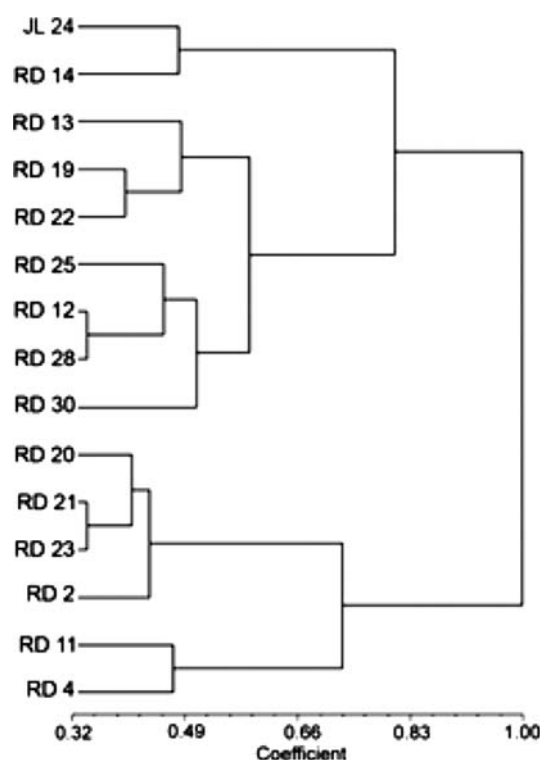
Fig. 6 Dendrogram showing relative similarities among 14 independent peanut transgenic events and wild type JL 24 based on FTSW threshold values and the number of days to end point (NTR = 0.1) under water deficit conditions

NTR–FTSW relationship

The transgenic events differed in the response of NTR to FTSW and were clearly distinguishable with respect to this from WT JL 24. Indeed, the transpiration of all the transgenic events started declining at lower FTSW values (drier soil) under drought stress than WT JL 24 across

both experiments (Table 3), even though the experiments were done at different times of the year. A "slow wilting" phenotype was observed toward the end of the dry down water stress treatment (Fig. 7b). Figure 8 shows a typical response of a transgenic event (RD2) compared to WT JL 24, thereby, indicating that the onset of transpiration decline occurred in drier soil in the transgenic events (about 0.28–0.31 FTSW) when compared to the WT JL 24 (about 0.55–0.65). This pattern was essentially the same for all events tested (data not shown), as indicated by their lower FTSW threshold values (Table 3).

Water extraction and cumulative transpiration under water deficit

At the end of the drought stress period, the total amount of water extracted from the soil by transpiration (TTSW) was measured by subtracting the final pot weight from the initial pot weight. Overall, none of the genotypes tested showed any difference in TTSW across the two experiments (Table 3). Similarly, the cumulative transpiration was computed as the sum of daily transpiration from initiation until the plant depleted all the soil water (FTSW = 0.1). Based on this, the transgenic event RD11 had a lower cumulative transpiration under drought stress than did WT JL 24 across the two experiments (Table 3). Overall, the TE of RD2 was consistently higher than that of WT JL 24 and all the other transgenic events across both experiments (Table 3).

Discussion

Agrobacterium-mediated transformation using the de-embryonated cotyledon explants of peanut variety JL 24

Table 1 Average shoot weight, root weight, and leaf area of five different transgenic lines and their wild type parent in two different greenhouse experiments

Events	Shoot weight (g plant^{-1})			Root weight (g plant^{-1})			Leaf area (cm^2 plant^{-1})		
	Initial	Final WW	Final DS	Initial	Final WW	Final DS	Initial	Final WW	Final DS
WT JL 24	5.35[ab]	12.32[b]	10.33[ab]	0.794[a]	1.628[ab]	2.031[a]	459.5[b]	1,345[b]	1,026[a]
RD19	4.03[b]	15.37[a]	10.45[a]	0.649[ab]	1.695[ab]	1.517[b]	484.3[b]	1,645[a]	975[ab]
RD12	4.69[ab]	15.29[a]	10.53[a]	0.791[a]	1.565[b]	1.432[b]	542.8[ab]	1,648[a]	1,012[a]
RD20	5.20[ab]	15.23[a]	11.20[a]	0.949[a]	1.945[a]	1.864[a]	633.6[a]	1,616[a]	1,084[a]
RD2	3.55[b]	14.77[ab]	11.23[a]	0.505[abc]	1.546[b]	1.489[b]	390.4[b]	1,387[b]	891[bc]
RD11	3.33[b]	13.16[b]	9.21[c]	0.602[ab]	1.534[b]	1.371[b]	412.2[b]	1,404[a]	895[b]
Grand mean	4.36	14.36	10.49	0.715	1.652	1.617	487	1,507	980
LSD	0.917	1.927	0.822	0.212	0.380	0.186	143.1	250	81.7

Data are those of the pre-treatment harvest at 28 DAS (initial) and those of the post-treatment harvest (final) of well watered (WW) and drought stressed (DS) plants. Data are the pooled mean across the two experiments that were carried out. Values followed by same letter are not significantly different at 5% level

Table 2 Pooled analysis for stomatal conductance (Gs), cumulative transpiration, and transpiration efficiency (TE) of five transgenic lines of peanut and their WT parent under well-watered conditions

Events	Cumulative transpiration (g plant^{-1})	Stomatal conductance	Transpiration efficiency (g biomass kg^{-1} water transpired)
JL 24 (untransformed)	2,952[a]	387[a]	3.05[b]
RD19	2,526[ab]	257[b]	4.76[a]
RD12	2,451[ab]	200[c]	5.23[a]
RD20	3,024[a]	289[b]	3.77[b]
RD2	2,751[ab]	156[c]	4.69[a]
RD11	2,049[b]	176[c]	4.89[a]
Grand mean	2,789	307	4.40
LSD	813.4	84.4	0.807

Cumulative transpiration data are computed over the 12-day period after imposing the water stress. Data are the pooled mean across the two experiments that were carried out, except for Gs that were measured only in experiment 2. Values representing mean ($n = 6$) followed by same letter are not significantly different at 5% confidence level

was carried out successfully following the transformation protocol reported earlier (Sharma and Anjaiah 2000). In the present study, the *DREB1A* gene was driven either by the CaMV 35S promoter for constitutive expression or by the promoter from *A. thaliana rd29A* gene for stress responsive expression. The progeny of putative transgenic plants generally followed Mendelian segregation ratios (3:1) for the transgenes in the T1 as well as T2 (data not shown), thus confirming the success of the transformation procedure. RT-PCR carried out for expression analysis of *DREB1A* from the transgenic plants revealed that *DREB1A* driven by the *rd29A* promoter was induced only by the fifth day after withdrawal of irrigation, and this corresponded to about 0.65 FTSW. This suggested that the *A. thaliana rd29A* gene promoter is an effective drought stress-inducible promoter in peanut. Earlier work on histochemical expression of *uid*A under the control of *rd29A* promoter also showed its induction by desiccation in almost all the organs and tissues of transgenic *Arabidopsis* rosettes (Shinwari et al. 1998).

Morphological observations showed that 35S : DREB1A plants exhibited stunted growth even under control conditions, while the peanut transgenics containing rd29A : DREB1A did not show any growth retardation either in vitro or in the greenhouse. Similar observations have been made earlier in *A. thaliana* where the constitutive expression of *DREB1A* in *Arabidopsis* transgenics resulted in improved stress tolerance at the expense of growth and productivity (Kasuga et al. 1999). However, in the present study, no differences in the growth pattern were

Fig. 7 a and **b** Progressive soil drying in pot experiments conducted in containment greenhouse conditions. **a** A typical randomized drying down set up in the greenhouse. **b** Phenotype of the wild type parent JL 24 (*left*) and a *DREB1A* containing event RD2 (*right*) after 12 days of progressive soil drying

observed in rd29A : DREB1A plants under non-stress conditions, which supports earlier observations indicating that the *rd29A* gene promoter is stress inducible (Shinwari et al. 1998). In contrast, a recent report on transgenic potato expressing *DREB1A* gene under the influence of the *rd29A* promoter showed growth retardation (Behnam et al. 2006). It was interesting to notice that amongst the five rd29A : DREB1A events selected for detailed observations based on the similarity index values, RD11, located in extreme position in the dendrogram showed lower growth even under WW conditions. This could possibly be due to gene expression modulation by the position of gene integration. Similar effects have been noted in other transformation systems (Jackson et al. 2004). However, this does indicate that our choice in the selection of a range of transgenic events from the dendrogram for detailed physiological evaluations was judicious when compared to most other
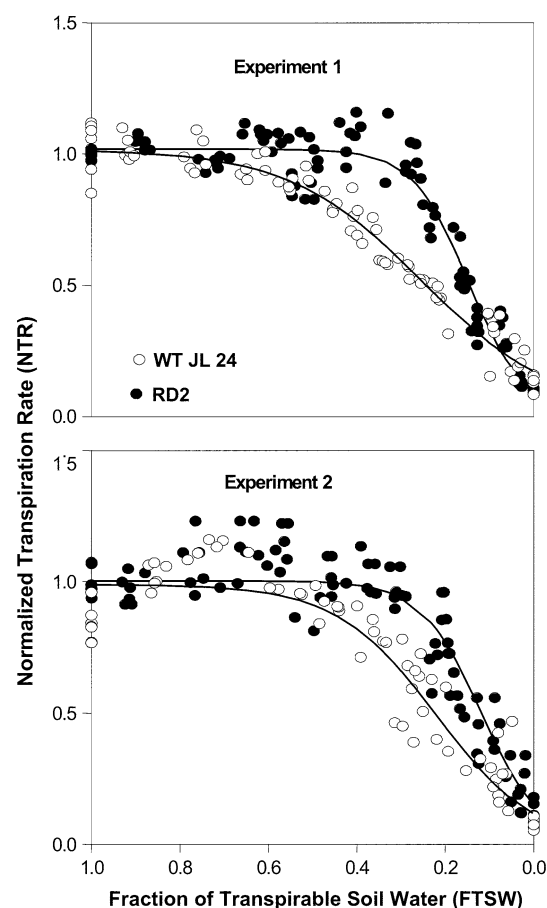
Table 3 Pooled analysis of average total transpirable soil water (TTSW), cumulative transpiration (T), transpiration efficiency (TE), and fraction of transpirable soil water (FTSW) at which the stomatal closure began to occur throughout the drying cycles of two greenhouse experiments for five transgenic lines of peanut and their wild type parent

Events	TTSW (g plant^{-1})	Cumulative transpiration (g plant^{-1})	TE (g biomass kg^{-1} water transpired)	FTSW-threshold
JL 24 (untransformed)	863[ab]	1,578[a]	4.25[bc]	0.667[a]
RD19	873[a]	1,490[ab]	5.11[b]	0.361[bc]
RD12	853[ab]	1,485[ab]	4.44[bc]	0.428[b]
RD20	909[a]	1,624[a]	4.77[b]	0.435[b]
RD2	887[a]	1,505[ab]	5.96[a]	0.300[c]
RD11	863[ab]	1,349[c]	5.09[b]	0.367[bc]
Grand mean	875	1,505	4.89	0.427
LSD	49.35	107.7	0.525	0.060

Data are the pooled mean across the two experiments that were carried out. Values representing mean ($n = 6$) followed by same letters are not significantly different at 5% confidence level

Fig. 8 Typical response of transpiration (NTR) to soil moisture (FTSW) in one of the *DREB1A* transformed events (RD2) and WT JL 24 under water deficit and respective curves fit in two different soil-drying experiments

studies that chose extremes based on phenotype or gene copy number (Behnam et al. 2006).

Although the PCR and RT-PCR analysis revealed that the *DREB1A* was integrated in these plants and was induced under water stress conditions, it is not clear as to why large differences were found in TE and Gs under WW conditions. It appears from previous work that the *DREB1A* transcripts have been detected only for a short period after stress imposition, whereas the transcripts from genes that are up-regulated by *DREB1A* have been detected for several days and even weeks after the imposition of drought stress (Shinozaki and Yamaguchi-Shinozaki 2000). Therefore, it may be possible that DREB1A was expressed even under WW conditions, although we were not able to capture it. In fact, even the expression of DREB1A occurred at FTSW = 0.65 in the DS plants, a soil moisture level where plants do not show any sign of stress, in particular not in its leaf gas exchanges. This is, therefore, a very intriguing and exciting question whether the stress responsive promoter may be able to respond to conditions that are not considered "stressful" in the common sense. This definitely deserves more investigations. In any case, it was intriguing to see that there were no overall differences in leaf area of transgenics under WW condition, except in RD2, whereas several events did show some differences in cumulated transpiration under WW conditions. Therefore, using data from Tables 1 and 2, we calculated the rate of water loss per unit leaf area by dividing cumulated transpiration over the entire period by the leaf area at harvest in WW conditions. Although this overlooks the fact

that leaf area increases over time, it provides an estimate of the rate of water loss per leaf area. We found that all transgenics had a lower rate of water loss per unit leaf area in WW conditions. We regressed this rate of water loss per leaf area against TE values under WW conditions and found a very close negative relation [$R = 0.8360$ (Fig 1: supporting information)]. This indicates that genotypes having lower rates of water loss per unit leaf area achieved higher TE. This result was in full agreement with the stomatal conductance measurements taken in experiment 2, where we showed that all transgenics had, indeed, a lower stomatal conductance than WT JL 24 under WW conditions [negative relation between TE and Gs data in experiment 2 where $R = 0.8694$ (Fig 2: supporting information)]. This is in agreement with the theory on TE (Farquhar et al. 1982). Whether the relation between the rate of water loss and TE under WW conditions also explains the differences in TE under water stress conditions is intriguing. In fact, this was likely not the case because the relationship between TE under WW conditions and TE

under water stress was poor [$R = 0.1337$ (Fig 3: supporting information)]. Further work is then required to elucidate the cause for the differences in Gs under WW conditions, and whether regulation of stomatal movements may be the cause for differences in TE under water stress in these transgenic events.

The most striking result of this study was that most transgenic events had higher TE than the WT under WW conditions, where some events showed up to 70% increase over WT JL 24. Further, transgenic event RD2 showed about 40% higher TE than WT JL 24 under water limiting conditions. These differences can be considered as very large when compared to the range of variation usually found for TE between germplasm accessions of peanut (Sheshshayee et al. 2006) and in many other crops (Krishnamurthy et al. 2007) report only a 20–30% difference in TE across a mapping population developed between high and low TE parents. This study is, as far as we know, one of the first to test the range of phenotypic variation for TE by using transgenic plants that can be considered as "near-isogenics". Such material is likely to be of enormous interest to re-explore the physiological mechanisms involved in the regulation of TE and drought responses in peanut.

In conclusion, we have been successful in developing transgenic events of peanut with the *DREB1A* transcription factor that is specifically expressed under a stress responsive promoter. The events exhibited a diversity of stress response patterns, especially with respect to the NTR-FTSW relationship. Various transgenic events exhibited increased TE, which is an important component of plant performance under limited moisture conditions (Passioura 1977). Our preliminary studies on differential display of transcripts in the selected transgenic events under water limiting conditions have enabled the detection of transcripts that were expressed differentially (results to be published elsewhere). Analysis of the newly expressed genes could indicate that their products might function co-operatively to protect the cells from dehydration and may also play an important role in plant adaptive mechanisms under different stress conditions (Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005). Future activities would involve the identification and functional analysis of these transcripts for use in both genetic engineering as well as marker-assisted selection for plant breeding. Further, the identification of other physiological traits linked to drought tolerance in our on-going studies with these transgenics is expected to provide more insights into the mechanisms of stress tolerance both under greenhouse and field conditions. This approach of engineering for abiotic stress tolerance by using the *DREB1A* gene of *A. thaliana* has the potential to contribute toward the development of more drought stress tolerant genotypes of peanut in the near future.

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