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Transgenic peanut overexpressing the *DREB1A* transcription factor has higher yields under drought stress

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Abstract Transgenic research using the DREB group of transcription factors has received much attention in developing drought-tolerant and climate-ready varieties of crop plants. While many reports have demonstrated increased tolerance to water deficits under laboratory and greenhouse conditions, only a few have tested possible effects under field conditions, with limited success in most cases. Here, we present evidence of a transgenic solution for enhanced drought tolerance in peanut (*Arachis hypogaea* L.), which is an important grain legume and a valuable cash crop for smallholder and resource-poor farmers occupying the largest portion of the farming systems in low income food deficit countries. The presence, integration, expression and inheritance of the transgene in advancing generations of the transgenic peanut plants were assessed using PCR, Southern blot, inverse-PCR, RT-PCR and q-PCR techniques. Four trials were conducted in various water stress regimes under varying vapour pressure deficits (VPDs), and drought tolerance was studied using various component traits of drought. A substantial yield improvement of up to 24 % in drought trials under field conditions was achieved across a wide range of stress intensities and was related to higher harvest indices (HIs). All transgenic events had significantly higher seed filling under drought and displayed 20–30 % lower pod yield reduction than their untransformed counterpart under drought stress. Two transgenic events showed yield advantage under drought stress that consistently had higher pod and seed yield than the untransformed parent under drought stress across all trials, without displaying any yield penalty under irrigated conditions.

Keywords *Arachis hypogaea* · *DREB1A* · Drought · Harvest index · Stress-inducible promoter · Transgenic peanut · Transcription factor · Yield

Introduction

Today, the world needs a second "green revolution" to increase crop yields and feed the projected world population of 9 billion by 2050 under situations of water challenge. Irrigation water has already reached its peak and the share of water for agricultural use decreases as societies develop, resulting in an ever-increasing pressure on producing more from less,

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especially under the ensuing climate change. Drought scenarios are also likely to worsen in the near future with the predicted climate change scenarios (Wassmann et al. 2009). Over the past 2 decades, transgenic research has received much attention and impetus to develop drought-tolerant and climate-ready varieties. While, globally, most transgenic work for drought tolerance involves major cereals crops with large cash markets such as rice, maize, tomato or tobacco, the dryland grain legume crops which are not very "attractive" to the private sector have been neglected, despite being critical to the livelihoods of over 650 million of the poorest and most food-insecure people living in the dryland areas of Africa and South Asia that constitute of the most marginal crop production environments.

Peanut or groundnut (*Arachis hypogaea* L.), an annual legume, is a valuable cash crop for smallholder and resource-poor farmers in the harsh rainfed environments, where its productivity is limited, mainly due to water deficits. Peanut occupies the largest portion of Low Income Food Deficit Countries (FAO 2009) farming systems, grown on 20.6 million ha in these regions, often fitted into underutilized crop niches. Globally, drought contributes to annual losses of over 6.7 million metric tons in the productivity of peanut (Subbarao et al. 1995), where breeding efforts for increased water use have been constrained due to the lack of genotypic variability (Gautami et al. 2011). This has led to the identification of only minor quantitative trait loci for this trait and its components, thus making it critical to attempt transgenic interventions for the drought tolerance trait (Varshney et al. 2009).

The complexity of drought response likely involves many genes that could be successfully regulated through the use of genes encoding transcription factors that control gene expression under abiotic stress conditions (Liu et al. 1998; Kasuga et al. 1999; Bhatnagar-Mathur et al. 2008). Although overexpression of transcription factors has been reported to enhance drought tolerance in several crops (Dubouzet et al. 2003; Pellegrineschi et al. 2004; Oh et al. 2005; Behnam et al. 2006; Xiao et al. 2006; Wang et al. 2008; Oh et al. 2009; Morran et al. 2011), most of these aimed at demonstrating gene expression responses to seemingly high stress levels under laboratory conditions (Yang et al. 2010). While most studies considered short-term stress acclimation/survival as tolerance, rather than the final productivity or yield, only a few have successfully tested their performance and productivity in the field (Xiao et al. 2006; Oh et al. 2009; Qin et al. 2011).

A major emphasis of our efforts to develop a transgenic solution for drought tolerance in peanut using stress-inducible *DREB1A* transcription factor (Bhatnagar-Mathur et al. 2007) was on selecting genotypes that yielded higher under drought stress while maintaining high yield potential under irrigation (Bhatnagar-Mathur et al. 2008). Our approach involved a thorough assessment of different component traits that potentially lead to better coping with drought, using protocols that closely mimic the target stress environments in which peanuts in the drylands of the world are grown, rather than stress extremes for the selection of best-bet transgenic events prior to their field testing (Bhatnagar-Mathur et al. 2004, 2007, 2009; Vadez et al. 2007a, b, 2008; Devi et al. 2011). Out of the 50 independent transgenic events thus screened, three with high transpiration efficiencies (TE; Bhatnagar-Mathur et al. 2004, 2007) and desirable root traits (Vadez et al. 2013) were selected for further evaluation of yield under greenhouse and field drought conditions over a period of 4 years.

Here, we present evidence that the stress-inducible expression of *DREB1A* in the transgenic peanut plants confers enhanced drought tolerance by contributing to higher yield and HI under water deficit without any penalty under normal irrigated conditions.

Materials and methods

Plant material

Homozygous progenies of the previously selected three transgenic events of peanut (*A. hypogaea* L.) including RD2, RD11, and RD33 in their T6 to T9 generations carrying the rd29A:DREB1A gene (Bhatnagar-Mathur et al. 2007) were used for yield evaluations under intermittent drought stress during four consecutive yield trials (2008–2011). These transgenic events were developed by *Agrobacterium*-mediated genetic transformation of a popular but drought-sensitive Spanish type peanut variety JL 24, grown mainly in the semi-arid tropics.

Molecular studies

The presence, integration, expression and inheritance of the transgene in advancing generations of the transgenic peanut plants was assessed using PCR, Southern blot and RT-PCR techniques. Since previous results from Southern blot analysis of T1 individuals have demonstrated a single copy of the *DREB1A* transgene in the genome of these transgenic events (Bhatnagar-Mathur et al. 2007), an integrative Southern blot analysis for transgene inheritance was carried out. Re-confirmation of the number of T-DNA integrations in the genome of the transgenic events was carried out using inverse PCR analysis (Chen et al. 2003). The primers for inverse PCR were designed to amplify the integration site of the right border of T-DNA, with forward primer (IP1: 5′-CGTTGCGGT TCTGTCAGTTCC-3′) designed from the *nos* promoter sequence and reverse primer (IP2: 5′-TTGT CAAGACCGACCTGTCCG-3′) from the *npt*II gene sequence.

The genomic DNA (5 µg) was digested with *Taq*I at 65 °C followed by phenol/chloroform extraction, ethanol precipitated and kept for an overnight ligation at 16 °C, and subsequently re-precipitated with three volumes of 100 % ethanol before dissolving in 100 µL of sterile distilled water. The ligated DNA solution was divided into two equal aliquots of 50 µL each for re-digestion with *Ssp*I or *Sst*II restriction enzyme for 2 h at 37 °C. The re-digested DNA was purified with phenol/chloroform, and the ethanol-precipitated pellet dissolved in 20 µL of sterile distilled water for further use. IPCR was carried out in a 30 µL reaction volume containing 200 ng of template DNA, 10 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 µM of each forward primer and reverse primer, 200 µM of each dNTP, and 1 U of *Taq* DNA polymerase (New England Biolabs). The amplification commenced at 95 °C for 5 min, followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C. The final extension was performed at 72 °C for 10 min. Following amplification, PCR products were analyzed on a 1 % TAE agarose gel.

Duplex RT-PCR and quantitative RT-PCR (qRT-PCR) analyses for transgene expression in the events was performed in a progressive drying-down pot experiment under a contained greenhouse as described earlier (Bhatnagar-Mathur et al. 2007). The leaf samples were collected 0, 1, 2, 3 and 5 days after imposition of drought stress, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions.

Duplex RT-PCR for *npt*II and *DREB1A* were performed as described earlier (Bhatnagar-Mathur et al. 2007). Quantitative cDNA amplification by qRT-PCR was carried out using SensiFAST SYBR No-ROX One-Step kit (Bioline), on Mastercycler ep realplex (Eppendorf). The peanut *actin1* was used as a reference gene for the normalization of *DREB1A* gene expression and the primers used for the *actin1* gene were:

GnAct-FP 5′-ATGCTAGTGGTCGTACAACTG G-3′

GnAct-RP 5′-CTAGACGAAGGATAGCATGTG G-3′

and for the *DREB1A* gene were:

DREB-FP 5′-AATCCCGGAATCAACTTGCGC T-3′

DREB-RP 5′-AAATAGCCTCCACCAACGTCT C-3′.

The reaction consisted of 100 ng of total RNA, SensiFAST SYBR RT mix (Bioline), and 300 nM of each primer using the following reaction conditions: 10 min at 45 °C; 2 min at 95 °C; 45 cycles of cDNA amplification for 10 s at 95 °C, 15 s at 62 °C and 10 s at 72 °C with fluorescent signal recording. At the end, a final step of 15 s at 95 °C and melting curve step was included. The qRT-PCR was performed with three technical replicates of each biological replicate and the mean values for the expression levels of the genes were calculated from three independent biological replicates.

Lysimetric evaluation under greenhouse and confined field conditions

A lysimetric evaluation system was used during the first three trials, comprising one in the greenhouse (indoors lysimetric trial, ILT) and two outdoors (first trial referred to as outdoors lysimetric trial OLT-1 and the second as OLT-2). These involved growing the test plants individually in long polyvinyl chloride (PVC) tubes of 120 cm length and 20 cm diameter. The lysimeters were filled with Alfisol that provided surface area and soil volume similar to the field conditions (Vadez et al. 2008; Ratnakumar et al. 2009).

Six replicates of each of the selected genotypes in ILT and nine replicates in OLT-1 and OLT-2 with two treatments sets, viz., drought-stressed (DS) and well-

watered (WW), were planted in the lysimeters with the soil surface covered with a 2-cm layer of plastic beads to prevent soil evaporation. The replicates were considered sufficient owing to the homozygous nature of these transgenic events. The cylinders were irrigated weekly with ~500 mL water and the germinated seedlings were maintained until flowering. The soil profile was brought to field capacity and weighed prior to initiating treatments followed by weekly weighing thereafter. The plants in the WW treatment were maintained at about 85 % field capacity by weekly replenishment of the transpirational water losses, while the DS plants were subjected to a cycle of drying and re-wetting. The decision to irrigate the DS plants was based on a leaf wilting score, i.e., when most plants in the trial had a majority of leaves wilted in the early afternoon. The DS plants were irrigated three times with 1 L of water under ILT, while in the outdoor trials (OLT-1 and OLT-2) 1.5 L water was added three times. These irrigation levels mimicked the field situation corresponding to 33 and 50 mm irrigation, respectively, which is very close to the 40 mm irrigation that is usually provided during the intermittent drought trials in dryland fields (Hamidou et al. 2012).

Confined field evaluation

A confined field evaluation trial was conducted in an isolated field on ICRISAT campus in Patancheru, Andhra Pradesh, India during the post-rainy season of 2010–2011. The field was surrounded by two rows of non-transgenic peanut (var. JL 24) followed by three rows of sorghum as border crops, as per the biosafety guidelines. The seeds were sown in 2-m rows, one-row plots, with a 60-cm distance between rows. Six replicates (blocks), each having 20 seeds per genotype (spaced at 10 cm; 120 seeds)/replicate per treatment (WW/DS) were sown according to the randomized complete block design (RCBD). Furrow irrigation was provided weekly until flowering. Thereafter, the WW plants received 50 mm irrigation weekly, while the DS plants were irrigated based on the wilting symptoms as described earlier, thereby receiving three irrigations of 50 mm until maturity (110 days).

Climatic conditions

The ILT trials were conducted during March–July, 2008, while the two outdoor trials were during the post-rainy season (January–May of 2009 and 2010). The confined field trial was carried out during the post-rainy season of 2011 (January–May). The daily air temperature (T) and relative humidity (RH) were recorded using data loggers to calculate the vapour pressure deficit (VPD), where VPD = VP saturation − VP air. The RH and temperature were recorded daily for each experiment from beginning to end, and daily VPD was calculated by averaging daily measured values.

The vapour pressure deficit (VPD) of GH was ~1.5 kPa with an average daytime temperature of 30 °C, while the average daytime temperature during the outdoor lysimetric trials was on average 32 °C with a VPD of 2.5–4.5 kPa. During the confined field trial, the average daily temperature was 25 °C with a VPD of up to 2.2 kPa (Supplementary Fig. S1).

Data recording and analysis

The test plants were harvested at maturity followed by the drying of shoots and pods for recording their dry weights. The cumulative transpiration (T) values were calculated from cylinder weighing and water additions. The dry weights of shoot, pod and seed were used to compute the transpiration efficiency (TE) (total biomass/cumulated transpiration), HI and yield (Y) as described earlier.

Mean C_T (threshold cycle) values of three technical replicates were taken for the calculation of change in target gene expression levels using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

$$\begin{aligned}&\text{Normalized expression ratio}\\&= 2^{-\Delta\Delta C_T}\Delta\Delta C_T = \Delta C_T(\text{test})\\&\quad -\Delta C_T(\text{calibrator})\Delta C_T(\text{test}) = C_T(\text{target, test})\\&\quad -C_T(\text{ref, test})\Delta C_T\ (\text{calibrator})\\&= C_T(\text{target, calibrator}) - C_T(\text{ref, calibrator})\end{aligned}$$

where the samples from irrigated plants (before the imposition of drought stress) were taken as calibrators; the samples from 3- and 5-day DS plants were taken as test; *Act1* was taken as a reference gene and the *DREB1A* transgene was taken as the target. The result obtained was the increase or decrease of the target gene in the test sample relative to the calibrator sample and was normalized to the expression of a reference gene. Normalizing expression of the target gene to that

of the reference gene compensated for any difference in the amount of sample tissue.

For the individual lysimeteric and field trial data, analysis of variance (ANOVA) was carried out to test the significance of different events by using PROC GLM procedure of SAS software version 9.2 for Windows. To have an overall picture of performance of events, pooled analysis was performed over three lysimeteric trials and also across all four trials. Before pooling data across trials, the Bartlett χ^2 test (Gomez and Gomez 1984) was used to test homogeneity of error variance of all trials. Data for the traits for which heterogeneity among the trial variances was confirmed was appropriately transformed and pooled analysis was carried out. To study the nature of significant trial $\times$ event ($T \times E$) interaction (crossover type/non-crossover), data was tested for presence of crossover $T \times E$ interaction (COI). A comparison-wise test of COI suggested that none of the traits had significant COI. Since there was no COI present, ranking of events and their comparison was possible based on pooled analysis across the trials (Yang 2007). Linear contrasts were estimated to compare transgenic events against the wild type (WT) for individual and pooled analysis. Pooled analysis helped to determine the contribution of trials (T), events (E) and their possible interaction ($T \times E$).

Results

Molecular analysis

Segregation analysis of the transgenic progenies in the T6–T9 generations was done by PCR using the *npt*II gene and rd29A:DREB1A junction-specific primers. Every single plant in these generations was found to be PCR-positive for both the transgenes (data not shown), thereby indicating the homozygous nature of these events. Since the selected transgenic events were known to contain single copy inserts (Bhatnagar-Mathur et al. 2007), an integrative Southern blot and inverse PCR analyses was carried out to confirm the inheritance and copy number in advanced generation progenies of the transgenic peanut events (Supplementary Fig. S2 a, b). Duplex RT-PCR analysis indicated expression of the *npt*II and *DREB1A* genes during the phenotyping experiments. While the *npt*II gene was constitutively expressed in both WW and DS test plants, *DREB1A* expression was observed only under stress, thereby indicating a tight promoter-gene regulation in the transgenic events. The induction of *DREBIA* gene expression was recorded only after 3 days of drought-stress treatment (Fig. 1a–c), indicating that *DREB1A* was expressed in transgenic plants only upon encountering water limitations. Quantitative real time-PCR (qRT-PCR) analysis also revealed significant accumulation of *DREB1A* transcript in the transgenic events under drought stress when compared to their WW counterparts (Fig. 1d). The increase or decrease of the expression of *DREB1A* in the transgenic events under drought stress was relative to their WW counterparts and was normalized to the expression of *actin1,* the internal reference gene. While no significant differences were observed in the normalized expression ratio (indicative of the increase/decrease expression of *DREB1A*) in the transgenic events until 3 days of stress imposition, the increase was multi-fold when compared between the 2 and 3 days of drought stress (8.5-fold–14-fold, respectively), indicating a strong expression of the *DREB1A* transgene during this period.

Contained-field evaluations

Water uptake and biomass accumulation

No significant differences were observed in the transgenic events and the untransformed controls (wild types; WT) for their total water uptake/cumulative transpiration under both WW and DS across all the lysimetric trials (Table 1a, b). Total biomass accumulated by the transgenic events RD2 and RD33 did not vary from the WT under both WW and DS (Table 1a, b). The transgenic event RD33 accumulated the highest shoot (aerial) biomass under irrigation (WW) when compared to the WT across all trials. The event RD11 had lowest shoot and total biomass under both WW and DS ($P < 0.01$) when compared to other events and the WT ($P < 0.01$) across the lysimetric trials (Table 1a, b). Again, biomass accumulation under irrigation in the field trial (SFT) for the event RD33 was higher ($P < 0.05$) than WT, while the event RD11 again had lower biomass ($P < 0.01$) than the WT (Table 1a).

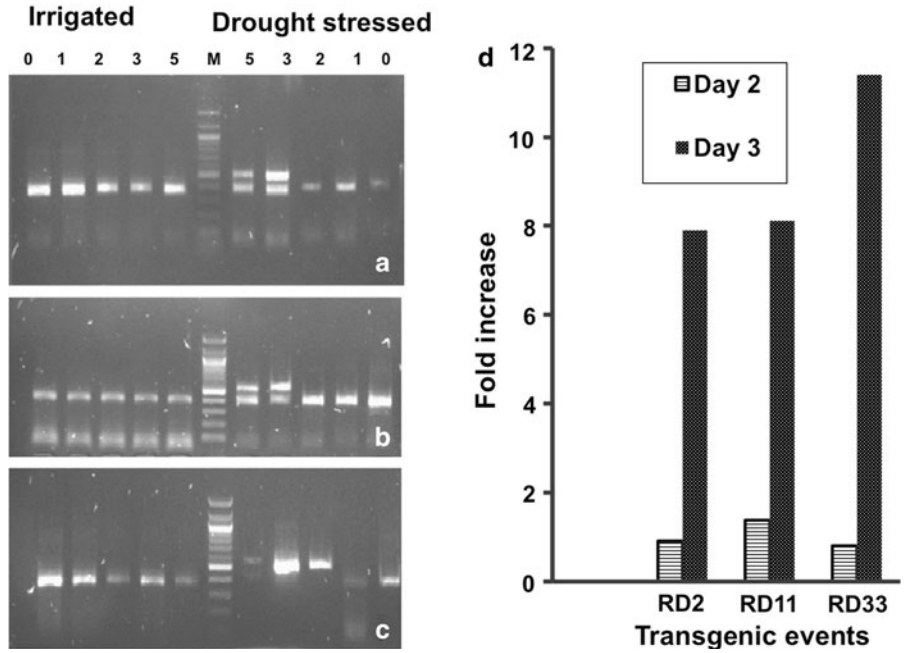


Fig. 1 *DREB1A* induction and expression in transgenic events under irrigated and drought-stress conditions in pot experiments. RT-PCR analysis for differential expression of the *npt*II (366 bp) and *DREB1A* (499 bp) genes in the transgenic events RD2 (**a**), RD11 (**b**) and RD33 (**c**) under progressive drought stress, *lanes 1–5* depict *npt*II amplification at 0, 1, 2, 3 and 5 days under irrigated conditions, *lane 6* carries 100 bp DNA ladder, *lanes 7–11* show *npt*II and *DREB1A* amplification after 5, 3, 2, 1, and 0 days after imposing drought stress. **d** Real time PCR (q-PCR) analysis for *DREB1A* expression under drought stress in the three transgenic events was determined using peanut *actin1* gene as internal control. Samples collected from the same plant at 0–2 and 2–3 days were compared, and mean values of three replicates presented

Yield and components under well-watered conditions

While the pod and seed weights (also referred as yield) of the transgenic events RD2 and RD33 were similar to the WT under irrigated lysimetric conditions (Fig. 2a, b), the transgenic event RD11 had a lower pod yield than the WT in these trials. In general, under WW, no significant differences were observed in the HIs of the transgenic events RD2 and RD11 and their WT across the three lysimetric trials, except that the event RD33 had a lower HI under WW across these trials (Fig. 2c). Although the 100-seed weight and seed number did not vary between the transgenics and the WT, the event RD2 had larger seeds under irrigation across the lysimetric trials, which also led to a higher shelling % (seed filling; Fig. 2d). Consistent with the lysimetric trials, there were no significant differences in the pod and seed yield of RD2, RD33 and the WT in the field under irrigation, indicating no yield penalty under normal conditions (Table 2; Fig. 2a, b). The 100-seed weights of the transgenic events were similar to the WT under irrigated conditions in the field (Supplementary Table S1). The seed filling and HI of event RD11 was significantly higher than the WT under irrigated conditions in the field (Table 2; Fig. 2c).

Yield and components under drought stress conditions

In contrast, the transgenic events RD2, RD11 and RD33 had 34–59 % higher pod yields ($P < 0.01$) than the WT under drought stress across all lysimetric trials (Fig. 2e). Differences in the yield benefit of the transgenics were explained by the differences in the stress intensities across trials, proxied by the ratio of mean pod yields of a trial under water stress and WW conditions. These benefits increased as the stress intensity decreased (data not shown). The superiority of these events was reflected in their ability to fill quality seeds under DS, where they had higher seed

Table 1 Details by trial on water uptake, vegetative and total biomass traits in the selected transgenic peanut events and the untransformed parent under (a) irrigated and (b) drought-stress conditions in the four trials during 2008–2011

ID	Transpiration Mean (kg/plant) ± SEM				Shoot dry weight Mean (g/plant) ± SEM				
	ILT	OLT-1	OLT-2	SFT	ILT	OLT-1	OLT-2	SFT	Across trials #
(a) Under irrigated conditions									
JL 24 (WT)	31.5 ± 1.8	29.3 ± 2.7	33.2 ± 2.6	ND	49.2 ± 4.5	28.9 ± 3.4	32.8 ± 3.6	20.1 ± 1.1	4.7 ± 0.2
RD11	27.8 ± 1.8	30.2 ± 2.3	35.7 ± 2.8	ND	30.7 ± 4.5**	25.9 ± 2.9	29.0 ± 3.8	12.9 ± 1.1***	3.1 ± 0.2***
RD2	30.4 ± 1.8	32.3 ± 2.5	37.4 ± 3.2	ND	42.2 ± 4.5	28.0 ± 3.1	33.2 ± 4.5	18.5 ± 1.1	3.4 ± 0.2
RD33	30.3 ± 1.8	36.4 ± 2.3	30.0 ± 2.7	ND	56.7 ± 4.5	35.5 ± 2.9	36.6 ± 3.8	24.3 ± 1.1**	4.4 ± 0.2**
(b) Under drought stress									
JL 24 (WT)	10.4 ± 0.4	17.3 ± 0.5	15.0 ± 0.4	ND	21.7 ± 2.1	23.3 ± 1.2	31.3 ± 0.7	15.5 ± 0.6	10.0 ± 0.2
RD11	10.4 ± 0.4	17.6 ± 0.5	15.5 ± 0.4	ND	15.6 ± 2.1	17.1 ± 1.2***	23.0 ± 0.8***	9.0 ± 0.6***	6.9 ± 0.2***
RD2	10.3 ± 0.4	16.1 ± 0.4	15.5 ± 30.1	ND	17.7 ± 2.1	18.5 ± 1.1***	29.7 ± 0.7	13.9 ± 0.6	9.0 ± 0.2**
RD33	10.4 ± 0.5	17.5 ± 0.5	15.3 ± 30.1	ND	22.7 ± 2.3	21.0 ± 1.2	31.0 ± 0.6	15.0 ± 0.6	9.8 ± 0.2

ID	Biomass Mean (g/plant) ± SEM				
	ILT	OLT-1	OLT-2	SFT	Across trials #
(a) Under irrigated conditions					
JL 24 (WT)	99.8 ± 7.7	50.1 ± 5.9	58.6 ± 5.0	38.20 ± 1.9	5.23 ± 0.2
RD11	68.6 ± 7.7**	43.8 ± 5.1	46.8 ± 5.4	27.2 ± 1.9***	3.9 ± 0.2***
RD2	87.8 ± 7.7	52.4 ± 5.5	56.2 ± 6.4	36.8 ± 1.9	4.0 ± 0.2
RD33	93.6 ± 7.7	64.5 ± 5.1	56.5 ± 5.4	44.1 ± 1.9*	5.0 ± 0.2
(b) Under drought stress					
JL 24 (WT)	31.4 ± 2.1	36.5 ± 1.4	34.8 ± 0.6	25.4 ± 0.9	12.4 ± 0.2
RD11	29.2 ± 2.1	32.0 ± 1.4*	27.7 ± 0.7***	18.8 ± 0.9***	10.1 ± 0.2***
RD2	34.1 ± 2.1	28.8 ± 1.2***	34.8 ± 0.6	25.6 ± 0.9	12.0 ± 0.2
RD33	33.8 ± 2.3	36.9 ± 1.4	34.7 ± 0.5	27.6 ± 1.1	12.8 ± 0.2

Each value represents the mean ($n > 6$) for the transgenics and the untransformed parent (WT)

ND not detected

*, ** and *** Significance at $P < 0.05$, 0.01 and 0.001 levels, respectively

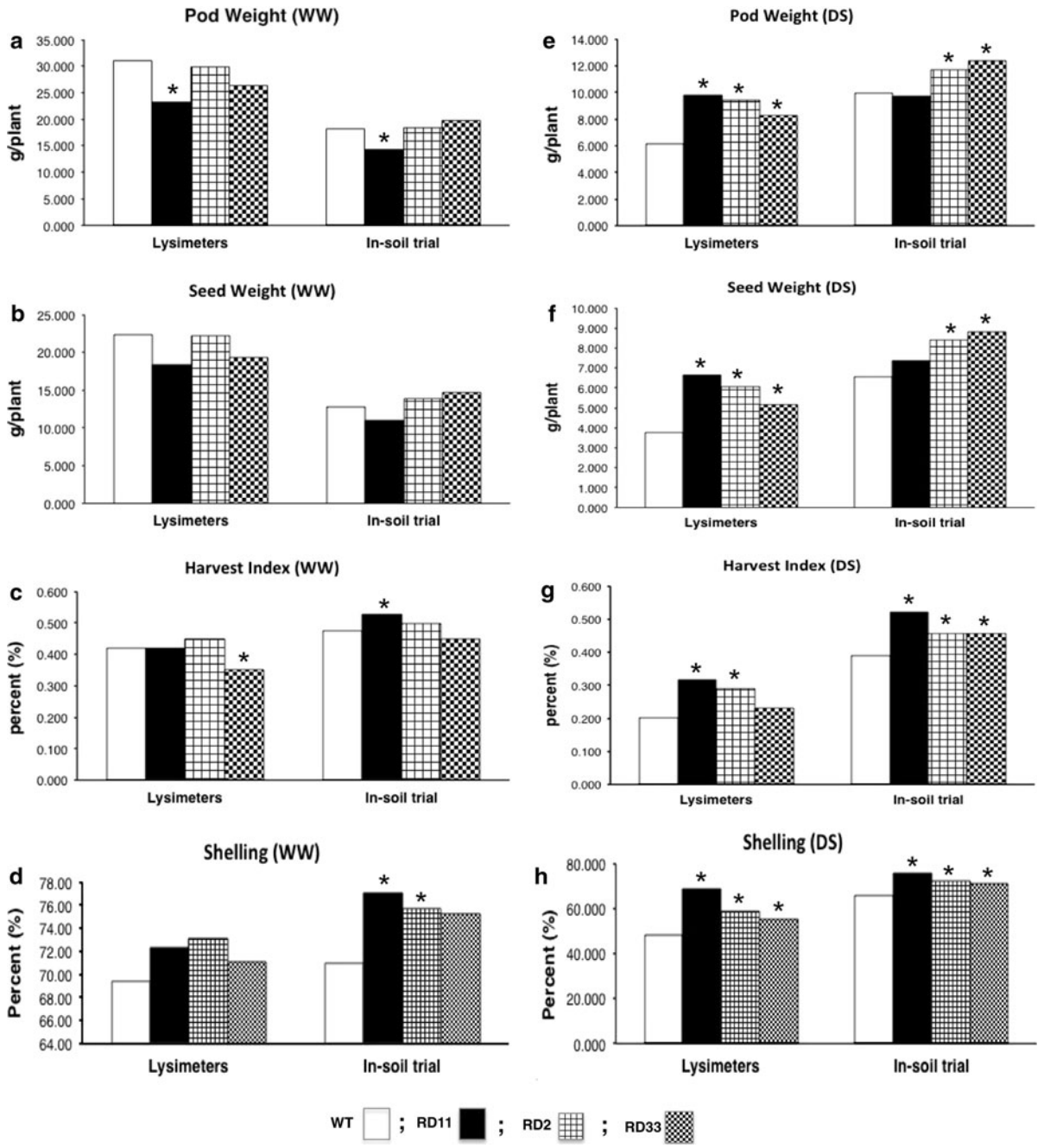


Fig. 2 Comparative yield data and relative ranking of the selected transgenic events along with the wild-type parent under irrigated and drought-stress conditions across four trials (pooled across the three lysimeter trials and one confined field trial). **a** Pod weight (g/plant) under irrigated conditions. **b** Seed weight (g/plant) under irrigated conditions. **c** Harvest index of the transgenic events under irrigated conditions. **d** Shelling % under irrigated conditions. (**e**) Pod weight (g/plant) under intermittent drought stress. **f** Seed weight (g/plant) under intermittent drought stress. **g** Harvest index under intermittent drought stress. **h** Shelling % under intermittent drought stress. $^{*}P < 0.01$

weight ($P < 0.05$) across the trials (Fig. 2f). The transgenic event RD11 had almost twofold higher seed yield than the WT under water deficits in the lysimetric trials. The transgenic events RD11 and RD2 had consistently higher HI ($P < 0.05$) than the WT under DS across all lysimetric trials (Fig. 2g; Table 2).

Table 2 Agronomic performance of the selected transgenic peanut events under well-watered and intermittent drought stress conditions in the individual trials during 2008–2011

Trait	Genotype	ILT 2008		OLT-1 2009		OLT-2 2010		SFT 2011	
		DS	WW	DS	WW	DS	WW	DS	WW
% of WW irrigation received in DS		12	100	25	100	30	100	43	100
VPD range (kPa)		0.75–1.5		1.5–2.5		1.5–4.5		1.2–2.2	
Pod yield (g/plant)	WT	8.01	50.66	7.02	16.60	3.47	25.8	9.95	18.14
	RD2	16.37**	45.62	6.82	20.72	5.06*	22.99	11.7*	18.37
	RD33	11.18	36.90*	9.92*	22.31	3.74	19.92	12.41**	19.76
	RD11	14.8*	37.86*	9.78**	14.11	4.74	17.77*	9.75	14.34**
Seed yield (g/plant)	WT	6.02	37.63	3.99	10.88	1.31	18.53	6.54	12.83
	RD2	11.66*	35.48	4.04	15.19	2.44*	15.80	8.40**	13.91
	RD33	7.11	28.90	6.85**	16.82*	1.55	13.20	8.83***	14.73
	RD11	9.89	27.86*	6.91**	9.49	3.11**	16.81	7.39	11.03
Harvest index	WT	0.31	0.51	0.19	0.31	0.1	0.43	0.39	0.47
	RD2	0.48*	0.52	0.24*	0.40	0.15*	0.43	0.46***	0.50
	RD33	0.31	0.39*	0.27**	0.34	0.11	0.34*	0.46***	0.45
	RD11	0.48*	0.56	0.31***	0.32	0.17**	0.39	0.52***	0.53*
Shelling %	WT	57.31	74.39	54.23	61.95	32.67	72.02	65.68	71.01
	RD2	70.76*	77.99	57.97	72.96	47.36*	68.26	72.14*	75.71*
	RD33	57.09	75.50	68.86**	73.31	40.15	64.45	71.20*	75.22
	RD11	72.30**	76.40	70.34***	65.07	63.41***	75.56	75.79***	77.10**

Each value represents the mean for transgenics and the untransformed parent (WT), where $n > 6$ in ILT, OLT-1 and OLT-2, $n = 120$ in SFT

*, ** and *** Significance at $P < 0.05$, 0.01 and 0.001 level, respectively

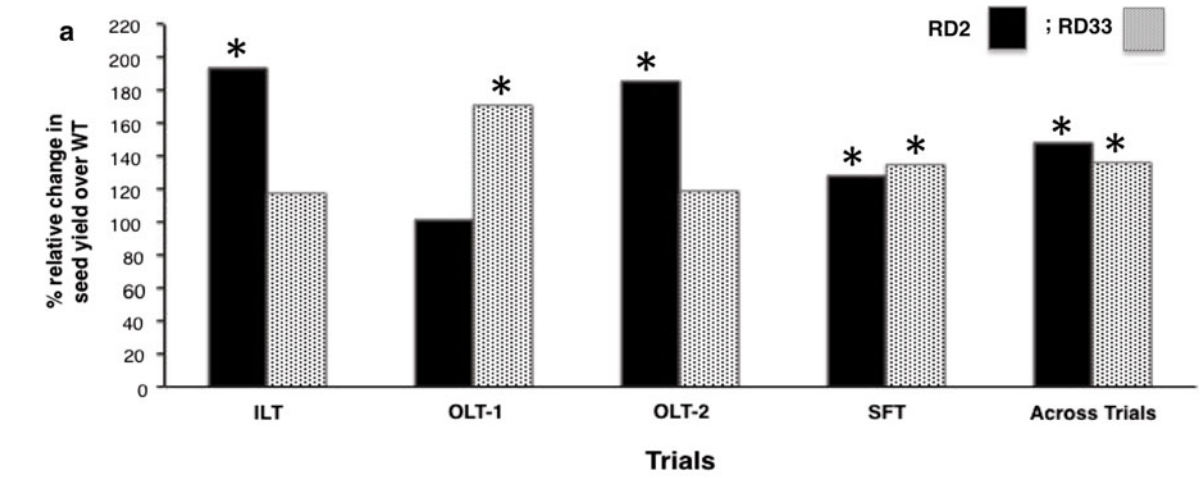
All the transgenic events also had higher seed filling, as indicated by a higher shelling % ($P < 0.05$) than the WT across the DS trials (Fig. 2h). Consistent with the lysimetric trials, the field trial also showed higher pod weight ($P < 0.05$), seed weight ($P < 0.01$) and seed number ($P < 0.05$) under DS in the transgenic events RD2 and RD33 than in the WT (Fig. 2e, f). These had up to 10 % higher seed filling under DS, which translated into a pod yield advantage of 18 and 24 %, respectively (data not shown), and a 28 and 39 % higher seed yield than the WT under drought (Fig. 3a). Likewise, the HI of RD2 and RD11 was significantly higher ($P < 0.01$) than the WT under DS (Table 2; Fig. 2g). All transgenic events had significantly higher seed filling (shelling %; $P < 0.05$) under drought (Table 2; Figs. 2h, 3a) where the events RD2, RD11 and RD33 displayed 20–30 % lower pod yield reduction under DS than the WT (Fig. 3b).

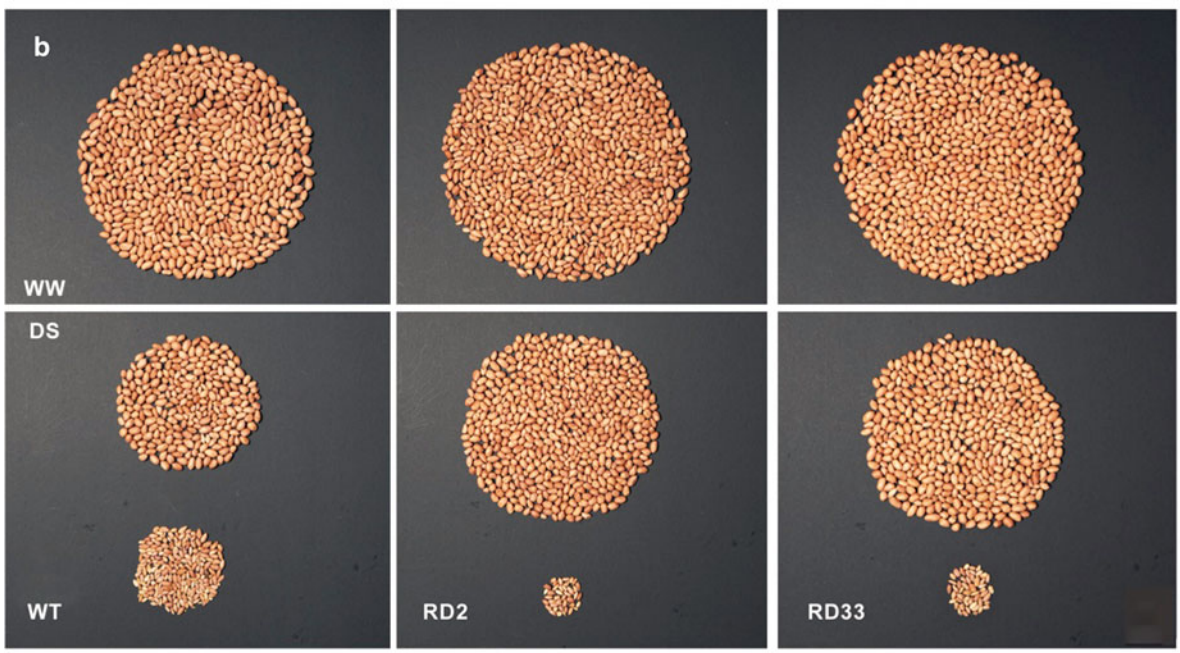
Predicted yield ($\hat{Y}_{ds}$) and drought tolerance indexes

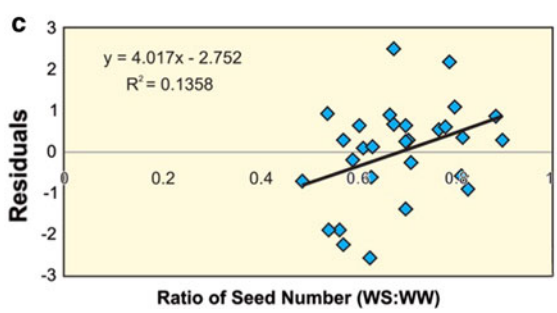
Since a significant linear relationship was observed between the seed yield under drought (Y_{ds}) and irrigation (Y_{ww}) ($R^2 = 0.20$) (Supplementary Fig. S3), a small portion of the seed yield under drought could be estimated from the yield potential component (Y_{ww}) using the equation

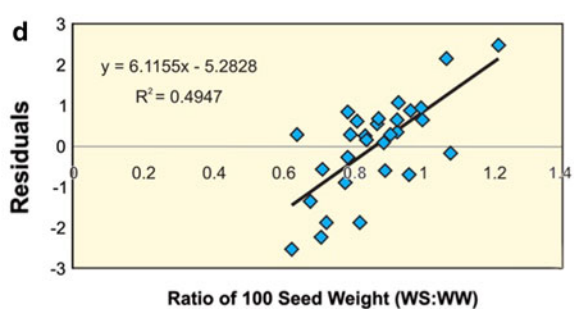
$$\hat{Y}_{ds} = 0.25 \times Y_{ww} + 4.17,$$

where $\hat{Y}_{ds}$ is the estimated yield under drought based on the yield potential. The residual seed yield variations under drought that were not accounted for by the yield potential could be estimated by the differences between Y_{ds} and $\hat{Y}_{ds}$ ($Y_{ds} - \hat{Y}_{ds}$) (Bidinger et al. 1987; Vadez et al. 2007a). These residuals (R) were used as a proxy for drought tolerance per se and were then


a
RD2
; RD33
% relative change in seed yield over WT
220
200
180
160
140
120
100
80
60
40
20
0
*
ILT
OLT-1
OLT-2
SFT
Across Trials
Trials


b
WW
DS
WT
RD2
RD33


c
y = 4.017x - 2.752
R² = 0.1358
Residuals
Ratio of Seed Number (WS:WW)


d
y = 6.1155x - 5.2828
R² = 0.4947
Residuals
Ratio of 100 Seed Weight (WS:WW)

◀**Fig. 3** **a** Relative change in seed weight of transgenic events RD33 and RD2 compared to wild-type (WT) parent JL 24 under drought stress in individual trials and across four trials. The change reflects the percent increase over WT, which is considered as 100 %. *Significance at $P < 0.05$ level. **b–d** Seed yield improvement and predicted yield ($\hat{Y}_{ds}$) and drought tolerance indexes in the transgenic peanut plants and their WT parent. **b** Yield potential and relative seed yield improvement in the best-bet transgenic events (RD2 and RD33) vis-a-vis untransformed parent under irrigated and drought-stress conditions in the field. The transgenic events RD2 and RD33 had significantly higher seed filling and 100-seed weight with lower number of shriveled seeds (lowermost seed heap). **c**, **d** Relationship between the residuals [difference between observed and predicted yield under drought ($Y_{ds} - \hat{Y}_{ds}$)] and **c** the ratio of seed number, **d** the ratio of 100-seed weight

regressed as dependent variables against: (1) the ratio of seed number per plant (seed number under DS/seed number under WW), (2) the ratio of 100-seed weight (100-seed weight under DS/100-seed weight under WW). While the residuals correlated significantly with both the ratios, the strength of the correlation with the relative seed number ($R^2 = 0.13$, $P = 0.04$; Fig. 3c) was much lower than that with the relative seed size ($R^2 = 0.49$, $P < 0.01$, Fig. 3d).

Discussion

We have previously demonstrated that these transgenic events of peanut had enhanced TE, an important component of plant performance under limited soil moisture conditions (Bhatnagar-Mathur et al. 2007). Transpiration declined in these events under dryer soil and maintained substantially higher TE (in g biomass produced/kg of water transpired), where the differences were considerably large when compared to the range of variation usually found for TE between germplasm accessions of peanut (Devi et al. 2011). Moreover, most of the biochemical parameters related to the anti-oxidative machinery appeared to "kick in" at fairly wetter soils (low FTSW values) in these peanut transgenics under progressive water stress, which appears to differ from the WT (Bhatnagar-Mathur et al. 2009). Nevertheless, since yield improvements under the highly changing tropical environments are an essential requirement, here we present data on thoroughly assessed and field-validated transgenics in a grain legume with improved drought tolerance. While many reports have demonstrated increased tolerance to water deficits under laboratory and greenhouse conditions in several crops (Dubouzet et al. 2003), very few have established the performance and productivity of transgenic lines in the field (Yang et al. 2010).

Various molecular analyses including PCR, Southern blot and inverse-PCR confirmed the transgene inheritance, copy number and homozygous nature of the transgenic events. The expression of *DREB1A* in the peanut transgenics did not show any morphological differences, which may be attributed to stress-inducible expression of the transgene, which was also observed previously during constitutive *DREB1A* expression in many other studies including ours (Kasuga et al. 1999; Bhatnagar-Mathur et al. 2007; Datta et al. 2012). Expression analysis of the transgenic plants revealed that *DREB1A* driven by the *rd29A* promoter was induced only after the third day following withdrawal of irrigation in the pot studies, thereby suggesting it to be an effective drought stress-inducible promoter for peanut. Earlier studies on histochemical expression of the *uid*A gene in transgenic *Arabidopsis* rosettes (Shinwari et al. 1998) and our previous work in peanut (Bhatnagar-Mathur et al. 2007) also indicated a tight regulation of *rd29A* promoter in all the tested organs and tissues. However, these results differ from those recently reported by Datta et al. (2012), where the DREB expression in transgenic rice events could be detected even on the first day of withholding water. This could not be explained, since the stress-inducible promoter is supposedly functional only after the stress is perceived by the plants, which in our experimental conditions occurred by the third day following the saturation of soil in the pots to field capacity. The qRT-PCR analysis was carried out using *Act* as a reference gene following the validation of different reference genes from peanut (data to be published elsewhere). The *Act* gene has also been reported to show stable and reproducible expression under abiotic stress in common bean when used in combination with the *Skip16* gene to validate *DREB* gene expression (Borges et al. 2012). While an induced expression of *DREB1A* in these transgenic events was detected only on the third day following exposure to water stress, a multi-fold induction of mRNA was recorded during 3–5 days after imposed water stress. Thereafter, the decrease in *DREB1A* expression in the transgenic events could either be attributed to reduced transcript abundance or to the rate of mRNA turnover under progressive drought stress in these pot studies.

Previously, we have reported differences in the TE under WW and DS conditions (Bhatnagar-Mathur et al. 2007; Devi et al. 2011), indicating that the regulation of stomatal movements might have been the cause for the observed relationships between TE and other surrogates SPAD chlorophyll meter reading (SCMR), specific leaf area (SLA) under drought stress. In this study, the four trials not only represented various water stress regimes but also varying VPDs, accompanied by high temperatures in some cases (OLT-2), resulting in multiple abiotic stresses, which is a usual phenomenon that crops experience under natural semi arid tropics (SAT) conditions. This would explain the differences in the relative yield reduction under drought stress across trials when compared to their WW counterparts. The phenotypic and agronomic data presented in this study clearly indicated that the *DREB* transgenic events adopted a more conservative, "risk-aversion", strategy that conferred a fitness advantage under drought stress in these drier conditions.

Nonetheless, the failure of earlier attempts to develop transgenic crops with acceptable yield under drought stress, while maintaining their yield potential under irrigated conditions is explained by selection of "extremely risk-averse" events that, although could survive severe seedling stress exposure, compromised their yield potential. Transgenic *DREB1A* wheat evaluated for survival and recovery under severe drought (SURV) as well as for water use efficiency (WUE) did not outperform the controls in terms of grain yield under water deficit in the field (Saint Pierre et al. 2012). In the present study, the transgenic event RD11 was the most "risk-averse" amongst the tested events.

We observed that the residuals which were not explained by the yield under fully irrigated conditions, which accounted for drought tolerance per se, were closely related to the relative decrease in seed size per plant, thereby indicating that these transgenic events had a better capacity to fill the seeds under drought stress. Previously, enhanced drought tolerance in transgenic rice plants was shown at the reproductive stage by increased grain yield (16–57 %) over the control under severe field drought conditions (Oh et al. 2009), although it was not clear whether this was caused by a decrease in the grain number or the filling of the seeds. In our case, it was clear that the seed yield differences were not caused by differences in the success of reproductive stages but rather by differences in the filling of the seeds.

The transgenic event RD11 had higher yield than the WT under drought stress across all the four trials, but had a lower yield potential under irrigation, owing to its characteristically smaller leaf canopy (Bhatnagar-Mathur et al. 2007; Vadez et al. 2007a, b; Devi et al. 2011). This possibly contributed to water saving under drought stress, resulting in its higher yield and thereby suggesting that genotypes like RD11 could also be targeted to specific environments where dry episodes are frequent, long and severe (Tardieu et al. 2010; Yadav et al. 2010). Overall, analysis of the yield variations that were independent of the yield potential clearly showed that yield losses, especially in the WT, were due to impaired seed filling rather than an effect on the seed number, thereby resulting in more shrivelled seeds.

The strength of the present work has been our approach to avoid plant survival as a criteria for the pre-selection of transgenic events, in contrast to many earlier studies on transgenics emphasizing selection on higher severity and longer duration of stress (Bhatnagar-Mathur et al. 2008). This was then followed by first carefully assessing a number of drought-related traits using protocols that would closely mimic the natural stress conditions (Bhatnagar-Mathur et al. 2004, 2007; Vadez et al. 2007a, b, Devi et al. 2011), prior to their evaluation for yield response under drought conditions. The fact that the traits leading to enhanced drought adaptation of these events also varied offers the possibility of using these for breeding for diverse target environments.

To our knowledge the present work is one of the few reports showing yield advantage under drought stress in any crop using the DREB family of transcription factors, with two events consistently having higher pod and seed yield than the untransformed parent under drought stress across all trials, without displaying any yield penalty under irrigated conditions. The outputs have the potential to realize stable yields under drought stress, besides maintaining maximum yield potential under optimal conditions. Targeting drought tolerance in peanut for marginal environments, where the poorest of the poor live, would potentially contribute towards food and nutritional security in the drylands.

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