Molecular Breeding
September 2013

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

Pooja Bhatnagar-Mathur, Jagana Sridhar Rao, Vincent Vadez, Srinivas Reddy Dumbala, Abhishek Rathore, Kazuko Yamaguchi-Shinozaki, Kiran K. Sharma

DOI: http://dx.doi.org/10.1007/s11032-013-9952-7

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

Pooja Bhatnagar-Mathur[1], Jagana Sridhar Rao[1], Vincent Vadez[1], Dumbala Srinivas Reddy[1], Abhishek Rathore[1], Kazuko Yamaguchi-Shinozaki[2] and Kiran K Sharma[1*]

[1]International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, AP 502 324, India

[2]Japan International Research Center for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki 305-0074, Japan

*For correspondence, Tel: +91-9849548540; Fax: +91-40-30713300; E-mail: k.sharma@cgiar.org

Running Title: Drought tolerant transgenic peanut

Abstract Transgenic research using 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 tested possible effects under field conditions with limited success in most cases. Here, we present evidence of 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 (VPD), and drought tolerance 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 related to higher harvest indices. 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 water challenged situation. Irrigation water has already reached its peak and the share of water for agricultural use decreases as societies develop, thereby, resulting in an ever-increasing pressure on producing more from less, 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 two decades, transgenic research has received much attention and push 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 (Reddy et al. 2003). Peanut occupies the largest portion of Low Income Food Deficit Countries (FAO 2008) 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 to 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 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 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 besides 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. 2007, 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 four years.

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

Material and methods

Plant material

Homozygous progenies of the previously selected three transgenic events of peanut (*Arachis 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 demonstrated a single copy of *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 RB of T-DNA, with forward primer (IP1: 5'- CGTTGCGGTTCTGTCAGTTCC-3') designed from the *nos* promoter sequence and reverse primer (IP2: 5'- TTGTCAAGACCGACCTGTCCG-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 was kept for an overnight ligation at 16 °C, 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 MgCl2, 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, 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 (q-PCR) analyses for transgene expression in the events was performed under progressive drying down pot experiment under contained greenhouse as described earlier (Bhatnagar-Mathur et al. 2007). The leaf samples were collected on 0, 1, 2, 3 and 5 d after

imposition of drought stress, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction done using TRIzol® reagent (Invitrogen) according to manufacturer 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 (Eppendrof). 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'-ATGCTAGTGGTCGTACAACTGG-3'

GnAct-RP 5'-CTAGACGAAGGATAGCATGTGG-3'

and for the *DREB1A* gene were:

DREB-FP :5´-AATCCCGGAATCAACTTGCGCT-3

DREB-RP 5´-AAATAGCCTCCACCAACGTCTC-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^{o}C; 2 min at 95 ^{0}C; 45 cycles of cDNA amplification for 10 s at 95 ^{0}C, 15 s at 62 ^{0}C, 10 s at 72 ^{0}C with fluorescent signal recording. At the end, a final step of 15 s at 95 ^{0}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 including one in the greenhouse (indoors lysimetric trial, ILT) and two outdoors (first trial referred to as outdoors lysimetric trial, OLT-1 and the second trial 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 genotype in ILT and nine replicates in OLT-1 and OLT-2 with two treatments sets viz., DS and 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 enough 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 replenishing 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 thrice with 1 L of water under ILT, while in the outdoor trials (OLT-1 and OLT-2) 1.5 L water was added thrice. 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 the fields of drylands (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) per replicate per treatment (WW/DS) were sown as per 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 3 irrigations of 50 mm till maturity (110 d).

Climatic conditions

The ILT trials were conducted during March-July, 2008, while the two outdoor trials were during the post-rainy season (Jan.-May of 2009 and 2010). The confined field trial was carried out during the post-rainy season of 2011 (Jan.-May). The daily air temperature (T) and relative humidity (RH) were recorded by 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 of the experiment till the 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 ^{0}C, while the average daytime temperature during the outdoor lysimetric trials was on an average 32 ^{0}C with the VPD of 2.5-4.5 kPa. During the confined field trial, the average daily temperature was 25°C with a VPD 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), Harvest Index (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).

Normalized expression ratio=$2^{-\Delta\Delta CT}$

$\Delta\Delta C_T = \Delta C_T(\text{test}) - \Delta C_T$ (calibrator)

ΔC_T (test) = C_T (target, test) – C_T(ref, test)

ΔCT(calibrator) = CT(target, calibrator) – CT(ref, calibrator)

Where, the samples from irrigated plants (before the imposition of drought stress) were taken as calibrators; the samples from 3 and 5 d drought stressed plants were taken as test; *Act1* was taken as a reference gene and the *DREB1A* transgene was taken as the target. The result obtained is the increase or decrease of the target gene in the test sample relative to the calibrator sample and is 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 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, Bartlett Chi-Square test (Gomez and Gomez, 1984) was used to test homogeneity of error variance of all trials. The traits for which heterogeneity among the trial variances was confirmed, data was appropriately transformed and pooled analysis was carried out. To study the nature of significant TxE interaction (crossover type /non crossover), data was tested for presence of crossovers TxE 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 (TxE).

Results

Molecular analysis

Segregation analysis of the transgenic progenies in T6-T9 generation 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 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 well-watered (WW) and drought-stressed (DS) test plants, the *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 d of drought stress treatment (Fig.1 a-c) indicating that the DREB1A was expressed in transgenic plants only upon encountering water limitations. Quantitative real time-PCR (q-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 the *Actin*, 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 d of stress imposition, the increase was multi-fold when compared between the 2 d and 3 d of drought stress (8.5 to 14-folds) 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 (Tables 1a,b). Total biomass accumulated by the transgenic

events RD2 and RD33 did not vary from the WT under both WW and DS (Tables 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 (Tables 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).

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 (Figure 2 a,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 harvest indices of the transgenic events RD2 and RD11 and their WT across the three lysimetric trials, except that the event RD33 had a lower harvest index (HI) under WW across these trials (Figure 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 percentage (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. 2 a,b). The 100 seed weight of the transgenic events were similar to the WT under irrigated conditions in the field (Supplementary Table S1). The seed filling and harvest index 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 well-watered 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 weight ($P<0.05$) across the trials (Fig. 2f). The transgenic event RD11 had almost 2-fold 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). All the transgenic events also had higher seed filling as indicated by a higher shelling percentage ($P<0.05$) 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. 2 e,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 indices

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:

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

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 $\hat{Y}_{ds}$ ($Y_{ds} - \hat{Y}_{ds}$)[21,22]. These residuals (R) were used as a proxy for drought tolerance *per se* and were then regressed as dependant variables against: (i) the ratio of seed number per plant (seed number under DS/seed number under WW); (ii) the ratio of 100 seed weight (100 seed weight under DS/100 seed weight under WW). While the residuals correlated significantly to both the ratios, the strength of the correlation to the relative seed number ($R^2 = 0.13$, $p = 0.04$; Fig. 3c) was much lower than that to 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 transpiration efficiency (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 per 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 imperious requirement, here we present data on thoroughly assessed and field validated transgenics in any 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 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 q-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 *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 d after imposed water stress. Thereafter, the decrease in *DREB1A* expression in the transgenic events could either be attributed to reduced transcript abundance or on 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 (SCMR, SLA) under drought stress. In this study, the four trials not only represented various water stress regimes but also varying vapour pressure deficits (VPD), accompanied by high temperatures in some cases (OLT-2) resulting in multiple abiotic stresses which is a usual phenomenon that crops experience under natural SAT conditions. This would explain the differences in the relative yield reduction under drought stress across trials when compared to their well watered 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.

Nontheless, 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, that 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 evidenced 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 weather 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 difference 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. 2007; 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 wild type 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. 2007, 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 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.

Acknowledgements The authors acknowledge JIRCAS, Japan for providing the gene constructs used in this study. We thank Dr. William D. Dar, Director General, ICRISAT for his keen interest and constant encouragement for this work. We thank Drs. Deborah Delmer and Thomas Sinclair for their critical comments on the manuscript. The excellent technical assistance from Kanaka Reddy, Md. Yousuf, C. Lakshminarayana and D. Pandary is acknowledged. We thank Ms. Roma Das for her help with the statistical analysis. This research was partly supported by a grant from the Department of Biotechnology (DBT), Government of India. This is to confirm that the authors have no Conflict of Interest with the work reported here.

Supplemantary data:

Supporting information in the form of Supplementary Tables S1 and S2, and Supplementary Figures S1, S2, and S3 are available online.

Supplementary Table S1. Trial-wise details on component traits of yield in the selected transgenic peanut events and the untransformed parent under well-watered conditions in the four conducted trials (2008-2011). Each value represents the mean±SEM (n > 6) for transgenics and the untransformed parent.

Supplementary Table S2. Trial-wise details on component traits of yield in the selected transgenic peanut events and their untransformed parent under drought stress conditions in the four trials during 2008-2011. Each value represents the mean±SEM (n >6) for transgenics and the untransformed parent.

Supplementary Figure S1. Mean vapour pressure deficit (VPD) during drought stress period across different lysimetric and confined field trials.

Supplementary Figure S2. Southern blot and inverse PCR analysis for gene inheritance and copy number validation in T7 generation transgenic events. (a) Southern blot analysis indicating DREB1A gene integration (b) Inverse-PCR (IPCR) analysis of the transgenic groundnut event for T-DNA integration in plant genome.

Supplementary Figure S3. Relationship between seed yields in the well-watered controls and under drought stress (g plant^{-1}). The regression equation was used to compute the estimated yield (Y ˆ ds).

References

Behnam B, Kikuchi A, Celebi-Toprak F, Yamanaka S, Kasuga M, Yamaguchi-Shinozaki K Watanabe KN (2006) The *Arabidopsis* DREB1A gene driven by the stress-inducible *rd29A* promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (*Solanum tuberosum*). Plant Biotechnol J 23: 169-177

Bhatnagar-Mathur P, Devi MJ, Serraj R, Yamaguchi-Shinozaki K, Vadez V, Sharma KK (2004) Evaluation of transgenic groundnut lines under water limited conditions. Int Arachis Newslett 24: 33–34

Bhatnagar-Mathur P, Devi, MJ, Vadez V, Sharma KK (2009) Differential antioxidative responses in transgenic peanut bear no relationship to their superior transpiration efficiency under drought stress**.** J Plant Physiol 166**:** 1207-1217

Bhatnagar-Mathur P, Reddy DS, Lavanya M, Yamaguchi-Shinozaki K, Sharma KK (2007) Stress-inducible expression of *Arabidopsis thaliana* DREB1A in transgenic peanut (*Arachis hypogaea L.*) increases transpiration efficiency under water-limiting conditions. Plant Cell Rep 26: 2071–82

Bhatnagar-Mathur P, Vadez V, Sharma KK (2008) Transgenic approaches for abiotic stress tolerance in plants: Retrospect and prospects. Plant Cell Rep 27: 411-424

Bidinger FR, Mahalakshmi V, Rao GDP (1987) Assessment of drought resistance in pearl millet *(Pennisetum americanum* (L.) Lecke). II. Estimation of genotype response to stress. Aust J Agric Res 38: 49-59

Borges A, Tsai SM, Caldas DGG (2012) Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stress. Plant Cell Rep 31: 827-838

Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11: 287-293

Datta K, Baisakh N, Ganguly M, Krishna S, Yamaguchi Shinozaki K, Datta SK (2012) Overexpression of *Arabidopsis* and Rice stress genes inducible transcription factor confers drought and salinity tolerance to rice. Plant Biotechnol J 10: 579–586

Devi JM, Bhatnagar-Mathur P, Sharma KK, Serraj R, Anwar SY, Vadez V (2011) Relationships between transpiration efficiency (TE) and its surrogate traits in the rd29A:DREB1A transgenic lines of groundnut. J Agron Crop Sci 197: 272-283

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J 33: 751-763

FAO (2009) Food and Agricultural Organization of the United Nation, FAO Statistical Database http://faostat.fao.org/faostat/collections? Subset =agriculture

Gautami B, Pandey MK, Vadez V, Nigam SN, Ratnakumar P, Krishnamurthy L, Radhakrishnan T, Gowda MVC, Narasu ML, Hoisington DA, Knapp SJ, Varshney RK (2011) Quantitative trait locus analysis and construction of consensus genetic map for drought tolerance traits based on three recombinant inbred line populations in cultivated groundnut (*Arachis hypogaea* L.). Mol Breed 30: 757-772

Gomez KA, Gomez AA (1984) In: *Statistical Procedure for Agricultural Research*, 2nd ed. John Wiley & Sons, New York

Hamidou F, Ratnakumar P, Halilou O, Mponda O, Kapewa T, Monyo E, Faye I, Ntare B, Nigam SN, Upadhyaya HD, Vadez V (2012) Selection of intermittent drought tolerant lines across years and locations in the reference collection of groundnut (*Arachis hypogeae* L.). Field Crop Res 126: 189-199

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress inducible transcription factor. Nature Biotechnol 17: 287-291

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA-binding domain separate two cellular signal transduction pathways in drought and low-temperature-responsive gene expression respectively in *Arabidopsis*. Plant Cell 10: 1391-1406

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using RealTime Quantitative PCR and the 2-ΔΔCT Method. Methods 25: 402–408

Morran S, Eini O, Pyvovarenko T, Parent B, Singh R, Ismagul A, Eliby S, Shirley N, Langridge P, Lopato S (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. . Plant Biotechnol J 9: 230–249

Oh SJ, Kim YS, Kwon C-W, Park HK, Jeong JS, Kim JK (2009) Overexpresson of the transcription factor AP37 in rice improves grain yield under drought conditions. Plant Physiol 150: 1368-1379

Oh SJ, Song SI, Kim YS, Jang HJ, Kim SY, Kim M, Kim YK, Nahm BH, Kim JK (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol 138: 341-351

Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. Genome 47: 493-500

Qin H, Gu Q, Zhang J, Sun L, Kuppu S, Zhang Y, Burow M, Payton P, Blumwald E, Zhang H (2011) Regulated expression of an isopentenyltransferase gene (IPT) in peanut significantly improves drought tolerance and increases yield under field conditions. Plant Cell Physiol 52: 1904-1914

Ratnakumar P, Vadez V, Nigam SN, Krishnamurthy L (2009) Assessment of transpiration efficiency in groundnut (*Arachis hypogaea* L.) under drought by lysimetric system. Plant Biology 11: 124–130

Saint Pierre C, Crossa JL, Bonnett D, Yamaguchi-Shinozaki K, Reynolds MP (2012) Phenotyping transgenic wheat for drought resistance. J Exp Bot 63: 1799-1808

Shinwari ZK, Nakashima K, Miura S, Kasuga M, Seki M, Yamaguchi-Shinozaki K, Shinozaki K (1998) An *Arabidopsis* gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. Biochem Biophys Res Commun 50: 161–170

Subbarao GV, Johansen C, Slinkard AE, NageshwaraRao RC, Saxena NP, Chauhan YS (1995) Strategies for improving drought resistance in grain legumes. Crit Rev Plant Sci 14: 469-523

Tardieu F, Parent B, Simonneau T (2010) Control of leaf growth by abscisic acid: hydraulic or non-hydraulic processes? Plant Cell Environ 33: 636–647

Vadez V, Krishnamurthy L, Serraj R, Gaur PM, Upadhyaya HD, Hoisington DA, Varshney RK, Turner NC, Siddique KHM (2007) Large variation in salinity tolerance in chickpea is explained by differences in sensitivity at the reproductive stage. Field Crop Res 104: 123-129

Vadez V, Rao JS, Bhatnagar-Mathur P, Sharma KK (2012) DREB1A promotes root development in deep soil layers and increases water extraction under water stress in groundnut. Plant Biology 15: 45-52

Vadez V, Rao S, Kholova J, Krishnamurthy L, Kashiwagi J, Ratnakumar P, Sharma KK, Bhatnagar-Mathur P, Basu PS (2008) Root research for drought tolerance in legumes: quo vadis? J Fd Legume 21: 77-85

Vadez V, Rao S, Sharma KK, Bhatnagar-Mathur P, Devi JM (2007) DREB1A allows for more water uptake in groundnut by a large modification in the root/shoot ratio under water deficit. Int Arachis Newslett 27: 27-31

Varshney RK, Bertioli DJ, Moretzsohn MC, Vadez V, Krishnamurthy L, Aruna R, Nigam SN, Moss BJ, Seetha K, Ravi K, He G, Knapp SJ, Hoisington DA (2009) The first SSR-based genetic linkage map for cultivated groundnut (*Arachis hypogaea* L.). Theor Appl Genet 118: 729-739

Wang Q, Guan Y, Wu Y, Chen H, Chen F, Chu C (2008) Overexpression of a rice OsDREB1F gene increases salt, drought, and low temperature tolerance in both *Arabidopsis* and rice. Plant Mol Biol 67: 589–602

Wassmann R, Jagadish SVK, Sumfleth K, Pathak H, Howell G, Ismail A, Serraj R, Redoña E, Singh RK, Heuer S (2009) Regional vulnerability of climate change impacts on Asian rice production and scope for adaptation. Adv Agron 102: 91-133

Xiao H, Siddiqua M, Braybrook S, Nassuth A (2006) Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. Plant Cell Environ 29: 1410-1421

Yadav RS, Sehgal D, Vadez V (2010) Using genetic mapping and genomics approaches in understanding and improving drought tolerance in pearl millet. J Exp Bot 62: 397–408

Yang RC (2007) Mixed model analysis of crossover genotype-environment interactions. Crop Sci 47: 1051–1062

Yang S, Vanderbeld B, Wan J, Huang Y (2010) Narrowing down the targets: towards successful genetic engineering of drought tolerant crops. Molecular Plant 3: 469–490

Table 1 Trial wise details 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. Each value represents the mean (n >6) for the transgenics and the untransformed parent (WT). *, ** & *** denote significance at p<0.05, 0.01 & 0.001 levels, respectively. ND= Not detected

ID	Transpiration mean (kg/plant)±SEM				Shoot dry weight mean (g/plant)±SEM					Biomass mean (g/plant)±SEM				
	ILT	OLT-1	OLT-2	SFT	ILT	OLT-1	OLT-2	SFT	Across trials #	ILT	OLT-1	OLT-2	SFT	Across
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	99.8 ± 7.7	50.1±5.9	58.6±5.0	38.20± 1.9	5.23±
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***	68.6 ± 7.7**	43.8±5.1	46.8±5.4	27.2 ± 1.9***	3.9± 0
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	87.8 ± 7.7	52.4±5.5	56.2±6.4	36.8± 1.9	4.0±
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**	93.6 ± 7.7	64.5±5.1	56.5±5.4	44.1 ± 1.9*	5.0±
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	31.4±2.1	36.5±1.4	34.8±0.6	25.4 ± 0.9	12.4±
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***	29.2±2.1	32.0±1.4*	27.7± 0.7***	18.8 ± 0.9***	10.1± 0
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**	34.1 ±2.1	28.8±1.2***	34.8± 0.6	25.6 ± 0.9	12.0±
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	33.8±2.3	36.9±1.4	34.7± 0.5	27.6 ± 1.1	12.8±

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. Each value represents the mean for transgenics and the untransformed parent (WT), where, n>6 for ILT, OLT-1 & OLT-2; n=120 in SFT. *, **& *** denotes significance at p<0.05, 0.01 & 0.001 level, respectively

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**

Figure Legends

Fig. 1 DREB1A induction and expression in transgenic events under irrigated and drought stress conditions in pot experiments (a-c) RT-PCR analysis for differential expression of the *npt*II (366 bp) and *DREB1A* (499 bp) genes in the transgenic event RD2 (a), RD11 (b) and RD 33 (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 d and 2-3 d were compared, and mean values of three replicates presented.

Fig. 2a-h 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 lysimeters 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 percentage (%) under irrigated conditions (e) Pod weight (g/plant) under intermittent drought stress (f) seed weight (g/plant) under intermittent drought stress (g) Harvest indices under intermittent drought stress (h) Shelling percentage (%) under intermittent drought stress. *, $P<0.01$

Fig. 3a-d (a) Relative change in seed weight of transgenic events RD33 and RD2 compared to wild type parent JL 24 under drought stress in individual trials and across four trials. The change reflects the percent increase over wild type (WT) which is considered as 100%. * denotes 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 wild type parent (b) Yield potential and relative seed yield improvement in the best bet transgenic events (RD2 and RD 33) vis-à-vis untransformed parent under irrigated and drought stress conditions in the field. The transgenic events RD 2 and RD 33 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 ($Yds-\underline{\hat{Y}ds}$) and: (c) the ratio of seed number, and (d) the ratio of 100-seed weight.

Fig. 1

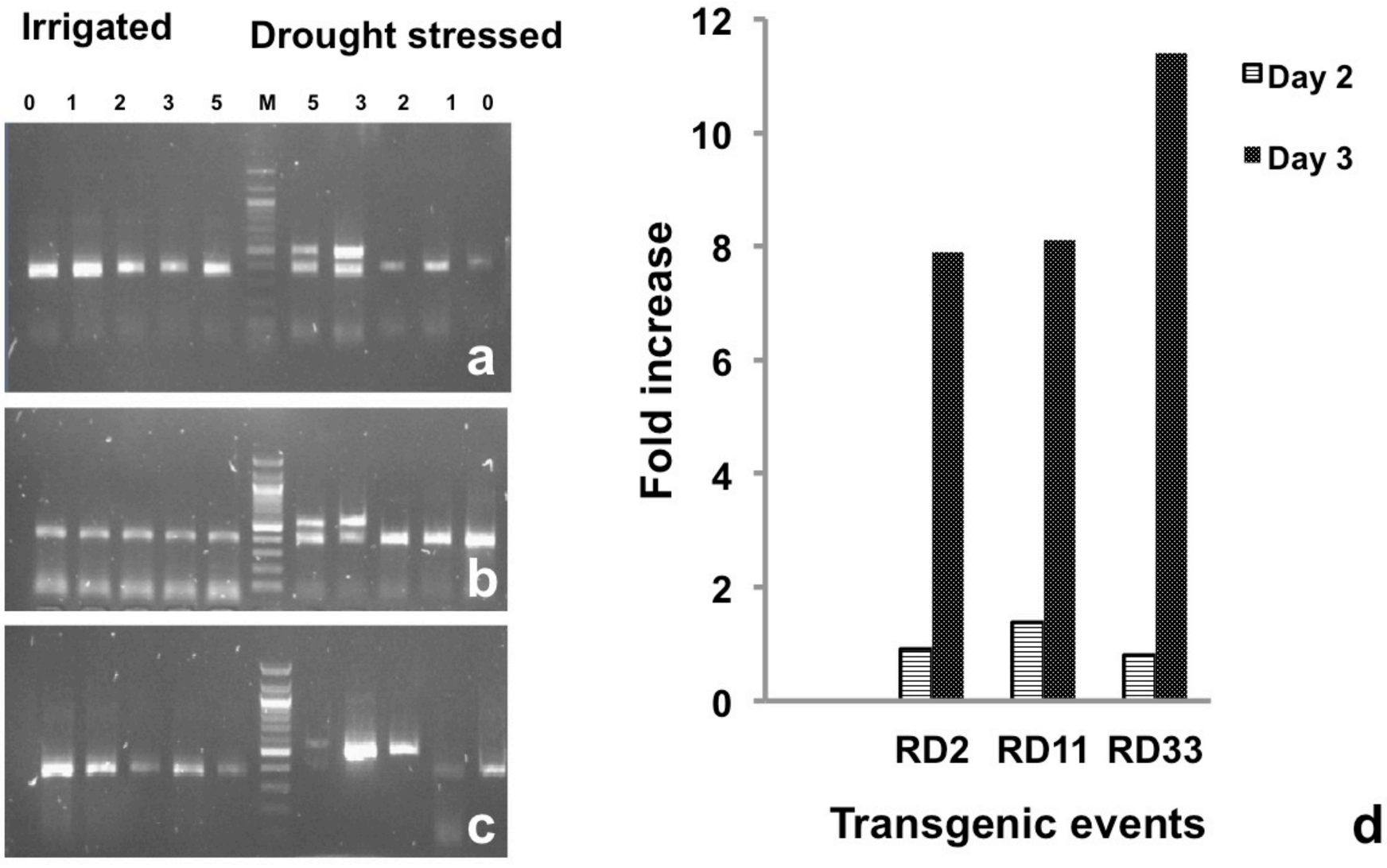
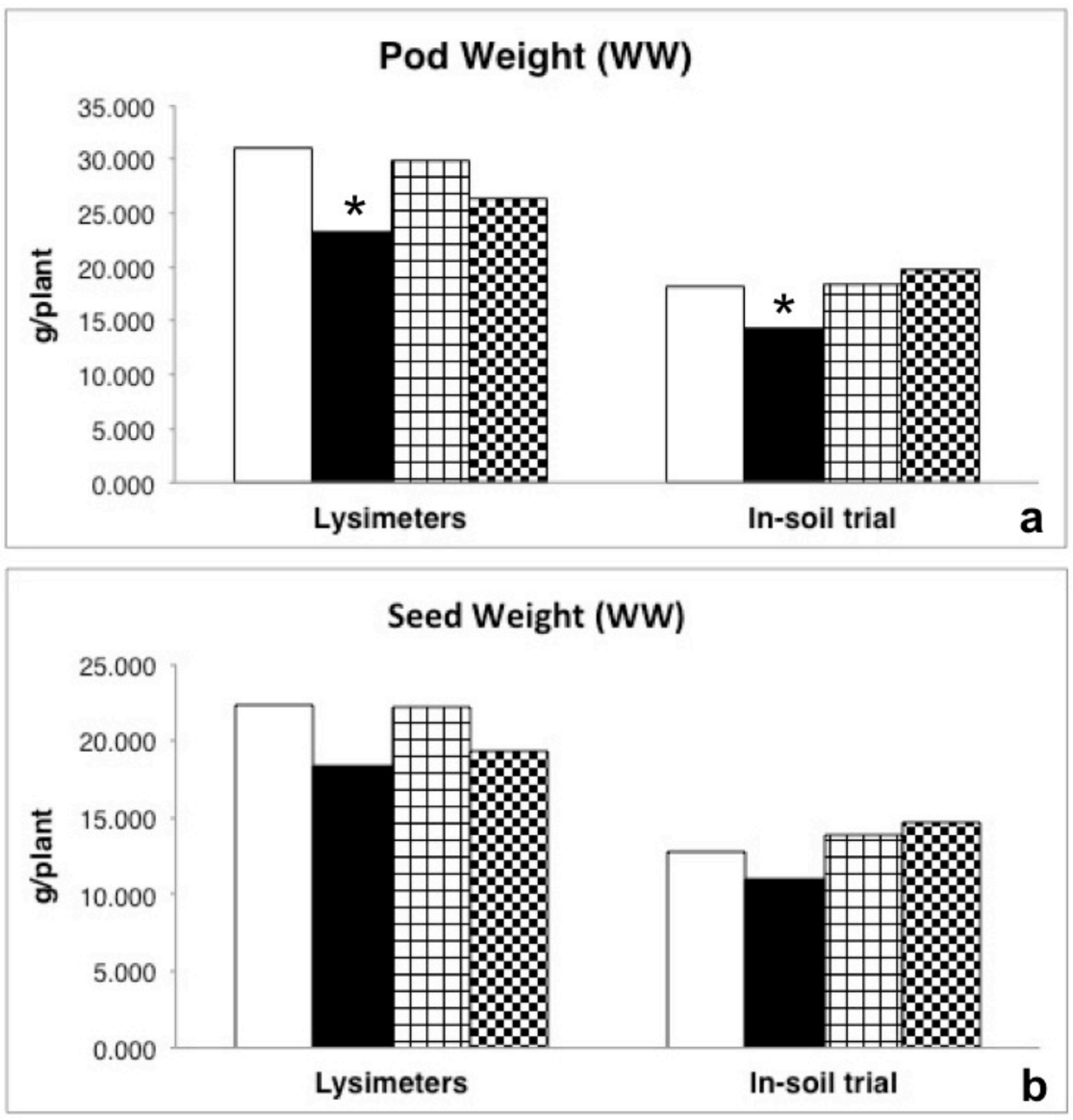
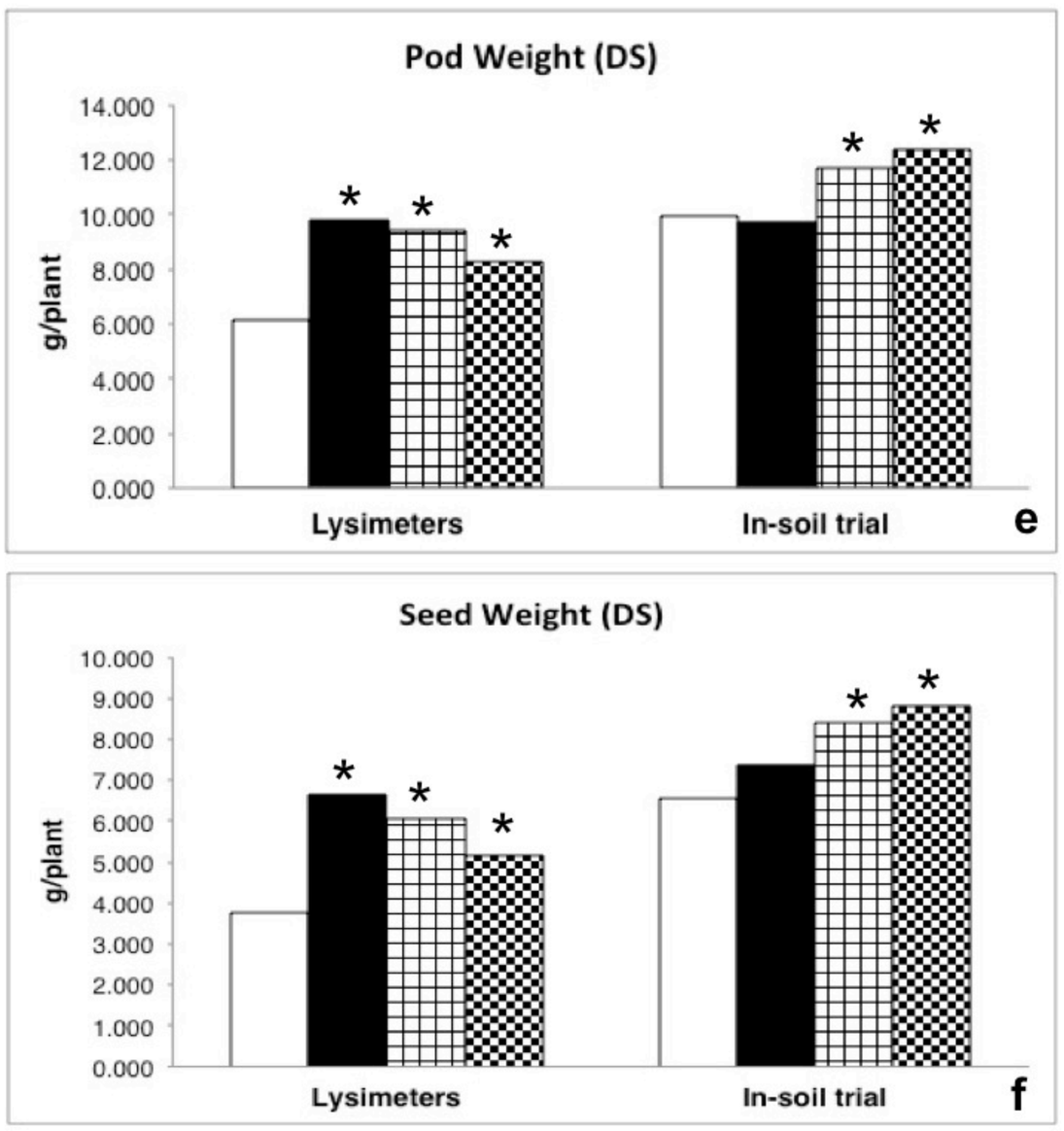
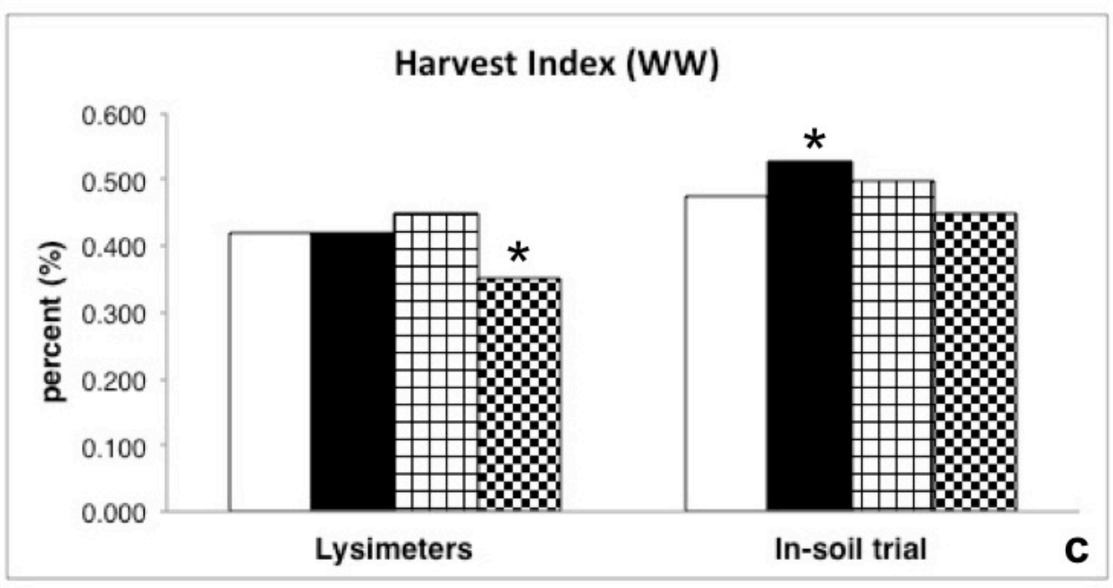
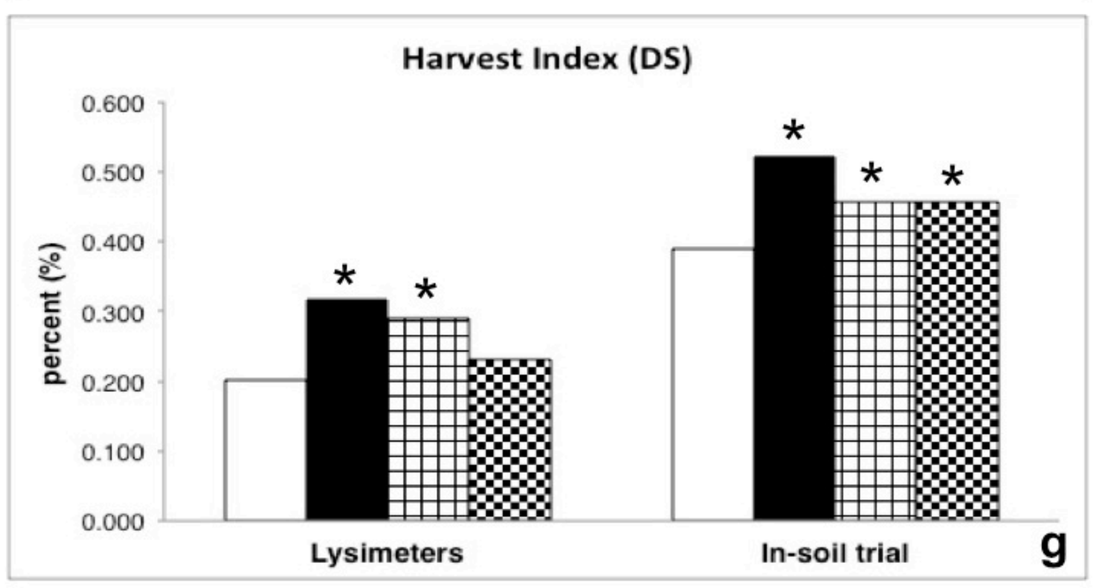

Irrigated
Drought stressed
0 1 2 3 5 M 5 3 2 1 0
a
b
c
Fold increase
12
10
8
6
4
2
0
Day 2
Day 3
RD2 RD11 RD33
Transgenic events
d

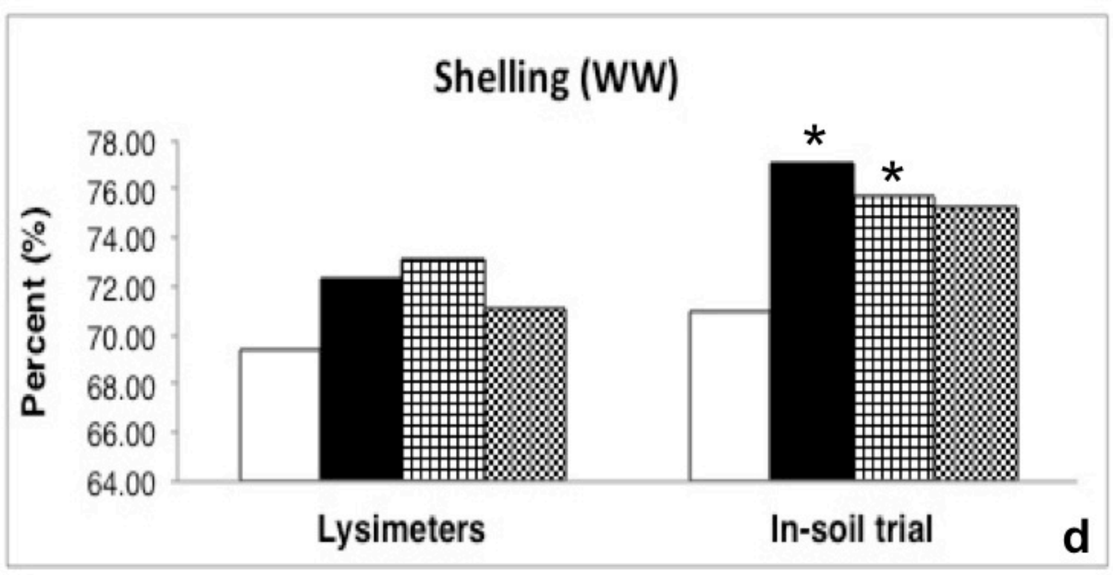
Fig. 2

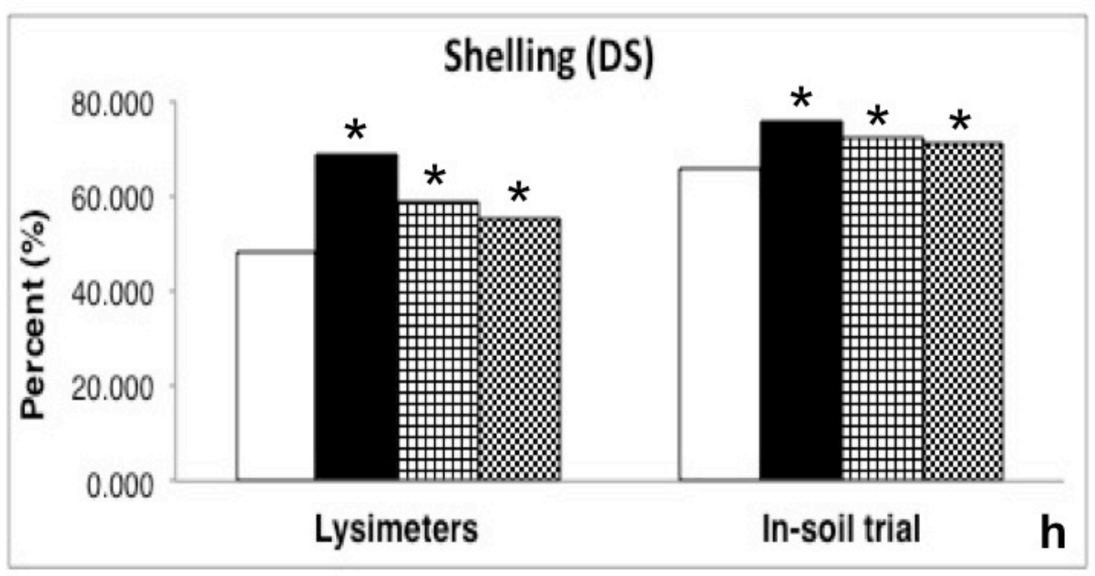






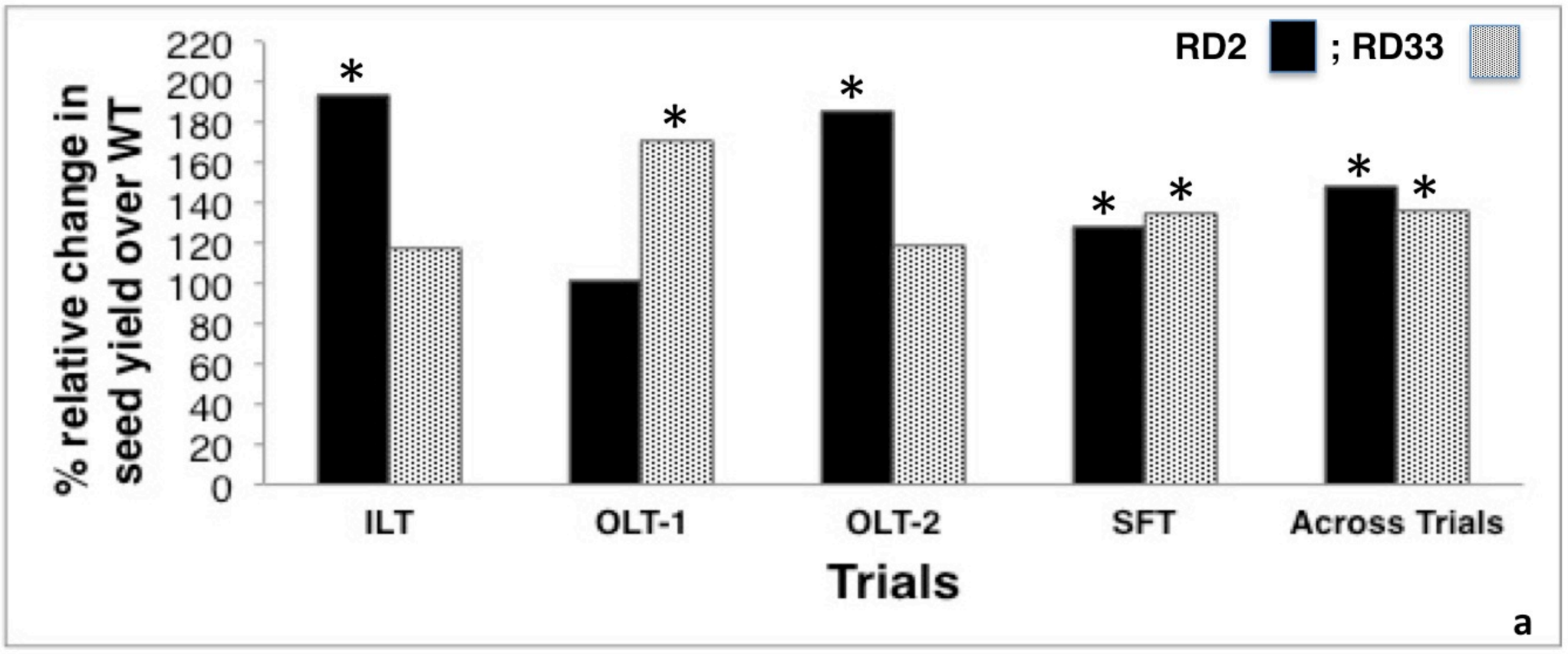


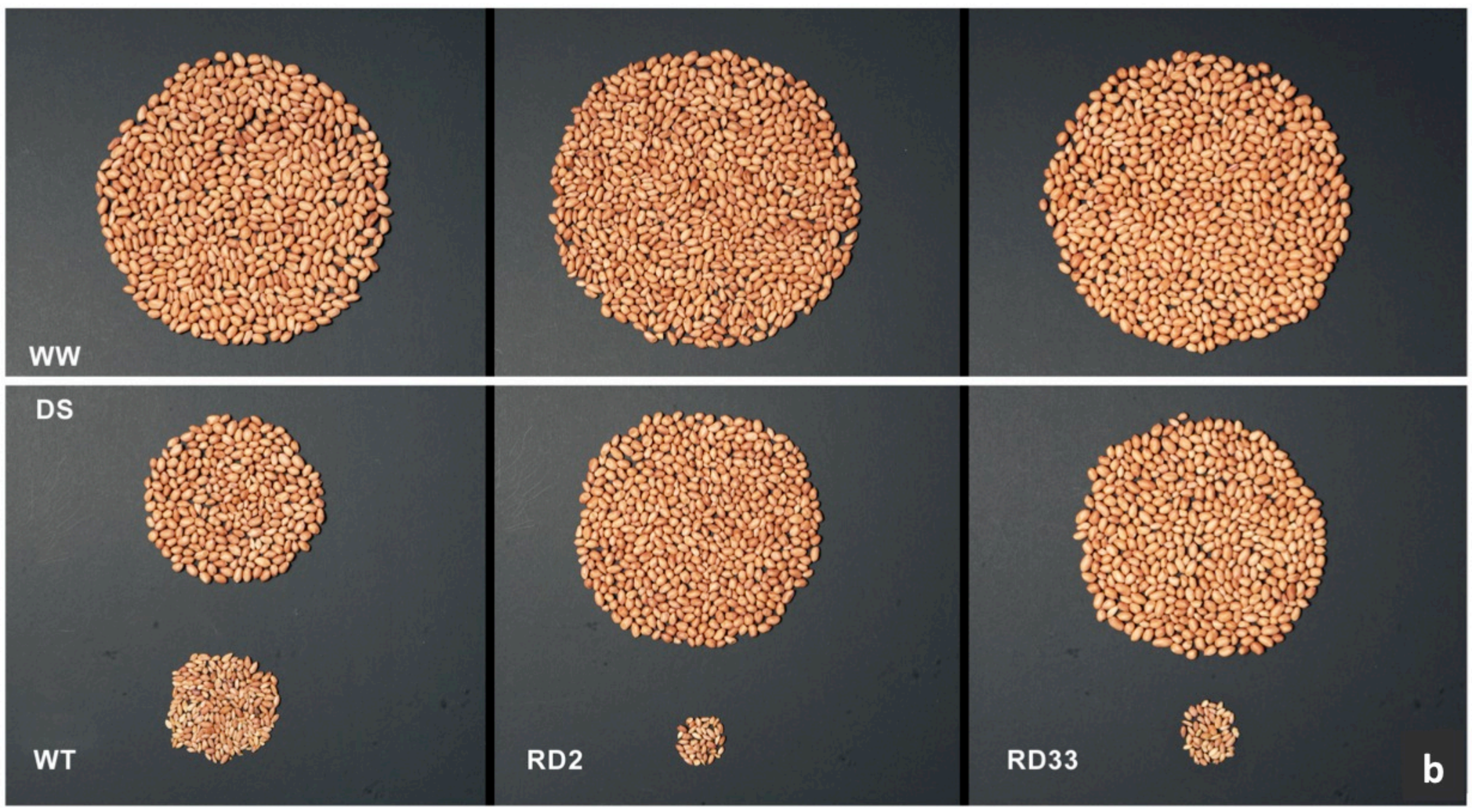


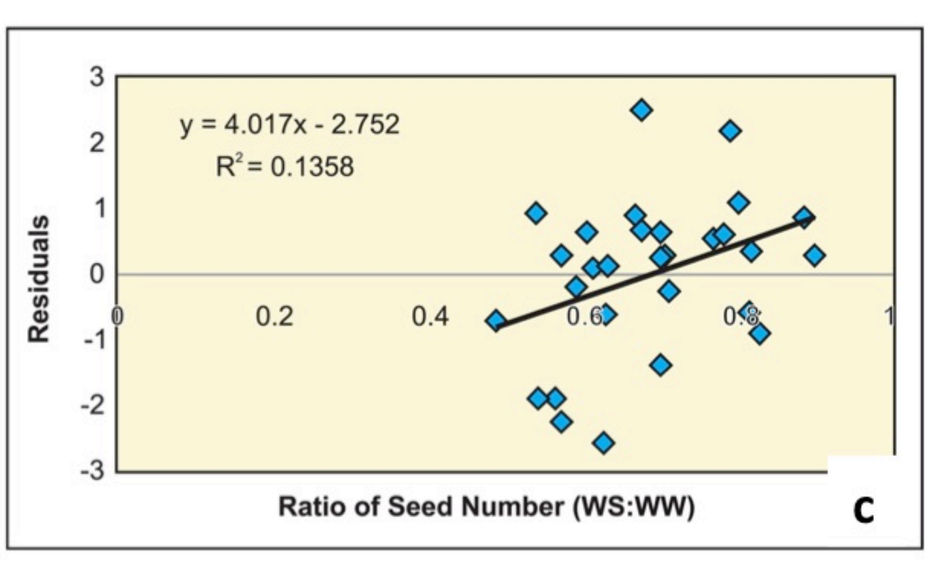


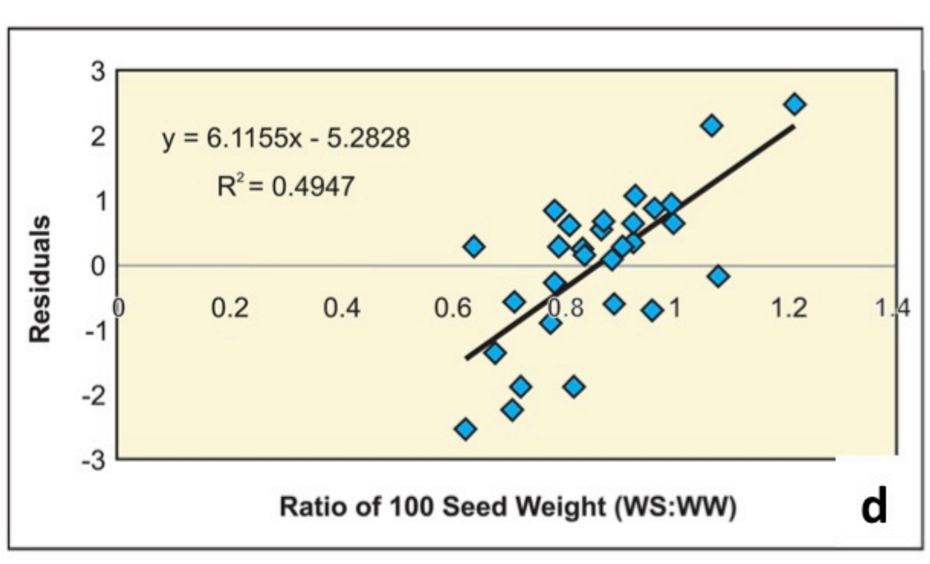
WT ; RD11 ; RD2 ; RD33

Fig. 3


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
a


WW
DS
WT
RD2
RD33
b


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


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