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Simultaneous expression of regulatory genes associated with specific drought-adaptive traits improves drought adaptation in peanut

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Summary

Adaptation of crops to drought-prone rain-fed conditions can be achieved by improving plant traits such as efficient water mining (by superior root characters) and cellular-level tolerance mechanisms. Pyramiding these drought-adaptive traits by simultaneous expression of genes regulating drought-adaptive mechanisms has phenomenal relevance in improving stress tolerance. In this study, we provide evidence that peanut transgenic plants expressing Alfalfa zinc finger 1 (*Alfin1*), a root growth-associated transcription factor gene, *Pennisetum glaucum* heat-shock factor (*PgHSF4*) and Pea DNA helicase (*PDH45*) involved in protein turnover and protection showed improved tolerance, higher growth and productivity under drought stress conditions. Stable integration of all the transgenes was noticed in transgenic lines. The transgenic lines showed higher root growth, cooler crop canopy air temperature difference (less CCATD) and higher relative water content (RWC) under drought stress. Low proline levels in transgenic lines substantiate the maintenance of higher water status. The survival and recovery of transgenic lines was significantly higher under gradual moisture stress conditions with higher biomass. Transgenic lines also showed significant tolerance to ethrel-induced senescence and methyl viologen-induced oxidative stress. Several stress-responsive genes such as heat-shock proteins (*HSPs*), RING box protein-1 (*RBX1*), Aldose reductase, late embryogenesis abundant-5 (*LEA5*) and proline-rich protein-2 (*PRP2*), a gene involved in root growth, showed enhanced expression under stress in transgenic lines. Thus, the simultaneous expression of regulatory genes contributing for drought-adaptive traits can improve crop adaptation and productivity under water-limited conditions.

Introduction

Peanut (*Arachis hypogaea* L.) (Leguminosae) is one of the most important legumes in India, China, the United States, Australia, South America, Central America and Africa. One-third of global peanut production is used for food, and the remaining is used for oil production. Globally, peanut is grown on an average of ~21 million hectares (ha) that produces ~33 million metric tons (Feng *et al.*, 2012). Two-thirds of the world's peanut is produced by India and China. In developing countries, peanut is largely cultivated as a rain-fed crop, and hence, the crop is exposed to intermittent moisture stress. Water deficit-induced desiccation results in loss of turgor affecting leaf expansion and hence decreases net carbon gain and pod yields. Peanut plants have evolved diverse adaptive strategies to cope with water deficit conditions (Nageswara Rao *et al.*, 1985; Nautiyal *et al.*, 1995). When tissue water status decreases under prolonged soil water deficit condition, cellular tolerance mechanisms help in maintaining the cell metabolic activities leading to the adaptation of plants to water deficit condition (Bandurska, 1998). Cellular tolerance mechanisms during water deficit conditions are many and mainly include osmotic adjustment (Laszlo and Arnould, 2009), cell cycle control, protein chaperoning (Kovacs *et al.*, 2008) and repair, removal of damaged proteins, production of scavenging enzymes and chromatin stabilization (Blum, 2005; Klutz, 2005). Diverse stress-responsive genes that regulate drought tolerance mechanisms have been shown to be up-regulated when tissue water potential decreases (Bernacchia and Furini, 2004; Govind *et al.*, 2009). Functional characterization of many of these stress-responsive genes has shown to sustain cellular metabolism under stress conditions (Amin *et al.*, 2012; Amuda and Balasubramani, 2011; Dang *et al.*, 2011). Genes that regulate DNA unwinding (Amin *et al.*, 2012; Luo *et al.*, 2009), stress-responsive transcription factors, chaperones involved in removal of secondary structures (Sanan-Mishra *et al.*, 2005) and chaperones involved in protein processing, folding and protection (Grover *et al.*, 2013) and genes that involved in the protein degradation (Chen and Hellmann, 2013) play a significant role in cellular tolerance mechanisms.

Protein turnover, stability and folding are the major factors that sustain cellular tolerance under stress. Although transcription and protein synthesis function in a coordinated manner, it has been shown that under stress, such an association significantly diminishes (Foss *et al.*, 2011). A few of the genes that regulate

these processes have been shown to improve stress tolerance in plants. In this regard, RNA helicases play an important role in translation process by unwinding the energetically stable mRNA secondary structure. Several reports have shown that stress-responsive RNA helicases improve stress adaptation (Patel *et al.*, 2002; Owttrim, 2006). Overexpression of a salt stress-responsive Pea DNA helicase (*PDH45*), which is a homologue of eukaryotic translation initiation factor *eIF-4A,* conferred salinity tolerance in tobacco plants (Sanan-Mishra *et al.*, 2005). Several heat-shock protein (HSP) chaperones have been reported to increase protein stability under stress (Liu *et al.*, 2013) and improve adaptation of cells with reduced turgor (Liu *et al.*, 2013). Stress-induced transcription factors such as heat-shock factors (HSFs) play a critical role in activating HSPs (Mittal *et al.*, 2011; Swindell *et al.*, 2007). Several reports clearly demonstrated improved tolerance of transgenic plants to diverse abiotic stress by overexpressing several HSFs (Zhu *et al.*, 2006). Transgenic rice overexpressing *OsHSFA7* showed higher survival rate under abiotic stresses (Liu *et al.*, 2013). Overexpression of *AtHSFA1* gene enhanced thermotolerance in *Arabidopsis*, soya bean and tomato (Zhu *et al.*, 2006). Expression analysis of *Pennisutum glaucum* heat-shock factor (*PgHSF4)* in *P. glaucum* showed maximum increase in transcript level in response to heat stress within 30 min of exposure (Reddy *et al.*, 2009).

Apart from improving cellular tolerance, it is also important to maintain plant water relations to sustain the growth under moisture stress. The plant mechanisms associated with traits such as better water mining by superior root architecture, water use efficiency (WUE) and water conservation traits have relevance in avoiding drought stress (Bartels and Sunkar, 2005). Many studies have shown that genotypes with superior root system maintained the leaf turgor and cooler leaf temperature for longer periods under continuous soil water-depleted conditions (Mohammadi *et al.*, 2012; Tuberosa, 2012). The regulatory networks involved in root growth and patterning (architecture) were elucidated in *Arabidopsis* by global gene expression studies (Jung and McCouch, 2013). Functional role of several of these genes in root growth and development has been demonstrated by mutant and overexpression studies (Hodge *et al.*, 2009; Ivashuta *et al.*, 2005; Winicov, 2000). The regulatory genes such as aberrant lateral root formation-4 (*ALF4), SOLITARY-ROOT (SLR)* and *NAC1 (NAM, ATAF1, 2, CUC2)* have been shown to play a role in regulating root growth and development (Celenza *et al.*, 1995; DiDonato *et al.*, 2004; Fukaki *et al.*, 2002; Xie *et al.*, 2002). *Alfin1* is a PHD finger protein with Cys4 and HisCys3 finger structures that have DNA-binding motif suggesting that they have a role in transcriptional regulation (Bastola *et al.*, 1998). The overexpression of *Alfin1* in alfalfa showed significant improvement in root growth (Winicov, 2000). Our studies also demonstrated improved root growth in *Alfin1* overexpressing tobacco plants (Nethra, 2010).

Intermittent moisture stress is a typical stress the crop faces in rain-fed agriculture scenario. Hence, besides improving cellular-level tolerance mechanisms leading to better recovery of plants upon stress alleviation, sustaining transpiration during the recovery period by efficient water mining is necessary to improve the carbon gain and growth rates. With this background, traits associated with higher water flux to shoot as well as cellular tolerance mechanisms are important not only to avoid initial stress but also for improved recovery growth upon stress alleviation. It is therefore desirable to bring together these traits in a single genetic background. Simultaneous expression of multiple genes is one of the options to introgress diverse stress-adaptive traits in one genetic background. Recently, we demonstrated that the expression of *AtbHLH17* and *AtWRKY28* transcription factor genes simultaneously in *Arabidopsis* resulted in enhanced tolerance to diverse abiotic stresses by up-regulating many downstream target genes (Babitha *et al.*, 2013). Similarly, the simultaneous expression of stress-specific transcription factors *EcNAC1*, *EcMYC57* and *EcbZIP60* that regulates the diverse downstream genes associated with protein stability substantially improved stress tolerance (Babitha, 2012). These reports clearly demonstrate that the expression of multiple genes regulating drought-adaptive traits can be a potential option to engineer plants for better adaptability to adverse environmental conditions.

The emphasis of this study is to improve the adaptation of peanut to drought stress by altering cellular tolerance mechanisms and root traits. The cellular tolerance mechanisms and higher root growth traits significantly contribute to improve the intrinsic tolerance of plant cell and increase the water uptake and mining abilities to sustain growth and development under drought stress. Therefore, genes involved in protein turnover, protein stability and protein folding (*PDH45, PgHSF4*) and root growth-associated gene (*Alfin1*) were simultaneously expressed in peanut resulting in improved tolerance to drought stress. The transgenic plants showed significantly low canopy temperature, improved root growth and higher productivity.

Results

Development of multigene construct and identification of putative peanut transformants

The multigene expressing binary vector *pKM12GW* harbouring three genes driven by individual promoter and terminator, *RD29A*:*PgHSF4*:*nos* (*RD29A* is a stress-inducible promoter (Kasuga *et al.*, 2004), *2X35SCaMV:PDH45:polyA and 35SCaMV:OsAlfin1*:*polyA,* with kanamycin selection marker was developed using modified Multisite Gateway technology (Vemanna *et al.*, 2013) (Figure 1a,b). The embryonal axes of peanut seeds were used to transform the multigene construct using *Agrobacterium*-mediated *in planta* transformation method (Rohini and Rao, 2000). The putative peanut transformants were identified in T_1 generation initially by screening the seedlings on kanamycin (Figure 1c). Many of these kanamycin-positive plants showed the amplification of marker gene *neomycin phosphotransferase* (*nptII*) indicating the integration of T-DNA into the transformed plants. The kanamycin-positive plants with confirmed integration of *nptII* were further screened for high temperature tolerance. Many *nptII*-positive plants showed significantly high thermotolerance when compared to wild type (Figure 1d). In these plants, the integration of *PgHSF4* was confirmed using *RD29A* promoter-specific forward primer and *PgHSF4* gene-specific reverse primer. Similarly, integration of *PDH45* and *OsAlfin1* genes was confirmed using gene-specific primers (Figure S1). The expression of all the three transgenes was assessed in a few selected plants, which showed high recovery growth upon high temperature treatment. The transcript levels of *PDH45*, *HSF4* and *OsAlfin1* genes were significantly high in these transgenic plants (Figure 1e). The accumulation of *Alfin1* and *PDH45* transcripts in nonstressed transgenic leaves indicates the constitutive expression of transgene in transformed peanut plants (Figure 1e). However, as *HSF4* was driven by *RD29A*, a stress-inducible promoter, the transcript levels were analysed in stress-induced

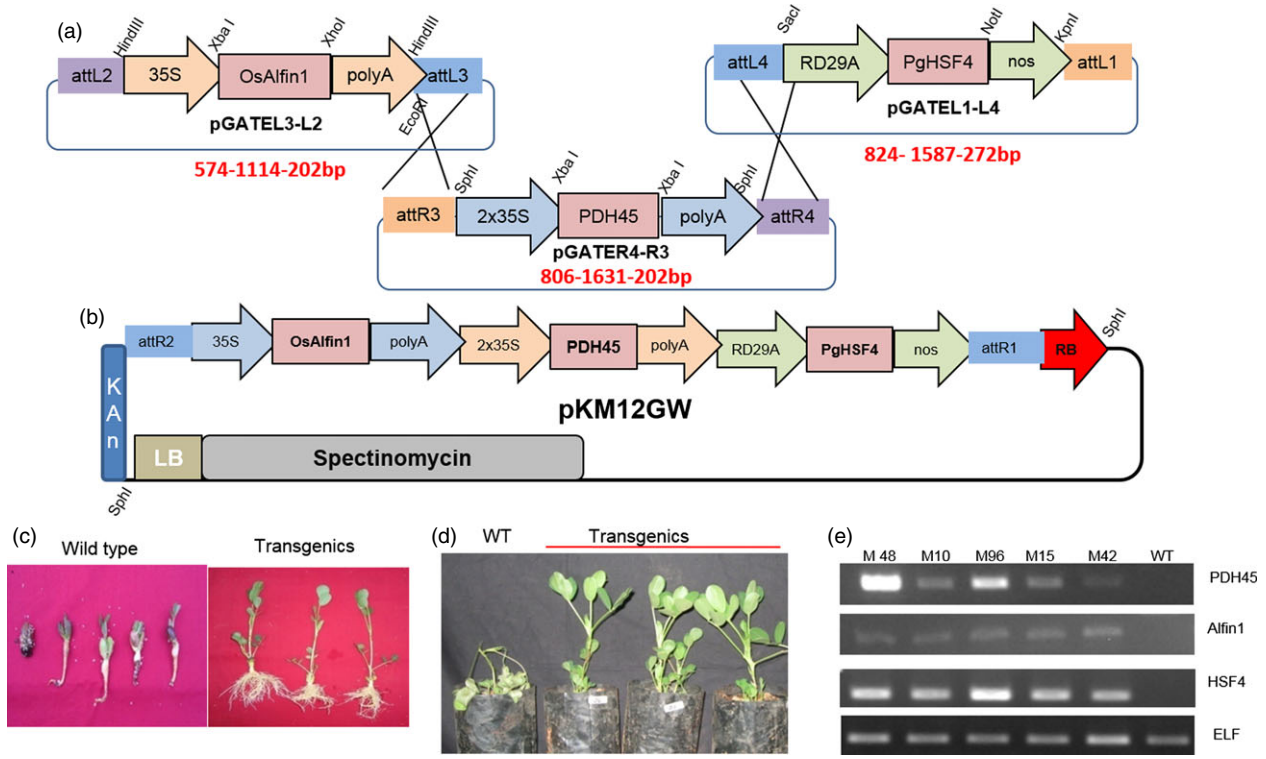

Figure 1 Development of multigene expressing transgenic plants and its response to kanamycin and temperature. Construction of multigene expression vector, (a) pGATE entry vectors with respective gene cassettes. (b) Destination vector pKM12GW after LR reaction harbouring three genes. (c) Response of putative transformants to 450 ppm of kanamycin. (d) Temperature stress response of kanamycin-positive plants. The plants were exposed to temperature induction response at 32–42 °C for 5 h and subsequently for 50 °C for 5 h. (e) Expression analysis of transgenic plants (M40, M42, M78, M86, M97,M100) by RT-PCR. The cDNA was amplified with gene-specific primers. *Elongation factor (ELF)* was used as housekeeping gene.

transgenic and wild-type plant tissues and higher expression of *HSF4* was observed in the transgenic plants. Based on the phenotype, kanamycin response, temperature tolerance and molecular characterization, 102 plants were further advanced to T_2 generation. In T_2 generation, 20 seedlings from each line were rigorously screened for kanamycin sensitivity (Figure S2). The lines that showed more than 90% of survived seedlings on kanamycin were considered for further advancement. The distinctly kanamycin-tolerant positive plants were established in pots and advanced to T_3 generation after confirming *nptII* amplification (Figure S3). Based on this analysis and phenotypic uniformity, 99 lines were advanced to T_3 generation.

Response of peanut transgenic plants to moisture stress

To study the moisture stress response of peanut transgenic plants, 99 lines from T_3 generation were grown in soil under contained glasshouse facility till 50th day, and then, gradual moisture stress was imposed for 20 days as described in Materials and Methods section. After 10 days of moisture stress imposition, canopy temperature of five plants from each line was measured using infrared (IR) thermometer. Many transgenic lines showed lower canopy temperature with more negative crop canopy air temperature difference (CCATD) values compared to wild-type plants. Based on CCATD, lines were classified into different categories. In these categories, seven lines showed +4 °C to +1 °C, 27 lines showed +1 °C to −1 °C, 44 lines showed −1 °C to −3 °C, and 22 lines showed −3 °C to −5 °C CCATD (Figure 2a). Amongst all the transgenic lines assessed, 18 lines were identified which showed higher negative CCATD (Figure 2b) with significantly higher relative water content (RWC) compared to wild-type plants (Figure 2c). These lines had low proline levels compared to wild-type plants (Figure 2d). In the selected transgenic events, a negative correlation was observed between RWC and proline content (Figure 2f) and also between RWC and CCATD (Figure 2e).

Growth and yield characteristics of transgenic plants

Peanut transgenic plants grown under drought conditions in soil were also analysed for phenotypic characters such as leaf size and stay green nature and also for pod yield. The cv. GPBD-4 used in this study is a Spanish bunch type with medium-sized elliptical leaf. Transgenic plants had bigger leaves, more leaf area and increased plant height as compared to wild-type plants (Table S1, Figure 3a). The average leaf size of 4th leaf in selected transgenic lines was 26.02 cm^2, whereas in wild type, it was 20.66 cm^2. A similar trend was seen in other leaves also. However, there was not much increase in the number of leaves between transgenic and wild type. Further, the leaves of transgenic plants maintained stay green characteristics even after 120 days, whereas wild-type plants showed senescence symptoms much earlier (Figure 3b). Under moisture stress conditions, productivity and yield losses occur; therefore, yield response of transgenic plants to sustain productivity is one of the criteria followed to characterize the transgenic plants (Figure 3c). Hence, the transgenic and wild-type plants were analysed for yield characters. Based on the pod dry weight, the transgenic lines were grouped into different categories. In the first category, 27 transgenic lines had pod weight similar to wild-type plants,

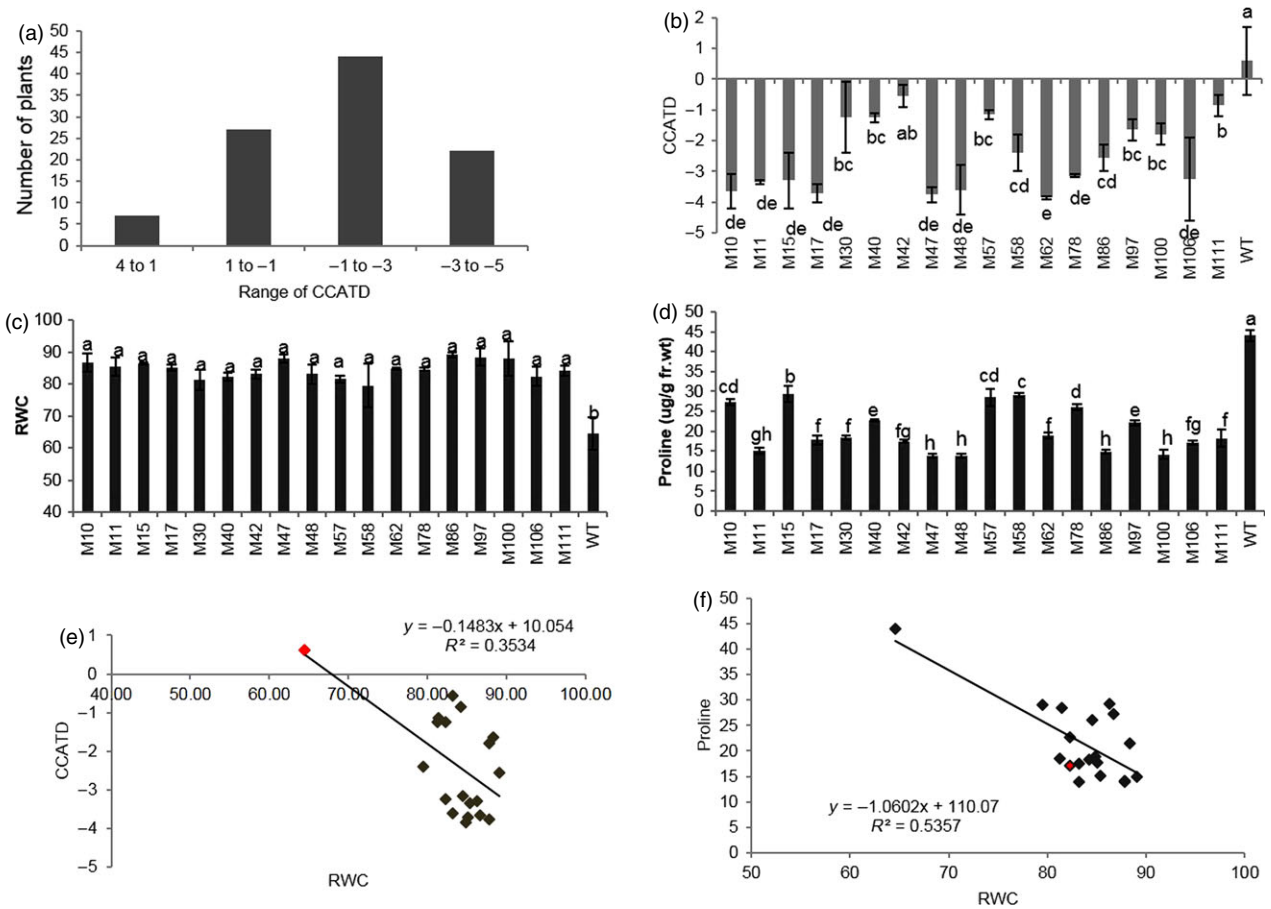

Figure 2 Stress response of peanut transgenic plants on exposure to mid-season moisture stress. (a) Frequency distribution of plants based on crop canopy air temperature difference (CCATD). (b) Variation in CCATD (n = 5 and P = 0.05). (c) Variation in relative water content (RWC) (n = 5 and P = 0.05). (d) Proline content in the selected lines (n = 5 and P = 0.05). (e) Correlation between RWC and CCATD. (f) Correlation between proline and RWC. Fifty-day-old transgenic and wild-type plants were exposed to mid-season moisture stress. CCATD was measured on 10th day, whereas RWC and proline were measured on 15th day after stress imposition.

whereas the pod yields of transgenic lines in 2nd, 3rd and 4th categories were 20, 43 and 56% higher than wild type. The mean pod weight of transgenic lines of 4th category was 41.6 g compared to 19 g in wild type (Figure 3d). The lines were also grouped based on total dry matter (TDM). Many lines showed higher accumulation of TDM; 34 lines showed 93–110 g in 1st category, 19 lines showed 111–125 g in 2nd category, and 17 lines showed more than 125 g of TDM in 3rd category. The biomass of remaining 29 lines was on par with wild type (Figure 3e). The biomass was consistently higher in transgenic lines and showed a positive correlation with pod yield (Figure 3f). Similarly, a positive correlation was seen between pod weight and pod number, indicating that observed increase in pod yield was due to higher pod number per plant (Figure 3g). Although differences existed in mean pod weight, there was no positive correlation between pod weight and mean pod size (Figure 3h). Based on yield responses and canopy temperature, 12 transgenic lines were selected for further characterization.

Transgenic plants showed tolerance to methyl viologen-induced oxidative stress and ethrel-induced senescence

To assess the effect of ethylene-associated senescence in these selected transgenic lines, leaf discs from 60-day-old plants grown in pots were exposed to 1500 ppm of ethrel. The extent of ethrel-induced chlorophyll degradation in transgenic plants was significantly less compared to wild-type plants (Figure 4a,b).

Another set of leaf discs was exposed to methyl viologen (MV) (10 μM) to induce oxidative stress. Transgenic plants showed improved tolerance to oxidative stress than wild type (Figure 4c). The production of reactive aldehydes was used as a biomarker to measure the level of damage caused by oxidative stress and to assess the oxidative stress-induced lipid peroxidation. Transgenic plants showed significantly reduced levels of malondialdehyde (MDA) compared to wild-type plants (Figure 4d). Some of the transgenic lines such as M86, M97, M100 and M111 showed relatively more chlorophyll stability and less MDA levels.

Root growth analysis and moisture stress response of selected transgenic lines

To study the variation in root growth and response to moisture stress, six transgenic lines with relatively more negative CCATD values and improved tolerance to ethrel were selected. The root growth was assessed at different developmental stages of plant growth. In addition, the intrinsic tolerance of these lines under defined moisture stress regimes was also examined based on recovery growth after stress.

Root growth of transgenic plants: The root characters of selected six transgenic lines were studied at different growth

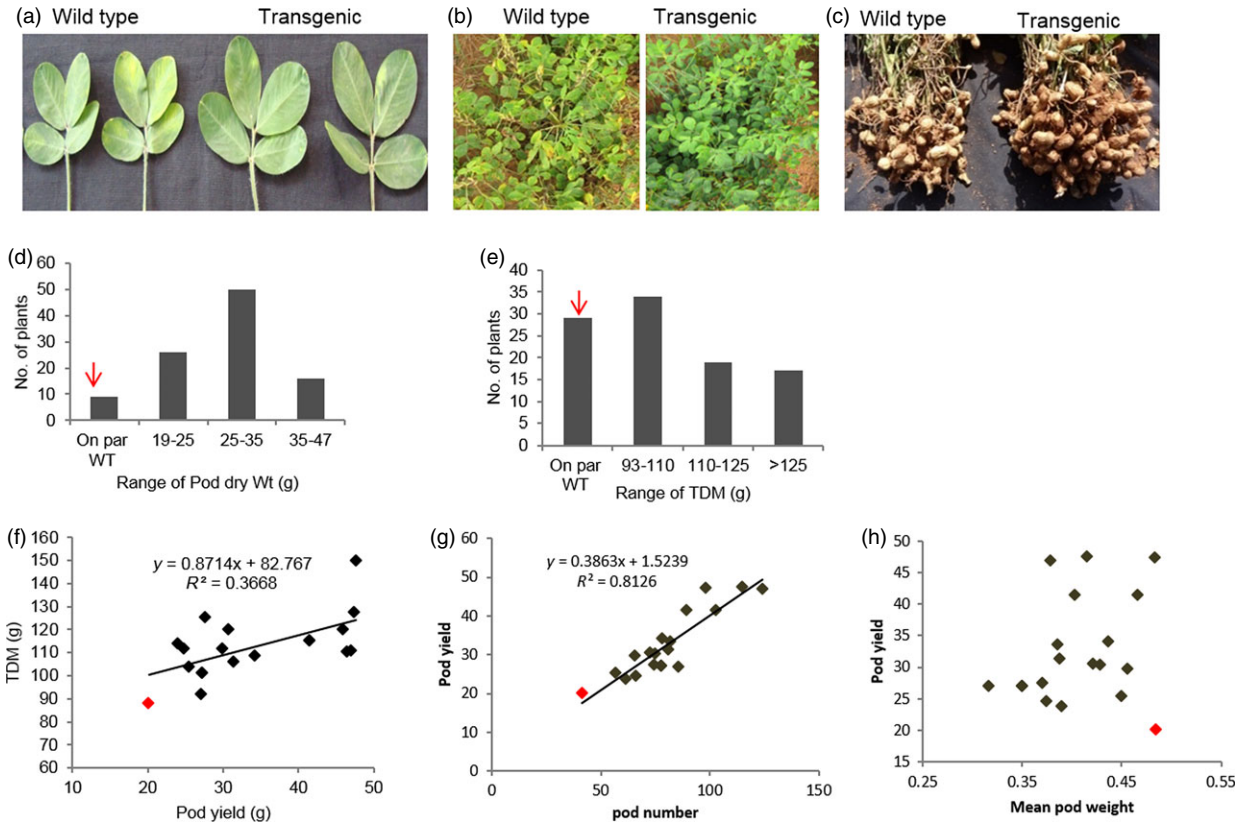

Figure 3 Growth and yield characteristics of transgenic plants. Photograph showing difference in (a) leaflet size. (b) Stay green phenotype. (c) Yield between wild-type and transgenic plants. (d) and (e) Frequency distribution of lines based on pod dry weight (yield) and total dry matter (TDM) per plant, respectively. Correlation analysis between (f) pod yield and biomass. (g) Pod yield and pod number. (h) Pod yield and mean pod weight.

stages under nonstress condition. At early stages of growth, the transgenic plants showed twofold increase in root growth compared to wild type. The transgenic plants maintained increased root growth at all growth stages and even at harvest (120 days) (Figure 5a–e). Besides root length, the lateral root number was significantly higher in transgenic plants (Figure 5f). The root to shoot ratio in 30-day-old transgenic plants was significantly higher than wild-type plants (Figure 5g).

Moisture stress recovery response of transgenic plants: The moisture stress response of 20-day-old transgenic plants was assessed by gradually imposing moisture stress gravimetrically. In each pot, one plant each from transgenic event and wild-type plants was interplanted to assess the relative performance under similar soil moisture status. At the end of stress period, transgenic plants showed less severe wilting symptoms compared to wild-type plants (Figure S4). The leaf RWC at the end of stress was significantly higher in many transgenic events compared to wild type, although both the plants were grown in the same soil moisture status. The improved leaf water status can be attributed to higher root growth and moisture retention capacity of the leaves of the transgenic plants upon stress alleviation. The recovery growth of transgenic lines was significantly superior over the wild-type plants and showed significantly higher root growth than wild-type plants (Figure 6a,b). The total biomass recorded at the end of the recovery period was substantially high in transgenic plants (Figure 6c). In addition, the statistical analysis showed significant variation in root growth between wild-type and transgenic plants (Table S2). Amongst the transgenic lines tested, M40, M86, M97 and M100 showed significantly higher root growth and biomass than other transgenic lines.

Further, to study the relevance of *Alfin1* overexpression in root growth, we compared the relative improvement of root growth of *Alfin1*, *PDH45* and *HSF4* overexpressing multigene transgenic plants with *HSF4 and PDH45* single gene overexpressing transgenic peanut plants that were developed earlier in our group. The multigene expressing transgenic plants showed significantly improved root growth compared to wild-type and transgenic plants individually overexpressing *PDH45* and *HSF4* (Figure S5).

Stable integration and expression of stress-responsive genes

The stable integration of transgenes was assessed by genomic Southern blot analysis in three transgenic lines that showed superior survival at the end of stress and high recovery growth on stress alleviation (Figure 7). Southern blot analysis of transgenic plants demonstrated that the transgenes were stably integrated into the genome of transgenic peanut lines (Figure 7a) with single copy of the transgene.

The transcript levels of a few stress-responsive genes were analysed by RT-PCR in two transgenic lines exposed to moisture stress for 4 days. Most of the genes (heat-shock proteins *HSP17.9* and *HSP60, aldehyde dehydrogenase (ADH1), Really interesting gene box-1 (RBX1)* and *Aldose reductase*) showed enhanced expression in transgenic plants than wild type under stress except for the transcript levels of *ADH1* which was less in the transgenic line M97. The transcript induction of pyrroline-5-carboxylate

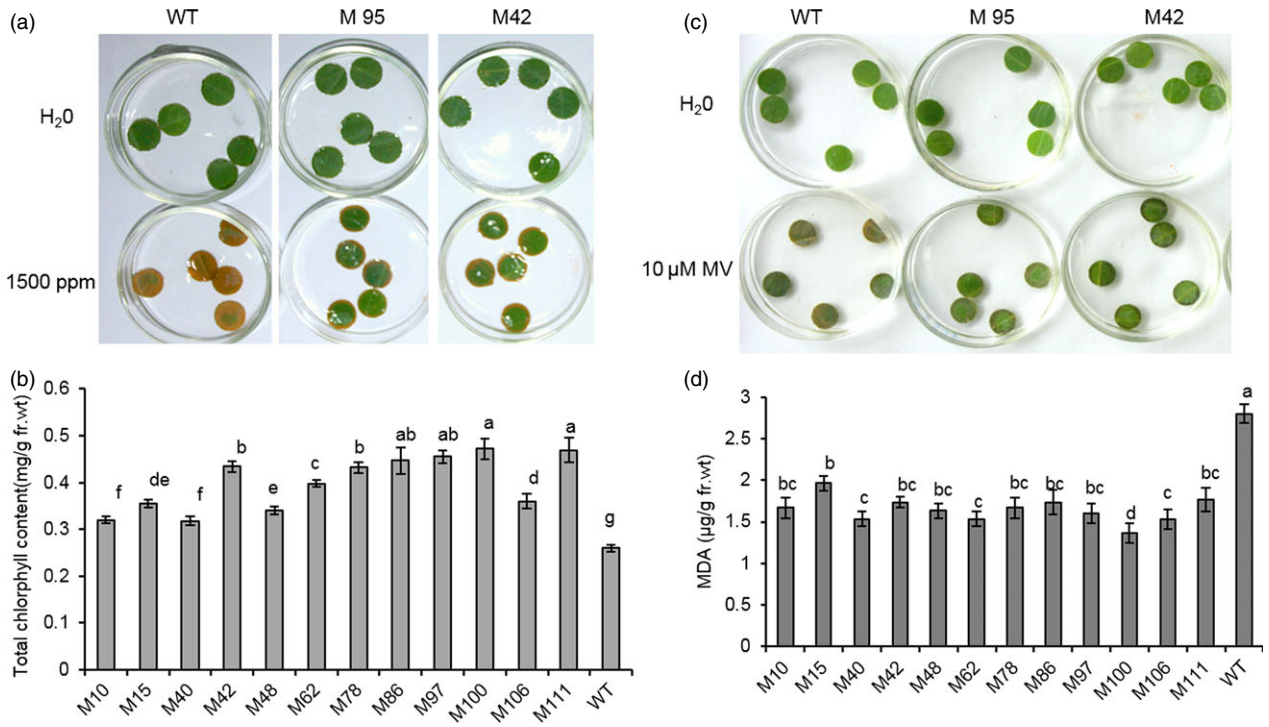

Figure 4 Stress response of peanut transgenic plants assessed by excised leaf disc assay. Leaf discs from 4th fully expanded leaf of transgenic and wild-type plants were exposed to 1500 ppm ethrel under dark and 10 µM methyl viologen at 200–300 µmol/m^2/s light intensity. Photographs showing response to (a) ethrel-induced senescence and (c) methyl viologen-induced oxidative stress, respectively. (b) & (d) Total chlorophyll content from the ethrel-treated leaf discs and malondialdehyde (MDA) content from MV-induced oxidative-stressed leaf discs, respectively (n = 5 replications for each line and P = 0.05).

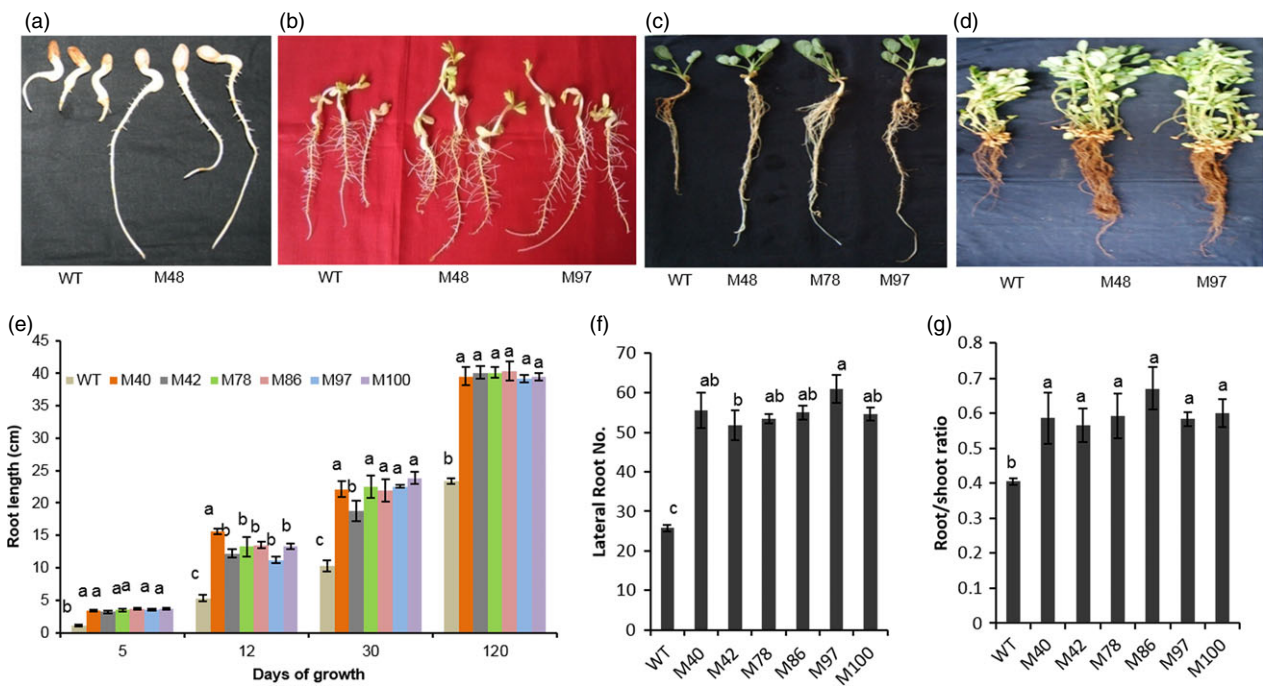

Figure 5 Variation in root growth of peanut transgenic plants expressing *OsAlfin1, PDH45 and PgHSF4* genes at different stages of plant growth. The plants were grown in pots, a subset of plants was harvested on 5, 12, 30 and 120 days of germination, and root growth was measured. Root growth variation at (a) initial stage of seed germination after 5 days, (b) at 12 days of growth, (c) at 30 days of plant establishment and (d) harvesting stage. (e) The root length measured at different stages of plant growth. (f) Variation in lateral root number in 12-day-old transgenic and wild-type plants. (g) Root to shoot ratio in 30-day-old plants (n = 5 seedlings per line and P = 0.05).

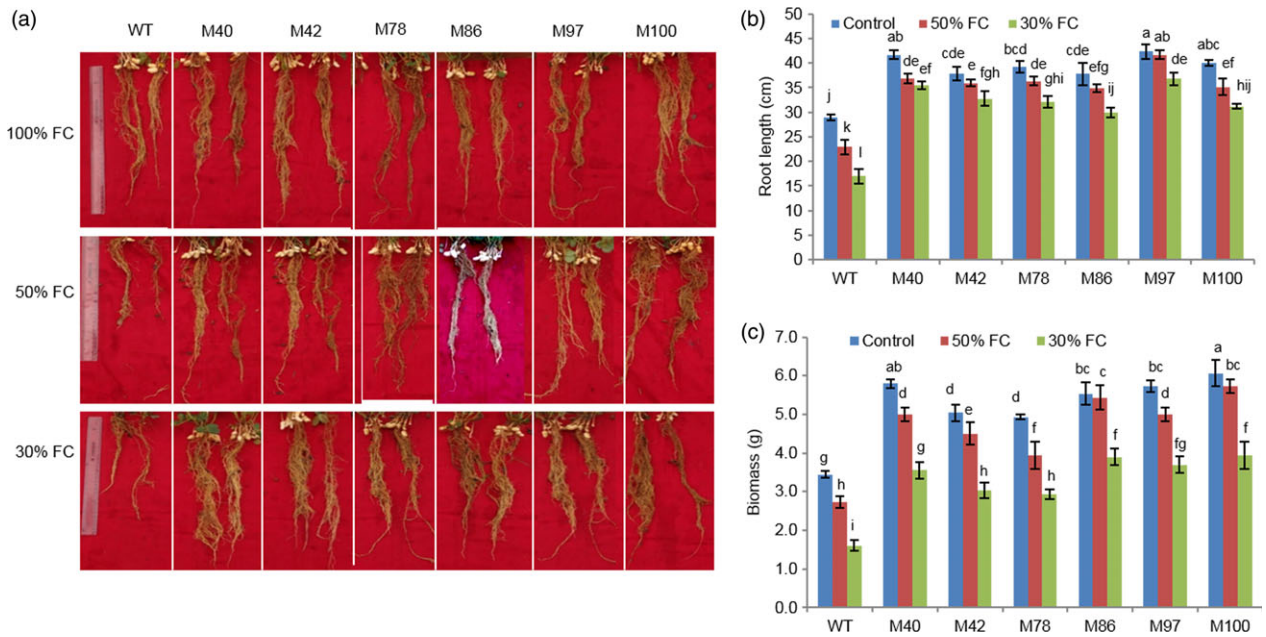

Figure 6 Response of *OsAlfin1, PDH45 and PgHSF4* expressing transgenic plants under simulated drought conditions. Peanut plants were subjected to gradual moisture stress with two different field capacities (50% and 30% FC) and later re-watered to recover. (a) Photographs of representative plants showing root growth after recovery. (b) Graph showing root length. (c) Biomass of transgenic plants under two different levels of moisture stress ($N = 5$ and $P = 0.05$; five replications from each line were maintained as described in M&M).

synthetase (*P5CS)* was less than other genes, but the transgenic plants still showed higher levels than wild type. However, late embryogenesis abundant-5 (*LEA5)* gene showed similar expression levels in wild-type and transgenic plants even after exposure to stress. Further, the root growth-associated proline-rich protein-2 (*PRP2)* showed significant up-regulation of transcripts in the transgenic plants (Figure 7b).

Discussion

Moisture stress experienced by plants under rain-fed conditions is complex as magnitude and duration is unpredictable and also affected by soil texture and vapour pressure deficit (VPD). Plants have evolved several adaptive mechanisms to cope up with diverse moisture stress situations. In response to a moderate

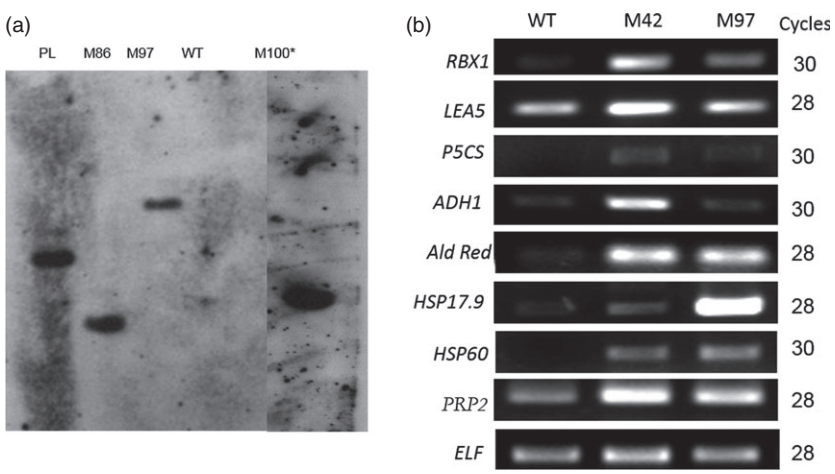

Figure 7 Integration and expression analysis of stress-responsive genes. (a) Southern blot analysis copy number and integration of multigene cassette in peanut transgenic plants. Thirty micrograms of genomic DNA from three independent plants from each line was digested with *EcoRI,* and Dig-labelled (GE-Amersham) *Alfin1* probe was used to hybridize the DNA (*- blot was done in a separate experiment). (b) Downstream target gene expression under moisture stress. Twenty-day-old transgenic and wild-type plants were exposed to moisture stress by withholding water for 4 days. The tissue was collected, total *RNA* was isolated, and cDNA that was prepared was used for *RT-PCR* analysis with gene-specific primers. Heat-shock proteins (HSP17.9, HSP60), aldehyde dehydrogenase (ADH1), Really interesting gene box 1 (RBX1), Aldose reductase (ALR), proline-rich protein-2 (PRP2), late embryogenesis abundant-5 (LEA5), pyrroline-5-carboxylate synthetase (P5CS), Elongation factor (ELF).

drought scenario, plants use strategies to reduce transpiration, conserve water and explore deeper soils to maintain water supply (Blum, 2005). Plants experience stress when transpiration exceeds water uptake, signifying the relevance of traits that maintain positive turgor. But when tissue water potential decreases under prolonged stress, the cellular-level-acquired tolerance mechanisms assume significance to sustain cell metabolic activities (Granier *et al.*, 2006; Spollen *et al.*, 2000). Because of the complex nature of drought stress, adaptation of the crops can be improved only by pyramiding drought-adaptive traits. From this context, plant traits associated with water relations and diverse cellular tolerance mechanisms are crucial to achieve field-level drought tolerance. Amongst the plant traits associated with water relations, superior root characters are important for better water mining under field conditions. In recent studies, emphasis has been given to characterize genes regulating root architecture. Several auxin-related genes have been reported to have regulatory role in root development (Overvoorde *et al.*, 2010). The role of ABA in stress-induced induction of root growth has also been well elucidated (Duan *et al.*, 2013; Erb *et al.*, 2011). Besides improving water mining, several tolerance mechanisms involved in sustaining metabolic activities in cells with decreased water status have phenomenal relevance in improving cell survival and recovery growth of plants. The genes/proteins that regulate the activity of cellular chaperones and others associated with sustaining transcription and translational machinery are important to improve cellular-level tolerance (Reddy *et al.*, 2009; Winicov, 2000).

As stress tolerance of crops can be improved by bringing together diverse adaptive traits/mechanisms, the approach in this study is to combine genes that improve root growth and cellular tolerance mechanisms. The simultaneous expression of relevant genes in a single locus necessitates the development of multigene cassette. We earlier developed modified vector system to engineer and stack multiple genes into plants to express two to three genes simultaneously (Vemanna *et al.*, 2013). Using this strategy, the simultaneous expression of *AtWRKY28* and *AtbHLH17* transcription factors substantially improved stress tolerance in *Arabidopsis* under different abiotic stress conditions (Babitha *et al.*, 2013). In the present study, by adopting this method, *OsAlfin1* that regulates root growth, *PDH45* and *PgHSF4* associated with protein turnover and protection were stacked and expressed in peanut. All the three transgenes were driven by different promoters. The emphasis was to express *PDH45* constitutively mainly to remove the RNA secondary structures and improve the protein turnover (Sanan-Mishra *et al.*, 2005) and hence expressed under 2XCaMV35S promoter. *Alfin1* gene has been shown to improve root growth (Nethra, 2010; Winicov, 2000), which is one of the important drought-adaptive traits, and hence expressed under constitutive CaMV35S promoter. The *HSF4* gene up-regulates *HSPs,* and the encoded HSPs are necessary to protect protein structure and folding under stress conditions (Swindell *et al.*, 2007), and hence, it was expressed under stress-induced *Rd29A* promoter. The expression of multiple transgenes is one of the limitations as the repetitive sequences of promoters, terminators or more copy number of the same gene may silence the transgene expression (Naqvi *et al.*, 2009). However, the expression of two insect-resistant genes and three genes contributing for abiotic stress tolerance was demonstrated in poplar (Su *et al.*, 2011). Similarly, the simultaneous expression of *Choline Oxidase*, *Superoxide dismutase* and *Ascorbate Peroxidase* genes in potato showed tolerance to abiotic stresses (Ahmad *et al.*, 2010). In addition, our previous studies in *Arabidopsis* overexpressing multiple genes that are important for biotic and abiotic stress tolerance (Babitha *et al.*, 2013; Vemanna *et al.*, 2013) showed the expression of all the transgenes. In this study, the integration of transgenes in peanut transgenic lines was confirmed through Southern blot analysis.

The peanut transgenic lines subjected to gradual drought stress showed significant variability in CCATD. Lower crop canopy temperature and larger negative CCATD values in transgenic lines reflect high transpiration rate and hence higher water mining abilities of these plants. However, the variation amongst the transgenic lines for CCATD could be due to the differential expression of transgenes or segregating population with some heterozygous plants. Genetic variability for canopy temperature has also been reported in many crops including peanut (Chakravarti *et al.*, 2010; Nautiyal *et al.*, 2008; Olivares-Villegas 2007). In the selected transgenic lines with cooler canopy temperatures, leaf RWC values were high and proline levels were low indicating the maintenance of high leaf water status (Figure 2c,d). A significant negative relationship was seen between proline and RWC in transgenic plants (Figure 2f). As the enhanced expression of *P5CS* and reduction in the levels of pyrroline-5-carboxylate reductase (*P5CR*) occur under stress resulting in higher levels of proline accumulation (Hare *et al.*, 1999; Savouré *et al.*, 1997), the transgenic lines that maintained higher RWC showed significantly lower levels of proline.

The negative CCATD and improved water relations in the selected transgenic lines could be mainly due to improved root characters. Under field conditions, especially with limited soil moisture status, root characters determine the crop canopy temperature (Balota *et al.*, 2012; Pinheiro *et al.*, 2005). The selected peanut transgenic lines showed enhanced root growth (primary and secondary roots) at all stages of the crop growth and also under moisture stress condition (Figures 5 and 6). The relative improvement in the root growth of peanut transgenic plants simultaneously expressing *Alfin1*, *PDH45* and *HSF4* genes was significantly higher than *PDH45* and *HSF4* expressing peanut transgenic plants (Figure S5). The improved root growth in multigene peanut transgenic plants could be due to the expression of *Alfin1*. The *Alfin1* gene has been shown to improve root growth in alfalfa and tobacco transgenic plants (Nethra, 2010; Winicov, 2000). The *Alfin1* silencing plants showed less root growth than *Alfin1* overexpression plants (Winicov and Bastola, 1999). Alfin1 cis-binding elements were found in promoter region of nitrate transporter 2.1 (*NRT2.1)* and nitrate-responsive promoters (*NRP)* (Udvardi *et al.*, 2007) that are involved in plant growth and nitrogen metabolism (Orsel *et al.*, 2004). *Alfin6* transcripts were induced under phosphate deficiency conditions and shown to play a role in root hair elongation (Chandrika *et al.*, 2013). The Alfin-like proteins interact with polycomb repressor complex (PRC) proteins, and these complexes regulate histone trimethylation at lysine 4 (H3K4me3) and lysine 27 (H3K27me3) positions on chromatin sites to activate or repress the genes involved in seed germination and development such as ABA insensitive-3 (*ABI3*), delay of germination-1 (*DOG1*), seed storage protein (crucifer1.3 [*CRU*]) and AP2 transcription factor CHOTTO1 (*CHO1*) (Lee and Lee, 2009; Molitor *et al.*, 2014). The multigene expressing peanut transgenic plants showed the enhanced expression of *Proline-rich protein (PRP2)* in roots under stress (Figure 7), which has been shown to be a downstream target of Alfin1 transcription factor (Winicov, 2000). PRPs are cell wall proteins with potential as an anchor to the membrane, and they

function as extensions that enable support and extension of cell wall structure. Increased utilization of cell wall proteins would be consistent with increased root growth (Deutch and Winicov, 1995). In *Arabidopsis, AtPRP*1 and *AtPRP*3 were highly expressed in roots and contributing to cell wall structure in root hair bearing epidermal cells (Bernhardt and Tierney, 2000).

Apart from having better water mining abilities with improved root growth, the peanut transgenic plants also showed improved cellular-level tolerance. The transgenic plants showed improved tolerance to methyl viologen-induced oxidative stress with minimal MDA accumulation and increased chlorophyll stability after ethrel treatment (Figure 4). The low MDA accumulation and higher chlorophyll stability are drought-tolerant traits. The improved cellular tolerance could be due to the expression of regulatory genes, *HSF4* and *PDH45*. Overexpression of *TaHsfA4a* and *OsHsfC1b* in rice provided tolerance to cadmium and salt stress, respectively (Donghwan *et al.*, 2009; Schmidt *et al.*, 2012). Overexpression of *AtHSFA1* in tomato provided tolerance to heat stress, and expression of *HSF4* in tobacco provided tolerance to salinity, oxidative and high temperature stress (Al-Whaibi, 2010; Nishizawa-Yokoi *et al.*, 2011). HSF4 transcription factor regulates diverse *HSPs* which act as molecular chaperones and play a crucial role in protein trafficking, folding, refolding and repair mechanisms under high temperature tolerance as well as other stresses (Wang *et al.*, 2004). The expression of *AtHSF41* in transgenic plants led to the constitutive expression of certain *HSPs* (Lee *et al.*, 1995). In this study, the peanut transgenic plants showed increased transcript levels of *HSP17.9* and *HSP60* under drought stress conditions (Figure 7), suggesting improved macromolecule protection in transgenic plants than wild-type plants. Under stress conditions, mRNA processing and translation is affected predominantly due to secondary structure, thus decreasing protein synthesis. From this context, RNA helicases are relevant in improving protein turnover, and hence, peanut transgenic plants expressing *PDH45* showed improved tolerance under stress conditions. The RNA helicases function as molecular motors that rearrange RNA secondary structures which function in RNA maturation or RNA proofreading (Owttrim, 2006). Overexpression of *PDH45* in tobacco and rice conferred tolerance to salinity stress by maintaining RNA stability, processing, transcription and translation initiation (Amin *et al.*, 2012; Sanan-Mishra *et al.*, 2005). In our earlier studies, peanut transgenic plants overexpressing *PDH45* showed higher water mining and WUE resulting in improved drought tolerance (Manjulatha *et al.*, 2011).

Further, enhanced levels of a few stress-responsive genes such as *RBX1*, *aldose reductase (ALR)* and *alcohol dehydrogenase-1 (ADH1*) were observed in transgenic plants under stress conditions (Figure 7). We previously showed that the overexpression of *AhRBX1* (having a role in removing unfolded proteins) in tobacco improved tolerance to moisture stress, NaCl and oxidative stress (Lakshmi *et al.*, 2010). Overexpression of aldose reductase from *Medicago sativa* in tobacco improved tolerance to different abiotic stresses including temperature and heavy metals (Hegedüs *et al.*, 2004). Similarly, *ADH1* from *Lotus japonicas* in yeast provided tolerance to abiotic stresses (Zeng *et al.*, 2011). Overall, this study provides evidence that the simultaneous expression of *OsAlfin1, PDH45 and PgHSF4* genes regulating diverse adaptive mechanisms improves drought tolerance and productivity in peanut. Maintenance of positive turgor by improved root growth substantially contributed for increased leaf size and positive carbon gain. Transgenic plants also showed improved cellular-level tolerance as evidenced by tolerance to drought and oxidative stress through up-regulation of many stress-responsive genes. Thus, we provide evidence that improved adaptation to moisture stress can be achieved by pyramiding traits associated with water mining and cellular-level tolerance mechanisms. Finally, our results confirm that transgenic technology is a potential approach to pyramid specific traits by the simultaneous expression of multiple genes.

Materials and methods

Construction of entry vectors for multigene stacking via gateway recombination

The whole gene cassettes with its respective promoters were subcloned to modified gateway entry vectors through conventional cloning method and subsequently used for recombination reaction with destination vector (Vemanna *et al.*, 2013). The *PgHSF4* (Accession No: EU492460) gene driving *RD29a* stress-inducible promoter cassette from binary vector *pBI121* was released by digesting with *SacI-KpnI* and ligated to *pGATEL1-L4* gateway entry vector. The *PDH45* (Accession No. Y17186*)* driving *2X35SCaMV* promoter from modified *pRT100* was subcloned at *SphI* site in *pGATER4-R3* vector. Similarly, *OsAlfin1* (Accession No: AK121400) driving *35SCaMVpromoter* from *pRT100* was released using *HindIII* and ligated to *pGATEL3-L2* vector.

Construction of multigene expressing binary vector

The entry vectors *pGATE L1L4-RD29A::PgHSF4:nos, pGATE R4-R3-2X35S CaMV::PDH45:polyA and pGATE L3-L2-CaMV35S::OsAlfin1: polyA* were subjected to LR clonase recombination reaction with *pKM12GW* destination vector [1 : 1 : 1 : 1.5 (P1: P2:P3:D)]. The recombination reaction mix was incubated overnight at 25 °C, and proteinase K was used to stop the reaction. The binary vector named *pKM12GWRD29A::PgHSF4-2xCaMV35S::PDH45-CaMV35S::OsAlfin1* has *nptII* as plant selection marker (Figure 1a).

Plant transformation

The binary vector *pKM12GW::PgHSF4:PDH45:OsAlfin1* was transformed into *Agrobacterium tumefaciens EHA105* by electroporation. The peanut cv. GPBD4 wild-type seeds were transformed using *in planta* transformation method (Rohini and Rao, 2000). The T_0 seeds were harvested, and T_1 seeds were screened on selective medium with kanamycin. The pregerminated seeds of both transgenic and wild type were treated with 400 ppm of kanamycin for 4 h at room temperature and subsequently transformed to quartz media. The putative transformants were identified based on their shoot and root growth compared to wild-type seedlings. The kanamycin-positive seedlings were transferred to polybags and screened for the amplification of *nptII* by PCR. The polybag that established *nptII*-positive plants were again screened for temperature tolerance by adopting temperature induction response technique (Senthil Kumar *et al.*, 2003). The plants were exposed to gradual induction response at 32–42 °C for 5 h and subsequently exposed to 50 °C for 4 h, and then, survival and recovery growth was recorded after 5 days. The transgenic plants that showed superior recovery growth to high temperature stress were further transplanted into pots and grown till harvest. PCR analysis was carried out in tolerant plants using gene-specific or promoter forward and gene-specific reverse primers. In subsequent generations, also the

kanamycin-positive plants were confirmed by PCR analysis (Table S2).

Southern blot analysis

Thirty micrograms of purified genomic DNA of transgenic and wild-type plant was digested overnight with *EcoRI* that cuts once within T-DNA region for the analysis of copy number by Southern hybridization. The digested DNA samples were electrophoresed on a 0.8% agarose gel in TAE buffer and blotted on to a positively charged nylon membrane (Amersham GE healthcare Pvt. Ltd., Bangalore, India). The 1 kb length of *Alfin1* fragment was restrict digested for labelling by Dig-labelled kit (Amersham, GE healthcare Pvt. Ltd.). Hybridization and washing was carried out according to user manual. The signal was developed using chemiluminescence substrate (CDP-star) reagent and exposed to X-ray and autoradiogram according to Sambrook *et al.* (1989).

Expression analysis

Total RNA was extracted using phenol–chloroform method according to Datta *et al.* (1989), and cDNA was synthesized by oligo(dT) primers using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT; MBI Fermentas, Hanover, MD). The cDNA pool was used as a template to perform RT-PCR analysis. The quantitative real-time RT-PCR was performed with the fluorescent dye SYBR Green (TAKARA SYBR Green qPCR Kit) following the manufacturer's protocol (Opticon 2; MJ research, USA & MJ Bioworks, Inc). The relative expression levels of the selected genes under a given stress condition were calculated using comparative threshold method. The PCR with SYBR dye and PCR conditions were 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 52–58 °C for 30 s, 72 °C for 40 s and a final extension of 72 °C for 5 min. Elongation factor (*ELF*) was used as internal control for normalization (for primer list, see Table S3). The expression of *Alfin1* and *PDH45* was examined in plants maintained under adequate soil moisture. Expression of *HSF4* was studied in both transgenic and wild-type plants subjected to moisture stress. Expression of downstream target genes was studied in 20-day-old plants (raised in 2 kg soil) after imposing moisture stress gravimetrically till the soil moisture content reached to 50% field capacity. The plant tissue was collected, RNA was extracted, and RT-PCR analysis was carried out.

Moisture stress imposition and measurement of CCATD

The T_3 generation transgenic lines were raised in containment facility and grown under well-watered condition till 50th day. Daily potential evapotranspiration (PET) values were arrived by E-pan and crop coefficient values. Plants were irrigated at PET of 1.0 once in 3 days to maintain near 100% field capacity (FC). Moisture stress was imposed from 50th day for a period of 20 days. The plants were exposed to the gradual moisture stress by irrigating the plants at diminishing PET from 1.0 to 0.1 at successive irrigations. At the end of the stress period, soil moisture content was measured at 20 cm depth ranged from 32% to 35% of field capacity (FC). Subsequently, the stress was alleviated by irrigating at 1.0 PET till harvest. During the stress period, canopy temperature measurement was taken using Canopy thermosensor (infrared gun). The canopy temperature was measured on 10th day after stress imposition, when the soil water status ranged between 45 and 48% of FC. The CCATD was calculated using the formula: CCATD (°C) = Crop Canopy Temperature (°C) − Air temperature (°C).

Drought stress imposition by gravimetric method

To impose moisture stress at 20-day-old seedling stage, the plants were raised in pots with 2.5 kg of soil. To arrive at relative comparison in each pot, one plant each of wild type and transgenic event was maintained. Adapting the gravimetric approach, the pots were weighed twice a day to measure the water loss in evapotranspiration and required amount of water was replenished to maintain the desired soil moisture status (FC). Altogether, three sets of plants were raised with five replications, one set of plants were maintained at 100% FC, the second and third set of plants maintained at 100% FC till 20 days, and subsequently, gradual moisture stress was imposed to arrive at desired soil moisture status. For the second set, the gradual stress was imposed till the soil FC reduced to 50% FC on 35th day, and in the third set, to 30% FC. At the end of the stress period, the plants were re-watered to 100% FC, and after a week, recovery growth was recorded.

RWC and proline measurements

Leaf discs were made and fresh weight was measured. Then, the leaf discs were floated on deionized water for 5 h to attain full turgidity and turgid weight was determined. Dry weight was recorded after oven-drying the samples to a constant weight. The RWC was calculated using the formula: RWC% = 100 × [(fresh weight − dry weight)/(turgid weight − dry weight)].

The colorimetric method of Bates *et al.* (1973) was used for proline estimation. Leaf samples of 300 mg (in triplicate) were crushed in liquid nitrogen and homogenized in 10 mL of 3% sulphosalicylic acid. The supernatant was collected by vacuum infiltration. Two millilitres of acid ninhydrin and 2 mL of glacial acetic acid were added to 2 mL of the filtrate and incubated for 1 h at 100 °C in a boiling water bath. The reaction was terminated on ice. The resulting reaction mixture was extracted with 4 mL toluene and mixed thoroughly for 15 s; the toluene phase was separated using the separating funnel. The absorbance of the solutions was determined at 520 nm using toluene as blank. The proline concentration was determined using a standard curve (1 OD = 36.2 μg proline) and expressed as μmol proline per gram fresh weight of the sample.

Ethrel-induced senescence assay

Leaves from 3-week-old transgenic and wild-type plants were excised and floated in Petri plates containing 1500 ppm of ethrel solution for 3 days under dark. At the end of incubation period, the leaf discs were removed and analysed for total chlorophyll content using acetone: DMSO (1 : 1) method. Total chlorophyll was expressed as mg/g fresh weight (Arnon, 1949).

Measurement of malondialdehyde (MDA) content

Malondialdehyde is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. About 0.5 g of tissue was homogenized in 5 mL of 5% (w/v) trichloroacetic acid, and the homogenate was centrifuged at 12 000 ***g*** for 15 min at room temperature. The supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroacetic acid], and the mixture was boiled for 25 min at 100 °C followed by centrifugation for 5 min at 7500 ***g*** to clarify the solution. Absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the A600.

Growth analysis of transgenic plants

The growth and productivity parameters were recorded at the time of harvest. Shoot and root lengths were measured, the leaf area of a tetra foliate leaves was measured, and after drying the shoot and root, total dry matter was measured. The pod number and pod weight were considered for the productivity.

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Conflict of interest

The authors have patent application filed at Indian Patent office File No. '2646/CHE/2014' entitled 'An expression construct and its use for increasing abiotic stress tolerance in a plant cell'. However, the authors declare no conflict of interest in the published data.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Genomic DNA PCR analysis of T_1 transgenic plants.
Figure S2 Response of T2 transgenic and wild type seedlings to kanamycin.
Figure S3 Genomic DNA PCR analysis of T_3 transgenic plants.
Figure S4 Moisture stress response of transgenic and wild type plants.
Figure S5 Comparative assessment of transgenic plants expressing individual genes and multiple genes.
Table S1 Phenotypic characters of transgenic and wild type plants.
Table S2 ANOVA for root growth and biomass under drought stress.
Table S3 Primers used for characterization and gene expression studies.