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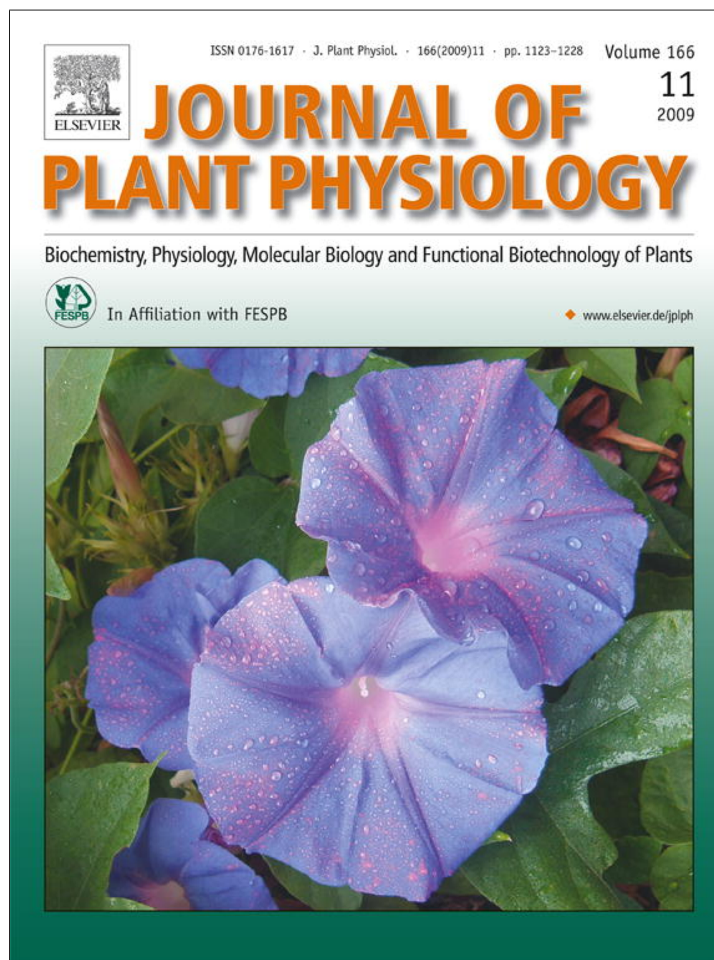

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Differential antioxidative responses in transgenic peanut bear no relationship to their superior transpiration efficiency under drought stress

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Transpiration efficiency

Summary

To counter the effects of environmental stresses, the plants must undergo detoxification that is crucial to avoid the accumulation of damaging free oxygen radicals (ROI). Here, we detail the oxidative damage, the antioxidant composition, and the osmoprotection achieved in transgenic plants of peanut overexpressing the *AtDREB1A* transgene, driven by a stress-inducible promoter (*Atrd29A*) when exposed to progressive water stress conditions. This study explored the biochemical mechanisms where (i) the antioxidants such as superoxide dismutase (SOD), ascorbate peroxidase (APOX), and glutathione reductase (GR) accumulated in the transgenic plants at comparably higher levels than their untransformed counterparts under dry soil conditions, (ii) a significant increase in the proline levels in the transgenic plants was observed in dry soils, and (iii) a dramatic increase in the lipid peroxidation in the untransformed controls in drier soils. Most of the biochemical parameters related to the antioxidative machinery in the tested peanut transgenics were triggered by the overexpression of *AtDREB1A* that appeared to differ from the untransformed controls. The antioxidants showed a negative correlation with the fraction of transpirable soil water (FTSW) thresholds, where the normalized transpiration rate (NTR) started decreasing in the tested plants. However, no significant relationship was observed between any of these biochemical indicators and the higher transpiration efficiency (TE) values found in the transgenic events. Our results show that changes in the antioxidative machinery in these transgenic peanut plants (over-expressing the *AtDREB1A* transcription factor) under water-limiting conditions played no causative role in improved TE.

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Abbreviations: DS, drought stress; FTSW, fraction of transpirable soil water; NTR, normalized transpiration rate; TE, transpiration efficiency; WW, well-watered.

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Introduction

Water deficit represents one of the most complex physiological phenomena that limits plant growth and productivity in water-limited environments where many of the world's poorest farmers live (Bartels, 2001). As of today, 80% of the agriculture worldwide is rainfed; food security could become unsustainable without dramatic yield increases in marginal environments, especially in drought-prone areas. The responses of plants to water deficit are observed in forms of phenological responses, morphological adaptations, physiological changes, and biochemical adaptations.

The complexity of signaling events associated with the sensing of stress and the activation of defense and acclimation pathways is believed to involve reactive oxygen intermediates (ROI), calcium, calcium-regulated proteins, mitogen-activated protein kinase (MAPK) cascades, and cross talk between different transcription factors (Liu et al., 1998; Xiong et al., 1999; Bowler and Fluhr, 2000; Kovtun et al., 2000; Knight and Knight, 2001; Chen et al., 2002). To counter the effects of stress, plants must undergo a process of stress acclimation to avoid the accumulation of free oxygen radicals during stress (Vierling, 1991; Dat et al., 2000; Mittler, 2002). The enzymatic detoxification mechanism involves the induction of various ROI scavenging enzymes that are components of the antioxidative defense system. Superoxide radicals generated in plant cells are converted to H_2O_2 by the action of superoxide dismutase (SOD); the accumulation of H_2O_2, a strong oxidant, is prevented in the cell, either by catalase or by the ascorbate–glutathione cycle where APX reduces H_2O_2 to H_2O.

The exploitation of cloned genes to alter the function of gene products in transgenic plants provides novel opportunities to assess their biological role in stress response. The complex drought-related responses in plants are believed to result from genomic re-organization and alterations in gene expression, witnessed as alteration of biochemical events and physiological functions that are very unlikely to be under the control of a single gene. Far beyond the initial attempts to insert "single-action" genes, engineering of the regulatory machinery involving transcription factors has emerged as a new tool for controlling the expression of many stress-responsive genes. Overexpression of regulatory genes resulting in the expression of several genes involved in response to abiotic stress, leading to an altered expression of a number of different downstream structural genes, and resulting in wide-array of altered response to stresses, seems adequately justified (Bartels and Sunkar, 2005; Chinnusamy et al., 2005). Further, transcription factors offer considerable insights into the signaling pathways, besides providing novel opportunities to assess their relative contributions to stress tolerance (Jaglo-Ottosen et al., 1998; Hsieh et al., 2002; Kasuga et al., 2004; Pellegrineschi et al., 2004). However, a major challenge is to develop transgenic plants not only with the ability to survive stress, but also to grow under adverse conditions and achieve an economic yield.

We have previously shown that in transgenic peanut (Bhatnagar-Mathur et al., 2007), the expression of *AtDREB1A* gene, driven by an *rd29A* (a stress-inducible promoter from of *Arabidopsis thaliana*), contributes to an enhanced transpiration efficiency (TE), an important component of plant performance under limited soil moisture conditions. Several transgenic events 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 and many other crops. Moreover, transpiration declined in these transgenic events under dryer soil. The enhanced TE in the transgenics relates to a lower stomatal conductance and an overall lower rate of water loss per unit leaf area.

The present study was undertaken (a) to determine the level of water stress at which the ROI(s) start accumulating in the previously reported high TE events, and whether there exist any genotypic differences and (b) to assess the relationship between the ROI and the level of the enzymes responsible for their scavenging and the range of TE previously reported. To fulfill these objectives, we have studied in detail the oxidative damage, the antioxidant composition, and osmoprotection achieved in these transgenics under progressive water stress, and related it to the TE value to assess whether changes at the biochemical level were followed by water productivity differences.

Material and methods

Genetic transformation

The regeneration and transformation system used for development of adventitious shoot buds in peanut (*Arachis hypogaea* L.) through the cotyledon explants of mature pre-soaked seeds was same as described earlier (Sharma and Anjaiah, 2000). The transgenic plants containing the *Arabidopsis thaliana*

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DREB1A driven by a drought responsive promoter *rd29A* (also from *A. thaliana*) were generated by means of the *Agrobacterium tumefaciens* transformation system as described earlier (Sharma and Anjaiah, 2000; Bhatnagar-Mathur et al., 2007). The transgenic events (T2–T6) were characterized molecularly using PCR, RT-PCR, and DNA gel blot analysis to confirm transgene presence, expression, and copy number (Sharma and Anjaiah, 2000; Bhatnagar-Mathur et al., 2007).

Analysis of transgenic plants under water stress conditions

Untransformed wild-type (WT) and transgenic peanut plants were grown under similar conditions in 20 cm pots containing sandy loam soil (red soil:sand:compost; 3:2:1) along with 1 g kg^{-1} of single super phosphate (SSP). Seeds were inoculated with 1 g L^{-1} *rhizobium* strain NC 92 (IC 7001) to ensure adequate nodulation. Day/night temperatures were maintained at 28/20 $\pm$ 2 °C with a relative humidity of 60 $\pm$ 5% and 16/8 h light (about 120 M m^{-2} s^{-1}). The experimental design adopted was Randomized Complete Block Design (RCBD), and the main possible gradients were taken into account, i.e., replication was organized from the window pane to inside the bay, and entries and treatments were randomized within each replicate.

Drought treatment

Twelve plants per genotype (all positives in the case of transgenics) were grown in the greenhouse with 28/20 ºC day/night temperatures under WW conditions until 28 days after sowing (DAS) which corresponds to late vegetative stage for the genotypes used. Thereafter, plants were divided into two groups: one set kept as well-watered (WW) control and the other set under drought stress (DS) treatment. Dry-down experiment was carried out in a biosafe containment facility as described earlier (Bhatnagar-Mathur et al., 2004, 2007). The irrigated control plants were maintained at about 80% field capacity throughout the experiment by daily re-watering. The pot soil was saturated with water and allowed to drain overnight prior to exposure to DS. The pots were sealed with polythene bags to prevent any water loss by direct evaporation from the soil surface. In order to expose the DS plants to a progressive water deficit, they were allowed to loose a maximum of 70 g water d^{-1}. Any transpiration in excess of 70 g d^{-1} was added back to the pot. Plant transpiration was then calculated as pot weight differences plus the water added. Normalized transpiration rates (NTRs) of DS plants were calculated to compare the DS to the WW plants and to minimize the effect of plant-to-plant variation (Ray and Sinclair, 1997, for details see Bhatnagar-Mathur et al., 2007). Pots were weighed and water adjustment done every day. The total cumulated water transpired was the sum of all transpiration values during the experimental period. The plants were finally harvested when NTR of DS plants dropped to a value of 0.1, defined as the end of the dry-down experiment, representing the stage where the transpiration of the drought-stressed plants fell below 10% of that in the well-watered plants.

The daily FTSW remaining in the pot was computed by using the equation

$$\text{FTSW} = 1 - \frac{\text{initial pot weight} - \text{daily pot weight}}{\text{initial pot weight} - \text{final pot weight}}$$

FTSW is the fraction of transpirable soil water that was used as an indicator of water remaining available for transpiration in the pot and was our index of stress.

The daily NTR was then plotted against daily FTSW and by using NTR as a function of FTSW. A plateau regression procedure using SAS (SAS Institute, 1996) was used to calculate the FTSW threshold value at which the stomatal closure initially occurred, i.e., where NTR begins to decline.

A subset of plants was harvested at 28 DAS before the imposition of stress to assess the pre-treatment biomass. The post-treatment biomass was recorded on plants exposed to DS or WW when these were harvested, i.e., when the transpiration of DS plants fell below 10% of than in WW. These biomass values were used for the calculation of TE. The plants were carefully separated into shoot and root components. The dry weight of the leaves, shoot, roots, nodules, and pods was determined by drying in a forced air oven at 80 °C for 3–4 d. The TE in DS and WW plants was estimated as the total biomass produced during the experimental period (from pot saturation to harvest, i.e., the post-treatment biomass minus pre-treatment biomass) divided by the cumulative water transpired during the same period. During the course of study, leaves were detached from each plant at different stages of stress and weighed for fresh weight, with sampling and measurements repeated five times for biochemical estimations.

Sampling and assays

Leaf tissues were harvested at five different stages of stress under a typical dry-down cycle.

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These stages corresponded to different stress intensity, based on the FTSW values that were back-calculated after the end of the experiment for each of these samplings. The decision on the stage of sampling was made from the estimation of the FTSW values that would have been reached at these stages, based on the rate of water loss by the plants. High value of FTSW indicated a soil that was relatively wet while a low FTSW value was indicative of a fairly dry soil and consequently a more severe water stress than at higher FTSW values. Since our observations on the relative water content of several peanut transgenics and adapted germplasm indicated that there were no significant differences across the lines (results not shown) and all measurements were done on a fresh weight basis. This was sufficient to compare the genotypes. The specific enzyme activities were expressed on the basis of the total soluble protein of the samples. Soluble protein of the extract was measured by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Ascorbate peroxidase (APOX; EC 1.11.1.11)

Total activity of APOX was measured spectrophotometrically by the method of Chen and Asada (1989). The extraction medium for leaf APOX contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 1% PVP-10, 1 mM ascorbate, and 0.1% Triton X-100. The leaf extract was filtered through Whatman filter paper no. 1 before being used for the enzyme assay. For assay of the enzyme activity, the rate of hydrogen peroxide-dependent oxidation of ascorbic acid was determined in a reaction mixture that contained 50 mM Hepes-KOH (pH 7.0) containing 1 mM ascorbate, 1% (v/v) Triton X-100 and enzyme extract. The reaction was initiated by the addition of 10 μL of 10% (v/v) H_2O_2 and the oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm (Beckman DU® 530) for 3 min.

Glutathione reductase (GR; EC 1.6.4.2)

Fresh leaf tissue (500 mg) was used for the extraction in an ice bath in 10 mL of 0.1 m potassium phosphate buffer (pH 7.5) containing 0.1 M EDTA, 200 mg PVP (MW = 25,000), 1% w/v BSA, and 200 μM β-mercaptoethanol. The leaf extract was filtered through Whatman filter paper no.1 and further used for assay. GR activity was estimated following the oxidation of NADPH at 340 nm as described by Schaedle and Bassham (1977). The assay buffer contained 0.5 mL phosphate buffer (pH 7.5), 0.1 mM NADPH, and 0.1 mM GSSG to a final volume of 1 mL. The reaction was initiated by adding 100 μL enzyme to the cuvette and the decrease in the absorbance at 340 nm was recorded. The enzyme activity was expressed as μM NADPH oxidized $min^{-1} g^{-1}$ of the tissue.

Total superoxide dismutase (SOD; EC 1.15.1.1) activity

SOD activity was measured according to Giannopolitis and Ries (1977). The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Each 3 mL reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 2 mM riboflavin, 75 mM NBT, 100 nM EDTA, and 100 μL of the enzyme extract. Careful monitoring of the increase in absorbance at 560 nm was carried out following the production of blue formazan. Identical tubes with the reaction mixture were kept in the dark that served as blanks. Reaction was carried out in test tubes at 25 °C under illumination from a fluorescent lamp (l5 W). Riboflavin was added at the last and the tubes were shaken and placed in the racks. The reaction was started by switching on the light and was run for 10 min, before being stopped by switching off the light. Under the experimental conditions, the initial rate of reaction, as measured by the difference in increase in absorbance at 560 nm in the presence and absence of extract, was proportional to the amount of enzyme. Enzyme activity (unit mL^{-1}) was proportional to ($V/v-1$), where V equals the change in absorbance per min in the absence of SOD, and v equals the change in absorbance per min in the presence of SOD. The unit of SOD activity was defined as the amount of enzyme that inhibits the nitro blue tetrazolium photoreduction by 50%. SOD activity values are given in units per mg of protein.

Lipid peroxidation

The concentration of malondialdehyde (MDA) as an end product of lipid peroxidation in the leaves was measured using the thiobarbituic acid (TBA) test (Dhindsa et al., 1981). In total 1 g of the leaf tissue was homogenized in 5 mL of 5% trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min at 25 °C. A 2 mL aliquot of the supernatant was mixed with equal volume of 20% TCA containing 0.5% TBA. The mixture was heated at 100 °C for 30 min, quickly cooled followed by centrifugation at 10,000*g* for 10 min. The absorbance of the supernatant was recorded at 532 nm (Beckman DU® 530). The non-specific turbidity was corrected by A_{600} subtracting from A_{532}. The concentration of MDA was calculated by using an extinction coefficient of 155 mM cm^{-1} (Heath and Packer, 1968).

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Proline

Free proline content in the leaves was determined following the method of Bates et al. (1973). In total 0.5 g of the leaf tissue was homogenized in 10 mL of 3% sulphosalicylic acid (w/v) using a pestle and mortar. The homogenate was filtered through Whatman filter paper no. 1 and the filtrate was further used for assay of proline. Totally 2 mL of the filtrate was added with equal volume of acid ninhydrin (50 mg of ninhydrin, 1.2 mL glacial acetic acid, and 0.8 mL 6 M o-phosphoric acid) and kept for 1 h at 100 °C in a boiling water bath. After color development, the tubes were placed on ice for 5–10 min for the termination of the reaction. Totally 4 mL of toluene was added to the reaction mixture and vortexed well for 5–10 s. Chromophore containing toluene was separated and absorbance read at 520 nm in UV visible spectrophotometer against a toluene blank. A standard curve was prepared using the commercial proline to calculate its concentration in the samples.

Statistical analysis

In all biochemical experiments, the data were scored and analyzed based on mean and standard error (SE) values using student's *t*-test.

Results

The present work was carried out to study the biochemical mechanisms that may have a role in water stress tolerance in the transgenic peanut overexpressing the *AtDREB1A* transcription factor under the control of a stress-inducible promoter from *Atrd29A* gene. This involved evaluating the effect of transpiration decline on key biochemical parameters and their correlation with the TE, a measure of plant's performance under a progressive dry-down cycle in greenhouse conditions as detailed below.

FTSW threshold values and TE

As shown in Table 1, the onset of transpiration decline occurred in drier soil in the all tested transgenic event (RD2) when compared to the WT JL 24 (0.54; high soil moisture content). Event RD2 had the lowest threshold for transpiration decline (0.28). Similarly, the TE of WT JL 24 (4.21) was the lowest when compared to all the transgenic events tested under water stress. The transgenic events RD2, RD11, and RD19 showed significant differences in their TE when compared to the untransformed WT under water-limiting conditions. RD2 showed a 37% higher TE than the untransformed control followed by 24% and 14% in RD19 and RD11, respectively.

Effect of water stress on SOD activity

The superoxide dismutase activity, which is responsible for the elimination of superoxide radicals in the cells in particular following stress imposition, did not statistically vary under WW conditions (data not shown). However, it increased significantly in the transgenics at FTSW below 0.6, compared to FTSW of 1 (wetter soil), whereas there was no increase between these two stages in the SOD level of WT. It should be noted that based on the FTSW thresholds where transpiration starts declining compared to the WW treatments, the NTR had not yet decreased in any of the transgenic events at FTSW $= 0.6$ (Table 1). Also, at FTSW of 0.6, a higher SOD activity was observed in the transgenic events (5.205–6.33 U mg^{-1} protein) when compared to the WT (4.562 U mg^{-1} protein). Thereafter, during stress progression, the transgenic events RD2 and RD11 continued to maintain a higher SOD activity as compared to WT JL 24 where RD2 showed a significantly higher activity at 0.4 FTSW in contrast to the WT. At higher DS levels (0.2–0.04 FTSW), all the transgenic events showed a significant increase in their SOD activity in contrast to the WT. It was also observed that almost all the transgenic events except RD20 showed a significantly higher ($P \leqslant 0.001$) enzyme activity at higher levels of stress (0.2–0.04 FTSW) in contrast to that at FTSW of 1 (Figure 1). However, there were no significant differences in the SOD activity in WT throughout the dry-down cycle. The SOD activity, however, showed no relation to the TE values in the tested plants (Table 2).

Effect of water stress on ascorbate peroxidase activity

Water stress did not bring about any clear pattern of change in the activity of ascorbate peroxidase in any of the events tested during the dry-down experiment. The differences in the APOX activity were not significant under WW conditions or at the beginning of the experiment. However, at FTSW of $\sim$0.6, APOX activity in the leaves of the transgenic plants exposed to progressive soil drying showed a 20% increase as compared to the untransformed control. The specific activity of APOX increased as the stress developed to 0.4 FTSW, and the APOX activity was significantly

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Table 1. Average values of proline, malondialdehyde (MDA), antioxidants [superoxide dismutase (SOD), ascorbate peroxidase (APOX), glutathione reductase (GR)], SOD/APOX ratios, transpiration efficiency, and FTSW threshold values where the transpiration started to decline upon exposure to water stress of the transgenic events and the WT parent throughout the drying cycle, where $n = 5$.

Transgenic event	Physiological and biochemical parameters tested							
	FTSW threshold	TE (g biomass kg^{-1} water)	MDA (U mg^{-1} protein)	GR (μM NADPH oxidised min^{-1} g^{-1} FW)	SOD (U mg^{-1} protein)	Proline (μg g^{-1} FW)	APOX (μM mg^{-1} protein)	SOD/APOX
RD11	0.39±0.02	4.59±0.23	24.9±0.44	0.42±0.09	6.88±0.85	787±135.56	0.58±0.08	11.8±0.47
RD12	0.44±0.02	4.25±0.19	25.18±0.47	0.68±0.08	7.07±0.85	773.52±136.74	0.57±0.06	12.11±0.41
RD19	0.36±0.01	5.24±0.17	23.80±1.03	0.65±0.07	6.63±0.77	766.28±133.76	0.57±0.11	12.21±1.12
RD2	0.28±0.01	5.79±0.11	23.96±0.64	0.70±0.08	7.78±1.01	800.08±137.12	0.66±0.08	11.79±0.64
RD20	0.49±0.01	4.43±0.23	25.19±0.95	0.66±0.06	5.98±0.27	731.74±121.23	0.47±0.01	12.72±0.42
WT	0.54±0.02	4.21±0.24	30.50±1.57	0.52±0.04	5.24±0.21	688.08±91.62	0.45±0.04	11.76±0.78

These average values were the means of samples (±SE) collected at several stages, whereby, at each stage of sampling, the FTSW values were not significant among the tested genotypes.

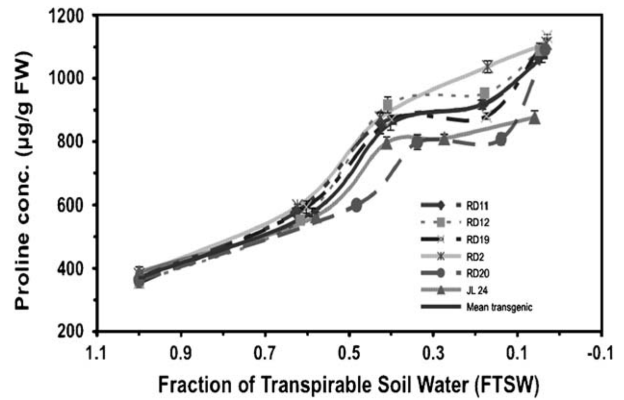

Figure 1. Effect of receding moisture in terms of FTSW values on superoxide dismutase (SOD) activity in the leaves of transgenic events and WT control across the dry-down cycle. Values represent mean activities ($n = 5$).

higher in RD2 (1.8-fold) and RD11 (1.6-fold) than the WT plants at 0.4 FTSW level. As the stress developed further, at FTSW 0.2, the APOX activity of RD2 was approximately 1.5-fold higher (0.7480 μM mg^{-1} protein) than the WT (0.4907 μM mg^{-1} protein). At very low FTSW (0.04), a significantly higher APOX activity was observed in RD19, RD2, and RD11, in contrast to WT. Unlike the observed SOD activity, the APOX activity in the transgenic events RD12, RD19, and RD20 did not show any significant increase when compared to their level observed at FTSW of ~1 (Figure 2). Again, the WT did not show any significant changes in APOX activity across different stages of water stress. TE did not correlate significantly with the APOX levels in these plants (Table 2).

Effect of water stress on glutathione reductase activity

Glutathione reductase, which catalyses the NADPH-dependent reduction of oxidized glutathione, did not show any significant increase in activity at FTSW of 0.6. As the stress started developing, the transgenic events RD11 and RD12 showed a significant increase ($P \leqslant 0.05$) in GR activity at FTSW of 0.4 compared to their value at FTSW = 1. The GR activity increased further at lower FTSW values and transgenic events RD11, RD19, and RD20 showed a higher activity that the WT at FTSW of 0.2. All of the transgenic events showed a significantly higher GR activity than in WT at FTSW 0.04 ($P \leqslant 0.001$ for RD2 and RD19) and ($P \leqslant 0.01$ for RD11, RD12, RD20). The GR activity in all the transgenic events was found to be significantly higher at FTSW $\leqslant$ 0.4 at least at $P \leqslant 0.05$

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Table 2. Regression values (r) drawn between different biochemical parameters of proline, malondialdehyde (MDA), antioxidants [superoxide dismutase (SOD), ascorbate peroxidase (APOX), glutathione reductase (GR)], and physiological traits (transpiration efficiency (TE), FTSW threshold) using average values of five groundnut transgenic events along with wild-type JL 24 under dry-down conditions using data from Table 1, where $n = 6$.

	MDA	GR	SOD	Proline	APOX	TE	FTSW threshold
MDA	1						
GR	0.40	1					
SOD	0.80	0.34	1				
Proline	0.86	0.18	0.97**	1			
APOX	0.72	0.23	0.97**	0.94**	1		
TE	0.61	0.39	0.68	0.63	0.75	1	
FTSW threshold	0.79	0.30	0.90	0.89	0.94**	0.91**	1

*Significant at $P = 0.05$; **significant at $P = 0.01$.

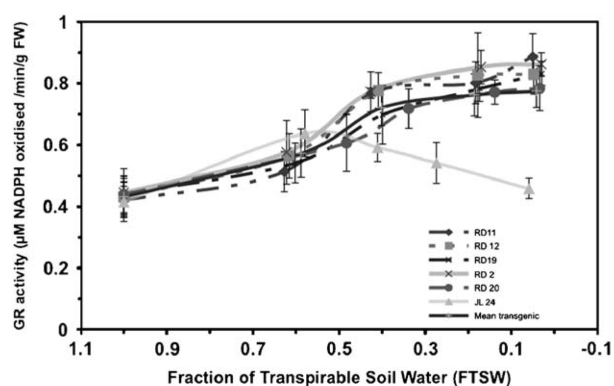

Figure 2. Effect of receding moisture in terms of FTSW values on ascorbate peroxidase (APOX) activity in the leaves of transgenic events and control across the dry-down cycle. Values represent mean activities ($n = 5$) at $p = 0.05$.

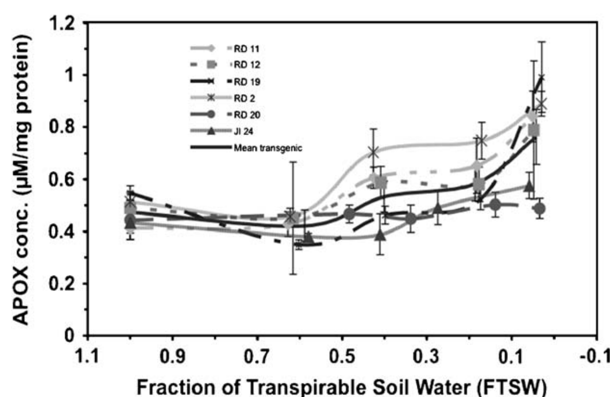

Figure 3. Effect of receding moisture in terms of FTSW values on glutathione reductase (GR) activity in the leaves of transgenic events and WT control across the dry-down cycle. Values represent mean activities ($n = 5$) at $p = 0.05$.

when compared to 0 DAS (FTSW 1.0; Figure 3). The change in GR activity in the WT was not significant across the stress period. Again GR activity had no relation with the TE values in the tested transgenic plants and their WT counterparts (Table 2).

Effect of water stress on proline levels

The proline level increased as a function of decreasing FTSW. At FTSW between about 0.6 and 1.0 (no stress based on normalized transpiration ratios), there were no significant differences in the proline content between the transgenic events and the WT. As the stress started developing, at FTSW < 0.6, there were significantly higher ($P \leqslant 0.01$) proline levels in all transgenic events (869.8–916.3 $\mu M\,g^{-1}$) than in the WT (797.2 $\mu M\,g^{-1}$), except in RD20 (Figure 4). The elevated proline levels in all transgenic events with the exception of RD20 were significantly higher than the WT (1.2–1.4-fold at $P \leqslant 0.01$) at FTSW 0.2. Similarly, significantly higher proline levels were observed in the transgenic events than in WT at FTSW 0.04 (1.25–1.3-fold, $P \leqslant 0.001$). A significant increase was observed in the proline level of all transgenic events as well as the WT at FTSW levels $\leqslant 0.6$, indicating that the proline accumulation had started even before the normalized transpiration ratios started decreasing (Bhatnagar-Mathur et al., 2007; Figure 4). However, no relationship was observed between proline levels and TE values of these plants (Table 2).

Effect of water stress on free radicals

An increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. In the present study, the malondealdehyde (MDA) levels did not change in the transgenic events as a function of decreasing FTSW. In the

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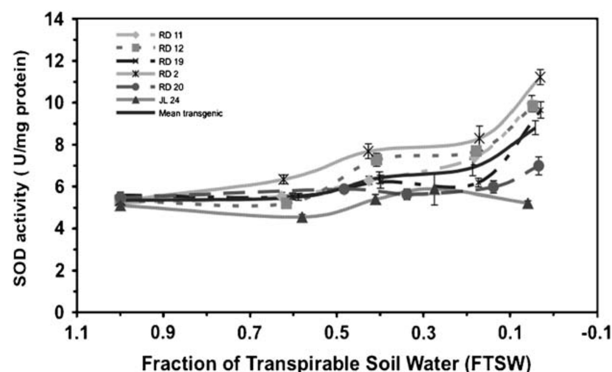

Figure 4. Effect of receding moisture in terms of FTSW values on proline content in the leaves of transgenic events and WT control across the dry-down cycle. Values represent mean activities ($n = 5$) at $p = 0.05$.

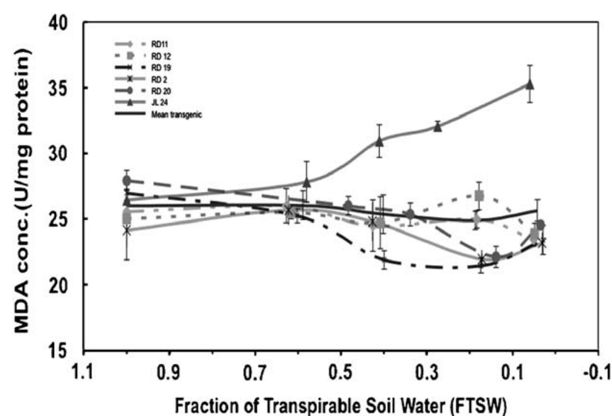

Figure 5. Effect of receding moisture in terms of FTSW values on MDA content in the leaves of transgenic events and WT control across the dry-down cycle. Values represent mean activities ($n = 5$) at $p = 0.05$.

WT, the MDA level did not increase significantly at FTSW of 0.6 but significantly did so at FTSW 0.4 when compared to the value at FTSW of 1; the MDA level of WT was higher than in the transgenic events RD2, RD11, RD12, RD19, and RD20. The MDA levels in the WT at FTSW$\leqslant$0.2 were further higher in the transgenic events ($P\leqslant0.001$). The MDA level in WT also increased significantly between FTSW $\sim$0.4 and FTSW$<$0.1. There were no significant differences in the MDA content in RD2, RD11, and RD12 throughout the drying cycle. However, MDA level in RD19 and RD20 even slightly decreased; only in WT did MDA significantly increased throughout the drying cycle (Figure 5). Again MDA levels in these tested transgenic plants had no correlation with their TE values (Table 2).

Ratio of antioxidants

The balance between SOD and APOX (and/or CAT) activity in cells is considered to be crucial for determining the steady-state level of O_2 and H_2O_2. Results based on the ratio of specific activities of SOD and APOX indicated that these were similar in the transgenic events (10.6–12.1) and WT (11.2–12.0) throughout until a FTSW of 0.2 was reached (data not shown). However, thereafter as stress progressed, a decrease in the SOD/APOX ratio was observed in WT (8.5) when compared to the average ratio of the transgenic events (11.2), thereby indicating that although the transgenic plants maintained a SOD:APOX ratio that was essentially identical to that in the WT plants under WW conditions, this ratio differed in the transgenics and the wild type plants at extreme water stress (FTSW 0.04; Table 1).

Discussion

The *A. thaliana rd29A* gene promoter used for developing these transgenics peanut plants has been found to be an effective DS-inducible promoter. As previously reported, RT-PCR studies carried out for expression analysis of *DREB1A* from the transgenic plants revealed that *DREB1A* driven by the rd29A promoter was induced only by the fifth day after withdrawal of irrigation, and this corresponded to an average of about 0.65 FTSW, i.e., at a soil moisture level where plants did not show any sign of stress, at least from the point of view of its leaf gas exchanges. The goal of this study was to investigate the possible difference in the antioxidant response of transgenic events and their putative relation with TE that can contribute to drought tolerance under certain conditions in peanut. As far as is known, no study has evaluated the kinetics of transpiration responses of the plants under water stress and antioxidant systems simultaneously.

Here we report that transgenic peanut plants carrying *DREB1A* transcription factor driven by a stress-inducible promoter under DS resulted in antioxidant accumulation where (i) SOD did not change much until FTSW 0.5 in all the tested plants, although transgenics had higher level thereafter as stress progressed, (ii) APOX showed a highly variable response with on an average higher APOX level in transgenics, (iii) GR showed a dramatic increase in all the tested transgenic events at FTSW$\leqslant$0.4 whereas it did not change much in the WT, (iv) proline levels increased in all

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plants and more so in the transgenics at FTSW < 0.6, (v) MDA level increased dramatically in the WT only at lower FTSW values, and (vi) there appeared to be no significant relationship between the average values of these biochemical indicators and TE, although there were, as expected, some relationship with the FTSW thresholds where transpiration started decreasing.

The observations revealed that the level of antioxidative enzymes associated with the glutathione–ascorbate cycle was greater in the transgenic peanut events under DS. This can be explained by the fact that expression of *DREB1A* also manifests in a differential gene expression under DS, thereby acting as a master switch to counter the effects of drought (Shinozaki and Yamaguchi-Shinozaki, 2000). As evident from the results, the up-regulation of genes responsible for antioxidants might be a consequence of the over-expression of *DREB1A* under DS.

Overall, all the tested transgenic events of peanut closed their stomata at lower FTSW values (drier soils) in comparison to WT controls. All the transgenic events excluding RD20 closed their stomata at lower FTSW threshold values (0.28–0.44); whereas the antioxidant levels started to increase much earlier during the stress (FTSW 0.6–0.5). These results are in accordance with accumulating evidences suggesting that the intra-cellular concentrations of ROS are controlled by the plant detoxifying system, which includes ascorbate and glutathione pools which are implicated in redox signal transduction, acting as secondary messengers in hormonal-mediated events (Foyer and Noctor, 2003), namely stomatal movements (Pei et al., 2000). Also, a significant negative correlation existed between the APOX levels and the FTSW threshold value of the tested plants under DS, indicating that APOX had a role to play in the stomatal regulation in these plants.

In the present study, it was observed that most of the transgenic events showed significantly higher antioxidant activities as the DS progressed beyond 0.5 FTSW values. The results clearly indicated that the transgenic events had higher antioxidant levels beginning at the early stages of phase II, i.e., representing the start of decline in transpiration in DS plants due to shortage of soil water (productive stage but under increasing rarity of water), and phase III, i.e., representing very low or no transpiration in DS plants due to nonavailability of soil water in the root zone and the transpiration levels falling below 10% of WW (unproductive but survival stage). It is also interesting to note that the transgenic events were capable of transpiring equal amount of water under WS as that of the WW plants in a relatively drier soil compared to the WT controls. Hence, we speculate that the antioxidant levels in the transgenics might have contributed to better osmotic adjustment, leading to sustained stomatal opening in the transgenics. Yet these antioxidant responses were not correlated to the difference in TE and may have been concurrent events.

Interestingly, the increased SOD and APOX activities in the DS plants coincided with enhanced proline content ($P \leqslant 0.01$) in the peanut plants. These results are in agreement with reports that concluded the role of proline as a hydroxyl radical scavenger in the antioxidative stress (Smirnoff and Colombé, 1988; Matysik et al., 2002), regulation of $NAD^+/NADH^+$ ratio (Alia et al., 1993), and as a protein-compatible hydrotrope (Srinivas and Balasubramanian, 1995). There have also been reports where the mRNA transcripts of *P5CS2* and proline content were found to increase in transgenic *Arabidopsis* and tomato plants expressing *CBF3* and *CBF1* (Gilmour et al., 2000; Hsieh et al., 2002), which implied that the antioxidant (*catalase*) and *P5CS* gene(s) may also be induced in transgenic plants overexpressing *DREB* genes. Besides, a negative correlation observed between the proline levels and MDA production ($P \leqslant 0.01$) in this study suggested that the elevated proline also reduced free radical levels in response to water stress (Figure 6). This confirms earlier observations on the role of proline in reducing oxidative stress induced by osmotic stress, in addition to its accepted role as an osmolyte (Alia et al., 1993; Hong et al., 2000).

In conclusion, *DREB1A* appeared to trigger most of the biochemical parameters related to the antioxidative machinery in these peanut transgenics that appears to differ from the WT. Further work is ongoing to elucidate if there exists a

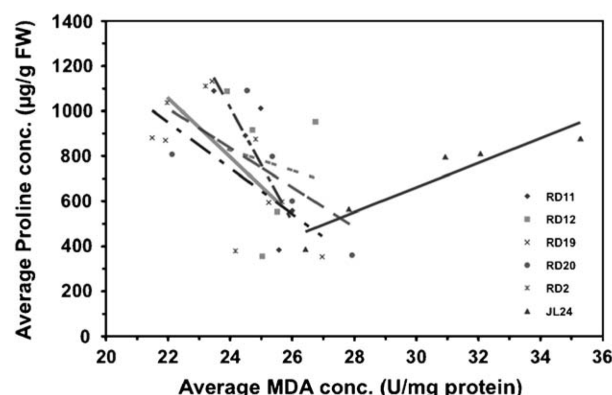

Figure 6. Correlation of proline and MDA levels under receding soil moisture conditions in the transgenic events and the WT control.

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correlation of DREB1A expression to the physiological and biochemical measurements made in these transgenics during this study. However, considering the data on early physiological or biochemical changes observed under DS conditions, a single and direct linear relationship between TE, an integrative measurement of plant performance, and antioxidative machinery underneath have not been proven in this study. The changes in the antioxidative machinery in the transgenics compared to the wild type under drought conditions appear to have no direct role on TE and are likely to be a concurrent response triggered by the over-expresssion of *DREB1A*. We did not test whether there could have been any non-linear relationship between TE and any of the components of the antioxidant machinery, because the antioxidant machinery response was very consistent across all events, whereas it was clear that the events showed a whole spectrum of TE values, from close to JL24 to much higher than JL24. In addition, the differences in the antioxidative machinery appeared to "kick-in" at fairly low values of FTSW. We speculate that the cause for TE differences is related to differences in the stomata regulation that are likely to take place from much higher FTSW level onwards. Since yield improvements under the highly changing tropical environments are an imperious requirement, identification of biochemical surrogate traits associated with various component traits (TE, Y, and harvest index) remains to be identified.

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