

## DROUGHT STRESS

# Relationships Between Transpiration Efficiency and Its Surrogate Traits in the *rd29A:DREB1A* Transgenic Lines of Groundnut

M. J. Devi<sup>1</sup>, P. Bhatnagar-Mathur<sup>2</sup>, K. K. Sharma<sup>2</sup>, R. Serraj<sup>3</sup>, S. Y. Anwar<sup>4</sup> & V. Vadez<sup>2</sup>

1 Agronomy Department, University of Florida, Gainesville, FL, USA

2 International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

3 International Rice Research Institute (IRRI), Los Banos, Laguna, Philippines

4 Department of Genetics, Osmania University, Hyderabad, Andhra Pradesh, India

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**Correspondence**

V. Vadez

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India

Tel.: 91-40 30 71 34 63

Fax: 91-40 30 71 30 74

Email: v.vadez@cgiar.org

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**Abstract**

Transpiration efficiency (TE) contributes to crop performance under water-limited conditions, but is difficult to measure. Herein, we assess the relationships between TE and surrogate traits and how these interact with water regimes, using isogenic materials: five transgenic events of groundnut and their wild-type (WT) parent JL 24, among which large variation in TE was previously reported. These five events came from the insertion of transcription factor *DREB1A* from *Arabidopsis thaliana*, driven by stress responsive promoter *rd29*. The events were in T3 generation and had been selected from a preliminary trial for having a large range of variation in the time needed to deplete soil moisture upon exposure to soil drying. Two experiments were conducted, in each case with plants exposed to well-watered (WW) and water-stressed (WS) conditions. Significant correlations were found between TE and soil plant analysis development chlorophyll meter readings (SCMR), TE and specific leaf area (SLA), and SLA and SCMR in both experiments. Nevertheless, these significant relationships were confined to the drought stress (DS) treatment. No correlation between TE and  $\Delta^{13}\text{C}$  (carbon isotope discrimination) was found in the present study, regardless of the water regime, and in none of the two experiments. A significant negative correlation was established between TE and the fraction of transpirable soil water (FTSW) threshold values where transpiration declined upon soil drying, in both experiments. The mean vapour pressure deficit in the two different seasons (1.47 kPa and 0.73 kPa) did not affect the ranking of genotypes for TE. It is concluded that surrogates for TE, when used, need careful consideration of the drought stress status of plants at the time of measurement, and that differences in TE might be closely related to how plants respond to soil drying, with high TE genotypes maintaining gas exchange until the soil is dryer than low TE genotypes.

**Introduction**

Transpiration efficiency (TE) i.e. biomass produced per water transpired, an important component of the crop water use efficiency (WUE), is a major trait contributing to tolerance to intermittent drought in groundnut (Ratnakumar et al. 2009). Efforts have been made over

the past two decades to breed groundnut genotypes for increased WUE. However, a trait-based approach using surrogate traits for TE has been compared with a conventional breeding approach based on yield selection, and both methods came up with lines performing equally under drought conditions (Nigam et al. 2005). Lines selected from the trait-based approach were assumed to

have high TE based on the values of the surrogate traits of TE, whereas lines selected from the conventional approach had high water use, and both traits could not be combined. From a breeding point of view, while it is important to assess whether and how WUE and water use are related, the priority is to ensure that the surrogate traits of TE truly represent TE, as they are widely used in breeding, and so it is critical to confirm that the relationships between TE and the surrogate traits of TE are robust, especially so under drought.

It has been indeed reported that TE is correlated with various easy and non-destructive traits like SPAD chlorophyll meter reading (SCMR), specific leaf area (SLA), specific leaf nitrogen (SLN) under well-watered (WW) conditions (e.g. Nageswara Rao et al. 2001, Bindu Madhava et al. 2003, Sheshshayee et al. 2006) and  $\Delta^{13}\text{C}$  (Farquhar et al. 1982, Wright et al. 1994, Udayakumar et al. 1998). These surrogate traits of TE have previously been used in breeding (Hubick et al. 1986, Wright et al. 1994) and are highly recommended (Sheshshayee et al. 2006). However, recent reports also showed that TE, measured gravimetrically, does not correlate with  $\Delta^{13}\text{C}$  in lentils, narrow-leaf lupin and chickpea under WW conditions (Kashiwagi et al. 2006, Turner et al. 2007). Under water-stressed conditions, these relationships were also reported (Wright et al. 1994), although with somewhat poorer correlation coefficient (Nageswara Rao et al. 1993). Poor relationships between TE and surrogates SCMR, SLA and  $\Delta^{13}\text{C}$  were also found in a large groundnut population developed to map quantitative trait loci (QTL) linked to TE (Krishnamurthy et al. 2007). A characteristic of some early studies reported above on the TE-surrogate traits relationship is the deliberate choice of entries having large variation in SCMR or  $\Delta^{13}\text{C}$  value. So, the question is whether the relationships would also be found if the variation in SCMR or  $\Delta^{13}\text{C}$  or SLA (e.g. Nautiyal et al., 2002) were not as large, and whether similar relationships are also found under water-stressed conditions.

In previous communication, we have reported large phenotypic variation for TE using five transgenic events of groundnut transformed with transcription factor *DREB1A* from *Arabidopsis thaliana*, driven by the stress inducible promoter *rd29A* from *A. thaliana*, and their untransformed control, JL 24, a popular groundnut variety in India and Africa, that has low-to-intermediate TE levels (Bhatnagar-Mathur et al. 2007), and is drought sensitive (Joshi et al. 1988, Ravindra et al. 1990). *DREB1A* has been shown to influence the response to drought in wheat (Pellegrineschi et al. 2004). In our previous study (Bhatnagar-Mathur et al. 2007), we have found a consistently 50–100 % higher TE in several transgenic events than in the wild-type parent JL 24 (WT JL 24), under both well-watered and water-deficit treatments. These

transgenics are lines that are nearly isogenic to WT JL 24 and differ only in the insertion of *DREB1A* element, although this transcription factor would be responsible for the expression of a cascade of genes. Indeed, DRE-binding gene *DREB1* is a transcription factor that binds to the promoter region of dehydration-responsive genes such as *rd29A*, thereby inducing their expression in response to different abiotic stresses (Shen et al. 1997). Therefore, the differences in TE observed in transgenic events offer an ideal material to test the strength of the relationships between different surrogate traits and TE.

Theoretically, differences in TE can either be obtained by increased carboxylation efficiency, or by a decreased stomatal conductance (Farquhar et al. 1989, Condon et al. 2002). Several authors have assumed that TE differences in groundnut would mostly be explained by differences in the carboxylation efficiency (Hubick et al. 1986, Nageswara Rao et al. 1993, Wright et al. 1994, Udayakumar et al. 1998), and then could be measured under WW conditions, with SCMR, SLA,  $\Delta^{13}\text{C}$  and SLN as relevant surrogates (Udayakumar et al. 1998). However, the importance of other physiological mechanisms to regulate TE, such as the control of stomatal movements cannot be excluded, in particular under conditions of water deficit, and this may affect the relationships between TE and surrogates. Such consideration has been recently used in wheat (Hui et al. 2008). In fact, a recently discovered gene, involved in the regulation of TE, appears to affect both stomatal conductance and photosynthetic efficiency in *A. thaliana* (Masle et al. 2005). Preliminary data indicated that the *DREB1A* transgenics usually have a more conservative use of water (Bhatnagar-Mathur et al. 2004), where all 14 transgenic events tested took a larger number of days to deplete the moisture of equally sized pots. This is consistent with what can be interpreted from other *DREB1A* transgenic materials like wheat (Pellegrineschi et al. 2004). It has also been found that the stomatal conductance in transgenic events under WW conditions was lower than that in the WT JL 24 (Bhatnagar-Mathur et al. 2007), which would suggest that the stomatal regulation is affected in the transgenics, even under WW conditions, thereby leading to higher TE. Our first hypothesis is that we may find weak relationships for those traits that proxy for mesophyll efficiency under WW conditions. Then, we also hypothesize that plant transpiration response to a progressive exposure to water deficit may be closely related to the TE differences found under drought.

It has been reported that the vapour pressure deficit (VPD) has an impact on the thresholds at which transpiration starts to decline due to its potential role in maintaining water flux among plant, soil and atmosphere (Tardieu et al. 1992) although previous studies show conflicting results with this respect (Turner et al. 1985). Ray

and Sinclair (1997), using a same experimental setup as ours in maize, found that the VPD had a minor effect on the fraction of transpirable soil water (FTSW, an index of soil moisture) thresholds where transpiration decline. Therefore, it would be important also to assess whether differences exist in the FTSW for transpiration decline at different VPDs in these transgenic materials and whether differences in VPD would lead to  $G \times E$  interaction for TE in these lines.

The objectives of this work were therefore threefold: (i) explore the relations between TE and its surrogate traits in transgenic materials and WT JL 24; (ii) compare the FTSW threshold values where transpiration start to decline and their relationships with TE; and (iii) study the changes in FTSW thresholds and TE with VPD levels.

## Materials and Methods

### Plant materials and experimental conditions

Transgenic events of groundnut variety WT JL 24 that carry the *DREB1A* transcription factor from *A. thaliana* and driven by the *A. thaliana rd29A* promoter were earlier produced through *Agrobacterium tumefaciens*-mediated transformation. Details of the transformation are reported in (Bhatnagar-Mathur et al. 2007). These transgenic events were at T3 generation. These came from an earlier selection from a set of 14 T1 generation events, tested for the soil moisture threshold where transpiration declines upon exposure to water deficit and for the number of days needed to deplete a set amount of soil moisture (Bhatnagar-Mathur et al. 2004). These T3 generation events, all with single copy gene insertion, were reported to have large variation in transpiration efficiency (TE, in kg biomass kg<sup>-1</sup> water transpired) pooled over two experiments (Bhatnagar-Mathur et al. 2007).

In short, the two experiments previously reported (Bhatnagar-Mathur et al. 2007) were set up in P2 level contained greenhouse facility (28 °C day/20 °C night temperature) and their purpose was to have an assessment of TE and of the FTSW threshold where the transpiration declines upon exposure to water-deficit stress (WDS), across two seasons (June–July, Exp1; October, Exp2) period, characterized by slightly different vapour pressure deficit (VPD) conditions (details provided below), and using a well-watered control treatment (WW). The additional objectives, reported here, were to assess surrogate traits of TE and assess their relationship to TE under different water regimes, and also to assess the possible effect of VPD on TE and on the FTSW thresholds. Light received and photoperiod differences between the two experiments were marginal.

The plants in excess number to those needed for the experiment were grown in pots of 20 cm diameter containing 5.0 kg of Alfisol : sand : compost mixture in 3 : 2 : 1; including SSP (single super phosphate; 300 mg kg<sup>-1</sup>), with one plant per pot. These plants were tested by polymerase chain reaction (PCR) as described earlier (Bhatnagar-Mathur et al. 2007) so that only the transgenic plants carrying the transgene were used for the experiments. These plants were separated in three subsets with equal number of plant per genotype in each subset. At 28 DAS, one subset was harvested and used to assess biomass at this stage. The other two sets were then arranged in a randomized complete block design with two watering treatments as main factors (well-watered (WW) conditions or water-deficit stress (WDS)), and genotype as subfactor, with six replicates within each treatment.

### Measurement of transpiration efficiency (TE)

Transpiration efficiency (in g biomass per kg of water transpired) was calculated as the ratio of biomass increase between 28 DAS (prior to treatment imposition) and the final harvest at the end of treatment imposition, divided by the total water transpired during the treatment period. Plants were harvested when the WDS plants had their transpiration below 10 % of the transpiration of WW plants (see below), and biomass weighed after drying to constant weight for 3 days at 70 °C. The biomass increase of each plant of a given genotype was the difference between the biomass at harvest minus the mean biomass of that same genotype at 28 DAS. Total water transpired or cumulated transpiration was the sum of the daily transpiration value between 28 DAS and the final harvest. How the gravimetric TE we measured here related to intrinsic TE was not the purpose of this study.

### Measurement of surrogate traits

Soil plant analysis development (SPAD) chlorophyll meter (SPAD-502) reading (SCMR) is a unit-less value, which corresponds to the relative amount of chlorophyll concentration in the leaves (Arunyanark et al. 2008). SCMR was recorded on the secondary leaf, which is fully expanded from the internodal position on two branches. Eight readings were made on each plant, one in each of the leaflets of secondary leaves in two branches, and then averaged. In Experiment 1, SCMR was evaluated in plants exposed to WDS conditions at 12 days after imposing the stress treatment ( $0.11 \pm 0.05$  FTSW), coinciding with a high level of stress and small differences between lines. SCMR was also evaluated at the same number of days in the WW plants. In experiment 2, readings were performed at three different times in plants exposed to

water-deficit conditions, i.e. after 7 and 12 days of stress imposition and at harvest. At these stages, the moisture availability (FTSW) in pots ranged between 0.37 and 0.48, 0.02 and 0.04, and 0 respectively. Under the WW conditions, the SCMR was recorded only at harvest.

Specific leaf area was measured from all the leaves from the whole plants, collected at harvest and whose leaf area was measured with the LI 3100-leaf area meter. After measuring the leaf area, leaves were immediately oven dried at 80 °C and dry weights were recorded. Specific leaf area of each plant was computed as the ratio of leaf area divided by the leaf dry weight. Specific leaf nitrogen (SLN) (g N cm<sup>-2</sup> leaf area) was calculated as N content per unit leaf area and was measured in the leaf samples used to calculate the SLA. Total N (nitrogen) was estimated in 150 mg of finely ground leaf sample, employing SKALAR Auto Analyzer, the Netherlands (Krom 1980).

#### Measurement of carbon and oxygen isotope discrimination

The isotopic composition of carbon ( $\delta^{13}\text{C}$ ) and oxygen ( $\delta^{18}\text{O}$ ) in total organic matter of leaf samples were determined by isotope ratio mass spectrometry (Farquhar et al. 1989). Carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) is a measure of the discrimination against  $^{13}\text{C}$  in leaf tissue and is assessed by the difference in carbon isotope ratios of the air ( $-7.6\text{‰}$  on the Pee Dee Belemnite scale (Hubick et al. 1986)) and of the leaf samples. A 10-mg subsamples of finely powdered leaves ( $<100\ \mu\text{m}$ ) from the harvested samples were combusted in an elemental analyser (Carlo Erba Instrumazione, Milan, Italy). The isotope ratios of the samples were estimated by comparison with a working standard of  $\text{CO}_2$  with an isotope ratio of  $-35.08\text{‰}$  relative to Pee Dee Belemnite. The carbon isotopic ratio was determined with an analytical uncertainty of  $<0.1\text{‰}$ . Carbon isotope discrimination values were computed as follows assuming the isotopic composition of atmospheric  $\text{CO}_2$  as 8 ppm following the formula

$$\Delta^{13}\text{C} = (\delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{product}}) / (1 + \delta^{13}\text{C}_{\text{product}}).$$

$\Delta^{18}\text{O}$  represents the enrichment in  $^{18}\text{O}$  of leaf sample over Vienna-standard mean ocean water (VSMOW) and was measured in experiment 2 only. The oxygen isotopic composition of the leaf samples was calculated by pyrolysing the dried leaf powder with glassy carbon catalyst at 1400 °C using a Temperature Conversion Element analyser (Thermo-Finnigan, San-Jose, CA, USA) interfaced with IRMS (Isotope Ratio Mass Spectrometers). The analytical uncertainty for oxygen isotope measurement was  $<0.4\text{‰}$ . The  $\Delta^{18}\text{O}$  over the irrigation water was computed as follows:

$$\Delta^{18}\text{O}_{\text{biomass}} = \delta^{18}\text{O}_{\text{biomass}} - \delta^{18}\text{O}_{\text{irrigation water}},$$

$\delta^{18}\text{O}_{\text{irrigation water}}$  was measured by  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium device (Gas-Bench III).

The whole leaves were sampled for ( $\Delta^{13}\text{C}$ ), and not only those leaves that were developed in the time course of the TE measurement. While this is, in strict term, a flaw for the evaluation of  $\Delta^{13}\text{C}$ , the amount of shoot tissues developed during the experimental period represented a large proportion of the total shoot biomass (60 % and 70 % on average across the lines, respectively under WDS and WW conditions).

#### Measurement of the FTSW-threshold for decline in transpiration

Prior to the imposition of stress, at 28 DAS, the pots of both WW and WDS treatments were saturated with water and left overnight to drain off the excess water. On the early morning of the following day, the pots were enclosed in polythene bags and wrapped around the stem to avoid any water loss by evaporation. A protruding tube was inserted in all the pots to facilitate watering of the pot. The pots were then weighed to record the initial weight followed by regular weighing every morning at the same time. The WW plants were maintained at about 80 % field capacity (about 200 g below the initial pot weight) throughout the experiment. To expose the WDS plants to a progressive water deficit, they were allowed to loose a maximum of 70 g of water per day, and any transpiration loss in excess of 70 g per day was compensated by the addition of water.

Daily transpiration of each drought-stressed plant was estimated as a difference between daily pot weights. Transpiration rate data of WDS plants were normalized to compare their transpirational rates relative to WW plants and to minimize the effect of plant-to-plant variation (Ray and Sinclair 1997). First, the transpiration ratio (TR) was calculated by dividing the daily transpiration data of each individual WDS plant by the average daily transpiration of WW plants for each genotype. Second, the normalized transpiration ratio (NTR) of each WDS was determined by dividing the TR of every single day by the mean of the TR over the first 3 days when WDS plants were still not stressed. The second normalization was performed to take care of the plant-to-plant variation in the transpiration ratio, in order to ease the comparison of the profile of transpiration response to drought stress between plants that vary in size. The Alfisol used in these experiments contains about 50 % of sand. Therefore, our alfisol : sand : compost mixture in 3 : 2 : 1 contained about 60 % of sand overall and would be within the sand-content limits in which the hydraulic conductance

of the soil should not limit water supply for the transpiration of the WW plants, and would then have no bearing on the normalized transpiration rate (Sinclair 2005).

The experiment was terminated when the daily NTR of WDS plants had decreased to <10 % of that in WW plants, indicative that there was no more available soil water to support transpiration (Sinclair and Ludlow 1986). Plants of an individual genotype under WW conditions were harvested when all WDS plants of that genotype had reached  $NTR < 0.1$ , to estimate the final biomass when WDS plants reached this point. The difference in pot weight between the initial recording and at the end point provided an estimate of the total transpirable soil water (TTSW) available in each pot. TTSW was subsequently used to back calculate the daily FTSW remaining in the pot where,  $FTSW = 1 - (\text{initial pot weight} - \text{daily pot weight})/TTSW$ . Values of daily NTR of each WDS plant were plotted against daily FTSW and a linear-plateau regression analysis (SAS Institute 1996) was employed to estimate the FTSW threshold value where NTR begins to decline (Ray and Sinclair 1997). The plateau regression procedure carries out iterations of the NTR data, starting at  $FTSW = 1$  (wet soil) and fits them to a  $y = 1$  equation. From the FTSW level onwards where  $y = 1$  is no longer the best fit for NTR, data are fitted to a linear decline equation. The FTSW threshold (with confidence interval) where NTR begins to decline is then taken as the intersection between the plateau ( $y = 1$ ) and linear decline equations.

#### Measurement of the vapour pressure deficit

The daily air temperature (T) and relative humidity (RH) in the contained greenhouse were recorded by using data loggers to calculate the vapour pressure deficit (VPD)

according to the method described by Prenger and Ling 2000 such as  $VPD = VP_{\text{saturation}} - VP_{\text{air}}$ .

The RH and temperature of the greenhouse were recorded daily for each experiment from beginning of the experiment (FTSW 1) till the end (FTSW 0) and daily VPD was calculated by averaging daily measured values (Fig. 1).

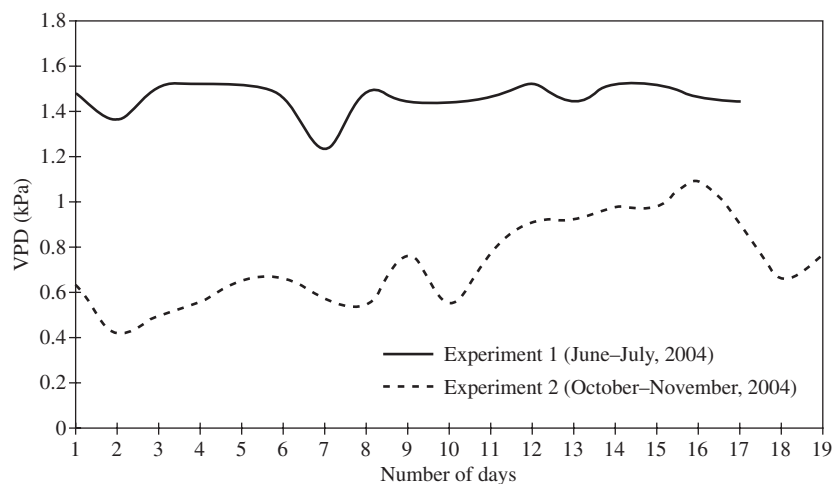
#### Data analysis

The data was analysed by analysis of variance (ANOVA) using GENSTAT version 8.0 (VSN International Ltd, Oxford, UK). A two-way ANOVA was used to test season and season-by-genotype interaction within water treatments. The average mean values were compared between the different transgenic events and the WT JL 24 with Tukey's test within treatment and season (experimental design details above). Mean regression analysis was derived for each trait by regressing the mean values of all lines for that particular trait. A plateau regression procedure using SAS (Cary, NC, USA) was used to determine the FTSW threshold values of transpiration decline (details provided above).

## Results

#### Variation in TE and surrogate traits

Under WDS significant genotypic variation was found in TE in both experiments ( $P < 0.01$ ), with RD2 having higher TE than WT JL 24 in experiment 1, and RD2, RD11, RD12, and RD19 having higher TE than WT JL 24 in experiment 2 (Table 1). There was no genotype-by-season interaction in neither of the water regimes. Under WW, there was also significant genotypic variation for TE in both experiments, with same events having higher TE than WT JL 24 (Table 2).



**Fig. 1** Daily mean vapour pressure deficit (VPD) during the experimental period in each experiment.

**Table 1** SCMR (SPAD chlorophyll meter readings) at 7 days (SCMR 7d), 12 days (SCMR 12d) after the onset of the stress treatment and at harvest (SCMR Hv), specific leaf area (SLA,  $\text{cm}^2 \text{g}^{-1}$ ), specific leaf nitrogen (SLN,  $\text{g N m}^{-2}$ ), transpiration efficiency (TE in  $\text{g biomass kg}^{-1}$  water transpired),  $\Delta^{13}\text{C}$  (‰) (carbon isotope discrimination),  $\Delta^{18}\text{O}$  (‰) (oxygen isotopic enrichment), FTSW (fraction of transpirable soil water) threshold for the transpiration decline and confidence interval for the FTSW thresholds, in the different transgenic lines and their wild-type parent under drought stressed (DS) conditions in two different experiments

Events	SCMR 7d	SCMR 12d	SCMR Hv	SLA	SLN	TE	$\Delta^{13}\text{C}$ (‰)	$\Delta^{18}\text{O}$ (‰)	FTSW	Confidence interval
Experiment 1										
WT JL 24	ND	44.12 b	ND	199.6 a	4.211 a	4.29b	21.67 c	ND	0.653 a	0.5574–0.7487
RD19	ND	46.33 ab	ND	191.3 b	4.235 a	4.99b	22.91 a	ND	0.3421 b	0.3207–0.3635
RD12	ND	45.98 ab	ND	195.9 ab	4.079 a	4.63ab	22.50 ab	ND	0.4414 b	0.4093–0.4736
RD20	ND	44.22 b	ND	200.1 a	4.222 a	4.51b	22.14 bc	ND	0.4501 b	0.4236–0.4767
RD2	ND	48.18 a	ND	184.6 b	4.329 a	6.12a	22.37 b	ND	0.3121 b	0.2860–0.3382
RD11	ND	45.34 ab	ND	192.3 b	4.193 a	5.59ab	22.61 ab	ND	0.3296 b	0.3085–0.3506
Grand mean	–	45.7	–	194.0	4.257	5.03	22.37	–	0.425	
Experiment 2										
WT JL 24	36.15 c	39.12 b	39.12 c	213.7 a	4.083 b	4.21c	21.57 a	31.16 a	0.5472 a	0.5053–0.5891
RD19	36.83 c	41.35 ab	46.85 ab	193.9 b	4.323 ab	5.24ab	21.50 a	27.82 b	0.3655 bc	0.3399–0.3910
RD12	36.47 c	41.35 ab	45.68 ab	202.1 ab	4.160 ab	4.25c	20.98 a	27.94 b	0.4461 abc	0.4043–0.4880
RD20	37.28 bc	41.33 ab	45.12 b	205.5 ab	4.167 ab	4.43bc	21.77 a	29.91 a	0.4935 ab	0.4595–0.5274
RD2	42.03 a	44.73 a	49.80 a	188.0 b	4.448 a	5.79a	21.39 a	25.89 c	0.2826 c	0.2529–0.3124
RD11	39.63 ab	42.32 ab	47.33 a	193.1 b	4.317 ab	4.59bc	21.38 a	26.26 bc	0.3981 abc	0.3565–0.4397
Grand mean	38.07	41.70	45.65	199.40	4.325	4.75	21.43	28.60	0.416	
F Prob (G)	<0.001	<0.001	<0.001	0.025	0.005	<0.001	0.021	<0.001	<0.001	
F Prob (season)	–	<0.001	–	0.145	0.359	0.141	<0.001	–	0.977	
F Prob (G*season)	–	0.776	–	0.929	0.385	0.489	<0.001	–	<0.001	

Mean data are the average of six replicated reading for each genotype.

Values followed by same letter are not significantly different at the 5 % level by Tukey's method ( $n = 6$ ).

SCMR, soil plant analysis development chlorophyll meter readings; SLA, specific leaf area; SLN, specific leaf nitrogen; ND, not determined.

The FTSW threshold where NTR declined upon WDS imposition also differed significantly, and were lower in all transgenic events than in WT JL 24 in experiment 1 and lower in RD2 and RD19 than in WT JL 24 in experiment 2 (Table 1). The FTSW thresholds showed significant genotype-by-season interaction. As reported in Bhatnagar-Mathur et al. (2007), the total amount of water extracted from the soil did not vary between the genotypes in any of the experiments (range: 808–917  $\text{ml plant}^{-1}$  and 800–956  $\text{ml plant}^{-1}$  in experiment 1 and 2). In experiment 1, the total water transpired (water extracted plus water added) of WT JL 24 and RD20 was about 150–200 ml above the other lines. In this experiment, it took 12 days for WT JL 24, 14 days in RD20 and 15 days in all other transgenics to fall below a NTR of 0.1. In experiment 2, the total water transpired under WDS was similar in all genotypes (circa 1600 ml), and it took 12 days for RD11, RD12 and WT JL 24, and 14 days for the other lines to fall below an NTR of 0.1. On each day of the dry down, in experiment 1 the FTSW of WT JL 24 was only slightly below that of the other genotypes, whereas it was similar in all genotypes in experiment 2.

So, the kinetics of stress imposition was very similar in all lines tested.

Under WDS, a significant genotypic variation ( $P < 0.01$ ) was found in the SCMR values in experiment 1 under WDS (Table 1), and SCMR of RD2 was higher than in WT JL 24. Under WDS conditions in experiment 2, there were also genotypic differences in the SCMR values at all sampling dates ( $P < 0.01$ ), with higher SCMR in RD2 and RD11 than in WT JL 24 after 7 days of WDS, higher SCMR in RD2 than in WT JL 24 after 12 days of WDS, and higher SCMR in RD2 and RD11 than in RD20 and WT JL 24 at harvest (Table 1). By contrast under WW conditions, there was neither genotype, nor genotype-by-season interaction effects on SCMR values (Table 2).

Under WDS, SLA varied significantly between genotypes in both experiments, SLA of RD2, RD11, and RD19 being lower than SLA of WT JL 24, and RD20. The genotype-by-season interaction was not significant under WDS (Table 1). Under WW conditions, none of the events had different SLA from WT JL 24 in any of the experiments (Table 2).

**Table 2** Average SCMR (SPAD chlorophyll meter readings) at harvest, specific leaf area (SLA,  $\text{cm}^2 \text{g}^{-1}$ ), specific leaf nitrogen (SLN,  $\text{g N m}^{-2}$ ), transpiration efficiency (TE in  $\text{g biomass kg}^{-1}$  water transpired),  $\Delta^{13}\text{C}$  (‰) (carbon isotope discrimination),  $\Delta^{18}\text{O}$  (‰) (oxygen isotopic enrichment), of the different transgenic lines and their wild-type parent under well-watered conditions in two different pot experiments

Events	SCMR	SLA ( $\text{cm}^2 \text{g}^{-1}$ )	SLN ( $\text{g N m}^{-2}$ )	TE	$\Delta^{13}\text{C}$ (‰)	$\Delta^{18}\text{O}$ (‰)
Experiment 1						
WT JL 24	37.32 a	233.1 ab	4.94 a	4.545b	21.51 b	ND
RD19	37.28 a	217.6 b	4.99 a	5.425ab	22.30 ab	ND
RD12	35.58 a	244.4 a	5.15 a	5.363ab	21.50 b	ND
RD20	36.70 a	229.2 ab	4.92 a	4.644b	21.82 b	ND
RD2	37.41 a	233.1 ab	4.99 a	5.603a	22.14 b	ND
RD11	37.63 a	212.6 b	4.69 a	4.775ab	23.08 a	ND
Experiment 2						
WT JL 24	36.30 a	201.0 a	4.97 a	2.05c	20.87 ab	26.92 a
RD19	35.48 a	202.5 a	4.87 a	4.31ab	21.92 a	24.94 a
RD12	36.73 a	202.2 a	5.01 a	5.13a	20.72 b	26.25 a
RD20	37.11 a	192.1 a	5.04 a	3.19bc	20.99 ab	26.33 a
RD2	37.70 a	187.9 a	4.92 a	4.09ab	20.69 b	24.67 a
RD11	37.10 a	187.1 a	5.03 a	4.96ab	20.47 b	25.03 a
F Prob (G)	0.867	0.607	0.607	< 0.001	0.002	0.079
F Prob (season)	0.84	<0.001	0.653	< 0.001	<0.001	–
F Prob (G*season)	0.955	0.169	0.180	0.074	<0.001	–

Mean data are the average of six replicated reading for each genotype.

Values followed by same letter are not significantly different at 5 % level by Tukey's method.

SCMR, soil plant analysis development chlorophyll meter readings; SLA, specific leaf area; SLN, specific leaf nitrogen; ND, not determined.

Under WDS, no significant variation for SLN was found among the genotypes in experiment 1, whereas in experiment 2, SLN showed significant variation, where WT JL 24 had a lower SLN than RD2 ( $P < 0.01$ ) (Table 1). The genotype-by-season interaction for SLN was significant ( $P < 0.01$ ) (Table 1). No significant variation was observed for SLN under WW conditions in both experiments (Table 2).

Under WDS conditions, a substantial variation ( $P < 0.05$ ) was observed for  $\Delta^{13}\text{C}$  in experiment 1 where WT JL 24 had lower  $\Delta^{13}\text{C}$  than in all the transgenic events, except RD20 (Table 1). By contrast, there was no genotypic difference for  $\Delta^{13}\text{C}$  in experiment 2. Under WW condition, genotypes differed significantly for  $\Delta^{13}\text{C}$  in experiment 1 ( $P < 0.001$ ), with RD11 having higher value than WT JL 24 (Table 2). The genotype-by-season interaction effect on  $\Delta^{13}\text{C}$  was significant ( $P < 0.001$ ). Under WDS,  $\Delta^{18}\text{O}$  varied significantly ( $P < 0.001$ ) among genotypes, with all transgenic events having significantly lower  $\Delta^{18}\text{O}$  than WT JL 24, except RD20. By contrast, there was no significant variation among genotypes under WW conditions (Table 2).

### Relationships between TE and its surrogates

In experiment 1, association between TE and SCMR measured at 12 days after stress imposition were positively and significantly related under WDS (Table 3). In experiment 2, with SCMR measured when FTSW reached 0.02

the correlation coefficient with TE was significant ( $r = 0.82$  at  $P < 0.05$ ), but measured at 7 days after stress imposition or at harvest, SCMR had no significant relationship with TE (data not shown). Under WW conditions, TE was not significantly correlated with SCMR in both experiments (data not shown). Under WDS, a significant negative correlation was established between TE and SLA both in experiment 1 ( $r = -0.93$ ,  $P < 0.01$ ) and experiment 2 ( $r = -0.82$ ,  $P < 0.05$ ) (Table 3). In contrast, under WW conditions TE was not significantly correlated with SLA in both the experiments (data not shown). Under WDS, TE and SLN were significantly correlated in experiment 2 only ( $r = 0.90$   $p < 0.05$ ) (Table 3). Under WW conditions, the relationship between TE and SLN was non-significant in both experiments (data not shown).

Association could not be established between TE and  $\Delta^{13}\text{C}$  in both the experiments either under WDS (Table 3) or WW conditions (data not shown).  $\Delta^{18}\text{O}$  did not exhibit any significant correlation with cumulated transpiration in experiment 2 under both WW and WDS regimes (data not shown). Under WDS there was a significant negative relationship between SCMR with SLA in both the experiments ( $r = -0.95$ ,  $P < 0.01$ ) (Table 3). Under WW conditions, SCMR and SLA were significantly correlated in experiment 2 only ( $r = 0.81$ ). Under WDS specific leaf nitrogen was positively related with SCMR ( $r = 0.89$ ,  $P < 0.05$ ) and negatively with SLA ( $r = -0.97$ ,  $P < 0.01$ ) in experiment 2 (Table 3), although such

**Table 3** Correlation values (r) drawn between TE and surrogate traits under drought-stressed conditions. The regression were drawn using mean values of six replicated plants in each genotype tested (n = 6). SCMR data used in the regressions are those collected at 12 days after beginning of stress imposition (FTSW = 0.02 in experiment 1 and between 0.02 and 0.04 in experiment 2)

	TE	SCMR	SLA	SLN	$\Delta^{13}C$
Experiment 1					
TE	1				
SCMR	0.82*	1			
SLA	-0.93**	-0.95**	1		
SLN	0.58	0.43	-0.56	1	
$\Delta^{13}C$	-0.46	-0.64	0.33	-0.65	1
Experiment 2					
TE	1				
SCMR	0.82*	1			
SLA	-0.82*	-0.95**	1		
SLN	0.89*	0.89*	-0.97**	1	
$\Delta^{13}C$	0.04	-0.75	0.42	-0.15	1

SCMR, soil plant analysis development chlorophyll meter readings; SLA, specific leaf area; SLN, specific leaf nitrogen; TE, transpiration efficiency;  $\Delta^{13}C$ , carbon isotope discrimination.

\*Significant at  $P < 0.05$ .  
\*\*Significant at  $P < 0.01$ .

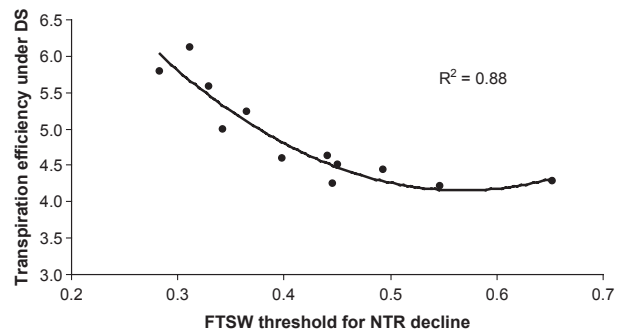
relations were not found in experiment 1. Under WW conditions, SLN was not significantly related to SCMR or SLA (data not shown). The relationship between carbon isotope discrimination and other traits such as SCMR, SLA and SLN was not significant in any of the treatment and experiments (Table 3) (data not shown for WW).

**Relation of TE with FTSW threshold for NTR decline**

The FTSW threshold values were significantly and negatively correlated with TE in both the experiments (data not shown). The data were then pooled across the two experiments and showed a significant negative relationship between the FTSW threshold value and TE ( $R^2 = 0.88$ ) (Fig. 2). TE declined sharply, close to 50 %, in the range of FTSW threshold 0.30–0.45, indicating that slight changes in the FTSW threshold where NTR decline within that range of FTSW value could lead to important differences in TE.

**Effect of season on the FTSW threshold where NTR declines and on TE**

The average VPD during experiment 1 was higher (1.47 kPa) than in experiment 2 (0.73 kPa) (Fig. 1). Under WDS the correlation coefficient ( $r = 0.81$ ,  $P < 0.05$ ) between TE values in the two experiments revealed that TE values for each genotype were consistent



**Fig. 2** Relationship between transpiration efficiency (TE, g biomass  $kg^{-1}$  water transpired) and the FTSW-threshold (fraction of transpirable soil water) where NTR (the normalized transpiration ratio) begins to decline of the five tested transgenic events and untransformed WT JL 24 under drought stressed conditions in experiment 1 and 2. Mean data used in the regression are the average of six replicated plants for each genotype and experiment.

across seasons. This agreed with the lack of a genotype-by-season interaction for TE (Table 1). The values of FTSW threshold where NTR declines for genotypes including WT JL 24 across the experiments were unaffected due to change in seasonal VPD, in the range of VPD that was tested in these experiments ( $r = 0.89$ ,  $P < 0.01$ ) (data not shown).

**Discussion**

Differences in TE were found under WW conditions, whereas surrogates did not vary between transgenic lines under these conditions and, contrary to previous observation, TE–surrogates relationship was not found. TE also differed under WDS and relationship between SCMR, SLA and TE were observed, but not between TE and  $\Delta^{13}C$ . A strong correlation was observed between TE and the FTSW threshold for NTR where transpiration begins to decline, whereby genotypes having higher TE under WDS also began to decline transpiration in dryer soils. Finally, in these two experiments, the ranking of genotypes for TE remained unchanged across two seasons under varying VPD (0.73 to 1.47 kPa).

Under WW conditions, there was no relationship between SCMR, SLA, or SLN and TE. This is inconsistent with previous reported data (e.g. Nageswara Rao et al. 2001, Bindu Madhava et al. 2003 and Sheshshayee et al. 2006). Difference in the photosynthetic efficiency is the main hypothesis proposed to explain genotypic differences in TE in groundnut (Hubick et al.1986, Nageswara Rao et al. 1993, Wright et al. 1994, Udayakumar et al. 1998). The physiological basis seems to be the more densely packed mesophyll cells, leading to higher photosynthetic pigments and Rubisco per unit of leaf area proxied



by a higher index of greenness (SCMR), N content (SLN), or leaf thickness (lower SLA). So, our data indicate that other mechanisms than the photosynthetic machinery may explain the TE differences under WW conditions in the material that we have used. Data reported previously (Bhatnagar-Mathur et al. 2007) suggest that the control of stomata movement under WW conditions may have a more important role in explaining TE differences in groundnut than previously assumed, at least in the plant materials that were used.

Under WDS conditions, significant positive relationship between TE and SCMR and TE and SLN, and significant negative relation between TE and SLA were observed, in agreement with previous reports (Nageswara Rao et al. 1993), and so the hypothesis of photosynthesis driving TE difference remains valid under WDS conditions. These observations are in agreement with our results in a groundnut population of recombinant inbred lines (RIL) that was developed to map QTL for TE (Krishnamurthy et al. 2007), although the relationships in that work were relatively weak. Hence, results suggest that the use of surrogate traits in selection for improved WUE should be confined to WDS conditions only. In the case of SCMR, a better correlation was found in SCMR measurement taken from plants at lower FTSW, in agreement with what was previously reported (Krishnamurthy et al. 2007). With regards to the gene action (Agarwal et al. 2006), this would also fit the hypothesis of having DRE-binding genes DREB1, as transcription factors, binding the promoter region of dehydration-responsive rd29A, and inducing the expression of a cascade of stress responsive genes (see discussion in Bhatnagar-Mathur et al. 2007). An interesting aspect is the fact that DREB1A is known to be inducing an ABA-independent gene expression. Moreover, recent evidence indicates that DREB1A action did involve modifications in the antioxidative machinery but these changes were not related to the differences in TE (Bhatnagar-Mathur et al. 2009).

In the present study, under WW conditions,  $\Delta^{13}\text{C}$  showed no significant correlation with TE. This result is different from previous reports where gravimetric TE and  $\Delta^{13}\text{C}$  were found to be closely related under WW conditions (Farquhar and Richards 1984, Udayakumar et al. 1998, Sheshshayee et al. 2006). In addition,  $\Delta^{13}\text{C}$  was not significantly related to SLA, in contrast to previous study (Nageswara Rao and Wright 1994). Under WDS conditions, although SCMR, SLA and SLN were related to TE, the expected relationship with  $\Delta^{13}\text{C}$  was not observed, inconsistent with the previous report (Nageswara Rao et al. 1993, Wright et al. 1994), but consistent with more recent findings (Kashiwagi et al. 2006, Turner et al. 2007). It is also in close agreement with the finding of a weak relationship between TE and  $\Delta^{13}\text{C}$  in a RIL popula-

tion of groundnut developed between high and low TE parents (Krishnamurthy et al. 2007).

Recent evidence indicates that the relationships involving  $\Delta^{13}\text{C}$  are highly dependent on the environment (Misra et al. 2006, Anyia et al. 2007). Therefore, the conditions in the glasshouse in the season that plants were tested must have changed enough to make the TE- $\Delta^{13}\text{C}$  relationships insignificant. We have proposed three other hypotheses for these findings, i.e. first, all leaves from the plant were used for the determination of  $\delta^{13}\text{C}$ , and not only those that developed during the experimental period. Therefore, the  $\delta^{13}\text{C}$  in the leaves developed during the experimental period may simply be 'diluted' in the overall carbon pool, putatively causing the lack of relation with TE. However, this hypothesis is unlikely as the biomass developed during the experimental period accounted for most of the biomass (58 % and 69 %). Second, a recent modelling study on sorghum showed that a maximum rate of transpiration per day, thanks to a partial stomata closure during the hours of the day having the highest VPD (and intrinsic TE the lowest), a phenomenon recently described in pearl millet (Kholová et al. 2010a), would contribute to an increased TE (Sinclair et al. 2005). We can speculate that such a mechanism may occur in DREB1A transgenics, which would lead to short periods of high gravimetric TE at certain times of the day that may not have any proportional relation to the  $\Delta^{13}\text{C}$  signature. Finally, the literature on carbon isotope discrimination expands little on the fact that the discrimination of Rubisco against  $^{13}\text{C}$  is in a factor of 2.7 % (Farquhar et al. 1989), whereas the discrimination of stomata against  $^{13}\text{C}$  is in a factor of 0.4 %, about seven times less. It is assumed that the carboxylation efficiency explains most differences in TE and the related differences in  $\Delta^{13}\text{C}$  in groundnut (Hubick et al. 1986, Nageswara Rao et al. 1993, Wright et al. 1994, Udayakumar et al. 1998). However, evidences from the current work indicated that the TE differences between the transgenics and the wild type would rather be explained by differences in the stomatal regulation as we found lower stomatal conductance in the transgenics (Bhatnagar-Mathur et al. 2007). The fact that the discrimination by the stomata has a less 'sensitive' signature would be part of the reason for this lack of relation, if TE differences are predominantly explained by differences in regulation of stomata opening.

The significant negative relationship between TE and FTSW-threshold for NTR found across experiments was a new finding, in contrast to the positive relationship found in Devi et al. (2009). Having lower FTSW values (transpiration upon WDS declining in dryer soil) led to higher TE, as it was the case in several transgenic events. The difference in TE between the transgenics and the wild

type were also found under WW conditions. Therefore, the relation TE–FTSW threshold for NTR decline might be explained by differences in the WW control itself. We previously reported that the rate of water loss per unit of leaf area under WW conditions (in g water transpired  $\text{cm}^{-2}$  per day), an index that reflects differences in stomatal conductance, was lower in the transgenics than in the WT JL 24, and was well correlated to TE values (Bhatnagar-Mathur et al. 2007). As the transpiration of WDS plant is normalized against the transpiration per day under WW conditions (giving the TR, transpiration ratio), a lower transpiration rate ( $\text{g cm}^{-2} \text{day}^{-1}$ ) in the WW transgenics would contribute to maintaining a relative rate of water loss (NTR) under WDS closer to the level of WW plants until dryer soil conditions are reached, thereby leading to lower FTSW thresholds where NTR declines. Similar results were recently found in pearl millet (Kholová et al. 2010b). This would result in a negative relation between these thresholds and TE. Therefore, more work is needed to clarify these aspects and confirm whether the groundnut transgenic events expressing the *rd29:DREB1A* gene had a lower rate of water loss per unit leaf area, and how this would result into differences in FTSW thresholds.

The higher VPD value in experiment 1 when compared to experiment 2 did not significantly influence the genotypes for TE under WDS conditions thus confirming that it did not induce any genotype-by-environment interaction effect on TE, within this range of VPD value. This is with the assumption that VPD was the only major change occurring between these two seasons. Similarly, the VPD also did not seem to affect the ranking of genotypes for the FTSW-threshold values where NTR declines, besides having no effect on the relationship between SCMR and SLA. As the VPD bears a direct relation with TE and it usually varies considerably during the day across environment and seasons, the fact that it did not affect TE in the range of VPD used is an important fact. However, the range of VPD that was tested was relatively narrow and more work would be needed to test the maximum range where the VPD-by-genotype interaction remains insignificant.

## Conclusions

Consistent and large significant variation for TE was found among the transgenic events expressing the drought-responsive element *DREB1A* and the untransformed WT JL 24 across VPD conditions. The surrogate traits such as SLA, SCMR were significantly associated with TE under drought stress conditions only. Then no relationship between TE and  $\Delta^{13}\text{C}$  was found in the present study, using two experiments and two water treatments within each experiment. In part in relation to the

lack of relationship between TE and  $\Delta^{13}\text{C}$ , we hypothesize that the control of stomatal conductance is likely to have more importance than previously reported to explain TE differences in groundnut, both under WW and WDS condition. On a practical side, our results show TE was increased in JL24, a genotype with limited drought adaptation and the challenge would now be whether TE can also be improved through transgenesis in genotypes that have high TE and/or drought adaptation. More importantly, our results clearly indicate that the current widespread use of surrogates such as SLA, SCMR and  $\Delta^{13}\text{C}$  as a proxy for transpiration efficiency (TE) should be considered with extreme care. SLA and SCMR may be used under water-stressed conditions only while these results and others (Krishnamurthy et al. 2007) indicate that  $\Delta^{13}\text{C}$  had poor or no relationship to TE in the case of groundnut and may not be useful in this crop.

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