

***DREB1A* overexpression in transgenic chickpea alters key traits influencing plant water budget across water regimes**

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Abstract

Key message We demonstrate the role of *DREB1A* transcription factor in better root and shoot partitioning and higher transpiration efficiency in transgenic chickpea under drought stress

Abstract Chickpea (*Cicer arietinum* L.) is mostly exposed to terminal drought stress which adversely influences its yield. Development of cultivars for suitable drought environments can offer sustainable solutions. We genetically engineered a *desi*-type chickpea variety to ectopically overexpress *AtDREB1A*, a transcription factor known to be involved in abiotic stress response, driven by the stress-inducible *Atrd29A* promoter. From several transgenic events of chickpea developed by *Agrobacterium*-mediated genetic transformation, four single copy events (RD2, RD7, RD9 and RD10) were characterized for *DREB1A* gene overexpression and evaluated under water stress in a biosafety greenhouse at T6 generation. Under progressive water stress, all transgenic events showed increased *DREB1A* gene expression before 50 % of soil moisture was lost (50 % FTSW or fraction of transpirable soil water), with a faster *DREB1A* transcript accumulation in RD2 at 85 % FTSW. Compared to the untransformed control, RD2 reduced its transpiration in drier soil and higher vapor pressure deficit

(VPD) range (2.0–3.4 kPa). The assessment of terminal water stress response using lysimetric system that closely mimics the soil conditions in the field, showed that transgenic events RD7 and RD10 had increased biomass partitioning into shoot, denser rooting in deeper layers of soil profile and higher transpiration efficiency than the untransformed control. Also, RD9 with deeper roots and RD10 with higher root diameter showed that the transgenic events had altered rooting pattern compared to the untransformed control. These results indicate the implicit influence of *rd29A::DREB1A* on mechanisms underlying water uptake, stomatal response, transpiration efficiency and rooting architecture in water-stressed plants.

Keywords Chickpea · *DREB1A* · Drought · Root length density · Transpiration efficiency · Vapor pressure deficit

Abbreviations

DAS	Days after sowing
DREB1A	Dehydration-responsive element binding factor 1A
FTSW	Fraction of transpirable soil water
NTR	Normalized transpiration ratio
<i>nptII</i>	Neomycin phosphotransferase II
RLD	Root length density
TE	Transpiration efficiency
VPD	Vapor pressure deficit
WW	Well-watered
WS	Water-stressed

Introduction

Chickpea (*Cicer arietinum* L.), a cool season legume crop is widely grown in the semi-arid tropics during the post-

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rainy season. With a crop cycle of three to 4 months, the crop begins with adequate soil moisture and experiences increasing water and temperature stress as it progresses into the reproductive phase (Turner 2003). While breeding drought-adapted chickpea cultivars is very important, chickpea has a narrow genetic base, and genetic engineering provides potential approach to introducing specific traits related to drought tolerance besides looking at the molecular determinisms of certain adaptation traits.

Using the ‘regulon technology’ in *Arabidopsis thaliana*, enhanced levels of several downstream drought-related genes, metabolites and proteins at cellular level were shown to improve drought tolerance at plant level under water, salt and cold stress (Liu et al. 1998; Kasuga et al. 1999; Nakashima and Yamaguchi-Shinozaki 2005, 2006). This work highlighted the overexpression of a dehydration-responsive transcription factor, DREB1A (dehydration responsive element binding factor 1A) driven by the drought-inducible *rd29A* promoter. Subsequently, the overexpression of *DREB1A* transgene was shown in different crops using both constitutive and drought-inducible promoters. Transgenic plants overexpressing *DREB1A* under the control of constitutive promoter in *Arabidopsis* (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000), tomato (Hsieh et al. 2002), tobacco (Kasuga et al. 2004; Cong et al. 2008), chrysanthemum (Hong et al. 2006), peanut (Bhatnagar-Mathur et al. 2007) and soybean (Polizel et al. 2011; Suo et al. 2012) showed stunted growth. Although, normal growth was observed in rice transgenics overexpressing *At DREB1A* under constitutive *Ubi1* promoter, use of stress-inducible promoters gained unanimous preference (Oh et al. 2005). Thus, the construct pBI29ApNot (Kasuga et al. 1999) harboring *At rd29A::DREB1A* along with selectable marker gene, *nptII* (neomycin phosphotransferase II) conferring kanamycin resistance was thereafter used in rice (Datta et al. 2012), tobacco (Kasuga et al. 2004), wheat (Pellegrineschi et al. 2004), potato (Celebi-Toprak et al. 2005; Behnam et al. 2006) and peanut (Bhatnagar-Mathur et al. 2007).

In *rd29A::DREB1A* transgenics, a wide range of “abiotic stress tolerance” has been reported depending on the crop, its growth stage, type of abiotic stress imposed and physiological traits measured. Phenotypic assessment showed crop survival due to a slower use of water by transgenic wheat seedlings contained in small 5 × 5 cm paper cups (Pellegrineschi et al. 2004). Under close-to-field water stress conditions, peanut *DREB1A* transgenics showed decreased stomatal conductance leading to differences in transpiration efficiency under progressively drying soil (Bhatnagar-Mathur et al. 2007; Devi et al. 2011) and showed increased rooting capacity and water extraction (Vadez et al. 2013) and yield (Bhatnagar-Mathur et al. 2014). Based on these findings, transgenic events of a *desi*-

type chickpea overexpressing the *Atrd29A::DREB1A* were developed and phenotypically characterized under greenhouse conditions for drought-related traits including crop growth, rooting architecture and soil moisture thresholds at which transpiration declines (fraction of transpirable soil thresholds, FTSW thresholds), transpiration efficiency (TE) and leaf conductance across vapor pressure deficit (VPD) conditions.

The objectives of this study were to generate transgenic events of chickpea overexpressing *DREB1A* gene under the control of stress-inducible *rd29A* promoter, their characterization and evaluation for drought-responsive traits under water-stressed (WS) and well-watered (WW) conditions in pot and lysimetric studies.

Materials and methods

Development of transgenic events

The previously used binary vector pBI29ApNot (Kasuga et al. 1999), harboring *DREB1A* gene under the control of stress-inducible *rd29A* promoter (both isolated from *Arabidopsis thaliana*) and a selectable marker *nptII* gene driven by the constitutive *nos* promoter were mobilized into competent cells of disarmed *Agrobacterium tumefaciens* C58-C1 strain. Single colony cultures selected with 10 mg/L kanamycin were thereafter used for transforming the axillary meristem explants of the *desi*-type chickpea variety, C235, using the protocol reported previously (Bhatnagar-Mathur et al. 2009).

Molecular characterization of transgenic events

Genomic DNA was extracted from 6 to 8 pinnules of leaves of 30-day-old plants using the reported protocol (Dellaporta et al. 1983). The DNA samples were quantified using NanoVue PlusTM (GE Healthcare, Germany) and normalized to 50 ng μ L with sterile distilled water. The plants were screened for the presence of *nptII* and *DREB1A* transgenes by PCR. The reactions were setup for 25 μ L with 100 ng of genomic DNA, 2.5 μ L of 10X PCR buffer (without $MgCl_2$), 1.5 μ L of 25 mM $MgCl_2$, 0.5 μ L of 10 pmol of each forward and reverse primers, 0.5 μ L of 10 mM dNTP mixture, 0.5 μ L of Taq DNA polymerase and the volume was made up with sterile distilled water. Control reactions were setup with sterile distilled water as negative control 1, with 100 ng of genomic DNA of untransformed C235 parent genotype as negative control 2, and with 25 ng of pBI29ApNot plasmid DNA as positive control. PCR reaction for *DREB1A* was setup using primers specific for a junction region between *rd29A* promoter (forward primer: 5'-TCAAGCCGACACAGACACGCG

TAG-3') and *DREB1A* gene (reverse primer: 5'-AAACCG AAGACTCGTAATCGGAGC-3') with a PCR program of initial denaturation of 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 54 °C for 40 s, 72 °C for 40 s and final extension 15 min at 72 °C. Whereas, for *nptII* fragment, primers and reaction program as described earlier was used (Bhatnagar-Mathur et al. 2007). The samples that showed desired amplicon size of 358 and 700 bp with primers specific for *rd29A::DREB* junction and *nptII*, respectively were advanced to T₁ generation and tested for transgene copy number using Southern blotting.

For Southern blotting, 30 µg of genomic DNA isolated from these selected transgenic events using CTAB method (Sambrook et al. 1989) was separately digested with 5 U/µg of *EcoRI* restriction enzyme. A positive control of 10 ng of plasmid DNA and a negative control of 30 µg of genomic DNA of untransformed C235 genotype was also processed simultaneously. The digested fragments were transferred onto the Hybond N+ nylon membrane using the alkaline transfer method. For detection, DNA was probed with a PCR amplicon of 760 bp *rd29A::DREB* junction fragment (forward primer: 5'-GGCCAATAGACATG GACCGACTAC-3' and reverse primer: 5'-GTTGATTC CGGGATTTCGGAGTCTC-3') and detected using CDP-starTM reagent using Alk-Phos Direct© kit (Amersham Pharmacia Biotech Ltd.). The events that showed single copy gene integration were advanced to further generations under greenhouse conditions.

At T₃ generation, the effect of progressive water deficit on plant transpiration was studied in seven transgenic events and their untransformed C235 parent genotype (Sharma et al. 2006) using the dry-down experiment described earlier (Bhatnagar-Mathur et al. 2007). Four transgenic events (RD2, RD7, RD9 and RD10) that showed contrasting soil moisture thresholds at which transpiration declines (NTR:FTSW value) were tested comprehensively for understanding the effect of AtDREB1A overexpression under WW and WS conditions.

Gene expression analysis in selected events

The selected transgenic events (RD2, RD7, RD9 and RD10) were subjected to progressive water stress in a dry-down experiment as described above. Leaf samples (6–8 pinnules) were collected in two biological replicates at 2-day intervals up to 15 day following the imposition of water stress. At these stages, the fraction of transpirable soil water (FTSW, see below for calculation) corresponded to 100, 85, 68, 53 and 35 %. Total RNA was isolated using TRIzol[®] reagent and the recommended protocol (Invitrogen, Carlsbad, CA, USA). The RNA samples were visualized by resolving on 2 % agarose gel and quantified using NanoVue PlusTM (GE Healthcare, Germany). The samples that showed intact

bands were selected for synthesizing cDNA from 4 µg RNA using Protoscript[®] First Strand cDNA synthesis kit (New England Bio Labs Inc., MA, USA). The samples were normalized to 200 ng µL using nuclease-free water.

Gene expression of *nptII* and *DREB1A* was ascertained by PCR (Bhatnagar-Mathur et al. 2007) using 1 µL of cDNA as template (as described above; data not shown). Based on the amplification profile, one biological replicate per genotype per treatment was selected for studying the real-time gene expression (qPCR) of *DREB1A* (forward and reverse primer: 5'-AAGCTGCGTTGGCGTTTCAG-3' and 5'-CAAACCTCGGCATCTCAAACA-3', respectively) using two reference genes *GAPDH* (forward and reverse primer: 5'-AGTTGTACCACCAACTGCCTTG-3' and 5'-GCCCATCAACAGTCTTCTGAGT-3', respectively) and *IF4* (forward and reverse primer: 5'-GTCTCAGCAACTCAT GGAGACA-3' and 5'-CACGTCAATACCACGAGCTA GA-3', respectively) for normalization. Using Eppendorf RealPlex (Eppendorf, Germany), a 20 µL reaction was setup with 10 µL of pre-mix 2X SensiMix[®] SYBR No-ROX (Bioline, UK), 2 µL of cDNA (diluted to 2:5 with sterile distilled water) and 0.4 µL each of forward and reverse primers, and the volume was made up with nuclease-free water. The reaction mixtures were run in two technical replicates per sample for the transgenic events (RD2, RD7, RD9 and RD10) across five treatments—WW; WS day 3 (WS3), day 5 (WS5), day 7 (WS7) and day 9 (WS9). Real-time reaction was carried out with initial denaturation of 95 °C for 2 min followed by 45 cycles of 95 °C for 15 s, 62 °C for 15 s, 68 °C for 15 s and a final cycle of 95 °C for 15 s, 58 °C for 15 s and a 20 min raise to 95 °C for melt-curve analysis. GenEx software (version 5.3.6.170, MultiD Analyses AB, Sweden) was used for analyzing the output. Using the treatment factor, the relative quantity (stress/control ratio; ddCt) in each WS sample was computed by dividing their normalized \log_2 :*DREB1A* to the geometric mean of the normalized \log_2 :*DREB1A* WW.

Transpiration response to progressive water stress

The standardized dry-down protocol (Bhatnagar-Mathur et al. 2009; Zaman-Allah et al. 2011) was used in the biosafety greenhouse facility of ICRISAT, Patancheru, India (17°30'N; 78°16'E; altitude 549 m). The selected four transgenic events (RD2, RD7, RD9 and RD10) along with their C235 untransformed parent genotype were grown in three sets—pre-treatment (PT), well-watered (WW) and water-stressed (WS) treatment. The experimental design was a complete randomized block design with each set of plants as the main factor and genotype as sub-factor randomized six times in each factor.

Three seeds per pot were sown in 8 inch pots filled with 4.5 kg Vertisol mixed uniformly with di-ammonium

phosphate and muriate of potash at the rate of 0.3 and 0.2 g/kg, respectively. At 25 days after sowing (DAS), the plants were screened for the presence of transgene by PCR and subsequently thinned to retain one PCR positive plant per pot by 40 DAS. The pots were watered to reach field capacity at 52 DAS and allowed to drain excess water overnight. On the following day, the pots of the WW and WS treatments were wrapped in a plastic bag and stapled close to the base of the stem to restrict evaporation from soil surface. The initial saturated weights were recorded by weighing each pot using a balance with 0.01 g precision (KERN and Sohn GmbH, Balingen, Germany). The plants were weighed every day between 0830 and 0930 hours to calculate their daily transpiration. The WW plants were brought back to 80 % of the field capacity by watering daily up to 200 g below the field capacity weight. The soil of the WS plants was allowed to dry progressively from 55 DAS. To ensure similar kinetics of stress imposition, WS plants were allowed to lose only a maximum of 70 g of water each day, and every transpirational loss above this limit was compensated by water addition. Data were normalized first to compare WS and WW plants in each genotype and then to minimize the effects of plant-to-plant variation in size (Zaman-Allah et al. 2011). It is considered that WS plants have same transpiration as WW plants as long as the normalized transpiration ratio (NTR) values remain centered on a 1.0 value, and that the stomata are fully closed once NTR values have dropped below 0.1 (i.e., the transpiration of WS plants is less than 10 % of that in WW plants). Graphs presenting non-linear regression (XY plateau decay) analysis of NTR and FTSW values of each genotype were composed using GraphPad Prism 5.0[®] (GraphPad Software Inc., San Diego, CA, USA). Using a plateau regression program in SAS (SAS Institute Inc., Cary, NC, USA), the FTSW threshold values at which the NTR started declining were computed (Ray and Sinclair 1998). When all plants within a genotype reached 0.1 NTR value in (68 DAS), the plants were cut from the base of the shoot and dried in an oven at 60 °C for 2 days. The shoot and leaf dry weights were recorded.

Transpiration response under increasing VPD conditions

For studying the influence of vapor pressure deficit (VPD) on transpiration rate in the transgenic events, the pre-treatment set of plants were watered to field capacity at 53 DAS and allowed to drain excess water overnight. The pots were, thereafter wrapped in plastic bags to avoid evaporation from soil surface, and transferred to a controlled growth chamber (Convicon CMPP4030; Convicon, Winnipeg, MB, Canada) preset at 18 °C and 60 % RH for 20 h acclimatization. Following this, the plants were subjected

to a gradient of increasing VPD conditions from 0.45 to 3.4 kPa programmed for an hourly alteration of temperature and RH ranging from 18 to 40 °C and 45 to 80 %, respectively. The transpiration of each plant at each VPD level was estimated gravimetrically. At the end of the assessment, the plants were harvested and the leaflets were separated carefully from the shoot, arranged on a transparent plastic sheet, scanned using Epson Expression 1640[®] (Seiko Epson corp., Japan) and the total leaf surface area was computed by analyzing the images using WinRhizo software (WinRhizo, Regent Ltd., Canada). The transpiration rate (g water loss cm⁻² h⁻¹), taken here as a proxy for the canopy conductance, was calculated for each time interval by dividing the respective transpiration by the total leaf area. The shoot and leaf dry weights were also recorded from the 2 d oven-dried plant material. The transpiration response to VPD was analysed with the XY regression plateau using GraphPad Prism 5.0[®] (GraphPad Software Inc., San Diego, CA, USA), which provided the values for slopes and breakpoints where the slope of the fitted regression significantly changed.

Water extraction pattern and root morphology under terminal water stress

A lysimetric trial was conducted in the biosafety greenhouse facility of ICRISAT, as described earlier (Vadez et al. 2013). The four transgenic events along and the untransformed C235 parent genotype were sown in lysimeters (PVC cylinders of 1.2 m length and 20 cm diameter) filled with approximately 35 kg Vertisol mixed uniformly with diammonium phosphate and muriate of potash at the rate of 0.3 and 0.2 g/kg soil, respectively. The plants were maintained in six replicates for each of the two treatments, i.e., WW and WS. An additional set of plants was grown in 9 inch pots (with approximately 5 kg of soil mixture) to measure the biomass accumulation in the genotypes prior to stress imposition. Four seeds per genotype were sown per lysimeter and pot. The plants were grown and maintained under WW conditions until treatment imposition. At 15 DAS, plants were screened for the presence of transgene and a single PCR positive plant was retained per lysimeter and pot. The lysimeters were watered to field capacity at 32 DAS and allowed to drain excess water for two nights. At 34 DAS, the lysimeters were prepared by covering the soil surface with polythene sheet and polythene beads to prevent direct loss of soil moisture and subsequently weighed for recording the saturated initial weight.

At 32 DAS, the potted plants were harvested by cutting the shoot from the base of the stem. The entire root system was extracted by carefully inverting the pots over a fine sieve, and gently washing the soil under running tap water.

The shoot and root samples were dried in 60 °C oven for 48 h and weighed for recording PT plant biomass.

For measuring water uptake, the lysimeters were weighed weekly during the 34–63 DAS period and thereafter, at a 3-day interval until 80 DAS. The water extraction data were the differences between consecutive weighing plus possible water addition after weighing. The dates associated to each water extraction data corresponded to the date of the second weighing; for instance water extracted at 49 DAS was the water extracted during the period of 43–49 DAS. The WW plants were maintained at 80 % soil capacity by replenishing water losses above 1 kg due to transpiration. The WS plants were exposed to terminal water stress by withholding water from 34 DAS.

The plants were harvested at 87 DAS when the normal-ized transpiration ratio (NTR) of most of the WS plants dropped to 10 % of the initial NTR. After harvesting, the aerial biomass was separated into shoots and pods. For extracting the roots, the lysimeters were placed horizontally on a bench and after removing the base plates the soil was gently washed away using a water hose. The detached root segments were trapped by placing 3 mm sieves at the ends of the lysimeters. When more than 80 % of the soil was washed away, the lysimeter was carefully erected on the sieve and the complete root system was slipped through. The root system was carefully washed by rinsing in clear water. Maximum root depth was measured after gently laying the root system on a bench, and then divided into segments of 30 cm length and imaged using the WinRhizo image analysis system. Root length density in each 30 cm layer was obtained by dividing the root length by the volume of a 30 cm section of the cylinder, assuming roots had colonized the entire volume at each depth. Dry weight of shoot, root and pod biomass was recorded for each plant. Since the experiment was terminated before maturity, the pods were included in the shoot biomass component.

Statistical analysis

Statistical analyses were performed using GenStat 10.1.0.72 (VSN International Ltd., UK) by one-way and two-way ANOVA. Differences between mean values within treatments were evaluated using least significant difference (LSD) at 0.05 and 0.1 significance levels.

Results

Development of transgenic events

Following the standardized chickpea tissue culture and transformation protocol, the axillary meristem explants were infected with recombinant *Agrobacterium* C58-C1

strain harboring pBI29ApNot for introducing the *DREB1A* and *nptII* genes. The healthy green shoots that survived selection pressure of 100 mg/L kanamycin were transferred for rooting. Plants that developed adventitious roots were suspended in a hydroponic system and gradually exposed to growth chamber conditions (26 °C; 40 % relative humidity; 12 h photoperiod) for further growth and hardening of roots. Well-rooted plantlets were transferred to a biosafety greenhouse facility (26 °C; 60 % relative humidity). The well-established putative transgenic events showed no morphological variations when compared to the untransformed parent genotype and were maintained under irrigated conditions until maturity.

Molecular characterization of transgenic events

Twenty-three independently transformed putative transgenic events were established in the greenhouse that showed the desired amplicon with primers specific for *rd29A::DREB1A* junction (358 bp) and *nptII* (700 bp) (data not shown) were advanced to T1 generation. Southern blot analysis of 12 putative transgenic events showed single copy of *rd29A::DREB1A* transgene integration (Fig. 1) and then advanced to further generations in the greenhouse with subsequent PCR screening for the transgenic plants.

Gene expression analysis in selected events

RNA was isolated from the WW and WS plants of the four-selected transgenic events (RD2, RD7, RD9 and RD10) subjected to a dry-down experiment (data not shown). Intact RNA bands were visualized in samples of all four genotypes (RD2, RD7, RD9, and RD10) under WW conditions on day 1 (WW1); and under WS conditions on day 3 (WS3), day 5 (WS5), day 7 (WS7) and day 9 (WS9), except for WS9 sample of RD10. PCR with *nptII* and *DREB1A* internal primers showed desired amplification of 700 and 499 bp fragments, respectively in the tested samples (data not shown). Relative quantities (stress/control: WS/WW; ddCt) of *DREB1A* transcript showed a quick accumulation in RD2 on WS3 (25 % higher) than WW (Fig. 2). All transgenic events had significantly lower expression intensities on WS5, and higher expression intensities (10–22 %) on WS7 compared to WW. Also, *DREB1A* expression on WS9 was slightly higher (8 %) than under WW in RD9 (Fig. 2).

Physiological characterization of transgenic events

Transpiration response to progressive water stress

Under both WW and WS conditions, RD7 had lower leaf biomass (5.4 and 2.9 g/plant, respectively) than C235 (6.6

Fig. 1 Southern blot hybridization of the putative transgenic events for copy number using 760 bp *rd29A::DREB1A* junction fragment as the probe. *P* plasmid, *C* untransformed C235 genotype, *M* DNA marker, 1–12 putative transgenic RD events 1–12

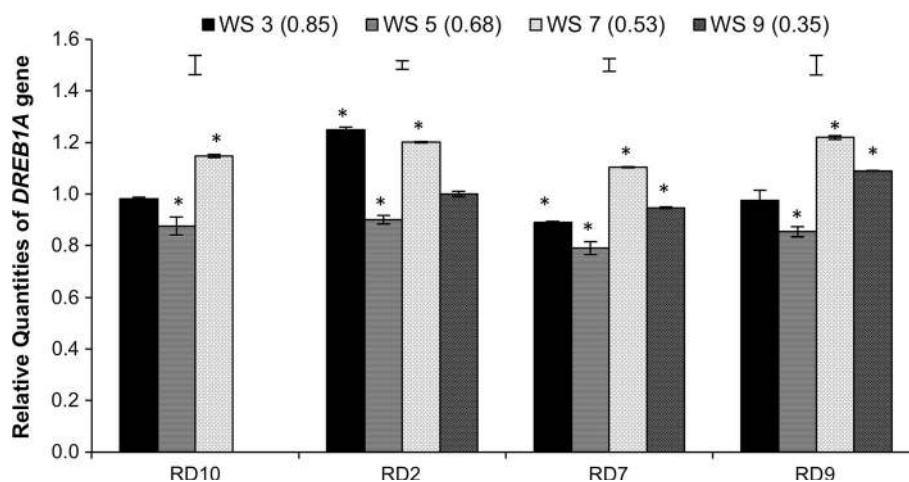
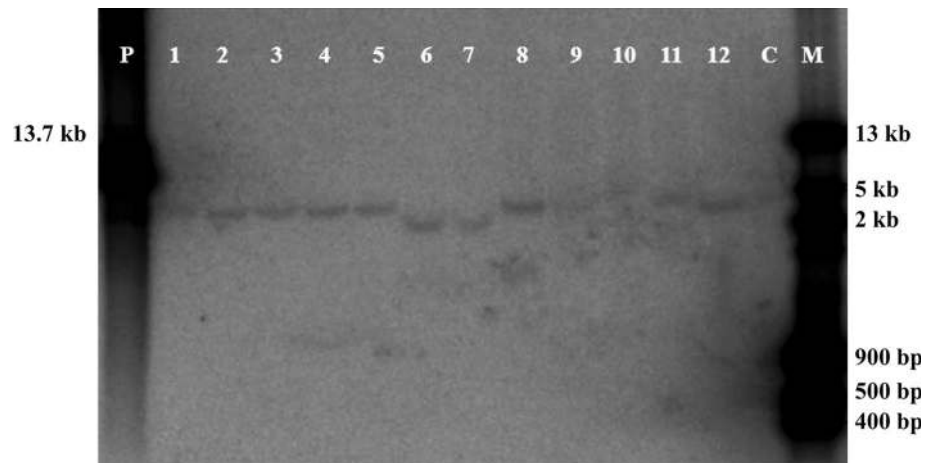


Fig. 2 qPCR analysis of relative quantity of *DREB1A* transcript in the transgenic events across different treatments. Error bars on the column indicate standard error and error bars in graph area indicate LSD value calculated for treatment factor. Significant differences are

indicated by asterisk mark on data points. WS3, WS5, WS7 and WS9—day 3, 5, 7 and 9 after water stress imposition, respectively; FTSW (fraction of transpirable soil water) values presented in brackets

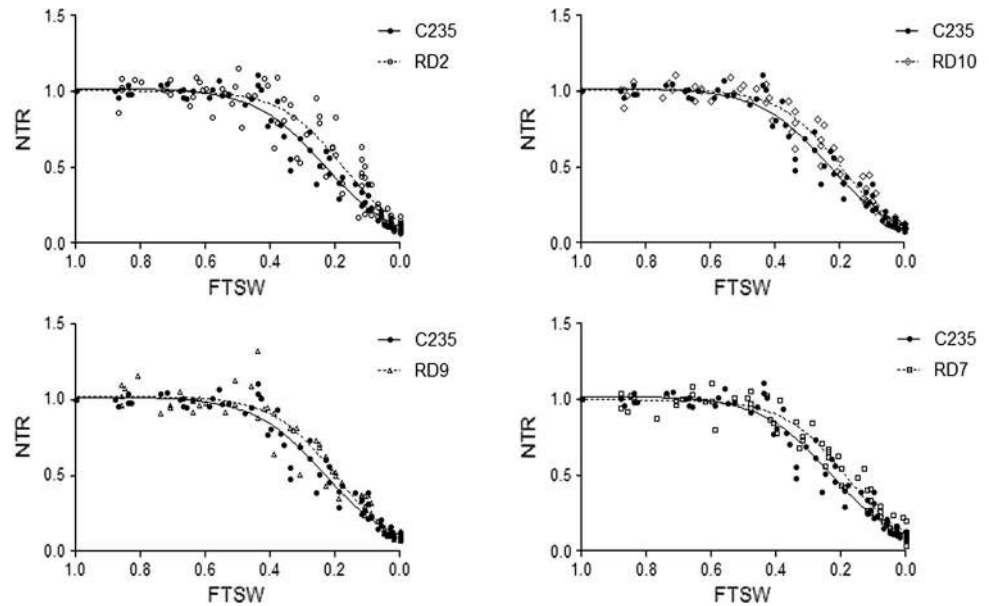
and 3.6 g/plant, respectively). The transgenic event RD2 had lower leaf biomass (3.3 g/plant) than C235 under WS condition. Despite these differences, total water extracted in WW and WS plants showed no genotypic differences (WW: 2.5–2.7 kg/plant; WS: 1.2–1.5 kg/plant) except for higher water uptake in RD2 (1.5 kg/plant) compared to C235 (1.3 kg/plant) under WS conditions.

Under WS, the NTR: FTSW regression plot showed the tendency of the transgenic events to lower their transpiration in drier soils than the C235 genotype (Fig. 3). The FTSW threshold value (*t* value) representing the soil moisture level beyond which the transpiration begins to decline was the highest in C235 (0.48) among the tested genotypes (Fig. 3). Confidence intervals for FTSW threshold values showed significantly lower threshold in the transgenic events RD2 and RD10 than in the untransformed C235 (Fig. 3).

Transpiration response to increasing VPD conditions

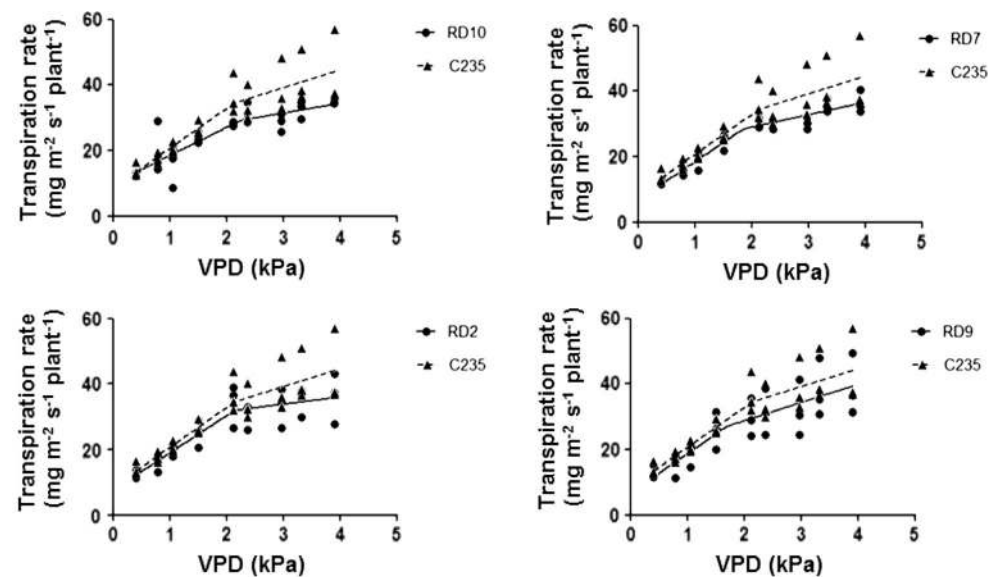
Upon increasing VPD conditions (0.45–3.4 kPa) all genotypes restricted their transpiration (Fig. 4). Significantly lower VPD breakpoint (i.e., the VPD value where the slope of the transpiration response to VPD significantly changes) was recorded in RD9 (1.69 ± 0.35) than in the untransformed C235 genotype (2.13 ± 0.38), although the rate of transpiration in RD9 was similar to C235 across the VPD conditions tested. At a VPD ranging between 0.45 and 2.2 kPa, the event RD10 had a lower slope of transpiration response (slope 1 = 8.6 ± 2.0) than the untransformed C235 (slope 1 = 12.2 ± 1.5), while the slope of the transpiration response in RD2 (slope 2 = 2.2 ± 1.1) was lower than C235 (slope 2 = 5.5 ± 1.2) at a VPD ranging between 2.2 and 3.4 kPa.

Fig. 3 Regression plot (XY plateau) showing decline in transpiration (*Y* axis-normalized transpiration ratio; NTR) across gradually drying soil profile (*X* axis-fraction of transpirable soil water; FTSW) of different WS transgenic events and the untransformed C235 genotype during the dry-down experiment conducted in P2 greenhouse facility along with values for FTSW threshold (*t*), confidence limits and standard error (SE)



Genotype	t value	NTR:FTSW threshold		s.e
		95% confidence limits		
C235	0.49	0.462	0.509	0.012
RD10	0.44	0.419	0.462	0.011
RD2	0.42	0.385	0.455	0.018
RD7	0.44	0.416	0.463	0.012
RD9	0.44	0.413	0.467	0.013

Fig. 4 Non-linear regression plot between vapor pressure deficit (VPD in kPa; *X*-axis) and transpiration rate (mg water loss $m^{-2} s^{-1} plant^{-1}$; *Y*-axis) in untransformed C235 genotype (dashed line and filled triangle symbols) and different transgenic events (solid line and filled circle symbols) along with the values for slope 1, slope 2 and breakpoint (BP)



	C235	RD10	RD2	RD7	RD9
SLOPE1	12.15 ± 1.48	8.60 ± 2.04	11.41 ± 1.73	11.70 ± 1.91	12.11 ± 1.98
BP	2.13 ± 0.38	2.24 ± 0.64	2.13 ± 0.29	1.84 ± 0.29	1.69 ± 0.35
SLOPE2	5.45 ± 1.19	2.99 ± 2.44	2.17 ± 1.10	3.84 ± 1.06	5.51 ± 1.10

Table 1 Amount of water extracted across the experimental duration of 35–80 DAS in different transgenic events and the untransformed C235 genotype tested under both well-watered (WW) and water-stressed (WS) conditions using lysimeters in the greenhouse

Treatment	Genotype	Water extraction (mL plant ⁻¹)								
		42 DAS	49 DAS	56 DAS	63 DAS	66 DAS	70 DAS	73 DAS	77 DAS	80 DAS
WW	C235	632	1440	1842	1592	816	1908	1208	2202	1288
	RD10	538	1295	1842	1675	917	1983	1295	2535	1628
	RD2	427	1218	1707	1405	725	1470	865	1787	1278
	RD7	536	1396	1836	1540	780	1628	832	1902	1190
	RD9	418	1134	1574	1384	688	1512	962	2002	1166
LSD $P < 0.05$		137	231	317	313	261	554	432	649	656
LSD $P < 0.1$		113	191	263	259	216	458	358	537	543
WS	C235	375	820	1073	1070	513	588	593	577	240
	RD10	408	982	1252	1144	490	532	520	474	198
	RD2	446	1088	1374	1156	540	558	490	442	152
	RD7	382	935	1268	1133	515	525	580	567	217
	RD9	362	930	1156	994	458	502	480	456	192
LSD $P < 0.05$		159	357	406	219	101	106	109	192	145
LSD $P < 0.1$		131	296	336	181	87	88	91	159	120

Bold values indicate significance at $P < 0.1$ for each parameter
DAS days after sowing

Water extraction pattern and root morphology under terminal water stress

With regards to water extraction under WW conditions, RD10 extracted similar amounts of water as the untransformed C235 over the experimental duration. Under WW conditions, water uptake in RD2 (35–49 DAS), RD9 (35–56 DAS) and RD7 (70–73 DAS) was lower than the untransformed C235 parent genotype (Table 1). Under WS conditions, no genotypic difference was recorded except the lower water uptake in RD2 and RD9 (70–73 DAS) than in C235 (Table 1). The total amount of water used under WW (11–14 kg/plant) and WS (5.6–6.2 kg/plant) conditions did not discriminate the genotypes tested (Table 2).

Under WW conditions, no genotypic difference in plant biomass (shoot, root and total plant) was recorded (Table 2). However, under WS, transgenic events RD7 and RD10 showed higher accumulation of biomass (shoot and total plant) compared to the untransformed C235 genotype (Table 2). Significantly higher TE was recorded in RD2 (WW conditions), and in RD7 and RD10 (WS conditions) than in C235 (Table 2).

Under WW conditions, the root/shoot ratio was significantly higher in RD7, RD9 and RD10 than in the untransformed C235. In contrast, under WS treatment RD7 and RD10 had lower root/shoot ratio than C235 (Table 2). The genotypes did not differ in the total root length and in the average root length density (RLD) under both WW and WS conditions. Average root diameter of genotypes tested differed only for RD10 under WS treatment (Table 2).

While the root depth was not significantly different under WW conditions, the root depth was higher in RD9 than in C235 under WS conditions (Table 2).

The root parameters were also assessed in different layers of soil profile to decipher genotypic differences in root growth pattern. Under WW conditions, RLD of RD2 in the 0–60 cm layers was lower than in C235, and the RLD of RD7 in the 0–30 cm layer was lower than in C235 (Fig. 5a). In deeper soil layer (90–120 cm), RD7 had higher RLD than C235. Under WS treatment, all the transgenic events showed a trend of a more profuse rooting in 30–90 cm layer of soil (Fig. 5b). Compared to C235 genotype significant differences were recorded in 30–60 cm layer for RD2 and in 60–90 cm layer for RD7, RD9 and RD10 (Fig. 5b).

In summary, under terminal WS condition, more profuse rooting was observed across different layers of soil profile in the transgenic events RD2 (30–60 cm), and in RD7, RD9 and RD10 (60–90 cm layer), as well as deeper rooting in RD9.

Discussion

Chickpea cultivar C235 that has a crop cycle of 120 days to maturity under post-rainy season cropping in the semi-arid tropics experiences terminal water stress. We hypothesized that a number of traits controlling plant water use could be improved in this cultivar, and could then be used to improve its drought adaptation, by introducing *DREB1A*

Table 2 Biomass accumulation (shoot, root and total), total water extracted, transpiration efficiency (TE) and root-related traits (root: shoot ratio, total root length, total rooting depth and average root diameter) of different transgenic events tested in lysimetric system in the greenhouse under both well-watered (WW) and water-stressed (WS) conditions

Treatment	Genotype	Shoot dry wt. (g plant ⁻¹)	Root dry wt. (g plant ⁻¹)	Total plant biomass (g plant ⁻¹)	Total water extracted (kg plant ⁻¹)	TE (g biomass accumulated kg ⁻¹ water extracted plant ⁻¹)	Root/Shoot ratio (plant ⁻¹)	Total rooting depth (m plant ⁻¹)	Total root length (m plant ⁻¹)	Avg. root diameter (mm plant ⁻¹)
WW	C235	19.0	1.58	20.6	12.9	1.67	0.08	108	254	0.63
	RD10	16.7	2.10	18.8	13.7	1.42	0.13	124	328	0.63
	RD2	21.7	1.98	23.7	10.9	2.17	0.09	112	226	0.59
	RD7	18.3	2.09	20.3	11.6	1.77	0.12	115	193	0.61
	RD9	20.1	2.14	22.2	10.8	2.04	0.11	105	204	0.61
LSD $P < 0.05$	5.7	0.82	6.2	2.7	0.55	0.04	29	91	0.07	
LSD $P < 0.1$	4.7	0.68	5.2	2.2	0.46	0.03	24	75	0.06	
WS	C235	15.1	2.46	17.5	5.85	2.94	0.17	108	208	0.60
	RD10	23.5	2.12	25.7	6.00	4.36	0.10	109	215	0.64
	RD2	17.6	2.31	19.9	6.25	3.20	0.13	128	255	0.58
	RD7	30.6	2.76	33.3	6.12	5.49	0.09	128	205	0.58
	RD9	16.2	2.06	18.2	5.53	3.29	0.14	137	226	0.63
LSD $P < 0.05$	7.3	1.02	7.6	0.84	1.33	0.06	33	99	0.05	
LSD $P < 0.1$	6.0	0.85	6.3	0.69	1.10	0.05	27	82	0.04	

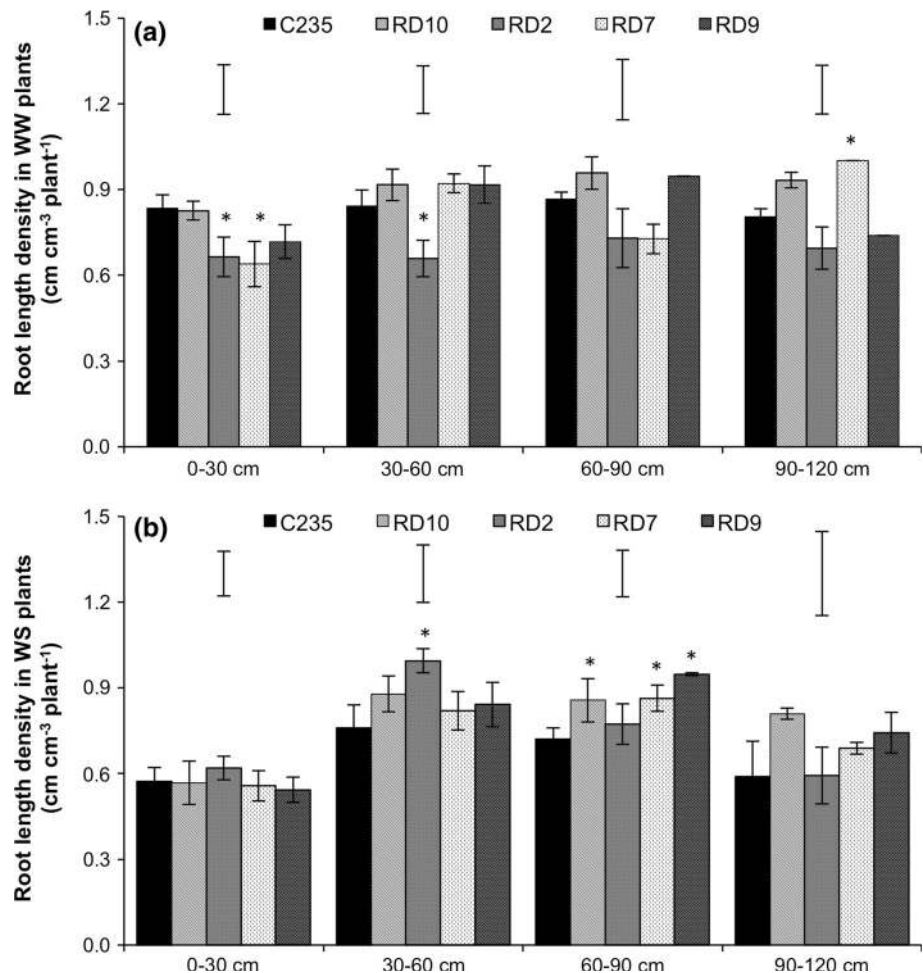
Bold values indicate significance at $P < 0.1$ for each parameter

transcription factor under the control of a stress-inducible *rd29A* promoter, both from *Arabidopsis thaliana*. The *rd29::DREB1A* transgenic events thus developed showed no morphological variations when compared to the untransformed parent genotype. The selected single copy transgenic events (RD2, RD7, RD9 and RD10) showed significant differences in *DREB1A* gene expression under water stress (WS) conditions compared to their well-watered (WW) counterparts. The transgenic events RD2 and RD10 initiated a decline in transpiration upon progressive soil drying at lower FTSW thresholds than the untransformed C235 as control. Stomatal conductance of the transgenic events varied from that of C235 upon exposure to increasing VPD conditions. Under terminal WS, transgenic events showed higher TE (RD7 and RD10), deeper rooting (RD9), increased average root diameter (RD10) and higher RLD at deeper layers of soil (RD7 and RD10) when compared to the untransformed control.

Rapid and high increase in *DREB1A* expression under WS conditions, and very low *DREB1A* expression under non-stressed conditions has been reported earlier (Kasuga et al. 1999, 2004; Behnam et al. 2006). Although, the *DREB1A* transcript accumulation was observed on day 2 of water withdrawal in wheat transgenics (Pellegrineschi et al. 2004), in the present study there was no assessment of the soil water available as an indicator of the stress intensity. Bhatnagar-Mathur et al. (2007) reported *DREB1A* expression on day 5 of WS that corresponded to 65 % of soil moisture available for transpiration (65 % FTSW) in *DREB1A* peanut transgenics. In our study, the expression of *DREB1A* varied amongst various transgenic events tested and also within each genotype across the period of water stress. The relative quantities of transcript showed rapid accumulation of *DREB1A* in RD2 on day 3 of WS (85 % FTSW) and all the events showed higher expression on day 7 of WS (53 % FTSW) compared to WW. These results suggest that changes in soil moisture levels directly influence *DREB1A* expression in the leaves of the stressed plants, although the enhanced expression at 85 % FTSW, a soil moisture level which is not far from field capacity and in principle not water-stressed, in RD2 suggests an alternative trigger than the soil moisture.

It has been hypothesized that the phenotype is holistically determined by two information systems—genetic and epigenetic (Trewavas and Malhó 1997; Trewavas 2009). Considering the genetic makeup, transgenic events and their untransformed parent genotype can be treated as a near-isogenic population. For characterizing the role of a transgene, we formulated a null hypothesis stating that overexpression of *At DREB1A* does not alter water stress response in transgenic events compared to their untransformed parent genotype, and tested for rejecting this hypothesis at 95 % confidence level using

Fig. 5 Layer-wise distribution of root length density (RLD) in different transgenic events and the untransformed C235 genotype under well watered (WW, **a**) and water-stressed (WS, **b**) conditions. *Standard error bars* are indicated on the columns and *error bar* in the graph area indicates LSD ($P < 0.05$) calculated for each layer of soil



physiological traits measured under both WW and WS conditions.

Considering that the stomatal activity is a function of plant water status (genotypic) and soil water status (environmental), the NTR: FTSW threshold value allows us to identify the soil moisture level beyond which the genotype begins to restrict its transpiration. The confidence interval of the FTSW threshold value where the transpiration begins to drop was lower in RD2 and RD10 than the untransformed genotype C235, suggesting that these transgenic events restricted their transpiration in slightly drier soil (less than 45 %) than the untransformed genotype, and therefore started saving water slightly later than C235. This trend was also observed in peanut *DREB1A* transgenics (Bhatnagar-Mathur et al. 2007; Devi et al. 2011) and in drought tolerant pearl millet (Kholová et al. 2010) and cowpea (Belko et al. 2012), although the contrary was found in chickpea (Zaman-Allah et al. 2011).

All five genotypes had similar total leaf area at 53 DAS but showed different responses in transpiration to increasing VPD conditions. The transgenic events RD2 and RD10 had lower slopes of response of transpiration rate to

increasing VPD than C235 at high and low VPD ranges, respectively. The transgenic event RD9 had a transpiration response to increasing VPD similar to that of C235, but had a lower breakpoint than C235. These variations under non-water limiting conditions indicate different stomatal regulation under increasing atmospheric VPD conditions in different transgenic events, and very likely a restriction in stomatal conductance (G_s), denoting the potential for saving water from the differences in these parameters (lower slope, lower breakpoint). Restricting G_s under high VPD would mechanically decrease the effective VPD driving transpiration under high VPD, and would lead to increases in TE, as discussed recently (Vadez et al. 2014). The data recorded is insufficient to explain the reasons for the changes in the transpiration response to VPD in these transgenic events, except that these relatively rapid responses may involve hydraulic signals. Structural and gene interaction studies with transcription factors of MYB family (MYB96, MYB44, MYB60 and MYB61A) and WRKY that are known to be associated with stomatal control and drought response (Saibo et al. 2009) might help in understanding these observations.

Earlier, lysimetric assessment of peanut *DREB1A* transgenics showed higher water uptake supported by increased root:shoot ratio (Vadez et al. 2007) and enhanced rooting (deeper and denser) (Vadez et al. 2013) under water limiting conditions. Similarly, in our present study, the transgenic event RD9 had deeper rooting than the untransformed C235 parent genotype under WS conditions. The transgenic events RD2, RD9 and the untransformed C235 genotype showed higher biomass partitioning into root component (root:shoot ratio) under WS than their WW counterparts. In the previous report on peanut over-expressing *DREB1A*, higher rooting capacity in deeper layers was associated with increased water uptake (Vadez et al. 2013). In the current experiment, although differences in root traits did not result in significant increases in water uptake, the RLD of entire soil profile (0–120 cm) correlated positively with water extracted in the initial weeks (35–63 DAS; $r = 0.48$; data not shown). In this off-season (April–June) experiment, WS plants were harvested at mid-flowering stage as their NTR dropped to 0.1, and we observed robust shoot development in RD7 and RD10 supported by root development, thereby resulting in higher TE in RD7 and RD10 than the untransformed C235 parent genotype.

In conclusion, the selected single copy transgenic events harboring *rd29A::DREB1A* showed quick accumulation of *DREB1A* transcripts in RD2 on day 3 of water stress. All transgenic events showed increased *DREB1A* expression on day 7 of WS (FTSW = 0.5) compared to the WW treatment. Using pot system, across progressively drying soil conditions, transgenic events RD2 and RD10 restricted their transpiration in drier soils than the untransformed parent. Using a lysimetric system, RD9 showed higher rooting depth, while the events RD7 and RD10 showed more profuse rooting in deeper layers and higher TE than the untransformed C235. The WW transgenic events showed variation in response in transpiration rate to increasing VPD conditions (0.45–3.4 kPa), either through lower slope of the transpiration rate response or lower VPD breakpoints. These results predicate that under dehydration, *DREB1A* overexpression influences cascades of events leading to increase water availability, either through water uptake, stomatal response and/or rooting. Also, phenotyping of these events in greenhouse environment across complete crop cycle revealed altered pattern of water uptake and crop phenology that resulted in yield gain (seed yield and harvest index) in transgenic events compared to C235 genotype under terminal water stress conditions (Anbazhagan et al. 2014). These results support the regulon technology approach for developing transgenic chickpea events for drought stressed environments. As the genomic position of the transgene directly influences the cellular and whole plant function and response, additional

expression studies of *DREB1A* up-regulated genes and protein interaction studies of *DREB1A* transcription factor and other regulatory proteins across periods of water stress will strengthen our hypothesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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