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Changes in timing of water uptake and phenology favours yield gain in terminal water stressed chickpea *At*DREB1A transgenics

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Abstract. Terminal drought causes major yield loss in chickpea, so it is imperative to identify genotypes with best suited adaptive traits to secure yield in terminal drought-prone environments. Here, we evaluated chickpea (*At*) *rd29A*:: (*At*) *DREB1A* transgenic events (RD2, RD7, RD9 and RD10) and their untransformed C235 genotype for growth, water use and yield under terminal water-stress (WS) and well-watered (WW) conditions. The assessment was made across three lysimetric trials conducted in contained environments in the greenhouse (2009GH and 2010GH) and the field (2010F). Results from the greenhouse trials showed genotypic variation for harvest index (HI), yield, temporal pattern of flowering and seed filling, temporal pattern of water uptake across crop cycle, and transpiration efficiency (TE) under terminal WS conditions. The mechanisms underlying the yield gain in the WS transgenic events under 2009GH trial was related to conserving water for the reproductive stage in RD7, and setting seeds early in RD10. Water conservation also led to a lower percentage of flower and pod abortion in both RD7 and RD10. Similarly, in the 2010GH trial, reduced water extraction during vegetative stage in events RD2, RD7 and RD9 was critical for better seed filling in the pods produced from late flowers in RD2, and reduced percentage of flower and pod abortion in RD2 and RD9. However, in the 2010F trial, the increased seed yield and HI in RD9 compared with C235 came along only with small changes in water uptake and podding pattern, probably not causal. Events RD2 (2010GH), RD7 (2010GH) and RD10 (2009GH) with higher seed yield also had higher TE than C235. The results suggest that DREB1A, a transcription factor involved in the regulation of several genes of abiotic stress response cascade, influenced the pattern of water uptake and flowering across the crop cycle, leading to reduction in the percentage of flower and pod abortion in the glasshouse trials.

Additional keywords: conservative water use, flower abortion, lysimeter, pod abortion, terminal drought stress.

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Introduction

Chickpea is world's second most important food legume largely cultivated in the arid and semiarid tropics (SAT), as well as the dry areas of near east and north Africa. Cultivation in both these environments is often challenged by terminal drought and heat stress as the crop is grown on receding soil moisture. It is estimated that the production could be improved by up to 50% if the soil water stress was alleviated (Ryan 1997). Breeding short duration varieties to escape terminal drought has been the major focus of chickpea breeding but early lines limit the overall light capture and potential yield. Medium duration varieties may then be suitable provided water is available through the entire cropping cycle. Simulation models have shown that higher yields may be achieved with traits such as deeper root system and higher transpiration efficiency (Jordan *et al*. 1983; Sinclair 1994). Several reports have proposed root development as the main contributor to achieve high seed yield under terminal drought conditions through extraction of large amounts of water, especially at depth (Ludlow and Muchow 1990; Subbarao *et al*. 1995; Turner *et al*. 2001; Kashiwagi *et al*. 2005).

However, recent studies have shown that root systems are important for crops grown with limited amounts of water in the soil profile as long as they allow water extraction at critical times for the plant growth (Boote *et al*. 1982; Boyer and Westgate 2004; Zaman-Allah *et al*. 2011*a*; Vadez *et al*. 2013*a*). Water deficits during flower and pod production have shown to impact negatively the final seed yield (Leport *et al*. 2006; Nayyar *et al*. 2006; Fang *et al*. 2010). Water deficits also suppress floral bud development, pollen viability, pod set and pod filling in legumes (Downes and Gladstones 1984; Ahmed and Hall 1993; Duc *et al*. 1994; Davies *et al*. 1999; Leport *et al*. 1999;

Croser *et al*. 2003; Clarke and Siddique 2004; Turner *et al*. 2005). Also, Eser *et al*. (1991) reported that a reduced seed filling time for the pods that are formed from the late flowers under terminal drought conditions also contributes to yield loss. Therefore, for crops grown with limited amounts of water in the soil profile, water uptake during the reproductive phases is critical to ensure its success (Merah 2001; Kato *et al*. 2008; Zaman-Allah *et al*. 2011*a*; Vadez *et al*. 2013*b*). It was recently shown that terminal drought tolerant chickpea genotypes limit water use at early stages with a subsequent significant amount of water left in the soil profile for the reproduction/pod filling stage (Zaman-Allah *et al*. 2011*b*).

In the past few years, genetic transformation has been used to introduce specific genes involved in functional and/or regulatory pathways (such as *IPT*, *HVA1*, *ABA3*, *DREB1A*, *AVP1*, *P5CSF*, *InsP*$_3$) with the hope of improving the 'drought tolerance' of crops (Bhatnagar-Mathur *et al*. 2008). On the same line of interest, chickpea transgenic events harbouring (*At*) *DREB1A* (Dehydration Responsive Element Binding factor 1A) gene under the control of stress inducible (*At*) *rd29A* promoter were developed. The single copy events were phenotypically characterised in dry-down experiments that measured transpiration efficiency (TE) and the soil moisture threshold where transpiration declines (Sharma *et al*. 2006). The events that were contrasting in those thresholds and in TE, i.e. RD7 and RD10, also showed variation in the transpiration rate under increasing vapour pressure deficit (VPD) conditions, and enhanced rooting and higher TE under terminal water stress conditions (K. Anbazhagan, P. Bhatnagar-Mathur, V. Vadez, D. Srinivas Reddy, P. B. Kavi Kishor and K. K. Sharma, unpubl. data).

To further understand if the observed traits facilitated in converting the water extracted into economic yield, several transgenic events were assessed under greenhouse and contained field conditions for (i) yield and growth, (ii) phenology, (ii) pattern of water uptake, and (iv) and their relationship under both well watered and terminal water stress conditions, using a lysimetric system that allowed both a precise assessment of plant water extraction over time and a relevant agronomic performance.

Material and methods

Plant material and molecular analysis of the transgenic plants grown in lysimeters

Chickpea transgenic events of C235 cultivar containing single copy *rd29A*::*DREB1A* transgene (RD2, RD7, RD9 and RD10) previously reported (Sharma *et al*. 2006; K. Anbazhagan, P. Bhatnagar-Mathur, V. Vadez, D. Srinivas Reddy, P. B. Kavi Kishor and K. K. Sharma, unpubl. data), advanced to T6 generation.

For the lysimetric experiment, genomic DNA was isolated from the transgenic events at 10 days after sowing (DAS) and screened for the presence of *DREB1A* transgene by PCR analysis. Plants that showed the desired amplicon of 358 bp with a PCR profile 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 of 72°C for 15 min with primers specific for a junction region between *rd29A* promoter and *DREB1A* gene, were used for the lysimetric experiment. No qualitative test was performed to assess the presence of the transgene. We used PCR only for amplifying the junction region between the promoter and the *DREB1A* gene.

Phenotypic evaluations of transgenic events in lysimeters

Using the methodology described by Zaman-Allah *et al*. (2011*a*), three yield trials were conducted under greenhouse (GH) and contained field (F) conditions at ICRISAT, Patancheru (17°30′N; 78°16′E; altitude 549 m above sea level) during the post-rainy season of 2009 (2009GH) and 2010 (2010GH and 2010F).

Two seeds of each genotype were sown in lysimeters consisting of PVC cylinders (20 cm diameter, 120 cm height) filled with ~35 kg of Vertisol mixed with di-ammonium phosphate and muriate of potash at the rate of 0.3 g kg^{-1} and 0.2 g kg^{-1} of soil respectively. There were two water treatments, i.e. a well-watered (WW) and a water stressed (WS) treatment. The lysimeters were arranged in a complete randomised block design with water treatment as the main factor and genotype as sub-factor randomised six times in each factor. The arrangement of the cylinders allowed a density of the chickpea crop of ~25 plants m^{-2}, which was close to field density. An additional set of plants were grown in ~23 cm pots (with ~5 kg of soil mixture) as pre-treatment (PT) plants to measure the shoot biomass accumulated in the genotypes before imposition of water treatment.

All plants were grown under fully irrigated conditions, by applying 500 mL of water every three to five days, until treatment imposition. Plants of the WW treatment were watered every week to maintain the soil above 80% field capacity until maturity. The cylinder weight at field capacity corresponded to the first weight when weighing started. This weight was taken as a target for re-watering the plants, assuming each tube would contain ~9 L of extractable water. This consisted in compensating water losses in excess of 1 kg below the weight at field capacity, which also prevented water drainage. The WS treatment was imposed by cessation of watering from 34, 42 and 37 DAS stages in the 2009GH, 2010GH and 2010F experiments, respectively until maturity. Before imposing the treatments, the cylinders were irrigated to reach the field capacity and allowed to drain the excess water over a 36 h period. The top of the cylinders were then covered with a round and slit plastic sheet, on top of which 2 cm of low density polyethylene granules were laid. These layers prevented more than 90% of soil evaporation, so that consecutive cylinder weighing allowed estimation of plant water uptake for transpiration. The cylinders were weighed every week from the day of stress imposition until maturity except the last two measurements were made with an interval of 2 weeks.

Follow up of reproductive organs and harvest procedure

For assessing flowering, coloured threads were attached to the stem just above the attachment point of each flower peduncle. Threads of a same colour were used to tag all flowers appearing during a one week period. The tagging started at the beginning of flowering and then every week threads of a different colour were used. This allowed a dynamic follow up of flower produced at different time and of the pods and seeds that originated from these flowers. As chickpea plants tend to shed their leaves during late reproductive stage, mostly the stems were accounted for the shoot

biomass component. At maturity, the plants were harvested and individually partitioned into pods and stems. The separated shoots were dried in the 60°C oven for 48 h before weighing. And pods in each plant was separated by thread colour coding and dried in 37°C oven for 48 h. The weight and number of pods and seeds was also recorded under each colour category. The total number of threads corresponded to the total number of flowers, and threads that were not related to any pod corresponded to either flower or pod abortion. These were then counted and divided by the total number of flowers to calculate a combine percentage of flowers and pods aborted.

Statistical analysis

Statistical analyses were performed using GENSTAT 10.1.0.72 by one-way and two-way ANOVA. Differences between mean values of treatments were evaluated using least significant difference (l.s.d.) at 0.05 and 0.1 significance levels.

Results

Growth, water uptake and transpiration efficiency

Across the three trials conducted under greenhouse (GH) and contained field (F) conditions, shoot biomass of the genotypes ranged widely from 5–14 g plant^{-1} and 3–7 g plant^{-1} under WW and WS conditions respectively (Table 1). Compared with C235, lower shoot biomass was recorded in the WW plants of RD2, RD7 and RD9 in the 2010GH trial and in the WS plants of RD9 in the 2010F trial (Table 1). There was no shoot biomass difference in the 2009GH trial. Total plant biomass (shoot and pod) showed genotypic differences in 2009GH and 2010GH trials (Table 1). In the 2009GH trial, the WS plants of RD10 had higher total plant biomass than the untransformed C235 genotype. In the 2010GH trial, event RD2 had lower plant biomass than the C235 genotype under WW conditions, whereas RD2 and RD7 had significantly higher plant biomass than C235 under WS treatment (Table 1).

Total water extracted by WW plants of RD10 was significantly higher than C235 genotype in the 2009GH trial. Whereas in the 2010GH trial, the WW plants of RD2, RD7 and RD9 extracted significantly lower amount of water than C235 genotype (Table 1). Under WS conditions, lower water uptake was recorded in the 2010GH trial for RD2 and RD7 than in C235 (Table 1).

Transpiration efficiency (TE), calculated as plant biomass accumulated per unit water extracted, was significantly higher in the WW plants of RD9 than C235 in the 2010GH trial (Table 1). Under WS, higher TE was recorded in RD2 (2010GH), RD7 (2010GH) and RD10 (2009GH) than in C235 (Table 1).

In summary, under terminal WS, events with higher total plant biomass had also higher TE than C235. In the 2009GH trial, RD10 with 18% higher total plant biomass and similar water uptake as C235, showed higher TE (19%) than C235. Whereas in the 2010GH trial, events RD2 and RD7 with ~25% higher plant biomass and 10% lower water uptake than C235, had higher TE (34 and 27% respectively) than C235.

Yield and harvest index

Under WW conditions, RD10 had higher yield components (pod weight, seed number and seed weight) than C235 in the 2010GH trial (Table 2). In the other trials, under WW conditions

Table 1. Dry mass of shoot and whole plant, water extracted (vegetative phase, reproductive phase and total) and transpiration efficiency (TE) of the different chickpea genotypes tested in P2 greenhouse facility (2009GH and 2010GH) and contained field conditions (2010F) under both well-watered (WW) and water stressed (WS) conditions

Numbers in bold represent values that are significantly different compared to the C235 untransformed parent; numbers in italics represent the calculated LSD values (at $P < 0.05$ and $P < 0.1$) for each parameter

Treatment	Genotype	Shoot biomass (g plant^{-1})			Total biomass (g plant^{-1})			Water extracted in vegetative phase (kg plant^{-1})			Water extracted in reproductive phase (kg plant^{-1})			Total water extracted (kg plant^{-1})				TE (g biomass accumulated kg^{-1} water extracted plant^{-1})		
Trial		*2009 GH*	*2010 GH*	*2010 F*	*2009 GH*	*2010 GH*	*2010 F*	*2009 GH*	*2010 GH*	*2010 F*	*2009 GH*	*2010 GH*	*2010 F*	*2009 GH*	*2010 GH*	*2010F*	*2009 GH*	*2010 GH*	*2010 F*
WW	C235	12.0	13.7	5.7	18.4	27.0	11.2	2.6	7.1	1.3	14.4	9.1	8.1	16.9	16.2	9.4	1.01	1.68	1.20
	RD10	13.9	13.0	5.2	22.2	31.2	10.3	**4.7**	**5.4**	1.4	**18.0**	9.5	7.6	**20.6**	14.9	9.0	1.08	2.10	1.14
	RD2	11.9	**8.0**	5.1	16.6	**21.1**	10.4	2.3	**2.6**	1.3	14.9	9.5	7.6	17.2	**12.0**	8.9	0.96	1.77	1.18
	RD7	11.6	**9.4**	5.9	18.0	26.9	12.5	**1.3**	**2.8**	1.4	16.8	**10.5**	8.6	18.1	**13.3**	10.0	0.98	2.03	1.28
	RD9	11.0	**9.0**	5.2	17.7	23.6	9.8	**1.2**	**2.5**	1.4	14.5	8.6	7.6	15.7	**11.1**	8.9	1.10	**2.18**	1.11
l.s.d. 0.05		*4.6*	*1.6*	*1.2*	*10.1*	*5.7*	*3.1*	*0.7*	*0.8*	*0.3*	*3.5*	*1.3*	*1.4*	*3.9*	*1.6*	*1.6*	*0.44*	*0.52*	*0.34*
l.s.d. 0.1		*3.8*	*1.3*	*1.0*	*8.4*	*4.7*	*2.6*	*0.6*	*0.7*	*0.2*	*2.9*	*1.1*	*1.1*	*3.2*	*1.3*	*1.3*	*0.37*	*0.43*	*0.28*
WS	C235	6.8	3.4	4.2	10.7	5.4	7.7	1.5	2.5	0.6	5.5	3.0	4.4	7.1	5.5	5.0	1.49	0.98	1.55
	RD10	6.9	4.0	4.0	**13.2**	5.4	7.3	1.4	**4.0**	**0.5**	5.6	**1.4**	4.6	7.1	5.3	5.1	**1.85**	1.02	1.43
	RD2	6.5	2.8	4.3	11.8	**6.8**	8.4	**1.0**	**0.9**	**0.2**	**5.9**	**3.8**	**5.0**	7.1	**4.7**	5.2	1.66	**1.49**	1.63
	RD7	6.2	3.0	4.0	12.3	**6.7**	8.2	**1.1**	2.1	0.6	**5.9**	3.0	4.6	7.2	**5.0**	5.1	1.70	**1.34**	1.61
	RD9	6.5	3.3	**3.5**	11.8	6.1	7.8	**1.2**	2.4	**0.5**	**6.0**	3.0	4.3	7.5	5.4	4.8	1.59	1.14	1.65
l.s.d. 0.05		*1.5*	*0.8*	*0.5*	*2.9*	*1.2*	*1.3*	*0.3*	*0.5*	*0.1*	*0.5*	*0.5*	*0.4*	*0.6*	*0.5*	*0.4*	*0.36*	*0.31*	*0.28*
l.s.d. 0.1		*1.2*	*0.7*	*0.4*	*2.4*	*1.0*	*1.1*	*0.2*	*0.5*	*0.1*	*0.4*	*0.4*	*0.3*	*0.5*	*0.4*	*0.4*	*0.30*	*0.25*	*0.23*

Table 2. Chickpea pod and seed number, pod and seed weight in the different genotypes tested in P2 greenhouse facility (2009GH and 2010GH) and contained field conditions (2010F) under both well-watered (WW) and water stressed (WS) conditions

Numbers in bold represent values that are significantly different compared to the C235 untransformed parent; numbers in italics represent the calculated LSD values (at $P < 0.05$ and $P < 0.1$) for each parameter

Treatment	Genotype	Yield components											
		Pod number (plant^{-1})			Pod weight (g plant^{-1})			Seed number (plant^{-1})			Seed weight (g plant^{-1})		
		2009 GH	2010 GH	2010 F	2009 GH	2010 GH	2010 F	2009 GH	2010 GH	2010 F	2009 GH	2010 GH	2010 F
WW	C235	50	116	58	6.4	13.3	5.6	58	99	35	5.7	10.6	4.0
	RD10	62	130	57	8.3	**18.1**	5.1	87	**134**	35	7.5	**14.6**	3.7
	RD2	46	104	63	4.8	13.2	5.4	48	94	34	4.0	10.5	3.8
	RD7	50	134	59	6.4	17.4	6.6	60	127	40	5.7	14.0	4.8
	RD9	46	106	53	6.7	14.6	4.6	63	108	27	5.9	11.8	3.3
	l.s.d. 0.05	*43*	*44*	*24*	*6.4*	*5.5*	*3.0*	*57*	*41*	*21*	*5.7*	*4.5*	*2.4*
	l.s.d. 0.1	*36*	*37*	*20*	*5.3*	*4.6*	*2.5*	*47*	*34*	*17*	*4.7*	*3.7*	*2.0*
WS	C235	33	20	48	3.9	2.0	3.6	33	15	27	3.6	1.6	2.4
	RD10	43	11	45	**6.3**	1.4	3.4	**53**	9	29	**5.9**	1.2	2.7
	RD2	37	**35**	43	5.3	**4.0**	4.1	39	**26**	31	4.9	**3.3**	3.2
	RD7	40	**30**	49	**6.1**	**3.7**	4.1	**51**	**26**	30	**5.6**	**2.7**	3.0
	RD9	38	29	45	5.3	2.8	4.4	44	**24**	34	4.9	**2.8**	**3.4**
	l.s.d. 0.05	*17*	*12*	*11*	*2.0*	*1.5*	*1.2*	*18*	*9*	*13*	*1.8*	*1.2*	*1.2*
	l.s.d. 0.1	*14*	*10*	*9*	*1.7*	*1.2*	*1.0*	*15*	*8*	*11*	*1.5*	*1.0*	*1.0*

there were no significant differences among genotypes for yield components. In contrast, there were many more variations under terminal WS conditions. Compared with C235 genotype, significantly higher seed weight was recorded in RD2 (2010GH), RD7 (2009GH and 2010GH), RD9 (2010GH and 2010F) and RD10 (2009GH) (Table 2). RD7 had consistently higher seed yield than C235 in the two greenhouse trials. By and large, the pod yield (pod weight) also followed the same trend as the seed yield results, except for RD9 (Table 2). In the 2009GH and 2010GH trials, genotypes that showed higher seed yield (1–2 g plant^{-1}) also had higher number of seeds (10–20 seeds plant^{-1}) than C235 (Table 2). Although the pod component followed the same trend, significant differences were recorded only in the 2010GH, where RD2 and RD7 had higher pod yield (2 g plant^{-1}) and pod number (10–15 pods plants^{-1}) than C235 (Table 2).

Harvest index (HI), calculated as the ratio of seed yield (seed weight) to total plant biomass, ranged around 0.33 (±0.15) under both WW and WS treatments across all three trials (Fig. 1). Under the WW treatment, all the transgenic events showed 16–26% higher HI than C235 in the 2010GH (Fig. 1). Under terminal WS conditions, higher HI was recorded in all the transgenic events (20–27%) in the 2009GH trial, in RD2 (38%) and RD9 (38%) in the 2010GH trial, and in RD9 (26%) in the 2010F trial, than in the untransformed C235 (Fig. 1).

In summary, under terminal WS conditions, several transgenic events gained 10–20 seeds plant^{-1} and 1–2 g seed yield plant^{-1} compared with C235 (Table 2). Also these events had significantly higher HI (all events in 2009GH, RD2 and RD9 in 2010GH, and RD9 in 2010F) than C235.

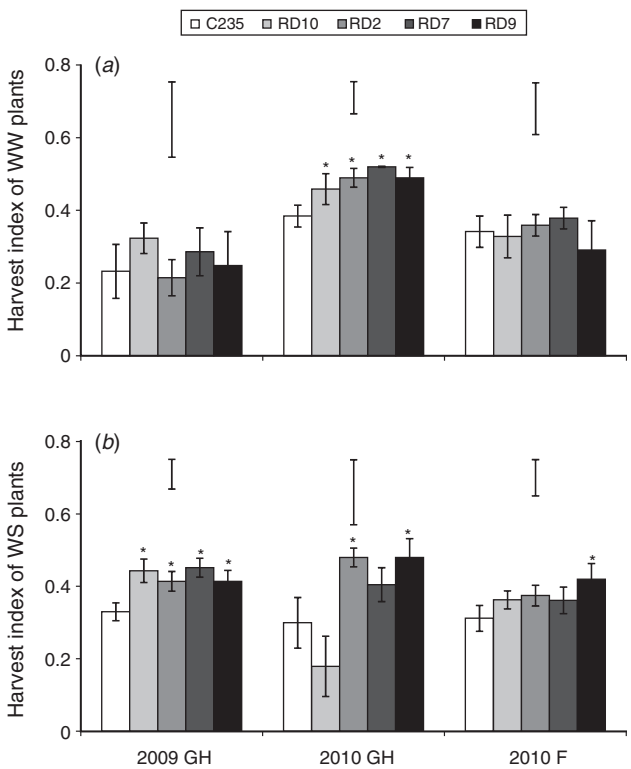

Fig. 1. Harvest index (HI) of the five chickpea genotypes tested under well-watered (WW, *a*) and water stressed (WS, *b*) conditions across three lysimetric trials, i.e. 2009GH, 2010GH and 2010F. Error bars on the columns represent s.e. for each genotype; error bars in the chart area represent l.s.d. value ($P < 0.05$) for individual trials; significant differences are indicated: *, $P < 0.05$.

Pattern of water extraction

Under WW and WS conditions, the pattern of weekly water uptake during the cropping cycle showed variations across the three trials conducted (Fig. 2). Under WW conditions the amount of water extracted across the cropping cycle in the 2009GH and 2010F trials were similar among the genotypes tested except for a higher water uptake in RD7 (97 DAS) and RD10 (84–97 DAS) in the 2009GH trial and lower water uptake in RD10 and RD9 (107

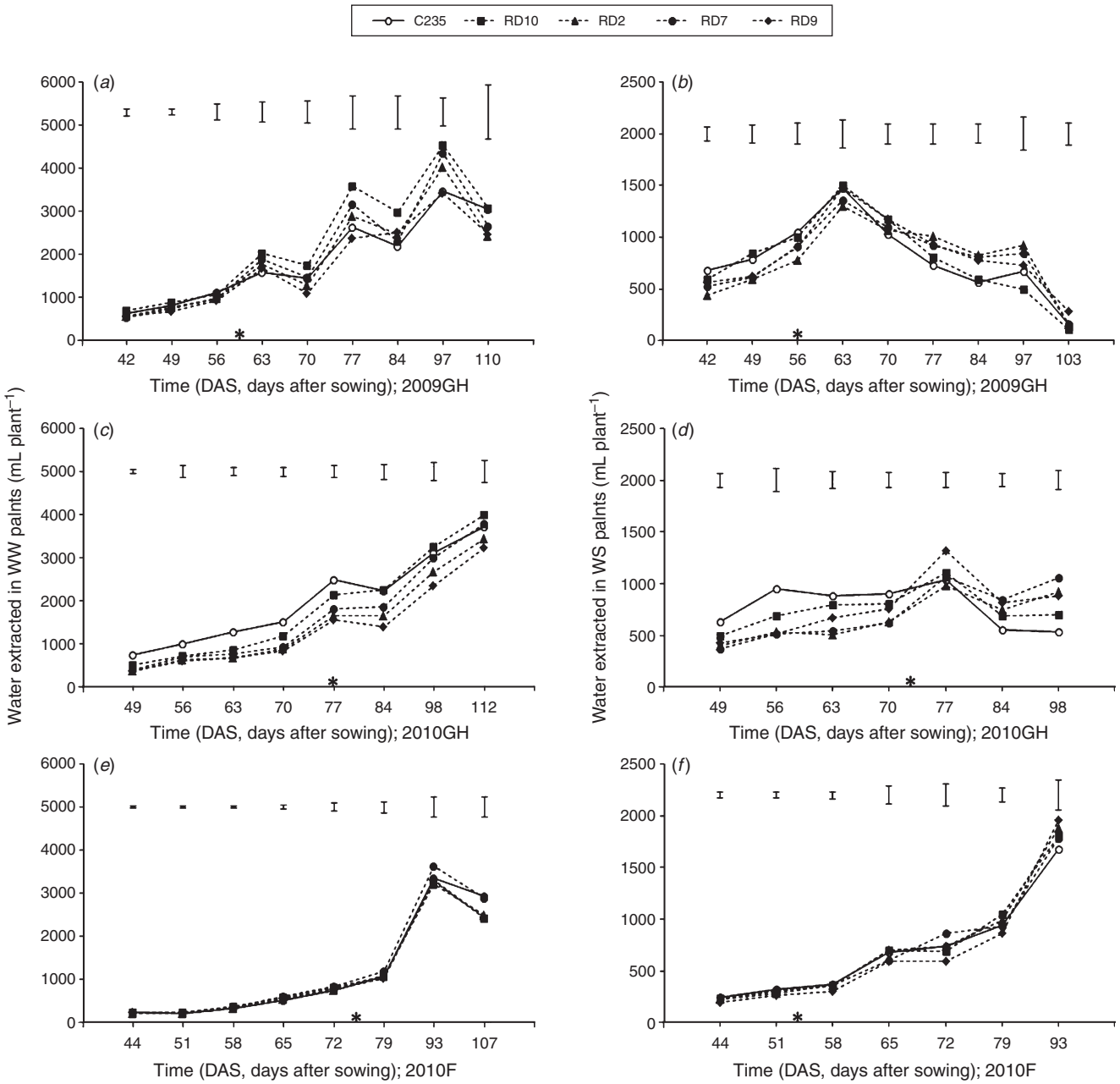

Fig. 2. Pattern of water extraction across the chickpea crop cycle of the five genotypes in the 2009GH (*a*, *b*), 2010GH (*c*, *d*), and 2010F (*e*, *f*) trials under well watered (WW; *a*, *c*, *e*; left panel) and water stressed (WS; *b*, *d*, *f*; right panel) conditions. Error bars in the chart area indicate l.s.d. value ($P < 0.1$) for water extracted during each time interval. Marker on the *x*-axis represents the transition point between vegetative and reproductive phase in the genotypes tested.

DAS) in 2010GH trial than in C235 (Fig. 2*a*, *c*). In contrast, in the 2010GH trial, all the transgenic events showed significantly lower water uptake during 49–98 DAS than C235 (Fig. 2). Under terminal WS conditions in the 2009GH and 2010GH trials, the transgenic events tended to extract less water during 42–63 DAS and increased water extraction during 77–98 DAS than C235 (Fig. 2). In the 2009GH trial, lower water extraction was found in RD2 (42–56 DAS) and RD7 (42–49 DAS), and higher water extraction was measured in RD2, RD7 and RD9 (77–84 DAS), than C235. Similarly, in the 2010GH trial, events RD2, RD7 and RD9 extracted less water during 49–70 DAS and extracted more water during 77–98 DAS (RD9), 84–98 (RD7) and 98 DAS (RD2) than C235. Also, RD10 showed significantly lower extraction than C235 during 49–56 DAS. By contrast under WS conditions in the 2010F, the pattern of water uptake was similar in all genotypes, except for RD9 at 58 DAS (Fig. 2).

Based on 50% flowering, the water extracted was summed up for the vegetative and reproductive stages of each genotype. Under WW conditions, in the 2009GH trial, events RD7 and RD9 had lower water extraction than C235 during vegetative

stage (Table 1). In contrast, RD10 had higher water extraction during both vegetative and reproductive stages than C235 (Table 1). In the 2010GH trial, all the transgenic events had lower water uptake (2.5–5.4 kg plant^{-1}) than C235 (7.1 kg plant^{-1}) during vegetative stage and RD7 had higher water uptake (10.5 kg plant^{-1}) than C235 (9.1 kg plant^{-1}) during reproductive stage (Table 1). In contrast, under WS conditions transgenic event tended to a lower water extraction during vegetative stage and higher water extraction during reproductive stages (Table 1), except RD10 in the 2010GH trial. Compared with C235, lower water extraction during vegetative stage was recorded in RD2 (all three trials), RD7 (2009GH) and RD9 (2009GH and 2010F) (Table 1). And higher water uptake during reproductive stage was recorded in RD2 (all three trials), RD7 (2009GH) and RD9 (2009GH) (Table 1).

In summary, except RD10 that had higher plant vigour, the other transgenic events under terminal WS seemed to conserve water during their vegetative stage making more water available for flowering and seed filling.

Pattern of flowering and seed filling

The onset and duration of flowering and seed set differed among the genotypes under WW and WS conditions across the three trials. Under WW conditions, the flowering period spanned over 7 weeks in 2009GH (49–97 DAS) and 2010F (51–93 DAS) trials, and up to 11 weeks in 2010GH (56–128 DAS) (see Table S1, available as Supplementary Material to this paper). In the 2009GH trial, the WW plants of C235, RD10 and RD9 started flowering a week earlier (49 DAS) than RD2 and RD7 (56 DAS). In the 2010GH trial, C235 flowered earlier (56 DAS) than the transgenic events (63–77 DAS). In contrast, in the 2010F trial, early flowering was seen in events RD2, RD7 and RD9 (51 DAS) than C235 and RD10 (58 DAS). Across the three trials, most of the flowers were produced during 77–84 DAS, 84–121 DAS and 79–93 DAS in 2009GH, 2010GH and 2010F, respectively, and successful seed set was derived from these flowers (Table S2). Late flowers (128 DAS) in the 2010GH trial did not set many seeds.

Under terminal WS conditions, the flowering period spanned over five weeks in 2009GH (49–77 DAS) and over 7 weeks in 2010GH (63–107 DAS) and in 2010F (51–93 DAS) trials (Table 3). In the 2009GH trial, the WS plants of C235, RD7 and RD10 showed an earlier onset of flowering (49 DAS) compared with RD2 and RD9 (56 DAS) (Table 3). A weekly account of the number of flowers produced showed no significant difference except for reduced flower number in RD10 compared with C235 at 70 DAS (Table 4). But the early flowers (49–56 DAS) produced in RD10 developed pods leading to significantly higher seed weight than C235 (Table 4). In contrast, in the 2010GH trial, RD2 and C235 started flowering a week earlier (63 DAS) than RD7, RD9 (70 DAS), and RD10 (77 DAS), although the flower number remained low in all genotypes until 77 DAS. Most flowering occurred during 84–100 DAS in the transgenics and contributed significantly to final seed yield, especially in RD2 (100–107 DAS), RD7 and RD9 (84 DAS), whereas in C235 most flowering occurred earlier (77–93 DAS). Hence, there was also a clear tendency of the transgenics to produce pod and seed weight from late flowers. These differences were significant at different stages between 84 and 107 DAS in RD2, RD7 and RD9. In the 2010F, flowering in all the transgenic events was a week earlier (51 DAS) than C235 (58 DAS). However, the onset of podding differed among the genotypes, with early pod set in RD7 (51 DAS), RD9 (58 DAS), RD10

Table 3. Week-wise data of percentage of chickpea plants that flowered and podded in the tested transgenic events and their untransformed C235 parent genotype under water stressed (WS) conditions in the three lysimetric trials conducted under greenhouse (2009GH and 2010GH) and contained field (2010F) conditions

Data represent the percentage at the end of a given week, e.g. onset at 63DAS represents the onset percentage in the week 56–63 days after sowing. Numbers in bold represent values that are significantly different compared to the C235 untransformed parent; numbers in italics represent the calculated LSD values (at $P < 0.05$ and $P < 0.1$) for each parameter

Experiment	Genotype	Onset of flowering (% plants)							Onset of podding (% plants)						
2009 GH	*DAS*	*49*	*56*	*63*	*70*	*77*	–	–	*49*	*56*	*63*	*70*	*77*	–	–
	C235	**20**	100	100	100	100	–	–	**20**	60	80	60	100	–	–
	RD10	**40**	80	80	60	100	–	–	**40**	80	80	20	100	–	–
	RD2	0	**60**	80	100	100	–	–	0	**40**	80	80	100	–	–
	RD7	**20**	60	60	100	100	–	–	0	**40**	60	80	100	–	–
	RD9	0	**67**	50	83	100	–	–	0	**17**	50	83	100	–	–
2010 GH	***DAS***	***63***	***70***	***77***	***84***	***93***	***100***	***107***	***63***	***70***	***77***	***84***	***93***	***100***	***107***
	C235	**17**	67	100	100	100	33	67	**17**	33	100	100	33	17	0
	RD10	0	0	**40**	100	100	20	80	0	0	**40**	100	20	0	20
	RD2	**40**	40	80	80	100	40	80	**20**	20	60	80	80	40	40
	RD7	0	**17**	67	100	100	83	83	0	0	**50**	100	83	67	17
	RD9	0	**20**	60	100	100	80	100	0	0	**60**	100	80	0	60
2010 F	***DAS***	***51***	***58***	***65***	***72***	***79***	***86***	***93***	***51***	***58***	***65***	***72***	***79***	***86***	***93***
	C235	0	**71**	71	86	100	100	100	0	0	0	**43**	100	100	71
	RD10	**13**	63	100	88	100	100	100	0	0	**13**	50	100	88	50
	RD2	**67**	100	100	100	100	67	83	0	0	0	**17**	100	67	50
	RD7	**25**	88	88	100	100	100	100	**13**	13	38	38	100	88	50
	RD9	**29**	86	100	100	100	100	100	0	**14**	0	71	100	86	29

Table 4. Week-wise data of chickpea flower number, pod and seed weight that arose from the flowers produced per transgenic event and the untransformed C235 genotype under water stressed (WS) conditions in three lysimetric trials conducted under greenhouse (2009GH and 2010GH) and contained field (2010F) conditions

Numbers in bold represent values that are significantly different compared to the C235 untransformed parent; numbers in italics represent the calculated LSD values (at $P < 0.05$ and $P < 0.1$) for each parameter

Genotype	Flower number (plant^{-1})							Pod weight (g plant^{-1})							Seed weight (g plant^{-1})						
2009GH/ DAS	*49*	*56*	*63*	*70*	*77*	–	–	*49*	*56*	*63*	*70*	*77*	–	–	*49*	*56*	*63*	*70*	*77*	–	–
C235	0.2	12	31	20	22	–	–	0.0	0.4	1.5	0.7	1.3	–	–	0.02	0.39	1.33	0.65	1.24	–	–
RD10	0.4	24	34	**5**	20	–	–	**0.1**	**2.6**	2.0	0.0	1.5	–	–	0.05	**2.44**	1.89	0.05	1.44	–	–
RD2	0.0	7	16	26	42	–	–	0.0	0.9	1.1	1.0	2.3	–	–	0.00	0.80	1.03	0.92	2.17	–	–
RD7	0.2	10	20	10	36	–	–	0.0	0.9	1.8	0.6	**2.7**	–	–	0.00	0.87	1.69	0.59	2.49	–	–
RD9	0.0	3	17	15	39	–	–	0.0	0.2	1.8	1.1	2.3	–	–	0.00	0.17	1.70	0.98	2.09	–	–
l.s.d. 0.05	*0.5*	*16*	*28*	*15*	*34*	–	–	*0.1*	*2.0*	*2.3*	*1.0*	*1.7*	–	–	*0.04*	*1.83*	*2.15*	*0.94*	*1.60*	–	–
l.s.d. 0.1	*0.4*	*13*	*23*	*12*	*28*	–	–	*0.1*	*1.6*	*1.9*	*0.8*	*1.4*	–	–	*0.04*	*1.51*	*1.78*	*0.78*	*1.32*	–	–
2010GH/ DAS	*63*	*70*	*77*	*84*	*93*	*100*	*107*	*63*	*70*	*77*	*84*	*93*	*100*	*107*	*63*	*70*	*77*	*84*	*93*	*100*	*107*
C235	0.3	3	12	24	13	1	3	0.1	0.0	0.9	1.0	0.1	0.0	0.0	0.05	0.00	0.74	0.84	0.02	0.00	0.00
RD10	0.0	0	5	14	16	1	9	0.0	0.0	**0.1**	1.0	0.4	0.0	0.0	0.00	0.00	0.06	0.80	0.32	0.00	0.00
RD2	0.6	**1**	9	25	22	**8**	**10**	0.1	**0.1**	0.7	1.4	0.7	**1.0**	**0.1**	0.05	**0.06**	0.55	1.17	0.58	**0.81**	**0.10**
RD7	0.0	0	5	25	**33**	**7**	7	0.0	0.0	**0.2**	**2.3**	**1.1**	0.1	**0.1**	0.00	0.00	0.16	**1.93**	0.51	0.05	0.05
RD9	0.0	**1**	6	30	21	1	7	0.0	0.0	0.6	1.9	0.3	0.0	0.0	0.00	0.00	0.63	**1.86**	0.30	0.00	0.00
l.s.d. 0.05	*0.6*	*3*	*10*	*18*	*14*	*6*	*9*	*0.1*	*0.1*	*0.8*	*1.3*	*1.0*	*0.8*	*0.2*	*0.08*	*0.07*	*0.68*	*1.03*	*0.71*	*0.67*	*0.12*
l.s.d. 0.1	*0.5*	*2*	*8*	*15*	*12*	*5*	*7*	*0.1*	*0.1*	*0.7*	*1.1*	*0.8*	*0.7*	*0.1*	*0.07*	*0.06*	*0.56*	*0.86*	*0.59*	*0.56*	*0.10*
2010F/ DAS	*51*	*58*	*65*	*72*	*79*	*86*	*93*	*51*	*58*	*65*	*72*	*79*	*86*	*93*	*51*	*58*	*65*	*72*	*79*	*86*	*93*
C235	0.0	4	2	10	65	38	15	0.0	0.0	0.0	0.3	2.4	0.8	0.1	0.00	0.00	0.00	0.23	1.59	0.58	0.03
RD10	0.0	2	**5**	9	52	34	16	0.0	0.0	0.0	0.1	2.8	0.4	0.0	0.00	0.00	0.00	0.05	2.37	0.28	0.00
RD2	**1.7**	3	4	11	61	24	14	0.0	0.0	0.0	0.1	**3.7**	0.3	0.1	0.00	0.00	0.00	0.10	**2.90**	0.17	0.04
RD7	1.1	4	5	14	62	27	14	0.0	**0.1**	**0.1**	0.3	3.3	0.3	0.0	0.00	0.08	**0.05**	0.27	2.40	0.22	0.01
RD9	0.5	4	5	15	**49**	26	13	0.0	**0.1**	0.0	0.8	3.2	0.3	0.0	0.00	0.05	0.00	0.60	2.51	0.20	0.03
l.s.d. 0.05	*1.8*	*4*	*4*	*9*	*18*	*23*	*9*	*0.0*	*0.2*	*0.1*	*0.6*	*1.4*	*0.7*	*0.1*	*0.00*	*0.13*	*0.05*	*0.50*	*1.19*	*0.52*	*0.06*
l.s.d. 0.1	*1.5*	*3*	*3*	*8*	*15*	*19*	*8*	*0.0*	*0.1*	*0.0*	*0.5*	*1.2*	*0.6*	*0.1*	*0.00*	*0.11*	*0.04*	*0.41*	*0.99*	*0.43*	*0.05*

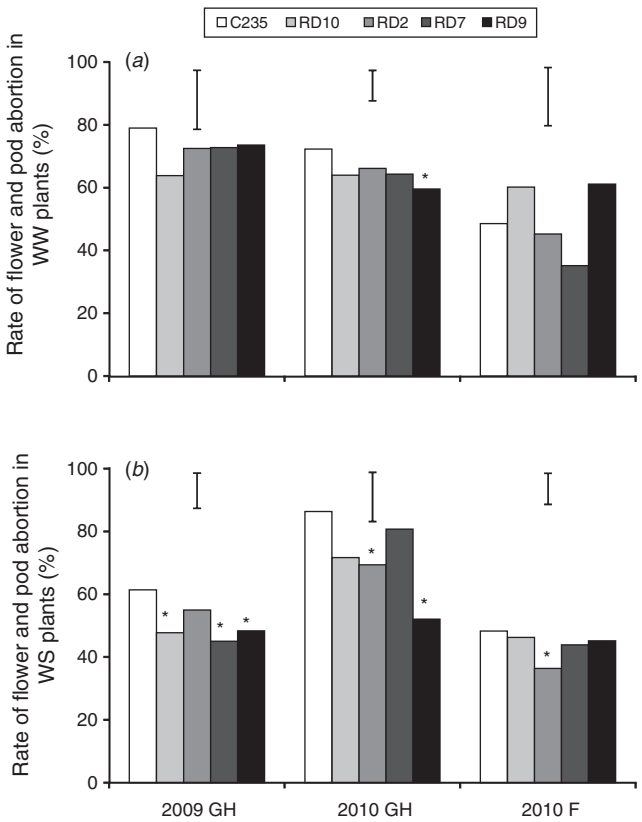

Fig. 3. Percentage of chickpea flower and pod abortion in different transgenic events and their untransformed C235 genotype under both well watered (WW, *a*) and water stressed (WS, *b*) conditions in three lysimetric trials, i.e. 2009GH, 2010GH and 2010F. Error bars in the chart area indicate l.s.d. value ($P < 0.05$) for the percentage of flower and pod abortion; significant differences are indicated: *, $P < 0.05$.

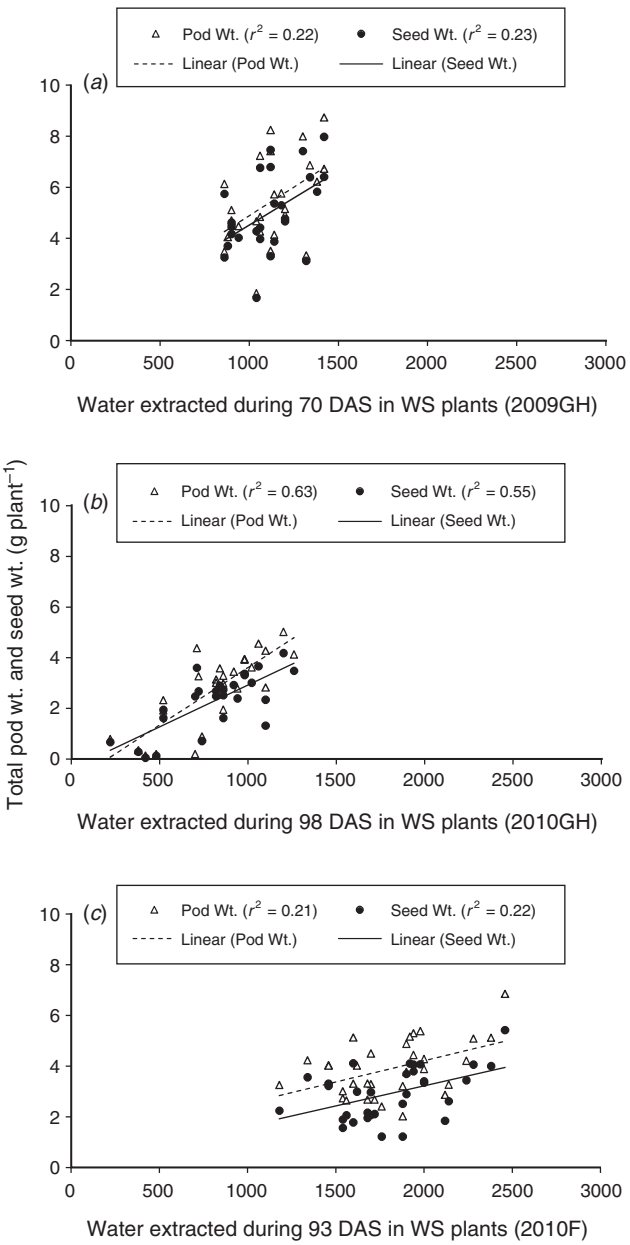

Fig. 4. Relationship between the water extracted during a few weeks of the reproductive phase and yield (pod and seed weight) produced in the water stressed (WS) chickpea plants across the three lysimetric trials, i.e. 2009GH (*a*), 2010GH (*b*), 2010F (*c*).

(65 DAS) than in RD2 and C235 (72 DAS). Over the reproductive phase, genotypic difference was recorded for significantly higher seed yield in RD2 for the seed set from pods produced at 79 DAS than C235 (Table 4).

The percentage of flower and pod abortion did not discriminate the genotypes tested under WW conditions except for RD9 in 2010GH and 2010F (Fig. 3). In contrast, several transgenic events under WS treatment had a lower percentage of flower and pod abortion than C235 genotype. Lower flower and pod abortion percentage were found in RD2 (2010GH and 2010F), RD7 (2009GH), RD9 (2009GH and 2010GH) and RD10 (2009GH) than in C235 (Fig. 3).

In summary, across the trials, the transgenic events under terminal WS conditions showed a trend of higher seed filling in pods developed from late flowers and had lower percentage of flower and pod abortion than the untransformed C235 genotype with significant differences for a few events.

Relationship between water uptake and yield components under terminal water stress

Across the 2009GH and 2010GH trials, shoot biomass was significantly related to the total water extracted (r = 0.53 and r = 0.46 respectively) and water extracted during vegetative stage (data not shown). In the 2010F trial, shoot biomass had significant correlation with total water extracted (r = 0.50) and water extracted during reproductive stage (r = 0.46) (data not shown). The relationship between the yield components (pod number and weight, seed number and weight) and water extraction varied in magnitude among three trials, but all suggested an increase in yield components with higher water extraction during period of the reproductive and grain filling stages. In the 2009GH trial, the yield components had significant positive correlation with the water extracted during 49 DAS

(onset of flowering, $r \geq 0.43$) and 70 DAS (mid-seed filling stage, $r \geq 0.49$, Fig. 4). In the 2010GH trial, the yield components significantly correlated with water extracted during reproductive stage ($r \geq 0.60$), 84 DAS ($r \geq 0.58$) (data not shown) and 98 DAS ($r \geq 0.66$, Fig. 4). In 2010F trial, the yield components (pod weight, seed number and seed weight) significantly correlated with the water extracted during 93 DAS ($r \geq 0.46$, Fig. 4).

In summary, both the amount of water extracted and the timing of water extraction (reproduction and seed filling stage) was critical for the final seed yield in the terminal WS plants.

Discussion

The transgenic events of chickpea C235 overexpressing *rd29A*::*DREB1A* (RD2, RD7, RD9 and RD10) were evaluated under greenhouse (2009GH and 2010GH) and contained field (2010F) conditions for water- and yield- related traits under terminal WS and WW treatments. Here, we identified four key plant attributes that contributed to a higher seed yield under terminal WS in transgenic events: (i) an early onset of flowering and podding in RD10 (2009GH trial); (ii) conserved water extraction during vegetative stage leading to increase water use during reproductive stage, as seen in RD2 (2010GH) and RD7 (2009GH); (iii) reduced flower and pod abortion as presented by RD2 (2010GH), RD7 (2009GH), RD9 (2010GH) and RD10 (2009GH); and (iv) successful pod set and seed filling from late flowers as seen in RD2 (2010GH) and RD7 (2009GH). RD7 in the 2010GH trial reached higher seed yield than C235 by efficient seed filling in the pods produced during the late but critical 84–93 DAS period. Events RD2 (2010GH), RD7 (2010GH) and RD10 (2009GH) with higher seed yield, plant biomass and lower total water extracted (except RD10) had higher TE than C235. By contrast, RD9 in the 2010F trial managed to secure higher yield and HI than C235 without any significant difference in any of the above mentioned traits, except for saving water during vegetative stage, and this may be due to small additive effects, insignificant individually, but making the overall effect significant. These differences in behaviour among the transgenic events could be the consequence of their insertion in different genome regions, with consequences on possible interactions with the recipient genome. In addition, the different behaviour across years and conditions also indicate a large degree of genotype × environment interactions. Therefore, further work using these events should consider closely the importance of the insertion site on possible genotype × genotype interactions, but also should consider what specific plant traits have been modified in each transgenics and especially whether any of these traits is responsive to the environment. Here we think in particular of traits controlling plant water use (Zaman-Allah *et al.* 2011*b*).

Reports suggest that reproductive success in chickpea is primarily dependent on the duration of the reproductive phase (Kumar and Abbo 2001), availability of water during seed filling stage (Zaman-Allah *et al.* 2011*a*, 2011*b*), biomass partitioning and nutrient remobilisation at reproductive stage (Leport *et al.* 1999; Davies *et al.* 2000), pattern of flowering (Zaiter and Barakat 1995; Berger *et al.* 2006) and reduced floral and pod abortion (Davies *et al.* 1999; Leport *et al.* 2006; Fang *et al.* 2010). In our study across three trials, total number of flowers produced did not discriminate the genotypes tested (data not shown), but their pattern of flowering and podding varied among the genotypes. The reproductive phase of WS plants in 2009GH seemed to be hastened by two weeks compared with that in 2010GH and 2010F trials. In the 2009GH trial, early onset of flowering was recorded in the WS plants of RD7, RD10 and C235 (49DAS). Of these genotypes, RD10 showed successful seed filling in the pods developed from the early flowers (56 DAS) which mainly contributed to the final yield (Tables 3, 4). However, this putative drought escape trait of securing reproduction soon after flowering in RD10 was recorded only in the late-sown 2009GH trial (November) and was not recorded in the following trials 2010GH and 2010F trials (October-sown). This feature of RD10 in the 2009GH seems to be influenced more by the environment than an inheritable genotypic character. In any case, it is known that drought affects the development of phenological stages by speeding up flowering. The difference in the onset of flowering between genotypes under WS conditions may suggest that each genotype perceives the stress at different time, and that early flowering transgenics (e.g. RD2 and RD7) would display water conserving mechanisms early on.

Another key mechanism identified across the greenhouse trials was the difference in the pattern of water uptake across the crop cycle under WS, where all the events (except RD10, 2009GH) had lower water extraction during the vegetative stage and then higher water uptake during the reproductive phase compared with C235 (Fig. 2). Event RD2 (across all three trials), RD7 (2009GH) and RD9 (2009GH) showed lower water extraction during vegetative stage and higher water uptake during their reproductive stages (Table 1), and this could have been related to the early stress response discussed in the previous paragraph. This tendency of saving water has also been reported in the tolerant genotypes of wheat (Moud and Yamagishi 2007), rice (Kato *et al.* 2008), pearl millet (Kholova *et al.* 2010), chickpea (Zaman-Allah *et al.* 2011*b*) and cowpea (Belko *et al.* 2012). In the chickpea study conducted by Zaman-Allah *et al.* (2011*a*, 2011*b*), tolerant genotypes were reported to have a lower water uptake during the vegetative stage compared with sensitive ones and that was related to a lower leaf/canopy conductance and a restricted shoot growth. With no canopy conductance data, the exact mechanism underlying the observed differences in the pattern of water uptake in this experiment remains unexplained. The lower water extraction in the greenhouse trials did not negatively influence the shoot biomass produced in RD2, RD7and RD9, but did have an influence in securing higher yield than C235 genotype (Table 1).

Changes in the water use pattern between vegetative and reproductive stages probably was the causal factor leading to reduced flower and pod abortion, and better seed filling in pods produced from late flowers, both mechanisms closely linked with soil moisture available during reproductive phase. In our study, the total flower number (over crop cycle and weekly) did not show any genotypic variations (data not shown), but the percentage of flower and pod abortion in transgenic events RD2 (2010GH and 2010F), RD7 (2009GH), RD9 (2009GH and 2010GH) and RD10 (2009GH) was significantly lower than C235 (Fig. 3). Further dissection of this parameter showed that apart from flower abortion, lower yield in the WS plants of C235 was due to failure of seed set rather than merely pod abscission (Table 2). This variation was further explained by the successful pod development and seed filling in the pods produced from late

flowers in the transgenic events as in the case of RD2 (2010GH) and RD7 (2009GH) (Tables 3, 4). Further, across the reproductive phase, weekly seed yield from a few critical weeks contributed maximally to the final yield. These weeks showed a significant positive correlation with the water extracted during that period (Fig. 4). In agreement with the previous reports on traits that are associated with yield gain, our results present conserved water uptake during vegetative stage, early flowering and reduced percentage of flower and pod abortion contributed majorly to the reproductive success in the DREB1A transgenics of chickpea under terminal water stress. These results further suggest that DREB1A, a dehydration- responsive transcription factor, influenced the pattern of water uptake and flowering across crop cycle, directly or indirectly, leading to reduction in the percentage of flower and pod abortion in the glasshouse trials.

Conclusion

Our results suggest that the kinetics of water extraction directly influenced flowering, pod development and seed filling and thereby contributed significantly to the final seed yield in the tested genotypes. Therefore, timing of water extraction, pattern of flowering and percentage of flower and pod abortion can be used as indicators of adaptation to terminal drought stress. The exact mechanism for this effect on the pattern of plant water use is still unclear. It could be, in part, derived from early stress onset in some transgenics, but could also be explained but lower leaf conductance as shown earlier in DREB1A peanut. Therefore, further research is needed to investigate how DREB1A influenced the kinetics of water extraction, alterations in flowering, pod development and seed filling, and nutrient mobilisation during late reproductive stage. Whether DREB1A interacted, directly or indirectly, with genes related to water and ion transport (e.g. via HVA1, H^+ pyrophosphatase), stomatal conductance (via ABA accumulation, MYB41, SNF1, ICE, HRD, HSPs etc.), photosynthetic efficiency (RAP2.4-related), circardian rhythm (e.g. via PIF4, CO), flowering (e.g. APETALA2, FTC, LFY), cell elongation and seed filling (e.g. ERECTA, LEA proteins, dehydrins) (Chew and Halliday 2010; Yang *et al*. 2010) is yet to be deciphered. Extensive molecular analysis of gene expression and protein interactions could help in identifying the molecular mechanism underlying the observed phenotypic variations.

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