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Genetic engineering of chickpea (*Cicer arietinum* L.) with the *P5CSF129A* gene for osmoregulation with implications on drought tolerance

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Abstract Abiotic stresses including water deficit severely limits crop yields in the semi-arid tropics. In chickpea, annual losses of over 3.7 million tones have been estimated to be due to water deficit conditions alone. Therefore, major efforts are needed to improve its tolerance to water deficit, and genetic engineering approaches provide an increasing hope for this possibility. We have used transgenic technology for the introduction of an osmoregulatory gene *P5CSF129A* encoding the mutagenized Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) for the overproduction of proline. A total of 49 transgenic events of chickpea were produced with the 35S:P5CSF129A gene through *Agrobacterium tumefaciens*-mediated gene transfer through the use of axillary meristem explants. Eleven transgenic events that accumulated high proline (2–6 folds) were further evaluated in greenhouse experiments based on their transpiration efficiency (TE), photosynthetic activity, stomatal conductance, and root length under water stress. Almost all the transgenic events showed a decline in transpiration at lower values of the fraction of transpirable soil water (dryer soil), and extracted more water than their untransformed parents. The accumulation of proline in the selected events was more pronounced that increased significantly in the leaves when exposed to water stress. However, the overexpression of *P5CSF129A* gene resulted only in a modest increase in TE, thereby indicating that the enhanced proline had little bearing on the components of yield architecture that are significant in overcoming the negative effects of drought stress in chickpea.

Keywords Chickpea · *Cicer arietinum* · Drought tolerance · Δ^1-Pyrroline-5-carboxylate synthetase · Transgenic plants · Transpiration efficiency

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Introduction

Abiotic stresses activate various metabolic and defense systems in the living cells when they are progressively exposed to stress. Osmotic stresses disturb the conserved intracellular ionic milieu in the cell due to H_2O efflux and consequently decrease its volume, disrupt its ion transport (such as uptake, extrusion and sequestration of ions) and metabolism (e.g., carbon metabolism, the synthesis of compatible solutes) in turn interfering with the cell function. The accumulation of osmolytes in response to stress is well documented (Molinari et al. 2004) and many studies have demonstrated that the manipulation of genes involved in the biosynthesis of low-molecular-

weight metabolites have improved the short term plant tolerance to drought and salinity stresses in a number of crops.

Amongst the low molecular weight metabolites, proline (Pro) is accumulated under drought and salinity stresses in a number of species. In addition to acting as an osmoprotectant (Christian 1955), Pro also serves as a sink for energy to regulate redox potentials (Blum and Ebercon 1976), as a hydroxyl radical scavenger (Smirnoff and Cumbes 1989), and as a means of reducing the acidity in the cell (Venekamp et al. 1989). Proline is also involved in the osmotic adjustment of plants under water stress whose putative role is to maintain cell turgor and to sustain growth processes, even under stress. The glutamate pathway of proline biosynthesis is predominant under osmotic stresses (Delauney and Verma 1993). In *Vigna aconitifolia* and *Arabidopsis*, the first two steps are catalysed by a single bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthetase (P5CS), with γ-glutamyl kinase (γ -GK) activities, and glutamic acid-5-semialdehyde (GSA) dehydrogensae (Hu et al. 1992; Yoshiba et al. 1995). P5CS plays a key role in the biosynthesis of Pro and catalyses the major regulated step under osmotic stress (Kiyosue et al. 1996).

There have been several attempts to obtain transgenic plants overexpressing proline for imparting tolerance to various abiotic stresses. Transgenic *Arabidopsis* plants with antisense suppression of proline degradation by proline dehydrogenase (antisense-AtProDH) accumulated higher levels of proline and conferred tolerance to freezing and high salinity conditions compared to wild type plants (Nanjo et al. 1999). Elevated proline levels due to the overexpression of *Vigna P5CS* in transgenic tobacco (Kavi Kishor et al. 1995); rice (Zhu et al. 1998), wheat (Sawahel and Hassan 2002) and potato (Hmida-Sayari et al. 2005) have been reported to confer enhanced tolerance to salt stress. However, most of these studies provide great details on the expression of the genes inserted and their effect on proline levels, much less information is given on the methods used to evaluate the stress response. A common feature in these studies is the exposure to fairly extreme levels of stress (high salt concentrations; complete water withholding) and a short term exposure. While this approach has the merit of elucidating some key aspects of gene expression under stress, it lacks some very important information on the effect of the expression of these genes on the physiological response of plant over longer period of time (several days or weeks) after exposure to stress that would be more representative of a production situation.

Only a few studies have focused on drought stress such as tobacco (Kavi Kishor et al. 1995) where the criteria of tolerance was simply based on the number of days the plants took for wilting under a 10-day water stress period. More recently, *P5CS* gene from both *Arabidopsis* and rice was shown to confer drought tolerance in transgenic petunia (Yamada et al. 2005) where the plants were grown without water for 10 days under culture room conditions; the level of tolerance in transgenics was based on the survival percentage of the revived plants after imposed water stress. Similarly, Vendruscolo et al. (2007) subjected 58–65 day-old (booting phase) transgenic wheat plants overproducing proline to a 14 days period of complete water withholding, and concluded that these transgenic plants had tolerance to water deficit based on total water, osmotic and membrane potentials, membrane stability and malondealdehyde (MDA) content. None of these studies, however, attempted to measure yield or any of its components such as water use or water use efficiency (Passioura 1977).

Chickpea (*Cicer arietinum* L.) is an important grain legume that plays a significant role in the nutrition of the poor in the developing world. Improving the adaptation of chickpea to drought is critical as it is a rainfed crop and more often exposed to terminal drought. In the light of previous reports suggesting the osmoprotective role of Pro and its relevance under stress conditions, but also the fact that there is no yield or yield component report showing the beneficial effect of osmoprotection, our objective was to evaluate the effect of proline, an osmoprotectant, on drought-related traits through genetic engineering. It is also in line with a recent report on the lack of beneficial effect of osmotic adjustment on yield in chickpea (Turner et al. 2007), and limited evidences in other crops (Serraj and Sinclair 2002). However, owing to the perceived recalcitrant nature of chickpea toward tissue culture, reliable regeneration and transformation protocols have not emerged. Although several regeneration and transformation protocols involving somatic embryogenesis and organogenesis have been reported with

varying success rates, asynchronous shoot bud production and low success rates for recovery of plants make these systems inefficient for genetic transformation (Kar et al. 1997; Krishnamurthy et al. 2000; Sarmah et al. 2004).

Here, we present an efficient and reproducible transformation procedure for high-frequency genetic transformation and the recovery of transgenic chickpea plants based on our earlier work (Jayanand et al. 2003; Sharma et al. 2006). The hypothesis for the present study was that the introduction of the *P5CSF129A* gene into chickpea might result in the accumulation of elevated amounts of endogenous proline, and consequently improve plant production into this important pulse crop. However, the overproduction of proline might contribute to a sustained stomatal opening, especially under water stress, with a possible negative effect on water use efficiency. So, the aim of this study was first to generate transgenic chickpea plants overexpressing the *P5CSF129A* gene, and second, to examine the plant gas exchange response to water deficit, in particular to evaluate how transpiration efficiency (TE) is affected in transgenics overproducing proline.

Materials and methods

Media and culture conditions

Unless mentioned otherwise, the following media were used during various stages of shoot regeneration. The shoot induction medium (SIM) consisted of MS (Murashige and Skoog 1962) along with 4 μM thidiazuron (TDZ), 10 μM 2-isopentenyladenine (2-iP) and 2 μM kinetin (pH adjusted to 5.8). The shoot elongation medium (SEM) consisted of MS along with 5 μM 2-iP and 2 μM kinetin (SEM1) and SEM2 consisting of MS + 2 μM gibberellic acid (GA3) with pH 5.5. The root induction medium (RIM) was modified MS containing 9.4 mM KNO_3 and 5 μM indolebutyric acid (IBA) at pH 6.0. The hydroponic system used had 1/2 strength Arnon's nutrient solution (pH 6.5) along with 3 μM IBA in 8 cm magenta jars. All culture media were used as liquid or solidified with 0.8% (w/v) Difco–Bacto® agar as required. All the tissue cultures were maintained at 26 ± 1°C under continuous light conditions (100 μMol m^{-2} s^{-1} light intensity provided by cool white fluorescent lamps).

Preparation of explants

Healthy and mature seeds of chickpea variety C 235 (desi type) were surface-sterilized with 70% (v/v) ethanol for 1 min, followed by treatment with 0.1% mercuric chloride for 10 min under aseptic conditions. Seeds were rinsed two to three times with sterile distilled water prior to soaking overnight. On the following day, the soaked seeds were decoated and placed for germination on SIM at a density of 10–15 seeds per plate. The seedlings were allowed to grow in the culture room for 5–7 days until the axillary buds were prominent. These axillary buds were carefully removed up to the base and two cuts were made through the axillary meristem in order to remove the shoot and root tips up to the hypocotyls and epicotyl regions, respectively. The axillary meristem explants (AMEs) thus obtained were subcultured on a petri plate containing SIM and incubated in the culture room. The shoot buds emerging from the enlarged base of the axillary bud were carefully removed by scraping with a sharp scalpel blade to obtain the explant (AM4) for genetic transformation (Sharma et al. 2006).

Plant transformation

The transformation experiments were conducted by using *Agrobacterium tumefaciens* strain C58 harboring the binary vector pBI:P5CSF129A (kind courtesy: Prof. D.P.S. Verma) carrying the mutagenized *Vigna aconitifolia P5CSF129A* cDNA under the control of the *CaMV 35S* promoter, where site-directed mutagenesis modified each of the six amino acid residues between positions 126 and 131 of the wild P5CS peptide. Besides, the vector contained selectable marker (*npt*II) and reporter (*uid*A) genes fused into a single gene again under the regulation of *CaMV 35S* promoter.

A single colony of *A. tumefaciens* strain C58 harboring binary vector pBI-P5CSF129A was grown overnight in a yeast extract broth (YEB) containing 50 mg ml^{-1} kanamycin on an incubator shaker at 100 rpm. The OD of the overnight-grown culture was ensured to be in the range of 0.6–1.0. Aliquots of 12.5 ml of the culture was taken in a 30 ml tube and

centrifuged at 5,000 rpm for 5 min. The supernatant was discarded and the precipitated cells were washed with 10 ml of sterile half-strength MS followed by centrifugation at 5,000 rpm to collect the cells that were resuspended in 25 ml of sterile half-strength MS and used for co-cultivation. The freshly prepared AM4 explants were briefly dipped individually into the bacterial culture poured in the petri plate for 1–2 s for *Agro*-infection followed by culturing five to seven explants on petri plates containing SIM with the base of the cotyledon embedded into the medium. The explants were co-cultivated with the bacteria for 48 h in the culture room.

Following co-cultivation, the explants were transferred to MS containing 250 mg l^{-1} cefotaxime for 4–5 days at 26 ± 1°C under continuous cool white light provided by fluorescent lamps (60 μE m^{-2} s^{-1}). The explants were transferred onto SIM containing 250 mg l^{-1} cefotaxime and a low selection pressure of 25 mg l^{-1} kanamycin for 1 week. The explants containing multiple shoot buds were further subcultured on the same medium containing a relatively higher selection pressure (50 mg l^{-1} kanamycin) for 7–10 days. Thereafter, the bunch of emerging shoot buds from the cotyledonary part were carefully separated with some intact basal callus and transferred to shoot elongation medium (SEM1) containing a higher selection pressure (75 mg l^{-1} kanamycin) for another 10 days. The elongated as well as unelongated shoots were transferred to SEM2 containing a stringent selection pressure of 100 mg l^{-1} kanamycin) for 2–3 passages at 7 days interval. Bunches of healthy shoots emerging from the elongating shoot were carefully separated and the untransformed bleached shoots are removed. The stunted shoots were sub-cultured on SEM2 for two to three passages of 1 week each for further elongation and increasing the length of internodes. The healthy green shoots (~5 cm long) were selected for the induction of adventitious roots. The basal stem segment of the elongated shoot was cut into half so that it did not contain any nodal meristem, and cultured on a filter paper bridge immersed in root induction medium (RIM) for 1–2 weeks. The plants were rooted and hardened as described previously (Jayanand et al. 2003) and transferred to a containment greenhouse with 24/18°C day/night temperatures where they were allowed to grow until maturity and subsequent progression of generations (T1, T2, and so on).

Molecular analysis of transformed plants

The genomic DNA was isolated from young leaves of the putative transformants using the CTAB–NaCl method (Sambrook et al. 1989). Initial screening of the transformants was done by PCR for the presence of *npt*II, *uid*A and *P5CS129A* genes. The 700 bp region of *npt*II gene was amplified by using 22-mer oligonucleotide primers (Sharma et al. 2000). Presence of the introduced *P5CSF129A* gene was detected by using 22-mer primers (FP 5′-ACA TTA TAC TCG TCT CCT CTG G-3′ and RP 5′-AAT TTT GTT TCC TTT CCT CTG A-3′) designed to obtain a 800 bp amplicon with a PCR profile of 30 cycles at (94°C for 45 s, 56°C for 1 min, and 72°C for 90 s) and a final extension at 72°C for 5 min. A 887 bp 35S:P5CSF129A region was amplified by using the junction primers 35_FP-5′-CAA GGC TTG CTT CAC AAA CC-3′ and p5_RP:5′-GAC GGG GCC AGA GGA GAC GAG TA-3′ with a PCR profile of 30 cycles at (94°C for 45 s, 60°C for 1 min, and 72°C for 90 s) and a final extension at 72°C for 10 min. Fidelity of the amplicons was verified by resolving the fragments on to a 1.2% agarose gel followed by transfer to Hybond +ve nylon membrane (Amersham Pharmacia Biotech) by Southern blotting (Sambrook et al. 1989) and probing with a 400 bp *Eco*RI fragment of the 35S:P5CSF129A region using Alk-Phos Direct® kit (Amersham Pharmacia Biotech).

Southern blot analysis was carried out according to Sambrook et al. (1989). The genomic DNA (25–30 μg) from each of the putative transformants was separately digested with *Hind*III that has two restriction sites within the plasmid DNA to ascertain the integration pattern based on size separation and number of copies of insert DNA. The blot was probed with a radio labeled 400 bp 35S:P5CSF129A region fragment. The copy number of the inserted gene was determined by digesting the genomic DNA samples with *Xba*I and probed with *npt*II for copy number.

RNA extraction and expression studies

Samplings were done at regular intervals throughout the dry-down cycle and tissues were freeze-dried for subsequent studies. RT-PCR analysis of the putative transformants was carried out by using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, Calif.) on the total RNA isolated with the TRIzol® reagent

(Invitrogen). The fraction of transpirable soil water (FTSW) values, an indicator of water stress intensity in this study at which the samples were taken was used to compare the evolution in proline content over the course of the dry-down regime. Quality of the RNA preparation was ascertained by loading it on to a 1.2% denaturing agarose gel while its integrity was confirmed with two bands of rRNA (28S and 16S). A 545 bp region of *P5CS129A* transcripts was amplified using 22 primers 5′-AAT CAA CAG CAG CTT CGC CGA C-3′ and 5′-CAA CAG GAA TGC CAG CTT CAG C-3′ with a PCR profile of 30 cycles at (94°C for 45 s, 59.6°C for 1 min, and 72°C for 90 s) and a final extension at 72°C for 10 min.

Stress treatments

PEG induced osmotic stress

Based on a previous screening for the overproduction of proline under well watered (WW) conditions, a set of transgenic events was tested for their response to PEG. The in vitro grown 3 week-old transgenic seedlings of 13 events (P10, P11, P12, P13, P16, P17, P26, P27, P28, P29, P30, P31and P32) in T1 generation along with the untransformed wild type (WT) C 235 were transferred to culture tubes containing a liquid medium composed of full strength basal MS including 6% PEG 6000 and maintained overnight at 25°C. Following the stress treatment, the plants were carefully removed and sampled for estimating the extent of lipid peroxidation under the osmotic stress.

Dry down procedure

Two experiments were conducted in a containment greenhouse during June–July 2006 and October–November 2006 by using 11 transgenic events (T3 generation) that were selected on the basis of their proline accumulation under normal growth conditions. In the first experiment, the response of leaf gas exchange under WW and water stress (WS) conditions was tested in seven transgenic events each carrying the *P5CS129A* gene (P10, P12, P13, P17, P26, P28, P31). Various parameters including the transpiration efficiency (TE; biomass produced per kg of water), photosynthetic activity, stomatal conductance and root length under WW and WS conditions were measured. In the second experiment, the transpiration responses and TE of another four transgenic events (P21, P6, P8, and P9) were assessed for their response to WW and WS conditions. The seeds inoculated with 1% rhizobium NC 92 (IC 7001) were sown in 6″ pots for experiment 1 and in 8″ pots for experiment 2 containing, respectively, 3 and 5.0 kg black soil: sand: compost mixture in 3:2:1 ratio.

Eighteen positive plants per transgenic event were grown under WW conditions until 36 days after sowing (DAS) under greenhouse conditions with 28/20°C day/night temperatures. The pots were divided into three subsets, i.e., initial harvest at 36 DAS while the other two were either used as WW or WS treatments. Prior to the initiation of WS treatment, the pots of both WW and WS treatments were saturated with water and left overnight to drain the excess water. On the following morning, the pots were enclosed in polythene bags to prevent any water loss by evaporation. Thereafter, the pots were weighed every morning between 900 and 1,000 h. The WW control plants were maintained at about 80% field capacity (about 200 g below the initial pot weight in both experiments) by daily compensating the water loss due to transpiration. To expose the WS plants to a progressive water deficit, they were allowed to loose a maximum of 70 g of water per day by adding the transpiration loss exceeding 70 g to the pots. The transpiration of each plant was then calculated as the difference in their weights on the successive days, plus water added on the previous day.

To ease the comparison of transpiration between WS and WW plants, a transpiration ratio (TR) was calculated between transpiration rate of an individual plant and the mean transpiration rate of their respective WW controls. To minimize the effect of plant-to-plant variation, normalization was carried out by dividing the TR data of an individual plant by the average TR of the first 3 days of the experiments, before the imposition of WS (Ray and Sinclair 1997). Drought stressed plants were considered to have extracted all the available water for transpiration from the pot when their normalized transpiration ratio (NTR) was <10% of the control NTR and were harvested. The difference between the initial and final pot weight provided an estimate of the total transpirable soil water (TTSW) available in each pot. However, here we were interested to investigate whether TTSW varied across genotypes differing in their proline production, since

the additional osmotic pressure conferred by the over production of proline may increase the water pressure gradients between the plant and the soil, leading to an increased extraction of water. Usually, TTSW does not vary across genotypes in the dry-down conditions we describe here.

The daily fraction of transpirable soil water (FTSW) remaining in the experimental pot was used to assess the stress intensity. For instance, FTSW was used to compare the evolution of proline across genotypes over the course of the dry-down treatment. The daily FTSW values were computed as:

$$\text{FTSW} = \frac{\text{Initial pot weight} - \text{Daily pot weight}}{\text{Initial pot weight} - \text{Final pot weight}}$$

The daily NTR was plotted against daily FTSW as previously described (Ray and Sinclair 1997). A plateau regression procedure with SAS (SAS Institute 1996), using NTR as a function of FTSW, was applied to calculate the FTSW value where NTR began its decline.

Harvest and transpiration efficiency measurements

The plants were carefully separated into shoot and root components. The dry weight of the leaves, shoot, roots, and pods was determined by drying them in a forced air oven at 80°C until constant weight. While the subset of plants harvested at 35 DAS before the imposition of treatments was used to assess the pre-treatment biomass, the post-treatment biomass was that of either WS or WW plants. The transpiration efficiency in WS and WW plants was calculated as the total biomass produced during the experimental period (from pot saturation to harvest) divided by the cumulative water transpired during the same period. The stomatal conductance, photosynthetic efficiency and leaf temperature of the WS and WW plants was also measured. Data were recorded using a LCA 4-leaf gas analyzer (ADC Bio-Scientific Ltd., UK) on the fully expanded leaf between 1,000 and 1,200 h, under saturating light conditions (PAR $>$ 1,200 μmol m^{-2} s^{-1}).

Biochemical analysis

Estimation of proline

The content of L-proline in the leaf samples was estimated by the photometric method described by Bates et al. (1973) at 520 nm absorbance. A standard curve was prepared by using the commercial proline to calculate the proline concentration in the samples.

Estimation of lipid peroxidation

An increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen species. To study the level of lipid peroxidation, MDA (malondealdehyde) level which is an indication of the free radicals were estimated as described by Dhindsa et al. (1981). The absorbance of the supernatant was recorded at 532 nm (Beckman DU® 530). The non-specific turbidity was corrected by A_{600} subtracting from A_{532}. The concentration of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Statistical analysis

The physiological data were analyzed by using Tukey's test for the comparison of different transgenic events with WT C 235.

Results

Regeneration of whole plants

Six-day-old seedlings were found to be ideal for removal of axillary bud and regeneration on the SIM. Inclusion of the cotyledon had considerable advantage for the regeneration of multiple shoot buds from the axillary meristem region. Explants with full cotyledon showed much earlier appearance of shoot buds when compared to the explants with half cotyledon followed by no cotyledon (data not shown). Sub-culturing of the regenerating shoot buds in clusters, however, showed an exponential increase in the number of multiple shoots per explants. This increase was found to be two to three times better in the explants with intact cotyledons.

Tissue culture regeneration of chickpea variety C 235 was optimized for high frequency synchronous shoot regeneration (>90%) from the axillary meristem explants (AM4). About 60% of the axillary buds showed multiple shoots originating from basal region of the axillary bud. The AM4 explant gave rise to multiple shoots as multiple clusters where the shoots

were formed from cut portions on the swollen part of axillary meristem. Histological studies of multiple shoot induction from these explants confirmed the formation of multiple meristemoids after the removal of axillary bud, which eventually resulted into multiple shoots from the basal part of the axillary bud (unpublished results). Prolonged culturing on SIM resulted in exponential increase in the number of shoots per explant where multiple branching of the regenerating shoots increased the number of shoots per explant with each shoot bud having the ability to develop into complete plant.

The shoots elongated best when initially cultured on SEM1 followed by their transfer to SEM2 in the later stages. The shoot bud clusters were cultured in SEM2 for two to three passages at 10–12 day intervals. GA_3 was found to play a crucial role in the second elongation phase resulting in increased internodal length and better leaf morphology. The shoots that elongated during each passage were transferred to the rooting medium, where an average of 70–80% of elongated shoots rooted well. Inclusion of any nodal meristem on the surface that was exposed to the rooting media drastically decreased the rooting frequency. Shoots, which did not root were pulse treated with concentrated 100 μM IBA. Rooting in this phase was adventitious with more number of roots originating from the basal cut end of the shoot.

Genetic transformation

The AM4 explants were used for genetic transformation following co-cultivation for 48–72 h with the *Agrobacterium* culture. Emerging shoot buds were cultured on the selection medium with an incremental kanamycin concentration (i.e., 25 mg l^{-1} initially, 50 mg l^{-1} in SEM1 followed by 75 and 100 mg l^{-1} in SEM2) that resulted in a stringent selection of the putative transformants. The non-transformed shoot buds bleached and showed no further growth. The selected transformed shoots with a healthy growth were transferred to rooting medium devoid of any selection pressure as it was found to decrease the rooting frequency.

Hardening and maintenance of transgenics

The transformants that were transplanted to the greenhouse after hardening showed normal growth and morphology that was comparable to the non-transgenic controls. The plants were allowed to grow vegetatively for an extended time by removing the terminal buds of some branches and the flower buds. Over 65 independently transformed plants with 35S:P5CS129A construct were successfully transplanted to the greenhouse, advanced to T3 generation and their seeds were collected.

Stable integration of the transgenes

Over 65 in vitro-regenerated plants were analyzed for the presence and expression of the *npt*II, *uid*A and *P5CSF129A* genes. Oligonucleotide primers specific to the coding regions of *npt*II and *P5CSF129A* gene amplified the expected size of the gene fragment from 75% of the analyzed putative tranformants. Fourty nine out of the 65 regenerants showed an expected amplification for the respective genes. Twenty-one transgenic events in the T0 generation were advanced to T1 generation and plants analyzed for their segregation pattern. PCR analysis for *npt*II and *P5CSF129A* genes showed that the transgenes segregated in a Mendelian ratio of 3:1 that is typical of single gene integration. The results based on PCR analysis were confirmed by southern hybridization using *npt*II, *uid*A and *P5CS129A* genes. Southern blot analysis of T1 individuals indicated that the number of copies of the transgene integration into the genome in the tested plants varied from one to three (Fig. 1a). RT-PCR analysis of cDNA from the putative transgenic plants showed positive amplification of *npt*II and *P5CS* transcripts in all the selected transgenic events under unstressed as well as stressed conditions (Fig. 1b).

Greenhouse evaluation of transgenic plants

The transgenic events accumulating high proline levels under normal growth conditions were further selected for subsequent gene expression and phenotyping studies. Two greenhouse experiments were conducted for the physiological and biochemical characterization of the 11 selected transgenic events in T3 generation under WS and WW conditions. The cumulative transpiration of almost all the tested transgenic events was similar to their WT controls with a few exceptions in the first experiment (P12, P13) in which the cumulated transpiration was significantly higher (Table 1).

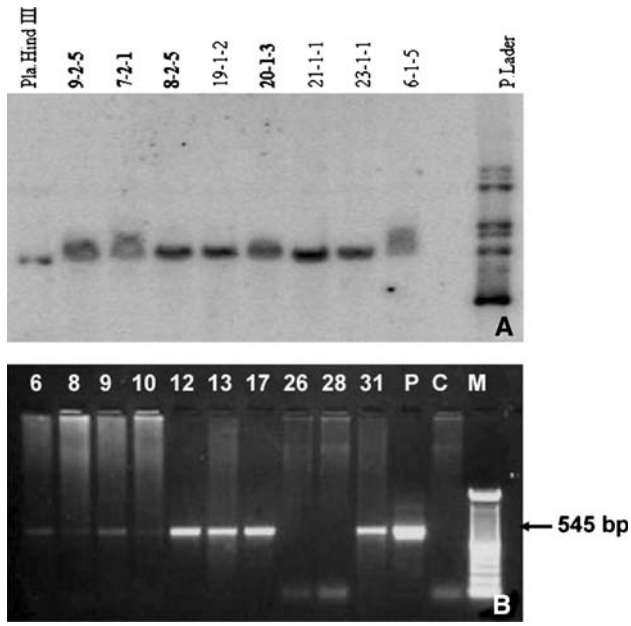


Fig. 1 Molecular analysis of transgenic events of chickpea carrying the P5CSF gene during T2 generation. **a** Southern blot analysis of the *P5CSF129A* gene in the genomic DNA of T2 transgenic plants transformed with the 35S:P5CSF129A gene construct. DNA was digested with *Hind*III that has two restriction sites within the plasmid pBI29NotAP DNA to ascertain the integration pattern based on size separation. The blot was probed with a radio-labeled 400 bp 35S:P5CSF129A region fragment, **b** A 545 bp *P5CSF129A* transcript is amplified where lanes 1–10 carry the RT-PCR amplicons from the total RNA isolated from transgenic plants, lane 11 is the plasmid, while 12 and 13 carry the untransformed control and marker, respectively

Similarly, under WS condition, the cumulative transpiration was computed as the sum of daily transpiration from initiation until the plant depleted all the soil water available for transpiration (FTSW = 0.1). All transgenic events had a significantly higher cumulative transpiration under drought stress than did WT in the first experiment, ranging from ~10 to 40% higher. In the second experiment, the total water use of event P9 only was significantly higher than in the other genotypes including WT (Table 1).

The transgenic events differed in their response of NTR to FTSW and were clearly distinguishable from the WT controls. Indeed, the normalized transpiration ratios of several transgenic events started declining at lower FTSW values (drier soil) under WS. (Table 1). Figure 2 shows a typical transpiration response of a transgenic event P28 compared to its WT, thereby, indicating that the onset of NTR decline occurred in drier soil in the transgenic event (0.53 FTSW) when compared to its WT (0.73). This pattern was essentially the same for all tested events (data not shown) as indicated by their significantly lower FTSW threshold values (Table 1). The transgenic events tested in the second experiment followed a similar pattern, i.e., their transpiration started declining in dryer soil, except for the event P9 that started closing its stomata at the same FTSW as that of the WT control.

The transpiration efficiency of WW plants from most of transgenic events was similar to that of WT in both the experiments. Under WS conditions in experiment 1, the biomass increase (delta-biomass) of plants exposed to progressive soil drying was significantly higher in P12, P26 and P28 than in the WT while no significant differences were observed in the biomass increase across the tested genotypes in the second experiment, except P8 whose biomass increase was higher than the other genotypes including the WT. Most of the transgenic events in experiment 1 showed no differences in TE except about 10–20% higher TE values in P12, P13 and P17 than in WT. Similarly, the TE of the all the transgenic events except P8 tested in second experiment showed no significant differences when compared to WT controls.

Table 1 Average transpiration efficiency (TE), total water use (cumulative transpiration), total transpirable soil water (TTSW), delta biomass and fraction of transpirable soil water (FTSW) at which the stomatal closure began to occur throughout the drying cycles for eleven transgenic lines of chickpea and their wild type parent under well watered (WW) and water stressed (WS) conditions of two greenhouse experiments

Genotype	TE (g biomass kg^{-1} water transpired)		Total water use (g $plant^{-1}$)		TTSW (g $plant^{-1}$)	Delta biomass (g)	FTSW-threshold
	WS	WW	WS	WW	WS	WS	
Experiment 1							
P10	3.083	2.529	508.8	1358	483.8	1.612	0.6167
P12	3.777	2.416	606.4	1742	584.5	2.338	0.6586
P13	3.36	2.411	503.1	1567	498.4	1.732	0.6609
P17	3.435	2.405	510.3	1324	500.4	1.74	0.6313
P26	3.344	2.419	539.3	1475	536.7	1.788	0.6235
P28	3.086	1.923	606.4	1381	609.2	1.893	0.5333
P31	2.738	1.981	508.8	1348	572.5	1.68	0.579
C 235	3.235	2.697	443.9	1478	447.3	1.49	0.7325
LSD	0.6125	0.407	47.82	187.2	36.82	0.2546	0.0939
Experiment 2							
C235	3.29	2.277	1473	2707	786.8	4.897	0.6115
P21	2.595	2.009	1433	2521	877	3.876	0.5372
P6	3.231	1.995	1326	2451	821.6	3.746	0.5442
P8	4.054	2.113	1493	2900	847.2	6.05	0.5539
P9	2.502	2.489	1601	3009	889.2	4.08	0.6418
LSD	1.069	0.475	256.3	724.3	55.06	1.673	0.076

The transgenic events tested in the first experiment were also studied for their leaf temperature, photosynthetic activity (*Gs*) and stomatal conductance under WW and WS conditions (Table 2). The stomatal conductance of the transgenic events measured under WW conditions in the first experiment was not significantly different between the transgenic events, although all the transgenics seemed to have higher *Gs* than the WT (Table 2). Leaf temperature followed a similar pattern with no significant differences between the tested events, but many had at least 1° less than WT. Similarly, Gs of the a few transgenic events was found higher than the WT, but again did not differ statistically.

Under WS at low FTSW values (<0.3), the stomatal conductance of most of transgenic events under WS was higher (0.42–0.71), although not significantly different to the WT (0.037). Interestingly, the transgenic event P28 which had the highest water use and lowest FTSW threshold, showed highest stomatal conductance under WS. The leaf temperature in all the transgenic events except one (P17) was similar with no significant differences when compared to the WT. In addition, the transgenic events had a high photosynthetic activity (3.32–5.36) where the events P10 and P28 exhibited significantly higher values than the WT control (2.42).

Rooting and water extraction under water stress

At the end of the WS period, the total amount of water extracted from the soil by transpiration (TTSW) was measured by subtracting the final pot weight from the initial pot weight. The TTSW of all transgenic events except P10 in experiment 1 and P6 in experiment 2 were significantly higher than for WT (Table 1) which clearly indicated the capacity of transgenic events to extract more water from the same soil volume (to an extent of 25% in Experiment 1 and 10% in Experiment 2). The root dry weights of transgenic events P17, P26 and P28 tested in the 1st experiment were lower than the WT at the end after WS imposition (Table 2) as was the total root lengths of events P13 and P31.

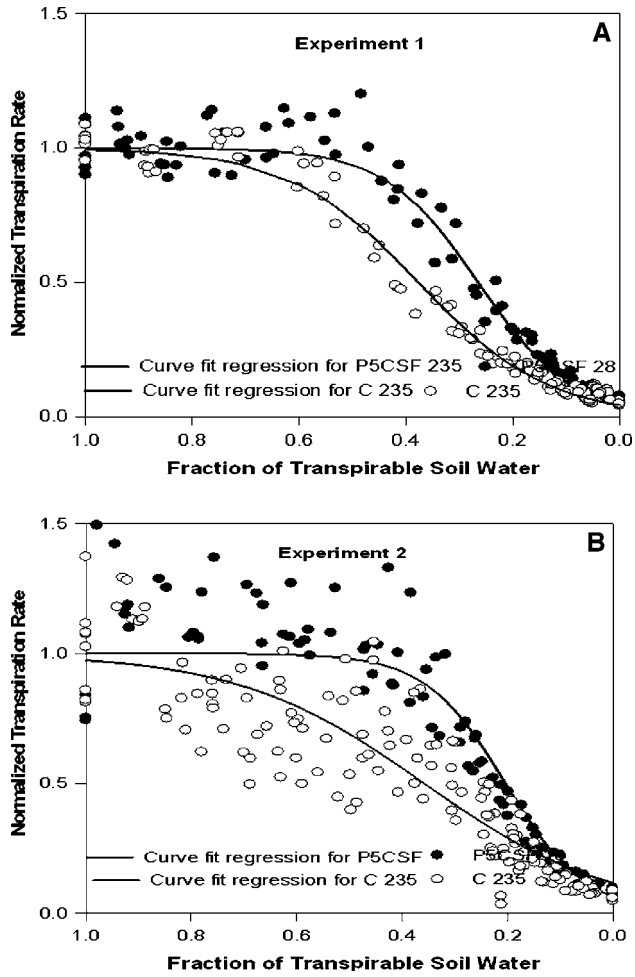


Fig. 2 A typical response of transpiration (NTR) to soil moisture (FTSW) in one of the *P5CSF129AF* transformed event and the untransformed control under water deficit and respective curves fit in two different soil-drying experiments

Proline and MDA assay

Proline assay was carried out in the T1 progeny of the *P5CSF129A* transgenic events to find the levels of proline content in a set of 3-week-old plants growing under normal growth conditions in the greenhouse. These events showed the overproduction of proline in the range of 2.0–6.0-fold when compared to the untransformed controls under normal growth conditions (Fig. 3a). MDA assay carried out on these transgenic events after undergoing 6% PEG induced osmotic stress overnight indicated a significant decrease in lipid peroxidation, as witnessed by the MDA assay that ranged from 11 to 41% decrease in the free radicals as compared to the WT (Fig. 3b). However, under the dry-down set up in the experiment 1, the proline concentration of the WW transgenic chickpea measured in 40-day-old plants was found to be only 1.3–2.9 fold higher than the WT (Fig. 3c). Also, the proline content of the plants undergoing water stress when sampled at low FTSW values (<0.1) showed a 2.0–5.4 fold increase compared to their WT counterparts. A significant negative correlation between proline content and the FTSW-threshold values of the WS plants was observed ($r = 0.80$**; Fig. 4a). Besides, a significant positive correlation was detected between the proline content and the TTSW value of the WS plants ($r = 0.88$**; Fig. 4b). However, no correlation was observed between the proline content and TE values.

Discussion

We report an efficient and reproducible transformation system for chickpea through *Agrobacterium*-

Table 2 Average root weight, root length, leaf temperature, photosynthetic capacity and stomatal conductance (*Gs*) of seven different T3 transgenic lines and their wild type parent under well watered (WW) and water stressed (WS) conditions in one of the greenhouse experiment

Genotype	Final root weight (g plant^{-1})	Final root length (cm)	Leaf temperature		Photosynthetic capacity		Stomatal conductance	
	WS	WS	WW	WS	WW	WS	WW	WS
P10	0.7217	5111	31.09	34.3	10.612	5.354	0.6938	0.054
P12	0.72	5595	31.49	34.71	11.625	1.87	0.8589	0.0296
P13	0.7217	4725	31.51	34.37	12.267	3.776	0.8482	0.056
P17	0.6317	4903	31.55	35.11b	6.615	3.318	0.7269	0.0365
P26	0.65	5178	31.65	34.09	7.93	3.636	0.6901	0.0615
P28	0.695	5417	32.06	34.43	8.73	5.131	1.0953	0.071
P31	0.7183	4858	32.19	34.45	7.322	3.645	0.7686	0.0426
C 235	0.8183	5459	32.95	34.82	9.366	2.429	0.2389	0.0372
Grand mean	0.71	5160	31.81	34.53	9.31	3.65	0.74	0.048
L.S.D.	0.101	576.5	2.007	0.969	7.645	3.186	0.7128	0.04085

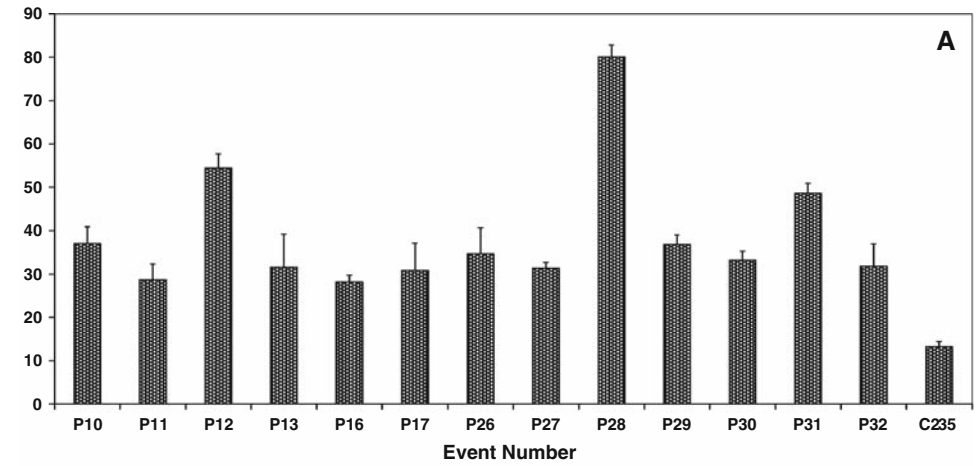
Data are the mean values, representing mean ($n = 6$)

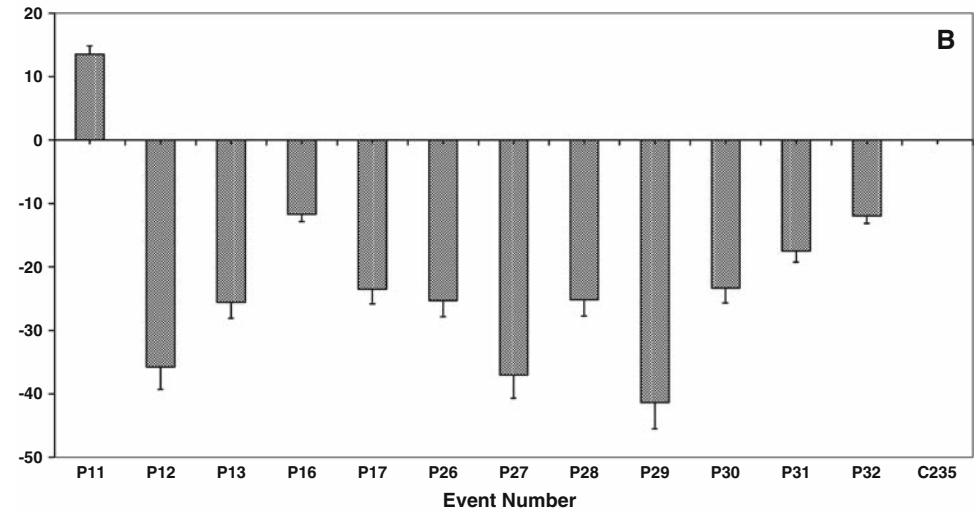
mediated genetic transformation using axillary meristem explants that result in a high frequency generation and recovery of transgenic plants (70%). The protocol used the axillary meristem explant produced by removing the axillary buds from germinated seedings and overcoming the apical dominance of the shoot buds. This is the first report of transgenic chickpeas for drought tolerance where several interesting observations were recorded as follows: (1) dramatic elevation of proline levels in the transgenic events transformed with the *P5CSF129A* gene, (2) exhibition of a diverse stress response patterns by the transgenic events including a decline in transpiration at lower FTSW values (dryer soil) and extraction of more water than their non-transgenic counterparts, (3) increased and pronounced proline accumulation in the leaves of all the tested transgenic events exposed to the osmotic stress, and (4) unchanged transpiration efficiency (TE) or rather a modest increase in some transgenic events existed, thereby indicating that differences in proline and MDA levels did not relate to differences in TE.

The formation of multiple shoots from different genotypes and the production of primary transgenic plants have been reported in chickpea (Kar et al. 1997; Krishnamurthy et al. 2000). Recently, different studies enabling the routine application of *Agrobacterium*-mediated transformation of chickpea have been carried out (Sarmah et al. 2004; Sanyal et al. 2005). Although, different research groups have reported successful transformation of chickpea, the overall frequency of transformation was still very low in these studies (0.1–1.0%). Besides, the reproducibility of transformation often has been quite low and limited in practical applicability.

Fig. 3 **a–c** Proline and MDA concentrations in the transgenic chickpea plants under normal growth conditions as well as under stress. **a** Free proline concentrations in transgenic chickpea plants (3-week-old) grown under normal conditions. **b** Percent increase or decrease in MDA concentration in the transgenic plants in comparison to the untransformed control under PEG induced osmotic stress in vitro. **c** Proline concentrations in 40 days-old transgenic chickpea plants and under well-watered (WW) as well as water stress (WS) conditions

The explant used here differ from those used previously for genetic transformation of chickpea such as apical meristems (shoot and root tips) and axillary buds that might be responsible for the higher rate of regeneration and transformation in the present study when compared to the previous reports. The AM4 explant was considered ideal since the number of shoots formed per explant was high which could be due to removal of the axillary bud that is devoid of apical dominance. Moreover, removal of the regenerating shoot buds provides wounding areas for successful infection of the tissue with *Agrobacterium*. Besides, there was a clear effect of the age of the seedlings from which the explants were derived,


A
90
80
70
60
50
40
30
20
10
0
P10 P11 P12 P13 P16 P17 P26 P27 P28 P29 P30 P31 P32 C235
Event Number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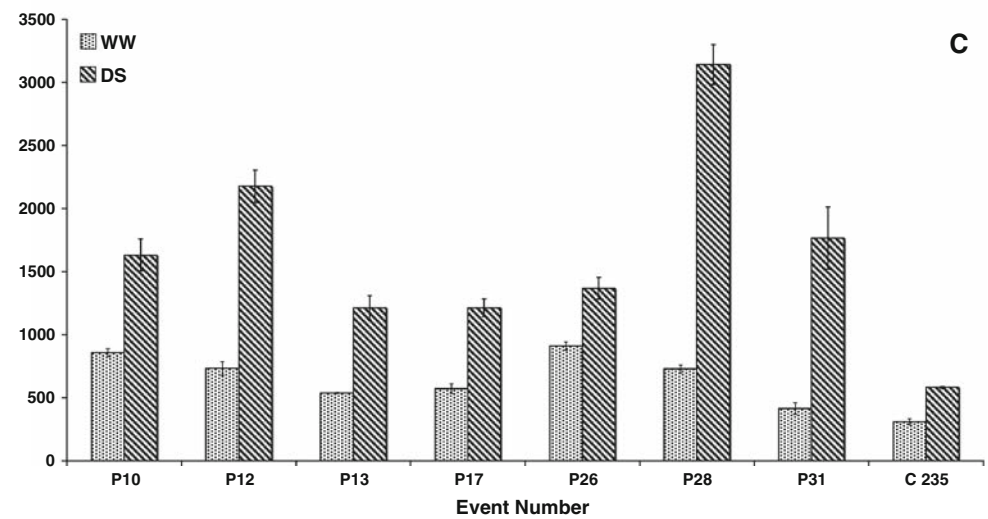
C
WW
DS
3500
3000
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P10 P12 P13 P17 P26 P28 P31 C 235
Event Number

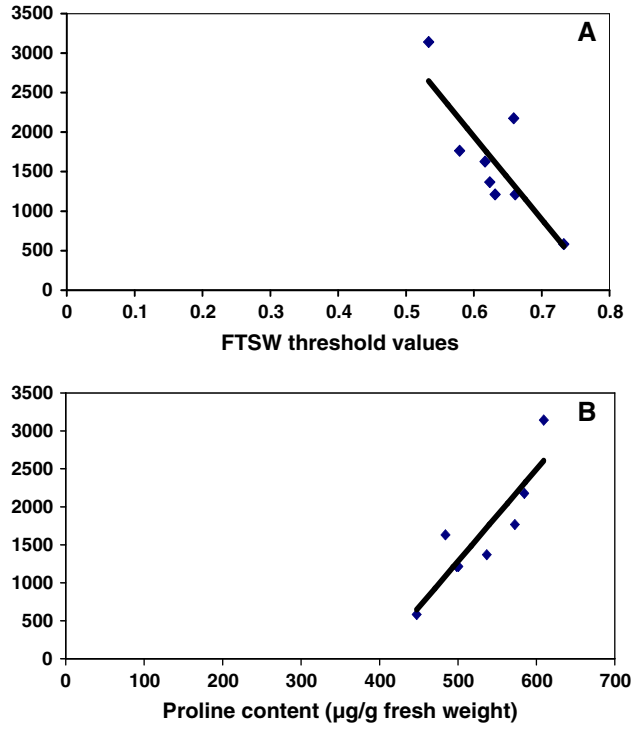

Fig. 4 Relationship between proline content with the FTSW-threshold (**a**), and TTSW (**b**) of the drought stressed transgenic events including the untransformed parent

indicating a gradual loss of multiple shoot regenerating capacity of the meristematic tissue with age.

The presence of cotyledonary tissue at the time of shoot bud induction has been shown to play an important role in induced morphogenesis of the target cells (Sharma et al. 1991). In the present study, a progressive selection system with stepwise increase in the concentration of the selective agent kanamycin at each stage has been found to be useful in obtaining stringently selected putative transformants in chickpea. Also, imposition of selection pressure was avoided at the rooting stage as it resulted in the decrease in the rooting frequency. These results were in accordance with a number of studies with chickpea, where the rooting medium lacked the selection pressure (Kar et al. 1997; Krishnamurthy et al. 2000). The protocol described here has been significant owing to its high reproducibility and recovery of the transgenics in a relatively short period (90–100 days).

The putatively transformed shoots that were tested for the incorporated genes showed a transformation frequency of 65%. Several independent transgenic events subjected to southern blot analysis contained a single or at most two transgene insertions. In earlier reports, Kar et al. (1997) noted multiple gene inserts in all their transgenic chickpeas, while Krishnamurthy et al. (2000) found 50% had single inserts and 50% had multiple (4–6) gene inserts. Low copy inserts in transgenic plants are preferred both from the point of view of regulation of gene technology, and from the perspective of plant breeders, since dealing with a trait conferred by a single, dominant gene is a convenient scenario in a plant breeding program (Sarmah et al. 2004). Inheritance of the inserted genes as demonstrated by PCR and southern blot analysis indicated that most of the events had stable incorporation of T-DNA into the nuclear DNA following the expected Mendelian ratio for a single gene insertion.

The transgenic chickpea plants showed significantly higher proline accumulation in the leaves when compared to the WT controls under stress-free conditions while a decrease in the free radicals in the transgenic plants was observed during PEG induced osmotic stress as measured by a decrease in the MDA levels, a lipid peroxidation product. This confirms earlier observations on the production of free radicals under salinity stress where a significant negative correlation was observed between the proline levels and MDA production (Alia et al. 1993). These observations suggest that the elevated proline also reduces the level of free radicals in response to

osmotic stress, thereby, significantly improving the ability of the transgenic plants to better survive under water stress. These results are in accordance with those reported earlier (Kavi Kishor et al. 1995; Hong et al. 2000) on the role of proline in reducing oxidative stress induced by osmotic stress, in addition to its accepted role as an osmolyte.

The onset of transpiration decline in the WT plants started at higher FTSW values (wetter soil) under WS than the transgenic events across both experiments, indicating the capacity of transgenic plants to maintain a rate of transpiration closer to the control up until the soil was fairly dry. This suggests a possible causal relation between having a high proline level and the capacity to maintain stomata open in dryer soils that very well fits the expected role of osmolytes such as proline in maintaining turgidity of the leaf cells, including stomatal guard cells. We did not measure the osmotic potential of leaves in transgenics, as we first wanted to evaluate the response of leaf gas exchanges in these transgenics that would be required for future studies. Indeed, osmolyte accumulation (OA) in plant cells has been shown to result in a decrease in the cell osmotic potential and thus in maintenance of water absorption and cell turgor pressure, which might contribute to sustaining physiological processes, such as stomatal opening, photosynthesis, and expansion growth (Blum et al. 1983; Morgan 1984; Ludlow and Muchow 1990; Blum 1996). These results are in line with a few other reports where the overproduction of proline led to a reported increased tolerance to osmotic stress based on one or more parameters like total water potential, osmotic and membrane potentials, membrane stability and MDA content, root length and weight, seed and capsule numbers etc. (Kavi Kishor et al. 1995; Liu and Zhu 1997; Vendruscolo et al. 2007).

The present study was designed to determine the relative importance of these mechanisms in the context of a "realistic" physiological response. The proline content in the chickpea transgenics exposed to progressive WS correlated well with their FTSW threshold values and TTSW, indicating that the putative role of proline under water stress might have been to sustain stomatal opening until the soil was dryer than in the WT. In fact, the proline data and the putative relation with lower FTSW thresholds for transpiration decline were also in agreement with the finding of higher TTSW values in most transgenics, which probably helped maintain high water potential differences between the plants and the soil, and may explain the higher water extraction rate of the transgenics from same volume of soil. Although the root length density in pots is likely to be largely higher than the root length density needed to extract all the available water, further work would be needed to confirm these differences in TTSW, and to test whether these differences would remain significant in a natural soil profile where the root length density would be naturally lower at each layer of soil depth.

Proline, as an osmoticum closely involved in the osmotic adjustment of plants, may sustain plant growth and stomatal opening under water stress, which may in turn decrease transpiration efficiency. Therefore, it was important to assess how TE was affected by the overproduction of proline in these transformants. The TE of the transgenic events was tested and apparently few of the events had either a similar or lower TE than the wild type parent. Hence, it appears that the overproduction of proline in these transgenic chickpeas probably resulted in a sort of osmotic adjustment by maintaining the cell turgor and stomatal opening, which could have meant a wasteful use of water especially at high vapor pressure deficit (VPD), did not contribute in an increased TE. In any case, the overexpression of proline appeared to have no beneficial effect on the biomass accumulation, since only a few events (P12, P13, P17, P26 and P28) showed a significant increase in the biomass production towards the end of the progressive drying period. These results agree with the previous reports where the osmolyte accumulation showed no or only marginal yield advantage in other crops (Turner and Jones 1980; Morgan 1984; Serraj and Sinclair 2002; Turner et al. 2007).

In conclusions, compared to low transformation frequency in earlier studies using *Agrobacterium*-mediated transformation of chickpea, the present study reports a high frequency transformation of axillary meristem explants of chickpea with a constitutively expressed *P5CSF129A* gene. Besides, it appeared that the genetic transformation involving a single gene having an osmotic effect had little bearing on transpiration efficiency, showing the lack of relation between transformation-related biochemical changes at the cell levels, and their integration in a production factor that is close to the yield architecture. However, these observations would be

conclusive when a contained field evaluation of the selected transgenic events of chickpea is carried out in the future.

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