Ann Microbiol (2017) 67:655–668
DOI 10.1007/s13213-017-1294-y

ORIGINAL ARTICLE

# Modulation of proline metabolic gene expression in *Arabidopsis thaliana* under water-stressed conditions by a drought-mitigating *Pseudomonas putida* strain

**Daipayan Ghosh**[1] · **Sunetra Sen**[1] · **Sridev Mohapatra**[1]

Received: 6 April 2017 / Accepted: 4 August 2017 / Published online: 24 August 2017


**Abstract** Although amelioration of drought stress in plants by plant growth promoting rhizobacteria (PGPR) is a well reported phenomenon, the molecular mechanisms governing it are not well understood. We have investigated the role of a drought ameliorating PGPR strain, *Pseudomonas putida* GAP-P45 on the regulation of proline metabolic gene expression in *Arabidopsis thaliana* under water-stressed conditions. Indeed, we found that *Pseudomonas putida* GAP-P45 alleviates the effects of water-stress in *A. thaliana* by drastic changes in proline metabolic gene expression profile at different time points post stress induction. Quantitative real-time expression analysis of proline metabolic genes in inoculated plants under water-stressed conditions showed a delayed but prolonged up-regulation of the expression of genes involved in proline biosynthesis, i.e., *ornithine-Δ-aminotransferase* (*OAT*), *$\Delta^1$-pyrroline-5-carboxylate synthetase1* (*P5CS1*), *$\Delta^1$-pyrroline-5-carboxylate reductase* (*P5CR*), as well as proline catabolism, i.e., *proline dehydrogenase1* (*PDH1*) and *$\Delta^1$-pyrroline-5-carboxylate dehydrogenase* (*P5CDH*). These observations were positively correlated with morpho-physiological evidences of water-stress mitigation in the plants inoculated with *Pseudomonas putida* GAP-P45 that showed better growth, increased fresh weight, enhanced plant water content, reduction in primary root length, enhanced chlorophyll content in leaves, and increased accumulation of endogenous proline. Our observations point towards PGPR-mediated enhanced proline turnover rate in *A. thaliana* under dehydration conditions.

**Keywords** *Arabidopsis thaliana* · *Pseudomonas putida* GAP-P45 · Water-stress · Proline · Gene expression

## Introduction

Plants are constantly facing various environmental stresses, and have evolved several mechanisms to counteract them to various degrees. While these mechanisms help plants in stress amelioration, severe and/or sustained stresses can lead to devastating injuries, leading to large-scale destruction of crops. One such important abiotic stress is water-stress, a form of osmotic stress caused by water-deprivation. Water-deprivation can be caused by environmental conditions such as drought—an adversity that is extremely prevalent in tropical and sub-tropical countries. An important cellular mechanism by which plants counteract osmotic stress is through internal osmotic adjustment, by accumulating several compatible osmolytes in their cells to prevent water loss (Kavi Kishor et al. 2005; Szabados and Savouré 2010; Zlatev and Lidon 2012; Krasensky and Jonak 2012; Reddy et al. 2015). Such compatible solutes include sugar alcohols (such as sorbitol), amino acids (such as proline) and amino acid derivatives (such as glycine betaine). These compatible solutes help decrease cell water potential, thus preventing the exosmosis of water, enabling the sustenance of turgor pressure and ensuring the plant metabolic activity and, therefore, growth and productivity (Zlatev and Lidon 2012; Krasensky and Jonak 2012; Liang et al. 2013; Reddy et al. 2015).

The role of proline as an important compatible osmolyte is well established. The cellular concentration of proline increases

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s13213-017-1294-y) contains supplementary material, which is available to authorized users.

✉ Sridev Mohapatra
sridev.mohapatra@hyderabad.bits-pilani.ac.in

[1] Department of Biological Sciences, Birla Institute of Technology and Science (Pilani), Hyderabad Campus Hyderabad, Telangana 500078, India

from 20%, under non-stressed conditions, to 80% under osmotic stress, of the total free amino acid pool in several plant species (Yancey et al. 1982; Kavi Kishor et al. 2005; Choudhary et al. 2005; Sharma and Verslues 2010; Liang et al. 2013). Similar observations have been made under salt and cold stress as well (Kaplan et al. 2007; Sharma and Verslues 2010). High proline accumulation in response to abiotic stress has been positively correlated with maintenance of optimum cell turgor pressure, cytosolic pH and intracellular redox potential (Kavi Kishor et al. 2005; Verbruggen and Hermans 2008; Sharma and Verslues 2010; Liang et al. 2013; Ben Rejeb et al. 2014; Reddy et al. 2015). The role of proline has also been established in the maintenance of cellular nitrogen content (Wu 2003). Proline has been suggested to protect proteins such as chaperones from dehydration-mediated mis-folding and/or degradation by forming a protective layer around their hydration shells and thus stabilizing their integrity (Szabados and Savouré 2010). Under stress, proline from leaves has been found to be transported to roots in maize (Verslues and Sharp 1999). Mitochondrial degradation of proline to produce glutamate yields energy in the form of $FADH_2$ and NAD(P)H for plant survival (Szabados and Savouré 2010). Proline accumulation in different plant tissues, both under stressed and non-stressed conditions, has been well studied, as well proline homeostasis during plant growth and development (Kavi Kishor and Sreenivasulu 2014). However, evidence from certain studies on proline metabolism under osmotic stress in *Arabidopsis thaliana* have critically challenged the conventional hypothesis of "more proline leads to better tolerance". Experiments using *Arabidopsis* mutants for proline metabolism (*p5cs1* and *pdh1*) under dehydration stress revealed that, not just accumulation of proline, but also its simultaneous catabolism is required for better growth and development under water stress (Sharma et al. 2011; Bhaskara et al. 2015).

The first committed step in proline biosynthesis is the production of glutamate-γ-semialdehyde (GSA), which gets converted into $\Delta^1$-pyrroline-5-carboxylate (P5C), the immediate precursor of proline. Glutamate-γ-semialdehyde can be made either from ornithine via the enzyme ornithine aminotransferase (OAT) in mitochondria (Delauney et al. 1993; Sharma and Verslues 2010; Liang et al. 2013; Sharma et al. 2013) or from glutamate via P5C synthase (P5CS) (Savouré et al. 1995; Székely et al. 2008; Mattioli et al. 2009; Sharma and Verslues 2010) in the cytosol and chloroplast. Many studies have reported the upregulation of *OAT* gene under salt and osmotic stress in *Arabidopsis* or increased tolerance via *OAT* overexpression (Roosens et al. 1998; Roosens et al. 2002; Wu 2003; Armengaud et al. 2004; Sharma and Verslues 2010). But, on the contrary, it has been reported by Funck et al. (2008) via mutant analysis that OAT does not take part in proline biosynthesis under drought stress conditions in *Arabidopsis*. The enzyme P5CS is reported to catalyze the rate-limiting step in proline biosynthetic pathway, and has a bifunctional activity, i.e., that of both γ-glutamyl kinase and glutamic- γ-semialdehyde dehydrogenase (reviewed by Kavi Kishor et al. 2005; Krasensky and Jonak 2012). The enzyme P5C reductase (P5CR) is responsible for the conversion of P5C to proline (Sharma et al. 2011; Funck et al. 2012; Giberti et al. 2014). The catabolism of proline (i.e., its conversion to glutamate) involves two important oxidation steps (1) proline is oxidized by the enzyme proline dehydrogenase (PDH) to form P5C (Funck et al. 2010; Sharma and Verslues 2010; Sharma et al. 2011), and (2) P5C is then oxidized to produce glutamate by the enzyme $\Delta^1$-pyrroline-5-carboxylate dehydrogenase (P5CDH) (Deuschle et al. 2004; Sharma and Verslues 2010; Rizzi et al. 2015).

Drought-mediated regulation of the proline metabolic genes is well established. It is well known that dehydration conditions upregulate gene expression leading to proline biosynthesis (Zhang et al. 1997; Choudhary et al. 2005; Szabados and Savouré 2010; Sharma and Verslues 2010; Sharma et al. 2011; Liang et al. 2013; Bhaskara et al. 2015; Reddy et al. 2015). The genes *P5CS1* (Yoshiba et al. 1995; Hong et al. 2000), *P5CR* (Zhang et al. 1997) and *OAT* (Hare and Cress 1996) are reported to be upregulated as a form of primary response of *A. thaliana* to dehydration stress. The enzyme P5CS1 has been shown to play a rate-limiting role in increased proline biosynthesis from glutamate (Székely et al. 2008). The other orthologue of this enzyme, P5CS2 is involved mainly in growth and development and biotic stress responses of *A. thaliana* (Fabro et al. 2004; Toka et al. 2010), but has not been reported to play any role in dehydration stress tolerance (Székely et al. 2008; Mattioli et al. 2009). Proline catabolic genes, *PDH1* and *P5CDH* have been shown to be downregulated under drought stress conditions (Verbruggen et al. 1996; Borsani et al. 2005; Verslues et al. 2007; Sharma and Verslues 2010), although several authors (Bhaskara et al. 2015; Fabro et al. 2004; Kaplan et al. 2007) have reported simultaneous upregulation of both proline biosynthetic and catabolic genes under dehydration conditions, indicating the importance of proline turnover in plants during drought for better survival. Sharma et al. (2011) have shown tissue-specific upregulation of *PDH1* in plants under drought stress. The orthologue, *PDH2*, was reportedly un-induced by drought (Sharma and Verslues 2010).

Plant growth promoting rhizobacteria (PGPR) constitute a group of soil bacteria that are well known to contribute positively towards alleviation of abiotic stress in plants (Hayat et al. 2010; Saharan and Nehra 2011; Bhattacharyya and Jha 2012; Liu et al. 2013; Timmusk et al. 2014; Bishnoi 2015; Ngumbi and Kloepper 2016). Although some of the physiological processes involved in plant-PGPR interaction for stress alleviation are known, the precise molecular mechanisms still remain unclear. Several species and strains of PGPR have been reported to enhance drought tolerance in

plants. Early studies reported enhanced drought tolerance in *A. thaliana* inoculated with *Paenibacillus polymyxa* (Timmusk and Wagner 1999). Volatile producing strains such as *Pseudomonas cholorophis 06* (Cho et al. 2008) and *Bacillus subtilis* GB03 (Zhang et al. 2010) have also been reported to ameliorate drought stress in *A. thaliana*. *Pseudomonas putida* GAP-P45 (Sandhya et al. 2009, 2010a, 2010b) is a strain of PGPR that has been reported to alleviate drought stress in crop plants. The aforementioned authors found that *P. putida* GAP-P45, growing under dehydration conditions, secretes IAA (auxin), gibberellins, cytokinin, exopolysaccharides, HCN and siderophores (Sandhya et al. 2010b). On inoculation of roots, this strain improved plant biomass, relative water content, and decreased leaf water loss in maize (Vardharajula et al. 2011) as well as sunflower (Sandhya et al. 2009). Inoculation of this strain to the aforementioned plants also significantly increased proline accumulation under drought conditions. In an attempt to elucidate some of the precise molecular mechanisms underlying PGPR-mediated drought amelioration in plants, we were interested in studying the regulation of the plant proline metabolic genes under dehydration conditions, in a time-dependent manner, after PGPR inoculation. For this purpose, we chose the above-described strain, *P. putida* GAP-P45 and the model plant *A. thaliana*. Since the impact of this strain on water-stress amelioration in *A. thaliana* had not been studied, we performed physiological experiments to confirm if it induces a similar stress-alleviating effect in *Arabidopsis* as in maize and sunflower. Afterwards, we conducted molecular studies focusing on *P. putida* GAP-P45 mediated regulation of the expression of proline metabolic genes under dehydrating conditions in a time-dependent manner. Our observations indicate that, under dehydrating conditions, *P. putida* GAP-P45 not only up-regulates the expression of proline biosynthetic genes, but, at the same time, up-regulates the expression of the genes involved in proline catabolism (i.e. its conversion to glutamate and ornithine), thus, possibly enabling enhanced proline turnover.

## Materials and methods

### Germination and growth of *A. thaliana*

Standard protocols were employed for the routine growth and maintenance of *A. thaliana*. Briefly, seeds (wild type; Columbia-0) were surface sterilized, stratified in the dark at 4 °C to break dormancy, and sown on square pieces of autoclaved, stainless-steel mesh (0.01 inch wire diameter, 0.015 inch clear opening) in Petri plates containing half strength, sterile Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 0.8% agar and 1% sucrose (modified from Zhang et al. 2010). The sown plates were then incubated in a controlled environment at 22 (±1) $^{o}$ C and a 16/8 h light/dark cycle with 350 μmol $m^{-2}$ $s^{-1}$ light intensity at 50–70% relative humidity. Seven days post germination, the individual meshes containing plantlets at the four-leaved stage were transferred to Magenta boxes containing agar-supplemented MS medium for various experiments. Each mesh contained five to seven seedlings and each Magenta box contained four of these meshes.

### Water-stress induction and PGPR inoculation

Water-stress induction was done by transferring 7-day-old seedlings (four-leaved stage) to Magenta boxes containing MS-agar medium (with 1% sucrose) supplemented with 25% polyethylene glycol (PEG-6000) (van der Weele 2000). Media was prepared by PEG-infusion method. Solidified MS-agar media (16 mL) was overlaid with 10 mL 80% PEG-6000 and incubated for 48 h. After incubation, the overlay was decanted completely. We observed that approximately 4 g PEG is retained by the MS-agar medium in the Magenta boxes, which makes it ~25% PEG-infused MS-agar medium. Before starting an experiment, *P. putida* GAP-P45 was grown overnight in Luria Bertani (LB) broth in a shaking incubator at 28 °C to an $OD_{600}$ of 0.6–0.8, sub-cultured, re-grown to the same OD and used for inoculating the plants. Prior to inoculation, bacterial cells were centrifuged and re-suspended in autoclaved, distilled water. Half of the control and drought-induced plants were subjected to bacterial inoculation by the addition of 200 μL of this aqueous suspension to the respective Magenta boxes. Thus, there were four experimental sets namely: (1) non-stressed, non-inoculated controls; (2) non-stressed + GAP-P45 inoculated; (3) water-stressed, non-inoculated; (4) water-stressed, GAP-P45 inoculated. For each experiment, at least three replicate Magenta boxes were used (as mentioned above, each Magenta box contained four meshes, each with five to seven seedlings). All experiments were repeated at least twice. To monitor the growth of *P. putida* GAP-P45 in Magenta boxes throughout the experiment, a loopful of culture from the surface of inoculated MS-agar medium (with or without PEG), at day-2, day-4 and day-7 post treatment, was scraped, streaked onto LB-agar plates and incubated at 28 °C for 12 h. In order to rule out any contamination, a similar action was performed from non-inoculated media as well. In order to prove that any drought-mitigation observed is not due to bacterial inoculation in general, a separate set of experiments replacing GAP-P45 with the common laboratory strain *Escherichia coli* DH5-α was also performed, using the same conditions as with GAP-P45. In order to assess if the 200 μL water (present in the inoculum) made any difference to the water potential of the medium, MS-agar medium with or without PEG supplementation and with or without addition of 200 μL water was subjected to water-potential measurements using PSYPRO water potential

system (Wescor, Logan, UT). These water potentials were measured in three replicate Magenta boxes per treatment and the values are given below:

(1) MS-agar (control): − 0.62 (± 0.029) MPa
(2) MS-agar with 200 μL water: − 0.6 (± 0.031) MPa
(3) PEG supplemented MS -agar medium: − 2.17 (± 0.046) MPa
(4) PEG supplemented MS-agar medium with 200 μL water: − 2.16 (± 0.049) MPa

Thus, adding 200 μL water made no significant change to the water potential of the medium.

### Physiological studies on plant responses to PGPR inoculation under water-stress conditions

Physiological studies were performed to assess the impact of the PGPR strain on water-stress alleviation of *A. thaliana* at different time-points (2 days, 4 days and 7 days) post treatment. Experiments included observations on overall plant health, fresh weight (FW), dry weight (DW), plant water content (PWC) of whole seedlings along with primary root length and chlorophyll content of leaves. For measurement of FW, 60 seedlings from three replicate Magenta boxes (20 seedlings from each box) were harvested. Following FW measurements, the seedlings were incubated at 80 °C for 48 h for measurement of DW. Plant water content was measured both on FW and DW basis, by using the formulae:

$PWC_{(DW\ basis)} = [(FW - DW) / DW] \times 100$, and $PWC_{(FW\ basis)} = [(FW - DW)/FW] \times 100$ (Turner 1981).

In order to measure primary root length, plants with intact root were placed on a glass plate, the tap root was straightened and the secondary roots separated using a fine needle. Length of the primary roots was measured using a centimeter ruler. A modification from the method of Hu et al. (2013) was used for the extraction and estimation of chlorophyll pigment from leaves of *A. thaliana* subjected to all treatments. Leaf samples (40 mg) were placed in a graduated tube containing 10 mL 80% buffered acetone (80 mL acetone made up to 100 mL with 20 mL 2.5 mM sodium phosphate buffer, pH 7.8). The leaves were incubated in the solvent in dark at 4 °C with occasional shaking to accelerate the extraction of the pigments. At the appropriate time of estimation, the extract was filtered to remove leaf pieces. The chlorophyll content was spectrophotometrically analyzed in the filtrate at 663 nm and 646 nm for chl *a* and *b*, respectively. Total chlorophyll content was assessed using the formula:

Chl $(a + b) = 7.49 \times A\ 663 + 18.21 \times A\ 646$ (Barnes et al. 1992).

The equation was derived from specific absorption coefficient of pure chl *a* and *b* in 80% acetone.

### Accumulated free proline content in plants under water-stress and PGPR inoculation

Proline estimation was done in whole seedlings, 2 days, 4 days and 7 days post treatment, using the method of Bates et al. (1973). Whole seedlings (100 mg) were homogenized in 5 mL 3% aqueous sulfosalicylic acid, and the homogenate was collected by filtering through Whatman no. 2 filter paper. Filtrate (2 mL) was treated with 2 mL glacial acetic acid and 2 mL acid ninhydrin (warm 1.25 g ninhydrin in 30 mL glacial acetic acid, and 20 mL 6 M phosphoric acid). The samples were incubated in a boiling water bath for 1 h, and the reaction was terminated by placing the reaction tubes on ice. To this reaction mixture, 4 mL toluene was added and stirred well for 20–30 s. A chromophore-containing toluene layer was separated and warmed to room temperature. Absorbance was read at 520 nm using a UV-visible spectrophotometer, blanked with toluene. Different concentrations of an aqueous solution of L-proline were used to plot a standard curve of absorbance vs. concentration and concentrations of proline in plant samples were extrapolated from it.

### Gene expression studies

For gene expression analysis, seedlings were harvested at different time periods post drought induction and GAP-P45 inoculation. Total RNA was isolated from whole seedlings by TRIZOL reagent (Invitrogen, Carlsbad, CA), followed by cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen). Semi-quantitative PCR was performed using appropriate primers (Sharma and Verslues 2010) of the following genes - *OAT*, *P5CS1*, *P5CR*, *PDH1* and *P5CDH*. The obtained data was corroborated using quantitative real-time PCR (Step One Plus, Applied Biosystems) using a SYBR green PCR master mix. Gene expression analysis was done using relative quantification by the $\Delta\Delta C_T$ method (Applied Biosystems, Foster City, CA). *β-actin 2* was used as endogenous control (Sharma and Verslues 2010), and gene expression was quantified relative to the non-stressed, non-inoculated controls (reference control).

### Statistical analysis

Wherever applicable, statistical analysis was performed by Student's t-test (level of significance, $P \leq 0.05$) using Microsoft Excel 2010 (Microsoft, Redmond, WA). As mentioned previously, each experiment was performed with at least three replicate Magenta boxes, each containing about 20 seedlings, distributed in four steel meshes, and each experiment was performed at least three times.

## Results

### Plant growth under water-stressed conditions

Prior to the treatments, all plants exhibited similar growth and developmental phenotypes (Fig. 1a–d). GAP-P45 inoculation under well-watered conditions did not cause any visible change in the size/growth of the plants at any time period of the study (Fig. 1e vs. f, i vs. j, m vs. n). By day-2 post transfer to PEG supplemented medium, the PEG treated, non-inoculated plants exhibited significant growth stunting as opposed to the all other treatments (Fig. 1g, k, o). Under PEG-treated conditions, inoculated plants exhibited much better growth compared to non-inoculated plants (Fig. 1h vs. g, l vs. k, p vs. o). With progression of days, the PEG treated, non-inoculated plants exhibited a gradual decline in health while those inoculated with GAP-P45 exhibited much better tolerance to dehydrating conditions.

The LB-agar plates used for monitoring bacterial growth showed discrete growth of GAP-P45 at all three time points of the study, while no contamination was detected in the non-inoculated medium (Fig. S1). No drought mitigation was observed with *E. coli* DH5-α inoculation (Fig. S2). Rather, this led to deterioration in plant health even under non-stressed conditions, probably because of the competition between the bacteria and the plants for nutrients.

### Fresh weight, dry weight, and plant water content

In order to quantify the impact of *P. putida* GAP-P45 on plant water status, we analyzed the FW, DW, and PWC at all time-periods of this study. As can be seen from Fig. 2a and b, GAP-P45 inoculation of *Arabidopsis* under non-stressed conditions did not significantly change FW and DW when compared to control plants. However, both FW and DW of water-stressed plants increased significantly on GAP-P45 inoculation at all three time periods of study. PWC was calculated both on DW and FW basis (Turner 1981). PWC (both FW and DW basis) followed similar trends in that the water-stressed, non-inoculated plants recorded the lowest PWC among all the treatments at all time points of the study (Fig. 2c, d). The PEG treated, inoculated plants exhibited significantly higher PWC, both on DW and FW basis, as opposed to the water-stressed, non-inoculated plants.

### Chlorophyll content

Chlorophyll content followed a similar trend as PWC (Fig. 3). While on day-2, marginal enhancement was seen in chlorophyll content by GAP-P45 inoculation without water-stress, on day-4 and day-7, GAP-P45 treatment under normal conditions did not cause any significant change in the chlorophyll content. As expected, PEG-treatment caused a significant decrease in chlorophyll content on all days of study, while GAP-P45 inoculation under water-stress conditions, significantly elevated the chlorophyll content in leaves.

### Primary root length and root structure architecture

It has been reported that length of the primary root tends to increase under drought conditions in many plants (Pace et al. 1999; Jacobs et al. 2004; Grossnickle 2005). Commensurate with these findings, we also observed similar trends in our plants (Fig. 4) at all time-periods of the study. Just before application of treatments (day-0), all plants showed similar root length (Fig. 4a, b). Water-stressed, non-inoculated plants exhibited the highest primary root length and enhanced branching at day-2, day-4 and day-7, while, in the case of water-stressed, GAP-P45 inoculated plants, primary root length and branching pattern were similar to control plants at most time periods of the study. Except for day-7 where GAP-P45 treatment caused a small dip in primary root length under non-stressed conditions, there was no significant difference in root length between control and GAP-P45 treated (without PEG) plants.

### Proline content

In order to analyze the level of proline accumulation under dehydrating conditions, proline content of the plants was measured in all treatments and at all time periods of this study. As can be seen from Fig. 5, PGPR inoculation alone (without water-stress) did not cause any change in proline content of the plants, as compared to the controls. It was observed that, post water-stress induction, both non-inoculated and inoculated plants exhibited enhanced proline levels as compared to the controls. Gradual, time-dependent increase in proline levels were seen in both these treatments from day-2 to day-7. Under water-stress, higher proline content was observed in the non-inoculated plants as compared to the inoculated plants, on day-2 and day-4 post treatments. At day-7, however, proline levels were similarly induced in both these treatments. This indicates that inoculation with GAP-P45 delayed proline accumulation in our plants under water-stressed conditions. The non-stressed plants (non-inoculated as well as inoculated) exhibited minor increase in proline levels from day-2 to day-7.

### Gene expression analysis

We analyzed the GAP-P45 mediated, time-dependent modulation of the expression of all important genes in the proline metabolic pathway, in response to osmotic-stress treatment. The candidate genes analyzed can be broadly classified into

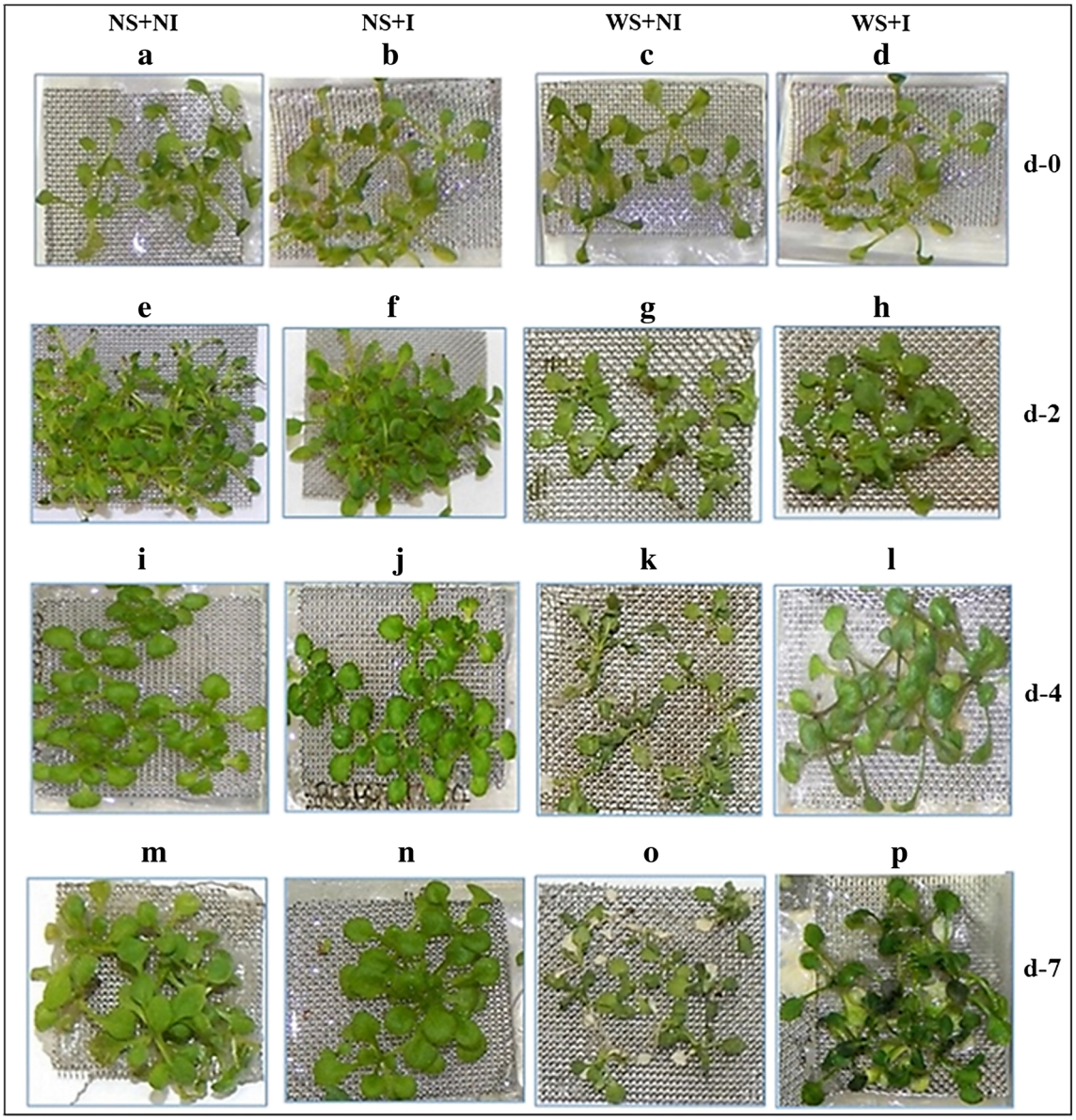


**Fig. 1a–p** Plant growth and development following *Pseudomonas putida* GAP-P45 inoculation in *Arabidopsis thaliana* under water-stressed conditions (25% PEG). **a–d** The period just before treatments, **e–h** day-2; **i–l** day–4; and **m–p** day–7 post treatments. *NS + NI* non-stressed, non-inoculated; *NS + I* non-stressed, inoculated; *WS + NI* water-stressed, non-inoculated; *WS + I* water-stressed, inoculated

proline biosynthetic genes (*OAT*, *P5CS1* and *P5CR*) and proline catabolic genes (*PDH1* and *P5CDH*). As can be seen from Figs. 6 and 7, except for minor inductions in a few genes at certain time periods (the biosynthetic genes on day-7 and *P5CDH* on day-2), PGPR treatment under non-stressed conditions did not cause any significant change in the expression of any of the above mentioned genes. Fluctuations were observed, though, in expression levels of all genes analyzed post stress induction with or without PGPR inoculation as compared to the non-stressed plants.

On day-2 post transfer to PEG-supplemented medium, the expression of all three biosynthetic genes was highest in water-stressed, non-inoculated plants as opposed to all other treatments (Fig. 6). In these plants, water-stress increased the expression of *OAT*, *P5CS1* and *P5CR* genes by 8-fold, 13-fold and 6-fold, respectively, with respect to the non-stressed, non-inoculated controls. Maximum expression was thus shown by *P5CS1* out of the three genes. In the water-stressed, inoculated plants, however, the same genes exhibited significantly lower increase in expression, i.e., 2-fold, 4-fold and 3-fold increase, respectively, relative to the non-stressed, non-inoculated controls. By day-4 post treatments, the expression of these genes had not changed much in the water-stressed, non-inoculated plants. However, by day-4, the expression of *OAT*, *P5CS1* and *P5CR* in water-stressed, inoculated plants had surpassed that of the water-stressed, non-inoculated plants, with the most dramatic increase

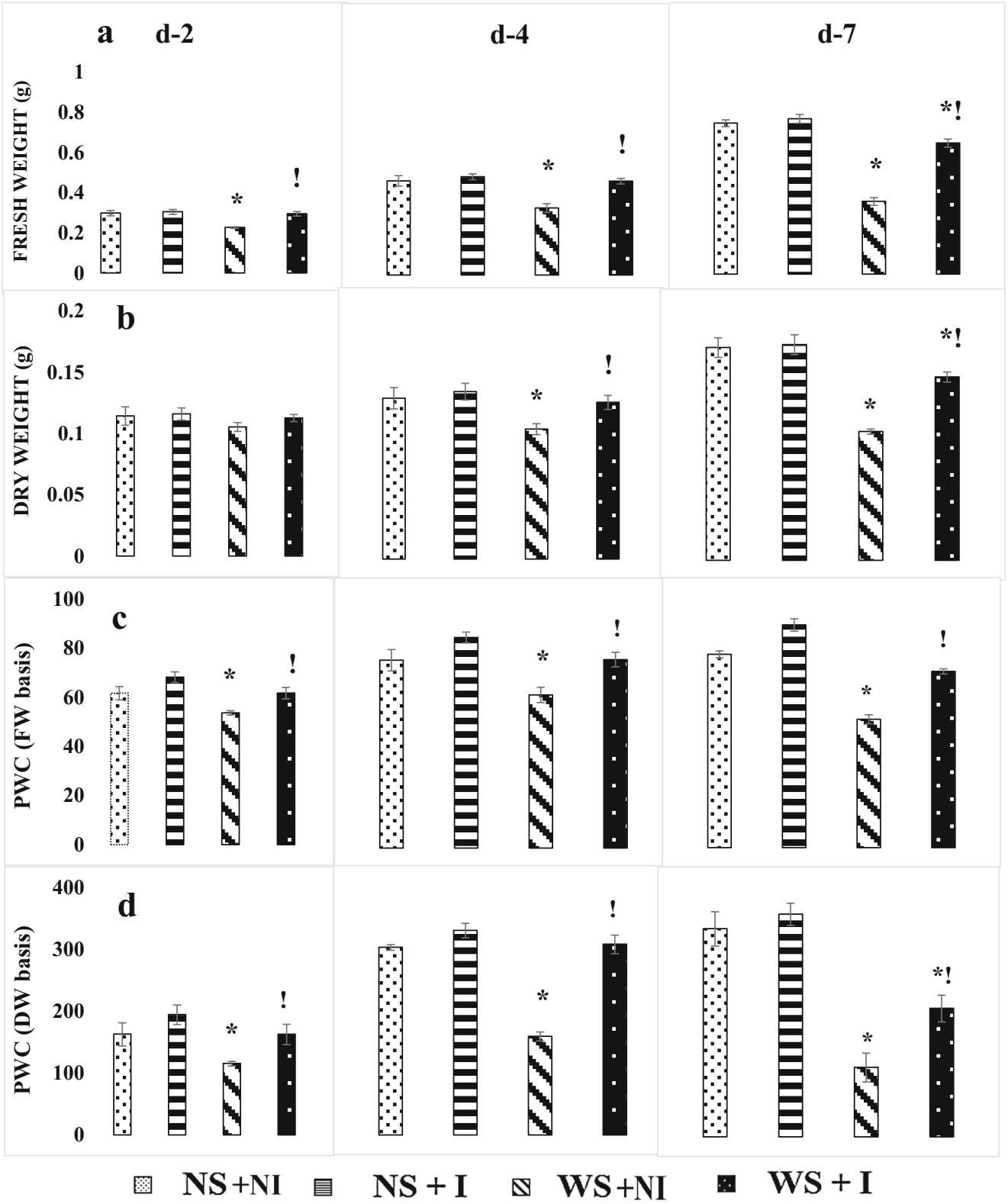


**Fig. 2a–d** Physiological studies on the impact of *P. putida* GAP-P45 on water-stress amelioration in *A. thaliana*. **a** Fresh weight (FW), **b** dry weight (DW), **c** plant water content (PWC) on FW basis, **d** PWC on DW of whole seedlings at day-2, day-4 and day-7 post treatment. Each bar represents the mean ± SE of 60 replicate plants. * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) in data between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's t-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010

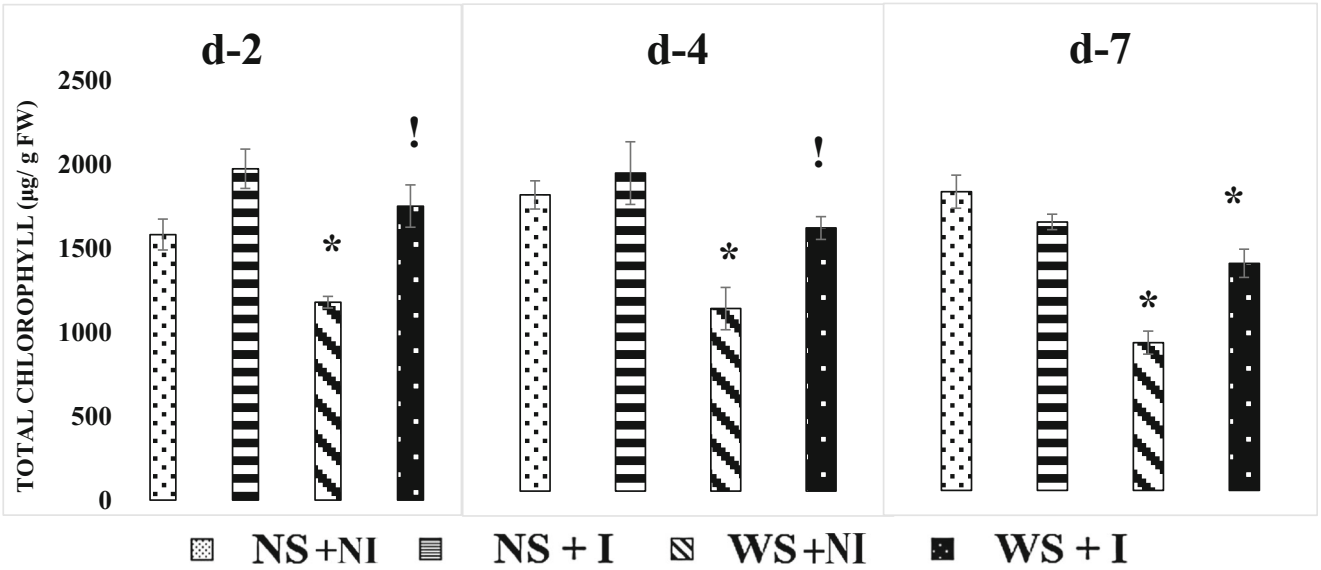


**Fig. 3** Chlorophyll content of *A. thaliana* at day-2, day-4 and day-7 post treatment. Each bar represents the mean ± SE of six replicate sets, each with 40 mg leaf sample. * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's t-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010

seen in the expression of the *P5CS1* gene. On day-7 water-stressed, GAP-P45-inoculated plants exhibited consistent high expression of *OAT*, *P5CS1* and *P5CR*, with minor fluctuations from day-4 data, whereas water-stressed non-inoculated plants exhibited substantial decrease in the expression of *P5CS1* and *P5CR* as opposed to day-2 and day-4. Thus, comparing across the different time-periods of study, we can see an overall reduction in the expression of proline biosynthetic genes in the PEG-treated, non-inoculated plants from day-2 and day-7. On the other hand, the water-stressed, inoculated plants exhibited an overall (and strong) increase in expression of the same from day-2 to day-7 post treatment.

As far as the catabolic genes (*PDH1* and *P5CDH*) are concerned (Fig. 7), on day-2 post treatments, their expression in the water-stressed, non-inoculated plants was similar to the non-stressed, non-inoculated controls, but significantly higher in the water-stressed, inoculated plants (an increase of ~ 3 fold as opposed to the controls). A partially time-dependent decrease was observed in the expression of these genes in the water-stressed, non-inoculated plants, while an overall increase (~ 5-fold) in their expression was observed in the water-stressed, inoculated plants.

## Discussion

### *P. putida* GAP-P45 improves water-stress tolerance in *A. thaliana*

Drought is one of the most devastating repercussions of world-wide climate change. In many agriculture-focused countries of the developing world, every year, thousands of livelihoods are affected due to the lack of adequate rainfall. Under these circumstances, it becomes imperative to develop strategies for sustainable agriculture under extreme climatic conditions. The use of drought-tolerant PGPR provide significant promise to overcome the challenges of sustainable agriculture in dehydrated soil. While many studies have identified several potential PGPR that help plants overcome abiotic stresses, few have reported the precise molecular mechanisms leading to such tolerance. Since proline is one of the most important compatible solutes whose accumulation has been widely studied under drought stress, we wanted to understand the time-dependent regulation of proline metabolic gene expression in plants in response to drought-mitigating PGPR. This study was carried out in vitro using PEG 6000 as inducer of osmotic stress by lowering water potential (the effects of which may not be identical to that of water deprivation in soil). As mentioned earlier, the strain of PGPR used in this study (*P. putida* GAP-P45) has been previously shown to confer drought tolerance to maize and sunflower (Sandhya et al. 2009, 2010a). We chose this strain to investigate its impact on the regulation of proline metabolic gene expression during water-stressed conditions in the model plant *A. thaliana*. Since there was no report on the impact of this strain on *A. thaliana* under drought conditions, we conducted experiments to ensure that the strain enhances water-stress tolerance in *A. thaliana* with respect to growth and physiological status of the plants. As can be seen from Figs. 1, 2, 3, 4, and 5, this strain has a profound positive impact on drought tolerance of *A. thaliana* with respect to plant growth and morphology, root structure architecture, fresh weight, dry weight, plant water content, chlorophyll content and proline

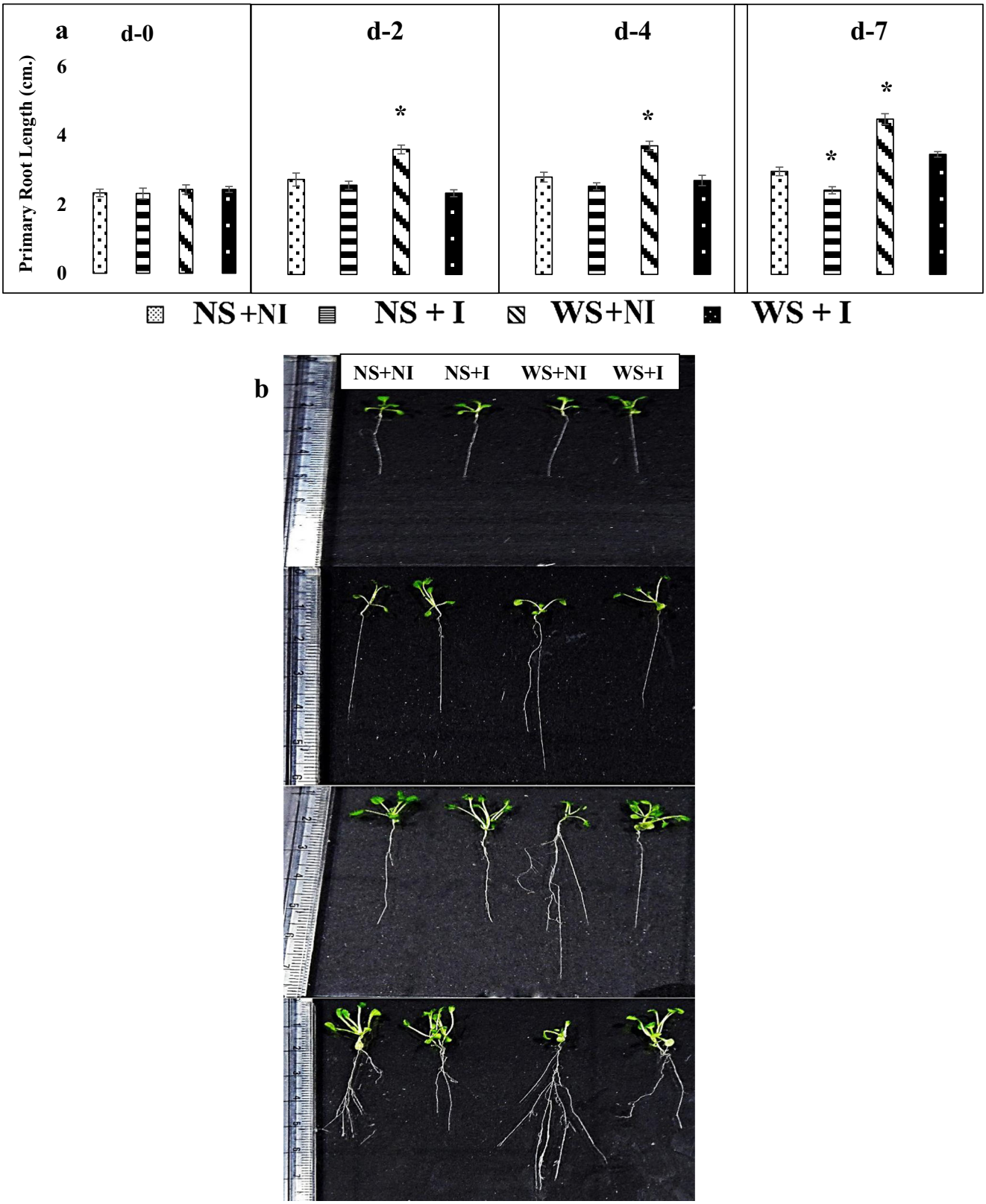


**Fig. 4** Primary root length of *A. thaliana* on day-2, day-4 and day-7 post treatments. **a** Each bar represents the mean ± SE of ten replicate plants, taken from three Magenta boxes. * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's t-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010. **b** Observation on primary root length of one replicate plant from each of the four treatments. In both **a** and **b**, *d-0* represents the time period just before treatments (the respective treatments in the *x*-axis indicate sets of plants thereafter transferred to Magenta boxes for those treatments)

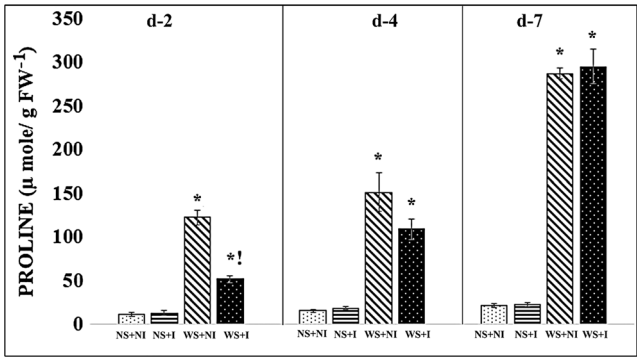


**Fig. 5** Content of accumulated free proline in *A. thaliana* on day-2, day-4 and day-7 post treatments. Each bar represents mean ± SE of six replicate samples (each sample represents 100 mg seedlings from one Magenta box). * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's *t*-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010

accumulation. These experiments were necessary to establish the compatibility of GAP-P45 with *A. thaliana* for osmotic-stress amelioration before moving on to molecular experiments. While many more assays could be done for quantitative estimation of drought alleviation by PGPR, we chose a subset of these experiments because our main focus was on gene expression analysis, and we wanted only to establish that GAP-P45 indeed exhibited drought ameliorating responses in *Arabidopsis* before moving on to the molecular analyses.

### *P. putida* GAP-P45 enhances expression of both proline biosynthetic as well as catabolic genes under water-stressed conditions in *A. thaliana*, possibly leading to better proline homeostasis

As previously mentioned, we wanted to study PGPR-mediated modulation in the expression of genes of the proline metabolic pathway (*OAT*, *P5CS1*, *P5CR*, *PDH1* and *P5CDH*) in *A. thaliana*, under water/osmotic-stress conditions, and to correlate them with stress-induced proline accumulation. Our objective was to observe the regulation pattern of these genes under PEG-induced water-stress with GAP-P45 inoculation. Though increased proline accumulation with PGPR inoculation under dehydration conditions has already been reported in several studies, regulation of both proline biosynthetic and catabolic genes with respect to PGPR has not been explored up to now.

In our study, under water-stressed conditions, proline accumulation followed an increasing trend from day-2 to day-7 in both non-inoculated and inoculated plants (Fig. 5). However, our data showed that GAP-P45 inoculation delayed proline accumulation under water stress. This delay is an intriguing phenomenon that deserves investigation. It could be argued that any of such PGPR-mediated delayed response to drought can be a consequence of enhanced hydration of the media/soil by PGPR themselves. However, in our case, we observed no change in the water potential of the medium following GAP-P45 inoculation under PEG-treated conditions. Hence, we hypothesize that the delayed proline accumulation is a more specific response caused by the bacterium. This hypothesis needs to be tested through in depth studies. We also observed that, although there was enhanced proline accumulation under water-stressed conditions, in both non-inoculated and inoculated plants, drought amelioration was better in inoculated plants than in non-inoculated ones. This observation can be explained by critically examining the gene expression data (Figs. 6 and 7), which not only correlates well with proline accumulation data (Fig. 5), but also provides interesting details into the pattern of regulation in the expression of the proline metabolic genes brought about by GAP-P45 inoculation under osmotic stress conditions. By day-2 post treatment, the PEG-treated, non-inoculated plants exhibited a 5- to 15-fold surge in the expression of proline biosynthetic genes

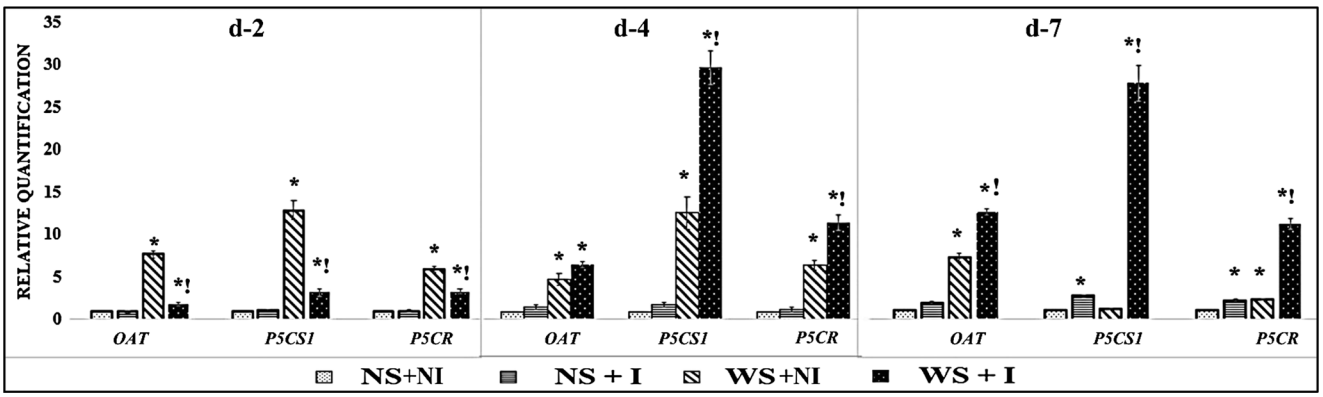


**Fig. 6** Quantitative RT-PCR analysis of the genes involved in proline biosynthesis in *A. thaliana* on day-2, day-4 and day-7 post treatment. Each bar represents the mean ± SE of six replicate samples (each sample represents 200 mg seedlings from one Magenta box). * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's t-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010

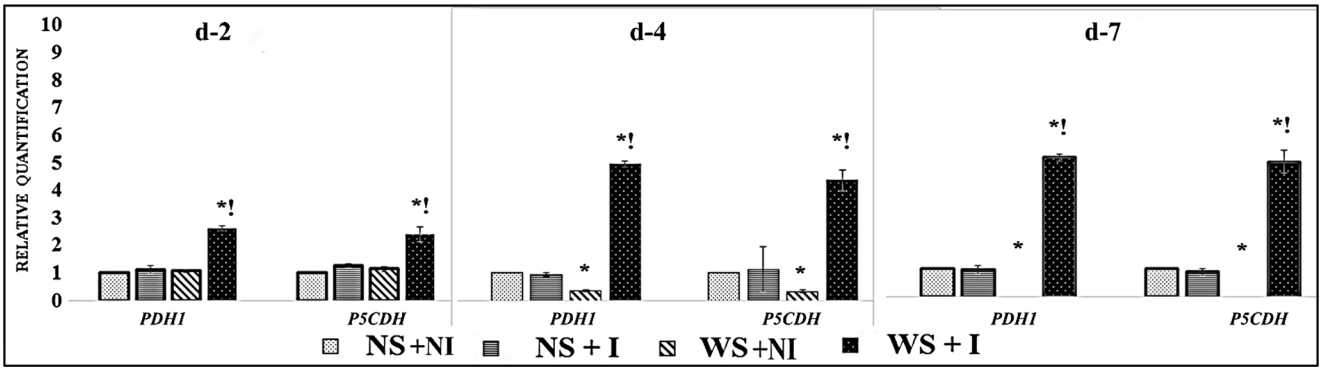


**Fig. 7** Quantitative RT-PCR analysis of the genes involved in proline catabolism in *A. thaliana* on day-2, day-4 and day-7 post treatment. Each bar represents mean ± SE of six replicate samples (each sample represents 200 mg seedlings from one Magenta box). * Significant difference ($P \leq 0.05$) in data between NS + NI and any other treatment within a particular day of analysis. ! Significant difference ($P \leq 0.05$) between WS + I and WS + NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by Student's t-test (two-tailed analysis, $P \leq 0.05$) using Microsoft Excel 2010

(expression of *P5CS1* being the highest) relative to the non-stressed, non-inoculated plants, whereas PEG-treated, GAP-P45-inoculated plants recorded only a 2- to 4-fold elevation in the expression of these genes. These data correlate well with the proline accumulation at day-2. By day-4 post treatment, the PEG-treated, non-inoculated plants did not show much change in the expression of the biosynthetic genes (*OAT*, *P5CS1* and *P5CR*), although, at day-7, an overall downregulation in the expression of *P5CS1* and *P5CR* was observed in these plants. However, the PEG-treated, inoculated plants exhibited a several-fold increase in the expression of the same genes at day-4, and maintained almost the same level of expression at day-7 post treatment, with *P5CS1* (which codes for an important and rate-limiting enzyme of the proline biosynthetic pathway) exhibiting the highest expression level. Hence, by day-7, while proline levels were similarly induced in both, non-inoculated and inoculated plants under water stress, the gene expression patterns were different in both these treatments. While the PEG-treated non-inoculated plants had undergone a marked downregulation in the expression of proline biosynthetic genes (except *OAT*), the water-stressed, inoculated plants exhibited a sustained upregulation of all three genes. One can argue that downregulation of proline biosynthetic genes in water-stressed, non-inoculated plants at day-7 could suggest accumulated proline-mediated feed-back inhibition of *P5CS1* (Hong et al. 2000; Sharma and Verslues 2010; Reddy et al. 2015) consequently leading to down-regulation of *P5CR*. However, such a feedback inhibition is not seen in the water-stressed, inoculated plants in spite of a similar surge in proline content. This suggests that GAP-P45 inoculation could somehow be suppressing the feedback inhibition caused by proline accumulation. Conversely, other than feedback inhibition, specific osmotic-stress responsive factors might be contributing towards the downregulation of these genes under water-stressed conditions. The expression pattern of the two proline catabolic genes (*P5CDH* and *PDH1*) exhibited some similarities with that of the biosynthetic genes. There was an overall, time-dependent downregulation in the expression of *P5CDH* and *PDH1* in the PEG treated non-inoculated plants, whereas in the PEG treated, GAP-P45 inoculated plants, there was an overall induction in the expression of both of these catabolic genes from day-2 to day-7. The high amount of proline accumulation in spite of downregulated *P5CS1* and *P5CR* in PEG-treated non-inoculated plants at day-7 could be the combined consequences of upregulated *OAT* and downregulated *PDH1* and *P5CDH*. Hence, we speculate that *OAT* may play an important role in proline biosynthesis in our non-inoculated plants under osmotic stress. As mentioned previously, there have been reports on *OAT* upregulation under salt and osmotic stress in *Arabidopsis* and enhanced stress tolerance via *OAT* overexpression (Roosens et al. 1998; Roosens et al. 2002; Wu 2003; Armengaud et al. 2004; Sharma and Verslues 2010). However, mutant analysis studies by Funck et al. (2008) have established that OAT does not take part in proline biosynthesis under drought stress conditions in *Arabidopsis*. Although our observations supports the former reports and contradicts the latter, specific experiments should be done to elucidate the specific role of upregulated *OAT* in our study.

Sustained induction of the proline catabolic genes, *PDH1* and *P5CDH* at day-4 and day-7 in the water-stressed, inoculated plants suggests that, although, both inoculated and non-inoculated plants accumulate proline under water-stressed conditions, there is probably higher proline turnover in the former vs. the latter. While, conventionally, abiotic stress amelioration in plants is associated with enhanced proline accumulation, several reports suggest that enhanced proline turnover could be a key player in this phenomenon. As mentioned in the Introduction, this has been established with studies using *p5cs1* and *pdh1* mutants of *A. thaliana* (Sharma et al. 2011 and Bhaskara et al. 2015) as well as studies showing simultaneous upregulation of both proline biosynthetic and

catabolic genes under dehydration conditions (Sharma et al. 2011; Bhaskara et al. 2015; Fabro et al. 2004; Kaplan et al. 2007). Our observations on stress alleviation by GAP-P45 treated plants, i.e., better morpho-physiological status under osmotic stress than that of PEG-treated non-inoculated plants, substantiate the hypothesis that increased proline turnover via simultaneous upregulation of both biosynthetic and catabolic genes, is more important for better sustenance of *A. thaliana* under osmotic-stress than mere accumulation of proline.

Thus, GAP-P45 inoculation initially delayed proline accumulation in *A. thaliana* and enhanced the expression of both proline biosynthetic as well as catabolic genes under dehydration conditions in a partially time-dependent manner, possibly leading to enhanced proline turnover. Kinetics of proline biosynthesis and degradation (i.e., its conversion to glutamate and/or ornithine) will have to be studied in order to gain greater insight into this process. As time of dehydration exposure increased, all drought treated plants, i.e., non-inoculated and GAP-P45-inoculated, accumulated enhanced proline, but in the inoculated plants, proline catabolism possibly kept pace with its biosynthesis, leading to enhanced turnover of proline. This indicates that the particular strain of PGPR used in this study, stimulates not only enhanced proline accumulation, but also its concomitant degradation, thus modulating proline homeostasis under drought conditions. The enhanced proline degradation could be a cause or an effect of better drought tolerance in the inoculated plants.

**Acknowledgements** The authors thank Minakshi Grover (Principal Scientist, Indian Agricultural Research Institute, New Delhi, India) for help in procuring the rhizobacterial strain used in this study.

**Funding** This work was funded by BITS-Pilani Seed Grant Scheme and Science and Engineering Research Board, Govt. of India.

## References

Armengaud P, Thiery L, Buhot N, Grenier-De-March G, Savouré A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant 120:442–450. https://doi.org/10.1111/j.0031-9317.2004.00251.x

Barnes JD, Balaguer L, Manrique E, Elvira S, Davison AW (1992) A reappraisal of the use of DMSO for the extraction and determination of chlorophylls a and b in lichens and higher plants. Environ Exp Bot 32:85–100. https://doi.org/10.1016/0098-8472(92)90034-Y

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207. https://doi.org/10.1007/BF00018060

Ben Rejeb K, Abdelly C, Savouré A (2014) How reactive oxygen species and proline face stress together. Plant Physiol Biochem 80:278–284. https://doi.org/10.1016/j.plaphy.2014.04.007

Bhaskara GB, Yang T-H, Verslues PE (2015) Dynamic proline metabolism: importance and regulation in water limited environments. Front Plant Sci 6:484. https://doi.org/10.3389/fpls.2015.00484

Bhattacharyya PN, Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350. https://doi.org/10.1007/s11274-011-0979-9

Bishnoi U (2015) PGPR Interaction: an ecofriendly approach promoting the sustainable agriculture system. Adv Bot Res 75: 81–113

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell 123:1279–1291. https://doi.org/10.1016/j.cell.2005.11.035

Cho SM, Kang BR, Han SH, Anderson AJ, Park JY, Lee YH, Cho BH, Yang KY, Ryu CM, Kim YC (2008) 2R,3R-butanediol, a bacterial volatile produced by *Pseudomonas chlororaphis* O6, is involved in induction of systemic tolerance to drought in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 21:1067–1075. https://doi.org/10.1094/MPMI-21-8-1067

Choudhary NL, Sairam RK, Tyagi A (2005) Expression of delta1-pyrroline-5-carboxylate synthetase gene during drought in rice (*Oryza sativa* L.) Indian J Biochem Biophys 42:366–370

Delauney AJ, Hu CA, Kishor PB, Verma DP (1993) Cloning of ornithine delta-aminotransferase cDNA from *Vigna aconitifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. J Biol Chem 268:18673–18678

Deuschle K, Funck D, Forlani G, Stransky H, Biehl A, Leister D, Van der Graaff E, Kunze R, Frommer WB (2004) The role of [Delta]1-pyrroline-5-carboxylate dehydrogenase in proline degradation. Plant Cell 16:3413–3425. https://doi.org/10.1105/tpc.104.023622

Fabro G, Kovács I, Pavet V, Szabados L, Alvarez ME (2004) Proline accumulation and *AtP5CS2* gene activation are induced by plant-pathogen incompatible interactions in *Arabidopsis*. Mol Plant-Microbe Interact 17:343–350. https://doi.org/10.1094/MPMI.2004.17.4.343

Funck D, Stadelhofer B, Koch W (2008) Ornithine-δ-aminotransferase is essential for arginine catabolism but not for proline biosynthesis. BMC Plant Biol 8:40. https://doi.org/10.1186/1471-2229-8-40

Funck D, Eckard S, Müller G (2010) Non-redundant functions of two proline dehydrogenase isoforms in *Arabidopsis*. BMC Plant Biol 10:70. https://doi.org/10.1186/1471-2229-10-70

Funck D, Winter G, Baumgarten L, Forlani G (2012) Requirement of proline synthesis during *Arabidopsis* reproductive development. BMC Plant Biol 12:191. https://doi.org/10.1186/1471-2229-12-191

Giberti S, Funck D, Forlani G (2014) $\Delta^1$ -pyrroline-5-carboxylate reductase from *Arabidopsis thaliana* : stimulation or inhibition by chloride ions and feedback regulation by proline depend on whether NADPH or NADH acts as co-substrate. New Phytol 202:911–919. https://doi.org/10.1111/nph.12701

Grossnickle SC (2005) Importance of root growth in overcoming planting stress. New For 30:273–294. https://doi.org/10.1007/s11056-004-8303-2

Hare PD, Cress WA (1996) Tissue-specific accumulation of transcript encoding Δ 1-pyrrolline-5-carboxylate reductase in *Arabidopsis thaliana*. Plant Growth Regul 19:249–256. https://doi.org/10.1007/BF00037798

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598. https://doi.org/10.1007/s13213-010-0117-1

Hong Z, Lakkineni K, Zhang Z, Verma DP (2000) Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol 122:1129–1136

Hu X, Tanaka A, Tanaka R (2013) Simple extraction methods that prevent the artifactual conversion of chlorophyll to chlorophyllide during pigment isolation from leaf samples. Plant Methods 9:19. https://doi.org/10.1186/1746-4811-9-19

Jacobs DF, Rose R, Haase DL, Alzugaray PO (2004) Fertilization at planting impairs root system development and drought avoidance

of Douglas-fir ( *Pseudotsuga menziesii* ) seedlings. Ann For Sci 61: 643–651. https://doi.org/10.1051/forest:2004065

Kaplan F, Kopka J, Sung DY, Zhao W, Popp M, Porat R, Guy CL (2007) Transcript and metabolite profiling during cold acclimation of *Arabidopsis* reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant J 50:967–981. https://doi.org/10.1111/j.1365-313X.2007.03100.x

Kavi Kishor PB, Sreenivasulu N (2014) Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant Cell Environ 37:300–311. https://doi.org/10.1111/pce.12157

Kavi Kishor PB, Sangam S, Amrutha RN, Sri Laxmi P, Naidu KR, Rao KRSS, Rao S, Reddy KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants : its implications in plant growth and abiotic stress tolerance. Curr Sci 88:424–438

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot 63:1593–1608. https://doi.org/10.1093/jxb/err460

Liang X, Zhang L, Natarajan SK, Becker DF (2013) Proline mechanisms of stress survival. Antioxid Redox Signal 19:998–1011. https://doi.org/10.1089/ars.2012.5074

Liu F, Xing S, Ma H, Du Z, Ma B (2013) Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. Appl Microbiol Biotechnol 97:9155–9164. https://doi.org/10.1007/s00253-013-5193-2

Mattioli R, Falasca G, Sabatini S, Attamura MM, Costantino P, Trovato M (2009) The proline biosynthetic genes P5CS1 and P5CS2 play overlapping roles in *Arabidopsis* flower transition but not in embryo development. Physiol Plant 137:72–85. https://doi.org/10.1111/j.1399-3054.2009.01261.x

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Planta 15:473–497

Ngumbi E, Kloepper J (2016) Bacterial-mediated drought tolerance: current and future prospects. Appl Soil Ecol 105:109–125

Pace PF, Cralle HT, El-Halawany SHM, Cothern JT, Senseman SA (1999) PHYSIOLOGY drought-induced changes in shoot and root growth of young cotton plants. J Cotton Sci 3:183–187

Reddy PS, Jogeswar G, Rasineni GK, Maheswari M, Reddy AR, Varshney RK, Kavi Kishor PB (2015) Proline over-accumulation alleviates salt stress and protects photosynthetic and antioxidant enzyme activities in transgenic sorghum [*Sorghum bicolor* (L.) Moench]. Plant Physiol Biochem 94:104–113. https://doi.org/10.1016/j.plaphy.2015.05.014

Rizzi YS, Monteoliva MI, Fabro G, Grosso CL, Laróvere LE, Alvarez ME (2015) P5CDH affects the pathways contributing to pro synthesis after ProDH activation by biotic and abiotic stress conditions. Front Plant Sci 6:572. https://doi.org/10.3389/fpls.2015.00572

Roosens NH, Thu TT, Iskandar HM, Jacobs M (1998) Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiol 117:263–271

Roosens NH, Bitar FA, Loenders K, Angenon G, Jacobs M (2002) Overexpression of ornithine-δ-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. Mol Breed 9:73–80. https://doi.org/10.1023/A:1026791932238

Saharan BS, Nehra V (2011) Plant growth promoting Rhizobacteria: a critical review. Life Sci Med Res 21:1–30

Sandhya V, Ali SKZ, Grover M, Reddy G, Venkateswarlu B (2009) Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-P45. Biol Fertil Soils 46:17–26. https://doi.org/10.1007/s00374-009-0401-z

Sandhya V, Ali SZ, Grover M, Reddy G, Venkateswarlu B (2010a) Effect of plant growth promoting *Pseudomonas spp*. on compatible solutes, antioxidant status and plant growth of maize under drought stress. Plant Growth Regul 62:21–30. https://doi.org/10.1007/s10725-010-9479-4

Sandhya V, Ali SZ, Venkateswarlu B, Reddy G, Grover M (2010b) Effect of osmotic stress on plant growth promoting *Pseudomonas spp*. Arch Microbiol 192:867–876. https://doi.org/10.1007/s00203-010-0613-5

Savouré A, Jaoua S, Hua XJ, Ardiles W, Van Montagu M, Verbruggen N (1995) Isolation, characterization, and chromosomal location of a gene encoding the delta 1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana*. FEBS Lett 372:13–19

Sharma S, Verslues PE (2010) Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. Plant Cell Environ 33:1838–1851. https://doi.org/10.1111/j.1365-3040.2010.02188.x

Sharma S, Villamor JG, Verslues PE (2011) Essential role of tissue-specific proline synthesis and catabolism in growth and redox balance at low water potential. Plant Physiol 157:292–304. https://doi.org/10.1104/pp.111.183210

Sharma S, Shinde S, Verslues PE (2013) Functional characterization of an ornithine cyclodeaminase-like protein of *Arabidopsis thaliana*. BMC Plant Biol 13:182. https://doi.org/10.1186/1471-2229-13-182

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15:89–97. https://doi.org/10.1016/j.tplants.2009.11.009

Székely G, Abrahám E, Cséplo A, Rigó G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, Koncz C, Szabados L (2008) Duplicated P5CS genes of *Arabidopsis* play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J 53:11–28. https://doi.org/10.1111/j.1365-313X.2007.03318.x

Timmusk S, Wagner EGH (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Mol Plant-Microbe Interact 12:951–959. https://doi.org/10.1094/MPMI.1999.12.11.951

Timmusk S, Abd El-Daim IA, Copolovici L, Tanilas T, Kännaste A, Behers L, Nevo E, Seisenbaeva G, Stenström E, Niinemets Ü (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. PLoS One 9:e96086. https://doi.org/10.1371/journal.pone.0096086

Toka I, Planchais S, Cabassa C, Justin AM, De Vos D, Richard L, Savouré A, Carol P (2010) Mutations in the hyperosmotic stress-responsive mitochondrial BASIC AMINO ACID CARRIER2 enhance proline accumulation in *Arabidopsis*. Plant Physiol 152: 1851–1862. https://doi.org/10.1104/pp.109.152371

Turner NC (1981) Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58:339–366. https://doi.org/10.1007/BF02180062

van der Weele CM (2000) Growth of *Arabidopsis thaliana* seedlings under water deficit studied by control of water potential in nutrient-agar media. J Exp Bot 51:1555–1562. https://doi.org/10.1093/jexbot/51.350.1555

Vardharajula S, Zulfikar Ali S, Grover M, Reddy G, Bandi V (2011) Drought-tolerant plant growth promoting *Bacillus spp.*: effect on growth, osmolytes, and antioxidant status of maize under drought stress. J Plant Interact 6:1–14. https://doi.org/10.1080/17429145.2010.535178

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35:753–759. https://doi.org/10.1007/s00726-008-0061-6

Verbruggen N, Hua XJ, May M, Van Montagu M (1996) Environmental and developmental signals modulate proline homeostasis: evidence

for a negative transcriptional regulator. Proc Natl Acad Sci USA 93: 8787–8791

Verslues PE, Sharp RE (1999) Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. Plant Physiol 119:1349–1360

Verslues PE, Kim YS, Zhu JK (2007) Altered ABA, proline and hydrogen peroxide in an *Arabidopsis* glutamate:glyoxylate aminotransferase mutant. Plant Mol Biol 64:205–217. https://doi.org/10.1007/s11103-007-9145-z

Wu L (2003) Over-expression of an *Arabidopsis* d -OAT gene enhances salt and drought tolerance in transgenic rice. Chinese Sci Bull 48: 2594. https://doi.org/10.1360/03wc0218

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217: 1214–1222

Yoshiba Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Wada K, Harada Y, Shinozaki K (1995) Correlation between the induction of a gene for delta 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J 7:751–760

Zhang C, Lu Q, Verma DPS (1997) Characterization of Δ1-pyrroline-5-carboxylate synthetase gene promoter in transgenic *Arabidopsis thaliana* subjected to water stress. Plant Sci 129:81–89. https://doi.org/10.1016/S0168-9452(97)00174-X

Zhang H, Murzello C, Sun Y, Kim MS, Xie X, Jeter RM, Zak JC, Dowd SE, Paré PW (2010) Choline and osmotic-stress tolerance induced in *Arabidopsis* by the soil microbe *Bacillus subtilis* (GB03). Mol Plant-Microbe Interact 23:1097–1104. https://doi.org/10.1094/MPMI-23-8-1097

Zlatev Z, Lidon FC (2012) An overview on drought induced changes in plant growth, water relations and photosynthesis. Emir J Food Agric Plant Sci 24:57–72