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Understanding the mode of regulation of proline biosynthesis for drought tolerance in transgenic rice overexpressing *PDH47* gene

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Plants respond to drought stress by inducing proline accumulation which would promote drought tolerance. During drought, several reports described activation/overexpression of genes encoding enzymes for glutamate and ornithine-based proline biosynthesis pathways that provide tolerance to the plant. Pea DNA helicase 47 (*PDH47*), involves in drought stress tolerance. However, the exact mechanism for drought tolerance is not known. The present study will provide insights into how overexpression of a *PDH47* in transgenic rice confers drought tolerance by further escalating the accumulation of proline. Here, the developed transgenic plants expressing *PDH47* showed increased tolerance to drought with increased expression of proline biosynthesis genes. Therefore, understanding the combined regulation for expression of proline metabolism genes during drought stress in rice overexpressing drought stress-responsive DEAD-box helicase like *PDH47* may result in engineering economically important crop plants for more tolerant to drought stress.

Keywords: Proline; drought; pea DNA helicase 47; DEAD-box helicases; *Agrobacterium*-mediated transformation.

Introduction

Environmental stresses such as drought results in immediate change of physiological and biochemical processes of plants. Plant survival and growth under such conditions result *via* adaptive processes involving altered ion uptake, stomatal regulation, and the accumulation of osmotic solutes. Changing concentration of osmotic solutes like proline is one of such immediate response to water deficit[1]. Prolines are secreted in response to drought stress and degraded after stress release[2]. During stress condition, proline maintains the stability of proteins, membranes, and sub-cellular structures by adjusting the cell's osmotic potential and maintaining cell turgor[3-4]. They also act as scavengers for reactive oxygen species[5].

Proline accumulation is the results of increased biosynthesis followed by decreased degradation of proline during abiotic stress[6-8]. The molecular basis of increasing accumulation of proline is due to up-regulation of proline biosynthetic genes and down-regulation of proline degrading enzymes[9]. Generally, proline in higher plants is synthesized from glutamate and ornithine[10-11]. In glutamate-based proline biosynthesis, glutamate-γ-semialdehyde (GSA) is formed from glutamate by the action of the enzyme [1]Δ-pyrroline-5-carboxylate (P5C) synthetase (P5CS) followed by cyclization of GSA to form P5C. P5C is then reduced to proline in NADPH dependent reaction catalyzed by P5C reductase (P5CR)[12]. P5CS is the rate limiting enzyme in glutamate based proline biosynthesis in higher plants[13]. Promoter analysis and transgenic studies have shown that P5CS is the major rate-limiting enzyme for proline accumulation[14]. During ornithine-based proline biosynthesis, the enzyme ornithine-δ-aminotransferase (OAT), converts ornithine into GSA[11]. OAT is the key enzyme of the ornithine biosynthetic pathway. Overexpression of rice *OsOAT* gene resulted in significantly increased osmotic stress tolerance with higher δ-OAT activity and proline accumulation[15]. Both the pathways follow the last reaction where P5C is reduced to proline in the presence of functional P5CR. On the other hand, two mitochondrial enzymes, flavin-dependent proline dehydrogenase (PRODH) and NAD^+-dependent P5C dehydrogenase (P5CDH) involves in the oxidation of proline. P5CDH plays a key role in controlling proline level in plant cells[16]. In the current study, for better understanding, the molecular regulation of proline metabolism, genes encoding P5CS, P5CR, OAT and P5CDH were analyzed during drought stress.

Many factors regulate proline metabolism. Many studies demonstrated proline accumulation, mediated

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through abscisic acid (ABA)-dependent and independent signaling pathways[17-18]. Apart from that, regulation of proline metabolism also associates with transcription factors (TFs). In rice, 24 different classes of TFs belonging to MYB, bZIP, and AP2/ERF TF families were detected to have a binding site in the promoter of *OsP5CS1*, *OsP5CS2*, and *OsP5CR* genes[19]. However, in rice, all possible regulatory mechanism involved in proline biosynthesis and degradation is not fully understood. Helicases are one such regulatory enzyme involved in nucleic acid metabolism via replication, repair, recombination, and transcription by utilizing the energy from nucleoside triphosphate (NTP) hydrolysis[20]. We hypothesized that helicases might have regulatory control over proline metabolism. In the current study, we investigated the role of helicases in proline accumulation during drought stress in transgenic rice overexpressing *PDH47* gene from pea. *PDH47* is a DEAD-box (Asp-Glu-Ala-Asp) family of helicase and are reported to be involved in diverse types of abiotic stresses. *PDH47* has a role in multiple abiotic stress conditions such as cold (4°C), salt stress and drought stress[21-22]. *PDH47* expression levels increased in root and leaves of water-starved transgenic rice depicting its involvement in drought stress[22]. Moreover, many endogenous stress responsive genes were regulated by the heterologous expression of *PDH47* gene thereby conferring drought tolerance in transgenic rice[22].

Materials and Methods

Vector Constructs and Plant Transformation

At *Nco*I site of the vector pRT100, the complete ORF of *PDH47* cDNA (1.2kb) was cloned. The whole cassette of *PDH47*, driven by constitutive 35S CaMV promoter was finally cloned into MCS region of pCAMBIA1301 at *Nco*I site and named as p1301-*PDH47*. The recombinant plasmid, p1301-*PDH47*, was mobilized into *Agrobacterium tumefaciens* strain AGL1 by electroporation and utilized for *Agrobacterium*-mediated genetic transformation. Following the protocol by Toki *et al*. (1997)[23], genetic transformation of *japonica* rice cv. Dongjin was performed using mature seeds with modifications. Callus was raised from the mature seeds in N6D medium and infected with *Agrobacterium* strain AGL1 in N6D-As medium (Fig. 1). The survived calluses after three rounds of selection in N6D medium supplemented with 35 mg/L hygromycin, regenerated in 2 mg/L kinetin and 0.02 mg/L NAA of MS medium supplemented with 35 mg/L hydromycin. The regenerated shoots are finally transferred for root

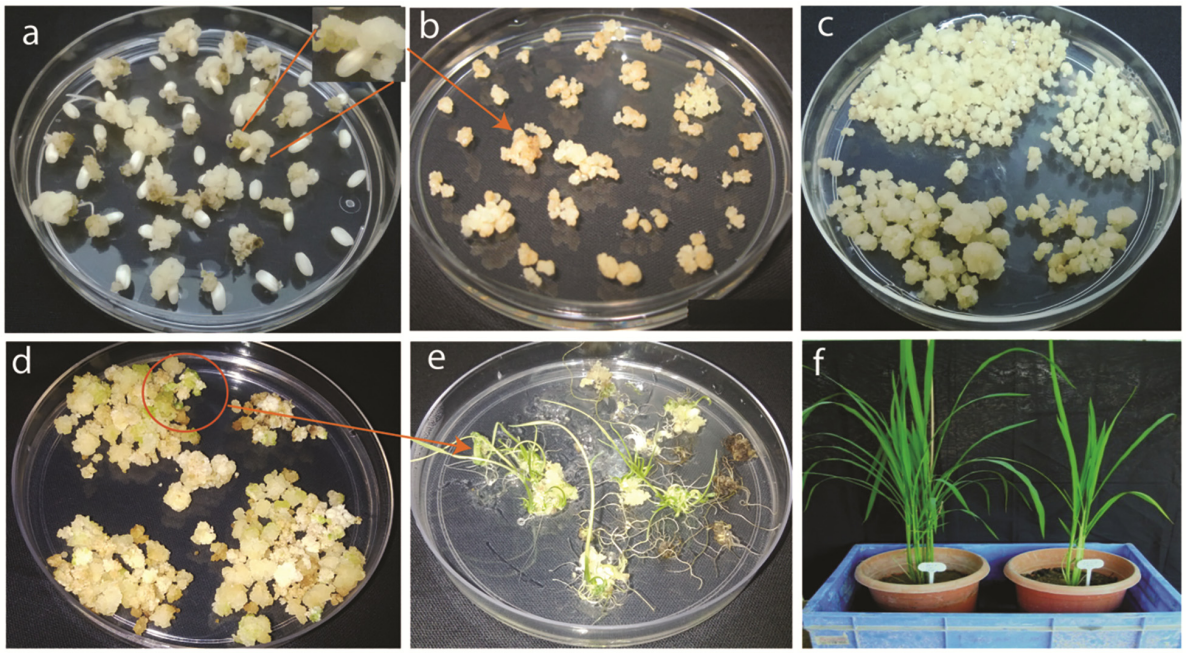

Fig. 1 — *Agrobacterium tumefaciens*-mediated genetic transformation of *japonica* rice cv. Dongjin using mature seeds. a. Raising of callus from the seeds in N6D medium; b. Co-cultivation of callus with *Agrobacterium* strain AGL1 harbouring p1301-*PDH47* for 2 days; c. Calluses in selection medium containing 30 mg/L hygromycin B; d. Induction of green embryoids on 2 mg/L kinetin and 0.02 mg/L NAA of MS medium supplemented with 50 mg/L hydromycin after 7 days of culture; e. Rooting of the *in vitro* regenerated shoots after 15 days of culture; f. Putative transgenic lines maintained in the transgenic green house.

regeneration in MS rooting medium supplemented with 35 mg/L hydromycin. Genomic DNA was isolated from the regenerated transgenic plants and subjected to PCR analysis with *PDH47* gene-specific primers (Table 1). The total RNA from the PCR positive transgenic lines were analyzed for expression of the transgene through qRT-PCR using *PDH47* primers. Total RNA was extracted from the young leaves of control and stress-treated plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After genomic DNA elimination, cDNA was synthesized followed by reverse-transcription reaction from 1 µg of total RNA, using the PrimeScript RT reagent Kit with gDNA eraser (Clonetech, USA). Quantitative real-time PCR reactions were carried out twice in three biological replicates using 30 ng of cDNA as per manufacturer's instruction. The experiment was carried out in StepOnePlusTM real-time PCR system (Applied Biosystems, USA) using the relative method with an annealing temperature of 60°C. The relative expression of each gene was calculated according to the method of $2^{-\Delta\Delta Ct}$. In the present study, three high expressing T_3 generation transgenic lines for *PDH47* gene were selected for further analysis.

Evaluation of Transgenic Rice for Drought Stress Tolerance

Three T_3 high expressing transgenic lines (Lines D3, D6 and D8) along with WT were selected for drought stress treatment. For drought stress treatment, several seeds each from D3, D6 and D8 high expressing T_3 transgenic lines were germinated in trays. PCR was done from the leaves of T_3 lines to select the positive plants for drought treatment. For each transgenic lines, three PCR positive plants were grown further in pots for 30 days in three replicates. During 30 days of growing, the pots were watered regularly to maintain 100% field capacity (FC). Finally, these plants were subjected to drought stress by withholding water. Samples were collected for further analysis from the transgenic lines and WT plants before stress (BS), medium drought (MD) and severe drought (SD) at 0, 15 and 30 days after drought treatment, respectively. The soil moisture content was measured through gravimetric analysis.

Determination of Leaf Water Status

Leaf relative water content (RWC) of transgenic and WT rice plants during WW, MD, SD were measured from the excised leaves. RWC was measured by taking the fresh weight (FW), turgid weight (TW) and dry weight (DW) of leaves of transgenic as well as WT rice plants. RWC was calculated from the equation as described by Schonfeld *et al* (1988)[24]:

$$RWC\ (\%) = (FW-DW)/(TW-DW)\times 100.$$

Biochemical Analysis: Determination of Chlorophyll Content, MDA Content and Proline Content

To determine chlorophyll, MDA and proline content, equal quantities of leaf samples were taken

Table 1 — Details of the primer sequences used in PCR, RT-PCR and qPCR analysis.

Sl. No.	Name of the gene present	Method	Forward (F) and reverse (R) primer	Size of the amplicon
1.	*PDH47*	PCR	F(5´CAACAGGCTCAGTCTGGAACGG3´) R(5´GCTGCACATCAATACCACGAGCC3´)	796 bp
2	*HPTII*	PCR	F(5´GGTCAAGACCAATGCGGAGC3´) R(5´GCTGCGCCGATGGTTTCTAC3´)	663 bp
3	*PDH47*	RT-PCR	F(5´CAACAGGCTCAGTCTGGAACGG3´) R(5´GCTGCACATCAATACCACGAGCC3´)	796 bp
4	*Actin*	RT-PCR	F(5´GCCAGTGGTCGAACATCTGG3´) R(5´CATGGATGCCAGGAGCTTCC3´)	520 bp
5	*PDH47*	qRT-PCR	F(5'GAGGGACCAGTGTTCGTGAG3') R(5'ACGACCAGGGGTACCTACAA3')	74 bp
6	*ACT1*	qRT-PCR	F(5'CTGCGGGTATCCATGAGACT3') R(5'GCAATGCCAGGGAACATAGT3')	118 bp
5	*P5CS*	qRT-PCR	F(5'TGTGGGAAGAGGTGGAATGC3') R(5'GAACAACCCCACTGGTTTGC3')	171 bp
6	*P5CR*	qRT-PCR	F(5'CCATGGCTGATGGTGGAGTT3') R(5'AGTTATGGTGGTTCCTGCCG3')	161 bp
7	*OAT*	qRT-PCR	F(5'GAACCCATTGGCAAGTGCTG3') R(5'TTAAGCAAACCCCTCCCACG3')	168 bp
8	*P5CDH*	qRT-PCR	F(5'GCCTGTGAAAGGATGAACGC3') R(5'TCTGTGGAGCACCAGTTGTC3')	139 bp

from the stressed and non-stressed transgenic and WT rice plants during WW, MD, SD. Chlorophyll pigments from the leaf tissues were extracted using 80% acetone. The absorbance by the chlorophyll pigments were analyzed spectrophotometrically at 645 and 663 nm and the contents determined by the method described by Arnon (1949)[25]. For MDA content estimation, leaf tissue powder was mixed with 20 ml of 0.1M potassium phosphate buffer (pH 6.8) and centrifuged at 10,000 × g for 15 min. The supernatant was collected and used for determination of MDA content according to Guidi *et al*. (2000)[26]. Proline content in the leaf samples of both stressed and non-stressed transgenic and WT rice plants was determined following the protocol described by Bates *et al*. (1973)[27]. Fresh leaf samples (0.5 g) were homogenized in 3% aqueous sulfosalicylic acid and centrifuged at 12,000 rpm for 10 min. The supernatant (2 ml) was mixed with glacial acetic acid (2 ml) and acid ninhydrin solution (2 ml). The final solution was boiled at 100°C for 1 hour and immediately transferred to the ice to stop the reaction. After cooling, the mixture was extracted with toluene (4 ml) and measured the absorption at 520 nm. The final concentration of proline was calculated using calibration curve developed with proline standard.

RNA Isolation and Proline Metabolism Gene Expression Analysis

Total RNA was isolated from the leaf and root samples of WT and transgenic lines at WW, MD and SD using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR reactions were set up using gene-specific primers (Table 1). The rice actin 1 gene (*ACT1*) was used as the endogenous reference for qRT-PCR analysis. The experiment was carried out in StepOnePlusTM real-time PCR system (Applied Biosystems, USA) using the relative method. For PCR assay, the annealing temperature was kept at 60°C. The relative expression of the *PDH47* transcript was calculated according to the method of 2-ΔΔCt[28].

Statistical Analysis

All experimental data were the means of at least three independent replicates. Analysis of variance (ANOVA) table was prepared using the R version 3.3.0[29].

Results

Development and Selection of Promising Transgenic Rice Overexpressing PDH47

The recombinant plasmid pCAMBIA1301-*PDH47* was transformed into *japonica* rice cv. Dongjin mediated through *Agrobacterium* using mature seeds as explants. A total of 51 independent T_0 transgenic lines were found to be PCR positive with the *PDH47* transgene. A total of 48 transgenic lines showed a varied level of expression of *PDH47* transcripts as evident by qRT-PCR analysis. Out of 48 expressing lines, 10 showed the highest expression of the transgene. Here, analysis of 10 lines is shown for PCR (Fig. 2a) and RT-PCR (Fig. 2b). Expression of the endogenous actin gene was similar in all the lines. Three T_3 transgenic lines (Lines D3, D6, and D8) were selected and analyzed further. The transgenic lines were selected based on their higher expression level of the *PDH47* transgene.

Overexpression of *PDH47* Due to Drought Stress

The three overexpressing transgenic lines (Lines D3, D6, and D8) with *PDH47* transgene were subjected to drought stress treatment by withholding water (Fig. 3a). The transgenic lines showed an enhanced expression of the *PDH47* transgene in both roots and shoot as compared to BS (0 days) after MD (15 days) and SD (30 days) stress treatment (Fig. 3b). Significantly higher expression level was observed in leaf tissues of lines D3 during SD followed by lines

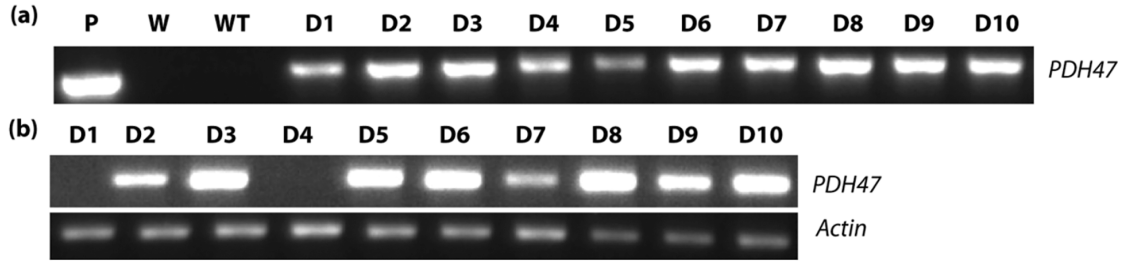

Fig. 2 — Molecular analysis of T_3 transgenic rice. a. PCR analysis of T_3 transgenic lines for the presence of *PDH47* transgene with gene specific primers b. Semi-quantitative expression analysis of *PDH47* transgene (upper lane) in T_3 transgenic lines; rice actin gene (lower lane) was used as internal control. (P: positive control - p1301-*PDH47* plasmid; W & WT: negative control with water and non-transformed rice respectively; Line D1-D10: transgenic lines).

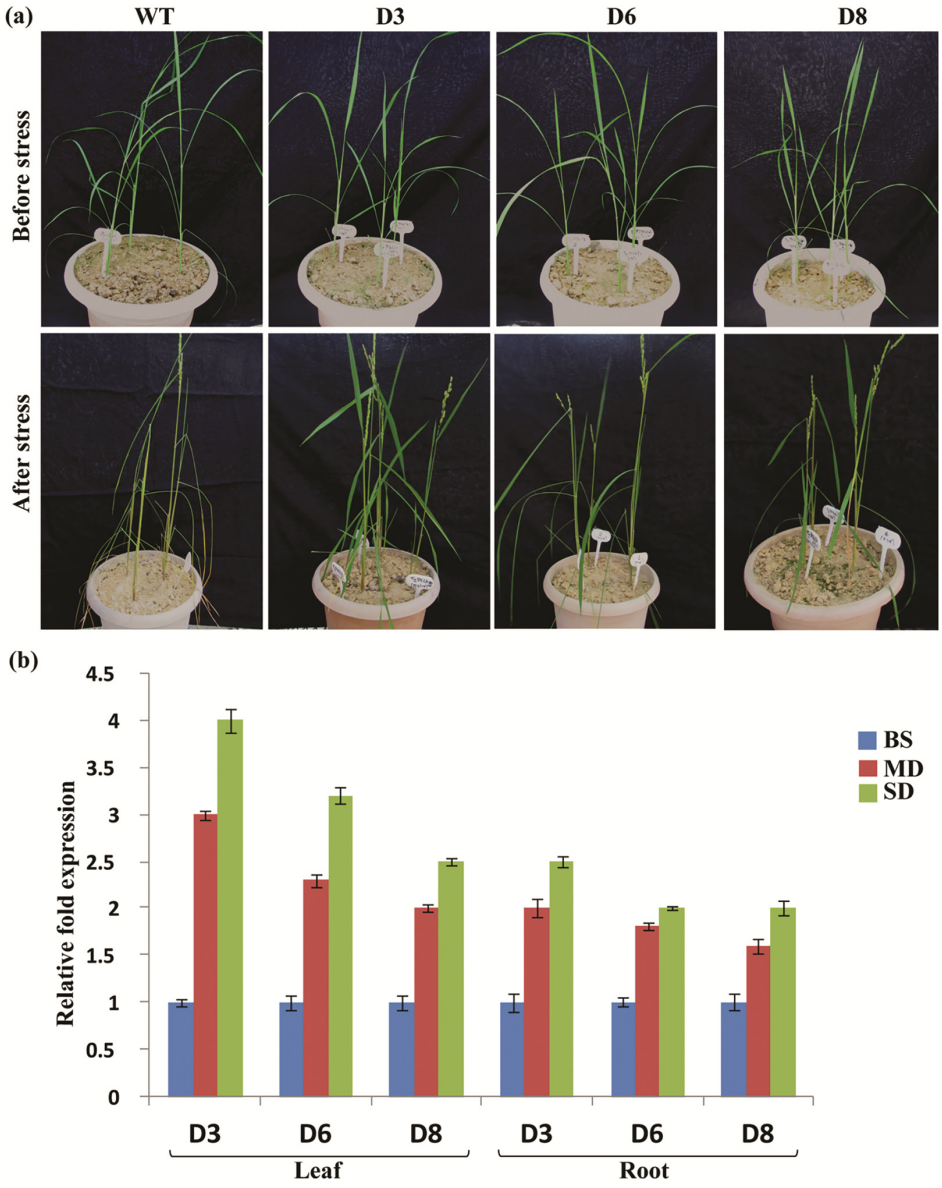

Fig. 3 — Drought stress analysis of T_3 transgenic rice overexpressing *PDH47* transcripts and WT. a. Drought stress analysis of 30 days old seedlings of T_3 transgenic rice (Lines D3, D6 and D8) and WT were subjected to drought stress by withholding water for 30 days. b. qRT-PCR analysis of T_3 transgenic lines over-expressing *PDH47* transcripts in leaf and root tissues before and after 30 days of drought.

D6 during SD. In root, highest expression was seen in line D3 during SD. However, collectively the expression level of *PDH47* transcripts was higher in leaf as compared to root in all the three transgenic lines.

Drought Tolerance by Transgenic Rice Overexpressing *PDH47*

The physiological function and mechanism of drought tolerance by the three T_3 transgenic lines were further analyzed. T_3 transgenic lines showed improved tolerance to drought stress as compared to WT even after SD, as leaves of WT plant severely rolled and dried (Fig. 3). Transgenic line D3 showed significantly higher tolerance as compared to D6 and D8. Moreover, the transgenic lines maintained normal growth and bear seeds after rehydration. After drought treatment, the leaf water status of transgenic lines and WT were determined at BS, MD and SD. The percent RWC of the transgenic lines were found to be higher than the WT at MD and SD, (Fig. 4a). Line D3 showed significantly highest RWC at SD whereas line D6 showed highest at MD. Transgenic lines exhibited higher levels of chlorophyll content

than its WT during drought stress conditions (Fig. 4b). Transgenic line D3 showed a higher content of chlorophyll at both MD and SD. Compared with WT, transgenic lines showed a significantly lower content of MDA at MD and SD suggesting an improved tolerance of reactive oxygen species (ROS) damage especially under drought environments (Fig. 4c). MDA level of D3 line was lowest both at MD and SD. The internal proline content in the leaf tissues of MD and SD transgenic lines increased by ~2 fold compared to WT (Fig. 5a). Among them, proline contents of transgenic line D3 at SD were significantly more as compared to BS and MD.

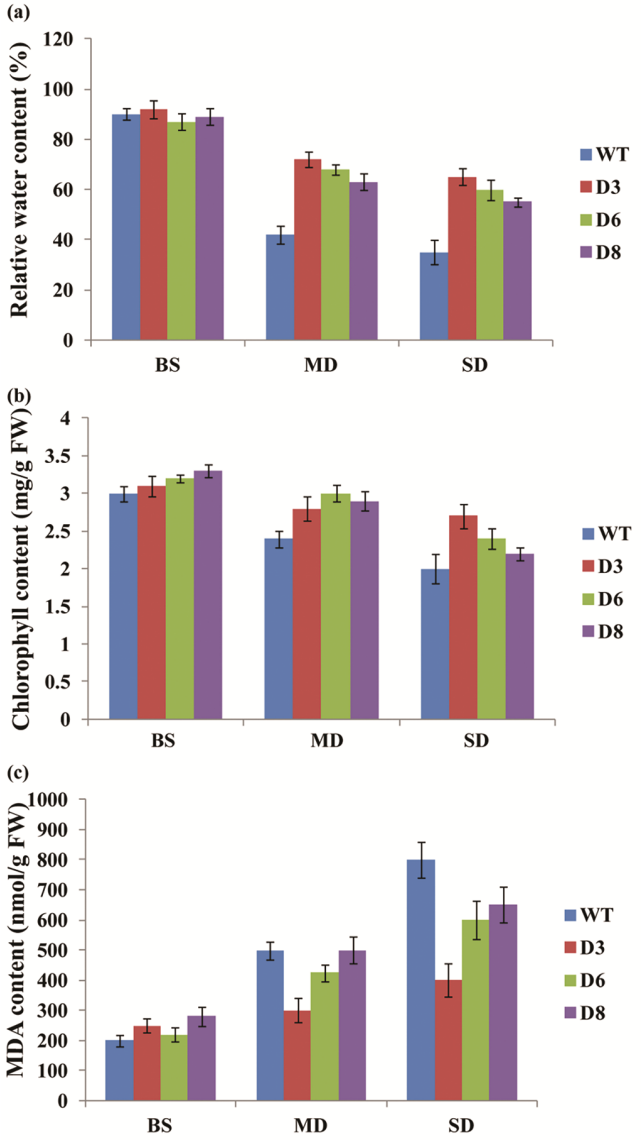

Fig. 4 — Physiological analysis of the T_3 transgenic lines and WT during drought stress. a. Relative water content of leaf tissues of T_3 transgenic lines (D3, D6 and D8) and WT before and 30 days of drought stress. b. Chlorophyll content in mg/g of FW. c. MDA content in nmol/g of FW. Bars represent means ± SE of three independent replicates and * indicates the values were significant at $P < 0.05$.

Differential Expression of Proline Metabolism Genes Under Drought Stress

The overexpression of *PDH47* in transgenic rice confers drought tolerance with improved leaf water status, chlorophyll content, reduced MDA content and increased accumulation of proline. To further investigate the regulation of *PDH47* through proline metabolism in transgenic rice during drought stress, the differential expression analysis of genes involved in proline metabolisms such as delta-1-pyrroline-5-carboxylate synthetase (*P5CS*), pyrroline-5-carboxylate reductase (*P5CR*), ornithine aminotransferase (*OAT*) and 1-pyrroline-5-carboxylate dehydrogenase (*P5CDH*) were studied under MD and SD stress conditions. *P5CS*, *P5CR*, and *OAT* genes were involved in proline biosynthesis while *P5CDH* gene is responsible for degradation of

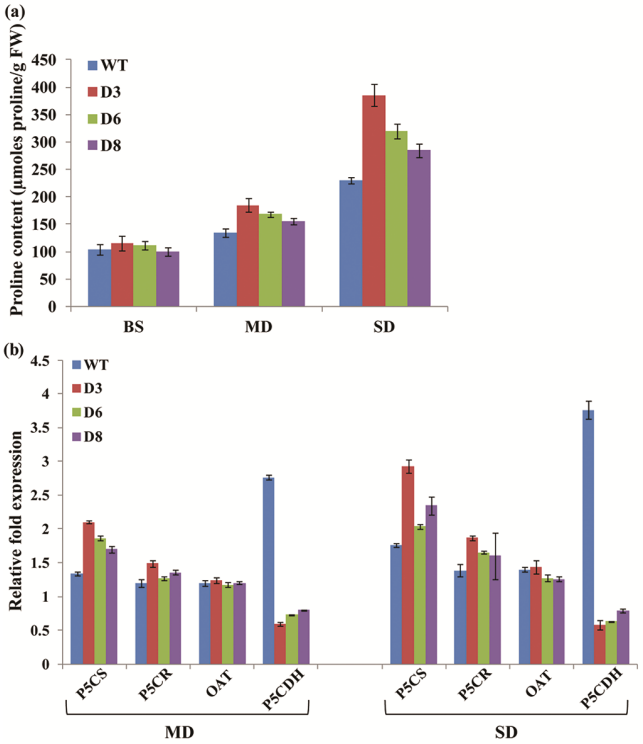

Fig. 5 — Accumulation of proline and expression analysis of proline metabolism genes in leaves of T_3 transgenic lines and WT. a. Proline contents in µmoles proline/g of fresh weight (FW) of leaf tissues of T_3 transgenic lines (D3, D6 and D8) and WT before and after 30 days of drought stress. Bars represent means ± SE of three independent replicates and * indicates the values were significant at $P < 0.05$. b. qRT-PCR analysis of the proline metabolism genes (*P5CS, P5CR, OAT* and *P5CDH*) after 30 days of drought stress.

internal proline content. qRT-PCR analysis revealed that expression level of 2 proline biosynthetic genes such as *P5CS* and *P5CR* were significantly upregulated in the three transgenic lines overexpressing *PDH47* transcripts as compared to WT and BS during MD and SD (Fig. 5b). Among the three proline biosynthetic genes, *P5CS* showed relatively ~3 fold up-regulation in transgenic line D3 as compared to WT during SD stress treatment (Fig. 5b). Although the expression level of *OAT* gene was upregulated in transgenic lines during drought stress but similar with the WT significantly. Interestingly, the expression level of proline degradation gene, *P5CDH* was found to be significantly downregulated in all the tested transgenic lines and highly up-regulated (~4 fold) in the WT under both MD and SD (Fig. 5b).

Discussion

Drought is one of the major limiting factors to crop production affecting plant growth and yield by inducing alteration to cellular, physiological and metabolic pathways[30]. To confer drought tolerance, we have generated several transgenic rice lines overexpressing varied levels of *PDH47* transcripts through *Agrobacterium*-mediated genetic transformation. However, mechanism of the transgene for conferring drought tolerance is not known. Understanding such hidden mechanisms will help in formulating new strategies to develop plants tolerant to various abiotic stresses.

Drought stress in plants results in alteration of physiological and biochemical mechanisms such as accumulation of osmolytes, the rate of lipid peroxidation and chlorophyll content etc.[31,32] In the present investigation, three T_3 transgenic lines (Lines D3, D6 and D8) overexpressing *PDH47* transgene were analyzed for drought stress treatment. Increased proline accumulation during drought stress is a regular physiological process that imparts drought stress tolerance. The increase accumulation results from the stress-induced increased synthesis and reduced catabolism[33]. The accumulated proline participate in many stress adaptation processes, such as regulation of shoot and root growth and limiting transpiration rate, thereby protect plants from wilting during drought stress[34]. The transgenic lines showed increased accumulation of proline with less wilting or rolling of leaves as compared to control. The proline accumulation under stress was higher (~2-fold) in the transgenic lines as compared to non-stressed transgenic lines. Higher accumulation of proline adjusts the processes of plant growth and development which in turn activates downstream regulation of drought-responsive genes[35]. Overexpression of *BrCIPK1* gene enhances abiotic stress tolerance by increasing proline biosynthesis in rice[36]. Sharma *et al.* (2011)[37] reported that in *Arabidopsis* both proline synthesis and catabolism were regulated under drought stress to maintain the growth of the plants. The transgenic lines also showed a lower level of MDA content with improved photosynthetic capacity. MDA is considered to be an indicator of membrane lipid peroxidation whereas drought results in the reduction of photosynthesis due to the prevention of entry of CO_2 (Zhang, 1999)

The authors suggested that further increase in proline biosynthesis could be the more effective strategy to enhance drought tolerance in plants. Therefore, it is important to know the regulatory pattern of the genes involved in proline biosynthesis. The regulatory pattern of the genes involved in proline metabolism is not fully understood. The genes may be regulated by the signaling pathways or at the post-translational level (Zarattini & Forlani, 2017). The signaling pathways such as abscisic acid (ABA)-dependent and ABA-independent pathways are reported to activate proline accumulation in an osmotic-dependent manner (Savoure *et al*, 1995; Abraham *et al*, 2003). Transcription factors (TFs) on the other hand are one of the regulators in plant growth, development, and responses to diverse biotic and abiotic stresses. Ectopic overexpression of *TaWRKY1* in tobacco conferred improved tolerance to drought[38]. DEAD-box helicases are involves in multiple abiotic stress processes. *PDH47,* a DEAD-box helicase involves in conferring abiotic stress tolerance in plants by regulating other stress-responsive pathways[39]. In the current study, we observed complete regulation of genes involved in proline metabolism in transgenic rice overexpressing *PDH47* transgene. The expression level of *OsP5CS* and *OsP5CR* genes significantly higher in leaf samples of transgenic lines as compared to wild type. However, the expression level of *OsOAT* transcript although higher but significantly lower than the expression level of *OsP5CS* and *OsP5CR* transcripts in transgenic plants as compared to wild type under drought stress. Therefore, the glutamate-based proline biosynthesis was more actively involves in drought tolerance in rice. Reports also suggested that the key enzyme, P5CS of glutamate-based proline biosynthesis

up-regulated during various abiotic stresses including salt [40], dehydration and ABA treatment[41] in *A. thaliana*. On the other hand, soybean plants overexpressing endogenous *P5CR* exhibited very rapid increase in proline content in leaves than the WT with least water loss during drought stress[42]. Overall, the increasing accumulation of proline by the down-regulation of proline catabolism genes such as *P5CDH* under drought stress is a common mechanism of regulation of proline biosynthesis[43]. In the current study, we also observed significant down-regulation of *OsP5CDH* transcript in transgenic lines and highly up-regulation in wild type. This might be one of the reasons for lower accumulation of proline in wild type as compared to transgenic lines. In conclusion, higher accumulation of proline in transgenic rice overexpressing *PDH47* transgene strongly correlated with increased level of proline biosynthesis genes and reduced expression of proline catabolism gene with significantly higher in low water, chlorophyll content with a reduced level of MDA.

Conclusion

The increased accumulation of proline under stress environment in plants is due to either increased biosynthesis or reduced catabolism or both. The proline synthesis and catabolism are regulated by multiple cellular mechanisms, which are not fully elucidated. Here, we showed that the two proline biosynthetic pathways in transgenic rice were osmotically stress-regulated at the transcriptional level due to the overexpression of *PDH47* transcripts. Furthermore, the expression of genes coding for proline degrading enzyme was highly up-regulated in wild type as compared to transgenic plants under drought stress which in turn lead to decrease accumulation of proline in wild type. Thus, up-regulation of proline biosynthesis and down-regulation of proline catabolism genes respectively, in transgenic rice overexpressing DEAD-box helicase (*PDH47*) results in drought tolerance. Hence, combined expression of proline metabolism genes and drought stress-responsive DEAD-box helicase like *PDH47* may result in engineering rice and other economically important crop plants for more tolerant to drought stress.

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