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RESEARCH

Introgression of *Δ*^{1}*-pyrroline-5-carboxylate synthetase* (*PgP5CS*) confers enhanced resistance to abiotic stresses in transgenic tobacco

Gothandapani Sellamuthu · Avijit Tarafdar · Rahul Singh Jasrotia · Minakshi Chaudhary · Harinder Vishwakarma · Jasdeep C. Padaria

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Abstract Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) is one of the key regulatory enzymes involved in the proline biosynthetic pathway. Proline acts as an osmoprotectant, molecular chaperone, antioxidant, and regulator of redox homeostasis. The accumulation of proline during stress is believed to confer tolerance in plants. In this study, we cloned the complete CDS of the *P5CS* from pearl millet (*Pennisetum glaucum* (L.) R.Br. and transformed into tobacco. Three transgenic tobacco plants with single-copy insertion were analyzed for drought and heat stress tolerance. No difference was observed between transgenic and wild-type (WT) plants when both were grown in normal conditions. However, under heat and drought, transgenic plants have been found to have higher chlorophyll, relative water, and proline content, and lower malondialdehyde (MDA) levels than WT plants. The photosynthetic parameters (stomatal conductance, intracellular CO_2 concentration, and transpiration rate) were also observed to be high in transgenic plants under abiotic stress conditions. qRT-PCR analysis revealed that the expression of the transgene in drought and heat conditions was 2–10 and 2–7.5 fold higher than in normal conditions, respectively. Surprisingly, only *P5CS* was increased under heat stress conditions, indicating the possibility of feedback inhibition. Our results demonstrate the positive role of *PgP5CS* in enhancing abiotic stress tolerance in tobacco, suggesting its possible use to increase abiotic stress-tolerance in crops for sustained yield under adverse climatic conditions.

Keywords *Pennisetum glaucum* · Proline · Gene *P5CS* · Transgenic · Functional validation

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G. Sellamuthu · A. Tarafdar · R. S. Jasrotia · M. Chaudhary · H. Vishwakarma · J. C. Padaria (✉)
National Institute for Plant Biotechnology, Pusa Campus, New Delhi 110012, India
e-mail: jasdeep_kaur64@yahoo.co.in

Present Address:
G. Sellamuthu
Forest Molecular Entomology Laboratory, Excellent Team for Mitigation (ETM), Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Prague, Czech Republic

Present Address:
A. Tarafdar
International Crops Research Institute for Semi-Arid Tropics, Patancheruvu, India

Present Address:
R. S. Jasrotia
Florida State University, Tallahassee, USA

Introduction

Abiotic stress in plants refers to the negative influence of non-living environmental factors on plant growth

and development. Rising temperatures, unexpected rainfall or water scarcity, and increased soil salinity are key factors limiting plant growth and productivity (He et al. 2018). Drought has become one of the most serious issues in global agriculture, substantially affecting crop yield, according to recent studies (Gang et al. 2016; Hendrawan et al. 2023). To overcome drought impact, plants have evolved several mechanisms by adapting their molecular, physiological, and metabolic levels to survive. Plants, for example, acquire several low molecular weight metabolites known as osmolytes, including quaternary amines, mannitol, glycine betaine, and proline under drought conditions (Hassan et al. 2019). Among these, proline is one of the most beneficial molecules in osmotic adjustment required for plant growth, development, and stress responses (Amini et al. 2015; Ma et al. 2022). It is a compatible osmoprotectant and free radical scavenger of reactive oxygen species that protects against oxidative damage in plants during the process of stress and supplies energy for resumed growth after stress (Ramachandra et al. 2004; Verslues and Bray 2006; Hayat et al. 2012). The proline contents of plant cells are crucial enzymes Δ1-Pyrroline-5-carboxylate synthetase (P5CS) in the glutamate pathway is the major route for proline synthesis during stress (Hu et al. 1992; Liang et al. 2013). Furthermore, it is predicted that increased proline accumulation under heat-stress conditions contributes to protein and membrane stability (Sung et al. 2003; Mirzaei et al. 2012). Proline levels in tissues can be elevated through either over-expression of the P5CS enzyme or inhibition of the enzyme proline dehydrogenase, which are two essential enzymes in the proline biosynthesis pathway.

Since P5CS is essential for proline biosynthesis, various attempts have been undertaken to increase plant stress tolerance by altering the P5CS gene. The use of genes coding for key regulatory enzymes of the osmoprotectant biosynthesis pathway has been shown to increase plant stress tolerance (Kido et al. 2013; Todaka et al. 2015). According to studies in *Oryza sativa*, *Gossypium hirsutum*, and other plants and trees, P5CS expression and activity levels are elevated under abiotic stress conditions, thus enhancing proline concentration (Igarashi et al. 1997; Parida et al. 2008; Chakraborty et al. 2012; Bandurska et al. 2017; Ma et al. 2022). The overexpression of P5CS in transgenic plants increased proline levels and was shown to lead to plants with increased resistant to drought and heat (Simon-Sarkadi et al. 2006; Kumar et al. 2010; Chen et al. 2013). Despite this, since P5CS is a rate-limiting enzyme in proline biosynthesis, it is subject to feedback inhibition by proline. Alternatively, site-directed mutation of feedback inhibition resulted in two times more proline accumulation in transgenics as compared to wild-type (Zhang et al. 1995; Hong et al. 2000). In addition, overexpression of P5CSF129A mutant enhanced proline content in chickpea under drought (Bhatnagar-Mathur et al. 2009).

Pennisetum glaucum (pearl millet) has been recognized as a promising climate-resilient crop in arid and semi-arid areas around the world, particularly in Africa, India, and South Asia, where circumstances are harsh (drought, low soil fertility, low soil moisture, high soil pH, and high temperature). As a result, the crop may be a rich source of genes for abiotic stress tolerance (James et al. 2015). The search for genes for abiotic stress tolerance from unrelated species or genera and their introduction in a specific crop by transgenesis seems to be an attractive option for the development of stress-tolerant crops (Padaria et al. 2015, 2016). Hence, in this study we have isolated and characterized the gene for the *P5CS* gene (PgP5CS; KJ459944) from *P. glaucum*, and functionally-validated it in transgenic tobacco lines under heat and drought conditions. The results of PgP5CS gene analysis provide insights into the functional role of this gene in pearl millet.

Materials and methods

Plant materials

This study used *Pennisetum glaucum* genotype 841B (drought and heat tolerant) seeds collected from the Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi, India. Seeds of *P. glaucum* were grown at the National Phytotron Facility IARI, New Delhi, India under a photoperiod of 16 h/8 h (day/night), the light intensity of 300 µmol m^{-2} s^{-1}, the temperature of 33 °C ± 2 °C and relative humidity of 50–60%. One-month-old seedlings from these plants were harvested and used for the isolation of RNA. Drought was given to 22-day-old seedlings by using 30% PEG. The

samples were collected at different periods i.e., 1/2 h, 2 h, 4 h, 8 h of drought treatment. Samples growing at normal conditions as mentioned above were used as a control. For the transformation studies, *Nicotiana tabacum* cv. Petit Havana was used.

qRT-PCR, isolation, and cloning of P5CS CDS from P. glaucum

Total RNA was isolated from pearl millet (*P. glaucum* genotype 841B) leaf tissue (control and treated) samples using the Spectrum Plant Total RNA isolation kit (Sigma Aldrich, USA) as per the manufacturer's instructions. DNA contamination was removed using the enzyme DNaseI. The isolated RNA was quantified using a NanoDrop (Thermo Scientific, USA) spectrophotometer. cDNA was synthesized from 1 µg of total RNA by the SuperscriptIII First-strand cDNA synthesis system (Invitrogen, USA). qRT-PCR was carried out in three technical replicates. *PgActin* was used as endogenous internal control and relative fold change expression was calculated based on Equation $2-^{\Delta\Delta Ct}$ (Livak and Schmittgen 2001). To get the complete CDS (Coding DNA Sequence), 5′ and 3′ ends were amplified by SMARTer® RACE 5′/3′ kit (Clontech, USA) according to the manufacturer's instructions. Nested PCR was carried out using the Universal Primer Mix (Clontech, USA) and *P5CS* specific primers (Supplementary Table 1). The RACE-PCR products obtained were ligated to the pGEM-T easy vector (Promega, USA) as per the manufacturer's instructions. The ligated products were transformed into *E. coli* DH5α cells. The cloned sequences were assembled using the software BioEdit Version 7.1.11 (Hall 1995) and identified using NCBI ORF finder (Rombel et al. 2002). Thereafter, CDS was amplified using *P5CS* gene-specific primers (Supplementary Table 1), and the polymerase chain reaction (PCR) was carried out in a thermal cycler (BioRad-USA), (Initial denaturation at 94 °C for 3 min; followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min; final extension at 72 °C for 7 min). Amplified product (CDS) was cloned into the pGEMT-Easy vector (Promega, USA) as per the manufacturer's protocol and sequenced using T7 and SP6 primers.

Bioinformatic analysis of PgP5CS

Protein Sequences of P5CS from plant species were retrieved from the NCBI database for in-silico analysis (https://www.ncbi.nlm.nih.gov/) (Supplementary Table S2). The MEGA11 software tool was used to construct a phylogenetic tree using the neighbor-joining method for the P5CS sequences retrieved from different plant species (bootstrap value 1500) (Tamura et al. 2021). The three-dimensional structure of PgP5CS protein was carried by Homology modeling using the Phyre^2 software tool based on human pyrroline-5-carboxylate synthetase (PDBID: c2h5gA) as a template (Webb and Sali 2014). For the stereochemical stability and quality of the predicted structure, Ramachandran plot analysis was performed using PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). Residues present at active sites were predicted using the 3DLigandSite server (http://www.sbg.bio.ic.ac.uk/3dligandsite/). To find out the potential glycosylation sites in the PgP5CS protein sequence, NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), YinOYang 1.2 (http://www.cbs.dtu.dk/services/YinOYang/) server was used for *N*-linked and *O*-linked glycosylation respectively. Protein-to-protein interaction of PgP5CS was done by string-based analysis (https://string-db.org). *P. glaucum* was not listed in the organism list, the string-based analysis was done based on *Setaria italica* due to its close relatedness to *P. glaucum*. Multiple sequence alignment was performed using the Clustal Omega software tool (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Construction of binary vector and generation of transgenic tobacco

The CDS of *PgP5CS* was amplified with primers having *Bam*HI and *Kpn*I sites respectively in forward and reverse primers (Supplementary Table 1). The amplicon was ligated to the corresponding sites of binary vector pCAMBIA1300 under the control of the maize (*Zea mays*) *ubiquitin* promoter. The recombinant binary vector was mobilized into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method (Chen et al. 1994). For validation of *PgP5CS* genetic transformation of *Nicotiana tabacum* cv. Petit Havana was carried out (Dhandapani et al. 2014). Seeds of wild-type (WT) tobacco plants were grown

in jam bottles having MS media (Murashige and Skoog) under sterile conditions in a growth chamber maintained at 22 ± 2 °C, 16/8 h of day/night photoperiod (300 µmol m^{-2} s^{-1}) and 60–70% relative humidity. The *Agrobacterium tumefaciens* strain EHA105 carrying desired construct pCAM1300Ubi-*PgP5CS* was transformed into wild-type plants (WT) of tobacco) by leaf disc method (Horsch et al. 1985). The putative transformants were selected on an MS agar medium containing hygromycin (30 mg L^{-1}) for selection. The putative transformants were regularly sub-cultured at an interval of 15 days. The well-rooted transgenic and WT untransformed plants were carefully transferred to soilrite mixture [peat moss and perlite (75:25)] and maintained under controlled glasshouse conditions (22 ± 2 °C, 16/8 h of day/night photoperiod).

Confirmation of transgene in putative transgenic tobacco plants

Genomic DNA was isolated from leaves of putative tobacco transgenic (TR) and wild (WT) plants using the CTAB method (Rogers et al. 1994). The presence of the transgene in T_0 TR plants was confirmed by PCR analysis using four different sets of primers i.e., *PgP5CS* full-length gene-specific, construct-specific, *PgP5CS* gene-specific internal, and *hpt*II primers (Supplementary Table 1). The PCR program followed was: Initial denaturation at 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 2 min. The final extension was given at 72 °C for 7 min. The final PCR products were resolved on a 1% agarose gel and visualized on a gel documentation system (Protein Simple, USA).

Southern hybridization was carried out to know the copy number and stable integration of *PgP5CS* in the tobacco genome. A total of 20 µg of genomic DNA from WT and TR tobacco plants was digested with the restriction enzyme *Eco*RI. The digested genomic DNA was resolved on 0.8% agarose gel and blotted on a positively charged nylon membrane (Millipore, USA) via the capillary method (Sambrook and Russell 2001). The probe was synthesized against gene *PgP5CS* using PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) as per the manufacturer's instructions. Pre-hybridization (for 3 h) and hybridization (16 h) were carried out in a hybridization oven (Major Science, India) at 50 °C. ~ 200 ng of the probe was used for the hybridization. Washing of membrane, blocking, incubation with Anti-DIG antibody, and further steps were carried out as per the manufacturer's instructions given in the DIG DNA Labeling and Detection Kit (Roche, Basel, Switzerland). The signals were detected on an X-ray film using CSPD as substrate, by incubating X-ray film with blot for 3 h.

The T-DNA insertion into the tobacco genome was analyzed using a commercially available GenomeWalker™ Universal Kit (Clontech, USA). Primers were designed based on the left T-DNA border of the pCAMBIA1300U-*PgP5CS* construct. Genomic DNA (2 µg) of Southern positive plant was digested using *Eco*RV enzyme and genome walk adaptors were ligated as per manufacturer's instructions. PCR amplification was carried out using the left border region of vector and adaptor-specific primers using a proofreading polymerase enzyme. The amplicon obtained was cloned in a pGEM-T easy vector (Promega, USA). Sequencing was carried out using T7 and SP6 primers.

Functional validation of transgenic tobacco plants for abiotic stress tolerance

To assess the drought tolerance ability of *PgP5CS* TR tobacco plants, drought, and heat was imposed on 30-day-old TR and WT tobacco plants. For drought imposition, 100 ml of 25% PEG-6000 (osmotic potential $\Psi = -1.0165$ MPa) solution was added to plants every alternate day for two weeks (Yang et al. 2008; Hazariak and Rajam 2011). Leaf samples were collected on the 0, 6th, and 12th days of drought. Similarly, for assessment of heat stress tolerance, 30-day-old TR and WT tobacco plants were exposed to heat stress for 2, 4, and 6 h in a growth chamber set at 35 °C and 45 °C by gradually increasing 1 °C per 10 min from 22 °C till to the desired temperature. After completion of the heat stress imposition, the temperature was lowered again in the same way. Drought and heat tolerance were measured by using biochemical assays such as relative water content (RWC) (Smart and Bingham 1974), malondialdehyde (MDA) content (Heath and Packer 1968), chlorophyll (Hiscox and Isaraelstam 1979) and proline (Bates et al. 1973). Photosynthetic parameters (Gas exchange parameters) such as photosynthetic rate (µmol m^{-2} s^{-1}), stomatal

conductance (μmol m^{-2} s^{-1}), intercellular CO_2 concentration, and transpiration rate were also measured in control and treated conditions plants using a portable infrared gas analyzer (IRGA) (LICOR 6400, USA) (Babitha et al. 2015; Senthil et al. 2007). The photosynthetic parameters were measured in tobacco plants (WT and TR) under control conditions and at the end of the stress period.

Expression analysis of PgP5CS in transgenic tobacco plants by qRT-PCR

Total RNA was isolated from WT and Southern positive TR tobacco plants using RNeasy Plant Mini-Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Contamination of genomic DNA was removed using the DNaseI enzyme. First-strand cDNA was synthesized from 1 μg of total RNA isolated from WT and TR tobacco plants using the SuperscriptIII First-strand cDNA synthesis system (Invitrogen, USA). Relative expression of *PgP5CS* in transgenic plants was measured using Roche Light Cycler 480™ (Roche, Germany), and the control plant Ct value was set as onefold for comparison. Three technical replicates were taken per sample. 10 μl reaction mixture contained 0.5 μl cDNA (50 ng), 5 pmol primers (Supplementary Table 1), and 5 μl 2X SYBR Green PCR Master Mix (Roche, Germany). Fold change was calculated by comparing the normalized transcript level of *PgP5CS* in transgenic plants to that of control (wild) plants using LC480 software (Roche, Germany) with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The *Nt*18S *rRNA* gene was used as an internal control. qRT-PCR was carried out under controlled conditions in all PCR positive transgenic plants. The Ct value of the lowest expressing transgenic plant was considered as a one-fold comparison. The qRT-PCR was also carried out in a few selected transgenic plants under heat and drought conditions. Further, five key genes (glutamate dehydrogenase, proline dehydrogenase, proline 5-carboxylate synthetase, pyrroline-5-carboxylate reductase, pyrroline-5-carboxylate dehydrogenase) are involved in the proline biosynthesis pathway were analyzed for differential expression in one selected transgenic tobacco plant.

Proteome analysis of WT and TR tobacco plants using Two-Dimensional SDS-PAGE

Leaf tissue from the one-month-old TR tobacco plant and WT tobacco plants was collected for proteomics analysis. The leaf tissue sample was finely powdered in liquid nitrogen with 1% PVP (Polyvinylpyrrolidone) and 200 mg of powdered tissue was resuspended in 1 ml of protein solubilization buffer (100 mM Tris–HCl pH-8, 0.3% SDS, 1X plant protease inhibitor, 1 mM PMSF, 0.2 M DTT). The solution was incubated at 22 °C for 30 min and thereafter centrifuged at 12,000 g (4 °C) for 15 min. The supernatant was collected in a fresh Eppendorf tube, and 1 ml of chilled precipitation buffer was added (10% TCA in acetone, 0.07% β-ME) and incubated overnight at − 20 °C. The solution was centrifuged at 12,000 g for 15 min at 4 °C. The further pellet was washed with prechilled wash buffer (90% acetone in water, 0.07% β-ME) 5 times to get a white clean protein pellet. The protein pellet was resuspended in rehydration buffer [(7 M Urea, 2 M Thiourea, 4% CHAPS, 2% IPG buffer (pI 3–10), and 61 mM DTT)]. The protein was quantified by Bradford reagents (BioRad, UDA), and 250 μg of protein sample (TR tobacco and WT plants separately) was loaded to 13 cm, pI 3–10 Immobiline dry strips (Amersham biosciences, USA) along with 1% DeStreak reagent. The samples were incubated overnight with strips in disposable reswelling trays (22 °C). The strips overlaid with mineral oil were focused on an IPG-phor III instrument (Amersham Biosciences, UK) at 100 V for 3 h, 2 h at 500 V, 2 h at 1000 V, 8000 Vh at 5000 V, 2500 Vh at 5000 V, 3000 Vh at 8000 V. The strips were equilibrated in 10 ml of solution I [(containing 6 M Urea, 75 mM Tris–HCl (pH 8.8), 30% glycerol, 2% SDS & 100 mg DTT (dithiothreitol)] for 15 min with gentle shaking. After discarding solution I, solution II (containing 6 M Urea, 75 mM Tris–HCl (pH 8.8), 30% glycerol, 2% SDS, and 125 mg iodoacetamide) was added and incubated for 15 min. The second dimension run was performed on the Hoefer SE 600 electrophoresis unit (Fischer Scientific, USA). The strips were placed on 12% SDS–polyacrylamide resolving gel, 1.5 mm thick along with a prestained protein marker. After the run was over, gels were stained with silver staining using the SilverQuest staining kit (Invitrogen, USA).

Protein gels were compared after scanning and digitization. Different parameters like protein spot densities, image smoothening, spot detection, spot quantification, image alignment, spot matching, molecular weight, and pI calculation were performed using the Melanie software tool (http://2d-gel-analysis.com/documentation/).

Statistical analysis

All data were obtained in three biological replicates. Data points represent the mean values ± standard deviation of three biological replicates. The statistical significance was analyzed using Two-way Analysis of variance (ANOVA) [Dunnett's multiple comparisons test (physiological and biochemical data) and Tukey's multiple comparisons test (relative expression data) comparing wild and transgenic plants with respective treatments] to estimate significant differences among the means within every treatment between TR lines and WT plants. Asterisk (*) was used to indicate the significant difference. For each ANOVA test residuals, we conducted Shapiro–Wilk tests for normality and Levene's tests for variance equality. Results did not justify rejecting the null hypotheses, affirming our use of parametric methods.

Results

Isolation and cloning of PgP5CS

The isolated total RNA from drought imposed and control *P. glaucum* plants revealed the presence of 28S and 18S rRNA bands. The prepared cDNA was observed as a smear in the range of 250 bp to 3 kb which showed that the preparation is of good quality (Supplementary Fig. 1A & B). The expression of *P5CS* in *P. glaucum* was observed to increase from ½ h to 4 h of drought and then it declined at 8 h after drought treatment (Supplementary Fig. 1C). The CDS (coding DNA sequence) of *PgP5CS* was 2151 bp, along with 81 bp 5′ UTR (Untranslated Region) and 354 bp 3′ UTR. The CDS (Accession number KJ459944) was successfully cloned in the pGEM-T vector and analyzed with restriction digestion (Supplementary Fig. 1D & E). The CDS encoded a protein of 716 amino acid residues, with a molecular mass of 79.58 kDa. The percentage of respective nucleotides in the coding region of the *PgP5CS* sequence (KJ459944) was deduced to be 28.17%, 27.90%, 24.97%, and 18.96% for adenine, thymine, guanine, and cytosine respectively. The *PgP5CS* amplified from genomic DNA of pearl millet showed a total of 5 exons and 4 introns, with a total length of 2861 bp (Supplementary Fig. 2).

Bioinformatic analysis of PgP5CS

PgP5CS showed a close phylogenetic relationship with P5CS of *Setaria italica*, *Oryza sativa, and O. brachyantha* (Supplementary Fig. 3), and showed a high level of sequence conservation with P5CS of other plant species. The 3D model was based on the template c2h5gA, which belongs to the crystal structure of human pyrroline-5-carboxylate synthetase (Supplementary Fig. 4A). 407 amino acid residues (57% coverage) of the sequence were modeled with 100% confidence (Supplementary Fig. 4B). Model dimensions given in angstrom as: X:78.635 (Å) Y:73.218 Å Z:50.024 Å. The residues present at active sites were LEU_{355}, GLY_{411}, PHE_{412}, GLU_{413}, ARG_{415}, ILE_{474}, ARG_{490}, GLY_{491}, SER_{492}, ASN_{493}, GLN_{494}, LEU_{495}, VAL_{496}, GLN_{498}, ILE_{499}, SER_{502}, HIS_{510}, ALA_{511}, CYS_{543}, GLU_{596}, HIS_{624}, GLY_{669}, ILE_{670}. The Ramachandran plot showed that 95.2% of amino acid residues were in the favored region, and 4.2% and 2% residues were in the allowed and outlier regions respectively (Supplementary Fig. 4B). Procheck and Verify3D software further predicted that the structure was fairly good. Two conserved domains namely glutamate-5-kinase (G5K) and γ-glutamyl phosphate reductase were observed in the PgP5CS protein structure. Further, the *N*-linked glycosylation was predicted to have 4 potential sites (amino acid residue position- 77, 176, 311, 651) and among them, two sites at positions 77 and 311 were the most potent. *O*-linked glycosylation revealed 8 potential sites (amino acid residue position—60, 117, 207, 260, 290, 402, 631, 645), among them, two sites at positions 117 and 207 were the most potent (Supplementary Fig. 4C & D). The predicted secondary structure along with the key color code and composed of 28 α-helices, 50 coils, and 23 β-strands (Supplementary Fig. 4E). The string-based analysis revealing protein-to-protein interaction has been shown (Supplementary Fig. 4F). Multiple sequence alignments revealed that our PgP5CS was most closely related

to the P5CS of *Setaria italica* and was categorized as isoform P5CS2 (Supplementary Fig. 3 and 5).

Construction of binary vector and generation of transgenic tobacco

The CDS of *PgP5CS* was successfully cloned in binary vector pCAMBIA1300U at *Bam*HI and *Kpn*I restriction sites. The developed binary vector was named pCAMBIA1300U-*PgP5CS* (Fig. 1A). Out of 10 putative transgenic tobacco plants obtained, 6 plants survived hygromycin selection and these were further maintained on hygromycin 30 mg L^{-1}, with regular subculturing at an interval of 15 days (Fig. 1B). The surviving healthy plants were transferred to soilrite media in pots under 16 h/8 h day/night conditions at 23 °C and allowed to grow till maturity.

Confirmation of transgenic tobacco overexpressing PgP5CS

The isolated genomic DNA from putative transgenics (TR) and wild-type (WT) tobacco plants were analyzed for the presence of the transgene. The PCR results revealed that all 6 putative tobacco plants were observed to show successful amplification with four different sets of primers i.e., *hpt*II specific (750 bp),

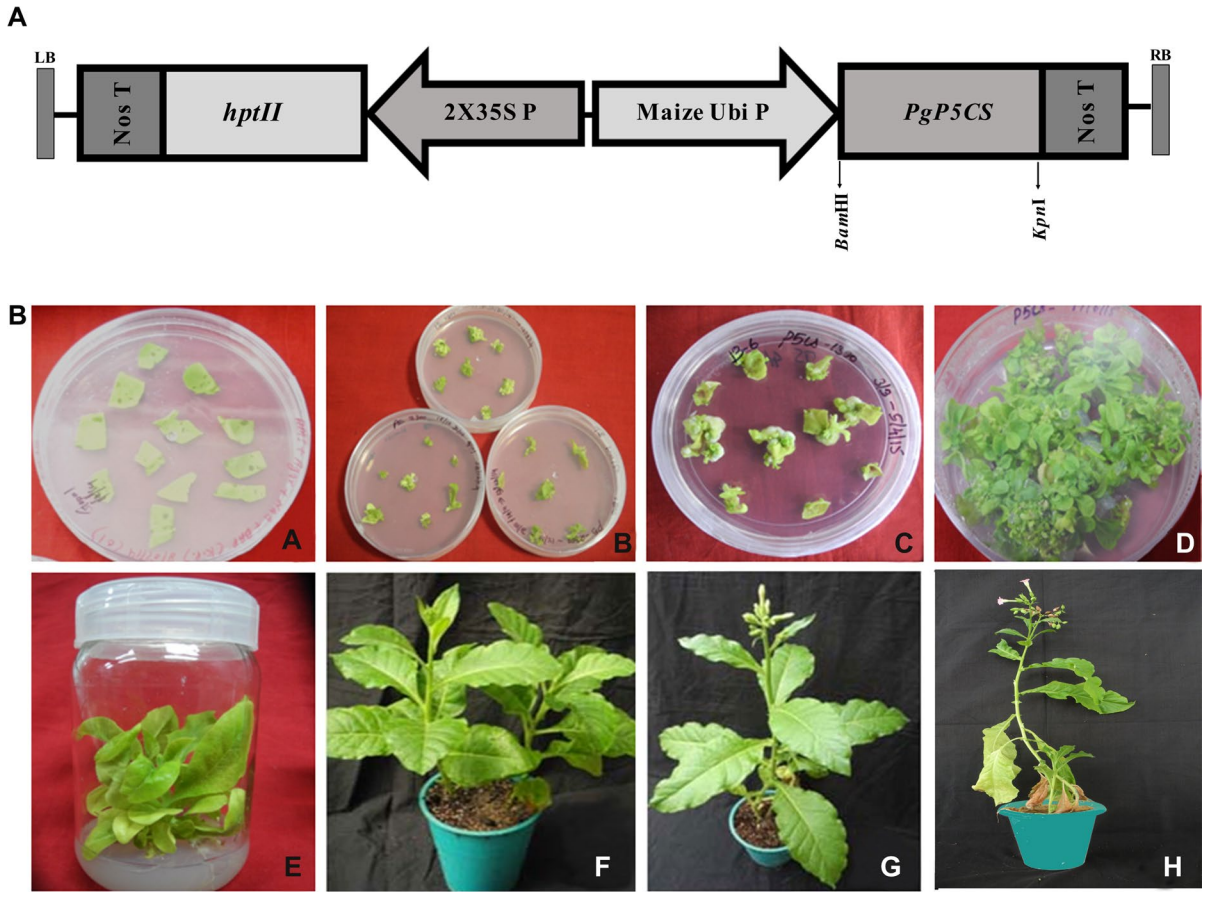

Fig. 1 Binary vector and stages for tobacco plant transformation and germination A- Binary vector (pCAMBIA1300U-*PgP5CS*) showing main components between left and right border termini, a vector carrying maize *ubiquitin* promoter and *hpt*II as plant selection marker. B- Different stages of germination after tobacco transformation **a** Tobacco leaf discs used for *Agrobacterium*-mediated transformation **b** Callus formation after hygromycin selection **c** Bud formation at 14th **d** Shoot formation at 20th and **e** Root and shoot formation at 25th day of culture **f** Hardening of putative transgenic tobacco plants **g** Mature putative tobacco transgenic plant (mother plant)

PgP5CS CDS specific (2151 bp), construct specific (2549 bp) & *PgP5CS* internal primers (1045 bp). WT plants & binary vectors carrying *P5CS* kept as negative & positive control respectively were observed to show results in the expected manner (Supplementary Fig. 6). Southern hybridization analysis of three selected plants (P2, P3, and P4) with probe *PgP5CS*, confirmed the integration of transgene as a single copy in all the three TR tobacco plants (Fig. 2A). The signals were observed at different independent locations in all three TR tobacco plants. The integration of transgene in one of the transgenic tobacco plants was confirmed by genome walking which revealed the presence of left border termini, tobacco genome sequence followed by adaptor sequence in an expected manner (Fig. 2B).

Physiological and biochemical characterization of PgP5CS TR tobacco plants

In response to drought, total chlorophyll content and relative water content (RWC) were shown to decrease in selected T_0 -TR plants (P2, P3, P4) and WT plants (Fig. 3A and B), whereas proline and MDA content increased. TR plants showed a substantial difference when compared to WT plants (Fig. 3C and D). Under control circumstances, the RWC in WT and transgenic plants ranged from 21.79 to 23.81%. In drought, the RWC in WT plants decreased to 17.51% and 8.17% after 6 d and 12 d of the drought. In the case of TR tobacco plants, RWC ranged from 20.19 to 21.46% and 13.12 to 15.70% after 6 d and 12 d of drought (Fig. 3A). The total chlorophyll content in tobacco plants (WT and TR) was 1.19 to 1.24 mg/g FW under normal conditions, which was reduced to 0.71 to 0.574 mg/g FW in WT plants. While in TR plants, the chlorophyll content was 0.91–0.98 mg/g FW and 0.69–0.88 mg/g FW after 6 d and 12 d of DS (Fig. 3B). The total proline content under control conditions (WT and TR) was in the range of 1.02–1.21 µmol/g FW. As a result of drought stress, a slight increment was observed in all tobacco plants. In the case of WT plants, it was 2.12 and 2.89 µmol/g FW after 6 d and 12 d of drought. In the case of TR tobacco plants, the proline content was 2.45–2.52 and 3.12–4.5 µmol/g FW after 6 d and 12 d of drought (Fig. 3C). Similarly, MDA content which relates to the membrane injury was observed to increase from 1.625 to 2.1 nmol/g FW in WT plants after 12 d of the drought. In the case of TR tobacco plants, the MDA content after 6 d of drought was 1.33–1.45 nmol/g FW, while it was 1.56–1.65 nmol/g FW after 12 d of drought

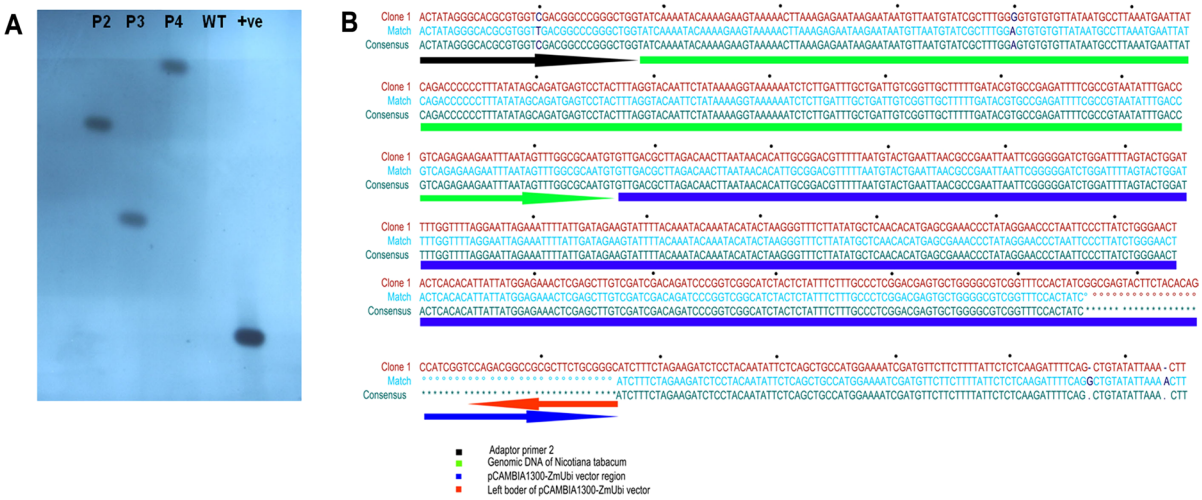

Fig. 2 Southern blot and T-DNA insertion analysis A-Southern blot analysis of transgenic tobacco, PCR purified *hpt*II amplicon was used as a positive control, and genomic DNA of wild type tobacco was used as a negative control. P2, P3, P4, WT &+ve indicate transgenic plants, negative control, and positive control respectively. B-T-DNA insertion analysis showing the integration of transgene in tobacco genome (orange marked arrow portion is adaptor sequence, green marked arrow portion is Tobacco genome, blue marked arrow portion is left border part of the vector)

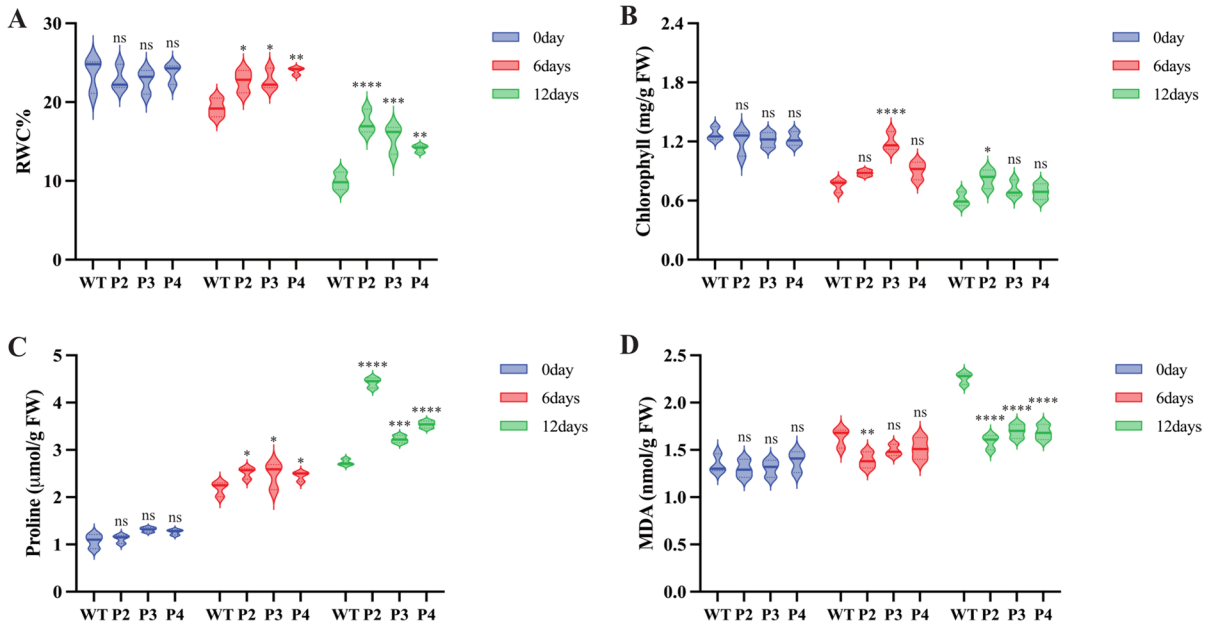

Fig. 3 Analysis of *PgP5CS* expressing transgenic plants under drought stress conditions A- Analysis of relative water content. B- Total chlorophyll C- Proline and D- MDA content. The data represent the mean of three replicates (n = 3), and the bar shows the SE. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0006$; **** $P < 0.0001$, NS- non-significant., comparing wild and transgenic plants with respective treatments. WT-wild type, P2, P3, and P4- transgenic plants expressing *PgP5CS*

(Fig. 3D). Overall, the TR tobacco plants could tolerate more drought as compared to WT plants.

Similar results were obtained in TR plants under Heat Stress (HS) conditions when compared to WT plants. The RWC, total chlorophyll content was observed to decrease in all the tobacco plants after HS, but TR tobacco plants showed a significant level of difference as compared to WT plants (Fig. 4A and B). The total proline content and MDA content were observed to be increased after HS treatment in TR and WT plants, but a significant increment was there in TR tobacco plants (Fig. 4C and D). Overall, it may be concluded that the TR tobacco plants could tolerate more HS as compared to WT plants.

The photosynthetic parameters (photosynthetic rate, stomatal conductance, intracellular CO_2 concentration, transmembrane conductance) measured using the IRGA instrument revealed that the physiological indexes decreased under abiotic stress conditions in all tobacco plants. It was observed that there was a significant difference between WT and TR tobacco plants (Fig. 5). Under control conditions, the photosynthetic rate in tobacco plants ranged from 2.337 to 2.516 μmol m^{-2} s^{-1}. After drought, the photosynthetic rate in WT plants was 1.011 μmol m^{-2} s^{-1}, while in TR tobacco plants it was in the range of 1.524–1.854 μmol m^{-2} s^{-1}. The stomatal conductance of tobacco plants under control conditions was 0.195–0.239 mmol m^{-2} s^{-1} (Fig. 5A). Under DS conditions, the stomatal conductance of the WT plant was 0.0129 mmol m^{-2} s^{-1}, while the TR tobacco plants were 0.0156–0.0185 mmol m^{-2} s^{-1} (Fig. 5C). The intracellular CO_2 concentration in tobacco plants under controlled conditions was 105–132.371 μmol mol^{-1}, while under drought the intracellular CO_2 concentration dropped to 50.308 μmol mol^{-1}. The TR tobacco plants maintained intracellular CO_2 concentrations in the range of 55.654–70.562 μmol mol^{-1} (Fig. 5E). Similarly, the transpiration rate under drought conditions was in the range of 0.248–0.275 mmol m^{-2} s^{-1}, which decreased to 0.118 mmol m^{-2} s^{-1} in WT plants. The TR tobacco plant's transpiration rate was in the range of 0.150–0.184 mmol m^{-2} s^{-1} (Fig. 5G). All these results concluded that the TR tobacco plants having *PgP5CS* maintained higher photosynthetic parameters than WT plants. Similarly, the TR tobacco *PgP5CS* plants could maintain high photosynthetic

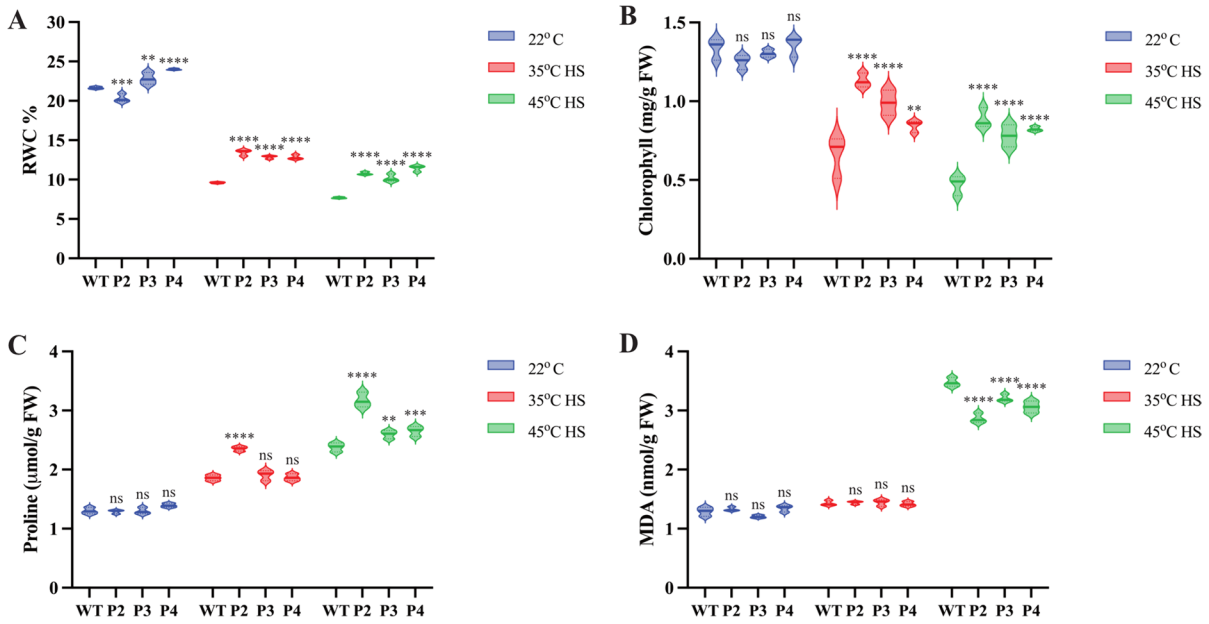

Fig. 4 Analysis of *PgP5CS* expressing transgenic plants under heat stress conditions **A**- Analysis of relative water content. **B**- Analysis of total chlorophyll content. **C**- Proline content. D- MDA content. The data represent the mean of three replicates (n = 3), and the bar shows the SE. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0006$; **** $P < 0.0001$, NS- non-significant., comparing wild and transgenic plants with respective treatments. WT-wild type, P2, P3, and P4- transgenic plants expressing *PgP5CS*

parameters under HS conditions as compared to WT plants (Fig. 5B, D, F and H). Despite this, no distinctions in morphology occur between transgenic and wild plants. However, no data on plant growth, morphology, or biomass were obtained for transgenic plants compared to wild-type plants.

Expression analysis of transgenic plants by qRT-PCR

Expression analysis of *PgP5CS* was carried out under drought and HS conditions. The Ct value of each transgenic plant under control conditions was set as onefold for comparison. The transgenic plants P2, P3, and P4 showed 5, 2, and 2.1 folds after 6 d of drought. While after 12 d of drought, the plants showed 10, 2.7, and 7.2 fold changes respectively (Fig. 6A). The transgenic plants P2, P3, and P4 showed 5, 5.1, and 2.0 folds at 37 °C of HS, while at 45 °C (HS) the plants showed 7.5, 5.2, and 5.3 fold changes respectively (Fig. 6B). Overall, it was observed that as the abiotic stress conditions increased, a slight fold change in the gene expression also increased. Out, of the three TR plants, P2 showed the highest fold change expression under drought and HS. The key genes involved in the proline biosynthesis pathway (*GDH, PDH, P5CDH, P5CS P5CR*) analyzed in one of the transgenic plant P2 revealed that under HS conditions, only gene *P5CS* was upregulated and other key linked genes were either downregulated or showed a basal level of expression (Supplementary Fig. 7).

2D SDS-PAGE gel analysis

The silver-stained SDS-PAGE gel image of transgenic plant P2 (control and HS conditions) was analyzed using the Melanie software tool (http://2d-gel-analysis.com/documentation/). A marked variation was observed in the transgenic sample as compared to wild type control. Superimposed gel images detected common spots as shown in Supplementary Fig. 8A & B., a total of 122 protein spots were detected in both the gels (61 in each gel) with common 46 spots. The unmatched gel spots were marked and considered unique spots. A total of 6 unique spots were found in both gels. The fold change expression varied from 0.0785 (minimum, Match ID 42) to 1.55 (maximum, Match ID 9) considered as up-regulated. Match ID 9,

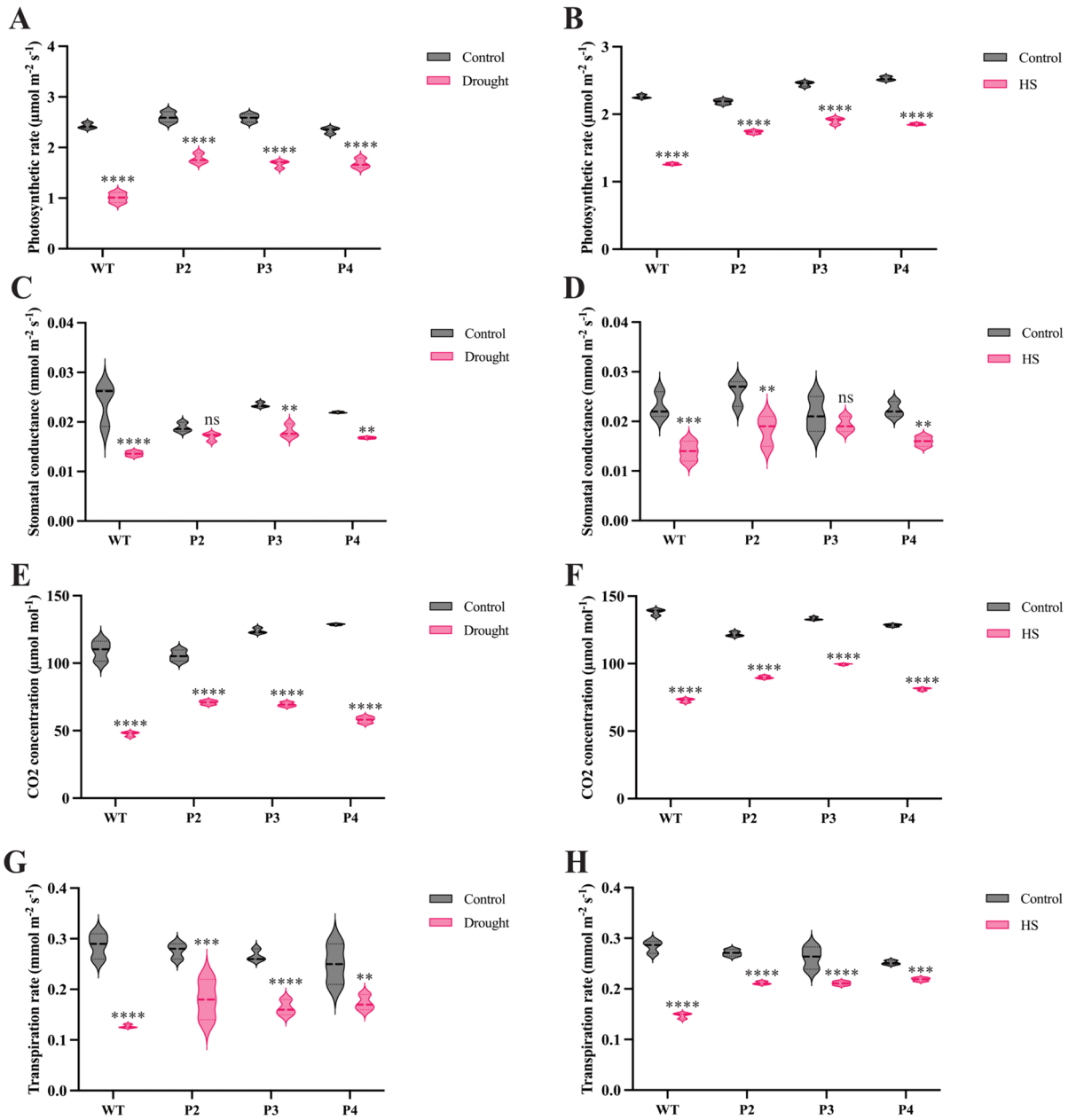

Fig. 5 Analysis of photosynthetic parameters in the control and transgenic plants under drought and HS conditions Photosynthetic measurements were made at the end of the stress period using IRGA (LiCOR, USA). **A–B** Photosynthetic rate measurement in WT and transgenic plants. **C–D** Stomatal conductance. **E–F** Intracellular CO_2 concentration measurement. **G–H** Transpiration rate measurement. The data represent the means of five replicates (n = 5), the bar shows the SE, significant difference is indicated by an asterisk (*) at * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0006$; **** $P < 0.0001$, NS- non-significant. WT-wild type, P2, P3, and P4- transgenic plants expressing *PgP5CS*

35, 19, 18, 22, 16, showed up-regulation while match ID 42, 20, 28, 29, 51 etc. were down-regulated (Supplementary Table S3).

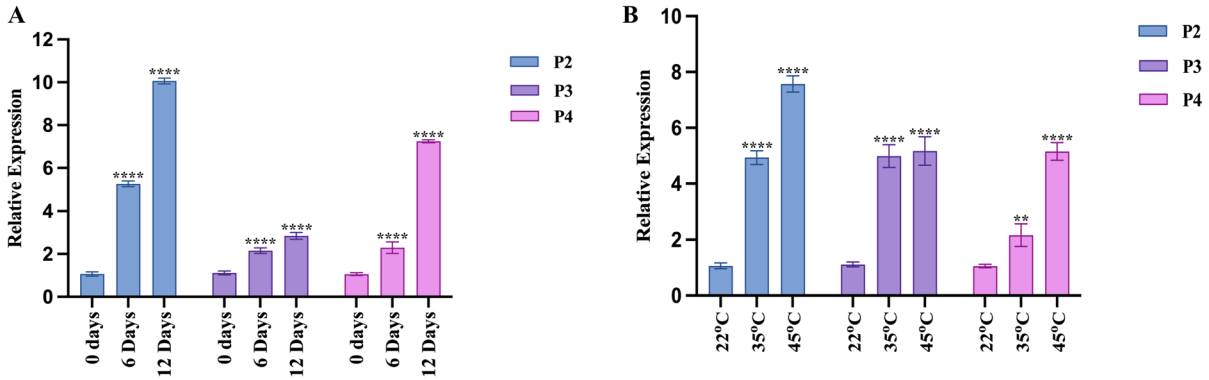

Fig. 6 Expression analysis of transgenic *PgP5CS* tobacco plants under control and abiotic stress conditions. **A**- qRT-PCR analysis of transgenic tobacco plants under DS conditions. **B**- qRT-PCR analysis of transgenic tobacco plants under HS conditions for XXXX days/h. The data represent the means of three technical replicates (n = 3), the bar shows the SE, and * represents the significance of the difference * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0006$; **** $P < 0.0001$, NS- non-significant

Discussion

In general, proline accumulation in plants exposed to drought is associated with the expression of P5CS, which is a key enzyme of proline biosynthesis. The P5CS gene has been isolated from several plants and studied for its physiological, biochemical, and functional properties. Nevertheless, in various species of plants, two forms of the P5CS gene (P5CS1 and P5CS2) have been found, and their functions are assumed to be distinct (Vendruscolo et al. 2007; Verdoy et al. 2006). In this study, the P5CS gene from *P. glaucum* was characterized for the first time at the molecular level. In an earlier study, the P5CS genes were reported from various plants like *Arabidopsis thaliana*, *Medicago truncatula*, *Oryza sativa*, and *Sorghum bicolor* where the gene length of two orthologs were 2154 and 2181 bp, 2301 and 2154 bp, 2208 and 2151 bp, and 2154 and 2181 bp respectively (Rai and Penna 2013). In this study, the length of *PgP5CS* gene was 2151 bp. As a result, though the length of the gene is almost near to P5CS1, at present it can be categorized as a PgP5CS2 isoform (Zhang and Becker 2015) as the gene was more similar (68.8%) to AtP5CS2 (At3g55610) than AtP5CS1 (At2g39800) (58.8%). In legume species *Lotus japonicus* three isoforms were identified (Signorelli and Monza 2017). However, additional investigation to isolate the other isoforms must still be carried out. A comparative investigation revealed that the PgP5CS is more comparable to the P5CS of *Setaria italica*. However, in this study PgP5CS has just four intron splicing sites, whereas *A. thaliana*, *M. truncatula, O. sativa,* and *S. bicolor* contain more than 18 intron splicing sites in both types of genes, P5CS1 and P5CS2 (Rai and Penna 2013). The secondary and tertiary structures, as well as predicted active sites have provided comprehensive understanding of how PgP5CS functions. Proteomics allows for global analysis of protein interactions, structural, functional, and abundance.

In this study, the induction of *PgP5CS* transcripts occurred in transgenic tobacco (TR) under heat and drought. The mRNA levels of *PgP5CS* in transgenic lines were higher when plants were stressed than in control under the maize ubiquitin promoter. Similarly, *AtP5CS* has been reported to be constitutively expressed under CaMV 35S promoter in transgenic potato plants and its accumulation is regulated by salt stress (Hmida-Sayari et al. 2005). Chen et al. (2009) reported that salt, drought, and cold stressors enormously triggered the *P5CS* gene in *Vigna aconitifolia* (mothbean). *OsP5CS1* expression increased in response to stress and accumulated during drought treatment in rice (Igarashi et al. 1997). Rapid increase of *AtP5CS1* mRNA has also been seen in Arabidopsis seedlings subjected to drought, NaCl, and ABA treatments (Yoshiba et al. 1995). *Chrysanthemum lavandulifolium ClP5CS1* and *ClP5CS2* expression increased in response to stress and was constitutively expressed in all organs (Zhang et al. 2014). In our study, *PgP5CS* expression increased in the TR

tobacco plants under heat and drought stress conditions. These results suggested that increased expression of *PgP5CS* not only occurred in response to drought but also response to heat stress. It is also reported that, during heat stress, the heat-inducible expression of the transgene *AtP5CS1* was capable of enhancing proline biosynthesis in transgenic *Arabidopsis* (Lv et al. 2011). In this study, over-expression of the *PgP5CS* gene in transgenic tobacco resulted in a higher accumulation of proline as compared to WT plants. Apart from *PgP5CS* expression, the current investigation determined the consequence of proline accumulation, MDA levels, chlorophyll content, RWC, photosynthetic gas exchange parameters (photosynthetic rate, stomatal conductance, intercellular CO2 concentration, transpiration rate) in transgenic versus non-transgenic *N. tabaccum* plants. The induction of higher *PgP5CS* expression leads to the accumulation of proline under the drought and heat stresses shown in this study. Similarly, proline accumulation increased in transgenic soybean overexpressing the L-Δ1-pyrroline-5-carboxylate reductase (P5CR) (Simon-Sarkadi et al. 2006) and chickpea and rice overexpressing P5CSF129A (Bhatnagar-Mathur et al. 2009; Kumar et al. 2010); wheat overexpressing P5CS (Pavei et al 2016). Other antioxidants, such as catalase (CAT), ascorbate peroxidase (APX), and malondialdehyde (MDA), increased in transgenic tobacco overexpressing P5CS (Zarei et al. 2012). Nevertheless, P5CS2 from *Phaseolus vulgaris* enhanced proline content in transgenic Arabidopsis when compared to P5CS1 (Chen et al. 2013). Proline content, water status, and the photosynthetic rate are positively related to plant abiotic stress tolerance while MDA production is negatively correlated to abiotic stress tolerance (Babitha et al. 2015; Panzade et al. 2020). In a similar study conducted by Babitha et al. (2015) the photosynthetic parameters (photosynthetic rate, stomatal conductance, intracellular CO2 concentration, transpiration rate) were observed to be high in TR tobacco plants under abiotic stress conditions (drought and HS) as compared to WT plants. Overall, transgenic tobacco plants harboring *PgP5CS* could maintain hydration status and photosynthesis, strengthening antioxidant activities and fewer membrane damages. Similar results have been obtained by Riahi and Ehsanpour (2013) in which tobacco plants overexpressing gene *P5CS* from *Vigna aconitifolia* could tolerate salinity stress. It is reported that overexpressing gene P5CS in transgenic petunia and tobacco provided drought tolerance by increasing proline levels (Kishor et al. 1995; Yamada et al. 2005).

Despite the knowledge of *P5CS* overexpression and the positive association of plant abiotic stress tolerance, few studies have concentrated on the status of other important proline biosynthesis pathway genes (*Glutamate dehydrogenase, Δ1-pyrroline-5-carboxylate dehydrogenase, Δ1-pyrroline-5-carboxylate reductase*, and *Proline dehydrogenase*) in transgenics. Here we have observed that in our study of TR tobacco plant P2, the expression of *PgP5CS* was highly upregulated while the expression of other genes was at a basal level. Eventually it could be due to a negative feedback inhibition mechanism of gene *P5CS*, prohibiting other proline biosynthetic pathway genes from overexpressing (Supplementary Fig. 7). Proline biosynthetic pathway involves five important genes i.e. *P5CS, P5CR, ProDH, GDH, P5CDH*, coding for the enzymes Pyrroline-5-carboxylate synthetase, Pyrroline-5-carboxylate reductase, Proline dehydrogenase, Glutamate dehydrogenase, Pyrroline-5-carboxylate dehydrogenase respectively (Kishor et al. 2005). For the enhanced production of proline in proportion to the expression of *P5CS* gene, all the other genes of the pathway must be also overexpressed. The qRT-PCR studies of genes of proline biosynthesis pathway in transgenic tobacco line showed that, with the exception of *PgP5CS*, all the other genes (*P5CR, ProDH, GDH, P5CDH*) were not overexpressed, which could be one of the reasons why proline production is not directly related to *P5CS* overexpression. Studies by other researchers have also shown that proline production is not enhanced unlimitedly. Transgenic wheat expressing the *VaP5CS* gene driven by maize ubiquitin promoter increased proline under stress conditions. Interestingly, the same gene under the control of a stress-inducible promoter did not significantly vary the proline levels in the plant (Pavei et al. 2016). Chen et al. (2013) have also shown that the overexpression of *PvP5CS* in transgenic *Arabidopsis* contributed to higher proline under stress conditions. Ibragimova et al. (2015) showed that T0 tobacco transgenic plants expressing the *AtP5CS* gene had four times the proline level found in control plants. However, the possibility of a 'proline feedback inhibition' mechanism controlling the expression of gene *PgP5CS* in TR tobacco plants cannot be ruled

out (Zhang et al. 1995). Further investigation in this direction is still needed. However, to our knowledge, this is the first report of TR tobacco plants overexpressing the PgP5CS gene, which will be used for functional confirmation of the proline biosynthesis pathways genes required.

Conclusions

Research on the *PgP5CS* gene in pearl millet has been associated with drought and heat tolerance. The *PgP5CS* gene is known to play a crucial role in the synthesis of proline, an amino acid that acts as a compatible solute and helps plants to cope with various environmental stresses, including drought and high temperatures. This increased proline content is believed to contribute to improved tolerance to drought and heat stress in plants, including pearl millet. Supported by a recent report of transcriptome analysis of heat tolerant *P. glaucum* genotype-841-B (Maibam et al. 2022) we suggest that, in our study, the P5CS from *P. glaucum* has a high level of upregulation in both drought and heat conditions, and it might play an important role in abiotic stress tolerance. It was concluded that alleviation of drought and heat stress was associated in part with enhanced expression of the *PgP5CS* gene and as a result proline accumulation. This proline accumulation could function as an osmolyte for intracellular osmotic adjustment and play a critical role in maintaining relative water content and water potential under drought and heat stress conditions. Thus, our results suggest that *PgP5CS* positively affects the growth of plants under abiotic stress, and it may thus constitute a candidate gene for breeding abiotic stress tolerant crops.

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Author contributions GS carried out tobacco transformation, physiological and biochemical assays, molecular analysis, and manuscript writing. MC, AT isolated *PgP5CS* gene. RSJ carried out the bioinformatic analysis. AT, RSJ involved in manuscript editing. HV was involved in the physiological and 2D-SDS PAGE. JCP conceptualized the research plan, designed experiments, involved in manuscript preparation and editing. All the authors have read and approved the manuscript.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors reported no potential conflict of interest.

Research involving human participants and/or animals Authors declare the research did not involve Human Participants and/or Animals.

Informed consent Authors consent to the publishing of this manuscript.

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