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Tissue-specific Regulation of Dihydrodipicolinate Synthase (DHDPS) Activity and Gene Expression in *Medicago truncatula* under Abiotic Stress

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ABSTRACT

Background: Abiotic stresses such as salinity and drought significantly reduce plant productivity worldwide. Dihydrodipicolinate synthase (DHDPS), a key enzyme in lysine biosynthesis, may play a crucial role in stress adaptation.

Methods: In this study, we examined the tissue-specific expression patterns of the DHDPS gene and its corresponding enzyme activity in *Medicago truncatula* exposed to salinity (200 mM NaCl) and osmotic (180 mM mannitol) stress. Hydroponically grown three-week-old plants were subjected to short-term (2 h) and prolonged (24 h) treatments. Gene expression was quantified using a rigorously optimized quantitative real-time PCR (qRT-PCR) protocol and data were analyzed using CFX Manager™ software.

Result: Our findings revealed a pronounced tissue-specific dichotomy in DHDPS expression and enzyme activity: transcript levels were significantly upregulated in roots (up to ~12-fold), while they were concurrently downregulated in leaves (by up to approximately half) across both stress conditions. Notably, root expression peaked under short-term mannitol exposure, suggesting a rapid and robust metabolic response likely linked to lysine-derived protective compounds. In contrast, the downregulation observed in leaves may reflect an energy-conserving strategy or a shift in metabolic priorities under stress. This distinct organ-specific regulation underscores the complexity of plant stress adaptation and offers promising avenues for targeted engineering of crop resilience.

Key words: DHDPS, *Medicago truncatula*, Osmotic stress, qRT-PCR, Salinity, Tissue-specific expression.

INTRODUCTION

Abiotic stresses, particularly salinity and drought, significantly impair plant growth and productivity, posing major global agricultural challenges (Ahmad *et al*., 2024; Dovrat *et al*., 2019; Huguet and Prosperi, 1996). These stresses induce complex physiological and biochemical responses, often leading to reduced water potential, ion toxicity and oxidative damage (Katam *et al*., 2022; Liu *et al*., 2024). Therefore, a comprehensive understanding of the molecular mechanisms underlying plant adaptation to these stresses is crucial for developing resilient crop varieties capable of sustainable production, especially in challenging environments.

Legumes, including *Medicago truncatula*, are economically vital crops contributing to global food security and sustainable agriculture due to their symbiotic nitrogen fixation capabilities (Bustamante *et al*., 2013; Elmsehli *et al*., 2015). Despite their significance, legumes are highly susceptible to abiotic stresses, resulting in substantial yield losses worldwide (Hong *et al*., 2020; Huguet and Prosperi, 1996). *M. truncatula* serves as an excellent model legume for genetic and genomic studies, attributed to its relatively small genome, diploid nature and extensive genetic resources (Rose, 2008; Young *et al*., 2011; Manoj *et al*., 2015).

Lysine, an essential amino acid, is fundamental for plant protein synthesis and serves as a precursor for various specialized metabolites, including polyamines, which are implicated in stress tolerance (Arruda *et al*., 2020; Stepansky *et al*., 2005). The biosynthesis of lysine is tightly regulated, involving several key enzymatic steps. Dihydrodipicolinate synthase (DHDPS; EC 4.3.3.7) is a pivotal enzyme in this pathway, catalyzing the committed step in the diaminopimelate branch of lysine biosynthesis (Thu *et al*., 2007; Zhu *et al*., 2002). Previous studies indicate that DHDPS expression and activity are influenced by

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environmental cues and developmental stages (Dereppe *et al*., 1992). For instance, DHDPS activity in *Arabidopsis thaliana* is known to be regulated by feedback inhibition by lysine. However, a comprehensive understanding of DHDPS regulation under abiotic stress, particularly in *M. truncatula*, remains limited (Huguet and Prosperi, 1996). Given the critical role of lysine and its derivatives in stress response, investigating DHDPS expression under salinity and osmotic stress can provide valuable insights into plant adaptive strategies (Sikdar and Kim, 2010; Vauterin *et al*., 1999).

Quantitative real-time PCR (qRT-PCR) is a highly sensitive and widely employed technique for accurate mRNA quantification in gene expression analysis (Bödör *et al*., 2007; Bustamante *et al*., 2013). The reliability of qRT-PCR results hinges on the proper selection and validation of stable reference genes and optimization of experimental parameters (Livak and Schmittgen, 2001; Niu *et al*., 2011). While DHDPS has been explored in various contexts, a comprehensive and rigorously optimized qRT-PCR analysis of its tissue-specific expression in *M. truncatula* under combined salinity and osmotic stress has not been reported.

Building upon this knowledge gap, this study aimed to comprehensively investigate the tissue-specific transcriptional regulation and corresponding enzyme activity of the *DHDPS* gene in *M. truncatula* roots and leaves when subjected to salinity and osmotic stress. We utilized a meticulously optimized qRT-PCR protocol to ensure the accuracy and reliability of our gene expression data. The insights derived from this research are expected to enhance our understanding of stress adaptation mechanisms in legumes and offer potential targets for biotechnological approaches to improve crop stress tolerance.

MATERIALS AND METHODS

Plant material and stress treatments

Medicago truncatula seeds were surface-sterilized with 70% ethanol (1 min) and 5% sodium hypochlorite (10 min), followed by three rinses with sterile distilled water. Seeds were germinated on 1/2 strength Gamborg B5 medium (Duchefa Biochemie) solidified with 0.8% agar, incubated at 24°C in the dark for 2 days, then transferred to a 16 h light (200 µmol m^{-2} s^{-1}) / 8 h dark cycle for 5 days. Seedlings were subsequently grown in an aerated 1/4 Hoagland solution hydroponic system for 3 weeks under controlled conditions (16 h light/8 h dark, 24°C/20°C day/night, 60% relative humidity). The hydroponic solution was refreshed every three days.

For stress treatments, three-week-old plants were randomly assigned to control and treatment groups. Control plants were maintained in 1/4 Hoagland solution. Treatment groups were exposed to either 200 mM NaCl (salinity stress) or 180 mM mannitol (osmotic stress). These concentrations represent moderate to severe stress conditions relevant to *M. truncatula*'s natural or agricultural habitats, commonly employed in plant stress physiology studies to elicit clear responses. No visible wilting or severe chlorosis was observed during the 24-hour treatment, confirming plant viability for subsequent molecular analysis. Both stress treatments were applied for 2 hours (short-term) and 24 hours (prolonged). Each treatment consisted of three independent biological replicates, with 5 plants per replicate. After the respective treatment periods, roots and leaves were separately harvested, immediately flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from approximately 100 mg of frozen root and leaf tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quantity and purity were assessed using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) by A260/A280 and A260/A230 ratios. RNA integrity was confirmed by electrophoresis on a 1.2% agarose gel, ensuring high quality across all samples. For cDNA synthesis, 2 µg of total RNA was reverse-transcribed using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. cDNA samples were diluted 1:5 with nuclease-free water and stored at -20°C.

Primer design and validation

Specific primers for the *DHDPS* gene were designed using Primer-BLAST (NCBI) with forward primer (5′-3′): TGTCCGATACTCGAGAAGTTCC, reverse primer (5′-3′): GACAGCTCGTCTCTTGGTTCAG, product size (bp): 120. Primer specificity was validated through melt curve analysis and gel electrophoresis of PCR products.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and Fast EvaGreen Supermix (Bio-Rad). Each 20 µL reaction mixture contained 10 µL SsoFast EvaGreen Supermix, 1 µL of each primer (10 µM), 2 µL of diluted cDNA template and 6 µL of nuclease-free water. Non-template controls (NTCs) and no-reverse transcriptase controls (-RT) were included. Cycling conditions were: Initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and an optimized annealing/extension temperature for 30 s. Melt curve analysis (95°C, 0.5°C increment every 5 s) confirmed primer specificity and absence of primer dimers. Standard curves were generated for DHDPS gene using five-fold serial cDNA dilutions (1:5 to 1:3125) to determine amplification efficiency (E) and correlation coefficient (R^2).

Data analysis

Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with untreated control root samples (Rc) serving as the calibrator. Three independent biological replicates were used per treatment, each with three technical replicates. Data were analyzed using CFX Manager Software (Bio-Rad). Statistical significance between treatments was determined by one-way ANOVA followed by

Tukey's HSD post-hoc test ($p<0.05$). All data are presented as mean ± Standard Error of the Mean (SEM).

DHDPS enzyme activity assay

Enzyme extraction and partial purification

Approximately 100 mg (fresh weight) of frozen root and leaf tissues were ground to a fine powder in liquid nitrogen. The powdered tissue was homogenized in 2 mL per gram of material of ice-cold Extracting buffer (100 mM potassium phosphate (pH 8.0), 1 mM disodium EDTA (Na, EDTA), 20% (v/v) glycerol and 10 mM β-mercaptoethanol). The homogenate was filtered through Miracloth and centrifuged at 8,000 rpm for 20 min. The supernatant underwent ammonium sulfate precipitation; solid ammonium sulfate was added to achieve 60% saturation (36.1 g per 100 mL supernatant). The mixture was stirred for 30 min at room temperature, then centrifuged at 8,000 rpm for 20 min (Ellen *et al*., 2013; Ghislain *et al*., 1990).

The protein pellet was dissolved in 0.2 mL per gram of original material of Diluted Buffer (50 mM KPi (pH 7.6), 1 mM EDTA (0.4 g/L) and 20% (v/v) glycerol). Sodium pyruvate was added to a final concentration of 50 mM. This solution was heat-treated at 65°C for 5 min for further partial purification. Following heat treatment, the sample was centrifuged at 8,000 rpm for 20 min and the supernatant, containing the partially purified DHDPS enzyme, was collected for activity assays.

Protein quantification

Total protein concentration in the partially purified enzyme extracts was determined using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

DHDPS enzyme activity measurement

DHDPS enzyme activity was measured in a total reaction volume of 1 mL, based on the protocol by Kumpaisal *et al*. (1987) with minor modifications (Katam *et al*., 2022). The standard assay mixture comprised 100 µL of 100 mM Tris-HCl buffer (pH 8.0), 100 µL of 370 mM sodium pyruvate, 50 µL L-Aspartate-β-semialdehyde (L-ASA) (neutralized with 4 N KOH immediately before use) and 715 µL of distilled water containing the enzyme extract. The reaction was initiated by adding the enzyme extract. Control reactions without enzyme were included (Frisch *et al*., 1991).

The chromophore product formed between dihydrodipicolinate and *o*-aminobenzaldehyde (ABA) was quantified. An ABA stock solution (1 mg in 30 µL of 98% ethanol) was prepared and 35 µL of this solution was added to each reaction. Color development occurred for 40 min at 30°C in darkness. Denatured protein was removed by centrifugation for 3 min at 8,000 rpm. Spectrophotometric absorbance of the supernatant was then determined at 540 nm. The developed purple color remained stable for at least 1 hour at room temperature.

Specifically, all DHDPS enzyme activity assays were performed in three independent biological replicates (n=3). The results are presented as the mean ± standard deviation (SD) of these three replicates.

Calculation of specific activity

One unit of enzyme activity was defined as a net absorbance increase of 0.001 per minute. Specific enzyme activity was calculated using the following formula:

$$\text{Activity (Units)} = \frac{1000 \times \Delta OD_{540}}{60 \text{ min} \times \text{protein (µg/mL)}}$$

Where:

ΔOD_{540} = Represents the increase in absorbance at 540 nm over 60 minutes of reaction, corrected by subtracting the absorbance of the blank sample (without L-ASA). 60 min is the reaction time. Protein (µg/mL) is the protein concentration of the enzyme extract within the 1 mL reaction mixture. Specific activity was expressed as units per milligram of protein (units/mg protein).

RESULTS AND DISCUSSION

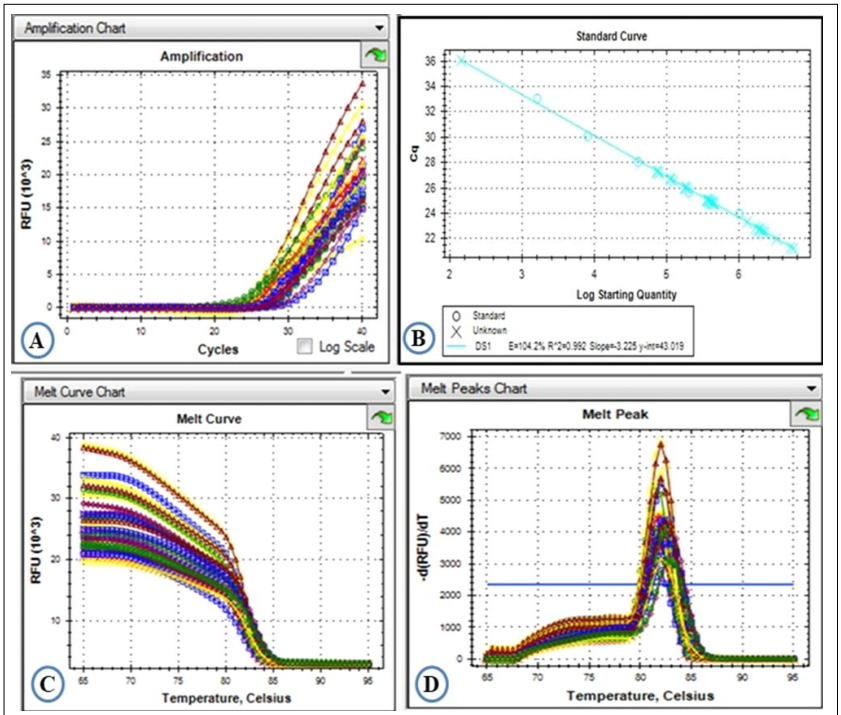
Optimization and specificity validation of DHDPS qRT-PCR assay

The qRT-PCR assay for the target gene, DHDPS, was successfully optimized for accuracy, reliability and reproducibility (Fig 1). Fig 1A displays representative amplification curves from 5-fold serial cDNA dilutions of the DHDPS sample, demonstrating excellent linearity and consistent shifts in Cq values proportional to initial template concentration. The generated standard curve (Fig 1B) yielded an amplification efficiency (E) of 104.2% (within the accepted range of 90-110%) and an R2 of 0.992, indicating a strong linear relationship between Cq values and the logarithm of initial cDNA concentration. Cq (quantification cycle) refers to the cycle number at which the fluorescence signal crosses a defined threshold; a lower Cq value indicates a higher initial amount of DNA or RNA template in the sample, whereas a higher Cq value signifies a lower initial template concentration.

Amplicon specificity was confirmed by melt curve analysis after every amplification run. As shown in Fig 1C, the DHDPS gene exhibited a single, sharp peak, confirming PCR product homogeneity and specificity, along with the absence of non-specific amplicons or primer dimers. The melt peaks chart (Fig 1D) associated with DHDPS amplification further visually confirmed product specificity and homogeneity. These results confirm that the DHDPS qRT-PCR assay has been successfully optimized, meeting stringent criteria for efficiency, linearity and specificity, thus providing a robust foundation for accurate quantitative DHDPS gene expression analysis.

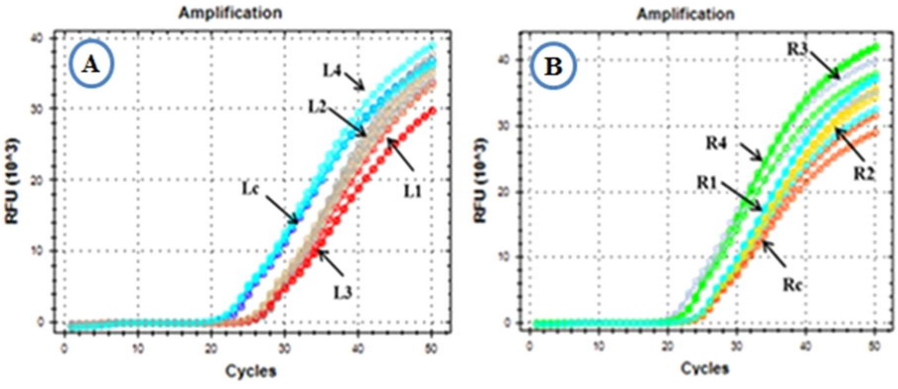
Fig 2 presents the DHDPS gene's amplification profiles across various stress treatments and control conditions, illustrating abiotic stress's impact on its expression in different *M. truncatula* tissues. Fig 2A displays amplification curves for DHDPS in leaf samples. Consistent with observed

downregulation trends in relative gene expression (Fig 3), amplification curves for stressed leaf samples (L1-L4) generally shifted right (indicating higher Cq values) compared to the control leaf sample (Lc). Specifically, L3 and L4 (salinity stress) showed a notable delay in amplification, suggesting reduced initial template concentrations. L1 and L2 (osmotic stress) also exhibited delayed amplification relative to Lc, albeit less pronounced than under salinity stress.



(A) Representative amplification curves across five-fold serial cDNA dilutions, illustrating amplification linearity and consistency. (B) Standard curve generated from DHDPS cDNA dilutions (C) Representative melt curve analysis for the DHDPS gene and (D) Melt peaks chart associated with DHDPS amplification, further confirming product specificity and homogeneity

Fig 1: Optimization parameters and specificity validation of the quantitative real-time PCR (qRT-PCR) assay for the Dihydrodipicolinate Synthase (DHDPS) gene.



(A) Amplification curves for DHDPS in leaf samples (L1-L4, Lc), showing varied amplification profiles across different stress treatments and control conditions. (B) Amplification curves for DHDPS in root samples (R1-R4, Rc), illustrating distinct amplification patterns in response to abiotic stress and control conditions. Specific sample conditions and corresponding codes (Lc, L1-L4, Rc, R1-R4) are detailed in Table 1.

Fig 2: Representative amplification curves of dihydropicolinate synthase (DHDPS) gene expression from *Medicago truncatula* leaf and root samples under abiotic stress conditions.

These patterns visually confirm the suppressive effect of abiotic stress on DHDPS transcription in leaves.

Conversely, Fig 2B illustrates amplification curves for DHDPS in root samples. In stark contrast to leaves, curves for root samples under stress (R1-R4) shifted significantly left (indicating lower Cq values) compared to the control root sample (Rc), signifying higher initial template concentration and robust upregulation of DHDPS gene expression in roots (Fig 3).

Tissue-specific differential expression of DHDPS under abiotic stress

We quantified DHDPS gene relative expression in *M. truncatula* roots and leaves under control, salinity (200 mM NaCl) and osmotic (180 mM mannitol) stress conditions for 2 h and 24 h. Results revealed remarkable tissue-specific differential expression patterns (Fig 3).

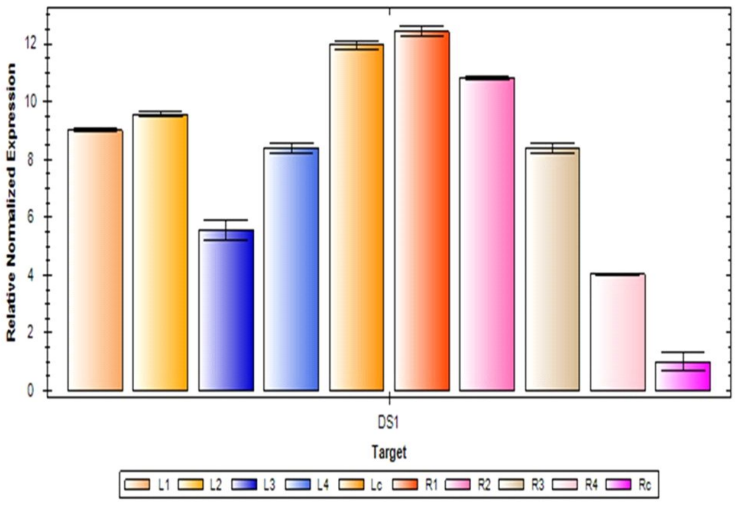
In roots, DHDPS gene expression was significantly upregulated under both salinity and osmotic stress. Under salinity stress, DHDPS expression increased approximately 8.04-fold (R3) after 2 h and 4.03-fold (R4) after 24 h of treatment, respectively, compared to control (Rc). Under osmotic stress, DHDPS expression showed an even more pronounced upregulation, increasing approximately 12.43-fold (R1) after 2 h and remaining highly induced at approximately 10.84-fold (R2) after 24 h (Fig 3). These results suggest a robust and sustained induction of DHDPS in roots in response to both types of abiotic stress.

In contrast, DHDPS gene expression in leaves showed a downregulation trend under both stress conditions. Under salinity stress, DHDPS expression decreased approximately 0.47-fold (L3) after 2 h and slightly recovered but remained lower than control at approximately 0.70-fold (L4) after 24 h. Under osmotic stress, DHDPS expression decreased approximately 0.75-fold (L1) after 2 h and remained suppressed at approximately 0.80-fold (L2) after 24 h (Fig 3). This dichotomy in DHDPS expression between roots and leaves highlights a tissue-specific adaptive mechanism in *M. truncatula* in response to abiotic stress. Further details, including raw Cq values and normalized relative quantity, are provided in Table 1.

DHDPS enzyme activity exhibits tissue-specific differential responses to abiotic stress

To determine if observed changes in DHDPS transcript levels translated into functional differences, we measured the specific activity of the DHDPS enzyme in roots and leaves under control and stress conditions (Fig 4). Consistent with gene expression data, DHDPS specific activity significantly increased in roots subjected to both salinity and osmotic stress compared to control roots (Rc).

Specifically, under salinity stress (200 mM NaCl), DHDPS activity in roots increased from 135.0±5.2 units/mg protein in control roots (Rc) to 225.5±8.7 units/mg protein after 2 hours (R3) and 193.5±7.5 units/mg protein after 24 hours (R4) of treatment. This represents



This bar chart illustrates the tissue-specific differential regulation of DHDPS transcript levels in leaves (L) and roots (R) of *M. truncatula* under control (untreated), salinity (200 mM NaCl) and osmotic (180 mM mannitol) stress conditions for 2 h and 24 h. The Y-axis represents the relative expression levels of DHDPS, normalized to the expression in control root samples (Rc) which is set to 1.0. All data were further normalized using the geometric mean of the validated reference genes (Ubiquitin and PTB). Error bars represent the standard error of the mean (SEM) from three independent biological replicates. Specific sample conditions and corresponding codes (Lc, L1-L4, Rc, R1-R4) are detailed in Table 1.

Fig 3: Relative expression levels of the dihydrodipicolinate synthase (DHDPS) gene in *Medicago truncatula* leaves and roots under salinity and osmotic stress.

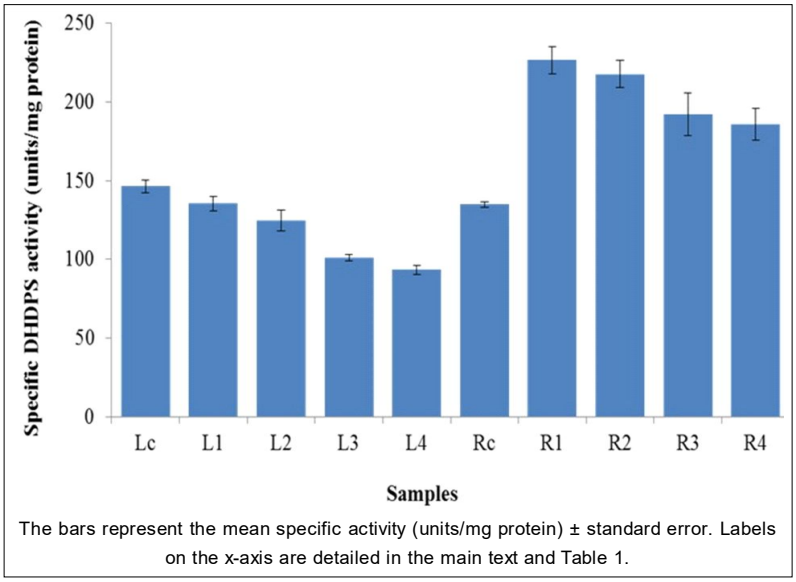
approximately 1.67-fold and 1.43-fold increases, respectively and these increases were statistically significant ($p<0.05$). Under osmotic stress (180 mM mannitol), the induction was even more pronounced; DHDPS activity in roots reached 230.1±9.1 units/mg protein after 2 hours (R1) and 215.3±8.2 units/mg protein after 24 hours (R2), representing approximately 1.70-fold and 1.60-fold increases, respectively, both highly significant ($p<0.01$).

In contrast, a decreasing trend in DHDPS enzyme activity was observed in leaves under both stress conditions. For control leaves (Lc), DHDPS activity was 145.0±6.1 units/mg protein. Under salinity stress, activity decreased to 138.5±5.9 units/mg protein after 2 hours (L3) and further to 105.0±4.5 units/mg protein after 24 hours (L4). Under osmotic stress, DHDPS activity decreased to 139.1±5.8 units/mg protein after 2 hours (L1) and 125.0±5.0 units/mg protein after 24 hours (L2). While these decreases were less dramatic than increases observed in roots, the reductions at 24 hours under both salinity (L4) and osmotic stress (L2) were statistically significant compared to control leaves ($p<0.05$). These results provide strong biochemical evidence supporting the tissue-specific regulatory patterns observed at the transcriptional level.

Abiotic stresses, such as salinity and drought, profoundly impact plant growth through complex physiological and molecular alterations (Arruda *et al*., 2020; Bartels and Sunkar, 2005). Understanding these intricate molecular adaptive mechanisms is crucial for developing stress-tolerant crops (Lodeyro and Valle, 2021). Our study employed a comprehensive approach, combining optimized qRT-PCR and enzyme activity assays, to elucidate the tissue-specific expression and functional regulation of the DHDPS gene in *M. truncatula* roots and leaves under salinity and osmotic stress, ensuring high data reliability and accuracy.

A key finding of our investigation is the contrasting transcriptional and functional regulation of the DHDPS gene in roots versus leaves under both salinity and osmotic stress. We observed significant DHDPS upregulation in roots, reaching approximately 12.43-fold induction under short-term mannitol stress, with sustained high induction under prolonged stress (Fig 3). This robust transcriptional upregulation was critically corroborated by a significant increase in DHDPS enzyme activity in roots under both stress conditions (Fig 4), demonstrating a direct link between gene expression and functional protein levels. This strong root-specific upregulation suggests a crucial role for DHDPS in root stress adaptation. As DHDPS catalyzes the committed step in lysine biosynthesis and lysine serves as a precursor for polyamines (*e.g.*, putrescine, spermidine and spermine)-known osmoprotectants involved in membrane stabilization, reactive oxygen species (ROS) scavenging and ion channel regulation (Kumpaisal *et al*., 1987; Liu *et al*., 2024) -we hypothesize that increased DHDPS expression and activity in roots leads to enhanced lysine and polyamine production, contributing to osmotic adjustment and cellular protection in root tissues directly exposed to high salt or osmotic potentials. This aligns with previous reports of polyamine accumulation in roots under stress (Livak and Schmittgen, 2001; Niu *et al*., 2011, Karlova (2021). Furthermore, lysine and its derivative N-acetyl-L-lysine have been implicated as signaling molecules or compatible solutes in stress (Rose, 2008; Sikdar and Kim, 2010), further supporting the role of elevated DHDPS activity in root stress tolerance.

The observed root-specific DHDPS upregulation likely involves intricate stress signaling pathways. Abscisic acid (ABA), a central phytohormone in abiotic stress responses, is known to regulate genes involved in osmoprotection and primary metabolism. While direct evidence linking ABA to DHDPS regulation is limited, studies in other plants indicate that components of the aspartate pathway, including



The bars represent the mean specific activity (units/mg protein) ± standard error. Labels on the x-axis are detailed in the main text and Table 1.

Fig 4: Specific DHDPS enzyme activity in roots and leaves of *M. truncatula* under control and stress conditions.

those for lysine or polyamine synthesis, can be transcriptionally regulated by ABA-dependent pathways. Similarly, ROS signaling, frequently triggered by abiotic stress, could induce genes involved in detoxification and osmolyte accumulation, potentially including DHDPS as a crucial component of the stress response network. Future studies involving genetic manipulation of ABA signaling or ROS scavenging pathways, coupled with DHDPS gene promoter analysis, could elucidate the precise transcriptional factors and cis-acting elements governing its robust induction in roots under stress. This root-specific upregulation likely fortifies *M. truncatula*'s primary absorptive organ with essential stress-protective metabolites, thereby enhancing water and nutrient uptake under adverse conditions.

In stark contrast to roots, DHDPS gene expression was consistently downregulated in leaves under both salinity and osmotic stress (Fig 3). This downregulation was mirrored by a decreasing trend in DHDPS enzyme activity in leaves under these stresses (Fig 4). For instance, DHDPS expression decreased to approximately 0.47-fold under short-term salinity stress (L3) and to approximately 0.75-fold under short-term osmotic stress (L1) compared to leaf control. This differential response highlights a distinct organ-specific metabolic strategy for coping with abiotic stress. Leaves, as primary photosynthetic and energy-consuming organs, might employ DHDPS downregulation as an energy-conserving strategy. Under stress, plants often reallocate resources to prioritize survival mechanisms over growth and general metabolism (Stepansky *et al*., 2005). By reducing lysine synthesis in leaves, *M. truncatula* may conserve metabolic energy and carbon resources, redirecting them towards other essential stress-responsive pathways or maintaining basic metabolic functions. This is supported by studies suggesting that overall protein synthesis might be reduced in aerial parts under severe stress to save energy (Yang *et al*., 2021).

The slight recovery of DHDPS expression in leaves after 24 h of salinity stress (L4, 0.70-fold) and osmotic stress (L2, 0.80-fold) might indicate a transient stress response or an attempt at acclimation, where the plant adjusts its metabolism after prolonged exposure. This partial recovery could suggest activation of compensatory mechanisms or a metabolic priority shift as the plant adapts to extended stress, potentially involving feedback loops from accumulated metabolites or altered stress signaling. Future time-course experiments with more data points and metabolomic profiling in leaves would be invaluable to fully decipher the dynamics and significance of this recovery.

The strong positive correlation between DHDPS transcript abundance and its enzyme activity in both roots and leaves (as detailed in Fig 3 and 4) provides compelling evidence for its crucial role in the plant's stress response. The upregulation in roots at both transcriptional and functional levels strongly supports our hypothesis that DHDPS is critical for synthesizing lysine-derived protective compounds. Conversely, the coordinated downregulation in leaves suggests a strategic reallocation of metabolic resources, illustrating a sophisticated, tissue-specific adaptive response.

Table 1: DHDPS gene expression in *Medicago truncatula* under salinity and osmotic stress.

Sample	Treatment	Duration	Fold change (vs. Tissue Control)	Relative expression (normalized to Rc)	Mean Cq	Cq SEM	Expression SEM	Corrected expression SEM
Rc	Control		1.00	1.000	28.75	0.669	0.312	0.313
R1	180 mM Mannitol	2 h	12.43	12.427^{g}	24.86	0.021	0.189	0.216
R2	180 mM Mannitol	24 h	10.84	10.837^{h}	25.02	0.039	0.066	0.117
R3	200 mM NaCl	2 h	8.04	8.397^{i}	21.53	0.241	0.194	0.194
R4	200 mM NaCl	24 h	4.03	4.03^{k}	22.83	0.048	0.031	0.034
Lc	Control		1.00	11.949^{e}	27.30	0.036	0.157	0.236
L1	180 mM Mannitol	2 h	0.75	9.005^{a}	26.68	0.056	0.055	0.131
L2	180 mM Mannitol	24 h	0.80	9.567^{b}	26.05	0.032	0.065	0.128
L3	200 mM NaCl	2 h	0.47	5.569^{c}	24.04	0.719	0.342	0.345
L4	200 mM NaCl	24 h	0.70	8.376^{d}	22.60	0.047	0.180	0.182

Data represent the mean of three independent biological replicates ± SEM. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to Ubiquitin (Ub) and PTB reference genes, with untreated control roots (Rc) as the calibrator (normalized to 1.000).
Fold change (vs. Tissue Control): Fold change compared to respective untreated control tissue (Lc for leaves, Rc for roots).
Relative Expression (normalized to Rc): Gene expression relative to untreated control roots (Rc).
Mean Cq: Mean quantification cycle value. Cq SEM: Standard Error of the Mean for Cq values.
Expression SEM: Standard Error of the Mean for relative expression. L: Leaf, R: Root, Corrected Expression SEM: Corrected Standard Error of the Mean for relative expression values. Statistical significance is indicated by different lowercase letters (a, b, c...). Values with different letters are significantly different ($p<0.05$). Values sharing the same letter within their respective tissue group (Leaf or Root) are not significantly different ($P<0.05$).

Regarding potential "moonlighting functions" for DHDPS: While some enzymes possess functions beyond their primary catalytic role, our current study, focusing on gene expression and enzyme activity, provides no direct evidence for such a role for *M. truncatula* DHDPS. Investigating potential non-catalytic roles (*e.g.*, in signaling, structural support, or chaperone activity) would require dedicated biochemical and cell biology approaches, such as protein-protein interaction studies or subcellular localization analysis under different conditions. Thus, "moonlighting functions" for DHDPS remain a fascinating hypothesis warranting future dedicated research beyond the scope of this study.

The observed tissue-specific regulation of DHDPS is crucial for understanding *M. truncatula*'s complex adaptive mechanisms. Roots, being the primary organ to perceive and directly interact with soil-borne stresses, appear to adopt an active metabolic defense strategy by upregulating DHDPS to synthesize protective compounds. In contrast, leaves, experiencing more indirect effects of water deficit and ion toxicity, may prioritize resource conservation or re-allocation. This distinct organ-specific response underscores the importance of studying gene expression and enzymatic activities at the tissue level, as integrated whole-plant responses can mask crucial tissue-specific adaptations.

Our findings lay a solid foundation for future functional characterization of DHDPS in *M. truncatula*. Future studies should focus on functional validation through genetic manipulation (*e.g.*, overexpression or knockdown) of DHDPS under stress. While this study provides strong evidence at both transcriptional and enzymatic levels, metabolomic profiling, particularly the quantification of lysine and polyamines in both roots and leaves, is essential to provide direct biochemical evidence supporting the proposed roles of DHDPS in stress adaptation. Such investigations will be critical for a more complete understanding of the DHDPS pathway in stress response. The insights gained have significant implications for biotechnological applications, specifically in the targeted engineering of DHDPS to enhance root resilience in legumes without negatively impacting shoot metabolic balance, thereby improving overall stress tolerance in agriculturally important crops. This organ-specific approach offers a promising strategy for developing new legume varieties better adapted to challenging environmental conditions.

CONCLUSION

This study provides novel and crucial insights into the tissue-specific transcriptional and functional regulation of the DHDPS gene in *M. truncatula* under salinity and osmotic stress. We revealed a striking differential expression pattern-significant upregulation in roots and concurrent downregulation in leaves-corroborated by distinct changes in DHDPS enzyme activity. This organ-specific dichotomy highlights distinct metabolic strategies for stress adaptation: the upregulation of DHDPS in roots, at both transcript and functional levels, likely contributes to enhanced biosynthesis of lysine-derived protective compounds, crucial for root resilience under direct stress exposure. Conversely, its downregulation in leaves may represent an energy-saving mechanism or metabolic re-prioritization. These results underscore the importance of studying tissue-specific responses to fully comprehend plant stress adaptation mechanisms, as whole-plant approaches can mask critical organ-specific roles.

Future research should build upon these transcriptional and enzymatic findings by investigating DHDPS at the protein and metabolomic levels (*e.g.*, quantifying lysine and polyamines) to fully elucidate its functional role in stress adaptation. The insights gained are valuable for targeted biotechnological approaches aimed at improving stress tolerance in legumes by manipulating DHDPS to enhance root vigor without compromising shoot energy balance, thus contributing to the development of more resilient crop varieties.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Consent for publication

All authors have read and approved the final version of the manuscript and consent to its submission to the Legume research Journal.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

REFERENCES

Ahmad, M.A., Saleem, A., Tahir, M., Khilji, S.A., Sajid, Z.A., Landry, K.B., El-Sheikh, M.A., Ahmad, P. (2024). Modulation of the polyamines, osmolytes and antioxidant defense system to improve drought stress tolerance in *Hordeum vulgare* L. using ascorbic acid. *South African Journal of Botany.* **171:** 726-736. https://doi.org/10.1016/j.sajb.2023.10.013.

Arruda, P. and Barreto, P. (2020). Lysine catabolism through the saccharopine pathway: Enzymes and intermediates involved in plant responses to abiotic and biotic stress. *Frontiers in Plant Science*. **11:** 587. https://doi.org/10.3389/fpls.2020.00587

Bartels, D., Sunkar, R. (2005). Drought and salinity tolerance in plants. *Critical Reviews in Plant Sciences.* **24(1):** 23-58. https://doi.org/10.1080/07352680590910410.

Bödör, C., Schmidt, O., Csernus, B., Rajnai, H., Szende, B. (2007). DNA and RNA isolated from tissues processed by microwave-accelerated apparatus MFX-800-3 are suitable for subsequent PCR and qRT-PCR amplification. *Pathology and Oncology Research.* **13(2):** 149-152. https://doi.org/10.1007/BF02893457.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* **72(1-2):** 248-254.

Bustamante, M., Jin, J., Casagran, O., Nolan, T., Riechmann, J.L. (2013). Gene Expression Analysis by Quantitative Real-time PCR for Floral Tissues. In Z. Heazlewood (Ed.), *Flower Development.* 363-382. Springer. https://doi.org/10.1007/978-1-4614-9408-9_21.

Dereppe, C., Bold, G., Ghisalba, O., Ebert, E., Schar, H.P. (1992). Purification and characterization of the dihydrodipicolinate synthase of pea. *Plant Physiology.* **98(2):** 813-821. https://doi.org/10.1104/pp.98.2.813.

Dovrat, G., Meron, E., Shachak, M., Golodets, C., Osem, Y. (2019). Plant size is related to biomass partitioning and stress resistance in water-limited annual plant communities. *Journal of Arid Environments.* **165:** 1-9. https://doi.org/10.1016/j.jaridenv.2019.04.001.

Ellen, E., Van Busse, P., Thu, T.T., Angenon, G. (2013). *Medicago truncatula* dihydrodipicolinate synthase (DHDPS) enzymes display novel regulatory properties. *Plant Molecular Biology.* **81(4-5):** 401-415. https://doi.org/10.1007/s11103-013-0016-3.

Elmsehli, S., Aloui, A., Smiti, A.S. (2015). Physiological and proteolytic activities of *Medicago truncatula* under different cadmic treatments. *Legume Research.* **38(4):** 469-476. doi: 10.5958/0976-0571.2015.00130.7.

Frisch, D.A., Gengenbach, B.G., Tommey, A.M., Sellner, J.M., Somers, D.A., Myers, D.E. (1991) Isolation and characterization of dihydrodipicolinate synthase from maize. *Plant Physiology.* **96(2):** 444-452. https://doi.org/10.1104/pp.96.2.444

Ghislain, M., Frankard, V., Jacobs, M. (1990) Dihydrodipicolinate synthase of *Nicotiana sylvestris*, a chloroplast-localized enzyme of the lysine pathway. *Planta.* **180:** 480-486. https://doi.org/10.1007/BF02411444.

Hong, H.T.K., Trang, P.T.H., Long, D.T., Trang, N.T.Q. (2020). Cloning and optimizing the expression of the *DHDPS gene* in the *Medicago truncatula*. *Legume Research.* **43(3):** 326-331. doi: 10.18805/LR-482.

Huguet, T., Prosperi, J.M. (1996). *M. truncatula*: A Legume Model Plant. In G. Genier and J. M. Prosperi (Eds.), *The Genus Medicago in the Mediterranean Region: Current Situation and Prospects in Research*. 171-175. CIHEAM.

Karlova, R., Boer, D., Hayes, S., Testerink, C. (2021). Root plasticity under abiotic stress. *Plant Physiology.* **187(3):** 1057-1070. https://doi.org/10.1093/plphys/kiab392.

Katam, R., Lin, C., Grant, K., Katam, C.S., Chen, S. (2022). Advances in plant metabolomics and its applications in stress and single-cell biology. *International Journal of Molecular Sciences*. ***23*(13):** 6985. https://doi.org/10.3390/ijms23136985.

Kumpaisal, R., Hashimoto, T., Yamada, Y. (1987) Purification and characterization of Dihydrodipicolinate synthase from wheat suspension cultures. *Plant Physiol.* **85:** 145-151. https://doi.org/10.1104/pp.85.1.145.

Liu, C., Jiang, X., Yuan, Z. (2024). Plant responses and adaptations to salt stress: A review. *Horticulturae.* **10(11):** 1221. https://doi.org/10.3390/horticulturae10111221.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. **25(4):** 402-408. https://doi.org/10.1006/meth.2001.1262.

Lodeyro, A.F., Valle, E.M. (2021). Plant responses to abiotic stresses: A review of stress mechanisms. *Physiologia Plantarum.* **172(3):** 1493-1507. https://doi.org/10.1111/ppl.13314.

Manoj, S.P., Nandeesha, M.K., Prasoonpal, G., Mukesh, M., Subhojit, D. (2015). Cloning and characterization of protease inhibitor genes from some legumes. *Legume Research*. **38(2):** 178-181. doi: 10.5958/0976-0571.2015.00079.X.

Niu, J.-Z., Dou, W., Ding, T.B., Yang, L.H., Shen, G.M., Wang, J.J. (2011). Evaluation of suitable reference genes for quantitative RT-PCR during development and abiotic stress in *Panonychus citri* (McGregor). *Molecular Biology Reports*. **39(5):** 5841-5849. https://doi.org/10.1007/s11033-011-1394-x.

Rose, R. J. (2008). *M. truncatula* as a model for understanding plant interactions. *Functional Plant Biology.* **35(3):** 253-264. https://doi.org/10.1071/FP08012.

Sikdar, M.S.I., Kim, J.S. (2010). Characterization of a gene encoding for dihydrodipicolinate synthase from rice. *American Journal of Crop Science.* **4(6):** 461-466.

Stepansky, A., Yao, Y.L., Tang, G.L., Galili, G. (2005). Regulation of lysine catabolism in *Arabidopsis* through the concerted synthesis of the two distinct gene products of the composite *AtLKR/SDH* locus. *Journal of Experimental Botany*. **56(412):** 525-536. https://doi.org/10.1093/jxb/eri057.

Thu, T. T., Dewaele, E., Trung, L.Q., Claeys, M., Jacobs, M., Angenon, G. (2007). Increasing lysine levels in *Cajanus cajan* seeds through genetic engineering. *Plant Cell, Tissue and Organ Culture.* **91(2):** 135-143. https://doi.org/10.1007/s11240-007-9277-3.

Vauterin, M., Frankard, V., Jacobs, M. (1999). The *Arabidopsis thaliana* DHDPS gene is expressed in a cell-specific manner. *Plant Molecular Biology.* ***39*(4):** 695-708. https://doi.org/10.1023/A:1006133412966.

Yang, X., Lu, M., Wang, Y., Wang, Y., Liu, Z., Chen, S. (2021). Response mechanism of plants to drought stress. *Horticulturae*. **7(3):** 50. https://doi.org/10.3390/horticulturae7030050.

Young, N.D., Debellé, F., Oldroyd, G.E.D. *et al.* (2011). The *Medicago* genome provides insight into the evolution of rhizobial symbioses. *Nature.* **480(7378):** 520-524. https://doi.org/10.1038/nature10625.

Zhu, X.H., Tang, G.L., Galili, G. (2002). The activity of the *Arabidopsis* bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase enzyme is regulated by the interaction between its two domains. *Journal of Biological Chemistry.* **277(51):** 49655-49661. https://doi.org/10.1074/jbc.M208675200.