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Baklagil Bitkilerinde qRT-PCR İçin Stres Belirteci Olarak Potansiyel Ortak Bir Pirolin-5-Karboksilat Sentetaz (P5CS) Geni

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ÖZ

Baklagiller olarak bilinen ve tarımsal, ekolojik öneme sahip bitkileri içeren *Leguminosae* (veya *Fabaceae*) ailesi, dünya çapında geniş bir dağılıma sahip önemli bir tarım bitkisi grubudur. Stres, tarımı yapılan bitkilerin büyümesini ve verimliliğini etkileyen ana çevresel faktörlerden biridir. Bitkiler, stresin etkilerini en aza indirmek ve hücre bileşenlerini korumak amacıyla prolin gibi osmolitler üreterek biyokimyasal düzeyde strese direnç gösterir. Pyrroline-5-carboxylate synthetase (P5CS), bitkilerde prolin biyosentez yolunda kilit bir enzim olup, prolin üretiminin başlangıç aşamasında yer alır. Bu çalışmada, prolin miktarındaki artışın belirlenmesinde biyokimyasal yöntemlere alternatif olarak qRT-PCR temelli çalışmalara olanak sağlayacak ortak bir primer belirlenmiştir. Bu amaçla, önemli tarım bitkileri (*Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L.) üç farklı strese (kuraklık, sıcaklık, tuz) maruz bırakılmıştır. Örneklerin biyokimyasal olarak prolin miktarları ölçülmüş, ardından ortak primer kullanılarak gen ekspresyon seviyeleri hesaplanmıştır. Sonuçlar, tüm bitki örneklerinde prolin miktarlarının önemli ölçüde arttığını göstermiştir. Gen ekspresyon analizleri de bu bulgularla korelasyon göstermektedir. Çalışmada, stres koşullarında tarımsal açıdan önemli bazı baklagil bitkilerinin strese maruz kaldığını gösteren ve önemli bir belirteç olan prolin seviyelerini gen ekspresyonu düzeyinde belirlemek için kullanılabilecek ortak bir marker tanımlanmıştır. Bulgularımız, prolin biyosentezinde görev alan genlerin ifadesinin araştırılmasının, bitkilerin stres tepkilerini moleküler düzeyde anlamaya yardımcı olduğunu ortaya koymaktadır. Ayrıca, moleküler yöntemlerle yapılan analizlerin daha hızlı ve hassas sonuçlar verdiği, prolin biyosentezinde aktif olan genlerin dinamik değişimlerini daha kısa sürede gözlemlemeye olanak sağladığı belirlenmiştir. Böylece, gen düzeyindeki değişiklikler sayesinde bitkinin strese karşı hazırlık aşamaları veya erken yanıtları daha etkili bir şekilde takip edilebilmektedir.

A Potential Common Pyrroline-5-Carboxylate Synthetase (P5CS) Gene As A Stress Marker For qRT-PCR In Legume Plants

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ABSTRACT

Leguminosae (or *Fabaceae*), commonly known as legumes, includes plants of agricultural and ecological importance, are important agricultural plant families with a very wide distribution worldwide. Stress is one of the main environmental factors affecting the growth and productivity of cultivated plants. Plants are

Keywords:
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designed to minimize the impact of stress and to develop resistance at the biochemical level by producing osmolytes such as proline to protect their cellular components. Pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme in the proline biosynthesis pathway in plants and is involved in the initial step of proline production. In this study, a common primer was identified to enable qRT-PCR-based studies as an alternative to biochemical methods for determining the increase in proline content. With this aim, important agricultural plants, *Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L., were exposed to three different stresses (drought, heat, salt). Proline amounts of the samples were determined biochemically and then gene expression levels were calculated using the common primer. In our results, proline levels were significantly increased in all plant samples. Relative gene expression analysis results also correlated with the biochemical results. In conclusion, this study revealed the existence of a common marker that can be used to determine proline levels at the gene expression level, which is an important marker indicating that some agricultural legume plants are under stress. Our findings show that investigating the expression of genes involved in proline biosynthesis can help to understand the stress responses of plants at the molecular level. In addition, molecular methods provide faster and more precise results compared to biochemical methods. This allows to observe the dynamic changes of genes active in proline biosynthesis in a shorter time and changes at the gene level allow us to observe the plant's readiness or early responses to stress.

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1. Introduction

Leguminosae (or *Fabaceae*), commonly known as legumes and includes plants of agricultural and ecological importance, is a family of plants with a very wide distribution worldwide. *Leguminosae* is a fairly large plant family, with about 19,325 species and 727 genera, and is widespread on almost every continent (Lewis et al., 2005). The *Leguminosae* family is usually divided into three subfamilies: *Papilionoideae* (*Faboideae*): which includes species such as peas, beans and clover. *Caesalpinioideae*: which consists of trees and shrubs more common in the tropical regions and *Mimosoideae* which includes species such as mimosa, robinia and is characterized by flowers arranged in spikes or panicles (Schrire et al., 2005). Plants in the *Leguminosae* family include trees, shrubs, grasses and vines. This family provides important food sources for both humans and animals. Widely cultivated *Leguminosae* species around the world include beans [*Phaseolus vulgaris* L.], lentils [*Lens culinaris* Medik.], chickpeas [*Cicer arietinum* L.], soybeans [*Glycine max* (L.) Merr.], peas [*Pisum sativum* L.], alfalfa [*Medicago sativa* L.] (Smýkal et al., 2015). These plants hold an important place in human nutrition and animal feed due to their high protein content. One of the most important characteristics of *Leguminosae* is their symbiotic relationship with nitrogen fixing bacteria such as *Rhizobium*, which live in their roots. These bacteria convert atmospheric nitrogen into a form usable by the plant, enriching the soil and serving as natural fertilizers for other plants. Legumes therefore enrich the soil and act as natural fertilizers for other plants. They are also used as green manure in organic farming and increase soil fertility (Giraud et al., 2004). Abiotic stress refers to adverse conditions that negatively impact plant growth, development and productivity (Oshunsanya et al., 2019). These stresses are caused by abiotic or biotic factors. Abiotic stresses include drought, salinity, extreme temperature, nutrient deficiency,

light and chemical stresses. Since the *Leguminosae* family includes agriculturally and ecologically important plants, the effects of abiotic stresses on these plants are of great importance for both yield and biodiversity. In the face of abiotic stresses, the rate of photosynthesis decreases, root development is limited, growth and development slow down, protein synthesis as well as metabolic balances may be disrupted in all plants including *Leguminosae* (Abdelrahman et al., 2018). Abiotic stresses pose serious threats to the agricultural production of legume crops and for this reason, breeding studies and genetic modification are trying to develop varieties that are more resistant to stresses. The amino acid proline is a small but critical molecule that plays an important role in plants against abiotic stress conditions (Hosseinifard et al., 2022). Under stress, plants respond by increasing proline production. In plant cells, proline acts as an osmoprotectant, maintaining intracellular fluid balance and preventing cellular water loss in stressed plants (Liang et al., 2013). It also protects cell membranes and proteins from harmful effects such as denaturation. Under stress conditions, proteins may degrade and cell membranes can be damaged, and proline stabilizes these structures and reduces the effects of stress (Kumar et al., 2012). Abiotic stresses create oxidative stress in plants, which leads to the accumulation of harmful reactive oxygen species (ROS) in cells (Banerjee et al., 2017). Proline scavenges free radicals, limiting cellular damage and combating oxidative stress. Pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme in the proline biosynthesis pathway in plants and involved in the initial step of proline production (Turchetto et al., 2009). P5CS converts the amino acid glutamate into the intermediate compound pyrroline-5-carboxylate (P5C), which is required for proline synthesis, and P5C is then converted to proline. P5CS, the checkpoint of proline synthesis, ensures that proline is produced rapidly and in sufficient quantities (Turchetto et al., 2009). Previous studies have reported that P5CS expression increases under abiotic stress (De Ronde et al., 2000; Zhang et al., 2014; Wang et al., 2015; Dai et al., 2018; Wei et al., 2022; Kijowska et al., 2024) and this helps to increase proline production resulted the plant to gain resistance to stress. Plants synthesize more proline by regulating P5CS gene expression depending on the severity of stress (Feng et al., 2016). Proline is one of the most effective defense mechanisms of plants against abiotic stresses and the enzyme Pyrroline-5-carboxylate synthetase (P5CS) is at the center of this process. Increased activity of P5CS under stress conditions accelerates proline production in plants, preventing water loss, scavenging free radicals and protecting cellular structures (Sripinyowanich et al., 2013). Therefore, P5CS is of great importance in plant stress tolerance and an important enzyme targeted in agricultural breeding studies (Kesari et al., 2012). In previous studies, the increase in P5CS gene activity has been associated with higher proline levels in transgenic tobacco and rice (Ma et al., 2022). Similarly, another study showed a significant positive correlation between P5CS expression, P5CS activity, and proline accumulation, suggesting this physiological trait as a promising index for developing osmotic stress-tolerant genotypes in rice (Sabbioni et al., 2021). However, the existing stress studies targeting the P5CS gene in the literature predominantly focus on gene expression research in a single plant species. In this context, the present study aims to fill this gap

by proposing the presence of a marker that can serve for common use in commercially important legume plants.

Based on all the information, in this study, a common P5CS gene expression marker based on qRT-PCR was identified, which can be used as an alternative to biochemical methods to understand that important agricultural legume plants (*Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L.) are under different abiotic stresses (drought, heat, salt). Conducting gene expression studies to determine the amount of proline in plants can be important and advantageous for understanding proline biosynthesis and metabolism at the molecular level. In particular, analyzing the expression of genes that play a critical role in proline biosynthesis (e.g. pyrroline-5-carboxylate synthetase (P5CS) gene) will help to understand plant response to abiotic stress conditions. Thus, it is believed that through genetic modifications, the preparation phases or early responses of the plant to stress can be monitored more effectively.

2. Material and Methods

2.1. Plant Materials

The seeds used as experimental material in this study were obtained from The Southeastern Anatolia Agricultural Research Institute, Eastern Mediterranean Agricultural Research Institute and Trakya Agricultural Research Institute. The following seed varieties were used Özmen for *Phaseolus vulgaris* L., Fırat for *Lens culinaris* Medik., Inci for *Cicer arietinum* L., Ilksoy for *Glycine max* (L.) Merr., Kurtbey for *Pisum sativum* L., Nimet for *Medicago sativa* L. were used.

2.2. Growth Conditions and Application of Stress

After surface sterilization, the seeds were kept in distilled water overnight to swell and were ready for sowing in pots with perlite. The next day, the seeds were sown in pots containing perlite and grown in climate chamber under long day conditions (25°C, humidity 10%, light intensity 16.000 lux). For 14 days, all experimental groups were irrigated with ½ Hoagland's nutrient solution. At the end of the 14th day, the control group was irrigated with ½ Hoagland's nutrient solution and the experimental groups were irrigated with ½ Hoagland's nutrient solution containing 15% PEG 6000 for drought and 100 mM NaCl for salinity (Farooq et al., 2017; Morgil et al., 2019). For heat stress, the plants were placed in climate chamber at 50°C with normal irrigation (Liu et al., 2019). The plants were exposed to stress for three days and then harvested.

2.3. Determination of Proline Amount

Proline content was determined according to Morgil et al., (2019). A standard curve was constructed to determine the proline concentration in the range 5-500 μm. Experiments were performed in three technical, three biological (n=6) replicates. The results of the study groups were expressed as mean ± standard deviation (mean ± SD). Student's t-test and one-way analysis of variance (ANOVA) were

applied for statistical analysis. Post-hoc Bonferroni test was applied to the groups with significant ANOVA results and a probability value of $p<0.05$ was considered statistically significant.

2.4. Determination of Common P5CS Gene Primer

To evaluate the response of *Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L. under different abiotic stresses (drought, heat, and salt), a common P5CS gene expression marker based on qRT-PCR was identified as an alternative to biochemical methods. The aim was to identify conserved regions within the P5CS gene sequences between these species and design a common primer. First, the complete sequence of the P5CS gene from each plant species was retrieved from the NCBI GenBank database (*Phaseolus vulgaris* L. (Genbank ID EU407263.1), *Lens culinaris* Medik. (Genbank ID: GT-622346.1), *Cicer arietinum* L. (Genbank ID: KC464462.1), *Glycine max* (L.) Merr. (Genbank ID: FM999730.1) *Pisum sativum* L. (Genbank ID: CAB63486.1), *Medicago sativa* L. (Genbank ID: GU180149.1). To identify conserved regions, P5CS gene sequences of plant species were aligned using the multiple sequence alignment program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Additional Figure 1). After conserved regions were identified, primer design was performed using Primer3Plus webtool, taking into account primer length, GC content, melting temperature, self-complementarity and amplification product size. Approximately ten primers were ordered according to the specifications and then primer checks were performed by BLAST analysis to check for matches with other genes. The selected primers were then tested by qRT-PCR and the results were normalized using the appropriate reference genes for each plant species and the common primer for qRT-PCR was determined for all samples (Table 1).

Table 1. Primers used for qRT-PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Product length
P5CS*	GTGGGAATGGGCTTCTCTTGA	ACTCCTACTGGACCCCGAG	763 bp
Ubiquitin_ Phaseolus vulgaris	AGAAAAGCCCCCAAGTGTTC	CTGCCATCTCCTTCTTCAGC	161 bp
Ubiquitin_Glycine max	TCCCCTCACACC CTTCCTC	CCATCCCAAGGGG TGTCAT	155 bp
Ubiquitin_Cicer arietinum	TCACCCTCGAGGTGGAGTCT	TGTCTTGGATCTTTGCTTTGACA	166 bp
GAPDH_Lens culinaris	TGGCAAAGTGCTTCCTGCTT	CAACAACGGAGACATCCACAGT	180 bp
GAPDH_Pisum sativum	GTGGTCTCCACTGACTTTATTGGT	TTCCTGCCTTGGCATCAAA	150 bp
GAPDH_Medicago sativa	CTGGAGAGGTGGAAGAGCTG	GGTTGGGACACGGAATGAC	160 bp

*The common primer identified as normalized among ten primers selected from conserved regions and found to work efficiently for qRT-PCR.

2.5. RNA Isolation and Reverse Transcription

RNA isolation was performed by harvesting leaf tissues from plant subjected to stress for three days. RNA isolations were performed using the RNeasy Mini Kit (Qiagen, Germany, Catalog No: 74106) according to the manufacturer instructions. No degradation of RNA samples and genomic DNA quality control was ensured by running on a 1% agarose gel to avoid contamination. NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific™, USA) was used to measure the quality and the quantity of the isolated total RNAs. Experiments were performed in three technical and biological (n=6) replicates. The isolated RNA samples were converted into cDNA using Revert Aid First Strand cDNA synthesis kit (Fermentas, Catalog No: K1622) according to the manufacturer instructions (Schmidt, 2004).

2.6. qRT-PCR Analysis

To examine the correlation between biochemically derived proline levels and gene expression in stressed plants, we performed qRT-PCR with a common primer for the pyrroline-5-carboxylate synthetase (P5CS) gene (Table 1). Amplification programs were performed using SYBR Green according to a protocol that included 35 cycles of 95°C for 4 min, 95°C for 8 s, 60°C for 10 s and 72°C for 20 s, and a thermal denaturing step to generate melting curves (95°C for 10 s, 70°C for 5 s). The Ubiquitin gene was used as an internal reference for *Phaseolus vulgaris* L., *Glycine max* (L.) Merr., *Cicer arietinum* L. and GAPDH gene for *Lens culinaris* Medik., *Pisum sativum* L. and *Medicago sativa* L.. This was accomplished by using the NormFinder tool and setting a MIQE guidelines value (M value) of 0.6, taking into account an average expression stability (Kakar et al., 2008). All reactions were repeated in biological and technical triplicates. Gene expression results were given as relative gene expression based on the transcription level of the internal reference gene in each sample (Seferoglu et al., 2013). The results of the amplified PCR product were checked on a 1% agarose gel.

2.7. Statistical analysis

The results were statistically analyzed to determine whether there was a statistical significance between the biochemical proline content results and gene expression analysis. The Shapiro-Wilk test was applied to the data set to examine whether the data fit a normal distribution. Afterwards, Pearson's correlation test was performed since both the proline and gene expression data fit the normal distribution. This test determines the linear relationship between two variables.

3. Results and Discussion

3.1. Accumulation of Proline

In this study, a common P5CS gene expression marker based on qRT-PCR was identified, which can be used as an alternative to biochemical methods to understand that important agricultural legume plants (*Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L., *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L.) are under different abiotic stresses (drought, heat, salt). For analyses,

plants grown in pots containing perlite for 14 days were subjected to different stresses for 3 days after 14 days. At the end of the 3-day stress period samples were harvested and proline content was measured (Figure 1). In general, our results show that proline levels significantly increased in plants exposed to stress conditions compared to the control group. This implies that proline is accumulated as a plant response to stress conditions and helps plants to maintain osmotic balance (Alagoz et al., 2023). Under control conditions, proline levels in the control group were quite low for each plant species. The lowest proline levels were determined in *Glycine max* (L.) Merr. control (0.24 µmol/mg) and *Lens culinaris* Medik. control (0.255 µmol/mg). Proline levels were slightly higher in as the control groups of *Pisum sativum* L. and *Cicer arietinum* L., but still low compared to stressed conditions. In all species, proline levels increased significantly in plants exposed to drought stress. Especially high proline accumulation was observed in *Pisum sativum* L. (1.4 µmol/mg) and *Phaseolus vulgaris* L. (1.37 µmol/mg). These species respond to drought stress by accumulating proline and that this is similar between species (Kijowska et al., 2023). Similarly, species such as *Glycine max* (L.) Merr. and *Cicer arietinum* L. also responded to drought stress with proline accumulation. In salt stress, proline levels increased in all plants compared to the control group. However, this increase was higher in some plants than in others. *Pisum sativum* L. (1.44 µmol/mg) and *Phaseolus vulgaris* L. (1.09 µmol/mg) showed a higher increase in proline accumulation than other plants.

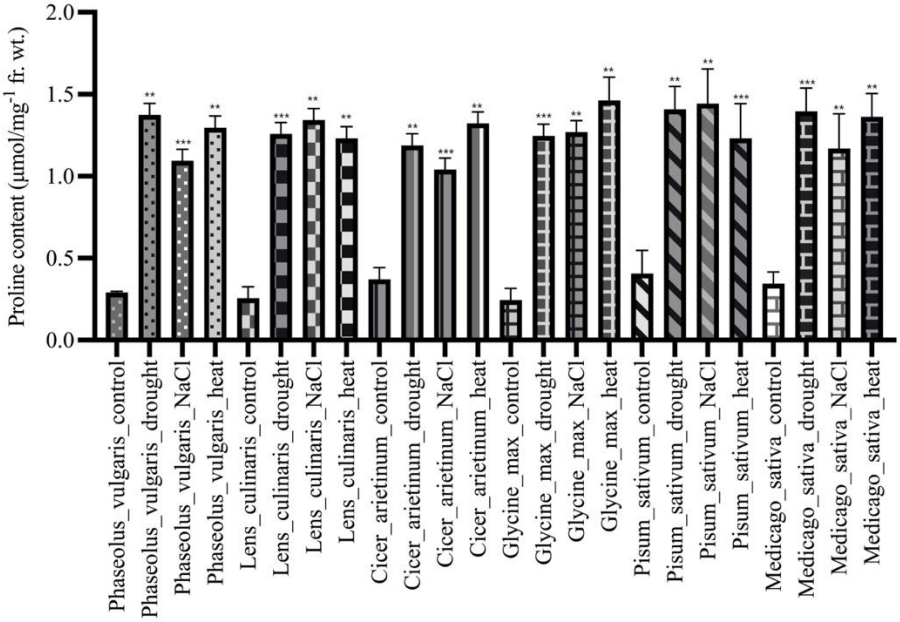

Figure 1. Proline content of all plant samples. Each plant was represented by a different pattern. Assays were done after 3 days of stress treatment. Stress treatments were conducted using 15% PEG 6000 for drought, 100 mM NaCl for salinity, and placing plants under normal irrigation in a 50°C climate chamber for heat stress. Means, standard errors of three biological and three technical replicates. The data were analyzed using a one-way ANOVA analysis of variance (Post-hoc Bonferroni Test). Asterisks indicate significant differences at **p<0.01, ***p<0.001 versus treated control samples at the relevant time point.

The accumulation of proline under salt stress acts as a mechanism of plant resistance to salt stress (Koc et al., 2024). Heat stress also increased proline levels compared to the control group, although the extent of this increase varied among species. High proline accumulation was observed in species such as *Phaseolus vulgaris* L. (1.29 μmol/mg) and *Glycine max* (L.) Merr. (1.46 μmol/mg). *Medicago sativa* L. (1.36 μmol/mg) and *Pisum sativum* L. (1.23 μmol/mg) also responded to heat stress by proline accumulation. All plants accumulate proline as an adaptation mechanism against stress conditions (Zulfiqar et al., 2023). In particular, drought and salt stress were found to cause more proline accumulation in plants. Some species (e.g. *Pisum sativum* L. and *Phaseolus vulgaris* L.) accumulated higher levels of proline, while others showed lower responses. Proline accumulation to heat stress was less pronounced than to other stressors. These results suggest that plant species develop different response mechanisms to different stress conditions and that these mechanisms are associated with proline accumulation (Mehta et al., 2023). The standard deviations between replicate samples compared to the control were approximately 0.07. These results show that proline amounts vary with stress conditions and reliable measurements were obtained for most of these changes. The effect of stress on plants has been consistently measured in different plant species and conditions.

3.2. qRT-PCR Analysis

To see the correlation between biochemically derived proline levels in stressed plants, we performed qRT-PCR with a common primer for the pyrroline-5-carboxylate synthetase (P5CS) gene. RNA isolations were performed by harvesting from the leaf parts at the end of the third day from plants subjected to stress. The quantity and quality of RNA samples were assessed using a Qubit® 2.0 spectrophotometer (Invitrogen, USA). In the results, the average amount of total RNAs was determined as 682.82 ng/μl and experiments were continued with RNAs with a spectrophotometric absorbance of 2.0 at 260/280 nm. The P5CS gene expression results show gene expression levels measured under control and stress conditions in various plant species (Figure 2).

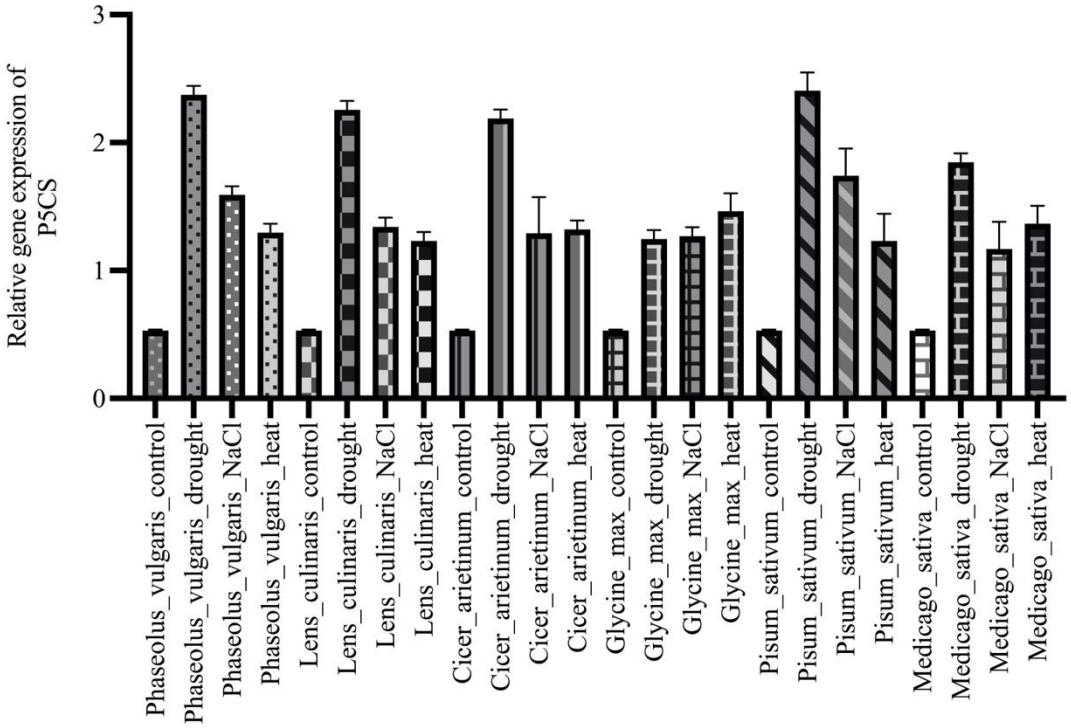

Figure 2. qRT-PCR relative gene expression results of all samples with common P5CS primer after 3 days stress application. Each plant was represented by a different pattern. Means, standard errors of three biological and three technical replicates. The results were first normalized to each plant-specific housekeeping gene and then the relative expression of the genes under various treatments was determined.

The P5CS gene plays an important role in proline synthesis and is therefore being studied in relation to proline accumulation. The level of P5CS relative gene expression in all plant species under control conditions was determined to be approximately 0.525. This suggests that plants maintain a basal level of gene expression against their healthy, stressed state (Georgieva et al., 2023). Under drought stress, a significant increase in P5CS gene expression was observed in all species. For *Phaseolus vulgaris*, the relative gene expression level reached its highest at 2.423920147. *Pisum sativum* L. (2.306768253) and *Lens culinaris* Medik. (2.206768253) also show high increases. These species significantly increased P5CS gene expression to increase proline synthesis under drought conditions. A similar increase is observed in other species, suggesting that plants increase proline production through the P5CS gene in response to drought stress. Under salt stress, P5CS gene expression was also increased compared to the control group, but remained lower compared to the drought condition. While a significant increase was observed in species such as *Pisum sativum* L. (1.892055467) and *Phaseolus vulgaris* L. (1.543983093), this increase was more moderate in other species. This implies that plants also respond to salt stress through P5CS, but less efficiently than in drought (Jamshidi et al., 2023). In heat stress, P5CS gene expression was generally increased compared to the control group, but still remained at lower levels compared to drought condition. *Glycine max* (L.) Merr. (1.56318) and *Pisum sativum* L. (1.381119173) show the highest levels. This indicates that plants increase P5CS gene expression in response to heat

stress but the effect of stress on species is variable (Duvnjak et al., 2024). P5CS gene expression shows a marked increase in plants subjected to drought stress. This indicates that proline synthesis is increased and is an important part of the survival mechanisms of plants under drought conditions. Although an increase was also observed in salt and heat stress, this increase was less pronounced than in drought condition. Overall, it appears that plants increase proline production through the P5CS gene in response to stress conditions and that this mechanism varies among plant species (Duvnjak et al., 2024). Our results showed similar results between relative gene expression levels and biochemically measured proline amounts.

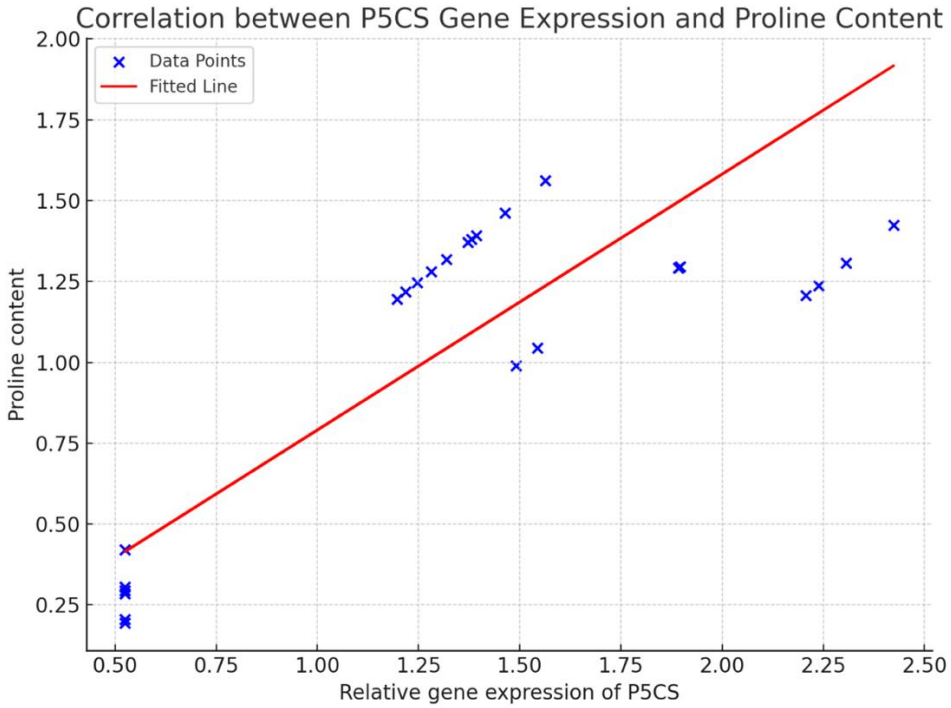

Figure 3. Correlation plot between proline content and P5CS gene expression. The blue dots represent the experimental data, while the red line is the curve showing the strong positive correlation between the two variables ($R^2 = 0.791$).

Biochemical proline content results were statistically analyzed to evaluate the relationships between biochemical proline levels and P5C5 gene expression analysis (Javed et al., 2024). The data appear under the headings “Relative gene expression of P5CS” and “Proline content” under control and stress conditions (drought, heat, salt) in different plant species (Figure 3). Statistical analysis was performed between these two variables to calculate the correlation. The results of the Shapiro-Wilk test showed a p-value of 0.031 for relative gene expression of P5CS, indicating that the data showed a significant deviation from a normal distribution ($p<0.05$). The p-value for proline content was also 0.001, indicating that the data also fit a normal distribution. Pearson correlation was then calculated for these two variables. Pearson correlation coefficient between proline content and P5CS relative gene expression was 0.791. Our results show a positive and strong linear relationship between the two

variables. Furthermore, this relationship is statistically significant as the p-value is 4.2×10^{-6} (0.0000042). This is well below the threshold value generally accepted as $p<0.05$, indicating that the findings are reliable.

4. Conclusion

Stress is an environmental condition that adversely affects plant growth, development and productivity. To cope with stress, plants undergo significant biochemical and molecular adjustments. The amino acid proline is a small but critical molecule that plays an important role in protecting plants under abiotic stress conditions. In plant cells, proline acts as an osmoprotectant and maintains intracellular water balance, preventing cellular water loss in stressed plants. Pyrroline-5-carboxylate synthetase (P5CS) is a key enzyme in the proline biosynthesis pathway in plants and is involved in the initial step of proline production. In this study, we successfully identified a common qRT-PCR-based P5CS gene expression marker that can be used as an alternative to biochemical methods in important agricultural legume crops (*Phaseolus vulgaris* L., *Lens culinaris* Medik., *Cicer arietinum* L.,, *Glycine max* (L.) Merr., *Pisum sativum* L., *Medicago sativa* L.) under different abiotic stresses. However, the experimental reproducibility of the selected common primer is plant-specific and open to further testing. Due to genetic diversity among different legume species, the P5CS gene may have different expression levels in different species. The specificity of the primer may only work with high accuracy in certain species and under specific conditions. The efficiency of the primer can vary depending on the genetic makeup of the legume species used and the stress conditions applied. Biological diversity in different plant samples can affect the expression of the P5CS gene, which can make the accuracy and reproducibility of the results more difficult. The applicability of the common primer depends on the proper selection of internal control genes and the use of appropriate reference standards. The expression of these control genes can vary across different plants, so special optimizations may be required for each species and condition.

Conducting gene expression studies to determine the amount of proline in plants may be important and advantageous for understanding proline biosynthesis and metabolism at the molecular level. Identifying and regulating genes involved in proline biosynthesis allows the development of plant species resistant to environmental stresses such as drought, salinity and heat. This is particularly important to mitigate the effects of climate change. In addition, developing plants that are more resilient to environmental stress conditions helps to avoid declines in agricultural yields. Plant species that increase proline biosynthesis through genetic manipulation or selective breeding can achieve high yields even under harsh environmental conditions. Using genes associated with proline biosynthesis as molecular markers facilitates early selection of individuals with high stress tolerance. This speeds up breeding processes, saving costs and time. Genetic modifications that increase proline production can be used in agricultural biotechnology crops, contributing to the development of sustainable plant varieties that are more resistant to stress conditions. It provides a great advantage for the continuation of agricultural

production, especially in saline soils or low rainfall areas. The efficient utilization of agricultural lands diminished by climate change and environmental stresses can be made possible through the applied outcomes of proline research. This would support food security on a global scale.

Conflict of Interest Statement

The author of the article declare that there is no conflict of interest.

Contribution Rate Statement Summary of Researchers

The author declare that she contributed 100% to the article.

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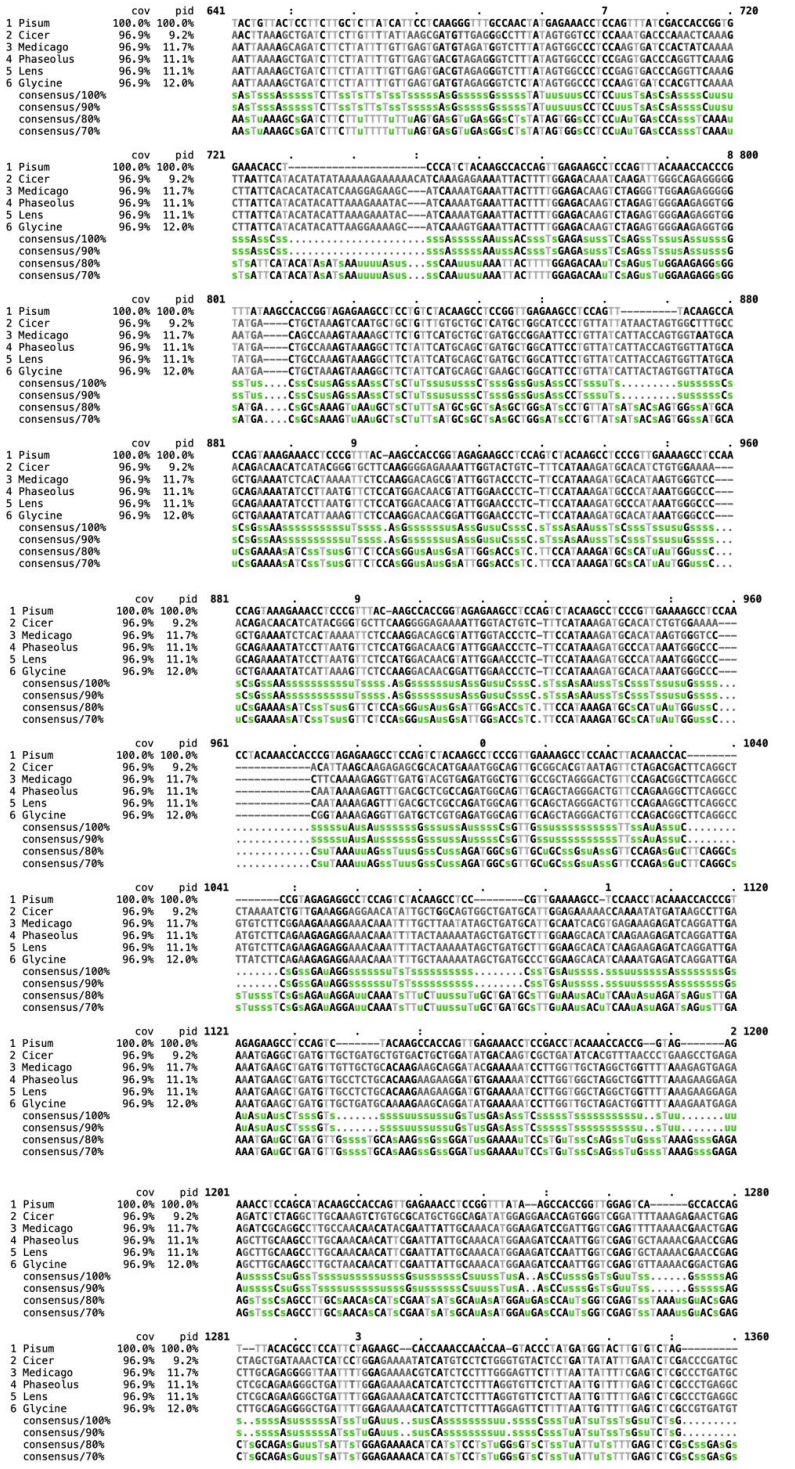

Additional Figure 1. Conserved regions of P5CS gene sequences of plant species aligned using the multiple sequence alignment program.