RESEARCH ARTICLE

IN SILICO PROFILING OF PROLINE BIOSYNTHESIS AND DEGRADATION RELATED GENES DURING FRUIT DEVELOPMENT OF TOMATO

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SUMMARY

Advancements in DNA sequencing technologies with decreasing costs have sparked the generation of larger gene expression datasets generated at an accelerating rate. The study aimed to visualize the spatiotemporal profiles of the tomato (*Solanum lycopersicum* L.) genes involved in L-proline biosynthesis and to show their potential functions. Increasing L-proline accumulation, through upregulation and downregulation of genes responsible for L-proline biosynthesis and degradation, plays an essential role in tomato plants suffering abiotic and biotic stress. Understanding the possible mechanism of L-proline biosynthesis and degradation needs an urgent study of the expression pattern and function of genes involved in these physiological processes. The study identified the genes governing the L-proline biosynthesis and degradation pathways and their expression profiles in 10 stages of tomato fruit development using the Tomato Expression Atlas (TEA) bioinformatic tool. The analysis showed that L-proline biosynthesis resulted from three pathways governed by six genes, while its degradation occurred in two pathways managed by three genes. The bioinformatics analysis also showed the expression of proline synthesis/degradation-related genes in fruit parts at various developmental stages. However, proline degradation-related genes showed higher expression levels than biosynthesis-related genes. This study sheds light on a recent bioinformatics tool, which will pave the way to detect early plant performance by analyzing the expression profiles of genes.

Keywords: Tomato, bioinformatics, L-proline accumulation genes, gene expression, proline biosynthesis genes, salinity, drought

Key findings: The recent study is a new alleyway to get fast *in silico* prediction of gene expression profiles related to proline biosynthesis and degradation during 10 developmental stages of a tomato fruit over *in vitro* experiments.

INTRODUCTION

Abiotic and biotic stress conditions negatively affect plant development and yield (Siripornadulsil *et al*., 2002; Moustafa *et al*., 2021). During stress conditions, plants tend to accumulate low-molecular-weight osmolytes, including L-proline (Sharma *et al*., 2019). Plants use L-proline to combat stress in many ways, including adjusting cell osmotic pressure and protein compound composition, contributing to reactive oxygen species (ROS) scavenging, and maintaining membrane integrity (Ashraf and Foolad, 2007; Hayat *et al*., 2012). The L-proline serves as an osmolyte, as well as, an antioxidative defense, and metal chelator molecules during stress conditions. The amino acid L-proline also contains an auxiliary amine necessary for metabolism (Verslues and Sharma, 2010; Meena *et al*., 2019). The accumulation of L-proline most commonly occurs in the cytoplasm, where it acts as a molecular companion, to resolve the protein structure, buffers the cytosol pH, and maintains the oxidation-reduction homeostasis of the cell. L-proline also acts as an osmolyte, radical scavenger, macromolecule stabilizer, and a cell wall component (Matysik *et al*., 2002).

Under normal conditions, plants have less than 5% L-proline content in the overall pool (Cara *et al*., 2020). The concentration of amino acids increases up to 80% in different plants under various stress conditions. In plants, L-proline synthesis occurs from glutamate, and its intracellular levels regulate production, degradation, and transport between cells. In higher plants, such as, tomatoes (*S. lycopersicum* L.), L-proline synthesis occurs through the glutamate or ornithine pathways (Kishor *et al*., 2005).

Exogenous L-proline ingestion proves to alleviate the plant abiotic stress found in many studies (Badiaa *et al*., 2020; Redha *et al*., 2021). However, plant stress tolerance can be improved by applying exogenous L-proline through spraying and soaking treatments (Boulahia *et al*., 2021). This is only advantageous with a sufficient amount or low concentration of L-proline, as greater concentrations cause toxicity (Badiaa *et al*., 2020; Redha *et al*., 2021).

Determining the L-proline biosynthesis and degradation genes and assessing their spatiotemporal expression profiles is important to enhance plants' stress tolerance to abiotic and biotic stresses by controlling their expression levels (Hassanin *et al*., 2017, 2020). *In silico* gene expression analysis uses the Tomato Expression Atlas (TEA). The TEA bioinformatic tool enables precise assessment of gene expression profiles using computed tomography gene expression of histological sections of tomato fruits at several development stages (Nelson *et al*., 2006). The TEA tool was performed to detect the expression profiles of several genes in tomatoes (Pattison *et al*., 2015). So far, a low number of reports about proline-related genes in *S. lycopersicum* L exists. Therefore, this study analyzed the expression profiles of six proline biosynthesis-related and three proline degradation-related genes in 10 stages of tomato fruit development to shed light on the genes that can be upregulated or downregulated in tomato plants to survive under abiotic and biotic stress conditions.

MATERIALS AND METHODS

During the study, all the bioinformatics analyses were carried out at the Sol Genomics Network (SGN; http://solgenomics.net/) tools that contain information on genomics, genetics, transcriptomics, and phenotypic data of solanaceous plants (tomato, potato, eggplant, pepper, and petunia), families Plantaginaceae (snapdragon), and Rubiaceae (coffee). The study on tomato (*S. lycopersicum* L.) was from 2021 to 2022 at the Department of Genetics, Faculty of Agriculture, Zagazig University, Egypt.

In silico gene expression analysis

Gene expression profiles of six proline biosynthesis-related genes, i.e., *Solyc08g043170.2* (gene 1), *Solyc00g026860.1* (gene 2)*, *Solyc02g068640.2* (gene 3), *Solyc12g089210.1* (gene 4), *Solyc04g080610.2* (gene 5), and *Solyc08g048450.2* (gene 6) (Table 1 and

Table 1. Genes involved in proline biosynthesis in tomato fruits.

| Gene name	Gene location	Length (bp)	Synonyms	Sol Genomics accession	Enzyme
Gene 1	Chromosome 8	12515	Solyc08g043170 SGN-U575897 pro2 tompro2 P5cs	Solyc08g043170.2 (LycoCyc)	δ1-pyrroline-5-carboxylate synthetase glutamate-5-semialdehyde dehydrogenase gamma-glutamyl kinase
Gene 2	Chromosome Pseudomolecule	1399	Solyc00g026860	Solyc00g026860.1 (LycoCyc)	Glutamate 5-kinase
Gene 3	Chromosome 2	6828	Solyc02g068640 SGN-U567857	Solyc02g068640.2 (LycoCyc)	Pyrroline-5-carboxylate reductase
Gene 4	Chromosome 12	4200	Solyc12g089210 SGN-U594401	Solyc12g089210.1 (LycoCyc)	Ornithine carbamoyltransferase
Gene 5	Chromosome 4	4311	Solyc04g080610 SGN-U575137	Solyc04g080610.2 (LycoCyc)	Ornithine carbamoyltransferase
Gene 6	Chromosome 8	11448	Solyc08g048450 SGN-U594785 Oat	Solyc08g048450.2 (LycoCyc)	Ornithine-oxo-acid transaminase ornithine aminotransferase
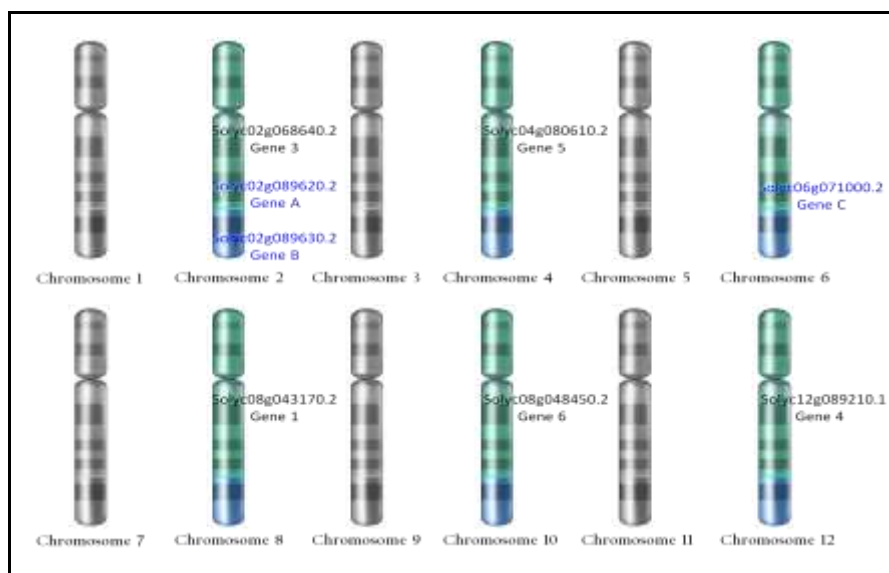
Figure 1. Illustration of tomato chromosomes with the position of genes involved in proline biosynthesis (in black) and proline degradation (in blue).

Figure 1) and three proline degradation-related genes. i.e., *Solyc02g089620.2* (gene A), *Solyc02g089630.2* (gene B), and *Solyc06g071000.2* (gene C) (Table 2 and Figure 1) were *in silico* analyzed in 10 tomato fruit development stages; anthesis (0DPA) (zero Days Post Anthesis), five DPA, 10 DPA, 20 DPA, 30 DPA, MG (Mature green), Br (Breaker), Pi (Pink), LR (Light red), and RR (Red ripe) to detect the expression levels of these genes in each stage within six parts of the fruit (Total pericarp, septum, locular tissue, placenta, columella, and seeds) using the bioinformatics tool TEA (http://tea.solgenomics.net/).

Visualization of gene expression TEA tool

Using the Expression viewer of TEA visualized the *in silico* expression data of proline biosynthesis and degradation-related genes based on the reads per million (RPM) in the various stages of fruit development in tomatoes. The TEA tool enables the determination of *in silico* expression profile of a gene using the blast the Gene ID and selecting the required developmental stages in tomato fruit and the tissue and cell types (https://tea.solgenomics.net/expression_viewe

Table 2. Genes involved in proline degradation in tomato fruits.

Gene name	Gene location	Length (bp)	Synonyms	Sol Genomics accession	Enzyme
Gene A	Chromosome 2	1753	Solyc02g089620 SGN-U581540 SGN-U592486	Solyc02g089620.2 (LycoCyc)	Proline dehydrogenase
Gene B	Chromosome 2	2235	Solyc02g089630 SGN-U578070 SGN-U592869 SGN-U592982	Solyc02g089630.2 (LycoCyc)	CIG1
Gene C	Chromosome 6	6203	Solyc06g071000 SGN-U571121 SGN-U588701 SGN-U588703 SGN-U588704	Solyc06g071000.2 (LycoCyc)	P5CD
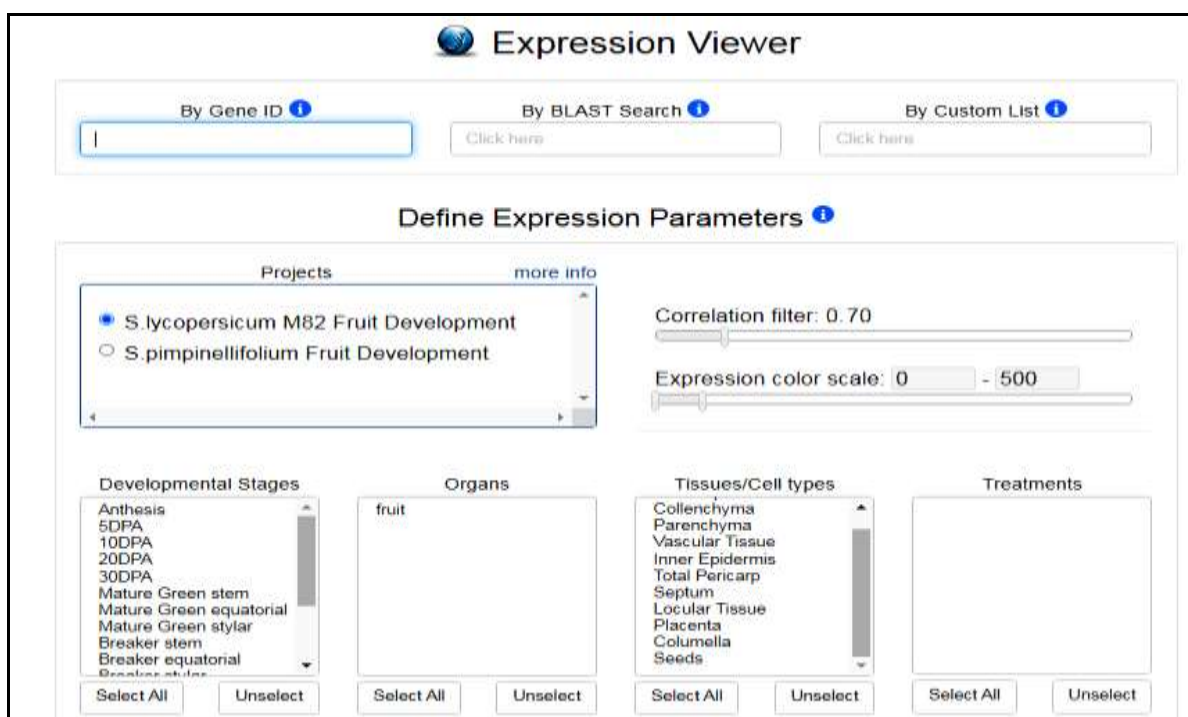
Figure 2. The expression viewer tool page indicates the required fields and the parameters to be selected.

r/input) (Figure 2). The *in silico* expression profiles were downloaded using the Sol Genomics database based on selected criteria (http://tea.solgenomics.net/) (Fernandez-Pozo *et al.*, 2017).

Extract sequences from BLAST databases

Using BLAST databases (https://solgenomics.sgn.cornell.edu/tools/blast/) the accession ID of the L-proline biosynthesis and degradation-related genes were extracted. Clicking on the "Extract sequences from BLAST databases" button and typing in the search string, an automatic search will commence in the selected database. Next steps include entering the sequence in the query text area and choosing the appropriate format from the format selector (e.g. nucleotide fasta), and the appropriate blast program (e.g. blastn for running a nucleotide query against a nucleotide database). The default is to autodetect, which should detect the format entered by the user and convert it to the correct sequence format to run blast automatically (Figure 3).
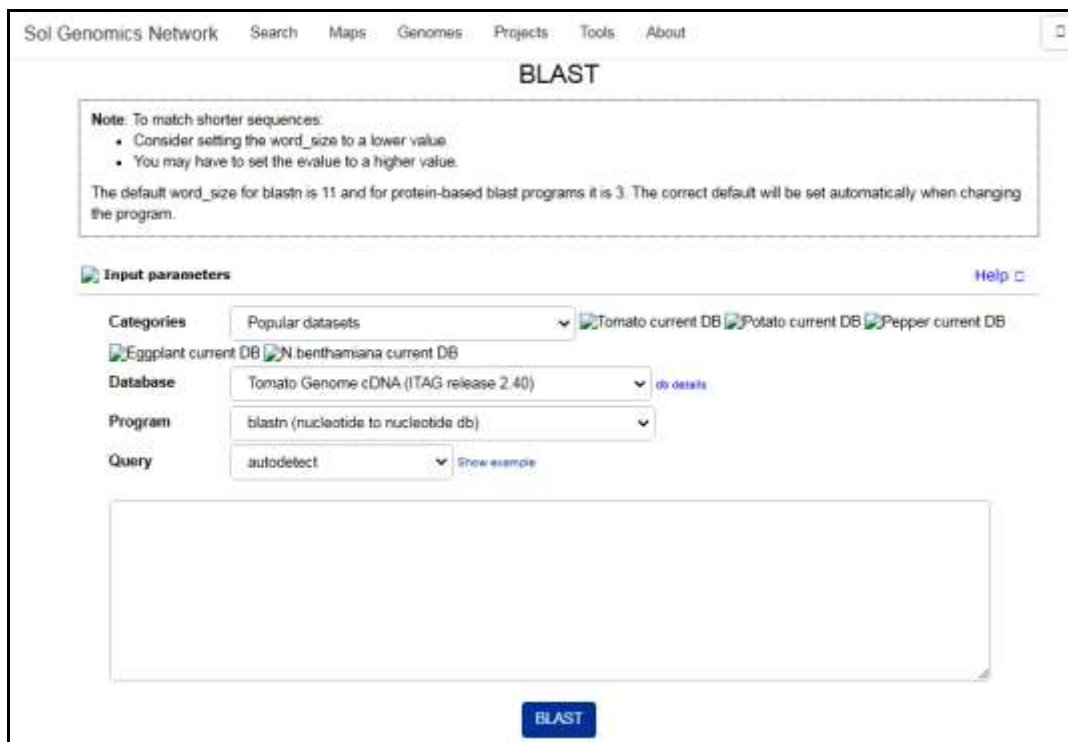
Figure 3. BLAST database window of Sol Genomics Network.

Pathway tools software

L-proline biosynthesis and degradation pathways were determined using pathway tools (https://biocyc.org/otherpgdbs.shtml). Pathway tools is a comprehensive bioinformatics software that spans enterprise genome data management, system biology, and omics data analysis. The software provides genome-informatics tools, such as, genome browser, sequence alignments, genome-variant analyzer, and comparative-genomics operation. It offers metabolic-informatics tools, i.e., metabolic reconstruction, quantitative metabolic modeling, prediction of reaction atom mapping, and metabolic route search. Pathway tools also provide regulatory-informatics tools, such as, the ability to represent and visualize a wide range of regulatory interactions.

RESULTS

L-proline biosynthesis pathways

Pathway tools indicated that the proline biosynthesis is performed through three pathways, i.e., pathway-I initiated from L-glutamate (Figure 4). This pathway is governed by three genes: gene 1 codes for δ1-pyrroline-5-carboxylate synthetase (P5CS), gamma-glutamyl phosphate reductase (GPR), and glutamate-5-semialdehyde dehydrogenase (G5SD), gene 2 codes for gamma-glutamyl kinase (GK), and gene 3 codes for pyrroline-5-carboxylate reductase (P5CR). The two enzymes, i.e., G5K and G5SD catalyzed the formation of L-glutamate-5-semialdehyde, which spontaneously formed (*S*)-1-pyrroline-5-carboxylate that is converted to L-proline in a single step, catalyzed by P5CR. Pathway-II of L-proline biosynthesis initiated from L-arginine (Figure 5), and this pathway is promoted by four genes, i.e., gene 3 codes for P5CR, gene 4 codes for ornithine carbamoyltransferase, gene 5 codes for ornithine carbamoyltransferase, and gene 6 codes for ornithine aminotransferase (OAT). Pathway-III of L-proline biosynthesis started from L-glutamate (Figure 6), and this pathway is catalyzed by three genes like those of pathway-I: gene 1 codes for P5CS, GPR, and G5SD, gene 3 codes for P5CR, and gene 6 codes for OAT.
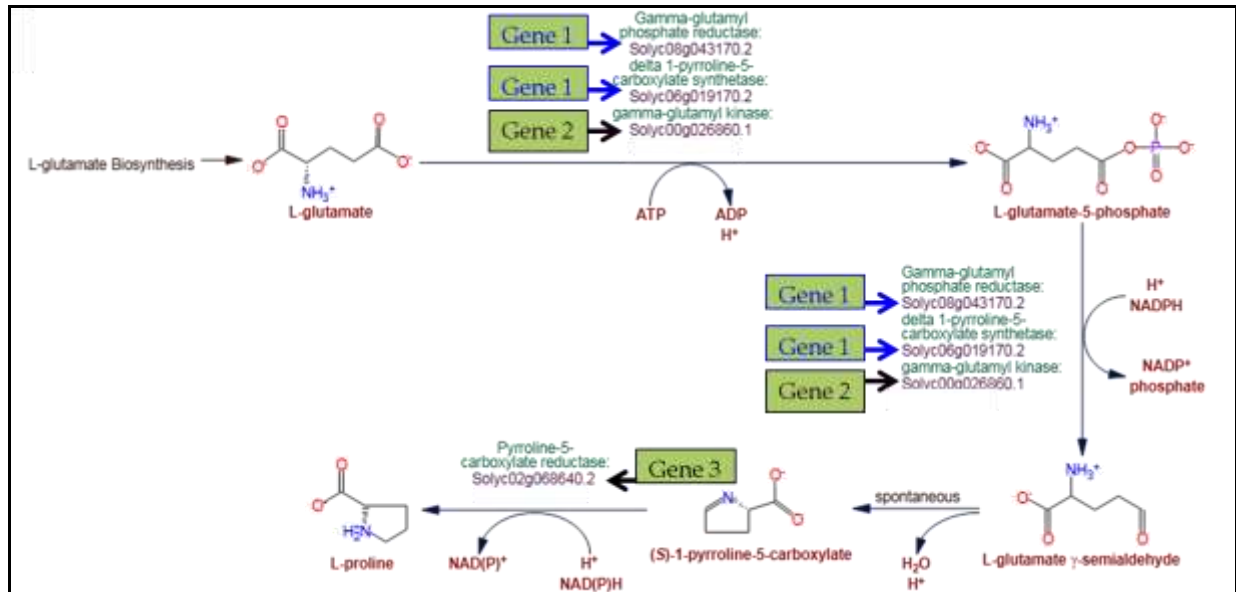
Figure 4. The metabolic pathway of L-proline through glutamate and ornithine. It also indicates the genes which catalyze the pathway I for L-proline synthesis.
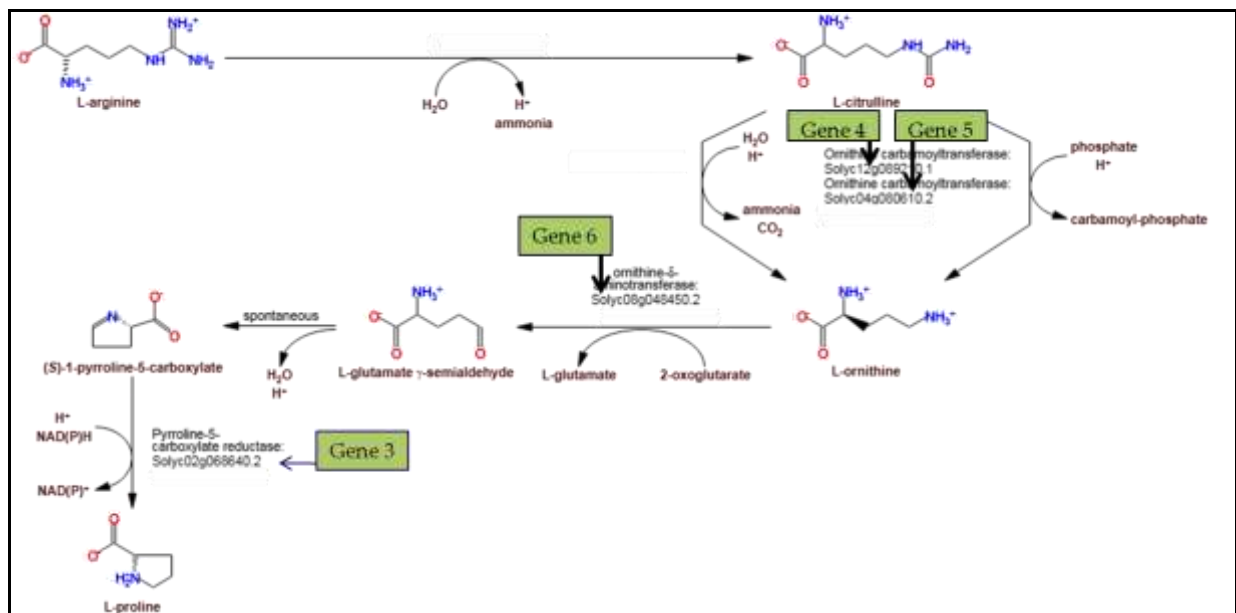

Figure 5. The metabolic pathway of L-proline through L-arginine. It also indicates the genes which catalyze the pathway II for L-proline synthesis.
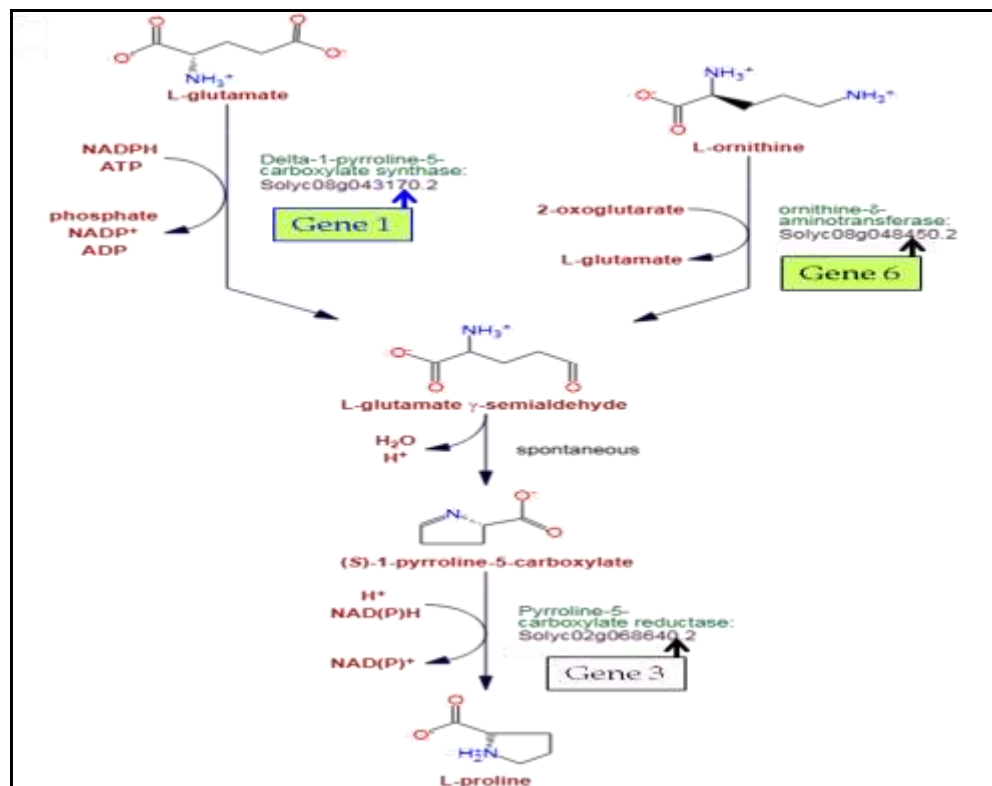
Figure 6. The metabolic pathway of L-proline through glutamate and ornithine. It also indicates the genes which catalyze the pathway III for L-proline synthesis.

L-proline degradation pathways

Degradation of L-proline. These pathways include degradation of L-proline to L-glutamate in tomatoes conducted by the action of two enzymes: proline dehydrogenase (PDH) encoded by gene A and L-glutamate γ-semialdehyde dehydrogenase (P5CDH) encoded by gene B, as well as, the action of gene C that codes for 1-pyrroline-5-carboxylate dehydrogenase (Figure 7 A and B).

L-proline is transformed to L-glutamate and then degraded to 2-oxoglutarate, which can be used as a complete supply of carbon, energy, and nitrogen. However, an insufficient supply of carbon and energy occurs when the transport of L-glutamate supplies exogenous L-glutamate at an insufficient rate, where L-glutamate is produced when L-proline is broken down. Given that L-glutamate-5-semialdehyde as an intermediary between (S)-1-pyrroline-5-carboxylate and L-glutamate, the route is presented in three steps where (S)-1-pyrroline-5-carboxylate hydrolyzes spontaneously to L-glutamate-5-semialdehyde.

***In silico* expression patterns of L-proline biosynthesis genes in fruits**

Gene expression profiles of six L-proline biosynthesis genes *in silico* were analyzed in 10 tomato fruit development stages to determine the expression levels of genes in each stage within six fruit parts (Figure 8A). BLAST results showed that the spatiotemporal expression of gene 1 reached the highest level at the zero DPA stage in all the fruit parts, whereas the lowest expression level was at the light red (LR) stage (Figures 8B and 10A). Gene 2 revealed no RPM profile and showed a lower expression compared with the other proline biosynthesis-related genes (Figure 8B). The expression of gene 3 showed varying levels of expression during tomato fruit development (Figures 8C and 10C). The expression in each total pericarp, septum, placenta, and columella reached its highest level at the 20 DPA stage, whereas the lowest level was at the light green stage. However, the anthesis stage showed the highest level of expression in both locular tissue and seeds.
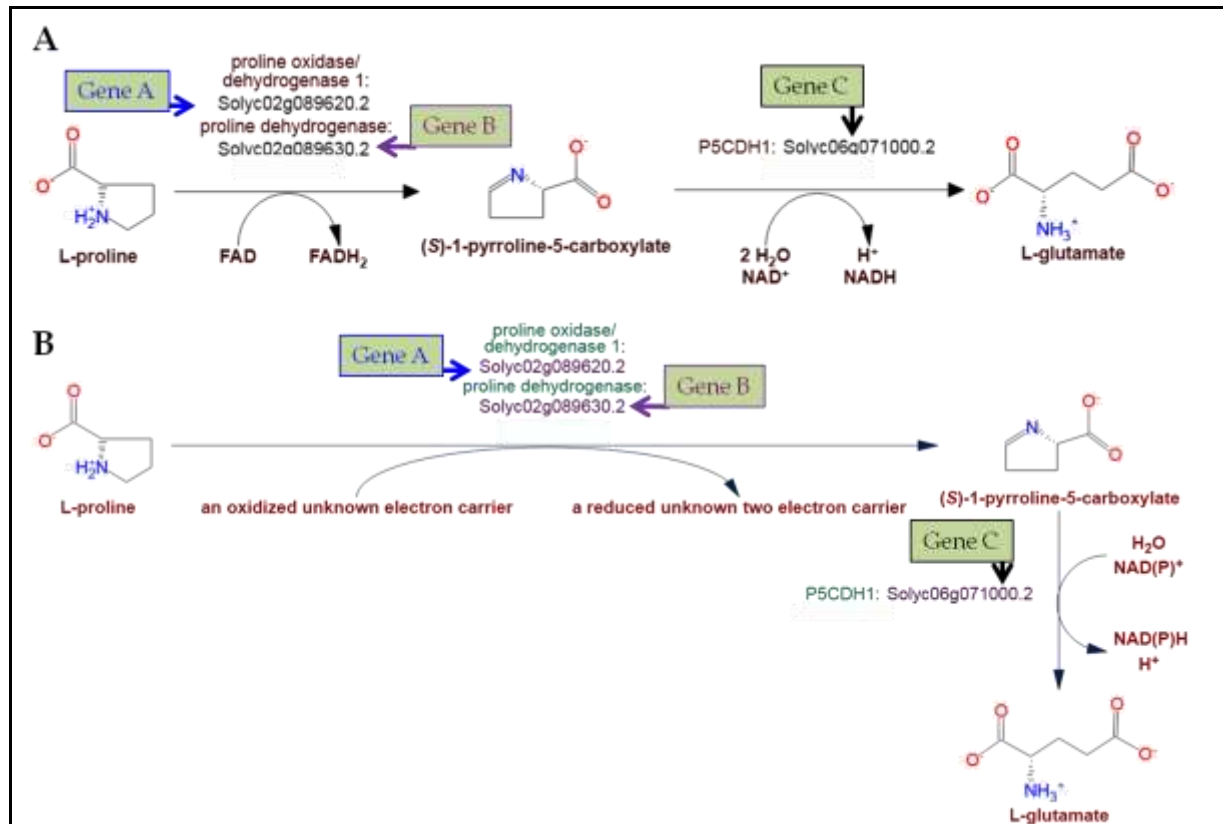
Figure 7. Proline degradation pathways. A. Proline degradation pathways I B. Proline degradation pathways II.

Gene 4 expression was enhanced in total pericarp and locular tissue at the red ripe stage and decreased at the mature green and anthesis stages (Figures 9A and 11A). In the septum, the highest expression level was at the pink stage but reached its lowest level at the anthesis stage. In the placenta, columella, and seeds, the highest expression level was at 10 DPA. The expression profile of gene 5 was slightly increased at the anthesis stage in all parts of the fruit and in each locular, placenta, and seed at 5 DPA (Figures 9B and 11B). In gene 6, the expression profile presented a regular trend. However, the gene showed a low expression level during the primary fruit development stages, then increased steadily in the later developmental stages (Figures 9C and 11C).

In silico expression pattern of proline degradation genes in fruits

Gene expression profiles of three L-proline degradation genes *in silico* indicated in 10 tomato fruit development stages within six fruit parts. *In silico* expression analysis showed that the spatiotemporal expression of gene A reached the highest level at the 10 DPA stage in all fruit parts, whereas lowest expression level was at pink, light red, and red ripe stages (Figures 12A and 13A). Gene A also presented the highest expression pattern in total pericarp at 5 DPA, 10DPA, 20DPA, and 30DPA. The gene B also showed the highest expression pattern in locular tissue and placenta, particularly at 5DPA, 10DPA, and 20DPA (Figures 12B and 13B). The expression of gene C reached its highest level at 10DPA and 20DPA in almost all the fruit parts (Figures 12C and 13C).
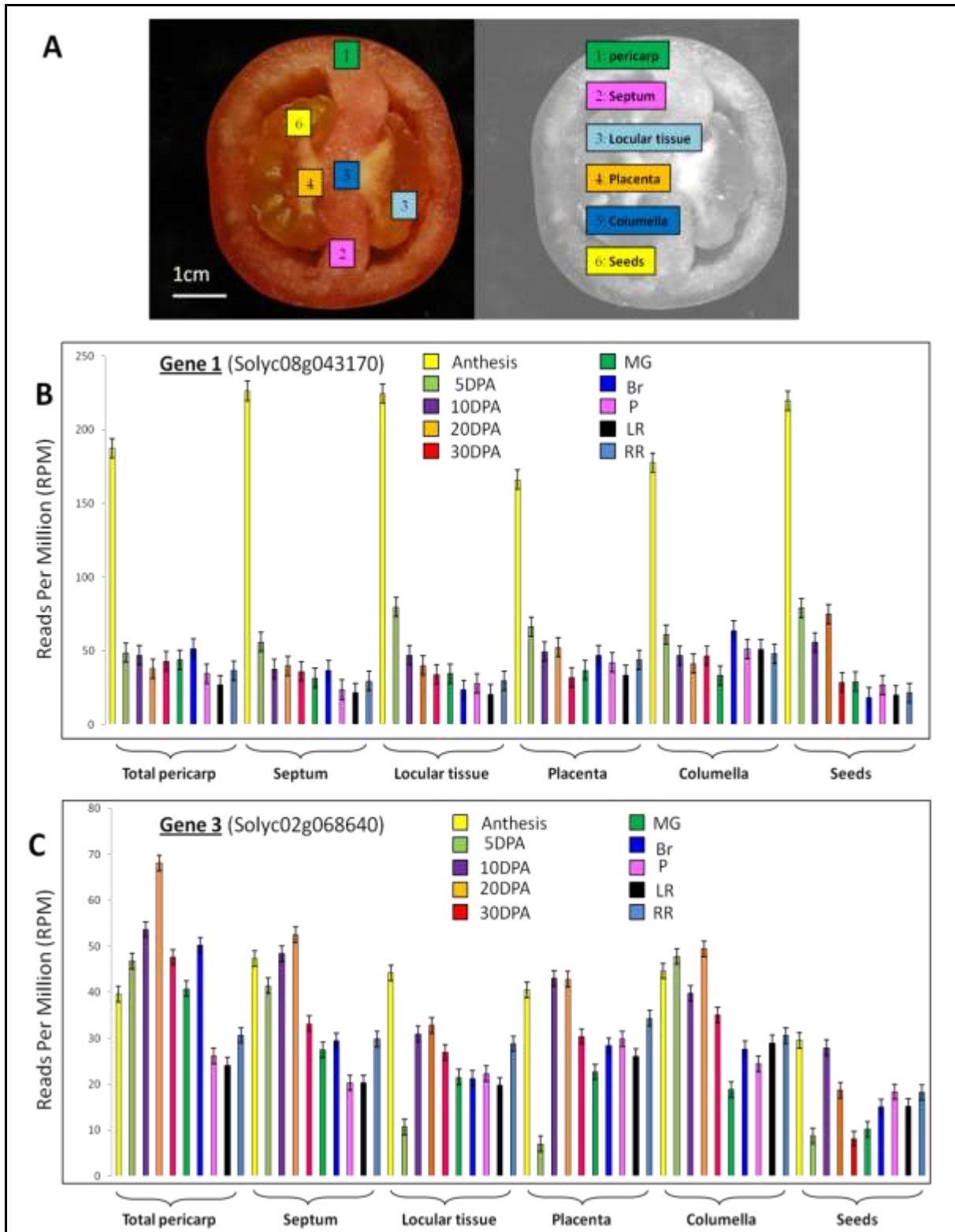
Figure 8. The expression profiles analysis of L-proline biosynthesis-related genes; *Solyc08g043170.2* and *Solyc02g068640.2* in fruits of tomato. (A) Illustration of tomato fruit parts involved in *in silico* expression analysis (B) Expression of *Solyc08g043170.2* gene (C) Expression of *Solyc02g068640.2* gene. Each data point represents the reads per million (RPM) with standard deviation bars.
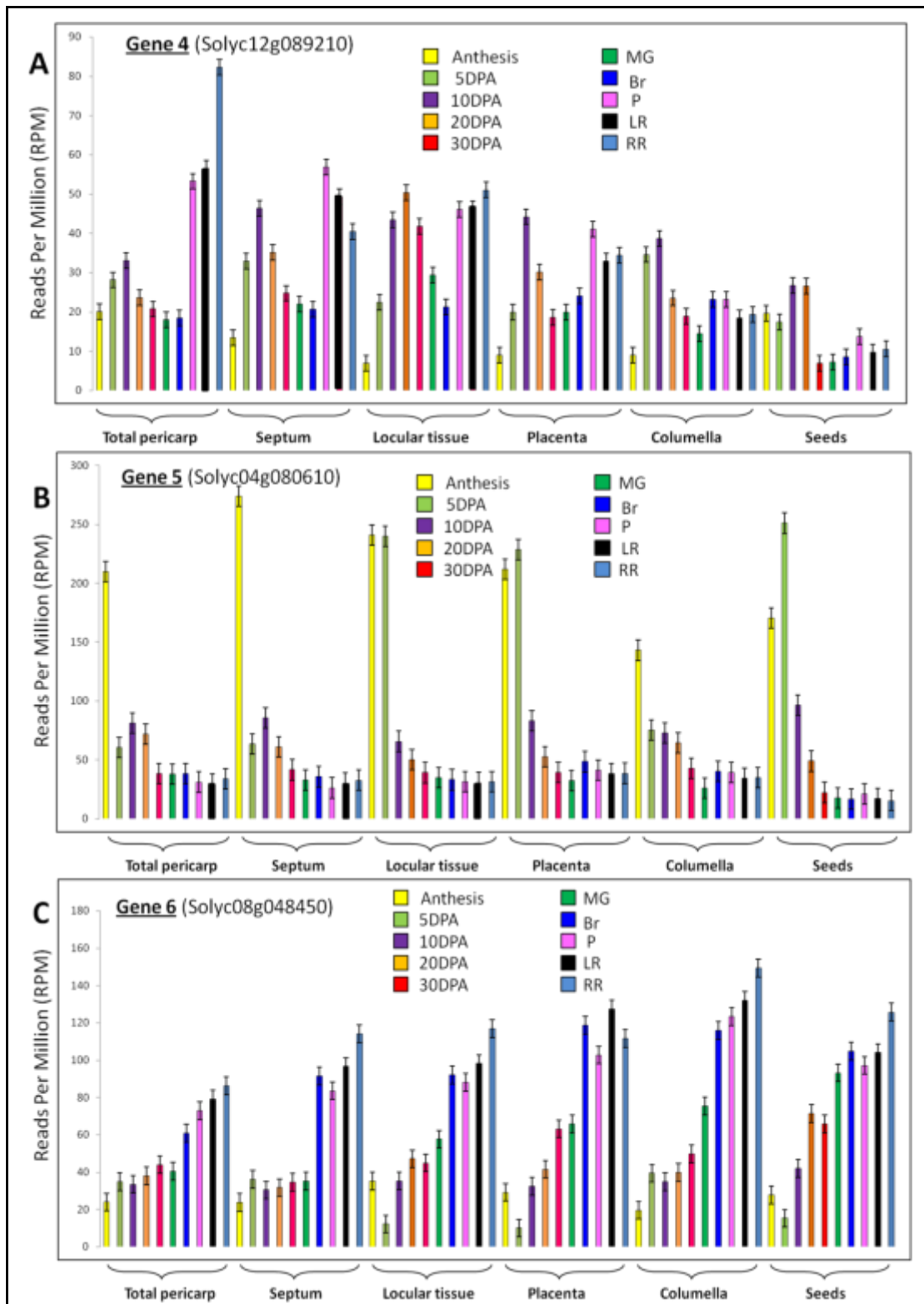
Figure 9. The expression profiles analysis of L-proline biosynthesis-related genes; *Solyc12g089210.1, Solyc04g080610.2, Solyc08g048450.2* in fruits of tomato. (A) Expression of *Solyc12g089210.1* gene (B) Expression of *Solyc04g080610.2* gene (C) Expression of *Solyc08g048450.2* gene. Each data point represents the reads per million (RPM) with standard deviation bars.
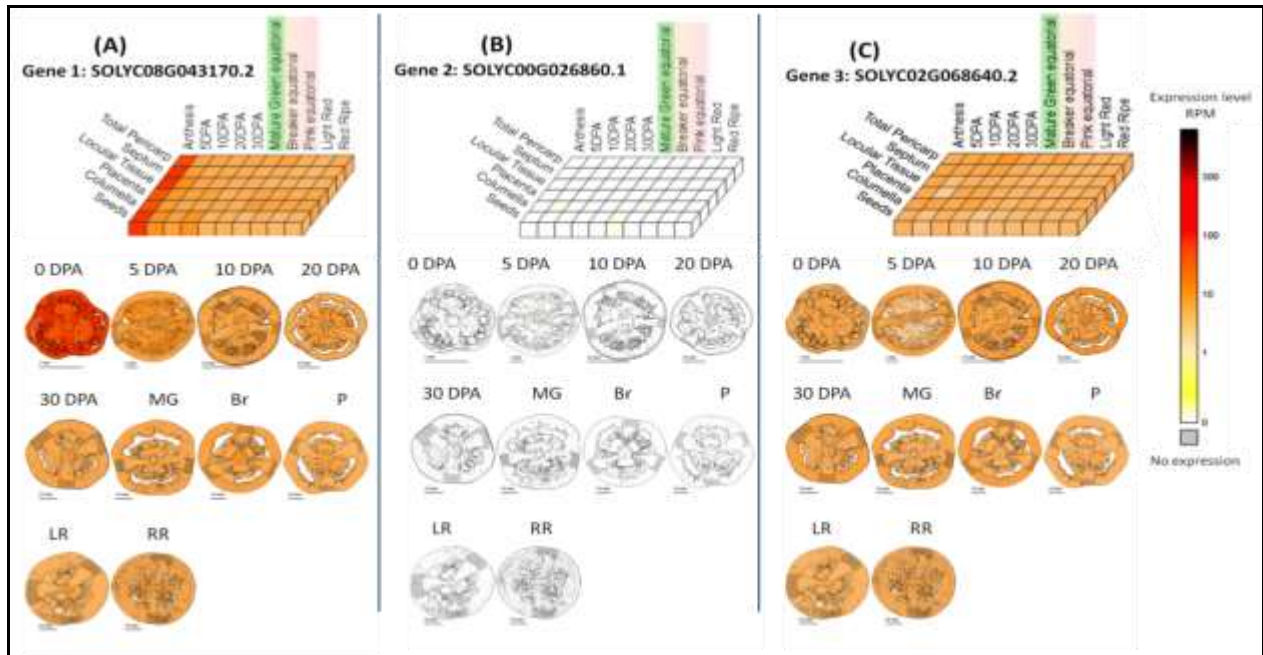
Figure 10. Computed tomography gene expression profiles of proline biosynthesis-related genes; *Solyc08g043170.2*, *Solyc00g026860.1*and *Solyc02g068640.2* genes during tomato fruit development. Note: Colors represent expression values in RPM. RPM=reads per million.
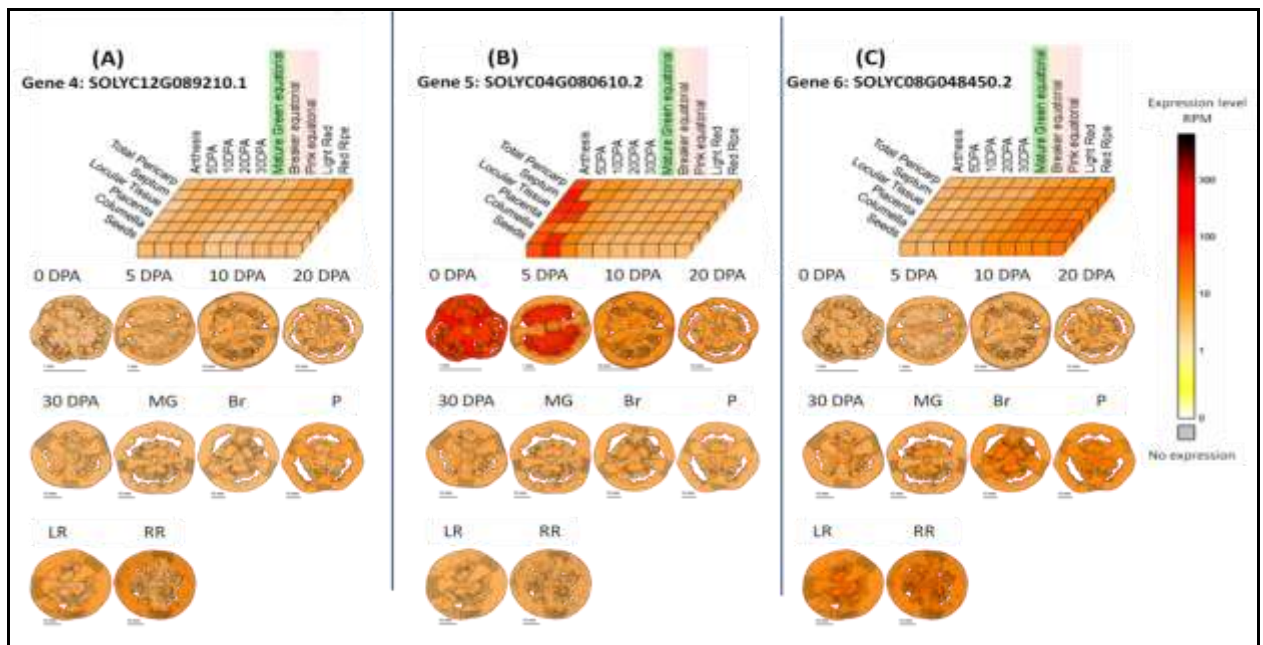

Figure 11. Computed tomography gene expression profiles of proline biosynthesis genes; *Solyc12g089210.1*, *Solyc04g080610.2*, and *Solyc08g048450.2* genes during fruit development of tomato. Note: Colors represent expression values in RPM. RPM=reads per million.
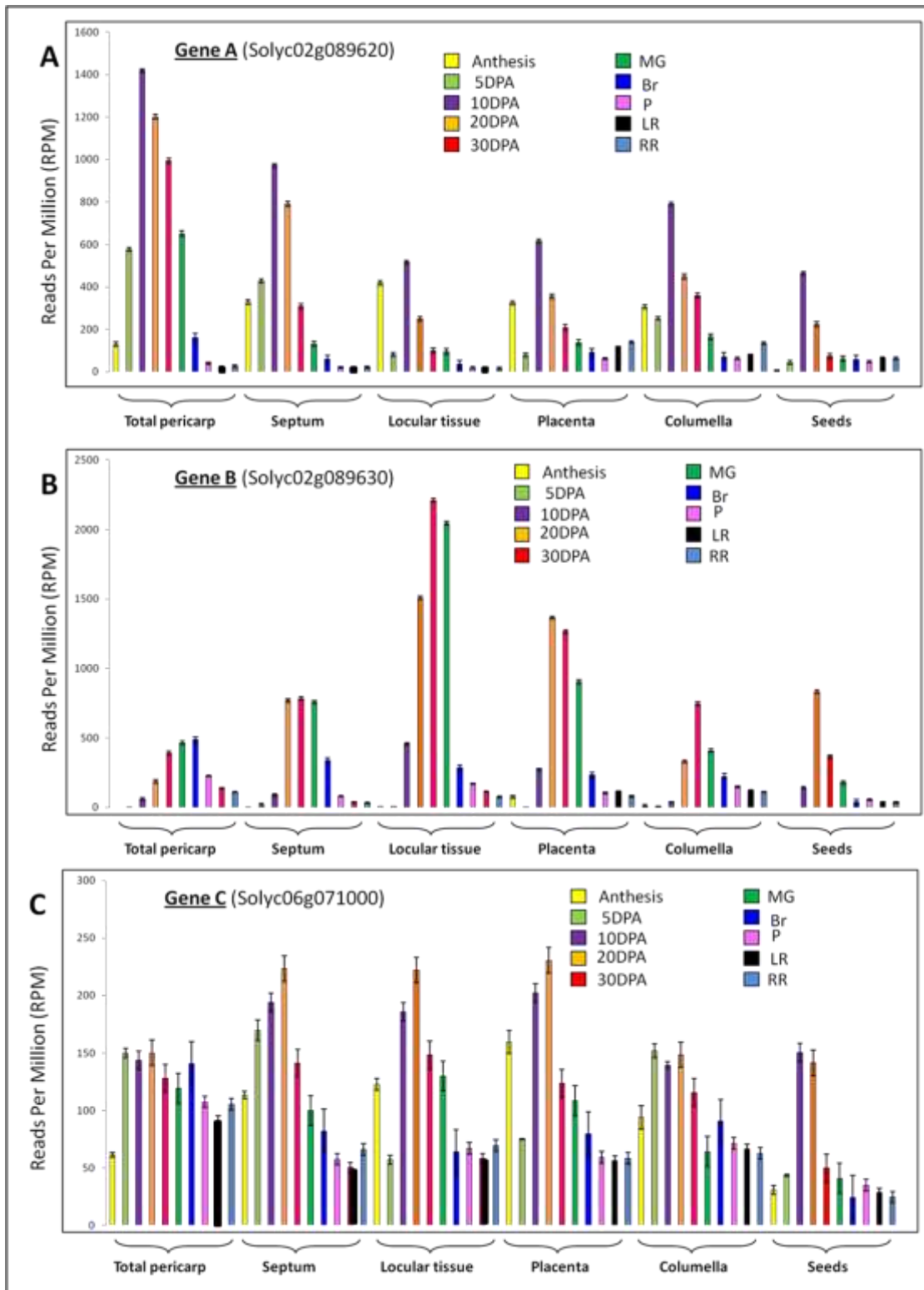
Figure 12. The expression profiles analysis of L-proline degradation-related genes; *SOLYC02G089620.2, SOLYC02G089630.2* and *SOLYC06G071000.2* in fruits of tomato. (A) Expression of *SOLYC02G089620.2* gene (B) Expression of *SOLYC02G089630.2* gene (C) Expression of *SOLYC06G071000.2* gene. Each data point represents the reads per million (RPM) with standard deviation bars.
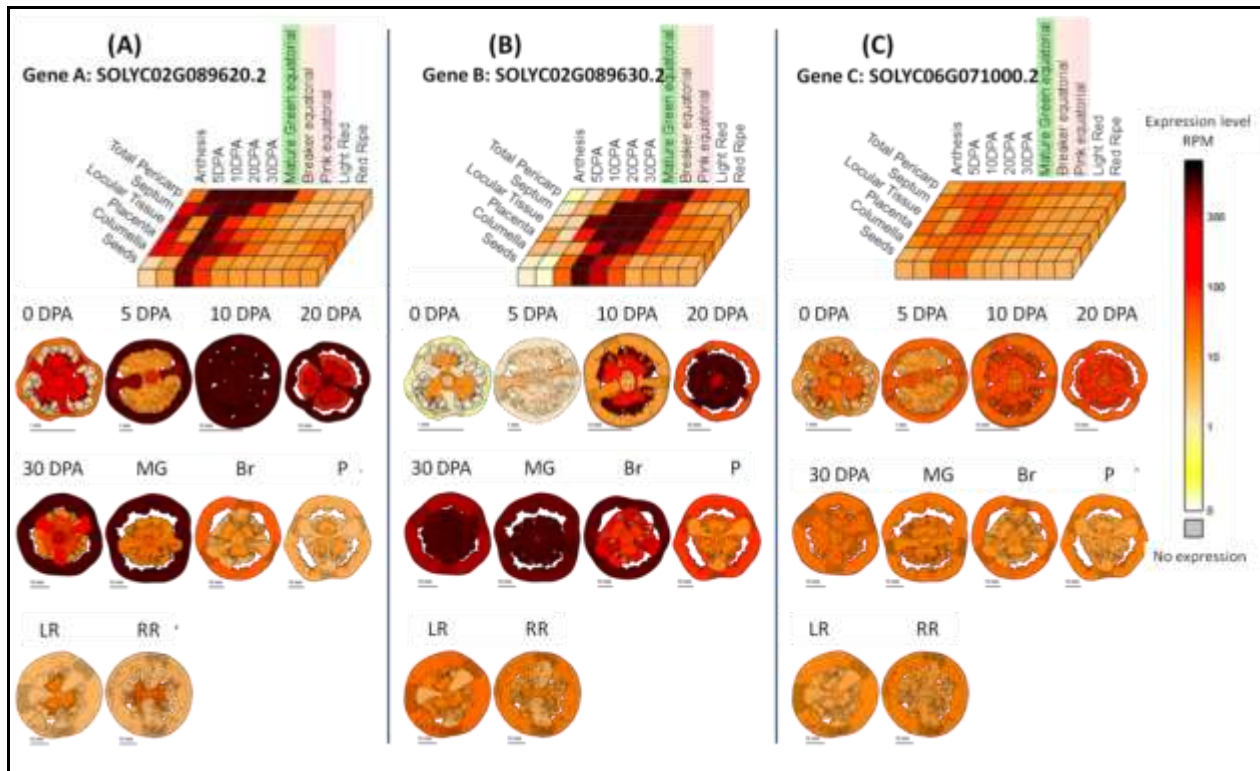
Figure 13. Computed tomography gene expression profiles of proline degradation genes; *SOLYC02G089620.2, SOLYC02G089630.2* and *SOLYC06G071000.2* genes during fruit development of tomato. Note: Colors represent expression values in RPM. RPM=reads per million.

DISCUSSION

In plants, the relative L-proline quantity is a result of the relative ratio of synthesis/degradation cycle. For this reason, there is the need to determine the expression of genes involved in these processes to figure out how L-proline metabolism works. In plants that are facing osmotic stress, L-proline acts as an osmotic protectant, in addition to being a major component of protein chains. Plants respond to abscisic acid (ABA) and salt stress by increasing proline biosynthesis to alleviate their harmful impact and scavenge the resulted free radicals (Abrahám *et al*., 2003).

During salt stress, proline can reach up to 20% of the total amino acids in *Arabidopsis* (Verbruggen *et al*., 1993). Both ornithine (L-proline biosynthesis pathway III (from L-ornithine)) and glutamate routes participate in the rise of L-proline levels in young Arabidopsis plants during osmotic stress, whereas only the glutamate pathway is involved in L-proline accumulation in adult plants (Roosens, 1998). In moth bean plants grown under salinity stress, a study found the glutamate pathway a substantial contributor to L-proline production in response to osmotic stress (Delauney *et al*., 1993). However, in *Medicago truncatula*, the ornithine and glutamate routes contribute to the proline upsurge caused by osmotic stress (Armengaud *et al*., 2004). Several studies determined the relative participation of glutamate and ornithine pathways in increasing L-proline accumulation in stressed plants. In determining L-proline biosynthesis and degradation, a study tested the salt-stressed plants for free proline concentration, gene expression, and enzyme activity of the glutamate pathway (Roosens (1998)). Their findings indicated that the salt-stress treatment markedly enhanced proline accumulation, mRNA levels, and enzymatic activity.

L-arginine is converted to L-proline by a single dual-function enzyme expressed by the *agrE* gene in *Nostoc* sp., a genus of cyanobacteria found in many terrestrial or freshwater backgrounds that form colonies. This enzyme's N-terminal part has arginine

dihydrolase, while the C-terminal part has ornithine cyclodeaminase. The enzyme is capable of converting L-arginine to L-proline by combining both activities, releasing three molecules of ammonium (Burnat *et al*., 2019).

Under normal conditions, the changes in proline content are accompanied by plants, especially in reproductive tissues (Lehmann *et al*., 2010). This study explored the extent of expression diversification of proline biosynthesis-related genes in tomatoes by investigating the expression profiles of six genes in different developmental stages of tomato plants. Results of proline expression patterns indicated that the proline accumulation is regulated in all growth and developmental stages of tomato fruit. For example, the proline biosynthesis is upregulated by gene 1 and gene 5 in the anthesis stage, followed by increased accumulation made by gene 3 in anthesis, 5 DPA, 10 DPA, and 20 DPA, respectively. Similarly, gene 4 controlled the proline biosynthesis in the later stages of tomato fruit development, while gene 6 presented a growing expression profile from anthesis to the red rope stage.

The glutamate route is the key pathway in L-proline formation during osmotic stress, based on the decreased OAT and increased P5CS transcript levels in moth bean plants under salt stress conditions (Delauney *et al*., 1993). In relation to the study, the glutamate pathway is enhanced when nitrogen is scarce, but the ornithine pathway is prominent when nitrogen is abundant. However, studies of P5CS and OAT transcript levels and their relationships with free proline content revealed that glutamate and ornithine routes play an important role in osmotic stress-induced L-proline buildup in *Medicago* (Armengaud *et al*., 2004).

The study revealed two important results. First is the integration of gene 3 in proline biosynthesis from L-arginine and the biosynthesis of proline from L-glutamate. Second is the integration of gene 6 in proline biosynthesis from L-arginine and the biosynthesis of proline from L-ornithine, which means that gene 6 is expressed in mitochondria where the ornithine pathway takes place. These results support that over-expression of gene 3 and gene 6 increased salt-, heat-, and drought-tolerance in tomatoes. Abscisic acid and salt stress conditions were considered positive regulators for the biosynthesis of L-proline (Abrahám *et al*., 2003). However, phospholipase D (PLD) is proved to be a negative regulator of L-proline

biosynthesis in *A. thaliana* (Thiery *et al*., 2004).

In tomatoes, the proline degrading enzymes, PDH and P5CDH, are expressed by two separate genes, and a single polypeptide expressed by the *putA* gene catalyzes both steps in most bacteria, including enteric bacteria (Menzel and Roth, 1981; Soto *et al*., 2000). Although the enzyme is substantially conserved across microorganisms, its genetic organization and expression control are widely diverse (Soto *et al*., 2000). The study revealed that the expression pattern of proline degradation genes reached its highest expression level in the intermediate stages of tomato fruit development—10 DPA, 20 DPA, and mature green. These findings are of great importance to avoid decreasing proline quantity inside plant tissues by proline exogenous treatment of tomato crop in these stages.

Saccharomyces cerevisiae can use L-proline as a sole source of nitrogen supply, which gets catalyzed within the mitochondrial matrix. Both enzymes (proline oxidase and delta 1-pyrroline-5-carboxylate [P5C] dehydrogenase) that are encoded in the nucleus and produced in the cytoplasm, are imported inside the mitochondria before being activated (Brandriss and Magasanik, 1979). To maximize the benefits of this study, phylogeny and alignment analysis must be applied to determine the same proline-related genes in different plant species for probable functions and gene expression profiles (Fang *et al*., 2010, 2011; Hassanin *et al*., 2017, 2021; Fathy *et al*., 2021; Raza *et al*., 2021a; Al-Khayri *et al*., 2022; Zhang *et al*., 2022). These genes can be genetically engineered to be upregulated to improve stress tolerance efficiency (Abdelnour *et al*., 2021; Raza *et al*., 2021b).

CONCLUSIONS

The study aimed to determine the expression profiles of the genes responsible for proline biosynthesis and degradation during tomato fruit development through *in silico* tools. The analysis concluded six genes found on chromosomes 2, 4, 8, 10, and 12 controlled proline biosynthesis, while three genes in chromosomes 2 and 6 governed proline degradation in tomato fruits. The bioinformatics analysis also showed the expression of the proline-related genes throughout the fruit developmental stages in six fruit parts at variable levels. Studying the

gene expression profiles enabled the assessment of the conditions resulting in specific gene expressions and how the cell functions at a specific time. Finally, *in silico* gene expression profiling can help to design a hypothesis to predict plant behavior in future studies. Future research is needed to identify the upregulate and downregulate proline biosynthesis-related and degradation-related genes, to develop stress-tolerant tomato varieties with high yields under severe abiotic and biotic stress conditions.

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