



Transcription expression dynamics of genes involved in L-ascorbic acid biosynthesis during fruit development and maturation in Kinnow mandarin

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ABSTRACT

Citrus fruits are major sources of vitamin C (L-ascorbic acid), a key antioxidant that protects human cells against reactive oxygen species (ROS) generated during metabolism and environmental stress. This study examined the transcriptional regulation of genes involved in ascorbic acid (AsA) biosynthesis and recycling during fruit development in Kinnow mandarin (*Citrus reticulata*). Fruits were collected at three developmental stages, and gene expression was analyzed in flavedo, albedo, and pulp tissues using quantitative real-time PCR. Thirteen genes representing L-galactose, myo-inositol, and D-galacturonate pathways, along with the recycling pathway, were evaluated. Distinct tissue-specific and stage-dependent expression patterns were observed. L-galactose pathway genes showed strong upregulation in flavedo during maturation, indicating its central role in AsA biosynthesis. In contrast, myo-inositol and D-galacturonate pathway genes were downregulated, suggesting reduced contribution at later stages. Recycling pathway genes also declined, particularly in pulp tissues. These findings reveal coordinated regulation of AsA metabolism and highlight the pivotal role of flavedo in vitamin C accumulation, providing targets for improving citrus fruit nutritional quality.

Key words: Flavedo, albedo, pulp, metabolic pathways, tissue-specific regulation.

INTRODUCTION

Kinnow mandarin (*Citrus reticulata* Blanco) is an important commercial citrus fruit widely cultivated in India, valued for its high juice content, characteristic flavour, nutritional quality, and substantial economic importance. Vitamin C is a major component of the antioxidant system protecting human cellular structure from reactive oxygen species (ROS) generated during normal metabolic activities and environmental stresses (Gallie, 10; Wang *et al.*, 15; Yadav *et al.*, 18; Yu *et al.*, 19), making citrus fruits an important dietary source of vitamin C. L-ascorbic acid is synthesized through several metabolic pathways in plants, including the L-galactose, myo-inositol and D-galacturonate pathways (Castro *et al.*, 6; Wang *et al.*, 14). Besides biosynthesis, tissue AsA levels are regulated through recycling mechanisms involving monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), which play important roles in maintaining AsA levels in plant tissues (Foyer and Noctor, 9).

Recent advances in molecular biology and genomics have revealed the involvement of key genes in AsA biosynthesis and their association with AsA accumulation in several plant species (Laing *et al.*, 4). Expression analyses have shown significant changes in transcriptional activity of

AsA-related genes during fruit development and maturation (Rodrigo *et al.*, 2; Caruso *et al.*, 5; Yadav A *et al.*, 18). Differential transcriptional profiling in citrus has also revealed tissue-specific regulation of AsA metabolism (Alós *et al.*, 1). Kinnow mandarin, a hybrid of *Citrus nobilis* and *C. deliciosa*, is an important citrus cultivar valued for its high juice and vitamin C content. However, limited information is available on transcriptional regulation of vitamin C biosynthesis genes in flavedo, albedo and pulp during fruit development. Therefore, the present study investigated the expression profiling of key genes involved in vitamin C biosynthesis during fruit development and maturation in Kinnow mandarin.

MATERIALS AND METHODS

Three biological replicates of fruits of Kinnow mandarin genotype were collected from the Experimental Farm of the Centre of Excellence for Citrus, Kota, Rajasthan and used for all analyses. Fruit samples were collected during 2025–2026 at three developmental stages: Stage 1 (April–June 2025), Stage 2 (July–September 2025) and Stage 3 (October 2025–January 2026). RNA was independently extracted from each biological replicate and cDNA synthesis was performed separately. Quantitative RT-PCR reactions were carried out in technical triplicates and the mean Ct value was used for statistical analysis.

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Total RNA from different tissues was extracted using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. First-strand cDNA was synthesized using 100 ng DNase-free RNA and Phi-Script™ cDNA Master Mix (DXBIDT, Bangalore, India) in a 20 µL reaction volume. Expression analysis was performed using a QIAquant 96 5-Plex Real-Time PCR System (QIAGEN, Hilden, Germany) with Polaris™ qMaster Mix with SYBR® (DXBIDT, Bangalore, India). Relative mRNA expression of 13 genes involved in AsA biosynthesis and recycling was calculated using the $\Delta\Delta C_t$ method, where Stage 1 was used as the biological calibrator and 18S rRNA served as the internal reference gene.

Expression profiles of 13 target genes were quantified in flavedo, albedo and pulp tissues. The selected genes represented the D-galactose pathway (GMP, GME, GGP, GPP, GDH and GLDH), myo-inositol pathway (MyoOX and MyoIPS), D-galacturonic acid pathway (GalUR8, GalUR10 and GalUR12) and recycling pathway (DHAR and MDHAR). Specific primers were designed using PRIMER3 software. Ct values of 18S rRNA showed minimal variation across tissues and developmental stages, confirming its suitability as a stable internal control. All the experiments were performed in triplicate and the results were expressed as (MEAN $\pm$ SEM). Following two-way ANOVA (factors: tissue type and developmental stage), Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$) was employed as the post hoc multiple comparison procedure to determine statistically significant differences among group means.

RESULTS AND DISCUSSION

In flavedo, all genes of the L-galactose pathway exhibited differential expression (Fig. 1A). GME decreased from 1.0-fold at Stage-1 to 0.25-fold at Stage-2 and increased sharply to 120-fold at Stage-3. GMP showed a similar pattern, declining from 1.0-fold to 0.10-fold between Stages 1 and 2 and increasing to 150-fold at Stage-3. GGP increased from 1.0-fold at Stage-1 to 4.5-fold at Stage-2 and remained stable at 4.6-fold at Stage-3. GPP exhibited the highest expression (200-fold) at Stage-3, while GDH and GLDH increased to 50-fold and 160-fold, respectively.

In albedo (Fig. 1B), GME decreased from 34.27-fold at Stage-1 to 27.00-fold at Stage-2 and increased to 30.11-fold at Stage-3. GGP showed a similar trend, whereas GPP exhibited a slight increase. GDH showed higher expression at Stage-3 (30.28-fold), while GLDH remained relatively stable.

In pulp (Fig. 1C), GME gradually declined from 28.31-fold at Stage-1 to 20.71-fold at Stage-3. GGP showed a similar trend, GPP exhibited higher

activation, GDH increased moderately towards Stage-3 and GLDH remained relatively constant across developmental stages.

The strong expression of six genes (GDP, GMP, GME, GGP, GPP and GDH) during Stage-3 of development of Kinnow fruit in flavedo suggests enhanced metabolic activity through L-galactose pathway which is the principal route of AsA biosynthesis in plants (Wheeler *et al.*, 13). Accumulation of higher level of ascorbic acid in flavedo then internal tissues, partly due to their direct exposure to external environment and oxidative stress (Alós *et al.*, 2; Fenech *et al.*, 8). The higher expression of GGP enzyme during Stage-2 often correlates with ascorbate biosynthesis and accumulation in many plant species (Bulley and Laing, 4; Mellidou and Kanellis, 12). Early induction of GGP suggests that it may play a central role in ascorbate biosynthesis during transition from early to late fruit development in kinnow mandarin.

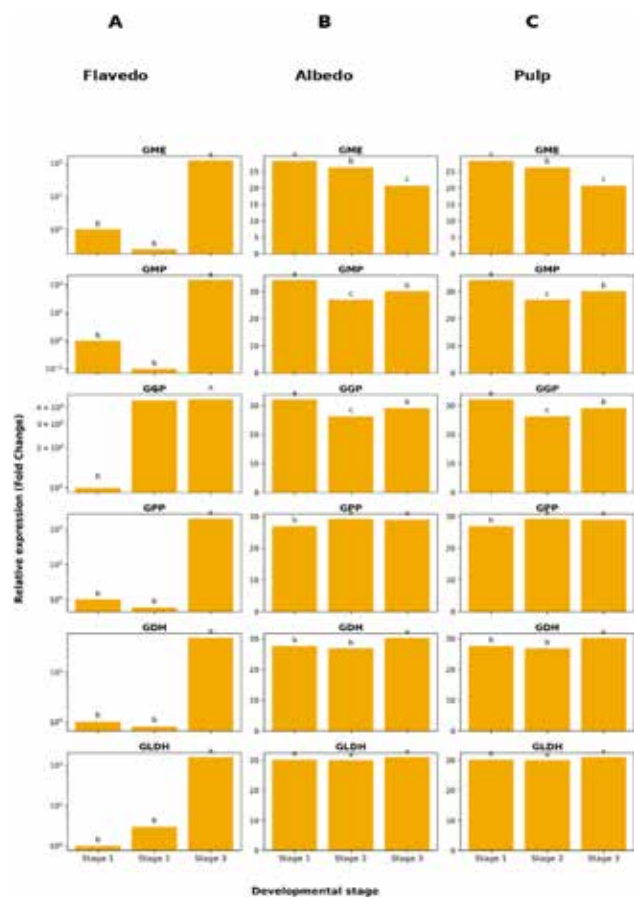


Fig. 1. Relative expression (fold change) of GME, GMP, GGP, GPP, GDH, and GLDH across three developmental stages in the A. flavedo, B. albedo, and C. pulp of Kinnow mandarin.

Furthermore, the strong transcriptional expression of GLDH gene at Stage-3 supports mitochondrial activity associated with ascorbate biosynthesis. The GLDH enzyme catalyses the oxidation of L-galactono-1-4 lactone to ascorbic acid is directly linked with the mitochondrial electron transport chain (Smirnov and Wheeler, 13). The strong GLDH expression during late development of fruit likely contributes to the enhanced ROS scavenging activity to maintain cellular redox balance in flavedo tissues. Similar studies on tissue specific regulation of L-galactose pathway genes have been reported in citrus and other fruit crops (Alós *et al.*, 2).

Overall, the combined analysis demonstrates a clear tissue specific activation of L-galactose pathway genes in Kinnow mandarin, with strong expression of flavedo tissues, while albedo and pulp exhibited stable expression profiles. The findings of this investigation provide important molecular insight into the regulation of vitamin C biosynthesis in Kinnow mandarin as GGP and GLDH may be the target genes for the improvement of citrus fruit nutritional quality through breeding and biotechnological approach (Bulley and Laing, 4).

The MyoOX gene showed a gradual and significant decreased expression in flavedo from 36.20-fold to 34.58-fold at Stage-1 and Stage -2 respectively and further to 33.46-fold at Stage-3. Similarly, MyoIPS exhibited relatively stable expression in Stages -1 (31.28-fold) and Stage-2 (32.27-fold) followed by a reduction in expression to 29.21-folds at Stage -3 (Fig. 2 A). The MyoOX and MyoIPS in albedo and pulp tissues exhibited significant decline expression compared with flavedo (Fig. 2 B). MyoOX expression decreased moderately in albedo from 33.07-fold at Stage -1 to 32.14-fold at Stage -2 followed by further reduction to 29.21-fold at Stage-3. MyoIPS remained stable during early stages 30.24-fold at Stage 1 and 30.28-fold at Stage 2) and further declined significantly to 23.55-fold at Stage -3.

The pulp tissue exhibited expression similar to those observed in albedo but comparatively lower expression (Fig 2 C). In pulp MyoOX expression decreased from 29.31-fold at Stage 1 to 26.26-fold at Stage 2 and 23.57-fold at Stage 3. Likewise, MyoIPS decreased from 28.37-fold at Stage 1 to 25.31-fold at Stage 2, and further to 24.06-fold at Stage 3.

The higher activation of myo-inositol pathway genes observed in flavedo tissues indicates the occurrence of strong metabolic activities in outer tissues of fruit which are directly exposed to external environmental stresses. The flavedo is known to exhibit high metabolic activity during early stage of fruit development supporting active vitamin C biosynthesis processes and structural growth of fruit

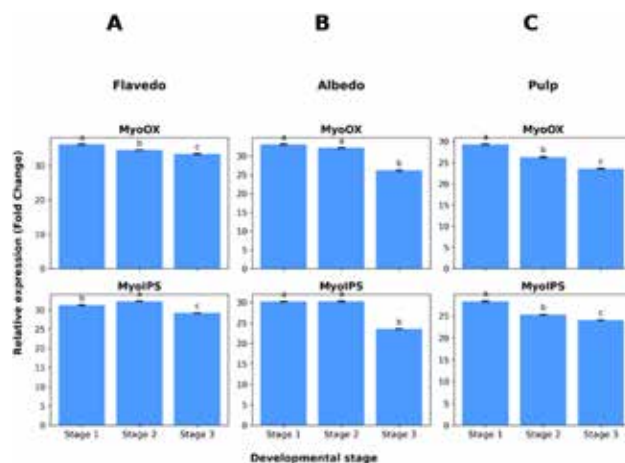


Fig. 2. Relative expression (fold change) of MyoOX and MyoIPS, genes involved in the myo-inositol pathway, across three developmental stages in the A. flavedo, B. albedo, and C. pulp of Kinnow mandarin.

in many plant species (Alós *et al.*, 2; Valluru and Van den Ende, 14). The relatively lower transcriptional expression in pulp tissue suggests the limited role of myo-inositol pathway in pulp during later development of fruit tissues in Kinnow where metabolic activities in fruit is largely involved in fruit maturation rather than structural growth (Fenech *et al.*, 8).

The observed transcriptional trends in Kinnow suggest functional importance of myo-inositol pathway during early fruit development in Kinnow. The myo-inositol is a central precursor which is essential for early fruit development (Loewus and Murthy, 11; Valluru and Van den Ende, 14). Similarly, the gradual decline in MyoOX expression suggests reduced conversion of myo-inositol to UDP-glucuronic acid, a precursor involved in cell wall biosynthesis, further supports the idea that structural carbohydrate synthesis decreases during late fruit development (Endres and Tenhaken, 7).

The gene expression profile in flavedo showed a marginal increase of GalUR18 from 27.28-fold in Stage -1 to 28.00-fold at Stage -2 followed by a significant decline to 23.78-fold at Stage-3 (Fig 3 A). In contrast, GalUR10 exhibited a strong decrease from Stage-1(30.37-fold) to Stage -2 (26.38-fold) and further to 21.44-fold at Stage-3. Similarly, expression of GalUR12 decreased from Stage-1 (30.37-fold) to Stage-3 (21.91-fold). Similarly, in albedo, the downregulation of GalUR8 was observed from 31.86-fold at Stage -1, 31.86-fold at Stage -2 and 26.00-fold at Stage-3 (Fig 3 B). GalUR10 remained stable between Stage 1 (26.84-fold) and Stage 2 (26.00-fold) and showed sharp decrease to 15.76-fold at Stage -3. Similarly, the GalUR12 gene

exhibited stable expression in Stage -1 (26.31-fold) and Stage-2 26.16-fold and decreased significantly to 20.12-fold at Stage-3.

The expression profile was same in pulp tissues followed the general pattern of fruit development. GalUR8 expression decreased gradually from 31.47-fold at Stage 1 to 28.71-fold at Stage 2, and further to 27.73-fold at Stage 3. GalUR10 displayed a more pronounced reduction from 29.85-fold at Stage 1 to 25.20-fold at Stage 2. The expression profile was same in pulp tissues followed the general pattern of fruit development. GalUR8 expression decreased gradually from 31.47-fold at Stage 1 to 28.71-fold at Stage 2, and further to 27.73-fold at Stage 3. GalUR10 displayed a more pronounced reduction from 29.85-fold at Stage 1 to 25.20-fold at Stage 2 and 21.24-fold at Stage 3. GalUR12 remained relatively stable during the early stages (27.81-fold at Stage 1 and 27.21-fold at Stage 2) before declining to 22.12-fold at Stage 3 (Fig 3 C). at Stage 3. GalUR12 remained relatively stable during the early stages (27.81-fold at Stage 1 and 27.21-fold at Stage 2) before declining to 22.12-fold at Stage 3 (Fig 3 C). The marked reduction in the of expression of D-galacturonic acid pathway genes during later stage of fruit maturation suggests a reduction of galacturonate metabolism as structural growth slows during fruit maturation. Compared with flavedo and albedo, the more gradual decline in

pulp suggests progressive re-modelling of cell wall polysaccharides associated with fruit softening during ripening (Atmodjo *et al.*, 3; Xu *et al.*, 17).

The recycling genes in flavedo and albedo exhibited gradual decline across the developmental stages. M-DHAR expression decreased significantly from 29.22-fold at Stage-1 to 27.71-fold in Stage-2 and 26.01-fold in Stage-3 in flavedo tissues. Similar pattern of expression in DHAR was observed as it showed sharp reduction from 36.50-fold at Stage 1 to 25.02-fold at Stage 2 and 25.35-fold at Stage-3 (Fig 4 A). Similar regulatory pattern was observed in albedo and pulp for both the genes. In albedo expression of M-DHAR decreased from 32.30-fold in Stage-1 and 26.57-fold in Stage -2 followed by significant enhancement to 30.13-fold in Stage -3 (Fig 4 B). The higher expression level of M-DHAR during late Stage-3 of fruit development indicates a redox balance in internal peel tissues, whereas stable expression of DHAR further suggests a constitutive antioxidant role to ensure a basal ascorbate recycling capacity in albedo tissues. M-DHAR in the pulp tissues showed sharp declined expression from 30.32-fold at Stage 1 to 25.92-fold at Stage 2 and further to 13.06-fold at Stage 3 (Fig 4 C). Further reduction was observed for DHAR from 33.34-fold and 27.21-fold in Stage -1 and Stage-2 respectively followed by stronger reduction 8.61-fold at Stage -3.

The developmental decline in expression of recycling pathway genes suggests a less dependency of ascorbate recycling mechanism in flavedo as fruit maturation progresses (Smirnoff, 13; Mellidou and Kanellis, 12). The coordinated regulation of M-DHAR

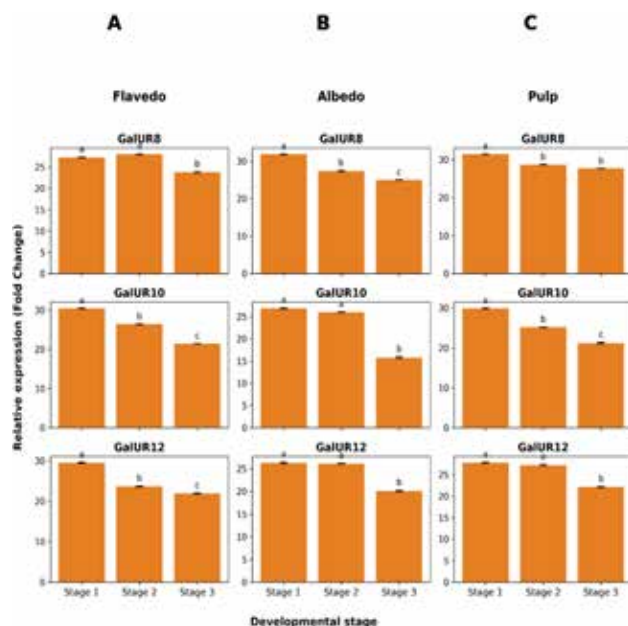


Fig. 3. Relative expression (fold change) of GalUR8, GalUR10, and GalUR12, genes involved in the D-galacturonic acid pathway, across three developmental stages in A. flavedo, B. albedo and C. pulp of Kinnow mandarin.

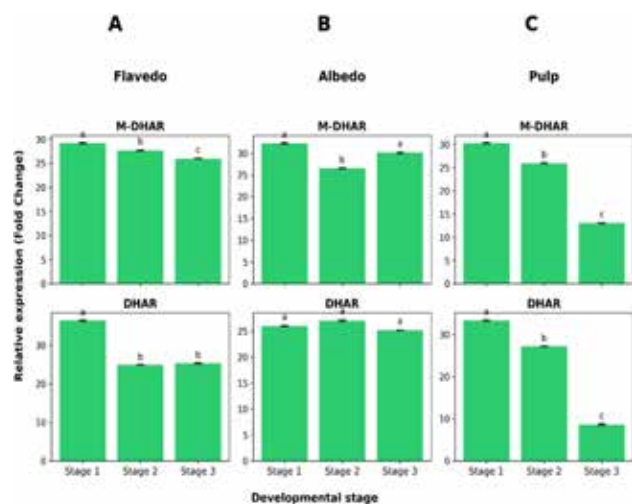


Fig. 4. Relative expression (fold change) of M-DHAR and DHAR, genes involved in the ascorbate recycling pathway, across three developmental stages in the flavedo, albedo, and pulp of Kinnow mandarin.

and DHAR across fruit tissues and developmental stages revealed that ascorbate recycling pathway plays a crucial role and the higher expression of these genes during early stage of fruit development suggests an increased requirement for antioxidant activity during cell division and metabolic activity in citrus fruits (Smirnoff, 13; Mellidou and Kanellis, 12; Wu *et al.*, 16).

As far as our information this is the first report in India on transcriptional expression of genes involved in vitamin C (L-ascorbic acid) biosynthesis, recycling during fruit development and maturation in Kinnow mandarin across three tissues: flavedo, albedo, and pulp. The results revealed strong tissue-specific and stage-dependent regulation of ascorbic acid metabolism genes.

Overall, the study demonstrates that vitamin C accumulation in Kinnow mandarin is regulated by coordinated expression of biosynthesis and recycling pathways with strong tissue specificity, particularly highlighting the dominant role of flavedo in ascorbic acid metabolism during fruit maturation. These findings provide important molecular insights that could support breeding and biotechnological strategies aimed at improving vitamin C content and nutritional quality in citrus fruits.

Principal component analysis (PCA) and Pearson correlation analysis revealed distinct coordinated expression patterns of genes involved in the L-galactose, Myo-inositol, D-galacturonic acid, and recycling pathways across citrus fruit tissues (Fig 5). PCA indicated clear variation in gene expression profiles among flavedo, albedo, and pulp samples, suggesting tissue-specific regulation of genes associated with ascorbate biosynthesis and recycling. Genes within individual pathways exhibited strong positive correlations, reflecting coordinated transcriptional regulation of enzymes functioning in the same metabolic routes. In particular, the recycling pathway genes M-DHAR and DHAR showed strong co-expression, consistent with their cooperative role in regenerating ascorbate from oxidized forms. Similarly, genes of the L-galactose and D-galacturonic acid pathways displayed correlated expression patterns, supporting their involvement in the biosynthetic network contributing to ascorbate accumulation. These findings suggest that multiple metabolic pathways act in a coordinated manner to regulate ascorbic acid metabolism during citrus fruit development, highlighting the integrated nature of biosynthesis and recycling processes in maintaining cellular antioxidant capacity.

Principal component analysis (PCA) and Pearson correlation analysis were performed using normalized expression values (Ct) of genes involved in four metabolic pathways associated with ascorbic acid metabolism in citrus tissues. L-galactose pathway genes showing

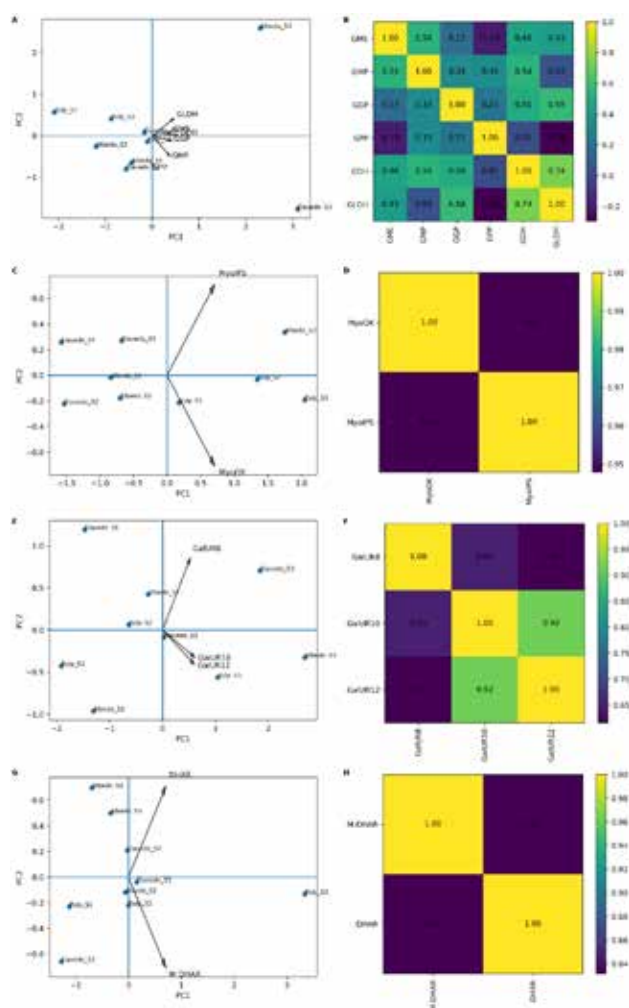


Fig. 5. Comparative PCA and correlation analysis of genes involved in ascorbate biosynthesis and recycling pathways.

PCA distribution of samples and gene-gene correlation patterns (Fig 5 A–B). Myo-inositol pathway genes illustrating sample clustering and correlation between MyoOX and MyoIPS (Fig 5 C–D). D-galacturonic acid pathway genes (GalUR8, GalUR10, GalUR12) displaying variation in gene expression across tissues and their correlation relationships (Fig 5 E–F).

Recycling pathway genes (M-DHAR and DHAR) showing PCA-based sample separation and strong co-expression patterns (Fig 5 G–H). Together, these analyses highlight coordinated regulation of genes associated with ascorbate biosynthesis and recycling pathways across citrus fruit tissues.

AUTHOR'S CONTRIBUTION

Conceptualization of research (RJ, VJ); Designing of experiments (AY, BA); Data collection (AY, HUB, AA, PS); Analysis of data and interpretation (RJ,

BA, VJ); Contribution of experimental materials (SS, IBS). Preparation of Manuscript (RJ, AY). All authors reviewed and approved the final manuscript.

DECLARATION

The authors declare that there is no conflict of interest.

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