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RESEARCH ARTICLE

Tissue-specific expression of vitamin C biosynthetic genes during fruit development in Nagpur sweet orange (*Citrus sinensis* L.)

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Abstract

Vitamin C (L-ascorbic acid, AsA) is an essential dietary antioxidant primarily obtained from fruits and vegetables, with *Citrus* serving as one of the most important natural sources. Despite extensive characterization of AsA content in citrus fruits, the molecular regulation of its biosynthesis, recycling and degradation across different tissues remains poorly understood. We analyzed tissue-specific expression of 16 biosynthetic genes in Nagpur sweet orange across three developmental stages. Results revealed that the L-galactose pathway, represented by *GMP*, *GGP*, *GPP*, *GME*, *GDH* and *GLDH*, was the predominant biosynthetic route, with early upregulation in all tissues, followed by tissue-specific modulation during maturation. Myo-inositol pathway genes (*MyoOX* and *MyoIPS*) displayed distinct tissue-dependent regulation, with sustained expression in pulp and late activation in albedo, suggesting a conditional role in AsA biosynthesis and cell wall metabolism. Members of the D-galacturonic acid pathway (*GalUR8, GalUR10* and *GalUR12*) exhibited biphasic expression, peaking during fruit maturation, consistent with pectin turnover and localized AsA biosynthesis. Ascorbic acid (AsA) degradation genes (*APX2, APX3* and *AO*) showed differential expression, with *APX2* strongly induced in flavedo and pulp during early and late stages, highlighting its role in ROS management. Recycling genes (*MDHAR* and *DHAR*) were dynamically regulated, with *DHAR* induction at maturity in flavedo. Collectively, these findings provide the first tissue-specific molecular framework of AsA metabolism in Indian sweet orange, offering insights for metabolic engineering to enhance vitamin C content and fruit quality.

Keywords: albedo; citrus; flavedo; gene expression; pulp; quantitative real-time polymerase chain reaction ; sweet orange; vitamin C

Introduction

Citrus fruits are widely recognized as one of the primary dietary sources of vitamin C (L-ascorbic acid, AsA) for humans. Ascorbic acid (AsA) is an essential micronutrient predominantly obtained from fruits and vegetables and is known for its potent antioxidant properties (1). It plays a key role in reducing the incidence of various health conditions including cancer and cardiovascular diseases (2). To understanding the mechanisms of AsA accumulation and the regulatory factors influencing its concentration in horticultural crops has gained significant attention and many studies have focused on assessing AsA levels in the edible tissues of plants (3, 4). In plants, four biosynthetic pathways have been identified for AsA production: the L-galactose, L-gulose, myo-inositol and D-galacturonic acid pathways. The predominance of each pathway varies depending on the plant species and developmental stage of the fruit (5).

Extensive research has been conducted on the AsA content in fresh fruits and juices of various *Citrus* species and cultivars (6, 7). However, insights into the molecular regulation of AsA metabolism in *Citrus* remain limited. Earlier studies proposed that alongside the L-galactose pathway, the D-galacturonic acid and myo-inositol pathways may significantly influence AsA accumulation in citrus tissues with D-galacturonic acid reductase 12 (*GalUR12*) and myo-inositol oxygenase (*MyoOX*) playing pivotal roles (8). Transcriptomic studies in orange and mandarin fruits indicate that the L-galactose pathway is the principal route of AsA biosynthesis in both the flavedo (the outer colored peel) and pulp tissues (9). Variability in AsA levels among species and cultivars has been linked to differential expression of key genes such as GDP-mannose pyrophosphorylase (*GMP*), GDP-L-galactose phosphorylase (*GGP)* and L-galactose-1-phosphate phosphatase (*GPP)*. More recently, previous researchers reported that the decline in AsA levels in blood orange juice during fruit maturation was associated with the downregulation of *MyoOX* and *GalUR12*, as well as genes of the L-galactose pathway such as GDP-mannose -3′, 5′-epimerase (*GME)* and L-galactose dehydrogenase (*GLDH*) (10). Interestingly, their findings suggest that the contribution of individual biosynthetic pathways varies throughout fruit development while the L-galactose and myo-inositol pathways are more active during early stages. For instance, previous researchers observed that differences in AsA content among citrus cultivars correlated with both gene expression in the L-galactose pathway and the activity of enzymes involved in AsA degradation (11).

Moreover, AsA accumulation is modulated by a complex interplay of biosynthesis, recycling, degradation and transport mechanisms (12–16). These pathways not only operate in different tissues or developmental stages but also function in concert to support *de novo* synthesis and other metabolic needs (17). Additionally, a transcriptomic comparison of sweet orange tissues (leaves, callus, flowers and fruits) revealed that certain members of the *GalUR* gene family were significantly upregulated in fruit, indicating a possible role of the D-galacturonic acid pathway in fruit-specific AsA biosynthesis (18–20).

Although whole-fruit analyses have been widely quantified in citrus fruits, no study from India has previously resolved the tissue-specific and stage-dependent transcriptional regulation of the complete AsA metabolic network in any Indian sweet orange cultivar, including Nagpur sweet orange (*Citrus sinensis* L.), which is the most commercially important cultivar in central India. The objective of present investigation is to elucidate the tissue-specific and developmental transcriptional regulation of vitamin C biosynthetic, recycling and degradation genes in Nagpur sweet orange to identify dominant pathways and regulatory transitions during fruit development.

This study provides the first tissue-specific transcriptional regulation of all major components of AsA metabolism (biosynthesis, recycling and degradation) across 3 defined fruit developmental stages in Nagpur sweet orange.

Materials and Methods

Plant material

One cultivar of sweet orange (Nagpur sweet orange) was selected for the study. Three biological replicates of the genotype were collected from the experimental farm of the Centre of Excellence for Citrus, Kota, Rajasthan and used for all the analyses. Fruit samples were collected at 3 distinct developmental stages: Stage 1 (April–June), stage 2 (July–September) and stage 3 (October–January). At each sampling time, fruits were randomly harvested from the same plant to ensure representative biological replicates. For each developmental stage, 3 independent biological replicates were used. Each biological replicate consisted of pooled tissue samples (flavedo, albedo, or pulp) collected from 3 individual fruits, harvested from different trees under uniform agronomic conditions. Thus, a total of nine fruits per developmental stage were used for gene expression analysis. RNA was independently extracted from each biological replicate and cDNA synthesis was performed separately. Quantitative real-time polymerase chain reaction (qRT-PCR) reactions for each cDNA sample were then carried out in technical triplicates and the mean Ct value of the technical replicates was used for subsequent statistical analysis. All samples were processed under sterile conditions. The fruit tissues; flavedo (outer colored part) albedo (white, spongy, inner layer of peel) and pulp (fleshy juice sacs) were carefully separated from each fruit and stored at -80 °C until further use for RNA extraction.

RNA extraction, cDNA synthesis and quantitative real-time PCR expression analysis

Total RNA from different tissues was extracted using the RNeasy plant mini kit (QIAGEN, Hilden, Germany), according to manufacturer's protocol. Briefly, approximately 100 mg fresh samples were homogenized in liquid nitrogen and subsequently 450 µL cell lysis and RNase inactivation buffer was added, then the lysate was vortexed vigorously. The lysate transferred to a QIA shredder spin column and then centrifuged at 8000 × g for 2 min. Further the sample was added with 0.5 volume of absolute ethanol for the RNA binding, after this it was transferred to an RNeasy mini spin column followed by the washing buffers (500 µL) to remove proteins, polysaccharides and other contaminants. After the final drying the RNA was eluted in 50 µL RNase-free water. The total RNA amount was measured by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the quality was verified by gel electrophoresis (0.8 % agarose in TAE).

First strand cDNA was synthesized according to the manufacturer's instructions using 100 ng of total DNase-free RNA using Phi-Script™ cDNA master mix DXBIDT, Bangalore, India, in a 20 µL final volume per PCR reaction. Expression analysis was performed through qRT-PCR, which was conducted using a QIA quant 96, 5 plex Real-Time PCR System (QIAGEN, Hilden, Germany) using 100 ng cDNA and Polaris™qmaster Mix with SYBR® (DXBIDT, Bangalore, India), according to the protocol provided by the manufacturer. The relative mRNA expression of 16 genes involved in AsA biosynthesis, recycling and degradation was calculated using $\Delta\Delta C_t$ method, where stage 1 (early fruit developmental stage) was used as the biological calibrator and 18S rRNA served as the internal reference gene for normalization.

Gene selection and primer design

All genes were selected from previous papers published on citrus after a search in the NCBI database (8). We used *C. sinensis* Annotation Project to find the corresponding "gene-ID" in the genome (21). Specific primers were designed with PRIMER3 software (Boston, MA, USA), Gene ID, primer sequences and product lengths are listed in Table 1 (22). In gene expression analysis, 18rRNA was used as the internal reference gene for normalization of qRT-PCR data. Before normalization, Ct values of 18S rRNA were examined across all samples (flavedo, albedo and pulp across 3 developmental stages) and showed minimal variation, confirming its suitability as a stable internal control under the experimental conditions of this study. Primer specificity for all target and reference genes was confirmed by melt curve analysis

Statistical analysis

All the experiments were performed in triplicate and the results were expressed as (Mean ± SEM). Following two-way ANOVA (factors: tissue type and developmental stage), Tukey's honestly significant difference (HSD) test was employed as the post hoc multiple comparison procedure to determine statistically significant differences among group means.

Results and Discussion

Tissue-specific transcriptional expression analysis of genes involved in ascorbic acid biosynthesis

To investigate the tissue-specific transcriptional regulation of key genes involved in AsA metabolism during fruit development in Nagpur sweet orange, the expression profiles of 16 target genes were quantified using qRT-PCR in 3 fruit tissues: flavedo, albedo and pulp.

Table 1. List of primers sequences, product lengths and gene IDs of 16 genes

S.No	Gene	Forward primer (5'–3')	Reverse primer (5'–3')	PL (bp)	Gene Id
1.	*GMP*	GGTGCATGAGAGTGCTCAAA	TACAGTGCAGCGAGAGAGTC	109	102608158
2.	*GME*	GCCTCCAGTGCCTGCATCT	GGCATCAGACTCCTTCAAGCTT	72	102614415
3.	*GGP*	AGTACGGGCATGTGCTGTTG	CAAGAAGCTATCACGGTCAATCC	74	102617187
4.	*GPP*	TCTTGGACACTGCAGTTGATG	TCTGTGACCAAATCCACCTG	109	102624075
5.	*GDH*	GGCGTTCCGAGAAATGAGTA	GGATTTTCCCTGCTTCCTTC	229	102621043
6.	*GLDH*	CACCGCCTCCTCCTCTTCAACAA	TGAGAGCGTACGGATTGAAGGT	69	102611546
7.	*MyoOX*	CTTATTCAGAAGGCCGTGGA	CCCGGTGTAAGGCATAGAAA	150	102618589
8.	*MyoIPS*	CTCTTTTGGCTGCTGCTCCAATC	GTTGTTCTCAGGAGCCAAGC	245	102629031
9.	*GalUR8*	TCCCTCCTTCCATCAATCAG	GACATTCATGCCCTTGACCT	89	102629204
10.	*GalUR10*	GGAGAAGCAATCGATGAAGC	TGAGCGTCATTCAACCAGAG	92	102625686
11.	*GalUR12*	GGTGGGTTTATCAGCAAGGA	TCGGCACTTAATTCCCAATC	100	102615776
12.	*MDHAR*	TCCCTCTGGTGTCCTCAGTC	TGAGTATGCGACGACAAAGC	93	102615220
13.	*DHAR*	CTGAGAAGGCTTCAGTCGGA	ATGCCTGCTCAGATCCATCA	90	102629685
14.	*AO*	CGGGTTCACTAAGTGGGCTA	TGGAGGTTTCATCACGTCATAC	151	127900242
15.	*APX2*	TCAGTTGGCTGGAGTTGTTG	TCAGATTTGTCCGGTCTTCC	81	102622372
16.	*APX3*	TTACCGGTCGTTGACACAGA	ACGCTAGACGGAGCATGATT	109	102607382

GMP - GDP-mannose pyrophosphorylase; *GME*- GDP-D-mannose 3',5'-epimerase; *GGP*- GDP-L-galactose phosphorylase; *GPP*- L-galactose-1-phosphate phosphatase; GDH- L-galactose dehydrogenase; *GLDH*- L-galactono-1,4-lactone dehydrogenase; *MyoOX*- Myoinositol oxygenase; MyoIPS- Myoinositol phosphate synthase; *GalUR8*-D-galacturonate reductase 8; *GalUR 10*- D-galacturonate reductase 10; *GalUR 12*- D-galacturonate reductase 12; *MDHAR*- Monodehydroascorbate reductase; *DHAR*- Dehydroascorbate reductase; *AO*- Ascorbate oxidase, *APX2*- Ascorbate peroxidase-2; *APX3*- Ascorbate peroxidase-3; PL- Product length.

The selected genes represent major steps across multiple AsA biosynthetic and metabolic pathways: D-Galactose pathway (6 genes): GDP-Mannose Pyrophosphorylase (*GMP*), GDP-Mannose-3,5 -Epimerase (*GME*), GDP-L-Galactose Phosphorylase (*GGP*), L-Galactose-1-Phosphate Phosphatase (*GPP*), L-Galactose Dehydrgenase (*GDH*) and L-Galactono-1,4Lactone Dehydrogenase (*GLDH*). Myo-Inositol Pathway (2 genes): Myo-inositol Oxygenase (*MyoOX*) and Myo-Inositol-1-Phosphate Synthase (*MyoIPS*). D-Galacturonic acid pathway (3 genes): D-Galacturonate reductase isoforms GalUR8, GalUR10 and GalUR12. Degradation pathway (3 genes): ascorbate peroxidase isoforms *APX2*, *APX3* and Ascorbate oxidase (*AO*). Recycling pathway (2 genes): Dehydroascorbate Reductase (*DHAR*), Monodehydroascorbate Reductase (*MDHAR*).

L-Galactose pathway is tightly coordinated with early regulation in all tissues of fruit

Transcript profiling of the L-Galactose pathway genes in *C. sinensis* revealed distinct tissue-specific and developmental stage-dependent expression patterns across flavedo, albedo and pulp during fruit development.

Flavedo

At the early developmental stage 1 (April–June), *GMP* and *GPP* transcripts were abundantly expressed, suggesting their active involvement in the initial biosynthesis of L-ascorbic acid (AsA) and cell wall metabolism during rapid fruit growth. By mid-development stage 2 (July–September), *GMP* expression declined significantly, whereas *GPP* remained relatively stable. Interestingly, *GME* and *GDH* were upregulated in stage 2, possibly reflecting a metabolic shift towards maintaining AsA homeostasis during chloroplast maturation and pigment accumulation. At full maturity stage 3, most pathway genes in flavedo exhibited stable expression (Fig. 1A), indicating that AsA biosynthesis is sustained but less dynamically regulated at this stage (Supplementary Table 1A). Similar tissue-specific regulation of L-galactose pathway genes in flavedo has been reported in citrus and other fruits, where the flavedo acts as a major site of antioxidant accumulation (23, 24).

Albedo

The *GME*, *GGP* and *GLDH* genes were strongly upregulated in stage 1, indicating an early role in precursor supply for AsA synthesis and cell expansion in the spongy mesocarp. In stage 2, these genes showed a transient down regulation, followed by a notable reactivation in stage 3 (Fig. 1B), likely associated with structural modifications and continued oxidative stress protection during late ripening. Conversely, *GMP*, *GPP* and *GDP* expression remained low through stages 1 and stage 2, but most L-galactose pathway genes showed a marked increase during fruit maturation (Supplementary Table 1B). This late-stage upregulation may reflect enhanced AsA transport or storage functions in the albedo during senescence, consistent with earlier findings in *Citrus reticulata* (25).

Pulp

In the juice vesicles, *GME*, *GGP*, *GPP* and *GMP* showed high expression at Stage 1, supporting active AsA biosynthesis during early cell proliferation and sugar accumulation. Expression declined sharply by stage 2, with only a slight further decrease at stage 3 (Fig. 1C). *GLDH* and *GDH* displayed a continuous downregulation throughout development, implying a reduced role of mitochondrial AsA synthesis during later pulp maturation (Supplementary Table 1C), possibly as sugar metabolism and flavor compounds dominate. This pattern is consistent with reports on *Actinidia deliciosa* and *Malus domestica*, where AsA biosynthetic gene activity in pulp is highest at early fruit set and declines as sugars accumulate (26).

Myo-inositol pathway is regulated in a tissue-specific manner during fruit development

Expression of genes from the myo-inositol pathway showed clear tissue-specific and stage-dependent patterns in Nagpur sweet orange. In the flavedo, transcripts of *MyoOX* and *MyoIPS* were elevated (Supplementary Table 2A) at the earliest sampling (Stage 1), dropped slightly at mid development (Stage-2) and rose again at fruit maturation (Stage 3) (Fig. 2A). In the albedo, *MyoOX* and *MyoIPS* strongly reduced in stage-1, then increased through stage-2 and remained higher at maturity stage-3 (Fig. 2B). The pulp displayed two general patterns: myo-inositol genes showed a progressive increase from stage-1 to stage-2 with a further small increase at maturity (Fig. 2C).

Elevate d *MyoOX* and *MyoIPS* in flavedo at stage-1 and again at maturity implies transient demand for myo-inositol catabolism in the peel at early cell expansion and during late maturation,

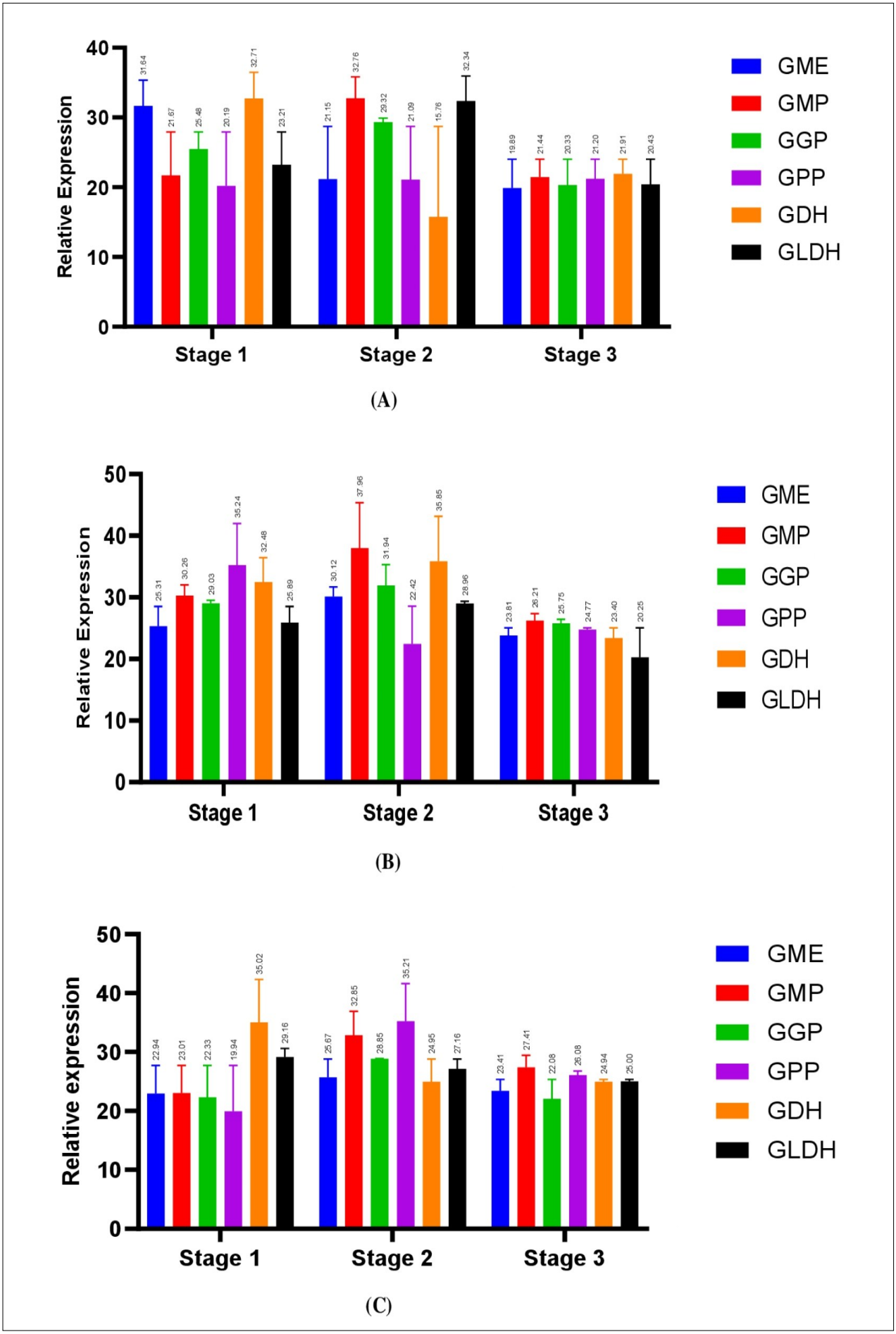

Fig. 1. Relative expression of AsA biosynthetic genes *GME*, *GMP*, *GGP*, *GPP*, *GDH* and *GLDH* of L-galactose pathway. A- Flavedo; B- Albedo; C- Pulp tissues of fruits collected in April to June (Stage1), July to September (Stage 2) and October to January (Stage 3) of Nagpur sweet orange (*Citrus sinensis*). Values represent mean ± SD (n = 3). Different error bars indicate statistically significant differences among stages within the same tissue (Two-way ANOVA followed by DMRT, $p \leq 0.05$).

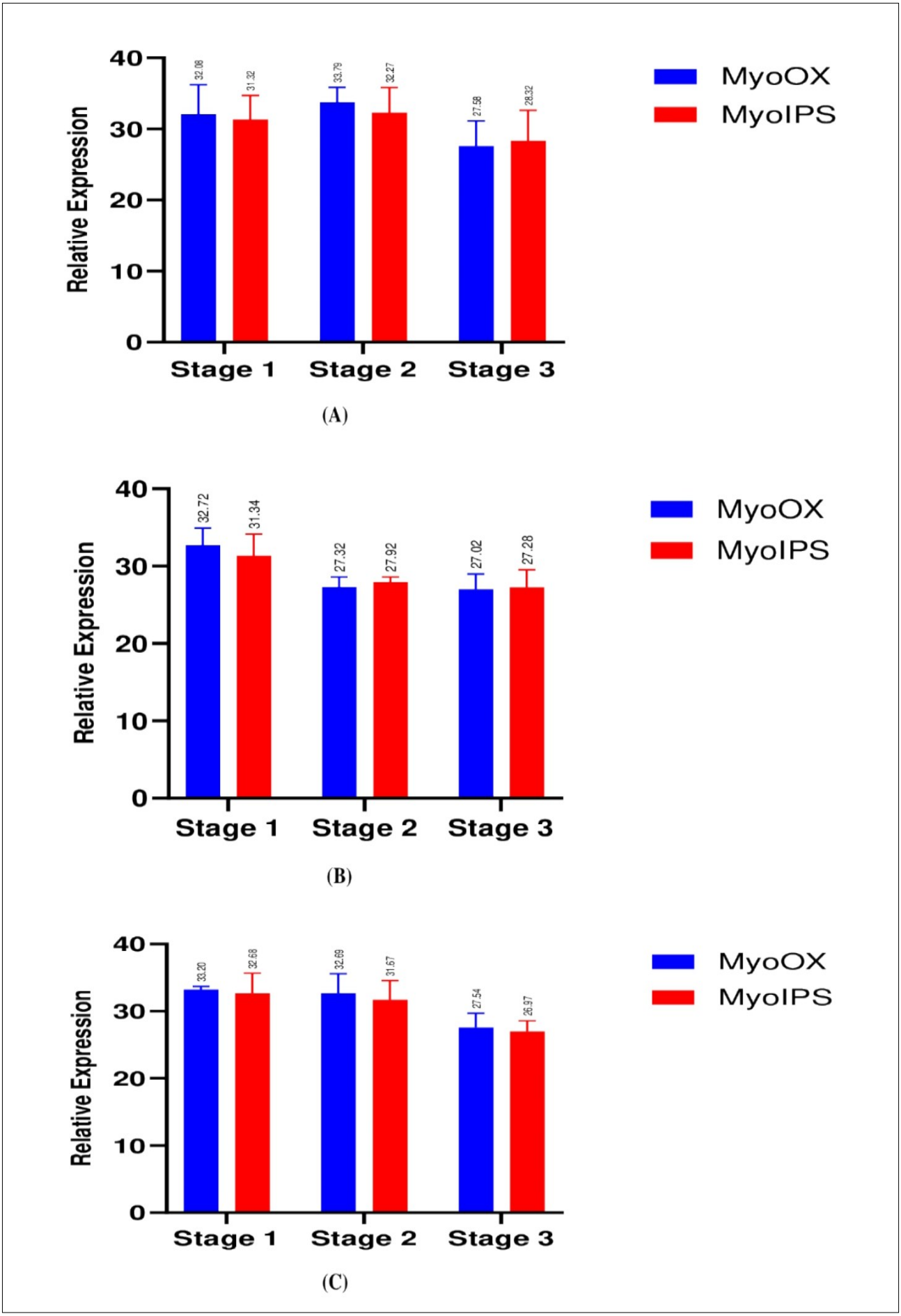

Fig. 2. Relative expression of AsA biosynthetic genes MyoOX and MyoIPS of Myo-inositol pathway. A- Flavedo; B- Albedo; C- Pulp tissues of fruits collected in April to June (Stage 1), July to September (Stage 2) and October to January (Stage 3) of Nagpur sweet orange (*Citrus sinensis*). Values represent mean ± SD (n = 3). Different error bars indicate statistically significant differences among stages within the same tissue (Two-way ANOVA followed by DMRT, $p \leq 0.05$).

potentially linked to cell wall modification, cuticle formation or protective antioxidant needs in the outer tissue. This interpretation is consistent with reports that *MyoOX* activity is tied to cell wall and glucuronate metabolism and that *MyoOX* expression responds to developmental stages of fruit. The marked repression of *MyoOX* and *MyoIPS* in albedo (Supplementary Table 2B) at stage-1 followed by upregulation toward maturity suggests either a developmental repression early on or a shift of carbon flux away from myo-inositol catabolism during early albedo expansion. Similar tissue-specific shifts for myo-inositol genes have been observed in other species where *MyoOX* family members are differentially expressed across organs and development (27, 28).

The steady increase of many myo-inositol transcripts in pulp (Supplementary Table 2C) together with the uniform upregulation of *AO* suggests the pulp increasingly engages the myo-inositol–derived glucuronate metabolism during fruit ripening (29). Our tissue-specific expression patterns are compatible with a role for myo-inositol metabolism in localized AsA biosynthesis (especially in pulp and flavedo at specific stages), or more broadly in supplying UDP-glucuronic acid for cell wall/pectin biosynthesis rather than bulk AsA production. This perspective aligns with recent reviews emphasizing the species- and tissue-specific variability in the functioning and importance of alternative AsA biosynthetic pathway (30).

D-Galacturonic acid route links gene expression to physiological changes in flavedo, albedo and pulp During fruit development and maturation

In the flavedo, transcripts of *GalUR8*, *GalUR10* and *GalUR12* showed a biphasic pattern (Supplementary Table 3A), their expression gradually declined from stage 1 (April–June) to stage-2 (July–September), then increased markedly at stage-3 (October–January) during fruit maturation (Fig. 3A). In the albedo, *GalUR8* and *GalUR10* followed a similar trend of downregulation through stages 1 and stage 2 (Supplementary Table 3B) with a pronounced increase in transcripts in mature fruit sampled in January (Fig. 3 B). In the pulp, *GalUR8, GalUR10* and*GalUR12* displayed an up-regulated profile at the initial (Stage-1) and maturation (Stage-3) points (Supplementary Table 3C), while *GalUR10* showed a modest reduction during mid development in stage-2 (Fig. 3C). Overall, the D-Galacturonic acid pathway genes examined exhibited clear tissue-specific and stage-dependent expression dynamics, with peak activity frequently associated with the mature fruit stage. Elevated *GalUR* expression in mature tissues is in line with reports that *GalUR* (D-Galacturonate reductase) can be upregulated during fruit ripening and is associated with changes in pectin metabolism and/or ascorbate (vitamin C) accumulation in several fleshy fruits (26).

The pulp shows relatively high *GalUR* expression in both early and late stages, which may reflect distinct roles of the pulp in carbon partitioning and cell-wall remodeling compared with flavedo and albedo tissues. In citrus and other fruits, the D-Galacturonic acid pathway has been proposed to contribute to ascorbate biosynthesis in a tissue-dependent manner (for example, a stronger contribution in peel/petals in some citrus cultivars), so the high transcript abundance of *GalURs* in mature flavedo and albedo could be functionally linked to localized ascorbate synthesis or recycling during ripening and stress protection (1).

The increase of *GalUR* transcripts at maturity therefore could reflect either (i) an upshift in pectin depolymerization yielding more substrate for *GalUR*, (ii) transcriptional control linked to ripening regulators, or (iii) a demand for localized ascorbate to counter oxidative stress associated with maturation and respiration changes. Similar patterns *GalUR* upregulation coincident with ripening and ascorbate accumulation have been documented in blueberry and other species, supporting the functional relevance of this pathway in fruit physiology (31).

Tissue-specific regulatory dynamics in the ascorbic acid degradation pathway

In the flavedo, the *APX2* isoform exhibited high transcript at the early developmental stage S 1 (April –June), followed by a marked decline in stage 2 (July–September) and a subsequent significant upregulation during the maturation stage 3 (October –January) (Fig. 4A). In contrast, *APX3* and *AO* transcripts displayed a consistent downregulation across all developmental stages (Supplementary Table 4A), suggesting limited involvement of these genes in maintaining AsA homeostasis in the outer peel during later fruit development. These observations agree with earlier studies reporting tissue-specific regulation of *APX* isoforms in citrus peel, where *APX2* is preferentially induced during oxidative stress peaks in early and late developmental windows (32, 33).

In the albedo, both *APX2* and *APX3* (Supplementary Table 4B) showed a progressive upregulation from stage-1 to stage-3 (Fig. 4B), implying a continuous role in reactive oxygen species (ROS) scavenging and possible AsA turnover during fruit enlargement and maturation. The sustained expression may help maintain the oxidative balance in this metabolically active tissue, which has been reported to influence fruit firmness and water retention (34).

In the pulp, *APX2* expression was low at stage 1, modestly increased at stage-2 and reached its peak during maturation (Stage 3), indicating a late developmental induction possibly associated with the rapid metabolic and respiratory activity during ripening (Fig. 4C). *AO* expression in pulp remained relatively uniform between stage 2 and stage 3 (Supplementary Table 4C) suggesting a stable but minor contribution to AsA degradation in this tissue. Similar late-stage induction of *APX2* in fruit flesh has been documented in kiwifruit and tomato, where it correlates with the onset of ripening-related oxidative metabolism (35, 36).

Overall, these patterns highlight tissue-specific regulatory dynamics in the AsA degradation pathway during development of fruit in Nagpur orange. These findings align with the hypothesis that AsA turnover is tightly linked to developmental stage-specific ROS management, ensuring fruit quality and stress tolerance.

Ascorbate recycling is dynamically regulated during fruit development

In the flavedo, genes of the ascorbate-recycling pathway showed two distinct expression patterns (Fig. 5A). *MDHAR* transcripts were relatively stable from early development through to mature fruit (Stage-3, January), whereas *DHAR* transcripts were low during early development and stage-2 but underwent a marked upregulation at stage 3 (Supplementary Table 5A). This pattern suggests that *MDHAR* may provide a steady basal capacity for monodehydroascorbate reduction in the peel, while the late surge in *DHAR* expression at maturation could reflect an increased demand for dehydroascorbate reduction to sustain the reduced AsA pool during fruit ripening and associated oxidative changes. Such tissue-specific and stage-dependent modulation of recycling enzymes is consistent with observations in citrus and other fleshy fruits where recycling enzymes contribute to maintaining AsA redox balance during

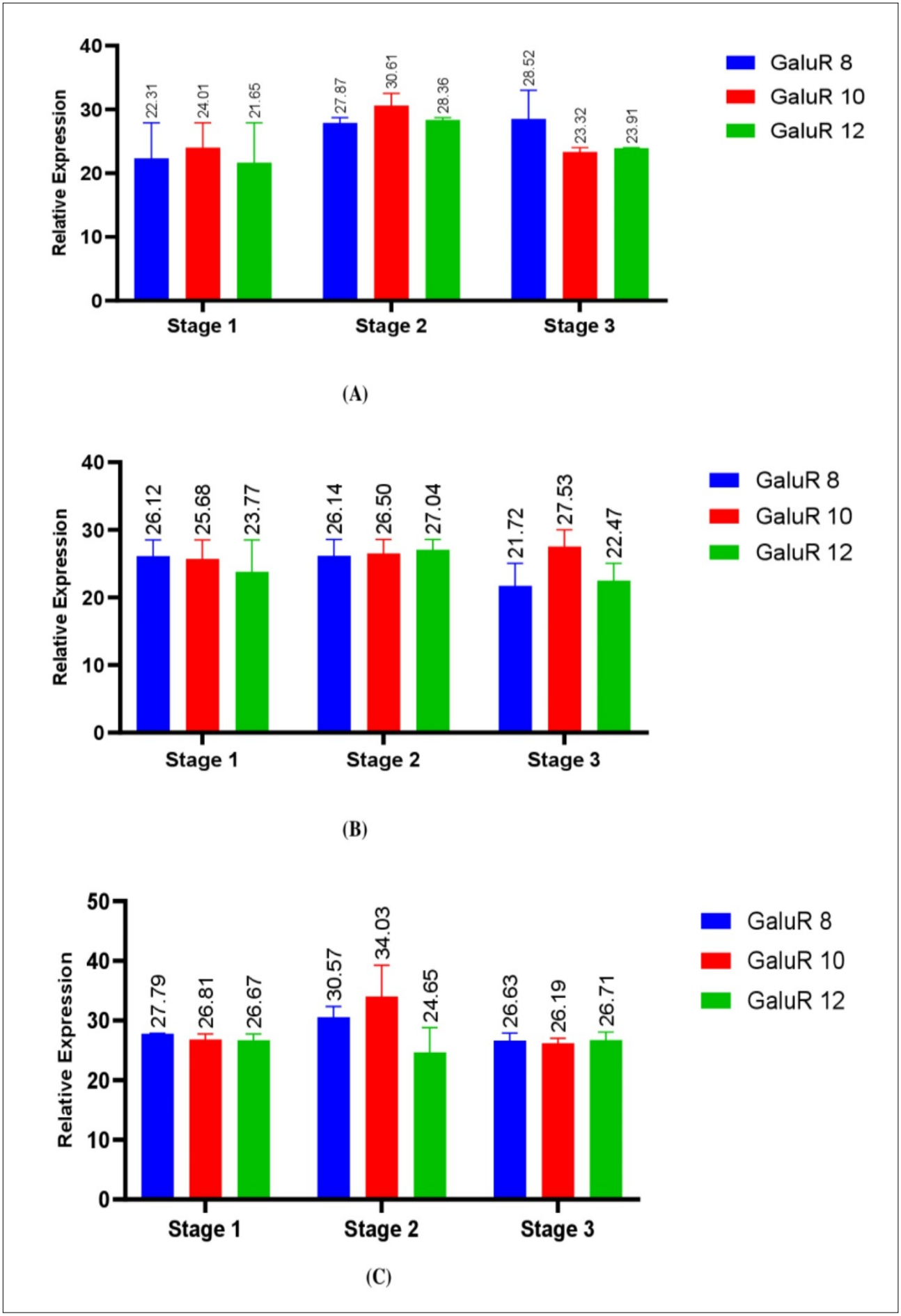

Fig. 3. Relative expression of ascorbic acid biosynthetic genes *GaluR8*, *GaluR10* and *GaluR12* of D- Galacturonic acid pathway. A- Flavedo; B- Albedo; C- Pulp tissues of fruits collected in April to June (Stage1), July to September (Stage 2) and October to January (Stage 3) of Nagpur sweet orange (*Citrus sinensis*). Values represent mean ± SD (n = 3). Different error bars indicate statistically significant differences among stages within the same tissue (Two-way ANOVA followed by DMRT, $p \leq 0.05$).

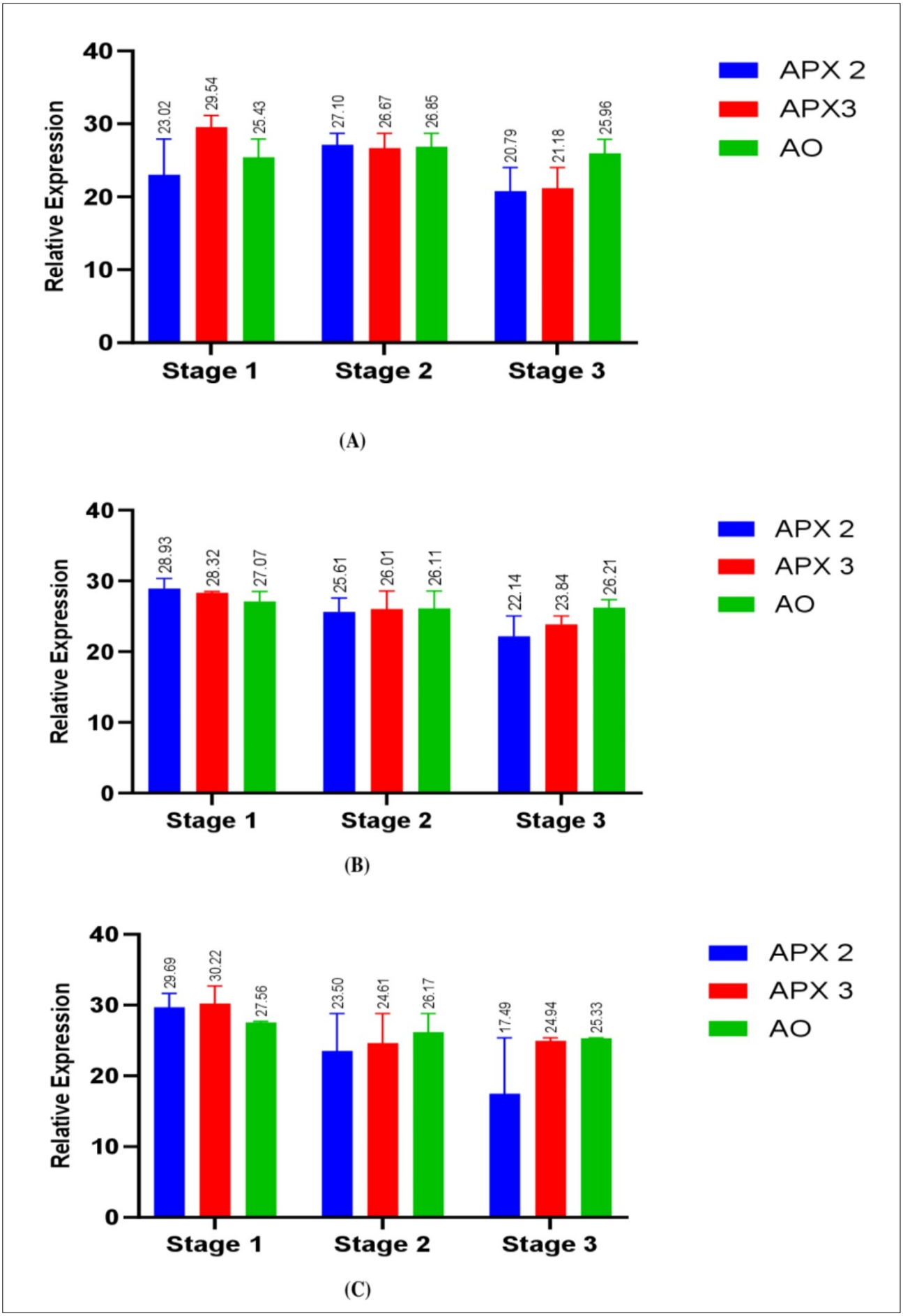

Fig. 4. Relative expression of AsA biosynthetic genes *APX2*, *APX3* and *AO* of Degradation pathway. A- Flavedo; B- Albedo; C- Pulp tissues of fruits collected in April to June (Stage1), July to September (Stage 2) and October to January (Stage 3) of Nagpur sweet orange (*Citrus sinensis*). Values represent mean ± SD (n = 3). Different error bars indicate statistically significant differences among stages within the same tissue (Two-way ANOVA followed by DMRT, $p \leq 0.05$).

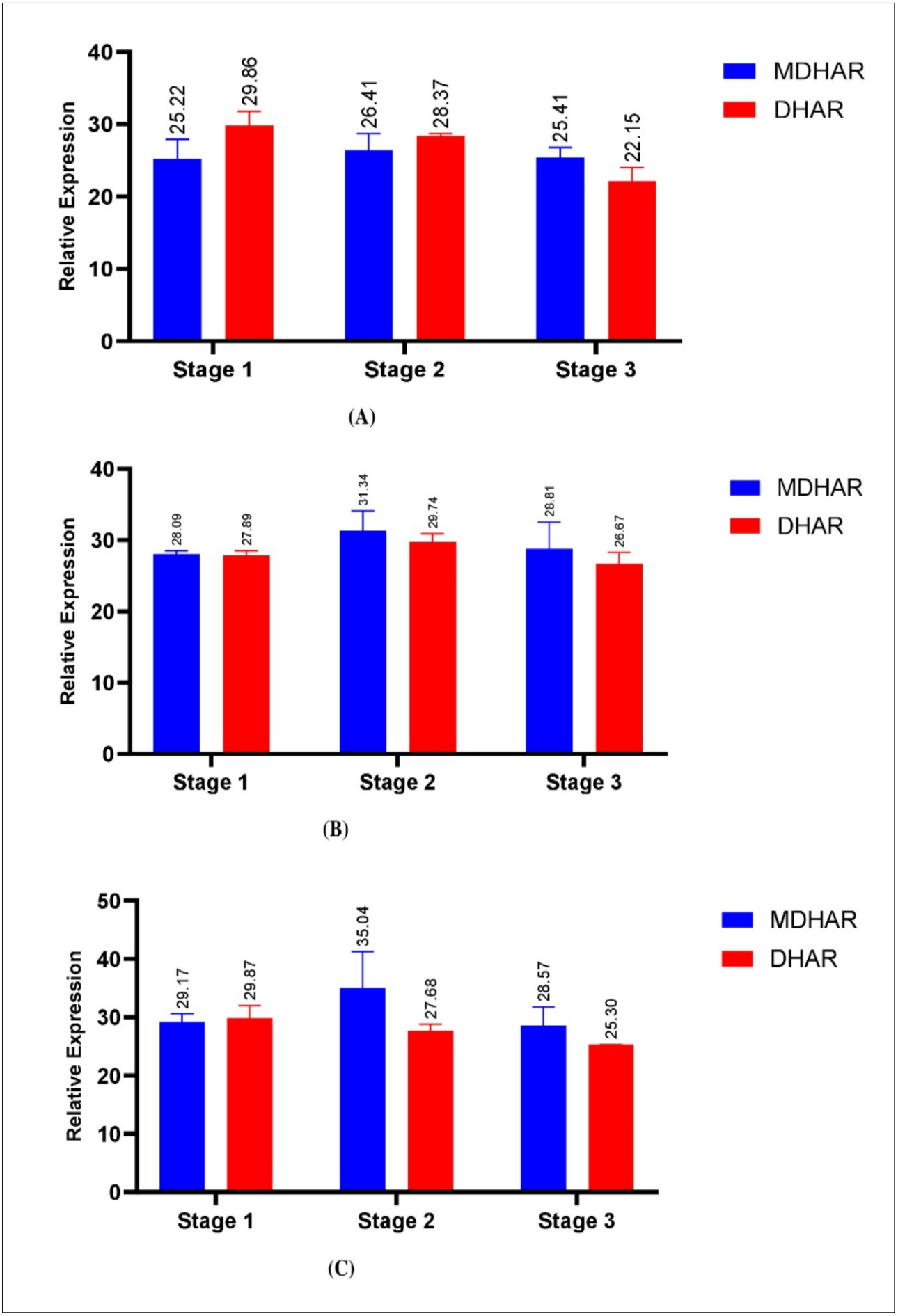

Fig. 5. Relative expression of AsA biosynthetic genes *MDHAR* and *DHAR* of recycling pathway. A- Flavedo; B- Albedo; C- Pulp tissues of fruits collected in April to June (Stage1), July to September (Stage 2) and October to January (Stage 3) of Nagpur sweet orange (*Citrus sinensis*). Values represent mean ± SD (n = 3). Different error bars indicate statistically significant differences among stages within the same tissue (Two-way ANOVA followed by DMRT, $p \leq 0.05$).

maturation (37).

In the albedo both *MDHAR* and *DHAR* were highly expressed in the initial phase stage-1 (Supplementary Table 5B) but declined significantly from stage-2 to stage 3 (Fig. 5B). The early high expression implies an active recycling system during early mesocarp development, possibly linked to rapid cell expansion and metabolic activity when reactive oxygen species (ROS) production is high. The subsequent decline toward maturation suggests reduced recycling demand in the albedo or a shift in the relative contributions of biosynthesis, degradation and transport to AsA homeostasis in this tissue as fruit growth slows. Comparable developmental down-regulation of recycling transcripts during maturation has been reported in other fruit tissues, indicating that the balance between synthesis and recycling shifts as fruits transition from growth to maturation (38).

In the pulp both *MDHAR* and *DHAR* showed progressive down regulation (Supplementary Table 5C) from the initial stage through to maturation Fig. 5C). This sustained decline in recycling transcripts in pulp coincides with the common observation that pulp often relies more on biosynthetic regulation and AsA import/compartmentation to determine final vitamin C content than on local recycling capacity. The downregulation may also reflect lower oxidative pressure or altered glutathione/thiol pools in pulp compared with peel tissues, which would reduce the need for DHA→AsA recycling via *DHAR*. Experimental manipulation of *MDHAR*/*DHAR* in tomato and other species has shown that changes in these enzymes alter fruit AsA levels and redox state, supporting the functional importance of observed expression differences among tissues (16). These findings align with recent reviews and experimental studies describing the central role of recycling enzymes in fruit AsA biosynthesis and the contribution of tissue context to overall vitamin C status (14, 15).

For further evaluation, a heatmap was generated depicting the differential regulation of all genes across the fruit tissues; Flavedo Albedo and Pulp, playing role in the AsA biosynthesis during the development and maturation of fruit in Nagpur sweet orange (Fig. 6). Overall, these results indicate that the L-galactose pathway is strongly coordinated across all the tissues with early upregulation in all tissues to support antioxidant protection and cell expansion, followed by tissue-specific adjustments as the fruit transitions from growth to ripening. Flavedo maintains higher steady-state expression at maturity, while albedo shows late induction and pulp progressively reduces biosynthetic activity. The myo-inositol pathway genes exhibit clear, tissue-specific developmental regulation in Nagpur sweet orange, with flavedo and pulp showing earlier and/or continuous engagement and albedo showing delayed activation. These patterns are consistent with roles in localized antioxidant support, cell-wall/pectin metabolism and possibly a minor or conditional contribution to AsA biosynthesis. *GalUR* family genes show a conserved pattern of reduced expression during mid-fruit development followed by strong induction at maturation, with tissue-specific differences between flavedo, albedo and pulp. These patterns support a role for the D-galacturonic acid pathway in late-stage pectin turnover and possibly localized ascorbate biosynthesis during citrus fruit maturation. Expression profiling of *APX2, APX3* and *AO* revealed distinct tissue-specific and stage-dependent regulation of the AsA degradation pathway. During the fruit development, *APX2* showed peak activity in flavedo during early and late stages, suggesting roles in oxidative stress response. In albedo, sustained *APX2* and *APX3* expression indicated continuous ROS scavenging, while in pulp, late-stage *APX2* induction corresponded with ripening-associated oxidative metabolism. These patterns underscore that AsA turnover is closely coordinated with ROS management to support fruit development, maturation and quality maintenance.

Gene co-expression network of all the tissues across the developmental stages based on Pearson correlation ($|r| \geq 0.70$) was analyzed. The results shown in Fig. 7 revealed distinct tissue-specific regulatory modules of AsA metabolism. In all tissues, strong positive correlations were observed among core biosynthetic genes, including GGP, GLDH, MYOOX and MyoIFS, indicating coordinated transcriptional control of AsA biosynthesis. Recycling-related genes such as APX2, APX3, DHAR and MDHAR formed tightly connected sub-networks, particularly in pulp tissue. Negative correlations between selected biosynthetic and recycling genes were also

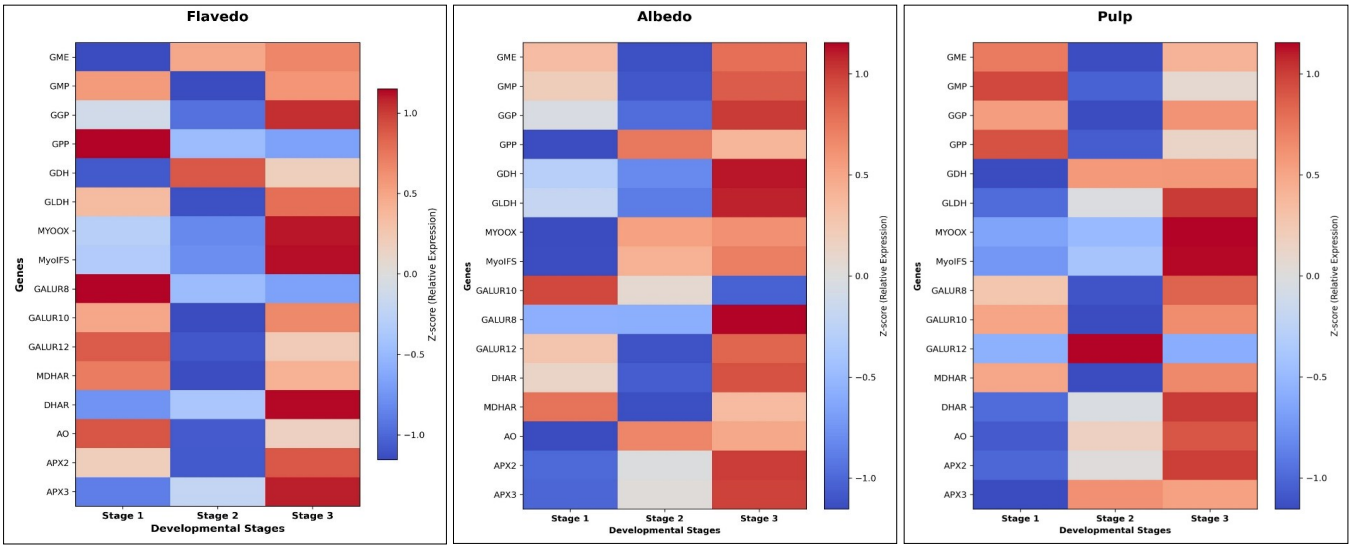

Fig. 6. Heat-map analysis illustrating stage-specific transcriptional regulation of genes involved in ascorbic acid metabolism across flavedo, albedo and pulp tissues. Quantitative real-time PCR data are expressed as mean Ct values (± SD) and were transformed to relative expression values (-Ct) prior to analysis. Gene-wise Z-score normalization was applied independently for each tissue to visualize standardized expression patterns across developmental stages. Color intensity represents relative transcript abundance, with warmer and cooler colors indicating higher and lower standardized expression levels, respectively.

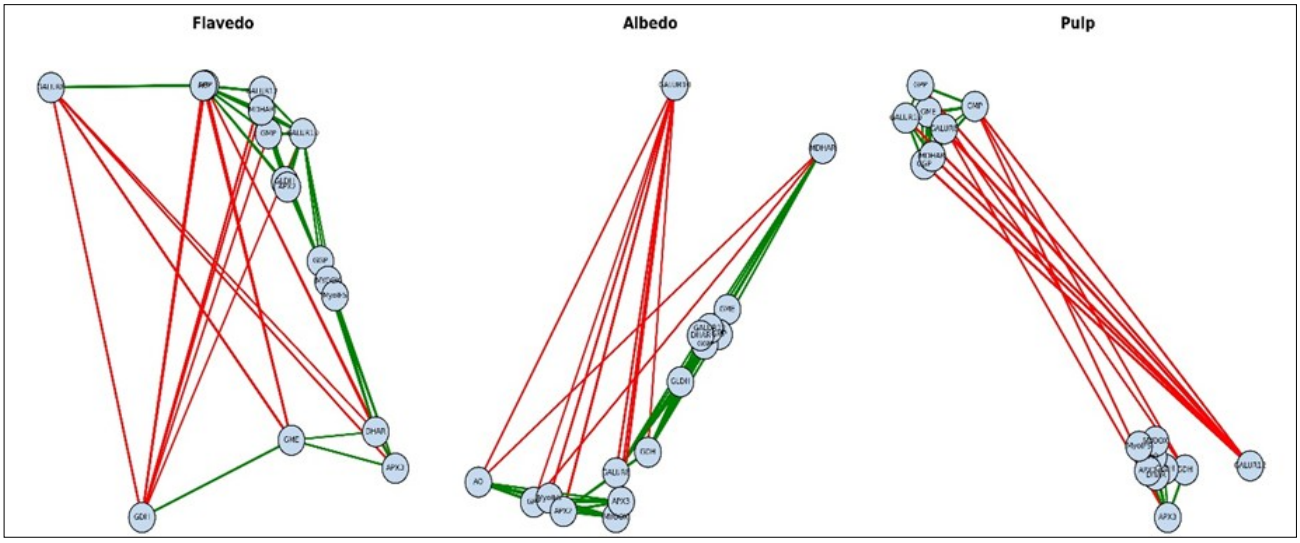

Fig. 7. Comparative gene co-expression networks of ascorbic acid metabolism-related genes across flavedo, albedo and pulp tissues. Networks were constructed using Pearson correlation analysis of qRT-PCR expression data after transformation of mean Ct values (± SD) to relative expression values (-Ct). Gene pairs exhibiting significant correlations (|r| ≥ 0.70) were included. Nodes represent individual genes, while edges denote significant positive (green) or negative (red) correlations, with edge thickness reflecting the magnitude of the correlation coefficient.

detected, suggesting stage-dependent redistribution of metabolic flux between AsA synthesis and recycling pathways.

The contrasting developmental regulation of *MDHAR* and *DHAR* across flavedo, albedo and pulp highlight a tissue-specific reprogramming of the ascorbate-recycling machinery during fruit maturation that likely contributes to differential AsA redox buffering and final vitamin C distribution in the fruit (39).

Conclusion

Our findings reveal that the L-galactose pathway is the predominant contributor to AsA biosynthesis with coordinated but tissue-specific regulation across fruit development. The myo-inositol and D-galacturonic acid pathways exhibit conditional, stage-dependent activation, highlighting their supportive roles during ripening and pectin turnover. Distinct regulation of *APX* isoforms and recycling enzymes (*MDHAR, DHAR*) underscores the close coupling of AsA metabolism with ROS management and redox homeostasis. Collectively, these insights emphasize a regulated tissue-specific coordination of AsA biosynthesis, degradation and recycling that supports vitamin C accumulation and fruit quality in Nagpur sweet orange. This investigation establishes a previously unavailable molecular framework of AsA regulation in Indian citrus, offering a scientifically robust foundation for cultivar-specific metabolic engineering, breeding strategies and nutritional enhancement programs in Indian citrus production systems.

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Authors' contributions

RJ conceived and designed the study. AY collected and prepared the samples and performed qRT-PCR gene expression data analysis. PS provided support in data analysis. VJ drafted the manuscript. BA provided technical support for qRT-PCR analysis. IBS prepared samples for RNA extraction. HUB provided the software for data analysis. AA edited and revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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