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Original Article

Trait-phenomics and omics integration reveal cultivar-specific anatomical, metabolic, and transcriptomic adaptation of tropical apple to contrasting highland environments

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Abstract

Climate change intensifies microclimatic variability in tropical highlands, posing a serious challenge to apple (*Malus domestica* Borkh) cultivation in Indonesia. An integrative analysis combining leaf anatomy, exploratory metabolomic profiling, and gene expression was conducted to identify two tropical cultivar-specific adaptive mechanisms in contrasting highland sites: Bumiaji (1123 m asl, 18.5°C) and Tutur (1325 m asl, 22.2°C). Leaf anatomical traits showed strong genotype × environment interactions, with Anna–Tutur having the thickest palisade (116.0 µm) and spongy mesophyll (112.6 µm), and the highest stomatal density (381 stomata/mm²). Metabolomic profiling revealed that Anna was enriched in fatty acid derivatives, including tetracosanoic and cis-eicosenoic acids. In contrast, Manalagi reprogrammed sugar and antioxidant pathways, particularly in Bumiaji, with a specific focus on fructose/mannose metabolism ($p = 0.0002$). Gene expression analysis of six MADS-box genes showed consistent induction of AP1, AP3, and SOC1 in Manalagi, contrasting with the site-dependent plasticity observed in Anna. Fruit quality also diverged: Manalagi accumulated higher soluble solids (11.7 °Brix) and vitamin C (44.9 mg/100 g), while Anna exhibited higher acidity (2.8%) and firmness (8.2 kgf). These findings suggest that 'Anna' employs responsive plasticity suited to variable microclimates, in contrast, 'Manalagi' uses constitutive resilience for stable performance, providing evidence-based guidance for cultivar deployment in tropical highland production systems under climate change.

Keywords: Gene expression, Malus domestica, Multi-omics integration, Phenotypic plasticity, Tropical apple

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Introduction

Apple (*Malus domestica* Borkh.) is a commercially significant fruit crop worldwide owing to its high nutritional value, wide genetic diversity, and strong market demand. Key fruit quality traits, such as total soluble solids (TSS), titratable acidity (TA), vitamin C, and skin color, are influenced by environmental factors, particularly temperature and light intensity (Akagić et al., 2019; Patocka et al., 2020; Akši´c et al., 2022; Argenta et al., 2022). Altitude markedly affects apple phenology, biochemical composition, and stress response by altering temperature, humidity, and the light regime (Kumar et al., 2019; Samudra et al., 2021; Li et al., 2021). Understanding cultivar-specific responses to such environments is vital for improving apple adaptability and productivity in tropical regions. In the tropics, such as Indonesia, apples are mainly cultivated in highlands where temperatures are cooler and suitable for fruit growth. However, tropical highland apple systems have been less studied than those in temperate production zones. Accordingly, there is a dearth of information on the physiological and molecular mechanisms governing the responses of tropical-adapted apple cultivars to divergent highland ecologies. This information is key to designing climate-adapted apple production systems.

Apple cultivars differ in anatomical features such as leaf thickness, mesophyll structure, and stomatal density, which influence photosynthetic efficiency and water-use regulation under altitude-related stress (Li et al., 2020; Wang et al., 2020). Nevertheless, integrated evaluations of these adaptive traits in tropical highland conditions remain scarce. With recent advances in metabolomics and transcriptomics, it is now possible to elucidate the molecular mechanisms underlying environmental adaptation. Plant metabolic processes are susceptible to altitude-related environmental variations, particularly shifts in atmospheric pressure, temperature, and solar radiation, making altitude an important driver of metabolic differentiation in apple. Metabolomic analyses reveal the reprogramming of secondary metabolic pathways related to stress tolerance, fruit maturation, and nutritional quality (Srednicka-Tober et al., 2020; Chen et al., 2023; Gao et al., 2025). Apple metabolite profiling studies employing GC–MS have also shown that apple fruit composition is susceptible to post-harvest factors, with sugars generally increasing and organic acids (including major TCA-cycle intermediates) decreasing during fruit development (Xu et al., 2020). By comparative metabolomic profiling, significant cultivar-specific differences between genotypes were observed with respect to major primary and secondary metabolites, including sugars (glucose, fructose, sucrose), asparagine, quinic acid, L-rhamnitol, phenylalanine, and some condensed polyphenols. Similarly, a high-altitude variety ‘Hongjiangjun’ has also higher malate content, suggesting that the influence of environment is dominant over malate metabolism

However, comprehensive omics studies of tropical apple cultivars remain underexplored, especially along altitudinal gradients in Southeast Asia. Closing this gap is critical to identify molecular and metabolite markers of such traits involved in adaptation to tropical environments. This research aims to assess morphophysiological, anatomical, metabolomic, and gene expression changes in two tropical apple cultivars (Anna and Manalagi) across different highland locations in East Java, Indonesia. We hypothesize that altitude-dependent cultivar-specific physiological and anatomical characteristics exhibit varying degrees of adaptation; these phenotypic changes are accompanied by specific metabolomic and transcriptomic profiles involved in environmental adaptation. The study provides new insights into the integrative adaptive responses of tropical apples, which will contribute to precision cultivar deployment and management schemes to improve fruit quality and yield stability in tropical highlands.

Material and Methods

Study sites, plant materials and climatic conditions

The study was conducted from February to October 2024 in two highland apple-producing regions of East Java, Indonesia: Bumiaji Subdistrict, Batu City (1,123 m above sea level), and Tutur Subdistrict, Pasuruan Regency (1,325 m above sea level), as shown in Table 1.

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Table-1. General climatic conditions of different locations.

No	Data	Bumiaji	Tutur
1	Geographical coordinates	-7º 49’ 36’’ S, 112º 32’ 13’’ E	-7º 54’ 38’’ S, 112º 50’ 14’’ E
2	Altitude (m asl)	1123	1325
3	Mean annual temperature (ºC)	18.5	22.2
4	Mean annual relative humidity (%)	86.5	85
5	Mean light intensity (Lux)	57,526	64,355

Mature (20-year-old) apple trees of the cultivars ‘Anna’ and ‘Manalagi′ were used in this study. These cultivars have been officially registered under Decree No. 513/Kpts/SR120/12/2005 (Anna) and No. 899/Kpts/TP. 240/11/1984 (‘Manalagi’). Site selection criteria included similar tree age, cultivation practices, and phenological stages. Standard orchard management at both locations consisted of organic fertilization (20 kg compost per plant per year), inorganic NPK fertilization (2 kg per plant per year), pest and disease control, and defoliation-induced dormancy breaking.

Leaf anatomical observation

Leaf samples were fixed in FAA for 24 hours, dehydrated in 70–100% ethanol, cleared in xylene, and embedded in paraffin at 57 °C. Sections (6–12 µm) were cut with a rotary microtome, mounted with glycerol–albumin, stained with Safranin O and Fast Green, and observed under an Olympus BX53 microscope (100×) (Metusala, 2017). Anatomical traits (leaf, epidermal, palisade, and spongy thickness) were measured using ImageJ. Stomatal density (per 0.015 mm²) was determined at 400× (Paul et al., 2017) after dissecting, cleaning, and mounting the lower epidermis, images were captured using an Optilab Advance Plus system for analysis.

Leaf untargeted metabolite profiling

Each leaf metabolomic sample represented a composite of leaf tissue pooled from 15 trees for each cultivar and location. Leaf samples were oven-dried at 50 °C for 48 h, ground, and sieved. One gram of powder was extracted with 1 mL of ethanol overnight at room temperature. After centrifugation (9,500 rpm, 3 min), the supernatant was collected for GC-MS analysis using a Thermo Trace™ 1310 ISQ system with an HP-5MS UI column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The oven temperature was increased from 60 to 280 °C within 32 min. The spectral data were collected in positive mode, full-scan mode (40–500 m/z). Since this GC–MS analysis was performed for qualitative, untargeted metabolite profiling, no calibration curve was applied. Compound identification was carried out by comparing spectra with the NIST Mass Spectral Library 17. Kovats retention indices were calculated using the n-alkane series, following the protocols for exploratory GC–MS metabolomics (Fiehn, 2016; Wishart, 2019; Salem et al., 2020). Internal standards Ribitol (Sigma-Aldrich Co., USA) was added for relative quantification and extraction efficiency normalization (Abadie et al., 2022). The metabolomic data should be interpreted as exploratory and hypothesis-generating, providing insights into pathway-level metabolic differences rather than absolute quantitative comparisons.

Gene expression analysis

The expression of each gene was assessed in full-bloom, young, and mature leaves at three phenological stages: full bloom (1 month after artificial defoliation/F1), young fruit (1-month post-pollination/F2), and mature fruit (3 months after flowering/F3). For gene expression analysis, composite sampling was intentionally used to obtain a robust, representative estimate of cultivar-level gene expression across different locations. Leaf samples were collected from 15 trees per cultivar per location. The total RNA was isolated using the Ribospin Plant Kit (GeneAll), and RNA quantification was performed using the NanoQuant M200 Pro (Tecan). First-strand cDNA was synthesized with iScript™ cDNA Synthesis Kit (Bio-Rad).

Seven floral development-related genes that were reported by (Kumar et al., 2016) were amplified with the primers in Table 2 for sequencing. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a housekeeping gene for normalization (Pfaffl et al., 2001). Gene expression data were obtained from single qPCR measurements

of each composite sample without technical replicates, and represent trend-level expression patterns rather than statistically validated fold-change estimates. The data should be interpreted as descriptive profiling to identify cultivar-specific transcriptional patterns across developmental stages and environments.

Table-2. Primer sequences and amplicon sizes used for qPCR.

No	Gene	GenBank Accession	Gene bank ID	Primer (5'–3')	Amplicon Size (bp)	Reference/ Validation
1	GAPDH	AT1G13440 (GAPC2)	837904	F: CAAGCCAGACATCCACATTCTTT R: ACCCTCAACAATTCCGAACCT	100	Arabidopsis_thaliana - Ensembl Genomes 62
2	AP3	AT3G54340 (AP3)	824601	F: TGGAGGAAAGAAGAGGAAACATG R: TCTCCCTCATTGTCCACATAACAA	85	Arabidopsis_thaliana - Ensembl Genomes 62.
3	SEP1	AT5G15800 (SEP1)	831436	F: TGTCGCTGAAGCAGATCAGATC R: CTTCGTTTAGCACGTGTTCATTTC	90	Arabidopsis_thaliana - Ensembl Genomes 62
4	SOC1	AT2G45660 (AGL20)	819174	F: GGGAACCATAGAGCGTTATCAGAA R: TTCAGATGCTGCATATTTTGTTCA	90	Arabidopsis_thaliana - Ensembl Genomes 62
5	AP1	AT1G69120	843244	F: CTTCTCTACCAAAGGCAAGCTTTT R: CACCAGCACTATACCCAGTCTGTCT	467	Arabidopsis_thaliana - Ensembl Genomes 62
6	AGL15	AT5G13790	831224	F: AGCTTCGATGTTTGTTTCCACAA R: GGTTTACAAGGGATTTGGTTTTCTC	90	Arabidopsis_thaliana - Ensembl Genomes 62
7	TM8	KF270624	270624	F: CGACATAACTAGGACCATTTC R: CCTCCAATACTCCATTGTTCT	100	Solanum lycopersicum cultivar Florida Petite TM8-like protein mRNA, co - Nucleotide - NCBI.

Source: Kumar et al., 2016.

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Fruit phenotyping and biochemical measurements

Fruit skin color was measured by a calibrated chromameter (Konica Minolta CR-400, Japan) using CIE Lab* mode. Uniform, clean fruits were sampled at four equidistant equatorial points per fruit. Parameters recorded were L* (lightness), a* (green-red), and b* (blue-yellow).

Total Soluble Solids (TSS) content (Brix) was determined using a handheld refractometer (Atago PAL-1, Japan) on fresh juice extracted from ripe fruits at 20°C; ten fruits per replicate were measured.

The titrimetric method determined total titratable acid (TTA) and vitamin C (Sallato et al., 2017).

Fruit firmness was measured using a penetrometer (Effegi FT 327, Italy) at two equidistant equatorial points per fruit; ten fruits were assessed per replicate.

Experimental design

The experiment employed a split-plot design with location (Bumiaji and Tutur highland sites) as the main plot factor and cultivar (Anna and Manalagi) as the subplot factor, with three biological field replications. This design was used for quantitative variables requiring statistical validation, including leaf anatomical traits and fruit quality parameters. For GC-MS untargeted metabolomics and qPCR-based gene expression analyses, a different sampling strategy was employed. Each metabolomic and transcriptomic sample consisted of a composite pool of tissue from 15 trees for each cultivar-location combination, without biological or technical replication. This composite sampling approach was chosen to figure out population-level metabolic and transcriptional signatures while minimizing individual tree variation, and is appropriate for exploratory, hypothesis-generating analyses.

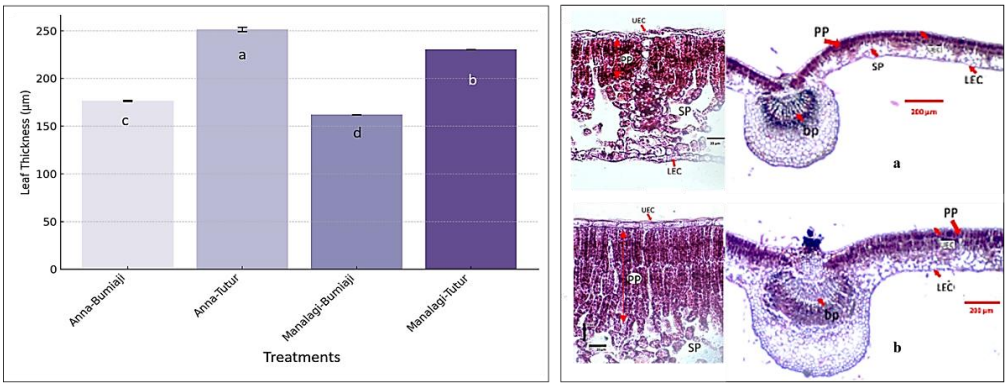
Statistical analyses

Data were analyzed using MINITAB 16 (Minitab Inc., USA), and significant differences ($p < 0.05$ and $p < 0.01$) were evaluated with Tukey's Honestly Significant Difference (HSD) test. For metabolomic data, heatmaps and hierarchical clustering were produced in R 4.3.2 using the pheatmap package after standardizing variables via Z-scores. Gene expression data were transformed using log10-transformed [log10(expression + 1)] to reduce skewness for heatmap visualization. Principal Component Analysis (PCA) was conducted using the MASS, ggplot2, and factoextra packages in R. MetaboAnalyst was employed to analyze the metabolomic pathway.

Results

Leaf anatomical responses

Leaf anatomical traits exhibited significant genotype × environment interactions, revealing cultivar-specific adaptive strategies to contrasting highland conditions (Figure 1a, Table 3). The 'Anna' cultivar demonstrated greater anatomical plasticity in response to environmental variation, particularly at Tutur, where leaves developed substantially thicker mesophyll tissues (total thickness $p < 0.01$) dominated by elongated, compacted palisade cells (116.0 µm) and enlarged spongy parenchyma (112.6 µm; Figure 1b). In contrast, 'Manalagi' at Bumiaji exhibited the most conservative anatomical phenotype with minimal mesophyll development (palisade: 55.5 µm; spongy: 83.4 µm).



a **b**

Figure-1. Leaf blade thickness (µm) of 'Anna' and 'Manalagi' apple cultivars grown in Bumiaji and Tutur highlands. Light microscopy images of leaf cross-sections stained with Safranin O and Fast Green (b) showing

anatomical features of (a) 'Manalagi-Bumiaji' and (b) 'Anna-Tutur'. Labels indicate upper epidermis cells (UEC), lower epidermis cells (LEC), palisade parenchyma (PP), spongy parenchyma (SP), and vascular bundle (bp). Scale bar = 200 µm. Different letters indicate statistically significant differences at $p \leq 0.05$ (Tukey's HSD test).

The epidermal features also separated the two cultivars, with 'Anna' having a thicker upper epidermis (13.7 µm) than 'Manalagi' (11.5 µm), which was consistently observed at both sites. Location (site) effect revealed that the two locations favored the formation of a thicker upper epidermis; Bumiaji was 14.0 µm, compared with Tutur at 11.2 µm, in lower epidermis thickness. The formation of 'Anna' was slightly higher than 'Manalagi' (Table 3). Stomatal density varied among cultivars and locations (337.5–381.3 stomata mm^{-2}), with 'Anna' at Tutur having the highest and 'Manalagi' at Tutur having the lowest, suggesting that genotypes have distinct regulatory mechanisms for gas exchange and water relations.

Table-3. Thickness of upper and lower epidermis, palisade and spongy mesophyll tissues (µm), and stomatal density (stomata/mm^2) of 'Anna' and 'Manalagi' apple leaves grown in Bumiaji and Tutur highlands.

Treatments	Upper epidermis	Lower epidermis	Palisade	Spongy	∑ of stomata/mm^2
Varieties					
Anna	13.7±1.8^{a}	11.0±0.4^{b}	88.9±28.6^{a}	100.3±13.3^{a}	373.4±11.5^{a}
Manalagi	11.5±1.3^{b}	11.4±1.1^{a}	81.1±27.0^{b}	92.1±9.3^{b}	343.8±10.4^{b}
Locations					
Bumiaji	14.0±1.4^{a}	10.8±0.5^{b}	58.7±3.4^{b}	85.7±2.7^{b}	357.8±11.5^{a}
Tutur	11.2±1.0^{b}	11.6±0.9^{a}	114.4±4.9^{a}	106.8±7.0^{a}	359.4±24.4^{a}
Interaction					
Anna-Bumiaji	15.4±0.4^{a}	11.2±0.4^{b}	61.9±0.8^{c}	88.1±1.4^{c}	365.3±8.6^{ab}
Manalagi-Bumiaji	12.7±0.1^{b}	10.4±0.1^{c}	55.5±0.5^{d}	83.4±0.6^{c}	350.0±8.6^{bc}
Anna-Tutur	12.1±0.5^{b}	10.8±0.3^{bc}	116.0±0.1^{a}	112.6±4.9^{a}	381.3±8.6^{a}
Manalagi-Tutur	10.3±0.1^{c}	12.5±0.4^{a}	106.8±0.5^{b}	100.9±1.1^{b}	337.5±8.6^{c}
R^2 (%)	98.4	94.5	99.9	98.3	91.1

Values represent mean ± standard error (n=3). Different letters within rows indicate significant differences by Tukey's HSD test ($p \leq 0.05$).

Leaf untergeting metabolomic profiling reveals distinct biochemical adaptation strategies

GC-MS analysis detected similar total metabolite numbers across all treatments (48–51 compounds per treatment), with approximately 18–19 confidently annotated metabolites consistently mapped to plant metabolic pathways, yet revealed substantial qualitative differences in metabolic composition. These comparable totals indicate that both 'Anna' and 'Manalagi' retain a broadly similar overall metabolic capacity in Bumiaji and Tutur.

Principal component analysis distinctly differentiated the samples based on both cultivar and location (Figure 2a–c); and PC1 and PC2 together accounted for variation of 45.0% and 29.3%, respectively (total: 74.3%). Metabolites with the highest loadings accounting for separation (PC1: 43.4%) were structural carbohydrate D-glycero-D-galacto-heptose and DL-arabinose block, as well as membrane-bound linoleic acid esters, and PC2 (31.6%) sugar derivatives, including polyunsaturated fatty acids, demonstrating that growth metabolism pathways and those linked to membrane integrity did not respond in the same way to the highland microclimate.

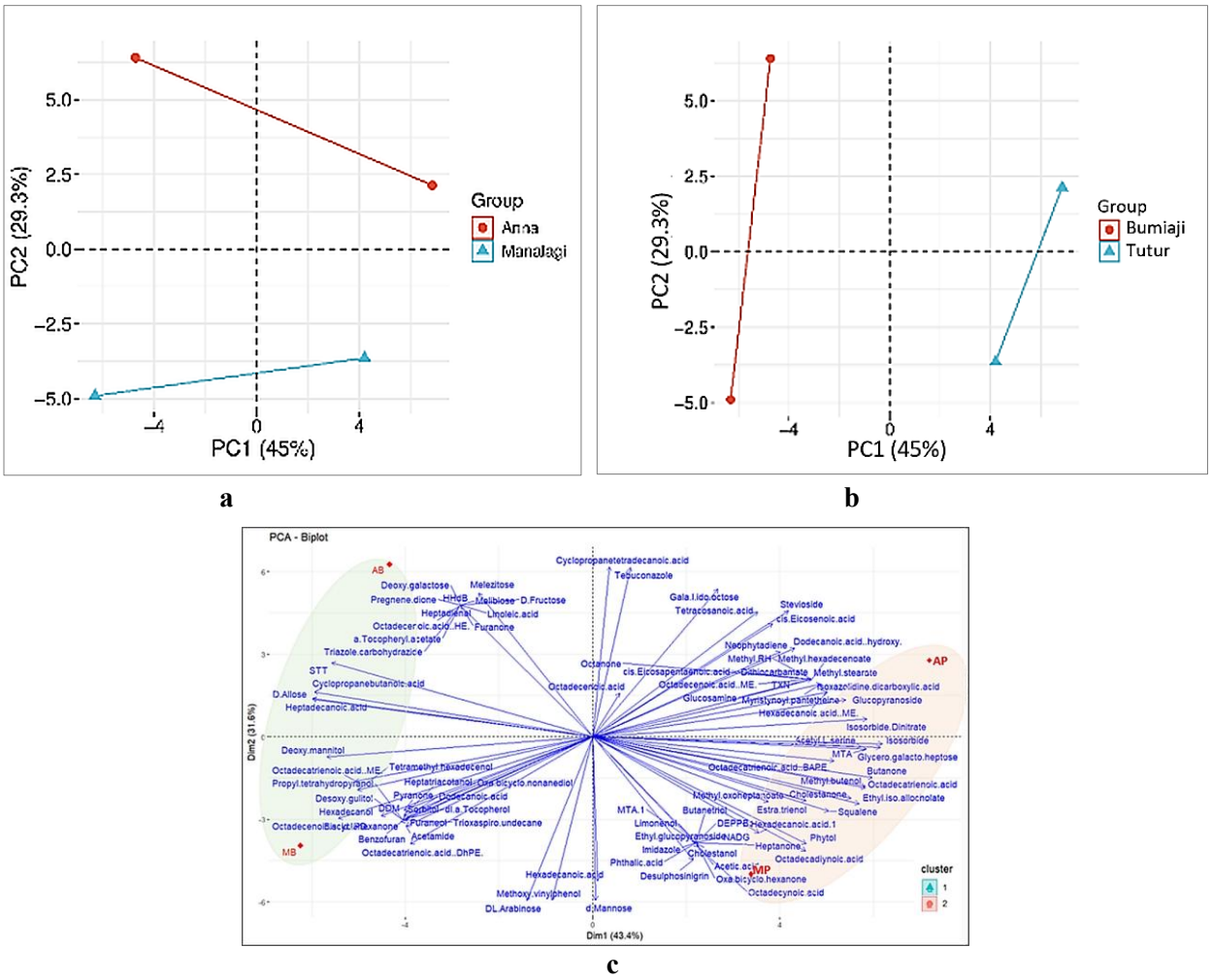


Figure-2. Principal component analysis (PCA) of metabolite profiles of tropical apple cultivars grown under contrasting highland environments. Each metabolomic sample represents composite tissue pooled from 15 trees per cultivar-location combination. Data reflect exploratory, and qualitative profiling analysis for pathway discovery. PCA score plot comparing *Malus domestica* cv. Anna and Manalagi, showing separation along PC1 (45.0% variance explained) and PC2 (29.3% variance explained) (a), PCA score plot comparing apple metabolomes from Bumiaji and Tutur, indicating location-driven metabolic divergence along PC1 (45.0%) and PC2 (29.3%) (b), and PCA biplot integrating the metabolite profiles of apple cultivars grown at two highland locations (c). Blue vectors represent metabolite loadings contributing to separation along PC1 (43.4% variance explained) and Dim2 (31.6%). Shaded ellipses indicate clustering patterns for each cultivar–location combination: AP (Anna–Tutur, Pasuruan), MP (Manalagi–Tutur, Pasuruan), AB (Anna–Bumiaji, Batu), and MB (Manalagi–Bumiaji, Batu).

The heatmap also showed pronounced metabolic differences among cultivars and locations (Figure 3). The greatest in general metabolite enrichment, especially lipid compounds (Sterol and Tetraterpenoids), was recorded from Anna grown at Tutur (AT). In contrast, AB (Anna from Bumiaji) showed general suppression across almost all compounds. A similar profile was observed for Manalagi, with plants growing in Tutur (MT) showing a moderate increase in the abundance of phenolics, fatty alcohols, and antioxidant metabolites. At the same time, Bumiaji-treated JM (MB) grouped with AB and showed lower overall metabolite abundance. These trends are indicative of cultivar- and site-specific differences in lipid metabolism, terpenoid biosynthesis, and antioxidant pathways.

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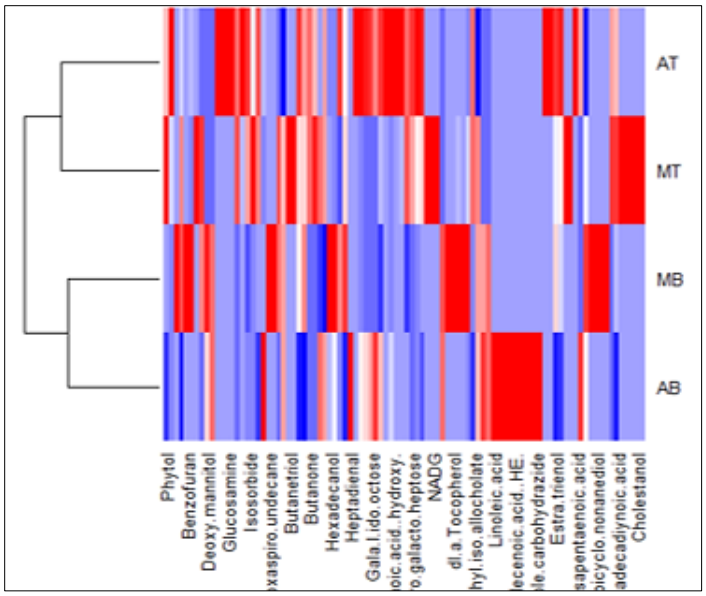

Figure-3. Heatmap of leaf metabolomic profiles of two apple cultivars grown at contrasting highland environments. The heatmap displays the relative abundance of GC–MS–detected metabolites in Anna and Manalagi cultivars grown at Tutur (AT, MT) and Bumiaji (AB, MB). Red and blue indicate higher or lower metabolite levels, respectively, compared to the mean value in the dataset. Hierarchical clustering of the two different genotypes exhibited a clear separation between Tutur and Bumiaji samples. Sample abbreviations: AB = *Anna* Bumiaji; AT = *Anna* Tutur; MB = *Manalagi* Bumiaji; MT = *Manalagi* Tutur. Each metabolomic sample represents composite tissue pooled from 15 trees per cultivar-location combination. Data reflect exploratory, and qualitative profiling analysis for pathway discovery.

Pathway enrichment analysis (Figure 4a–d; Table 4) highlighted unsaturated fatty acid biosynthesis and amino sugar/nucleotide sugar metabolism as consistently activated across treatments. ‘Anna’ for both sites was significantly enriched in biosynthesis of unsaturated fatty acids (Bumiaji: $p = 0.0029$; Tutur: $p = 0.0001$), galactose metabolism (Bumiaji: $p = 0.0043$), and amino sugar metabolism (Bumiaji: $p = 0.0104$; Tutur: $p = 0.0282$). 'Manalagi' showed a more extensive metabolic reprogramming and with the highest impact at Bumiaji, where five pathways were up-regulated in a significant way: fructose and mannose metabolism ($p = 0.0002$), unsaturated fatty acid biosynthesis ($p = 0.0010$), galactose metabolism ($p = 0.0033$), amino sugar/nucleotide sugar metabolism ($p = 0.0082$) and sulfur metabolism ($p = 0.091$). These metabolomic profiles suggest that 'Anna' utilizes focused metabolic activation, possibly related to stress tolerance, whereas 'Manalagi' exhibits broad metabolic reprogramming, especially in sugar-antioxidant pathways, at the lower elevation site.

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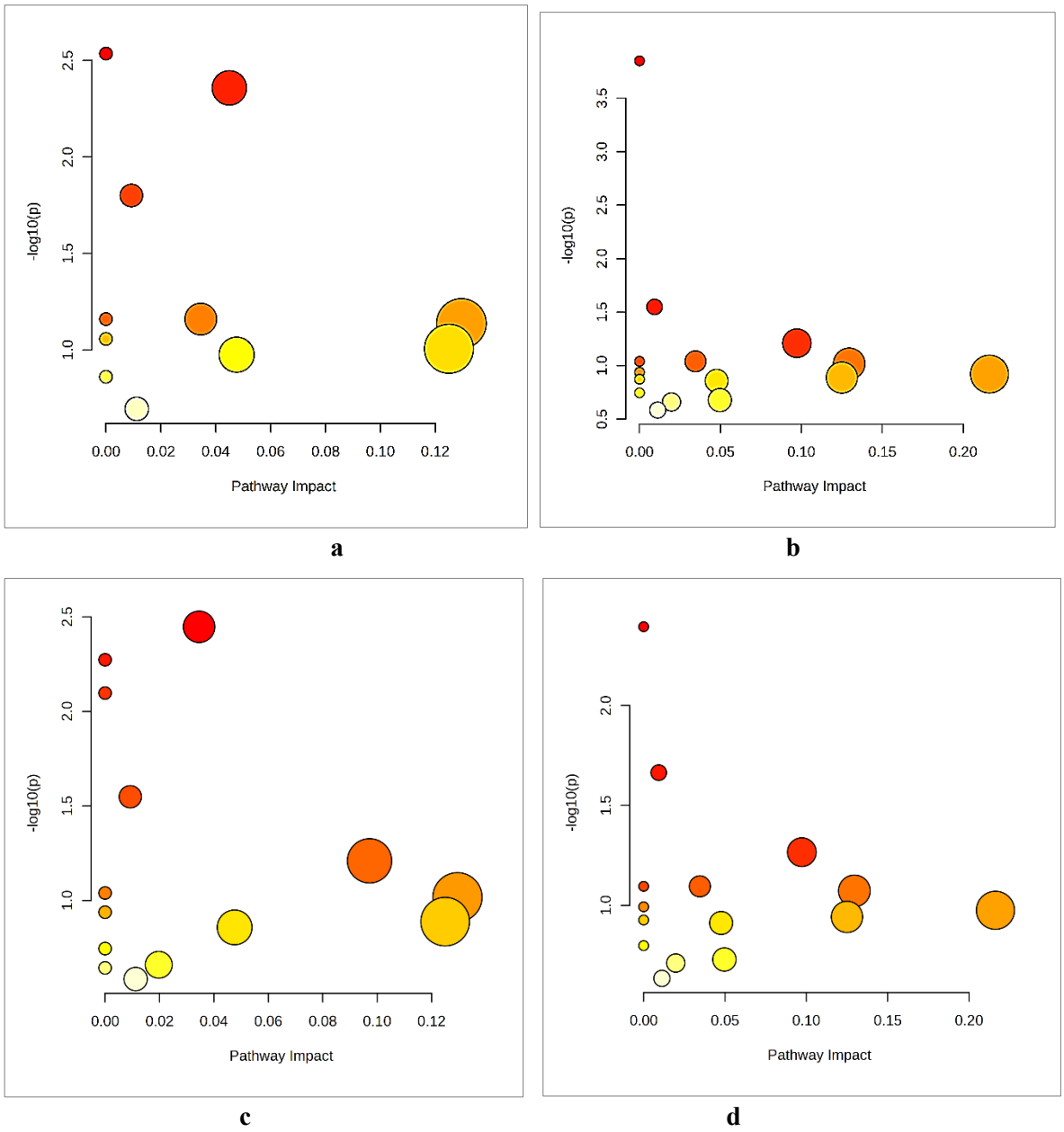

Figure-4. Integrated pathway analysis of leaf metabolites in two tropical apple cultivars ('Anna' and 'Manalagi') grown under contrasting highland environments. Bubble plots present significantly enriched pathways for each combination of cultivar–location, being Anna–Bumiaji (a), Anna–Tutur (b), Manalagi-Bumiaji (c), and Manalagi-Tutur (d). The pathway impact value from the pathway topology analysis is plotted on the x-axis, and $-\log_{10}(p)$ on the y-axis. Bubble size indicates the number of identifications matched to metabolites, while bubble color corresponds to pathway significance. Each metabolomic sample represents composite tissue pooled from 15 trees per cultivar-location combination. Data reflect exploratory, and qualitative profiling analysis for pathway discovery.

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Table-4. Summary of significantly enriched metabolic pathways ($p < 0.05$) in leaf tissues of 'Anna' and 'Manalagi' grown in Bumiaji and Tutur.

Cultivar-Location	Significantly Enriched Pathways ($p < 0.05$)	Biological Interpretation
Anna–Bumiaji	• Unsaturated fatty acid biosynthesis ($p = 0.0029$) • Galactose metabolism ($p = 0.0043$) • Amino sugar and nucleotide sugar metabolism ($p = 0.0104$)	Lipid remodeling; monosaccharide turnover; cell-wall precursor metabolism
Anna–Tutur	• Unsaturated fatty acid biosynthesis ($p = 0.0001$) • Amino sugar metabolism ($p = 0.0282$)	Focused membrane lipid adaptation under Tutur conditions
Manalagi–Bumiaji	• Fructose/mannose metabolism ($p = 0.0002$) • Unsaturated fatty acid biosynthesis ($p = 0.0010$) • Galactose metabolism ($p = 0.0033$) • Amino sugar and nucleotide sugar metabolism ($p = 0.0082$) • Sulfur metabolism ($p = 0.091$)	Broad activation of carbohydrate and lipid pathways; strong metabolic plasticity
Manalagi–Tutur	• Unsaturated fatty acid biosynthesis ($p = 0.00404$) • Amino sugar and nucleotide sugar metabolism ($p = 0.02168$)	Conserved lipid remodeling; moderate sugar-derived pathway activation

Anna–Bumiaji showed enrichment in the biosynthesis of unsaturated fatty acid, galactose metabolism, and amino sugar/nucleotide sugar metabolism, while Anna–Tutur was only enriched in the biosynthesis of unsaturated fatty acid. Manalagi–Bumiaji showed the most significant pathway-level activation: fructose/mannose metabolism, unsaturated fatty acid biosynthesis, galactose metabolism, and amino sugar/nucleotide sugar metabolism. At the same time, Manalagi-Tutur only showed less distinctive activated pathways in mainly unsaturated fatty acid synthesis and amino sugar/nucleotide sugar metabolism.

Developmental stage- and environment-dependent mads-box gene expression patterns

Quantitative RT-PCR analysis (Figure 5) revealed genotype- and site-specific expression patterns of six MADS-box genes (*AP1, AP3, SEP, TM8, AGL15,* and *SOC1*) during three flowering and fruit developmental stages (F1: full bloom, F2: young fruit, F3: mature fruit) in 'Anna' and 'Manalagi' apple cultivars.

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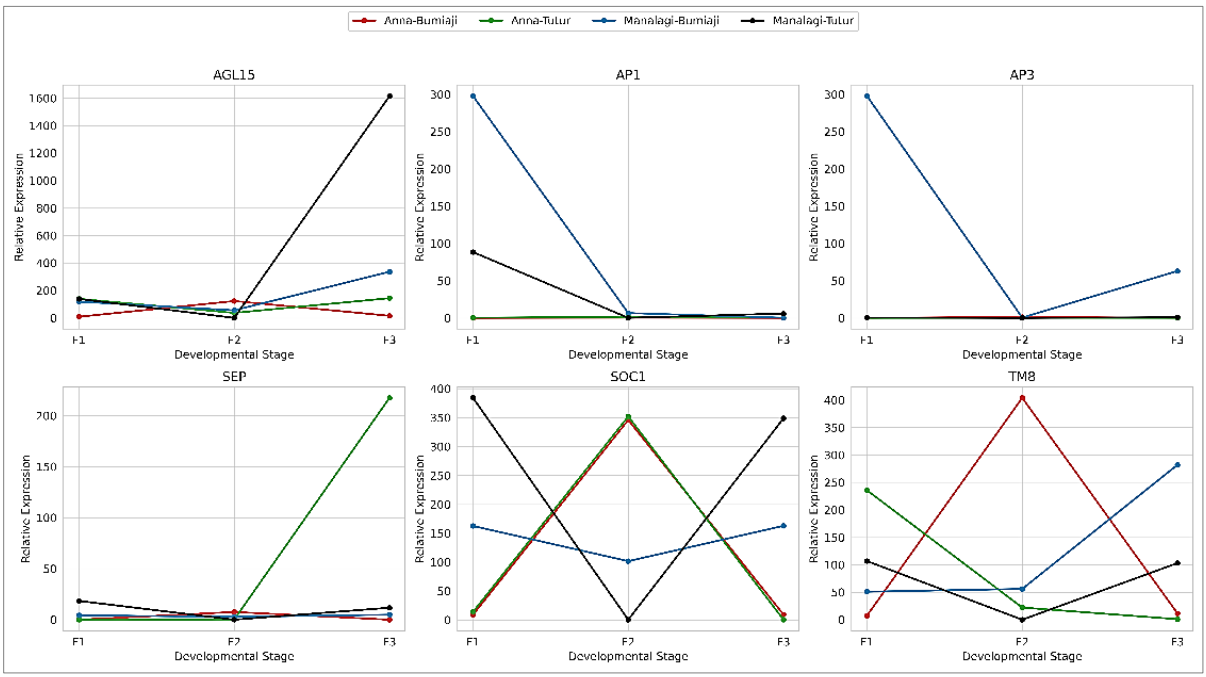

Figure-5. Relative expression profiles of *AGL15*, *AP1*, *AP3, SEP, SOC1*, and *TM8* genes across three developmental stages (F1–F3) in two apple cultivars ('Anna' and 'Manalagi') grown at contrasting highland environments (Bumiaji and Tutur).

Note: Each line represents a unique cultivar–location combination: Anna–Bumiaji (red), Anna–Tutur (green), Manalagi–Bumiaji (blue), and Manalagi–Tutur (black). Data are presented as relative normalized expression values obtained from qPCR. Clear separation in gene activation patterns highlights genotype-specific and site-dependent regulatory responses during floral developmental progression. Gene expression data were obtained from composite RNA samples pooled from 15 trees per cultivar-location-stage combination. Data represent trend-level expression patterns rather than statistically validated fold changes.

In 'Anna', grown at Bumiaji, *AP1*, *SOC1*, and *AGL15* reached peak expression at the young fruit stage (F2), then declined at maturity (F3). The expression of *SEP* genes was increased during the F1-F3 stages, which might be involved in fruit ripening. *TM8* was continuously expressed during all phases. In contrast, 'Anna' at Tutur showed the highest expression of TM8 at F1, but with a gradual increase in *SEP* up to maturity and crisp spike for *SOC1* at F2.

'Manalagi' displayed contrasting transcriptional trends. In Bumiaji, the expression of *AP1*, *AP3*, *SEP*, *AGL15*, and *SOC1* increased until the F1 stage, then decreased during the F2 stage, and recovered slightly at the F3 stage. *TM8* remained consistently expressed. In Tutur-grown Manalagi, the majority of genes exhibited very low expression at the early stage, and only the expression level of *AGL15*, *SEP*, *SOC1*, and *AP3* significantly increased at F3. Most striking is the expression of *AGL15* in Manalagi-Tutur, which opens the hypothesis that gene activity is environmentally sensitive.

AP1 and *AP3* were expressed more highly in Manalagi than in Anna at F1. *TM8* was consistently expressed in both cultivars, in particular in Bumiaji-Anna. Altogether, the cultivar 'Manalagi' showed a stronger and more consistent transcriptional pattern, while 'Anna' tended to have a more variable response across sites and growth stages.

Fruit quality traits reflect complex genotype-environment-trait interactions

The fruit morphology and coloration are presented in Figure 6a,b. It shows the different types for both cultivars. These apples are distinguished by their bright red, conical fruit, as seen in Anna, and round, yellow-green skin, as seen in Manalagi. These phenotypic differences are indicative of underlying genetic variation and are influenced by factors such as

the reliability of the highlands, temperature, and light intensity. In terms of the skin colour parameters (L*, a*, b*), 'Anna' and 'Manalagi' apples, as well as growing location, showed significant variations (Figure 6c). 'The L*, a*, and b* value indicating lightness, red-green, and yellow-blue chromatic axis, respectively, was consistently higher in 'Anna' apples as compared to 'Manalagi' in both locations. In terms of location effect, irrespective of cultivar, Bumiaji consistently exhibited better skin colour development than those from Tutur.

For quality parameters, including total soluble solids (TSS), titratable acidity (TA), vitamin C content, and fruit firmness, significant interactions between cultivar and location were observed (Figure 6d). Biochemical analysis revealed that the majority of quality attributes were cultivar-specific but also varied by location, with fruits from Manalagi having significantly higher TSS (11.7 °Brix) and vitamin C (44.9 mg/100 g) than those from Anna. On the other hand, Anna exhibited higher TA (2.8%) and better firmness level (8.2 kgf). Vitamin C and firmness were significantly affected by location, with the highest values of both parameters observed in Tutur for 'Manalagi ' (Figure 6e). It shows that Bumiaji offers the best microclimatic conditions for producing high-quality apples across the cultivars Anna and Manalagi. Bumiaji had a relatively lower elevation (1123 m asl) than Tutur (1325 m asl). However, its significantly lower mean annual temperature (18.5°C) was associated with higher values of fruit skin color, total soluble solids, titratable acidity, and vitamin C.

Figure-6. Representative fruit traits of 'Anna' and 'Manalagi' apples grown in Bumiaji and Tutur highland locations. 'Anna' apples exhibit bright red, conical fruits characteristic of the cultivar (a), 'Manalagi' apples display round fruits with yellowish-green skin (b). Fruit skin color parameters (L, a*, b*) (c), TSS and TA (d),

and Vitamin C and fruit hardness (e). Bars are presented as mean ± standard error (n=3). Different letters indicate significant differences at $p \leq 0.05$ according to Tukey's HSD test.

Discussion

Leaf anatomical characteristics exhibited significant G × E interactions, and the two cultivars showed different structural strategies in response to contrasting highland environments. 'Anna' showed greater anatomical plasticity, particularly in Tutur, where clear palisade and spongy mesophyll tissue thickening indicate a larger surface area for increased CO_2 diffusion and better light capture at higher irradiance (Locatelli et al., 2019). On the other hand, the greater palisade cell length and thickness of mesophyll tissue for 'Manalagi'-Bumiaji could be related to a less conservative water-use strategy for genotypes that are adapted to lower temperatures with minimal variability (Liu et al., 2020). Because of these divergences in epidermal features, additional differences were interpreted as further support for these adaptations to the environment. The thicker upper epidermis in 'Anna' should deliver higher photoprotection under high radiation conditions. At the same time, the thicker lower layer in both cultivars at Tutur may also imply a putative role in UV screening. The opposite stomatal patterns (more 'Anna'-Tutur leaves, but less in 'Manalagi'-Tutur) show that the two scions have contrary strategies for gas exchange and water use efficiency (Paudel et al., 2019; Sun et al., 2021). Overall, these morphological changes show that 'Anna' utilizes a flexible structure, with greater changes in the plant from an architectural perspective to cope with environmental variation, while 'Manalagi' shows a more conservative plant architecture aimed at stability.

Another anatomical change in Anna is also incorporated: a thicker leaf blade and an increased palisade layer. It is associated with higher stomatal densities rather than denser, conservative leaf anatomy in Manalagi, an example of genotype-environment-specific adaptation. Mesophyll and stomatal arrangements are organized in a manner that enhances photosynthetic capacity and adaptation to elevated irradiance and warmer or colder environments; hence, more effort is put into optimizing its structural traits for water conservation, at least in such mild environments (Shen et al., 2021; Xiang et al., 2024). Anatomical flexibility may be required to optimize physiological performance across a broad range of tropical highland microclimates. Overall, these results suggest that Anna's higher anatomical flexibility probably gives it a competitive advantage in changing environments, and will be a key morphological indicator used to improve photosynthesis capacity, stress tolerance, and productivity of tropical apple production systems (Locatelli et al., 2019; Karagiannis et al., 2020; He et al., 2021).

These anatomical responses were well coordinated with metabolomics divergence among cultivars and sites. PCA and heatmap distinguished groups based on cultivars and microclimate, primarily due to structural carbohydrates, membrane lipids, and sugar derivatives (Karagiannis et al., 2020; Li et al., 2021). It suggests that differences in mesophyll structure play a meaningful role in downstream biochemical composition. Pathway analysis also showed that 'Anna'-Bumiaji activated lipid remodelling and sugar turnover pathways in the promotion of growth, while the pathway for amino sugar and α-linolenic acid metabolism took over how to deal with abiotic stress adaptation. It seems that linoleic and α-linolenic acids serve critical roles by maintaining membrane fluidity under temperature fluctuations characteristic of tropical highlands (Jia et al., 2017; Shen et al., 2021). In contrast, 'Manalagi'-Bumiaji appeared to be more metabolically coordinated (broad-based activation of both carbohydrate and lipid pathways), suggesting that when grown under cooler conditions, an improvement in metabolic integration contributed to reduced temperature stress, while 'Manalagi'-Tutur underwent a narrower scope of stress-related perturbation. This dissimilarity shows a noticeable difference: 'Anna' features strong metabolic plasticity and environmental sensitivity, whereas 'Manalagi' exhibits more stable, defence-associated biochemical profiles (Akši´c et al., 2022; Chen et al., 2023). Altogether, PCA with heatmap results suggest environment-induced convergence among cultivars at Tutur (exhibiting metabolite upregulation under cooler, higher-elevation conditions) and cultivar-based divergence in metabolic approaches for Anna, boosting lipid/terpenoid pathways, versus Manalagi, which involves enhanced antioxidant/phenolic metabolism. This metabolic integration enables 'Manalagi' to strengthen cellular architecture that protect macromolecules and maintain turgor pressure under water stress conditions at lower temperatures and higher humidity levels in Bumiaji. These results

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strengthen the contribution of microclimate to regulating leaf biochemical pathways and underscore the different physiological adaptations of Anna and Manalagi across contrasting highland locations.

Transcriptomic profiles of key MADS-box genes also supported the structural and metabolic bases of reproductive development. 'Manalagi' exhibited marked early induction of AP1 and AP3 at full bloom, suggesting a closely orchestrated flowering program and an environment-independent stable fruit set (Kumar et al., 2016; Urrestarazu et al., 2017). By contrast, 'Anna's' expression of SOC1, AGL15, and SEP was delayed and variable, particularly under warmer and brighter Tutur conditions, indicating a greater dependence on environmental signals for the control of developmental time. Up-regulated AGL15 transcripts in 'Anna'-Tutur—a known floral repressor—are probably responsible for these developmental delays (Chen et al., 2018; Joshi et al., 2022). TM8 expression was consistently maintained across different cultivars (Kagaya et al., 2020), supporting its conserved function in fruit ripening, while the gradual up-regulation of SEP expression during fruit ripening is consistent with its well-established roles in organ identity and later developmental processes (Li et al., 2022). These transcriptional patterns indicate that 'Manalagi' has a genetically stable regulation of flowering and ripening. In contrast, the plastic responses are significant for the expression of these genes in 'Anna', resulting from the effects of desirability or environmental instability. It seems that this would be more evident in 'Manalagi', in which transcriptional activation at the mature stage may be associated with fruit quality improvement by better development and size under stress (McAtee et al., 2015). Such flexible molecular timing likely enables a highly sophisticated blend of developmental cues and environmental signals necessary for maximizing reproductive success in variable tropical highlands.

The interaction among anatomical, metabolomic, and transcriptional responses accounts for the opposite fruit-quality characteristics in both cultivars. In 'Anna', thicker mesophyll and better-developed light-intercepting capabilities provide a complement to metabolite enrichment in membrane lipids and carbohydrate intermediates that underpin active growth metabolism (Théroux-Rancourt et al., 2023; Bechynska et al., 2024). This results in a hard texture, increased acidity, and a darker red colour, all of which are supported by Myeloblastosis (MYB) regulation of anthocyanin biosynthesis under varying light and temperature conditions (Karagiannis et al., 2020; Migicovsky et al., 2021). On the other hand, 'Manalagi' took advantage of a conservative leaf structure and a metabolome rich in nitrogenous and antioxidant compounds (Li et al., 2021). Supporting constant sucrose accumulation, increased vitamin C content, and more stable yellow–green skin pigmentation, notably when exposed to cooler Bumiaji climate. It has been proposed that these compatible solutes play a role in plant tolerance by stabilizing proteins and membranes, and as osmolytes (Dhyani et al., 2018; Monago-Maraña et al., 2021; Kumar et al., 2023). Following, 'Anna' also developed greater acidity and firmness, which are indicative of differences in the ratio of cell wall components and organic acids that affect flavour and postharvest life (Zhao and Wang, 2015). The robust *AP1*/*AP3* induction in 'Manalagi' is associated with firm flowering and added to the stable fruit sweetness, whereas the more environmentally connected *SOC1*/*AGL15* control of 'Anna' results in raised acidity and stiffness (Urrestarazu et al., 2017; Li et al., 2022).

Further, the higher temperatures and light intensities in Tutur may partly explain the selective induction of heat-stress- and light-responsive transcription factor genes. Nevertheless, this flexibility comes at the expense of higher transcriptional noise as indicative for oscillation-activities of central flowering and fruit development genes (*AP1, SOC1*, *AGL15*) parallel to *SEP* and *TM8* expression during fruity maturation supporting Anna's transgressing firmness and acidity peaks also points towards Anna's susceptibility toward sensual input from the environment or postharvest instability (Paudel et al., 2019; Kagaya et al., 2020). Moreover, genes involved in different signalling pathways related to stress response and fruit development and ripening were also found to be induced by temperature variations. This has a significant impact on fruit taste and nutrient content, as well as on the synthesis and accumulation of specific metabolites, including organic acids, volatiles, antioxidants, and total sugar content (Liu et al., 2020; Geană et al., 2021; Seyednami et al., 2023). Similar to 'Manalagi', the high levels of transcription of early-flowering genes (*AP1*, *AP3*, *SOC1*), which were also directly activated by these floral identity genes, contributed to uniform flower and fruiting formation.

Our results indicate that metabolic and transcriptional responses work together as integrated networks. The Anna genotype appears to be metabolically more versatile, as it accumulates in Bumiaji terpenoids and steroid biosynthesis pathways, as well as in nitrogen metabolism. The activation of the alpha-linolenic pathway in Tutur, however, suggests that nutrient supply can interfere with the preference from growth-state to stress-state in response. Moreover, these lipids can activate mitogen-activated protein kinase (MAPK) pathways at the membrane level, which then regulate gene expression including certain MADS-box transcription factors, in response to temperature fluctuations (Jia et al., 2017; Kumar et al., 2023; Chen et al., 2023). The metabolic and molecular malleability driving Anna's phenotype indicates a strong, fit state in optimal conditions. On the contrary, 'Manalagi' and 'Anna' have a stable and balanced secondary metabolite profile at various sites, though showing a similar response of the upregulation of biosynthesis of unsaturated fatty acid (alkene), which makes the species more adaptive (Shen et al., 2021). This finding is consistent with that of Tang et al. (2022), who suggested that secondary metabolite accumulation is responsible for the plant's high-altitudinal adaptation.

In practical terms, these results are of great value for breeding, orchard management, and climate adaptation. Genotypes such as 'Manalagi', which are structurally and metabolically stable, offer strong prospects as genetic material for breeding programs aimed at improving stress tolerance and stable fruit quality in a warmer tropical highland environment. The high plasticity of 'Anna' could, on the other hand, provide an opportunity to breed targeted varieties with moderate tolerance to stress or adaptation for cultivation in microenvironments with reduced heat and irradiance. Differential expression emergence points to the evolution of more refined control over flowering and fruit set to stabilize systems in the face of projected climate change in highland apple production systems that are responsive, more regenerative, and adaptive. Nevertheless, in this study, the metabolomic were generated using qualitative rather than quantitative methods; as a result, the GC–MS data reflect relative metabolite patterns rather than absolute quantitative differences. In addition, the qPCR profiles represent trend-level gene expression rather than statistically validated fold-change estimates. These exploratory datasets are valuable for identifying major pathway shifts and for generating hypotheses in tropical highland apple systems. Therefore, future studies should incorporate quantitative metabolomics and qPCR or RNA-seq workflows to strengthen the reproducibility and statistical robustness of multi-omics integration under contrasting environments. Such improvements will enhance confidence in the biological interpretation and promote stronger trait–marker associations for breeding and management applications.

Conclusion

This study shows that different apple cultivars respond differently to tropical highland environments, with `Anna' displaying substantial anatomical and metabolic plasticity, shown by the activation of lipid-remodeling, amino sugar, α-linolenic acid pathways, and environment-dependent expression of *SOC1*, *AGL15*, *SEP*, allowing for fast growth but increased susceptibility to heat and irradiance. In contrast, 'Manalagi' possesses a conservative leaf architecture and is characterized by an enriched nitrate-rich and antioxidant-driven metabolome, with slight variation in early induction of *AP1* and *AP3* or in floral gene regulation, and more uniform fruit sweetness and nutritional quality across sites. These characteristic metabolomic and transcriptomic fingerprints situate the cultivars along a stability–plasticity gradient and reveal pivotal markers (e.g., mesophyll thicknesses) and many antioxidant metabolites, as well as MADS-box expression profiles for cultivar improvement. This study has practical implications for breeding, spotlighting the importance of 'Manalagi'- type resilience traits in the development of climate-ready apples and the need to improve stress tolerance in plastic cultivars like 'Anna'.

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Ethical Approval Statement

We ensure that all research conducted respects participant confidentiality, follows ethical guidelines, and contributes to the academic community with honesty and integrity.

Use of Generative AI Tools Statement

During the preparation of this work, the author(s) used QuillBot, Chat GPT-4, and Claude-3.5sonnet to check grammar and paraphrase. After using this tool/service, the author(s) reviewed and edited the content as needed and take full responsibility for the publication's content.

Contribution of Authors

Hardiyanto & Devy NF: Conceptualization, data curation, methodology, investigation, formal analysis, software, funding acquisition, writing, review and editing.

Yulianti F, Sugiyatno A, Triwiratno A, Dwiastuti ME, Istianto M, Sutanto A & Sukartini: Data curation, investigation, formal analysis, writing, review and editing.

All authors read and approved the final draft of manuscript.

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