



Metabolomic and transcriptomic insights into nitrogen-responsive flavonoid biosynthesis in red lettuce varieties

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ABSTRACT

Nitrogen (N) is an important macronutrient for plant growth and development, significantly influences nitrogen use efficiency (NUE) and modulates metabolic pathways when applied at varying concentrations. Despite its importance, the mechanistic effects of N on biomass accumulation and polyphenolic biosynthesis in two distinct red lettuce cultivars (*Lactuca sativa* L. cv. H20K7044 and H20K3447) under differential N concentrations remain unexplored. This study employed integrated metabolomics and transcriptomic analyses to elucidate these dynamics. This study used three different N treatments including high_N (10 mmol L⁻¹), low_N (2.5 mmol L⁻¹) and normal_N (5 mmol L⁻¹) concentrations. Elevated N supply (10 mmol L⁻¹) enhanced growth parameters in H20K7044, including chlorophyll *a*, *b*, total chlorophyll contents and fresh/dry biomass, whereas H20K3447 exhibited a higher nitrogen balance index (NBI) under high N conditions. Intriguingly, H20K7044 showed high relative flavonol contents under low N treatment. By mapping polyphenolic metabolic pathways, we identified biosynthetic modification reactions through a multi-omics framework, correlating transcriptomic profiles with metabolite networks across N treatments. Comparative analysis revealed cultivar-specific divergence in secondary metabolism regulation. Key structural genes in flavonoid biosynthesis: *PAL* (phenylalanine ammonia lyase), *CHI* (chalcone isomerase), *ANS* (anthocyanidin synthase), *DFR* (dihydroflavonol 4 reductase), *FLS* (flavonol synthase), and *F3'H* (flavonoid 3'-hydroxylase) were significantly upregulated under low N conditions in both red lettuce cultivars. Metabolomic profiling highlighted differential accumulation of polyphenolic derivatives, including kaempferol 4'-glucoside, quercetin 3-O-(6"-acetyl-glucoside), cyanidin-3-O-glucuronide, and epicatechin-3-O-gallate, which varied with N availability and genotypic background. This study establishes a novel framework for interrogating N-mediated regulation of polyphenolic biosynthesis, linking transcriptional activity to metabolic flux in red lettuce cultivars. The findings underscore the utility of multi-omics integration in dissecting genotype-environment interactions, offering actionable insights for optimizing N fertilization strategies to enhance phytochemical richness and nutritional quality in lettuce cultivation. Such advances hold promises for precision agriculture and enabling tailored nutrient management to improve crop resilience and values.

1. Introduction

In plant phenotype, plants show substantial morphological characteristics including leaf and stem architectures; major variability has been

reported across horticultural crop by variation in color and shapes [1–3]. Lettuce (*Lactuca sativa* L.) is a member of the Asteraceae family, is a globally cultivated annual leafy vegetable, predominantly consumed raw in salads. Mostly lettuce cultivars exhibit in green color, however,

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anthocyanin-rich red-leaf varieties have gained attention for their enhanced phytochemical profiles and consumers' acceptability [4]. Lettuce contains different types of bioactive metabolites including flavonoids and phenolic acids, which are also potentially associated with many health-promoting properties including anti-inflammatory and anti-cardiovascular diseases in humans [5,6]. These metabolites serve dual roles in plant physiology: primary metabolites sustain growth and development, while secondary metabolites mediate adaptive responses to abiotic stresses, including nutrients, temperature, salinity, drought and heavy metals etc [7].

In nutrients, nitrogen (N) is an important macronutrient, profoundly influences lettuce yield and phytochemical composition [5,8]. Intensive N fertilization is widely practiced to meet global food demands, yet excessive or deficient application disrupts metabolic homeostasis, impairing photosynthesis, biomass accumulation, and secondary metabolite biosynthesis [9–11]. Optimal N management enhances nitrogen use efficiency (NUE), balancing yield with nutritional quality [12]. Nitrogen modulates amino acid metabolism, directly impacting protein synthesis and indirectly regulating secondary metabolic pathways. For instance, N deficiency elevates phenolic compounds in *Cyclocarya paliurus* and anthocyanins in *Vitis vinifera* [11,13], whereas excess N suppresses flavonoid biosynthesis in *Brassica juncea* and *Calendula officinalis* L. [14–16]. Similarly, N applications affect secondary metabolites including phenolic acids and total phenolic contents in lettuce [17–19]. The N applications on lettuce for flavonoid biosynthesis metabolism is not fully explored. So, this study will help to understand the polyphenolic biosynthesis mechanism in red lettuce varieties under different N application levels. This study will also give insights on biomass accumulation and flavonoid biosynthesis response under different N applications in red lettuce varieties.

Recent advances in multi-omics technologies (e.g., metabolomics and transcriptomics) have enabled systematic exploration of genotype-phenotype interactions under environmental stress [20,21]. However, the genetic and molecular mechanisms governing metabolic regulation in lettuce under N imbalance remain poorly understand. However,

transcriptomics identifies stress-responsive genes (e.g., MYB transcription factors and phenylpropanoid pathway enzymes etc.) and metabolomics reveal dynamic shifts in specialized metabolites in *Axonopus compressus* and wheat [22,23]. Integrative analysis of these datasets can elucidate regulatory networks linking N availability to flavonoid biosynthesis, as demonstrated in *Arabidopsis thaliana*, wheat and *Oryza sativa* [22–26].

This study employs a multi-omics approach to investigate the regulatory mechanisms underlying polyphenolic biosynthesis in genetically diverse lettuce cultivars under different N concentrations. By correlating transcriptomic profiles with metabolomic signatures, we aim to identify key genes (e.g., CHS, DFR, ANS) and metabolic hubs modulated by N availability. Findings will inform precision agriculture strategies for optimizing N fertilization and guide the development of nutrient-efficient and phytochemically enriched red lettuce varieties.

2. Material and methods

2.1. Plant materials

Two red lettuce varieties (H20K7044 and H20K3447) (Fig. 1) were cultivated at School of Agriculture and Biology, Shanghai Jiao Tong University to check the metabolomic and transcriptomic changes in red lettuce cultivars under different N applications. Seeds were provided by Shanghai Agro-biological Gene Center, Shanghai, China. Lettuce seeds were grown in trays for seedlings and after 2 weeks, lettuce seedlings were transferred at two leaves stage in the hydroponic system for N applications. Lettuce was grown in growth chamber from May to July. Following growing conditions were remained for whole growing season in growth chamber: 24–18 °C with 12 h in light and 12 h in dark with 60 % relative humidity and light intensity ranging from 180 to 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Three different N treatments 5 mmol L^{-1} (normal_N), 10 mmol L^{-1} (high_N) and 2.5 mmol L^{-1} (low_N) were applied after 3 days of transplanting. 5 mmol L^{-1} (normal_N) was considered as normal and optimum concentration for lettuce production as previously reported

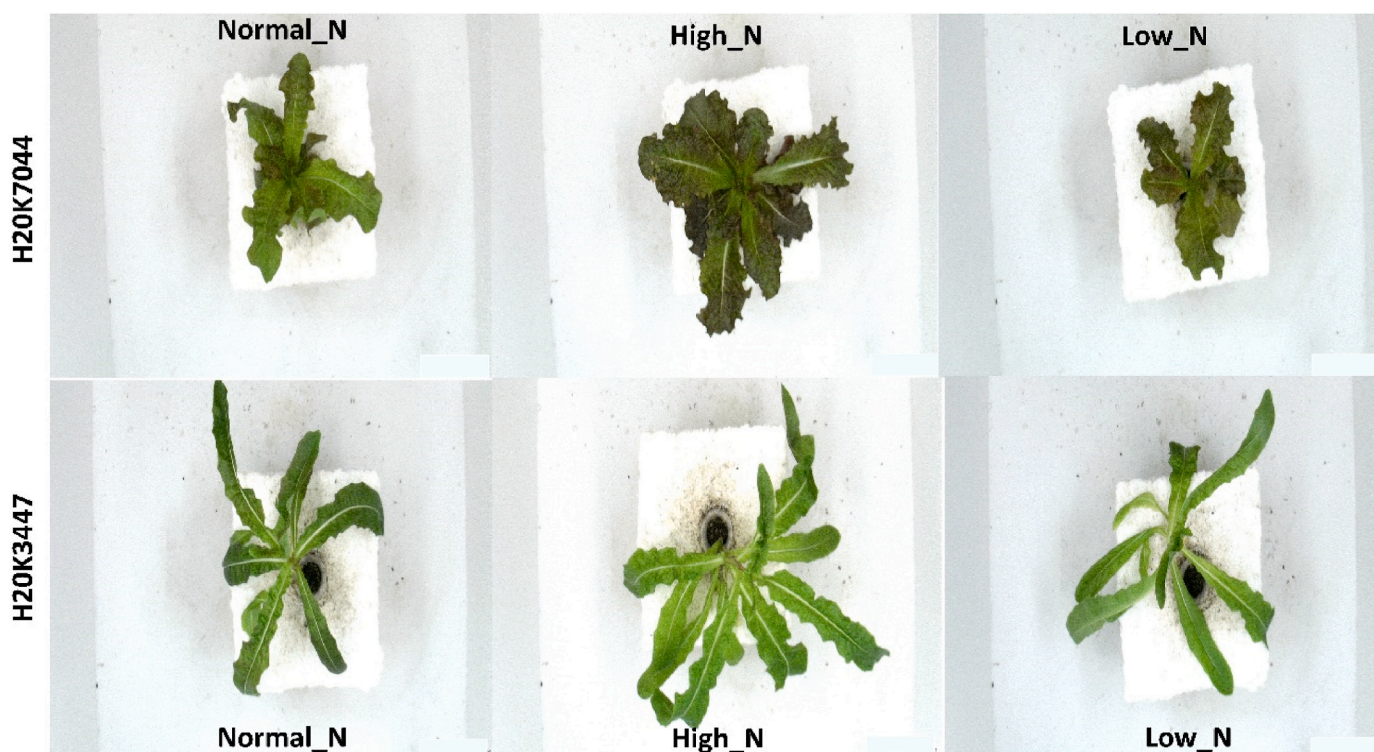


Fig. 1. Two red lettuce varieties (H20K7044 and H20K3447) under different nitrogen applications (Normal_N = 5 mmol L^{-1} , High_N = 10 mmol L^{-1} and Low_N = 2.5 mmol L^{-1}).

[27]. The application of N was applied at the ratio of 25 % ammonium (NH_4) and 75 % for nitrate (NO_3). Ammonium sulphate and calcium nitrate were used for making NH_4 and NO_3 ratio. Other nutrients were applied according to Hoagland's solution as previously reported study [10] and maintained the pH 5.5–6.5 and EC 2 dS/m of Hoagland's solution during the whole lettuce growing season. The nutrition solution was renewed every two days with proper aeration in hydroponic cultivation system. Lettuce plants were harvested after 28 days and measured the physiological and morphological data of plant (Fig. 2).

2.2. Physiological and growth parameters

After harvesting, the plant fresh weight was measured by weighing

balance. For plant dry weight, the plant samples were placed in oven at 65 °C for constant weight and measured the dry weight by weighing balance.

Chlorophyll and carotenoids contents were determined as described by Wellburn and Lichtenthaler [28]. Briefly, 0.1 g of fresh plant leaves were taken into 10 mL centrifuge tubes. Add 8 mL of 95 % ethanol and put it in dark place for 24–48 h. Later, the supernatant (200 μL) of samples was collected and transferred into 96-well plate for further analysis. Tecan Infinite 200 Pro Microplate Reader (Männedorf, Switzerland) was used for determination of chlorophyll *a*, *b* and carotenoids at 665 nm, 649 nm and 470 nm absorbance. Following formulas were used for determination:

$$\text{Chlorophyll } a \text{ (mg L}^{-1}\text{)} = 13.95A_{665} - 6.88A_{649}$$

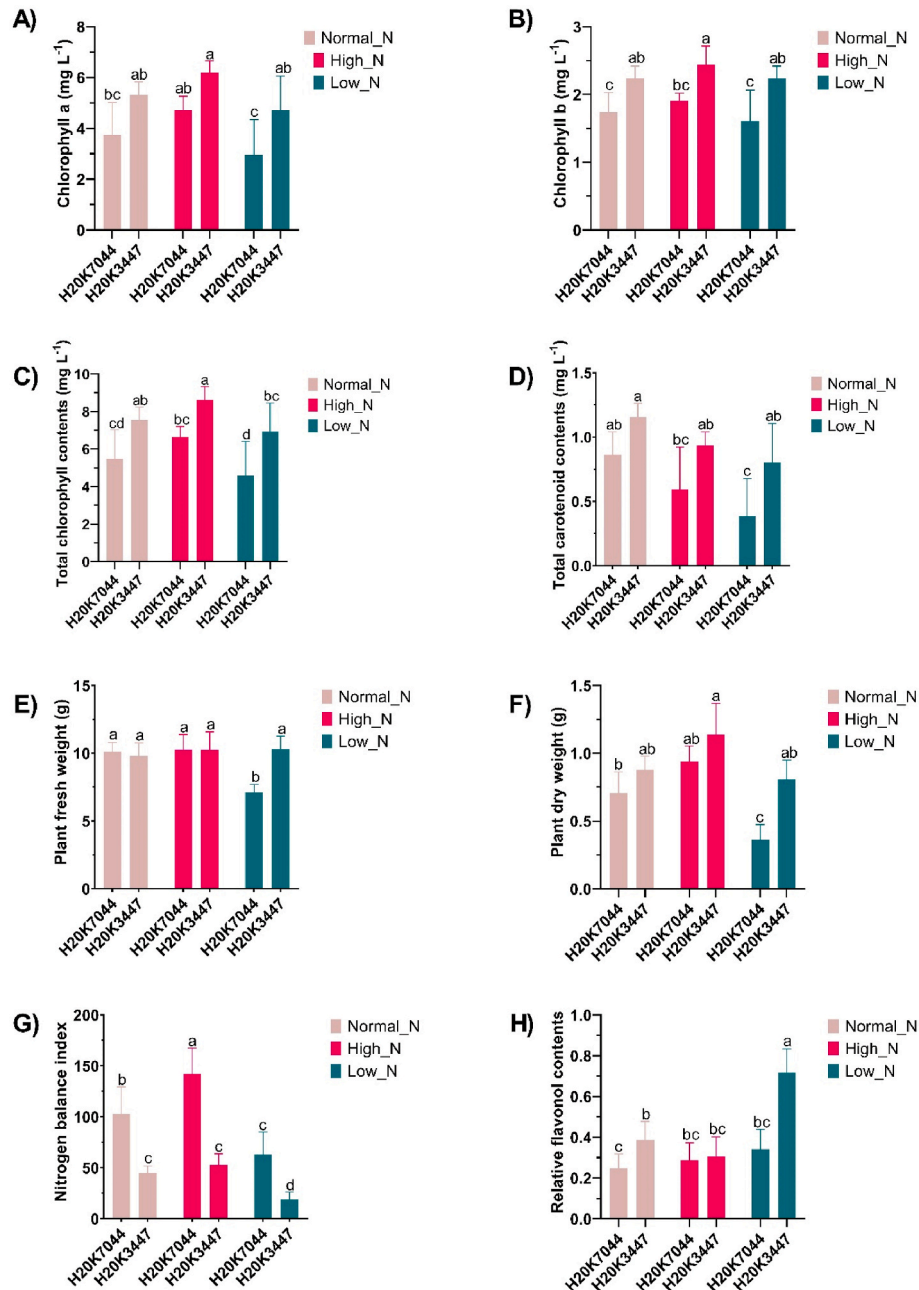


Fig. 2. Physiological and growth parameters: (a) chlorophyll *a*, (b) chlorophyll *b*, (c) total chlorophyll contents, (d) total carotenoid contents, (e) plant fresh weight, (f) plant dry weight, (g) nitrogen balance index and (h) relative flavonol contents, under different N applications in H20K7044 and H20K3447 red lettuce varieties. Means $\pm$ standard deviations are presented and different letters show a significant difference ($p < 0.05$). Treatments were followed as: Normal_N = 5 mmol L⁻¹, High_N = 10 mmol L⁻¹ and Low_N = 2.5 mmol L⁻¹.

$$\text{Chlorophyll b (mg L}^{-1}\text{)} = 24.96A_{649} - 7.32A_{665}$$

$$\text{Total chlorophyll contents (mg L}^{-1}\text{)} = \text{chlorophyll a} + \text{chlorophyll b}$$

$$\text{Carotenoid Contents (mg L}^{-1}\text{)} = (1000 \times A_{470} - 2.05 \times \text{chlorophyll a} - 114.8 \times \text{chlorophyll b}) / 245.$$

Dualux 4 Scientific™ (FORCE-A, Orsay, France) was used to measure the nitrogen balance index (NBI) and relative flavonol contents in red lettuce varieties [29]. Readings were taken from 4 different leaves of one plant in one replication and repeated this process for 4 replications in one treatment.

2.3. Metabolite profiling

Frozen liquid N lettuce plant samples (300 mg) were used for metabolomic analysis by mixing water to methanol (20:80 v/v) in 1 mL. After mixing, 30 min sonication was performed at 25 °C and later placed the plant samples at 4 °C for 12 h. The plant samples centrifuged at 12000 g for 15 min and supernatant of plant samples (0.5 mL) used for metabolomic analysis through ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC-MS/MS) [3].

For metabolomic analysis, the Acquity UPLC-MS/MS (Waters Corp., Milford, MA, USA) used. The sample separation by using water to acetonitrile solution (0.4 mL min⁻¹) at 40 °C with 100 % gradient elution for 0–3 min. The 90–100 % with water as linear gradient for 3–5 min. Similarly, 65–90 % for 5–11.5 min, 1–65 % for 11.5–14 min, 1 % for 14–17 min and 1–100 % for 17–17.1 min were used [10]. The preparation of the QC samples involved combining all of the samples and injecting them before and after every eight samples. In each sample, 5 µL of internal standards were added. Using the inbuilt software of the instruments, peak alignment, deconvolution, denoising, baseline correction was performed on the raw LC/MS data.

The precise mass and MS² fragment data compared with bibliographies, internal databases, and internet reference sources (HMDB, Lipidmaps, Metlin and ReSpect) for metabolite identification. At least two major fragments were found, and the MS and MS² limits were established at 3 mDa. The instrument's integrated software produced and outputted a data matrix that included the chemicals' names and their raw abundances with four replications of each sample.

2.4. Extraction and sequencing of RNA in lettuce

The total RNA of lettuce leaves was extracted using TRIzol reagent kits (Invitrogen, Carlsbad, CA, USA). Lettuce sample (100 mg) fine powder was taken and transferred to 1 mL of TRIzol reagent and vigorously vortex the samples until homogeneous solution formed. Added 0.2 mL of chloroform per 1 mL of TRIzol reagent and shaken for 15–30 s for thoroughly mix of samples and later incubated the mixture at room temperature for 2–3 min. Later, centrifuged at 12000 g for 15 min at 4 °C. After centrifugation, separate the RNA containing colorless aqueous phase and transferred to a new tube. For isolation of RNA, added an equal volume of 100 % isopropanol to the aqueous phase and mixed it and placed at room temperature for 10 min. After that centrifuged again at 12000 g for 10 min at 4 °C. The RNA formed a gel-like pellet at the bottom of the tube. Carefully discarded the supernatant and washed the RNA pellet by adding 1 mL of 75 % ethanol per 1 mL of TRIzol reagent which used initially for lysis. Vortex the sample briefly and centrifuged at 7500 g for 5 min at 4 °C. Discarded the ethanol wash and let the pellet air dry for 5–10 min and finally dissolved the pure RNA pellet in 20–50 µL of RNase free water, 0.1 mM EDTA or 0.5 % SDS solution by gently pipetting up and down. Incubated the tube in a water bath or heat block set at 55–60 °C for 5–10 min to aid dissolution and later stored the RNA at –70 °C. Diluted sample in RNase free water, then measured absorbance at 260 nm and 280 nm and calculated the RNA concentration using the formula: A₂₆₀ x dilution x 40 = µg RNA/mL. Calculated the A₂₆₀/A₂₈₀ ratio. A ratio of ~2 is considered pure. An

Agilent 2100 Bioanalyzer was used to analyze the integrity and quality of total RNA in each sample. The Illumina TruSeq™ RNA sample preparation kit (Illumina, USA) was used to prepare the RNA sequence transcriptomic library of each sample. The library was used to the Illumina HiSeq (Illumina NovaSeq 6000) platform to get the paired end 2x125 bp reads length of each samples (Majorbio, China). The expression level of transcripts in each sample was determined using the fragments per kilobase of exon per million (FPKM) mapped read technique. Each sample contained more than 46,407,124 bp reads. From raw datasets, removed the poor quality and adaptor sequences reads and kept the clean datasets by fastp with Q20, Q30, and GC contents for additional downstream studies (Supplementary data table 1). The clean reads were aligned to the lettuce reference genome source (<http://genomevolution.org/coge/GenomeInfo.pl?gid=37106>) (version v8) in orientation mode via HISAT2 software. The clean reads of each sample were sequenced with the specified reference genome via StringTie, and the alignment rate ranged from 94.04 % to 95.24 %. A total of 101299 expressed genes were detected in this analysis, including 96511 known genes and 4788 new genes. There are 125893 expression transcripts, including 95470 known transcripts and 30423 new transcripts. Functional database annotation analysis (NR, Swiss-prot, Pfam, COG, GO and KEGG) was performed for the genes and transcripts expressed at this time (Supplementary data table 2). Based on the quantitative results of expression, the differential gene analysis between groups was carried out to obtain the differentially expressed genes between the two groups, the difference analysis software was: DESeq2, and the screening threshold was $|\log_2\text{FC}| \geq 1$ and p value < 0.05 by Bonferroni's method. The sample identity was designated based on the RNA sequencing data with multiple and unique mapped (Supplementary data table 3). Significantly expressed genes were screened in each applied treatments and in each cultivar using the parameter of an absolute log₂ ratio of 2 and a false discovery rate with significant score less than 0.05. Additionally, significantly expressed genes were submitted to the KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>) and Goatools (<https://github.com/tanghaibao/Goatools>) for Kyoto Encyclopedia of genes and Genomes (KEGG) and GO (Gene Ontology) functional enrichment pathway analysis.

2.5. Quantitative real time PCR (qRT-PCR) analysis

The expression levels of 13 vital genes in lettuce were determined via qRT-RNA analysis to confirm the reliability of sequencing-based transcriptome profiling. The forward and reverse primers were constructed by using Primer 5.0 (Premier Bio-soft International, CA, USA) (Supplementary data table 4). For internal reference gene, actin was used. The quantitative reverse transcription PCR (qRT-PCR) was performed using the Perfect-Start Green qPCR SuperMix (Trans-Gen Biotech, China) in a 20 µL reaction volume on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). The thermal cycling protocol consisted of an initial denaturation at 95 °C for 3 min, followed by 33 cycles of 95 °C for 10 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 10 s (elongation). Following amplification, a melt curve analysis was conducted to verify amplification specificity. Gene expression levels were calculated using the comparative cycle threshold (2^{−ΔΔCT}) method [30].

In order to identify the polyphenolic biosynthesis pathways by integration of metabolomic and transcriptomic analysis, all differentially expressed metabolites and genes in all applied N treatments in H20K7044 and H20K3447 red lettuce cultivars were concurrently mapped to the KEGG pathway database. To assess the differentially expressed metabolites and genes shared statistical enrichment KEGG pathways, we utilized the cluster Profiler R package in R software (v 3.4.3) and Python (v 3.5.0). With a p value < 0.05, the combined analysis of the metabolomic and transcriptomic data was combined and developed flavonoid biosynthesis pathways.

2.6. Statistics analysis

The SPSS Statistics 26.0 software (SPSS, Inc., Chicago, IL, USA) was used for two-way analysis of variance (ANOVA) in H20K7044 and H20K3447 red lettuce varieties under different N applications. The Tukey's HSD test ($p < 0.05$) was used for significant difference in applied N application and between the red lettuce varieties. Multivariate analysis (PCA and OPLS-DA) was used for determining the contrast under applied N applications in both red lettuce varieties by using SIMCA-14.1 (Umetrics, Umeå, Sweden). The heatmaps and Venn diagrams were produced with R software (v 3.4.3) and metaboAnalyst 5.0 (www.metaboanalyst.ca).

3. Results

3.1. Physiological and growth parameters

High nitrogen (high_N) application increased the most of growth parameters and physiological parameters in H20K7044 and H20K3447 red lettuce varieties (Fig. 2A–H). In physiological parameters, high_N increased chlorophyll *a* (6.19 and 4.72 mg L⁻¹), *b* (2.43 and 1.90 mg L⁻¹) and total chlorophyll contents (8.63 and 6.63 mg L⁻¹) (Fig. 2A–C) followed by normal nitrogen (normal_N) application and minimum chlorophyll *a* (4.70 and 2.96 mg L⁻¹), *b* (2.23 and 1.61 mg L⁻¹) and total chlorophyll contents (6.94 and 4.57 mg L⁻¹) were found under low nitrogen (low_N) application in both red lettuce varieties (H20K7044 and H20K3447) respectively. Total carotenoid contents (1.15 and 0.86 mg L⁻¹) increased with normal nitrogen (normal_N) in both red lettuce varieties (H20K7044 and H20K3447) respectively (Fig. 2D). Overall in variety comparison, H20K3447 showed significant difference as compared to H20K7044 for physiological parameters (chlorophyll *a*, *b*, total chlorophyll contents and total carotenoid contents).

Similarly, shoot fresh weight (11.50 g and 11.15 g) (Fig. 2E) and dry weight (0.94 g and 1.13 g) (Fig. 2F) of both red lettuce varieties (H20K7044 and H20K3447) increased with high_N application, respectively. The highest fresh weight found by high_N application followed by normal_N in both red lettuce varieties (H20K7044 and H20K3447) (Fig. 2E), while lettuce fresh weight did not show significant difference with high_N and normal_N in both H20K7044 and H20K3447 red lettuce varieties but high_N showed significant difference in fresh weight as compared to low_N application in H20K7044 red lettuce variety (Fig. 2E). Although, lettuce dry weight showed significant difference with high_N application in H20K3447 followed by H20K7044 as compared to other applied nitrogen applications in both red lettuce varieties (Fig. 2F). Lowest dry weight found in H20K7044 red lettuce variety. Overall in variety comparison, H20K3447 showed significant difference in fresh and dry weight as compared to H20K7044 red lettuce variety.

Nitrogen balanced index (NBI) increased with high_N application (142.16 and 52.54) followed by normal_N (102.66 and 44.82) in H20K7044 and H20K3447 red lettuce varieties, respectively (Fig. 2G). Lowest NBI found under low_N application in both red lettuce varieties. Similar trend was found in N application in both red lettuce varieties. However, NBI found higher in H20K7044 as compared to H20K3447 red lettuce variety.

However, relative flavonol contents increased by low_N application (0.71) in H20K3447 red lettuce variety as compared to normal_N and high_N applications (0.38 and 0.30), respectively (Fig. 2H). Similar trend was found for relative flavonol contents by low_N application (0.34) but followed by high_N (0.28) and normal_N (0.24) in H20K7044 red lettuce variety, respectively. The highest relative flavonol contents found overall in low_N application followed by normal_N and high_N applications in both red lettuce varieties. Overall in variety comparison, H20K3447 showed significant difference in relative flavonol contents as compared to H20K7044 red lettuce variety.

The results showed that physiological and growth parameters

significantly increased in H20K3447 as compared to H20K7044 red lettuce variety. Application of high_N increased all physiological and growth parameters which demonstrated that increasing N concentration could have beneficial effects on the plant growth and yield in both red lettuce varieties.

3.2. Metabolomic profiling in lettuce

The untargeted metabolomic profiling of different two red lettuce varieties (H20K7044 and H20K3447) were investigated in leaves through UPLC-MS/MS under normal, high and low_N applications. In this study, total 1352 metabolites, including amino acids, carbohydrates, organic acids, phenolic compounds, flavonoids, alkaloids, lipids, terpenoids, lignan, coumarins and tannins were putatively annotated by comparing the *m/z* and MS² with inhouse built database and online databases HMDB, Lipidmaps, Metlin and ReSpec. Tentative identified metabolites in H20K7044 and H20K3447 red lettuce varieties under different N applications were presented (Supplementary data table 5). The relative contents of all annotated metabolites were measured using the instrument's inbuilt software and studied further through multivariate analysis. A robust PCA (Supplementary Fig. 1A and 1C) and OPLS-DA analyses (Supplementary Fig. 1B and 1D) were performed and clear separation was found among all applied N treatments between H20K7044 and H20K3447 red lettuce varieties, respectively. Additionally, clear separation showed a significant difference among all comparisons of different applied N treatments in H20K7044 (Supplementary Fig. 2A–F) and H20K3447 (Supplementary Fig. 3A–F) and also showed a significant difference in metabolites of both (H20K7044 and H20K3447) red lettuce varieties (Fig. 3A–D).

A robust PCA (Fig. 3C) and OPLS-DA analyses (Fig. 3D) were performed on two red (H20K7044 and H20K3447) lettuce varieties irrespective of applied N treatments. For this analysis, total 24 samples were used for PCA and OPLS-DA analysis in SIMCA-14.1 software. The results showed that differential metabolites were found in PCA and OPLS-DA analyses in both red (H20K7044 and H20K3447) lettuce varieties. In PCA analysis, R2X (0.536 %) and Q2 (0.404 %) variation was found (Fig. 3A) while OPLS-DA analysis R2X (0.744 %), R2Y (0.907 %) and Q2 (0.42 %) (Fig. 3B) variation was found for comparisons of different N applications in H20K7044 and H20K3447. However, R2X (0.663 %) and Q2 (0.529 %) variation in PCA analysis (Fig. 3C) for H20K7044 and H20K3447 red lettuce varieties comparison while R2x (0.575 %), R2Y (0.993 %) and Q2 (0.981 %) variation in OPLSDA analysis (Fig. 3D) for H20K7044 and H20K3447 red lettuce varieties comparison.

Similarly, for different comparisons of applied N treatments in H20K3447 showed upregulation (110) and down regulation (106) metabolites in High_N_vs_Low_N comparison, upregulation (595) and down regulation (391) metabolites in High_N_vs_Normal_N comparison and upregulation (297) and down regulation (220) metabolites in Low_N_vs_Normal_N comparison (Supplementary Table 6). This trend showed that all treatments showed significant difference in both H20K7044 and H20K3447 red lettuce varieties for metabolites variations. In H20K7044 red lettuce variety, the maximum metabolites variations were observed in High_N_vs_Normal_N comparison while minimum metabolites variations were observed in Low_N_vs_Normal_N comparison (Supplementary Table 6). In contrast, H20K3447 red lettuce variety showed minimum metabolites variations in High_N_vs_Low_N comparison and maximum metabolites variations were observed in High_N_vs_Normal_N comparison (Supplementary Table 6).

Candidate metabolites with VIP values > 1.0 , *p* values > 0.1 or -0.1 , and *p* (corr) > 0.5 or -0.5 were chosen for further investigation in order to find potential metabolites that help in differentiate among all applied N treatments in red (H20K7044 and H20K3447) lettuce varieties. Different comparisons of metabolites in H20K7044 and H20K3447 lettuce varieties showed differential up-regulated and down-regulated metabolites under different N applications.

Additionally, Venn diagram analysis showed that intersection of

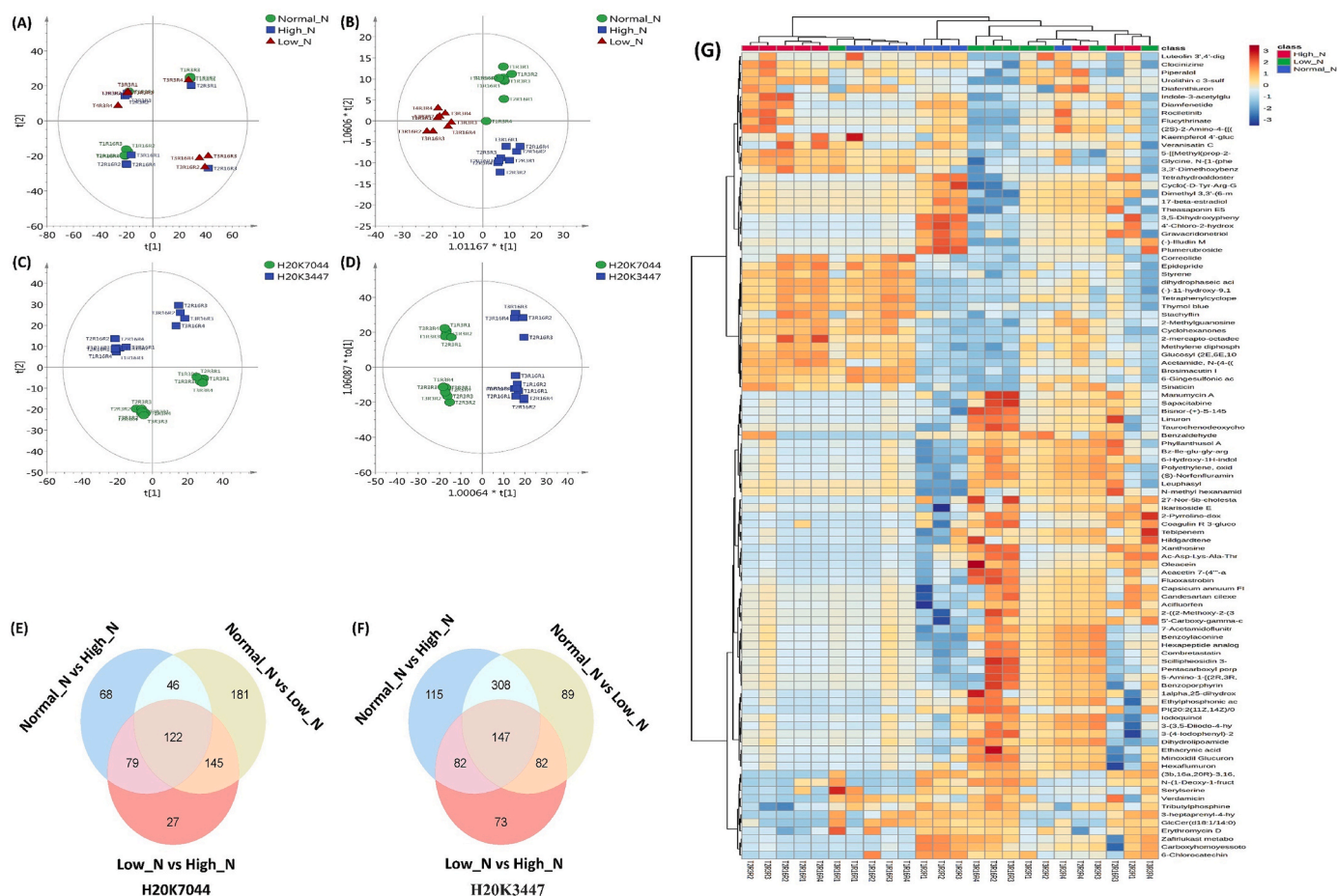


Fig. 3. (A and C) PC score plot of differential identified metabolites in H20K7044 and H20K3447 red lettuce varieties, respectively (B and D) OPLS-DA score plot of differential identified metabolites in H20K7044 and H20K3447 red lettuce varieties, respectively. In figure (3A-D), green color represents normal_N = 5 mmol L⁻¹, blue color represents high_N = 10 mmol L⁻¹ and red color represents low_N = 2.5 mmol L⁻¹, (E and F) Venn diagram represents comparison among applied different N treatments in H20K7044 and H20K3447 red lettuce varieties for metabolites variation, (G) Heatmap of differentially expressed metabolites among applied different N treatments in H20K7044 and H20K3447 red lettuce varieties. Variation of color in heatmap chart from red to blue represents highest to lowest p values of metabolites relative contents under different applied N treatments in H20K7044 and H20K3447 red lettuce varieties.

three paired comparisons of red (H20K7044 and H20K3447) lettuce varieties irrespective of applied N treatments (Fig. 3E and F). A total of 643 and 651 differential metabolites were identified from H20K7044 and H20K3447 lettuce varieties, respectively. In Venn diagram, 285 discriminatory metabolites were found in H20K7044 (Fig. 3E) and H20K3447 (Fig. 3F) red lettuce cultivars. These metabolites include amino acids, organic acid, flavonoids, isoflavonoids, phenolic compounds, lipids and terpenoids. The relative abundance of top 100 metabolites was shown in heat maps (Fig. 3G). Variation in heatmap color from red to blue represents more differentiation of metabolites in applied N treatments as well as in H20K7044 and H20K3447 red lettuce varieties (Fig. 3G).

In addition, low_N (2.5 mmol L⁻¹) application showed highest variation and upregulation of following metabolites: vitexin 6"-O-malonyl 2"-O-xyloside, quercetin-4'-glucuronide, linocinnamarin, piceatannol, isoferulic acid, linocinnamarin, galocatechin-3-gallate, cyanidin 7-glucoside, kaempferol 3-(2"-acetylramnoside), kaempferol 7-O-neohesperidoside, p-coumaric acid, isosakuranetin 7-xyloside, feruloyltartaric acid, isoferulic acid, piceatannol, isorhamnetin and pinoresinol-4'-O-β-D-glucoside, as compared to other high_N and normal_N application in H20K7044 red lettuce variety (Fig. 3G).

However, normal_N (5 mmol L⁻¹) showed significant difference and upregulation of following metabolites: p-coumaric acid glucuronide, epigallocatechin 3-cafeate, quercetin 3-O-(6"-acetyl-glucoside), rhamnetin 3-rhamnoside, taraxastane-3β,20β-diol, luteolin 3'-glucuronide,

gallic acid, chlorogenic acid, isoferulic acid, shikimic acid, chicoric acid, isochlorogenic acid C, as compared to high_N and low_N application in H20K7044 red lettuce variety (Fig. 3G).

Furthermore, high_N (10 mmol L⁻¹) showed significant difference and up-regulation of following metabolites: daidzin 4'-O-glucuronide, syringic acid glucuronide, methyl ferulate, secoisolariciresinol, sinapic acid, octyl gallate, lariciresinol, taxifolin 3-arabinoside, myricetin 3-arabinoside, vanillic acid 4-beta-D-glucoside and secoisolariciresinol 9,9'-diglucoside as compared to low_N and normal_N application in H20K7044 red lettuce variety (Fig. 3G).

In addition, there was no significant difference among following metabolites: quercetin 3-O-(6"-malonyl-glucoside) 7-O-glucoside, isolariciresinol 9-O-beta-D-glucoside, myricetin 3-(3',4"-diacetylramnoside), catechin 7-glucuronide, gomphrenol 3-methylether 4'-glucuronide and quercetin 3-(6"-malonyl-glucoside) in H20K7044 red lettuce variety under different N applications (Fig. 3G).

In H20K3447 red lettuce variety, high_N (10 mmol L⁻¹) showed upregulation in the following metabolites: gallic acid, isoferulic acid, quercetin, lariciresinol, galocatechin-3-gallate, syringic acid glucuronide, linocinnamarin, 2-hydroxybenzaldehyde O-[xylosyl-(1->6)-glucoside], (+)-7-epi-syringaresinol 4'-glucoside, myricetin, taxifolin 3-arabinoside as compared to low_N and normal_N application (Fig. 3G).

Meanwhile, low_N (2.5 mmol L⁻¹) showed upregulation in the following metabolites: isorhamnetin, secoisolariciresinol, daidzin 4'-O-glucuronide, luteolin 3'-glucuronide, p-coumaric acid, 4-

(butoxymethyl)-phenol, phenylalanine, glycitein 7-O-glucuronide, isopeonidin 3-xyloside, cis-resveratrol-3,4'-o-beta-diglucoside, 4-tert-butylcatechol, quercetin 3-O-(6''-acetyl-glucoside), quercetin-4'-glucuronide as compared to normal_N and high_N application in H20K3447 red lettuce variety (Fig. 3G).

Similarly, normal_N (5 mmol L⁻¹) also upregulated polyphenolic compounds in H20K3447 red lettuce variety, in which kaempferol 7-O-neohesperidoside, taraxinic acid glucosyl ester, 3-(4-Hydroxybenzoyl) epicatechin, 4-p-Coumaroyl-1,5-quinolactone, sinapic acid, D-linalool 3-glucoside, naringin 6''-rhamnoside, vanillin, phloretin 2'-O-

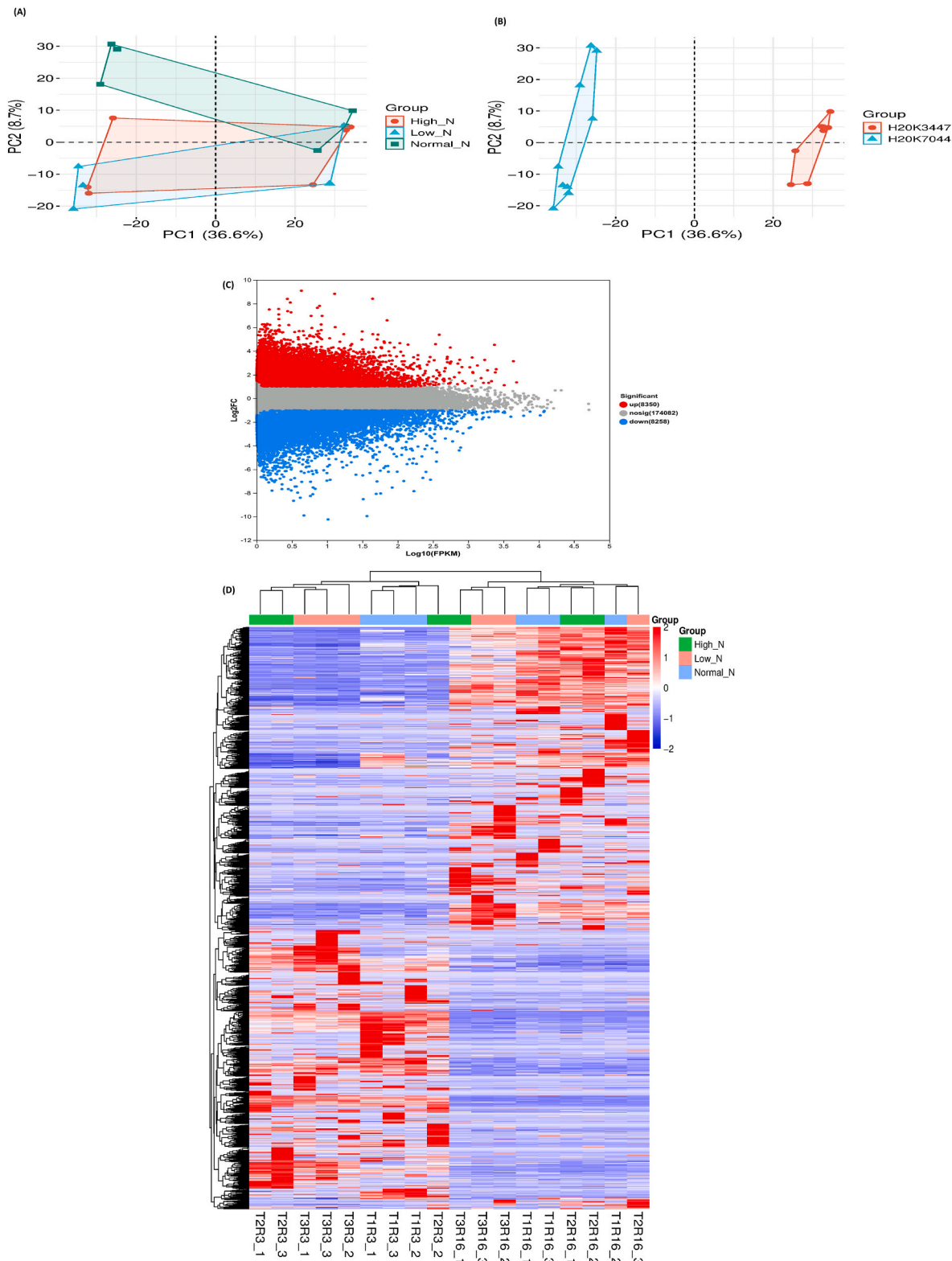


Fig. 4. (A) PC score plot of differentially identified genes under different N applications (B) PC score plot of differentially identified genes in two red lettuce varieties (H20K7044 and H20K3447). (C) Up- and down-regulated genes (D) Heatmap of differentially expressed genes in red lettuce varieties (H20K7044 and H20K3447) under Normal_N = 5 mmol L⁻¹, High_N = 10 mmol L⁻¹, and Low_N = 2.5 mmol L⁻¹ conditions.

glucuronide, pinoreosin-4'-O- β -D-glucoside, isolaricresinol 9-O- β -D-glucoside, secoisolaricresinol 9,9'-diglucoside, tetrahydrofurfuryl cinnamate, vanillic acid 4- β -D-glucoside were prominent as compared to low_N and high_N application in H20K3447 red lettuce variety (Fig. 3G).

Furthermore, the polyphenolic compounds including feruloyltartaric acid, catechin 7-glucuronide, trans-resveratrol 4'-O-glucuronide, epigallocatechin 3-caffeate, galocatechin, gomprenol 3-methylether 4'-glucuronide, quercetin 3-(6"-malonyl-glucoside), scutellarein 6-xyloside, apigenin 7,4'-dimethyl ether were upregulated with different N applications in H20K3447 red lettuce variety.

Red color represented highest *p* values of metabolites relative abundance while blue color represented lowest *p* values of metabolites relative abundance in H20K7044 and H20K3447 red lettuce varieties under different N applications. In H20K3447 red lettuce variety, treatments (normal_N and high_N) showed the highest *p* values of metabolites relative abundance as compared to low_N application. However, low_N and high_N application showed more variation in *p* values of metabolites relative abundance as compared to normal_N application in H20K7044 red lettuce variety. In both H20K7044 and H20K3447 red lettuce varieties, all applied N treatments showed significant difference in *p* values of metabolites relative abundance (Fig. 3G). The relative contents and metabolites' types changed in both H20K7044 and H20K3447 red lettuce varieties may be due to more variation in phenotypic and genotypic characteristics of both H20K7044 and H20K3447 red lettuce varieties under different N applications.

3.3. Transcriptomic analysis in lettuce varieties

Total 18 samples were used for RNA sequence analysis and found more than 46 million clean reads of expressed data with more than 44.04 % GC contents in each sample. The reference genome of *L. sativa* was mapped using the clean reads from each sample [31]. The average mapping rate was found more than 94 %, with a range of 94.04–95.24 % for each sample (Supplementary data table 1). In PCA analysis for differential expressed genes, PC1 explained 36.6 % while PC2 explained the 8.7 % of total variance (45.3 %) (Fig. 4A). The results showed a clear separation of differential expressed genes under different N applications between H20K7044 and H20K3447 red lettuce varieties. The applied N treatments showed a significant variation and positive correlation in PCA analysis on y-axis under applied N treatments while lettuce varieties also found significant variation in PCA analysis (Fig. 4B). In H20K7044 and H20K3447 red lettuce varieties showed a significant variation irrespective of applied N treatments and PC1 explained 36.6 % while PC2 explained the 8.7 % of total variance (45.3 %) (Fig. 4B). However, all low_N and high_N applied treatments showed a significant difference as compared to normal_N application in H20K7044 and H20K3447 red lettuce varieties (Fig. 4A).

In H20K7044 and H20K3447 red lettuce varieties, total 8350 upregulated and 8258 down regulated differentially expressed genes through fold change were found while 174082 non-significant genes were reported irrespective of applied N treatments in H20K7044 and H20K3447 red lettuce varieties (Fig. 4C).

In heatmap of differentially expressed genes showed different type of genes related to different functions in H20K7044 and H20K3447 red lettuce varieties under different N applications (Fig. 4D). The up-regulated genes were presented in red color while blue color presented down-regulated genes functions in H20K7044 and H20K3447 red lettuce varieties under different N applications. Similarly, for different comparisons of applied N treatments in H20K3447 showed upregulation (1651) and down regulation (1711) genes in High_N_vs.Low_N comparison, upregulation (674) and down regulation (452) genes in High_N_vs.Normal_N comparison and upregulation (1780) and down regulation (1324) genes in Low_N_vs.Normal_N comparison (Supplementary Table 7). This trend showed that all treatments showed significant difference in both H20K7044 and H20K3447 red lettuce varieties for genes variations. In H20K7044 red lettuce variety, the

maximum genes variations were observed in Low_N_vs.Normal_N comparison while minimum genes variations were observed in High_N_vs.Low_N comparison. In contrast, H20K3447 red lettuce variety showed minimum genes variations in High_N_vs.Normal_N comparison and maximum genes variations were observed in Low_N_vs.Normal_N comparison (Supplementary Table 7).

The KEGG enrichment pathway analysis was used for differentially expressed genes in both H20K7044 and H20K3447 red lettuce varieties under different N applications. In KEGG pathway enrichment analysis, different number of genes were found ranging from 1 to 203 with having 0–1 *p* adjusting values for different pathways in all types of comparison of H20K7044 and H20K3447 red lettuce varieties under different N applications (Fig. 5A–F). In comparisons of different N applied treatments in H20K7044 and H20K3447 red lettuce varieties found different number of compounds with different *p*-adjusting values and sharing different rich-factors for different secondary metabolites biosynthesis.

Furthermore, H20K7044 red lettuce variety showed significant difference in results of KEGG enrichment pathways analysis for different comparisons including high_N vs low_N comparison (rich factor = 0.0170, *p*-adjust = 0.157 and 16 identified genes for phenylpropanoid biosynthesis while rich factor = 0.0687, *p*-adjust = 0.000029 and 11 identified genes for flavonoid biosynthesis), high_N vs normal_N comparison (rich factor = 0.05556, *p*-adjust = 0.0000216, and 52 identified genes for phenylpropanoid biosynthesis while rich factor = 0.1875, *p*-adjust = 4.12×10^{-15} and 30 identified genes for flavonoid biosynthesis), low_N vs normal_N comparison (rich factor = 0.05128, *p*-adjust = 0.0134 and 48 identified genes for phenylpropanoid biosynthesis while rich factor = 0.1687, *p*-adjust = 2.49×10^{-10} and 27 identified genes for flavonoid biosynthesis) (Fig. 5A–C).

Similarly, H20K3447 red lettuce variety also showed different results in KEGG enrichment pathways analysis in different comparisons including high_N vs low_N comparison (rich factor = 0.0405, *p*-adjust = 0.0000572 and 38 identified genes for phenylpropanoid biosynthesis while rich factor = 0.0375, *p*-adjust = 0.3461 and 6 identified genes for flavonoid biosynthesis), high_N vs normal_N comparison (rich factor = 0.0170, *p*-adjust = 0.022, and 16 identified genes for phenylpropanoid biosynthesis while rich factor = 0.0125, *p*-adjust = 0.81056 and 2 identified genes for flavonoid biosynthesis), low_N vs normal_N comparison (rich factor = 0.03632, *p*-adjust = 0.000662 and 34 identified genes for phenylpropanoid biosynthesis while rich factor = 0.0625, *p*-adjust = 0.00498 and 10 identified genes for flavonoid biosynthesis) (Fig. 5D–F).

Results showed that pathways include anthocyanin biosynthesis, carotenoid biosynthesis, citrate cycle (TCA cycle), diterpenoid biosynthesis, flavone and favonol biosynthesis, flavonoids biosynthesis, glucosinolate biosynthesis, isoflavonoid biosynthesis, nitrogen metabolism, phenylalanine metabolism, phenylpropanoid biosynthesis, photosynthesis, sesquiterpenoid and triterpenoid biosynthesis, stilbenoid, diarylheptanoid and gingerol biosynthesis and terpenoid backbone biosynthesis were found for all types of comparisons under different applied N treatments in H20K7044 and H20K3447 red lettuce varieties. All these biosynthesis pathways were found common in comparison of H20K7044 and H20K3447 red lettuce varieties irrespective of applied N applications (Supplementary Fig. 4). These results mostly represented the identified genes to the polyphenolic biosynthesis mechanism in red lettuce varieties. The highest KEGG enrichment was found related flavonoid biosynthesis for both H20K7044 and H20K3447 red lettuce varieties under different comparisons of N applications.

Comparison of differently expressed genes in H20K7044 and H20K3447 red lettuce varieties under different N application were expressed (Fig. 5A–F). Minimum number of gene sets (1810) were found in comparison of High_N vs Low_N in which 205 gene sets were significantly found differently in H20K7044 red lettuce variety. In H20K7044 red lettuce variety, 1346 gene sets were found differently as compared to other comparisons of applied N treatments. Total 45 gene sets were expressed common in comparison of all applied N treatments in

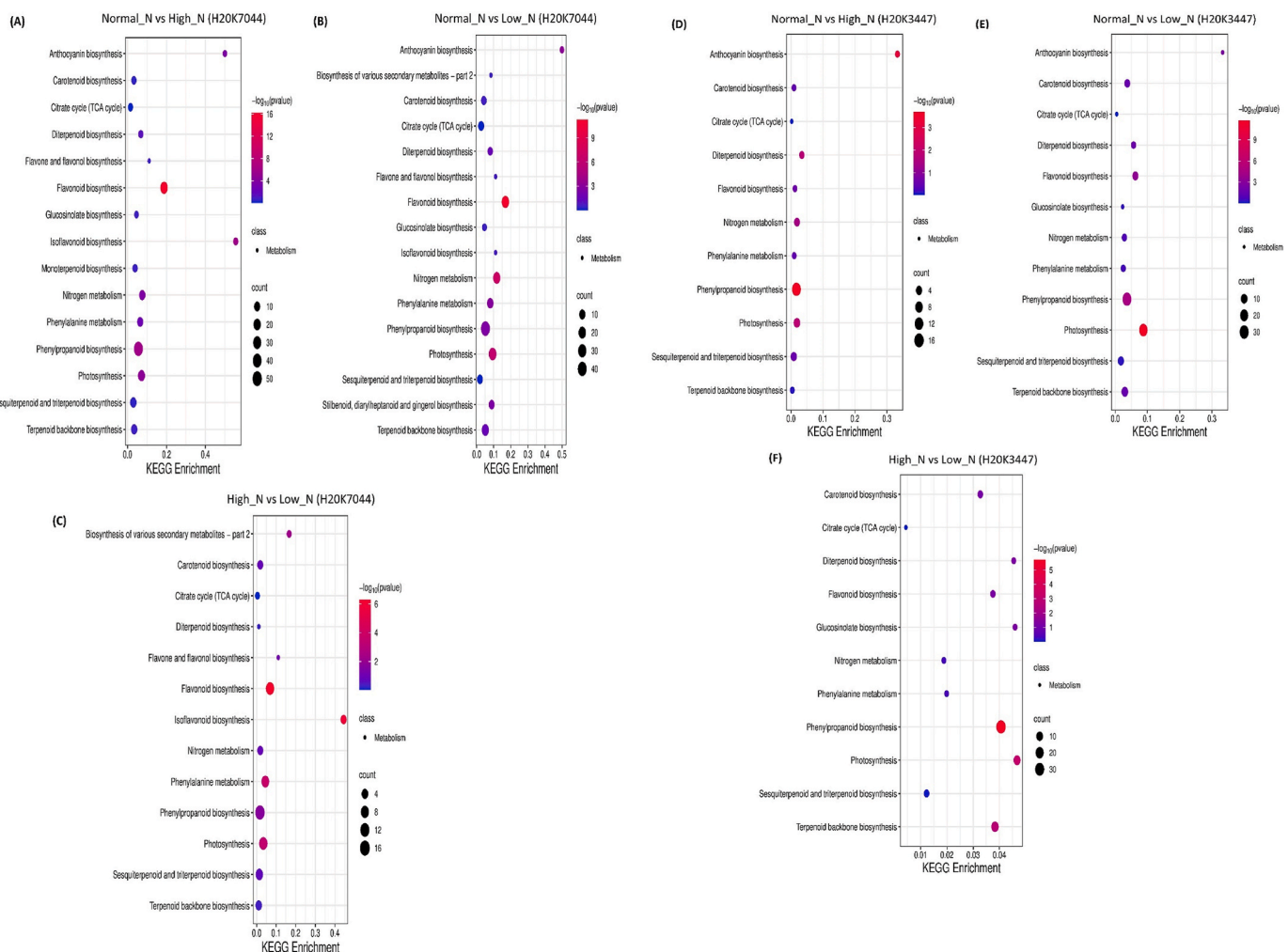


Fig. 5. Differentially expressed genes for KEGG enrichment pathways in all possible comparisons of H20K7044 and H20K3447 red lettuce varieties under different N applications. Normal_N = 5 mmol N L⁻¹, High_N = 10 mmol N L⁻¹ and Low_N = 2.5 mmol N L⁻¹ application.

H20K7044 red lettuce variety (Fig. 5A–C). In H20K3447 red lettuce variety, minimum number of gene sets (1126) were reported in comparison of High_N vs Normal_N in which 223 gene sets were expressed differently (Fig. 5D–F).

Moreover, in H20K7044 red lettuce variety, treatment wise comparisons gave different response for up and down-regulated genes related to polyphenolic biosynthesis pathways, total 26 genes in normal_N vs high_N comparison (up = 16 and down = 10), total 73 genes in normal_N vs low_N comparison (up = 19 and down = 54), total 67 genes in high_N vs low_N comparison (up = 22 and down = 45) (Fig. 5A–C). Similarly, H20K3447 red lettuce variety, treatment wise comparisons gave different response for up and down-regulated genes related to polyphenolic biosynthesis pathways, total 39 genes in normal_N vs high_N comparison (up = 24 and down = 15), total 19 genes in normal_N vs low_N comparison (up = 12 and down = 7), total 37 genes in high_N vs low_N comparison (up = 25 and down = 12) (Fig. 5D–F). In varieties-wise comparisons, different genes related to polyphenol biosynthesis pathways were found up- and down-regulated in which total 139 genes in H20K7044 and H20K3447 red lettuce varieties comparison (up = 62 and down = 77). The results showed that different number of gene sets found in different comparisons of applied N treatments in H20K7044 and H20K3447 red lettuce varieties. Additionally, the number of gene sets were also found differently in different lettuce varieties. Differently expressed gene sets showed variation in all varieties as well as in all applied N treatments which showed an effect of applied N treatments in H20K7044 and H20K3447 red lettuce varieties

(Fig. 5A–F).

In Venn diagram (Fig. 6A–B), shared and unique differentially expressed metabolites and genes were presented in H20K7044 and H20K3447 red lettuce varieties. The unique metabolites 227 and 247 in H20K7044 and H20K3447 respectively while shared metabolites (285) in both H20K7044 and H20K3447 red lettuce varieties were presented (Fig. 6A). Similarly, unique genes 1355 and 662 in H20K7044 and H20K3447, respectively and shared genes (412) in both H20K7044 and H20K3447 red lettuce varieties were presented (Fig. 6B).

To investigate the further polyphenolic biosynthesis mechanism in H20K7044 and H20K3447 red lettuce varieties, integration of metabolomics and transcriptomics data analysis was used for better understanding the impact of N applications on metabolites and genes expression in H20K7044 and H20K3447 red lettuce varieties (Fig. 7). Different types of metabolites were found in H20K7044 and H20K3447 red lettuce varieties under different N applications in which majority identified metabolites were found related to polyphenol biosynthesis pathways including cinnamic acid, caffeoylquinic acid, chlorogenic acid, naringenin and their derivatives, kaempferol and their derivatives, luteolin and their derivatives, quercetin and their derivative, cyanidin-3-O-glucuronide, epigallocatechin-3-O-gallate and pelargonidin 3-O-[2-O-(beta-D-xylopyranosyl)-6-O-(methyl-malonyl)-beta-D-galactopyranoside]. Metabolites were up-regulated and down-regulated in both H20K7044 and H20K3447 red lettuce varieties. It showed much variation and different responses in each red lettuce variety under different N applications (Fig. 7).

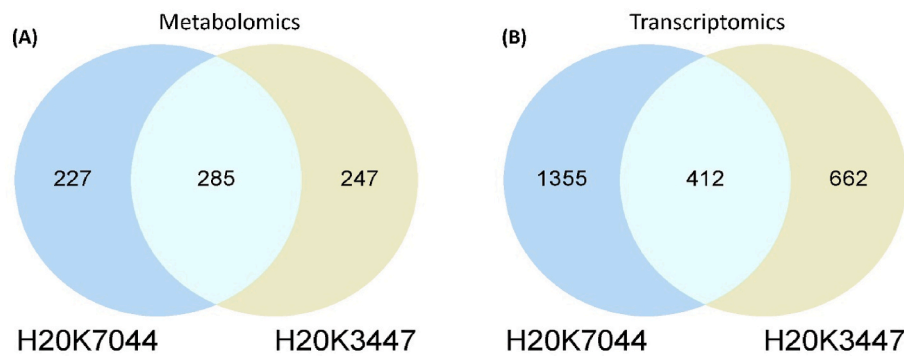


Fig. 6. Venn diagram of shared and unique differentially expressed metabolites and genes in comparison of irrespective different applied N treatments. (A) metabolomic and (B) transcriptomics variations in red lettuce varieties (H20K7044 and H20K3447). Note: The figures show the number of common and unique metabolites and genes in red lettuce varieties (H20K7044 and H20K3447).

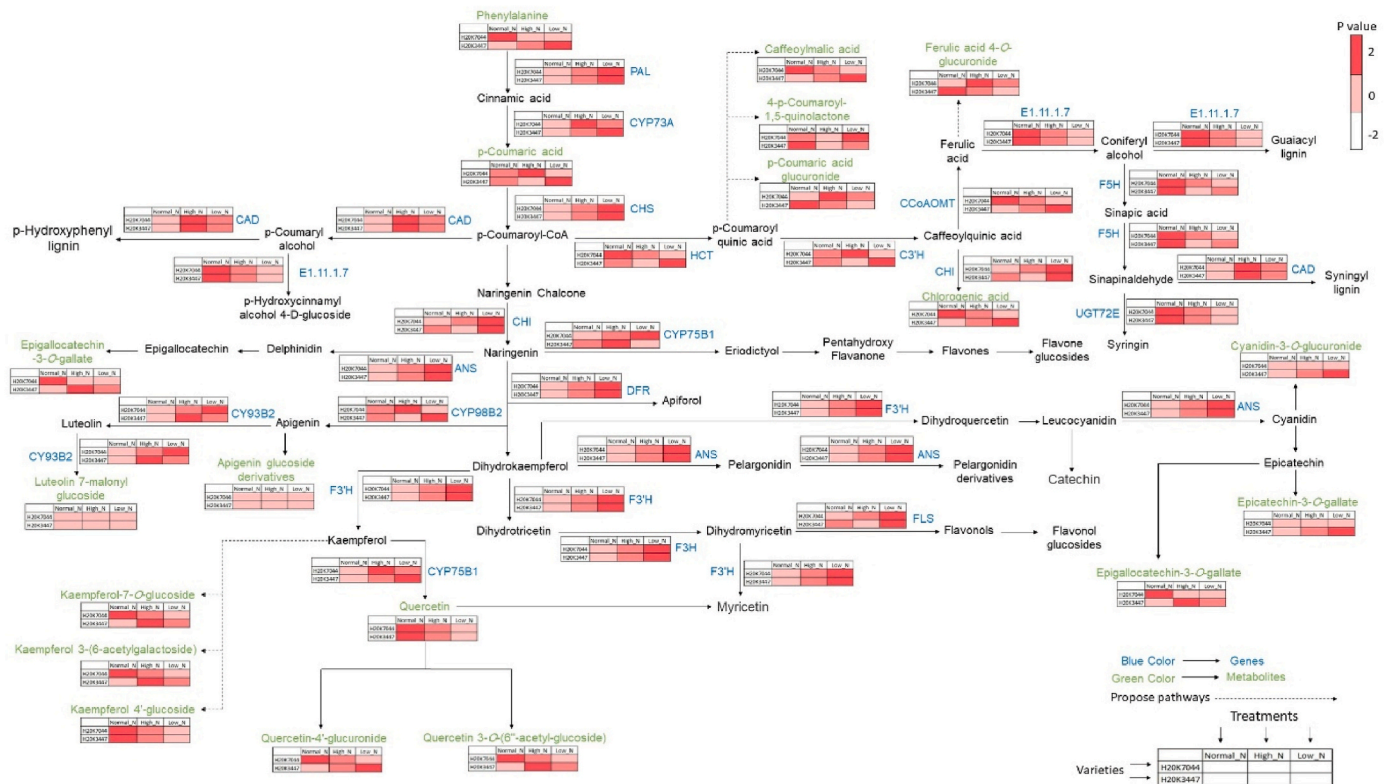


Fig. 7. Metabolomic and transcriptomic profiling in different (H20K7044 and H20K3447) red lettuce varieties under different N applications for polyphenol biosynthesis pathways. Light red to dark red color boxes are candidate genes and metabolites with *p* adjusting values involved for polyphenol biosynthesis pathways. phenylalanine ammonia-lyase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavanoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FLS, flavonol synthesis and ANR, anthocyanin reductase.

In different comparisons of H20K7044 and H20K3447 red lettuce varieties under different N applications found different genes related to polyphenol biosynthesis mechanism including phenylalanine ammonia-lyase (PAL) (Lsat_31000; Lsat_106540; Lsat_31080; Lsat_106520), cinnamic acid (Lsat_99521), 4CL (Lsat_63841; Lsat_9201), CYP73A (Lsat_99521), CHS (Lsat_76880; Lsat_42860; Lsat_147220), CHI chalcone-flavanone isomerase (Lsat_66221; Lsat_11480), CYP81E1 (Lsat_24061), CCoAMTs (Lsat_67821, Lsat_83900), E2.1.1.104 (Lsat_85100, Lsat_85000, Lsat_85040, Lsat_83940, Lsat_67881) E2.3.1.133, HCT (Lsat_63761, Lsat_116280, Lsat_51680, Lsat_56241, Lsat_14521, Lsat_8960, MSTRG.12776), DFR (Lsat_77261), FLS (Lsat_117441), CYP75B1 (Lsat_23101), E3.2.1.21 (Lsat_60560), E5.5.1.6 (Lsat_60701), CYP98A, C3'H (Lsat_63400), CAD (Lsat_66380), CYP93B2

(Lsat_70860), F3H (Lsat_74560), ANS (Lsat_97280) and BZ1 (Lsat_66261, Lsat_100200) and all these genes were triggered differently under different N applications in both H20K7044 and H20K3447 red lettuce varieties (Fig. 7).

In H20K7044 red lettuce variety, in comparison of high_N vs normal_N application, the following polyphenolic biosynthesis genes including Lsat_23101, Lsat_76880, Lsat_11480, Lsat_100200, Lsat_74560, Lsat_42860, Lsat_7261, Lsat_97280, Lsat_117441, Lsat_66221 and Lsat_66261 were upregulated with high_N application as compared to normal_N application. Similarly, the following polyphenolic biosynthesis genes including Lsat_23101, Lsat_74560, Lsat_76880, Lsat_11480, Lsat_42860, Lsat_100200, Lsat_77261, Lsat_97280, Lsat_66221, Lsat_70860 and Lsat_117441 were upregulated

in comparison of low_N vs normal_N application. In addition, Lsat_63761 and Lsat_24061 were upregulated in comparison of high_N vs low_N application in H20K7044 red lettuce variety (Fig. 7).

The H20K3447 red lettuce variety also showed a significant variation for polyphenolic biosynthesis genes under different N applications. In comparison of high_N vs normal_N application, the following polyphenolic biosynthesis genes including Lsat_100200, Lsat_117441, Lsat_99521, Lsat_74560, Lsat_67821, Lsat_77261, Lsat_70860, Lsat_83900 and Lsat_97280 were upregulated. Similarly, the following polyphenolic biosynthesis genes including, Lsat_99521, Lsat_74560, Lsat_67361, Lsat_67821, Lsat_66221, Lsat_66261, Lsat_76880, Lsat_11480, Lsat_14521, Lsat_67881 and Lsat_97280 were upregulated in comparison of low_N vs normal_N application. In addition, the following polyphenolic biosynthesis genes including Lsat_70860, Lsat_100200, Lsat_23101, Lsat_42860 and Lsat_117441 were upregulated under high_N vs low_N application in H20K3447 red lettuce variety (Fig. 7).

Mostly polyphenolic biosynthesis genes including CHS (Lsat_147220), E2.1.1.104 (Lsat_85000), FLS (Lsat_117441), E5.5.1.6 (Lsat_40701, Lsat_11480), E2.3.1.133, HCT (Lsat_116280, Lsat_8960, Lsat_56241, Lsat_63761) and CYP73A (Lsat_99521) were significantly upregulated in H20K3447 red lettuce variety (Fig. 7). Mostly were upregulated with low_N and high_N applications in lettuce varieties. Mostly up-regulated polyphenolic biosynthesis genes were found in H20K3447 as compared to H20K7044 red lettuce variety. Different variation was observed in both H20K7044 and H20K3447 red lettuce varieties but the variation was not constant for all types of compounds and genes for flavonoid biosynthesis mechanisms under different N applications in both red lettuce varieties (Fig. 7). Taken together, integration of metabolomics and transcriptomic analysis revealed that most of metabolites and differentially expressed genes explored the secondary metabolites biosynthesis related pathways in which flavonoids, lignan, isoflavonol and anthocyanin biosynthesis pathways were prominent for both H20K7044 and H20K3447 red lettuce varieties under different N applications (Fig. 7).

In plant signal transduction pathways, transcription factors (TFs) are essential in adaptation against N applications for H20K7044 and H20K3447 red lettuce varieties production. Total 531 identified TFs related genes that were differentially expressed under N applications in both red lettuce varieties (Supplementary data table 8). The ethylene responsive factor (ERF) family related genes were found the most differentially expressed in both red lettuce varieties with 100 (18.83 %) followed by the MYB and bHLH family with 52 (9.79 %) and 39 (7.34 %) differentially expressed genes respectively. Furthermore, minimum number of TFs TALE, RAV, Nin-like, GRF and EIL with 1 (0.18 %) differentially expressed genes were found under different N applications in H20K7044 and H20K3447 red lettuce varieties. Transcriptional factors of differentially expressed genes showed a variation of expressions in both H20K7044 and H20K3447 red lettuce varieties under different N applications which showed that these transcriptional factors showed different functions in both H20K7044 and H20K3447 red lettuce varieties under different N applications. The variation in polyphenolic metabolites and genes concentrations might be linked with these transcriptional factors functions as well in H20K7044 and H20K3447 red lettuce varieties under different N applications.

Total 13 polyphenolic biosynthesis related genes were analyzed through qRT-PCR analysis to confirm the accuracy of transcriptional data of H20K7044 and H20K3447 red lettuce varieties under different N applications. The relative gene expression of 13 genes revealed that polyphenolic biosynthesis related genes showed variation under different N applications in H20K7044 and H20K3447 red lettuce varieties (Fig. 8A–M). The results showed a similar trend and verified the credibility and reproducibility of the transcriptional data (Fig. 8A–M).

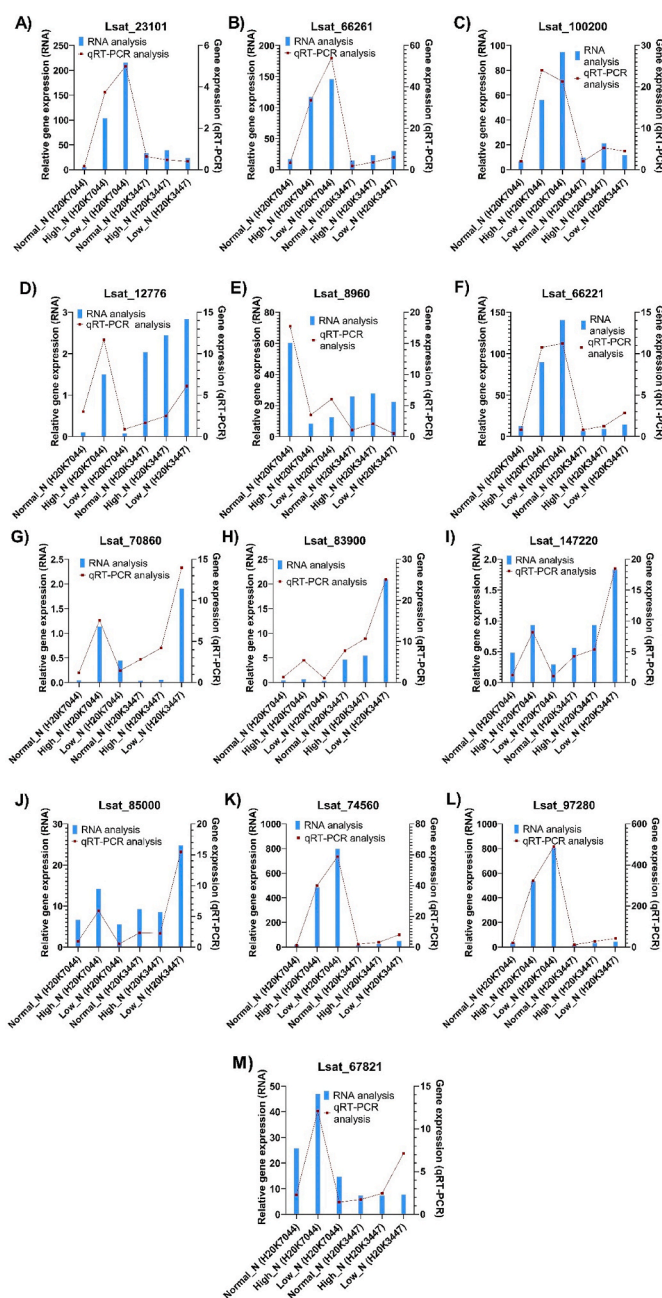


Fig. 8. Expression of representative genes through RNA sequencing and qRT-PCR analysis in H20K7044 and H20K3447 red lettuce varieties under different N applications. Normal_N = 5 mmol L⁻¹, High_N = 10 mmol L⁻¹ and Low_N = 2.5 mmol L⁻¹.

4. Discussion

Nitrogen (N) is essential macro nutrient and applied to the plants in ammonium (NH₄⁺) and nitrate (NO₃⁻) form [32]. The N application in balanced form increases the plant growth and development including plant fresh and dry weight [10]. The stresses of N influence the plant physiological, growth, quality, metabolic activities and ultimately yield of the plants. Increasing or decreasing N application is influenced the plant fresh and dry biomass in plants such as in pakchoi [33]. The N application directly influences the physiological growth parameter including chlorophyll *a*, *b* and total chlorophyll contents in plants [34]. In this study, high_N (10 mmol L⁻¹) application influenced more on chlorophyll *a*, chlorophyll *b* and total chlorophyll contents followed by normal_N (5 mmol L⁻¹) application as compared to low_N (2.5 mmol

L^{-1}) application in both H20K7044 and H20K3447 red lettuce varieties (Fig. 2A–C). However, carotenoid contents in our study were found highest under normal_N (5 mmol L^{-1}) application followed by high_N (10 mmol L^{-1}) as compared to low_N (2.5 mmol L^{-1}) application in both H20K7044 and H20K3447 red lettuce varieties (Fig. 2D). Similar trend was observed under different N applications in previously reported lettuce production [34,35].

The lower N application reduced total chlorophyll contents which ultimately reduced the fresh and dry biomass while in contrast higher N application increased the fresh and dry biomass due to high total chlorophyll contents as compared to normal_N application in H20K7044 and H20K3447 red lettuce varieties. High and normal_N applications increased the plant physiology and growth parameters as compared to low_N in both H20K7044 and H20K3447 red lettuce varieties (Fig. 1). Similar results have been reported in olive [36], basil [37], banana [38], spearmint [39], maize [40], lettuce [35] and peppermint [41]. The results revealed that H20K7044 and H20K3447 red lettuce showed different contents of physiological and growth attributes and exhibited maximum growth parameters including biomass productions which could be due to high efficiency of light capture and N uptake in H20K3447 as compared to H20K7044 red lettuce variety. In earlier studies, N deficiency influenced the plant growth and reduced the protein and chlorophyll contents in woody plants [42,43] due to which reduce the physiological process by decreasing the photosynthetic rate and CO_2 assimilation for plant growth production [10,44,45]. Our results showed similar response of previous reported studies in which different genotypes respond differently for N uptake and biomass production under changing physiological growth including chlorophyll *a*, *b* and total chlorophyll contents [46,47].

Modulating secondary metabolite production and accumulation is a crucial strategy employed by plants to withstand different environmental stresses. The multiomics analyses conducted in our study elucidate how different N application concentrations selectively influenced secondary metabolite production especially flavonoid biosynthesis in red lettuce varieties. In accordance with prior studies, we discovered that N application concentrations exerted the most potent positive effect on phenylpropanoids, inducing the majority of phenylpropanoid compounds. Furthermore, N application concentrations stimulatory effects on flavonoids biosynthesis were not more widespread and pronounced than those on organic and amino acids at both the transcription and metabolic profiling levels.

This study was based on multi-omic study through metabolomics and transcriptomic analysis under different N applications in H20K7044 and H20K3447 red lettuce varieties. The results revealed that changing N concentrations impacted the primary and secondary metabolites including amino acids, organic acids, phenolic compounds, flavonoids, alkaloids, lignan, lipids, terpenoids and many other specialized metabolites. In metabolomic analysis, the secondary metabolites including flavonoids, flavones and isoflavonoids (Fig. 3G) showed significant difference under low, high and normal_N applications in H20K7044 and H20K3447 red lettuce varieties. Different secondary metabolites showed different relative contents of metabolites including flavonoids, isoflavonoids, flavonol, flavones and anthocyanins under different applied N applications in H20K7044 and H20K3447 red lettuce varieties which exhibited different phenotypic characteristics (Fig. 3A–G). Major regulated metabolites under different N applications belonged to polyphenol biosynthesis pathways including flavonoids (Supplementary Table 5). The low_N (2.5 mmol L^{-1}) application showed highest variation and upregulation of quercetin-4'-glucuronide, gallic acid, cyanidin 7-glucoside, kaempferol 3-(2'-acetylramnoside), kaempferol 7-O-neohesperidoside, *p*-coumaric acid, isoflavonol, as compared to other high_N and normal_N application in H20K7044 red lettuce variety (Fig. 3G). However, normal_N (5 mmol L^{-1}) showed upregulation of *p*-coumaric acid glucuronide, epigallocatechin 3-caffeate, quercetin 3-O-(6'-acetyl-glucoside), and luteolin 3'-glucuronide, as compared to high_N and low_N application in H20K7044 red lettuce variety (Fig. 3G).

Furthermore, high_N (10 mmol L^{-1}) showed up-regulation of daidzin 4'-O-glucuronide, syringic acid glucuronide, sinapic acid, octyl gallate, laticresinol, taxifolin 3-arabinoside, myricetin 3-arabinoside, vanillic acid 4-beta-D-glucoside and secoisolaricresinol 9,9'-diglucoside as compared to low_N and normal_N application in H20K7044 red lettuce variety (Fig. 3G). In H20K3447 red lettuce variety, high_N (10 mmol L^{-1}) showed upregulation in gallic acid, isoflavonol, quercetin, gallic acid-3-gallate, syringic acid glucuronide, myricetin, and taxifolin 3-arabinoside as compared to low_N and normal_N application (Fig. 3G). Meanwhile, low_N (2.5 mmol L^{-1}) application showed upregulation in luteolin 3'-glucuronide, *p*-coumaric acid, glycitein 7-O-glucuronide, isoflavonol 3-xyloside, and quercetin-4'-glucuronide as compared to normal_N and high_N application in H20K3447 red lettuce variety (Fig. 3G). Similarly, normal_N (5 mmol L^{-1}) application also upregulated polyphenolic compounds in H20K3447 red lettuce variety, in which kaempferol 7-O-neohesperidoside, sinapic acid, D-linalool 3-glucoside, and naringin 6'-rhamnoside, were prominent as compared to low_N and high_N application in H20K3447 red lettuce variety (Fig. 3G). Plant growth and defence mechanisms against various stresses reported on flavonoid compounds and biosynthesis pathways [48]. Derivative of phenylpropanoids are precursor of flavonoids biosynthesis in plants under different stresses conditions with involvement of malonyl CoA activity for production of different heterocyclic structural forms such as isoflavones, isoflavonols, flavanones, flavones and anthocyanins in plants [49]. Metabolomics based techniques such as targeted and untargeted metabolomics are successful way to determine the metabolites in numerous plant parts including cassava [50], loquat [51], apple [52], and strawberry [53] under different conditions and different genotypes and also helpful to understand the mechanism of plant responses under different stress conditions. In this study, H20K7044 and H20K3447 red lettuce varieties were examined through untargeted metabolomic profiling under different N applications and found different metabolites were regulated differently under different N concentrations in H20K7044 and H20K3447 red lettuce varieties. Total annotated metabolites were found 1286 compounds in which 135 flavonoids, 101 phenolic acids, 65 lipids, 59 amino acids and derivatives and 926 others compounds including nucleotide and derivatives, alkaloids, terpenoids, organic acids, lignan, coumarins and tannins etc. Similarly, H20K7044 and H20K3447 red lettuce varieties showed different response to high_N, low_N and normal_N concentrations (Fig. 3A–G). Under low_N and high_N application in both H20K7044 and H20K3447 red lettuce varieties triggered secondary metabolites mostly related to polyphenolic compounds including phenylpropanoid and flavonoid biosynthesis pathways. Both red lettuce varieties showed different responses to up-regulation and down-regulation of metabolites and their relative abundance under different N applications which may contribute to color, genotypes and contains strong antioxidant activities in red lettuce varieties [54,55]. These findings showed that different metabolites with evident variations showed much quality variances under different N applications in both H20K7044 and H20K3447 red lettuce varieties. This mechanism has been demonstrated in various plant species with Protein Competition Model [56,57]. The biosynthesis of flavonoids may be hindered with the less accumulation of phenylalanine, and the generation of secondary metabolites derived from phenylalanine might compete with protein synthesis for phenylalanine production. The accumulation of different amino acids and their derivatives under high N application offered additional evidence in the current investigation. The high contents of different amino acids are influenced the other metabolic pathways like tricarboxylic acid pathway in which 2-oxoglutaric acid shows intermediary for the production of nucleotide, amino acids, sugar and flavonoid compounds in apple [52]. The 2-oxoglutaric acid was found in lettuce varieties which could be considered as for the production of flavonoid compounds in both H20K7044 and H20K3447 red lettuce varieties under different N applications [50]. Similar results are found in lettuce under different abiotic stresses [58]. Production of different secondary metabolites in

plants especially anthocyanins and other phenolic compounds during nutrient-stress situations for delaying senescence in plants [59]. Similarly, low N applications are influenced H20K7044 and H20K3447 red lettuce varieties and significantly enhanced the total polyphenol, flavonoid and different metabolites including luteolin, quercetin, chlorogen acid and chicoric acid and decreased the carotenoid in lettuce [60,61]. Furthermore, an adequate N application is negatively influenced on polyphenol production, however, the impact was found differently in our study especially for H20K7044 and H20K3447 red lettuce varieties. The photosynthetically active carbon molecules produced the flavonoid and phenolic compounds due to imbalance of mineral concentration in H20K7044 and H20K3447 red lettuce varieties under different N applications [19,62]. Similarly, previous study showed that increasing the N application (12 mM) are also negatively impacted the flavonoid, caffeic acids and their derivatives while carotenoid contents are increased with increasing the N application including isomers of violaxanthin, luteoxanthin, neoxanthin and β -carotene [19]. The luteolin and quercetin glycosides contents are reduced with increasing the N application in our study for red lettuce varieties. The increasing of N application impacted the luteolin 7-glucuronide, quercetin 3-glucuronid, quercetin 3-(6"-malonyl)-glucoside, cyanidin 3-(6"-malonyl)-glucoside in red lettuce varieties [19]. Relative abundance of different metabolites under different N applications was shown different and same results as reported above mentioned studies for different plants species under different stresses conditions.

Lettuce species constitute an attractive paradigm for deciphering the diversity in polyphenol pathways because the lettuce clade encompasses a group of species with a richness of genomic and metabolomic resources that diverged recently but still depict great variations at the phenological and physiological levels [63,64]. Transcription modifications coincide with variations in N availability to the plants under different stressful conditions. Additionally, alterations in the underlying transcriptional regulatory network were likely responsible for the differences in transcription factor evolved for secondary metabolite productions in H20K7044 and H20K3447 red lettuce varieties under different N applications. Phenylalanine ammonia-lyase (PAL), the initial enzyme in the phenylpropanoid biosynthesis pathway, converts phenylalanine to cinnamic acid as a source of energy for the synthesis of other phenolic compounds. As a result, phenylalanine metabolism is crucial for polyphenol biosynthesis mechanism including flavonoids [65]. Hydroxybenzoic and hydroxycinnamic acids are the two sub-categories of phenolic acids. The most well-known hydroxycinnamic acids include chlorogenic acid, quinic acid, sinapic acid, ferulic acid and caffeic acid. Phenolic compounds contain a C6-C3 structure, which is the ancestor of other common lignin and phenolic compounds [66]. The PAL and hydroxycinnamic acids are precursor to several polyphenol biosynthesis under different stressful conditions including mineral nutrition [67]. In PAL contents, H20K7044 and H20K3447 red lettuce varieties showed different response to N application and up-regulated with low N application in both H20K7044 and H20K3447 red lettuce varieties (Fig. 4D). The results indicated that different genes PAL, E5.5.1.6 and CHS showed different respond to applied N applications in H20K7044 and H20K3447 red lettuce varieties which are responsible for production of different type of secondary metabolites in flavonoid biosynthesis (Fig. 5A-F and Fig. 7). Improving the high level of expressed genes for flavonoids biosynthesis are responsible for antioxidant properties in H20K7044 and H20K3447 red lettuce varieties. The production of PAL, CHS, F3H, C4H and CHI are helpful for flavonoid biosynthesis in plants [68,69]. The F3H is responsible for conversion of naringenin to eriodictyol in plants. The FLS is involved in biosynthesis of different flavonol derivatives including myricetin, kaempferol and quercetin [69]. The proanthocyanins including epicatechin and catechin are produced with the help of LAR and ANR in plants for polyphenol biosynthesis mechanism [70]. The DFR is responsible for the production of catechins and anthocyanidins in tea and tomato plants [71,72]. In plant quality and development, DFR and ANS transcript

levels are played important role and responsible for the production of anthocyanin in plants [73–76]. The ANS were found in cherry for the production of polyphenol biosynthesis pathways including flavonoid and anthocyanin pathways [76]. The identified genes showed same results in both H20K7044 and H20K3447 red lettuce varieties under different N applications for the production of flavonoid biosynthesis, however, the expression level was not same in H20K7044 and H20K3447 red lettuce varieties under different N applications as above reported studies. So, different N concentrations, different composition and different genotype respond differently for flavonoid biosynthesis mechanism under transcriptomic and metabolomics integration in different plants.

It indicated that low or high N application influenced the secondary metabolites but mostly these are related to phenotypic and genotypic characteristics of the plant species. The genes and metabolites connected to the physiological and metabolic reactions of several plant species to N stresses and have been studied in different plants [77,78]. A significant difference in metabolomic profiling and gene expressions are observed for different N application in rapeseed [79]. Changes of metabolomic profiling and gene expressions, which influences the biochemical and physiological mechanisms in plants under different N stresses and also influences the primary and secondary metabolites in plants under different N stresses [79,80]. Similar respond was observed in our study, different metabolites and gene were influenced for different secondary metabolites biosynthesis mechanism under different N applications in H20K7044 and H20K3447 red lettuce varieties (Fig. 5A–F). The poplar plants are also showed similar results under different N applications [43].

Transcription factors act as a switch that may either activate or deactivate a gene by recognizing specific patterns and function in plants' part. In this study, the major transcription factors of ERF, MYB, bHLH, NAC, WRKY and bZIP were found for H20K7044 and H20K3447 red lettuce varieties under different N applications. The ethylene responsive factors (ERF) family of transcription factors respond to several plant nutrient treatments and play a crucial part in the control of a number of abiotic stresses [81–83]. The transcription factors have reported different DNA binding preferences and are activated in response to particular stresses, which enables these transcription factors to integrate responses to various stimuli, nutrient stresses and take part in regulatory processes [84]. In this study, the high transcriptional factor of ERF, MYB and bHLH showed that these transcripts were influenced with different N applications and influenced the polyphenol biosynthesis pathways especially flavonoid biosynthesis (Supplementary Table 8). In previous study, seven ERF transcriptional factors are identified in cucumber under N application [85]. In tobacco and Arabidopsis plants, the transcription factor WRKY are shown to regulate phenolic compounds productions such tannin, lignin and flavanols [86,87]. In the same way, basic leucine zipper (bZIP) transcriptional family are also influenced by different type of abiotic stresses and performed different biological and metabolic processes in plants [88,89]. Similarly, overexpression of bZIP transcriptional factor of Arabidopsis under N deficiency induced the drought tolerance and N accumulation [90]. For the H20K3447 as compared to H20K7044 red lettuce varieties, there were more down-regulated basic helix-loop-helix domain (bHLH) transcriptional factors and these were responsible for plant growth and development under stress conditions [91,92]. The MYB and NAC transcriptional factors are also influenced with abiotic stresses and controlled the different molecular and biochemical pathways to survive the plants in different stresses. This higher expressions of these transcriptional factors in H20K7044 and H20K3447 red lettuce varieties under different N applications indicated that these transcriptional factors influenced under different N applications and produced different biochemical pathways which were responsible for the production of polyphenols in H20K7044 and H20K3447 red lettuce varieties. These all above mentioned transcriptional factors were incorporated with N application in H20K7044 and H20K3447 red lettuce varieties for the production of polyphenol

biosynthesis pathways. Overall, these transcriptional factors influenced polyphenol biosynthesis pathways in H20K7044 and H20K3447 red lettuce varieties and differentially expressed of distinct transcription factors were responded differently under different N applications in H20K7044 and H20K3447 red lettuce varieties. Our integration of metabolomic and transcriptomic analysis results indicated that there was distinct variations in metabolites and genes analysis for polyphenol biosynthesis under different N applications in H20K7044 and H20K3447 red lettuce varieties and this study can provide a theoretical evidence to improve the nutritional quality under different N applications by using different multi-omics techniques. With the integration of metabolomic and transcriptome analyses, the results give trustworthy evidence and confirming the scientific validity and efficiency of diverse N applications on the red lettuce production for polyphenol biosynthesis pathways. This study can help for choosing and producing the high-quality polyphenol lettuce varieties.

5. Conclusion

This study elucidates the nitrogen (N)-mediated regulatory mechanisms governing polyphenolic biosynthesis in two red lettuce cultivars (*Lactuca sativa* L. cv. H20K7044 and H20K3447) through integrated metabolomic and transcriptomic analyses. High N application (10 mmol L⁻¹) enhanced chlorophyll content and biomass in H20K7044, while H20K3447 exhibited superior nitrogen balance indices under high N application. Notably, low N triggered upregulation of key flavonoid biosynthetic genes (*PAL*, *CHI*, *ANS*, *DFR*, *FLS*, *F3H*) and elevated flavonol accumulation in H20K7044. Metabolomic profiling revealed genotype- and N-dependent differential accumulation of polyphenolic derivatives, including quercetin-3-O-(6"-acetyl-glucoside), cyanidin-3-O-glucuronide, and epicatechin-3-O-gallate. Transcriptomic analysis identified overall 389,734 differentially expressed genes (206,695 up-regulated, 183,039 down-regulated), with 36 linked to flavonoid biosynthesis (31 up-regulated, 5 down-regulated). H20K3447 demonstrated significantly higher polyphenolic gene expression and metabolite abundance compared to H20K7044, underscoring cultivar-specific metabolic pathways. Multi-omics integration delineated molecular networks connecting N availability to transcriptional regulation and metabolic flux, revealing candidate genes (e.g., *ANS*, *DFR*, *FLS*, *F3H*) critical for enhancing phytochemical diversity to secondary metabolic pathways. These findings advance precision agriculture strategies by linking genotype-specific N responses to phytochemical quality, offering actionable insights for optimizing N fertilization to improve red lettuce nutritional values and stress resilience. This work establishes a foundational framework for leveraging multi-omics approaches in crop improvement, emphasizing the role of tailored nutrient management in sustainable horticulture for red lettuce production.

CRedit authorship contribution statement

Muhammad Khalid Hameed: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Wajid Umar:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation. **Ali Razzaq:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Muhammad Hasham Anwaar:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Shiwei Wei:** Writing – review & editing, Writing – original draft, Software, Resources. **Qingliang Niu:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Danfeng Huang:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Pengli Li:** Writing – review & editing, Writing – original draft, Resources, Project administration,

Methodology, Investigation. **Liying Chang:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jafr.2025.102444>.

Data availability

All data has been presented in manuscript and additional data is presented in supplementary data.

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