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Article

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***RcDHD/SDH* gene family differentially drive gallic and ellagic acid biosynthesis in *Rubus chingii* fruit: insights from transient overexpression and metabolic profiling**

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Abstract: The 3-dehydroquinic acid dehydrogenase/shikimate dehydrogenase (*DHD/SDH*) gene, which encodes a protein, acts as a dual-enzyme capable of catalyzing both shikimate and gallic acid synthesis. This dual-enzyme is playing a pivotal regulatory role in the gallic acid metabolic pathway. **Objective:** To investigate the functional roles of *DHD/SDH* in the gallic acid and ellagic acid synthesis pathways within the fruit of Chinese raspberry (*Rubus chingii* Hu). **Method:** Using *Rubus chingii* Hu (*R. chingii*) as experimental material, Agrobacterium-mediated transformation was utilized to introduce over-expression vectors carrying the target gene. Specifically, Agrobacterium cultures harboring the *R. chingii RcDHD/SDH* target gene over-expression vectors ranging from pCambia1300-*RcDHD/SDH1* to *RcDHD/SDH4*-GFP were injected into raspberry fruit tissue for transient expression. To evaluate the impact, fluorescent quantitative PCR was employed to assess *DHD/SDH* gene expression levels, while HPLC was used to determine gallic acid and ellagic acid content, providing preliminary insights into the biological roles of the *RcDHD/SDH* gene family. **Results:** Subcellular localization experiments revealed that these gene products are localized to the nucleus. This gene cluster is pivotal in controlling the synthesis of phenolic acid in *R. chingii* through temporal expression patterns, with its activity aligning closely with the level of ellagic acid and gallic acid. In the case of thornless cultivars, *RcDHD/SDH1*-*RcDHD/SDH2* take center stage, enabling an early rapid accumulation, whereas thorny cultivars activate *RcDHD/SDH3*-*RcDHD/SDH4* to implement a sustained synthesis approach during late-stage, highlighting gene functional differentiation in secondary metabolic strategies between cultivars. **Conclusion:** *RcDHD/SDH* stimulates biosynthesis and

accumulation of gallic and ellagic acid in *R. chingii*, providing a theoretical basis for molecular breeding to enhance fruit ellagic acid content.

Keywords: *Rubus chingii* Hu; gallic acid; ellagic acid; *RcDHD/SDH* gene family; transient expression

Introduction

Ellagic acid (EA) is a natural polyphenolic compound derived from gallic acid that's a crucial secondary metabolite in plants. It can be found across a wide variety of botanical species, with particularly high concentrations in fruits like pomegranates, grapes, strawberries, and *R. chingii*^[1-4]. As a typical plant polyphenolic secondary metabolite, EA performs a vital biological function within plants, effectively scavenging reactive oxygen species, endowing plants with antioxidant, antibacterial, and stress-resistant capabilities, and serving as a unique and crucial component of the plant defense systems^[5-7]. Furthermore, extensive research has revealed that ellagic acid also offers potential health benefits for humans, exhibiting notable pharmacological activities including anti-inflammatory, antibacterial, antitumor, hypoglycemic, and immunomodulatory effects, which collectively suggest its broad application prospects in both the prevention and treatment of various chronic diseases^[8-10].

The biosynthesis of ellagic acid in plants depends on the shikimate pathway within the plant tissues^[11]. Its biosynthesis can be divided into three stages: the first stage involves synthesizing the precursor gallic acid (GA) through the shikimate metabolic pathway; the second stage synthesizes the precursor ellagitannin (ET) via the galloylation-degalloylation cycle metabolic pathway; the third stage synthesizes ellagic acid through the hydrolysis metabolic pathway of ellagitannin (ET)^[12-15]. The inaugural stage features shikimate dehydrogenase (SDH) catalyzing the conversion of 3-dehydroshikimate (3-DHS) to gallic acid, serving as the pivotal rate-limiting enzyme in this biochemical cascade. Notably, SDH frequently teams up with 3-dehydroquinase dehydratase (DHD) to form a bifunctional enzyme complex (DHD/SDH) which collaboratively executes the key reaction steps of the shikimate pathway^[16]. In most microorganisms, DHD and SDH as separate enzymes, but in plants, these two enzymes can fuse to create a single bifunctional protein with dual capabilities^[17]. The bifunctional 3-dehydroquinase dehydratase/shikimate dehydrogenase (DHD/SDH) serves as a key regulatory enzyme in the gallic acid metabolic pathway, catalyzing both the dehydration of 3-dehydroquinase to form 3-dehydroshikimate and the reversible reduction between 3-dehydroshikimate and shikimate^[18]. While 3-Dehydroquinic acid can be interconverted with quinic acid through SDH catalysis, it can also be further transformed into 3-dehydro-shikimic acid via DHD/SDH^[19]. SDH facilitates the conversion of 3-dehydro-shikimic acid to gallic acid for entry into the hydrolyzable tannin metabolic pathway, or to shikimic acid for subsequent synthesis of chorismic acid^[20]. The *DHD/SDH* gene family in plants represents a

multigene clan that play distinct roles in the reduction of 3-DHS, the oxidation of shikimic acid (SA), and the synthesis of GA ^[21]. Existing research has indicated that the expression of *DHD/SDH* gene in various species correlates closely with the accumulation of gallic acid and its derivatives. For example, the high expression of *VvSDH3* and *VvSDH4* in grape coincides with gallic acid accumulation^[22]; in tea plants, the expression levels of *CsDHD/SDH3* and *CsDHD/SDH4* show significant positive correlations with gallic acid and its derived epigallocatechin content, while over-expression of these genes enhances the accumulation of related products^[23]; In pomegranate peel, *PgSDH3* and *PgSDH4* gene expression is upregulated under osmotic stress, accompanied by increased gallic acid and hydrolyzable tannin content^[14]. Collectively, these studies indicate that *DHD/SDH* enzymes are considered key regulatory enzymes in the gallic acid biosynthesis pathway ^[24], which also serves as the primary source for GA production in plants ^[25], positioning the *DHD/SDH* gene family at the heart of precursors for ellagic acid.

Rubus chingii Hu is a perennial shrub belonging to the genus *Rubus* within the Rosaceae family. Primarily cultivated in eastern China, particularly in Zhejiang, Fujian, Jiangsu, and Anhui provinces. This remarkable dual-purpose plant—valued equally for its medicinal and culinary applications—holds immense potential for commercial development and boasts a promising economic future. The unripe fruit, known medicinally as “*Rubus chingii* Hu” is one of Zhejiang Province’s “Eight New Zhejiang Herbs” and is officially documented in the Chinese Pharmacopoeia (2025 Edition)^[26]. *R. chingii* are aggregate fruits composed of numerous small drupes. The dried unripe fruit serves as a medicinal herb, and the plant is also earned its common name “Zhangye Fupenzi” due to its palmately lobed leaves. The unripe *R. chingii* fruits pack a significant concentration of secondary metabolites, including ellagitannins, flavanols, and anthocyanins, which act as antioxidants and confer various health benefits ^[27-28]. The primary bioactive compounds in the immature fruits of this species are ellagic acid, tannin, and kaempferol-3-*O*-rutinoside, with the Chinese Pharmacopoeia stipulates that the content of ellagic acid and kaempferol-3-*O*-rutinoside must not be less than 0.20% and 0.03%, respectively. However, commercially available *R. chingii* medicinal materials from the current harvest year often fails to meet this standard for ellagic acid, which significantly compromises their quality and puts a hamper on downstream sales. This makes identifying the key regulatory enzymes for ellagic acid synthesis an urgent priority. Although the *DHD/SDH* gene has been studied in the synthesis of gallic acid and its derivatives in various crops, to date, no functional studies on the *DHD/SDH* gene family in *R. chingii* regarding ellagic acid synthesis have been reported.

Transient genetic transformation has emerged as an increasingly popular alternative to stable transformation. This method offers a faster approach to assessing gene function, yielding results within days ^[29-30]. In plants, transient expression serves as a valuable tool in

various aspects of functional genomics and promoter screening. It enables both over-expression and silencing of candidate genes. Since being independent of the integration of heterologous DNA into chromosomes, the process is relatively straightforward and can lead to high levels of transgenic expression. Recombinant DNA can be introduced into plant cells via physical methods, *Agrobacterium*, or viral vectors [31]. *Agrobacterium*-mediated transient transformation has been successfully employed to assess gene function in numerous plant species [32-35]. In addition, fruit injection represents a novel method for verifying transient gene expression using fleshy fruits as transformation material, characterized by its simplicity and short turnaround time. Previous researches have already employed this technique to conduct successful gene function studies in fleshy fruits like apple, pear, tomato, peach, strawberry, and orange [36], although there's no record of its application in *R. chingii*.

Therefore, to investigate the role of the *RcDHD/SDH* gene in the synthesis and accumulation of gallic and ellagic acid in raspberry fruit, this study employed fruit samples from the prickly-stemmed (HT) and smooth-stemmed (NT) varieties of *R. chingii*, which are predominant in Chun'an District of Hangzhou City. The expression levels of the *RcDHD/SDH* gene family in both HT and NT raspberry varieties were analysed using fluorescent quantitative PCR (qRT-PCR). Additionally, *Agrobacterium*-mediated transient expression technology was employed, where *Agrobacterium* suspensions containing the over-expression vectors pCAMBIA1300-*RcDHD/SDH1*-GFP, pCAMBIA1300-*RcDHD/SDH2*-GFP, pCAMBIA1300-*RcDHD/SDH3*-GFP, and pCAMBIA1300-*RcDHD/SDH4*-GFP, along with wide-type empty vector (WT), were injected into immature fruits to enable transient expression. Catalytic products, gallic and ellagic acid, were detected via HPLC-MS to preliminarily validate *RcDHD/SDH* gene function. Additionally, the constructed over-expression vectors were analyzed for subcellular localization in tobacco via transient transfection. This study contributes to elucidation of the ellagic acid biosynthesis pathway, aims to accumulate valuable data for establishing stable transformation systems in raspberries. It also provides a reference framework for functional validation of other genes using the raspberry transient expression system.

1. Materials and Methods

1.1 Plant materials

Fruits samples were harvested from the *R. chingii* cultivation base in Linqi Village, Chun'an County, Hangzhou City (29°50'N, 119°5'E). The raspberry plants were identified as *R. chingii* by Associate Professor Zhuoni Hou from the School of Life Sciences and Medicine at Zhejiang Sci-Tech University. As shown in Figure 1, fruits from the HT (thorny) and NT (thornless) varieties were collected at four distinct growth stages, with NT fruits generally exhibiting larger dimensions compared to their HT counterparts at equivalent time points. Transient over-expression experiments were conducted using raspberry fruits from the green

stage of both HT and NT varieties of *R. chingii*. The fruit samples were collected between April and May 2025, immediately frozen in liquid nitrogen post-harvest, and subsequently stored at -80°C for future analysis.

The plant over-expression vector utilized was pCAMBIA1300-GFP, while DH5 α competent cells served as the strain for transformation strain for transformation and GV3101 competent cells were employed as the *Agrobacterium* strain for tobacco infection. Both Kanamycin (Kan) and Rifampicin (Rif) were obtained from the laboratory stock at the School of Life Sciences and Medicine, Zhejiang Sci-Tech University.

Seeds of *Nicotiana benthamiana* preserved in the laboratory were sown in a mixed substrate (nutrient soil: vermiculite: perlite = 2:1:1). After covering with plastic wrap, the trays were placed in an artificial climate chamber (day/night temperature: 22°C/16°C, light:dark cycle of 16 h/8 h, relative humidity 72%, light intensity 11,000 Lux) for 28 days. Healthy plants were selected as *Agrobacterium*-mediated transient transformation material for tobacco.

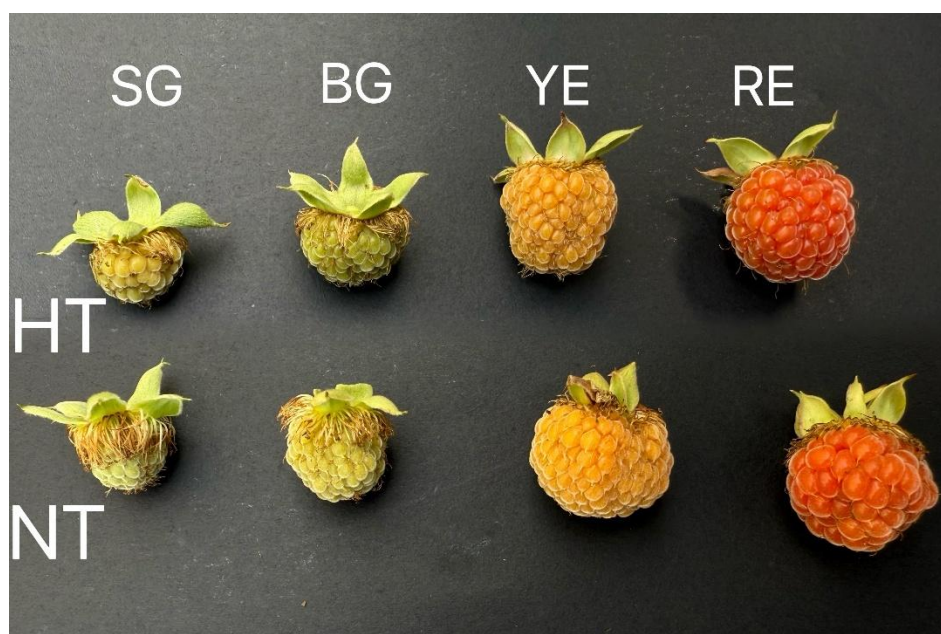


Figure 1: Four growth stages of the HT and NT varieties of the *Rubus chingii* Hu

1.2 Construction of *RcDHD/SDH* Gene Vectors in *Rubus chingii* Hu

The raspberry *RcDHD/SDH1~RcDHD/SDH4* gene sequences were obtained from the *R. chingii* genome database [37]. Primers were also designed for the laboratory-stored pCAMBIA1300-GFP vector based on SmaI (forward) and XbaI (reverse) restriction sites and their flanking homologous arm sequences (with the stop codon removed from the reverse primer) [38-39] (Table 1). Using *R. chingii* cDNA as a template, the full-length sequence of the *RcDHD/SDH* target gene CDS region was amplified with the synthesized cloning primers.

The pCAMBIA1300-GFP empty vector was double-digested with restriction enzymes SmaI and XbaI to obtain a linearized vector, followed by a 30 min incubation at 37°C using the SuperScript™ III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA). The target gene bands were excised from a 1% agarose gel, and the DNA fragment were recovered and purified according to the FastPureGel DNA Extraction Mini Kit (Nanjing Novogene Biotechnology Co., Ltd., Nanjing, China) protocol. The concentration of the DNA was determined by measuring the absorbance of 1 µL using a NanoDrop UV spectrophotometer.

Following the protocol of the ClonExpress® II One Step Cloning Kit (Novogene Bio-Tech Co., Ltd., Nanjing, China), a recombination reaction between the purified *RcDHD/SDH* gene products and the linearized vector was performed to construct the pCAMBIA1300-RcDHD/SDH1~RcDHD/SDH4-GFP over-expression vectors.

To initiate the transformation process, 10 µL of the prepared pCAMBIA1300-RcDHD/SDH1-GFP, pCAMBIA1300-RcDHD/SDH2-GFP, pCAMBIA1300-RcDHD/SDH3-GFP, and pCAMBIA1300-RcDHD/SDH4-GFP were transformed into 100 µL of *E. coli* DH5α competent cells, then gently mix and incubate on ice for 30 min. Heat-shock the bacterial suspension at 42°C for 45 s, then immediately transfer to ice for 5 min cooling. Add 800 µL LB liquid medium to the bacterial suspension and incubate at 28°C for 3 hours. The bacterial should be spread an appropriate amount of bacterial suspension onto LB plates containing 50 mg/L rifampicin and 50 mg/L kanamycin, and then the plates should be incubated at 28°C for 2 days. The isolate of single colonies from the plates is necessary for the purpose of expansion culture. The subsequent steps entail performing PCR identification and amplification, followed by plasmid extraction and restriction enzyme digestion for identification. The transfer of 100 µL of *Agrobacterium* culture into 3 mL of LB liquid medium (containing 50 mg/L of rifampicin and 50 mg/L of kanamycin) is to be conducted, followed by incubation for a period of 2 days until the OD600 value reaches 0.6-0.8. Subsequently, perform plant transformation is to be performed using *Agrobacterium* containing the corresponding plasmid. Positive colonies that were identified as containing the target gene pCAMBIA1300-GFP fragment were subsequently sent to Youkang Biotechnology Co., Ltd. (Hangzhou) for the purpose of sequencing. Colonies with confirmed sequencing results were purified using a plasmid extraction kit to obtain pCAMBIA1300-RcDHD/SDH1~RcDHD/SDH4-GFP plasmids for subsequent use.

1.3 Subcellular Localization of the *RcDHD/SDH* Gene Family

In order to facilitate subcellular localisation analysis, the coding sequences (CDS) of *RcDHD/SDH1* to *RcDHD/SDH4* were integrated into the expression vector pCAMBIA1300-GFP, in conjunction with g with the empty pCAMBIA1300-GFP vector lacking the stop codon. The transformation of the recombinant vectors were transformed into *Agrobacterium*

tumefaciens GV3101 (Weidi Biotechnology, Shanghai) was conducted via the freeze-thaw method [40]. Positive clones were then cultivated on LB solid medium, containing rifampicin and kanamycin, for a period of 2-3 days. Following this, the culture should be adjusted to an A600 of 0.8-1.0, and the cells should be collected by centrifugation at 8000 r/min for 15 min. The cells should then be resuspended in prepared buffer (10 mmol/L MgCl₂, 10 mmol/L MES, 100 µmol/L AS) to an A600 of 0.8. The initial phase of the procedure is to incubate at room temperature in the dark for a period of 2-3 hours. The subsequent stage involves the selection of young, vigorous tobacco leaves, which are then treated with the nuclear localization dye *mCherry*, thus enabling the visualization of the A600 to 0.8 is to be performed using the L⁻¹ AS. The initial phase of the process is to incubate at room temperature in the dark for a period of 2-3 hours. It is also imperative to select young, healthy tobacco leaves. The injection of the inject of the suspension containing the nuclear-localising dye *mCherry* and the constructed plasmid into the lower epidermis of 28-day-old tobacco leaves is to be conducted using a needle-free syringe. Following a 24-hour period in the absence of light, the photoperiodic culture for should be resumed for a duration of 48 hours. A disposable sharp blade was utilised to meticulously slice the expressed tobacco leaf tissue (1 cm²) into the microscope slide, which had been previously moistened with water droplets, thereby facilitating for direct observation. Subsequently, signals were detected under a Confocal scanning imaging microscope (CSIM110, Sunny Technology, China) at 488 nm excitation (for GFP) and 610 nm emission (for *mCherry*). It is imperative to observe the distribution of green fluorescent protein (GFP) fluorescence. The utilisation of tobacco leaves infected with pCambia1300-GFP empty vector-carrying *Agrobacterium* constitutes a control mechanism for the analysis of the subcellular localization of the pCambia1300-RcDHD/SDH1~RcDHD/SDH4-GFP fusion protein.

1.4 Transient Over-expression in *R. chingii*

The ligation product must then be transformed into *Agrobacterium* GV3101. Positive clones were cultivated in YEB liquid medium containing 50 mg/L kanamycin and 50 mg/L rifampicin. The culture was subjected to agitation at 220 rpm at 28°C until the optical density (OD) at 600 nm reached approximately 0.6-0.8. The cells were subjected to a centrifuged process, after which they were and resuspended in a buffer specifically designed for tobacco infection buffer (10 mM MgCl₂, 10 mM MES, 100 µM acetophenone, pH 5.6). The bacterial density of the cells was then adjusted to an A₆₀₀ bacterial density of 0.6-0.8, then incubated in the dark for period of 2-3 hours.

The selection of green fruits of similar maturity is paramount. Using a 1 mL ultra-fine needle syringe, inject 100 µL of the *Agrobacterium* suspension containing the aforementioned plasmid into the green raspberry fruit. The following incubation times are to be set: 0 h, 12 h, 24 h, 48 h, and 60 h. A negative control is to be established by injecting the permeate of an

empty *Agrobacterium* strain carrying pCAMBIA1300-GFP. An investigation must be conducted into the effect transient transformation efficiency for gene over-expression. Approximately 100 fruits were utilised in each experiment.

1.5 Fluorescent Quantitative Reverse Transcription PCR (RT-qPCR) Analysis

Total RNA was isolated from raspberry fruits using the RNeasy Pure Plant Kit (Qiagen, China). The RNA content was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) [41-42]. Transcriptional levels of the *RcDHD/SDH* gene family were analysed via SYBR Green real-time PCR on the CFX96™ Real-Time System (Bio-Rad) [43-44]. Primers for the *RcDHD/SDH* genes are shown in Table 1. Real-time PCR reactions were then performed using SYBR Premix ExTaq™ (Invitrogen) in accordance with the procedures delineated in the product manual. The reaction volume was determined to be 9 µL of Real Master Mix (SYBR Green TIANGEN), 2 µL of cDNA, 1 of µL each primer (10 µM), and 7 µL of RNase-free water, yielding a total volume of 20 µL. Transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method with raspberry *EEF1A* as the internal control via relative quantification. Real-time PCR data were obtained from three biological replicates. the specific primer pairs for pCAMBIA1300-*RcDHD/SDH1~RcDHD/SDH4-GFP* in RT-qPCR were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). See Table S1. The experimental design comprised three biological replicates and three technical replicates. The relative gene expression levels were calculated using the $2^{-(\Delta\Delta C_t)}$ method as outlined by [45-46].

Table 1 Primers of *RcDHD/SDH* used for RT-qPCR analysis, gene cloning and function verification.

Gene name	Primer name	Upstream primer (5'→3')	Downstream primer (5'→3')
<i>RcDHD/SDH</i>	DHD/SDH1	ATGGACTATGAAAACATTAC	AGTTCTCAATGAGAGGTGTT
	DHD/SDH2	ATGGGGAGTTCAACCCTTAT	AGTTCTCGATAGCAGGTCTTGA
	DHD/SDH3	ATGACTCTCAGCAGTGTTCCG	AATCAGATAGTGTTAACCAAGT
	DHD/SDH4	ATGGAGAAGATGGATTCCAAAG	AATACGTACAAAGAGGTACTCG
	DHD/SDH1	GGGACgagctcggtaccgggATGGAC TATGAAAACATTACGCTTGCTTC	CTTGCTCACCATCTGCAGCTgAG AGTTACTCTCCACAACCTTTCCTG AAGAGTTC
<i>OE- RcDHD/SDH</i>	DHD/SDH2	TCATTTGGAGAGAACACGGGGG ACgagctcggtaccgggATGGGGAGT TCAACCC	CTTGCTCACCATCTGCAGCTgAG AGCTATCGTCCAGAACTTTCCTA AAGAGTTC
	DHD/SDH3	GGGACgagctcggtaccgggATGAC TCTCAGCAGTGTTCCGTTGGCT AC	CTTGCTCACCATCTGCAGCTgGTC TATCACAATTGGTTCATCGTTGAG GTTGCC
	DHD/SDH4	GGGACgagctcggtaccgggATGGA GAAGATGGATTCCAAAGTACCG GA	CTTGCTCACCATCTGCAGCTgTGC ATGTTTCTCCATGAGCTCCCTAAA CAATTC

qPCR- RcDHD/SDH	EEFIA	CCTTTCGCGCTCAGCCTTGA	AAGTCGACCACCACGGGTCA
	DHD/SDH1	GTGTTCGGTATAATTGGAAAGC	CTATTGACTTGGCAACTGGAT
	DHD/SDH2	GATCCGGTTGCCAAGTCCATAG	GCAGAAATAGCCCCAAAATAGTC
			C
	DHD/SDH3	TTGCCAACTCTTGTCACCTTAC	ATATAATCTGCTCCCAATTCCAT
	DHD/SDH4	GTCACAGCAAAAGTCCTCTA	TGGCAATGGAATCAATCTCA

1.6 HPLC Determination of Gallic Acid and Ellagic Acid Content

HPLC Analysis: Quantitative analysis of raspberry constituents (gallic acid and ellagic acid) was performed using reverse-phase high-performance liquid chromatography (LC-20A HPLC system with SPD-20A detector; Shimadzu Corporation, Kyoto, Japan)^[47-48], with extraction and processing methods referenced from the Chinese Pharmacopoeia. Approximately 0.5 g of raspberry fruit powder, passed through a No. 4 sieve, should be taken, precisely weighed it, and placed it in a stoppered conical flask. The precise volume of 70% methanol required is 50 mL. the flask should then be weighed. The substance should then be placed in a water bath at 60°C for one hour, after which it should be cooled to room temperature and reweigh. The addition of 70% methanol is necessary to compensate for weight loss, and the solution should be shaken well and filtered. The filtrate should be measured out with a precision of 1 mL and transferred into a 5 mL volumetric flask. It should then be diluted to the mark with 70% methanol, shaken well, and the extracted juice filtered through a 0.22 µm membrane filter. The substance must be stored in a 1.5 mL amber vial for the purpose of HPLC analysis. The following conditions are required for HPLC detection conditions: Column: The mobile phase was composed of Thermo ODSC 18 (250 mm × 4.6 mm, 5 µm). Acetonitrile (A) - 0.2% phosphoric acid aqueous solution (B), gradient elution (0-8 min, 15% A; 8-23 min, 15% → 33% A; 23-39 min, 33% → 42% A; 39-45 min, 42% → 28% A; 45-60 min, 28% → 15% A); Flow rate: 1.0 mL·min⁻¹; Column temperature: The operating temperature was set at 30°C and the detection wavelengths were 254 nm (ellagic acid) and 271 nm (gallic acid). The injection volume was set at 10 µL. The content was determined by comparison with gallic acid and ellagic acid standards (source: leaves). All samples were analysed in triplicate biological replicates.

1.7 Statistical Analysis

The statistical significance of the observed differences between the treatments was determined by employing IBM SPSS Statistics 27.0 software (SPSS, Chicago, USA) and GraphPad Prism 10. The collected data underwent a multifactorial analysis of variance, followed by least significant difference (LSD) test. A P-value of less than 0.05 indicating significant difference. All data are expressed as mean ± standard deviation (Mean ± SD). The generation of all figures and tables in this paper was conducted utilising GraphPad Prism 10,

TBtools II, Image, and Microsoft Excel 2023.

2 Results and Discussion

2.1 Cloning of the *Rubus chingii* Hu *RcDHD/SDH* Genes and Construction of Expression Vectors

The utilisation of raspberry fruit cDNA as a template, was instrumental in facilitating PCR amplification, a process that was pivotal in the successful cloning of the *RcDHD/SDH1* to *RcDHD/SDH4* genes. The resulting gene fragments were 1626 base pairs (bp), 1335 bp, 3990 bp, and 3834 bp in size, respectively. The obtained nucleotide sequences were found to match the target gene sequences, thus confirming the successful cloning of the raspberry *RcDHD/SDH* genes. Following the ligation of the plant expression vector pCAMBIA1300-*RcDHD/SDH1*~*RcDHD/SDH4*-GFP into *E. coli* competent cells DH5 α , a single colony was selected for bacterial PCR analysis (Figure 2). Subsequent sequencing by the company confirmed sequence identity with the target gene, thereby verifying the successful construction of the pCAMBIA1300-*RcDHD/SDH1*~*RcDHD/SDH4*-GFP vector.

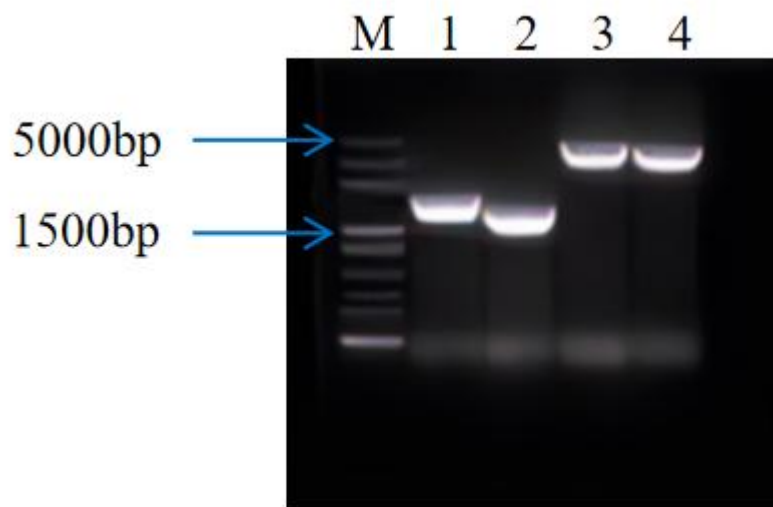
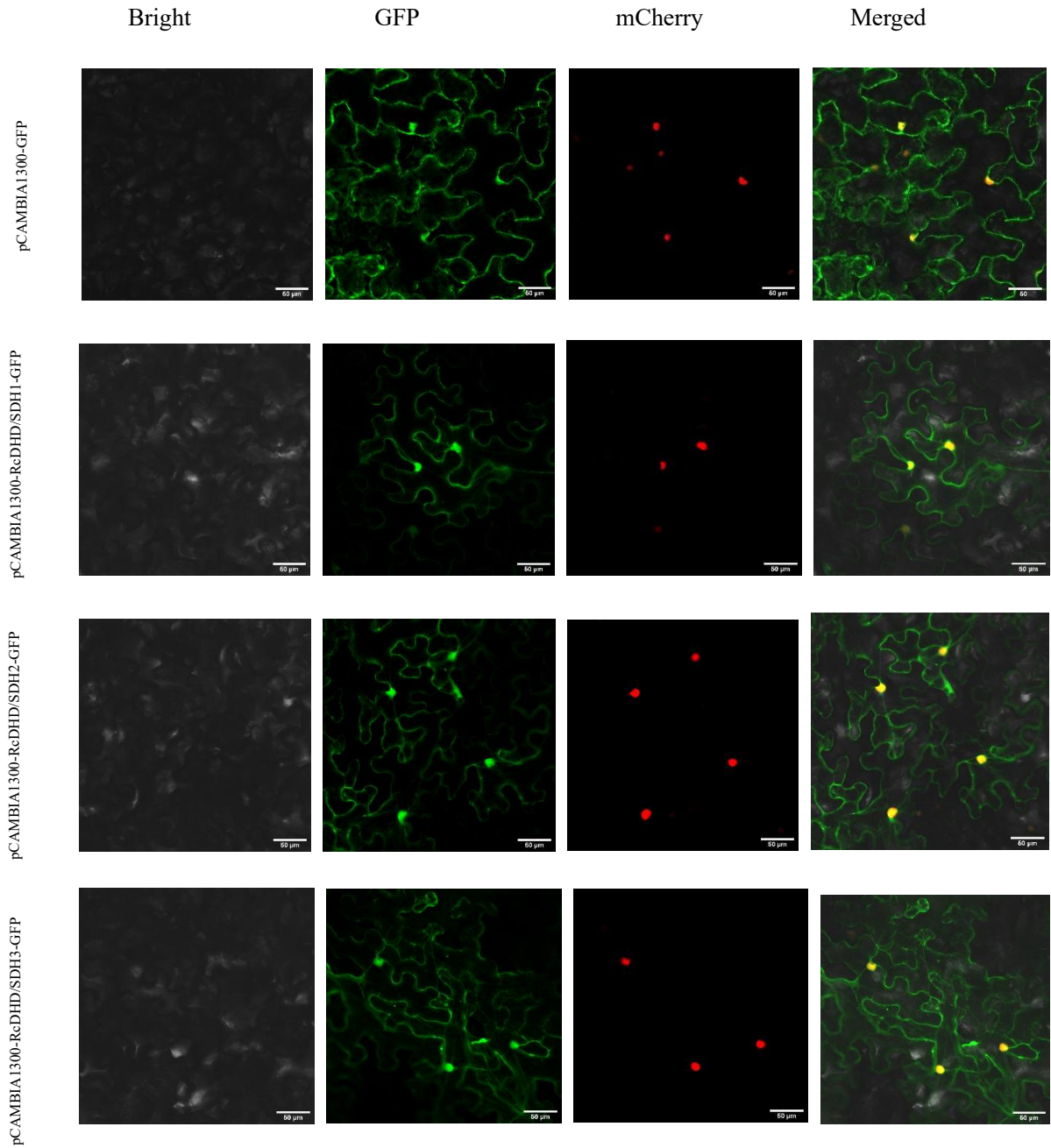


Figure 2 PCR Detection Gel of pCM1300-*RcDHD/SDH1*~*DHD/SDH4*-GFP Bacterial Culture

2.2 Subcellular Localization of pCAMBIA1300-*RcDHD/SDH1*~*RcDHD/SDH4*-GFP in *Rubus chingii* Hu

In order to ascertain the precise subcellular localisation of *RcDHD/SDH*, the successfully constructed pCAMBIA1300-*RcDHD/SDH1*~*RcDHD/SDH4*-GFP over-expression vectors and the pCAMBIA1300-GFP empty vector were transformed into GV3101 *Agrobacterium* via *Agrobacterium*-mediated transient expression in tobacco. These vectors were then

introduced into tobacco leaves, which serve as the model plant in this study. Fluorescence localization was observed using laser confocal microscopy. The results demonstrated that under different excitation and emission light conditions, the influence of chloroplast autofluorescence could be excluded. The nuclear localization markers that have been previously identified were found to exhibit red fluorescence, which coincided with the green fluorescence of the GFP marker. This finding suggests that RcDHD/SDH colocalizes with the nucleus (Figure 3).



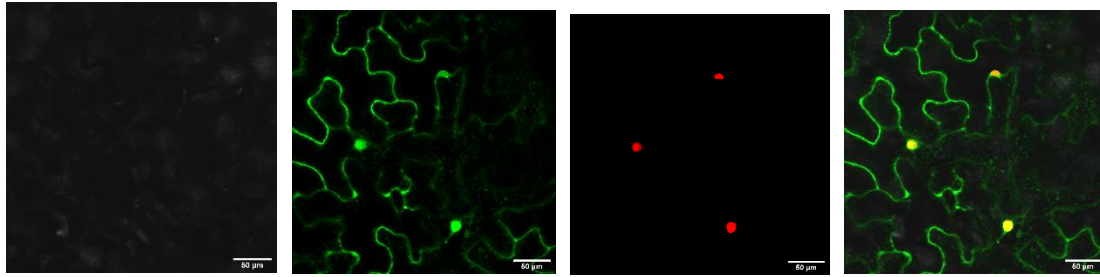


Figure 3 Subcellular localization of *RcDHD/SDH* in tobacco leaf epidermis

2.3 Expression Analysis of the *RcDHD/SDH* Gene Family in Raspberries

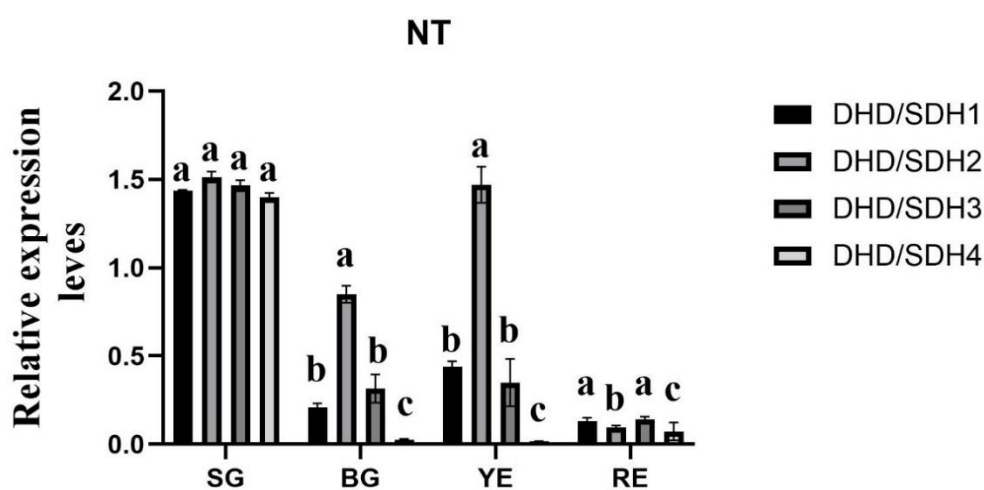
In order to investigate the expression characteristics of the *RcDHD/SDH* gene family during raspberry fruit development, the relative expression levels were analysed across four developmental stages (SG: small green fruit; BG: big green fruit; YE: yellow fruit; RE: red fruit) in both NT and HT cultivars (Figure 4). The results demonstrated that the *RcDHD/SDH* gene family exhibited elevated expression levels during the SG stage, followed by a gradual decline as fruit matured, thereby indicating its crucial role in early fruit metabolic activities.

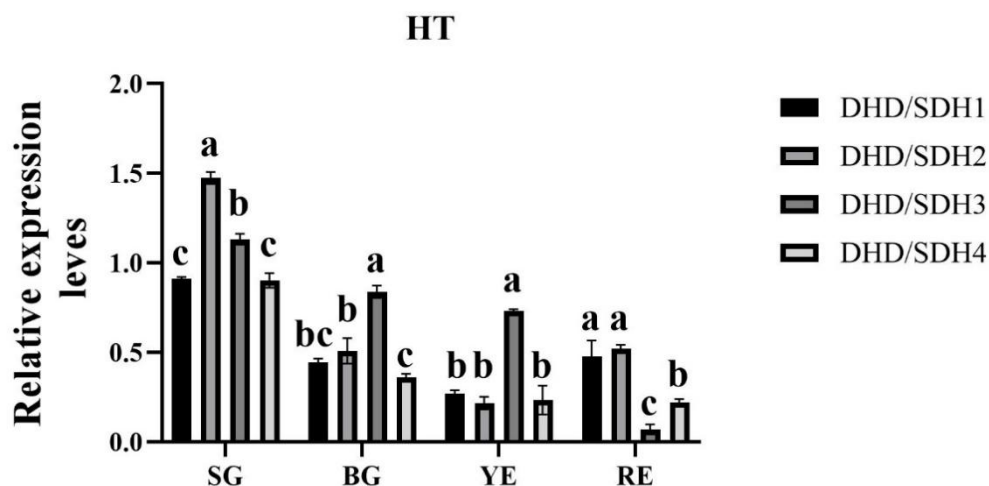
In the NT cultivar, all four members of the *RcDHD/SDH* gene family attained peak expression levels during the SG stage with no significant differences among them. The average expression values of the former were significantly higher than those of the latter, which correspond to the mid-to-late stages of NT. As fruit development progressed into the BG and YE stages, there was an overall decline in expression. However, *RcDHD/SDH3* demonstrated a significant expression peak during the YE stage, which was markedly higher than that of the other members ($P < 0.05$). This finding suggests the potential involvement of *RcDHD/SDH3* may participate in specific metabolic processes during fruit coloration, such as the regulation of phenylpropanoid or phenolic acid synthesis. By the RE stage, expression of all four genes decreased to lower levels, indicating that the *RcDHD/SDH* gene family is primarily expressed during the early to mid-stages of fruit development, with transcriptional activity regulated after ripening.

In the HT variety, the expression of all four genes was found to be relatively high during the SG stage. As fruit ripened, the decline in expression for each gene was smaller, exhibiting a relatively flat dynamic change curve. *RcDHD/SDH1* and *RcDHD/SDH2* exhibited reduced expression during the YE stage and increased expression during the RE stage, although overall expression remained lower than during the SG stage. *RcDHD/SDH3* and *RcDHD/SDH4* exhibited a comparatively stable downward trend across all four stages.

In summary, the *RcDHD/SDH* gene family exhibits distinct expression patterns across different stages of raspberry fruit development (Figure 4). In summary, the expression of all genes reached its zenith during the SG stage, subsequently declined as fruit matured, and attained its nadir during the RE stage. The four genes in NT demonstrated no significant

expression differences during the SG stage, indicating synchronous activation of this family during the initial phases of fruit development. *RcDHD/SDH3* exhibited a significant expression peak in NT during the YE stage, substantially higher than other genes at the same stage ($P < 0.05$), suggesting that this gene may participate in regulating the synthesis of phenolic metabolites such as gallic acid and ellagic acid. In HT, the overall expression levels of the four genes were lower than in NT, and no similar mid-stage peak of a similar nature was observed, instead showing a more gradual decline. It was evident that the expression levels of *RcDHD/SDH1* and *RcDHD/SDH3* in the HT sample were notably lower in comparison to the NT sample, particularly during the SG stage. This finding suggests that the activation of these genes was less pronounced in the early fruit stage. The results of this study suggest that the *RcDHD/SDH* gene family is predominantly active during the initial to mid-stage of fruit development. In comparison with HT, NT demonstrates heightened expression activity of this gene family during this particular period. The observed differences in expression between the two varieties may be indicative of variations in metabolic pathway activity and transcriptional regulatory mechanisms, thereby providing a foundation for further elucidating the role of the *RcDHD/SDH* gene family in fruit development and phenolic compound accumulation.





b

Figure 4: Expression Analysis of the *RcDHD/SDH* Gene Family in *Rubus chingii* Hu at Different Growth Stages

2.4 Analysis of Gallic Acid and Ellagic Acid Content in Raspberries via *RcDHD/SDH* Genes

The present study investigates the relationship between *RcDHD/SDH* gene expression and phenolic metabolite accumulation during raspberry fruit development. To this end, gallic acid and ellagic acid contents were measured at four developmental stages (SG, BG, YE, RE) of two varieties: HT and NT.

As demonstrated in Figure 5a, gallic acid accumulated in both varieties throughout the process of fruit development. In general, the NT sample demonstrated a higher level of gallic acid in comparison to the HT sample at all stages of the experiment. A number of significant differences were observed between the two varieties at the SG and YE stages ($P < 0.01$ and $P < 0.0001$, respectively), with a significant difference also observed at the RE stage ($P < 0.05$). However, no significant difference was detected at the BG stage ($P > 0.05$). This finding suggests that the thornless variety displays increased accumulation of gallic acid during the initial and medial phases of fruit development, a phenomenon that may be associated with elevated expression levels of DHD/SDH genes within its phenolic synthesis pathway.

The trend in ellagic acid content differed from that of gallic acid (Figure 5b). In general, the level of ellagic acid in NT was found to be considerably higher than in HT, with highly significant variations observed, especially during the SG, BG, and YE stages. However, no significant differences were detected at the RE stage. In NT varieties, ellagic acid levels peaked early in fruit development and then gradually declined; in HT varieties, the overall content remained lower with smaller fluctuations. It is hypothesised that, in accordance with

the observed trends in gallic acid, ellagic acid may be synthesised from its precursor gallic acid through a process of oxidation and dimerisation. It is hypothesised that higher expression of the *RcDHD/SDH* genes enhances the activity of this metabolic pathway during the early to mid-development stages, thereby promoting ellagic acid accumulation.

In summary, the *RcDHD/SDH* gene may participate in phenolic metabolism pathways during raspberry fruit development by regulating the synthesis of gallic acid and ellagic acid. The NT cultivar exhibited higher metabolic activity, suggesting a potential close association with defense and antioxidant mechanisms during early fruit development.

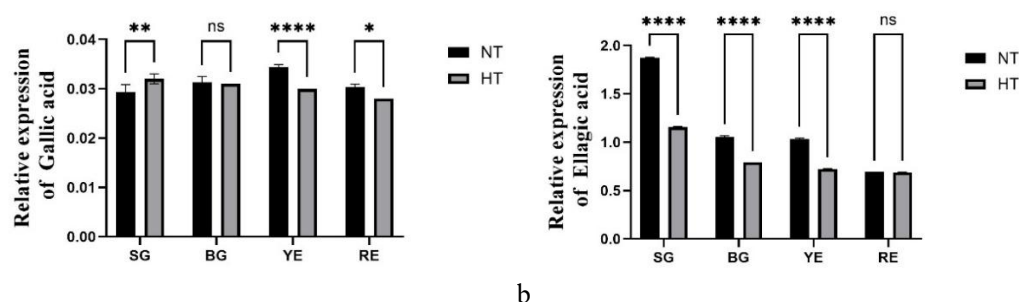


Figure 5: Gallic Acid and Ellagic Acid Content in NT and HT *Rubus chingii* Hu at Different Developmental Stages

2.5 Analysis of Transient Expression of *RcDHD/SDH* Genes in *Rubus chingii* Hu

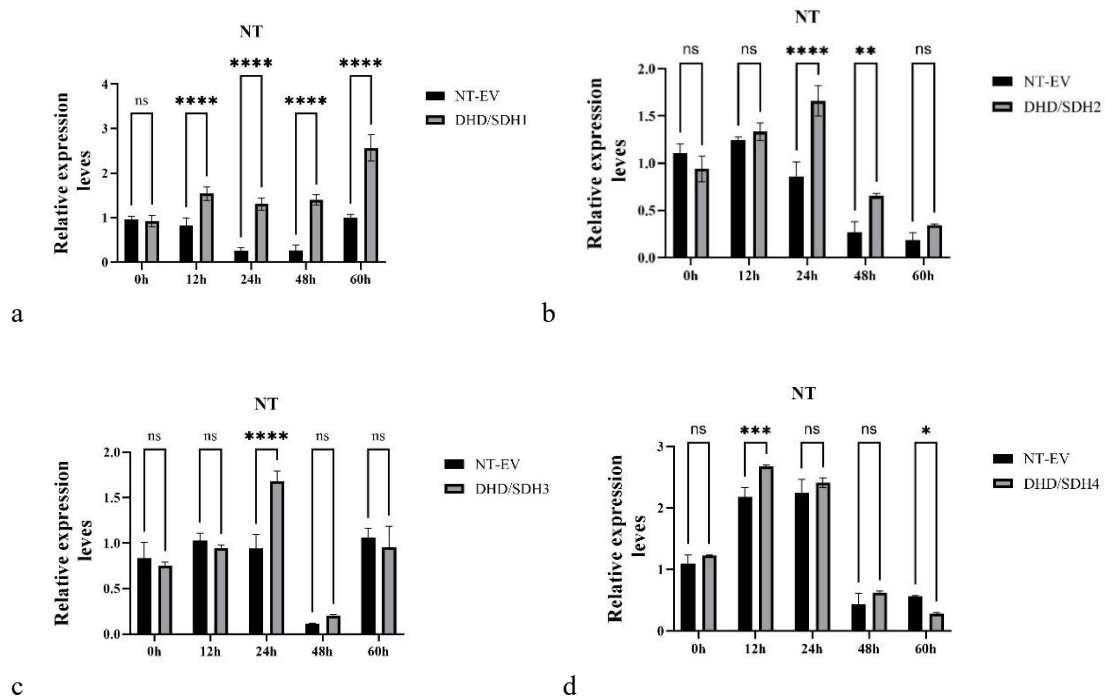
In order to provide further clarification regarding the potential influence of *RcDHD/SDH* on changes in gallic acid and ellagic acid, this study employed a transient injection approach. In this method, pCAMBIA1300-GFP recombinant vectors carrying *RcDHD/SDH1-RcDHD/SDH4*, in addition to the empty vector pCAMBIA1300 (EV), which lacked the target gene, were injected into HT and NT palmate raspberry fruits. The temporal framework encompassed the 0-hour, 12-hour, 24-hour, 48-hour, and 60-hour time points post-injection. The results are presented in Figure 6 (a-h). All data are presented as mean $\pm$ standard deviation, with significant differences determined by Tukey's multiple comparison test ($p < 0.05$).

In the case of NT raspberries, the transient expression patterns of *RcDHD/SDH* family members (see Figure 5) demonstrated that *RcDHD/SDH1* expression rapidly increased at 12 hours and peaked between 24 hours and 48 hours, being significantly higher than the empty vector control ($p < 0.0001$). This finding indicates its dominant role in the early stages of the metabolic pathway. *RcDHD/SDH2* demonstrated significant induction during the 24-48 h phase, exhibiting a mid-stage response characteristic. In contrast, *RcDHD/SDH3* was only transiently upregulated at 24 h. *RcDHD/SDH4* exhibited a biphasic increase at both 12 h and 60 h, suggesting its potential involvement in metabolic feedback regulation. The results indicate that *RcDHD/SDH1* and *RcDHD/SDH2* exhibit stronger transcriptional activity in NT

raspberries, thus serving as key components in the promotion of gallic acid and its derivative synthesis. Conversely, *RcDHD/SDH3* and *RcDHD/SDH4* are likely to play auxiliary roles in the regulation of pathways and metabolic homeostasis. The present findings indicate that the expression of the *RcDHD/SDH* gene family in NT raspberries undergoes temporal dynamic regulation. The assumption of specialized functions by different members at distinct stages within the phenolic metabolism pathway reflects a collaborative transcriptional regulatory pattern. This pattern is characterised by division of labour within this metabolic pathway.

Following the extraction of the four *RcDHD/SDH* genes from HT raspberry fruits, transient expression exhibited varying degrees of induction. Overall, *RcDHD/SDH1* and *RcDHD/SDH4* exhibited significantly elevated expression levels, peaking between 24 and 48 hours, indicating high expression efficiency. *RcDHD/SDH3* exhibited sustained upregulation, indicative of stable, high expression. In contrast, *RcDHD/SDH2* demonstrated a comparatively modest upregulation, predominantly at the 12-hour and 48-hour stages, suggesting a potential role in early induction.

In summary, the results demonstrate a significant induction of all four *RcDHD/SDH* genes upon their expression in HT fruits. Among them, *RcDHD/SDH4* exhibited higher expression levels, while *RcDHD/SDH3* exhibited a sustained upregulation trend. In addition, *RcDHD/SDH1* and *RcDHD/SDH2* likely participate in metabolic regulation at different time points. This finding indicates that these genes may collectively contribute to the regulation of ellagic acid and related polyphenolic compound synthesis in HT raspberries through time-specific expression patterns.



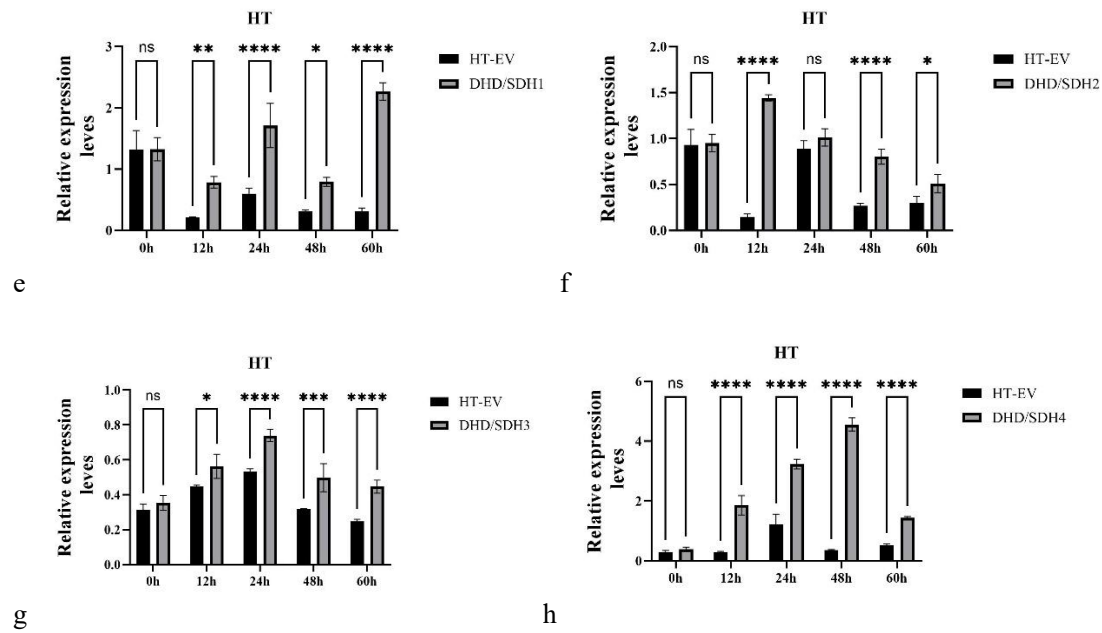


Figure 6. Transient expression analysis of the *RcDHD/SDH* gene family in NT and HT *Rubus chingii* Hu

2.5 Analysis of *RcDHD/SDH* Genes on Ellagic Acid and Gallic Acid Content in *Rubus chingii* Hu

A HPLC-MS/MS quantitative analysis was conducted in order to examine the effects of transiently expressed *RcDHD/SDH* gene family members (*RcDHD/SDH1*-*RcDHD/SDH4*) on the content of ellagic acid and gallic acid in *Rubus chingii* Hu. Furthermore, a comparison was made between the differences in secondary metabolite regulation observed between the HT and NT cultivars. The results obtained demonstrate that different genes exhibit significant differences in the way they regulate phenolic acid content, with distinct variety-specific response patterns observed between HT and NT cultivars.

The *RcDHD/SDH* gene family was found to significantly regulate ellagic acid and gallic acid synthesis in raspberry fruit, with distinct expression patterns between HT and NT varieties. In both varieties, *RcDHD/SDH1* and *RcDHD/SDH2* were identified as the major regulatory genes, with the capacity to promote the synthesis of ellagic acid and gallic acid. In contrast, *RcDHD/SDH3* and *RcDHD/SDH4* were found to be significantly activated in the HT variety, suggesting a potential role in regulating late-stage phenolic acid synthesis. Furthermore, HT varieties were observed to exhibit enhanced late-stage phenolic acid accumulation capacity through transient expression over brief periods. This finding supports the hypothesis that the *RcDHD/SDH* gene family may play a pivotal role in the raspberry ellagic acid biosynthesis pathway by enhancing the conversion efficiency of 3-dehydrokaempferol to gallic acid, thereby enhancing the downstream ellagic acid biosynthetic efficiency.

2.5.1 Expression Patterns of Gallic Acid and Ellagic Acid in NT Varieties

As demonstrated in Figure 7a, the over-expression of all four *RcDHD/SDH* genes in NT resulted in a significant increase in gallic acid accumulation. In comparison with the control group (NT-EV), *DHD/SDH1* and *DHD/SDH2* attained peak expression at 24 hours, while *DHD/SDH3* and *DHD/SDH4* sustained elevated levels at 48 hours. In general, all four genes demonstrated swift increases within the initial 12 hours, followed by modest decreases after attaining mid-stage peaks (24-48 hours), suggesting that their regulatory functions primarily occur during the early-to-mid metabolic phases. The extent of influence exhibited by the genes under investigation varied, with *RcDHD/SDH1* exhibiting the strongest promotion effect, followed by *RcDHD/SDH3*. These changes suggest that in NT varieties, *RcDHD/SDH* genes may enhance downstream phenolic metabolism flux by accelerating gallic acid production and accumulation, thereby providing more precursor substances for ellagic acid and its derivative synthesis.

As demonstrated in Figure 7b, ellagic acid also exhibited an upward trend across the four overexpressed samples, though its overall change was less pronounced than gallic acid's, with a slightly delayed peak time. *RcDHD/SDH1* and *RcDHD/SDH2* exhibited the highest levels of ellagic acid at 24 hours, whereas *RcDHD/SDH3* and *RcDHD/SDH4* maintained elevated levels of this compound even after 48 hours of expression, indicating their sustained role in the later stages of ellagic acid accumulation. The results of this study indicate that different members of the gene family may participate in distinct metabolic stages of the ellagic acid synthesis pathway. This suggests the existence of a temporal division of labour and functional complementarity.

It is evident that the *RcDHD/SDH* family exerts a discernible positive regulatory effect on NT raspberries, as evidenced by the combined trends of gallic acid and ellagic acid. *RcDHD/SDH1* and *RcDHD/SDH2* appear to be predominantly responsible for the promotion of early gallic acid synthesis, with a rapid acceleration in its conversion to ellagic acid. In contrast, *RcDHD/SDH3* and *RcDHD/SDH4* are likely to play a pivotal role in maintaining product stability during the later metabolic stages, thereby contributing to the sustained accumulation of ellagic acid. This finding suggests that the DHD/SDH gene family exerts synergistic and stage-specific regulatory functions in raspberry phenolic metabolism. Its role may involve modulating the conversion between gallic acid and ellagic acid pathways, thereby influencing the synthesis efficiency of downstream phenolics such as ellagitannins.

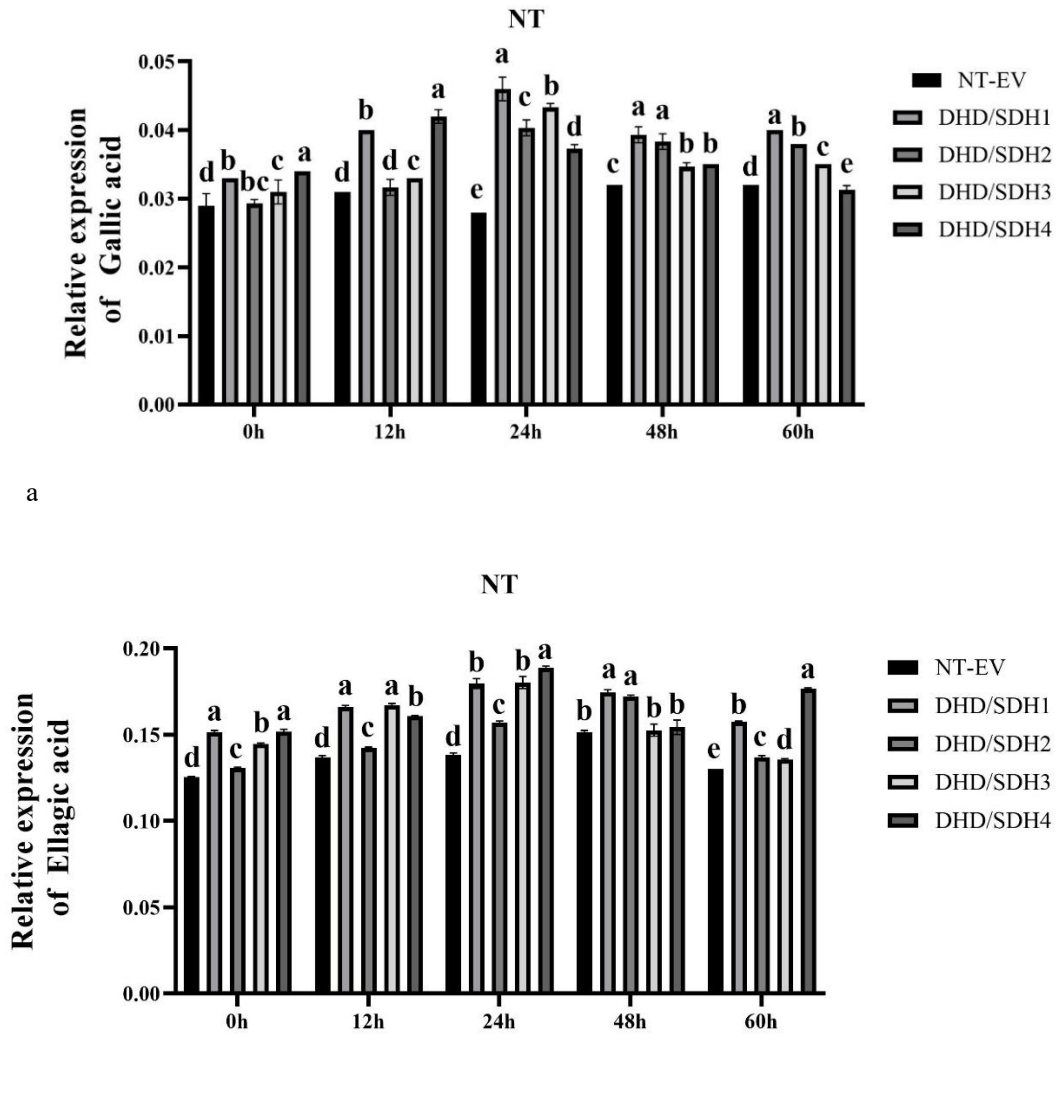


Figure 7 Analysis of gallic acid and ellagic acid content in NT *Rubus chingii* Hu during transient expression

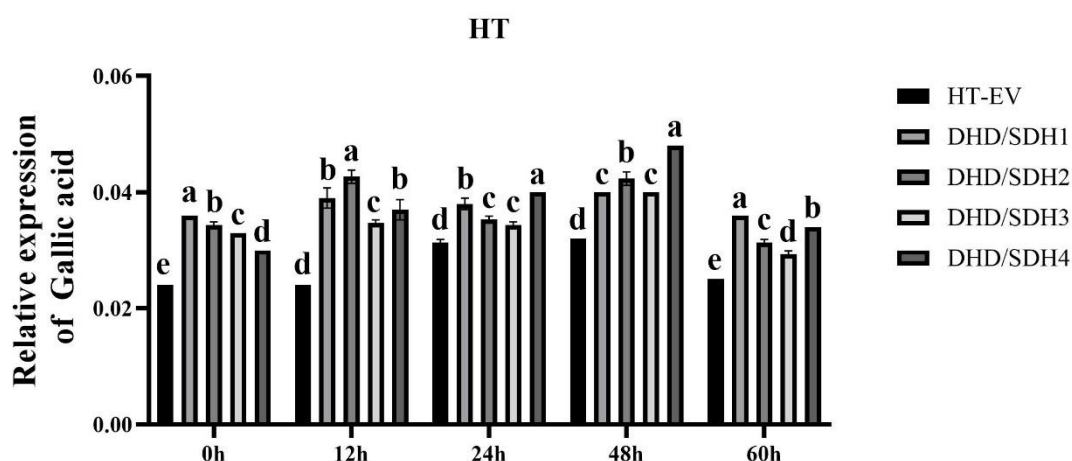
2.5.2 Expression Patterns of Gallic Acid and Ellagic Acid in HT Varieties

In raspberry fruits in which the *RcDHD/SDH* gene family is overexpressed under HT conditions, the relative expression levels of gallic acid were consistently higher than those in the control group (HT-EV), with the most significant differences observed at 12 h and 48 h. Specifically, *RcDHD/SDH1* reached its peak at 12 h, while *RcDHD/SDH2* and *RcDHD/SDH4* exhibited the highest levels of expression at 48 h. *RcDHD/SDH3* showed smaller overall fluctuations but remained elevated compared to the control. This finding suggests that transient expression of all four genes promotes gallic acid accumulation, with *RcDHD/SDH1* and *RcDHD/SDH4* exhibiting more pronounced effects.

The content of ellagic acid was found to be significantly higher than that of the control at all time points, and its accumulation pattern exhibited a certain time lag relative to gallic acid accumulation. *RcDHD/SDH1* and *RcDHD/SDH2* exhibited peak ellagic acid accumulation at

12 hours, followed by a gradual decline; whereas *RcDHD/SDH3* and *RcDHD/SDH4* maintained elevated ellagic acid levels at 24 hours and 48 hours post-overexpression. This finding is consistent with the observed accumulation patterns of ellagic acid in the engineered strains, further substantiating the hypothesis that distinct *DHD/SDH* members may function at discrete stages of the ellagic acid synthesis pathway.

When considered in conjunction with the results of transient expression, the over-expression of *RcDHD/SDH1* and *RcDHD/SDH2* appears to result primarily in the early accumulation of gallic acid and ellagic acid. By contrast, *RcDHD/SDH3* and *RcDHD/SDH4* appear to contribute to the maintenance of stable levels during the middle and late stages. In conclusion, the expression of all four *RcDHD/SDH* genes was found to positively regulate the synthesis of the two phenolic metabolites in question. This finding indicates their synergistic roles in raspberry phenolic biosynthesis pathways, albeit with differences in regulatory intensity and temporal phases. These findings further validate the pivotal role of *DHD/SDH* genes in the conversion of gallic acid to ellagic acid, providing a basis for elucidating their functional differentiation and metabolic regulatory mechanisms.



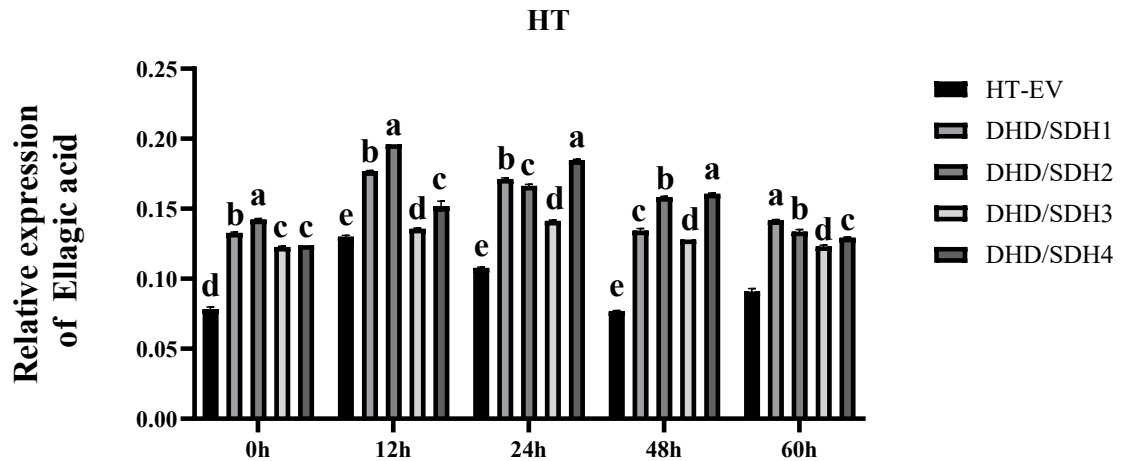


Figure 8 Analysis of gallic acid and ellagic acid content in HT *Rubus chingii* Hu during transient expression

2.6 Expression Patterns of *RcDHD/SDH* Genes Following Over-expression in NT and HT *Rubus chingii* Hu and Their Relationship with Metabolite Accumulation

In order to further investigate the functional differences among *RcDHD/SDH* family members in different raspberry cultivars, transient expression analysis of four *RcDHD/SDH* genes was conducted in both NT and HT cultivars. The results demonstrated that all four genes exhibited significant induced expression at various time points, with notable variations in expression intensity and response time (Fig. 6). In the present study, the expression levels of *RcDHD/SDH1* and *RcDHD/SDH4* were found to rapidly increase after 12 hours, reaching a peak between 48 and 60 hours. Although *RcDHD/SDH2* and *RcDHD/SDH3* exhibited lower overall expression levels, both showed significant enhancement around 24 hours (Fig. 7). Conversely, the expression levels of all four genes were found to be comparatively reduced in HT, with *RcDHD/SDH4* exhibiting the most significant induction between 24-48 hours (Fig. 8). The expression levels of the other members gradually declined in subsequent stages. This finding suggests that NT varieties exhibit higher transcriptional responsiveness to exogenous *DHD/SDH* genes, while HT varieties maintain relatively conservative regulatory mechanisms

This study constitutes the initial systematic analysis correlating the dynamic expression of *RcDHD/SDH* genes with alterations in ellagic acid and gallic acid content. A thoroughgoing comparison of metabolic responses between NT and HT varieties discloses that the metabolic promotion effect of *DHD/SDH* genes in NT raspberries is, in general, higher than in HT varieties. This phenomenon can be attributed to the presence of more active secondary metabolic pathways and a sufficient substrate supply in NT fruits. Furthermore, the accumulation patterns of gallic acid and ellagic acid, in conjunction with the observed

differences in expression among various genes, suggest the presence of distinct regulatory sites within this metabolic pathway. *RcDHD/SDH1* and *RcDHD/SDH2* appear to be more inclined to catalyse the early accumulation of gallic acid, while *RcDHD/SDH3* and *RcDHD/SDH4* may participate in the sustained formation of ellagic acid and ellagitannins in later stages. This results in the following core conclusion: The *RcDHD/SDH* gene family has been demonstrated to regulate phenolic acid synthesis in raspberry fruits through temporal expression patterns, which have been shown to exhibit significant positive correlations with ellagic acid and gallic acid content. It is evident that, in the case of NT varieties, there is a predominant reliance on *RcDHD/SDH1-RcDHD/SDH2* for the purpose of rapid early accumulation. In contrast, the activation of *RcDHD/SDH3-RcDHD/SDH4* by HT varieties is indicative of the establishment of a sustained late-stage synthesis mechanism. This phenomenon is a manifestation of the functional differentiation that is evident in the secondary metabolic strategies employed by these two varieties.

At the metabolic pathway level, the *DHD/SDH* gene family plays a crucial role in the sequential conversion of gallic acid to ellagic acid to ellagitannin (see Figure 9). Within this pathway, gallic acid functions as a pivotal intermediate, capable of yielding ellagic acid through either esterification or oxidative condensation reactions. Subsequent reactions between gallic acid and ellagic acid yield hydrolyzable tannins^[49]. In the presence of *DHD/SDH* catalysis, 3-dehydroquinic acid (3-DHS) and NADPH have been observed to generate SA. Conversely, SA and NADP⁺ have been shown to undergo a reverse reaction, resulting in the formation of 3-DHS under *DHD/SDH* catalysis^[50-51]. The *DHD/SDH* gene, by regulating NADP-dependent dehydrogenase enzymes, is likely involved in electron transfer and substrate catalysis during both gallic acid formation and its conversion to ellagic acid. This, in turn, has a direct effect on the rate of ellagic acid and its polymer formation.

A comparison of the results obtained from HT and NT raspberry types suggests that the differences in expression levels and metabolite contents among varieties are indicative of variations in transcriptional regulation and substrate utilisation efficiency. The hypothesis is that NT varieties may possess higher substrate supply capacity and more active phenylpropanoid metabolic pathways, leading to significant accumulation of phenolic compounds.

In summary, the *RcDHD/SDH* gene family plays a central role in the metabolic regulation of gallic acid and ellagic acid. Furthermore, significant functional differentiation and temporal synergistic effects are exhibited among its members. The differential expression of these genes across different varieties may represent a key molecular mechanism underlying the variation in phenolic accumulation in raspberry fruits. These findings provide crucial theoretical support for further elucidating the *DHD/SDH*-mediated synthesis mechanism of ellagic tannins.

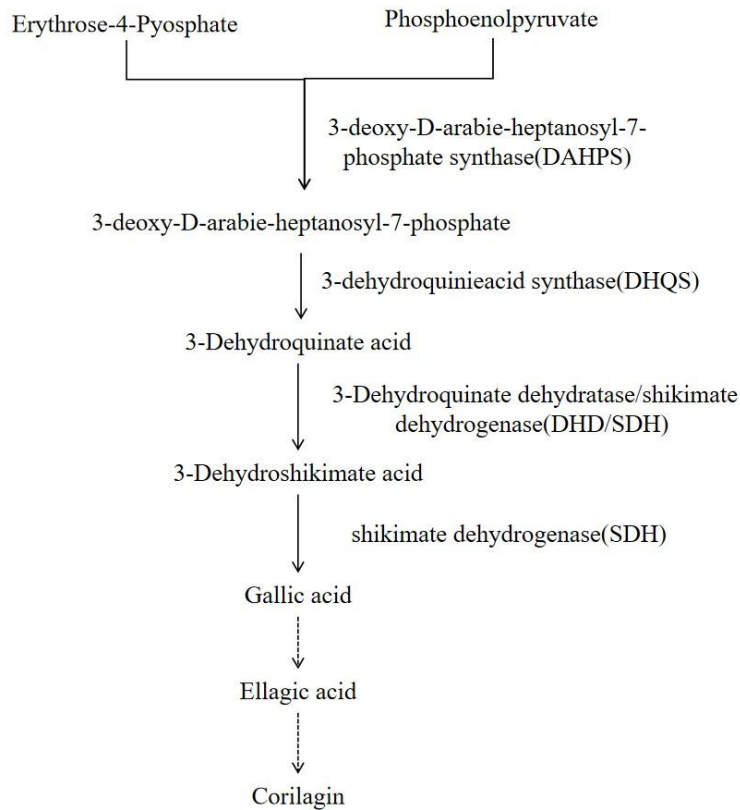


Figure 9: Metabolic pathway of gallic acid-ellagic acid-ellagitannin in *Rubus chingii* Hu

4. Discussion

The advantage of the *DHD/SDH* bifunctional enzyme is that it enhances metabolic flux efficiency within the shikimate pathway by limiting the accumulation of intermediates in competing pathways ^[52]. It is clearly that plant *DHD/SDH* enzymes are belong to a multigene family and that they may play distinct roles in 3-DHS reduction, SA oxidation, and GA synthesis ^[40]. Enzymes belonging to the *DHD/SDH* family play a pivotal role in the synthesis of plant secondary metabolites, with a particular emphasis on the biosynthesis of gallic acid and its derivatives. A further study has indicated that 1-aminocyclopropane-1-carboxylic acid (ACC), which is produced through *DHD/SDH*-catalysed reactions involving dehydrin and dehydration, is synthesized in both the shikimate and phenylpropanoid metabolism pathways ^[53]. As essential precursors for ellagic acid and ellagitannin synthesis, GA and its derivatives accumulate extensively in plants. The fluctuations in the concentration of these substances have been demonstrated to exert a direct influence on the antioxidant capacity of fruit, as well as on their antiviral and anti-inflammatory properties, and the development of their flavour ^[54-55].

This study, by synthesizing the findings from both HT and NT fruit types, revealing that the *DHD/SDH* gene family exhibits distinct variety-specific regulatory characteristics in

raspberry phenolic metabolism. In the case of HT fruits, the early expression of *RcDHD/SDH1* and *RcDHD/SDH2* has been demonstrated to promote gallic acid accumulation. However, the delayed response of *RcDHD/SDH3* and *RcDHD/SDH4* in later stages restricts the accumulation of ellagic acid. Conversely, the NT fruits, the coordinated expression of these gene family members, thereby creates a stable dynamic equilibrium that results in a substantial increase in both gallic acid and ellagic acid levels. over-expression of the *RcDHD/SDH* genes significantly boosts gallic acid accumulation, accompanied by a delayed increase in ellagic acid content, suggesting that these genes may be upstream in the regulatory cascade of ellagic acid synthesis. Particularly, *RcDHD/SDH1* and *RcDHD/SDH4* exhibited the most pronounced transcriptional responses, leading to the hypothesis that they might encode isoenzymes with higher substrate affinity or catalytic efficiency, thereby accelerating the conversion rate of 3-dehydroxypyruvic acid to gallic acid. As gallic acid accumulates, a sufficient supply of substrates for ellagic acid synthesis becomes available, thereby promoting subsequent formation of ellagitannins. This metabolic trend aligns with previous findings in polyphenol-rich plants, such as grapes and pomegranates, further confirming the role of *DHD/SDH* in phenolic acid synthesis.

Furthermore, the disparities in expression among various varieties serve to underscore the intricacy of the regulatory mechanisms that govern them. Research has identified a correlation between *DHQ/SDH* gene expression and gallic acid content in pomegranate fruits across various cultivars. This provides a theoretical foundation for elucidating the metabolic mechanisms of gallic acid in pomegranate fruits at the molecular level ^[56]. A plethora of studies have indicated that *R. chingii* has been found to demonstrate superior overall evaluations with regard to plant traits, fruit characteristics and component measurements when compared to thorny varieties ^[57]. In the case of NT raspberry varieties, *RcDHD/SDH* expression levels were found to be elevated in comparison to those observed in HT varieties, resulting in a heightened accumulation of gallic acid and ellagic acid. This phenomenon may be associated with higher expression activity of upstream genes in the shikimic acid pathway (e.g., *DHQ/SDH*, *EPSPS*, *PAL*) or a metabolic flux allocation favouring the polyphenol synthesis pathway. Conversely, the over-expression effects of this gene family appear diminished in HT varieties, potentially indicating the presence of negative regulatory mechanisms or feedback inhibition that limit metabolite accumulation. This variation may also be closely related to differences in antioxidant capacity and stress resistance among varieties. The results of this study demonstrate that the synthesis and accumulation of gallic acid and its derivatives is promoted

This study demonstrates the pivotal function of *RcDHD/SDH* in the metabolic pathway of gallic acid, ellagic acid and ellagitannin in raspberry as evidenced by dual approaches of gene expression and metabolite accumulation. The present study provides fresh insights into

the molecular mechanisms by which *DHD/SDH* regulates polyphenolic compound synthesis, thereby laying a theoretical foundation for future molecular breeding approaches to enhance the functional quality of raspberry fruits.

Abbreviations

GA	gallic acid
EA	ellagic acid
TCM	traditional Chinese medicine
DHD/SDH	3-dehydroquinate dehydratase/shikimate dehydrogenase
DHQ	3-dehydroquinate
DHS	dehydroshikimate
SDH	shikimate dehydrogenase
ET	Ellagitannin
SA	shikimic acid

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None

Authors' contribution

L.X.A: Formal Analysis, Data Curation, Investigation, Methodology, Software, Validation, Writing-Original Draft. Z.Y.L: investigation. F.D.Y: Supervision, Validation. H.J.C: Resources. L.J: Resources. S.L.W: Resources. N.Z.H: Conceptualization, Data curation, Funding acquisition, Methodology, Writing-Review and Editing.

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Ethics approval and consent to participate

The authors declare that all methods were carried out in accordance with relevant guidelines and regulations. These plant materials do not include any wild species at risk of extinction. No specific permits are required for sample collection in this study. We comply with relevant institutional, national, and international guidelines and legislation for plant study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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