Comparative Expression Profiling of Flavonoid Biosynthetic Genes Associated with Tricin Biosynthesis in Medicinal and Cultivated Released Rice Varieties of Kerala

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Submitted by

AHZIN N.

Candidate code: 63824142001

Exam code: 63822403

Subject code: BC 544

Under the Guidance of

Prof. (Dr.) R. Rajalakshmi & Dr. Nadiya F.

Inter University-Centre for Genomics & Gene Technology (IU-CGGT)
University of Kerala, Kariavattom, Thiruvananthapuram



DEPARTMENT OF BIOCHEMISTRY
T.K.M. COLLEGE OF ARTS AND SCIENCE, KARICODE, KOLLAM

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Department of Biochemistry
T.K.M. College of Arts and Science
(Affiliated to University of Kerala)
Re-accredited by NAAC with "A++" Grade
Kollam – 691005, Kerala.
www.tkmcas.ac.in

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CERTIFICATE

This is to certify that the dissertation entitled **'Comparative Expression Profiling of Flavonoid Biosynthetic Genes Associated with Tricin Biosynthesis in Medicinal and Cultivated Released Rice Varieties Of Kerala'** submitted to the University of Kerala in partial fulfilment of the requirements for the award of the degree of Master of Science in Biochemistry, is a record of original research work carried out by **AHZIN N. (Candidate code: 63824142001)** of the Department of Biochemistry, T.K.M. College of Arts and Science, Kollam under the guidance and supervision of **Prof. (Dr.) R. Rajalakshmi** and **Dr Nadiya F.,** in the Inter University Centre for Genomics and Gene Technology (IU-CGGT), University of Kerala, Kariavattom. It is also certified that no part of this work has been presented for the award of any other degree, diploma, or similar title at any other university.

Dr. Hari Sankar H. S.
Internal Guide

Dr. Ajinza A.
Head, Department of Biochemistry

Examiners: **1.**

2.

Declaration

I hereby declare that the thesis titled **"Comparative Expression Profiling of Flavonoid Biosynthetic Genes Associated with Tricin Biosynthesis in Medicinal and Cultivated Released Rice Varieties of Kerala"** is based on original research work carried out by me under the supervision of **Prof. (Dr.) R. Rajalakshmi and Dr. Nadiya. F,** in the Inter University Centre for Genomics and Gene Technology (IUCGGT), University of Kerala. I further declare that no part of this work has been presented for the award of any other degree, diploma, or similar title from any other university.

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List of Contents

Chapter	Title	Page No.
	List of figures	
	List of tables	
	Abbreviations	
	Abstract	
1	Introduction	1 – 10
2	Review of literature	11 – 21
3	Aims and objectives	22
4	Materials and methods	23 – 31
5	Results	32 – 45
6	Discussion	46 – 48
	Summary and Conclusion	49
	References	50 – 65

List of Figures

Figures	Title	Page No.
1	Structure of major classes of flavonoids in plants	4
2	Exemplary structure of tricin	5
3	General phenylpropanoid pathway and early flavonoid biosynthetic pathway	7
4	Tricin biosynthetic pathway	9
5	Grain morphology of the rice varieties used in the study.	23
6	12-day old seedlings of selected rice varieties used for the present study.	24
7	Agarose gel electrophoresis of total RNA isolated from selected rice varieties.	33
8	Representative qRT-PCR Amplification and melt curve analysis of the βactin reference gene used for cDNA quality assessment.	34
9	Representative amplification plot showing the typical sigmoid amplification curves and melt curve data for the primers used for the study	35
10	Relative expression profile of the Phenylalanine Ammonia-Lyase (PAL) gene	37
11	Relative expression profile of the Chalcone Synthase (CHS) gene	39
12	Relative expression profile of the Flavanone 3-Hydroxylase (F3H) gene	40
13	Relative expression profile of the Dihydroflavonol 4-Reductase (DFR) gene	42
14	Relative expression profile of the Anthocyanidin Synthase (ANS) gene	44

List of Tables

Table	Title	Page No.
1.	Details of rice varieties used in the study, including their classification, medicinal status, and source of seed procurement	23
2.	Primers used for quantitative real-time PCR analysis	29
3.	Quality Assessment of total RNA isolated from selected rice varieties based on RNA concentration and spectrophotometric purity ratios.	32

Abbreviations

4CL	: 4-Coumarate: Coenzyme A Ligase
ANR	: Anthocyanidin Reductase
ANS	: Anthocyanidin Synthase
C4H	: Cinnamate 4-Hydroxylase
cDNA	: Complementary Deoxyribonucleic Acid
CHS	: Chalcone Synthase
COMT	: Caffeic Acid *O*-Methyltransferase
DEPC	: Diethyl Pyrocarbonate
DFR	: Dihydroflavonol 4-Reductase
DHFs	: Dihydroflavonols
DNA	: Deoxyribonucleic Acid
dNTPs	: Deoxynucleotide Triphosphates
ECQ	: Eating and Cooking Quality
EDTA	: Ethylenediaminetetraacetic Acid
EtBr	: Ethidium Bromide
F3H	: Flavanone 3-Hydroxylase
FLS	: Flavonol Synthase
FNSI	: Flavone Synthase I
FOMT	: Flavonoid *O*-Methyltransferase
GTS	: Glycosyltransferases
HYVs	: High Yielding Varieties
IFS	: Isoflavone Synthase
LAR	: Leucoanthocyanidin Reductase
LDOX	: Leucoanthocyanidin Dioxygenase
NADPH	: Nicotinamide Adenine Dinucleotide Phosphate
PAL	: Phenylalanine Ammonia-Lyase
PCR	: Polymerase Chain Reaction
PKS	: Polyketide Synthase
PTAL	: L-Phenylalanine/L-Tyrosine Ammonia-Lyase
RNA	: Ribonucleic Acid
TAE Buffer	: Tris–Acetate–EDTA Buffer

Abstract

Traditional medicinal rice varieties of Kerala, particularly Njavara and Raktasali, are renowned for their therapeutic properties and are widely used in Ayurveda due to their rich antioxidant and health-promoting compounds. Among these bioactive metabolites, tricin, a rice-specific flavonoid, has attracted considerable attention for its antioxidant, anti-inflammatory, antimicrobial, and anticancer properties. However, information on the molecular regulation of tricin biosynthesis in medicinal and released rice varieties remains limited. Therefore, the present study was undertaken to evaluate the expression profiles of key genes associated with the tricin biosynthetic pathway in selected medicinal (Njavara and Raktasali) and released (Pratyasa and Uma) rice varieties of Kerala. Total RNA was isolated from leaf tissues using the TRIzol method, followed by cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis to determine the relative expression of PAL, CHS, F3H, DFR, and ANS, using Njavara as the reference genotype.

Comparative gene expression analysis revealed significant genotype-dependent variation among the studied rice varieties. Raktasali exhibited relatively higher expression of F3H, DFR, and ANS, indicating enhanced transcriptional activity of the downstream flavonoid biosynthetic pathway, whereas Uma showed the lowest transcript accumulation for most of the analysed genes. Njavara displayed comparatively higher expression of PAL, suggesting greater activity of the phenylpropanoid pathway, while Pratyasa exhibited intermediate expression levels for most genes. These findings demonstrate differential regulation of tricin biosynthetic genes among medicinal and released rice varieties and suggest that medicinal cultivars possess a greater genetic potential for flavonoid biosynthesis. The study provides valuable molecular insights into tricin biosynthesis in rice and establishes a foundation for future investigations correlating gene expression with tricin accumulation and antioxidant potential, thereby supporting the conservation and genetic improvement of nutritionally and medicinally important rice germplasm.

Chapter 1

Introduction

Rice (*Oryza sativa* L.) is one of the most important agricultural crops in the world, serving as the staple food for more than half of the global population (Agricultural Statistics Division [ASD], 2017). It is a vital cereal crop because a large proportion of the world's population depends on it for daily caloric intake and basic nutrition (Awika et al., 2011). Rice is particularly significant in developing Asian countries, where it constitutes a major component of the daily diet. Approximately half of the world's population consumes rice, and nearly 95% of global rice production occurs in Asian countries. It serves as the primary source of dietary energy for populations in 17 countries across Asia and the Pacific, nine countries in North and South America, and eight countries in Africa (ASD, 2017). Rice is cultivated across diverse geographical regions and climatic conditions, ranging from the terraced rice fields of Southeast Asia to the paddy fields of Africa and the Americas (Asma et al., 2023). During the 1960s, the introduction of high-yielding varieties, coupled with increased use of irrigation, fertilizers, and agrochemicals, resulted in a dramatic increase in agricultural productivity, a phenomenon known as the Green Revolution. This increase in productivity and profitability contributed significantly to enhanced food security and poverty reduction among farmers with access to irrigated land (Khush, 1995).

India once possessed approximately 110,000 rice varieties; however, the emphasis on monoculture and hybrid crops during the Green Revolution led to a substantial erosion of this rich genetic diversity. Today, only about 6,000 varieties remain (The Hindu, 2012). The loss of rice diversity is not only an agricultural concern but also a nutritional and pharmacological one, as many high-yielding varieties lack the unique secondary metabolites present in traditional landraces. Historically, India has been recognized as a centre of rice diversity, harbouring more than 200,000 rice varieties, a richness unmatched by any other country (Mohammed Ashraf & Subbalakshmi, 2017). While the Green Revolution prioritized high-yielding monocultures, contemporary research has renewed interest in the health-promoting properties and diverse phytochemical profiles of traditional rice varieties (The Hindu, 2012).Traditional rice varieties possess several desirable traits, including adaptability to adverse environmental conditions, resilience to biotic and abiotic stresses, and substantial nutritional and medicinal value. Many traditional varieties have a lower glycemic response and are therefore preferred by individuals

seeking to manage diabetes, obesity, and other metabolic disorders. They are also rich in dietary fibre, vitamins, and amino acids such as glutamic acid (Bhat & Riar, 2015).

In India, the medicinal properties of rice have long been recognized in both Ayurvedic and Unani systems of medicine. Traditional rice varieties have been used for the management of hypertension, digestive disorders, childhood diarrhoea, skin inflammations, blood glucose regulation, and several other ailments (Pushpam et al., 2019). These medicinal rice varieties are rich sources of essential nutrients and bioactive compounds. Their heterogeneous genetic background and adaptability to diverse agroecological conditions contribute to significant variations in their nutritional composition (Manikandan et al., 2025). Certain traditional rice varieties are particularly rich in micronutrients. For example, Himalayan red rice, Raktasali rice, and red rice from Assam are notable sources of iron, whereas brown rice varieties generally contain higher levels of zinc (Sangeetha et al., 2022). Traditional rice varieties also possess elevated concentrations of minerals such as potassium, calcium, phosphorus, and magnesium (Kowsalya et al., 2022; Noreen et al., 2009; Singh et al., 2022).

Njavara is a unique medicinal rice variety belonging to the genus *Oryza* (Deepa et al., 2008). It has been cultivated exclusively in Kerala, South India, since ancient times and is widely used in Ayurvedic treatments and formulations (Deepa et al., 2008). Njavara is primarily cultivated as an upland crop under relatively low-water conditions (Menon, 2004). Although it has a comparatively low yield, it exhibits moderate productivity and a reasonable degree of resistance to pests (Deepa et al., 2008). The dehusked grains of Njavara are red in colour and have gained considerable attention as a functional food due to their high concentrations of anthocyanins and polyphenols (Itani & Ogawa, 2004; Ling et al., 2001; Perera & Jansz, 2000). Morphologically, Njavara resembles conventional rice varieties; however, its husk colour may vary from brownish and golden yellow to black depending on environmental and soil conditions (Menon, 2004). Despite its medicinal significance, Njavara is currently considered at risk of extinction because of its low yield, high production cost, and limited cultivation. Its use is largely restricted to Ayurvedic medicines and specialized preparations. According to Ayurvedic literature, Njavara offers numerous health benefits for the respiratory, digestive, and circulatory systems. It has traditionally been used in the treatment of arthritis, muscle wasting disorders, cervical spondylitis, skin diseases, and certain neurological conditions (Deepa et al., 2008).

Raktasali, a pigmented rice variety belonging to the genus *Oryza* and native to Kerala, is highly valued for its nutritional composition and medicinal properties, particularly among tribal communities and in Ayurvedic medicine. Similar to Njavara, pigmented rice varieties such as Raktasali have attracted considerable scientific interest due to their high concentrations of polyphenols, vitamins, and minerals, as well as their diverse biological activities. Traditionally, Raktasali is believed to promote skin health and vision, act as a diuretic, and provide various pharmacological benefits, including anti-inflammatory, antioxidant, antitumour, and free radical scavenging activities. It has also been used in the management of conditions such as fever and ulcers (Girijan et al., 2020).Rice consumption has been associated with several health benefits, including the provision of instant energy, improved bowel function, enhanced metabolism, regulation of blood glucose levels, reduction of hypertension, delayed ageing, and protection against certain chronic diseases and dysentery (Kolahdouzan et al., 2013). The nutritional and phytochemical composition of rice is influenced by a range of genetic and environmental factors. These include the rice variety, ranging from conventional white rice to pigmented red and black rice, as well as soil characteristics, cultivation practices, post-harvest processing, and cooking methods (Dunna & Roy, 2013). Morphologically, the rice grain is a caryopsis enclosed within a protective outer husk. Removal of the husk exposes the grain, which consists of several maternal tissues, including the pericarp, seed coat, nucellus, and aleurone layer, surrounding the starchy endosperm and embryo. Each of these tissues possesses a distinct chemical composition and physiological function. The endosperm primarily serves as a storage tissue for carbohydrates and proteins, whereas the embryo and aleurone layer are rich in proteins, lipids, vitamins, and minerals. Importantly, pigments and flavonoids, which are of particular interest in phytochemical studies, are predominantly localized in the pericarp layer (Juliano & Bechtel, 1985).

Starch is the principal component of rice grains, accounting for approximately 75–80% of the grain's dry weight, while moisture and protein constitute about 12% and 7%, respectively. Although rice contains a relatively low protein content compared to other cereals, its proteins are considered nutritionally valuable because of their balanced amino acid composition (Oko et al., 2012). The nutritional profile of rice is characterized by the presence of carbohydrates, proteins, moisture, dietary fibre, lipids, vitamins, and minerals. As one of the world's most important staple foods, rice is primarily valued for its high carbohydrate content, which contributes substantially to its caloric value and accounts for nearly 87% of the grain's total composition (Devi et al., 2015). On a 100 g basis, raw rice provides approximately 1510 kJ of

energy, largely derived from about 79 g of carbohydrates. In addition, it contains approximately 12 g of moisture, 7 g of protein, 0.6 g of fat, and trace amounts of vitamins, including vitamin B6 (Dababneh & Abu-Rumman, 2008).

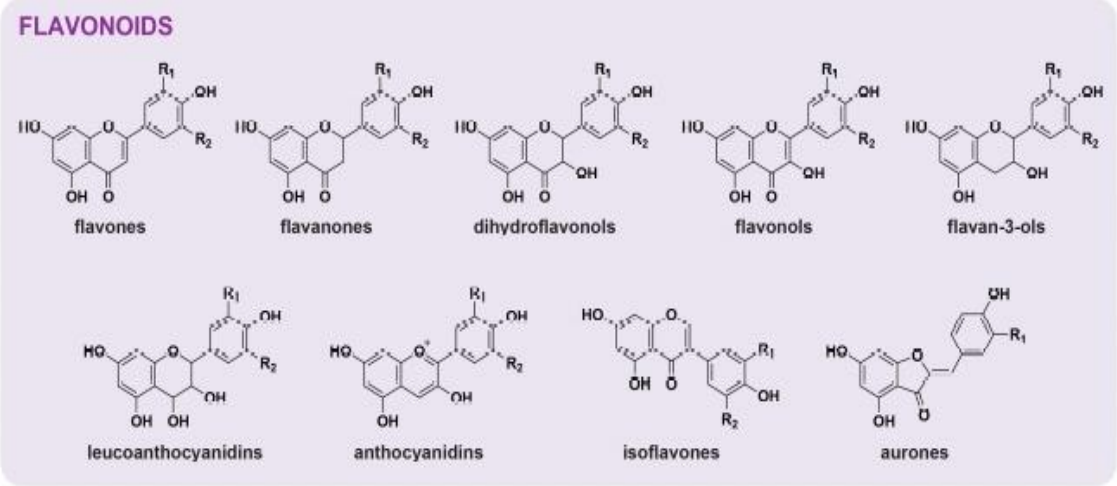


Figure 1: Flavonoids in plants. Structure of major class of flavonoids in plants. (Yang et al., 2018); Nakayama et al., 2019).

The therapeutic potential of traditional rice landraces is largely attributed to their diverse secondary metabolite profiles, particularly flavonoids (Figure 1) (Koes et al., 1994). Flavonoids are among the most abundant classes of plant secondary metabolites and perform a wide range of physiological functions, including the regulation of auxin transport, pigmentation of flowers and fruits, attraction of pollinators, interactions with microbes and animals, and protection against ultraviolet-B (UV-B) radiation (Dixon & Paiva, 1995; Lepiniec et al., 2006; Ryan et al., 2001; Winkel-Shirley, 2001).Structurally, flavonoids possess a characteristic diphenylpropane (C6–C3–C6) skeleton consisting of two aromatic benzene rings (A and B) linked by a heterocyclic pyran ring (C ring) (Alseekh et al., 2020). Based on the degree of oxidation and substitution patterns of the C ring, flavonoids are classified into several subclasses (Schijlen et al., 2004). These compounds are synthesized through the phenylpropanoid pathway and are broadly categorized into nine major groups: flavanones, flavones, dihydroflavonols, flavonols, flavan-3-ols, leucoanthocyanidins, anthocyanidins, isoflavones, and aurones (Nakayama et al., 2019; Yang et al., 2018). The remarkable structural diversity of flavonoids arises from various modification reactions, including glycosylation, methylation, acylation, and hydroxylation (Lepiniec et al., 2006; Stobiecki, 2000).

Rice, like many other cereal crops, predominantly accumulates flavone C-glycosides, whereas numerous dicotyledonous plants mainly produce flavonols (Yun et al., 2008). Consequently,

the flavone biosynthetic pathway assumes particular importance in cereals because it generates several biologically active compounds, including apigenin, luteolin, chrysoeriol, tricetin, and especially tricin, a flavonoid of considerable pharmacological significance that is abundant in many traditional rice varieties (Cavaliere et al., 2005; Wojnarowski et al., 2013).Flavones constitute the predominant class of flavonoids accumulated in members of the Poaceae (grass) family, including rice (Harborne & Hall, 1964; Tohge et al., 2017). This accumulation pattern is largely attributed to a unique metabolic characteristic of rice, namely the limited activity or expression of flavanone 3-hydroxylase (F3H), an enzyme that diverts flavonoid metabolism towards the synthesis of flavonols and anthocyanidins in many other plant species. The reduced flux through this branch pathway in rice effectively channels metabolic intermediates toward flavone biosynthesis (Deboo et al., 1995; Shih et al., 2008; Wang et al., 2020). This distinctive metabolic framework may contribute to the enhanced accumulation of specialized bioactive flavonoids such as tricin in medicinal rice varieties including Njavara and Raktasali.

Tricin [IUPAC name: 5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4H-chromen-4-one] is a naturally occurring flavonoid belonging to the flavone subclass (Harborne et al., 1999). Structurally, tricin possesses the characteristic flavonoid C6–C3–C6 skeleton composed of two aromatic rings (A and B) connected through a heterocyclic pyran ring (C ring). The A ring corresponds to the benzoyl system, whereas the B ring represents the cinnamoyl system. The presence of hydroxyl and methoxy substituents on these rings contributes to the unique biological and pharmacological properties of tricin (Figure 2).

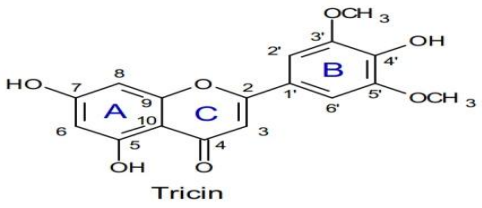


Figure: 2 Exemplary structure of tricin (Zhou and Ibrahim, 2010)

Tricin biosynthesis occurs through a series of enzymatic reactions involving both the phenylpropanoid and flavonoid pathways. Initially, the flavanone backbone is synthesized through the condensation of intermediates derived from the phenylpropanoid and polyketide pathways, a process catalysed by chalcone synthase (CHS) and chalcone isomerase (CHI). Subsequent hydroxylation and methylation reactions lead to the formation of flavones and

ultimately tricin (Zhou & Ibrahim, 2010). Tricin generally accumulates in the leaves, stems, and other aerial tissues of cereal and herbaceous plants, although its concentration varies considerably among species and developmental stages (Williams et al., 1973; Wollenweber et al., 2008). In rice, tricin has been implicated in plant defence mechanisms. Studies have shown that pest-resistant rice cultivars accumulate significantly higher levels of tricin in their leaves than susceptible cultivars, suggesting a protective role against biotic stress (Zhang et al., 2015). In addition to its importance in plant physiology, tricin has attracted considerable interest as a potential nutraceutical compound due to its broad spectrum of biological activities. Numerous studies have reported its anticancer (Hudson et al., 2000; Yue et al., 2020), antiviral (Akuzawa et al., 2011; Yazawa et al., 2010), antihistaminic (Jiang et al., 2020; Lan et al., 2016, 2019; Zhou & Ibrahim, 2010), anti-inflammatory (Shalini et al., 2016), and antioxidant properties (Ajitha et al., 2012). Furthermore, tricin has demonstrated low toxicity and significant pharmacological efficacy, making it a promising candidate for clinical development (Cai et al., 2009; Verschoyle et al., 2006). Although its bioavailability is relatively limited, structural modification and prodrug development strategies have been proposed to overcome this constraint (Ninomiya et al., 2011). Like other flavonoids, tricin is synthesized through the phenylpropanoid pathway (Figure 3). The pathway begins with the deamination of L-phenylalanine by phenylalanine ammonia-lyase (PAL), producing cinnamic acid (Camm & Towers, 1973; Elkind et al., 1990). Subsequently, cinnamate 4-hydroxylase (C4H) catalyses the para-hydroxylation of cinnamic acid to generate *p*-coumaric acid (Russell, 1971; Russell & Conn, 1967; Schilmiller et al., 2009). The resulting *p*-coumaric acid is then converted into *p*-coumaroyl-CoA by 4-coumarate: CoA ligase (4CL). This activated intermediate serves as a key precursor for the biosynthesis of numerous phenylpropanoid-derived compounds, including flavonoids and lignin (Gui et al., 2011; Li et al., 2015). The early stages of flavonoid biosynthesis are highly conserved throughout the plant kingdom. Chalcone synthase (CHS), a member of the type III polyketide synthase superfamily, catalyses the sequential condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone, the first committed intermediate in flavonoid biosynthesis (Figure 3). Naringenin chalcone is subsequently converted into the flavanone naringenin through the action of chalcone isomerase (CHI) or, less frequently, by spontaneous isomerization. Naringenin serves as the central precursor for the biosynthesis of diverse flavonoid classes, including flavones, flavonols, anthocyanins, and ultimately tricin (Lam et al., 2021).

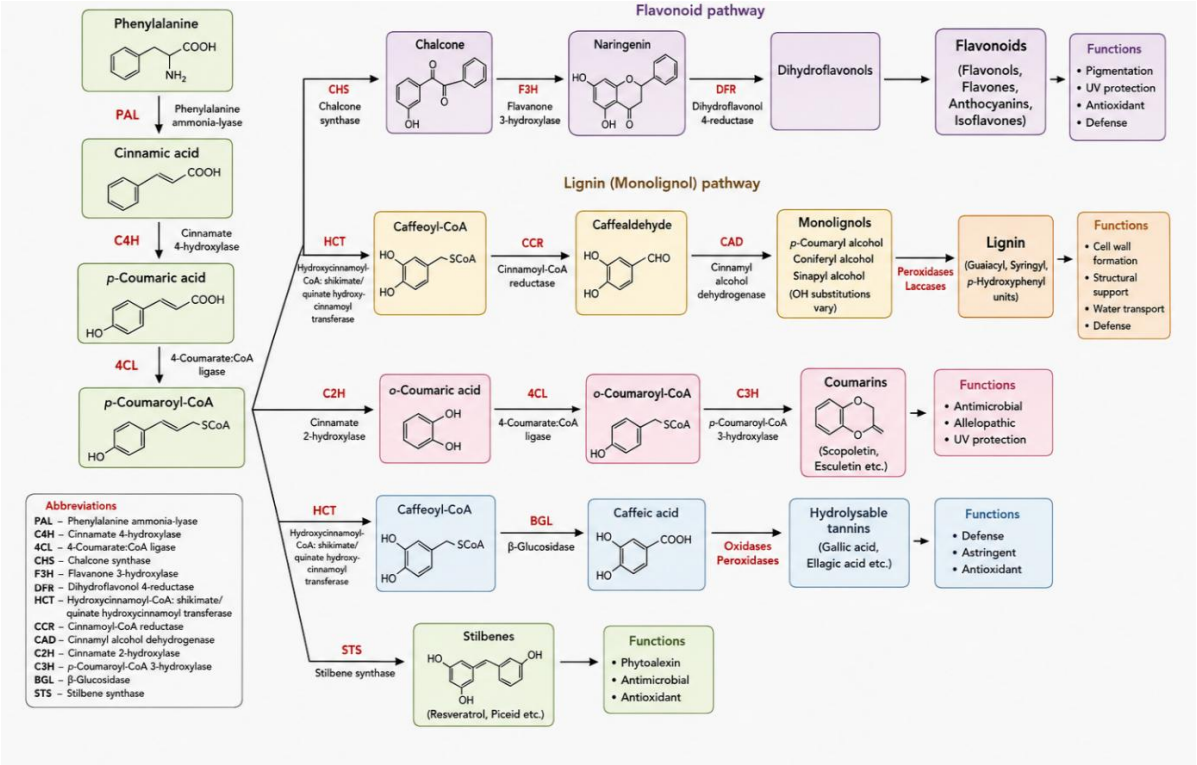


Figure 3: Phenyl Propanoid Pathway, PAL, L-phenylalanine ammonia-lyase; PTAL, L-phenylalanine/L-tyrosine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-hydroxycinnamate: CoA ligase; CHS, chalcone synthase; and CHI, chalcone isomerase. The general phenylpropanoid pathway is indicated in green, the flavonoid biosynthetic pathway in purple, and the monolignol biosynthetic pathway in blue (Lam et al., 2021)

Studies have demonstrated that suppression or loss of chalcone synthase (CHS) activity in rice results in a substantial reduction in soluble flavonoids and tricin incorporation into lignin, highlighting the critical role of CHS in tricin biosynthesis (Eloy et al., 2017; Wang et al., 2020). Using *Oryza sativa* as a model system, flavone synthase II (FNSII) was identified as the key enzyme responsible for the formation of the flavone nucleus during tricin biosynthesis (Lam et al., 2014).

A critical branch point in flavonoid metabolism is controlled by flavanone 3-hydroxylase (F3H), which directs metabolic flux toward the biosynthesis of flavonols and anthocyanins. In grasses, including rice, it has been proposed that flavonoid metabolism is preferentially channelled toward flavone production, particularly tricin biosynthesis, rather than through the F3H-dependent pathway. Recombinant *OsFNSII* directly catalyses the conversion of flavanones such as naringenin and eriodictyol into the flavones apigenin and luteolin, respectively (Brazier-Hicks & Edwards, 2013; Lam et al., 2014). These flavones subsequently undergo a series of hydroxylation and methylation reactions to produce tricin aglycone. A major breakthrough in understanding tricin biosynthesis was the identification of cytochrome

P450 CYP75B4, which functions as a unique chrysoeriol 5′-hydroxylase and plays a crucial role in completing the tricin biosynthetic pathway in rice (Lam et al., 2019). Alternatively, naringenin may be converted by flavanone 3-hydroxylase (F3H) into dihydroflavonols such as dihydrokaempferol, dihydroquercetin, and dihydromyricetin. These intermediates can undergo further hydroxylation by flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), enzymes that contribute significantly to anthocyanin structural diversity and pigmentation patterns (Ahn et al., 2015).

The resulting dihydroflavonols serve as direct substrates for dihydroflavonol 4-reductase (DFR), leading to the formation of leucoanthocyanidins, or for flavonol synthase (FLS), resulting in flavonol biosynthesis. Subsequently, anthocyanidin synthase (ANS) catalyses the oxidation of colourless leucoanthocyanidins to form coloured anthocyanidins. These anthocyanidins are then glycosylated by glycosyltransferases (GTs) to produce stable anthocyanin pigments that accumulate in plant tissues (Holton & Cornish, 1995). Tricetin (3′,4′,5′-trihydroxylated flavone) has been proposed as an important intermediate and direct precursor in the biosynthesis of tricin (Figure 4) Subsequent O-methylation reactions convert tricetin into tricin, thereby completing the flavone biosynthetic pathway in rice (Chummins et al., 2006; Zhou & Ibrahim, 2010).

Dihydroflavonol 4-reductase (DFR; EC 1.1.1.219) is a key branch-point enzyme in the anthocyanin biosynthetic pathway. It catalyses the NADPH-dependent reduction of dihydroflavonols (DHFs) to leucoanthocyanidins, which serve as immediate precursors for anthocyanin production (Diharce et al., 2016; Lepiniec et al., 2013; Saito et al., 2013). The metabolic fate of dihydroflavonols is largely determined by the competitive activities of DFR and flavonol synthase (FLS). While DFR channels dihydroflavonols toward anthocyanin biosynthesis through the formation of leucoanthocyanidins, FLS converts the same substrates into flavonols. Therefore, the relative activities and expression levels of these two enzymes play a crucial role in regulating carbon flux partitioning between anthocyanin and flavonol biosynthesis (Davies et al., 1997, 2003; Holton et al., 1993; Luo et al., 2016; Nielsen et al., 2002).

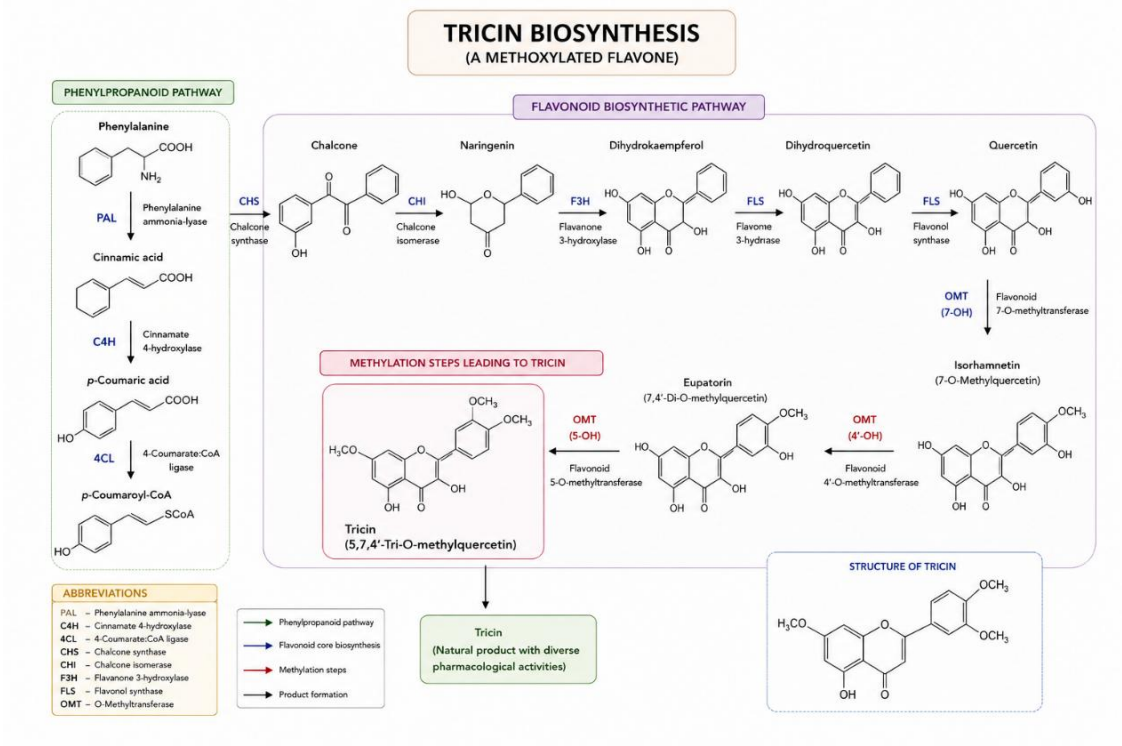


Figure 4: Tricin biosynthetic pathway (Originally proposed tricin biosynthetic pathway; Lam et al.,2021)

Chalcone synthase (CHS) is considered the gateway enzyme of the flavonoid and anthocyanin biosynthetic pathways, catalysing the first committed step in flavonoid biosynthesis (Dao et al., 2011). CHS belongs to the type III polyketide synthase (PKS) superfamily, a class of enzymes unique to plants that plays a central role in the production of diverse secondary metabolites (Austin & Noel, 2003; Schröder, 1997). In addition to its biosynthetic function, CHS expression has been shown to correlate strongly with flavonoid accumulation during plant responses to heat stress, highlighting its importance in stress adaptation and defence mechanisms (Glagoleva et al., 2019).Flavanone 3-hydroxylase (F3H) catalyses the conversion of flavanones into dihydroflavonols, which serve as colourless precursors of anthocyanins and other flavonoid compounds (Britsch &Grisebach, 1986). In rice, three F3H homologues have been identified: *OsF3H-1*, *OsF3H-2*, and *OsF3H-3*. Among these, *OsF3H-1* exhibits the highest enzymatic activity, whereas *OsF3H-2* shows the highest level of gene expression (Gong et al., 2017). Dihydroflavonol 4-reductase (DFR) catalyses the reduction of dihydroflavonols to their corresponding leucoanthocyanidins, which are direct precursors of anthocyanins (Huits et al., 1994). DFR represents the first enzyme of the so-called “late” flavonoid biosynthetic pathway and plays a pivotal role in determining the accumulation of anthocyanins, flavan-3-ols, and related flavonoid metabolites (Gosch et al., 2014).

Despite extensive research on the roles of key flavonoid biosynthetic genes, including *PAL*, *CHS*, *F3H*, *DFR*, and *ANS*, in model plant species and major cereal crops, a comprehensive understanding of their expression patterns in Kerala's indigenous medicinal rice landraces remains limited. Traditional medicinal rice varieties such as Njavara (Black) and Raktasali are renowned for their therapeutic properties and rich phytochemical composition; however, the molecular mechanisms underlying these attributes have not been fully elucidated. Therefore, the present study focuses on the comparative molecular profiling of selected flavonoid biosynthetic genes in the medicinal rice landraces Njavara and Raktasali in comparison with the high-yielding non-medicinal rice varieties Uma and Pratyasa. By analysing the relative expression patterns of *PAL*, *CHS*, *F3H*, *DFR*, and *ANS*, this study aims to investigate the molecular regulation of flavonoid biosynthesis and its association with phytochemical accumulation. Particular emphasis is placed on understanding the role of *CHS* in initiating flavonoid biosynthesis, the function of *F3H* as a critical branch-point enzyme influencing metabolic flux, and the contribution of *DFR* toward directing metabolite partitioning into anthocyanin biosynthesis. Furthermore, the study seeks to determine whether metabolic resources are preferentially channelled toward tricin biosynthesis or diverted into competing flavonoid pathways, including anthocyanin and flavanol biosynthesis. The findings are expected to provide valuable insights into the molecular basis of phytochemical diversity and medicinal properties in traditional rice landraces and may facilitate the identification of candidate molecular markers associated with enhanced bioactive compound accumulation.

Chapter 2

Review of literature

2.1 Traditional Rice Diversity in India and Kerala

According to the Food and Agriculture Organization (FAO, 2006), India is recognized as one of the primary centres of origin and diversity for rice, with archaeological evidence indicating its cultivation as early as 1500–1000 BC. The FAO further reported that, among the approximately 140,000 rice landraces conserved worldwide, India possesses a substantial share, comprising nearly 86,330 accessions. Gopi and Manjula (2018) defined specialty rice varieties as a broad category of traditional cultivars that have been preserved and cultivated over generations in different parts of the world. Within India, Kerala is particularly rich in rice diversity, harbouring approximately 2,000 traditional rice varieties (Leena Kumari, 2012). These landraces have evolved through natural selection and farmer-led conservation practices, enabling them to adapt to the diverse agroecological conditions of the region.

The remarkable adaptability of rice contributes significantly to its global importance. FAO (2006) emphasized that rice can thrive across a wide range of ecosystems and climatic conditions, from flooded lowland paddies to dry upland terraces. This ecological flexibility has enabled rice cultivation to expand across diverse geographical regions, ranging from humid tropical environments to arid plains, thereby establishing rice as a staple food for much of the world's population.

2.2 Phytochemical and Nutritional Profile of Rice

Recent studies have demonstrated that grain quality is a major determinant of consumer preference and market acceptance of rice. Eating and cooking quality (ECQ) has been identified as one of the most important attributes influencing consumer satisfaction (Zhao et al., 2020). In addition, nutritional quality and grain composition play crucial roles in determining the overall value of rice (Yang et al., 2018). Appearance and milling quality are also important parameters affecting consumer acceptance and commercial marketability (Zhao et al., 2018). Rice grains are primarily composed of carbohydrates, proteins, lipids, fibre, vitamins, and minerals. Juliano (1985) reported that rice typically contains approximately 80% carbohydrates and 8% protein, along with smaller quantities of fat and dietary fibre. The nutritional composition of rice varies depending on the variety and degree of processing.

White rice is produced through extensive milling, during which the hull, bran, and germ layers are removed. As a result, substantial amounts of dietary fibre, vitamins, minerals, and bioactive compounds are lost during processing (Pengkumsri et al., 2015). In contrast, pigmented rice varieties, including red, black, purple, and brown rice, retain many of these nutritionally valuable components and are therefore considered healthier alternatives.

Pigmented rice varieties have long been valued for their nutritional and therapeutic properties and continue to command premium prices in regional markets (Saikia et al., 2012). The distinctive colours of these rice varieties arise from the accumulation of various bioactive compounds, including polyphenols, anthocyanins, carotenoids, and chlorophylls. Among these compounds, flavonoids and anthocyanins are primarily responsible for the pigmentation observed in rice grains (Hou et al., 2013). Anthocyanins impart the characteristic red, purple, and blue colours to pigmented rice and possess potent antioxidant and anti-inflammatory activities. These bioactive compounds contribute significantly to the health-promoting properties associated with pigmented rice consumption (Hou et al., 2013).

Natural phenolic compounds are widely utilized in the food and beverage industries because of their strong antioxidant properties (Galanakis, 2012). These compounds can extend product shelf life by delaying oxidative deterioration, preventing the development of off-flavours, and reducing rancidity. Consequently, natural phenolics are increasingly being used as safer alternatives to synthetic preservatives. The antioxidant compounds present in rice have been associated with a reduced risk of several chronic diseases (Hudson et al., 2000). Populations with high rice consumption, particularly those consuming pigmented rice varieties, often exhibit lower incidences of certain chronic disorders, highlighting the potential health benefits of rice-derived phytochemicals. The antioxidant capacity of pigmented rice is largely attributed to its diverse array of bioactive constituents. These include phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid, all of which contribute to the nutritional and therapeutic value of the grain (Goufo & Trindade, 2014). Several studies have reported that pigmented rice varieties contain substantially higher concentrations of phenolic acids, proanthocyanidins, and anthocyanins than non-pigmented white rice varieties (Deng et al., 2013). Furthermore, the composition of phenolic compounds differs according to pericarp colour. Rice varieties with lighter pericarp colours, such as brown rice, generally contain phenolic compounds of lower molecular weight, whereas red and black rice varieties with darker pericarps are characterized by the presence of higher molecular weight phenolic compounds (Bergman & Goffman, 2004).

2.3 Pharmacological Significance of Specialized Secondary Metabolites

Phenolic compounds present in rice have attracted considerable attention due to their diverse pharmacological properties. Through cell culture studies, Hu et al. (2003) demonstrated that rice-derived phenolic compounds exhibit significant antimutagenic, anticarcinogenic, and antimetastatic activities. These compounds contribute to human health by protecting against DNA damage, regulating cell proliferation, and inhibiting the progression and spread of cancer cells.In addition to their anticancer properties, rice phenolics play an important role in cardiovascular health. Xia et al. (2003) reported that phenolic compounds can reduce total blood cholesterol levels and inhibit the oxidation of low-density lipoprotein (LDL) cholesterol. Furthermore, Ling et al. (2001) demonstrated that these compounds contribute to a reduction in the LDL-to-HDL cholesterol ratio, thereby supporting cardiovascular health. Collectively, these findings suggest that pigmented rice varieties may serve as valuable dietary components for the prevention of cardiovascular diseases and the maintenance of healthy lipid profiles.

Numerous studies have demonstrated that the consumption of pigmented rice is associated with a broad range of health-promoting effects. Hu et al. (2003) reported potent antioxidant and anticarcinogenic activities, whereas Morimitsu et al. (2002) identified significant anti-inflammatory, α-glucosidase inhibitory, and antidiabetic properties. In addition, Shao et al. (2014) reported hepatoprotective effects, further supporting the role of pigmented rice as a functional food with substantial nutritional and therapeutic value. Vogt (2010) reported that flavonoid biosynthesis originates from the general phenylpropanoid pathway. Flavonoids are characterized by a common 15-carbon (C15) skeleton composed of two aromatic rings (A and B) connected by a three-carbon heterocyclic ring (C), resulting in the characteristic C6–C3–C6 structure. Rice contains several classes of flavonoids, including flavones, flavonols, flavan-3-ols, isoflavones, dihydroflavonols, and flavanones, which commonly occur as O-glycosides or C-glycosides. Among the flavonoids reported in rice, tricin is the predominant flavone present in rice bran (Goufo & Trindade, 2014). Allan et al. (2008) noted that most of the genes and enzymes involved in flavonoid biosynthesis have been extensively characterized in model plant systems, providing a strong foundation for understanding these pathways in rice. The accumulation of flavonoids varies according to plant species, tissue type, and developmental stage. Environmental factors also exert a significant influence on flavonoid biosynthesis. Various stress conditions, including ultraviolet radiation, drought, temperature fluctuations, nutrient deficiencies, and pathogen infection, have been shown to stimulate flavonoid production (Chalker-Scott, 1999; Shan et al., 2009). The expression of flavonoid biosynthetic

genes is tightly regulated by complex genetic regulatory networks. Koes et al. (2005) reported that the activity of structural genes involved in flavonoid biosynthesis is controlled by specific regulatory factors that determine the spatial and temporal accumulation of flavonoids within plant tissues. Furthermore, Chen et al. (2013) observed that the expression patterns of flavonoid biosynthetic genes vary considerably among tissues, developmental stages, and genotypes. Such variation may explain the differences in flavonoid accumulation observed between medicinal rice landraces and conventionally cultivated rice varieties.

2.4 Tricin Biosynthetic Pathway

Cereal grains constitute the primary source of dietary energy for more than half of the global population. However, the most commonly consumed cereal products are polished grains, which predominantly consist of the starchy endosperm and contain relatively low concentrations of essential micronutrients, minerals, and bioactive phytochemicals (Awika, 2011). The widespread reliance on such nutrient-poor staple foods, particularly in developing countries, has been identified as a major contributor to micronutrient malnutrition, often referred to as "hidden hunger" (Bhullar & Gruissem, 2013). This nutritional challenge is further aggravated by limited dietary diversity and inadequate consumption of fruits, vegetables, and other nutrient rich foods.

To address micronutrient deficiencies, significant efforts have been directed toward biofortification of cereal crops through metabolic engineering. Biofortification involves enhancing the nutritional quality of crops by increasing the concentration and bioavailability of essential nutrients and health-promoting compounds in edible plant tissues. Among the most successful examples is Golden Rice, a genetically engineered rice variety developed to accumulate β-carotene, a precursor of vitamin A, in the endosperm. The development of Golden Rice represented a landmark achievement in plant biotechnology and demonstrated the feasibility of engineering complex biosynthetic pathways into cereal endosperm to combat vitamin A deficiency, a major public health problem in many developing nations (Ye et al., 2000; Paine et al., 2005; Owens, 2018).

Following the success of Golden Rice, numerous biofortification strategies have been employed to enhance the nutritional composition of cereal grains. Transgenic rice and other cereal crops enriched with iron, zinc, folate, and various carotenoids have been successfully developed through genetic engineering approaches (Wirth et al., 2009; Johnson et al., 2011; Saltzman et al., 2013; Blancquaert et al., 2015; Singh et al., 2017; Zhu et al., 2008). These

advancements have demonstrated the potential of metabolic engineering to improve the nutritional value of staple foods and contribute to global food and nutritional security.

More recently, attention has shifted toward the enrichment of cereal grains with phenolic compounds, particularly flavonoids, anthocyanins, stilbenoids, and related secondary metabolites that possess significant antioxidant and therapeutic properties. Several studies have successfully engineered rice endosperm to accumulate flavonoids and anthocyanins, thereby transforming cereal grains into functional foods with enhanced health-promoting potential (Baek et al., 2013; Ogo et al., 2013; Zhu et al., 2017). These bioactive compounds have been associated with reduced risks of chronic diseases, including cardiovascular disorders, diabetes, and certain forms of cancer.

Among cereal-derived flavonoids, tricin (3′,5′-dimethoxyflavone) has attracted considerable interest because of its diverse biological activities. Tricin exhibits strong antioxidant, anti-inflammatory, antimicrobial, anticancer, and chemo preventive properties, making it a promising candidate for functional food development (Cai et al., 2004; Duarte-Almeida et al., 2007; Yazawa et al., 2010; Jung et al., 2014, 2015; Lee et al., 2015; Shalini et al., 2016). Despite its beneficial effects, tricin is generally absent from the edible endosperm of cereal grains because the genes responsible for its biosynthesis are not expressed in this tissue (Ogo et al., 2013).

Naturally occurring tricin is primarily found in the vegetative tissues of grasses and in the bran layers of cereal grains, including rice, wheat, barley, and oats. Small quantities of tricin are retained in the pericarp, testa, aleurone layer, and embryo of whole grains but are largely removed during milling and polishing processes (Poulev et al., 2018, 2019). Additional dietary sources include grass-derived food products such as sugarcane juice and barley leaf powder (Duarte-Almeida et al., 2007; Zeng et al., 2018). Consequently, the average human diet contains only limited amounts of tricin. The development of tricin-enriched cereal grains through genetic engineering has therefore emerged as an attractive strategy to increase the dietary intake of this valuable flavonoid. Ogo et al. (2013) successfully generated transgenic rice seeds capable of accumulating tricin by introducing a combination of genes involved in the flavonoid biosynthetic pathway, including rice phenylalanine ammonia-lyase (PAL), rice chalcone synthase (CHS), parsley flavone synthase I (FNSI), soybean flavone synthase II (FNSII), Viola-derived flavonoid 3′,5′-hydroxylase (F3′5′H), and rice caffeic acid O-

methyltransferase (COMT/CAldOMT). This study demonstrated the feasibility of reconstructing the complete tricin biosynthetic pathway in rice endosperm.

Subsequent research has substantially advanced the understanding of tricin biosynthesis in grasses and legumes. Detailed elucidation of the endogenous tricin biosynthetic pathway in grasses (Lam et al., 2014, 2015, 2019a) and Medicago species (Lui et al., 2020), together with increasing knowledge of the regulatory mechanisms controlling flavonoid biosynthesis, has provided new opportunities for metabolic engineering. These developments are expected to facilitate the production of tricin-rich crops and other plant-based functional foods with enhanced nutritional and pharmaceutical value. Such advances highlight the potential of combining molecular genetics, metabolic engineering, and nutritional science to develop next-generation cereal crops capable of addressing both food security and human health challenges.

Studies on tissue distribution in murine and human models further suggest that tricin possesses considerable potential as a stable and effective chemo preventive agent (Cai et al., 2004). Tricin is one of the most abundant flavones present in brown rice and rice bran. Hudson et al. (2000) reported that tricin exhibits potent chemo preventive activity by inhibiting the growth of human breast and colon cancer cells. Owing to its substantial medicinal value, tricin has become a major focus of phytochemical and pharmacological research, particularly in traditional rice varieties. Goufo and Trindade (2014) identified tricin as the predominant flavonoid present in rice bran, indicating that it is not only biologically active but also a major contributor to the phytochemical profile of pigmented rice varieties. The anti-inflammatory activity of tricin, particularly that isolated from Njavara rice bran, has also been extensively investigated. Shalini et al. (2016) demonstrated that tricin exerts its anti-inflammatory effects through modulation of the nuclear factor-kappa B (NF-κB) signalling pathway. Using lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (hPBMCs) and carrageenan-induced rat models, the authors observed significant suppression of pro-inflammatory gene expression. These findings suggest that tricin derived from traditional medicinal rice varieties may possess considerable therapeutic potential for the management of inflammatory disorders. Beyond its specialized therapeutic applications, tricin is increasingly recognized as a multifunctional nutraceutical due to its broad spectrum of biological activities. Zhou and Ibrahim (2010) reported that tricin exhibits strong antioxidant properties by preventing lipid peroxidation and oxidative degradation. Furthermore, tricin contributes to cellular protection by preserving vitamin E levels within erythrocyte membranes. These properties classify tricin as an important

bioactive compound capable of enhancing cellular health and maintaining oxidative homeostasis.

2.5 Comparative Molecular Profiling of Flavonoid Biosynthetic Genes

Beyond its catalytic role, the genetic basis of flavonoid biosynthesis provides valuable insights into plant evolution and the regulation of secondary metabolism. Chalcone synthase (CHS) is one of the most extensively studied genes involved in flavonoid biosynthesis and is generally present as a low-copy-number nuclear gene family that is highly conserved across vascular plants. Zimmer and Wen (2012) reported that CHS encodes the enzyme responsible for catalysing the first committed step in flavonoid biosynthesis, thereby initiating the production of a wide range of secondary metabolites. Owing to the occurrence of multiple CHS isoforms in many plant species, molecular characterization of the CHS gene family often requires advanced genomic and phylogenetic approaches.

Most plant species possess one or several CHS gene copies, making the cloning and sequencing of PCR-amplified products necessary for distinguishing among different isoforms and constructing accurate phylogenetic relationships (Zimmer & Wen, 2012). Early phylogenetic studies established the usefulness of CHS as a molecular marker for evolutionary investigations. For example, Koch et al. (2000) successfully amplified a CHS fragment of approximately 1200 bp from species belonging to the genera *Arabis* and *Arabidopsis*. The authors reported that nearly 80% of the amplified sequence consisted of coding regions, providing approximately 300 phylogenetically informative sites. Consequently, the CHS gene has become an important molecular marker for investigating the evolutionary diversification of flavonoid biosynthesis and secondary metabolic pathways in plants. Flavanone 3-hydroxylase (F3H) is regarded as a key regulatory enzyme within the flavonoid biosynthetic pathway. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG), F3H occupies a strategic position at a major branch point connecting flavone, flavonol, and anthocyanin biosynthesis. The enzyme catalyses the hydroxylation of flavanones, such as naringenin, at the C3 position of the heterocyclic ring to produce dihydroflavonols. This reaction represents a critical metabolic step because it determines the availability of intermediates for multiple downstream flavonoid classes. The biological significance of F3H lies in its ability to regulate carbon flux within the flavonoid pathway. Through its catalytic activity, F3H influences the synthesis of pigments, antioxidants, and other protective secondary metabolites. Consequently, the expression of F3H is often considered an indicator of overall flavonoid pathway activation.

Furthermore, F3H expression has been associated with plant responses to various environmental and physiological stimuli.

Several studies have reported the cloning and functional characterization of F3H genes in diverse plant species, including *Petunia hybrida*, *Hordeum vulgare*, *Medicago sativa*, *Zea mays*, *Arabidopsis thaliana*, *Medicago truncatula*, and *Ascocenda* spp. (Britsch et al., 1992; Charrier et al., 1995; Deboo et al., 1995; Khumkarjorn et al., 2017; Meldgaard, 1992; Pelletier & Shirley, 1996; Shen et al., 2010). Studies have further demonstrated that F3H expression is regulated by environmental and hormonal signals. In *Epimedium*, gibberellic acid and sucrose treatments significantly increased *EsF3H* expression and flavonoid accumulation (Zeng et al., 2013). Similarly, jasmonic acid and abscisic acid were shown to influence *AtF3H* expression in *Arabidopsis thaliana* (Lewis et al., 2011; Loreti et al., 2008). Moreover, overexpression of *F3H* from *Pohlia nutans* enhanced tolerance to salt and oxidative stress in *Arabidopsis thaliana* (Li et al., 2017).

Dihydroflavonol 4-reductase (DFR) is another key regulatory enzyme in flavonoid biosynthesis. Miyagawa et al. (2015) reported that DFR belongs to the NAD(P)-dependent epimerase/dehydratase protein family and plays a central role in directing metabolic flux toward anthocyanin biosynthesis. The authors further demonstrated that DFR activity is associated with flower colour intensity in *Delphinium* species. Similarly, Cheng et al. (2013) reported that DFR catalyses the conversion of dihydrokaempferol, dihydroquercetin, and dihydromyricetin into their corresponding leucoanthocyanidins, thereby serving as a key regulatory enzyme in the anthocyanidin biosynthetic pathway of *Ginkgo biloba*. Recent advances in rice flavonoid research have improved our understanding of the molecular basis of tricin biosynthesis. Lam et al. (2015) identified CYP75B4 as a unique chrysoeriol 5′-hydroxylase involved in the final stages of tricin biosynthesis and elucidated critical steps in the rice tricin biosynthetic pathway. Their findings established the molecular framework underlying tricin production in rice and highlighted the importance of cytochrome P450-mediated reactions in flavone biosynthesis. Furthermore, the authors reported tissue-specific expression patterns of tricin biosynthetic genes, particularly in vegetative tissues, indicating their contribution to flavonoid accumulation. These findings provide a valuable molecular basis for investigating tricin biosynthesis in medicinal rice landraces such as Njavara and Raktasali. The study also demonstrated the utility of gene expression profiling approaches for characterizing pathway-specific enzymes and evaluating flavonoid biosynthesis in rice tissues.

Recent studies have also revealed a close relationship between antioxidant activity and flavonoid biosynthesis in rice. In a comparative study of cultivated rice (*Oryza sativa*) and Chinese wild rice (*Zizania latifolia*), Yu et al. (2021) reported that pigmented rice varieties contained significantly higher levels of phenolic compounds and flavonoids, accompanied by enhanced antioxidant activity. The authors suggested that key flavonoid biosynthetic genes, including *CHS* and *PAL* (phenylalanine ammonia-lyase), play important roles in the accumulation of health-promoting phenolic compounds, thereby contributing to the nutritional and medicinal value of pigmented rice. To investigate the molecular basis of flavonoid accumulation, Yu et al. (2021) employed gene expression analyses involving RNA extraction and quantitative real-time PCR (RT-qPCR). Their results demonstrated the effectiveness of transcript profiling approaches for elucidating the regulatory mechanisms governing flavonoid biosynthesis and antioxidant activity in different rice genotypes. These findings further support the use of comparative gene expression analysis as a powerful tool for understanding the molecular factors underlying phytochemical diversity in medicinal and non-medicinal rice varieties. Using transcriptomic approaches, Cai et al. (2020) investigated the roles of lignin and flavonoid biosynthetic pathways in rice thermotolerance. Their study demonstrated that several structural genes involved in flavonoid biosynthesis, including *PAL*, *CHS*, and *CHI*, exhibited differential expression under heat stress conditions, highlighting the importance of flavonoid metabolism in rice stress responses. The authors further employed TRIzol-based RNA extraction and quantitative real-time PCR (RT-qPCR) to validate transcriptomic data and analyse gene expression patterns in rice tissues. Their findings support the application of transcriptomic and quantitative gene expression analyses for investigating flavonoid biosynthesis in medicinal rice landraces such as Njavara and Raktasali. Moreover, the study revealed tissue-specific transcriptomic responses, indicating that actively growing tissues, including leaves and sprouts, are important sites for flavonoid biosynthesis and secondary metabolite accumulation.

To elucidate the molecular basis of flavonoid accumulation, integrated transcriptomic and proteomic approaches have been increasingly employed in rice research. Chen et al. (2021) performed comparative transcriptomic and proteomic analyses of rice varieties with different grain pigmentation patterns and identified significant physiological and molecular differences associated with flavonoid biosynthesis. Their study demonstrated that flavonoid accumulation and grain pigmentation are regulated through the coordinated expression of key structural genes, including *CHS*, *F3H*, and *DFR*. The authors concluded that differential expression of

flavonoid biosynthetic genes constitutes an important molecular mechanism underlying the variation observed between pigmented and non-pigmented rice cultivars. The relationship between gene expression and phytochemical accumulation has also been supported by integrated multi-omics studies. Chen et al. (2019) further demonstrated that coordinated regulation of flavonoid biosynthetic genes plays a crucial role in determining flavonoid content in rice. These findings support the application of molecular profiling approaches for evaluating the metabolic potential and phytochemical diversity of medicinal rice landraces such as Njavara and Raktasali. Comparative transcriptomic studies in medicinal plants have similarly highlighted the importance of molecular regulation in flavonoid biosynthesis. Wang et al. (2025) conducted a comprehensive transcriptome analysis of *Orthosiphon aristatus* and identified numerous differentially expressed genes involved in flavonoid biosynthesis, including *CHS*, *CHI*, *F3H*, and *FLS*. The study revealed tissue-specific expression patterns of flavonoid-related genes and demonstrated the value of molecular profiling for understanding the biosynthesis of bioactive compounds in medicinal plants. These findings suggest that comparative expression analyses may facilitate the identification of molecular markers associated with the therapeutic potential of medicinal rice varieties.

Similarly, Fang et al. (2022) investigated flavonoid biosynthesis in *Agriophyllum squarrosum* using an integrated metabolomic and transcriptomic approach. By analysing six ecotypes grown under common environmental conditions, the authors identified strong correlations between flavonoid accumulation and the expression of flavonoid biosynthetic genes. Their results indicated that variations in flavonoid composition were closely associated with differences in gene expression profiles. The study emphasized the importance of combining phytochemical analysis with transcriptomic profiling to gain a comprehensive understanding of the molecular mechanisms regulating flavonoid biosynthesis.

One of the major challenges in plant molecular biology is the extraction of high-quality RNA from tissues rich in polysaccharides, polyphenols, and other secondary metabolites. Chaparro-Encinas et al. (2020) developed a modified RNA extraction protocol that combined cetyltrimethylammonium bromide (CTAB) and TRIzol® methods to effectively remove interfering compounds from plant tissues. The study demonstrated that hybrid extraction protocols can significantly improve RNA yield, purity, and integrity, thereby enhancing the reliability of downstream applications such as RT-qPCR and transcriptomic analyses. These methodological improvements are particularly relevant for medicinal rice varieties, which

often contain high concentrations of phenolic compounds and other secondary metabolites that can interfere with RNA isolation and gene expression studies.

The extraction of high-quality nucleic acids from plant tissues rich in polysaccharides, polyphenols, and other secondary metabolites remains a significant challenge in plant molecular biology. Liu et al. (2018) developed an optimized RNA extraction protocol for *Dendrobium huoshanense*, a medicinal plant characterized by high concentrations of secondary metabolites. Their study demonstrated that modifications to conventional extraction procedures could effectively reduce contamination by polysaccharides and polyphenols, thereby improving RNA quality, yield, and suitability for downstream applications such as RT-qPCR and transcriptomic analyses.

In conclusion, previous studies have demonstrated the importance of flavonoids and other secondary metabolites in determining the antioxidant, nutritional, and therapeutic properties of rice. Numerous investigations have employed molecular, transcriptomic, and multi-omics approaches to elucidate the functions and regulatory mechanisms of flavonoid biosynthetic genes. Although substantial progress has been made in understanding flavonoid biosynthesis in model plants and major cereal crops, the comparative expression patterns of key flavonoid biosynthetic genes in traditional medicinal rice landraces of South India, particularly Njavara and Raktasali, remain insufficiently explored. Therefore, the present study aims to investigate the comparative expression of selected flavonoid biosynthetic genes and examine their relationship with phytochemical accumulation in medicinal and non-medicinal rice varieties. By integrating gene expression analysis with phytochemical characterization, this research seeks to provide insights into the molecular mechanisms underlying the medicinal significance of traditional rice landraces and to identify potential molecular markers associated with enhanced bioactive compound production

Chapter 3
Aim and objectives

Aim

To perform comparative molecular profiling of tricin and other flavonoid biosynthetic genes in medicinal and released rice varieties and to evaluate variations in their gene expression patterns associated with flavonoid biosynthesis.

Objectives

1. To isolate high-quality RNA and synthesize cDNA from rice leaf tissues.
2. To analyse the relative expression of *PAL, CHS, F3H, DFR,* and *ANS* genes using RT-qPCR.
3. To compare tricin and other flavonoid biosynthetic gene expression between medicinal (Njavara and Raktasali) and released (Uma and Pratyasa) rice varieties.
4. To identify genes associated with enhanced flavonoid biosynthesis and medicinal potential in traditional rice landraces.

Chapter 4

Materials and methods

4.1 Materials

4.1.1 Rice Varieties Selected for the Study

Four rice genotypes (*Oryza sativa* L.) were selected for comparative molecular analysis of flavonoid biosynthetic genes. Morphology of the selected rice varieties were shown in Figure 5.

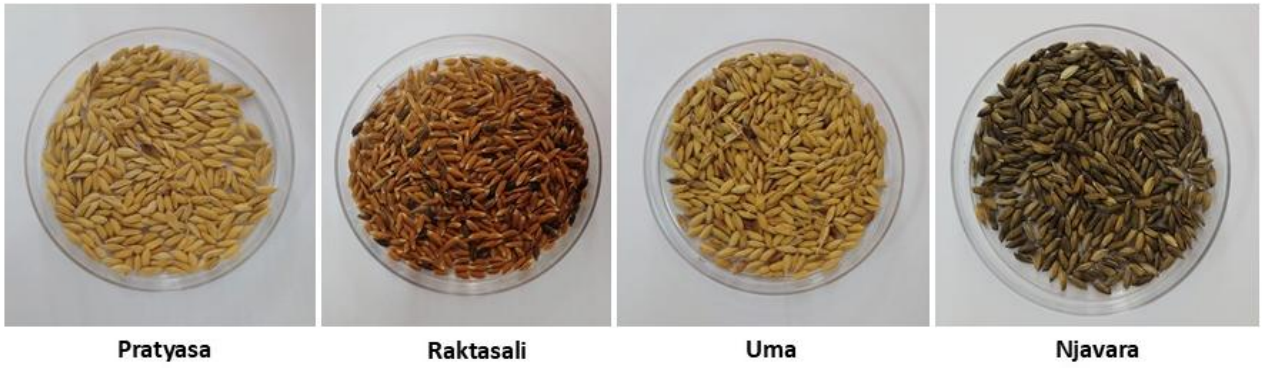


Figure 5: Grain morphology of the rice varieties used in the study. Representative images of varieties showing variations in grain size, shape, colour, and overall appearance, indicating their distinct genetic and phenotypic characteristics

The grain morphology of the selected rice varieties such as medicinal varieties Njavara, Raktasali, along with the cultivated released variety Pratyasa and Uma.

Table1: Details of rice varieties used in the study, including their classification, medicinal status, and source of seed procurement

Rice variety	Type/classification	Medicinal status	Source of seed	Location
Njavara (Black)	Traditional rice landrace	Medicinal	Njavara Ecofarm	Chitoor, Palakkad, Kerala
Raktasali	Traditional rice landrace	Medicinal	Farmer's plot	Chirayinkil, Thiruvananthapuram, Kerala
Uma	High-yielding improved variety	Non-medicinal (Control)	Rice Research Station, Kerala Agricultural University	Mankombu, Kerala
Pratyasa	High-yielding improved variety	Non-medicinal	Rice Research Station, Kerala Agricultural University	Mankombu, Kerala

Variations in grain size, shape, colour, and overall appearance were observed among the varieties, reflecting their distinct genetic backgrounds and agronomic characteristics. These morphological differences contribute to varietal diversity and may influence grain quality attributes such as cooking behaviour, texture, and nutritional properties.

4.1.2 Sample preparation for total RNA isolation from leaves:

For RNA isolation, rice grains of the selected varieties were first subjected to germination under controlled conditions. Healthy and uniform grains were washed thoroughly with distilled water and placed in a clean, moist cotton cloth. The grains were wrapped securely and incubated in darkness at room temperature for three days to facilitate uniform sprouting. To prevent microbial contamination and maintain adequate moisture, the grains were washed with sterile distilled water on alternate days during the germination period.

After three days, the sprouted grains were transferred to pots containing sterilized paddy soil and maintained under suitable growth conditions. The seedlings were watered regularly to ensure healthy growth. On the twelfth day after sowing, young, actively growing leaves were collected for RNA extraction Figure 6.



Figure 6: Twelve-day-old seedlings of the selected rice varieties used for the present study

4.2 Reagents and Chemicals

4.2.1 RNA Isolation

- TRIzol® Reagent (Invitrogen, USA)
- Chloroform
- Isopropanol
- Ethanol (75%)
- Nuclease-free water
- DEPC-treated water
- RNaseZap™ solution

4.2.2 cDNA Synthesis

- iScript™ cDNA Synthesis Kit (Bio-Rad, USA)

4.2.3 Gradient PCR and Agarose gel Electrophoresis

- Emerald Green PCR Master mix (Takara, USA)
- Ethidium bromide
- 1X TAE buffer (Tris-Acetic acid-EDTA)
- 6x loading dye (40% sucrose, Xylene cyanol, Bromophenol blue)
- 100 bp DNA ladder (NEB, USA)
- Agarose (1.2% gel for RNA isolation and PCR products)

4.2.4 Quantitative Real-Time PCR

- SYBR Green Master Mix (Bio-Rad, USA)
- Gene-specific and Reference/Housekeeping gene primers
- Nuclease-free water

4.3 Instruments and Equipment

The following instruments were used for the present study:

- Refrigerated centrifuge (Eppendorf, Germany)
- Tabletop centrifuge (Neuation Technologies, India)
- Thermal cycler (Thermo Fisher Scientific, USA)
- CFX96 Real-Time PCR System (Bio-Rad, USA)
- GelDoc GO Imaging System (Bio-Rad, USA)
- Electrophoresis unit (Bio-Rad, USA)

- BioPhotometer (Eppendorf, Germany)
- Analytical balance (Shimadzu, Japan)
- Autoclave
- Microwave oven
- Deep freezer (-20°C and -80°C)
- Liquid nitrogen

4.4 Seed Germination, Seedling Establishment and Sample Collection

Approximately twenty seeds from each rice variety were surface-cleaned and wrapped in sterile moist cotton cloth to facilitate germination. The wrapped seeds were placed in centrifuge tubes containing water up to three-fourths of their volume and maintained under ambient laboratory conditions. Water was replaced every 24 h to prevent microbial contamination and ensure adequate aeration. After four days, uniformly germinated seeds exhibiting radicle emergence were transferred to paper cups containing moist local soil and maintained under natural environmental conditions. Seedlings were watered regularly and allowed to grow for two weeks. Fresh young leaves were harvested from healthy seedlings, immediately frozen in liquid nitrogen, and immediately used for RNA extraction. Young leaf tissues were selected because of their high metabolic activity and active expression of flavonoid biosynthetic genes.

4.5 RNase-Free Precautions for RNA Isolation

To prevent RNA degradation, strict RNase-free conditions were maintained throughout the experiment. All glassware, plasticware, and solutions used for RNA extraction were treated with 0.01% diethyl pyro carbonate (DEPC) and incubated overnight at room temperature. Subsequently, materials were autoclaved to eliminate residual DEPC and ensure RNase inactivation. Mortars, pestles, spatulas, and other reusable laboratory tools were treated with DEPC, wrapped in aluminium foil, and sterilized at 150ºC for 1hr before use. Work surfaces, pipettes, centrifuges, and laboratory benches were cleaned thoroughly with RNaseZap™ solution before and after RNA isolation. Gloves were worn throughout the procedure and changed frequently to minimize RNase contamination.

4.6 Isolation of Total RNA

Total RNA was isolated from leaf tissues using the TRIzol® reagent method with minor modifications optimized for rice tissues.

- 500 mg of frozen leaf tissue was ground into a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. The powdered tissue was immediately homogenized in 1 mL of TRIzol® reagent.
- The homogenate was incubated at room temperature for 10 min to permit complete dissociation of nucleoprotein complexes. Samples were centrifuged at 13,000 rpm for 10 min at 4°C to remove insoluble cellular debris.
- Subsequently, 200 µL of chloroform was added to each sample, followed by vigorous shaking for 15 s and incubation at room temperature for 3 min. The samples were centrifuged at 12,000 × g for 15 min at 4°C, resulting in phase separation. The upper aqueous phase containing RNA was carefully transferred to a fresh RNase-free microcentrifuge tube.
- RNA was precipitated by adding 500 µL of isopropanol and incubating at room temperature for 10 min. Following centrifugation at 12,000 × g for 10 min at 4°C, the RNA pellet was recovered and washed with 1 mL of 75% ethanol. Samples were vortexed briefly and centrifuged at 8,000 rpm for 4 min at 4°C.
- The ethanol was discarded carefully, and the RNA pellet was air-dried for approximately 5 min at room temperature. The dried pellet was dissolved in RNase-free water and stored at −80°C until further analysis.

4.7 Determination of RNA Quality

RNA concentration and purity were determined using a Bio-Photometer (Eppendorf, Germany). Purity was assessed by measuring absorbance ratios at A260/A280 and A260/A230. It measures nucleic acid concentration and purity based on the absorbance of ultraviolet (UV) light by RNA molecules. RNA absorbs UV light maximally at 260 nm due to the presence of aromatic nitrogenous bases. According to the Beer–Lambert law, absorbance is directly proportional to the concentration of nucleic acids in the sample.

The purity of RNA is assessed by measuring absorbance at 260 nm, 280 nm, and 230 nm:

- A260: Measures nucleic acid concentration.
- A280: Indicates protein contamination (mainly aromatic amino acids).
- A230: Indicates contamination by phenol, carbohydrates, polysaccharides, guanidine salts, or other organic compounds.
- A260/A280 ratio should be between 1.8 and 2.1.
- A260/A230 ratio should be approximately 2.0–2.2.

The concentration of RNA is calculated using the conversion factor:

$$\text{RNA concentration } (\mu g/mL) = A_{260} \times 40 \times \text{Dilution Factor}$$

4.8 Integrity assessment of RNA using Agarose Gel Electrophoresis

The integrity of total RNA isolated, DNA contamination and the extend of degradation were assessed through 1.2% agarose gel. It was prepared by dissolving 1.2 g agarose in 100 mL of 1× TAE buffer. The solution was heated until complete dissolution and allowed to cool before adding ethidium bromide (intercalating dye). The molten agarose was poured into a gel casting tray and allowed to solidify. RNA samples mixed with 6X- gel loading dye were loaded into the wells and electrophoresed at 70 V for about 40 minutes when the separation was achieved. The gel was visualized using a GelDoc GO Imaging System (Bio-Rad, USA), and images were documented for quality assessment.

4.9 First-Strand cDNA Synthesis

First-strand complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) according to the manufacturer's protocol.

The 20 µL reaction mixture consisted of:

- 4 µL 5× iScript Reaction Mix
- 1 µL iScript Reverse Transcriptase
- 1 µg total RNA
- Nuclease-free water to 20 µL

The reaction was carried out in a thermal cycler using the following conditions:

- 25°C for 5 min (primer annealing)
- 46°C for 20 min (reverse transcription)
- 95°C for 1 min (enzyme inactivation)

Synthesized cDNA was stored at −20°C and diluted ten-fold prior to quantitative real-time PCR analysis.

4.10 Gene-Specific Primers

Working primer solutions (10 pmol/µL) were prepared from 100 pmol/µL stock solutions by a 1:10 dilution using nuclease-free water. For each PCR reaction, 10 pmol/µL of both forward and reverse primers were used. All primers were standardised for annealing temperature using

gradient PCR (Temperature range 55ºC to 62ºC). The primers used for amplification of flavonoid biosynthetic genes are presented in Table 2.

Table 2. Primers used for quantitative real-time PCR analysis

Gene	Primer	Sequence (5′–3′)
PAL	Forward (PAL-F)	AGCTCCGTCAAGAACTGCGTC
	Reverse (PAL-R)	CGATGGCGGTGAGGAGGT
CHS	Forward (CHS-F)	GTTCACACCGCTGGGGATCT
	Reverse (CHS-R)	TCCTCTCCTTGTCCAGCCCA
F3H	Forward (F3H-F)	AGCACAGAAGCCCAAGTCTC
	Reverse (F3H-R)	CTTCGATTTTCGACGGAAGA
DFR	Forward (DFR-F)	GACATCGACTTCTGTCGCCG
	Reverse (DFR-R)	TGACGCTGATGAGGTCCAGC
ANS	Forward (ANS-F)	AGCTGCTCGCCATCCTCTCC
	Reverse (ANS-R)	GCTGACGTCGGTGTGTGCC
Actin	Forward (Actin-F)	GAACAACTGGGACGACAT
	Reverse (Actin-R)	CCTTTGGGTTCAGAGGAG

4.11 Quantitative Real-Time PCR (RT-qPCR)

Quantitative real-time PCR was performed to check the relative gene expression pattern using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with SYBR Green chemistry.

Each 20 µL reaction contained:

- 10 µL 2× SYBR Green Master Mix (Bio-Rad, USA)
- 1 µL forward primer (10 pmol)
- 1 µL reverse primer (10 pmol)
- 2 µL diluted cDNA template
- 6 µL nuclease-free water

The amplification program consisted of:

- Initial denaturation: 95°C for 3 min
- 40 amplification cycles:

- 95°C for 10 s
- 60°C for 30 s
- 72°C for 30 s

A melt curve analysis was performed following amplification to verify product specificity and confirm the absence of primer-dimer formation.

4.12 Analysis of Relative Gene Expression using Real-Time PCR Data Analysis

The relative expression level of flavonoid biosynthetic genes was quantified using the comparative threshold cycle (2^−ΔΔCt) method described by Livak and Schmittgen (2001). This method enables the determination of changes in gene expression levels between experimental samples and a control sample after normalization against an internal reference gene. In the present study, β-Actin was used as the endogenous reference gene because of its constitutive and stable expression across different rice genotypes and experimental conditions. The reference gene served to normalize variations arising from differences in RNA quantity, RNA quality, and cDNA synthesis efficiency among samples.

Quantitative real-time PCR reactions were carried out in triplicate for each biological sample. The cycle threshold (**Ct**) values obtained from the qRT-PCR instrument were recorded, and the mean Ct value of the three technical replicates was used for expression profiling analysis.

The relative expression of all selected genes was calculated using the following steps:

Step 1: Calculation of ΔCt

For each sample, the Ct value of the reference gene (β-Actin) was subtracted from the Ct value of the target genes:

$$\Delta Ct = Ct_{(target\ gene)} - Ct_{(\beta\text{-}Actin)}$$

This normalization step corrects for sample-to-sample variations in template concentration.

Step 2: Calculation of ΔΔCt

The ΔCt value of each test sample was compared with the ΔCt value of the control sample to determine the relative change in expression:

$$\Delta\Delta Ct = \Delta Ct_{(Test)} - \Delta Ct_{(Control)}$$

The control sample served as the calibrator (Njavara) against which all other samples were evaluated.

Step 3: Calculation of Relative Gene Expression

The fold change in candidate gene participating in flavonoid biosynthesis related expression relative to the control was determined using the following equation:

$$\text{Relative Expression (Fold Change)} = 2^{-\Delta\Delta Ct}$$

A fold-change value greater than 1 indicated upregulation of gene expression relative to the control, whereas a value less than 1 indicated downregulation. A fold-change value equal to 1 represented no significant change in expression. The calculated relative expression values were used to compare gene transcript abundance among the selected rice varieties. The results were expressed as mean fold-change values derived from three technical replicates and were subsequently used for statistical analysis and graphical representation.

4.13 Statistical Analysis

All RealTime-PCR reactions were performed in triplicates. Mean Ct values were used for the calculation of ΔCt, ΔΔCt, and fold-change values. Results were expressed as mean ± standard error (SE). Statistical analyses and graphical representations were performed using Microsoft Excel.

Chapter 5

Results

5.1 Total RNA isolation

Good quality total RNA was isolated from the leaf tissues of all selected rice varieties Njavara, Raktasali, Pratyasa, and Uma using the TRIzol reagent method. The isolated RNA exhibited satisfactory yield and quality to be continued with cDNA synthesis and gene expression profiling.

5.1.1Quality analysis of isolated RNA

The quantity and quality of total RNA isolated from the selected rice varieties were assessed using spectrophotometric analysis (Table3).

Table 3: Quality assessment of total RNA isolated from selected rice varieties based on RNA concentration and spectrophotometric purity ratios

Rice variety	Classification	RNA concentration (ng/µl)	A260/a280 ratio	A260/a230 ratio
Njavara	Traditional-Medicinal	385.4	2.02	1.68
Raktasali	Traditional-Medicinal	412.8	1.97	1.94
Uma	Released High-Yielding Variety	450.2	2.03	1.93
Pratyasa	Released High-Yielding Variety	445.6	2.00	1.90

Among the four varieties, Uma recorded the highest RNA concentration (450.2 ng/µL), followed closely by Pratyasa (445.6 ng/µL), Raktasali (412.8 ng/µL), and Njavara (385.4 ng/µL). The RNA yields obtained from all varieties were sufficiently high for downstream molecular analyses such as cDNA synthesis and quantitative real-time PCR (qRT-PCR). The A260/A280 ratio, which reflects RNA purity with respect to protein contamination, ranged from 1.97 to 2.03 across the samples. These values are close to the ideal value of 2.0 for pure RNA, indicating minimal protein or phenolic contamination. Uma exhibited the highest A260/A280 ratio (2.03), suggesting excellent RNA purity, while Raktasali showed the lowest value (1.97), which is still within the acceptable range for molecular biology applications.

The A260/A230 ratio, an indicator of contamination from carbohydrates, phenols, salts, or other organic compounds, ranged from 1.68 to 1.94. Raktasali showed the highest A260/A230

ratio (1.94), followed by Uma (1.93) and Pratyasa (1.90), indicating relatively clean RNA preparations. Njavara recorded a comparatively lower ratio (1.68), suggesting the presence of few residual contaminants, which is common in medicinal rice varieties due to their higher levels of secondary metabolites such as phenolics and polysaccharides. Nevertheless, this value remains suitable for gene expression studies.

5.1.2 Integrity of isolated RNA

The agarose gel electrophoresis confirms that the RNA isolated from Njavara, Raktasali, Uma, and Pratyasa is of high integrity and suitable quality for downstream molecular analyses, including reverse transcription, PCR amplification, and quantitative real-time PCR (qRT-PCR).

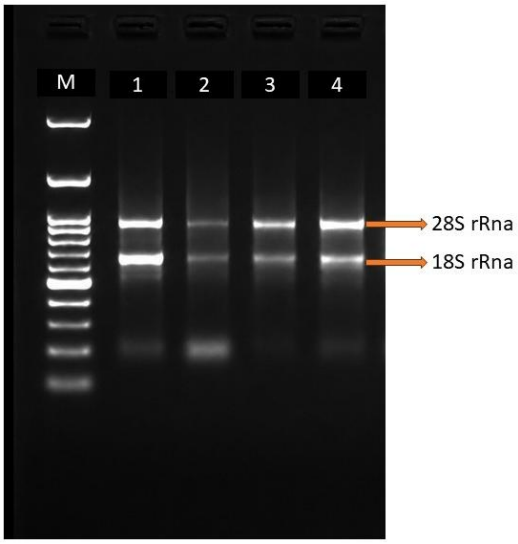


Figure:7 Agarose gel electrophoresis of total RNA isolated from selected rice varieties. Lane M: 100bp ladder, Lane 1: Njavara, Lane 2: Raktasali, Lane 3: Pratyasa, Lane 4: Uma

Distinct and well-resolved ribosomal RNA (rRNA) bands corresponding to 28S rRNA, 18S rRNA, and 5.8S rRNA were observed in all samples (Figure 7). The presence of sharp and intense 28S and 18S rRNA bands indicates that the RNA was intact and had not undergone significant degradation during extraction. The 28S rRNA band appeared more intense than the 18S rRNA band in all lanes, which is characteristic of high-quality eukaryotic RNA. No extensive smearing was observed throughout the gel, suggesting minimal RNA degradation and the absence of significant nuclease activity. The consistency of banding patterns among all four varieties further demonstrates the reproducibility of the RNA isolation procedure. Slight variations in band intensity among lanes may be attributed to differences in RNA concentration, which is in agreement with the spectrophotometric quantification results. The clear visualization of the three rRNA bands and the absence of substantial degradation products

confirm that the isolated RNA possessed good integrity and purity. Therefore, the RNA samples were considered suitable for downstream molecular applications, including cDNA synthesis, reverse transcription, and quantitative real-time PCR (qRT-PCR) analysis

5.2 cDNA Quality Assessment Using β-Actin Reference Gene

The quality and integrity of the synthesized cDNA were evaluated by quantitative real-time PCR (qRT-PCR) using the β-actin reference gene. The amplification profile exhibited a characteristic sigmoid-shaped curve with a Ct value of 24.3, indicating successful cDNA synthesis and adequate template quantity for downstream gene expression analysis. Melt curve analysis revealed a single, sharp melting peak, confirming the amplification of a specific PCR product without any non-specific amplification or primer-dimer formation (Figure 8). These results demonstrate that the synthesized cDNA was of high quality and suitable for reliable and reproducible quantification of target gene expression in the selected rice varieties.

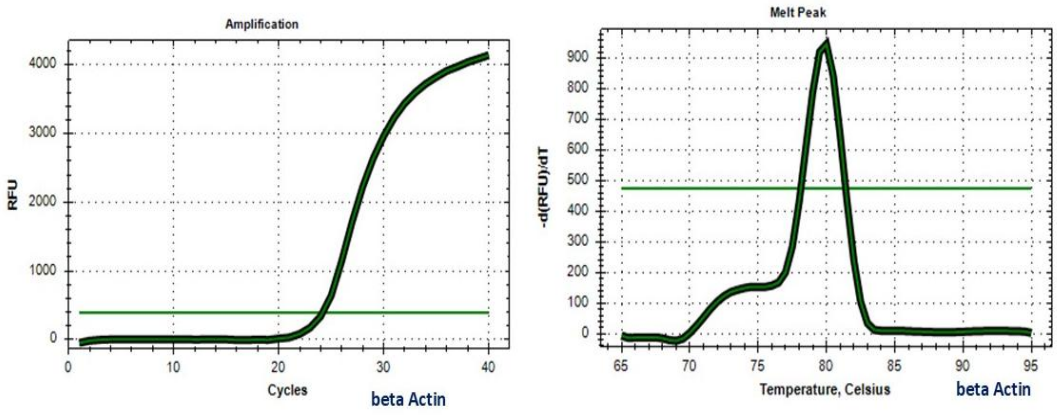


Figure 8: Representative qRT-PCR amplification and melt curve analysis of the β-actin reference gene used for cDNA quality assessment

The reliability of quantitative real-time PCR (qRT-PCR) data is fundamentally dependent on the stability of the internal reference (housekeeping) gene used for normalization. In this study, the β-actin (ACT) gene was tested across all biological replicates and experimental runs, ACT demonstrated highly stable and consistent amplification, yielding an average Cycle threshold (Ct) value of 24.3. The tight grouping of these replicates confirms high-quality total RNA isolation from the seedling tissues and validates the efficiency of the cDNA synthesis protocol. Consequently, the relative expression of the target genes of flavonoid biosynthetic pathway were mathematically normalized against ACT using the standard Livak method $(2^{-\Delta\Delta C_q})$.

5.3 Gene Expression Profiling

Quantitative real-time PCR (qRT-PCR) analysis was performed to investigate the expression patterns of five key genes involved in the flavonoid biosynthetic pathway, namely Phenylalanine Ammonia-Lyase (PAL), Chalcone Synthase (CHS), Flavanone 3-Hydroxylase (F3H), Dihydroflavonol 4-Reductase (DFR), and Anthocyanidin Synthase (ANS), in the rice varieties Raktasali, Pratyasa, and Uma, using Njavara as the reference genotype.

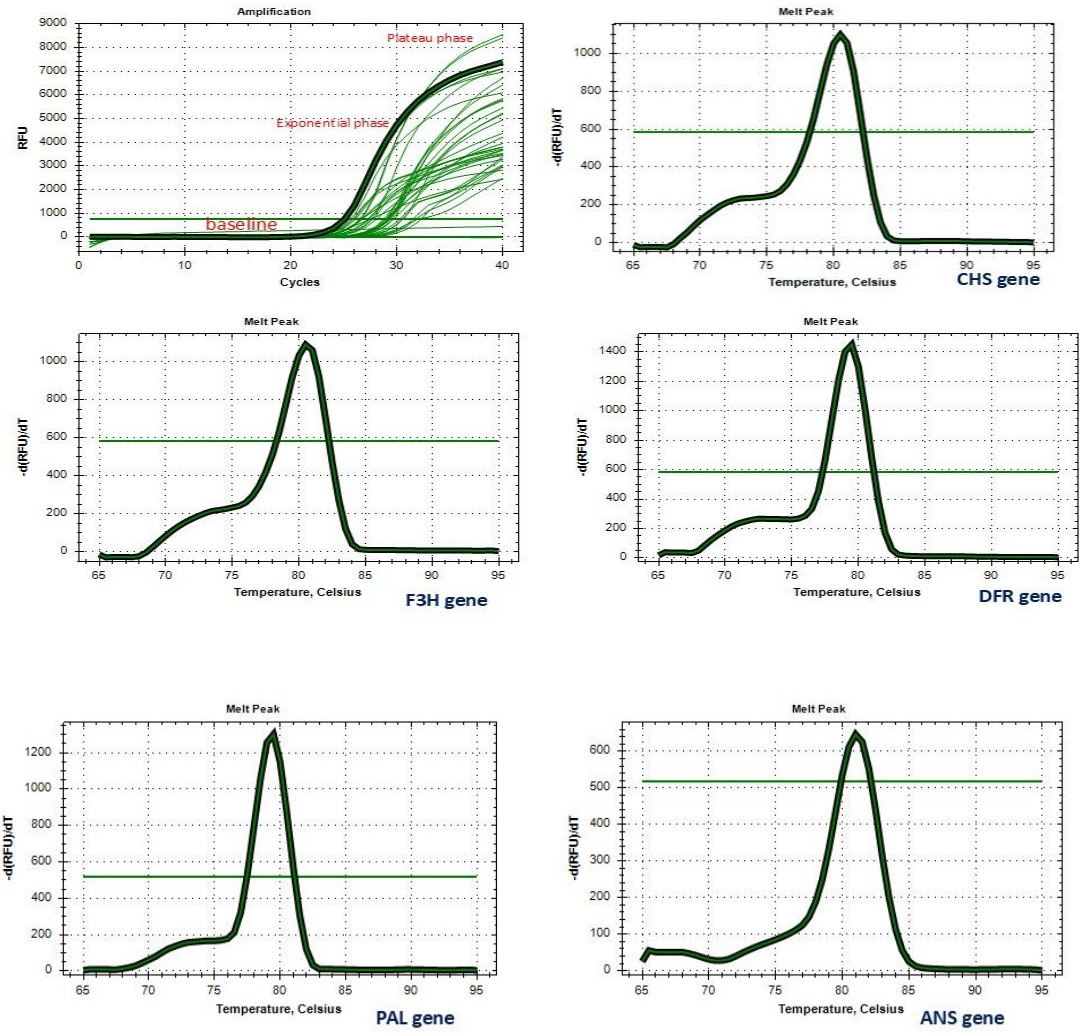


Figure:9 Representative amplification plot showing the typical sigmoid amplification curves and melt curve data for the primers used for the study

The analysis was undertaken to compare the expression profiles of flavonoid biosynthetic genes between medicinal rice varieties (Njavara and Raktasali) and non-medicinal/high-yielding rice varieties (Pratyasa and Uma), thereby providing insights into the molecular basis

underlying their differences in flavonoid accumulation and associated medicinal properties. All gene-specific primers used in the study exhibited efficient and specific amplification during qRT-PCR analysis, with no evidence of non-specific amplification or primer-dimer formation. Representative amplification plots displayed characteristic sigmoid-shaped curves, indicating efficient exponential amplification and consistent fluorescence signal accumulation throughout the PCR cycles. The absence of aberrant amplification patterns confirmed the high specificity and reliability of the designed primers, ensuring accurate quantification of target gene expression. A representative amplification plot showing the typical sigmoid amplification curves is presented in the corresponding figure (Figure 9).

The relative transcript abundance of each target gene was determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Gene expression levels were normalized against the internal reference gene β-actin to account for variations in RNA quantity and cDNA synthesis efficiency among samples. Fold-change values obtained from the analysis were used to assess the upregulation or downregulation of the selected flavonoid biosynthetic genes relative to the control genotype, Njavara. The expression profiles of the analysed genes are presented in the following sections.

5.3.1 Expression Profile of Phenylalanine Ammonia-Lyase (PAL) Gene

PAL is a key regulatory enzyme that catalyses the first committed step of the phenylpropanoid pathway by converting L-phenylalanine into trans-cinnamic acid. This reaction initiates the biosynthesis of a wide range of secondary metabolites, including flavonoids, lignins, phenolic acids, phytoalexins, and other phenylpropanoid-derived compounds. As the entry-point enzyme of the pathway, PAL plays a critical role in controlling the availability of precursors required for downstream flavonoid biosynthesis.

The qRT-PCR analysis revealed that all three test varieties exhibited downregulation of PAL expression relative to the Njavara control (Figure 10). The non-medicinal variety Pratyasa showed a marked reduction in transcript accumulation, while Raktasali and Uma also displayed substantial suppression of PAL expression. Overall, the expression levels of PAL were considerably lower in all tested varieties compared to the control genotype, indicating reduced transcriptional activity of this key phenylpropanoid pathway gene. The reduced expression of PAL observed in Pratyasa, Raktasali, and Uma suggests a lower activation of the phenylpropanoid biosynthetic pathway relative to Njavara. Since PAL catalyses the first committed step leading to the formation of cinnamic acid and subsequent phenylpropanoid

intermediates, decreased transcript abundance may limit the availability of substrates required for the synthesis of downstream flavonoids and related secondary metabolites.

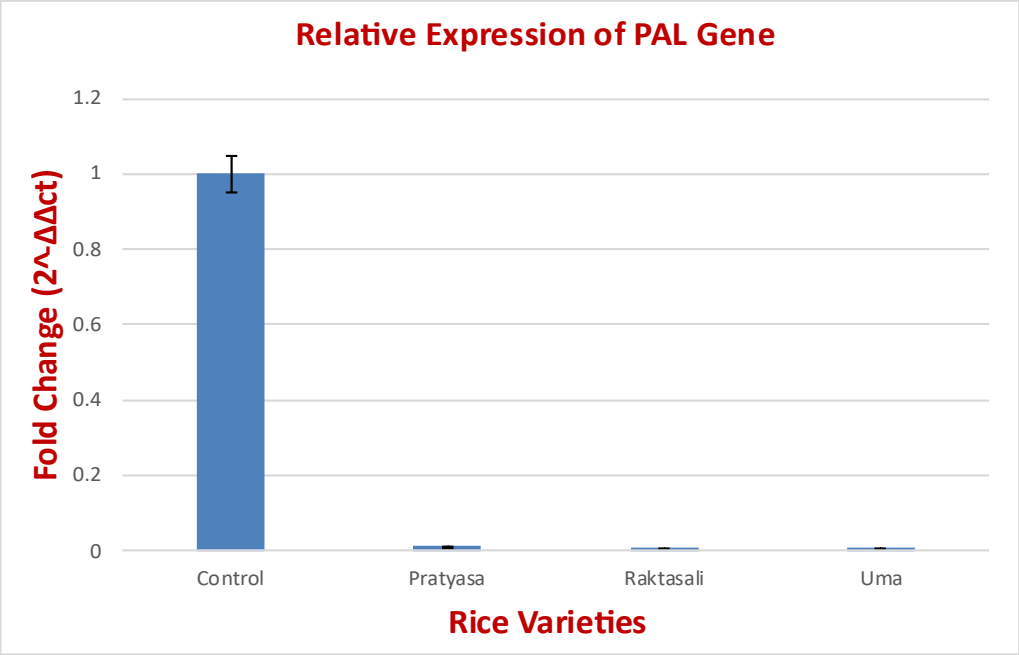


Figure 10: Relative expression profile of the Phenylalanine Ammonia-Lyase (PAL) gene in Pratyasa, Raktasali, and Uma rice varieties relative to the reference genotype Njavara, Relative transcript levels were calculated using the 2^−ΔΔCt method and normalized against the β-actin housekeeping gene. Error bars indicate the standard error of the mean (SEM) from biological replicates

PAL is particularly important in tricin biosynthesis because it provides the initial phenylpropanoid precursors from which the flavonoid pathway is derived. The products generated through PAL activity are subsequently converted through a series of enzymatic reactions involving cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), and other downstream enzymes to produce flavones, including tricin. Therefore, PAL functions as a fundamental upstream regulator of the entire biosynthetic network leading to tricin formation. Reduced PAL expression may consequently affect the metabolic flux entering the flavonoid pathway and influence the accumulation of tricin and other phenolic compounds.

The comparatively higher PAL transcript accumulation in Njavara indicates a greater activation of the phenylpropanoid pathway in this medicinal rice variety. Enhanced expression of this upstream regulatory gene may support increased biosynthesis of flavonoids, phenolic acids, and tricin-related metabolites, which are often associated with the nutritional and medicinal properties of traditional rice landraces. In contrast, the lower PAL expression observed in

Pratyasa, Raktasali, and Uma suggests reduced precursor availability for downstream phenolic metabolism.

The expression analysis demonstrated significant downregulation of PAL in all tested rice varieties relative to the Njavara control. The highest PAL transcript accumulation was observed in Njavara, highlighting its enhanced phenylpropanoid pathway activity. As PAL represents the primary entry point into flavonoid and tricin biosynthesis, the observed differences in gene expression indicate substantial varietal variation in the regulation of secondary metabolite production among the rice genotypes studied.

5.3.2 Expression Profile of Chalcone Synthase (CHS) Gene

The relative expression of the Chalcone Synthase (CHS) gene was analysed in the rice varieties Pratyasa, Raktasali, and Uma using quantitative real-time PCR (qRT-PCR), with Njavara serving as the reference genotype. Relative transcript levels were calculated using the 2^−ΔΔCt method, and the results are presented as fold changes relative to the control. Error bars represent the standard error of the mean calculated from biological replicates. CHS is the first committed and rate-limiting enzyme of the flavonoid biosynthetic pathway. It catalyses the condensation of one molecule of p-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone, the precursor for a wide range of flavonoid compounds including flavones, flavonols, anthocyanins, and tricin. Since tricin biosynthesis originates from the general flavonoid pathway, CHS plays a crucial role in determining the metabolic flux toward the production of tricin and other flavonoid-derived metabolites.

The qRT-PCR analysis revealed that all three test varieties exhibited reduced CHS expression relative to the Njavara control (Figure 11). Among the varieties analysed, Pratyasa and Raktasali displayed comparable levels of downregulation, indicating a similar reduction in CHS transcript abundance. In contrast, Uma showed the lowest CHS expression level, representing the greatest reduction among the tested varieties. The observed decrease in CHS transcript accumulation suggests a reduced activation of the early flavonoid biosynthetic pathway in Pratyasa, Raktasali, and Uma compared with Njavara. As CHS catalyses the initial committed step leading to the formation of flavonoid precursors, lower expression of this gene may limit the availability of intermediate compounds required for the synthesis of downstream metabolites, including tricin. Since tricin biosynthesis proceeds through successive enzymatic modifications of flavonoid intermediates derived from the CHS-catalyzed reaction, the reduced

expression of CHS may influence the overall biosynthetic capacity for tricin production in these varieties.

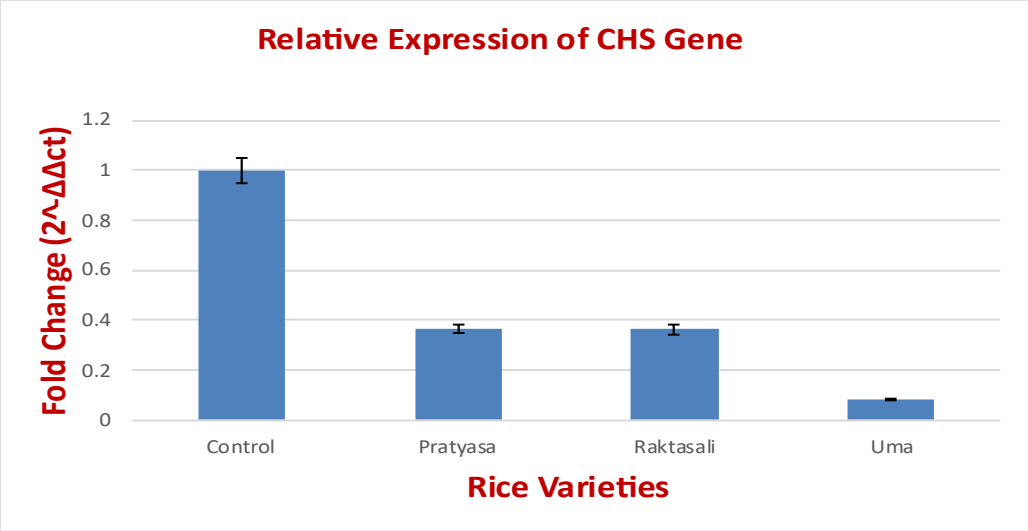


Figure 11: Relative expression profile of the Chalcone Synthase (CHS) gene in Pratyasa, Raktasali, and Uma rice varieties compared with the reference genotype Njavara. Relative transcript levels were calculated using the 2^−ΔΔCt method and normalized against the β-actin housekeeping gene. Error bars represent the standard error of the mean (SEM) from biological replicates

The highest CHS transcript accumulation observed in Njavara indicates a comparatively greater activation of the flavonoid biosynthetic pathway in this medicinal rice variety. This enhanced expression may contribute to increased synthesis of flavonoid-derived compounds, including tricin and other phenolic metabolites, which are associated with the nutritional and medicinal properties of traditional rice landraces. The differential expression pattern observed among the varieties demonstrates variation in the regulation of flavonoid biosynthesis and highlights the importance of CHS as a key regulatory gene controlling the entry of carbon flux into the tricin biosynthetic pathway.

5.3.3 Expression Profile of Flavanone 3-Hydroxylase (F3H) Gene

The relative expression of the Flavanone 3-Hydroxylase (F3H) gene was evaluated in the rice varieties Pratyasa, Raktasali, and Uma with Njavara serving as the reference genotype. F3H encodes a 2-oxoglutarate-dependent dioxygenase that catalyses the stereospecific hydroxylation of flavanones, such as naringenin, to form dihydroflavonols. This reaction represents an important branch point in the flavonoid biosynthetic pathway, supplying precursors for the synthesis of flavonols, anthocyanins, proanthocyanidins, and other specialized flavonoid metabolites. In grasses, the products generated through F3H-mediated

reactions contribute to the formation of intermediates required for the biosynthesis of flavone compounds, including tricin.

The qRT-PCR analysis revealed distinct differences in F3H expression among the rice varieties examined (Figure 12). Compared with the Njavara control, the medicinal rice variety Raktasali exhibited significant upregulation of the F3H gene, indicating enhanced transcript accumulation. In contrast, both non-medicinal varieties, Pratyasa and Uma, showed reduced expression levels relative to the control. Pratyasa displayed moderate downregulation, whereas Uma exhibited the lowest expression level, indicating substantial suppression of F3H transcription among the tested varieties.

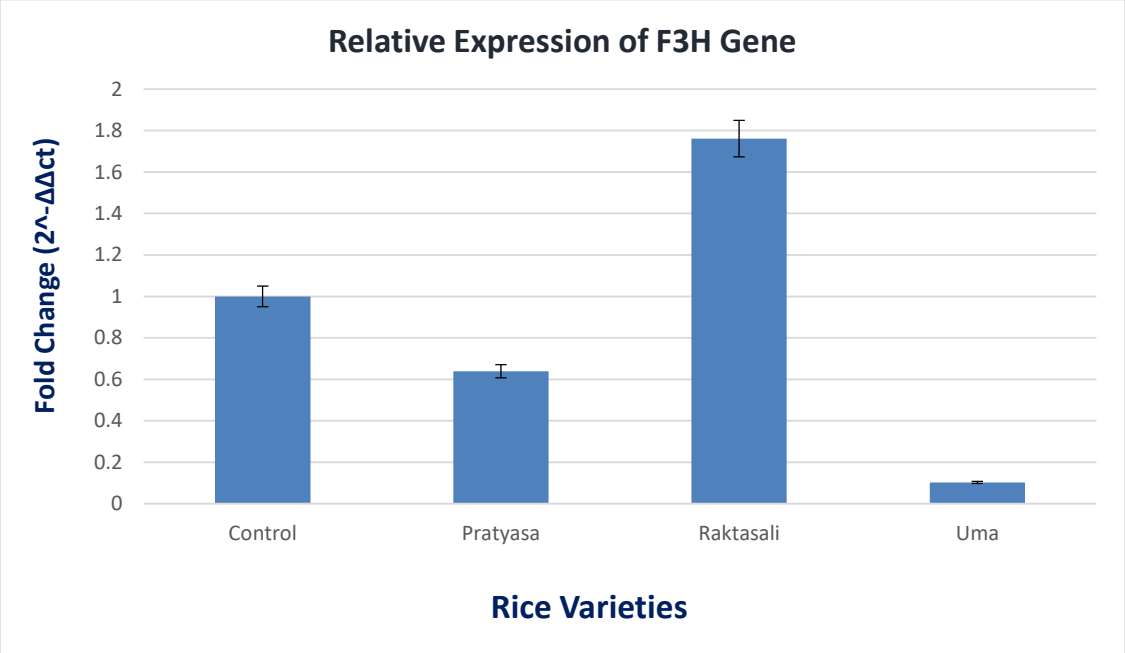


Figure 12: Relative expression profile of the Flavanone 3-Hydroxylase (F3H) gene in Pratyasa, Raktasali, and Uma rice varieties relative to the reference genotype Njavara. Expression levels were calculated using the 2^−ΔΔCt method with β-actin as the internal control. Error bars indicate the standard error of the mean (SEM) from biological replicates

The elevated expression of F3H in Raktasali suggests increased metabolic activity through the central flavonoid biosynthetic pathway. Since F3H catalyses the conversion of flavanones into di-hydroflavonols, enhanced transcript accumulation may facilitate greater production of downstream flavonoid intermediates required for the synthesis of diverse phenolic compounds. In grasses such as rice, these intermediates contribute indirectly to the formation of tricin through subsequent hydroxylation, methylation, and flavone biosynthetic reactions. Therefore, the increased expression of F3H observed in Raktasali may support a greater metabolic flux

toward tricin biosynthesis and the accumulation of other flavonoid-derived secondary metabolites.

Conversely, the reduced expression of F3H in Pratyasa and Uma indicates a lower level of activation of the flavonoid pathway. Reduced transcript abundance of this gene may limit the formation of di-hydroflavonol intermediates and consequently decrease the availability of substrates required for downstream biosynthetic reactions. The markedly lower expression observed in Uma suggests a comparatively reduced capacity for flavonoid biosynthesis and may influence the accumulation of tricin and related phenolic compounds in this variety.

The contrasting expression patterns observed between the medicinal and non-medicinal rice varieties highlight the differential regulation of the F3H gene. The significant upregulation of F3H in Raktasali, together with its role in generating key flavonoid intermediates, indicates the active operation of flavonoid metabolic pathways in this medicinal rice genotype. These findings suggest that F3H may contribute to varietal differences in flavonoid composition and could play an important role in regulating the biosynthetic network leading to tricin production in rice. Altogether, the expression analysis demonstrated that F3H transcripts accumulated at the highest level in Raktasali, whereas Pratyasa and Uma exhibited reduced expression relative to the control. This differential expression pattern indicates variation in flavonoid pathway activity among the rice varieties and underscores the importance of F3H as a key gene involved in the biosynthesis of tricin and other flavonoid metabolites.

5.3.4 Expression Profile of Di-hydro flavonol 4-Reductase (DFR) Gene

DFR encodes a key enzyme of the flavonoid biosynthetic pathway that catalyses the NADPH-dependent reduction of di-hydro flavonols to leucoanthocyanidins. These compounds serve as important intermediates in the biosynthesis of anthocyanins, proanthocyanidins, and other flavonoid-derived metabolites. As DFR functions at a downstream stage of the flavonoid pathway, its expression reflects the extent to which flavonoid precursors are channelled toward the synthesis of specialized phenolic compounds. The qRT-PCR analysis revealed distinct expression patterns of DFR among the rice varieties examined (Figure 13). Compared with the Njavara control, the medicinal rice variety Raktasali exhibited a slight increase in DFR transcript accumulation, indicating moderate upregulation of the gene. In contrast, both non-medicinal varieties showed reduced expression levels relative to the control. Pratyasa displayed moderate downregulation, with transcript levels reduced to approximately half of those

observed in Njavara, whereas Uma exhibited the lowest expression level, indicating strong suppression of DFR transcription.

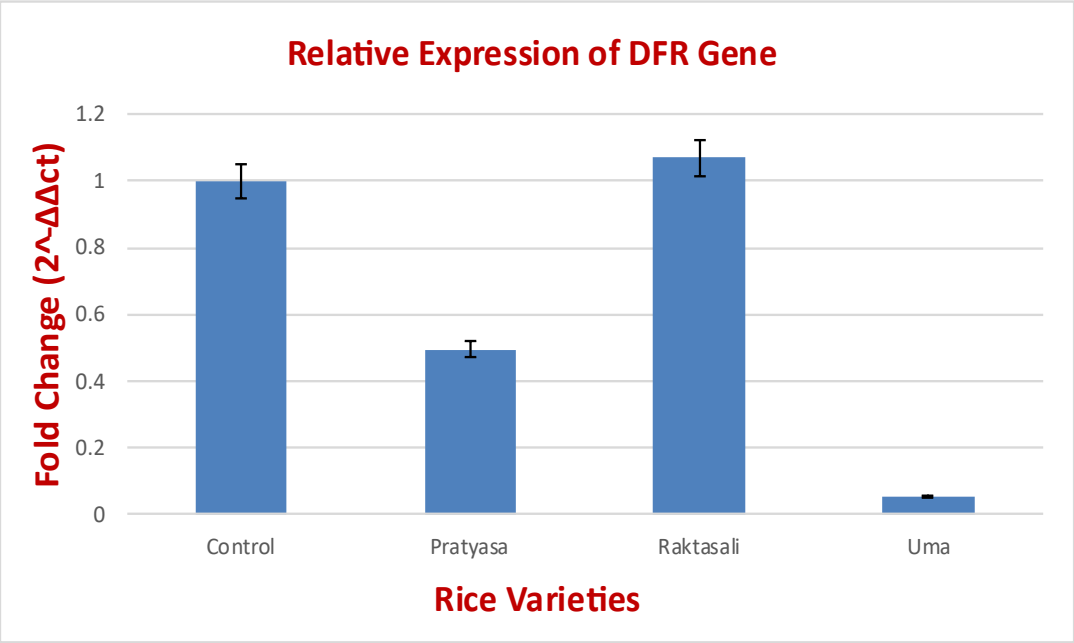


Figure 13: Relative expression profile of the Di-hydroflavonol 4-Reductase (DFR) gene in Pratyasa, Raktasali, and Uma rice varieties compared with the reference genotype Njavara. Relative transcript levels were calculated using the 2^−ΔΔCt method and normalized against the β-actin housekeeping gene. Error bars represent the standard error of the mean (SEM) from biological replicates

The elevated expression of DFR observed in Raktasali suggests enhanced activity of downstream flavonoid biosynthesis in this medicinal rice variety. Increased transcript accumulation of DFR may facilitate greater conversion of di-hydroflavonols into leucoanthocyanidins, thereby supporting the production of anthocyanins and other flavonoid-derived metabolites. Although DFR is not directly involved in the tricin-specific biosynthetic branch, its expression serves as an indicator of the overall activation of flavonoid metabolism. Enhanced DFR expression therefore reflects a greater metabolic commitment toward flavonoid biosynthesis and may be associated with increased accumulation of phenolic compounds in Raktasali.

In contrast, the reduced expression observed in Pratyasa and Uma indicates a lower level of downstream flavonoid pathway activity. Suppression of DFR expression may reduce the conversion efficiency of di-hydroflavonol intermediates into downstream products, thereby limiting the biosynthesis of anthocyanins and related flavonoids. The markedly lower expression detected in Uma suggests a comparatively weaker activation of flavonoid metabolic processes in this variety. The expression pattern observed for DFR further highlights the

differences between medicinal and non-medicinal rice varieties with respect to flavonoid pathway regulation. The slight upregulation of DFR in Raktasali, together with the downregulation observed in Pratyasa and Uma, indicates varietal variation in the expression of genes involved in secondary metabolite biosynthesis. Such differences may contribute to the distinct phytochemical profiles and medicinal properties associated with traditional rice landraces. Overall, the results demonstrated that DFR expression was highest in Raktasali and lowest in Uma, while Pratyasa exhibited intermediate transcript levels. The differential expression of DFR among the rice varieties suggests variation in the regulation of downstream flavonoid biosynthesis and supports the existence of enhanced flavonoid metabolic activity in medicinal rice genotypes.

5.2.5 Expression Profile of Anthocyanidin Synthase (ANS) Gene

The relative expression of the Anthocyanidin Synthase (ANS) gene was evaluated in the rice varieties Pratyasa, Raktasali, and Uma using quantitative real-time PCR (qRT-PCR), with Njavara serving as the reference genotype. Relative transcript levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method and normalized against the β-actin housekeeping gene. Error bars represent the standard error of the mean obtained from biological replicates.

ANS, also known as leucoanthocyanidin dioxygenase (LDOX), is a key enzyme in the late stages of the flavonoid biosynthetic pathway. It catalyses the conversion of leucoanthocyanidins into anthocyanidins, which subsequently give rise to anthocyanin pigments responsible for red, purple, and blue coloration in plant tissues. In addition to their role in pigmentation, anthocyanins contribute to antioxidant activity and protection against various environmental stresses. The qRT-PCR analysis revealed distinct patterns of ANS expression among the rice varieties examined (Figure 14). Compared with the Njavara control, the medicinal rice variety Raktasali exhibited a marked upregulation of ANS expression, with transcript levels increasing to approximately 3.5-fold relative to the control. This represented the highest ANS expression among the varieties analysed. In contrast, the non-medicinal varieties displayed different expression responses. Pratyasa maintained expression levels comparable to those of the control genotype, indicating little or no significant change in ANS transcript accumulation. Conversely, Uma exhibited downregulation of ANS expression, showing reduced transcript levels relative to Njavara.

The substantial increase in ANS expression observed in Raktasali suggests enhanced activation of the late flavonoid biosynthetic pathway in this medicinal rice variety. Elevated transcript

accumulation of ANS may promote increased conversion of leucoanthocyanidins into anthocyanidins, thereby facilitating the biosynthesis of anthocyanin pigments and related flavonoid compounds. The strong upregulation of ANS is consistent with the enhanced flavonoid metabolism often associated with pigmented and medicinal rice genotypes.

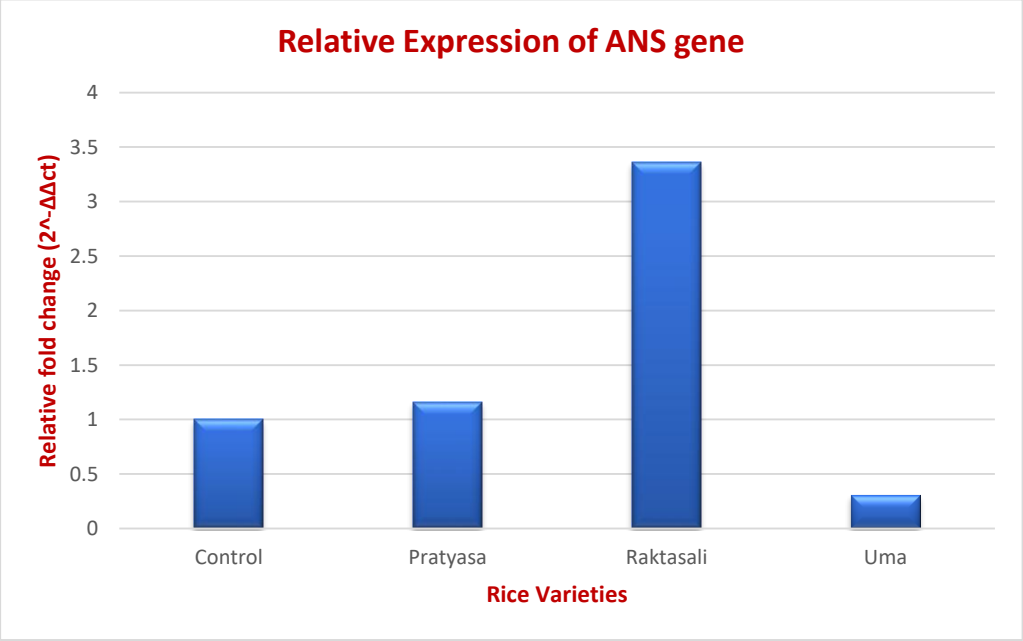


Figure 14: Relative expression profile of the Anthocyanidin Synthase (ANS) gene in Pratyasa, Raktasali, and Uma rice varieties relative to the reference genotype Njavara. Relative transcript levels were calculated using the 2^−ΔΔCt method and normalized against the β-actin housekeeping gene. Error bars indicate the standard error of the mean (SEM) from biological replicates

Although ANS is not directly involved in the tricin-specific biosynthetic pathway, its expression provides valuable information regarding the overall activity of flavonoid metabolism. Tricin biosynthesis in rice primarily proceeds through the flavone branch of the flavonoid pathway, whereas ANS functions in the anthocyanin branch. Nevertheless, both branches originate from common upstream phenylpropanoid and flavonoid intermediates. Therefore, elevated ANS expression may reflect increased flux through the broader flavonoid biosynthetic network and indicate enhanced production of diverse flavonoid-derived secondary metabolites in Raktasali. The stable expression observed in Pratyasa suggests maintenance of basal anthocyanin biosynthetic activity, while the reduced expression detected in Uma indicates lower activation of the anthocyanin branch of flavonoid metabolism. These differences demonstrate considerable variation in the regulation of downstream flavonoid biosynthetic genes among the rice varieties studied.

The pronounced upregulation of ANS in Raktasali, together with the expression patterns of other flavonoid biosynthetic genes, suggests that this medicinal rice variety possesses a more active flavonoid metabolic system than the non-medicinal varieties. Such enhanced expression may contribute to the accumulation of anthocyanins and other bioactive phenolic compounds that are characteristic of traditional medicinal rice landraces. Collectively, the expression analysis demonstrated that ANS transcripts accumulated at the highest level in Raktasali, remained relatively unchanged in Pratyasa, and were downregulated in Uma. These findings indicate significant varietal differences in the regulation of the anthocyanin biosynthetic pathway and further support the enhanced activation of flavonoid metabolism in the medicinal rice genotype Raktasali.

Chapter 6

Discussion

The present study was undertaken to evaluate and compare the transcriptional expression profiles of key genes associated in flavonoid biosynthesis associated with tricin biosynthesis among selected medicinal and non-medicinal rice varieties of Kerala. The expression patterns of five major genes, namely CHS, F3H, DFR, PAL and ANS, were analysed using quantitative real- time PCR (qRT -PCR) in the rice varieties Raktasali, Pratyasa, and Uma, with the medicinal rice Njavara serving as the control genotype. Understanding the differential expression of these genes provide insights into the molecular mechanisms underlying the variation in flavonoid biosynthesis and unique phytochemical potential associated with different rice genotypes.

To ensure the reliability of gene expression analysis, the quality and integrity of the isolated RNA were carefully assessed prior to cDNA synthesis. Total RNA extracted from leaf tissues using the TRIzol- based method showed distinct and intact ribosomal RNA bands. The successful synthesis of first -strand cDNA further confirmed the quality of the isolated RNA and provide reliable template for subsequent qRT- PCR analysis, thereby ensuring accurate and reproducible gene expression.

The present study revealed the genotype-dependent differences in the expression of genes associated with the tricin biosynthetic pathway among the selected rice varieties. The traditional red rice variety Raktasali exhibited relatively higher expression of key downstream pathway genes, particularly F3H and DFR and ANS, compared with the other tested genotypes, suggesting enhanced transcriptional activation of the later stages of the pathway. These enzymes participate in the conversion of intermediate metabolites associated with tricin biosynthesis. In contrast, Pratyasa exhibited reduced expression of tested genes relative to the control, Njavara. However, the ANS expression was maintained at a level comparable to that of control. In contrast, Uma exhibited the lowest transcript levels for the analysed genes. Furthermore, the expression of the upstream gene PAL was lower in all three test varieties relative to the control genotype, Njavara. Collectively, these findings indicate differential regulation of genes associated with the tricin biosynthetic pathway among the studied rice genotypes under the specific developmental stage and tissue conditions examined in the present study. The observed variation in transcript accumulation provides insight into the molecular

regulation of tricin biosynthesis and highlights genotype-specific differences in the expression patterns of the selected pathway genes.

The transcriptional variations observed among the selected medicinal and non-medicinal rice varieties of Kerala are consistent with several reports describing genotype-dependent regulation of genes associated with tricin biosynthesis in rice and other cereal crops. In the present study, Raktasali exhibited relatively higher expression of the downstream genes F3H, DFR and ANS compared with the other tested varieties. Similar differential regulation of structural genes has been reported by Shih et al., 2008, who demonstrated that genes involved in flavonoid biosynthesis may exhibit distinct expression profiles depending on the genetic background of the rice genotype. Furthermore, chen et al.,2019 reported considerable variation in the expression of biosynthetic genes among pigmented rice varieties, suggesting that individual genes within the pathway are subject to complex transcriptional regulation rather than controlled coordinated expression.

The findings of the present study suggest that the expression of genes associated with tricin biosynthesis is regulated through multiple transcriptional sites. Lam et al. (2014) reported that tricin biosynthesis in rice proceeds through unique enzymatic steps that diverge from the general flavonoid pathway, highlighting the complexity of pathway regulation. The differential expression patterns observed in the present investigation further support the view that individual genes within the pathway may be regulated independently depending on the genetic background of the rice genotype.

A prominent observation in the present study was the comparatively higher PAL expression observed in the control genotype Njavara relative to the tested varieties. This finding is consistent with the established role of PAL as the rate limiting enzyme of the phenylpropanoid pathway. According to Vogt (2010), PAL plays a significant role in directing carbon flux from primary metabolism towards the synthesis of diverse phenolic compounds. Similarly, Grotewold (2006) reported that increased precursor availability through the phenylpropanoid pathway does not necessarily result in a proportional increase in all downstream flavonoid biosynthetic genes, as intermediates may be utilized by multiple competing metabolic branches. This may explain the absence of a corresponding increase in all downstream genes in Njavara despite its comparatively higher PAL expression.

The lower transcript accumulation observed in the commercial variety Uma compared with the traditional rice varieties is in agreement with previous studies demonstrating the influence of

genetic background on secondary metabolite biosynthesis. Matsuda et al. (2012) reported that the expression of genes involved in specialized metabolic pathways may be influenced by domestication and selection for agronomic traits. In contrast, traditional landraces often retain unique genetic characteristics associated with the production of secondary metabolites. The differential expression of several tricin biosynthetic genes in Raktasali and the distinct expression profile observed in Njavara suggest the presence of genotype-specific regulatory mechanisms that may contribute to their unique phytochemical characteristics.

Moreover, the relationship between upstream pathway activation and downstream structural gene expression is often non-linear, which is a well-recognized feature of plant secondary metabolism. Dixon et al. (2002) reported that enzyme interactions, substrate availability and metabolic channelling can significantly influence metabolic flux within biosynthetic pathways. Therefore, transcript abundance should be considered an indicator of potential pathway activity rather than a direct measure of final metabolite accumulation. This observation further emphasizes the need for metabolite profiling to establish a direct relationship between gene expression patterns and tricin content in the studied rice varieties.

Summary and Conclusion

The present study provides a comprehensive molecular evaluation of selected medicinal (Njavara and Raktasali) and non-medicinal released rice varieties (Uma and Pratyasa) of Kerala by investigating the transcriptional expression of key genes involved in the tricin and flavonoid biosynthetic pathways. Gene expression analysis revealed clear genotype-dependent differences in the expression of PAL, CHS, F3H, DFR, and ANS, indicating differential regulation of flavonoid biosynthesis among the studied rice varieties. The medicinal variety Raktasali exhibited relatively higher expression of the downstream biosynthetic genes F3H, DFR, and ANS, while Njavara showed higher expression of PAL, the gateway enzyme of the phenylpropanoid pathway. In contrast, the released high-yielding variety Uma displayed comparatively lower transcript levels for most of the genes analysed, suggesting reduced metabolic activity towards flavonoid biosynthesis. The enhanced expression of flavonoid biosynthetic genes in the medicinal rice varieties indicates their greater potential for the synthesis of tricin and other phenolic compounds, which may contribute to their reported therapeutic properties and superior antioxidant potential.

These findings highlight the importance of indigenous rice germplasm as a valuable source of genetic variation for secondary metabolite biosynthesis and emphasize their potential use in breeding programmes aimed at improving grain nutritional quality, antioxidant capacity, and stress tolerance. However, transcriptional expression alone does not necessarily reflect the accumulation of tricin and other flavonoids, as metabolite production is also influenced by post-transcriptional regulation, enzyme activity, substrate availability, developmental stage, and environmental factors. Therefore, future studies integrating metabolomic profiling (HPLC/LC-MS/MS), enzyme activity assays, and functional characterization of candidate genes are required to establish a direct relationship between gene expression and metabolite accumulation. Furthermore, the identification of regulatory transcription factors and allelic variations controlling these biosynthetic pathways will facilitate marker-assisted breeding and genomic improvement for the development of nutritionally enhanced and functionally superior rice varieties.

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