Biochemical and Molecular Characterization of Traditional Rice Varieties Through GBSS Gene Expression and Protein Profiling

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CERTIFICATE

This is to certify that the dissertation entitled **"Biochemical and Molecular Characterization of Traditional Rice Varieties Through GBSS Gene Expression and Protein Profiling"** 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 **RAISHA RIYAS P. (Candidate code: 63824142008)** 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.

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Declaration

I hereby declare that the thesis titled "**Molecular and Biochemical Characterization of Traditional Rice Varieties Through GBSS Gene Expression and Protein Profiling."** 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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Abbreviations

AGE : Agarose Gel Electrophoresis

AK : Aspartate kinase

APS : Ammonium Persulfate

BCA : Bicinchoninic Acid

BME : Beta mercaptoethanol

BSA : Bovine serum albumin

cDNA : Complementary DNA

CT value : Cycle threshold

CVD : Cardiovascular Disease

DEPC : Diethyl Pyrocarbonate

DHPS : Dihydro dipicolinate synthase

EtBr : Ethidium Bromide

GBSS : Granule Bound starch synthase

GI : Glycemic Index

GPC : Granule protein content

HCL : Hydrochloric acid

LKR/SDH : lysine ketoglutarate reductase /saccharopine dehydrogenase

qRT-PCR : Quantitative real time polymerase chain reaction

RNA : Ribonucleic Acid

rRNA : Ribosomal Ribonucleic Acid

RS : Resistant starch

SDS : Slowly digestable starch

SDS-PAGE : Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SDW : Sterile distilled water

SNP : Single nucleotide polymorphism

TAE : Tris-Acetic acid-EDTA

TEMED : N,N,N′,N′-Tetramethylethylenediamine

Tris-HCl : Tris(hydroxymethyl)aminomethane hydrochloride.

Wx : Waxy

Abstract

Rice (*Oryza sativa* L.) is one of the world's most important cereal crops, and its grain quality is largely influenced by the regulation of starch biosynthesis and seed protein composition. The present study aimed to characterize selected rice varieties, namely Ponmani, Jeerakasala, Basmati, Njavara, and Uma, through integrated molecular and biochemical analyses. Total soluble proteins were extracted and quantified using the Bicinchoninic Acid (BCA) assay, while seed protein profiles were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Total RNA was isolated, reverse transcribed into cDNA, and the relative expression of starch biosynthesis-related genes (*pre-GBSS1, GBSS1,* and *GBSS2*) was quantified by quantitative real-time PCR (qRT-PCR).

The results revealed significant genotype-dependent variation in both gene expression and protein characteristics among the rice varieties. Jeerakasala exhibited the highest relative expression of *pre-GBSS1* and *GBSS1*, whereas Basmati showed the highest *GBSS2* transcript abundance. Njavara displayed relatively high expression of both *GBSS1* and *GBSS2*. Soluble protein estimation showed that Njavara possessed the highest protein concentration, while Basmati recorded the lowest. SDS-PAGE analysis revealed distinct seed protein profiles among the rice varieties, including a prominent protein band of approximately 55 kDa corresponding to granule-bound starch synthase (GBSS), with differences in band intensity indicating variation in protein accumulation across genotypes.

The combined molecular and biochemical analyses demonstrated clear varietal differences in the expression of starch biosynthesis-related genes, soluble protein content, and seed protein profiles. Since transcript abundance and protein accumulation represent different levels of biological regulation, these datasets provide complementary information for characterizing genetic diversity rather than indicating a direct correlation between gene expression and protein abundance. The findings provide valuable baseline information for understanding molecular and biochemical variation among rice genotypes and may support future studies aimed at improving grain quality, nutritional attributes, and rice breeding.

CHAPTER 1

Introduction

Oryza sativa L. (rice) belongs to the genus *Oryza* within the family Poaceae. The genus *Oryza* comprises approximately 22 wild species (2n = 24, 48) and two cultivated species, Asian rice (*Oryza sativa* L.; 2n = 24, AA genome) and African rice (*Oryza glaberrima*Steud.; 2n = 24, AA genome) (Singh, 2000; Vaughan et al., 2003). Based on eco-geographical distribution and morphological characteristics, Asian rice has differentiated into three major subspecies: *indica*, *japonica*, and *javanica* (Singh, 2000; Vaughan et al., 2003). Southeast Asia is widely regarded as the center of origin and domestication of *Oryza sativa* (Vaughan, 1994).

Rice exhibits diverse functional and cooking properties primarily due to variations in the proportions of its two major starch components, amylose and amylopectin. Amylose, a predominantly linear polysaccharide, generally constitutes 15–30% of rice starch and contributes to a firmer and less sticky texture in cooked rice. In contrast, amylopectin, a highly branched polysaccharide that accounts for approximately 70–99% of rice starch, imparts a soft, cohesive, and sticky texture upon cooking (Juliano & Tuaño, 2019; Wang et al., 2020). The concentrations of amylose and amylopectin vary among rice types and are closely associated with the two major subspecies, *indica* and *japonica*. *Indica* rice varieties typically contain higher amylose levels (20–30%), resulting in cooked grains that are firmer, drier, and less adhesive. Conversely, *japonica* varieties generally possess lower amylose contents (10–20%), producing softer and stickier cooked rice (Fitzgerald et al., 2009).

Amylose content also influences the digestibility of rice starch. High-amylose starches are generally less susceptible to enzymatic digestion because amylose molecules form compact and tightly packed structures that restrict enzyme access. Consequently, rice varieties with higher amylose content tend to exhibit a lower glycemic index (GI) and produce a slower postprandial rise in blood glucose levels (Boers et al., 2015). For example, high-amylose rice varieties such as Basmati rice are often associated with a lower GI and may be beneficial for glycemic management. Rice is commercially classified into several grain types, including white, brown, black, red, and glutinous rice. Both white and brown rice can belong to either the *indica* or *japonica* subspecies, depending on the cultivar. Red rice is generally associated with *indica* types and is characterized by its non-sticky texture and pigmented bran layer. Glutinous (waxy) rice is predominantly classified within the *japonica* group and is

distinguished by its extremely low amylose content and highly sticky texture after cooking (Farooq & Yu, 2025).

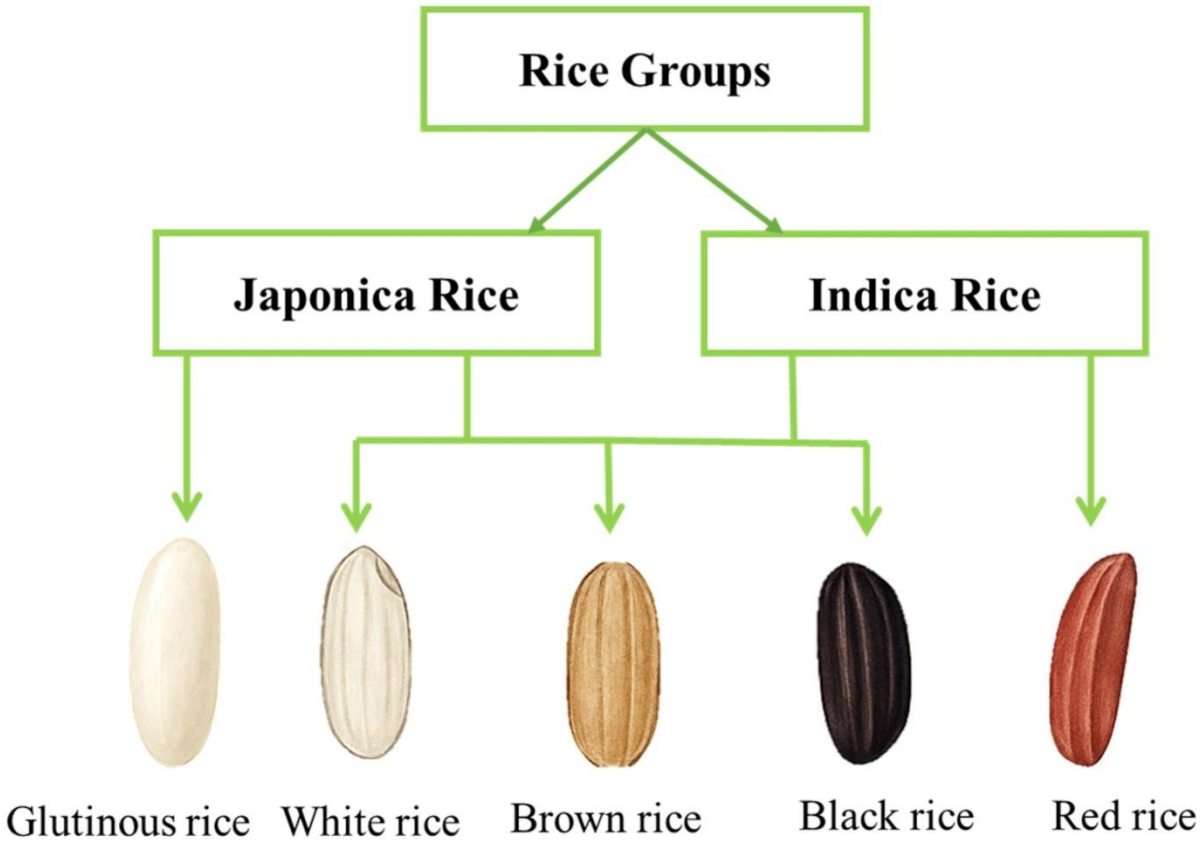


Figure 1: Types of rice varieties; General view

From its origin, rice spread to many regions of the world and has become the staple food for a large proportion of the global population. Approximately half of the world's population depends on rice as a primary source of nourishment. Although rice is cultivated in about 114 countries, Asia accounts for nearly 90% of global rice production. India is the second-largest producer of rice after China and possesses the largest area under rice cultivation. Rice is grown in almost every state of India; however, the middle and lower Gangetic plains and the coastal lowlands of Peninsular India constitute the major rice-producing regions (Melchinger et al., 1991). Annual per capita rice consumption ranges from 100 to 140 kg in several Asian and African countries (Shallan et al., 2010a, 2010b; Fahad et al., 2019). Rice is one of the most important agricultural commodities worldwide, providing more than 21% of the total caloric intake of the global population and up to 76% of daily caloric requirements in some developing countries, particularly in Southeast Asia (Zhao et al., 2020; Elkatry et al., 2023). The prevalence of chronic non-communicable diseases is increasing at an alarming rate in many developing countries. India has one of the largest populations of individuals with

diabetes, with an estimated 50.8 million affected people in 2009, and this number was projected to increase to approximately 87 million by 2030 (International Diabetes Federation, 2009). Indians are known to have a higher susceptibility to diabetes, partly due to genetic predisposition (Radha et al., 2006). However, genetic factors alone cannot explain the rapid increase in diabetes prevalence observed in urban India over the past few decades (Mohan et al., 2006), indicating the significant role of environmental and lifestyle factors.

Economic development is often accompanied by rapid urbanization. Along with reduced physical activity, unhealthy dietary patterns, and increased exposure to environmental pollution, urbanization is believed to contribute substantially to the growing burden of diabetes and cardiovascular disease (CVD) in India (Cropper et al., 1997). The association between physical inactivity, obesity, and the development of type 2 diabetes has been well established (Cropper et al., 1997). Cereals constitute a major component of the Indian diet, with carbohydrates contributing the largest proportion of total energy intake. Since 1980, the proportion of carbohydrates in Indian diets has remained relatively stable, accounting for approximately 55–65% of total caloric intake (Mohan et al., 2005; Mohan et al., 2008). These values are only slightly higher than those recommended by the World Health Organization for the prevention of chronic diseases (World Health Organization, 2003). Nevertheless, despite relatively stable carbohydrate consumption, the prevalence of diabetes in urban India increased from approximately 8% in 1980 to 16% in 2006, particularly in Chennai (Mohan et al., 2006).Consumption of whole grains has been associated with several health benefits, whereas refined grains, which primarily consist of the starchy endosperm, have been linked to adverse cardiovascular and metabolic outcomes, including impaired glucose tolerance and type 2 diabetes (Radhika et al., 2009). A positive association has been reported between the risk of diabetes and higher intake of refined carbohydrates or foods with a high glycemic impact. Excessive consumption of rice, particularly refined white rice, has been associated with an increased risk of type 2 diabetes because of its high glycemic potential (Hu et al., 2004).

Refined rice, commonly known as white rice, is produced through a series of processing steps, including dehusking and milling, which remove the bran and germ layers, leaving mainly the starch-rich endosperm (Miller et al., 2002). White rice is the most widely consumed form of rice globally (Villegas et al., 2007; Nanri et al., 2010; United States Department of Agriculture, 2010). Although the glycemic index (GI) of white rice varies depending on factors such as the degree of milling, cooking conditions, and amylose content,

its average GI is generally higher than that of whole grains (Foster-Powell et al., 2002). In contrast, whole grains provide dietary fiber along with a range of bioactive compounds, vitamins, minerals, and other essential nutrients that contribute to improved metabolic health (Deepa et al., 2008).

Resistant starch (RS), often referred to as undigested or unhydrolyzed starch, comprises the fraction of starch that escapes digestion in the small intestine and reaches the large intestine, where it functions similarly to dietary fiber (Marquart et al., 2002; Qi et al., 2006). Although resistant starch contributes only a small proportion of total dietary energy intake, it exerts physiological effects comparable to those of dietary fiber, including modulation of gut health and glucose metabolism (Qi et al., 2006). Several studies have highlighted the importance of dietary energy density in relation to obesity, metabolic syndrome, and cardiovascular disease risk (Howarth et al., 2006; Yoshita et al., 2010; Wilks et al., 2011; Schusdziarra et al., 2011). Dietary fiber is one of the primary determinants of energy density in foods. Because white rice undergoes polishing, which removes much of the dietary fiber, vitamins, and minerals present in the bran layer, it can be considered a relatively energy-dense food (Azadbakht & Esmaillzadeh, 2012; Bahadoran et al., 2014).

The relationship between rice consumption and chronic diseases has been investigated extensively, although findings remain inconsistent. With respect to cardiovascular disease, some studies have reported a positive association between rice consumption and disease risk (Yu et al., 2013), whereas others have found no significant relationship (Eshak et al., 2014). Similarly, most epidemiological studies examining rice intake and cancer risk have reported no significant associations (Abe et al., 2014; Giles et al., 2006; Kato et al., 1992; Severson et al., 1989). However, a few studies have suggested potential associations between rice consumption and the risk of prostate cancer and upper aerodigestive tract cancers (Chyou et al., 1995; Drake et al., 2012). Evidence regarding rice consumption and mortality is also inconclusive. Several studies have reported no significant association between rice intake and all-cause mortality (Eshak et al., 2014; Oba et al., 2010; Rebello et al., 2014). In contrast, other investigations have observed an inverse association between rice consumption and mortality among men, although similar effects were not observed among women (Eshak et al., 2011; Iso & Kubota, 2007). Furthermore, some studies have suggested that replacing refined rice with fruits, vegetables, or whole-grain products may reduce the risk of cardiovascular mortality (Rebello et al., 2014). Conversely, a prospective cohort study conducted in Japan reported that higher rice consumption was associated with reduced

mortality from coronary heart disease, heart failure, and overall cardiovascular disease among men (Eshak et al., 2011). Collectively, the available evidence regarding rice consumption, chronic disease development, and mortality remains inconsistent. To address these discrepancies, comprehensive systematic reviews and meta-analyses have been conducted to evaluate the relationship between white rice consumption and the risk of chronic diseases and mortality (Saneei et al., Year). Rice is increasingly recognized as a functional food due to the presence of various bioactive compounds, particularly in pigmented rice varieties. These bioactive constituents include phenolic compounds, γ-oryzanol, tocotrienols, and other phytochemicals that possess antioxidant, antidiabetic, anti-inflammatory, and cardioprotective properties (Goufo & Trindade, 2014; Patel & Naik, 2004; Sohail et al., 2017). Consequently, the health-promoting effects of rice extend beyond its basic nutritional value.

Starch is the major macronutrient in rice, accounting for approximately 77.6% of the grain composition, and is therefore the principal determinant of both rice quality and dietary energy supply. Based on the rate and extent of enzymatic digestion, starch is commonly classified into three categories: rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Chang et al., 2014). Resistant starch consists of starch molecules and starch degradation products that resist digestion in the small intestine and subsequently reach the colon (Chang et al., 2014). The digestibility of starch is an important determinant of postprandial blood glucose response because different rice varieties exhibit varying rates of starch digestion and absorption. Studies have demonstrated that differences in starch digestibility contribute significantly to the variation in glycemic index (GI) observed among rice cultivars (Chang et al., 2014; Kaur et al., 2016). Resistant starch can be classified into five major types. RS1 consists of physically inaccessible starch trapped within intact cellular structures, commonly found in whole grains and seeds. RS2 comprises ungelatinized starch granules that are naturally resistant to enzymatic digestion. RS3 is formed through starch retrogradation following cooking and cooling, resulting in reduced digestibility. RS4 includes chemically or physically modified starches produced through industrial processing. RS5 consists of amylose–lipid complexes that exhibit resistance to enzymatic hydrolysis (Nugent, 2005; Yi et al., 2021).

In recent years, increasing attention has been directed toward RS4 because of its potential for modification through physical, chemical, and enzymatic methods to produce starches with enhanced functional properties and broader industrial applications (Alcázar-Alay & Meireles, 2015; Yi et al., 2021).The resistant starch content of rice generally ranges from approximately

0.1% to 3.2%, depending on the variety and processing conditions (Deepa et al., 2010; Frei et al., 2003; Hu et al., 2004; Kumar et al., 2018). Several intrinsic characteristics of rice starch, including granule size, crystalline structure, amylose-to-amylopectin ratio, and gelatinization properties, influence starch digestibility and glycemic response (Chang et al., 2014; Hu et al., 2004; Kaur et al., 2016; Kumar et al., 2018; Toutounji et al., 2019). Environmental conditions during crop growth also affect starch composition and functionality. Factors such as temperature, soil conditions, and cultivation practices influence the interactions of starch with proteins and lipids, which may alter susceptibility to enzymatic digestion (Lian et al., 2013; López-Barón et al., 2017; Ye et al., 2018). Furthermore, environmental factors can significantly affect the accumulation of bioactive compounds in rice grains, thereby influencing their nutritional and functional properties (Anacleto et al., 2019; Hu et al., 2004). The impact of these bioactive compounds on human health will be discussed in subsequent sections.

Protein is the second most abundant nutrient in rice after starch. White rice contains approximately 4.5–10.5% protein, whereas rice bran contains substantially higher protein levels, ranging from 11.3% to 14.9%, nearly double that of polished white rice. Rice proteins have attracted considerable attention because of their influence on starch digestibility through interactions with starch granules (Toutounji et al., 2019; Ye et al., 2018). Studies have demonstrated that rice proteins can modulate the rate of starch digestion and consequently affect the glycemic response. Ye et al. (2018) reported that the removal of intrinsic proteins from rice flour significantly increased starch digestibility, indicating that protein–starch interactions restrict enzymatic access to starch granules. This finding suggests that rice varieties with higher protein contents may contribute to a lower glycemic index (GI) by slowing starch digestion.Rice grain proteins are broadly classified into four major fractions: glutelins (60–80%), albumins (4–22%), globulins (5–13%), and prolamins (1–5%). In contrast, the protein composition of rice bran differs considerably and consists of albumins (24–37%), globulins (15–36%), glutelins (11–38%), and prolamins (2–5%) (Hoogenkamp et al., 2017).

In addition to their nutritional value, rice proteins are recognized for several health-promoting properties. Rice proteins are naturally gluten-free and hypoallergenic, making them suitable ingredients for infant foods, specialized dietary products, and health beverages. Furthermore, their functional properties can be enhanced through enzymatic hydrolysis, leading to the generation of bioactive peptides with potential health benefits (Amagliani et al., 2017; Zheng

et al., 2019). Among the different protein fractions, albumin is particularly important because of its high solubility in water and superior digestibility. Rice albumin is predominantly concentrated in the bran layer and is present at levels of approximately 3.2–4.9 g per 100 g of rice bran (Adebiyi et al., 2009). Several studies have reported that rice albumins possess inhibitory activity against starch-hydrolyzing enzymes, particularly α-amylase, thereby reducing starch digestion and potentially lowering the glycemic index of foods (Uraipong & Zhao, 2015). Rice albumins, with molecular weights ranging from approximately 14 to 20 kDa, are recognized as natural α-amylase inhibitors. Through inhibition of α-amylase activity, these proteins may delay carbohydrate digestion, reduce postprandial glucose responses, and contribute to improved glycemic control (Champagne, 2004; Uraipong & Zhao, 2015).

Rice bran contains substantially higher lipid levels (15.0–19.7%) than polished white rice. Lipids in rice grains are generally classified into two categories: starch lipids, which are located within starch granules, and non-starch lipids, which are present outside the starch granules (Champagne, 2004). Triacylglycerols constitute the major non-starch lipids in rice grains, whereas free fatty acids and lysophospholipids are the predominant starch lipids (Khatun et al., 2019). Although starch lipids occur in relatively small quantities, they play a critical role in determining starch functionality and quality by influencing swelling, gelatinization, pasting properties, and starch digestibility (Luo et al., 2021). During starch biosynthesis, starch lipids interact with amylose molecules to form amylose–lipid complexes that are stabilized by hydrogen bonding (Khatun et al., 2019; Luo et al., 2021). These complexes restrict starch granule swelling and reduce the accessibility of starch to digestive enzymes, thereby decreasing starch digestibility. Ye et al. (2018) demonstrated that the removal of endogenous lipids from rice flour significantly increased starch digestibility, providing evidence for the important role of starch–lipid interactions in regulating starch hydrolysis. In addition to their influence on starch digestibility, rice lipids contain several bioactive compounds with potential health-promoting properties. These include γ-oryzanol, tocopherols, tocotrienols, ferulic acid, and triterpene alcohols, which exhibit antioxidant, hypoglycemic, and cardioprotective activities. These compounds have been reported to contribute to the prevention and management of oxidative stress-related disorders, including diabetes and cardiovascular diseases (Ali & Devarajan, 2017; Lu et al., 2011; Sohail et al., 2017).

Dietary fiber consists of non-digestible carbohydrate polymers that resist digestion and absorption in the human small intestine and undergo partial or complete fermentation in the

large intestine. Dietary fibers are commonly classified according to their solubility, viscosity, and fermentability. Soluble dietary fibers include compounds such as pectin and inulin, whereas insoluble dietary fibers primarily comprise resistant starch, cellulose, hemicellulose, and certain oligosaccharides (Khatun et al., 2019; Sohail et al., 2017).Adequate dietary fiber intake is associated with numerous health benefits, including improved glycemic control, reduced serum cholesterol levels, enhanced gastrointestinal health, and a lower risk of chronic diseases such as type 2 diabetes, obesity, and cardiovascular disease (Qureshi et al., 2002; Sohail et al., 2017; Ullah et al., 2019).Rice bran is a rich source of dietary fiber, containing approximately 19–29% fiber, whereas polished white rice contains only trace amounts because most of the fiber-rich bran layer is removed during milling and polishing. Consequently, brown rice and rice bran products generally possess superior nutritional and functional properties compared with refined white rice (Khatun et al., 2019).

India is recognized as one of the major centers of rice diversity, with numerous traditional and improved cultivars cultivated across diverse agroecological regions (Singh et al., 2000). Kerala, in particular, possesses a rich heritage of indigenous rice varieties, including Njavara, Jeerakasala, Uma, and Ponmani, which are valued for their unique agronomic, nutritional, and cultural attributes (Shylaraj et al., 2018). Njavara is a short-duration medicinal rice variety characterized by short to medium bold grains, a reddish pericarp, and a coarse texture (Deepa et al., 2009; Gopinath et al., 2008). Owing to its reputed therapeutic properties, Njavara has been widely used in Ayurvedic preparations and traditional medicinal practices.Jeerakasala is a fine-grained aromatic rice variety with short, slender grains resembling cumin seeds, from which its name is derived. It is highly valued for its distinctive aroma, excellent cooking quality, and premium culinary applications (Shylaraj et al., 2018). Among the improved rice cultivars developed in Kerala, Uma and Ponmani are notable for their high yield potential and adaptability. Uma produces medium-bold grains with good milling quality and is widely cultivated for regular consumption (Kerala Agricultural University [KAU], 2016). Ponmani is another high-yielding variety characterized by medium-sized grains, a golden-yellow husk, and desirable cooking quality (KAU, 2016).

Although primarily cultivated in northern India, Basmati rice is internationally renowned for its exceptional grain quality. Basmati grains are extra-long and slender and exhibit remarkable elongation during cooking. Its characteristic aroma is attributed to the presence of 2-acetyl-1-pyrroline, a volatile compound responsible for its unique fragrance (Singh et al., 2000). Compared with many Kerala rice varieties, Basmati demonstrates superior grain

elongation and fluffiness after cooking, making it highly valued in domestic and international markets. The wide variation in grain morphology, texture, aroma, and cooking quality among rice varieties reflects substantial genetic diversity. These differences significantly influence nutritional composition, starch characteristics, digestibility, consumer preference, and market value. Traditional rice varieties are often reported to possess superior nutritional quality, higher dietary fiber content, and greater concentrations of bioactive compounds compared with modern cultivars (Shylaraj et al., 2018; Sohail et al., 2017). In contrast, improved varieties have primarily been developed to achieve higher productivity, resistance to pests and diseases, and enhanced adaptability to environmental stresses (Khush, 2005; Fitzgerald et al., 2009; Zhao et al., 2020). However, breeding for yield and agronomic performance may occasionally alter grain nutritional composition and starch quality. Therefore, comparative studies of traditional and improved rice varieties are essential for evaluating their nutritional, functional, and health-promoting properties (Frei et al., 2003).

The present study was undertaken to determine and compare the protein content of selected rice varieties, namely Njavara, Uma, Ponmani, Jeerakasala, and Basmati, and to relate these findings to their physical and grain quality characteristics. Recent advances in rice genomics have led to the identification of numerous genes associated with grain quality traits, including grain appearance, cooking quality, texture, and eating characteristics. Among these, the granule-bound starch synthase (GBSS) gene plays a central role in amylose biosynthesis. GBSS proteins are targeted to starch granules through interactions with GBSS-binding proteins, similar to those first identified in *Arabidopsis* (Seung et al., 2015; Wang et al., 2020). In cereals, GBSS genes are expressed in tissues such as the endosperm, pollen, and embryo sac. Two major isoforms of GBSS have been identified: GBSSI and GBSSII. GBSSI, encoded by the Waxy (Wx) gene, is predominantly expressed in storage tissues, particularly the seed endosperm, and is primarily responsible for amylose synthesis. In contrast, GBSSII is mainly expressed in leaves and other non-storage tissues, including the seed coat (Li & Liu, 2019). The GBSSI gene plays a pivotal role in regulating amylose accumulation within the rice caryopsis. Numerous studies have demonstrated that allelic variation and differential expression of GBSSI significantly influence amylose content and, consequently, rice grain quality (Chen et al., 2008; Wang et al., 2018).

Rice grain quality is largely determined by starch composition, particularly the relative proportions of amylose and amylopectin (Juliano, 1992). The ratio of these two polysaccharides strongly influences cooking behavior, texture, digestibility, and consumer

acceptance (Bao, 2012). Among the enzymes involved in starch biosynthesis, GBSSI, the product of the Waxy (Wx) gene, is principally responsible for the elongation of amylose chains within the rice endosperm (Nakamura et al., 2005; Smith et al., 1997). Variations in GBSSI expression led to differences in amylose content among rice cultivars, resulting in diverse textural properties ranging from highly sticky to non-sticky cooked rice (Bao, 2012; Wang et al., 1995). At the molecular level, GBSSI is localized within starch granules, where it catalyzes the elongation of α-1,4-glucan chains using ADP-glucose as the substrate (Tetlow, 2006). Consequently, the abundance of GBSSI protein directly influences starch structure and functionality.

Differences in GBSSI protein accumulation among rice varieties can be analyzed using biochemical techniques such as SDS–PAGE and Western blotting. These approaches provide valuable insights into the relationship between gene expression, protein abundance, and grain quality characteristics (Denyer et al., 2001; Mahmood & Yang, 2012). Understanding GBSSI expression is particularly important when comparing traditional and improved rice varieties because it helps explain differences in amylose content, cooking quality, starch digestibility, and nutritional properties. Such knowledge is valuable for rice breeding programs aimed at developing cultivars with improved physicochemical properties, enhanced nutritional quality, and greater health benefits (Frei et al., 2003; Sweeney & McCouch, 2007).

Understanding the relationship between protein content and starch quality is important for improving the nutritional and functional characteristics of rice. The present study focuses on selected traditional rice varieties of Kerala to evaluate variations in total protein content and their association with starch quality traits. Total proteins were extracted and analysed using SDS–PAGE to identify differences in protein profiles among the varieties. Furthermore, the relative expression of the Granule-Bound Starch Synthase (GBSS) genes, which encodes the enzyme responsible for amylose synthesis in rice endosperm, was quantified using qRT-PCR. By correlating total protein content, protein expression patterns, and GBSS gene expression levels, this study aims to better understand their influence on amylose accumulation and starch quality. The findings may contribute to the identification of nutritionally superior rice varieties and provide valuable information for rice quality improvement programs.

CHAPTER 2

Review of literature

2.1 Rice Protein Content and Nutritional Significance

Rice (*Oryza sativa* L.) is one of the most important food crops in the world and serves as the primary source of nutrition for nearly half of the global population (Fitzgerald et al., 2009). Based on pericarp coloration, rice is commonly classified into white, red, black, and green rice, among which white rice is the most widely cultivated and consumed. In addition to being consumed as steamed rice, it is used in the preparation of various food products, including fried rice, parched rice, rice cakes, noodles, dumplings, and rice flour-based products. Annual per capita rice consumption in rice-eating countries ranges from approximately 62 to 190 kg (Graham et al., 1999). Polished or milled rice, the form most commonly consumed, consists primarily of the endosperm after the removal of the bran and germ during milling. Consequently, the nutritional composition of polished rice is largely determined by the endosperm, which contains approximately 70–80% starch, 7–10% protein, and less than 1% lipid (Yang et al., 2019). However, rice endosperm is relatively poor in several micronutrients and phytochemicals because these compounds are concentrated in the bran and germ layers that are removed during processing. Proteins in rice grains are predominantly stored in protein bodies within endosperm cells, with approximately 80% of the total grain protein located in the endosperm and the remaining 20% distributed in the embryo and aleurone layers (Yang et al., 2015). Protein content in polished rice generally ranges from 5% to 17% on a dry weight basis, making rice an important source of dietary protein for populations that rely heavily on rice as a staple food. Grain protein content (GPC) varies considerably among rice cultivars, ranging from 4.9% to 19.3% in *indica* rice and from 5.9% to 16.5% in *japonica* rice (Yang et al., 2019).

Several genetic studies have been conducted to elucidate the molecular mechanisms underlying grain protein accumulation in rice (Peng et al., 2014; Yang et al., 2019). Through map-based cloning, Peng et al. (2014) identified OsAAP6, an amino acid transporter gene that positively regulates grain protein content by enhancing amino acid uptake and transport. Natural variation within the 5′ untranslated region of *OsAAP6* has been associated with differences in grain protein content among rice cultivars, particularly within *indica* germplasm (Peng et al., 2014). Subsequently, Yang et al. (2019) identified another positive regulator of grain protein content, OsGluA2, which encodes a glutelin type-A2 precursor protein. A single nucleotide polymorphism (SNP) within the promoter region of *OsGluA2*

was found to influence gene expression and contribute to variation in grain protein content. These studies demonstrate that the identification of key genes and naturally occurring allelic variants can facilitate the development of rice varieties with enhanced nutritional quality through conventional breeding approaches (Peng et al., 2014; Yang et al., 2019). In addition to total protein content, amino acid composition is an important determinant of rice protein quality. Essential amino acids, particularly lysine, play a critical role in human nutrition. Lysine is considered the primary limiting essential amino acid in rice and is present at relatively low concentrations, especially in polished rice (Galili, 1995, 2002). Consequently, rice protein is often regarded as nutritionally incomplete with respect to essential amino acid balance.The biosynthesis and metabolism of lysine in plants have been extensively studied. Lysine is synthesized from aspartate through a branched metabolic pathway that is tightly regulated by feedback inhibition and degradation mechanisms. Early attempts to increase lysine levels in rice through the constitutive expression of a feedback-insensitive maize dihydrodipicolinate synthase (DHPS) gene resulted in only modest increases in free lysine accumulation (Lee et al., 2001).

Few studies demonstrated that reducing lysine degradation is a more effective strategy for enhancing lysine content. Long et al. (2013) reported that the simultaneous expression of feedback-insensitive aspartate kinase (AK) and DHPS genes, combined with endosperm-specific suppression of lysine ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), increased free lysine concentrations in rice grains by approximately 60-fold. Field evaluations indicated that these high-lysine rice lines exhibited minimal alterations in agronomic performance, except for changes in plant height and grain coloration (Yang et al., 2016a). Interestingly, elevated lysine accumulation in rice endosperm has been associated with the development of a dark-brown grain phenotype. Yang et al. (2018) demonstrated that excessive lysine accumulation activates jasmonate signalling pathways and stimulates serotonin biosynthesis, resulting in the observed pigmentation changes. Feeding trials further revealed that lysine-biofortified rice improved growth performance, feed conversion efficiency, and lysine bioavailability in experimental animals (Yang et al., 2017).The growing prevalence of lifestyle-related disorders such as diabetes, obesity, and cardiovascular diseases has increased interest in identifying functional foods with health-promoting properties. Rapid urbanization, globalization, and changing dietary habits have contributed to increased consumption of energy-dense processed foods, leading to a higher incidence of metabolic disorders (Chaudhari et al., 2018). Consequently, considerable research efforts are being

directed toward identifying food-based strategies for disease prevention and health promotion.

Rice has attracted significant attention as a functional food because of its nutritional value and the presence of bioactive compounds in certain traditional and pigmented varieties. Researchers have increasingly focused on evaluating the mineral composition, antioxidant properties, glycemic index, and health-promoting potential of different rice cultivars. Given its widespread consumption and nutritional importance, rice represents an ideal target for the development of nutritionally enhanced and health-promoting food products. According to Juliano (1985), more than two billion people in Asia obtain nearly 80% of their dietary energy from rice. Rice grain typically contains approximately 80% carbohydrates, 7–8% protein, 2–3% lipids, and small amounts of dietary fiber and micronutrients, highlighting its importance as a staple food and a major source of dietary energy worldwide.

2.2 Health Benefits of Rice

2.2.1 Source of Carbohydrates and Energy

Rice is an excellent source of complex carbohydrates and serves as a major energy provider for a large proportion of the world's population. Carbohydrates in rice are hydrolysed to glucose, which functions as the primary energy source for bodily activities and brain metabolism (Dolson et al., 2009). The rate of starch digestion varies among rice varieties and is influenced by factors such as amylose content, starch granule structure, and processing methods. Rice varieties with higher amylose content generally exhibit a lower glycemic index (GI) and slower starch digestion than low-amylose varieties. Brown rice and other whole-grain rice products contain higher levels of dietary fiber and resistant starch than polished white rice. A portion of the resistant starch escapes digestion in the small intestine and reaches the colon, where it functions similarly to dietary fiber. Epidemiological studies have shown that replacing white rice with brown rice or other whole grains is associated with a reduced risk of type 2 diabetes (Qi et al., 2010). Similarly, stabilized rice bran has been reported to improve glycemic control and lipid profiles in individuals with diabetes (Qureshi et al., 2002). However, carbohydrate intake should still be appropriately managed in diabetic individuals as part of a balanced dietary plan (Frei & Becker, 2004). The digestion and absorption of carbohydrates vary among individuals according to their physiological and energy requirements. Rice is best consumed as part of a balanced diet that includes

vegetables, fruits, legumes, fish, and meat to ensure adequate intake of proteins, vitamins, minerals, and other essential nutrients (FAO, 1997; Chaudhari et al., 2018).

2.2.2 Low Fat, Low Sodium, and Cholesterol-Free

Rice is naturally low in fat, sodium, and cholesterol, making it a suitable component of a healthy diet (Frei & Becker, 2004). Most lipids in rice are concentrated in the bran layer and are largely removed during milling and polishing. Rice bran oil is rich in unsaturated fatty acids, which may account for up to 80% of its total fatty acid content, depending on the variety (Frei & Becker, 2004). Oleic acid and linoleic acid are the predominant unsaturated fatty acids present in rice bran oil. These essential fatty acids play important roles in maintaining cell membrane integrity, supporting nervous system function, and promoting cardiovascular health (Chaudhari et al., 2018).

2.2.3 Rice as a Source of Protein

Rice serves as an important source of dietary protein, particularly in populations where it constitutes a major staple food. Rice protein is generally characterized by good digestibility and a relatively balanced amino acid composition compared with many other cereal proteins (Chaudhari et al., 2018). Although lysine remains the limiting essential amino acid in rice, its protein quality is considered superior to that of several other cereal grains. Rice proteins contribute to growth, tissue repair, enzyme synthesis, and the maintenance of normal physiological functions.

2.2.4 Source of Vitamins and Bioactive Compounds

Brown rice and rice bran are important sources of B-complex vitamins, including thiamine (vitamin B1), riboflavin (vitamin B2), and niacin (vitamin B3), which play essential roles in energy metabolism and nervous system function (Lloyd et al., 2000). Rice bran also contains several bioactive compounds, including tocopherols, tocotrienols, γ-oryzanol, and phenolic compounds, which possess antioxidant properties (Kline et al., 2004; Lloyd et al., 2000). Numerous studies have investigated the potential health benefits of these bioactive compounds. Tocopherols and tocotrienols have been associated with antioxidant, anti-inflammatory, and cardioprotective effects, while γ-oryzanol has been reported to contribute to cholesterol reduction and cardiovascular health (Frei & Becker, 2004; Lloyd et al., 2000). Pigmented rice varieties, such as red and black rice, are particularly rich in anthocyanins, tannins, and other phenolic compounds that exhibit strong antioxidant and anti-inflammatory

activities. These compounds have attracted considerable interest because of their potential roles in reducing the risk of chronic diseases, including cardiovascular disease, diabetes, and certain forms of cancer (Chaudhari et al., 2018).

2.2.5 Source of Essential Minerals

Rice, particularly pigmented and whole-grain varieties, provides several essential minerals, including iron, zinc, potassium, phosphorus, manganese, and copper (Ahuja et al., 2000). Iron is required for hemoglobin synthesis and oxygen transport, while zinc functions as a cofactor for numerous enzymes and plays important roles in immune function and cellular metabolism.Deficiencies of iron and zinc remain significant nutritional concerns worldwide and may lead to anemia, impaired growth, weakened immunity, and other health complications. Rice can contribute to the dietary intake of these minerals, especially when consumed as whole grain or pigmented varieties (Ahuja et al., 2000). Potassium contributes to fluid balance, muscle function, nerve transmission, and cardiovascular health, whereas phosphorus is essential for bone development and energy metabolism. Trace elements such as manganese and copper are also involved in enzymatic reactions and normal physiological functions. Therefore, rice serves not only as a major source of dietary energy but also as a valuable contributor to micronutrient intake and overall nutritional well-being (Chaudhari et al., 2018).

2.2.6 Rice Protein Composition and Variability

Rice protein is recognized for its high nutritional quality, digestibility, and hypoallergenic properties. The total protein content of the edible portions of rice grains is approximately 16%, although considerable variation exists among cultivars and growing environments (Hoogenkamp et al., 2017). During milling and polishing, the bran and germ layers, which contain a substantial proportion of the grain protein, are removed, resulting in a reduction in the protein content of white rice.Like other cereal proteins, rice proteins are rich in glutamine and asparagine. The major storage proteins in rice endosperm are glutelin (oryzenin) and prolamine, which account for approximately 80% and 20–25% of the total endosperm protein, respectively. Glutelin is nutritionally important because it contains a higher proportion of lysine than other rice proteins. Albumins and globulins are mainly concentrated in the outer layers of the grain and contribute approximately 4–22% and 5–13% of the total protein content, respectively (Hoogenkamp et al., 2017). The distribution of protein within rice grains is not uniform. Most proteins are concentrated in the aleurone and subaleurone

layers, while smaller quantities are associated with starch granules in the endosperm. Juliano et al. (1973) reported that protein distribution becomes more uniform as grain protein content increases. Their study also demonstrated that the Kjeldahl method provides a reliable estimate of rice protein content, particularly when compared with histological observations of individual grains. Protein content in rice is influenced by both genetic and environmental factors. Gomez and De Datta (1975) reported that protein content in brown rice of the variety IR8 ranged from 4.8% to 12.1% under different environmental conditions in the Philippines. Similarly, protein content in the variety BPI-76 varied between 9% and 15% when grown at different planting dates (IRRI, 1963). These findings indicate that environmental conditions significantly influence grain protein accumulation. Several studies have confirmed that grain protein content is a heritable trait (Gupta et al., 1988; Kaul, 1983; Kambayshi et al., 1984; Mosina, 1988; Shenoy et al., 1991; Singh & Singh, 1982; Singh et al., 1977; Sood & Siddiq, 1986). Estimates of heritability have varied considerably, reflecting the influence of genotype environment interactions. Hillerislambers et al. (1973) reported heritability values ranging from 0.13 to 0.37, whereas Tsuzuki and Furusho (1986) and Shenoy et al. (1991) estimated heritability values of approximately 58.8% and 71.0%, respectively. Further genetic studies demonstrated that protein content is influenced by the combined effects of the triploid endosperm, maternal plant, and cytoplasmic inheritance (Shi et al., 1996a, 1996b). Recent advances in molecular genetics have improved the understanding of grain protein accumulation in rice. Peng et al. (2014) identified the amino acid transporter gene *OsAAP6* as a positive regulator of grain protein content, while Yang et al. (2019) identified *OsGluA2*, a glutelin precursor gene associated with variation in protein accumulation. These findings provide valuable opportunities for improving rice nutritional quality through marker-assisted breeding and biofortification programs.

2.3 Protein Content and Quality in Waxy Rice

Waxy or glutinous rice is characterized by its opaque grain appearance and extremely low amylose content, generally less than 2%, with amylopectin accounting for nearly all of the starch fraction (Palmiano& Juliano, 1972). Waxy rice is widely cultivated in several Asian countries and is extensively used in the preparation of rice cakes, puddings, fermented foods, and other traditional products (Kwon, 2012). The unique starch composition of waxy rice confers distinct functional and processing properties, making it suitable for specialized food applications (Kum et al., 2010). In addition to starch composition, protein content has been reported to influence the cooking, pasting, and textural properties of waxy rice. Xie et al.

(2008) demonstrated that variations in protein content significantly affect the physicochemical characteristics and processing quality of waxy rice products.

2.4 Nutritional Significance of Njavara Rice

Njavara is a unique medicinal rice landrace indigenous to Kerala and is widely recognized in Ayurvedic medicine for its therapeutic and nutritional properties. Deepa et al. (2008) reported that Njavara rice contains higher levels of protein, dietary fiber, minerals, and antioxidant compounds than many conventional rice varieties. The same study revealed elevated concentrations of essential minerals, including calcium, potassium, magnesium, phosphorus, sodium, and iron, indicating the superior nutritional quality of Njavara rice (Deepa et al., 2008). In addition, Njavara exhibited distinctive physicochemical properties, such as enhanced water absorption capacity and unique starch characteristics, which may contribute to its medicinal value. A physicochemical and genetic analyses demonstrated that Njavara is genetically distinct from popular South Indian rice cultivars such as Jyothi and IR64 (Deepa et al., 2009). The authors reported that Njavara possesses unique grain morphology, cooking quality, and nutritional attributes that differentiate it from conventional rice varieties. These distinctive genetic and nutritional characteristics support its recognition as a special medicinal rice variety with potential health-promoting benefits.

2.5 Nutritional and Quality Characteristics of Basmati and Jeerakasala Rice Varieties

Basmati rice is a premium aromatic long-grain rice cultivated predominantly in India and Pakistan. It is internationally recognized for its distinctive aroma, superior cooking quality, grain elongation, and desirable sensory characteristics. The unique quality attributes of Basmati rice are largely attributed to its moderate amylose content, favourable starch composition, and excellent gelatinization properties, which contribute to its soft texture and pronounced kernel elongation during cooking (Singh et al., 2000). The characteristic aroma of Basmati rice is primarily due to the presence of 2-acetyl-1-pyrroline (2AP), a volatile aromatic compound responsible for its distinctive fragrance (Bradbury et al., 2005). Genetic studies have revealed that mutations in the *BADH2* gene play a major role in fragrance development in aromatic rice varieties, including Basmati. These findings have facilitated the use of molecular markers in breeding programs aimed at improving aroma and grain quality traits (Bradbury et al., 2005).

In addition to its aromatic qualities, Basmati rice exhibits excellent sensory attributes, including desirable texture, flavour, appearance, and consumer acceptability (Champagne et

al., 2004). Grain quality parameters such as aroma, amylose content, grain appearance, and cooking behaviour significantly influence consumer preference and market value, making Basmati one of the most commercially important specialty rice types worldwide (Fitzgerald et al., 2009). Furthermore, image analysis and multivariate statistical approaches have been successfully employed to classify Basmati cultivars based on grain morphology and surface characteristics (Kambo & Yerpude, 2014). The nutritional quality of Basmati rice has also attracted considerable attention. Boers et al. (2015) reported that rice varieties differ significantly in their postprandial glycemic responses, largely due to variations in starch composition and digestibility. Because of its moderate amylose content, Basmati rice generally produces a lower glycemic response than many conventional polished rice varieties. Recent studies have further demonstrated that processing methods such as germination and parboiling can improve the nutritional quality of Basmati rice while reducing starch digestibility and maintaining desirable sensory characteristics.

Pigmented rice varieties, including red rice, contain substantially higher levels of phenolic compounds, flavonoids, vitamins, minerals, and antioxidant molecules than polished white rice (Zhao et al., 2020). The red bran layer serves as a major reservoir of bioactive phytochemicals, contributing significantly to antioxidant activity and nutritional quality. The presence of dietary fiber, resistant starch, and bioactive compounds in pigmented rice varieties contributes to slower starch digestion and lower postprandial glycemic responses (Boers et al., 2015). Studies have shown that whole-grain and pigmented rice varieties generally contain higher levels of protein, minerals, and dietary fiber than polished rice products (Shallan et al., 2010). These characteristics may contribute to improved glucose regulation and lipid metabolism. Furthermore, traditional rice varieties with higher fiber and amylose contents often exhibit lower glycemic indices than refined rice varieties (Farooq & Yu, 2025).

Physicochemical studies have revealed considerable variation among South Indian rice cultivars with respect to amylose content, gel consistency, cooking quality, and sensory attributes (Febina et al., 2023). Pigmented rice varieties generally display unique textural properties, water absorption behavior, and cooking characteristics compared with non-pigmented cultivars. Jeerakasala is a traditional aromatic rice variety indigenous to the Wayanad region of Kerala. It is characterized by its small grain size, distinctive aroma, excellent cooking quality, and high consumer preference. The variety is widely used in the preparation of premium traditional dishes and possesses significant cultural and economic

importance within Kerala.George et al. (2006) reported that Jeerakasala exhibited grain yields comparable to those of Pusa Basmati-1 under the agroclimatic conditions of Wayanad. In addition to its favourable yield performance, Jeerakasala demonstrated excellent aromatic properties and desirable grain quality characteristics. Its adaptability to upland cultivation systems and local environmental conditions has contributed to its continued cultivation and popularity among farmers and consumers. The combination of unique aroma, grain quality, and regional adaptation makes Jeerakasala an important traditional rice variety and a valuable genetic resource for future rice improvement programs.

2.6 Protein Profiling in Rice

Protein is one of the most important nutritional components of rice and contributes significantly to grain quality, nutritional value, and functional properties. Several studies have reported considerable variation in protein content among rice genotypes, indicating the influence of both genetic and environmental factors on protein accumulation in rice grains Komatsu et al. (1998) employed two-dimensional polyacrylamide gel electrophoresis to characterize proteins extracted from rice suspension-cultured cells. Their study identified numerous proteins involved in cellular growth, development, stress responses, and metabolic activities. The authors demonstrated the usefulness of electrophoretic techniques in protein identification and functional characterization in rice. Mohanty et al. (2011) evaluated high-protein rice cultivars from the Assam Rice Collection and reported grain protein contents ranging from approximately 15–16.4% on a dry weight basis. Comparative protein profiling revealed differences between high- and low-protein cultivars, including the presence of additional slow-moving protein bands and increased accumulation of glutelin polypeptides in high-protein genotypes. These findings suggested that variation in protein composition contributes to differences in grain protein content among rice varieties.

Likittrakulwong et al. (2021) investigated protein extraction from paddy rice and germinated paddy rice using alkaline, sodium dodecyl sulfate (SDS), and enzymatic extraction methods. The study demonstrated that both extraction method and germination significantly influenced protein yield and protein fraction composition. SDS-based extraction was found to be effective for recovering rice proteins and facilitating subsequent protein analysis. Rice protein has gained increasing attention because of its high nutritional quality, digestibility, hypoallergenic nature, and potential health-promoting properties. Zheng et al. (2024) reported that rice proteins possess several bioactive functions, including antihypertensive and

cholesterol-lowering activities. The authors further noted that extraction efficiency can be improved through combinations of physical and enzymatic treatments, although enzymatic methods may involve certain limitations such as prolonged processing time and incomplete hydrolysis. Banerjee et al. (2010) examined genetic variability for grain protein and micronutrient content in diverse rice genotypes and observed substantial variation in grain protein content, ranging from 6.19% to 10.75%. The study identified several high-protein genotypes and emphasized the importance of genetic factors and genotype × environment interactions in determining grain nutritional quality. Molecular analyses further revealed genetic markers and candidate genes associated with nutrient accumulation, highlighting their potential application in breeding nutritionally enhanced rice varieties. Collectively, these studies demonstrate significant variability in rice grain protein content and composition among genotypes. Protein profiling techniques such as SDS-PAGE have proven valuable for detecting differences in protein expression patterns and identifying proteins associated with grain quality traits. Understanding variations in total protein content and protein expression among rice varieties is therefore important for improving nutritional quality and for developing rice cultivars with enhanced functional and health-promoting properties.

Among the genes involved in starch biosynthesis, Granule-Bound Starch Synthase I (GBSSI), encoded by the *Waxy (Wx)* gene, plays a central role in amylose synthesis and is a major determinant of rice cooking and eating quality. Variations in the *GBSSI* gene and its expression have been associated with differences in amylose content, starch characteristics, and grain quality among rice genotypes (Dixit et al., 2024). Studies have also demonstrated that the expression of starch biosynthesis-related genes contributes significantly to variations in grain quality traits among different rice cultivars (Jayhoon et al., 2024). In addition, molecular investigations have identified several genetic factors associated with nutritional quality, highlighting the importance of integrating biochemical and molecular approaches for rice improvement (Banerjee et al., 2010; Zhao et al., 2020). Biochemical characterization of rice grains, particularly protein analysis, provides valuable information regarding nutritional quality and functional properties. Techniques such as protein extraction, protein quantification assays, and SDS-PAGE have been widely used to evaluate variations in protein content and protein expression patterns among rice varieties. These approaches enable the identification of genotype-specific protein profiles and contribute to understanding the biochemical basis of grain quality traits.

Although numerous studies have investigated rice grain quality, protein composition, starch biosynthesis, and quality-related genes, most have focused either on biochemical characterization or molecular analysis independently (Juliano & Tuaño, 2019; Wang et al., 2020). Limited information is available on studies that combine protein profiling with the analysis of *GBSS*, particularly in traditional rice varieties of Kerala. Furthermore, comparative studies evaluating total protein content, SDS-PAGE protein profiles, and *GBSS* gene variation in medicinal, traditional, and improved rice cultivars remain scarce. Traditional rice varieties of Kerala, such as Njavara and Jeerakasala, possess unique nutritional, medicinal, and grain quality attributes. However, systematic information regarding their protein composition and its relationship with starch biosynthesis is limited. Understanding these relationships is important for evaluating the nutritional and functional significance of these valuable genetic resources.

Therefore, the present study aims to integrate biochemical and molecular approaches to evaluate selected traditional and improved rice varieties of Kerala. Total protein content will be determined following protein extraction, protein expression patterns will be analysed using SDS-PAGE, and the pre-GBSSI, GBSS1 and GBSS2 gene will be examined through molecular analysis. The results will help elucidate the relationship between protein content, protein profiles, and a key starch biosynthesis gene, thereby contributing to a better understanding of grain quality characteristics in diverse rice genotypes.

CHAPTER 3
Aim and Objectives

Aim

To evaluate the relationship between total protein content, protein expression profiles, and GBSS gene expression in selected traditional rice varieties of Kerala and to assess their association with rice quality characteristics.

Objectives

1. To estimate and compare the total protein content of the selected rice varieties.
2. To extract and profile rice grain proteins using SDS–PAGE and identify variations in protein banding patterns among the varieties.
3. To isolate RNA and analyse the relative expression of the Granule-Bound Starch Synthase (pre-GBSS1, GBSSI and GBBS2) gene using quantitative real-time PCR (qRT-PCR).
4. To compare the gene expression levels among the selected rice varieties.
5. To correlate total protein content and protein expression profiles with GBSS gene expression.

CHAPTER 4

Materials and Methods

4.1 Materials

4.1.1 Plant Materials

The present study was conducted using six rice (*Oryza sativa* L.) genotypes, namely Njavara, Ponmani, Uma, Jeerakasala, and Basmati (Figure2). The details of samples collected were given in the table (Table 1).

Table 1: Rice varieties used for study

Sl. No.	Rice Variety	Type	Place of Collection	Source
1	Njavara	Traditional medicinal rice	Chittoor, Palakkad, Kerala	Njavara Eco Farm
2	Uma	Improved rice variety	Rice Research Station, Mankombu, Alappuzha, Kerala	Kerala Agricultural University
3	Ponmani	Traditional red rice variety	Wayanad, Kerala	Farmers' field
4	Jeerakasala	Traditional aromatic rice variety	Wayanad, Kerala	Farmers' field
5	Basmati	Aromatic rice variety	Wayanad, Kerala	Farmers'field (seed)/Local source (rice)

4.1.2 Sample preparation for total Protein Extraction: The grains were thoroughly cleaned to remove dust and other extraneous materials. The samples were subsequently ground into a fine powder using liquid nitrogen in a mortar and pestle. The powdered samples were stored in sterile airtight containers at 4ºC for protein extraction.



Figure 2: Morphology of rice varieties selected for the study

4.1.3 Samples for total RNA isolation: 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 (SDW) 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 3).



Figure 3: Samples used for the study

Leaf samples were harvested under sterile conditions using RNase-free scissors and forceps to minimize contamination and RNA degradation. Immediately after collection, the leaf tissues were frozen in liquid nitrogen and used for total RNA isolation.

4.1.4 Reagents Used

A. Reagents for RNA Isolation

- TRIzol reagent (Invitrogen, USA)
- Chloroform
- Isopropanol
- Ethanol (75%)
- RNase-free water

B. cDNA Synthesis

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

C. Gradient PCR and Agarose gel Electrophoresis

- Emerald Green 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)

D. Quantitative Real-Time PCR

- SYBR Green Master Mix (Bio-Rad, USA)
- Gene-specific primers and House-keeping gene (pre-GBSS1, GBSS1, GBSS2 and beta Actin)
- Nuclease-free water

E. Reagents for Protein Isolation

Tris-Urea Extraction Buffer (100 mL)

Component	Quantity
Urea	30.3 g
0.5 M Tris-HCl (pH 6.8)	10 mL
10% SDS	20 mL
β-Mercaptoethanol	100 µL
Distilled water	To make 100 mL

Preparation

1. Dissolved 6.06 g Tris base in approximately 80 mL distilled water and adjusted the pH to 6.8 using HCl. The volume was made up to 100 mL.
2. Transfer 10 mL of 0.5 M Tris-HCl (pH 6.8) into a beaker.
3. Add approximately 30–40 mL of distilled water.
4. Added 30.3 g urea gradually to the Tris solution and stirred until completely dissolved.
5. Prepared 10% SDS solution by dissolving 2 g SDS in distilled water and making the final volume to 20 mL.
6. Add 20 mL of 10% SDS and mix thoroughly.
7. Added 100 µL β-mercaptoethanol (BME).
8. Made up the final volume to 100 mL using distilled water.

9. Store the extraction buffer at room temperature for short-term use or at 4°C for longer storage.

10. The extraction buffer was stored at room temperature for short-term use.

4.1.5 Reagents and Buffers for SDS–PAGE

The reagents and buffers used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared according to standard protocols and are described below.

4.1.5.1 Running (Electrophoresis) Buffer (1× Tris–Glycine–SDS Buffer)

The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS. To prepare 1 L of 1× running buffer, 3.03 g of Tris base and 14.4 g of glycine were dissolved in approximately 800 mL of distilled water. Subsequently, 1 g of SDS was added and dissolved completely. The volume was then adjusted to 1 L with distilled water and mixed thoroughly. The buffer was stored at room temperature until use.

4.1.5.2 Resolving Gel (10%)

A 10% resolving gel was prepared by mixing the following components:

Component	Volume
30% Acrylamide/Bis-acrylamide (29:1)	3.33 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL
10% SDS	100 µL
Distilled water	4 mL
10% Ammonium Persulfate (APS)	100 µL
TEMED	4 µL

The acrylamide solution, Tris-HCl buffer, SDS, and distilled water were mixed thoroughly. Polym4 erization was initiated by adding APS and TEMED immediately before casting the gel.

4.1.5.3 Stacking Gel (4%)

A 4% stacking gel was prepared using the following composition:

Component	Volume
30% Acrylamide/Bis-acrylamide (29:1)	0.67 mL
0.5 M Tris-HCl (pH 6.8)	1.25 mL
10% SDS	50 µL
Distilled water	3.03 mL
10% APS	50 µL
TEMED	5µL

The components were mixed thoroughly, and APS and TEMED were added immediately before pouring the stacking gel onto the polymerized resolving gel.

4.1.5.4 Laemmli Sample Buffer (2×)

The protein samples were mixed with 2× Laemmli sample buffer prior to electrophoresis. The composition of the buffer was as follows:

Component	Final Concentration
Tris-HCl (pH 6.8)	125 mM
SDS	4% (w/v)
Glycerol	20% (v/v)
Bromophenol blue	0.02% (w/v)
β-Mercaptoethanol	10% (v/v)

4.1.5.5 Coomassie Brilliant Blue R-250 Staining Solution

Protein bands were visualized using Coomassie Brilliant Blue R-250 staining solution prepared with the following composition:

Component	Concentration
Coomassie Brilliant Blue R-250	0.25% (w/v)
Methanol	50% (v/v)
Glacial Acetic Acid	10% (v/v)
Distilled Water	40% (v/v)

4.1.5.6 Destaining Solution

The destaining solution used for removing excess stain from the gel consisted of:

Component	Concentration
Methanol	50% (v/v)
Glacial Acetic Acid	10% (v/v)
Distilled Water	40% (v/v)

4.2 Instruments Used

- Centrifuge (Eppendorf, Germany)
- GelDoc GO Imaging System (Bio-Rad, USA)
- Thermal Cycler (Thermo Fisher Scientific, USA)
- Electrophoresis Apparatus (Bio-Rad, USA)
- SDS-PAGE Unit (Bio-Rad, USA)
- Microplate Reader (BioTek 800 TS)
- Tabletop Centrifuge (Neuation Technologies, India)
- Biophotometer (Eppendorf, Germany)
- UV-Visible Spectrophotometer (Thermo Scientific, USA)

- Analytical Balance (Shimadzu, Japan)
- Water Bath (Grant Instruments, UK)
- pH Meter (Thermo Scientific, USA)
- Autoclave
- −20°C Freezer (Elan-Pro, India)
- Microwave Oven

4.3 Methods

4.3.1 Isolation of Total Protein

Total protein was extracted from rice flour following a modified Tris–urea extraction method. Approximately 25 mg of powdered rice sample was mixed with 500 µL of Tris–urea extraction buffer. The mixture was vortexed thoroughly for 5 min and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant containing soluble proteins was collected and stored at −20°C until further analysis.

4.3.2 Estimation of Protein Content by BCA Assay

Protein concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay.The working reagent was prepared by mixing BCA Reagent A and BCA Reagent B in a ratio of 50:1. Ten microlitres of each BSA standard and protein sample were pipetted into separate wells of a 96-well microplate. Subsequently, 80 µL of BCA working reagent was added to each well.The plate was mixed gently and incubated at 37°C for 30 min. After cooling to room temperature, absorbance was measured at 560 nm using a BioTek 800 TS microplate reader. Protein concentration was calculated from the BSA standard curve.

4.3.3 SDS-PAGE Analysis of Protein Profiles

After polymerization of the resolving gel, the stacking gel solution was poured and a Teflon comb was inserted. Polymerization was allowed for 20 min.

Sample Preparation and Electrophoresis

- Thirty microlitres of protein extract was mixed with 10 µL of sample loading buffer and heated at 95–100°C for 5 min. The samples were immediately cooled on ice.
- Following polymerization, the comb was removed and wells were rinsed with running buffer. The gel cassette was assembled in the electrophoresis tank containing Tris-Glycine-SDS running buffer.

- Twenty microlitres of each protein sample and molecular weight marker were loaded into individual wells. Electrophoresis was performed initially at 80 V until samples entered the resolving gel and subsequently at 120 V until the bromophenol blue dye front reached the bottom of the gel.
- Following electrophoresis, carefully removed gel and placed in staining tray.
- The gel was washed 3 times with distilled water stained using Coomassie Brilliant Blue R-250 for 2 h and destained using destaining solution for 4 h. The gel was then washed with distilled water and documented using a GelDoc GO Imaging System (Bio-Rad, USA) and compared with protein standard (10-250 kDa) (NEB, USA).

4.3.4 Isolation of Total RNA

Total RNA was isolated from 100 mg of young rice leaf tissue using TRIzol reagent.

- Leaf tissues were ground into a fine powder using liquid nitrogen and homogenized in 1 mL TRIzol reagent. The homogenate was incubated at room temperature for 5 min. Chloroform (200 µL) was added and mixed vigorously for 15s, followed by incubation for 3 min.
- The mixture was centrifuged at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and mixed with 500 µL isopropanol. After incubation for 10 min, samples were centrifuged at 12,000 × g for 10 min.
- The RNA pellet was washed with 75% ethanol, air-dried, and dissolved in 50 µL RNase-free water.

4.3.6 Determination of RNA Quality and Integrity

The concentration and purity of the isolated RNA were determined using a Bio-Photometer (Eppendorf, Germany). RNA quality was evaluated by measuring the absorbance ratios at 260/280 nm and 260/230 nm.

The Bio-Photometer estimates nucleic acid concentration and purity based on the absorption of ultraviolet (UV) light by RNA molecules. RNA exhibits maximum absorbance at 260 nm due to the presence of nitrogenous bases. Following the Beer–Lambert law, the absorbance recorded at this wavelength is directly proportional to the RNA concentration in the sample.

RNA purity was assessed using absorbance values at three wavelengths:

- A260: Indicates the concentration of nucleic acids.

- A280: Detects protein contamination, mainly from aromatic amino acids.
- A230: Indicates the presence of contaminants such as phenol, carbohydrates, polysaccharides, guanidine salts, and other organic compounds.

High-quality RNA typically shows an A260/A280 ratio of 1.8–2.1 and an A260/A230 ratio of approximately 2.0–2.2.

The RNA concentration was calculated using the following equation:

RNA concentration (μg/mL) = A260 × 40 × Dilution Factor

4.3.7 Agarose Gel Electrophoresis

The integrity of the isolated total RNA, along with the presence of genomic DNA contamination and RNA degradation, was examined by 1.2% agarose gel electrophoresis. The gel was prepared by dissolving 1.2 g of agarose in 100 mL of 1× TAE buffer. After complete dissolution by heating, the solution was cooled slightly, and ethidium bromide (EtBr) was added as a nucleic acid staining dye. The molten agarose was poured into a casting tray and allowed to solidify.

RNA samples were mixed with 6× gel loading dye and carefully loaded into the wells. Electrophoresis was carried out at 70 V for approximately 40 minutes until sufficient separation of RNA bands was achieved. The gel was then visualized using a GelDoc GO Imaging System (Bio-Rad, USA), and the images were recorded to evaluate RNA quality and integrity.

4.3.8 First-Strand cDNA Synthesis

First-strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA).

The reaction mixture (20 μL) contained:

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

Thermal cycling conditions for cDNA synthesis were: 25°C for 5 min followed by 46°C for 20 min and 95°C for 1 min

The synthesized cDNA was stored at −20°C and diluted ten-fold before qPCR analysis.

4.4 Primers used to amplify the target gene

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 list of primers used for the study were included in table 2.

Table:2 Primers Used for qRT-PCR Analysis of Rice GBSS and Reference Genes.

Gene	Primer name	Primer sequence (5′–3′)
GBSS1	GBSS1F	AACGTGGCTGCTCCTTGAA
GBSS1	GBSS1R	TTGGCAATAAGCCACACACA
GBSS2	GBSS2F	AGGCATCGAGGGTGAGGAG
GBSS2	GBSS2R	CCATCTGGCCCACATCTCTA
Pre GBSSI	Pre-GBSS1F	GAAGAACATCTGCAAGTGCAGAG
Pre GBSSI	Pre GBSS1R	GAGCTGGGACGTGGTGAGAG
Actin (Reference Gene)	Act F	GAACAACTGGGACGACAT
Actin (Reference Gene)	Act R	CCTTTGGGTTCAGAGGAG

4.5 Quantitative Real-Time PCR Analysis

Gene expression analysis was performed using a Bio-Rad CFX96 Real-Time PCR System.

Each 20 µL reaction contained:

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

The PCR program consisted of:

- Initial denaturation: 95°C for 3 min
- 40 cycles of:
    - 95°C for 10 s

- 60°C for 30 s
- 72°C for 30 s

A melt-curve analysis was performed after amplification to verify product specificity.

4.5.1 Analysis of Relative Gene Expression through Real-Time PCR Data Analysis

The relative expression level of the Granule Bound Starch Synthase (GBSS) gene 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 designated control sample after normalization against a stable 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 the GBSSI gene 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 gene (pre-GBSS1, GBSSI, GBSS2):

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

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 (Uma) against which all other samples were evaluated.

Step 3: Calculation of Relative Gene Expression

The fold change in GBSS gene 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 GBSS 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 GBSS 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.

4.6 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 Isolation of Total RNA from Rice Leaf Tissues using TRIzol method

The standard TRIzol reagent method yielded high quality total RNA from the metabolically active leaf tissues of the five selected rice varieties (Ponmani, Jeerakasala, Njavara, Basmati, Uma). This method proved to be effective in breaking down the rigid plant cell walls and inhibiting the endogenous ribonucleases thus enabling the successful partitioning and recovery of pure RNA from the complex cellular matrix.

5.2 Quality Assessment of RNA

5.2.1 Quality and Quantity estimation

The quantity and quality of total RNA isolated from the selected rice varieties were assessed using a biophotometer. RNA concentration was measured by recording the absorbance at 260 nm (A260), while RNA purity was evaluated using the A260/A280 and A260/A230 absorbance ratios (Table 3).

The RNA concentration obtained from the different rice genotypes ranged from 385.4 to 450.2 ng/µL, indicating successful extraction of adequate amounts of RNA suitable for downstream molecular analyses such as cDNA synthesis and quantitative real-time PCR (qRT-PCR). Among the varieties studied, Njavara exhibited the highest RNA concentration (450.2 ng/µL), followed by Basmati (445.6 ng/µL), Uma (440.3 ng/µL), Jeerakasala (412.8 ng/µL), and Ponmani (385.4 ng/µL). The high RNA yields obtained from all samples suggest efficient extraction from the young leaf tissues. The A260/A280 ratio, which indicates protein contamination in RNA preparations, ranged from 1.98 to 2.05. Values close to 2.0 are generally considered indicative of highly pure RNA. Njavara showed the highest A260/A280 ratio (2.05), while Jeerakasala recorded the lowest (1.98). All samples fell within the acceptable range for high-quality RNA, indicating minimal protein contamination.

The A260/A230 ratio, an indicator of contamination by polysaccharides, phenolic compounds, carbohydrates, and residual extraction reagents, ranged from 1.78 to 1.98. Jeerakasala exhibited the highest A260/A230 value (1.98), followed by Njavara (1.97), Basmati (1.93), Uma (1.89), and Ponmani (1.78). Although the A260/A230 ratio of Ponmani

was slightly lower than the ideal value of 2.0, it remained within an acceptable range and did not indicate severe contamination.

Table 3: Quantitative estimation of total RNA extracted from traditional and released rice leaf tissues.

Rice Variety	Classification	Concentration (ng/µL)	A260/A280 Ratio	A260/A230 Ratio
Ponmani	Traditional	385.4	2.02	1.78
Jeerakasala	Traditional	412.8	1.98	1.98
Njavara	Traditional	450.2	2.05	1.97
Basmati	Aromatic	445.6	2.01	1.93
Uma	Released, High yield Variety	440.3	2.00	1.89

The spectrophotometric measurements confirmed that the isolated RNA from all five rice varieties was of high quality and sufficient quantity, with minimal contamination from proteins, polysaccharides, or phenolic compounds. The RNA samples were therefore considered suitable for subsequent cDNA synthesis and gene expression analysis by qRT-PCR.

5.2.2 Integrity assessment of isolated RNA using Agarose Gel Electrophoresis

The integrity of the isolated total RNA from the five rice varieties was evaluated by agarose gel electrophoresis (AGE) (Figure 4).

Distinct and well-resolved ribosomal RNA (rRNA) bands corresponding to 28S rRNA, 18S rRNA, and 5.8S rRNA were observed in all samples. 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 five 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.

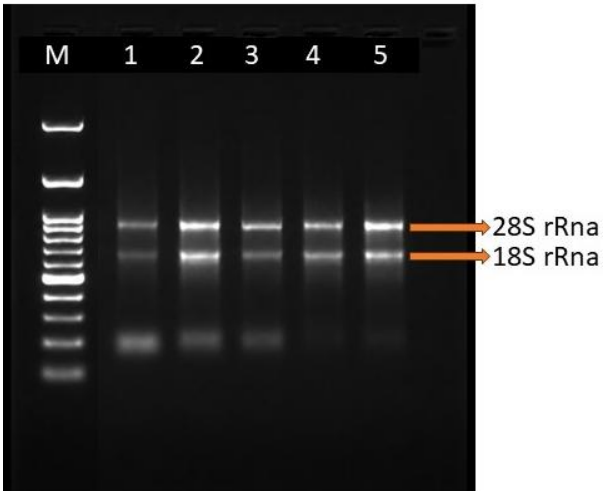


Figure 4: Agarose gel electrophoresis showing the integrity of total RNA isolated. M: 100bp DNA ladder, 1 to 5: RNA from Ponmani, Jeerakasala, Njavara, Basmati, Uma

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 of starch biosynthesis genes.

5.3 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 5).

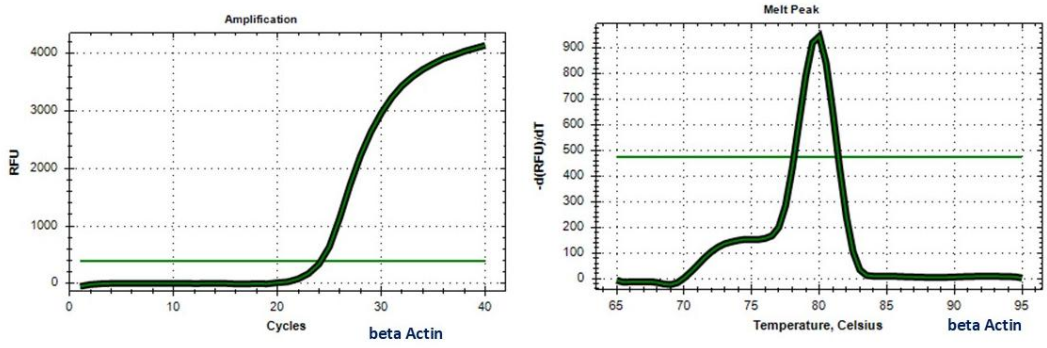


Figure 5: Representative qRT-PCR amplification and melt curve analysis of the β-actin reference gene used for cDNA quality assessment

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. 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 pre-GBSS1, GBSS1 and GBSS2 were mathematically normalized against ACT using the standard Livak method $(2^{-\Delta\Delta C_q})$. The widely cultivated released variety, Uma, was established as the baseline calibrator (fold change = 1.00) for all comparative analyses.

5.5 Expression Profiling of Granule Bound starch synthase Genes (GBSS)

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. A representative amplification plot showing the typical sigmoid amplification curves is presented in the corresponding Figure 6. The absence of aberrant amplification patterns confirmed the high specificity and reliability of the designed primers, ensuring accurate quantification of target gene expression.

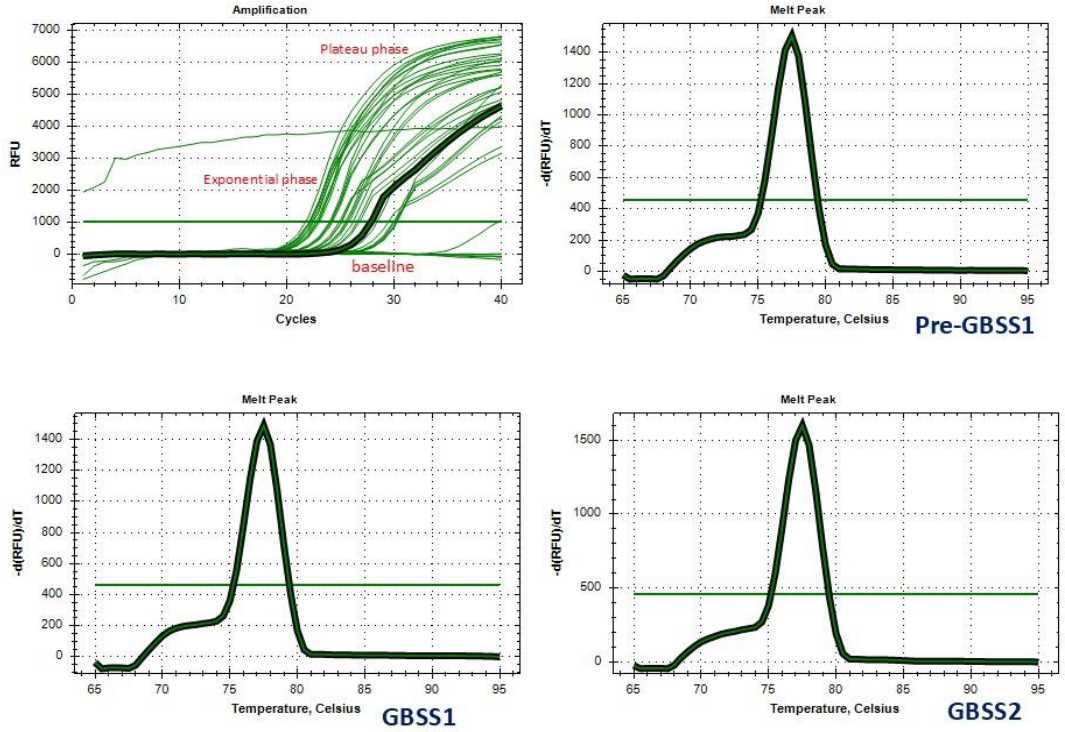


Figure 6: Representative amplification plot showing the typical sigmoid amplification curves and melt curve data for the primers used for the study

5.5.1 Relative Expression of Pre-GBSS1 Gene in Rice Varieties

The relative expression analysis of the Pre-GBSS1 (Granule Bound Starch Synthase I) gene revealed significant variation among the rice varieties studied.

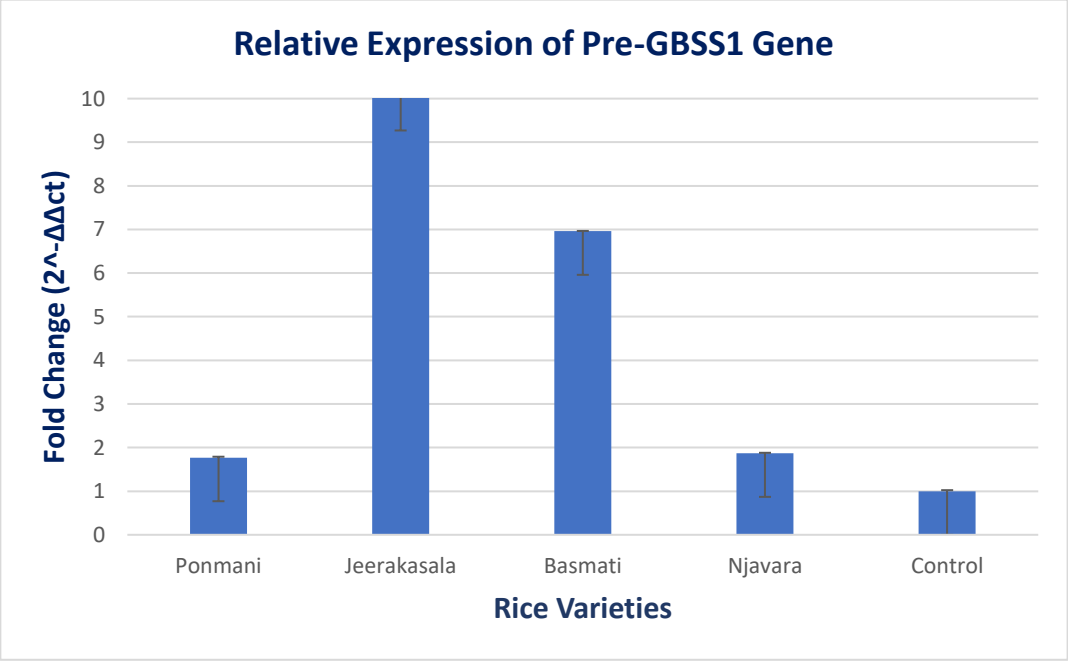


Figure 7: Relative expression levels of the Pre-GBSS1 (Granule Bound Starch Synthase I) gene in selected rice varieties determined by qRT-PCR using the 2^-ΔΔCt method, with Uma (Control)

Expression levels were calculated using the 2^-ΔΔCt method, with the control variety Uma normalized to a value of 1.0 (Figure 7). Among the genotypes evaluated, Jeerakasala exhibited the highest expression of the pre-GBSS1 gene, showing approximately 10-fold upregulation compared to the control. Basmati also demonstrated substantially elevated expression, with nearly 7-fold higher transcript accumulation. In contrast, Ponmani and Njavara showed only moderate increases in expression, approximately 1.8-fold relative to the control. These results indicate that the aromatic rice varieties (Jeerakasala and Basmati) possess markedly higher transcriptional activity of the pre-GBSS1 gene than the non-aromatic varieties.

The GBSS1 gene encodes Granule Bound Starch Synthase I, a key enzyme responsible for the synthesis of amylose, the linear component of starch granules in rice endosperm. GBSS1 catalyzes the elongation of α-(1→4)-linked glucan chains using ADP-glucose as the substrate. Therefore, increased expression of the GBSS1 gene is generally associated with enhanced amylose biosynthesis and altered starch composition. The high expression observed in Jeerakasala and Basmati suggests greater production of GBSS1 enzyme, which may lead to increased amylose accumulation during grain development. Elevated amylose content influences several grain quality traits, including cooking characteristics, texture, grain firmness, and glycemic response. Aromatic rice varieties are often characterized by distinctive starch properties, and the enhanced expression of GBSS1 observed in these varieties may contribute to their unique grain quality attributes.

Conversely, the relatively low expression levels recorded in Ponmani and Njavara indicate reduced GBSS1 transcript abundance and potentially lower enzyme production. Reduced GBSS1 activity can result in decreased amylose synthesis and a proportionally higher amylopectin content, thereby affecting starch structure and functional properties. Gene expression at the mRNA level is generally reflected in the synthesis of the corresponding protein. Thus, the elevated pre-GBSS1 transcript levels observed in Jeerakasala and Basmati are expected to translate into increased accumulation of the GBSS1 protein, resulting in enhanced enzymatic activity for amylose biosynthesis. Although post-transcriptional and post-translational regulatory mechanisms may influence the final protein abundance, the strong upregulation of GBSS1 transcripts strongly suggests higher levels of GBSS1 enzyme in these varieties.

5.5.2 Relative Expression of GBSS1 Gene

The expression profile revealed significant differences among the rice varieties. Jeerakasala exhibited the highest GBSS1 expression level, showing approximately 14.5-fold upregulation relative to the control (Figure 8). Njavara also displayed strong expression, with about 13.7-fold higher transcript accumulation, followed by Basmati (9.1-fold) and Ponmani (7.3-fold).

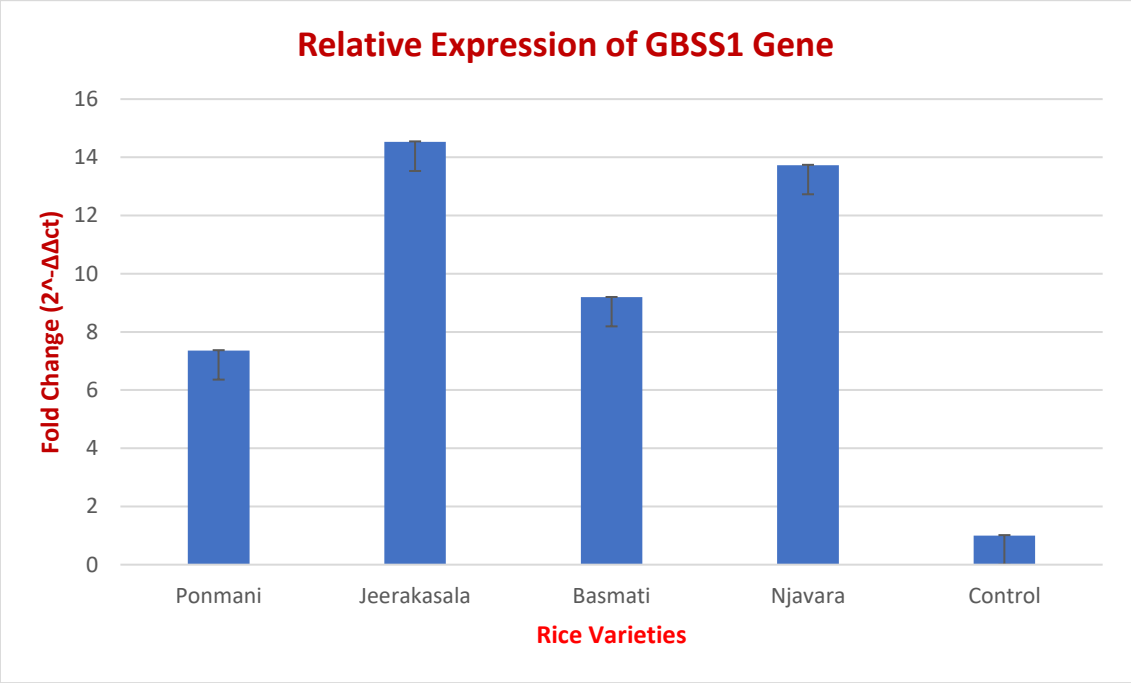


Figure 8: Relative expression of the GBSS1 gene in different rice varieties determined by qRT-PCR. Relative expression values were calculated using the 2^-ΔΔCt method, with β-actin as the internal reference gene and Uma as the control

All tested varieties showed substantially higher GBSS1 expression than the control, indicating enhanced transcriptional activity of this key starch biosynthetic gene. GBSS1 encodes the Granule Bound Starch Synthase I enzyme, which is primarily responsible for the synthesis of amylose, the linear polysaccharide component of starch granules. The elevated expression observed in Jeerakasala and Njavara suggests increased production of GBSS1 protein and enhanced enzymatic activity, leading to greater amylose biosynthesis. Since amylose content significantly influences rice grain quality, cooking behaviour, texture, gelatinization characteristics, and glycemic response, the differential expression of GBSS1 among varieties may contribute to their distinct physicochemical and nutritional properties.

The higher GBSS1 expression levels in aromatic varieties such as Jeerakasala and Basmati support the hypothesis that starch biosynthetic genes are actively regulated in these

genotypes. Interestingly, Njavara also exhibited strong GBSS1 expression, indicating a high genetic potential for amylose synthesis despite its classification as a traditional medicinal rice. The comparatively lower expression observed in Ponmani, although still significantly higher than the control, suggests a relatively reduced capacity for GBSS1-mediated starch synthesis.Comparison of the pre-GBSS1 and GBSS1 expression profiles indicates that varieties showing high precursor transcript levels also exhibited elevated mature GBSS1 expression. Jeerakasala consistently showed the highest expression in both analyses, suggesting efficient transcription and processing of the GBSS1 gene. The substantial increase in GBSS1 expression compared with pre-GBSS1 levels further indicates active gene regulation and accumulation of transcripts required for starch biosynthesis.

5.5.3 Relative Expression of GBSS2 Gene

The results revealed considerable variation in GBSS2 gene expression among the rice varieties studied. Basmati exhibited the highest expression level, showing approximately 7.6-fold upregulation relative to the control. This was followed by Njavara, which displayed nearly 5.9-fold higher expression, and Jeerakasala, which showed about 3.8-fold upregulation.

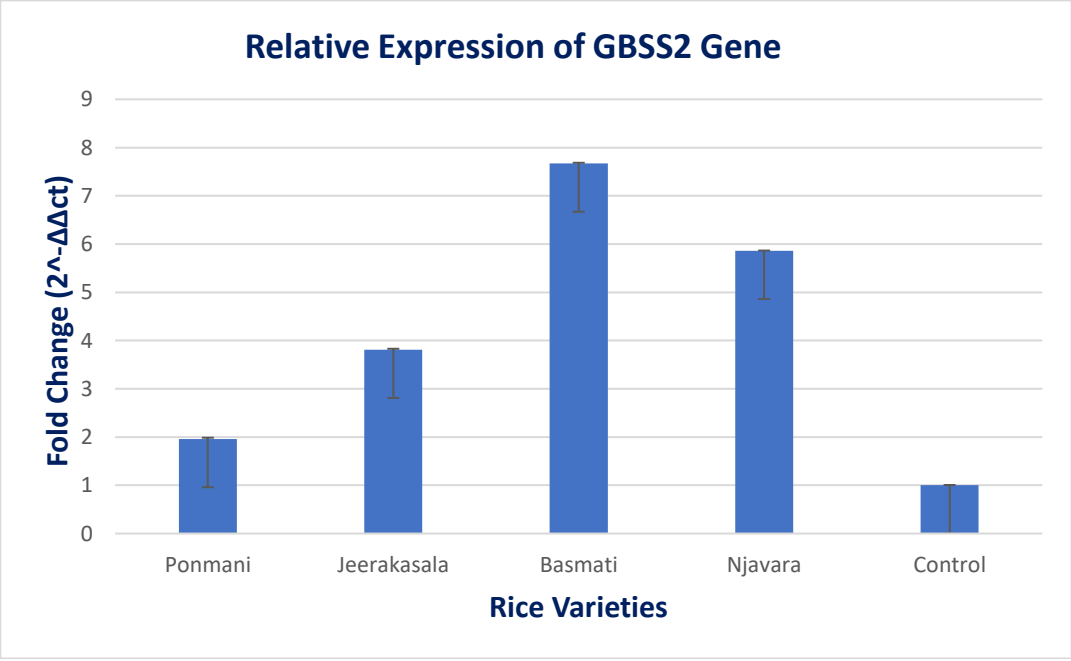


Figure 9: Relative expression of the GBSS2 gene in different rice varieties determined by qRT-PCR. Relative expression values were calculated using the 2^-ΔΔCt method, with β-actin as the internal reference gene and Uma as the control

Ponmani exhibited the lowest expression among the test varieties, with approximately 2-fold higher expression than the control. The control variety maintained a normalized expression value of 1.0 (Figure 9). GBSS2 belongs to the starch synthase gene family and is involved in the synthesis and elongation of glucan chains during starch biosynthesis. Although GBSS1 is the principal enzyme responsible for amylose production in rice endosperm, GBSS2 contributes to starch metabolism in specific tissues and developmental stages and may influence the structure and organization of starch granules. Therefore, increased expression of GBSS2 may enhance starch biosynthetic activity and contribute to variations in starch composition and grain quality. The elevated expression observed in Basmati and Njavara suggests a greater accumulation of GBSS2 transcripts and potentially higher synthesis of the corresponding protein. This may facilitate increased starch polymer formation and contribute to the unique physicochemical properties of these varieties. The moderate expression observed in Jeerakasala indicates active participation of GBSS2 in starch synthesis, while the lower expression in Ponmani suggests comparatively reduced involvement of this gene in starch biosynthetic processes.

When the expression profiles of pre-GBSS1, GBSS1, and GBSS2 were compared, distinct patterns of regulation were observed among the rice varieties, indicating genotype-dependent differences in starch biosynthesis. Jeerakasala exhibited the highest expression of both pre-GBSS1 and GBSS1, suggesting a strong capacity for amylose synthesis through increased production of granule-bound starch synthase I. In contrast, Basmati showed the highest expression of GBSS2, while Njavara exhibited high expression levels of both GBSS1 and GBSS2, indicating active starch biosynthetic processes. These findings suggest that individual starch synthase genes are differentially regulated among rice genotypes and may play complementary roles in determining starch composition. The coordinated upregulation of these genes, particularly in aromatic varieties such as Jeerakasala and Basmati, reflects enhanced transcriptional activity of the starch biosynthetic pathway. Collectively, the results demonstrate that variation in the expression of key starch synthesis genes is associated with differences in amylose biosynthesis, starch granule development, and grain quality characteristics, including texture, cooking properties, and nutritional attributes. The elevated expression of these genes in aromatic and traditional rice varieties highlights their potential contribution to desirable starch quality traits.

5.6 Estimation of total protein

The total soluble protein content of five selected rice varieties was quantified using the Bicinchoninic Acid (BCA) assay. The assay is based on the reduction of Cu^{2+} to Cu^{+} by peptide bonds under alkaline conditions, followed by the formation of a purple-coloured complex between Bicinchoninic acid and Cu^{+}. The intensity of the colour produced is directly proportional to the protein concentration and was measured spectrophotometrically at 562 nm. A standard calibration curve was generated using bovine serum albumin (BSA) standards of known concentrations, and the protein concentrations of the rice samples were calculated using the regression equation obtained from the standard curve (Figure 10).

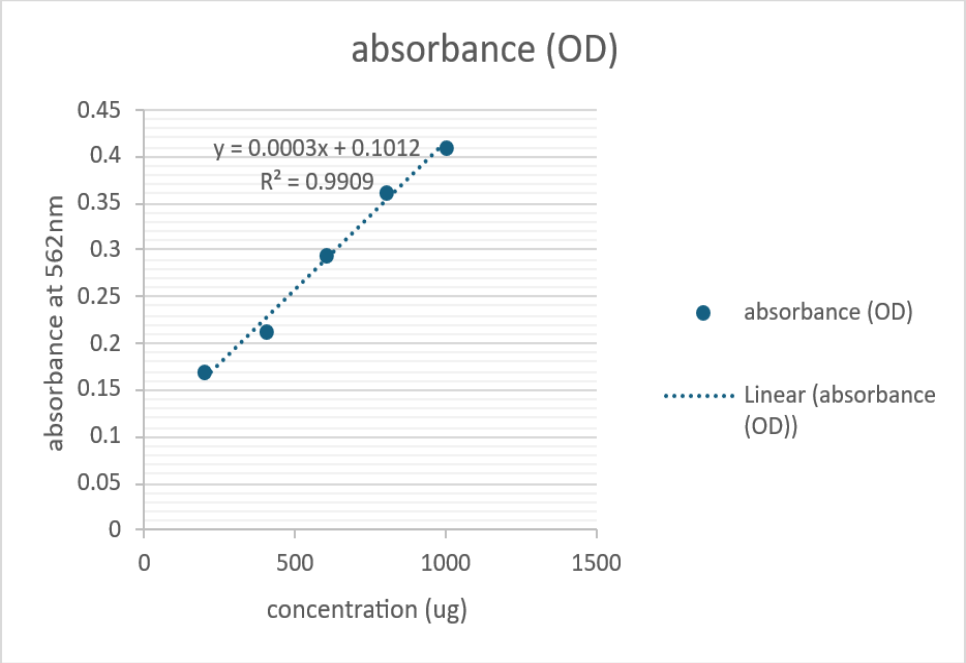


Figure 10: Standard curve for total protein concentration determination

The results demonstrated measurable variation in total protein content among the selected rice varieties, with protein concentrations ranging from 0.86 to 1.263 mg/mL (Figure11). The highest protein concentration was recorded in Njavara rice (1.263 mg/mL), followed closely by Uma (1.249 mg/mL), while the variety Ponmani exhibited a protein concentration of 1.136 mg/mL. These varieties showed comparatively higher levels of soluble protein, suggesting their potential nutritional advantage as sources of dietary protein. In contrast, Basmati exhibited the lowest protein concentration (0.86 mg/mL), indicating a comparatively lower accumulation of soluble proteins in the grain.

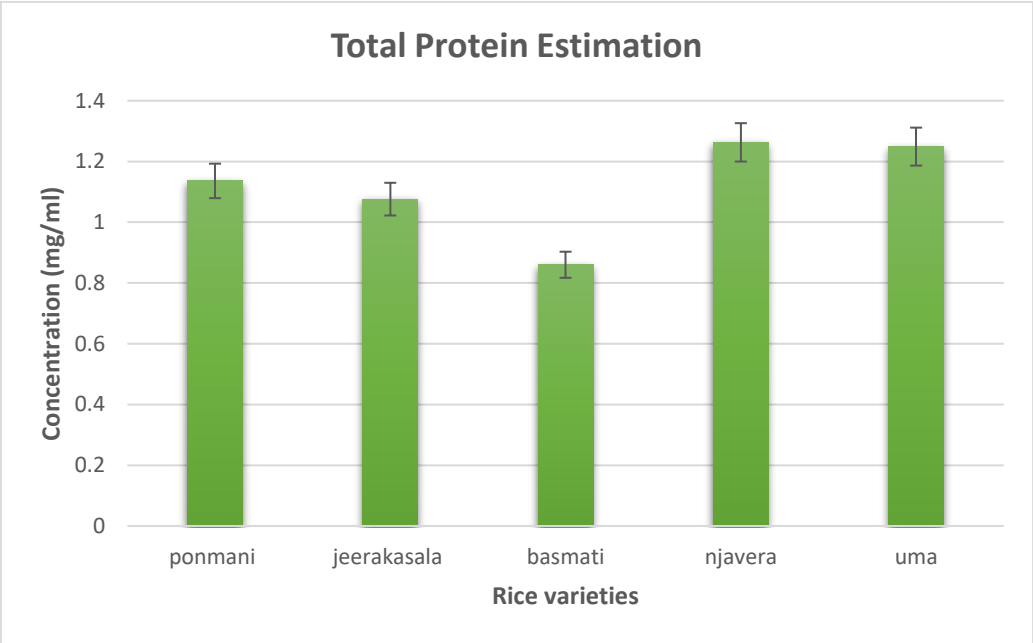


Figure 11: Total Protein Concentration in selected rice varieties

The observed differences in protein content among the rice varieties may be attributed to genotypic variations influencing protein synthesis, storage protein accumulation, and nitrogen utilization efficiency. Such variation is commonly reported among rice cultivars and contributes significantly to differences in nutritional quality. Varieties with higher protein content may provide enhanced nutritional benefits and could be considered valuable genetic resources for breeding programs aimed at improving grain protein content. Overall, the results indicate that the selected rice genotypes possess distinct protein accumulation profiles, reflecting their genetic diversity and potential differences in grain nutritional composition.

5.7 Analysis of Rice Seed Proteins by SDS–PAGE

The total proteins extracted from five selected rice varieties were separated according to their molecular weight using Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The electrophoretic profiles revealed distinct protein banding patterns across all varieties, indicating the successful extraction and resolution of seed proteins with varying molecular weights (Figure 12). The SDS–PAGE profile revealed distinct protein banding patterns among the five rice varieties, indicating variation in total seed protein composition. Lane M contained the molecular weight marker ranging from approximately 34 to 250 kDa. Lanes 2–6 represented the rice varieties Basmati, Njavara, Jeerakasala, Ponmani, and Uma,

respectively. Multiple protein bands were observed across all varieties, demonstrating the presence of diverse protein fractions with different molecular weights. Particular attention was given to the protein band around 55 kDa, which corresponds to the Granule Bound Starch Synthase (GBSS) protein, a key enzyme involved in amylose biosynthesis. The intensity of the 55 kDa band varied among the rice varieties, suggesting differential accumulation of GBSS protein.

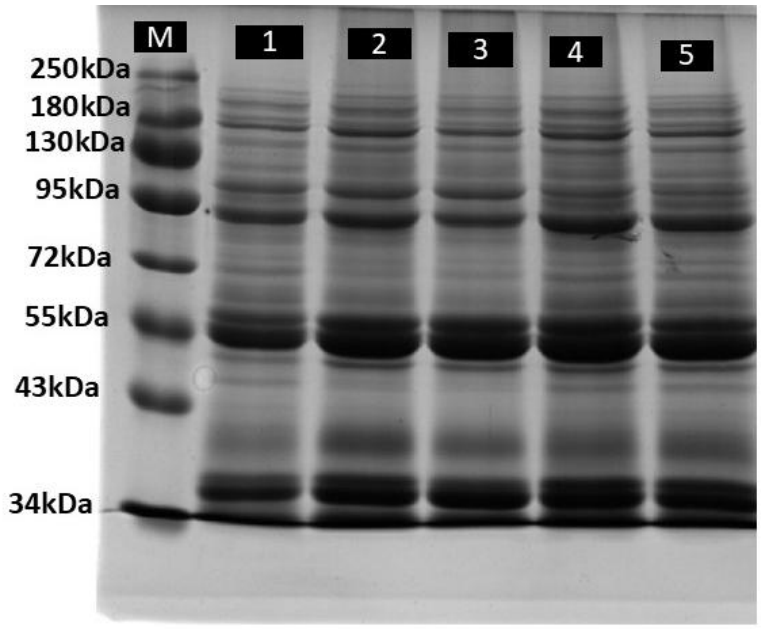


Figure 12: Separation of total protein and variation of GBSS protein content in rice varieties using SDS-PAGE Lane 1 (M): Protein Marker, Lane 2 to 6: GBSS protein, 1: Ponmani ,2: Jeerakasala, 3: Basmati, 4: Njavara, 5: Uma

Among the analysed genotypes, basmati and Njavara exhibited comparatively stronger 55 kDa bands, indicating higher GBSS protein abundance. Jeerakasala showed moderate expression, while Ponmani and Uma displayed relatively lower band intensity, suggesting reduced GBSS protein levels. The observed variation in GBSS protein accumulation is consistent with the genetic diversity among rice varieties and may contribute to differences in amylose content, starch structure, grain texture, cooking quality, and glycemic properties. The stronger expression of the GBSS protein in certain traditional varieties suggests enhanced starch biosynthetic activity, whereas lower expression in other varieties may reflect differences in the regulation of starch synthesis pathways. Hence the SDS–PAGE analysis confirms varietal variation in GBSS protein expression, particularly at the 55 kDa molecular weight region, highlighting its potential role in determining rice grain quality traits.

Chapter 6
Discussion

The present study investigated biochemical and molecular variations among selected rice (*Oryza sativa* L.) varieties through comparative analysis of total protein content, protein expression profiles, and transcriptional regulation of key starch biosynthesis genes, namely pre-GBSS1, GBSS1, and GBSS2. The observed differences among Ponmani, Jeerakasala, Njavara, Basmati, and Uma demonstrate the existence of substantial genetic diversity influencing grain composition, starch biosynthesis, and quality-related traits.

Protein content is an important nutritional parameter in rice and contributes significantly to grain quality. In the present study, Njavara recorded the highest soluble protein concentration (1.263 mg/mL), followed closely by Uma (1.249 mg/mL), whereas Basmati exhibited the lowest protein concentration (0.860 mg/mL). These findings agree with previous reports indicating that grain protein content is highly genotype-dependent and influenced by the genetic regulation of storage protein synthesis and accumulation (Juliano and Tuaño, 2019; Fitzgerald et al., 2009). The elevated protein concentration observed in Njavara may be attributed to enhanced synthesis and deposition of storage proteins within the grain. Traditional rice varieties are often characterized by greater biochemical diversity and may possess superior nutritional attributes compared to modern cultivars. Similar observations have been reported by Zhao et al. (2020), who demonstrated that genetic variation among rice genotypes significantly affects nitrogen assimilation, protein biosynthesis, and nutrient accumulation. Therefore, the high protein content recorded in Njavara suggests its potential nutritional advantage and supports its traditional medicinal importance.

The SDS-PAGE analysis further revealed varietal differences in seed protein composition. Multiple protein bands were detected across all rice varieties, confirming the presence of diverse protein fractions including glutelins, globulins, prolamins, and albumins, which collectively contribute to grain nutritional quality (Juliano and Tuaño, 2019). Although the overall banding patterns were broadly similar, indicating conservation of major seed storage proteins among rice varieties, considerable variation was observed in band intensity. Such differences suggest differential protein accumulation resulting from genotype-specific regulation of protein synthesis pathways.

Particular emphasis was placed on the protein band observed at approximately 55 kDa, corresponding to Granule Bound Starch Synthase (GBSS), the key enzyme responsible for

amylose biosynthesis in rice endosperm. The intensity of the 55 kDa band varied among the rice varieties, indicating differences in GBSS protein accumulation. Jeerakasala and Ponmani exhibited relatively stronger bands, whereas Njavara showed moderate expression and Basmati and Uma displayed comparatively lower intensities. Since GBSS catalyses the elongation of amylose chains within starch granules, variation in its abundance may directly influence amylose content and starch properties. Similar varietal differences in rice protein profiles have been documented by Amagliani et al. (2017), who reported that genetic diversity among cultivars contributes to differential accumulation of grain proteins associated with nutritional and functional characteristics. The stronger GBSS protein expression observed in certain traditional varieties suggests enhanced starch biosynthetic activity, while lower expression may reflect reduced synthesis or altered regulation of starch metabolism.

The molecular analysis of starch biosynthesis genes provided further insight into the genetic mechanisms underlying these biochemical differences. The pre-GBSS1 gene represents the precursor transcript involved in the transcriptional regulation of the waxy (Wx) locus, which ultimately encodes GBSS1. Significant variation in pre-GBSS1 expression was observed among the rice varieties. Jeerakasala exhibited the highest expression, followed by Basmati, while Ponmani and Njavara showed comparatively lower transcript levels. These findings indicate varietal differences in the transcriptional activation of the Wx gene region. Previous studies have demonstrated that variation in Wx gene expression significantly influences amylose synthesis and grain quality characteristics (Wang et al., 1995; Fitzgerald et al., 2009). The elevated pre-GBSS1 expression observed in Jeerakasala and Basmati therefore suggests enhanced transcriptional activity associated with starch biosynthesis.

Gene expression at the mRNA level is generally reflected in the synthesis of the corresponding protein. Consequently, the elevated pre-GBSS1 transcript abundance observed in Jeerakasala and Basmati is expected to result in increased GBSS1 protein synthesis and enhanced enzymatic activity during grain development. Although post-transcriptional and post-translational regulatory mechanisms may influence final protein accumulation, the substantial upregulation of precursor transcripts strongly indicates active GBSS1 gene expression. The consistency between elevated precursor transcript levels and increased mature gene expression further supports efficient transcriptional regulation of starch biosynthesis in these varieties.

Analysis of GBSS expression revealed a similar pattern of varietal variation. Jeerakasala recorded the highest GBSS1 expression, followed by Njavara and Basmati, whereas Ponmani exhibited comparatively lower expression levels. GBSS1 encodes Granule Bound Starch Synthase I, the principal enzyme responsible for amylose biosynthesis in rice endosperm (Wang et al., 1995; Liu et al., 2014). The high expression observed in Jeerakasala and Basmati suggests increased production of the GBSS1 enzyme, which may ultimately lead to greater amylose accumulation during grain development. Amylose is a major determinant of rice grain quality and influences cooking behaviour, texture, grain firmness, water absorption characteristics, and glycemic response. Aromatic rice varieties such as Jeerakasala and Basmati are known for their unique grain quality attributes, and the enhanced GBSS1 expression observed in these varieties may contribute significantly to their distinctive starch characteristics.

On the other hand, the lower GBSS1 expression observed in Ponmani indicates reduced transcript abundance and potentially lower enzyme production. Reduced GBSS1 activity may decrease amylose synthesis while increasing the relative proportion of amylopectin, thereby altering starch granule architecture and functional properties. Such variations in amylose-to-amylopectin ratio have profound effects on cooking quality, digestibility, and consumer preference. Interestingly, Njavara exhibited strong GBSS1 expression despite being classified as a traditional medicinal rice variety. This finding suggests that Njavara possesses considerable genetic potential for amylose synthesis and may combine enhanced nutritional value with desirable starch quality characteristics. Comparison between pre-GBSS1 and GBSS1 expression profiles revealed a positive relationship between precursor transcript abundance and mature GBSS1 expression. Varieties exhibiting elevated pre-GBSS1 expression also demonstrated increased GBSS1 transcript levels. Jeerakasala consistently showed the highest expression in both analyses, indicating efficient transcription, processing, and accumulation of GBSS1 transcripts. The marked increase in mature GBSS1 expression relative to precursor transcript abundance suggests active gene regulation mechanisms supporting enhanced starch biosynthesis.

Unlike GBSS1, GBSS2 exhibited a distinct expression pattern. Basmati recorded the highest GBSS2 expression, followed by Njavara and Jeerakasala, while Ponmani displayed the lowest expression. Previous studies have shown that GBSS2 differs functionally from GBSS1 and is involved in regulating starch metabolism in a tissue- and genotype-dependent manner (Wang et al., 2020; Lim et al., 2024). Although GBSS2 is not considered the primary enzyme

responsible for amylose synthesis, it contributes to the overall regulation of starch biosynthesis and starch granule formation. The elevated GBSS2 expression observed in Basmati suggests active participation of this gene in starch metabolic pathways and may contribute to the unique grain quality characteristics associated with aromatic rice.

When the expression profiles of pre-GBSS1, GBSS1, and GBSS2 were compared collectively, distinct genotype-dependent regulatory patterns became evident. Jeerakasala exhibited the highest expression of both pre-GBSS1 and GBSS1, indicating strong transcriptional activation of the amylose biosynthetic pathway. In contrast, Basmati demonstrated the highest GBSS2 expression, suggesting enhanced regulation of starch metabolism through alternative starch synthase pathways. Njavara showed elevated expression of both GBSS1 and GBSS2, reflecting active starch biosynthesis despite its traditional classification. These observations indicate that members of the GBSS gene family are differentially regulated among rice genotypes and may perform complementary functions in determining starch composition. The observed molecular variations are likely to influence starch granule development, amylose accumulation, grain texture, cooking quality, and nutritional properties. Enhanced expression of starch biosynthetic genes in aromatic varieties such as Jeerakasala and Basmati supports the hypothesis that transcriptional regulation plays a crucial role in determining their desirable grain quality traits. Furthermore, the correspondence between GBSS transcript levels and the 55 kDa GBSS-protein band detected through SDS-PAGE provides evidence that transcriptional regulation is translated into functional protein accumulation.

Altogether, the present study demonstrates substantial biochemical and molecular diversity among the selected rice varieties. Njavara exhibited superior protein accumulation, indicating enhanced nutritional quality, whereas Jeerakasala showed the strongest activation of the GBSS1-mediated amylose biosynthetic pathway. Basmati displayed elevated GBSS2 expression, highlighting differences in starch metabolic regulation. The integration of protein profiling and gene expression analyses suggests that genotype-dependent regulation of starch biosynthesis genes contributes significantly to varietal differences in amylose synthesis, starch structure, grain texture, cooking behaviour, and nutritional quality. These findings provide valuable insights into the molecular basis of grain quality traits and may facilitate the selection of rice genotypes for breeding programs aimed at improving nutritional and functional characteristics.

Summary and Conclusion

The present study successfully characterized the selected rice varieties, namely Ponmani, Jeerakasala, Njavara, Basmati, and Uma, through integrated biochemical and molecular analyses. The study evaluated the transcript expression of key starch biosynthesis genes (pre-GBSS1, GBSS1, and GBSS2), soluble protein content, and seed protein profiles to investigate variation in starch biosynthesis-related traits among the selected genotypes.

Gene expression analysis revealed significant genotype-dependent differences in the transcription of starch biosynthesis genes. Jeerakasala exhibited the highest expression of pre-GBSS1 and GBSS1, indicating enhanced transcriptional activity of these genes, whereas Basmati showed the highest GBSS2 expression. Njavara displayed relatively high expression of both GBSS1 and GBSS2, suggesting active transcription of genes associated with starch biosynthesis. Since GBSS1 and GBSS2 encode granule-bound starch synthase (GBSS), their transcript abundance reflects differences in the regulation of genes involved in amylose synthesis among the rice varieties. Biochemical analysis demonstrated considerable variation in soluble protein content, with Njavara recording the highest protein concentration, followed by Uma and Ponmani, while Basmati exhibited the lowest protein content. SDS-PAGE analysis further revealed differences in seed protein profiles, including the presence of a prominent protein band of approximately 55 kDa corresponding to GBSS in all the rice varieties. Variations in band intensity among the genotypes indicated differences in the accumulation of this protein, with Jeerakasala and Ponmani exhibiting relatively stronger GBSS bands.

The differences observed in gene expression, protein content, and protein profiles demonstrate that the selected rice varieties possess distinct biochemical and molecular characteristics. As transcript abundance and protein accumulation are regulated at different biological levels, variations in gene expression are not necessarily reflected by proportional changes in protein abundance. Therefore, the molecular and biochemical datasets should be considered complementary approaches for characterizing varietal differences rather than as evidence of a direct correlation between transcript and protein levels. These findings contribute to a better understanding of the molecular and biochemical diversity among rice genotypes and provide useful baseline information for future studies on grain quality, starch composition, and the development of rice varieties with improved nutritional and functional properties.

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