Salt Stress Induced Regulation of Secondary Metabolite Biosynthetic Pathway in Indigenous Rice Varieties

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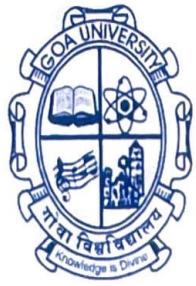

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, **"Salt Stress Induced Regulation of Secondary Metabolite Biosynthetic Pathway in Indigenous Rice Varieties"** is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of **Dr. Siddhi K. Jalmi** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **"Salt Stress Induced Regulation of Secondary Metabolite Biosynthetic Pathway in Indigenous Rice Varieties"** is a bonafide work carried out by **Mr Allan Jose Almeida** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of M.Sc. in the Discipline of Botany at the School of Biological Sciences and Biotechnology, Goa University.

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CONTENTS

ABBREVIATIONS

KC: Korgut Control

JC: Jaya Control

IC: IR64 Control

KT: Korgut 300mM NaCl Treated

JT: Jaya 300mM NaCl Treated

IT: IR64 300mM NaCl Treated

CHS: Chalcone synthase

CHI: Chalcone isomerase

NOMT: Naringenin 7-O-methyltransferase

F2H: Flavanone 2-hydroxylase

FNSII: Flavone synthase II

F3'H: Flavonoid 3'-hydroxylase

F3H: Flavanone 3-hydroxylase

DFR: Dihydroflavonol 4-reductase

LAR: Leucoanthocyanidin reductase

ANS: Anthocyanidin synthase

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ABSTRACT

Secondary metabolites are metabolic intermediates produced in plants required for their interaction with the environment and produced in response to stress. Flavonoids are class of polyphenolic secondary metabolite compounds that naturally occur in plants. and are involved in various functions like growth, development and stress defense. However, little is known about the roles of the key enzymes of the flavonoid biosynthesis pathway in response to salt stress in Goan rice cultivars (Korgut and Jaya). Here, we investigated the expression level of flavonoid biosynthesis genes and accumulation of the secondary metabolites in the leaves of these rice varieties under salt stress. Total phenolic content, total flavonoid content and anthocyanin content in rice leaves were enhanced during salinity stress. Quantitative real-time analysis showed that there was a rapid increase in expression levels of *OsLAR, OsNOMT, OsF3H, OsFNS2* and *OsF2H* genes in the rice cultivar Jaya under salt-stress. The cultivar Korgut showed enhanced expression of *OsLAR, OsNOMT* and *OsF3H* genes during salt-stress. The salt-sensitive IR64 used as control showed enhanced expression of *OsNOMT,* and *OsFNS2* whereas the other genes were not upregulated under salt-stress. In-silico analysis of these genes showed their possible interactions with many salt-stress tolerance genes. These results suggest that the flavonoids pathway gene expression and accumulation of flavonoid compounds maybe closely related to salinity tolerance in the Korgut and Jaya rice cultivars.

INTRODUCTION

1. Introduction

Rice (*Oryza sativa*) belonging to the Gramineae family is the major staple food crop of the world, being a major source of carbohydrate feeding more than half of the world's population. 85% of the rice consumed by the population is the white and polished rice, which has lower phytochemical content than the pigmented rice (Devi and Badwaik, 2022). Pigmented rice varieties possess significant number of secondary metabolites which imparts colour to the grain and also gives various nutritional benefits to the consumers (Mbanjo et al., 2020). Primary and secondary metabolites primarily constitute phytochemical content of the plant, which are important in plant's growth and defence. The phytochemical compounds produced in plants are divided into primary and secondary metabolites. Primary metabolites like carbohydrates, proteins and lipids are essential for plant growth and development and are common in most of the plants. Secondary metabolites are chemically diverse and are required by the plants for various roles like defence and protecting the plant in adverse conditions (Patra B et al., 2013).

Biosynthesis of secondary metabolites begins from precursors formed during glycolysis of carbohydrate. The main pathways involved in formation of the precursors of secondary metabolites are the mevalonic acid pathway (MVP) in the cytosol and the methylerythritol 4-phosphate pathway (MEP) in the plastids and shikimic acid pathway (Santos-Sánchez et al., 2019, Vranová et al., 2013). The precursors for the biosynthesis of phenols are obtained by the shikimic acid pathway and precursors for terpenes are derived from MVP and MEP pathway. The synthesis of types of metabolites diversifies depending on various factors like the cell types, developmental stages and environmental conditions (Carrington et al., 2018).

Secondary metabolites are divided into 2 groups based on the chemical composition viz.

i) Alkaloids: nitrogen containing molecules

ii) Terpenoids and phenolics: nitrogen deficient molecules

Alkaloids are nitrogen-containing basic compounds derived from amino acids such as tryptophan, tyrosine, phenylalanine and lysine. Alkaloids like vinblastine, vincristine, cocaine, caffeine help in chemical defence against herbivores, play role in antimicrobial defence and also protect plants from UV radiation (Patra B et al., 2013).

Amongst the nitrogen deficient metabolites terpenoids are derived from universal five-carbon precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Terpenoids are oxygenated hydrocarbons synthesised in the cytosol, through the mevalonate (MVA) pathway, or in the chloroplasts, through the methylerythritol phosphate (MEP) pathway (Nagegowda., 2010). Some terpenes act as phytohormones (e.g., gibberellin-diterpene, brassinosteroids- triterpenes) and play roles in growth and development (Patra B et al., 2013). Terpenes such as β-carotene and chlorophyll, are plant pigments helping in photosynthesis.

Phenolics belonging to nitrogen deficient molecules, are characterised by the presence of an aromatic ring bearing one or more hydroxyl groups. The biosynthesis of these phenolics depends on two pathways viz. the shikimic acid pathway and the malonic acid pathway. Plant phenolics are involved in defence against UV. Flavonoids, tannins and lignins belong to this group of secondary metabolites (Patra B et al., 2013). Flavonoids provide beautiful pigmentation in flowers thereby attracting pollinators and fruit dispersers. Condensed tannins are compounds formed by polymerization of flavonoid units. They give anthocyanidins in the presence of strong acids thus called pro-anthocyanidins.

Flavonoids are naturally occurring phenylpropanoid derived, polyphenolic compounds which are abundantly found across the plant kingdom. Flavonoid molecules have a basic carbon skeleton with the flavan nucleus, containing 15 carbons arranged in two aromatic rings connected by a three carbon-bridge (C_6-C_3-C_6) which is composed of a benzo-γ-pyrone structure and a phenyl ring. The benzene and phenyl ring are denoted as the A and B ring respectively, while the oxygen containing γ-pyrone ring is referred to as the C ring (Fig1.1). Flavonoids can be divided into different groups, primarily on the basis of the degree of oxidation of the three-carbon bridge. These are isoflavones, flavones, flavonol, flavanone, flavanonol, flavanol, and anthocyanidin (Ku Yee-Shan et al., 2020).

Isoflavone is one distinctive sub-group of flavonoids, having the B ring attached at position 3 on the C ring. Daidzin, genistin and glycitin belong to this group of flavonoids found in soybean, red clover, alfalfa, kudzu. Flavones shows the presence of a double bond between the carbons at position 2 and 3, and a ketone group at position 4 on the C ring. Apigenin and luteolin are examples of flavones. Chamomile, cabbage, carrot, wheat sprout, citric fruits are few food sources of flavones.

Flavonols (e.g., quercetin, kaempferol or myricetin) have a structure similar to flavones, except there is an extra hydroxyl group substituted at C-3 (Zhang, Q., 2013; Ku Yee-Shan et al., 2020). Mostly occurring in berries, apple, grapes, tomatoes, red wine, citric fruits. Flavanone have a saturated C ring as it lacks the double bound between C-2 and C-3 and are mostly found in citrus fruits and are the source of the bitter taste in citrus juices. They usually act as intermediate precursors (e.g., naringenin) of other groups of flavonoids or even found as the end products in the flavonoid biosynthetic pathway (Ku Yee-Shan et al., 2020). Flavanonols, also known as dihydroflavanonols are the 3-hydroxy derivatives of flavanones. Taxifolin, a flavanonol, has beneficial pharmaceutical properties. Like other groups of flavonoids, it is abundant in citrus fruits (Tsao R., 2010 and Ku Yee-Shan et

al.,2020). Flavanols lack a double bond between C-2 and C-3, and the ketone group at position 4 of the C ring. They are also known as flavan-3-ols due to the attachment of a hydroxyl group at position 3 on the C-ring. Citrus fruits, tea and rice are rich in flavanols.

Anthocyanidin appear as a form of flavylium ion, where the first oxygen atom on the C ring carries a positive charge. They differ from flavonols and flavanones, as they lack a ketone group at position 4 on the C ring. Anthocyanidins are unstable and commonly not found in fresh plants. Instead, the glycosylated, water-soluble pigment anthocyanin occurs more frequently in plants like blueberry, grapes, eggplant. Cyanidin, malvidin, pelargonidin are few examples of anthocyanidins. (Ku Yee-Shan et al., 2020).

Plants being sessile are exposed to varied environmental stresses mostly classified as biotic and abiotic stresses. Plant responds to these stresses by the use of intricate signalling pathways which activate defence or stress response genes and proteins, thereby allowing the plants to cop up with different stresses. Extensive work has been done on these signalling pathways and stress responsive genes in context with stress response, however, the involvement of plant secondary metabolite pathways in stress response is not explored fully in various abiotic stresses. Some of the studies suggest that the biosynthesis of plant secondary metabolites is induced and regulated by various biotic and abiotic stresses (Wang W. et al., 2018). The shikimic acid pathway is activated in stress conditions to produce aromatic acids like tryptophan, tyrosine and phenylalanine. The various biotic stresses known to affect secondary metabolites biosynthesis in various living organisms like bacteria, fungi, insect pests, nematodes, viruses, other plants etc. However, involvement of the abiotic stresses like salinity, drought, heat, cold, heavy metals, UV radiations (Jan R. et al., 2021) in inducing and regulating secondary metabolite biosynthetic pathways remains elusive.

Agriculture in Goa is mostly affected by salinity stress because of its geographical location on coastal line. In Goa, rice occupies around 39% of the total cultivated land. The area is estimated to be around 18,000 ha, out of which 12,000 ha are utilised for cultivation of rice. It is grown in the lateritic uplands (morod lands), midlands (kher lands) and coastal saline lands (khazan lands). These Khazan lands are subjected to sea water during high tides and are thereby rendered saline (B.L. Manjunath et al., 2009). Salinity is an important abiotic stress in such areas which also affect the plant yield drastically due to the reduction of different phenotypic characters such as leaf area, leaf number, height, root volume etc (Jan R. et al., 2021). When the plant is exposed to salt stress, the absorption of sufficient water by the roots reduces, which leads to water scarcity and osmotic pressure caused by high accumulation of salt. As a result of osmotic pressure, membrane functional stability, nutrient balance, and redox homeostasis are disturbed, which in turn affects the primary metabolites that are precursors of SMs (Ramakrishna and Ravishankar, 2011; Hossain et al., 2017).

The rice varieties which can be grown in these saline fields are salt tolerant varieties. In Goa many of such salt tolerant varieties are grown in Khazan fields and one of the important varieties is Korgut. *Oryza sativa* var. Korgut (which means small coloured grains) grown in the Khazan lands or coastal saline lands in Goa is 100% salt-tolerant variety. The husk colour ranges from dark brown to light brown and kernel colour is red to dark brown (Fig 1.2), sub-transparent. It has 76% total carbohydrates, 17.55% amylose content and total protein of 7.37%. it contains minerals viz. Iron (9.38mg/kg), zinc (37.45mg/kg), potassium (0.20%) and calcium (0.04%) (Bhonsle and Krishnan, 2012). Jaya is another high yielding and salt sensitive rice variety mostly grown in midlands and low lands of Goa (Srivastava and Sharma, 2020; Khatoon and Gopalakrishna, 2004).

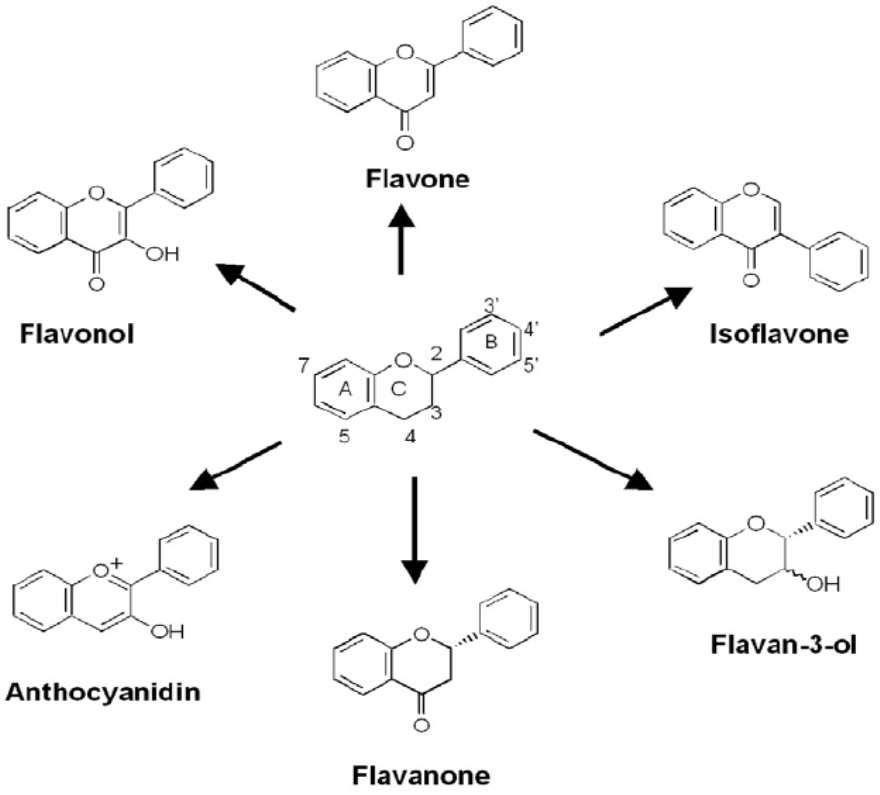


Fig1.1: Chemical structures of some flavonoids (Nishiumi, S et al., 2011): A ring: benzene ring; B ring: phenyl ring; C ring: oxygen containing γ-pyrone ring

Fig 1.2: Dehusked rice grains of Korgut, Jaya and IR64

Korgut being a salt-tolerant, pigmented, nutritive rice variety, is presumed to possess more secondary metabolites. Being salt tolerant pigmented variety, it represents the best model plant to study the regulation of rice secondary metabolite biosynthetic pathways under salt stress. Since scientifically and molecularly very little is known about indigenous rice variety Korgut, it is very important to understand the biosynthesis of different secondary metabolites in this rice variety at molecular level. Also, it is worth to study the production of different secondary metabolites under salt stress in Korgut and other salt susceptible varieties. Further the analysis of expression of genes of biosynthetic enzymes will give the idea about possible involvement of these metabolite biosynthetic pathways in salt stress tolerance in Korgut. This study will be a base to many other studies to explore the link between plant secondary metabolites and salinity stress. Hence this work proposes to explore the role of the secondary metabolites' biosynthesis pathway under salt stress in indigenous rice varieties like Korgut and Jaya. It further suggests the expression of genes of the key enzymes of secondary metabolite biosynthetic pathway in these rice varieties under salinity.

REVIEW OF LITERATURE

2. Review of literature

2.1 Secondary metabolites in crop plant rice

Rice is the staple food crop in most of countries around the world feeding the major group of people, with highest production in Asia. There is more than lakh of varieties grown in countries of Asia that vary in quality and nutritional content. Brown rice is reported to be healthier rice with more minerals, vitamins and bioactive compounds than white rice. Scientific studies have provided evidence to support the hypothesis that pigmented rice grain (black, purple, red orange or brown) possesses anti-oxidant, antidiabetic, anti-hyperlipidaemic and anti-cancer activity. Pigmented rice has been associated with anti-inflammatory and diuretic activity. (Umadevi. et al., 2012). Red rice is known to have 2-3 times higher zinc and iron content than that of white rice (Ahuja et al., 2007). The pigmented rice is known to have higher antioxidant activity than white rice (Muntana N and Prasong S, 2010; Jun HI et al., 2012). According to the indigenous knowledge, pigmented rice varieties have also been recommended for treatment of diarrhoea, vomiting, fever, jaundice as well as addressing various liver and kidney disorders. (Hedge et al., 2013; Sathya, 2013). The nutritional quality of white rice is poor as compared to the pigmented variants. Pigmented rice varieties are thus gaining consumer interest because of its numerous health benefits. The compounds responsible for these nutritional benefits are the secondary metabolites such as flavonoids, anthocyanins and pro-anthocyanidins (Mbanjo et al., 2020). The major flavonoids present in pigmented rice grains include pro-anthocyanidins and anthocyanins.

The flavanones present in red and brown rice are luteolin, tricetin, tricin, quercetin and myricetin. Tricin-O-rhamnoside-O-hexoside and apigenin-6-C-glucosyl-8-C-arabinoside

are predominantly found in white rice grains (Table 2.1) (Wang et al., 2018; Mbanjo et al., 2020).

Pro-anthocyanidins are oligomers and polymers of flavan-3-ols. These pro-anthocyanidins and catechins make up the bulk of the phenolic compounds found in red rice, being responsible for red pigmentation of the pericarp. White rice does not show the presence of pro-anthocyanidins however, some black rice varieties have been reported to contain them (Table 2.2) (Wang et al., 2018; Mbanjo et al., 2020).

Anthocyanins which are responsible for purple to blue pigmentation represent the bulk of the flavonoids present in black and purple rice. These include cyanidin-3-O-glucoside and peonidin-3-O-glucoside being the most prominent, but also represented are cyanidin-3,5-diglucoside, cyanidin-3-O-(6”-O-p-coumaroyl) glucoside, pelargonidin-3-O-glucoside, peonidin-3-O-(6”-O-p-coumaroyl) glucoside, and cyanidin-3-O-arabidoside. Red and white grains have been classified as lacking anthocyanins (Table 2.3) (Wang et al., 2018; Mbanjo et al., 2020).

Pigmented rice grain also contains a higher level of phenolic acids as compared to white grains. White rice contains p-coumaric acid, pinellic acid and ferulic acid. Black rice shows the presence of vanillic acid, hydroxybenzoic acid and protocatechuic acid whereas caffeic acid is present in minor quantities in red rice. Syringic acid is found in the extracts of brown, red and black rice. The red rice also shows the presence of pinellic acids. The extracts of the red rice mutant AM-45 have shown the presence of gallic acid (Mbanjo et al., 2020).

The major secondary metabolites present in pigmented rice are flavonoids which include pro-anthocyanidins and anthocyanins. The biosynthesis of flavonoids begins with the amino

Table 2.1: Flavonoids identified in rice and their roles

	Flavanones	**Rice**	**Role**
1.	Apigenin	Red and brown rice	Antifeedant activity, antioxidant activity
2.	Luteolin	Red and brown rice	-
3.	Tricetin	Red and brown rice	-
4.	Tricin	Red and brown rice	DPPH radical
5.	Quercetin	Red and brown rice	Antioxidant activity
6.	Myricetin	Red and brown rice	-
7.	Tricin-O-rhamnoside-O-hexoside	White rice	-
8.	Apigenin-6-C-glucosyl-8-C-arabinoside	White rice	-

Table 2.2: Pro-anthocyanidins identified in rice and their roles

	Pro-anthocyanidins	Rice	Role
1.	Pro-anthocyanidins	Red rice and some black rice varieties	-
2.	Catechins	Red rice and some black rice varieties	Antioxidant activity (ROS scavengers and metal ion chelators)

Table 2.3: Phenolic acids identified in rice and their roles

	Phenolic acids	Rice	Role
1.	p-coumaric acid	White Rice	-
2.	Ferulic acid	White Rice	Antioxidant activity
3.	Vanillic acid	Black rice	Allelopathic activity
4.	Protocatechuic acid	Black rice	Antioxidant activity
5.	Caffeic acid	Red rice	Antioxidant activity, allelopathic effect
6.	Syringic acid	Black, red and brown rice	Antioxidant activity, allelopathic effect
7.	Pinellic acids	White and red rice	-
8.	Hydroxybenzoic acid	Black rice	-
9.	Gallic acid	Present in red rice mutant AM-425	-

acid phenylalanine and end products include anthocyanins, flavones/isoflavones and condensed tannins (pro-anthocyanidins) as shown in Fig 2.1. Phenylalanine undergoes the deamination reaction catalysed by phenylalanine ammonia lyase to yield cinnamic acid. Cinnamic acid is oxidised by cinnamate-4-hydroxylase to 4-coumaric acid (phenolic acid), which is in turn converted to 4-coumaroyl-CoA by the action of the enzyme 4-coumaroyl-CoA ligase.

Flavanones: One coumaroyl-CoA molecule combines with three malonyl CoA molecules to produce naringenin chalcone by the action of the enzyme chalcone synthase (CHS). Naringenin chalcone undergoes isomerization to give naringenin catalysed by the enzyme chalcone isomerase. This naringenin then forms flavones in the tricin pathway. FLAVONE SYNTHASE II (FNSII) enzyme converts naringenin to apigenin (flavanones). FLAVANONE-2-HYDROXYLASE (F2H) catalyses the conversion of naringenin to 2-hydroxyflavanone, this is the first step in the synthesis of C-glycosylated flavanones.

Pro-anthocyanidins: are oligomers and polymers of flavanol-3-ols (Gunaratne et al., 2013). Flavonoid-3'-hydroxylase (F3'H) converts naringenin to eriodictyol, which is then converted to dihydroquercetin by the action of FLAVONE-3-HYDROXYLASE (F3H). Dihydroquercetin is then converted leucoanthocyanidins by the action of enzyme dihydroflavonol-4-reductase. LEUCOANTHOCYANIDIN REDUCTASE (LAR) is responsible for catalysing the conversion of leucocyanidin into flavan-3-ol catechin. This catechin monomers are then polymerised by yet another unknown pathway to form pro-anthocyanidin.

Anthocyanidins: Leucoanthocyanidin undergoes oxidization by the enzyme anthocyanin synthase (ANS) to produce various anthocyanidins like cyanidin, pelargonidin and

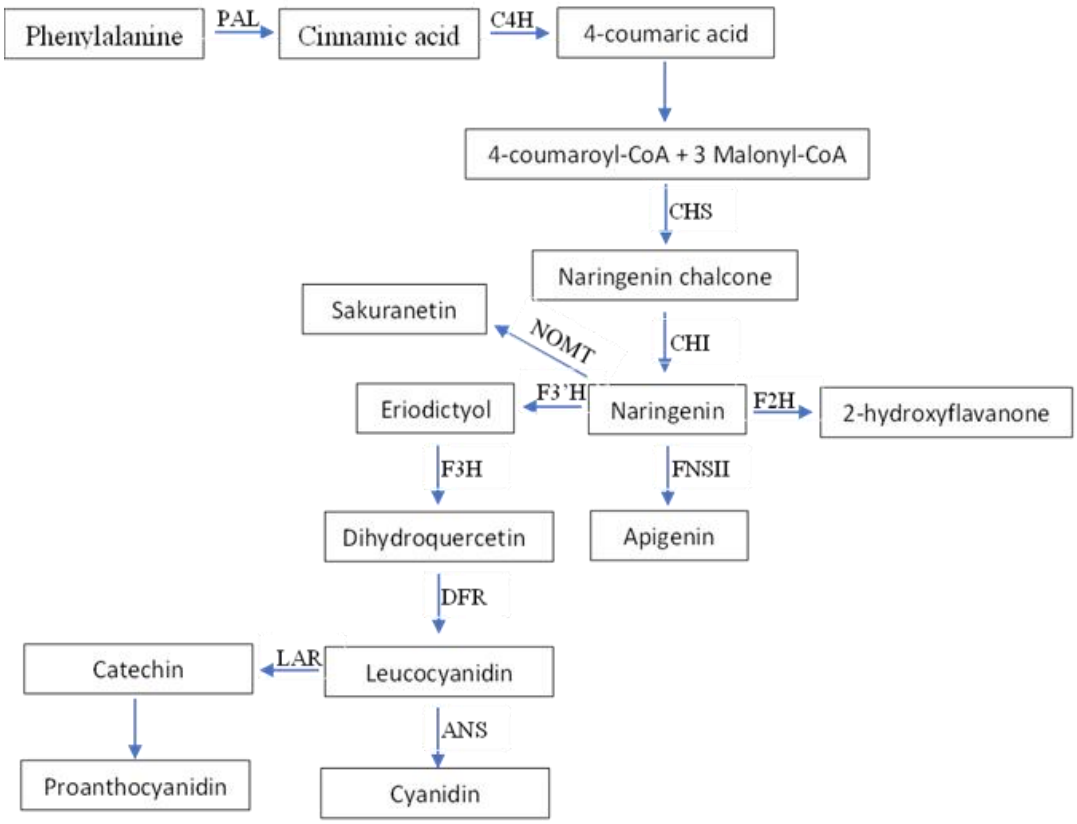


Fig 2.1: Flavonoids Biosynthesis Pathway in Rice

Abbreviations: CHS, Chalcone synthase; CHI, Chalcone Isomerase; NOMT, Naringenin 7-O-methyltransferase; F2H, Flavanone 2-hydroxylase; FNSII, Flavone synthase II; F3'H, Flavonoid 3'-hydroxylase; F3H, Flavanone 3-hydroxylase; DFR, Dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, Anthocyanidin synthase.

delphinidin. Naringenin forms Sakuranetin by the action of NARINGENIN-7-O-METHYLTRANSFERASE (NOMT). Sakuranetin is a flavone-type phytoalexin mostly found in the rice leaves.

2.2 Secondary metabolites role in alleviating biotic stress

CHS produces naringenin-chalcone that is further metabolised to produce different secondary metabolites in plants (Hahlbrock and Grisebach, 1979; Ebel, 1986; Heller and Forkmann, 1988). These secondary metabolites function as UV protectants, signal molecules, pigments and phytoalexins. Plants lacking the CHS activity are known to be acyanic and cannot produce anthocyanins. CHS activity is essential for the production of floral pigments called aurones that function as phytoalexins in some species (Harbourne, 1988; Pare et al., 1992). Lijuan et al., 2015 demonstrated that *CHS* gene is involved in regulating plant adaptation to salt stress by maintaining ROS homeostasis. CHS is the gatekeeper of flavonoid biosynthesis pathway as it plays an important role in regulating the pathway. The gene expression of *CHS* is influenced by environmental factors and stress like UV, wounding, herbivory, light or pathogen attack. These factors like UV light, phytopathogens, elicitors and wounding induce *CHS* gene expression and enhance the production of flavonoids. (Koes et al. 1987; Ryder et al. 1984, 1987; Bell et al. 1986; Burbulis et al. 1996; Chappell and Hahlbrock 1984; Feinbaum and Ausubel 1988; van Tunen et al. 1988; Taylor and Briggs 1990). *CHS* gene expression is induced by light stimuli specifically in the epidermal cells thereby leading to the accumulation of flavonoids in the epidermal cells of the leaves.

Chalcone Isomerase (CHI) is the next enzyme in the pathway that uses the substrate naringenin chalcone to catalyse the conversion of chalcones to flavanones (naringenin)

providing precursors for biosynthesis of the flavonoids (Chao, N et al., 2021). The CHI mRNA was reported to increase during *Colletotrichum lindemuthianum* infection in bean hypocotyls (Mehdy M C et al., 1988). Gharibi S et al., (2019) reported that the *CHI* gene expression remains unchanged in *Achillea pachycephala* under drought stress. However, it was reported to be increased in wheat leaves under drought stress (D. Ma et al., 2014). The transcription level of *CHI* in *Millettia pinnata* leaf was confirmed to be enhanced after being treated by seawater or NaCl (500mM). The salt-sensitive Saccharomyces cerevisiae also showed improved salt-tolerance when transformed with *CHI* (H Wang et al., 2013). Ozone fumigation and cold acclimatisation increased the CHS and CHI enzyme in variety of plant species (Kangasjarvi et al. 1994; Paolacci et al. 2001; Hannah et al. 2006; Di Ferdinando et al., 2012).

NOMT is important to catalyse the production of the major flavonoid phytoalexin in rice, Sakuranetin from naringenin. Sakuranetin is a potential pharmaceutical agent, exhibiting anti-inflammatory and antimutagenic activity (Saito, T., Abe, D., & Sekiya, K., 2008) The production of sakuranetin was induced by biotic and abiotic stressor such as the pathogen *M. oryzae*, $CuCl_2$, and UV irradiation and jasmonate hormone (Yamane, H. 2013). Gene expression of *OsNOMT* was upregulated by jasmonic acid treatment and *M. oryzae*-infected rice leaves indicating the defense responses through elicitor-induced production of sakuranetin in rice (Shimizu, T et al., 2012). However, not much is known about its role in abiotic stress.

FNSII enzyme is present at an important junction of the flavonoid biosynthesis pathway leading to different flavonoids, such as flavones, isoflavones, flavonols, proanthocyanidins and anthocyanidins. Therefore, the synthesis of these compounds can be controlled by

changing the *FNSII* gene activity (Martens, S., & Forkmann, G., 1999). FNSII converts naringenin to a major flavone aglycone, apigenin that functions as defence compound, signalling molecules and regulators of gene expression during pathogen attack (Martens and Mithofer 2005). The expression of the *FNSII* gene was enhanced in the transgenic plant roots by methyl jasmonate (MeJA) treatment and in soybean cell cultures in the presence of phytohormone jasmonate (Zhang et al. 2007; Fliegmann et al. 2010). Glucose acts as an osmotic stressor and signalling molecule that induced the Soybean *FNSII* due to osmotic stress (Kochs et al. 1987, Jiang et al. 2010). Apigenin produced by FNSII has been reported to limit Na^+ accumulation and regulate gene expression of *CNGC1*, *HKT2-1, SOS1*, inhibit lipid peroxidation, eliminate free radicals and enhance endogenous antioxidant defence (Heim et al. 2002, Prince Vijeya Singh et al. 2004). Apigenin is suggested to improve rice tolerance to salinity stress by increasing accumulation of chlorophylls, carotenoids and flavonoids as well as enhanced antioxidant activities. It also prevents toxic level accumulation of Na^+ in the shoots particularly leaves (Mekawy, A. M. M., Abdelaziz, M. N., & Ueda, A. 2018).

No significant studies have been made with respect to gene expression of *F2H* in response to biotic and abiotic stress. However, the product of F2H, C-glycosylated flavones are well studied. They are found in almost all plant phyla. The difference in the C-glycosylated flavones found in *indica* and *japonica* subspecies are consistent with the genomic data of these subspecies indicating a possible role of C-glycosylated flavones as biomarkers for discrimination of *indica* and *japonica* subspecies and has the potential link between the genotype and morphological and physiological traits (W. Chen et al., 2013). Large differences between the two rice subspecies has made it possible to dissect the flavonoid

biosynthetic pathway in rice using genetic and/or natural populations. C-glycosylated flavones may play a potential role in indica rice to provide higher resistance to rice blast (Liu et al., 2010). They also exhibit analgesic activity (Gorzalczany S et al., 2011). C-glycosylflavone isoschaftoside acts as an allelochemical that inhibits radicle growth of parasitic witchweed *Striga hermontica*, thus reducing and preventing maize parasitism (Hooper AM et al., 2010).

Dihydroquercetin is produced by F3H using naringenin as the substrate. *F3H* overexpression provide resistance to whitebacked planthopper in rice (Kim E. G. et., 2021). Overaccumulation of Kaempferol and quercetin helps scavenge reactive oxygen species in response to bacterial blight pathogenic infection in rice. *F3H* also promotes the SA accumulation that leads to SA-dependent nonexpressor pathogenesis-related (NPR1) that increases tolerance to bacterial leaf blight (BLB) stress (Jan et al., 2021). *F3H* expression is increased in response to salt in rice, *A. thaliana, Dendrobium officinale* and in the roots and stem of *Medicago truncatula*. Dihydroquercetin is a bioactive flavonoid. It is a very rare and best antioxidant. It has anti-inflammatory roles, antimicrobial, anti-cancer, and anti-Alzheimer and anti-toxoplasmosis effects (Sunil, C et al., 2019; G. T. Gao et al., 2019; Mitsunaga T et al., 2009; Razak S et al., 2018). Dihydroquercetin is reduced to leucoanthocyanidins by the action of the enzyme dihydroflavonol-4-reductase (DFR) that then act as precursor the production of flavan-3-ols. This *DFR* showed enhanced resistance against insect herbivory in the cDNA overexpressing lines of transgenic tobacco. Enhanced DFR expression was also linked to antioxidant potential of the plant (V Kumar et al., 2013). JA, cold, freezing stress, UV radiation and light have also proved to modulate the expression of *DFR* (NU Ahmed et al., 2014)

The expression of the *LAR* gene in tobacco and *M. notabilis* increased the resistance to *Botrytis cinerea* (Xin, Y et al., 2020). *M. notabilis* under UV light, PEG-6000 or methyl-jasmonate stress showed enhanced expression of the *LAR* transcript indicating the possible roles of *LAR* to stress tolerance. LAR produces a proanthocyanidin catechin. Catechins are ROS scavengers and metal ion chelators. They induce the antioxidant enzymes like SOD, GSH and CAT. It also modulates the activities of redox-sensitive transcription factors (NF-κB and AP-1) that help respond to pathogenesis-related oxidative stress. Pro-anthocyanidins are known to improve human health via their effects against cardiovascular disease and cancer. PAs are known to limit bacterial growth by their metal-chelating activities. (Braicu, C et al., 2013; Bernatoniene, J., & Kopustinskiene, D. M., 2018).

2.3 Secondary metabolites role in alleviating abiotic stress

As the secondary metabolites are considered to be involved in defence, signalling and communication, their biosynthesis generally increases when plant is under stress. Under normal conditions the synthesis of secondary metabolites is often low as plants do not have any defence strategies due to lack of any stress (Indrajeet K & Rajesh K S., 2018). Few reports suggest that secondary metabolites are involved in mitigating abiotic stresses such as temperature, drought, salinity and UV light stresses. Secondary plant products like glycine, carotenoids, proline, total phenolic contents, total flavonoids increased with increase in salinity up to 200mM NaCl concentration in *Carthamus tinctorius* L. plant (Pooran Golkar & Marzieh Taghizadeh., 2018). N Xu et al., (2021), reported that the flavonoid biosynthesis gene expression as well as the flavonoid content increased significantly after treatment with 100mmol/L NaCl in *G. biloba* seedlings. Coordination between the enhanced expression anthocyanin biosynthesis genes and accumulation of

anthocyanin was been reported in pigmented rice seeds and leaves of salt-tolerant rice cultivar (Kim, B.G et al.,2007; Chunthaburee et al., 2016).

Phenylpropanoids and phenolic compounds accumulation increases when the plant is subjected to stress. The expression level of genes governing the production of secondary metabolites have also showed considerable increase in response to various abiotic stresses (Dixon & Paiva, 1995). The results of D. Ma et al. (2014) exhibited increase in the flavonoid pathway gene expression of *TaCHS, TaCHI, TaF3H, TaFNS, TaFLS, TaDFR*, and *TaANS* and the flavonoid content was also increased indicating a close relation between the both, under drought stress in two wheat cultivars. Flavonoid biosynthesis is up-regulated in response to wide range of abiotic stresses like cold, drought and salinity (Ithal et al., 2004).

2.4 Salt stress affecting rice cultivation

The global rice production is affected by reduced arable land, climate change, biotic and abiotic stresses. Salinity is one of the major contributors of reducing global rice yields. High salt stress is proved to have several negative effects on rice plants, including stunted growth, germination interference, reduced photosynthetic activity etc. The expression of proteins encoded by the stress-related genes such as *SOS3, NHX1, HKT1, PAL* and *CHS* are upregulated under salinity stress and played a role in salt tolerance of the plant. (Shakri et al., 2022). Salt stress enhanced the ABA levels thereby causing a hypersensitive response leading to anthocyanin accumulation in rice (Ende and El-Esawe, 2014). Chunthaburee et al., 2016 reported that salt plays a regulatory role in anthocyanin synthesis pathway by increasing the expression of *CHS, DFR* and *ANS*.

Rice plants are very sensitive to salinity stress at young seedling stages and less so at reproduction (Flowers and Yeo, 1981; Lutts et al., 1995). From an agronomic point of view, tiller number and number of spikelets per panicle have been reported to be the most salinity-sensitive yield components in one genotype (Zeng and Shannon, 2000). These components are determined at vegetative and panicle initiation stages, respectively (Hoshikawa, 1989). In contrast, rice is considered to be relatively more salt tolerant at germination (Heenan et al., 1988; Khan et al., 1997). Seed germination is not significantly affected up to 16.3 dS m^{-1} (Heenan et al., 1988). Hence, improvement of salt tolerance of rice should target the specific growth stages that are more sensitive to salinity stress and can substantially affect grain yield.

Oryza sativa var. Korgut is a salt-tolerant Goan variety that has red grain colour. Jaya variety is believed to be a salt-sensitive variety and the grain colour varies from light-brown to brown. Previous studies have only focussed on lipid peroxidation, proline content, antioxidant enzymes like ascorbate and APX w.r.t salinity stress on these varieties (Sankhalkar and Vernekar 2016). However, there is no data about the secondary metabolites present in these two Goan rice varieties and the effect of salinity stress on these secondary metabolite biosynthesis pathways.

According to this background four objectives were formulated for this study. Here, we investigated the production of different secondary metabolites in indigenous rice varieties of Goa and studied expression profiling of flavonoids biosynthesis genes (*CHS, FNSII, F2H, F3H, NOMT and LAR*) in response to salt stress in the leaves of Korgut and Jaya rice varieties and compared them to that of the moderately salt-sensitive IR64 variety.

OBJECTIVES

Objectives:

1) To determine the presence of secondary metabolites, present in Korgut, Jaya and IR64 rice varieties
2) To study the change in the accumulation of total phenolic content, total flavonoid content and anthocyanin content in these varieties under salinity stress.
3) To study the effects of salt-stress on the expression of flavonoid biosynthesis genes in the leaves of the rice seedlings.
4) To determine the potential role of these genes in providing salt-tolerance to these rice varieties.

MATERIALS AND METHODS

3. Materials and methods

Table 3.1: List of Chemicals and stocks used

Type	Materials	Source
Fine Chemicals	NaCl, bismuth carbonate, sodium iodide, glacial acetic acid, ethyl acetate, acetic acid, sodium nitrite, glycerine, ferric chloride, ammonium hydroxide, Folin Ciocalteu reagent, sodium bicarbonate, gallic acid, methanol, HCl, AlCl3.6H2O, NaOH, Quercetin, TRIzol, chloroform, isopropanol, ethanol, DEPC, EtBr, EDTA, TE buffer, agarose, TBE Electrophoresis Buffer, SYBR® green master mix	Himedia; DuchefaBiochemie; Thermo Scientific; Bio-Rad
Kits	RevertAid First Strand cDNA synthesis kit	Thermo Scientific
Enzymes	RNase free DNase	Thermo Scientific
	Revert Aid reverse transcriptase	Thermo Scientific
	Taq DNA polymerase	Thermo Scientific
Oligonucleotide/Primers	Gene specific	Eurofins Genomics

3.1. Plant materials

Seeds of rice varieties viz. Korgut, Jaya were procured from the local farmers and IR64 were procured from National Institute of Plant Genome Research, New Delhi. Rice seeds were soaked overnight in sterile distilled water and germinated on moist Whatman filter paper inside the petridishes. The germinated seeds were then planted in the pots filled with autoclaved potting mix containing soil and compost (3:1) and transferred to the growth room where the temperature was maintained between 20-25°C. The plants were allowed to grow for 7 days. On the 7^{th} day the seedlings were subjected to salt stress of 300mM NaCl solution. Both the control and treated seedlings were harvested 24hours after treatment and frozen immediately in liquid nitrogen to facilitate nucleic acid extraction.

3.2. Localisation of secondary metabolites

Histochemical analysis was performed on transverse sections of fresh leaf samples obtained from control and salt-treated Korgut, Jaya and IR64 rice varieties. Transverse sections were treated with different solutions to precipitate out the secondary metabolites present within the plant tissue and observed under the Olympus light microscope and photographed using NIS software (Fig. 3.1. A).

3.2.1. Localisation of alkaloids

Dragendorff's test

Preparation of reagent: the stock solution was prepared by boiling 5.2g bismuth carbonate and 4g sodium iodide with 50mL of glacial acetic acid for few minutes. After 12 hours, the precipitated sodium-acetate crystals were filtered off using a sintered glass funnel. The clear, red-brown filtrate was mixed with 160mL of ethyl acetate and 1mL of water and

stored in an amber-coloured bottle. The working solution was prepared by mixing 10mL of stock solution with 20mL acetic acid and made up to 100mL with distilled water.

Protocol: Sections were placed in Dragendorff's reagent for 20minutes. These were then rinsed briefly in 5% sodium nitrite for 1minute and then mounted with glycerine. The yellow colour in the section indicates the presence of alkaloids.

3.2.2. Localisation of Phenolic compounds

The transverse sections of the plant were treated with 10% ferric chloride for 30min. Wash twice with distilled water to remove surplus ferric chloride and mount in glycerine. A dark colour, usually black, dark green or sometimes brown indicates the presence of tannins.

3.2.3. Localisation of anthocyanins

The transverse sections of the plant were treated with ammonium hydroxide solution to precipitate out anthocyanins. The presence of green colour staining in plant cells indicates the presence of anthocyanins.

3.3. Phytochemical properties of rice leaves

3.3.1. Determination of total phenolic content

The phenolic content of the rice leaves was extracted using a modified method based on Cai et al., (2004). The leaves were air dried and were ground to fine powder using liquid nitrogen. 5g of the powdered sample was extracted using 100mL distilled water at 80ºC for 20min in a water bath shaker. After cooling the extract was centrifuged at 5000rpm for 10min and filtered using Whatman Filter Paper No.1. The filtrate was stored at 4ºC until use within 24hr. 200µL of the extract was pipetted out in a test tube and followed by

addition of 1mL of Folin-Ciocalteu reagent (FCR) diluted to 1:10 distilled water. The dispersion was shaken vigorously and addition of 1mL of 10% Na_2CO_3 solution was done and the solution was stirred and diluted it with distilled water to final volume of 5mL. The dispersed solution was kept to stand in the dark at room temperature for 2h and the absorbance was measured at 765nm using a spectrophotometer. Gallic acid (5mg/100mL) was used for measurement of standard. The calibration curve was generated plotting concentration of standard (0.2, 0.4, 0.6, 0.8 and 1mL of gallic acid) along X-axis and the corresponding absorbance values at 765nm along Y-axis, resulting in a straight line.

3.3.2. Determination of total flavonoid content

The extracts were prepared using the modified method described by Bao et al., (2005). The leaf samples were ground using liquid nitrogen. 10g of the ground powder was extracted with 30mL of methanol containing 1% HCl at room temperature for 24h. The procedure was repeated twice. The methanolic extracts were filtered using Whatman filter paper No.1 and centrifuged at 20000g for 8min. The supernatant was used to determine the total flavonoid content using the colorimetric method. Aliquots of 0.5mL of appropriately diluted extracts or standard solutions were pipetted into a tube containing 2mL double distilled water and mixed with 0.15ml 5% $NaNO_2$. After 5min, 0.15mL 10% $AlCl_3.6H_2O$ solution was added, and the mixture was allowed to stand for an additional 5min, followed by addition of 1M NaOH. The reaction solution was mixed well and incubated for 15min, and the absorbance was then determined at 510nm using a spectrophotometer. Quercetin (5mg/100mL) was used as standard. Quantification was expressed by reporting the absorbance in the calibration graph of quercetin. The calibration was obtained using the concentration 0.2, 0.4, 0.6, 0.8, 1.0mL of quercetin vs corresponding absorbance.

3.3.3 Determination of anthocyanin content

Extracts were prepared using the method followed by D. Ma et al., (2014). Leaf samples were ground to fine powder using liquid nitrogen. 0.5g samples were extracted with 1.5mL mixture of methanol/hydrochloric acid/water (25:5:70, v/v/v) in shakers (32ºC, 150r/min) for 4h. The supernatant was collected after centrifuged at 10,000g for 20min. The absorbance of the supernatant was then determined at 525nm and 657nm. The anthocyanin content was calculated as: An = (OD525 – 0.25 x OD657)/FW.

3.4 RNA extraction and cDNA synthesis

Total RNA was extracted from control and salt treated leaf samples of Korgut, Jaya and IR64 using the Trizol method. 0.1g leaf tissue was homogenised in a mortar and pestle using 1ml RNA-Xpress Reagent following the manufacturer's instructions. The resultant mixture was centrifuged (Eppendorf® Centrifuge 5804R) at 12000g for 15min at 4ºC. To permit the phase separation of nucleoprotein complexes 500µL of chloroform was added to the supernatant, mixed vigorously and allowed to stand for 5min at room temperature. This was followed by centrifugation at 12000g for 10min at 4ºC. The colourless upper phase containing RNA was transferred to a fresh tube and 500µL of isopropanol was added, mixed and allowed to stand at room temperature for 15min. The mixture was later centrifuged at 12000g for 15min at 4ºC to facilitate RNA precipitation. The pellet obtained was then washed using 1ml 75% ethanol and centrifuged at 7500g for 5min at 4ºC. The washing was repeated twice, the supernatant was discarded without disturbing the pellet. The RNA pellet was then air dried for 5-10min at room temperature and dissolved in in 25µL DEPC-treated water. This was followed by quantitative estimation of the total RNA using ultraviolet (UV) visible spectrophotometer (UV 2450, Shimadzu) (Fig. 3.1. B) at

260nm and 280nm. Qualitative estimation was performed using 1% (w/v) agarose gel electrophoresis containing EtBr and visualised under UV transilluminator.

The First-strand cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit according to the specified protocol. To a RNase-free tube 1µL of 10x Reaction Buffer with $MgCl_2$, 1µg equivalent of the isolated RNA was added and treated with 1µL DNase I, RNase-free (Thermo Scientific) to remove the genomic DNA from the isolated RNA. Nuclease-free water was added to make the final volume to 10µL. The mixture was incubated at 37ºC for 30min. 1µL of 50mM EDTA Stop solution was added and incubated at 65ºC for 10min. EDTA acts as a chelating agent preventing RNA hydrolyses during heating. This was followed by adding 1µL Oligo $(dT)_{18}$ primer. Incubated at 65ºC for 5min to prevent secondary structures formation. Immediately snap chilled on ice and 8µL of master mix containing – 4µL 5x Reaction Buffer, 1µL RiboLock RNase Inhibitor (20U/µL), 2µL 10mM dNTP Mix and 1µL RevertAid M-MuLV RT (200U/µL) was added. The mixture was then mixed gently followed by incubation at 42ºC for 60min for the primers to anneal and begin extension (reverse transcription). The reaction was then terminated by heating at 70ºC for 5min which deactivates the reverse transcriptase. The product of the first strand cDNA synthesis was either used directly for sqRTPCR/qRTPCR or was stored at -20ºC until further use.

3.5. Primer design for real-time PCR analysis

Specific primers were designed for CHS, NOMT, F3H, F2H, FNSII and LAR genes on the basis of the genomic sequences of *Oryza sativa* based on the sequences available on National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/).

All primers were designed using Primer3 Plus software and synthesised by Eurofins Genomics.

Table 3.2: Primer sequences for real-time PCR analysis

Gene	Locus ID (Rice Genome Annotation Project)	Forward primer	Reverse primer
Actin		CGGTGTGATGGTTGGTATGG	GCCTCAGTCAGCAACACAGG
CHS	LOC_Os11g32650.1	GTCCAACACCTCCATCATCC	CTTCTTCCGCATCCTAGCC
NOMT	LOC_Os12g13800.1	CAAGCTGCTCCAATTCTTCC	CTCCATATGCTCCACACCT
F2H	LOC_Os06g01250.1	CTGCTGGAGACCATCATCG	GAAGATGTCGAGGACGAAGG
FNSII	LOC_Os04g01140.1	GTCCAACACCTCCATCATCC	CCTTGTGCCTGATCATCTCC
F3H	LOC_Os04g56700.1	GTACCATCACGCTCCTCCTC	TCTTGAACCTCCCATTGCTC
LAR	LOC_Os03g15360.1	GTGGTGATCTCGGTGATGG	GATGGAGTTGCAGCAGATGC

3.5.1. Primer stock solution

Tris-EDTA (TE) Buffer was prepared dissolving 15.8mg TE buffer powder (DuchefaBiochemie) in 10mL sterile distilled water which was used to prepare 100µM primer stock solutions. The stock solutions were stored at -20ºC until future use. The primers were diluted to 10µM (5µL of 100µM primer stock solution in 45µL sterile distilled water) to use for RTPCR and qRTPCR analysis.

3.6. Semi-quantitative Reverse Transcription Polymerase Chain Reaction (sq-RTPCR)

sq-RTPCR was performed to check the extraction efficiency, quality and amplification efficiency of each sample by comparing its amplification band density with that of the internal reference gene. To set up parallel reactions and minimize the possibility of pipetting errors, a sq-RTPCR master mix was prepared by mixing water, buffer, dNTPs, primers and DreamTaq DNA polymerase. Sufficient master mix was prepared for the number of reactions plus one extra. The sq-RTPCR was conducted in a thin-walled PCR tube on ice and the following components were added: 5µL 10x DreamTaq Buffer, 0.5µL dNTP Mix, 1µL Forward primer, 1µL Reverse primer, 0.5µL DreamTaq DNA polymerase, 1µL template cDNA. The final volume was adjusted to 20µL using sterile distilled water. The samples were vortexed and placed in a MiniAmp^{TM}Plus Thermal Cycler (Fig. 3.1. C). The sq-RTPCR was performed using the thermal cycling conditions as follows:

Table 3.3: Thermal cycling conditions for semi quantitative-RTPCR

Step	**Temperature ºC**	**Time**	**Number of cycles**
Initial denaturation	95	02 min	1
Denaturation	95	30 s	25-40
Annealing	Tm - 5ºC	30 s	
Extension	72	30 s	
Final extension	72	5 min	1

After the reaction is completed, the tubes were removed and stored at -20ºC. Agarose gel electrophoresis was performed to check the integrity of the amplified PCR products by comparing it with the housekeeping gene (actin) bands. The ladder with known DNA lengths were used for comparison of the cDNA bands.

3.7. Real-time PCR analysis (quantitative RTPCR)

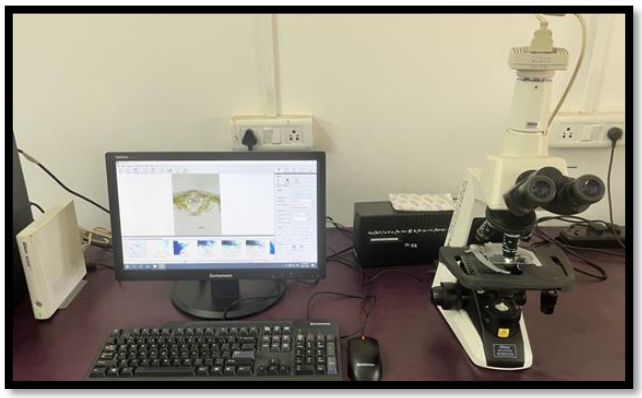
Real-time analysis of gene expression involved in the secondary metabolite pathway (CHS, NOMT, F3H, F2H, FNSII and LAR) and the housekeeping gene (Actin) was performed using CFX96^{TM} Real-Time System (Fig. 3.1. D). All real-time PCR runs were performed in triplicates. Real-time PCR was conducted in a 10µL reaction volume containing, 0.5 µL primers (final concentration µM), 3.5 µL Nuclease-free water, 5 µL SYBR® green master mix (Bio-Rad) and 1 µL of the diluted synthesised cDNA template (1:8 ratio). The PCR reaction was performed as follows: Initial denaturation at 95°C for 3 min followed by 39 cycles of denaturation at 95ºC for 15 sec, annealing at 48°C for 30 sec and extension at 65ºC for 5 sec, followed by melting curve analysis: 95ºC for 5 min.

3.8. In-silico analysis

In-silico PCR was done to determine the results of the PCR reaction computationally using the set of selected primers. “STRING” database (https://string-db.org/) was used to check the primers against the genome of our interest. The rice protein sequences of the flavonoid biosynthesis genes used in this study were obtained from the Rice Genome Annotation Project (RGAP) (http://rice.uga.edu/index.shtml) using the Locus ID of the primer. This protein sequence was fed to the STRING 9.0 database and the information about the possible interactions of the gene of interest with other genes was obtained. PlantRegMap

Database (http://plantregmap.gao-lab.org/) was used to identify the transcription factors interacting with genes used in our study.

(A)



(B)

(C)

(D)

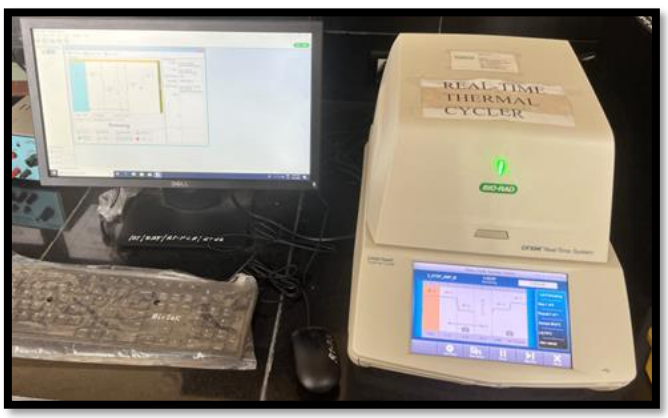


Fig 3.1: Instruments used : (A) Olympus light microscope; (B) UV-Vis Spectrophotometer (UV 2450, Shimadzu); (C) MiniAmp™ Plus Thermal Cycler; (D) CFX96™ Real-Time System.

RESULTS

4. Results

Pigmented rice is known to consist of greater pool of secondary metabolites, these phytochemicals play a very important role in plant defence mostly against pest and herbivores. Very few reports suggested the importance of secondary metabolites in imparting tolerance against abiotic stresses. Salinity being one of the abiotic stresses impacting the Goan agriculture, it is interesting to study the role of secondary metabolites in imparting tolerance against salt stress. Korgut rice being important indigenous brown rice salt tolerant variety, it is proposed to be a promising model plant for studying the above question. We also used another indigenous rice variety Jaya for our study and salt susceptible rice IR64 was used as control for all the experiments. In pigmented rice, the metabolites studied to be prominent are flavonoids (pro-anthocyanidins and anthocyanins). The production of these metabolites can be studied either by detecting directly the levels of phenols, flavonoids or by studying the levels of the biosynthetic enzymes involved in their production. The production of phenols and flavonoids were detected by staining the leaf sections with stains that specifically detects them. The total phenolic and flavonoid content, and anthocyanin content was determined using phytochemical analysis. The production of specific class of secondary metabolite was studied by gene expression analysis of those gene coding for biosynthetic enzymes of secondary metabolites.

4.1. Localisation of secondary metabolites

To detect the production of secondary metabolites in rice varieties Korgut, Jaya and IR64 in control and saline conditions, the 7 days old rice seedlings were treated with 300mM NaCl (Fig 4.1). Transverse sections of the control and salt treated leaves were stained using different secondary metabolites precipitating solutions to localise the secondary

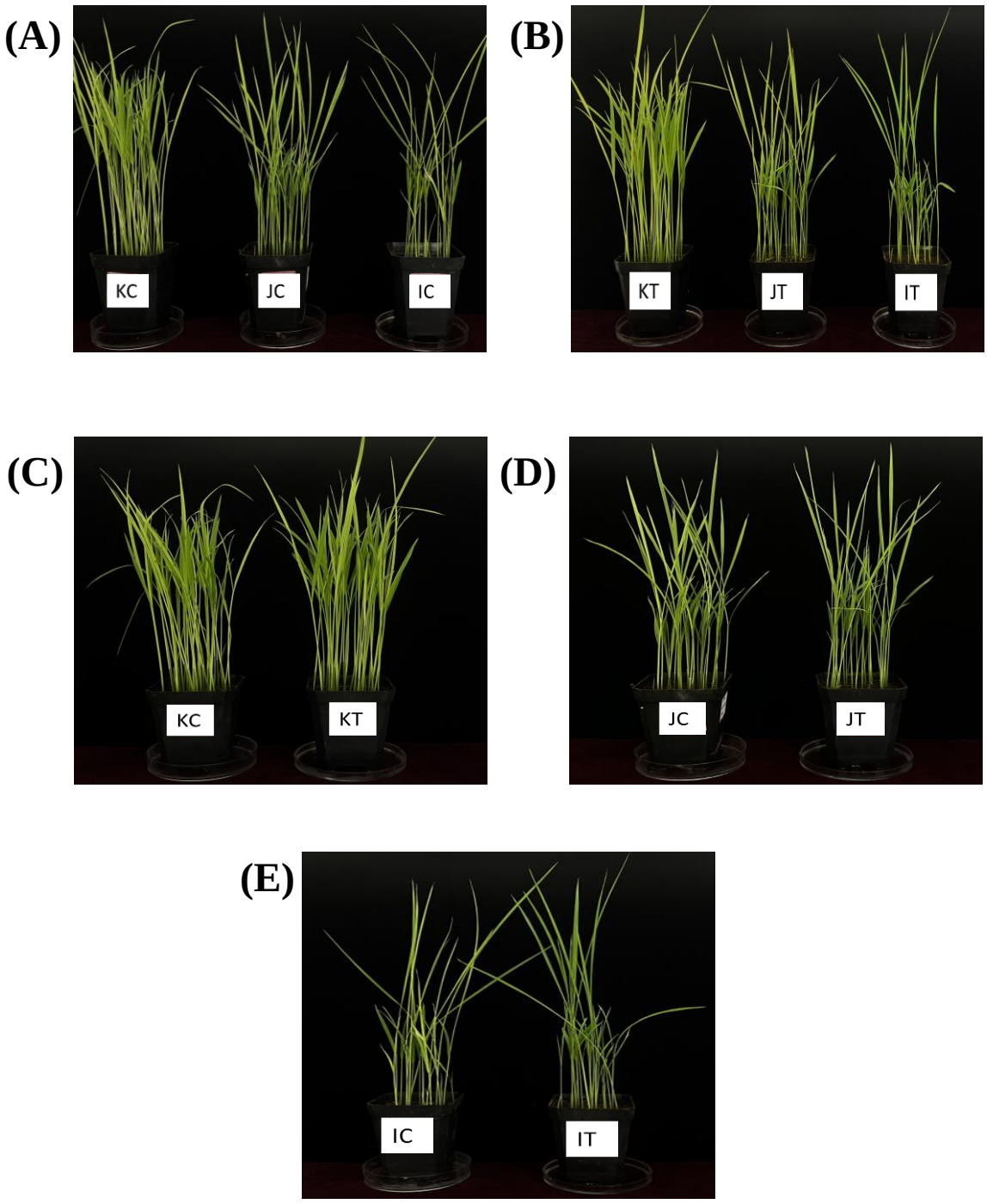


Fig 4.1: Growth of Korgut, Jaya and IR64 Rice seedlings under salt stress

(A) Control seedlings **(B)** Seedlings 24hr after 300mM NaCl treatment **(C)** Korgut control and treated seedlings 24hr after 300mM NaCl treatment **(D)** Jaya control and treated seedlings 24hr after 300mM NaCl treatment **(E)** IR64 control and treated seedlings 24hr after 300mM NaCl treatment. **KC:** Korgut Control seedlings; **KT:** Korgut seedlings 24h after 300mM NaCl treatment; **JC:** Jaya Control seedlings; **JT:** Jaya seedlings 24h after 300mM NaCl treatment; **IC:** IR64 Control seedlings; **IT:** IR64 seedlings 24h after 300mM NaCl treatment

metabolites in vivo in plant cells. Histochemical localisation of secondary metabolites suggested that the secondary metabolites get accumulated in the specific layer of leaves.

4.1.1 Localization of Alkaloids in leaves of salt tolerant and susceptible rice varieties

Alkaloids present in the leaves of Korgut, Jaya and IR64 were precipitated using Dragendorff's Reagent. The heavy metal ions present in the reagent combine with the nitrogen present in the alkaloid normally as tertiary amine or secondary amines to form an ion pair. Depending on the nature of alkaloid (or tertiary amine), this ion pair has a yellow to orange to red to brown colour. The sections of the leaves showed yellow to brown colour staining in cells containing alkaloids. No distinct yellow-coloured precipitation was observed in plant tissues under analysis. Control Korgut and Jaya leaves (Fig 4.2 and Fig 4.3) showed light yellow colour staining in the phloem region below the lower epidermis which was not observed in control IR64 (Fig 4.4) without any NaCl treatment, which suggestspresence of alkaloids in Korgut and Jaya leaves as compared to IR64. However, after NaCl treatment the yellow colouration was not observed in Korgut and Jaya rice leaves while it was observed in IR64 leaves.

4.1.2. Localization of Phenolics in leaves of salt tolerant and susceptible rice varieties

Tannins are phenolic secondary metabolites in plants which possess toxic or anti-nutritional effects on herbivores, insects hence play important role in plant defence. In this study we wanted to study their involvement in salt stress. Hence, we first studied their presence in Korgut, Jaya and IR64 in presence or absence of NaCl stress. Tannins present in the rice leaves were determined using $FeCl_3$ solution. Tannins form dark green precipitates with iron (Fe) compounds. Hydrolysable tannins are derived from gallic acid

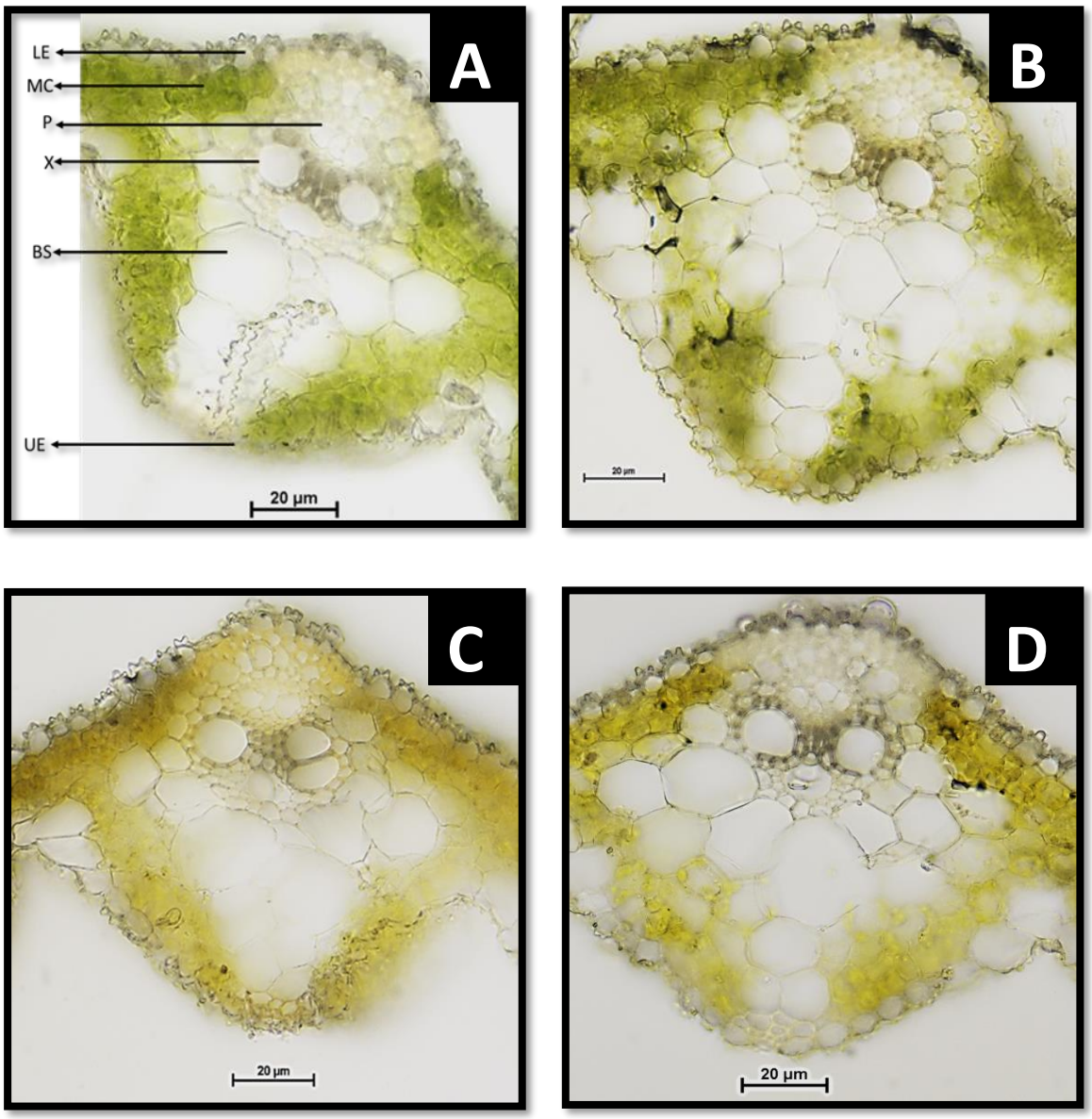


Fig 4.2: **Localisation of alkaloids**

A: T.S of unstained control Korgut leaf; **B:** T.S of unstained salt treated Korgut leaf; **C:** T.S of stained control Korgut leaf; **D:** T.S of stained salt treated Korgut leaf . LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

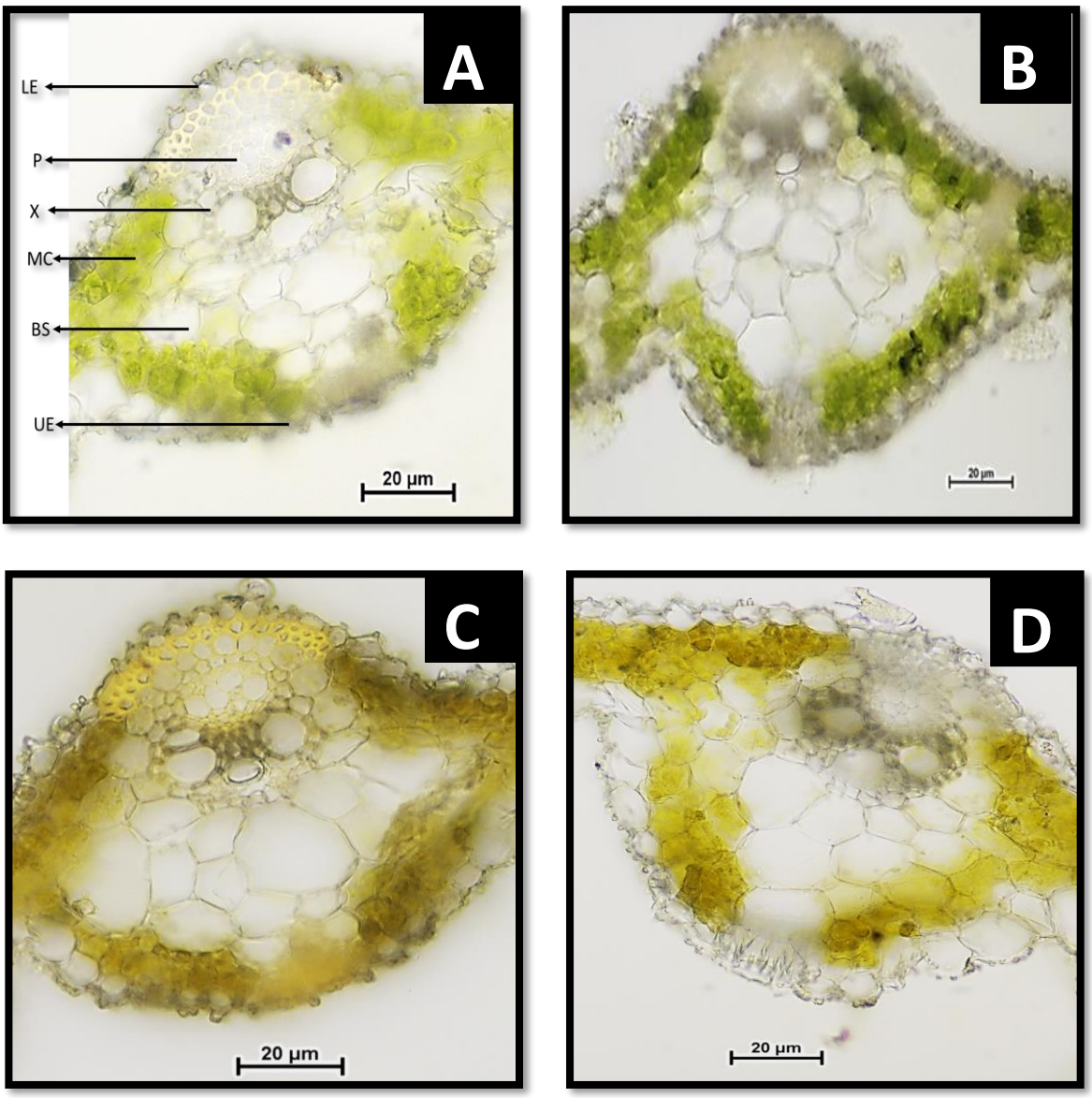


Fig 4.3: **Localisation of alkaloids**

A: T.S of unstained control Jaya leaf; **B:** T.S of unstained salt treated Jaya leaf; **C:** T.S of stained control Jaya leaf; **D:** T.S of stained salt treated Jaya leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

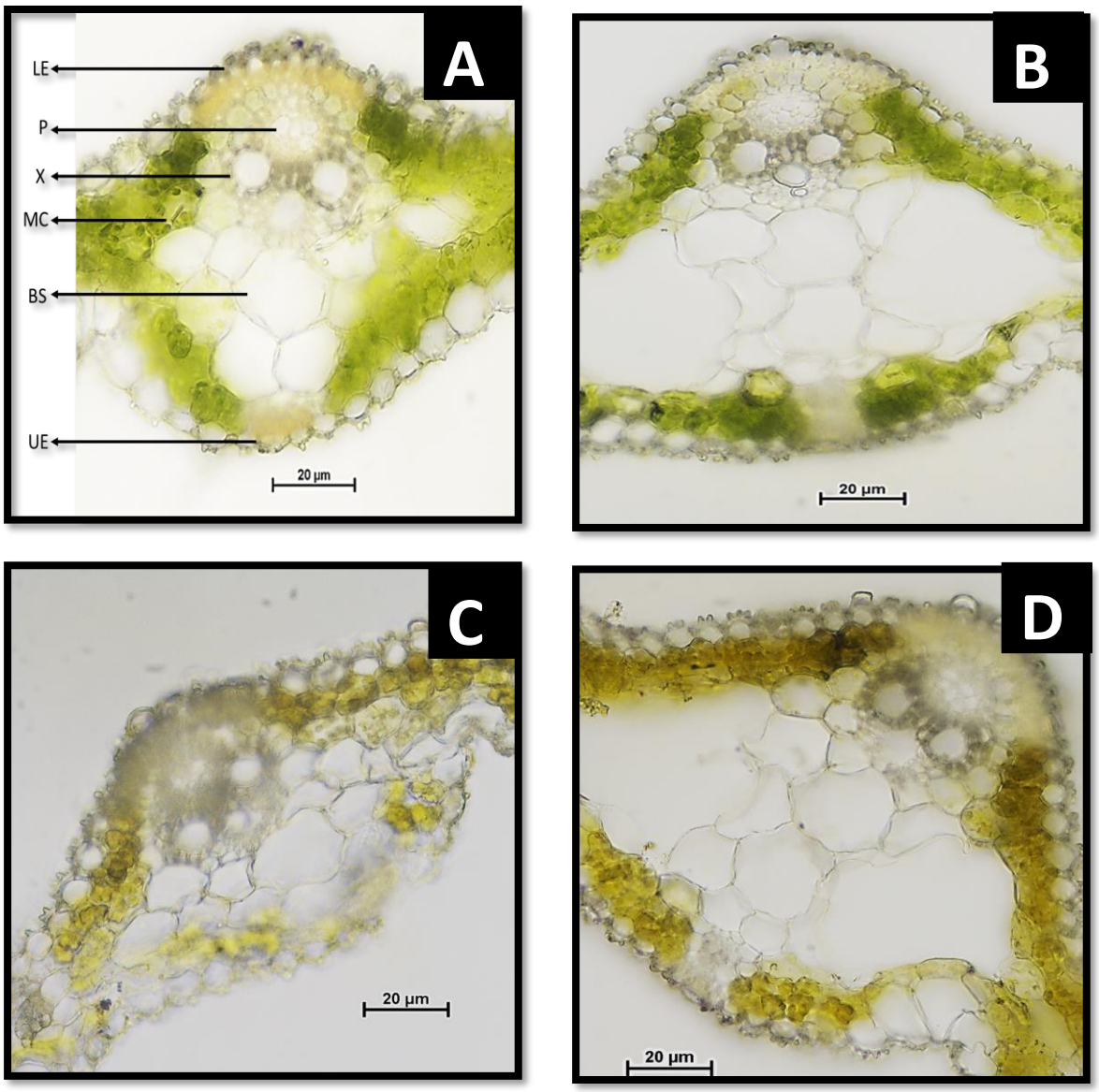


Fig 4.4: **Localisation of alkaloids**

A: T.S of unstained control IR64 leaf; **B:** T.S of unstained salt treated IR64 leaf; **C:** T.S of stained control IR64 leaf; **D:** T.S of stained salt treated IR64 leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

and when treated with ferric chloride produces brown colour. Condensed tannins are derived from flavonols, catechins and flavan-3,4-diols. These tannins when treated with ferric chloride produces dark green colour. Therefore, when transverse section of the leaves of NaCl untreated and treated rice leaves of Korgut, Jaya and IR64 cultivars were treated with ferric chloride solution, phenolic compounds were precipitated forming dark-green to brown colour staining in the plant cell layers. It was observed that dark green to light brown coloured salts were found in the mesophyll cells of the control leaf tissues. In salt treated leaves, there was additional light brown colouration observed in the region below the lower epidermis and the xylem cells of all the 3 varieties (Fig 4.5, 4.6, 4.7).

4.1.3. Localization of Anthocyanin in leaves of salt tolerant and susceptible rice varieties

Anthocyanins are known to be widely distributed in black rice and they show antioxidant capacity due to free radical scavenging capacity. To study the presence of anthocyanins in indigenous rice varieties Korgut and Jaya under salt treatment, their localization was detected histochemically using ammonium hydroxide solution. Anthocyanins when interacts with ammonium hydroxide precipitates to give a green colour staining in the plant cells. As evident from Fig 4.8, 4.9 and 4.10 the rice leaves when tested with ammonium hydroxide solution did not show prominent green colour. It did not show considerable difference as compared to the unstained sections indicating that anthocyanins are either absent or present in very minute amounts that cannot be detectable using histochemical staining.

4.2. Phytochemical properties

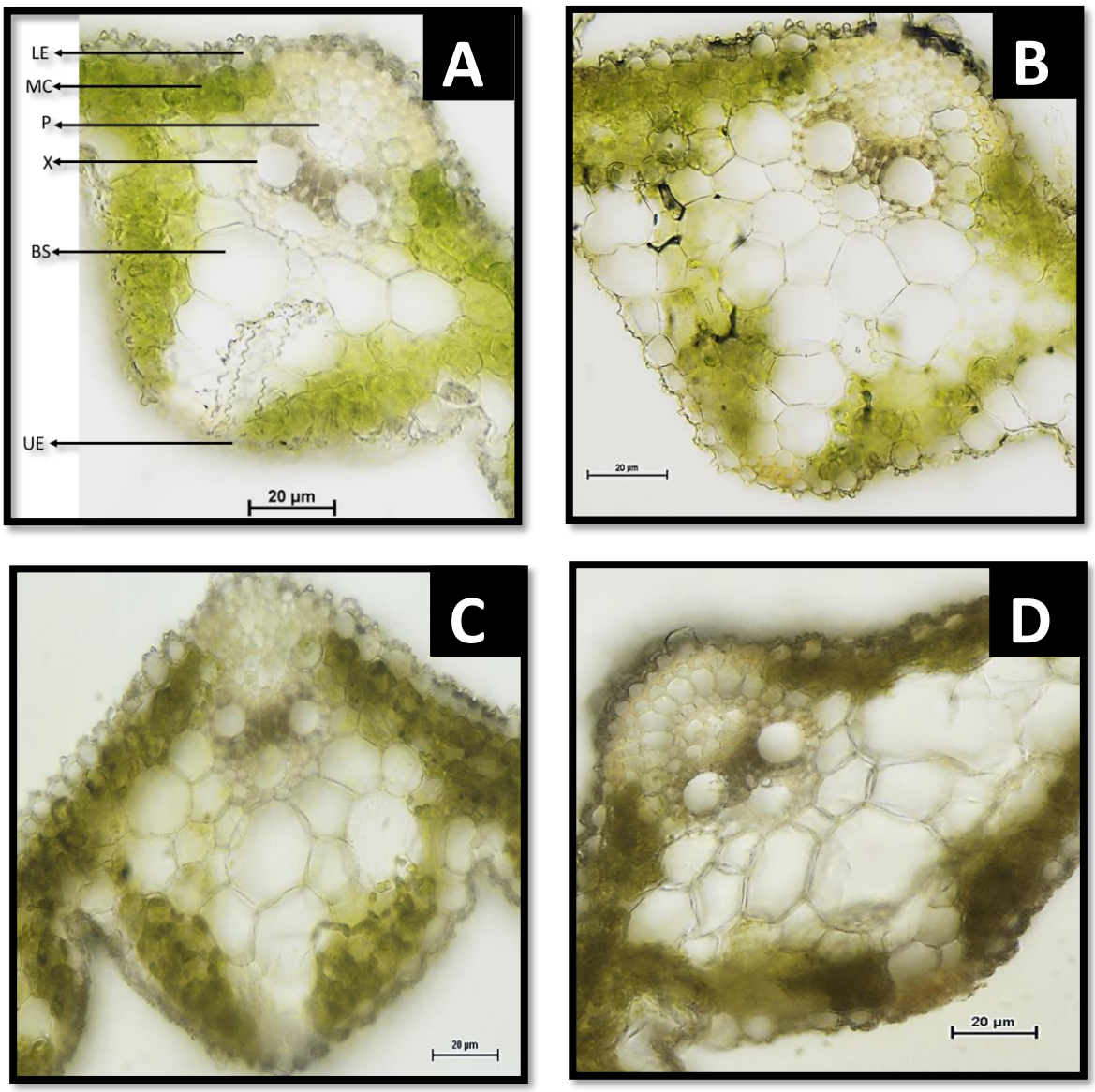


Fig 4.5: **Localisation of phenolics**

A: T.S of unstained control Korgut leaf; **B:** T.S of unstained salt treated Korgut leaf; **C:** T.S of stained control Korgut leaf; **D:** T.S of stained salt treated Korgut leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

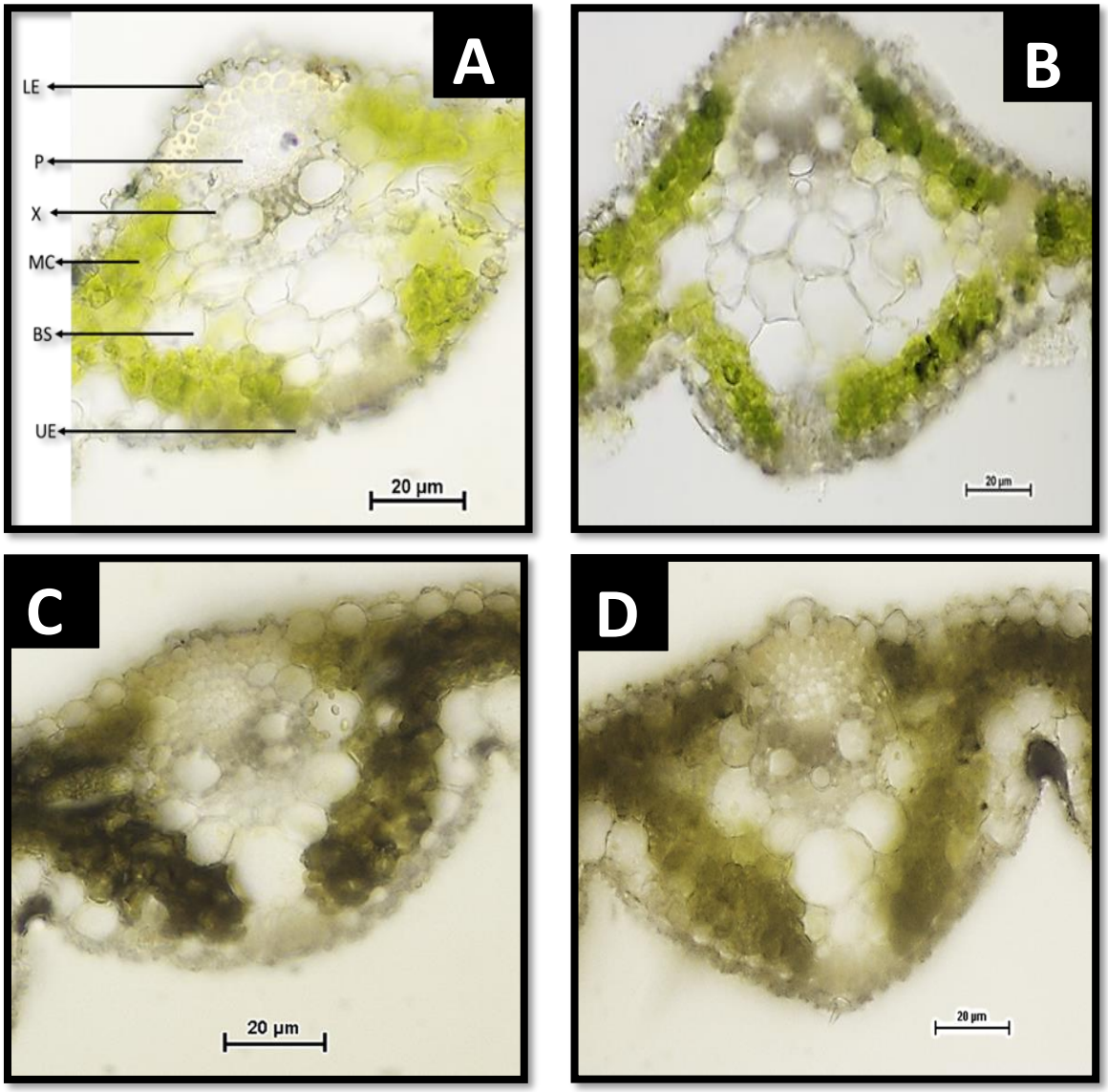


Fig 4.6: **Localisation of phenolics**

A: T.S of unstained control Jaya leaf; **B:** T.S of unstained salt treated Jaya leaf; **C:** T.S of stained control Jaya leaf; **D:** T.S of stained salt treated Jaya leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

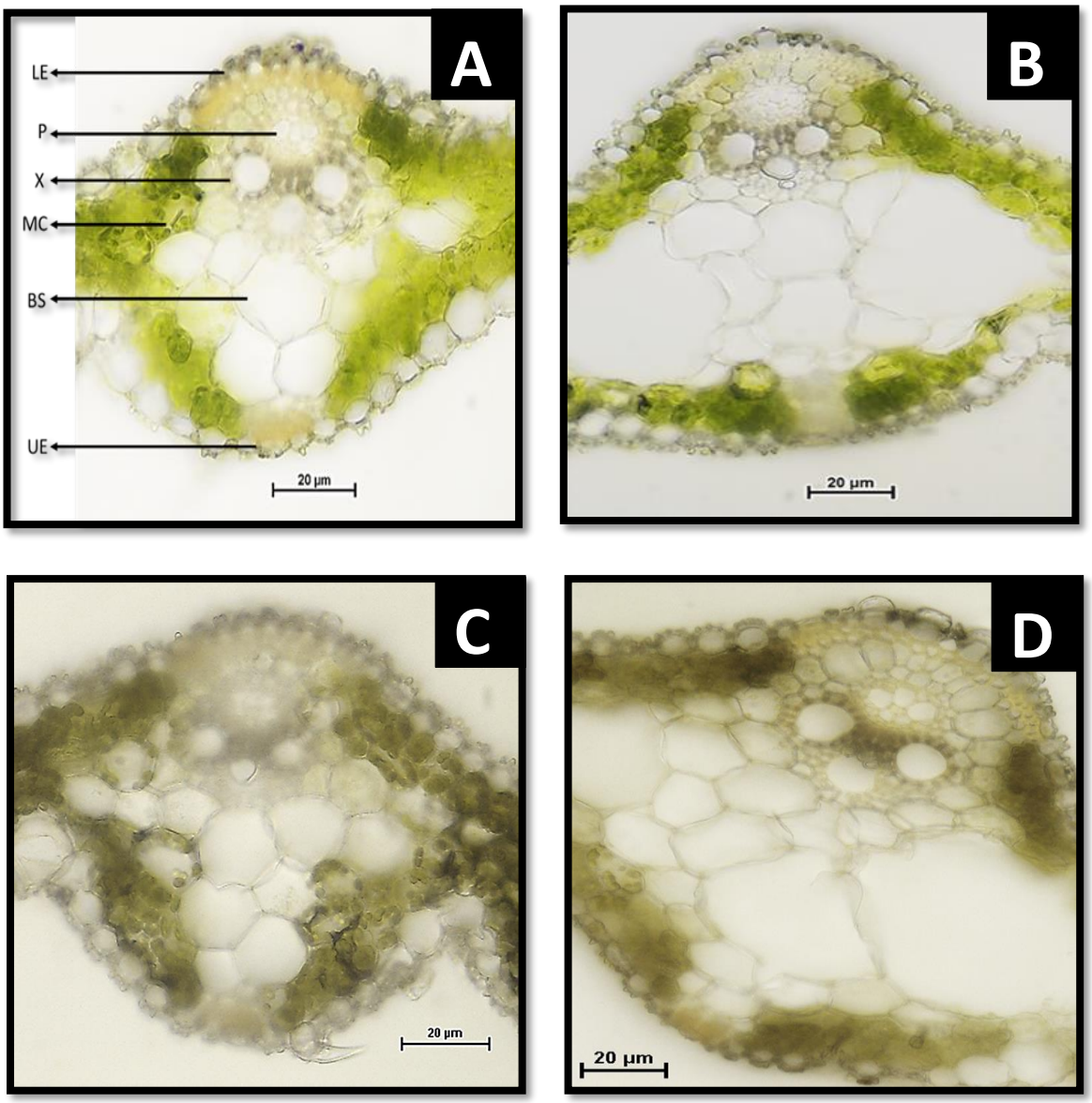


Fig 4.7: **Localisation of phenolics**

A: T.S of unstained control IR64 leaf; **B:** T.S of unstained salt treated IR64 leaf; **C:** T.S of stained control IR64 leaf; **D:** T.S of stained salt treated IR64 leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

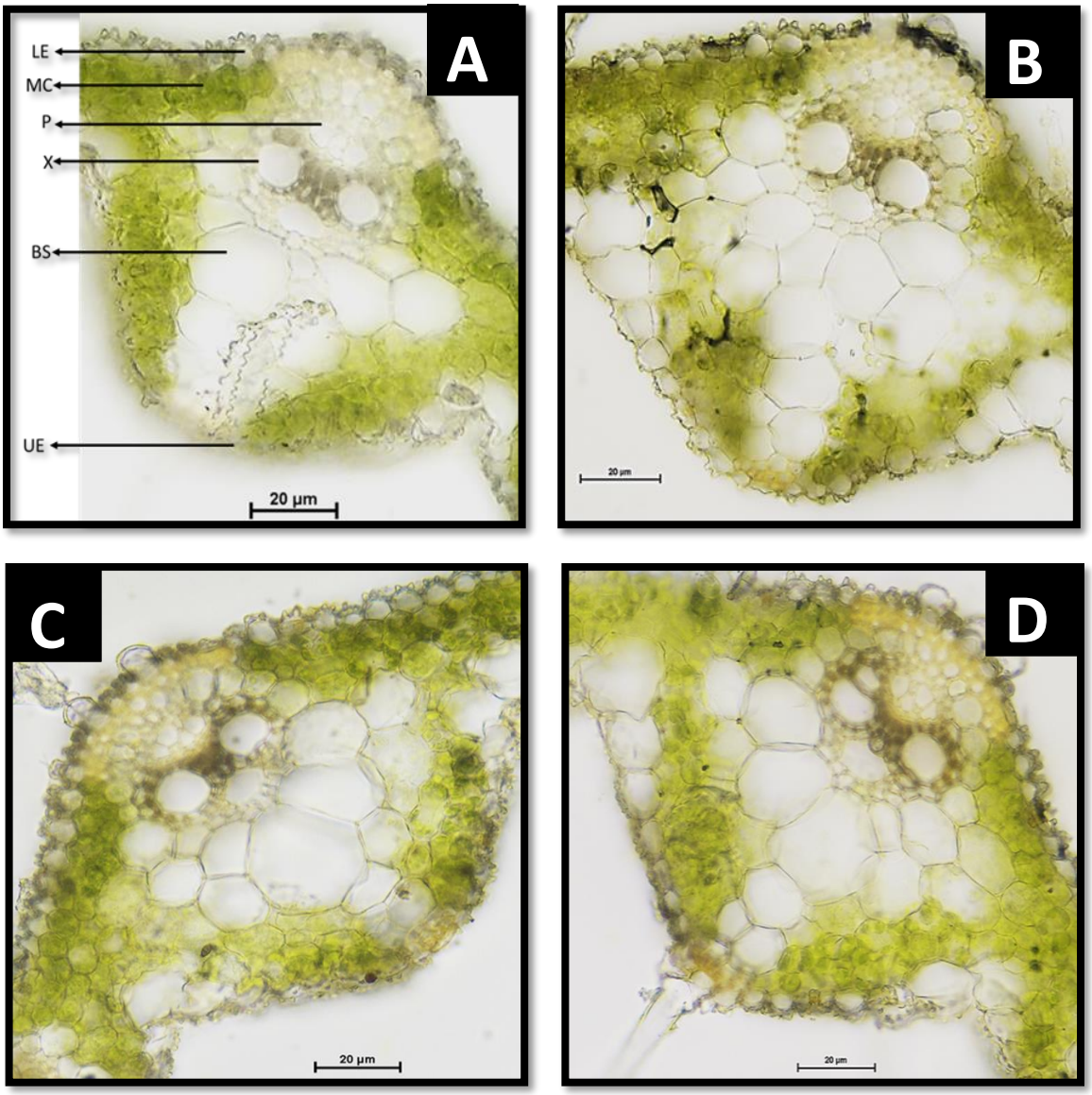


Fig 4.8: **Localisation of anthocyanins**

A: T.S of unstained control Korgut leaf; **B:** T.S of unstained salt treated Korgut leaf; **C:** T.S of stained control Korgut leaf; **D:** T.S of stained salt treated Korgut leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

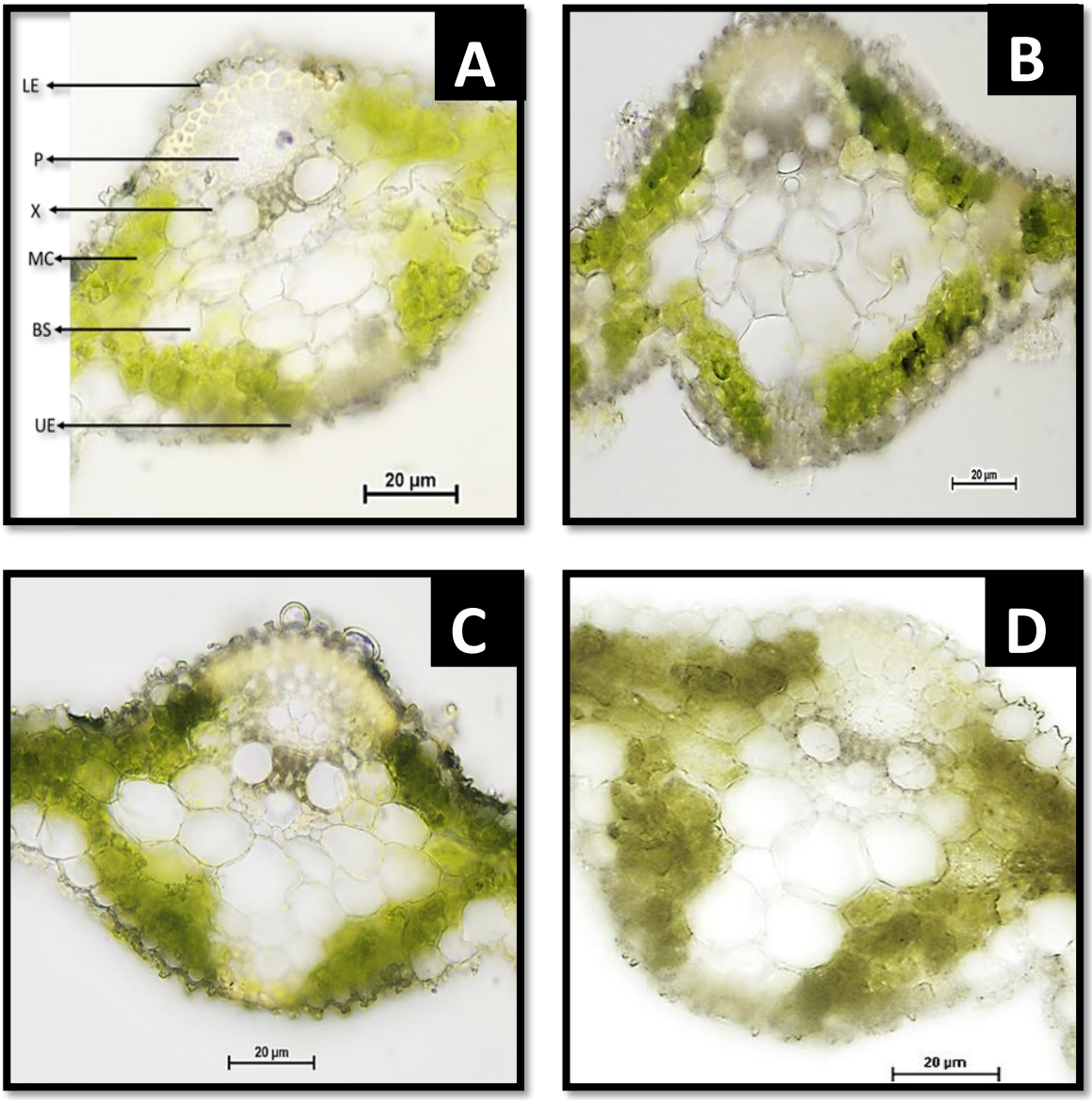


Fig 4.9: **Localisation of anthocyanins**

A: T.S of unstained control Jaya leaf; **B:** T.S of unstained salt treated Jaya leaf; **C:** T.S of stained control Jaya leaf; **D:** T.S of stained salt treated Jaya leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

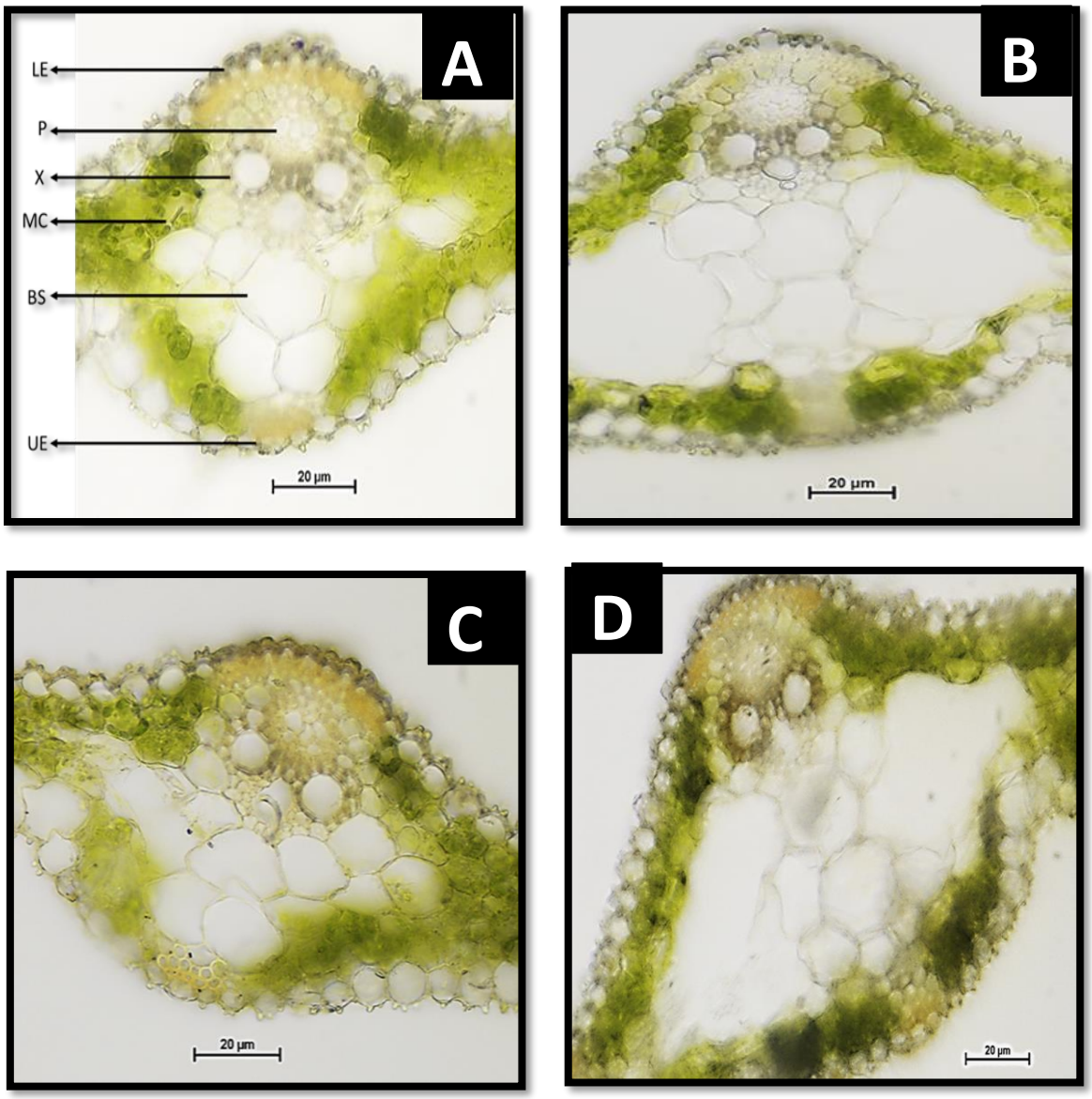


Fig 4.10: **Localisation of anthocyanins**

A: T.S of unstained control IR64 leaf; **B:** T.S of unstained salt treated IR64 leaf; **C:** T.S of stained control IR64 leaf; **D:** T.S of stained salt treated IR64 leaf. LE: Lower Epidermis; P: Phloem; X: Xylem; MC: Mesophyll Cells; BS: Bundle Sheath cells; UE: Upper Epidermis

Phytochemical content of the leaves of all 3 rice varieties were identified using standard solutions (Fig 4.11 and 4.12) and the outcomes are presented in Table 4.1. In the control rice plants, the total phenolic content (TPC) ranged from 0.0348 to 0.1035 (mg GAE/g). Under controlled conditions (without any salt stress), the highest TPC content was observed in the IR64 variety followed by Jaya and Korgut (Fig 4.14). However, after subjecting the plants to 300mM NaCl treatment the TPC content was found to be the highest in Korgut (0.2171 mg GAE/g) followed by Jaya (0.1068 mg GAE/g) and the least in IR64 plants (0.0714mg GAE/g).

Total flavonoid content of the controlled and treated plants varied from 0.3354 mg quercetin/g to 1.4176 mg quercetin/g. The maximum TFC as evident from Fig 4.16 under controlled conditions was found in Jaya (0.6695 mg quercetin/g) followed by IR64 (0.6276 mg quercetin/g) whereas the minimum in Korgut (0.3354 mg quercetin/g). However, this was not the case in salt treated plants. The trend was similar as that of TPC. Korgut showed the highest amount of TFC (1.4176 mg quercetin/g) and the least was seen in IR64 (0.7730mg quercetin/g).

The anthocyanin content was estimated as described by D. Ma et al., (2014). The anthocyanin content was very less in all the 3 varieties and ranged from 0.0171 mg cyanidin-3-glucoside/g to 0.0876 mg cyanidin-3-glucoside/g. As compared to their respective control plants, all 3 varieties showed an increase in the anthocyanin content in the treated plants (Fig 4.17). IR64 control showed the highest anthocyanin content 0.0667 mg cyanidin-3-glucoside/g while the Jaya and Korgut control showed 0.0551 mg cyanidin-3-glucoside/g and 0.0171 mg cyanidin-3-glucoside/g, respectively. In the treated plants,

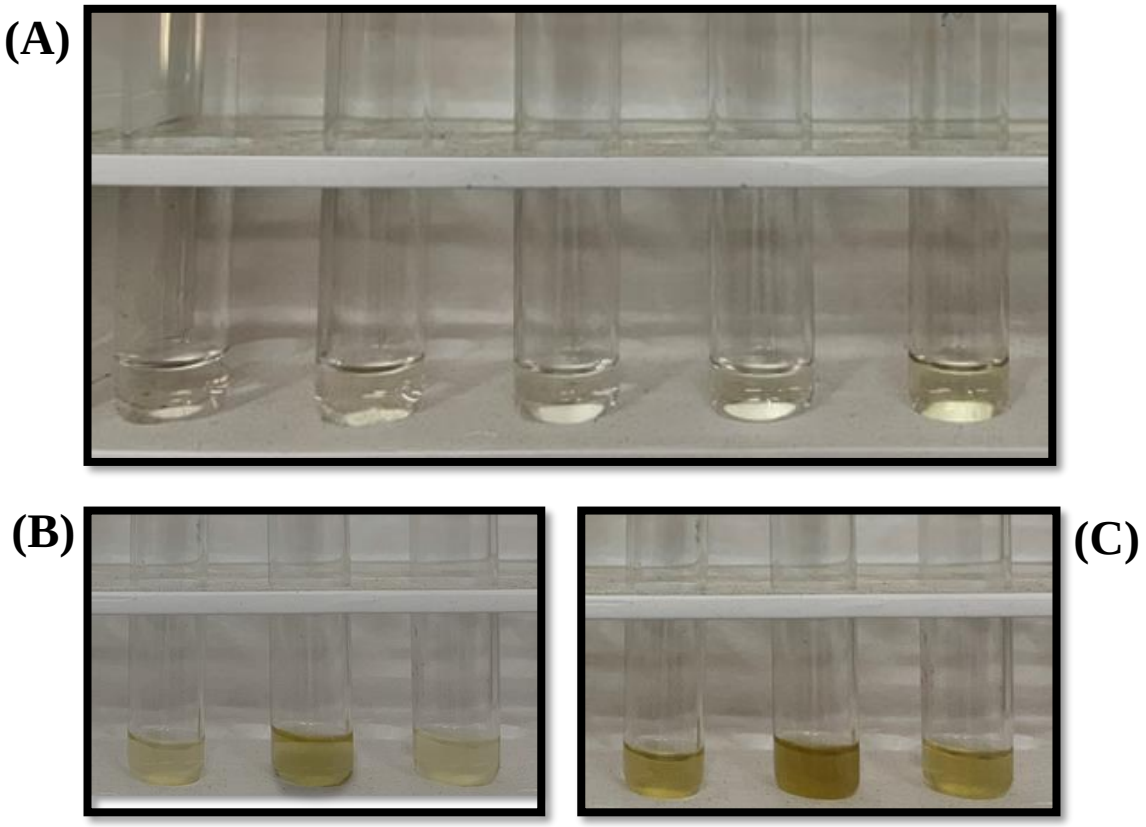


Fig 4.11: Determination of Total Flavonoid Content (TFC) of **(A)** Quercetin standard **(B)** Methanolic extracts of control seedlings **(C)** Methanolic extracts of 24h 300mM NaCl treated seedlings

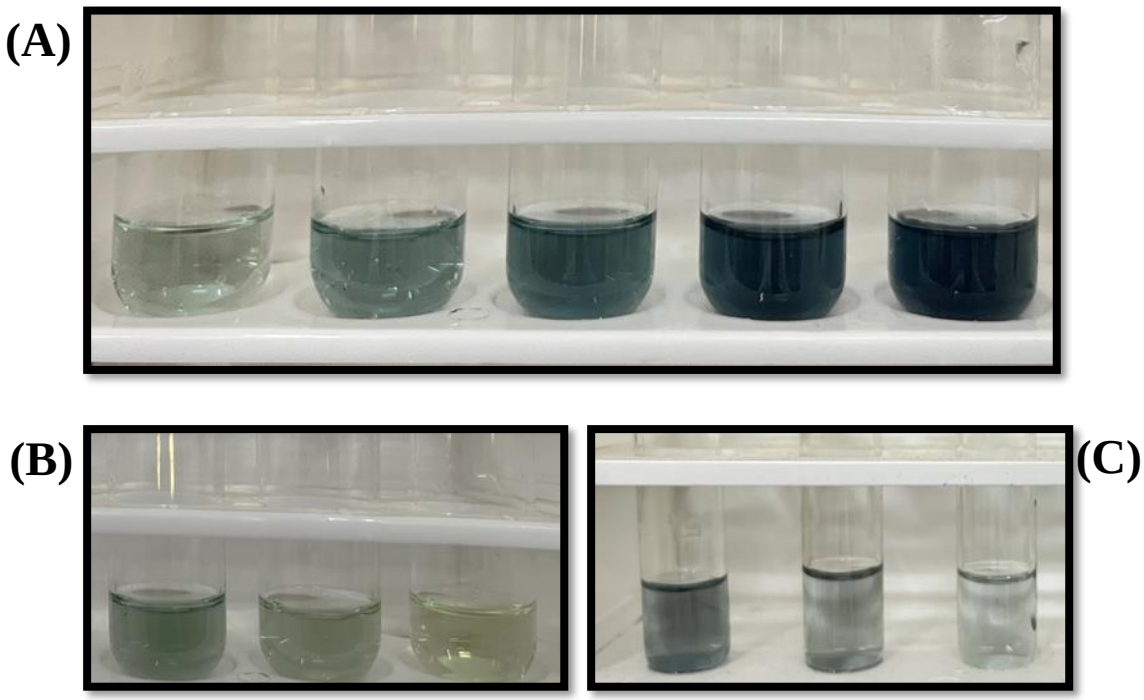


Fig 4.12: Determination of Total Phenolic Content (TPC) of **(A)** Gallic acid standard **(B)** Extracts of control seedlings **(C)** Extracts of 24h 300mM NaCl treated seedlings

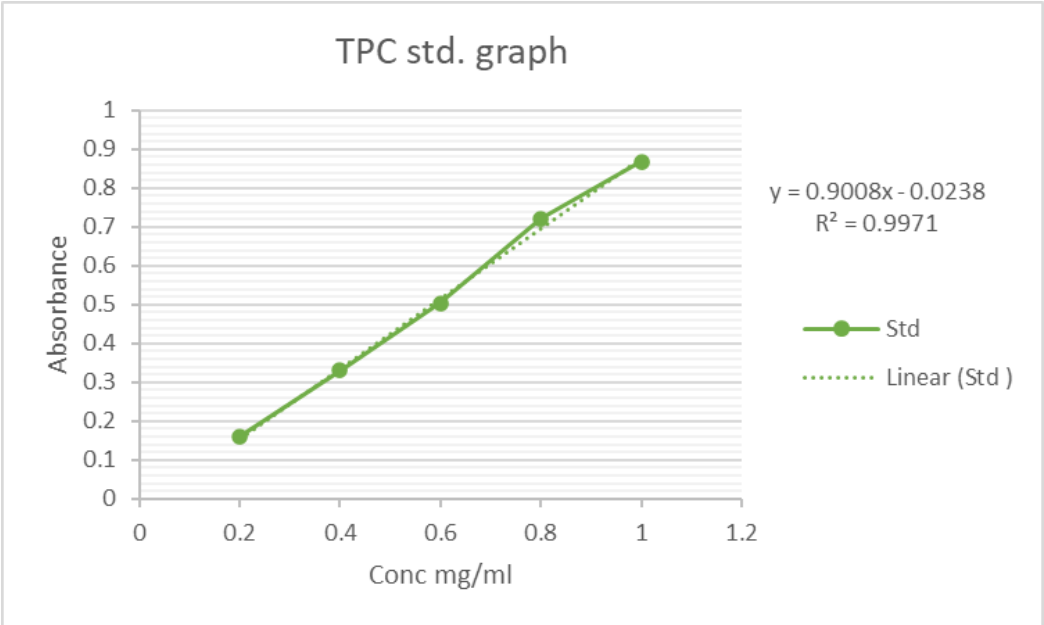


Fig 4.13: Standard graph for Total Phenolic content (TPC)

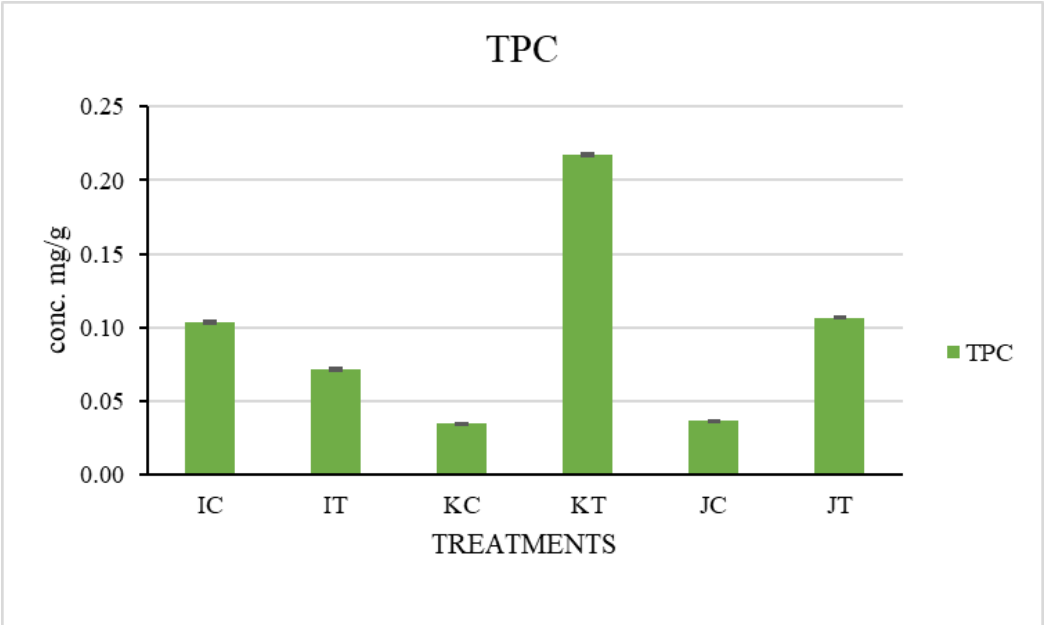


Fig 4.14: Relative accumulation of total phenolic content (TPC) in control and salt-treated rice varieties

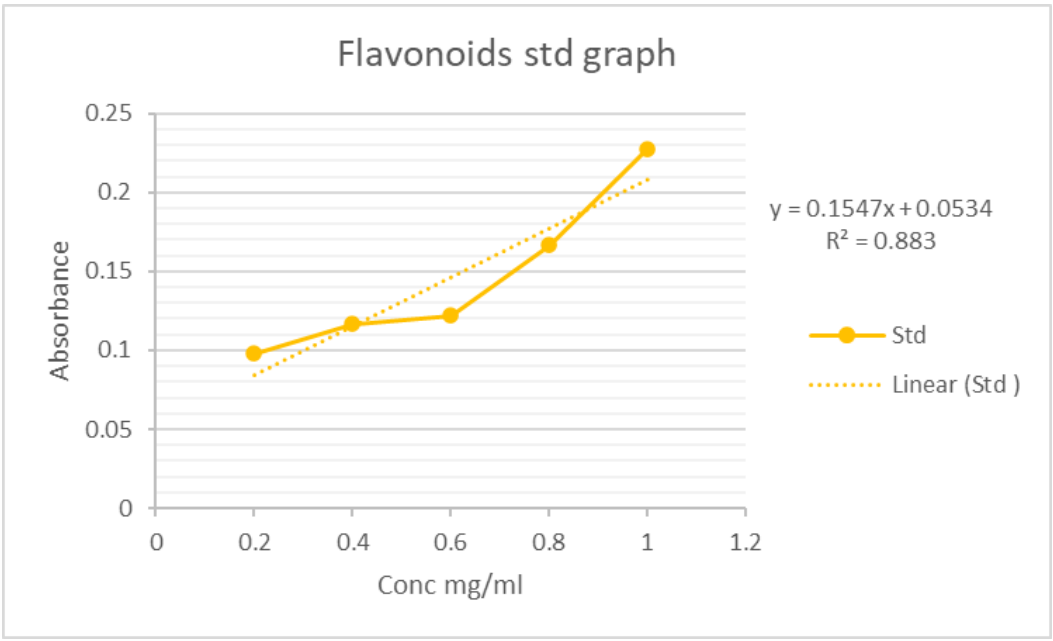


Fig 4.15: Standard graph for Total Flavonoid content (TFC)

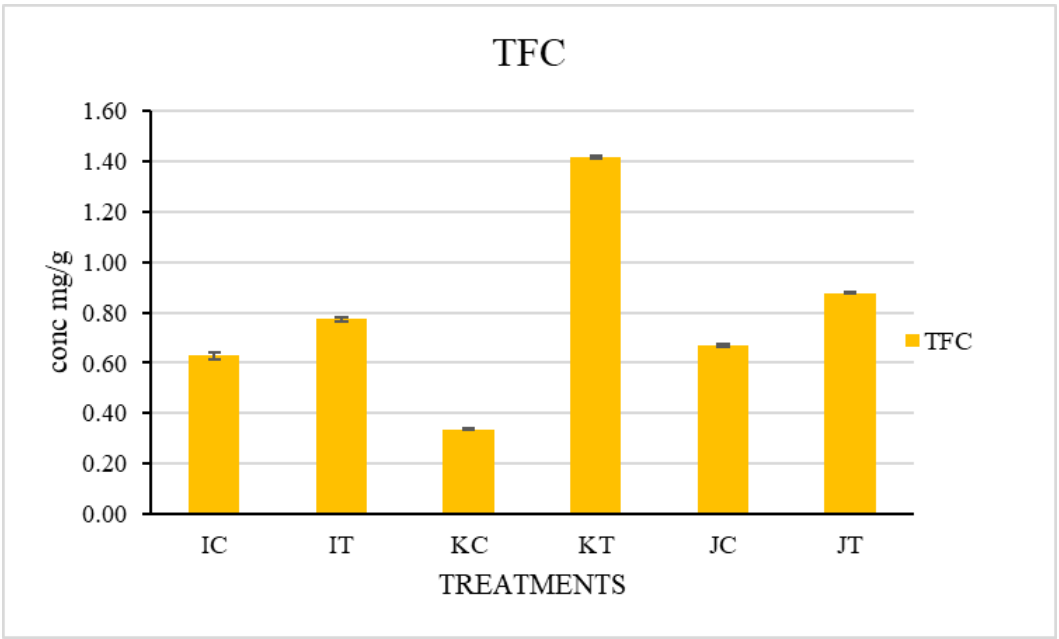


Fig 4.16: Relative accumulation of total flavonoid content (TFC) in control and salt-treated rice varieties

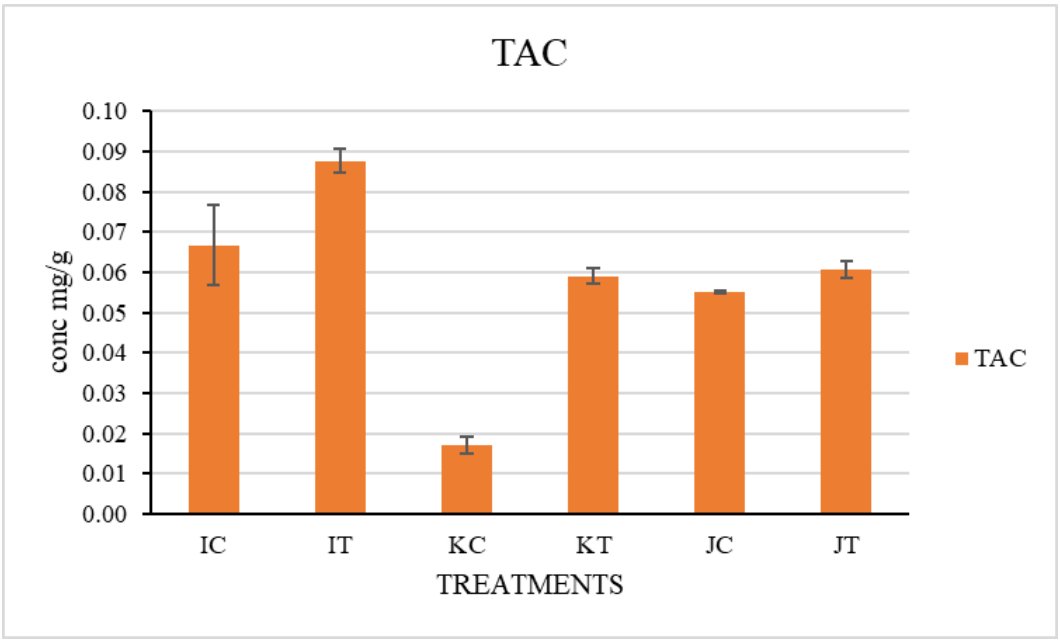


Fig 4.17: Relative accumulation of anthocyanin content in control and salt-treated rice varieties

the anthocyanin content was highest in IR64 0.0876 mg cyanidin-3-glucoside/g and least in Korgut 0.0591 mg cyanidin-3-glucoside/g.

4.3 Gene expression analysis of secondary metabolite biosynthetic pathway genes in rice

In order to understand the role of secondary metabolites in tolerance towards salinity stress, it was important to understand their biosynthesis at molecular level. Hence the genes encoding biosynthetic enzymes of secondary metabolite biosynthetic pathway was analysed in Korgut, Jaya and IR64 rice varieties.

For this the total RNA was isolated from the three varieties that were treated with 300mM of NaCl solution for 24hours. Analysis of total RNA from the 3 rice varieties on agarose gel electrophoresis (shown in Fig 4.18) revealed that the ribosomal RNA bands of 28S, 18S and 5.8S were intact and intense, demonstrating that the total RNA extracted was not degraded. The ratios of A260/A280 were close to or above 1.8 for all the RNA samples extracted (Table 4.2). This demonstrated that the RNA extracted was free from protein contaminants, organic solvents, and other plant products. The results of sq-RTPCR did not show distinct bands corresponding to the ladder bands in agarose gel electrophoresis as seen in Fig 4.19. Thus, qRT-PCR analysis was performed to determine the gene expression of the flavonoid biosynthesis genes.

4.4. Expression of flavonoid biosynthesis genes in rice leaves

Since flavonoids are widely distributed in rice, we selected important genes of flavonoid biosynthetic pathway for the gene expression study. Naringeninchalcone produced by CHS is further catalysed by CHI to produce naringenin that act as a substrate for the production

Table 4.1: Phytochemical properties for control and salt-treated rice varieties

Varieties	TPC (mg GAE/g)	TFC (mg quercetin/g)	Anthocyanin content (mg cyanidin-3-glucoside/g)
IR64 control	0.1035 ± 0.0010	0.6276 ± 0.015	0.0667 ± 0.01
IR64 treated	0.0714 ± 0.0010	0.7730 ± 0.008	0.0876 ± 0.003
Korgut control	0.0348 ± 0.0004	0.3354 ± 0.002	0.0171 ± 0.002
Korgut treated	0.2171 ± 0.0010	1.4176 ± 0.007	0.0591 ± 0.002
Jaya control	0.0364 ± 0.002	0.6695 ± 0.004	0.0551 ± 0.0003
Jaya treated	0.1068 ± 0.003	0.8782 ± 0.001	0.0607 ± 0.002

Table 4.2: A260/A280 ratios of RNA extracted from the control and salt-treated rice leaves

Sample	A260/A280
Korgut control	1.52
Korgut treated	1.67
Jaya control	1.85
Jaya treated	1.82
IR64 control	1.44
IR64 treated	2.36

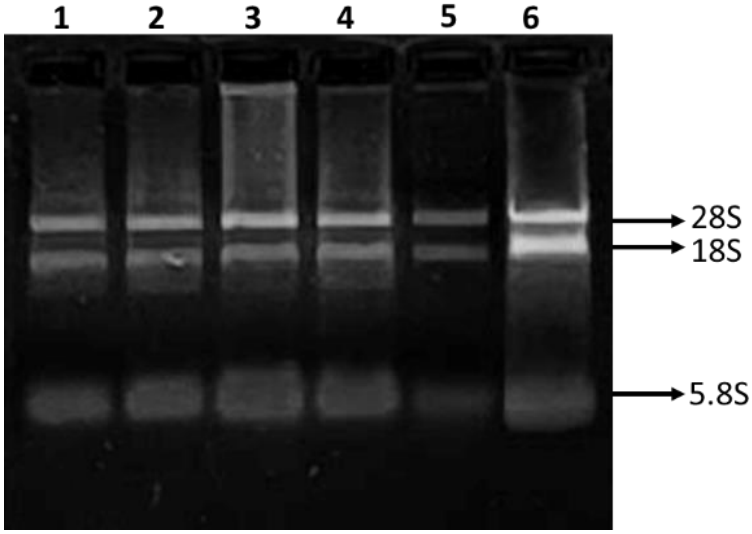


Fig 4.18: Agarose gel electrophoresis of total RNA from rice cultivars. 1: Korgut control; 2: Korgut treated; 3: Jaya control; 4: Jaya treated; 5: IR64 control; 6: IR64 treated

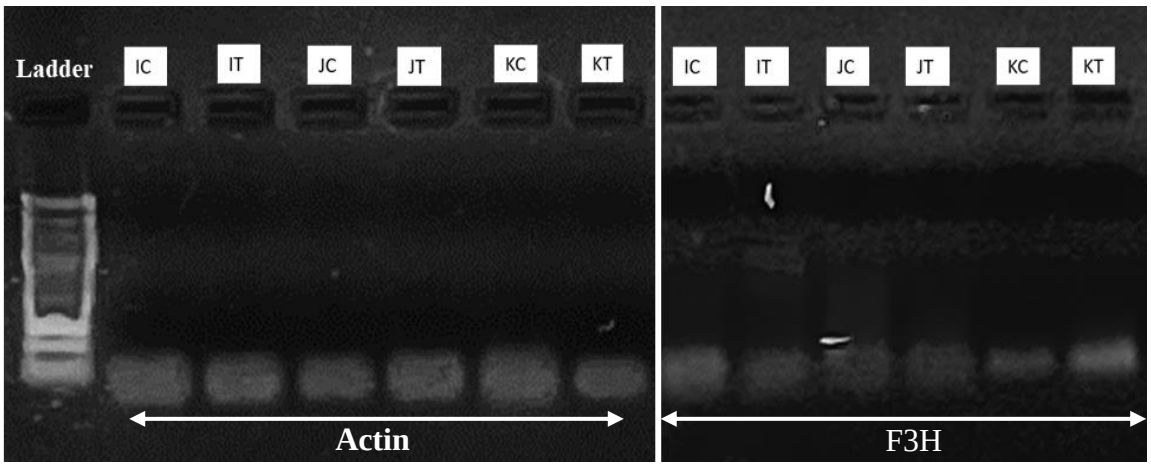


Fig 4.19: Agarose gel electrophoresis of sq-RTPCR, showing expression of actin gene and F3H gene

of various flavonoids. *NOMT, FNS2, F2H, F3H* and *LAR* genes which encode for the enzymes involved in converting the Naringenin to Sakuranetin, Apigenin, 2-hydroxyfalvanone, Dihydrokaempferol, and Catechin respectively were selected for the study. The upregulation or downregulation of these genes will give us the idea whether which specific pathway in flavonoid biosynthesis is activated in response to salt stress in Korgut rice as compared to IR64. The expression level of CHS, NOMT, FNS2, F2H, F3H and LAR genes involved in flavonoids biosynthesis were analysed by qRT-PCR in rice leaves with and without salt treatment.

Firstly, it was studied whether these genes for flavonoid biosynthetic enzymes are either differentially expressed in all the three varieties in salinity stress. In Korgut it was observed that upon NaCl treatment the expression of LAR, NOMT, F3H was increased in salt treatment. In the salt-tolerant Korgut variety, the gene expression of F3H and LAR was the highest after subjecting the plants to 24h salt treatment (Fig. 4.20.A). F3H gene responsible for converting eriodictyol to dihydroquercetin, exhibited 4.65 times increase in gene expression after 24h salt stress. Similarly, the gene expression of LAR increased 4-folds after 24h salt treatment. This was followed by the NOMT gene responsible for producing the sakuranetin metabolite which acts as an important phytoalexin in rice. The NOMT gene expression increased 2-folds as compared to the control plants. However, the gene expression of CHS, F2H and FNSII did not show a considerable increased in salt treated Korgut plants.

Gene expression analysis in Jaya variety as seen in Fig. 4.20.B showed that on 24h salt-treatment, LAR gene responsible for producing catechin the precursor for pro-anthocyanidin showed a 2-fold increase. This was followed by 1.98 times increase in gene

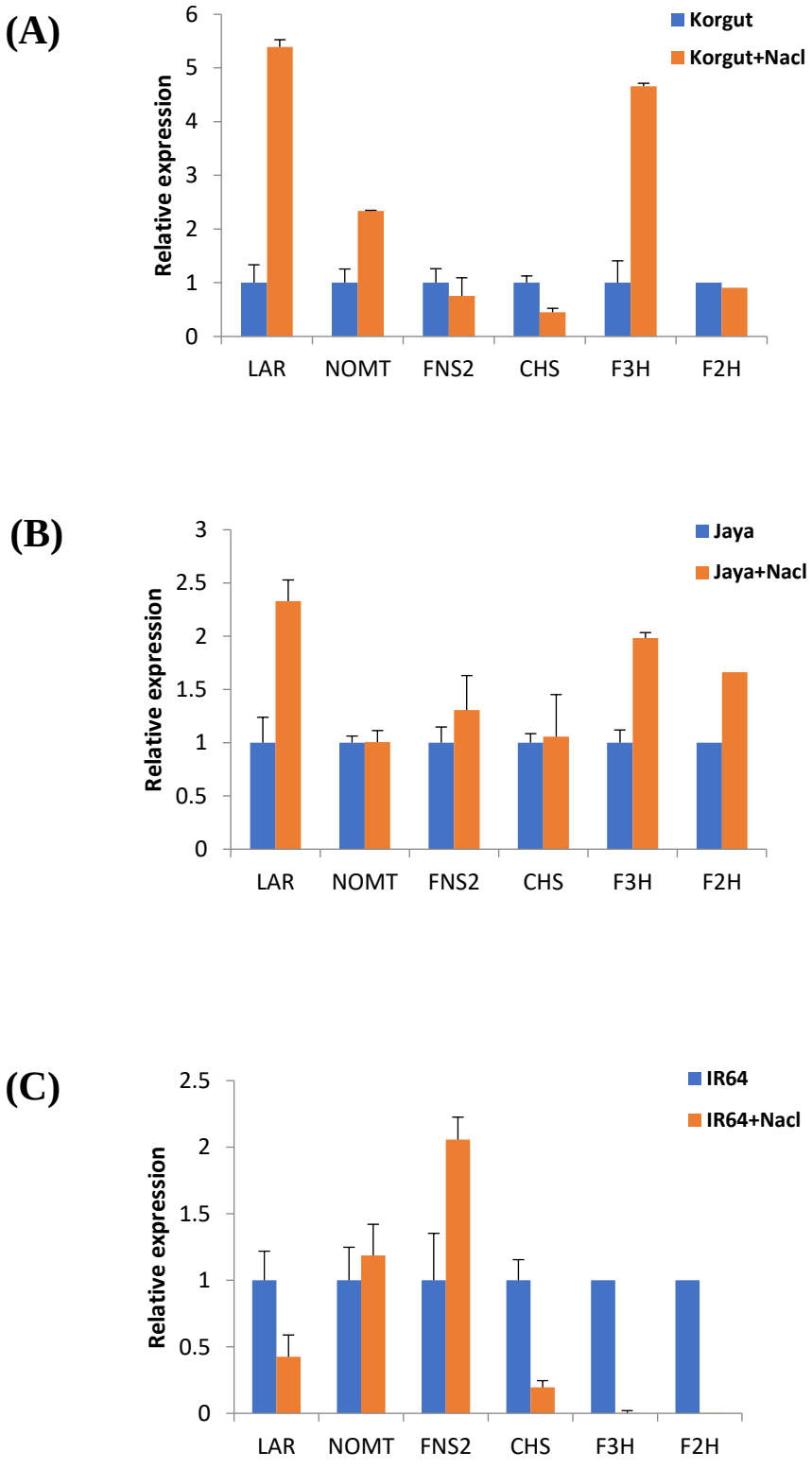


Fig 4.20: Relative expression of flavonoid-biosynthesis genes in rice leaves under control and 24hr salt stress. Transcript levels are indicated as relative to those from rice actin gene. (A) Korgut; (B) Jaya; (C) IR64

expression of F3H in salt-treated Jaya plants. F2H and FNS2 are competing enzymes using the same flavanone substrates. The qRT-PCR results for these genes showed 1.66 times and 1.3 times increase after 24h salt treatment respectively. CHS and NOMT genes did not show considerable increase in gene expression on 24h salt treatment in this rice cultivar.

The data in Fig. 4.20.C indicates that IR64, a salt-sensitive variety showed least gene expression on 24h salinity stress as compared to the other two varieties. The gene expression of LAR and CHS showed a considerable decrease (0.42 and 0.19 times) in the salt-treated plants as compared to the control plants. F3H and F2H genes did not express in the 24h salt-treated plants. NOMT did not show a considerable increase in gene expression as compared to the control plants. However, the expression of FNS2 gene increased 2-folds when subjected to salt stress.

The relative expression of these genes in the Goan cultivars Korgut and Jaya was compared with the salt-sensitive IR64 variety as seen in Fig. 4.21. In the absence of salt stress (Fig.4.21. C), it was observed that the gene expression of LAR and CHS gene in the Korgut and Jaya varieties was lower than the IR64 variety. Relatively lower transcript amounts were detected for F3H and F2H in Korgut and Jaya. For the NOMT gene, the expression was 6 times more in Korgut and 5.85 times more in Jaya as compared to the IR64 variety. Similar trend was observed for FNS2 gene expression (Fig.4.21. A); the increase was superior in Korgut (6 times of the IR64 variety) compared to Jaya (3.37 times of the IR64 variety).

Results of the analysis of flavonoid biosynthesis gene expression in 24h salt-treated Korgut and Jaya plants relative to the expression in IR64 treated plants using qRT-PCR is shown in Fig 4.21.B. Expression of F2H and F3H was enhanced dramatically (36-fold and 13-

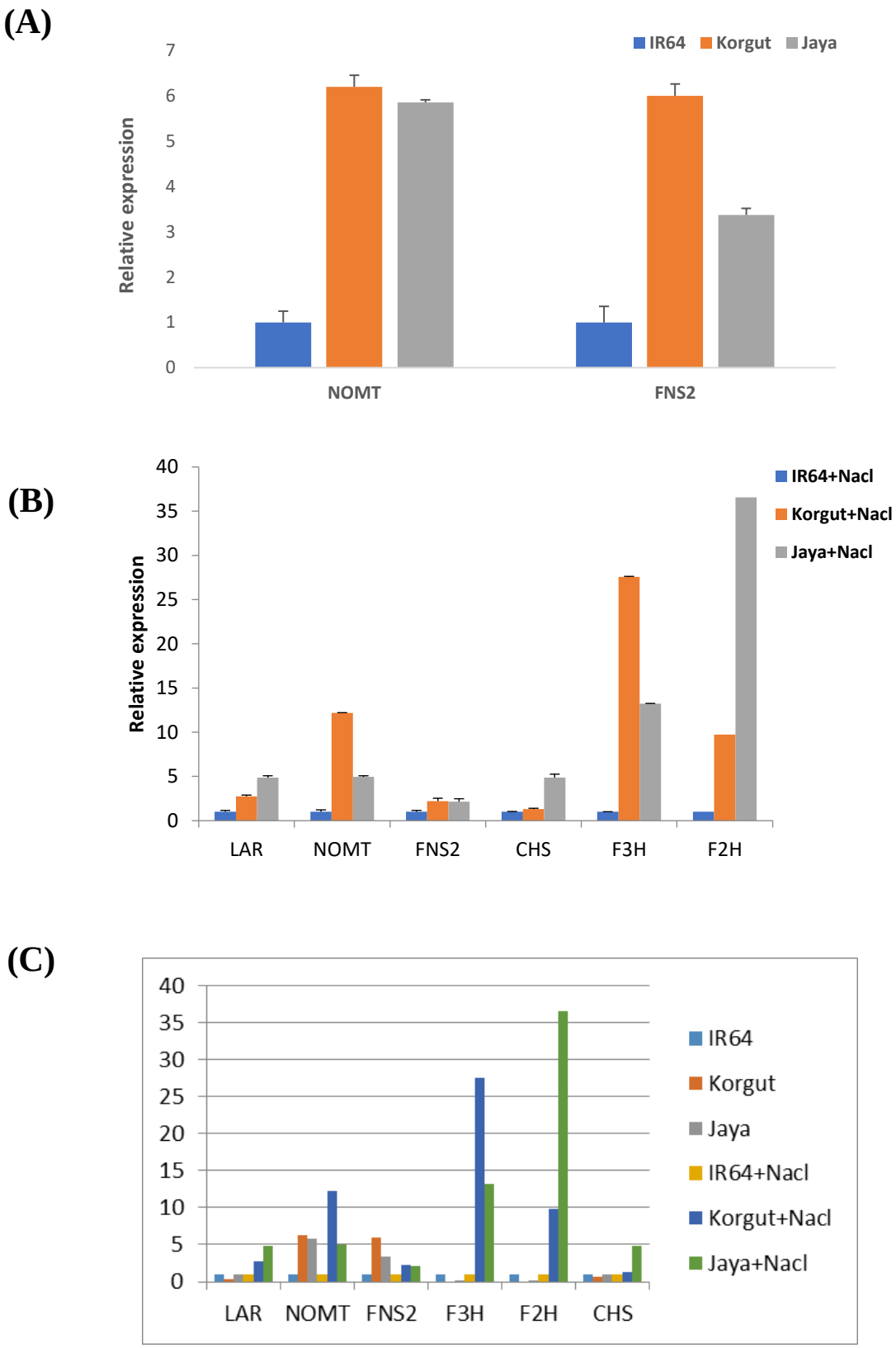


Fig 4.21: Relative expression of flavonoid-biosynthesis genes in the three rice varieties Transcript levels are indicated as relative to those of IR64 flavonoid biosynthesis gene expression. (A) Upregulated genes in untreated Korgut and Jaya rice plants as compared to IR64; (B) Upregulated genes in 300mM NaCl treated Korgut and Jaya rice plants as compared to IR64 plants; (C) Differential expression of genes in untreated and 300mM NaCl treated rice plants.

fold, respectively) in Jaya variety as compared to IR64 variety under salt-stress. Salt-treated Jaya variety also showed an enhanced gene expression of LAR, NOMT and CHS (about 4 times more) and FNS2 (2 times more) as compared to salt-treated IR64.

The expression level of F3H, NOMT and F2H genes in the salt-tolerant Korgut variety when compared to the salt-sensitive IR64 variety showed significant increase (27-fold, 12-fold and 9-fold, respectively) after 24h treatment. The expression of LAR and FNS2 in salt-treated Korgut was twice than that of salt-treated IR64 plants. CHS, responsible to catalyse the first

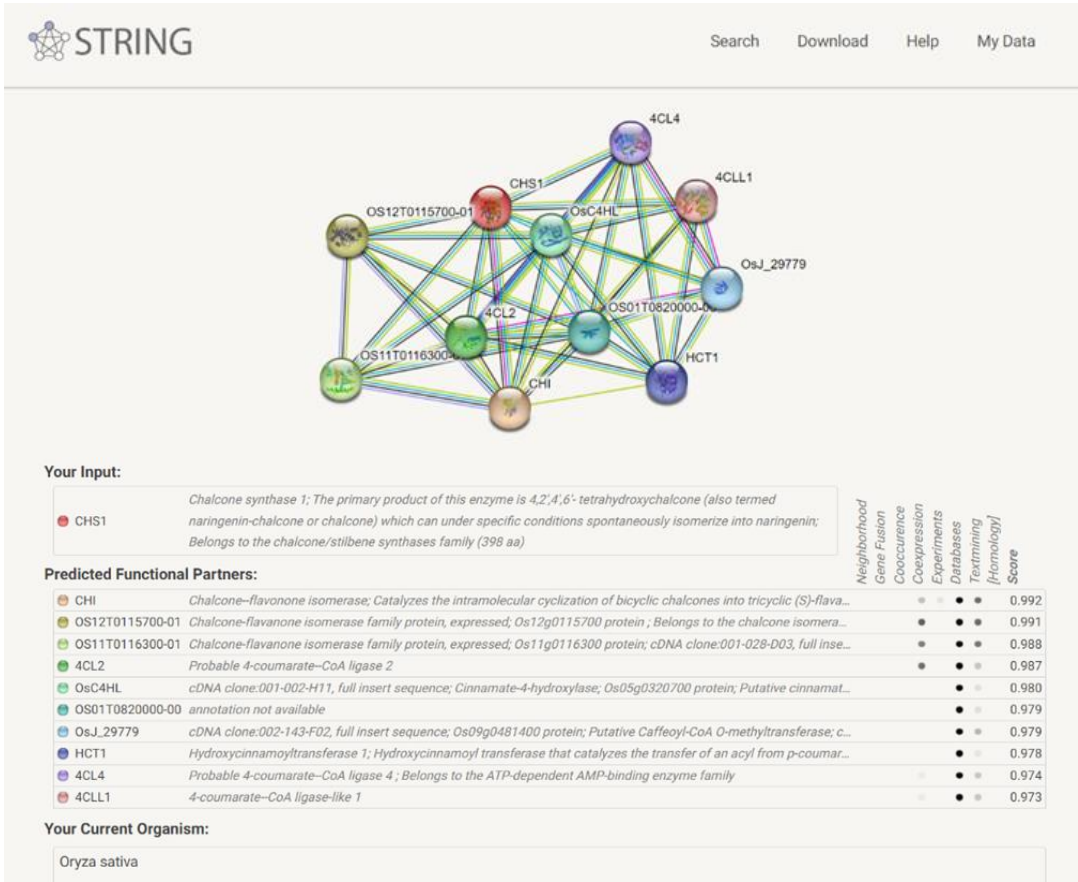
committed step of flavonoid pathway showed no difference in expression in Korgut and IR64on 24h salt stress.

4.5 *In-silico* analysis

The gene expression analysis gave us idea about the regulation of secondary metabolites biosynthetic genes under salt stress. The further question was which possible transcription factor is regulating the expression of these genes encoding enzymes of secondary metabolites pathway. Hence *in silico* analysis (Fig.4.22-4.27) was performed to check if the genes of the flavonoid biosynthesis pathway are activated by any of the transcription factors involved in mitigating salt stress. It was observed that some of the important transcription factors like DREB2A which is very well studied to have role in mitigating abiotic stresses like drought and salt stress were found to involved in regulating the expression. Table 4.3 shows the list of the transcription factors interacting with the genes used in our study. It was interesting to note that DREB2A interacted with CHS and F2H. LBD interacted with NOMT and F3H. Transcription factors (TF) of the ERF family

interacted with FNSII, F3H, F2H and LAR. TFs of the C2H2 and MYB TF family interacted with F3H, F2H and LAR. The involvement of salt and drought responsive transcription factors in upregulating secondary metabolites biosynthetic genes suggest the crosstalk and the involvement of secondary metabolites secondary metabolites in alleviating the salt stress in rice. Particularly the indigenous salt tolerant rice varieties like Korgut elevates the production of secondary metabolites especially flavonoids under salt stress in order to help itself in coping up with the stress.

(A)



(B)

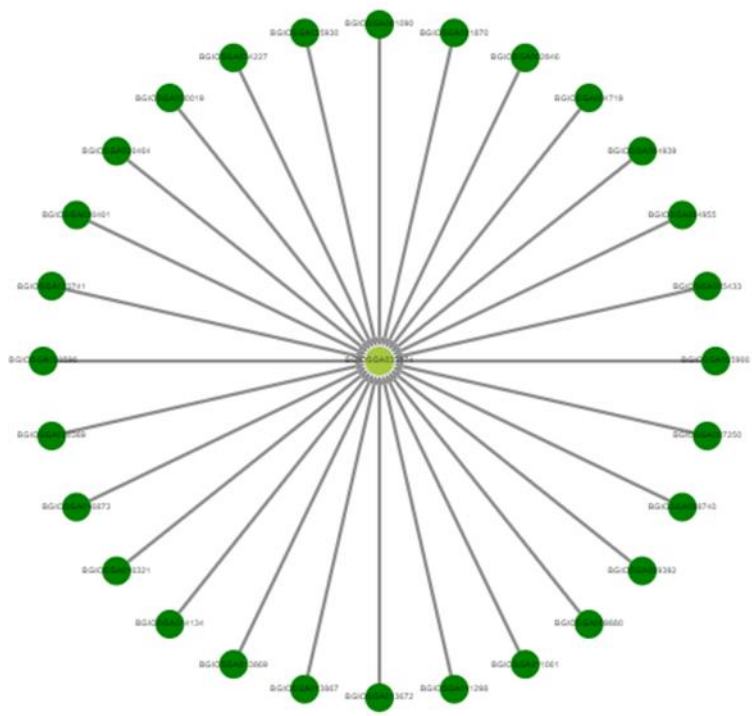
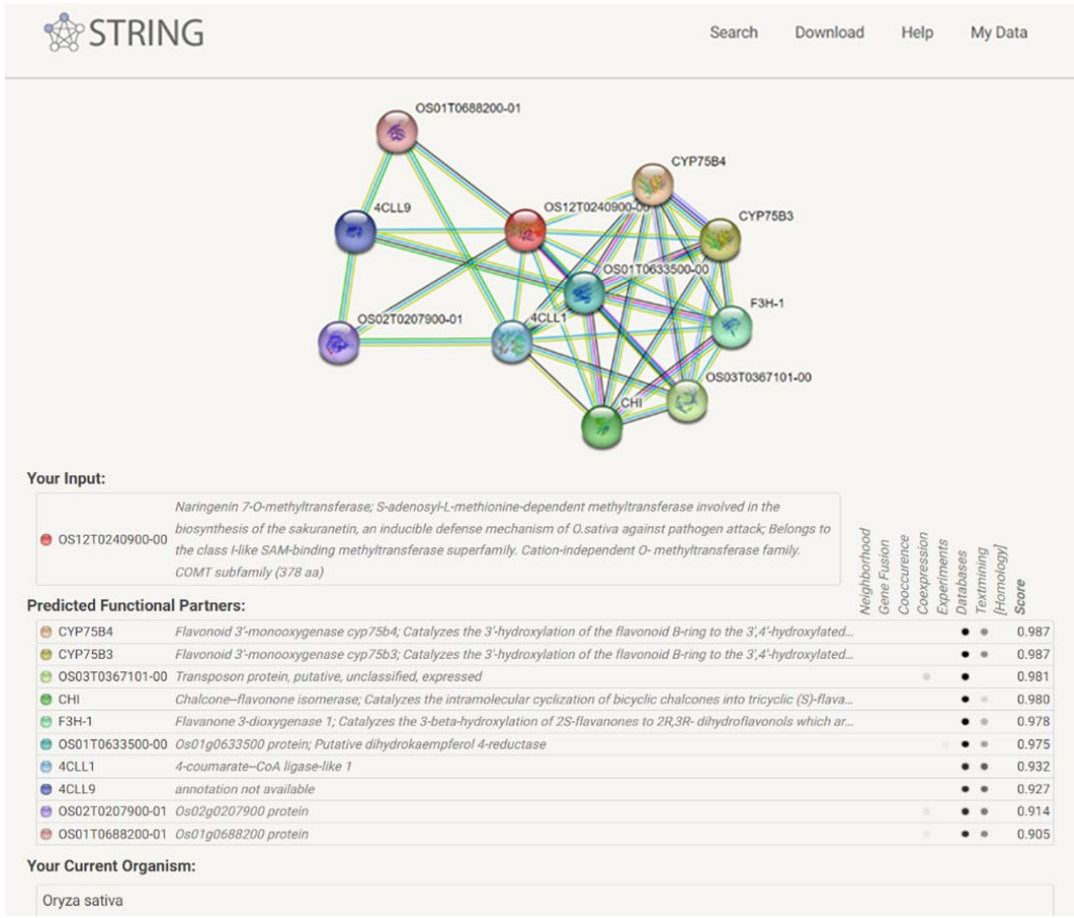


Fig 4.22: Protein-Protein and Protein DNA interaction of CHS: (A) Suggest the proteins interacting with CHS enzyme, (B) suggest transcription factors interacting with *CHS* gene promoter

(A)



(B)

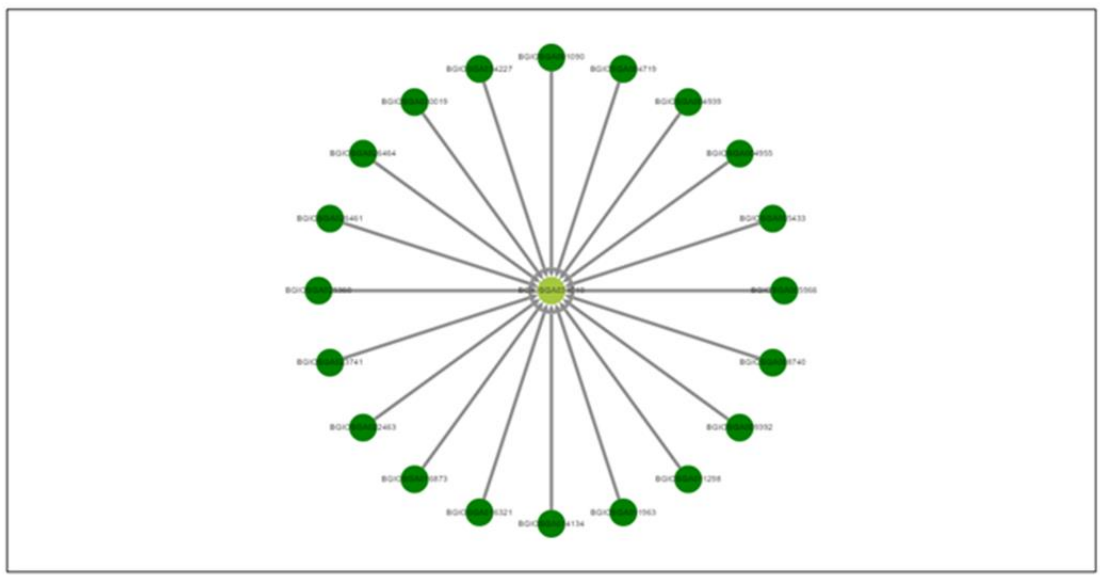
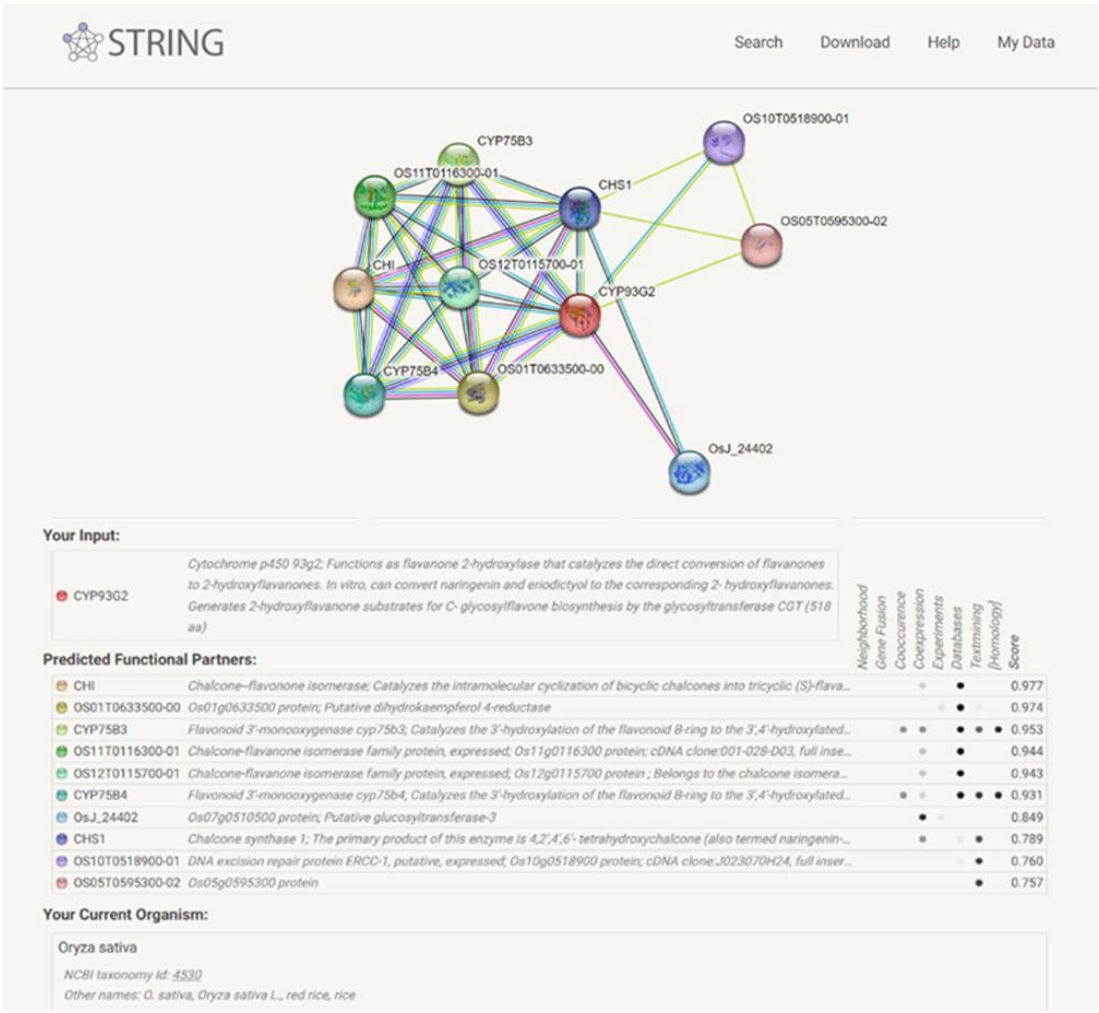


Fig 4.23: Protein-Protein and Protein DNA interaction of NOMT: (A) Suggest the proteins interacting with NOMT enzyme, (B) suggest transcription factors interacting with *NOMT* gene promoter

(A)



(B)

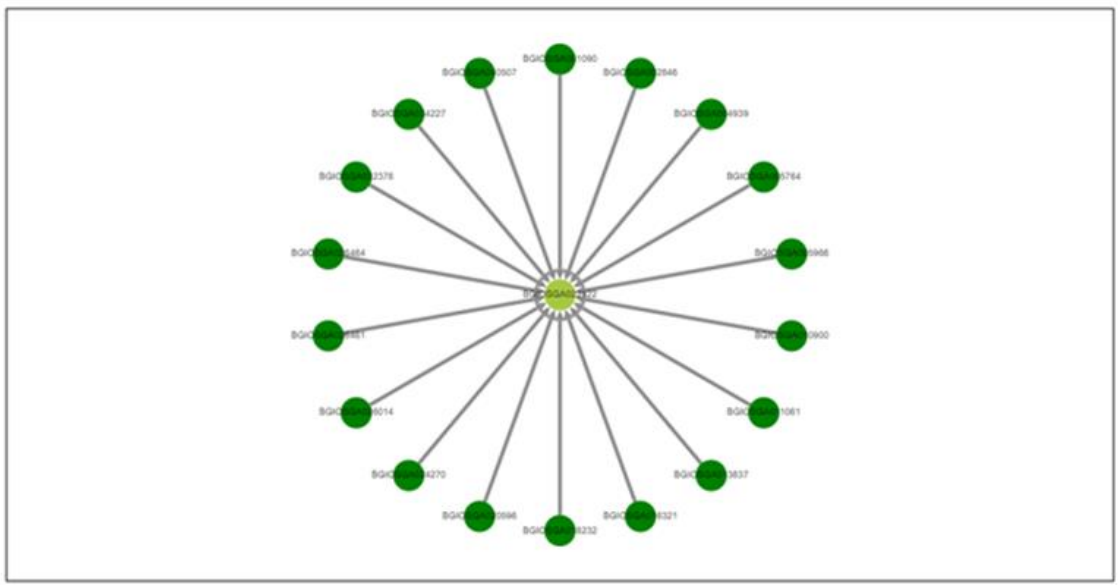
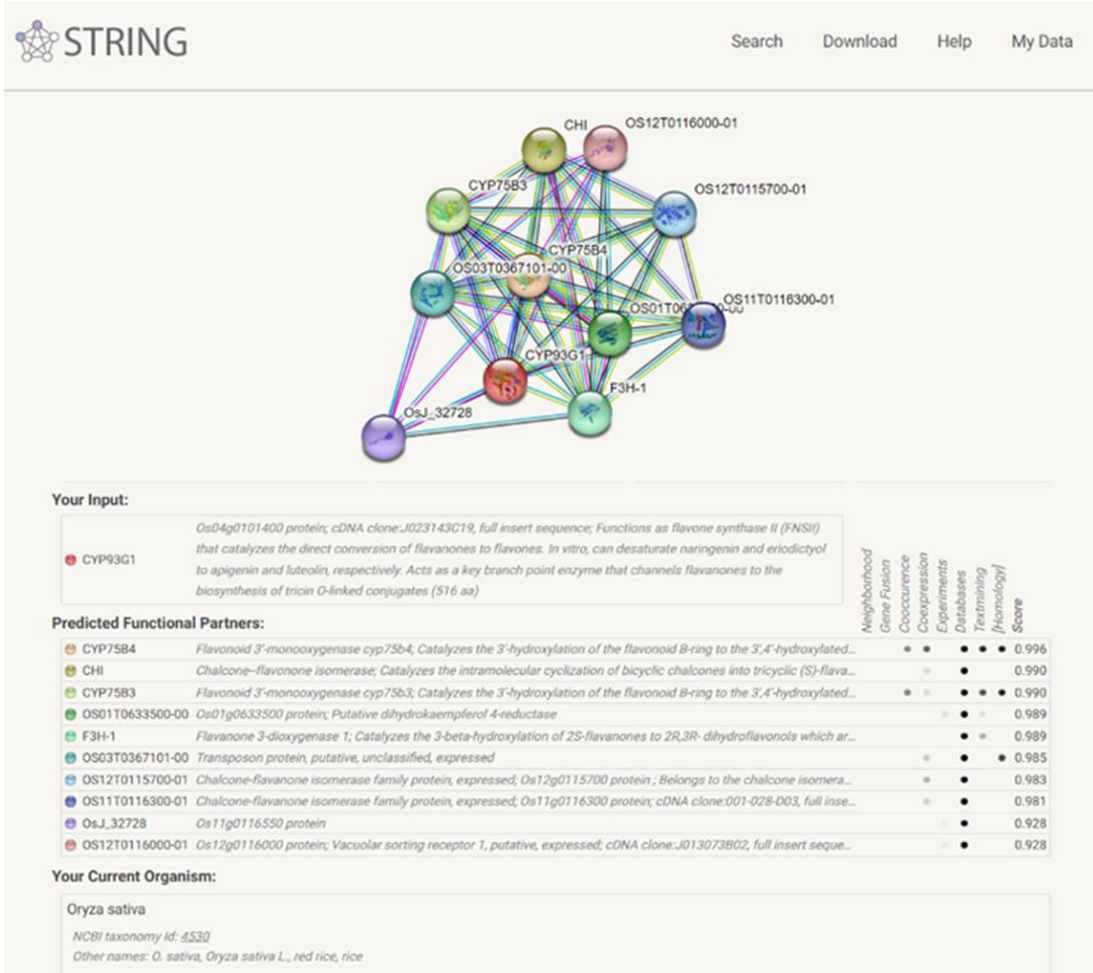


Fig 4.24: Protein-Protein and Protein DNA interaction of F2H: (A) Suggest the proteins interacting with F2H enzyme, (B) suggest transcription factors interacting with *F2H* gene promoter

(A)



(B)

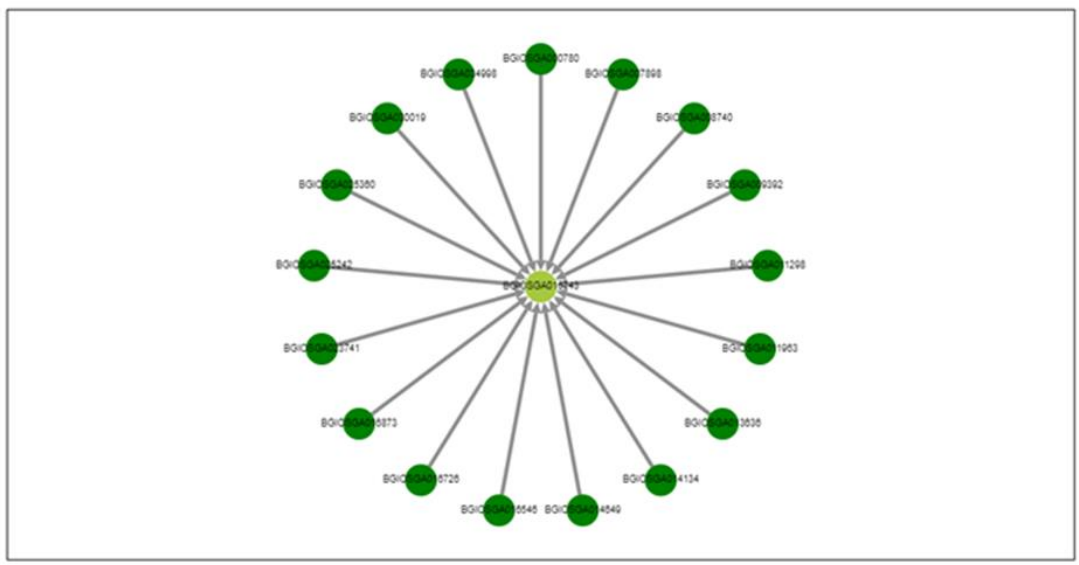
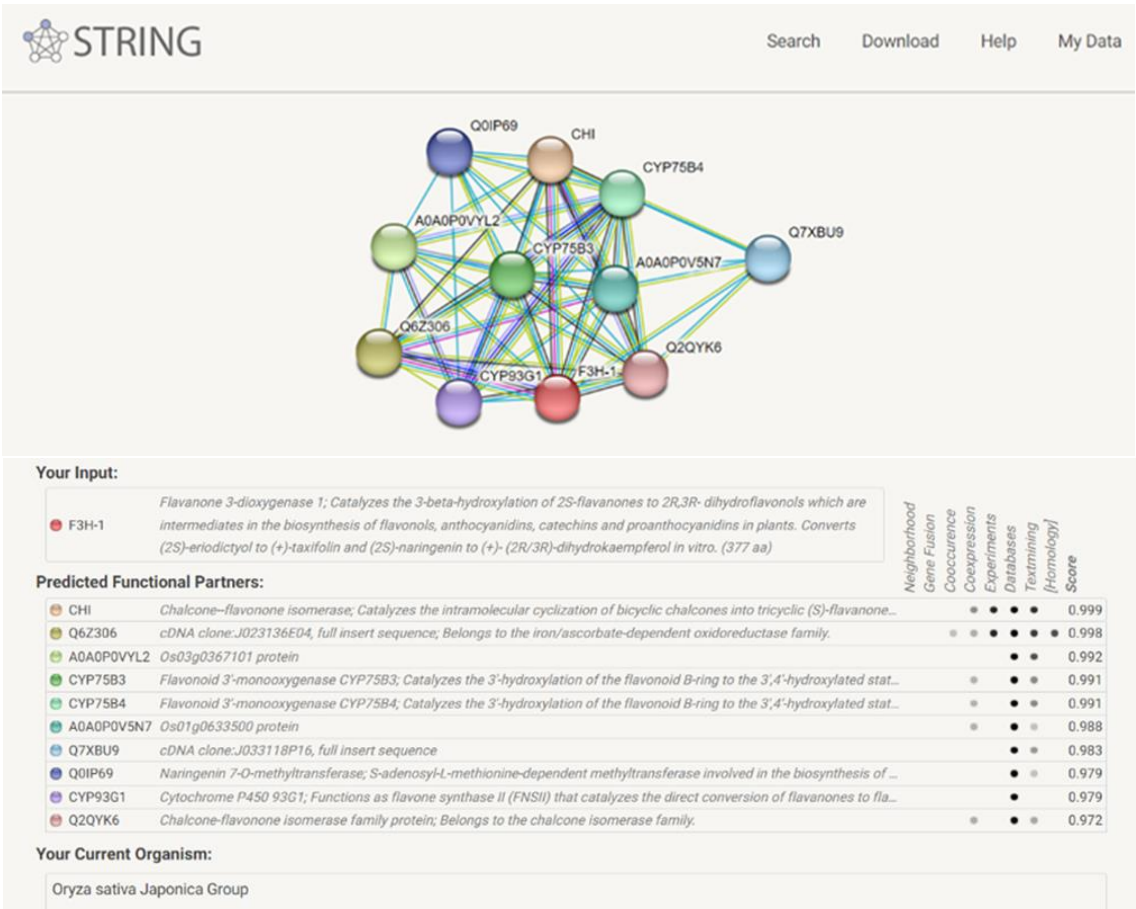


Fig 4.25: Protein-Protein and Protein DNA interaction network of FNSII: (A) Suggest the proteins interacting with FNSII enzyme, (B) suggest transcription factors interacting with *FNSII* gene promoter

(A)



(B)

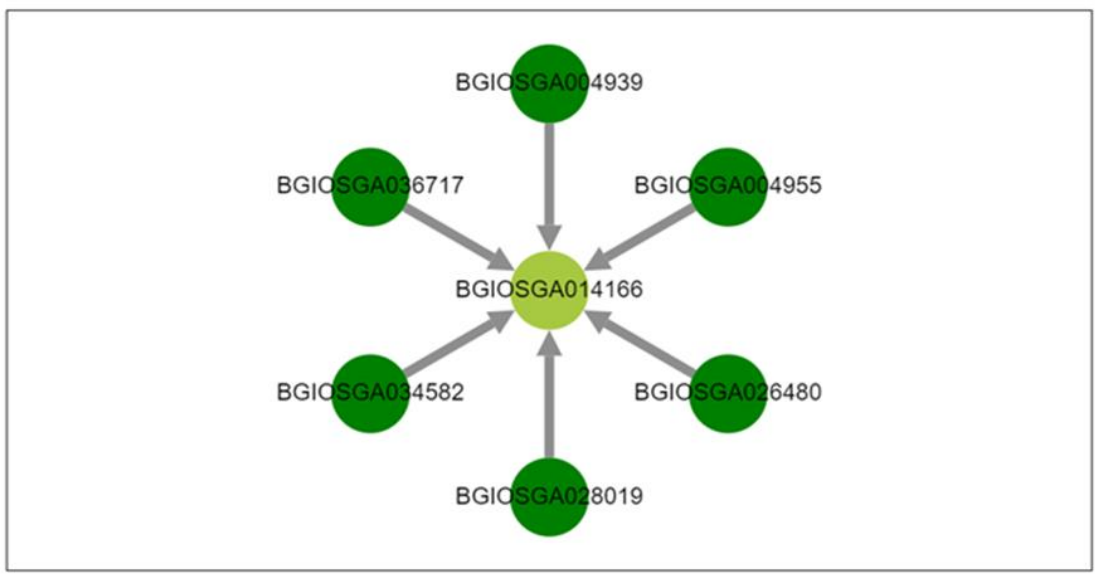
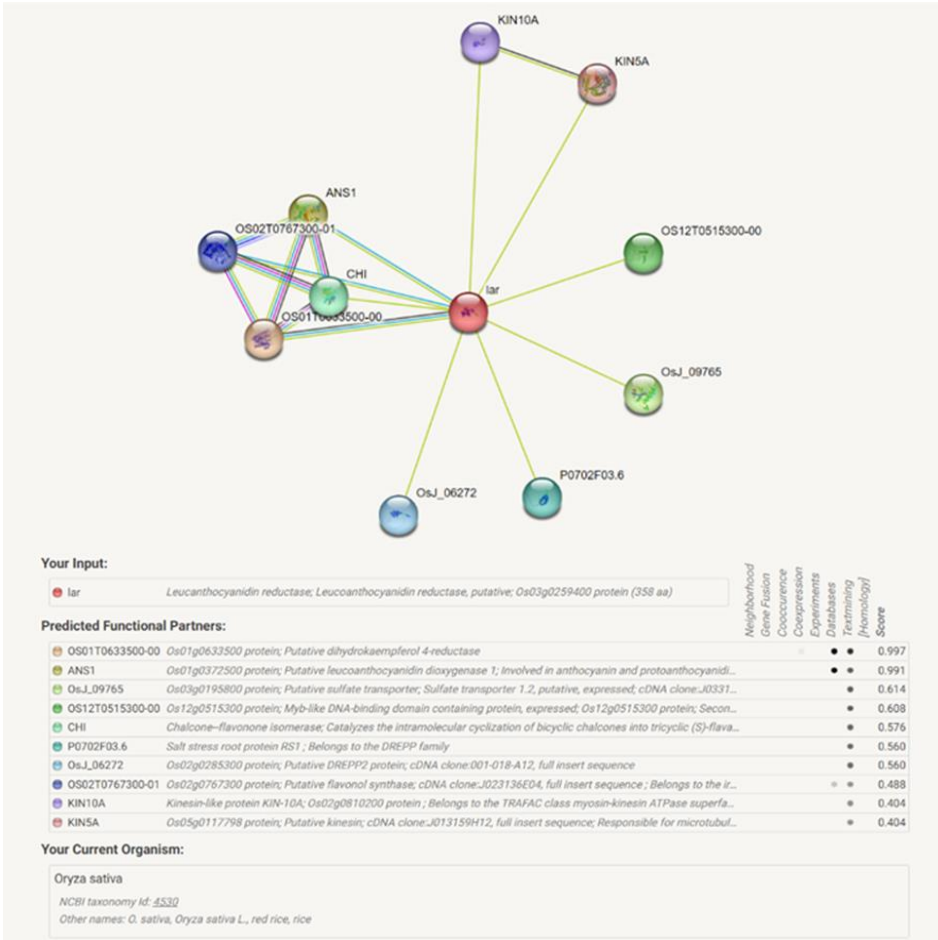


Fig 4.26: Protein-Protein and Protein DNA interaction network of F3H: (A) Suggest the proteins interacting with F3H enzyme, (B) suggest transcription factors interacting with *F3H* gene promoter

(A)



(B)

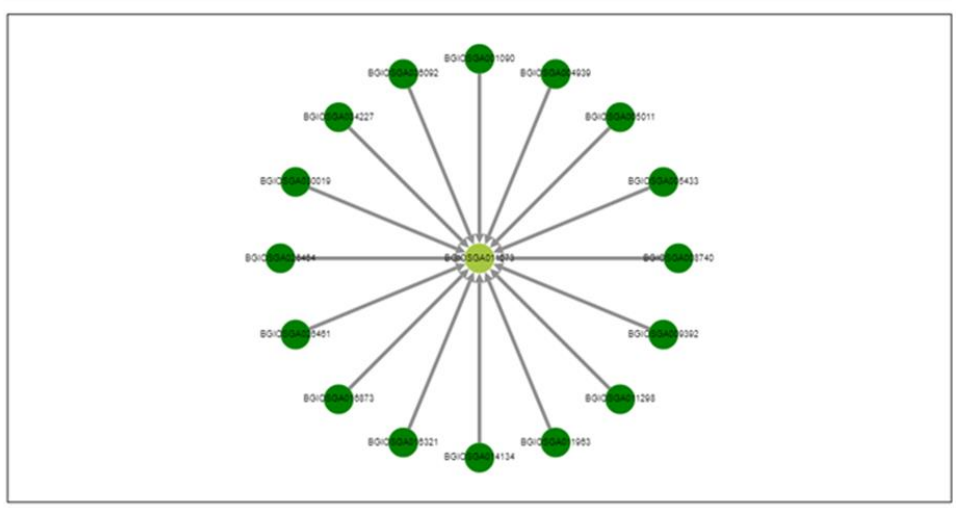


Fig 4.27: Protein-Protein and Protein DNA interaction network of LAR: (A) Suggest the proteins interacting with LAR enzyme, (B) suggest transcription factors interacting with *LAR* gene promoter

Table 4.3: *In silico* analysis showing Transcription Factors (TF) binding to the promoters of secondary metabolite biosynthetic genes

Target	TF	TF Family
CHS	DREB2A	ERF
NOMT	LBD6	LBD
FNSII		CPP
FNSII		NAC
FNSII		ERF
F3H	BGIOSGA004939-PA	ERF
F3H	LBD6	LBD
F3H	OsI_30289	C2H2
F3H	BGIOSGA028019-PA	MYB-related
F3H	BGIOSGA034582-PA	MYB
F3H	BGIOSGA036717-PA	MYB
LAR	BGIOSGA001090-PA	ERF
LAR	BGIOSGA004939-PA	ERF
LAR	BGIOSGA005433-PA	ERF
LAR	BGIOSGA008740-PA	ERF
LAR	BGIOSGA036092-PA	MYB
LAR	BGIOSGA005011-PA	C2H2
F2H	BGIOSGA001090-PA	ERF
F2H	DREB2A	ERF
F2H	BGIOSGA005764-PA	DOF
F2H	BGIOSGA010900-PA	MYB
F2H	BGIOSGA020596-PA	GATA
F2H	BGIOSGA026014-PA	HD-ZIP
F2H	BGIOSGA032378-PA	BBR-BPC

DISCUSSION

5. Discussion

Histochemistry is the branch of histology that deals with the identification and distribution of chemical compounds within and between biological cells, using stains, indicators and light and electron microscopy. Histochemistry can provide valuable support to downstream the chemical analyses of secondary metabolism in plants, by localising, at the cellular/tissue level, the sites of production/accumulation of secondary metabolites. Hence in the present study attempt was done to identify secondary metabolites present in rice plant through histochemical localization process. These secondary metabolites were selected as they play various physiological and ecological roles (i.e., antimicrobial, insecticidal, growth regulatory, and allelopathic activities). They are also beneficial to humans, including cytotoxic, anti-tumor, anti-inflammatory, antioxidant, and neuroprotective properties (Wang et al., 2018). Biological activities of phenolic acids mostly include antioxidant activity and allelopathy. 2-hydroxy 5-[(3S)-3-hydroxybutyl] phenyl β-D-glucoside (HHPG) is known to have inhibitory activity on tunicamycin-induced retinal damage (Tanaka, J et al., 2013; Wang et al., 2018). Most of the alkaloids like N-Benzoyltryamine, N-Benzoylserotonin, N-Benzoyltryptamine found in the rice leaves have been reported to possess antimicrobial activity. Alkaloids are also known to possess antibacterial, allelopathic and antifungal activities (Morimoto, N et al., 2018; Thi, H.L et al., 2014; Wang et al., 2018). Tannins found in the plants are anticarcinogenic, antioxidant, antimutagenic and antimicrobial in action (Chung et al., 1998, Patale and Tank, 2021). Anthocyanins are mostly found in black rice. They act as antioxidants in plants due to their free radical scavenging capacity. (Hao, J., 2015; Wang et al., 2018). In humans, anthocyanins are used in remedies for liver disfunction, hypertension, vision

disorders, diarrhoea and other ailments. (Lila, 2004; Patale and Tank., 2021).Histochemical localisation of secondary metabolites such as alkaloids, phenolics and anthocyanins were localised in the transverse sections of the leaves. However, the results were inconclusive in this study hence this experiment can be repeated to obtain accurate results. Phytochemical analysis was performed to differentiate between the secondary metabolite content in the control and salt-treated varieties and to find out the coordination between the gene expression of secondary metabolite biosynthesis genes and accumulation of secondary metabolites.

The content of some phenolics may increase under stress conditions such as UV radiation, infection by pathogens and parasites, wounding, air pollution and exposure to extreme temperatures (L.M. Devi and L.S. Badwaik., 2022). In our study the total phenolic content in both Korgut and Jaya cultivars was observed to increase under salt stress. However, the TPC was higher in IR64 under controlled conditions whereas it decreased when subjected to salt-stress. This could be a possible reason for the salt-sensitivity of IR64 variety and enhanced tolerance of Korgut and Jaya variety.

The TFC was found to increase in all the salt treated plants as compared to that of the control plants. These results were similar to the findings of Liu et al., (2013), Yuan et al., (2012) and D. Ma et al., (2014). Salt-treated Korgut plants had the highest total flavonoid content followed by Jaya and IR64, indicating that the flavonoids have a protective role when plants are exposed to salinity stress. The increase in the TFC correlated with the enhanced gene expression of most of the genes in the flavonoid biosynthesis pathway. The protective role of flavonoids is due to the special structure of flavonoids, e.g., hydroxyl

groups, double carbon bonds and modifications like glycosylation, prenylation and methylation (Rice-Evans et al., 1997; D. Ma et al., 2014).

The anthocyanin content was found to be comparatively low than the TPC and TFC in these varieties. However, after salt treatment the anthocyanin content was found to be higher than the control plants which was consistent to the findings of D. Ma et al., (2014) and Liu et al., (2013).

Salt stress causes hyperosmotic stress and ionic stresses at the cellular level by increasing the toxic ions such as Na^+ and Cl^-. It is also known to enhance the abscisic acid (ABA) levels which is associated with the increased levels of Reactive Oxygen Species (ROS) and nitrogen oxide (NO) (Wim Van den Ende and El-Esawe, 2014). Flavonoids are a major class of secondary metabolites involved in mitigating such free radicals, thereby preventing the oxidative damage and protecting the plant (Xu et al., 2020). The flavonoid biosynthetic pathway and the key genes involved in this pathway have been investigated in response to environment stress (Lenka et al., 2011; Vasquez-Robinet et al., 2008; D. Ma et al., 2014).

CHS catalyses the first step for the biosynthesis of flavonoids. Lijuan et al., 2015 demonstrated that CHS gene is involved in regulating plant adaptation to salt stress by maintaining ROS homeostasis. In our experiment, it has been found that CHS gene expression was upregulated in Korgut and Jaya cultivars under salt stress, compared to the IR64 salt-sensitive variety. However, this gene was down regulated under salt-stress in Korgut and IR64 as compared to their controls.

NOMT is a key enzyme in the biosynthesis of the flavonoid phytoalexin, Sakuranetin in rice. Sakuranetin has high antimicrobial activity and is known to be induced in response to phytopathogenic induction, ultraviolet (UV) irradiation, treatment with $CuCl_2$, JA and methionine (Shimizu T et al., 2012; Wang et al., 2018). In the present study we noticed that the expression levels of rice NOMT was enhanced by salt stress, the gene expression increased 2-folds in the salt-tolerant variety. As compared to the salt-sensitive IR64 variety the gene expression of increased 12-folds in the tolerant variety and 4-folds in the Jaya salt-sensitive variety. It is possible to hypothesise that *NOMT* has a potential role in salt tolerance, but the response mechanism may be different from other flavonoid pathway genes. As *NOMT* plays a key role in sakuranetin biosynthesis, determination of the sakuranetin content in the rice plants under salt stress, could provide useful hints about the role of Sakuranetin in salt-tolerance.

F2H and FNSII are competing enzymes using the same flavanone substrates. There are few reports about the response of these two genes to abiotic stress. D. Ma et al. (2014) reported that FNS was induced by drought stress in wheat and hinted on its possible roles in mitigating abiotic stress. There are similarities in our findings where the FNSII gene expression increased in the salt-sensitive varieties (Jaya and IR64) under salt stress. C-glycosylated flavanones act as phytoalexins against insects and fungi in a number of plants (Du et al., 2010; Morohashi et al.,2012; Chen et al., 2013). F2H catalyses the first step in the synthesis of these phytoalexins. Findings in the present study did not show enhanced gene expression of this gene.

F3H is a key enzyme that can convert (2S)-flavanones to (2R, 3R)-dihydroflavonols and play an important role in biotic and abiotic stress (D. Ma et al., 2014; Can Si et al., 2023).

Shen et al., (2010) reported that the expression of MtF3H was greatly induced in stem and roots of *Medicago truncatula* when treated with 200mmol/L NaCl, but weakly expressed in leaves. *DoF3H* mRNA were significantly induced by salt and cold stresses (Can Si et al., 2023). Transgenic *A. thaliana* were also conferred tolerant to salt-stress by the F3H protein from *Pohlia nutans* (Li et al., 2017). *F3H* was overexpressed during salt and heat stress in rice plants that was involved in regulating the physiological, biochemical and molecular machinery (Jan R. et al., 2021). Findings in the present study are consistent with the previous findings, we found that Jaya and Korgut cultivars had the increase expression of *F3H* gene under salt stress. However, this was not the case in the salt-treated IR64 variety and can be one of the possible reasons for the salt-sensitive behaviour of this variety.

Nemesio-Gorriz et al. (2016) indicated that the regulation of *LAR* expression is responsible for the higher resistance to *H. parviporum* in *P. abies.* However, there is relatively less amount of work done on the expression of leucoanthocyanidin reductase (*LAR*) gene is response to abiotic stress in plants. In our study we report the considerable increase in the gene expression of *LAR* in the Goan rice cultivars under salt-stress. These findings suggest that the *LAR* gene responsible for production of catechin, the monomers of pro-anthocyanidins may have potential role in mitigating salt-stress in rice plants.

In silico analysis showed the interaction of various TFs with the flavonoid biosynthesis genes under study. The ERF (Ethylene Responsive Factor) was reported to interact with most of the flavonoid biosynthesis genes in this study. ERF belongs to the Arabidopsis APETALA2 (AP2)/ERF family transcription factor. It was reported that ERF34 promoted salt stress tolerance at different stages of the plant cycle such as seed germination and

vegetative growth. It is also known to negatively regulate leaf senescence induced by age, dark and salt stress. Under salinity stress, IR64 did not show enhanced expression of *LAR, F3H* and *F2H* which are found to be interacting with the transcription factor ERF, being a possible reason for the salt sensitivity of IR64.

Lateral organ boundaries (LOB) domain (*LBD*) genes, encodes for plant-specific transcription factors that play important roles in plant growth and development. Class II LBD proteins are known to regulate anthocyanin synthesis and nitrogen responses. In rice, *OsLBD3-7* was reported to be in involved in regulation of rice leaf rolling as its overexpression leads to narrow and adaxially rolled leaves. Genome-wide expression profiles identified that LBD members can respond to abiotic stress, the *SbLBD32* of *Sorghum bicolor*, *CsLOB_3* and *CsLBD36_2* of *Camelia sinensis* were found to be highly induced by salt and drought stress. Which was in turn found to improve the promoter activity of some of the key genes of the flavonoid biosynthesis pathway (Zhang et al.,2019; Wang et al., 2021). *LBD* interacting with *NOMT* and *F3H* may show similar enhancement of the promoter activity of these 2 genes under salt stress owing to the increase in their gene expression under salt stress.

MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factor family is one of the largest transcription factors in plants and get its name because its structure has a conserved DNA binding region, known as MYB domain. MYB transcription factors have been characterised for their regulatory roles in response of plants to abiotic stress (Hu et al., 2008; Takasaki et al., 2010; Yang et al., 2012). *OsMYB6* was reported to be strongly induced by drought and salt stress (Huang et al., 2014; Zhou et al., 2016; Y. Tang et al., 2019). *DcMYB6* is found to be a potential key regulator involved in regulation of

anthocyanin biosynthesis in purple carrots and *EsMYB9* participates in modulating the flavonoid biosynthesis pathway in Epimedium (Xu et a., 2017; Huang e al., 2017; Y. Tang et al., 2019). In silico analysis showed the interaction of MYB TFs with *LAR, F2H* and *F3H*. The increase in gene expression of these genes in Korgut and Jaya after salt treatment, indicate that the improved salt tolerance of these varieties maybe due to the interaction of MYB TFs with these 2 genes. Whereas the *LAR* and *F3H* gene expression was not enhanced under salt-stress in IR64, being one of the possible reasons for its salt-sensitivity.

DREB2A (Dehydration-Responsive Element Binding) transcription factor was found to be interacting with the *CHS* and *F2H* gene. DREB2A have been reported to be abiotic stress inducible (Matsukuru et al., 2010). *DREB2A* gene expression was higher in rice plants under salt-stress and it is reported that the transgenic overexpression of *OsDREB2A* in rice positively correlates with stress tolerance. The gene expression of *CHS* and *F2H* was increased under salinity stress in the Jaya variety and in the Korgut plants the gene expression did not differ much in control and salt-treated plants. However,*F2H* gene expression was very less in salt-treated IR64 plants. The interaction of the DREB2A with *F2H*, can be a reason for the enhanced gene expression in Jaya and Korgut leading to their salt-tolerant behaviour. The gene expression of *CHS* in Jaya and Korgut varieties under salinity stress as compared to IR64 was much higher. So it is possible to hypothesise that the DREB2A-CHS interaction in Korgut and Jaya maybe one of the reason for their enhanced salt-tolerance as compared to IR64.

Dof (DNA-binding with one finger) is a plant specific TF family that plays a substantial role in resistance mechanisms against biotic and abiotic stress tolerance in plants

(Yanagisawa, 2004; Noguero et al., 2013). These TFs are indirectly involved in abiotic stress tolerance either as activators or repressors of several abiotic stress-responsive genes (M Waqas et al., 2020). In *Gossypium hirsutum*, the *GhDof1* upregulated the salt and cold stress-responsive genes such as *GhMYB, GhSOD, GhP5CS* (Su et al., 2017; M Waqas et al., 2020). The DofTFs was involved in drought and salt tolerance in *Arabidopsis* and *Triticum aestivum* (Shaw et al., 2009; Massange-Sanchezz et al., 2016; M Waqas et al., 2020).

The GATA family of TFs recognize the GATA motif and specifically bind to the promoter sequence (A/T)GATA(A/G) (Hannon et al., 1991). The *SlGATA17*overexpression in tomato is known to enhance its drought resistance by promoting the phenylpropanoid pathway. The *OsGATA8* has a role in salinity stress in rice as it is known to increase the biomass, RWC and electrolyte leakage, proline content, and K^+/Na^+ ratio. *OsGATA8* maintained yield under stress by regulating the expression of critical genes involved in stress tolerance, scavenging ROS and chlorophyll biosynthesis (Nutan et al., 2019).

Homeodomain leucine zipper (HD-ZIP) proteins are plant-specific transcription factors that contain a homeodomain (HD) and a leucine zipper (LZ) domain. The highly conserved HD binds specifically to DNA and the LZ mediates homodimer or heterodimer formation. HD-ZIP transcription factors control plant growth, development, and responses to abiotic stress by regulating downstream target genes and hormone regulatory pathways. HD-ZIP proteins mainly mediate plant stress tolerance by regulating the expression of downstream stress-related genes through abscisic acid (ABA) mediated signalling pathways, and also by regulating plant growth and development.

CONCLUSION

6. Conclusion

The secondary metabolite content and gene expression of the flavonoid biosynthesis genes were investigated under salt stress in 3 Goan rice cultivars. Salt stress induced greater accumulation of phenolics, flavonoid and anthocyanins in the leaves of salt-tolerant cultivar than the salt-sensitive one (IR64). The changes in the flavonoid content were closely related to the changes in the expression levels of the flavonoid biosynthesis genes. In-silico analysis showed that various transcription factors involved in salt-stress tolerance interact with the flavonoid biosynthesis genes. The qRTPCR and in silico analysis results obtained suggest that higher flavonoid production in the rice seedlings under salinity stress was one of the mechanisms responsible for protection of the plant from salt-induced damage during the early vegetative stage and provides salt-tolerance to these varieties. This investigation can help in identifying the various secondary metabolite compounds using HPLC and other techniques. This data also provides opportunities to investigate the cross talk of these secondary-metabolite genes and the salt-stress tolerance genes.

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