Doctoral Thesis

Molecular Physiological Study on the Underlying Mechanisms of Riboflavin Pretreatment to Alleviate Salinity Stress in Rice

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Graduate School of Integrated Sciences for Life

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Abbreviations

ARAP	5-amino-9-ribityl-amino-2, 4 (1H, 3H) pyrimidinedione
cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
DEGs	Differentially expressed genes
DHBP	3,4-dihydroxy-2-butanone-4-phosphate
DHBPS	3, 4-dihydroxy-2-butanone-4-phosphate synthase
DRL	6,7-dimethyl-8-(d-ribityl) lumazine
DW	Dry weight
EC	Electrical conductivity
ELR	Electrolyte leakage ratio
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GCHII	GTP cyclohydrolase II
GTP	Guanosine-5'-triphosphate
H_2O_2	Hydrogen peroxide
HAL3	Halotolerance 3

HKTs	High-affinity potassium transporters
LC-MS	Liquid chromatography-mass spectrometry
LS	Lumazine synthase
LUC	Lumichrome
LUF	Lumiflavin
MDA	Malondialdehyde
MGT1	Magnesium transporter 1
NHX	Na^+/H^+ antiporter
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RIB	Riboflavin
RNA-seq	Ribonucleic acid-sequencing
ROS	Reactive oxygen species
RP	Ribulose 5-phosphate
RS	Riboflavin synthase
ΔC_T	Change in threshold cycle

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Chapter 1

General introduction

1.1 Salinity stress

The sessility of plants, which indicates that their lives may be challenged by environmental disturbances, is undoubtedly their most unfavorable trait when compared to other living species. Plants have developed mechanisms enabling rapid sensing of changing environmental conditions as well as complicated chemical reactions, leading to phenotypic plasticity (Dubois et al., 2018). Abiotic stress caused by deficiencies or excesses in environmental factors such as water, salt, light, and temperature can significantly reduce plant growth and productivity and even threaten their survival (Zhang et al., 2020).

The onset of the 21st century is marked by the limitation of water resources worldwide, environmental pollution, and a notable rise in salinized soil and water (Shrivastava and Kumar, 2015). The challenges of agricultural sustainability encompass the dual concerns of escalating human populations and the concomitant decrease in fertile land (Shahbaz and Ashraf, 2013). Salinity stress is one of the prominent abiotic factors threatening food security, while arable lands are degraded on a global scale which causes major lowering in cultivated land area, crop productivity and quality (Yamaguchi and Blumwald, 2005; Julkowska and Testerink, 2015). According to estimates, around 20% of total cultivated and 33% of irrigated agricultural lands globally are affected by excessive salinity. In addition, the expansion of salinized regions is occurring at a rate of 10% annually due to several factors, for example, the worsening of climate change and agricultural malpractices (Jamil et al., 2011).

1.2 The effect ROS overproduction on plant growth

When a plant cell detects stress, it leads to the stimulation of second messengers such as calcium, reactive oxygen species (ROS), phospholipids, nitric oxide, and different types of protein kinases (Kudla et al., 2018; Testerink and Munnik, 2011). ROS are derived from photosynthesis and photorespiration in plants, which are generated in the chloroplasts, mitochondria, and peroxisomes (Choi et al., 2016). Plants exhibit responses to low osmotic potential conditions by decreasing the aperture of stomata, thereby minimizing water consumption. This phenomenon gives rise to a sequence of adverse consequences for the photosystem (Gururani et al., 2015). Initially, stomata closure, also known as reduced stomatal conductance, along with the decreased mesophyll conductance of carbon dioxide (CO_2), which refers to the diffusion rate of CO_2 through mesophyll cells, collectively restrict the internal CO_2 concentration (Lawson and Blatt, 2014). Consequently, these factors contribute to a decline in the rate of photosynthesis (Flexas et al., 2006). Furthermore, a reduction in CO_2 in the mesophyll cells results in a decrease in the energy consumption of the Calvin-Benson cycle (Dusenge et al., 2018). The reduction in CO_2 availability in the mesophyll cells also leads to an over-reduction of the photosynthetic electron transport chain due to excess light energy (Lawlor and Tezara, 2009). These resulted in ROS formation, encompassing singlet oxygen, superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals (Tripathy and Oelmüller, 2012). Cell death occurs as a consequence of increased ROS production, leading to photooxidative damage to DNA, proteins, and lipids (Mittler, 2017). The understanding of oxidative stress has changed in

the past decades. Initially, ROS were perceived as detrimental agents that non-selectively damaged diverse molecules and structures. However, this perception has evolved to recognize the idea of ROS signaling (Foyer and Noctor, 2005). Based on contemporary knowledge, it is widely accepted that the symplastic compartments possess efficient antioxidative mechanisms that effectively maintain low levels of ROS, even in the presence of ROS overproduction (Noctor et al., 2016). It has been well established that H_2O_2 overproduction in plant cells entails oxidative stress arising from an imbalance between the generation of ROS and their elimination through enzymatic and non-enzymatic reactions (Gill and Tuteja, 2010). Plants have evolved intricate defense mechanisms that encompass a range of enzymatic antioxidants, such as catalase, glutathione reductase, superoxide dismutase, ascorbate peroxidase, and guaiacol peroxidase (Nath et al., 2016). Additionally, they employ non-enzymatic antioxidants, including ascorbate, glutathione, carotenoids, flavonoids, and other phenolics, to alleviate the harmful consequences of oxidative damage (Zandi and Schnug, 2022). H_2O_2 has the potential to render enzymes inactive through the process of oxidizing their thiol groups. Tewari et al. (2005) observed a significant increase in H_2O_2 accumulation inside the central region of trichomes in *Morus alba* cv. Kanva 2 leaves in Cu-deficient plants as compared to plants grown under Cu-excess conditions. H_2O_2 has a dual function in plants; at low concentrations, it functions as a signaling molecule that participates in acclimatory signaling processes, hence promoting tolerance to a range of biotic and abiotic stressors. Conversely, at high concentrations, it induces programmed cell death (Quan et al., 2008). H_2O_2 has been demonstrated to function as a significant regulator in several physiological processes, including stomal movement

(Bright et al., 2005), the cell cycle (Mittler et al., 2004), growth and development (Foreman et al., 2003). It is noteworthy that H_2O_2 is increasingly being recognized as a viable second messenger for signaling pathways mediated by ROS, owing to its comparatively extended lifespan and notable ability to traverse cellular membranes (Quan et al., 2008). The previous result of Tanou et al. (2009) demonstrated that 10 mM H_2O_2 and 100 mM sodium nitroprusside pretreated in citrus roots induced antioxidant defense responses under 150 mM salinity stress. In addition, the signal transduction pathways triggered by ROS are governed by two discrete protein families, namely the Mitogen Activated Protein Kinase (MAPK) and the redox-sensitive kinases (Tripathy and Oelmüller, 2012). ROS are used as biological stimuli and signals that stimulate and regulate various genetic stress-response programs due to cell evolution strategies (Dalton et al., 1999).

1.3 Na^+ and K^+ accumulation and Na^+ transporter genes

The negative impact of NaCl on plants is attributed to two main factors: osmotic stress and ionic stress, which are considered to be temporally and spatially separated, with water availability rapidly reduced due to the high Na^+ concentration in the soil and slow accumulation of Na^+ in the shoots, limiting photosynthesis (Munns and Tester, 2008). Furthermore, the similarity in physicochemical properties, such as ionic radius and energy of ion hydration, between Na^+ and K^+ leads to competition in ion absorption in the root zone (Marschner, 2011; Véry and Sentenac, 2003). Hence, some roles of K^+ in the plant

cell are replaced by Na^+ under salinity stress, which is exclusively attributable to disturbed protein synthesis and enzyme activities (Schachtman and Liu, 1999).

Plants have evolved a specific network of cation channels across the cellular and vacuolar membranes to effectively control the transport of Na^+ and K^+ ions, ensuring their balanced availability for various cellular processes (Blumwald, 2000). Na^+ accumulation and the genes encoding Na^+ transporters and channels are the primary determinants of salinity stress and are the main targets for engineering salt stress tolerance (Assaha et al., 2017). Extensive studies have been performed in the past decades to investigate the physiological, biochemical, and molecular mechanisms underlying salt stress (Hasegawa et al., 2000; Ueda et al., 2001). Thus, the control of Na^+ movement has an enormous influence on salinity tolerance. Proton gradients play a vital role in facilitating the movement of ions and solutes across various cellular membranes in plants. Plant cells contain three main proton transport proteins: (1) plasma membrane (PM) proton transporters; (2) vacuolar H^+-ATPases that facilitate proton transport through adenosine triphosphate (ATP) hydrolysis; and (3) PM and vacuolar H^+-PPases, which couple pyrophosphate hydrolysis with proton transport (Gaxiola et al., 2007). Several genes related to membrane proteins were well-characterized, including salt overly sensitive 1 (SOS1) (Ji et al., 2013) and vacuolar membrane (tonoplast) localized sodium/hydrogen antiporter (NHX) proteins (Jiang et al., 2010; Fukuda et al., 2010), which are two Na^+/H^+ antiporters related to Na^+ exclusion back to the soil and Na^+ compartmentalization in the vacuole, respectively. Additionally, high-affinity potassium transporters (HKT) are responsible for the reabsorption of Na^+ from

xylem sap into the root cells, controlling Na^+ long-distance transport and inhibiting Na^+ overaccumulation in the shoots (Sunarpi et al., 2005). Notably, the Na^+ retrieval mechanism from the transpiration stream governed by HKT1 has been considered a strong trait in salt tolerance in rice (Ren et al., 2005) and durum wheat (*Triticum turgidum* L. subsp. *durum*) (James et al., 2006).

Tissue tolerance has been identified as a prominent salt tolerance mechanism in wild rice (Munns et al., 2008). Tissue tolerance refers to the ability of plants to tolerate elevated levels of NaCl inside their tissues, simultaneously maintaining chlorophyll synthesis, leaf water potentials, photosynthetic activity, and other essential cellular activities (Assaha et al., 2017). To achieve this kind of tolerance, extra Na^+ can be stored in the vacuole, which prevents too much Na^+ from building up in the cytoplasm (Munns et al., 2016). Sequestered Na^+ is a cheap osmoticum for sustaining cell turgor pressure and facilitating shoot expansion and growth under salinity stress conditions (Flowers and Colmer, 2008). NHX transporters facilitate the removal of Na^+ from the cytosol by actively pumping H^+ into the vacuole, mediated by vacuolar H^+-inorganic pyrophosphatase (V-PPase, E.C. 3.6.1.1) and vacuolar H^+-ATPase (V-ATPase, E.C. 3.6.1.3) (Fukuda et al., 2010). Vacuolar Na^+ sequestration is not only essential for Na^+ detoxification in the cytoplasm, but it is also a crucial mechanism of osmotic adjustment to balance water uptake under salinity stress (Bassil et al., 2012). Numerous studies conducted on Arabidopsis and rice have shown the main roles NHX plays in salinity tolerance, in which plants overexpressing NHXs have the

ability to maintain K^+ in the cytosol under salinity stress, resulting in increasing tolerance (Jiang et al., 2010; Barragán et al., 2012; Jiadkong et al., 2022).

2.2 The pretreatment applications alleviate stresses in plant

Halophytes are known as well-adapted plants with specialized strategies to withstand salinity stress (Flowers et al., 2014). Nevertheless, the majority of crop species are salt-sensitive, known as glycophytes (Van Zelm et al., 2020). The classification of plants as halophytes and glycophytes is based on the efficiency of Na^+ mitigation strategies together with Na^+ compartmentalization into the vacuoles and the restriction of Na^+ overaccumulation in the cytoplasm (Blumwald, 2000). Rice is sensitive to salinity stress and is recognized as the most salt sensitive cereal crop with a threshold of 3 dSm^{-1} especially the cultivated varieties (Qin et al. 2020). Thus, one potential approach to enhancing crop productivity on agricultural salinized land is to focus on improving these glycophytes to tolerate more salinity stress.

Pretreatment (priming) is a stage of equipping the plant to be ready for an enhanced and inducible response to stress in its re-occurrence (Conrath et al., 2015). Priming and the capacity to retrieve the stress memory upon encountering the same or different stress caused by the longevity of the stress memory are pivotal, especially when there is a long gap between the two stress events (Johnson and Puthur, 2021). Changes in the phenological or genomic level are unassociated with primed states. However, the memory towards stress

tolerance is conserved either by transcriptional, translational, and epigenetic means or by all three means (Chen and Arora, 2011). The pretreatment is divided into cis-priming or stress tolerance when the stimulus and the stress are the same, whereas when the stimulus is different from the stress, it is called “trans-priming or cross-tolerance” (Liu et al., 2022). Abiotic stimuli having priming effects are commonly applied as cis-priming, for example, high temperatures, cold, drought, salinity, and chemical compounds (Johnson and Puthur, 2021). Trans-priming is composed of osmo-priming (Farooq et al., 2020), chemical priming (Jiadkong et al., 2022), nutrient priming (Majda et al., 2019), ultraviolet priming (Thomas and Puthur, 2019), etc.

1.5 Riboflavin biosynthesis pathway

Riboflavin (RIB) serves as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are essential cofactors in energy metabolism involving a multitude of oxidation and reduction activities in all aerobic organisms (Abbas and Sibirny, 2011). Microorganisms, plants, and fungi are capable of synthesizing RIB; however, RIB biosynthesis does not exist in vertebrates or some prokaryotic and eukaryotic microorganisms (Dmytruk et al., 2011).

The two initial steps of RIB biosynthesis in plants started with the bifunctional RIBA enzyme, which consists of peptide domains for GTP cyclohydrolase II (GCHII) and 3,4-dihydroxy-2-butanone-4-phosphate synthase (DHBPS) activity (Haase et al., 2014). The

release of pyrophosphate occurs subsequent to the hydrolytic cleavage of the C8 position of guanosine triphosphate (GTP), resulting in the formation of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione 5'-phosphate (Fischer and Bacher, 2006). The three enzymatic reactions related to pyrimidine intermediate are channeled through: 1) deamination of the pyrimidine ring to form 5-amino-6-ribosylamino-2,4(1H,3H)-pyrimidinedione by the pyrimidine deaminase (PYRD); 2) reduction of the ribosyl side chain to form 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate by the pyrimidine reductase (PYRR); and 3) dephosphorylation to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione by the elusive pyrimidine phosphatase (PYRP). The process catalyzed by PYRP results in the formation of 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, which is then combined with 3,4-dihydro-2-butanone 4-phosphate under the influence of lumazine synthase (LS). The required 3,4-dihydro-2-butanone 4-phosphate molecule is provided by the 3,4-dihydro-2-butanone 4-phosphate synthase (DHBPS, E.C.4.1.99.12) using ribulose-5-phosphate as a substrate. The substrates of riboflavin synthase (RS) are two molecules of 6,7-dimethyl-8-ribityllumazine, which is the result of LS. Through a dismutation process involving the exchange of a four-carbon unit, these substrates undergo synthesis to produce equimolar quantities of riboflavin and 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione. The second product is fed back into the condensation reaction by the LS (Fischer et al., 2002; Fischer and Bacher, 2006; Hiltunen et al., 2012).

1.6 Study rationale

Salinity stress has caused harm to more than 20% of the entire irrigated land, salinized soil covers an area of over 800 million hectares, which is more than 6% of the world's total land (Pitman and Läuchli, 2002). In the northeastern region of Thailand, there are 2.3 million hectares of salinized soil negatively impacts the rice productivity (Arunin and Pongwichian, 2015). The Indica rice cultivar IR29, which is a salt-sensitive variety and has a high yield, has been predominantly utilized as a model for studying various perspectives related to salinity stress (Kruasuwan et al., 2023). Numerous strategies have been revealed to ameliorate salinity stress, such as the more frequent application of salinity-tolerant transgenic lines (Hirayama and Shinozaki, 2010). Nevertheless, salinity tolerance is involved with many different genes (Munns and Tester, 2008), and the application of transgenic plants is highly criticized due to the lack of evaluation methodology, which leads to the limitation of transgenic plants under field conditions in some countries (Flowers, 2004). The constitutive expression of a particular transgenic typically results in a reduction in crop productivity (Heil, 2002); therefore, the expression of defense genes only in response to stress conditions would be considered highly desirable. Plants have developed numerous defense strategies to mitigate salinity stress. In addition, researchers have proposed various unsophisticated methodologies to readily approach farmers, including pretreatment such as acclimation (Sriskantharajah et al., 2020), foliar application (Deng et al., 2014), and root priming (Jiménez-Arias et al., 2019). Pretreatment is characterized as the genetic or metabolic alterations triggered by an initial exposure to

stress, resulting in an increased capacity to withstand subsequent stresses. (Conrath et al., 2015). Due to this rationale, pretreatment represents an effective approach to addressing salinity stress since the defensive mechanisms in primed plants stay inactive until they are activated by the upcoming stresses. It is worth noting that pretreatment does not incur higher fitness expenses when optimal development circumstances are present (Van Hulten et al., 2006).

In the previous study, the different concentrations of beta-aminobutyric acid, coumarin, gamma-aminobutyric acid, and RIB were studied under salinity stress (data not shown). However, the salinity tolerance mechanisms were obviously observed in the RIB pretreatment, so RIB was selected for further studies. In addition, vitamins have been applied as priming agents that alleviate different unrelated stresses (Boubakri et al., 2016). Previous works have unraveled that RIB is capable of mitigating biotic (Azami-Sardooei et al., 2010) and abiotic stresses (Deng et al., 2014; Wang et al., 2022). These previous studies suggest that RIB may act as a slight oxidative burst that triggers the ROS-dependent signaling network, which is related to the accumulation of latent defense proteins such as ROS-scavenging and transcription factors, leading to a primed state and an improved stress response (Borges et al., 2014). Up to date, vitamin K3 and menadione sodium bisulfite (MSB) have practical applications in field conditions, including the stimulation of plant tolerance to salinity stress (Borges et al., 2014), and various MSB-based formulations have been marketed for crop improvement and protection (Jiménez-Arias et al., 2019). However,

the availability of RIB applications and RIB-based formulations in the agricultural sector remains limited.

RIB foliar application has been reported for drought stress amelioration (Deng et al., 2014) and pathogen resistance (Azami-Sardooei et al., 2010). Soaking seeds in RIB solution has shown results in alleviating salinity stress (Jiadkong et al., 2022). Hence, RIB is considered a potentially beneficial pretreatment for use under field conditions. Although salinity stress alleviation has shown compromising results, it is important to consider the practical implications of this strategy, particularly in relation to seed storage. Previous studies have documented that seed storage causes a decrease in seed longevity and the loss of the primed state (Chiu et al., 2002). Due to these reasons, the application of seed priming is limited (Paparella et al., 2015). However, the advantages of priming triggered by RIB through root pretreatment to alleviate salinity stress have not been studied in depth. Therefore, understanding the underlying mechanisms of RIB pretreatment in response to salinity stress in plants under both hydroponic and soil-based conditions is important and can therefore be recommended to farmers.

1.7 Study objectives

1. To elucidate the physiological responses of IR29, which has been pretreated with tap water (non-pretreated), and RIB-pretreated seedlings under salinity stress by

comparing the plant growth, Na^+, and K^+ accumulation patterns under hydroponic and soil-based conditions.

2. To investigate the underlying mechanisms of Na^+ accumulation and RIB biosynthesis by analyzing the expression profiles of the genes that encode Na^+ transporter and RIB biosynthesis-related genes in the non- and RIB-pretreated seedlings.
3. To unravel the biochemical mechanisms of RIB-pretreated seedlings in salinity stress alleviation that minimize oxidative stress under both hydroponic and soil-based conditions.

Chapter 2

Exogenous riboflavin (vitamin B2) application enhances salinity tolerance through the activation of its biosynthesis in rice seedlings under salinity stress

2.1 Introduction

Increasing global population has resulted in increased demand for food production despite a predicted decrease in the total fertile area (Ausubel et al., 2013). Improving the ability of plants to grow under stressful conditions is essential to meet the growing economic demand. Soil salinity is a major constraint on crop production in the agricultural sector. Salinity stress has two toxic types: osmotic stress, which initially decreases the root water uptake from the soil, and ionic stress, which due to the high accumulation of Na^+ in the shoots negatively affects the plant growth by reducing the photosynthetic rate in the absence of Na^+ compartmentalization at the cellular or intercellular level (Munns & Tester, 2008; van Zelm et al., 2020; Julkowska and Testerink, 2015)

ROS molecules, such as H_2O_2, singlet oxygen, superoxide, and hydroxyl radicals, are drastically generated under high salinity conditions and induce oxidative stress. In addition, the complex ROS production network plays a key role in the early salinity stress response (van Zelm et al., 2020). Bidirectional regulation of H_2O_2 is observed in plants; low concentrations of ROS function as intermediate signaling molecules involved in physical responses, such as gene expression and stomatal movement (Mittler, 2017). However, excessive H_2O_2 concentrations in plant cells lead to the inactivation of enzymes due to oxidative stress (Tripathy and Oelmüller, 2012). ROS defense machinery consists of enzymatic components, such as superoxide dismutase (SOD), catalase, and ascorbate peroxidase, and non-enzymatic components, such as ascorbic acid (AA), carotenoids, phenolics, vitamins, and flavonoids, which alleviate oxidative stress and promote plant

growth and development (Das and Roychoudhury, 2014). Plant adaptation to salinity stress requires both ROS detoxification and ion homeostasis (Ueda et al., 2004; Assaha et al., 2017). Maintenance of a low Na^+/K^+ ratio in shoots is critical for withstanding salinity stress, which is related to Na^+ retrieval (*OsHKT1;4, OsHKT1;5*) (Wangsawang et al., 2018; Sriskantharajah et al., 2020), Na^+ exclusion (*OsSOS1*) (Mekawy et al., 2018), inhibition of Na^+ uptake (*OsHKT2;1*) (Miyamoto et al., 2015) and Na^+ compartmentalization (*OsNHXs*) (Jiadkong et al., 2022).

Pretreatment, which refers to the biochemical modification triggered by the first stress or non-stress exposure that leads to enhanced resistance to future stress stimuli, is often used to cope with salinity stress (Conrath et al., 2015). Potent substances are applied to various plants to stimulate different salinity stress-mitigating mechanisms, especially seed priming (Shalata and Neumann, 2001; Liu et al., 2020). NaHS root priming enhances endogenous H_2S content in *Malus hupehensis* seedlings and antioxidant enzyme activities minimizing alkaline salt stress-induced growth inhibition, Na^+/K^+ ratio, and oxidative damage (Akbar et al., 2021). Water-soluble vitamin K3 induces the upregulation of *SlSOS1*, *SlNHX4*, and *SlHKT1;2* and a non-enzymatic antioxidant, alleviating salinity stress in tomato root-primed seedlings (Jiménez-Arias et al., 2019). AA treatment of roots ameliorates salinity stress in tomato seedlings due to the antioxidant activity of AA (Shalata and Neumann, 2001). Thus, we hypothesize that the underlying mechanisms of RIB in salinity stress alleviation may be involved in antioxidant activity and root priming is an effective strategy to ameliorate salinity stress in plants.

Vitamin B group, involving cobalamin, niacin, and RIB, can mitigate both biotic and abiotic stresses (Deng et al., 2014; Rhaman et al., 2021). RIB (vitamin B2) is a vital component required for fundamental metabolism and a precursor of the coenzymes, FAD and FMN, which are important flavoenzymes and flavoproteins, respectively (Fischer and Bacher, 2008; Haase et al., 2013). Flavin-dependent proteins function in redox capacity by taking up electrons from a donor substrate and transferring them to an acceptor substrate and are included in the family of flavoenzymes and flavoproteins (Macheroux, 2021). Flavoenzymes are involved in abscisic acid biosynthesis in plants (Seo et al., 2000) and enhance plant growth and development via flavin and hormonal regulation (Barrero et al., 2005). RIB is synthesized by Arabidopsis (Hiltunen et al., 2012), *Ashbya gossypii* (Averianova et al., 2020), and *Azospirillum brasilense* (Palacios et al., 2021) via a similar biosynthetic pathway (Haase et al., 2013; Bacher et al., 2000). RIB metabolism includes antioxidant activity, cell signaling, and coenzyme function (Pinto and Zempleni, 2016). RIB biosynthesis and m^6A alteration also targeted miRNA, regulating cell division and RNA homeostasis in longan early somatic embryogenesis (Xu et al., 2023). RIB is derived from ribulose 5-phosphate (RP) and guanosine triphosphate (GTP) is converted into 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) and 5-amino-9-ribityl-amino-2, 4 (1H, 3H) pyrimidinedione (ARAP) respectively to generate 6,7-dimethyl-8-(d-ribityl) lumazine (DRL), which is then dismutated to produce RIB (Hümbelin et al., 1999; Haase et al., 2014). The first committed substrates of the RIB biosynthetic pathway are 2,5-diamino-6-ribosyl amino-4(3H)-pyrimidinone 5'-phosphate and 3, 4-dihydroxy-2-butanone-4-phosphate synthase (DHBPS) (Richter et al., 1993; Fischer and Bacher, 2008). Meanwhile, several

catalyzes are involved in the reaction from GTP to ARAP and have been considered to be the longest step in the RIB-biosynthesis reaction (Sa et al., 2016).

The relation among vitamin B, enzyme cofactors, and abiotic stresses was reported (Hanson et al., 2016). Also, the roles of RIB under biotic stress have been identified as pathogen defense in Arabidopsis (Zhang et al., 2009) and resistance against *Fusarium* wilt and charcoal rot diseases of chickpeas (Saikia et al., 2006). Additionally, lumiflavin (LUF) and lumichrome (LUC) are the potent photodegradation forms of RIB (Crocker et al., 2022). Pholo et al. 2018 suggested that the potential for growth increase in Arabidopsis treated with LUC through mechanisms including hormone crosstalk, intracellular signaling cascades, and mitotic cell differentiation and expansion. In our previous study, we revealed that RIB seed priming triggers the upregulation of *OsNHXs* family expression in Koshihikari rice seedlings, resulting in better plant growth and a low Na^+/K^+ ratio in the shoots under salinity stress (Jiadkong et al., 2022). This study aimed to assess how NaCl-induced salinity stress influences RIB biosynthesis in IR29 rice salinity sensitive cultivar, also, to elucidate the roles of RIB under salinity stress. Our findings suggest that RIB pretreatment stimulates the upregulation of the rate-limiting step (*OsRIBA1*) in the RIB-biosynthesis pathway resulting in RIB concentration enhancement in which RIB acts as a non-enzymatic antioxidant reducing the oxidative in rice under salinity stress to withstand salinity stress.

2.2 Materials and Methods

2.2.1 Seed preparation and plant material

Rice seed stock (*Oryza sativa* L. cv. IR29) was obtained from the Plant Nutritional Physiology Laboratory of Hiroshima University, Japan. Seeds were sterilized with 5% (v/v) sodium hypochlorite for 30 min and washed thoroughly with running tap water for 10 min. The seeds were then incubated at 30 °C and soaked in tap water for 24 h. Then, the tap water was renewed, and the seeds were re-soaked in tap water for another 24 h.

2.2.2 Growth conditions

The germinated seeds were grown in a glasshouse using half-strength Kimura B solution as control (0.18 mM $(NH_4)_2SO_4$, 0.27 mM $MgSO_4 \cdot 7H_2O$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 0.09 mM KH_2PO_4, 20 µM NaEDTA-$Fe \cdot 3H_2O$, 6.7 µM $MnCl_2 \cdot 4H_2O$, 9.4 µM H_3BO_3, 0.015 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 µM $ZnSO_4 \cdot 7H_2O$, and 0.16 µM $CuSO_4 \cdot 5H_2O$). The two-week-old seedlings were then divided into two groups: non-pretreated seedlings that were soaked in the½ Kimura B solution and RIB-pretreated seedlings that were soaked in the½ Kimura B solution supplemented with 0.75 µM RIB for 24 h. RIB concentration was selected based on the preliminary experiments (data not shown). The roots of non- and RIB-pretreated seedlings were thoroughly washed with deionized water and grown under control and salinity stress conditions. The control group was grown in ½ Kimura B solution alone, whereas the salinity stress group was grown in½ Kimura B solution under salinity stress induced by 50 mM NaCl. Four-week-old seedlings

were harvested when salinity stress damage was evident. The solutions were replaced twice a week, and the pH was adjusted daily to 5.0–5.5. The growth chamber was maintained at the following conditions: 70% relative humidity at 28/25 °C (8/16 h dark/light period) and 400/0 μmol m^{-2} s^{-1} (day/night) photosynthetic photon flux density.

2.2.3 Plant biomass

One month after cultivation, the seedlings were harvested and separated into leaf blades, leaf sheaths, and roots. Seedlings, especially the roots, were washed thoroughly with deionized water and wiped gently with paper towels. To determine the plant biomass, separated seedlings were dried in the oven at 70 °C for 72 h prior to being weighed. The remaining fresh separated seedlings from each treatment were immediately frozen in liquid nitrogen and stored at −80 °C until biochemical analyses.

2.2.4 Malondialdehyde (MDA) and H_2O_2 concentration analysis

To determine the MDA concentration in the fresh leaf blades, 100 mg of the fresh leaf blades was homogenized in 5 mL of extraction buffer (10 mM HEPES, pH 7, 15.0% [w/v] trichloroacetic acid, 0.375% [w/v] thiobarbituric acid, 0.25 M HCl, 0.04% [v/v] butylated hydroxyl toluene, and 2.0% [v/v] ethanol) and incubated at 95 °C for 30 min, as previously described (Assaha et al., 2015). The mixture was then immersed in an ice bath to stop the reaction. The mixture was centrifuged at 10,000 × *g* for 20 min and the absorbance of the

supernatant was measured at 532 and 600 nm using a UV-1850 spectrophotometer (Shimadzu, Japan). MDA concentration was calculated using 155 mM^{-1} cm^{-1} as the extinction coefficient (Dionisio-Sese and Tobita, 1998).

Frozen leaf blades (100 mg) were homogenized with 4 mL of cold acetone to measure the H_2O_2 concentration. The homogenate was centrifuged at 8000 × *g* for 15 min at 4 °C. Then, 1 mL of reaction buffer (0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 125 μM xylenol orange, and 10 mM sorbitol) was added to 100 μL sample of each homogenate and allowed to stand for 1 h at 25 °C. H_2O_2 levels were determined at 560 nm using a spectrophotometer. H_2O_2 concentration was calculated with reference to the standard (Suharsono et al., 2002).

2.2.5 LC-MS/MS verification of RIB, LUF, and LUC concentrations

Sample preparation protocol was as described (Hiltunen et al., 2012). Fresh shoots and roots (150 mg) were homogenized in liquid nitrogen. Extraction buffer (methanol: methanol chloride: Milli-Q-water = 7.5: 10: 2.5) was added to the samples and vortexed immediately. The homogenates were centrifuged at 10,000 × *g* at 4 °C for 15 min and 50 μL supernatant was collected. The supernatant was diluted with 200 μL milli-Q-water, centrifuged again, and filtered using the 0.22-μm TORAST Disc Syringe filter and kept on ice until LC-MS analysis.

Liquid chromatographic separation was performed on a Shim-pack Velox C18 column (100×2.1 mm, 2.7 μm, Shimadzu, Japan) using an Acquity UPLC system. Elution was conducted using 10 mM ammonium acetate (pH 6) in 10% acetonitrile as mobile phase A and 100% acetonitrile as mobile phase B, which was introduced at a flow rate of 0.2 mL/min. Gradient elution was programmed as follows: 0–5 min, 95–47.5% A; 5–6 min, 47.5–5% A; 6–8 min, 5% A; 8–8.5 min, 5–95% A; 8.5–9.5 min, 95% A. The column temperature was set at 40 °C.

Mass spectrometry was performed using an Acquity TQD triple quadrupole mass spectrometer with electrospray ionization in positive mode. The capillary voltage was set at 3.0 kV and the source and desolvation temperatures were 120 °C and 350 °C, respectively. The cone voltage was 30V. RIB detection was performed using multiple reaction monitoring with m/z 377.2>243.1, and a collision energy of 22 eV (Sa et al., 2016).

2.2.6 RIB scavenging activities assay

The protocol for H_2O_2 enzyme activity *in vitro* was adapted from the previous study (Aebi, 1984). H_2O_2 (30 mM) was prepared in phosphate buffer (50 mM, pH 7.4). The concentration of H_2O_2 was determined by measuring the absorption at 240 nm using a UV-1850 spectrophotometer.

2.2.7 Elements concentration

The dried leaf blades, leaf sheaths, and roots were homogenized using a freeze crusher (μT-48, TAITEC) and weighed to 100 mg in 50 mL polypropylene tubes. Consequently, 2 mL nitric acid and 500 μL H_2O_2 were added to the samples and they were incubated overnight at room temperature for digestion. Subsequently, a heat block was used to heat the sample at 80 °C for 30 min, releasing the pressure by loosening the lids and increasing the temperature to 125 °C for 2 h. The heated samples were cooled and filtered, and 25 mL of Milli-Q water (Millipore, Direct-Q 3 UV) was added (Wheal et al., 2011). The concentration in the roots, leaf sheaths, and leaf blades of each element Na^+, K^+, magnesium (Mg), and calcium (Ca) was analyzed using inductively coupled plasma optical emission spectrophotometry (SPECTROGREEN-FMD46, Hitachi).

2.2.8 RIB-biosynthesis-related genes, flavoprotein, and Na^+ transporter genes expression analysis

Leaf blades, leaf sheaths, and roots (200 mg) were homogenized to extract total RNA in both non- and RIB-pretreated seedlings under control and salinity stress conditions using RNA extraction Kit Mini (RBC Bioscience, Birmingham, UK). The concentration and purity of RNA were measured using Nanodrop Spectrophotometer (Thermo Fisher Scientific) at A260 and A280. After digestion with DNase I, one μg of total RNA was reverse transcribed to cDNA using reversed transcription Master Mix (5X) (Toyobo Co., Ltd., Osaka, Japan). To generate cDNA, reversed transcription was conducted at 37 °C for

15 min and then reverse transcriptase was denatured at 98 °C for 5 min. Quantitative polymerase chain reaction (qPCR) was conducted using Thunderbird SYBR qPCR mix (Toyobo Co., Ltd., Osaka, Japan) on an Applied Biosystems StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) (Ueda et al. 2013). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the following profile: initial denaturation at 95 °C for 1 min, followed by denaturation at 95 °C for 15 s, and the extension at 60 °C for 1 min. Forty cycles of denaturation were achieved before performing melting curve analysis, and the melting curve was used to verify the PCR products by adjusting the temperature from 60 °C to 95 °C. *Os25SrRNA* was used as an initial control to normalize the relative expression level of the genes. The relative abundance of the gene transcripts was calculated using the comparative $2^{-\Delta\Delta}$CT method (Livak and Schmittgen 2001). The relative expression levels of each gene were calculated based on non-treated seedlings under control conditions. The details of forward and reverse primers used are described in Table 2.1. The relative expression levels of each gene were analyzed in non-pretreated seedlings under control conditions.

2.2.9 RNA extraction and sequencing

Two-week-old seedlings were pretreated with 0.75 µM RIB or without (non-pretreatment) for 24 h. Salinity stress induced by 50 mM NaCl and control conditions were applied to both non- and RIB-pretreated seedlings for 24 h, and then the roots were sampled for RNA extraction. Total RNA was extracted from the 400 µg roots fresh weight using

Sepasol-RNA I Super G (Nacalai Tesque Co.). RNA Extraction Kit Mini in combination with DNase I (Nippon gene) was used to purify 50 μg total RNA to obtain DNA-free high-quality RNA. The quality of total RNA was evaluated using an Agilent 2100 Bioanalyzer, and RNA samples with more than 7.0 RIN values were used for RNA sequencing analysis. RNA sequencing was performed using DNBSEQ Technology (BGI, China). The fragment per kilobase per million reads (FPKM) method was used to analyze the differentially expressed genes (DEGs) (Li and Dewey, 2011). DEGs were detected using two replicates of the NaCl/control, RIB + NaCl/control, and RIB + NaCl/NaCl treatments. The assessment of overlap between two lists of DEGs is typically performed by comparing the observed number of DEGs that are present in both lists to the expected number of DEGs that would be anticipated to occur by chance. This expected number is calculated based on the total number of DEGs and non-differentially expressed genes in each circumstance.

2.2.10 Statistical analyses

Three biological replicates were used for each analysis. Statistical significance was calculated using a one-way analysis of variance with IBM SPSS Statistics 23.0. The statistical significance of differences between datasets and treatment was compared using Duncan's multiple comparison tests which were considered statistically significant at $P \leq 0.05$.

2.3 Results

2.3.1 Effects of RIB root pretreatment on plant biomass

Understanding how physiological and metabolic processes respond to non- and RIB-pretreated rice-sensitive cultivars is pivotal to establishing a potential method for salinity stress alleviation in rice seedlings. Rice seedlings were grown under control conditions for two weeks, then, the non-pretreated seedlings were subjected to 1/2 Kimura B solution, meanwhile, the RIB-pretreated seedlings were subjected to 1/2 Kimura B solution together with 0.75 μM RIB for 24 h prior to salinity stress induced by 50 mM NaCl applied for another two weeks. Fig. 2.1A illustrates that shorter heights, smaller leaf blades, and leaf sheaths were clearly observed in the non-pretreated seedlings in comparison with the RIB-pretreated seedlings under salinity stress. Simultaneously, the height of the non-pretreated seedlings under control conditions was slightly greater than that of the RIB-pretreated seedlings (Fig. 2.1A). RIB-pretreated seedlings exhibited better plant biomass accumulation in the roots, leaf sheaths, and leaf blades compared to non-pretreated seedlings under salinity stress (Fig. 2.1AB). No seedling death was observed in the non- and RIB-pretreated seedlings under control conditions (Fig. 2.1A).

2.3.2 Lipid peroxidation and ROS-scavenging activity assays

Lipid peroxidation refers to the accumulation of H_2O_2 and MDA concentration in plants, which disturb cellular membranes; H_2O_2 is the main ROS produced during oxidative stress

(Li et al., 2020). The leaf blades of the RIB-pretreated seedlings accumulated lower H_2O_2 concentrations than those of the non-pretreated seedlings (Table 2.2). MDA is an oxidative stress indicator that was analyzed using the TBA reaction to determine the concentration of MDA in the leaf blades. As shown in Table 2.2, exposure to salinity stress showed a significant increase in MDA concentration by 112% in non-pretreated seedlings compared to non-pretreated seedlings under control conditions. The MDA concentration in the leaf blades of non-pretreated and RIB-pretreated seedlings under control conditions remained unchanged. RIB-pretreated seedlings showed reduced H_2O_2 and MDA concentrations compared with non-pretreated seedlings under salinity stress (Table 2.2). These results suggest that RIB pretreatment alleviates salinity stress-induced oxidative stress in rice seedlings.

2.3.3 The alteration of RIB biosynthesis-related genes in the RIB biosynthesis pathway

RIB and its derivatives concentrations was slightly reduced by salinity stress compared to that in the control conditions, especially in non-pretreated seedlings (Fig. 2.2ABC). RIB-pretreated seedlings showed significantly increased RIB concentrations in the roots compared to non-pretreated seedlings under both control and salinity stress conditions. However, a remarkable increase was observed in the shoots of RIB-pretreated seedlings compared with those of non-pretreated seedlings under salinity stress (Fig. 2.2A). Crocker et al. (2022) reported that LUF and LUC are the photodegradation forms of RIB. We also

analyzed LUF and LUC concentrations, the results manifested that no significantly difference was found in the LUF and LUC concentrations in RIB-pretreated seedlings compared to the non-pretreated seedlings (Fig. 2.2BC). RIB biosynthesis pathway of microorganisms fungi, and plants have been clarified to have similar biosynthesis pathways (Bacher et al., 2000; Haase et al., 2014). The heatmap represents the RIB biosynthesis-related gene expression in non- and RIB-pretreated seedlings under both control and salinity stress conditions in the RIB biosynthesis pathway (Fig. 2.3). The DHBPS and guanosine 5'- triphosphate cyclohydrolase II (GCHII) activities are crucial for increasing RIB productivity (Hümbelin et al., 1999; Hedtke et al., 2011). The RP is catalyzed by DHBPS to form DHBP, a bifunctional RibA protein with an N-terminal DHBPS region (Richter et al., 1993). *OsRIBA1* expression was upregulated in the roots and leaf blades of RIB-pretreated seedlings compared to non-pretreated seedlings under salinity stress (Fig. 2.3A). Consequently, GTP is catalyzed by GCHII. *OsGCHII* expression was upregulated in the roots of non-pretreated seedlings compared to RIB-pretreated seedlings under salinity stress (Fig. 2.3B). Lumazine synthase (LS) is the penultimate-related enzyme in the RIB biosynthesis pathway (Haase et al., 2014). A significant upregulation of *OsLS* expression was found in the roots of RIB-pretreated seedlings compared to non-pretreated seedlings under salinity stress (Fig. 2.3C). RIB is a byproduct of DRL and is catalyzed by riboflavin synthase (RS). A higher upregulation of *OsRS* expression was found in the roots of non-pretreated seedlings than in RIB-pretreated seedlings under salinity stress (Fig. 2.3D). Notably, the upregulation of *OsRIBA1*, *OsGCHII*, *OsLS*, and *OsRS* expressions was

found in the roots, leaf sheaths, and leaf blades of RIB-pretreated seedlings, respectively, compared to non-pretreated seedlings under salinity stress (Fig. 2.3).

2.3.4 H_2O_2 *in vitro* assays

Based on the results shown in Table 2.2 and Fig. 2.2, lower MDA and H_2O_2 concentrations may correlate with higher RIB concentrations. Therefore, various RIB concentrations were used to determine the H_2O_2 scavenging activity *in vitro* assay to clarify this hypothesis. As shown in Fig. 2.4, the H_2O_2 concentration decreases as the RIB concentration increases. The H_2O_2 concentration in the control refers to H_2O_2 concentration that was triggered by 50 mM NaCl in the leaf blades of non-pretreated seedlings (Table 2.2). The H_2O_2 concentration dramatically decreased at 50 μM RIB compared with that at 25 μM RIB (Fig. 2.4).

2.3.5 Elements concentration and Na^+/K^+ ratio

The concentration of elements in plants is a major factor influencing plant growth and development. The accumulation of harmful ions or the overaccumulation of useful ions has drawbacks in plant physiology and metabolism. A significant increase in Na^+ concentration was observed in non-pretreated and RIB-pretreated seedlings under salinity stress compared with that in rice seedlings under control conditions (Fig. 2.5A). However, the Na^+ concentration under salinity stress was significantly higher in the leaf blades of non-

pretreated seedlings than in those of RIB-pretreated seedlings. In contrast, the non- and RIB-pretreated seedlings possessed higher K^+ concentration under control conditions than rice seedlings under salinity stress. The K^+ concentration in the non-pretreated seedlings was significantly lower in the roots and leaf blades than in the RIB-pretreated seedlings (Fig. 2.5B). Therefore, the RIB-pretreated seedlings had lower Na^+/K^+ ratio in the roots, leaf sheaths and leaf blades (Fig. 2.5C). A significant increase in Mg concentration in leaf blades was found in RIB-pretreated seedlings compared to that in non-pretreated seedlings under both control and salinity stress conditions (Fig. 2.5D). The Ca concentration in the roots remained unchanged regardless of the treatments and conditions. Salinity stress reduced the Ca concentration in the leaf sheaths and blades of both non- and RIB-pretreated seedlings compared with rice seedlings under control conditions (Fig. 2.5E).

2.3.6 Relative expression of flavoprotein and Na^+ transporter genes

qRT-PCR was used to analyze the expression of genes encoding flavoproteins (*OsHAL3*) and Na^+ transporters (*OsHKT1;4*, *OsHKT2;1*, and *OsNHX1*). *OsHAL3* expression was highly upregulated in the roots of RIB-pretreated seedlings compared to non-pretreated seedlings under salinity stress (Fig. 2.6A). *OsHAL3* is a highly conserved flavoprotein regulating Na^+ and K^+ uptake. Therefore, *OsHAL3* overexpression may enhance ion homeostasis in rice grown under salinity conditions (Sun et al., 2009).

RIB-pretreated seedlings showed decreased Na^+ accumulation in leaf blades under salinity stress (Fig. 2.5A), implying that RIB may activate Na^+ transport systems. Na^+

exclusion, Na^+ retrieval, Na^+ compartmentalization, and inhibition of Na^+ uptake are well-known determinants of salinity stress tolerance (Munns and Tester, 2008; Assaha et al., 2017). Each contributes differently to the plant-developed mechanisms of osmotic tolerance, ionic tolerance, and tissue tolerance. Salinity stress induces upregulation of Na^+ transporter genes, especially in salt-sensitive rice cultivars. *OsHKT1;4* expression is recognized as an alternative candidate for Na^+ exclusion, which is effective in the leaf sheaths, thereby decreasing Na^+ overaccumulation in leaf blades (Wangsawang et al., 2018). Simultaneously, *OsHKT1;4* expression was significantly upregulated in the leaf sheaths of RIB-pretreated seedlings compared to non-pretreated seedlings. The root epidermis-located protein OsHKT2;1 facilitates Na^+ uptake by root cells. As shown in Fig. 2.6C, non-pretreated seedlings upregulated *OsHKT2;1* expression in roots higher than that in the RIB-pretreated seedlings. When the cytosol accumulates more toxic ions, Na^+ compartmentalization is critical to sequester toxic ions into vacuoles, which are functioned by *OsNHXs* expression (Fukuda et al., 2010). Upregulation of *OsNHX1* expression and higher Na^+ concentrations were observed in the acclimatized rice variety (Sriskantharajah et al., 2020). Significantly higher upregulation of *OsNHX1* in the roots and leaf blades was observed in RIB-pretreated seedlings than in non-pretreated seedlings under salinity stress (Fig. 2.6DE).

2.3.7 Transcriptional responses to RIB in roots

To ascertain the effect of RIB-pretreated seedlings on transcriptome responses, we performed RNA-seq analysis of rice roots under salinity stress. The Venn diagram illustrates that the expression levels of 1,191 and 459 genes were affected in the non- and RIB-pretreated seedlings, respectively, compared with the control conditions (Fig. 2.7A). The non-pretreated seedlings induced more upregulated and downregulated genes than the RIB-pretreated seedlings under control conditions (Fig. 2.7B). Ten upregulated and downregulated genes involved in salinity stress tolerance were selected for further determination of their expression levels using qRT-PCR. The results showed that the expression of *Os07g41340.1*, *Os09g20260*, *Os05g0139100*, *Os09g36680*, *Os07g34006*, *Os06g07220*, *Os04g48200*, *Os03g45619.1*, *Os04g27060*, and *Os01g06310* genes was consistent with the RNA-seq results (Table 2.3). Non-pretreated seedlings showed upregulated *Os05g0139100* gene under salinity stress, delineating the salinity-responsive gene. In addition, *Os09g36680*, *Os07g34006*, and *Os06g07220* genes were upregulated in the RIB-pretreated seedlings under control conditions, indicating the presence of RIB-responsive genes. Importantly, the two outstanding genes in the roots of RIB-pretreated seedlings shown in the Venn diagrams were also found to be upregulated by qRT-PCR (*Os04g48200* and *Os01g06310* genes) in RIB-pretreated seedlings compared to non-pretreated seedlings under salinity stress (Fig. 2.7A, Table 2.3). These two genes may be RIB salinity-responsive.

Table 2.1 Primers used for qRT-PCR.

Gene/LOC	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
Os25SrRNA	AAGGCCGAAGAGGAGAAAGGT	CGTCCCTTAGGATCGGCTTAC	Jain et al. 2006
OsHAL3	TGATAGAACGTCTCTGCCTAGCA	ATACCTAGCAGGTTGATTGTCTCA	Sun et al. 2009
OsHKT1;4	GTCGAAGTTGTCAGTGCATATGG	TGAGCCTCCCAAAGAACATCAC	Suzuki et al. 2016
OsHKT2;1	ATGGCAGTGAACGCAAGG	GTGCAAATGTTGTCGATGGTG	Miyamoto et al. 2015
OsMGT1	GGCGCGTGCAGAAGATTAGGG	CGCGTATTCACGGATATGGTACAGGG	Chen et al. 2017
OsNHX1	TGGCTGCTGCTAATGAGTTG	ACCAATCATCCCGAACCAT	Jiadkong et al., 2022
Os07g41340.1	ACGACGAAGACGAAGACGAT	GCCCGCATTAACTTCTCATT	
Os09g20260	GCGCTACAATGGCTATGTCC	CACAACGCACACTTGAGCA	
Os09g36680	CTGAAACTGTGCTGGCCTAA	ATTCAGAGGGCACAACAATG	
Os07g34006	TCAGGTAGATTTTCCGCACA	AAAACGGGTGCCATTTCC	
Os06g07220	CTGAACAAACGAAGCAGCAG	CTTGGATTAAAACCGGAGGT	
Os04g48200	CCCAATAACTTGTTGCGTATG	TTTTCCACCCTTTGTTCCAA	
Os03g45619.1	CCCAAAACCTTGAAGTACCG	GCATAGCTCATTCAGCAGTGA	
Os04g27060	CGTGGAGATCAGGCAATTA	CACATGGCAAAAACACTGGA	
Os01g06310	AAGTGGAAGTGAAGCGGCTA	GTCTGCCTGCATGCACTTTA	

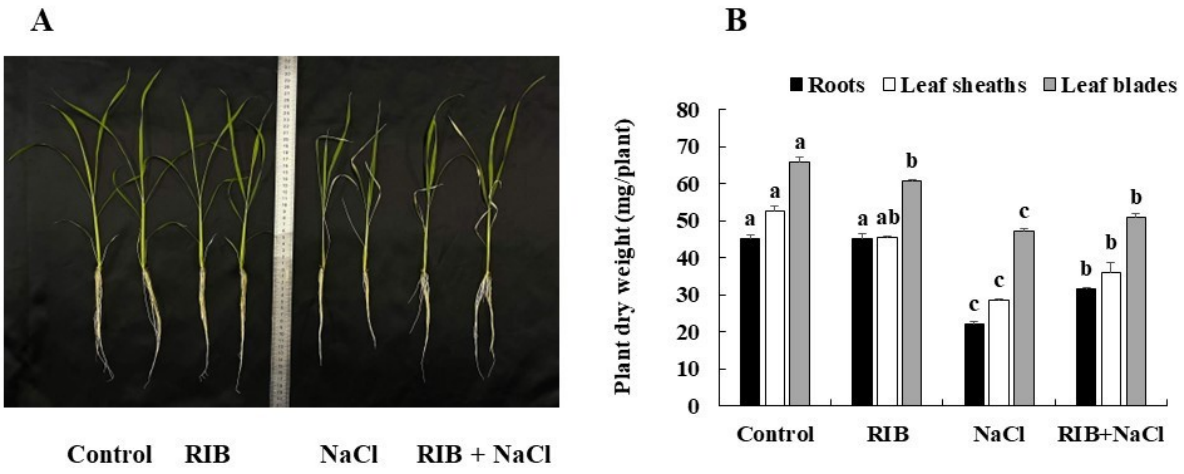


Figure 2.1 (A) Plant growth and (B) plant DW response of non- and RIB-pretreated seedlings subjected to 50 mM NaCl treatment for two weeks. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

Table 2.2 MDA and H_2O_2 concentrations response of non- and RIB-pretreated seedlings subjected to 50 mM NaCl treatment for two weeks. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

Treatment	H_2O_2 (µmol/g FW)	MDA (nmol/g FW)
Control	10.30 ± 0.54^{c}	10.75 ± 0.32^{c}
RIB	10.87 ± 0.17^{c}	10.99 ± 0.56^{c}
NaCl	15.33 ± 0.36^{a}	22.91 ± 0.23^{a}
RIB+NaCl	12.31 ± 0.48^{b}	17.72 ± 0.63^{b}

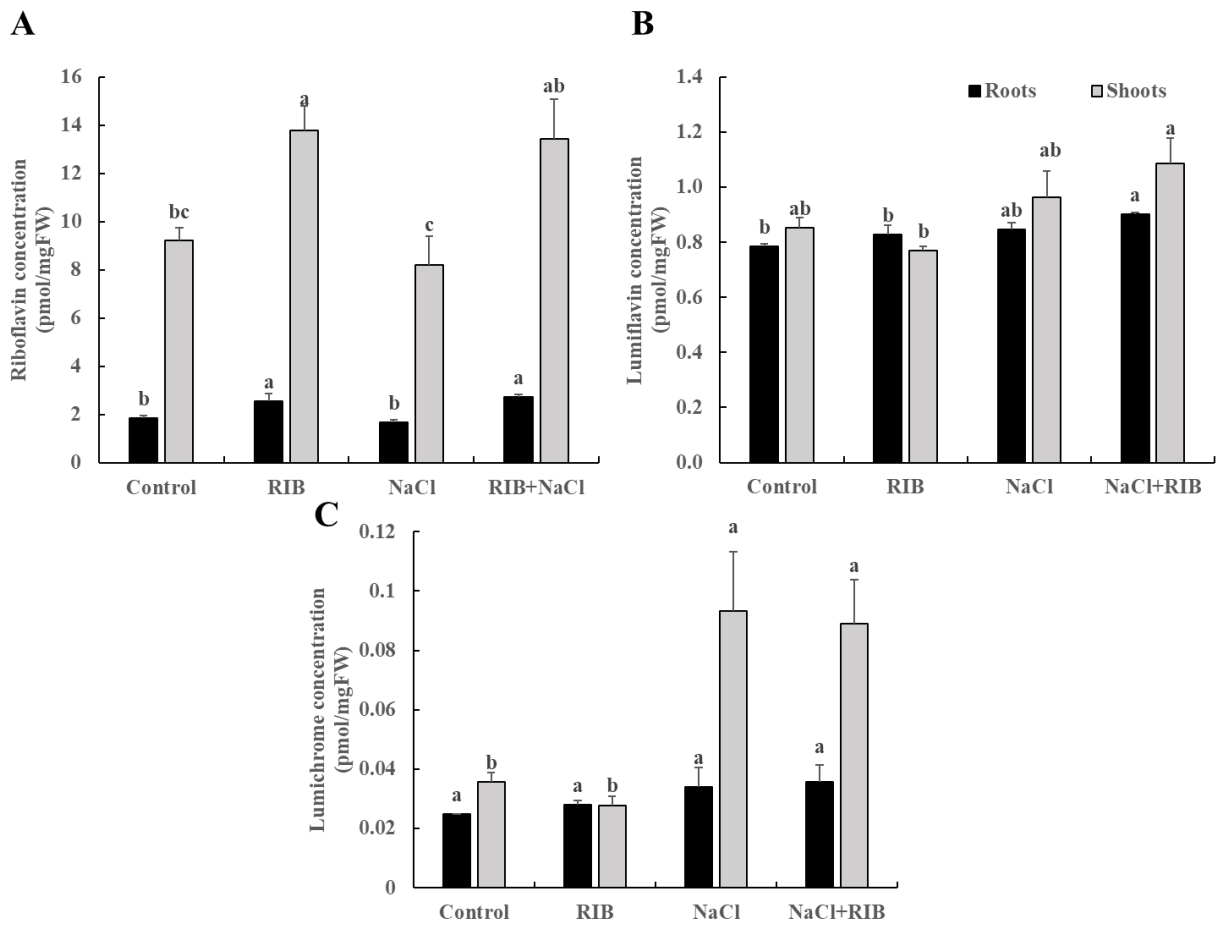


Figure 2.2 (A) RIB, (B) LUF, and (C) LUC concentrations in the roots and shoots responses of non- and RIB-pretreated seedlings subjected to 50 mM NaCl treatment for two weeks using LC-MS/MS. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

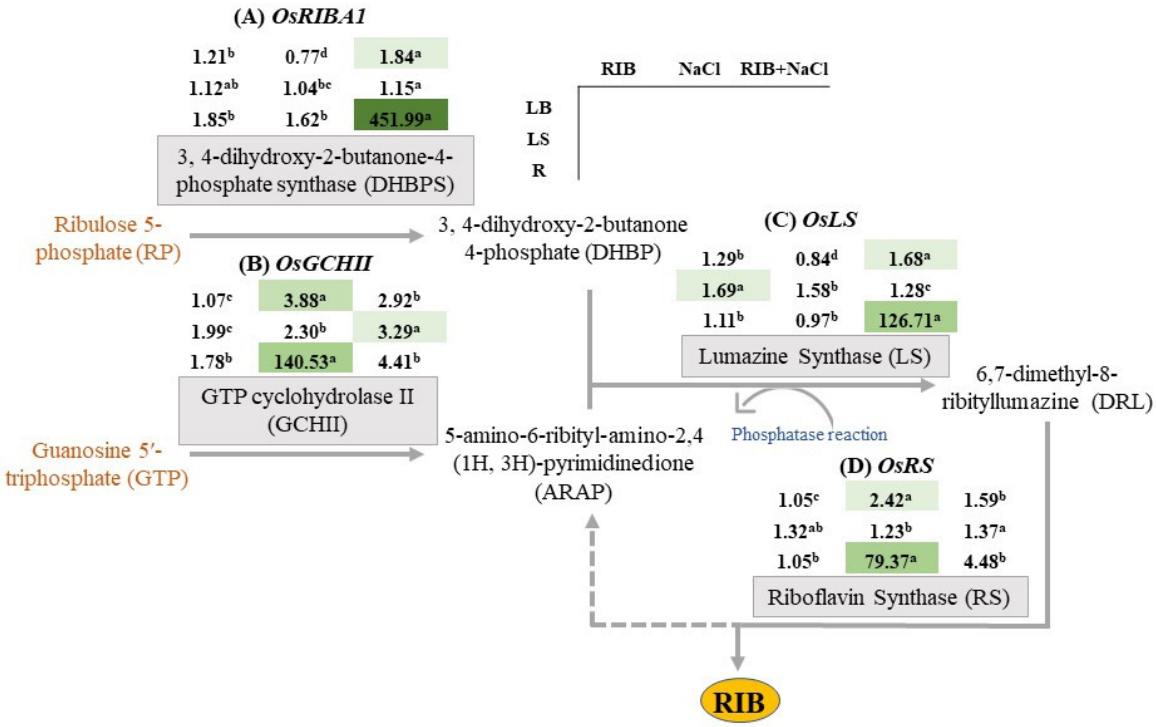


Figure 2.3 The heatmap represented the fold-change (FC) of RIB biosynthesis-related genes together with the RIB biosynthesis pathway in plants. The relative expression levels of each gene were analyzed based on the non-pretreated seedlings under control conditions (FC=1) (data not shown). The green color indicates the significant difference in each treatment. The same letter represents no significant difference ($P \leq 0.05$). RIB biosynthesis initially starts from the first (A) *OsRIBA1*(DHBPS), (B) *OsGCHII* (GCHII) enzymes that catalyze (ARAP), and (DHBP) which are catalyzed by (C) *OsLS* LS (a byproduct of RIB) becoming (DRL). (D) *OsRS* RS then catalyzes (DRL) to RIB and RIB yields back to (ARAP) to generate one molecule of RIB. This figure was based on RIB biosynthesis in Arabidopsis by Hiltunen et al., (2012).

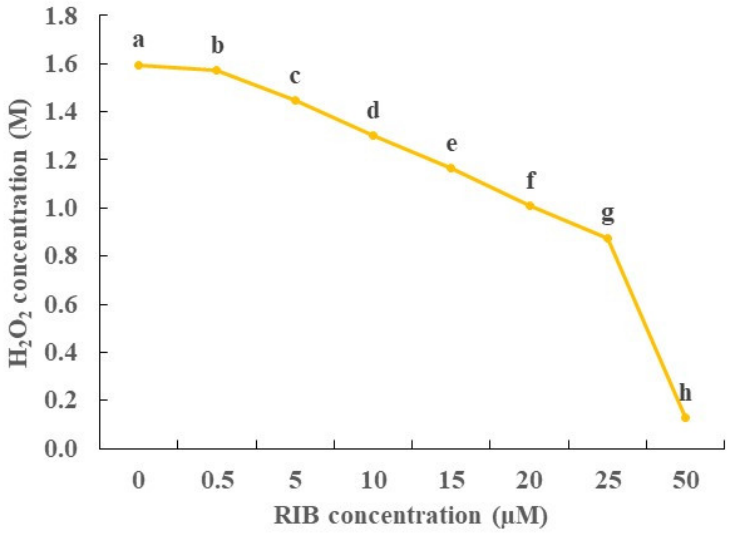


Figure 2.4 The scavenging ability of RIB *in vitro* assay, H_2O_2 is reacted with RIB in phosphate buffer. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

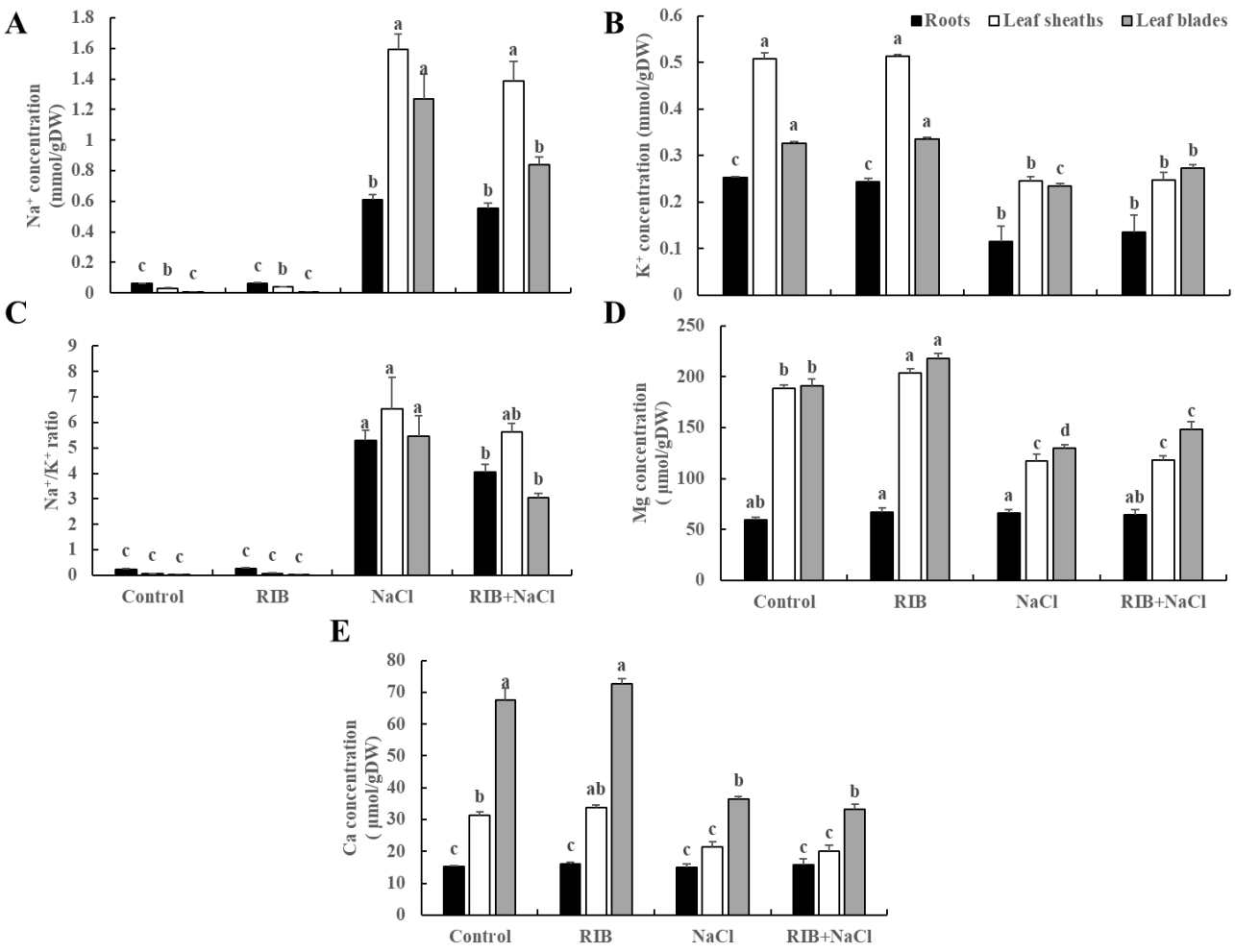


Figure 2.5 Elements concentration in the leaf blades, leaf sheaths, and roots response of non- and RIB-pretreated seedlings subjected to 50 mM NaCl treatment for two weeks (A) Na^+, (B) K^+, (C) Na^+/K^+ ratio, (D) Mg, (E) Ca. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

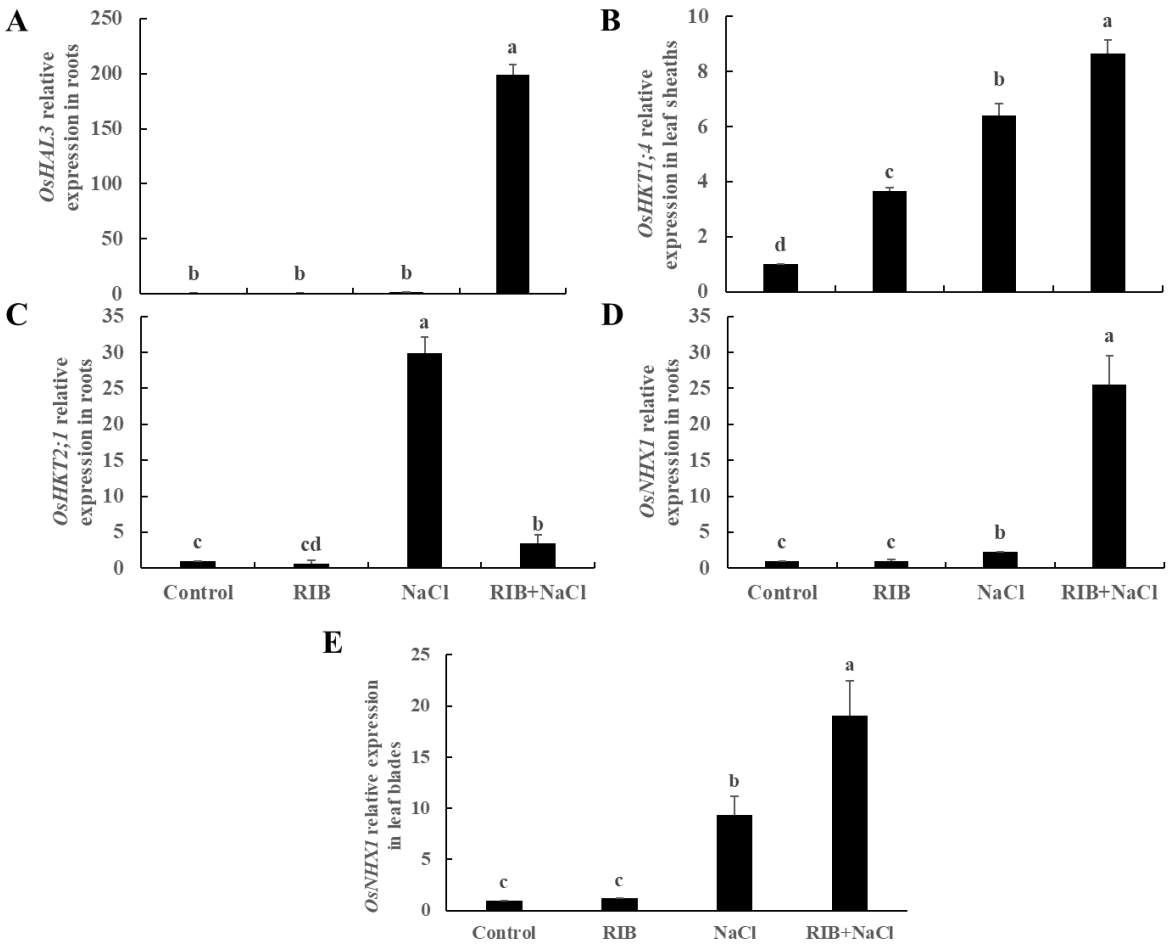


Figure 2.6 Effect of RIB on the expression of (A) flavoprotein *OsHAL3* in roots and the relative expression of Na^+ transporter proteins (B) *OsHKT1;4* in leaf sheaths, (C) *OsHKT2;1* in roots and (D) *OsNHX1* in roots after two weeks subjected to 50 mM NaCl treatment. Data are means ± standard error of three replicates. The same letter represents no significant difference ($P \leq 0.05$).

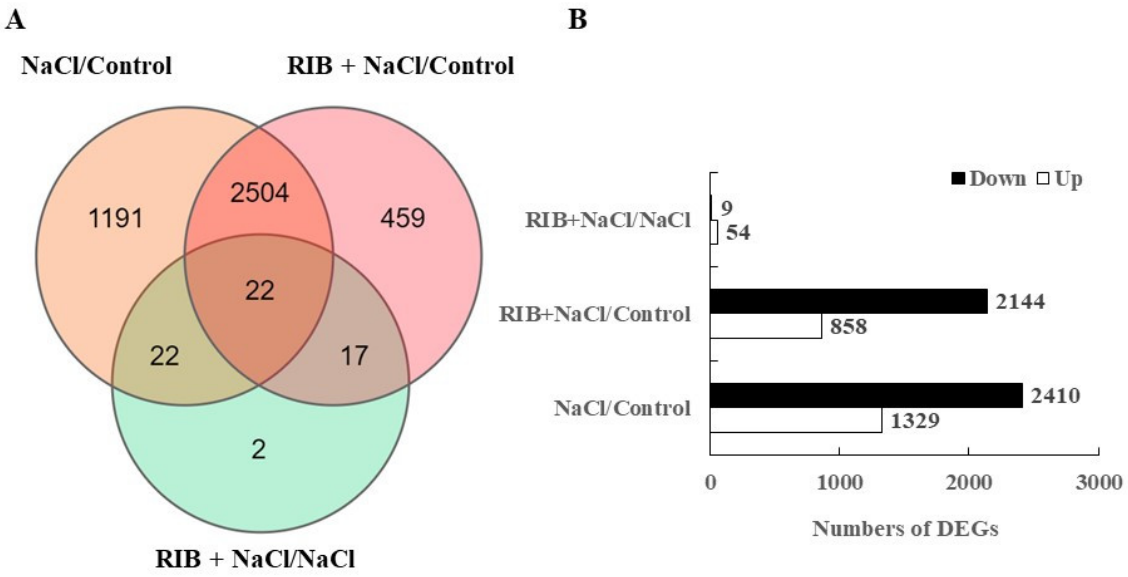


Figure 2.7 Transcriptome response of the non- and RIB-pretreated in the roots subjected to 50 mM NaCl for 24 h. (A) Venn diagram illustrating numbers of overlapping differentially expressed genes (DEGs) in the transcriptome data. (B) Numbers of DEGs regulated by non- and RIB-pretreated seedlings compared to control conditions.

Table 2.3 The values mean fold changes of RNA-seq vs qRT-PCR and the description of the responsive genes related to salinity stress in each treatment comparison.

Treatment	LOC	RNA-seq	RT-PCR	Description
Non-pretreated/Control	Os07g41340.1	-5.59	0.12	NADH-ubiquinone reductase complex 1 MLRQ subunit
	Os09g20260	-3.62	0.55	Flavin containing amine oxidoreductase
	Os05g0139100	3.49	2.96	Typical DNA-binding bHLH protein
Non-pretreated/Control	Os09g36680	23.21	2.64	Similar to Ribonuclease 2 precursor
	Os07g34006	22.45	2.11	Casein kinase II alpha subunit.
	Os06g07220	4.05	4.31	Lipid transfer protein
RIB-pretreated/non-pretreated	Os04g48200	5.86	3.08	Similar to RCc3 protein
	Os03g45619.1	3.88	4.13	Similar to Cytochrome P450 99A2
	Os04g27060	1.80	1.3	NADP-dependent oxidoreductase domain
	Os01g06310	1.86	1.46	Glycine-rich cell wall structural protein precursor

2.4.4 Discussion

The importance of RIB biosynthesis in plants has received little attention, despite the fact that RIB biosynthesis influences plant somatic embryogenesis (Xu et al., 2023) and RIB biosynthesis induces ferric reduction activity enhancing Fe uptake under Fe deficiency (Wang et al., 2022). Nevertheless, the involvement of RIB biosynthesis and salinity stress remains unknown. In our previous study, RIB seed priming was shown to stimulate the *OsNHXs* family as a tissue tolerance mechanism (Jiadkong et al., 2022). The susceptibility of IR29 to salinity stress is well documented (Walia et al., 2005; Cartagena et al., 2021; Fang et al., 2023). However, the mechanisms by which salinity stress is mitigated by chemical pretreatment in IR29 remain elusive. In our attempt to elucidate how RIB concentration alteration regulates salinity stress responses, we reported that RIB root pretreatment alleviated salinity stress in IR29 rice seedlings by improving RIB biosynthesis, which may function as a non-enzymatic antioxidant scavenging ROS. In addition, we were able to parse transcriptome responses in the roots of RIB-pretreated seedlings, indicating that multiple salinity tolerance-related genes may contribute to salinity stress alleviation in RIB-pretreated seedlings.

Salinity stress stimulates ROS overaccumulation, leading to lipid peroxidation in the cell membrane, which affects protein and DNA malfunction. MDA is the main product, and its concentration represents the degree of cell membrane damage (Farmer and Mueller, 2013). Thus, H_2O_2 concentration was correlated with MDA concentration. ROS production is permanently initiated in plastids, peroxisomes, and mitochondria (Tripathy and Oelmüller,

2012). IR29 accumulated higher H_2O_2 and MDA concentrations in comparison with the Haihong11 (salt-tolerant rice variety) (Fang et al., 2023). In this study, RIB pretreatment decreased H_2O_2 and MDA concentrations and increased RIB concentration under salinity stress (Table 2.2, Fig. 2.2). Several researchers elucidated that RIB biosynthesis occurs in plastids (Sandoval et al., 2008; Haase et al., 2014). The green fluorescent protein result of the *ZmRIBA1* overexpression line illustrated that RIB was localized in the root plastids and leaf chloroplasts (Tian et al., 2022). RIB accumulation was observed in the roots of *Medicago truncatula* in response to iron deficiency (Rodríguez-Celma et al., 2011). As shown in Table 2.2 and Fig. 2.2, these results prompted us to hypothesize that H_2O_2 and MDA concentration reductions are correlated with RIB concentration increases. RIB pretreatment minimized ROS production and lipid peroxidation in tobacco plants under drought stress (Deng et al., 2014). *In vitro* assay was conducted to test this hypothesis, and the results showed that H_2O_2 concentration gradually decreased as RIB concentration increased (Fig. 4), suggesting that RIB pretreatment increases RIB concentration in rice plants, which acts as a non-enzymatic antioxidant that minimizes ROS overaccumulation and lipid peroxidation.

Numerous genes related to flavonoid biosynthesis were up-regulated in IR29 under salinity stress (Walia et al., 2005). Our results showed that RIB-pretreated seedlings induced RIB biosynthesis-related genes under salinity stress. The upstream regulation of RIB biosynthesis and the roles of RIB and its derivatives have been characterized in *M. truncatula* (Wang et al., 2022). The process of riboflavin biosynthesis in plants follows the

identical reaction steps as in eubacteria (Fischer and Bacher, 2006). RIB biosynthesis in plants is initiated by the involvement of two enzymes, DHBPS and GCHII, which are catalyzed by RP and GTP (Richter et al., 1993). The combination of these two enzymes related to pyrophosphate release appears to be the rate-limiting step in the RIB biosynthesis reaction, which is followed by the opening of the imidazole ring and formate elimination. As shown in Fig. 2.2, rice synthesizes RIB, despite the RIB concentration being increased by RIB pretreatment under salinity stress, which may be influenced by the overexpression of *OsRIBA1* (Fig. 2.3). However, *OsRIBA1* expression was not observed in RIB-pretreated seedlings under control condition while it also possessed high RIB concentration. Bacteria encode numerous paralogs of the RIB biosynthesis pathway enzymes and as for other micronutrient supply pathways, biosynthesis and uptake functions usually coexist. It is suggested that bacteria cease RIB biosynthesis and would rather uptake RIB when the vitamin is present in the environment (García-Angulo, 2016). Jaehme and Slotboom (2015) reported that RIB uptake necessitates less ATP compared to the endogenous RIB biosynthesis. This may delineate that RIB-pretreated seedlings under control condition uptake and store RIB in the pretreatment period, so that the RIB biosynthesis pathway is not activated. *AtRIBA1* expression is considered to be a rate-limiting step in the RIB biosynthesis pathway (Hümbelin et al., 1999). Dramatic downregulation of *AtRIBA1* reduces flavin content in *planta* of the *AtRIBA1* mutant *rfd1* (Hedtke et al., 2011). *ZmRIBA1* overexpression line possessed remarkably higher RIB concentration compared to the wild types (Tian et al., 2022). ARAP is condensed with DHBP by LS, with DRL as the reaction product, along with specific phosphatase reactions required in Arabidopsis (Sa et al., 2016).

RIB production in all organisms uses the same final reaction steps catalyzed by LS and RS (Ladenstein et al., 2013). *OsLS* expression was also been observed in RIB-pretreated seedlings under salinity stress, and LS plays a pivotal role in the penultimate reaction of the RIB biosynthesis pathway (Abbas and Sibirny, 2011). In addition, the expression of *GCHII*, *LS*, and *RS* in the RIB biosynthetic pathway is activated by an unidentified transcription factor (TF) that is negatively regulated by CIPK12, leading to increased RIB accumulation in *cipk12* under Fe conditions (Wang et al., 2022). As shown in Fig. 2.2, the RIB concentration is higher in the shoots compared to the roots, regardless of the conditions. However, the genes related to RIB biosynthesis were activated in the roots under salinity stress (Fig. 2.3). These imply that RIB may synthesize in the roots and then translocate to the shoots of RIB-pretreated seedlings responsible for H_2O_2 scavengers under salinity stress. Also, it is conceivable that RIB-pretreated seedlings trigger the expression of *OsRIBA1* (the rate-limiting step) in RIB biosynthesis, which in turn increases RIB concentration to mitigate salinity stress.

Na^+ overaccumulation affects the yellow-brownish color of the lower leaves and eventually leads to lethality (Munns and Testers, 2008). Under salinity stresses, IR29 accumulated high Na^+ in the shoots and low Na^+ in the roots in comparison with the salt-tolerance rice varieties (Cotsaftis et al., 2011; Chuamnakthong et al., 2019). In the present study, we demonstrated that the Na^+ concentration of RIB-pretreated seedlings was low in the leaf sheaths and leaf blades, and high in the roots, resulting in a low Na^+/K^+ ratio in the shoots especially in the leaf blades (Fig. 2.5). The expression of *OsHKT1;4*

(Chuamnakthong et al., 2019) and *OsNHX1* (Cartagena et al., 2021) expressions was not observed in IR29 under salinity stresses. However, RIB-pretreated seedlings upregulated *OsHKT1;4* and *OsNHX1* expressions under salinity stress (Fig. 6BCE). *OsHKTs* expression was analyzed in both HKT1;4 and HKT2;1, where HKT1 was responsible for reducing the high Na^+ concentration in the leaf blades and HKT2 negatively affected the uptake of Na^+ into the root cell (Miyamoto et al., 2015; Golldack et al., 2002). *OsNHX1* expression is found in plants that accumulate high Na^+ regardless of plant metabolism disturbances, owing to the compartmentalization of Na^+ from the cytosol to vacuoles (Assaha et al., 2017). Na^+ accumulation in the roots, instead of the shoots, is one of the key factors for plants under salinity stress (Munns and Testers, 2008). Several chemical pretreatments have been reported to induce the upregulation of ion transporter genes to withstand salinity stress (Mekawy et al., 2018; Jiménez-Arias et al., 2019; Liu et al., 2020). It can be concluded that RIB-pretreated seedlings reduce Na^+ concentrations in shoots by regulating related Na^+ transporter genes.

Mg is necessary for chlorophyll and protein synthesis and enzyme activation in various fundamental physiological and biochemical processes in plants. The distinctive functions of Mg in plants can be attributed to its exceptional chemical characteristics, including its great hydrated radius and preference to interact with oxygen (Moomaw and Maguire, 2008). The Mg transporter gene (*OsMGT1*) plays a pivotal role in conferring salt tolerance in rice by inducing HKT1 expression under salinity stress (Chen et al., 2017). FAD and FMN serve as RIB precursors and function as cofactors for multiple flavoproteins containing Mg

and other metals (Haase et al., 2014). The previous study reported that *OsHAL3*; a highly conserved flavoprotein, plays a key role in balancing Na^+/K^+ under salinity stress (Sun et al., 2009). Besides, *AtHAL3* overexpression positively alters plant growth and enhances salinity and osmotic tolerance (Espinosa-Ruiz et al., 1999). The higher Mg concentration was congruent with the expression of *OsHAL3* in RIB-pretreated seedlings (Fig. 2.5D, 2.6A). This implies that higher RIB concentrations are correlated with flavoproteins, which may contribute to Mg accumulation and salinity stress amelioration in RIB-pretreated seedlings.

Additionally, transcriptome analysis by RNA-seq showed that the number of DEGs was 54 up- and 9 down-regulated in the roots of RIB-pretreated seedlings compared to the non-pretreated seedlings, encoding different enzyme TF and transporter genes (Fig. 2.7B). Ten outstanding up- and downregulated salinity tolerance genes were selected to confirm the results of qRT-PCR analysis. The regulatory role of MdbHLH24 in enhancing plant stress tolerance is achieved through the regulation of gene expression within the ABA signaling pathway (Mao et al., 2017). Ribonucleases (RNases), ubiquitous components of living cells, catalyze the hydrolysis of the 3', 5'-phosphodiester bond between two adjacent nucleotides, which is involved in a variety of processes other than the turnover of cellular RNA (Irie, 1999). *OsRNS4*-overexpression regulates ABA responses and improves rice salinity tolerance (Zheng et al., 2014). The enhancement of plant growth, root system, and salinity stress tolerance of rice plants was observed with RCc3 overexpression (Li et al., 2018). As shown in Table 2.3, these results suggest that salinity stress induced some salinity-related

TF while RIB pretreatment also triggered some components and proteins that alleviated salinity stress.

2.5 Conclusion

In the present study, RIB supplementation mitigated the negative effect of salinity stress in rice seedlings by ameliorating oxidative stress and inducing RIB biosynthesis. RIB root pretreatment could regulate RIB concentration by mediating related gene expression and enzymes in the RIB biosynthesis pathway. Notably, we identified that RIB functions as a non-enzymatic antioxidant by scavenging H_2O_2 and exerting positive regulatory effects on genes encoding Na^+ transporters. Which leads to a greater Na^+ concentration in the roots compared to the shoots in RIB-pretreated seedlings. Alteration of multiple salinity stress-responsive genes at the transcriptional level indicates that RIB-pretreated seedlings may stimulate proteins and components to ameliorate salinity stress. Nevertheless, further studies to observe the downstream regulation of the RIB biosynthesis and ROS signaling pathways, and ion homeostasis are crucial to understanding the molecular mechanism in salinity-alleviated plants. Also, the experiment testing the potential of RIB to alleviate salinity stress in rice under field conditions is pivotal for future RIB utilization in improving production under salinized soil condition.

Chapter 3

Effect of riboflavin application on rice growth under salinized soil conditions

3.1 Introduction

The accumulation of greenhouse gases, carbon dioxide (CO_2), and methane in the atmosphere has led to increased planetary heat-trapping and global warming resulting in ongoing climate change and led to more frequent and worsened abiotic stresses (Eckardt et al., 2023; Verslues et al., 2023). In addition, the manner in which plants react to these stresses can be directly affected by rising CO_2 levels. Excessive soluble salts and exchangeable Na^+ are present in salinity-affected soils, which affect plant root systems and metabolism (Anwar and Kim et al., 2020). Salinity stress initially begins with osmotic stress, which inhibits water uptake, followed by ionic stress caused by the overaccumulation of Na^+ (Munns and Tester, 2008). These stresses inevitably lead to oxidative damage due to excessive accumulation of ROS and lipid peroxidation (Roy et al., 2014; Hasanuzzaman et al., 2021). ROS are important signaling molecules involved in stress responses, and plants have a variety of mechanisms that enable them to perceive and transduce external signals to trigger adaptive responses (Miller et al., 2011). Furthermore, it is widely acknowledged that Na^+ overaccumulation interferes with cell wall integrity, inhibits enzymatic activity, decreases photosynthetic efficiency, and disturbs ion balance (Colin et al., 2023).

Salinity stress hinders plant growth and reduces crop yield and quality of the staple food, rice (*Oryza sativa* L.), which is consumed by two-thirds of the world's population (Horie et al., 2012). Rice can withstand salinity stress at a threshold soil electrical conductivity (EC) of 3 dS m^{-1}, and on average, a 12% yield loss occurs with every dS m^{-1} increase in

soil EC (Machado and Serralheiro et al., 2017). The indica rice cultivar IR29, known as a salt-sensitive and high yielding variety, has been prominently utilized as a model for researching salinity stress in rice (Kruasuwan et al., 2023). Several attempts have been made to enhance rice salinity tolerance and improve rice growth in higher EC soil conditions (Linh et al., 2012). Therefore, salt-sensitive plant varieties are commonly pretreated to ameliorate salinity stress using organic and inorganic compounds, biostimulants, and nutrients that have been reported to trigger different salinity stress tolerance mechanisms (Mekawy et al., 2018; Mohsin et al., 2020; Desoky et al., 2018). As it is an unsophisticated and effective application, it can be recommended to farmers. Effective strategies to withstand salinity stress involve the selecting/breeding crop genotypes with better performance on salinized soil and managing the soil properties by adding optimal nutrients or agent that uplift the salinity stress tolerance in plant (Zhao et al., 2021). Although rice breeding is a potent method for mitigating salinity stress, the main barriers to this are the complexities of the multigenetic traits of salinity and the limitations on the use of transgenic plants in some countries (Qin et al., 2020).

To combat ROS and prevent the cells from oxidative damage, rice plants have developed various enzymatic and non-enzymatic detoxification systems in response to salinity, including catalase, glutathione reductase, phenolic compounds, and compatible solutes (Sairam and Tyagi, 2004). Tartary buckwheat responses to salinized soil by increasing the osmolyte concentrations (Zhang et al., 2023). Compatible solutes play a pivotal role as signaling molecules in maintaining cell turgidity, preventing oxidative damage to cell

compartments, and increasing the osmotic pressure (Tang et al., 2011). Proline is a compatible solute required for osmotic adjustment and osmolyte profiles may differ between species under salinity stress (Van Zelm et al., 2020). Proline is important for protecting cells from ROS overproduction under salinity stress. Exogenous proline induces the upregulation of P5C5, increasing proline concentrations that may regulate cytokinin metabolism in cucumber (*Cucumis sativus* L.) to mitigate salinity stress (Zhu et al., 2020). In addition, exogenous proline reduces oxidative stress in rice by stimulating antioxidant enzymes under salinity stress (Hasanuzzaman et al., 2014). Proline has thus been identified as an osmoprotectant (Szabados and Savouré, 2010), a potent nonenzymatic antioxidant (Rejeb et al., 2014), and ROS detoxifier (Alia et al., 2001). The exogenous ABA induced the *OsP5CS1* which in turn improved proline concentration under salinity stress (Sripinyowanich et al., 2013). Maintaining the favorable element concentrations improve salinity stresses tolerance in plant (Mohsin et al., 2020; Chuamnakthong et al., 2019). Mg is an important mineral for the growth of plants. Approximately 75% of the magnesium found in leaf blades is engaged in metabolic activities, whereas 15% to 20% of the total magnesium is associated with chlorophyll pigments (Pogłodziński et al., 2021). Furthermore, even a slight Mg deficiency affect the overall plant growth and plant susceptibility to external challenges stressors by interfering with their biochemical and physiological functions (Senbayram et al., 2015). Salicylic acid pretreatment enhanced potassium (K), calcium (Ca), Fe, and proline concentrations in the leaf blades of a salt-sensitive tomato variety (Souri and Tohidloo, 2019).

RIB is a water-soluble vitamin B2 consisting of flavin adenine dinucleotide and flavin mononucleotide and is involved in various redox processes that affect plant defense responses (Abbas and Sibirny, 2011). RIB pretreatment in tobacco (*Nicotiana benthamiana*) decreased H_2O_2 and thiobarbituric acid reactive substance concentrations, which were correlated with an increase in antioxidant enzymes, resulting in drought stress alleviation in soil-based experiments (Deng et al., 2014). Salinity stress alleviation by reducing lipid peroxidation and Na^+ uptake has also been studied in tomatoes (*Lycopersicon esculentum* Mill.) using exogenous ascorbic acid (Shalata and Neumann, 2001). In our previous study, RIB seed priming triggered the upregulation of the *OsNHXs* family, alleviating salinity stress in the Koshihikari salt-sensitive rice variety grown hydroponically (Jiadkong et al., 2022). However, the mechanisms of RIB-pretreated plants under salinized soil condition in different rice varieties are not completely understood yet. Due to the differences in RIB application, growth medium, and rice cultivar compared to the previous studies, it is valuable to elucidate the response of RIB-pretreated seedlings in soil-based conditions. This prompted us to further analyze the effects of direct RIB application and changes caused in the physiological and biochemical properties of the salt-sensitive rice variety (IR29) under salinized soil condition. In the current study, we demonstrated that the mechanism of direct RIB application under salinized soil condition is related to improving the rate-limiting step of proline biosynthesis, which in turn increases proline concentration in the shoots to minimize oxidative stress. In addition, the expression of *OsIRT2* and *OsMGT1* in the roots was congruent with the higher Fe and Mg concentration and regulating the expression of Na^+ transporter genes *OsNHX1* and

OsHKT1;5 resulted in high Na^+ concentration in the roots and low Na^+ concentrations in the leaf blades. Notably, RIB pretreatment may be recommended to farmers and applied to salinized fields.

3.2 Materials and Methods

3.2.1 Seed and soil preparation and plant growth conditions

The rice seeds (*O. sativa* L. cv. IR29) were obtained from the Plant Nutritional Physiology Laboratory of Hiroshima University. After sterilizing the seed surface with 5% sodium hypochlorite for 30 min, the seeds were washed thoroughly under running water for 10 min. Seeds were then soaked in tap water and incubated at 30°C. After 24 h, the water was renewed and the seeds were re-soaked in tap water for an additional 24 h. Five kilograms of commercial soil (NPK; 0.2:0.25:0.325 g) was placed in four containers (13.3 L, 386 L × 256 W × 135 H). Germinated seeds were sown in the soil, watered to 5.2 kg weight, and maintained under identical conditions. A week after sowing, water was maintained 2 cm from the top of the containers (up to 5.7 kg) and remained in this condition throughout the experiment. Two-week-old seedlings were divided into two groups: RIB-pretreated seedlings; 10 mM RIB was applied directly to make 0.5 μM RIB in each treatment (RIB, RIB + NaCl), and non-pretreated; water was used as the control (control, NaCl). RIB concentration was selected based on the preliminary experiments (data not shown). After 24 h of pretreatment, seedlings from the first group were grown under controlled conditions with soil and tap water, whereas the salinity stress group was grown

in salinized soil induced with 50 mM NaCl solution. NaCl solution was added every three days for two weeks. Seedlings were harvested when salinity stress damage was evident. The growth chamber was maintained at the following conditions: 70% relative humidity at 28/25°C (8/16 h dark/light period) and 400/0 μmol m^{-2} s^{-1} (day/night) photosynthetic photon flux density.

3.2.2 Plant dry weight

The protocol described in 2.2.3 was followed for plant dry weight.

3.2.3 Percentage of ELR, MDA, and H_2O_2 concentration analysis

The third leaf from the top was cut and placed in polypropylene tubes containing 30 ml of deionized water to determine the ELR. The tubes were then covered with plastic caps and agitated gently for 24 h. An electrical conductivity meter (CM31P; Kyoto Electronics, Kyoto, Japan) was used to measure the initial electrical conductivity (EC1) of the medium. The samples were autoclaved at 121°C for 20 min to completely deactivate the tissues and release all electrolytes. The samples were then cooled to room temperature and the final electrical conductivity (EC2) was determined. ELR was calculated using the following formula: ELR (%) = (EC1/EC2) × 100.

The protocol described in 2.2.4 was followed to measure MDA and H_2O_2 concentrations.

3.2.4 Proline concentration

Leaf blades (400 mg) were homogenized in 5 mL of 3% (w/v) sulfosalicylic acid and the extract was then centrifuged at 10,000 *g* for 5 min at 4°C. Later, 2 mL of the supernatants were added into 2 mL each of acid ninhydrin and acetic acid in the glass tubes and were then incubated in the water bath for 1 h at 100°C. The samples were cooled in an ice bath, and toluene (4 mL) was added and vigorously mixed for 20 s for chromophore development. The absorbance of the chromophores was measured at 520 nm using a spectrophotometer. Proline standards were used to estimate the free proline concentration in roots and leaf blades (Bates et al. 1973).

3.2.5 Chlorophyll concentration

The dissected second leaf was weighed (100 mg) and immersed in a glass bottle containing 10 ml *N, N*-Dimethyl formamide (DMF). The bottles were closed tightly with a plastic cap, covered with aluminum foil, and kept at room temperature for 24 h. Chlorophyll content was measured at 646.8 nm and 663.8 nm using a spectrophotometer. Total chlorophyll concentration was calculated based on the FW described by (Porra et al. 1989; Wellburn et al. 1994).

3.2.6 Macro- and micro-element concentration

The protocol described in 2.2.7 was followed to measure the macro- and micro-element concentration.

3.2.7 Na^+ transporter genes expression analysis

The protocol described in 2.2.8 was followed to determine genes encoding Na^+ transporter genes *OsHKT1;5* and *OsNHX1*. The details of forward and reverse primers used are described in Table 3.1.

3.2.8 Statistical analyses

The protocol described in 2.2.10 was followed for statistical analysis.

3.3 Results

3.3.1 Effect of RIB direct application on plant growth

Plants possess diverse survival mechanisms under unfavorable conditions that alter the balance of energy use in them. Munns and Tester (2008) reported that, under salinity stress, plants suffer from limited water availability and excessive accumulation of Na^+ and chloride. It is essential to increase the ability of plants to grow under salinity stress, as the soil salinity tends to increase annually. Our study directly applied RIB to a soil-based experiment using commercial soil and salinity stress induced by 50 mM NaCl for two weeks. The seedlings were harvested when salinity damage was evident. Under control conditions, no yellowish leaves were observed in non- and RIB-pretreated seedlings. In contrast, non-pretreated seedlings exhibited brownish, smaller leaves and shorter roots compared to RIB-pretreated seedlings under salinity stress (Fig. 3.1AB). Under control conditions, the non- and RIB-pretreated seedlings showed no significant differences in plant dry weight (Fig. 3.1C). However, under salinity stress, RIB-pretreated seedlings possessed significantly higher leaf sheath and leaf blade dry weights than non-pretreated seedlings (Fig. 3.1C).

3.3.2 Percentage of ELR, H_2O_2, and MDA concentrations

ELR was analyzed for salinity stress-induced injury in plant tissues as a determinant of plant stress tolerance (Bajji et al. 2002). Table 3.2 indicates that ELR remained unchanged between the non- and RIB-pretreated seedlings under control conditions. A significantly

higher percentage of ELR was observed in the non-pretreated seedlings than in the RIB-pretreated seedlings under salinity stress. Oxidative stress refers to an imbalance between ROS production and detoxification or scavenging and is minimized by enzymatic or non-enzymatic antioxidants (Miller et al., 2010). H_2O_2 is the key ROS generated during oxidative stress and is a precursor of lipid peroxidation. The H_2O_2 and MDA concentrations showed a trend similar to that of the ELR (Table 3.2). This implies that non-pretreated seedlings suffered more cellular damage from salinity stress than RIB-pretreated seedlings.

3.3.3 Proline concentration and proline biosynthesis-related genes

Proline is a compatible solute required for osmotic adjustment and osmolyte profiles may differ between species under salinity stress (Van Zelm et al., 2020). In our study, proline concentration was analyzed based on the reaction of proline with the acid ninhydrin. Fig. 3.2 shows that no significant difference was observed in proline concentration in the roots and shoots of non- and RIB-pretreated seedlings under control conditions. Proline concentration in the shoots of non- and RIB-pretreated seedlings under salinity stress was significantly higher than that in non-pretreated seedlings under control conditions (Fig. 3.2). However, a significant increase was observed in the shoots of RIB-pretreated seedlings compared to those of non-pretreated seedlings under salinity stress (Fig. 3.2). To gain further insight into proline biosynthesis in RIB-pretreated seedlings under salinity stress, the expression profiles of the proline biosynthesis-responsive genes *OsP5CS1* and *OsP5CS2* were analyzed in the roots, leaf sheaths, and leaf blades. *OsP5CS1* was significantly upregulated in the roots, leaf sheaths, and leaf blades of RIB-pretreated

seedlings compared with those of non-pretreated seedlings under salinity stress (Fig. 3.3ABC). *OsP5CS2* was upregulated in the leaf sheaths of RIB-pretreated seedlings compared to that in non-pretreated seedlings under salinity stress (Fig. 3.3D). A similar trend was observed in the leaf blades of RIB-pretreated seedlings (Fig. 3.3E). However, the upregulation of *OsP5CS2* was not observed in the roots (data not shown).

3.3.4 Chlorophyll concentration

Rice grown under control conditions had more green leaves and higher chlorophyll concentrations (Fig. 3.1AB, Fig. 3.4). Significantly higher Chl_b and Chl_{a+b} concentration was observed in the leaf blades of RIB-pretreated seedlings, and no significant differences were found in Chl_a under salinity stress (Fig. 3.4). In contrast, non- and RIB-pretreated seedlings reduced Chl_b and Chl_{a+b} concentration by 66%, 36% and 51%, 17% respectively in comparison with the non-pretreated seedlings under control conditions (Fig. 3.4).

3.3.5 Element concentration, Mg and Fe transporter-responsive genes

Salinity stress can impair plant growth by affecting the intricate relationships between nutrient uptake and accumulation, hormonal imbalances, and oxidative stress (Anwar and Kim, 2020). Fig. 3.5A shows that RIB-pretreated seedlings possessed higher P concentrations in the leaf sheaths than non-pretreated seedlings under control conditions. A similar trend was observed in the leaf blades under salinity stress (Fig. 3.5A). The Mg concentration in the leaf sheaths, roots, and leaf blades of the RIB-pretreated seedlings under control conditions was significantly higher than that in the non-pretreated seedlings

(Fig. 3.5B). While a significantly higher Mg concentration of the roots and leaf blades was observed in RIB-pretreated seedlings compared to non-pretreated seedlings under salinity stress (Fig. 3.5B). Under salinity stress, Ca concentration was also found to be significantly higher in the roots, leaf sheaths, and leaf blades of RIB-pretreated seedlings than in those of non-pretreated seedlings (Fig. 3.5C). The Mn concentration in the leaf blades of RIB-pretreated seedlings was significantly higher than that of non-pretreated seedlings under salinity stress (Fig. 3.5D). The Fe concentration greatly increased in the roots, regardless of the treatments and conditions (Fig. 3.5E). However, a significant decrease in the Fe concentration was observed in the leaf blades of non-pretreated seedlings under salinity stress (Fig. 3.5E). To further explore the relationship between the ion transporter genes and RIB-pretreated seedlings in rice seedlings under salinity stress, the expression level of *OsMGT1*, *OsYSL15*, and *OsIRT2* in the roots was analyzed. Under salinity stress, RIB-pretreated seedlings significantly upregulated *OsMGT1* and *OsIRT2* expressions in the roots (Fig. 3.6AC). Whereas, the expression of *OsYSL15* was observed in the roots of non-pretreated seedlings under salinity stress (Fig. 3.6B).

3.3.6 Na^+, K^+ concentration and Na^+/K^+ and the relative expression of Na^+ transporter genes

The Na^+ concentration significantly increased under salinity stress compared to that in non- and RIB-pretreated seedlings under control conditions (Fig. 3.7A). However, the Na^+ concentration in the RIB-pretreated seedlings was significantly lower in the leaf sheath and

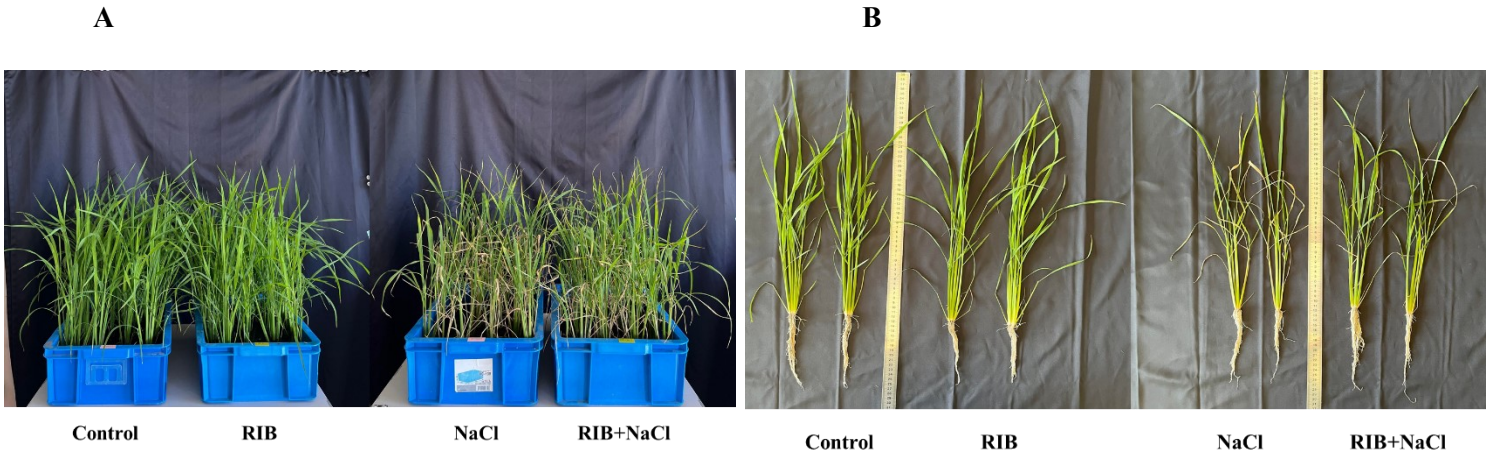
leaf blades and higher in the roots than in the non-pretreated seedlings (Fig. 3.7A). In contrast, a significantly higher K^+ concentration was observed in the roots of RIB-pretreated seedlings than in those of non-pretreated seedlings under salinity stress (Fig. 3.7B). No significant difference was observed in the K^+ concentration in the leaf sheaths and leaf blades under salinity stress (Fig. 3.7B). These results inevitably led to a significantly lower Na^+/K^+ ratio in the leaf sheaths and leaf blades of RIB-pretreated seedlings than in non-pretreated seedlings under salinity stress (Fig. 3.7C), suggesting that RIB-pretreated seedlings may have regulated Na^+ accumulation in the shoots. Significantly higher Na^+ concentrations were found in the roots of RIB-pretreated seedlings than in those of non-pretreated seedlings (Fig. 3.7A). It is suspected that Na^+ transporter genes are positively regulated in RIB-pretreated seedlings. qRT-PCR was used to determine the expression of Na^+ transporter genes. Fig. 3.7DE showed that *OsNHX1* and *OsHKT1;5* expressions significantly upregulated in the roots of RIB-pretreated seedlings in comparison with the non-pretreated seedlings under salinity stress which are responsible for Na^+ compartmentalization and Na^+ retrieval respectively (Assaha et al., 2017). It is possible that the contributions of these two Na^+ transporter genes led to higher Na^+ concentrations in the roots, preventing plant metabolic disturbances in the shoots. The proposed mechanisms of RIB-pretreated seedlings illustrated in Fig. 3.8.

Table 3.1 Primers used for qRT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
Os25SrRNA	AAGGCCGAAGAGGAGAAAGGT	CGTCCCTTAGGATCGGCTTAC	Jain et al. 2006
OsNHX1	TGGCTGCTGCTAATGAGTTG	ACCAATCATCCCGAACCAT	Jiadkong et al., 2022
OsP5CS1	TGAGGTTGGCATAAGCAC	TTGCTCTCAGAACTGATGAAT	
OsP5CS2	AACTCGATGCATTTTACGAG	GCCAGATCTTTATCATGGAA	
OsHKT1;5	CCCATCAACTACAGCGTCCT	AGCTGTACCCCGTGCTGA	Ueda et al., 2013
OsMGT1	AACACGCATCTAAAAGTTTCACC	TTCGATTATTATTGCTCCCACA	Chuamnakthong et al., 2019
OsYSL15	ACTGGTACCCTGCAAACATAC	GCAATGATGCTTAGCAAGAAG	Chuamnakthong et al., 2019
OsIRT2	GGGCACTGTCACGCTCAC	GCGCCTTGGTGGTGTTACC	

Table 3.2 H_2O_2, MDA concentrations, and ELR indicating RIB-pretreated seedling reduced lipid peroxidation under salinity stress. Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated) or without (non-pretreated) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

Treatment	H_2O_2 (μmol/gFW)	MDA (nmol/gFW)	ELR (%)
Control	11.97 ± 0.54^{c}	142.46 ± 4.79^{c}	19.54 ± 2.90^{c}
RIB	12.11 ± 0.71^{c}	142.63 ± 5.66^{c}	16.91 ± 2.24^{c}
NaCl	17.24 ± 0.21^{a}	199.21 ± 7.01^{a}	42.68 ± 2.10^{a}
RIB+NaCl	15.50 ± 0.67^{b}	175.90 ± 1.84^{b}	32.72 ± 4.93^{b}



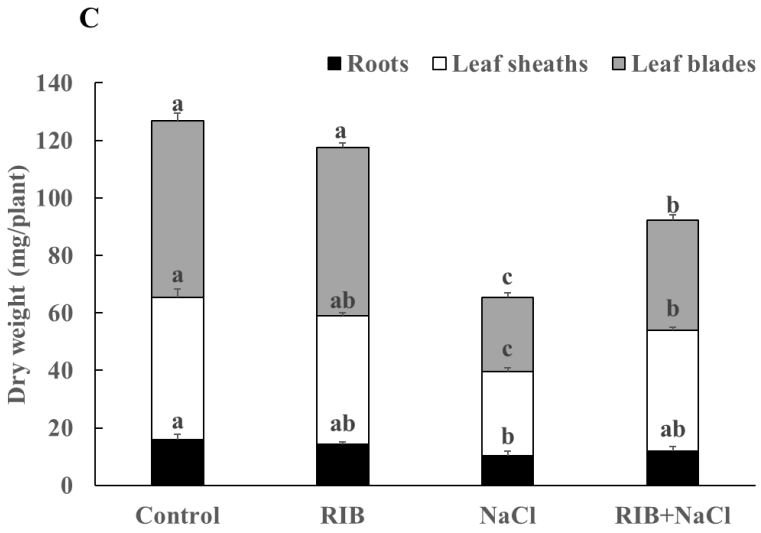


Figure 3.1 RIB pretreatment rescued rice seedlings from wilting and death under salinity stress. Image of seedlings growth (A), (B), and plant dry weight (C). Two-week-old rice seedlings were pretreated with 0.5 µM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

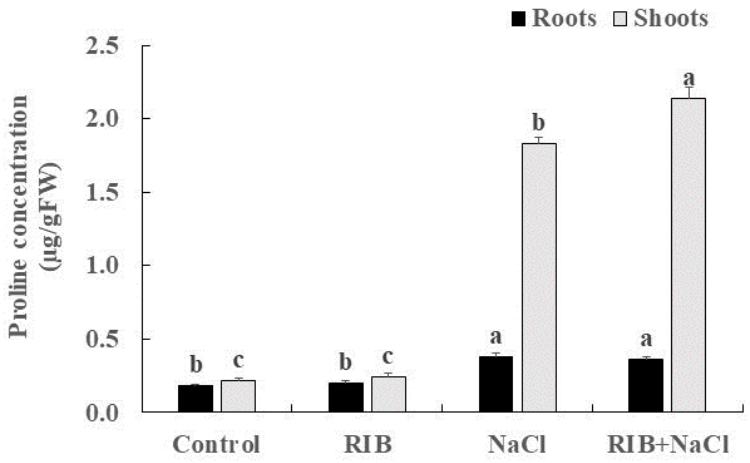


Figure 3.2 RIB-pretreated seedlings enhanced proline concentration under salinity stress. Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

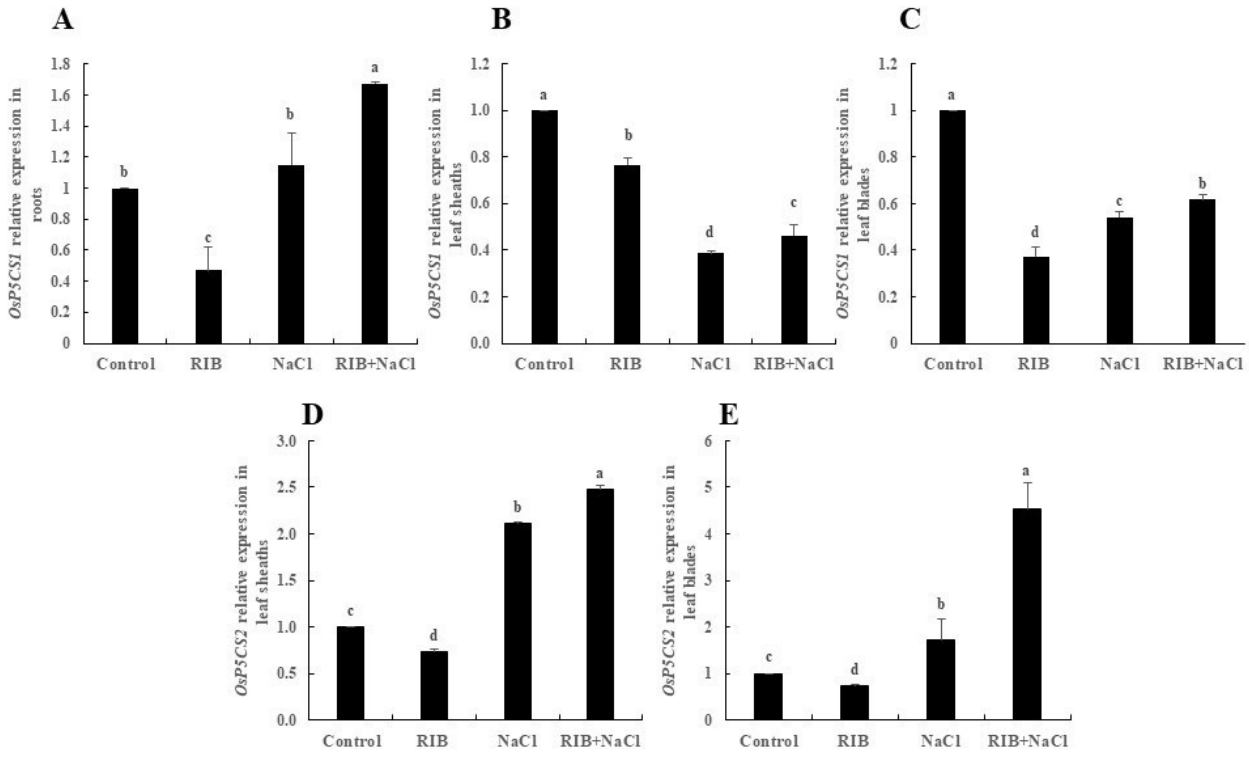


Figure 3.3 RIB-pretreated seedlings upregulated proline biosynthesis-responsive genes under salinity stress. The upregulation of *OsP5CS1* in the roots (A), leaf sheaths (B), and leaf blades (C). The upregulation of *OsP5CS2* in the leaf sheath (D) and leaf blades (E). Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

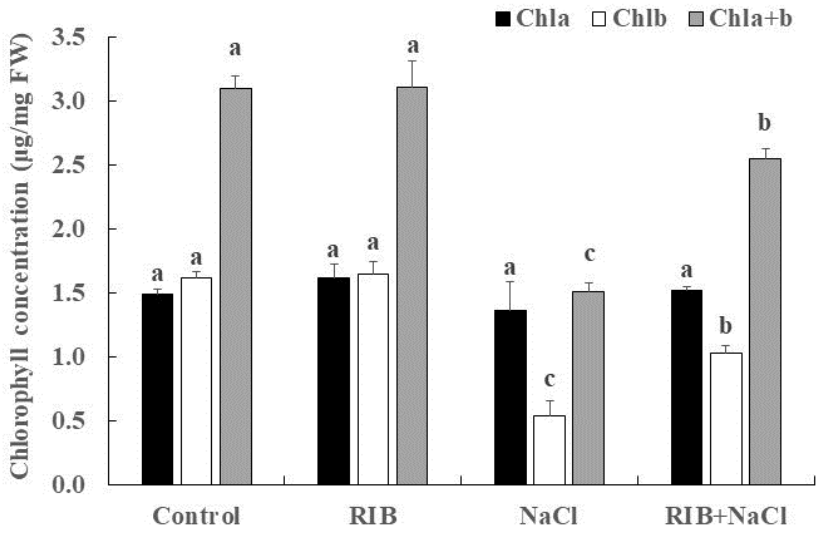


Figure 3.4 RIB-pretreated seedlings improved chlorophyll concentration under salinity stress. Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

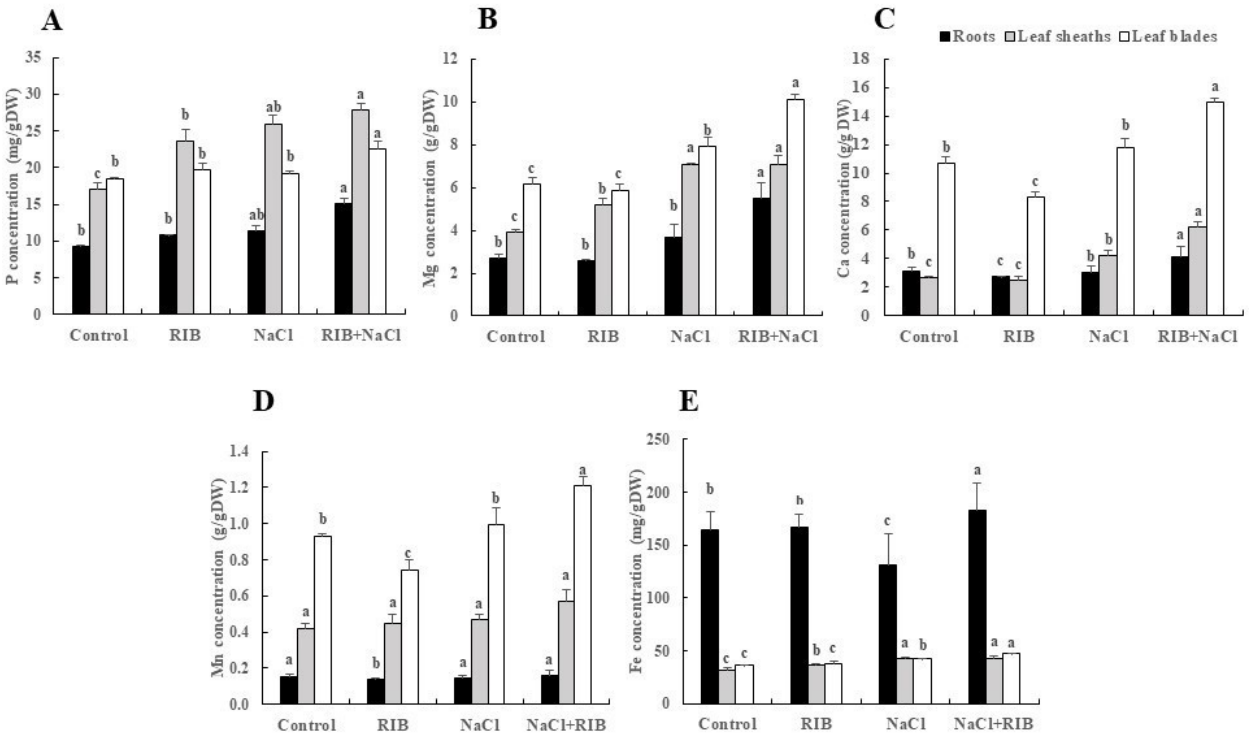


Figure 3.5 RIB-pretreated seedlings increased element concentrations under salinity stress. The concentration of *P* (A), Mg (B), Ca (C), Mn (D), and Fe (E). Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

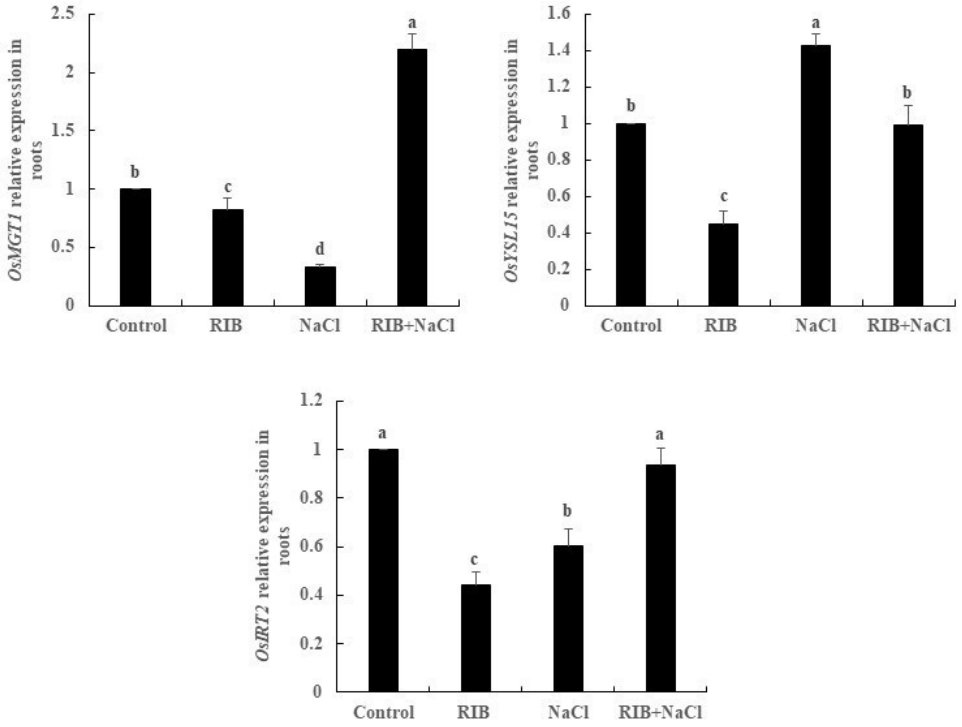


Figure 3.6 RIB-pretreated seedlings upregulated Mg and Fe transporter genes under salinity stress. The upregulation of *OsMGT1* (A), *OsYSL15* (B), and *OsIRT2* (C) in the roots. Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

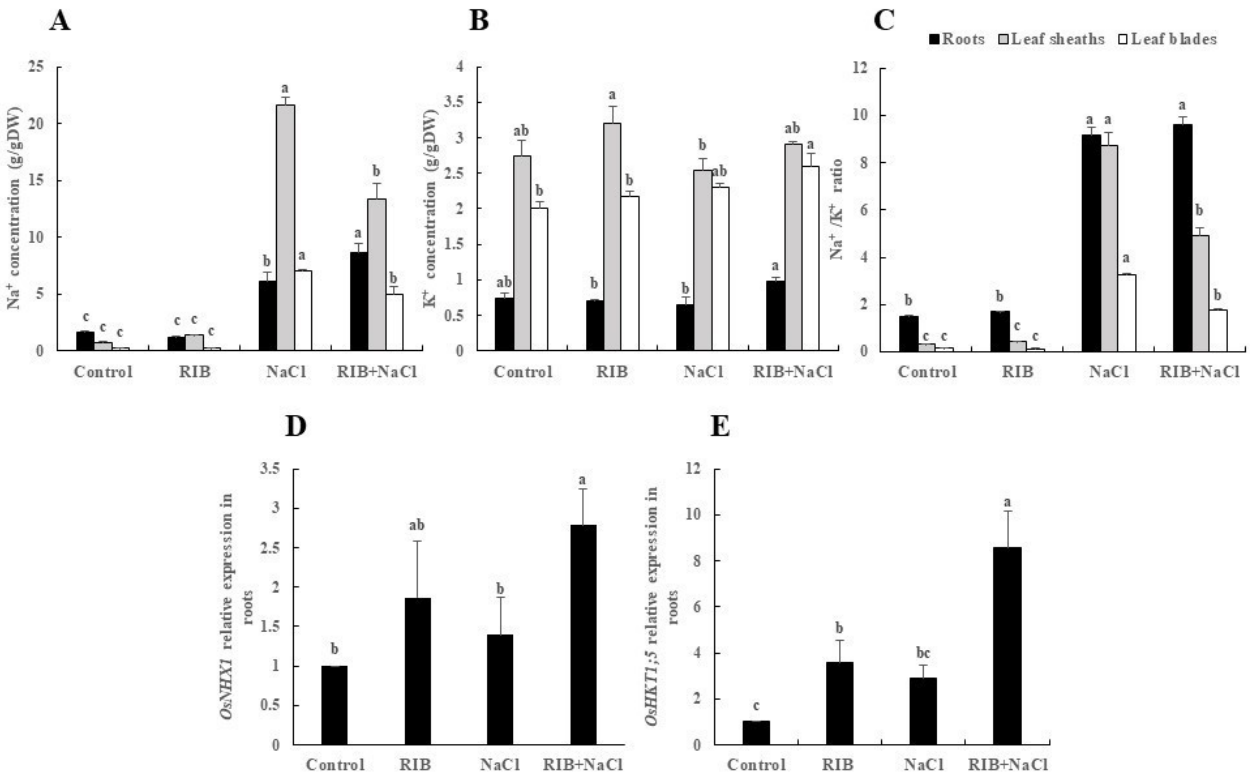


Figure 3.7 RIB-pretreated seedlings maintained favorable Na^+ (A), K^+ (B) concentrations, and Na^+/K^+ ratio (C). The upregulation of *OsNHX1* (D) *OsHKT1;5* (E) under salinity stress. Two-week-old rice seedlings were pretreated with 0.5 μM RIB (RIB-pretreated seedlings) or without (non-pretreated seedlings) for 24 h and then subjected either to control or salinity stress (NaCl) conditions. Values are means ± SE, n=3. The different letter represents a significant difference ($P \leq 0.05$).

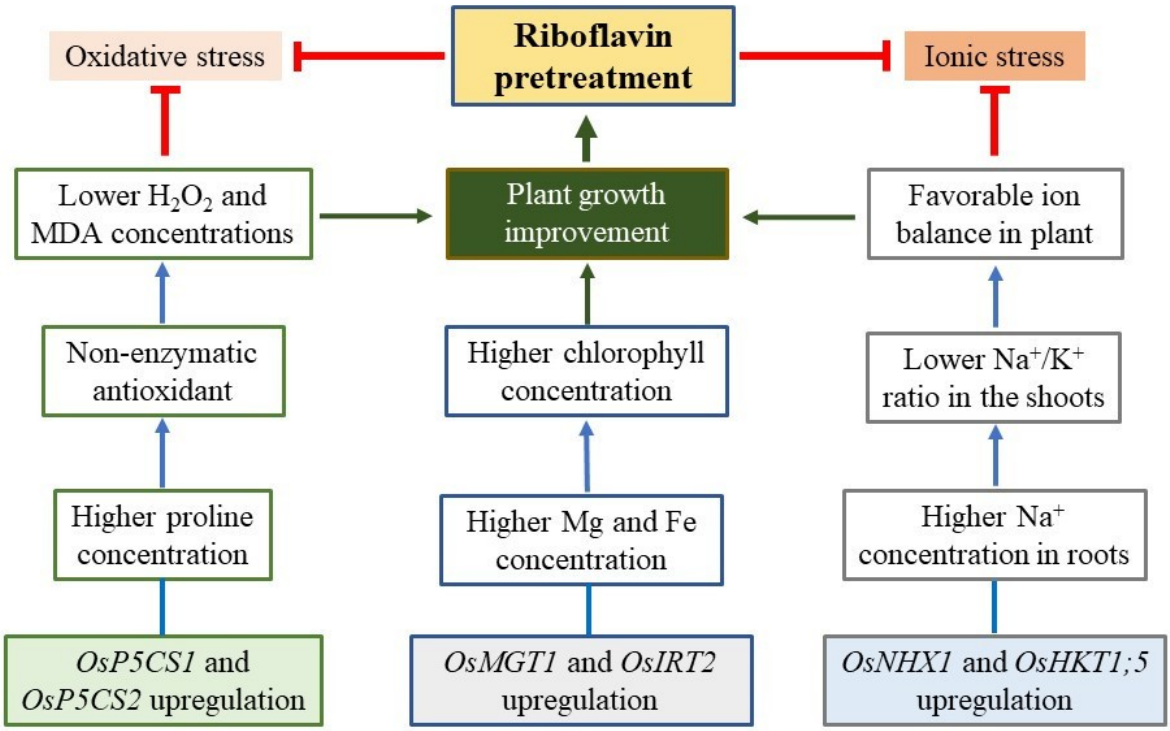


Figure 3.8 Proposed mechanisms of RIB-pretreated seedlings under salinized soil condition.

3.4 Discussion

The consequences of salinity stress in plants include metabolic malfunction, irregular cell division and expansion, reduced photosynthetic activity, increased ion toxicity, and enzymatic disorders that eventually lead to cell death (Munns and Tester, 2008; Assaha et al., 2017). ROS detoxification and ion balance are key indicators of plant survival under salinity stress. To utilize salinized soil, it is important to understand how pretreatment ameliorates salinity stress in plants. In our attempt to understand the role of RIB-pretreated in salinized soil condition, we found that RIB-pretreated seedlings improved plant biomass, proline concentration, and essential element concentrations involved in photosynthesis. Simultaneously, RIB-pretreated seedlings showed reduced lipid peroxidation and Na^+ accumulation in the shoots and retained lower ELR and higher chlorophyll concentrations under salinized soil condition.

ROS, including H_2O_2, superoxide radicals, and hydroxyl radicals, are typically formed in the apoplast and are constitutive components of lipid peroxidation (Mittler et al., 2004). H_2O_2 concentration correlates with MDA concentration, which is an indicator of oxidative stress under biotic and abiotic stresses (Miller et al., 2010). A previous study reported that salinity stress causes cellular damage owing to an increase in H_2O_2 and MDA concentrations and ELR in *Populus cathayana* Rehder (Yang et al., 2009). Electrolyte leakage was used to quantify the amount of damage inflicted on plant tissues under unfavorable conditions (Demidchik et al., 2014). The salt-tolerant rice variety exhibited higher plant biomass and lower ELR and Na^+ concentrations in the leaf blades under

salinity stress (Wangsawang et al., 2018). As shown in Table 3.2, lower H_2O_2 and MDA concentrations and ELR were observed in RIB-pretreated seedlings under salinity stress, implying less cellular damage in RIB-pretreated seedlings under salinity stress. Simultaneously, proline has been reported to be a non-enzymatic antioxidant that limits the oxidative stress caused by ROS overproduction (Alia et al., 2001; Rejeb et al., 2014). The enhancement of proline concentration was observed in the co-culture of *Azospirillum brasilense* the bacteria produce RIB with *Chlorella sorokiniana* under salinity stress (Palacios et al., 2021). *P5CS* upregulation is one of the mechanisms used to overcome osmotic stress in barley (Ueda et al., 2004). Plants use glutamate, which is converted to proline by two successive reductions catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR), as the primary pathway for proline biosynthesis during osmotic stress (Szabados and Savouré, 2010). P5CS, the rate-limiting enzyme in proline biosynthesis, may increase in response to stress, resulting in proline accumulation (Sharma and Verslues, 2010). This is consistent with our findings of higher proline concentrations and upregulation of *OsP5CS1* and *OsP5CS2* expressions (Fig. 3.3ABCDE). This implies that proline acts as a non-enzymatic antioxidant that reduces oxidative stress in RIB-pretreated seedlings under salinity stress.

Furthermore, ROS decreases membrane fluidity and selectivity by causing membrane lipid peroxidation and chlorophyll degradation (Verma and Mishra, 2005). Our results indicate a significant increase in Chl_b and Chl_{a+b} in the RIB-pretreated seedlings under salinity stress (Fig. 3.4), which agrees with the results of Mekawy et al. (2018) on apigenin-

pretreated rice seedlings under salinity stress. Salinity stress affects the light-absorbing structures and governs the state transition of photosynthesis (Chen and Hoehenwarter, 2015). Porphyrin formation is limited by salinity stress, which may decrease the production of proteins that bind to chlorophyll (Abdelkader et al., 2007). The main factors involved in the reduced chlorophyll concentration are not only the inhibition of chlorophyll synthesis but also the stimulation of its degradation by the enzyme chlorophyllase (Santos, 2004). However, either slow synthesis or fast breakdown suggests the existence of a photoprotection mechanism that minimizes light absorbance by reducing the chlorophyll content (Elsheery and Cao, 2008). Notably, the retention of higher chlorophyll concentrations may be related to the reduced oxidative damage in RIB-pretreated seedlings under salinity stress.

Kabata-Pendias (2011) reported that N, P, K, Ca, sulfur (S), Mg, Mn, copper (Cu), and Fe are crucial for physiological processes and plant growth. However, the availability of these elements depends on the soil pH, hydrolytic acidity, granulometric composition, and organic matter content (Kalaji et al., 2018). To further understand this phenomenon in rice, we investigated the P, Mg, Ca, Mn, and Fe concentrations in the roots, leaf sheaths, and leaf blades of non- and RIB-pretreated seedlings under control and salinity stress conditions. Plant growth and development are intertwined with the physiological responses that result from the ion accumulation, macro and micro nutrients are required for plants to adapt to salinity stress (Wang et al., 2003). The accumulation of P, Mg, Mn, and Fe plays a crucial role in the chlorophyll components and photosynthetic reactions (Taiz et al., 2015;

Ebrahimi et al., 2023). Cobalamin (vitamin B12) contributes to photosynthesis as a ring-contracted modified tetrapyrrole containing cobalt (Osman et. al., 2021). Significantly higher chlorophyll concentrations are correlated with higher Mg, Mn, and Fe concentrations in the leaf blades of RIB-pretreated seedlings under salinity stress (Fig. 3.4, Fig. 3.5BDE). RIB pretreatment improves photosynthesis by increasing the concentration of related elements. Two distinct roles of Ca have been well studied: a structural/apoplastic role and a signaling role that initiates plant responses to environmental stimuli (Taiz et al., 2015). ROS and Ca signal work together to maintain cellular pH and ion homeostasis in the early stage of salinity stress in which 3'5'-cyclic guanosine monophosphate triggers Ca import, reduces Na^+ influx, and reduces K^+ efflux (Van Zelm et al., 2021). $MgSO_4$ and $CaSO_4$ applications enhanced plant biomass and chlorophyll concentration in the red clover (*Trifolium pratense*) and tall fescue (*Festuca arundinacea*) under long-term salinity stress (Sharavdorj et al., 2022). Fig. 3.5C showed that RIB-pretreated seedlings under salinity stress had higher Ca concentrations in the leaf blades, implying that Ca may have played a signaling role in maintaining a favorable ion balance. Despite the abundance of Fe in soils, it is mostly found in its oxidized and insoluble form, Fe^{3+}, under aerobic conditions which is generally inaccessible to plants (Ishimaru et al., 2006). This is consistent with our results that the Fe concentration in the roots greatly increased, regardless of the conditions and treatments (Fig. 3.5E). The significantly higher concentration of Mg and Fe prompted us to further analyze the expression of ion transporter genes. *OsMGT1* and *OsIRT2* expressions were upregulated in the roots of RIB-pretreated seedlings (Fig. 3.6AC) which is correlated with the higher Mg and Fe concentrations in the leaf blades of RIB-pretreated

seedlings under salinity stress (Fig. 3.5BE). OsMGT1 is a plasma membrane-localized protein facilitating Mg uptake in plants (Chen et al., 2017). Rice plants have a distinct Fe^{2+} absorption mechanism which associated with *OsIRT1* and *OsIRT2* expressions (Ishimaru et al., 2006). Wang et al. (2019) reported that *OsIRT1* expression was downregulated and *OsIRT2* expression was upregulated in Nippobare rice cultivar implying its role in Fe uptake under flooded conditions. These results suggest that RIB-pretreated seedlings improved the Mg and Fe uptake which in turn increased Mg, Fe, and chlorophyll concentrations under salinized soil condition.

The benefit of Na^+ at low concentrations is that it improves water use efficiency, and stomatal diffusion augments water uptake efficiency (Mateus et al. 2019). Sriskantharajah et al. (2020) reported that 1 mM NaCl is a potent concentration for acclimatizing rice to salinity stress. However, Na^+ overaccumulation causes detrimental cell growth. Under salinity stress, the RIB-pretreated seedlings accumulated lower Na^+ and higher K^+ concentrations in their shoots, which led to higher plant biomass (Fig. 3.1B, Fig. 3.4A). Suppression of Na^+ enhanced apoplastic flow to reduce Na^+ uptake has been observed in rice plants under salinity stress (Sobahan et al., 2009). Lower maintenance of the Na^+/K^+ ratio involves the regulation of Na^+ and K^+ transporter genes, which are critical determinants of salt tolerance (Assaha et al., 2017). It is suggested that RIB-pretreated seedlings may regulate the Na^+ transporter to reduce Na accumulation in the shoots resulting in a lower Na^+/K^+ ratio in shoots. The function of NHXs has been clarified in Arabidopsis (Fukuda et al., 2010). The expression of *OsNHX1* and *OsHKT1;5* was

observed in RIB-pretreated seedlings under salinity stress (Fig. 3.6DE). High Na^+ concentrations and the expression of *OsNHX* are commonly observed together, contributing to Na^+ compartmentalization from the cytosol to vacuoles without detrimental effects on plant growth (Rodríguez-Rosales et al., 2009). RIB seed priming activates the expression of *OsNHXs* family in the roots, resulting in high Na^+ concentration in the roots of the Koshihikari rice-sensitive cultivar under hydroponic conditions (Jiadkong et al., 2022). OsHKT1;5 plays a key role in retrieving Na^+ from the transpiration stream to the xylem parenchyma, and is localized in the plasma membrane (Golldack et al., 2002). The reduced Na^+ concentration in young leaf blades was attributed to the expression of *OsHKT1;5* in rice (Kobayashi et al., 2017). The expression of *OsHKT1;5* was correlated with the expression of *OsMGT1* (Mg transporter), which restricts Na^+ uptake in the shoots (Chen et al., 2017). This is in agreement with our finding that higher Mg concentrations and *OsHKT1;5* expression were observed in RIB-pretreated seedlings under salinity stress (Fig. 3.5B, Fig. 3.6E). This implies that the high Na^+ concentration in the roots is caused by the expression of *OsNHX1* and *OsHKT1;5*, which prevented toxic effects on cellular molecules in the leaf sheaths and blades of RIB-pretreated seedlings.

3.5 Conclusion

The direct application of RIB as a pretreatment for rice seedlings under salinity stress resulted in substantial protection from oxidative and ionic stress injuries. It is worth noting that the mechanisms of RIB-pretreated seedlings under salinized soil condition upregulate

the expression of responsive genes in the rate-limiting step of proline biosynthesis, resulting in an enhancement of proline concentration in the leaf blades, which acts as a non-enzymatic antioxidant. Also, RIB-pretreated seedlings upregulate the expression of Mg and Fe transporter genes, inducing the Mg and Fe concentrations in the leaf blades to retain a higher chlorophyll concentration. Furthermore, RIB-pretreated seedlings upregulate *OsNHX1* and *OsHKT1;5* expressions to positively manage Na^+ overaccumulation by accumulating Na^+ in the roots instead of translocating to the leaf blades to maintain regular enzyme activities. However, further elucidation is needed to ascertain whether the initial trends observed in this study are repeatable at the field scale, and therefore could be advised for soil amendment under salinity stress.

Chapter 4

General discussion

The purpose of this study is (1) to elucidate the physiological responses of IR29 which has been pretreated with tap water (non-pretreated) and RIB-pretreated seedlings under salinity stress by comparing the plant growth, Na^+ and K^+ accumulation patterns under hydroponic and soil-based conditions, (2) to investigate the underlying mechanisms of Na^+ accumulation and RIB biosynthesis by analyzing the expression profiles of the genes that encode a Na^+ transporter and RIB biosynthesis-related genes of the non- and RIB-pretreated seedlings, (3) to unravel the biochemical mechanisms of RIB-pretreated seedlings in salinity stress alleviation that minimizing oxidative stress under both hydroponic and soil-based conditions.

4.1 Exogenous riboflavin (vitamin B2) application enhances salinity tolerance through the activation of its biosynthesis in rice seedlings under salinity stress

The inhibition of plant growth under salinity stress is caused by the limitation of water uptake and excess Na^+ accumulation, as well as oxidative stress (Assaha et al., 2017). Na^+ accumulation and preferable Na^+ management are pivotal under salinity stress. Rice possesses well-developed mechanisms for managing excessive Na^+ concentration in the plant cells under salinity stress conditions; (1) oxidative stress tolerance; the overaccumulation of non- or enzymatic antioxidants to manage the ROS overaccumulation (Van Camp et al., 1996); (2) ionic stress tolerance; the well-regulation of Na^+ or K^+ transporter genes maintaining Na^+ or K^+ homeostasis (Yang and Guo, 2018); and (3) tissue tolerance; Na^+ sequestration from the cytosol into vacuoles to conserve plant metabolism

processes (Munns and Tester, 2008). In the present study, lower H_2O_2 and MDA concentrations were found together with the higher RIB concentration in RIB-pretreated seedlings, indicating that less oxidative damage may be caused by the RIB concentration improvement. Hümbelin et al. (1999) reported that *AtRIBA1* plays a vital role and is recognized as a rate-limiting step in the RIB biosynthesis pathway. The upregulation of *ZmRIBA1* led to an increase in RIB concentration in *Zea mays* (Tian et al., 2022). This is consistent with our result: *OsRIBA1* expression was overexpressed in the roots of RIB-pretreated seedlings, which in turn enhanced RIB concentration under salinity stress. *In vitro* assays extrapolated that the concentration of H_2O_2 was reduced as RIB concentration rose, suggesting the role of RIB under salinity stress as a non-enzymatic antioxidant scavenging H_2O_2 concentration. This is consistent with our result: *OsRIBA1* was overexpressed in the roots of RIB-pretreated seedlings, which in turn enhanced RIB concentration under salinity stress. These results suggested that RIB-pretreated seedlings contribute to oxidative stress tolerance. Na^+ gets into plant cells through HKT2 (Golldack et al., 2002). However, plants have several strategies to deal with the toxic ion overaccumulation (Munns and Tester, 2008). Na^+ exclusion from the leaf sheath, reducing Na^+ transport to the leaf blades, is the contribution of *OsHKT1;4* expression (Wangsawang et al., 2018). Na^+ compartmentalization from cytosol to vacuole, allowing the plant to continue its metabolism under salinity stress, which is known to be the function of the NHX family (Fukuda et al., 2010). It is delineated that RIB-pretreated seedlings possessed high Na^+ in the roots and low Na^+ in the leaf blades, which were attributed to downregulate the expression of *OsHKT2;1* and upregulate the expression of *OsHKT1;4*, *OsNHX1*. These

results indicated that RIB-pretreated seedlings activated ionic stress and tissue tolerance mechanisms.

4.2 Effect of riboflavin application on rice growth under salinized soil conditions

Global sea-level rise is an environmental condition resulting from ongoing climate change. According to Machado and Serralheiro (2017), salts can affect up to 50% of irrigated land. However, salinized soil is caused by climate change as well as agricultural malpractice, which inevitably leads to tremendous losses in the agricultural sector (Roy et al., 2014). Thus, the study on understanding plant responses under salinized soil condition and the strategies to improve crop production are essential to meet increasing future demands. The present study aimed to analyze the effect of direct application on non- and RIB-treated seedlings under salinized soil condition. Salinity stress involves multigenetic traits and has multidimensional effects, including osmotic stress and ionic stress, which lead to oxidative stress involving the overaccumulation of ROS (Van Zelm et al., 2020). A number of studies showed that maintaining low ROS accumulation is one of the keys to withstand salinity stress (Assaha et al., 2015; Mekawy et al., 2018). Proline is a notable osmo-protectant response to salinity stress and has been considered a non-enzymatic antioxidant that limits the oxidative stress caused by ROS overproduction (Alia et al., 2001; Ueda et al., 2004). Sharma and Verslues (2010) suggested that P5CS is known as a rate-limiting step of proline biosynthesis. In this study, we found that RIB pretreatment

minimized oxidative damage by activating the rate-limiting step of proline biosynthesis, enhancing proline concentration, which is also considered oxidative stress tolerance. Simultaneously, ionic stress and tissue tolerance were also triggered in RIB-pretreated seedlings under salinized soil condition by upregulating *OsHKT1* and *OsNHX1* expressions. Preferable physiological processes and plant growth require the optimal accumulation of macro- and micronutrients (Kabata-Pendias, 2011). The main factor decreasing chlorophyll concentration is the inhibition of chlorophyll synthesis (Santos, 2004). Taiz and Zeiger (2014) reported that Mg and Fe are responsible for chlorophyll synthesis. In the present study, higher Mg and Fe concentrations were observed together with a higher chlorophyll concentration in the leaf blades of RIB-pretreated seedlings. Simultaneously, *OsMGT1* and *OsIRT2* expressions were observed in the roots of RIB-pretreated seedlings. It is suggested that the enhancement of element concentration contributed to not only oxidative stress and ionic stress tolerance but also chlorophyll synthesis. Taken together, the better plant growth of RIB-pretreated seedlings under salinized soil condition is also associated with the stimulation of oxidative stress, ionic stress, and tissue tolerances.

4.3 Conclusion

This study aimed at investigating the physiological and biochemical responses of the salt-sensitive rice variety (IR29) under hydroponic and soil-based conditions. In comparison between non- and RIB-pretreated seedlings, the growth parameters, Na^+, K^+, and other element concentrations, ROS and lipid peroxidation concentrations, and gene expression

of Na^+ transporter genes, RIB, and proline biosynthesis-related genes were analyzed. It was demonstrated that RIB-pretreated seedlings are relatively salinity-alleviated compared to the non-pretreated seedlings, and this was evident in higher plant biomass, a lower Na^+/K^+ ratio in the leaf blades, and lower H_2O_2 and MDA concentrations. As well as the ability to manage Na^+ uptake from roots by downregulating *OsHKT2;1* and Na^+ accumulation in the leaf blades by upregulating *OsHKT1;4*, *OsHKT1;5,* and *OsNHX1* expressions. Notably, RIB-pretreated seedlings induced the rate-liming step (*OsRIBA1*) in the RIB biosynthesis pathway under hydroponic conditions. Under soil-based conditions, RIB-pretreated seedlings upregulated the rate-limiting steps (*OsP5CS1* and *OsP5CS2*) in proline biosynthesis pathways and the Mg and Fe transporter-responsive genes (*OsMGT1* and *OsIRT2*) which in turn improving chlorophyll concentration under salinity stress. Whereas, RIB and proline were recognized as non-enzymatic antioxidants attributing to ROS detoxification. Overall, these studies extrapolated that the salinity-alleviated effect of RIB pretreatment relies on its ability to activate mechanisms of oxidative stress, ionic stress, and tissue tolerance.

The potential of RIB pretreatment may be adopted as a standard practice, fortifying the crops against the uncertainties of climate change and soil salinity by mitigating oxidative stress, maintaining ion homeostasis, and enhancing tissue tolerance. This study paves the way for a more resilient global food supply chain, holding the promise of increased food security, especially in regions where salinity stress.

Summary

Salinity stress is a major abiotic stress that leads to economic loss in the agricultural sector. With the challenge of an increasing global population and the limitation of food production, several researches have been conducted to alleviate and solve this problem. Nowadays, pretreatment has gained more attention due to its efficiency and simple methodology, as well as its lower time consumption in comparison with plant breeding. RIB is a vital component required for fundamental metabolism, a precursor of the coenzymes, and is known for biotic and abiotic stress alleviation. However, the information on RIB pretreatment in rice seedlings to confer salinity stress remains limited. Thus, the present study aimed to evaluate the underlying mechanisms of RIB pretreatment in the IR29 salt-sensitive variety under both hydroponic and soil-based conditions. Also, to investigate the physiological and biochemical responses in plant biomass, the Na^+/K^+ ratio, the accumulation of ROS detoxification compounds, element and chlorophyll concentrations, and the expression profiles of some genes encoding Na^+ transporter genes. Further, to identify the alteration of RIB and proline biosynthesis-related genes, which could also contribute to salinity-alleviated mechanisms of RIB pretreatment.

1. Exogenous riboflavin (vitamin B2) application enhances salinity tolerance through the activation of its biosynthesis in rice seedlings under salinity stress

To elucidate the effect of salinity stress on RIB biosynthesis and the role of RIB pretreatment in the IR29 rice salinity-sensitive cultivar Two-week-old IR29 were pretreated with tap water as non-pretreated seedlings, and 0.75 μM RIB was used for RIB-

pretreated seedlings cultivating under 50-mM NaCl-induced salinity stress for two weeks. Our results indicated that RIB-pretreated seedlings were relatively salinity-alleviated in comparison with the non-pretreated seedlings. It was evident in its higher plant biomass, lower ROS and lipid peroxidation concentrations, maintaining favorable Na^+, K^+, and other element concentrations, and positive regulation of the gene-encoding Na^+ transporter genes, RIB, and proline biosynthesis-related genes. On the contrary, the non-pretreated seedlings accumulated higher Na^+ in the leaf blades, with reduced plant growth and higher H_2O_2 and MDA concentrations in the leaf blades. Therefore, the adaptation of RIB-pretreated seedlings to salinity stress is associated with a reduced H_2O_2 and MDA concentration, which might be due to the higher RIB concentration that functions as a non-enzymatic antioxidant scavenging ROS overproduction. In addition, *OsRIBA1* expression was highly upregulated in RIB-pretreated seedlings, which is known as the most significant gene in the RIB biosynthesis pathway influencing RIB concentration. Also, RIB-pretreated seedlings possessed a higher Na^+ concentration in the roots instead of the leaf blade, which may intertwine with the upregulation of *OsNHX1* expression in the roots and *OsHKT1;4* expression in the leaf sheaths, playing a vital role in minimizing Na^+ accumulation in the leaf blades. These findings suggested that RIB pretreatment ameliorates salinity stress in rice by improving (1) oxidative stress tolerance, as increased RIB concentration may function as a non-enzymatic antioxidant; (2) ionic stress tolerance, as RIB pretreatment limits Na^+ accumulation in the leaf blades and maintains a favorable Na^+/K^+ balance; and (3) tissue tolerance, as root Na^+ concentration is high, implying that Na^+ may be accumulated in the vacuole instead of the cytoplasm.

2. Effect of riboflavin application on rice growth under salinized soil conditions

To further analyze the effects of RIB direct application and changes caused in the physiological and biochemical properties under salinized soil condition. Two-week-old IR29 were pretreated with tap water as non-pretreated seedlings, and 0.5 μM RIB was used for direct application as RIB-pretreated seedlings cultivating under a 50-mM NaCl-induced salinized soil condition for two weeks. Lower plant biomass, chlorophyll concentrations, a higher Na^+/K^+ ratio in the leaf blades, and H_2O_2 and MDA concentrations were observed in the non-pretreated seedlings. In contrast, RIB-pretreated seedlings possessed higher plant biomass, Mg, Fe, and chlorophyll concentrations, together with a lower Na^+/K^+ ratio in the leaf blades and H_2O_2 and MDA concentrations. Additionally, higher proline concentrations were correlated with the upregulation of the rate-limiting step of the proline biosynthesis pathway in the leaf blades of the RIB-pretreated seedlings, which may act as a non-enzymatic antioxidant, reducing oxidative damage. As well as the higher Mg, Ca, Mn, and Fe concentrations in the leaf blades, which led to better chlorophyll concentration improvement. It is congruent with the *OsMGT1* and *OsIRT2* expressions which function in Mg and Fe transports in RIB-pretreated seedlings. The upregulation of *OsHKT1;5* and *OsNHX1* expressions in the root was associated with the lower Na^+ concentration in the leaf blade of RIB-pretreated seedlings, decreasing the Na^+ in the xylem sap and accumulating Na^+ in the root's vacuoles. Collectively, the mechanisms of RIB pretreatment that improve plant growth under salinized soil condition are achieved by (1) the improvement of proline concentration, minimizing oxidative stress; (2) the enhancement

of related chlorophyll synthesis element concentration, maintaining a higher chlorophyll concentration; and (3) *OsHKT1;5* and *OsNHX1* expressions, reducing Na^+ concentration in the leaf blades.

This study has revealed that RIB pretreatment ameliorated salinity stress in rice salinity-sensitive varieties under both hydroponic and soil-based conditions by uplifting the plant's developed mechanisms of oxidative stress tolerance, ionic stress tolerance, and tissue tolerance. Nevertheless, observing the downstream regulation of RIB and proline biosynthesis, ROS signaling pathways, and the relation between RIB and ion homeostasis is also crucial to understanding the molecular mechanism in salinity-alleviated plants. In addition, further elucidation is needed to ascertain the underlying mechanisms and whether the initial trends observed in this study are repeatable at the field scale and therefore could be advised for soil amendment under salinity stress.

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