Doctoral Thesis

Molecular Physiological Characterization of Tissue Tolerance in Rice under Salt Stress

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List of Abbreviations

DEGs	Differentially expressed genes
DW	Dry weight
EC	Electrical conductivity
ELR	Electrolyte leakage ratio
FAO	Food and Agriculture Organization
FW	Fresh weight
ICP	Inductively coupled plasma
MDA	Malondialdehyde
PCR	Polymerase chain reaction
qRT-PCR	Qualitative real-time polymerase chain reaction
RNA-Seq	RNA sequence
ROS	Reactive oxygen species
SES	Standard evaluation score
WC	Water content

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Chapter 1

General Introduction

1.1 Soil salinity

Soil salinity is the accumulation of salts into levels that impact natural assets such as plants, animals, and aquatic ecosystems (Brouwer and Jenkins, 2005). Soil is defined as saline when the electrical conductivity (EC) exceeds 4 dS/m (deciSiemens per meter), which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPs (Munns and Tester, 2008). Accumulation of excess salt damages the soil structure and causes particle aggregation due to slaking, swelling, and dispersion effects (Machado and Serralheiro, 2017). High Na^+ concentration in soils also caused the increase of soil pH (alkalization), leading to decreased nutrient availability.

The underlying salinization process can be natural or primary and human-induced (anthropogenic) or secondary. The main causes of primary salinity are natural processes such as the parent rock from which the soil was formed, seawater intrusion, and mobility of salts from the soil to the surface through capillary action and rainfall (Ashraf and Munns, 2022). Climate change, which leads to global warming, also contributes to salinization by increasing evaporation in periods of drought (Van Zelm et al., 2020). When the water evaporates, the salts remain and accumulate in the soil. This condition mostly occurs in arid and semi-arid areas with a high evaporation rate and temperature.

Secondary salinization is caused by agricultural practices such as irrigation and the application of organic/inorganic soil amendments (Ondrasek et al., 2011). Irrigation combined with poor drainage is the most serious salinization, representing the loss of once-productive agricultural land (Zhu, 2001). Irrigation water quality is an essential factor in determining the sustainability of agriculture on salt-impaired lands (Grieve et al., 2012). Most of the irrigation water contains salts. After irrigation, the water is either used by the crop or evaporates. When the

water evaporates, the salt accumulates in the soil. Primary salinity is more widespread, while secondary salinity continues to grow (Munns, 2005).

Over 800 million hectares of land worldwide are affected by salt (Munns and Tester 2008), making it a global concern reducing agricultural productivity. Salt-affected soil also causes economic loss. It has been estimated to incur $27.3 billion in agricultural damages annually (Qadir et al., 2014). Compared to normal soils, saline soils have excessive soluble salts such as NaCl, Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl, and Na_2CO_3, each of which contributes to salt stress, but NaCl is the most prevalent salt and has been the focus of much work on salinity to date (Munns and Tester, 2008; Tavakkoli et al., 2010). Many of the salt ions are toxic to plant cells when present at high concentrations externally or internally (Zhu, 2001).

1.2 Plant responses to salt stress

Salt-affected soils have a high content of soluble salts/or high amounts of Na^+. The toxic mineral of salt causes the soil to undergo a nutrient imbalance and lessens water availability. The salts hold water in the soil at high osmotic potential, which limits the easy exchange of water and nutrients with plant roots (FAO, 2020). These conditions negatively affect the growth and development of the plants. The destructive effects of salt stress on plants result from a complex interaction between morphological, molecular, physiological, and biochemical processes, including the inhibition of nutrient uptake, which disrupts ion homeostasis. High saline conditions inhibit nitrate absorption and can be deleterious for Ca^{2+} nutrition, as Ca^{2+} deficiency can affect membrane function and plant growth (Grieve et al., 2012; Läuchli and Grattan, 2007).

Plant responses to salt stress occur in two distinct phases: osmotic stress—a rapid response to an increase in external osmotic pressure—and ionic stress—a slower response due to the

accumulation of Na^+ in leaves (Munns and Tester 2008). Osmotic stress starts immediately after the salt concentration around the roots increases to a threshold level, significantly reducing shoot growth. Within minutes, the cellular turgor is reduced, and cell expansion is decreased (Almeida et al., 2017). Osmotic stress is also known as water stress because water is unavailable to plants owing to the high solutes in the soil or medium, negatively affecting the root's water uptake. In this condition, the plant faces a dilemma because it needs to restrict transpiration by stomatal closure to reduce the water lost, but it will reduce the amount of CO_2 that can be fixed and used for growth. Consequently, the growth rate is reduced, resulting in smaller leaves, shorter stature, and fewer leaves. The cellular and metabolic processes involved in the initial stage of the salt stress are in common to drought-affected plants (Munns, 2005).

Ionic stress begins when salt accumulates to toxic concentrations in leaves, leading to nutritional disorders, alteration of metabolic processes, membrane disorganization, and reduced cell division and expansion (Hasegawa et al. 2000; Munns 2002; Munns and Tester 2008; Zhu 2001). During this stage, the salt taken up by the plant accumulates in old leaves, continues transport into transpiring leaves over a long period, and eventually results in very high Na^+ and Cl^- concentrations, then the leaves die (Munns, 2005). In these conditions, the leaf tissue also encounters injury, which is probably caused by the salt load exceeding the ability of cells to compartmentalize salts in the vacuole, then flow through the cytoplasm and inhibit enzyme activity (Munns, 2005). Over days or weeks, Na^+ and Cl^- accumulate into toxic levels in the cytosol, slowing down metabolic processes and inhibiting enzyme activity, causing early flowering, premature senescence, and cell death (Roy et al., 2014).

Root-to-shoot signaling upon salt stress induces the synthesis of abscisic acid (ABA). This plant hormone reduces stomatal conductance, causing decreased CO_2 uptake, lower carbon

assimilation rate, and biomass production (Hasegawa, 2013; Munns, 2005; Munns and Tester, 2008). Treating salt-stressed plants with increased CO_2 concentrations to overcome stomatal initiation did not improve photosynthesis rates (Bose et al., 2017). Therefore, a non-stomatal component probably reduces photosynthetic performance under salt stress. Both oxidative stress due to the production of reactive oxygen species (ROS) and the excessive accumulation of Na^+ and Cl^- in the cytosol disrupting K^+ homeostasis are likely responsible for the non-stomatal limitations of photosynthesis (Munns and Tester, 2008).

The underlying cause for the ion toxicity of Na^+ is not only the increased Na^+ content but also the disrupted Na^+/K^+ ratio in the cytosol. K^+ is an essential macronutrient, as it is involved in enzyme activation, turgor generation, osmotic adjustment, cytoplasmic pH homeostasis, and regulation of membrane potential (Almeida et al., 2017; Barragán et al., 2012). Na^+ is similar to K^+ in chemical properties so that Na^+ can act as a competitive inhibitor of K^+ and block K^+ binding sites of enzymes (Hasegawa et al., 2000). The effect of this competitive inhibition increases with increasing Na^+/K^+ ratio because more Na^+ ions are present and more likely to bind to an open binding site, impairing enzymatic activity (Munns et al., 2006). Over 50 different cytoplasmic proteins, including pyruvate kinase, phosphofructokinase, membrane-bound ATPases, and vacuolar pyrophosphatase, require K^+ for activation. Moreover, K^+ is involved in tRNA binding to ribosomes, stomatal regulation, and maintaining a pH gradient over the thylakoid membrane for ATP synthesis. Therefore, a disrupted K^+ homeostasis causes severe impairment of metabolism and protein biosynthesis (Marschner and Marschner, 2012). Due to the similar chemical properties, a high concentration of Na^+ can also disrupt the transport of K^+ through plant cell membranes. Enhanced Na^+ uptake leads to depolarization of the plasma membrane, which activates

depolarization-activated outward rectifying K^+ channels, leading to K^+ efflux (Shabala and Cuin, 2008).

Plants can be primarily divided into two groups based on the effect of salt on plant growth: glycophytes, which are plants sensitive to salt stress, and halophytes, which are plants that grow naturally under high salinity and generally tolerate high salt concentrations (Hasegawa et al. 2000; Reddy et al. 2017a; Zhu, 2001). Most crop plants, including rice, the most widely consumed grain worldwide, are glycophytes that cannot tolerate salt stress. All major metabolic activity of rice, including cell wall damage, cytoplasmic lysis, and damage to endoplasmic reticulum in leaf blades within 1 d of salt treatment, increase in proline concentrations by 4- to 20-fold, decrease in Fv/Fm ratio, reduction in photosynthesis, and overall decline in germination and seedling growth (Sahi et al., 2006).

1.3 Salt tolerance mechanism in plants

Plants are salt-tolerant when they can maintain good growth and metabolism under salt-stress conditions. Munns (2005) describes that salt tolerance comes from genes that limit the rate of salt uptake from the soil and the transport of salts throughout the plant, adjust the ionic and osmotic balance of cells in roots and shoots, and regulate leaf development and the onset of senescence. Moreover, Hasegawa et al. (2000) define determinants of salt stress tolerance as effector molecules (metabolites, proteins, or components of biochemical pathways) that lead to adaptation and as regulatory molecules (signal transduction pathway components) that control the amount and timing of these effector molecules.

Plants employ plenty of strategies to cope with salt stress. The most common include a reduction of the quantity of salt gathered by the roots, and its apportion at the tissue and cellular

levels to prevent the accumulation of toxic concentrations in the cytosol. Ion homeostasis, osmotic adjustments, redox equilibrium, and growth regulation are among the distinct mechanisms that plants employ to deal with salt stress (Rodríguez Coca et al., 2023). These mechanisms are attained through adapting physiological and biochemical modifications enabled by the expression of several salt-responsive genes. These involved activations of functional protein-coding genes comprising osmoregulatory genes, antioxidant proteins, transporters/antiporters, transcription factors (TFs), and signal-associated protein kinases. These strategies and mechanisms provide diverse levels of tolerance to different plants. Plants considered salt-tolerant can show effective mechanisms of stress sensing, signal transduction, and gene expression or feasibly disparate metabolic pathways. Understanding the salinity tolerance mechanism in rice can be important information for developing plant resistance to salt stress.

Plants experience a series of adaptations to cope with salt stress, including morphological, physiological, and biochemical changes (Acosta-Motos et al., 2017). Specific morphological and anatomical changes accompany most salinity adaptive mechanisms in plants. Often found in coastal salt marshes, tidal zones, and saline deserts, halophytes developed various strategies to survive in highly salty environments. Some halophytes have salt glands to secrete excess salts, or bladder cells, which accumulate salt and then abscise (Dassanayake and Larkin, 2017). Unlike the halophytes, glycophytes, which are very sensitive to saline conditions, rarely have morphological and anatomical changes.

In terms of glycophytes, there are three different mechanisms of salt tolerance, i.e., 1) tolerance to osmotic stress, 2) Na^+ exclusion from leaf blades, and 3) tissue tolerance (Munns and Tester, 2008). The reduced rate of photosynthesis due to less availability of CO_2 during the osmotic stress induces the formation of ROS and increases the activity of enzymes such as superoxide

dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and various peroxidase to detoxify these species (Apel and Hirt, 2004; Foyer and Noctor, 2005; Munns and Tester, 2008). The ability of plants to detoxify ROS is one of the mechanisms to achieve osmotic stress tolerance. An increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) is also considered a coping strategy for osmotic stress. The extracellular addition of Na^+ is apparently able to activate the flux of Ca^{2+} into the cytosol across the plasma membrane. $[Ca^{2+}]_{cyt}$ activates the synthesis of salt overly sensitive (SOS) protein, which facilitates the Na^+ exclusion in the root (Kader and Lindberg, 2010; Zhu, 2002).

Na^+ exclusion and tissue tolerance are two mechanisms to deal with ionic stress. Na^+ exclusion by roots will prevent Na^+ from accumulating into toxic concentrations within leaves. The inability to exclude Na^+ leads to its accumulation in leaves, disrupting many physiological processes and causing premature death of older leaves. However, Na^+ and Cl^- cannot be excluded entirely from shoot and leaf cells, as plants accumulate both for osmotic adjustment. Thus, ion uptake must be highly regulated to avoid cytotoxic effects. Plants developed several mechanisms to reduce Na^+ concentration in the transpiration stream and prevent Na^+ accumulation in metabolically active cells. Those mechanisms are 1) strictly regulating ion uptake by the root cells, 2) restricting xylem loading and preferential loading of K^+ over Na^+, and 3) unloading or removing Na^+ from the xylem in the upper root parts and retaining Na^+ in the root (Munns, 2002).

Under salt stress conditions, Na^+ enters the roots via passive transport, mainly mediated by non-selective cation channels (NSCCs) (Blumwald, 2000). Most of the Na^+ is then transported out via a Na^+/H^+ antiporter called salt overly sensitive 1 (SOS1) (Tester and Davenport, 2003). SOS1 is localized in epidermal cells in the root apex, where it actively extrudes Na^+ from the cytosol into the rhizosphere (Shi et al., 2002). SOS 1 also affects the partitioning of Na^+ between the plant organs (Olías et al., 2009). However, in susceptible plants, SOS1 may not work effectively, and

the remaining Na^+ could be transported to the xylem stream via symplastic flow across plasma membranes or apoplastic flow without crossing the plasma membrane (Almeida et al., 2017). Na^+ in the apoplastic flow is restricted by the Casparian strip, a hydrophobic barrier located at the root endodermis, which avoids Na^+ movement into the stele (Flowers and Colmer, 2015). Subsequently, Na^+ in the symplastic flow enters the xylem vessels. Na^+ can accumulate in the pericycle and xylem parenchyma cells instead of being transported into the xylem vessels. Within these cells, Na^+ mainly accumulates in the vacuole, and Na^+/H^+ antiporters (NHX) at the tonoplast are presumed to be responsible for this process (Apse and Blumwald, 2007; Hasegawa, 2013). Na^+ in the xylem vessels can be reabsorbed from the xylem sap into the root cells to prevent Na^+ accumulation in the above-ground tissue. This Na^+ retrieval from the xylem is facilitated by the HKT1, family of high-affinity K^+ transporters (HKTs) (Hasegawa, 2013; Munns and Tester, 2008). HKT transporters can retrieve Na^+ from the xylem and contribute to Na^+ exclusion from leaves when expressed in the xylem parenchyma cells. Rice (O. *sativa* cv. Nipponbare) possesses nine *HKT* genes in its genome, with two considered pseudogenes (Garciadeblás et al., 2003).

The HKT1;4 and HKT1;5 transporters were considered particularly important in retrieving Na^+ from the transpiration stream, a process that prevents further transport of Na^+ to leaves (Davenport et al., 2005). Cotsaftis et al. (2012) explained a two-staged model of Na^+ exclusion in rice. OsHKT1;5 present in xylem parenchymal cells pump Na^+ back into the root to minimize the amount of Na^+ reaching the shoot. This root-to-shoot Na^+ transfer mechanism represents the first stage of a Na^+ exclusion model in rice. The remaining Na^+ ions that arrive into the shoot are diverted into different leaves. There, OsHKT1;4 proteins load the sheath tissues with Na^+ before they can reach the photosynthetic part of the shoot, i.e., the blades. This sheath-to-blade Na^+ transfer mechanism represents the second stage of the Na^+ exclusion model in rice.

1.4 Tissue tolerance mechanism

Tissue tolerance is the tolerance of tissue to accumulated Na^+, which requires compartmentalization of Na^+ at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm, especially in mesophyll cells in the leaf (Munns and Tester, 2008). In this process, Na^+ is sequestered into the vacuoles to maintain its low concentration in the cytosol, where most cellular metabolism occurs. Munns et al. (2016) explained in more detail that tissue tolerance is the capacity of the tissue to function while containing high levels of internal Na^+ or Cl^-. Tolerance of leaf tissue to high Na^+ concentrations is an adaptive mechanism, as exemplified by most halophytes (Flowers and Colmer, 2015) and glycophytes such as barley (James et al., 2006) and wild rice (Prusty et al., 2018).

Tissue tolerance is a complex physiological trait composed of multiple 'sub-traits', which include Na^+ compartmentalization, K^+ retention, and osmotic tolerance (Niu et al., 2018). The main component of tissue tolerance is the compartmentalization of ions within vacuoles, which involves ion transporters in the tonoplast, particularly vacuolar Na^+/H^+ antiporters (NHXs) (Flowers and Colmer 2015). NHXs catalyze the exchange of Na^+ for H^+ across membranes and regulate the cytoplasm's internal pH, cell volume, and Na^+ levels (Fukuda et al., 2011). NHXs not only play a role in Na^+ compartmentalization but also mediate K^+ accumulation in the vacuole, thus regulating osmotic adjustment, cell expansion, and intracellular K^+ homeostasis (Almeida et al., 2017; Hasegawa, 2013). Na^+ is partitioned either within specialized cells (e.g., leaf epidermis and epidermal bladder cells in halophytes (Shabala et al., 2014)) or is sequestered in the vacuoles of the leaf mesophyll (Munns and Gilliham, 2015).

Fukuda et al. (2011) identified five NHX-type antiporter genes in rice (*OsNHX1* through *OsNHX5*). Studies in Arabidopsis and rice shown that *ATNHX1* and *AtNHX2,* as well as *OsNHX1, OsNHX 2, OsNHX 3,* and *OsNHX 5,* are induced by hyperosmotic stress, ABA treatment, and salt stress, thus strongly suggesting that NHXs plays a crucial role in salinity stress response (Almeida et al., 2017; Fukuda et al., 2011). Intracellular NHX proteins are also involved in K^+ homeostasis; for example, NHX1 and NHX2 are essential for active K^+ transport into the vacuole (Barragán et al., 2012).

Another aspect of tissue tolerance is K^+ retention. Enhanced K^+ retention and the ability of a cell to maintain cytosolic K^+ homeostasis correlate with salinity tolerance in a broad range of plant species and are essential for preventing salinity-induced programmed cell death (Niu et al., 2018). High cytosolic K^+ levels are also necessary to maintain high vacuolar H^+-PPase activity, thus enabling the operation of tonoplast NHX proteins that mediate vacuolar Na^+ sequestration (Shabala, 2003). K^+ is also the major inorganic osmolyte for tissue osmotic adjustment under stress conditions.

The last component of the tissue tolerance mechanism is an osmotic tolerance (Munns et al., 2016). In plants processing tissue tolerance, accumulating Na^+ can be used as a low-energy-cost osmolyte to adjust cell turgor and tissue growth under salt stress (Munns and Gilliham, 2015). In addition, the immediate constraint imposed by salinity is an osmotic stress. Increased vacuolar Na^+ concentrations would require a coordinated increase in the osmotic pressure of the other subcellular compartments, including the cytosol, to maintain their volume. This can be achieved by increasing the concentration of K^+ to sub-toxic levels and the concentration of compatible solutes (Munns and Tester, 2008). Organic solutes compatible with metabolic activity must accumulate in the cytosol and organelles to balance the osmotic pressure of the ions in the vacuole

(Munns et al., 2016). To facilitate osmotic adjustment, plants synthesize osmoprotectants such as proline, glycine betaine, polyamines, and sugar alcohols (polyols). The synthesis of compatible solutes requires high energy and hence involves a potential growth penalty.

1.5 Rice sensitivity to salt stress

The sensitivity of rice to salt stress varies with growth stage and cultivars. Rice is relatively salt-tolerant at the germination stage and very sensitive at the young seedling stage (Walia et al., 2007). Several rice cultivars and landraces have been reported to be salt-tolerant. Pokkali and Nona Bokra, two indica rice landraces originally from India known to be salt-tolerant. These two genotypes have been frequently used as a donor of salt-tolerance traits in breeding programs. Zeng et al. (2003) identified one of the most salt-tolerant genotypes, IR63731, an indica line developed from a cross between tolerant landrace Nona Bokra and sensitive cultivar IR28. This genotype had the most favorable combination of salt tolerance at early vegetative and panicle initiation stages (Walia et al., 2007). Another genotype, Agami, a salt-tolerant japonica subspecies cultivar grown in Egypt, was ranked high for its ability to yield high under salt stress (Walia et al., 2007). A recombinant inbred population developed at the IRRI (International Rice Research Institute) using Pokkali and IR29 identified a salt-tolerant inbred line (RIL) FL478, a F2-derived F8 which has salt tolerance higher than or comparable to the tolerant parent Pokkali (Walia et al., 2005). FL478 has been widely used as the salt-tolerant check for comparative study.

Several screening and comparative studies of salt tolerance between rice genotypes identified rice landraces and cultivars with higher salt tolerance levels than the elite cultivars. Lee et al. (2003) suggested that the tolerance level of indica (Pokkali from India, TCCP 266 and IR45427 from the Philippines) was higher than that of japonica (Namyang 7 from Korea and

Agami M1 from Egypt). Screening the salt tolerance of 116 Asian rice cultivars by Alkahtani and Dwiningsih (2023) also suggested that Pokkali, TCCP 266, and IR45427 are recommended as valuable germplasm resources for Asian rice breeding programs in saline agricultural areas. Chakraborty et al. (2012) compared four indica rice genotypes (FL478, Kamini, AC847, and IR29) and suggested that Kamini, a genotype originally from mangrove regions of Sunderbans India, has a unique mechanism that could effectively balance both ionic selectivity and tissue tolerance to achieve considerable salt tolerance. Wangsawang et al. (2018) screened japonica rice cultivars and identified a highly salt-tolerance cultivar (Oukan383) with an effective Na^+ exclusions mechanism at the leaf sheath to prevent Na^+ accumulation in the leaf blades.

1.6 Study rationale

The world population has increased rapidly over the last few centuries (Ritchie et al., 2023), creating a major socioeconomic challenge: how to sustainably feed this rapidly growing population. Increasing the productivity of crop plants is essential to meet the increased food demand in the future. Salt-affected soils in arid and semi-arid regions caused considerable agronomic problems. Rice is one of the most important crops that suffered productivity decline due to salinity as it is considered the most salt-sensitive cereal crop. Accumulation of excess Na^+ disrupts the growth and development of rice plants. Therefore, improving rice resistance to salt stress is urgently needed.

Identifying and developing rice varieties with high tolerance to salt stress is one of the attempts to overcome the decrease in rice production. Rice genotypes with high salt tolerance can provide donor alleles to develop high-salt-tolerant varieties through rice breeding programs. Although breeding is a conventional technique to develop a plant with a superior trait, it still

becomes a major goal in crop plants to ensure food sustainability. Most of the identified salt-tolerant rice is an indica rice subspecies. However, only a few studies identified japonica rice as tolerant to salt stress. Therefore, screening japonica rice varieties and landraces for salt tolerance will improve the genetic resources' potential for salt tolerance traits.

Understanding the mechanism of salt tolerance based on physiological and molecular approaches can accelerate the development of rice varieties with high salt tolerance by genetic engineering techniques. Hence, this study attempts to elucidate the physiological and molecular characterization of salt-tolerant japonica landrace. Furthermore, transcriptomic analysis was conducted to understand the molecular changes associated with salt tolerance in the japonica landrace. Identifying genes associated with salt tolerance will give a better understanding in molecular mechanisms underlying salt tolerance and further develop a salt-tolerant rice variety.

1.7 Study objectives

1) To identify rice genotypes from several japonica rice landraces tolerant to salt stress conditions during the vegetative stage.
2) To characterize physiological and molecular mechanisms underlying the salt-tolerance in the selected rice variety (Shuzenji-kokumai, SZK) by comparing its physiological parameters and expression profiles of some essential genes for salt-tolerance with FL478, a salt-tolerant indica and Kunishi-jinjya-mai (Kunishi), a salt-sensitive japonica variety.
3) To elucidate transcriptomic profile and identify genes associated with tissue tolerance in SZK.

Chapter 2

Screening of Salt-tolerant Japonica Rice Varieties

2.1 Introduction

Soil salinity is a significant abiotic stress in agricultural crop productivity worldwide. One crop that has suffered a decline in productivity due to high salinity is rice. Rice is a glycophyte known as a sensitive plant to high Na^+ environments. On the other hand, rice is the most widely consumed food crop, and due to population growth, the global rice demand is increasing. Therefore, improving rice resistance to salt stress is essential to meet the yearly increase in rice demand. Increasing rice's salt tolerance requires new genetic sources and more efficient techniques for identifying salt-tolerant germplasm.

Many rice varieties and cultivars are growing worldwide, especially in Asian countries, where rice is a staple food. Two main subspecies of Asian cultivated rice are indica and japonica. Although rice is categorized as a glycophyte that is highly sensitive to salt stress, rice salt sensitivity varies across cultivars (Li et al., 2017; Munns and Tester, 2008). Not only in rice, plant response to salt stress is generally multifactorial. There are cultivar-specific variations and significant genetic variations for salinity tolerance within species (Mekawy et al., 2020; Melino and Tester, 2023). Lee et al. (2003) suggested that among the ten varieties of indica and japonica rice tested, the tolerance level of indica was higher than that of japonica. Four rice genotypes (FL478, Kamini, AC847, and IR29) showed differential salt sensitivity when subjected to 12 dS/m of salt stress for seven days (Chakraborty et al., 2012). Wangsawang et al. (2018) screened japonica rice cultivars and identified a highly salt-tolerance cultivar with an effective Na^+ exclusions mechanism at the leaf sheath. This variation can be exploited to discover genes and proteins contributing to salt tolerance.

Some traditional cultivars and rice landraces are more tolerant to various stresses than many elite cultivars (Walia et al., 2005). These resistant genotypes are considered a good source

of tolerance traits and can be used to improve plant resistance to salt stress. Classification of tolerance for various crops is helpful for farmers and agricultural advisors to improve plant resilience. Several traditional cultivars, landraces, and wild varieties such as Pokkali, CSR type, and *Porteresia coarctata* appear to be prospective sources for donating requisite salt tolerance genes (Sahi et al., 2006). Exploration of rice landraces or traditional cultivars with salt tolerance traits can be one potential approach to overcoming the decline in rice productivity due to high salinity. Screening rice genotypes with potential for salt tolerance is important to identify rice genotypes that have the potential for salt tolerance. Therefore, this study aimed to identify rice genotypes that are tolerant to salt stress by screening several japonica landraces during the vegetative stage using FL478 and IR29 as the salt-tolerant and salt-sensitive checks, respectively.

2. Materials and Methods

2.2.1 Plant materials and growth conditions

This study was conducted in a greenhouse of the Laboratory of Plant Nutritional Physiology, Graduate School of Integrated Sciences for Life, Hiroshima University, in 2021. The rice varieties used in this study are listed in **Table 2.1**, obtained from the collection of the same laboratory mentioned previously. The seeds were incubated in a water bath at 60℃ for 10 minutes, then surface-sterilized with 0.1% benlate (fungicide) solution for 24 hours at 30℃, followed by soaking in the tap water for 24 hours for germination. The germinated seeds were then transferred onto a floating nylon mesh. After one week of cultivation, Kimura B nutrient solution was added with the following nutrient composition in μM: 365 $(NH_4)_2SO_4$, 547 $MgSO_4$ $7H_2O$, 183 KNO_3, 365 $Ca(NO_3)_2$ $4H_2O$, 182 KH_2PO_4, 19 Fe-EDTA, 48.7 H_3BO_3, 9 $MnSO_4$ $5H_2O$, 0.3 $CuSO_4$ $5H_2O$, 0.7 $ZnSO_4$ $7H_2O$, 0.1 Na_2MoO_4. At day 21, the 4-5 leaf stage rice seedlings were transferred to a

180 L water container with holes. After a week in the holes container, treatment conditions were conducted as follows: controls (0 mM NaCl) and stepwise treatment (25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 150 mM for three days intervals). pH changes were monitored daily using a pH meter (AS700, As One Corp., Osaka, Japan) and were adjusted to 5 – 5.5 using 2 N HCl and 2 N NaOH throughout the cultivation period. The nutrient solution was changed every seven days, and water lost through evapotranspiration was replaced by the addition of tap water.

2.2.2 Determination of plant fresh and dry weight

Seedlings were harvested in replicates of four. Plants were dissected into roots and shoots and immediately measured for fresh weight (FW). Dry weights (DW) were obtained after oven-drying the samples for 72 hours at 70℃.

2.2.3 Determination of Na^+ and K^+ concentration

The Na^+ and K^+ concentrations in roots and shoots were performed using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan) as described previously (Mekawy et al., 2015). Dried samples were gently agitated in 0.1 N HCl overnight, and the concentrations of Na^+ and K^+ ions were calculated from the Na^+ and K^+ standard curves.

2.3 Results

2.3.1 Effect of salt stress on plant growth

The 46-week-old seedlings of 15 rice genotypes were subjected to salt-stress conditions (stepwise from 25-150 mM NaCl) at three days intervals. After six days in salinized solution (50 mM NaCl), initial signs of salt stress damage were observed in most seedlings' oldest leaves, which

started to desiccate and roll inward. When the salt concentration was increased to 100 mM, the signs of salt-stress damage were observed. The leaf blades of most seedlings were curled and wilted. After the 150 mM NaCl treatment, most leaf blades of the japonica landraces (**Figure 2.1**) and IR29 (**Figure 2.1B**) had died, with only the youngest leaf blades of some seedlings remaining green. In contrast, some leaf blades in FL478 (**Figure 2.1A)** and SZK (**Figure 2.1C**) remain green.

The standard evaluating system (SES) for scoring the visual symptoms of salt toxicity established at IRRI (Gregorio et al., 1997) was used to discriminate the sensitive and tolerant varieties (**Table 2.2**). Scoring was conducted on the last day of cultivation (after 18 days of salt stress treatment) when four tolerance categories could be visually distinguished (**Table 2.3**). The salt-tolerant variety FL478 showed the lowest score (2.75) as these seedlings look nearly normal. Of the 13 japonica varieties, SZK had the lowest score (3.25). In both FL478 and SZK, only the old leaves were wilted and rolled, while the younger leaves remained green and healthy (**Figure 2.1A** and **2.1C**). On the contrary, most plants like Kunishi had died, with only the youngest leaves of some seedlings remaining green. According to the visual symptoms under salt stress, FL478 and SZK are categorized as salt-tolerant, while Kunishi is salt-sensitive.

2.3.2 Effect of salt stress on biomass production

The effect of salt stress on the fresh weight and dry weight of 15 rice varieties is shown in **Figures 2.2** and **2.4**, respectively. The salt stress treatment resulted in severe decreases in root and shoot fresh weight and dry weight in most of the the varieties. However, there was no significant decrease in root and shoot fresh weight and dry weight of FL478 and SZK (**Figures 2.2** and **2.4**). In addition, the root and shoot relative fresh weight of the SZK is the highest among the other varieties (**Figures 2.3A** and **B**), while the shoot relative dry weight is almost the same as FL478.

These results indicate that SZK has a tolerance similar to FL478 and can maintain high biomass under salt stress.

2.3. Effect of salt stress on Na^+ and K^+ accumulation

Effects of salt stress on Na^+ and K^+ accumulation in 15 rice varieties are shown in **Figures 2.6** and **2.7**. Salt stress treatment increased Na^+ accumulation in the root and shoot of all the varieties. However, the salt-tolerant variety FL478 has the lowest Na^+ concentration in the shoot, while SZK has a high Na^+ concentration, similar to the sensitive varieties (**Figure 2.7**). The remarkable difference in the Na^+ concentration of these two varieties indicates that they have different mechanisms for avoiding Na^+ toxicity. FL478 is known to have the ability to exclude Na^+ from the shoot. SZK can maintain high biomass while having a high Na^+ concentration in the shoot, indicating that it may have a tissue tolerance ability.

Salt stress significantly decreased the K^+ concentration in the roots and shoots of all varieties (**Figure 2.7**). Notably, SZK showed less decrease of K^+ concentration in the root compared to other varieties (**Figure 2.7A**). The increase in Na^+ accumulation was followed by the decrease in K^+ concentration, resulting in an increase in the Na^+/K^+ ratio (**Figure 2.8**). FL478 showed the lowest Na^+/K^+ ratio in the shoot, while SZK had a high Na^+/K^+ ratio in the shoot, similar to the highly sensitive variety such as Kunishi. These findings suggest that the japonica varieties SZK can maintain high biomass while having low K^+ and accumulate high Na^+.

Table 2.1 Rice varieties used in the study

No.	Name/code	Japanese name	Type
1.	FL478		Salt-tolerant indica
2.	IR29		Salt-sensitive indica
3.	340	Shuzenji-kokumai (SZK)	Japonica landrace
4.	355	Kunishi-jinja-mai (Kunishi)	Japonica landrace
5.	265	Shikyo-ine	Japonica landrace
6.	266	Murasaki koya bozu	Japonica landrace
7.	277	Shitan	Japonica landrace
8.	287	Shin-oukan	Japonica landrace
9.	289	Kasenkoumai	Japonica landrace
10.	307	Benisome-mochi	Japonica landrace
11.	314	Kanniho	Japonica landrace
12.	324	Musashino-murasaki	Japonica landrace
13.	330	Fukuizumi	Japonica landrace
14.	338	Akamai mochi	Japonica landrace
15.	357	Tsutsu	Japonica landrace

Table 2.2 Modified standard evaluation score (SES) of visual injury at the seedling stage (Gregorio et al., 1997)

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of a few leaves whitish and rolled	Tolerant
5	Growth is severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants are dead or dying	Highly susceptible

Table 2.3 Standard Evaluation Score on rice (SES) of 15 varieties under salt stress treatment.

Rice Varieties	SES	Tolerance
FL478	2.75 ± 0.25	Tolerant
IR29	5.75 ± 0.25	Susceptible
SZK	3.25 ± 0.25	Tolerant
Kunishi	8.25 ± 0.25	Highly susceptible
Shikyo-ine	8.75 ± 0.25	Highly susceptible
Murasaki koya bozu	8.50 ± 0.29	Highly susceptible
Shitan	8.25 ± 0.25	Highly susceptible
Shin-oukan	7.00 ± 0.00	Susceptible
Kasenkoumai	3.75 ± 0.48	Tolerant
Benisome-mochi	7.25 ± 0.25	Susceptible
Kanniho	8.00 ± 0.00	Highly susceptible
Musashino-murasaki	7.50 ± 0.29	Susceptible
Fukuizumi	6.50 ± 0.29	Susceptible
Akamai mochi	8.50 ± 0.29	Highly susceptible
Tsutsu	8.75 ± 0.25	Highly susceptible

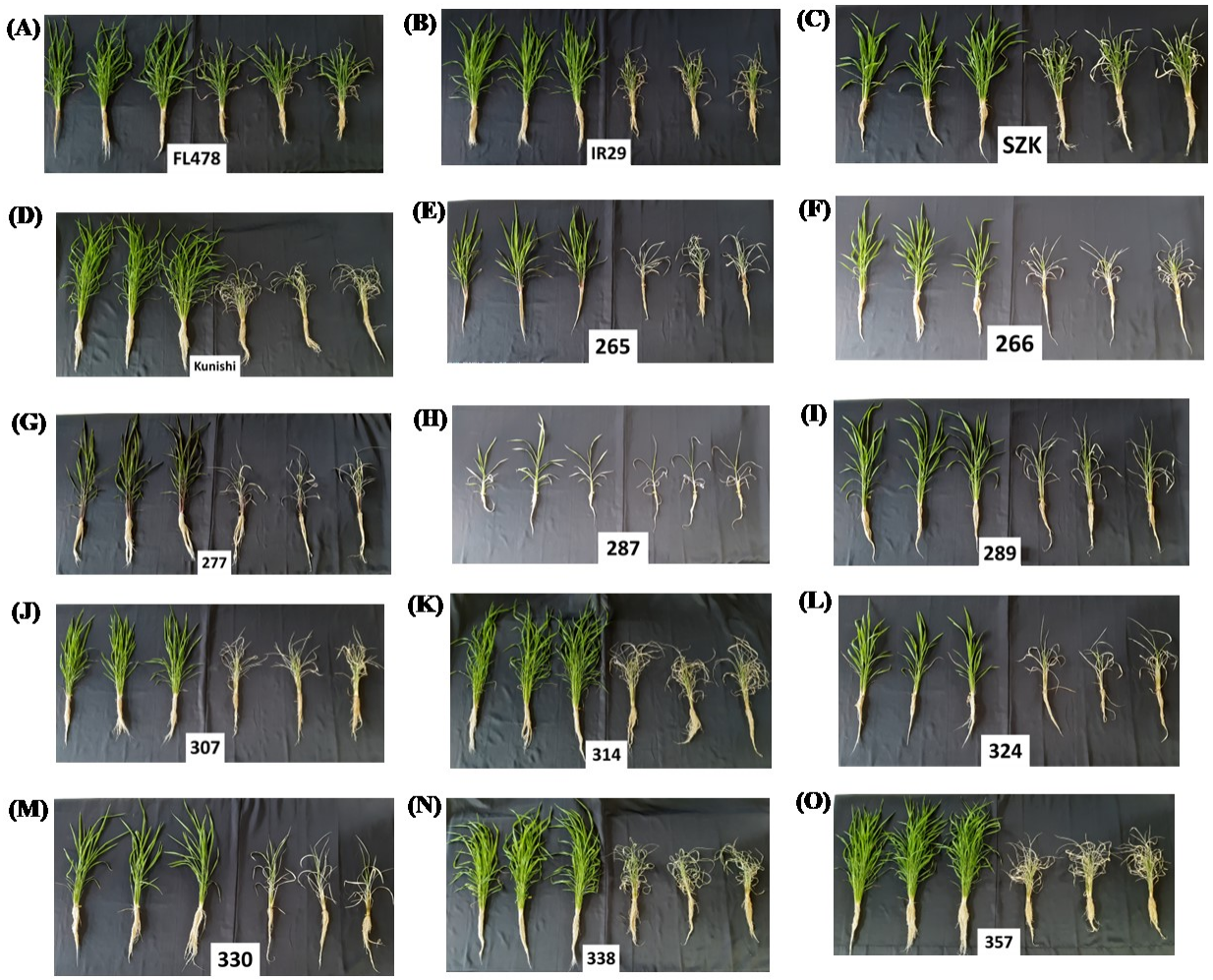

Figure 2.1 Representative images of the 46-day-old rice seedlings under control (left) and salt stress treatment (right). The picture was taken after 150 mM NaCl treatment for 3 days. (A) FL478, (B) IR29, (C) SZK, (D) Kunishi (E) 265, (F) 266, (G) 277, (H) 287, (I) 289, (J) 307, (K) 314, (L) 324, (M) 330, (N) 338, and (O) 357

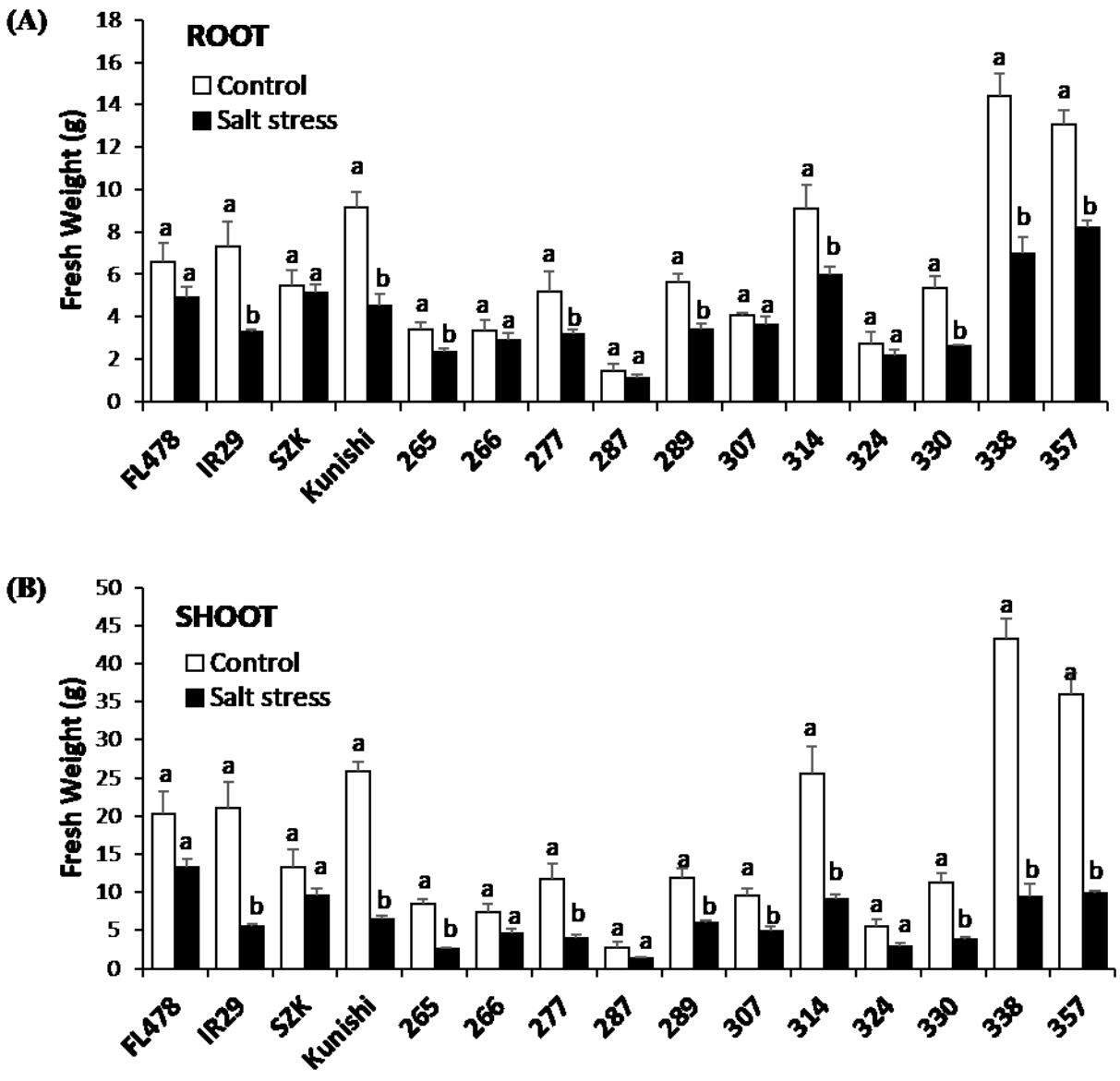

Figure 2.2 Effects of salt stress on (A) root and (B) shoot fresh weight of 15 rice varieties. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

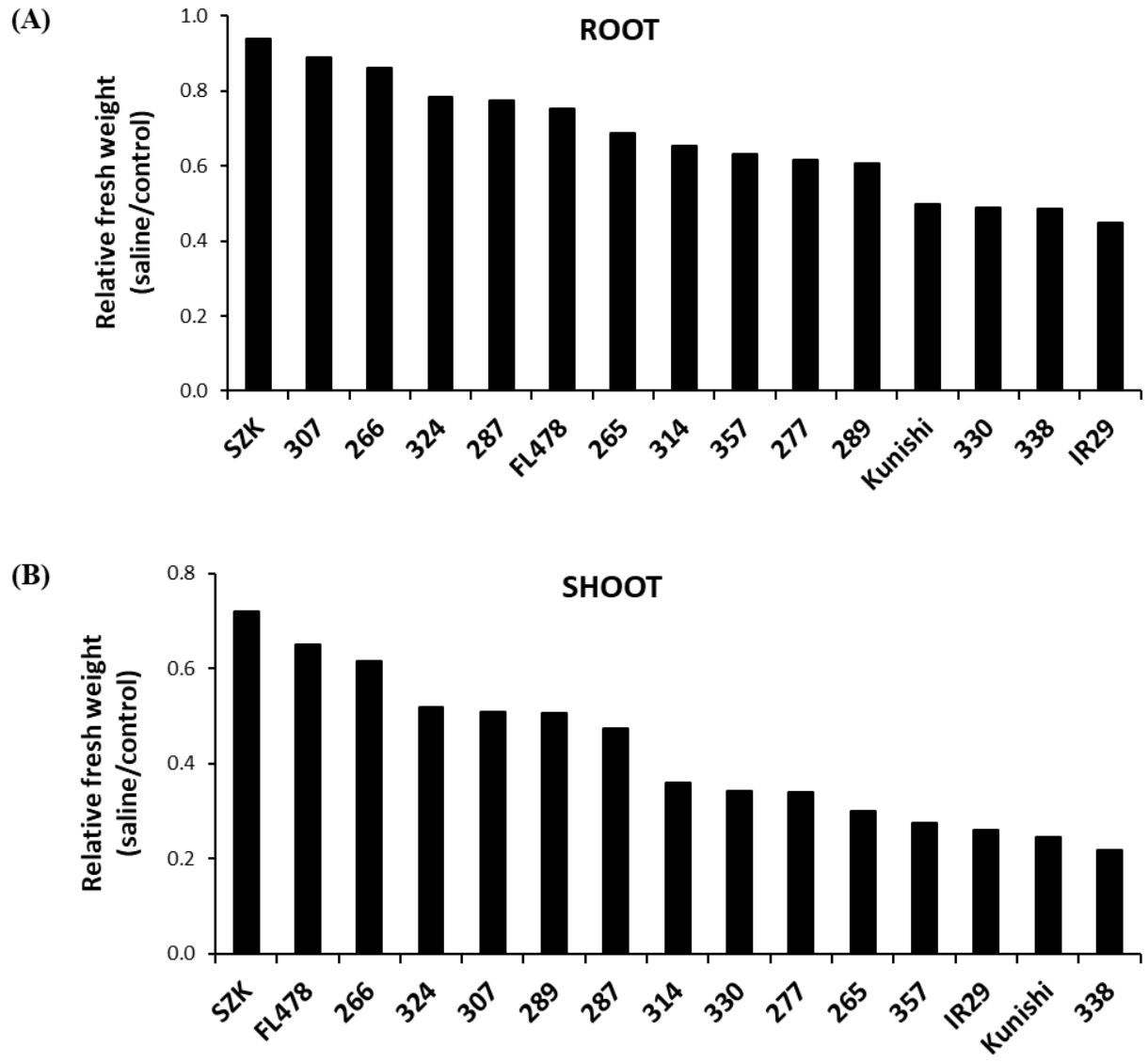

Figure 2.3 Relative fresh weight of (A) root and (B) shoot of 15 rice varieties under salt stress treatment.

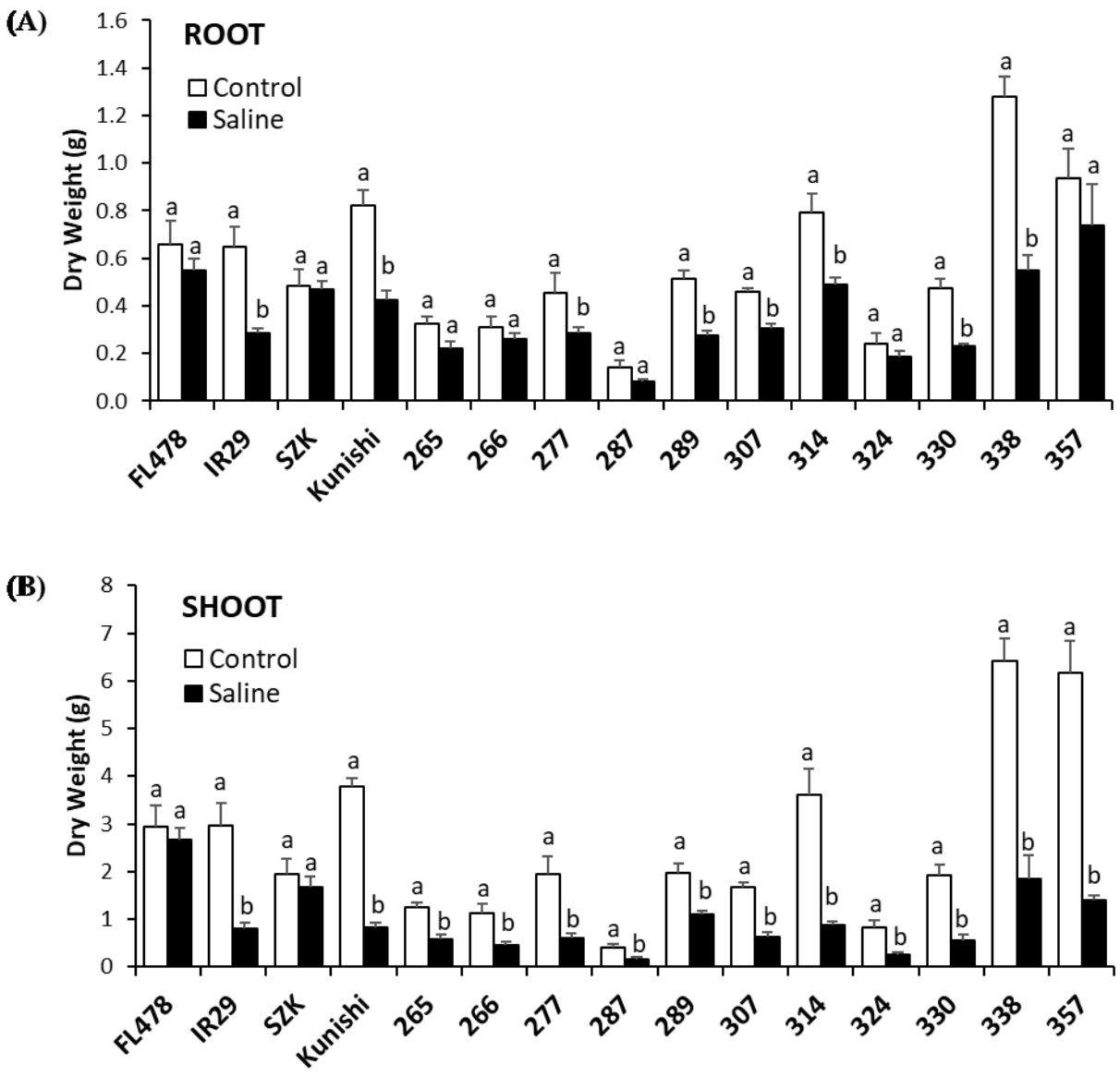

Figure 2.4 Root (A) and shoot (B) dry weight of 15 rice varieties under control and salt stress treatment. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

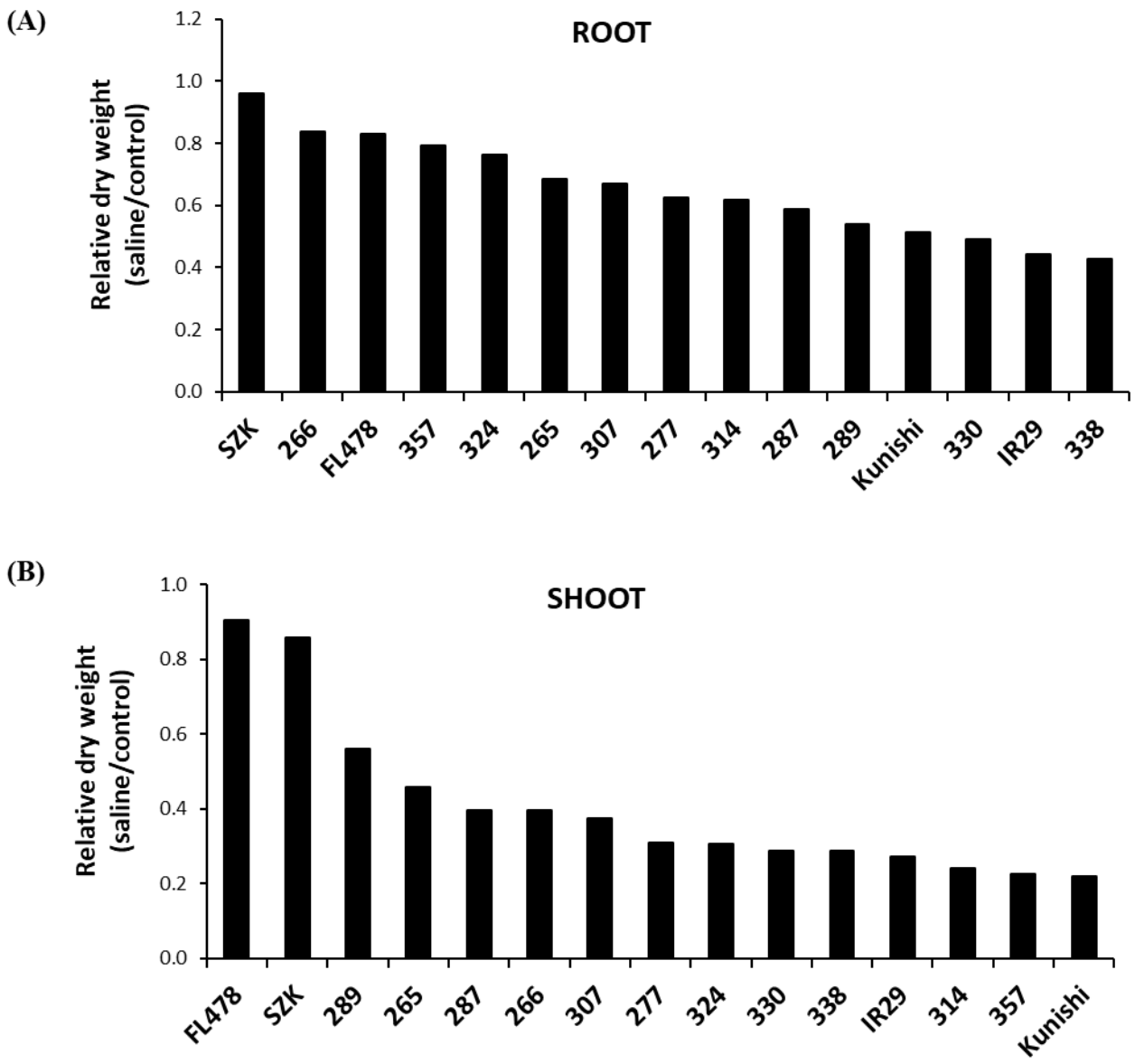

Figure 2.5 Relative dry weight of the root (A) and shoot (B) of 15 rice varieties.

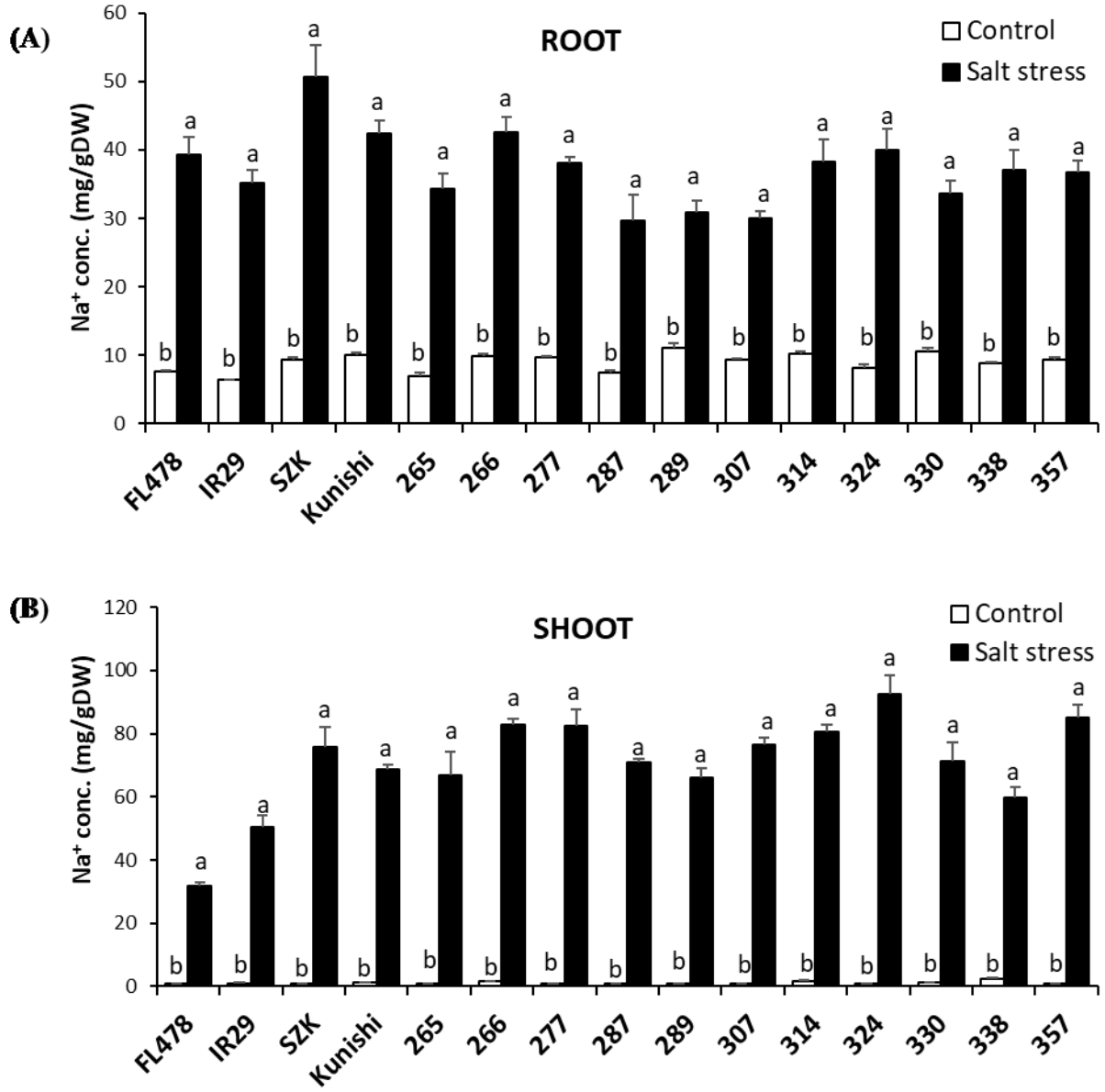

Figure 2.6 Na^+ concentration in the root (A) and shoot (B) of 15 rice varieties under saline condition. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

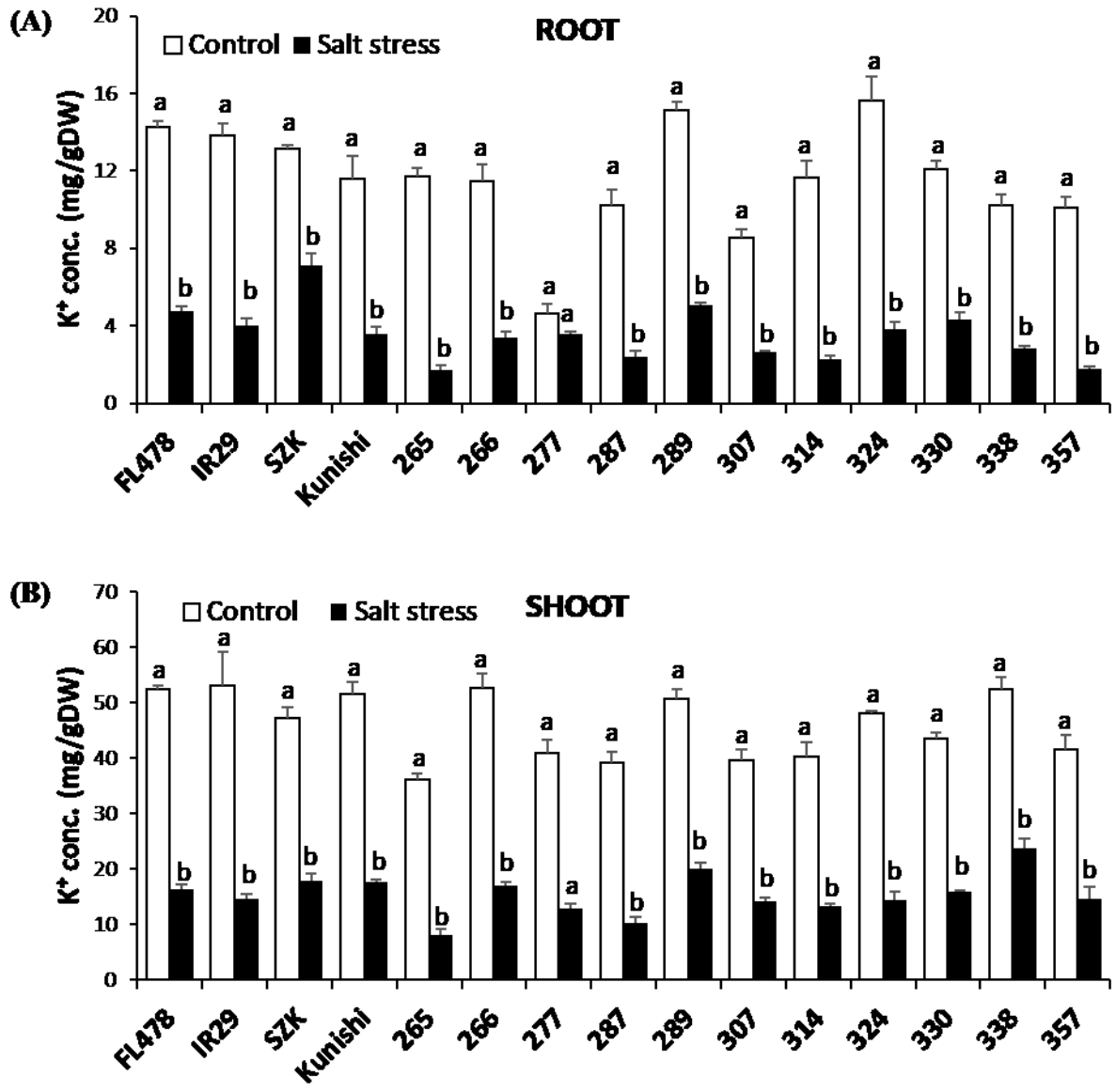

Figure 2.7 K^+ concentration in the root (A) and shoot (B) of 15 rice varieties under saline condition. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

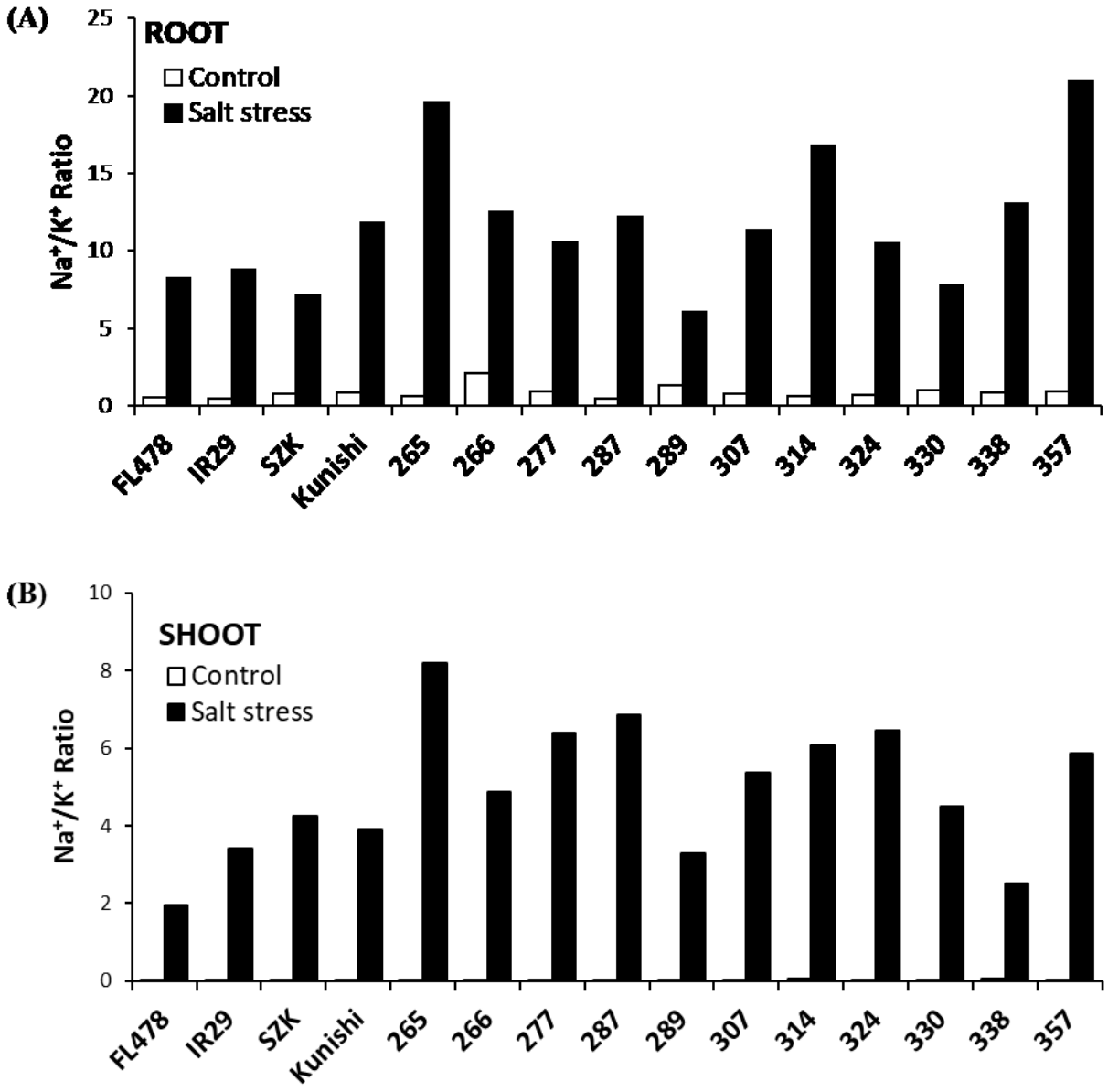

Figure 2.8 Na^+/K^+ ratio in the root (A) and shoot (B) of 15 rice varieties under saline condition.

2.4 Discussions

Rice is the most widely used staple food worldwide and is very sensitive to salt. Hence, increasing rice growth and production in such an environment is crucial. Screening of salt-tolerant rice varieties and landraces is important to identify salt-tolerant genotypes. These genotypes have a potential source of tolerance traits and can be used to improve plant resistance to salt stress. In this study, screening of japonica rice varieties and landraces for salt tolerance was conducted in hydroponic cultivation. A stepwise salt stress treatment (25-150 mM NaCl) was applied to the 15 rice varieties, including indica rice variety FL478 and IR29, as the tolerance and sensitive checks, respectively. After the salt stress treatment, almost all rice varieties showed a reduction in fresh and dry weight (**Figures 2.2 and 2.4**).

The most significant effect of salt stress is the reduction of plant biomass. Salt tolerance can be assessed by comparing the percentage of biomass production under salt stress and control conditions over a prolonged period (Munns, 2002). From the screening experiment, a salt-tolerant japonica rice variety, namely Shuzenji-kokumai (SZK), was identified. SZK has a similar tolerance to FL478, as the reduction of the fresh and dry weights in the roots and shoots under salt stress was not significant (**Figures 2.2** and **2.4**). In addition, the relative fresh weight of SZK was the highest (**Figure 2.3**), even higher than FL478, suggesting that SZK can maintain high biomass under salt stress. Moreover, the SES categorized SZK as a tolerant variety, the same as FL478 (**Table 2.3**). Identifying salt-tolerant rice genotypes has been done in several studies. However, most studies focus on the indica rice varieties (Chakraborty et al., 2020). A comparative study on indica and japonica suggested that the tolerance level of indica was higher than that of japonica (Lee et al., 2003). Li et al. (2017) identified an elite japonica rice genotype that can grow in saline-alkaline soils in northeast China and is tolerant to moderate salt stress. However, little is known

about tolerant japonica genotypes, particularly for traditional cultivars and landraces (Wangsawang et al., 2018).

Salt stress causes nutrient imbalance and leads to Na^+ accumulation, which is toxic to plants by disrupting cellular processes (Van Zelm et al., 2020). As shown in **Figure 2.6**, the salt stress treatment significantly increased Na^+ concentration in the roots and shoots of all varieties tested. However, the increase of Na^+ concentration in the shoot of FL478 was the lowest, indicating that FL478 can exclude Na^+ from the shoots to avoid Na^+ toxicity. Salt tolerance in rice is usually associated with low Na^+ transport to the shoots and employs the Na^+ exclusion mechanism (Assaha et al. 2017). FL478 is a well-known japonica rice variety with a superior Na^+ exclusion mechanism. On the contrary, SZK has a high Na^+ concentration in the shoot, almost as high as the sensitive IR29 and Kunishi (**Figure 2.6**). The K^+ concentration in the shoot of all varieties was significantly decreased under salt stress (**Figure 2.7B**). Consequently, higher Na^+/K^+ ratios were observed in the salt-sensitive varieties. However, this condition did not happen in the salt-tolerant FL478, which has the lowest Na^+/K^+ ratio in the shoot as it has a low Na^+ concentration.

To persist in saline soil, adverse effects of Na^+ and Cl^- on plant metabolism must be avoided. Na^+ exclusion and compartmentalization are the two mechanisms by which plants cope with ion toxicity. Avoiding excess Na^+ in the shoots is a well-characterized mechanism of salt tolerance (Munns and Tester 2008) and is associated with tolerance in rice. Nevertheless, SZK is considered a "tissue-tolerant" variety as it accumulates high Na^+ concentrations in shoots, almost the same as the salt-sensitive IR29 and Kunishi, while maintaining a similar level of salt tolerance to FL478. Tissue tolerance is the capacity of the tissue to function while containing high levels of internal Na^+ or Cl^- (Munns et al. 2016). The main component of tissue tolerance is the compartmentalization of ions within vacuoles, which involves ion transporters in the tonoplast,

particularly vacuolar Na^+/H^+ antiporters (Flowers and Colmer 2015). Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membranes and regulate the cytoplasm's internal pH, cell volume, and Na^+ levels (Fukuda et al., 2011). Although SZK and FL478 were considerably tolerant of salt stress, a distinctive mechanistic difference was observed in their tolerance strategies. Further investigation into the physiological and molecular characterization of these two varieties will give a better understanding of the tissue tolerance mechanism in SZK.

2.5 Conclusions

This study demonstrated that SZK has a different mechanism in response to salt stress compared with FL478. Under 150 mM NaCl, FL478 has a high Na^+ concentration in the roots but low in the shoots. In contrast, although SZK has a tolerance similar to FL478, as it can maintain high biomass, it has a high Na^+ concentration. This variety is considered to have a tissue tolerance ability that compartmentalizes Na^+ in the vacuole so that Na^+ toxicity in the cytosol can be avoided.

Chapter 3

Physiological and Molecular Characterization of Tissue Tolerance Mechanism

3.1 Introduction

Salt-affected soils have become a global concern because they reduce agricultural productivity. Over 800 million hectares of land worldwide are affected by salt (Munns and Tester 2008). Salt accumulation in the soil can be caused by the parent rock from which the soil is formed, the intrusion of seawater, and water irrigation (Morton et al., 2018). In addition to naturally occurring soil salinity, salinization increases owing to climate change, which acts through increased evaporation during periods of drought (Van Zelm et al., 2020). Compared to normal soils, saline soils have excessive soluble salts such as sodium, chloride, and sulfate. These minerals cause nutrient imbalance in the soil and reduce water availability. These conditions negatively affect the plant growth and development. Plants can be primarily divided into two groups based on the effect of salt on plant growth: glycophytes, which are plants sensitive to soil salinity, and halophytes, which are plants that grow naturally under high salinity and generally tolerate high salt concentrations (Hasegawa et al. 2000; Reddy et al. 2017a; Zhu, 2001). Most crop plants, including rice, the most widely consumed grain worldwide, are glycophytes that cannot tolerate salt stress (Assaha et al. 2017). The demand for rice has increased in recent years due to the rapidly growing global population. Therefore, there is an urgent need to address salinity problems in rice plants. Characterizing the salt tolerance mechanisms is critical for further experiments to increase crop yield on salinized agricultural land (Chuamnakthong et al., 2019).

For plants to persist in saline soil, adverse effects of Na^+ and Cl^- on plant metabolism must be avoided. Na^+ exclusion and compartmentalization are the two mechanisms by which plants cope with ion toxicity (Jiadkong et al., 2023). The latter is the plant's strategy to sequester Na^+ and Cl^- in vacuoles and maintain a low concentration in the cytosol, where most cellular metabolism occurs, resulting in a situation known as tissue tolerance. Tissue tolerance is the capacity of the

tissue to function while containing high levels of internal Na^+ or Cl^- (Munns et al., 2016). The main component of tissue tolerance is the compartmentalization of ions within vacuoles, which involves ion transporters in the tonoplast, particularly vacuolar Na^+/H^+ antiporters (Flowers and Colmer, 2015). Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membranes and regulate the cytoplasm's internal pH, cell volume, and Na^+ levels (Fukuda et al., 2011).

Many rice varieties and cultivars are growing worldwide, especially in Asian countries, where rice is a staple food. The two main subspecies of Asian cultivated rice are indica and japonica. Although rice is categorized as a glycophyte that is highly sensitive to salt stress, salt sensitivity varies across cultivars (Mekawy et al., 2018b; Sriskantharajah et al., 2020). Lee et al. (2003) suggested that the tolerance level of indica was higher than that of japonica rice tested. Four rice genotypes (FL478, Kamini, AC847, and IR29) showed differential salt sensitivity when subjected to 12 dS m^{-1} of salt stress for seven days (Chakraborty et al., 2012). Wangsawang et al. (2018) screened japonica rice cultivars and identified a highly salt-tolerant cultivar with an effective Na^+ exclusion mechanism in the leaf sheath. These variations can be exploited to identify genes and proteins contributing to salt tolerance. Some traditional cultivars and rice landraces are more tolerant of various stresses than elite ones (Walia et al., 2005). These resistant genotypes are considered good genetic sources of further breeding.

There are apparent differences in salinity tolerance mechanisms among rice varieties. The previous study on the screening of salt tolerance rice identified a Japanese rice variety, Shuzenji-kokumai (SZK), which is salt-tolerant with a unique response to salt-stress conditions. This variety can maintain high biomass while having a high Na^+ concentration in the shoot. These manners differ from FL478, which can exclude Na^+ from shoots in response to salt stress, so FL478 shows low Na^+ concentration in shoots. Based on those findings, this variety is considered to have a tissue

tolerance ability. To understand the mechanism underlying the salt tolerance in SZK, the physiological and molecular characteristics of SZK under salt stress were evaluated. Therefore, the objectives of this study are 1) to characterize physiological and molecular mechanisms underlying the salt-tolerance in SZK by comparing its physiological parameters and expression profiles of some essential genes for salt-tolerance with FL478 and Kunishi, 2) to elucidate transcriptomic profile and identify genes associated with tissue-tolerance in SZK.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

This study was conducted in a greenhouse of the laboratory of Plant Nutritional Physiology, Graduate School of Integrated Sciences for Life, Hiroshima University, during 2021-2022. The study included a series of three cultivations, which are described below. All experiments were arranged in a completely randomized design with three replications.

Experiment 1: Cultivation for physiological and molecular analysis of three rice varieties

This study used two japonica rice varieties, Shuzenji-kokumai (SZK) and Kunishi-jinja-mai (Kunishi), and one indica variety, FL478. The seeds of SZK and Kunishi were obtained from the Hiroshima University Plant Nutritional Physiology Laboratory collection**.** The seeds were incubated in a water bath at 60℃ for 10 minutes, then surface-sterilized with 0.1% benlate (fungicide) solution for 24 hours at 30℃, followed by soaking in the tap water for 24 hours for germination. The germinated seeds were then transferred onto a floating nylon mesh. After one week cultivation, Kimura B nutrient solution was added to water with the following nutrient composition in μM: 365 $(NH_4)_2SO_4$, 547 $MgSO_4$ $7H_2O$, 183 KNO_3, 365 $Ca(NO_3)_2$ $4H_2O$, 182 KH_2PO_4, 19 Fe-EDTA, 48.7 H_3BO_3, 9 $MnSO_4$ $5H_2O$, 0.3 $CuSO_4$ $5H_2O$, 0.7 $ZnSO_4$ $7H_2O$, 0.1

Na_2MoO_4. After three weeks of cultivation, the 4-5 leaf stage rice seedlings were transferred to a 180 L water container with holes. After a week in the holes container, treatment conditions were conducted as follows: controls (0 mM NaCl) and stepwise treatment (25 mM, 50 mM, 75 mM, 100 mM for five days intervals). pH changes were monitored daily using a pH meter (AS700, As One Corp., Osaka, Japan) and were adjusted into 5 – 5.5 using 2 N HCl and 2 N NaOH throughout the growth period. The nutrient solution was changed every seven days, and water lost through evapotranspiration was replaced by the addition of tap water.

Experiment 2: Cultivation for analysis of Na and K in time course

This study used two japonica rice varieties, Shuzenji-kokumai (SZK) and Kunishi-jinja-mai (Kunishi), and one indica variety, FL478. The seeds of SZK and Kunishi were obtained from the Hiroshima University Plant Nutritional Physiology Laboratory collection**.** The seeds were incubated in a water bath at 60℃ for 10 minutes, then surface-sterilized with 0.1% benlate (fungicide) solution for 24 hours at 30℃, followed by soaking in the tap water for 24 hours for germination. The germinated seeds were then transferred onto a floating nylon mesh. After one week cultivation, Kimura B nutrient solution was added to water with the following nutrient composition in μM: 365 $(NH_4)_2SO_4$, 547 $MgSO_4$ $7H_2O$, 183 KNO_3, 365 $Ca(NO_3)_2$ $4H_2O$, 182 KH_2PO_4, 19 Fe-EDTA, 48.7 H_3BO_3, 9 $MnSO_4$ $5H_2O$, 0.3 $CuSO_4$ $5H_2O$, 0.7 $ZnSO_4$ $7H_2O$, 0.1 Na_2MoO_4. At day 21, the 4-5 leaf stage rice seedlings were transferred to a 180 L water container with holes. After a week in the holes container, treatment conditions were conducted as follows: stepwise treatment (0 mM, 25 mM, 50 mM, 75 mM, 100 mM for six-day intervals). Sampling times were conducted every three days (before treatment, day 3, day 6, day 9, and so on until the endpoint on day 24). pH changes were monitored daily using a pH meter (AS700, As One Corp., Osaka, Japan) and were adjusted into 5 – 5.5 using 2 N HCl and 2 N NaOH throughout the growth

period. The nutrient solution was changed every seven days, and water lost through evapotranspiration was replaced by the addition of tap water.

Experiment 3: Cultivation for RNA sequencing

The rice variety used in this study was salt-tolerant SZK. The seeds were incubated in a water bath at 60℃ for 10 minutes, then surface-sterilized with 0.1% benlate (fungicide) solution for 24 hours at 30℃, followed by soaking in the tap water for 24 hours for germination. The germinated seeds were then transferred onto a floating nylon mesh. After one week of cultivation, Kimura B nutrient solution was added to water with the following nutrient composition in μM: 365 $(NH_4)_2SO_4$, 547 $MgSO_4$ $7H_2O$, 183 KNO_3, 365 $Ca(NO_3)_2$ $4H_2O$, 182 KH_2PO_4, 19 Fe-EDTA, 48.7 H_3BO_3, 9 $MnSO_4$ $5H_2O$, 0.3 $CuSO_4$ $5H_2O$, 0.7 $ZnSO_4$ $7H_2O$, 0.1 Na_2MoO_4. At day 21, the 4-5 leaf stage rice seedlings were transferred to a 180 L water container with holes. After a week in the holes container, treatment conditions were conducted as follows: control (0 mM NaCl) and salt stress treatment (100 mM NaCl). Sampling was conducted 24 h after treatment. pH changes were monitored daily using a pH meter (AS700, As One Corp., Osaka, Japan) and were adjusted into 5 – 5.5 using 2 N HCl and 2 N NaOH throughout the growth period. The nutrient solution was changed every seven days, and water lost through evapotranspiration was replaced by the addition of tap water.

3.2.2 Determination of plant fresh and dry weight

Seedlings were harvested in replicates of four. Plants were dissected into roots, leaf sheaths, and leaf blades and immediately measured for fresh weight (FW). Dry weights (DW) were obtained after oven-drying the samples for 72 hours at 70℃.

3.2.3 Measurement of water content

Leaf blade samples were dried at 70℃ for three days before being weighed. The water content was calculated using the equation (FW-DW)/FW.

3.2.4 Measurement of electrolyte leakage ratio (ELR)

The ELR was determined according to the method described by Murray et al. (1989) with minor modifications. The second leaves from the top of the plants were cut into 5 mm length and placed in 50 ml tubes containing 30 ml deionized water. The initial electrical conductivity of the medium (EC1) was measured using an electrical conductivity meter (CM-31p, Kyoto Electronics, Kyoto, Japan). The samples were boiled in a heat block at 100℃ for 20 minutes then cooled to 25, and the final electrical conductivity (EC2) was measured. The ELR was calculated as the ratio of the conductivity before boiling to the conductivity after boiling using the following formula: ELR (%) = (EC1/EC2) × 100.

3.2.5 Measurement of proline concentration

The proline concentrations determined using a rapid method developed by Bates et al., (1973), based on Ninhydrin reaction. Fresh leaf blades (1-2 g) were extracted in 5 mL of aqueous 3% (w/v) sulfosalicylic acid. The extract was centrifuged at 10,000 × g for 5 min at 4°C. For colorimetric determination of proline concentration, 2 mL of the supernatant was reacted with 2 mL of acid Ninhydrin and 2 mL of glacial acetic acid, and then incubated at 100ºC for 1 h. The reaction was stopped by placing the mixture in an ice bath. Finally, the chromophore in each sample was extracted with 4 mL of toluene. The absorbance of the sample was measured at 520

nm using a Hitachi U-2001 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Proline (0, 10, 25, 50, and 100 μM) was used to estimate the free proline concentration.

3.2.6 Measurement of lipid peroxidation/malondialdehyde (MDA)

Malondialdehyde (MDA) concentrations in the leaf blades were analyzed according to Hodges et al. (1999). The sample (100 mg FW) was ground with liquid nitrogen, then 3 mL of extraction buffer (80:20 (v/v) ethanol/deionized water) was added. The mixture was incubated at room temperature for 20 min before centrifuging at 3,000 × g for 10 min. One mL of supernatant was transferred into 1 mL of TBA (-) or TBA (+) solutions and mixed well; TBA (-) solution containing 20% (w/v) trichloroacetic acid and 0.01% (w/v) butyl hydroxyl toluene (BHT), and TBA (+) solution containing the same chemicals as TBA (-) solution together with 0.65% (w/v) thiobarbituric acid (TBA). Then, the mixture was incubated at 95°C for 30 min, and the reaction was stopped by placing the mixture in an ice bath. After centrifuging at 3,000 × g for 10 min, the absorbance of the sample was measured at 440, 532, and 600 nm using a Hitachi U-2001 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The following formula was used to calculate MDA concentration in each sample (nmol mL^{-1}):

$$X = A_{532}\ (\text{TBA}\ (+)) - A_{600}\ (\text{TBA}\ ((+) - [A_{532}\ (\text{TBA}\ (-)) - A_{600}\ (\text{TBA}\ (-))]$$

$$Y = [A_{440}\ (\text{TBA}\ (+)) - A_{600}\ (\text{TBA}\ (+))] \times 0.0571$$

$$\text{MDA (nmol mL}^{-1}\text{)} = [(X-Y)/\ 157{,}000] \times 10^6$$

3.2.7 Determination of element concentration (Na^+, K^+, Mg, Ca, Cu, Mn, Zn, Fe, P)

Element concentration in the samples was analyzed according to Wheal et al. (2011). Dried plant samples (roots, leaf sheaths, and leaf blades) were crushed into a fine powder using a crusher

(Shake Master, BMS, Bio medical science). The samples were weighed to 100 mg in 50 ml tubes. Subsequently, 2 ml HNO_3 and 500 µl H_2O_2 were added, and the samples were incubated overnight at 23 ℃ for digestion. Afterward, the samples were heated at 80 ℃ for 30 minutes, the lids were loosened to release the pressure, and the temperature was increased to 125 ℃ for 2 hours. The samples were cooled overnight, filtered using filter paper (Double Ring Quantitative Filter Paper, Grade: FAST 201), and 25 ml of Milli-Q water (Millipore, Direct-Q UV) was added. The concentration of each element (Na^+, K^+, Ca, Mg, Fe, Cu, phosphorus (P), manganese (Mn), and Zinc (Zn)) was measured using inductively coupled plasma optical emission spectrophotometry (SPECTROGREEN-FMD46, Hitachi).

3.2.8 Expression analysis of the genes encoding Na transport

Total RNA was extracted from the roots, leaf blades, and leaf sheaths of control and salt stress treatments using RNA extraction Kit Mini (RBC Bioscience, Birmingham, UK) with digestion using DNaseI. The concentration and purity of RNA were measured using a nanodrop spectrometer (Thermo Fisher Scientific) at A_{260} and A_{280}. One µg of total RNA was reverse transcribed to cDNA using reversed transcription Master Mix (5X) (Toyobo Co., Ltd., Osaka, Japan). To generate cDNA, reverse transcription was conducted at 37 °C for 15 min, and then reverse transcriptase was denatured at 98 °C for 5 min. Quantitative-Real Time polymerase chain reaction (qRT-PCR) 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) according to Ueda et al. (2013). qRT-PCR was performed using the following conditions: 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. *OsUBQ5* 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 (fold change) were calculated based on that under control conditions. The details of forward and reverse primers used are described in **Table 3.1**

3.2.9 RNA sequencing

Total RNA was extracted from the 400 µg leaf blades and leaf sheaths fresh weight using Sepasol-RNA I Super G. RNA Extraction Kit Mini with DNA digestion using DNase I (Nippon gene) to purify 50 µg total RNA, obtaining DNA-free high-quality RNA (the ratio of A260/A280 and A260/A230 must be more than 2). RNA quality control, preparation of cDNA libraries, sequencing, and raw reads processing were performed on the DNBSeq platform (BGI, Kobe, Japan) in 150-bp paired-end mode. Low quality, adaptor-polluted, and high content of unknown base reads were filtered, and 41-44 million clean reads were obtained from each library. Differentially expressed genes (DEGs) were identified using DESeq2 (Love et al., 2014) and RaNA-seq (Prieto and Barrios, 2020). Upregulated DEGs were tested for GO (Gene Ontology) term enrichment and KEGG pathway analysis using ShinyGO v0.80 (http://bioinformatics.sdstate.edu/go/) (Ge et al., 2020; Kanehisa et al., 2021; Luo and Brouwer, 2013). The raw data of RNA seq were deposited to the NCBI Gene Expression Omnibus under accession No. GSE266657 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE266657).

3.2.10. Functional characterization of novel genes using yeast mutant

The coding sequences of the genes (*OsCYP76M2, OsLAC28, OsLOX-L2, OsCI2c, OsHsp18*, and *OsHsp20*) were subcloned into yeast expression vector pYES2 using the In-Fusion Snap Assembly Master Mix (Takara Bio, Japan). The constructed vectors were transformed into the Na^+-sensitive yeast strain AB11c, which lacks Na^+-ATPases and Na^+/H^+ antiporters (W303, *ena1Δ::HIS3::ena4Δ, nha1Δ::LEU2, nhx1Δ::TRP1*) and an osmotic sensitive strain BY22935, which lacks *hog1* gene (W303, *ade2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1 can1-100 hog1::TRP1*) using the LiAC/ssNDA/PEG method, as described by Gietz and Schiestl (2007). Growth spot assays on synthetic minimal glucose medium (SD) lacking uracil were performed as described previously (Marešová and Sychrová 2007).

3.3 Results

3.3.1 Effect of salt stress on growth

Salts were gradually applied at levels of 25-100 mM NaCl at five-day intervals to the three rice varieties (FL478, SZK, Kunishi). As shown in **Figure 3.1A**, the salt stress-treated seedlings had a smaller size than the control seedlings, particularly in the salt-sensitive Kunishi variety. Most of the leaves of Kunishi were markedly wilted and rolled, whereas only a small number of leaves of FL478 and SZK were wilted (**Figure 3.1A**). Salt stress reduced the overall biomass of all the varieties. The dry weights of the roots, leaf blades, and leaf sheaths in Kunishi decreased significantly, whereas the reduction in FL478 was only significant in the leaf sheath (**Figure 3.1B**). Importantly, no significant decrease was observed in SZK. These results confirm that FL478 and SZK are salt-tolerant, whereas Kunishi is salt-sensitive.

3.3.2 Effect of salt stress on physiological parameters

To understand the physiological responses of the three varieties to salt stress, physiological parameters related to salt stress were examined (**Figure 3.2**). High salinity resulted in osmotic imbalance, leading to a loss of water content in the leaves (Wangsawang et al. 2018). The water content of the leaf blades was measured to estimate the amount of water lost due to salt stress. The results showed that salt stress reduced the water content of all varieties (**Figure 3.2A**). This reduction was significant in all varieties other than FL478, indicating that FL478 exhibited a greater potential to maintain tissue water than SZK and Kunishi. However, the reduction in water content in Kunishi (4.4%) was higher than in SZK (3.2%).

Excess accumulations of Na^+ in plant cells can disrupt cellular ion homeostasis and metabolism, including reactive oxygen species (ROS) generation, which is the primary cause of oxidative stress (Panda et al. 2019). Accumulation of ROS can damage cellular membranes and cause leakage of cellular components, such as electrolytes (Murray et al. 1989; Ueda et al. 2013). ELR was measured to examine the cell membrane stability under salt stress. As shown in **Figure 3.2B**, ELR increased significantly in Kunishi from 14 % to 49 %, whereas it increased slightly from 16.5% to 26% in FL478. Interestingly, ELR in SZK remained unchanged, suggesting that SZK maintains membrane stability under salt stress. This result was verified by measuring MDA concentrations. MDA is one of the main products of cellular oxidation that represents cell oxidative damage. A significant increase in MDA was observed in Kunishi treated with salt stress, whereas the increase in FL478 and Kunishi was not substantial (**Figure 3.2D**).

High salt accumulation in the soil inhibits water uptake and reduces turgor pressure, leading to wilting. To overcome water deficit, plants synthesize various osmoprotectants, such as glycine betaine, proline, and soluble sugar (Qureshi et al. 2013). Proline concentration was

measured in the leaf blades to understand the involvement of osmoprotectants in the salt adaptation of the three varieties. As shown in **Figure 3.2C**, significantly higher proline concentrations were observed in all three varieties after salt stress treatment. FL478 showed a 10-fold increase in proline concentration relative to the control. Kunishi showed the highest increase of 16.9-fold, and SZK showed a slight increase of only 3.5-fold. These results suggested that proline accumulations may not be one of the mechanisms by which SZK adapts to salt stress.

3.3.3 Effect of salt stress on Na^+ and K^+ concentration

Salt stress causes nutrient imbalance and leads to Na^+ accumulation, which is toxic to plants by disrupting cellular processes (Van Zelm et al. 2020). As shown in **Figure 3.3A**, the salt stress treatment significantly increased Na^+ concentration in the roots and leaf sheaths of all three varieties. However, the Na^+ concentration remained unchanged in the leaf blades of FL478, whereas the increase in Na^+ concentration was nearly five-fold higher in SZK and Kunishi than in FL478. The K^+ concentration in the roots and leaf blades of all three varieties increased significantly (**Figure 3.3B**). On the contrary, K^+ concentration decreased significantly in the leaf sheaths of SZK and Kunishi, whereas it remained unchanged in FL478. Consequently, higher Na^+/K^+ ratios were observed in SZK and Kunishi than in FL478 (**Figure 3.3C**).

3.3.4 Effect of salt stress on Na^+ concentration in time course

Avoiding excess Na^+ in the shoots is one of the well-characterized mechanisms of salt tolerance (Munns and Tester 2008). Nevertheless, SZK accumulated high Na^+ in leaf sheaths and leaf blades, indicating a tissue-tolerance mechanism. Therefore, we investigated the initiation of Na^+ accumulation. We conducted a time-course experiment to monitor the increase in Na^+

concentration (**Figure 3.4**). The results showed that SZK immediately started accumulating Na^+ in the roots, leaf sheaths, and leaf blades after the salt-stress treatment was started (three days after treatment) without any symptoms or injury observed. In contrast, FL478 and Kunishi started to accumulate Na^+ six days after treatment (**Figure 3.4**), indicating that SZK accumulates Na^+ faster than FL478 and Kunishi.

3.3.5 Effect of salt stress on element concentration

Mg and Ca concentrations of the three varieties showed similar trends, significantly decreasing under salt-stress conditions (**Figures 3.5** and **3.6**). P concentrations decreased in the roots, leaf sheaths, and leaf blades of FL478; however, it increased in SZK and Kunishi. Fe concentrations in FL478 and SZK in roots, leaf sheaths, and leaf blades remained unchanged, whereas the concentration increased in the roots and leaf sheaths in Kunishi. SZK and Kunishi showed similar trends for other micronutrient concentrations, such as Mn, Cu, and Zn.

3.3.6 Effect of salt stress on gene expression of Na transporters

Transcriptomic changes of genes encoding Na^+ transporters were analyzed to understand the mechanisms underlying the differences in Na^+ concentrations among the three varieties. In response to salt stress, FL478 showed high up-regulation of *OsHKT1;5* expression in the roots (**Figure 3.7A**). However, expression of this gene in the roots of SZK and Kunishi was much lower than in FL478. Moreover, only Kunishi showed up-regulation of *OsHKT1;5* expression in leaf sheaths and leaf blades. Salt stress also induced expression of *OsSOS1* in the root, leaf blades, and leaf sheaths of FL478 by 1.3-, 1.6-, and 2.0-fold, respectively (**Figure 3.7B**). These results

suggested that *OsHKT1;5* and *OsSOS1* may contribute to the low Na^+ concentrations in FL478 shoots.

Vacuolar Na^+/H^+ antiporter, OsNHX1, plays an essential role in the compartmentalization of excess Na^+ in the cytoplasm into vacuoles (Fukuda et al. 2011) without any impact on plant growth, even when high Na^+ accumulation occurs (Assaha et al. 2017). Relative expression of *OsNHX1* was up-regulated in the leaf sheaths and leaf blades of SZK by 1.2- and 1.18-fold, respectively (**Figure 3.8A**), indicating that this gene may contribute to the tissue tolerance of SZK. The expression of other OsNHX genes was analyzed, and it was found that *OsNHX2* expression in the leaf sheaths of SZK was highly up-regulated to 5.07-fold (**Figure 3.8B**). At the same time, the up-regulation was only 1.48-fold in Kunishi, and the expression was repressed in FL478. *OsNHX3* expression was significantly up-regulated in the root of SZK compared to the other two varieties (**Figure 3.8C**). *OsNHX4* expression was up-regulated in the roots of SZK and Kunishi and in the leaf sheaths and leaf blades of FL478 (**Figure 3.8D**). *OsNHX5* expression was up-regulated only in Kunishi leaf sheaths (**Figure 3.8E**).

3.3.7 Transcriptomic analysis

Genome-wide transcriptome analysis was performed by using RNA-seq to ascertain the molecular changes associated with tissue tolerance in SZK. As a result, 1998 and 4623 DEGs were identified in the leaf sheaths and leaf blades of SKZ, respectively. Among them, 415 and 917 were up-regulated, and 1583 and 3706 were down-regulated, respectively (**Figure 3.9A**). A Venn Diagram of the up-regulated genes showed that 207 genes overlapped between the leaf sheaths and leaf blades. To identify the genes responsible for salt tolerance in SZK, we selected genes from the 207 genes in the leaf sheaths, in which the expression was up-regulated to more than 2-

fold and obtained 64 genes. We aligned these genes with those in the leaf blades (**Figure 3.9B**) and found that expression of the genes encoding heat shock proteins (OsHSP), such as *OsHSP90, OsHSP18, OsHSP70, OsHSP101,* and *OsHSP24.1,* were up-regulated. Heat shock transcription factors such as *OsHsfA2a*, small heat shock proteins such as *OsHSP17.3* and *OsHSP18,* and cytosolic HSP70 (*OsctHSP70*) were up-regulated in both the leaf sheaths and leaf blades. Subsequently, qRT-PCR was performed to confirm the RNA-seq results. *OsHSP90* expression was highly up-regulated by 7.24- and 20.36-fold in leaf blades and leaf sheaths, respectively (**Figure 3.10A**). *OsHSP90* was found to be specific to SZK, indicating that this gene may play an essential role in SZK under salt stress. Moreover, expressions of *OsHsp20, OsHsp24.1,* and *OsHsfA2a* (**Figures 3.10B-D**) were also found to be up-regulated specifically in the leaf sheaths of SZK, indicating that these genes may be associated with tissue tolerance in SZK. Besides *OsHsps* genes, *OsCI2c* was found to be highly up-regulated in the leaf sheaths of SZK by 290.93-fold (**Figure 3.10F**), indicating that *OsCI2c* which is a protease inhibitor may play a role in tissue tolerance in SZK.

GO enrichment analysis revealed that up-regulated DEGs in the leaf sheaths of SZK were involved primarily in response to abiotic stresses and protein folding, including chaperone-mediated protein folding, *de novo* post-translational protein folding, and chaperone cofactor-dependent protein folding (**Figure 3.11A**), which manage by HSP protein. These results were verified by the KEGG pathway analysis, which found that protein processing in the endoplasmic reticulum is the most significant pathway (**Figure 3.11A, C).** GO terms of up-regulated DEGs in the leaf blades sample were predominantly associated with response to abiotic stresses such as response to hydrogen peroxide, heat, and salt stress. KEGG pathway analysis of this sample showed significance in carotenoid biosynthesis.

3.3.8 Yeast complementary assay

Functional analysis of several genes was conducted to understand the molecular mechanisms underlying tissue tolerance in SZK. Six up-regulated genes from the RNA-seq results (*OsCYP76C2, OsLAC28, OsLOX-L2, OsCI2c, OsHsp18, and OsHsp20*) were selected to be expressed in the Na^+ sensitive yeast strain AB11c and osmotic sensitive yeast strain BY22935. Subsequently, their effect on yeast growth under salt stress and osmotic stress conditions was observed. As shown in **Figure 3.13**, the growth of the AB11c cells transformed with empty pYES2 decreased in a medium containing 150 mM NaCl. In contrast, the growth of the AB11c cells expressing *OsCYP76C2, OsLAC28,* or *OsLOX-L2* was enhanced. The growth of the BY22935 cells transformed with pYES2 decreased in a medium containing 300 mM and 600 mM sorbitol, while the growth of the BY22935 cells expressing *OsCI2c, OsHsp18*, and *OsHsp20* was enhanced (**Figure 3.14**). These results indicate that these six genes may play a role in the tissue tolerance of SZK.

Table 3.1 Primers used for quantitative real-time PCR

Genes	Forward Primer (5'-3')	Reverse Primer (5'-3')
OsUBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
OsHKT1;5	CCCATCAACTACAGCGTCCT	AGCTGTACCCCGTGCTGA
OsSOS1	ATACTGAGTGGGGTTGTTATTG	AAAGGTAAATTTCAAAAGGTACATGG
OsNHX1	TGGCTGCTGCTAATGAGTTG	ACCAATCATCCCGAACCAT
OsNHX2	TTCATGAGGCCGGTGTTT	GCACGCTCCTTTGAAAAAGTT
OsNHX3	AGGCTTCGTTCCTTTCGTG	ATGCTTGCACCTTCTGAACC
OsNHX4	GACTTCATCATGCCGTTCCT	CATGAACCTGTCGTCGTCGAACTT
OsNHX5	CCACCTGACAGCCACATACA	GCCGACGAATCAAGTTGTGT
OsHSP90	AGATGGAGGAGGTGGACTGA	ACACGACCACACACTGTTGA
OsHsp20	CTGATCAAGCCCGACCCTTT	TCGCCCTTCACAACATTCGA
OsHsp24.1	CTCGCGTCGTGGTCAAATTC	AGAGCAAAATGAAGGACGCC
OsHsfA2a	GGGTAGCACTGCACTTGTCT	AGAACATTCGAAGCCACCGT

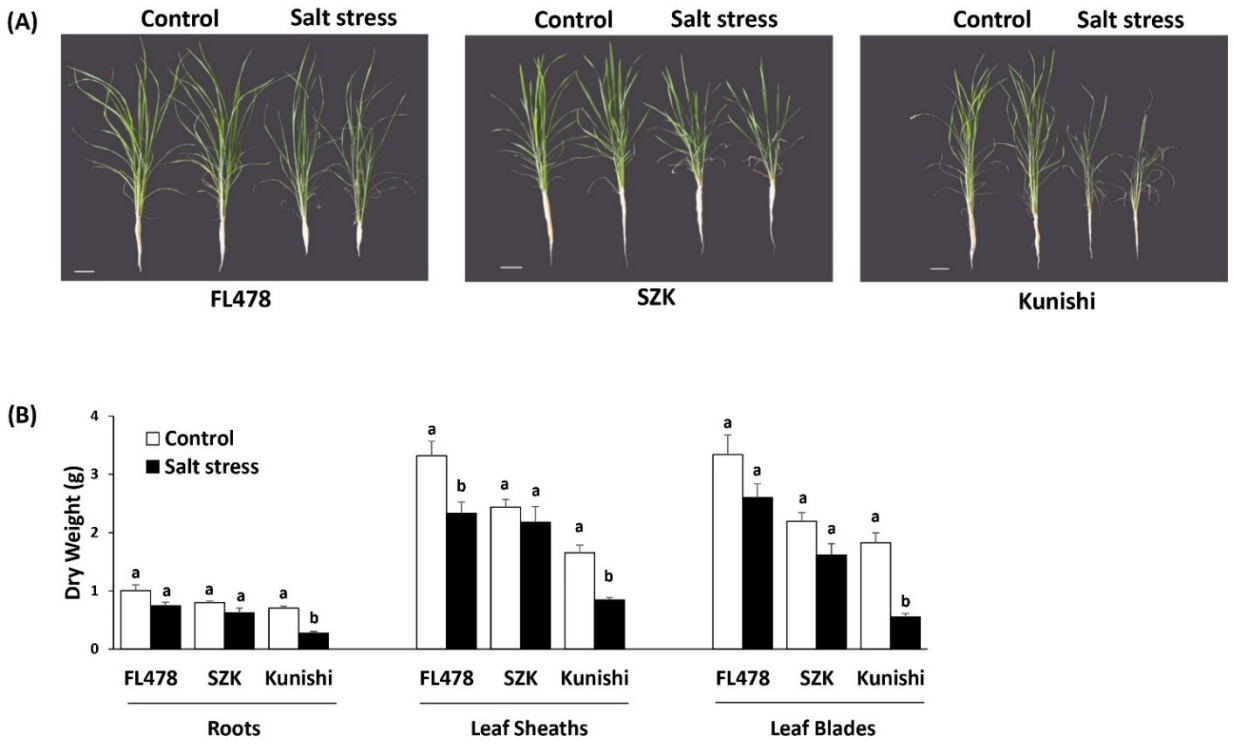

Figure 3.1 Effect of salt stress (100 mM NaCl) on the growth of three varieties. (A) Representative images of 6-week-old rice seedlings under control and salt stress conditions. White bar=10 cm. (B) Dry weight of the roots, leaf sheaths, and leaf blades of the three varieties. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

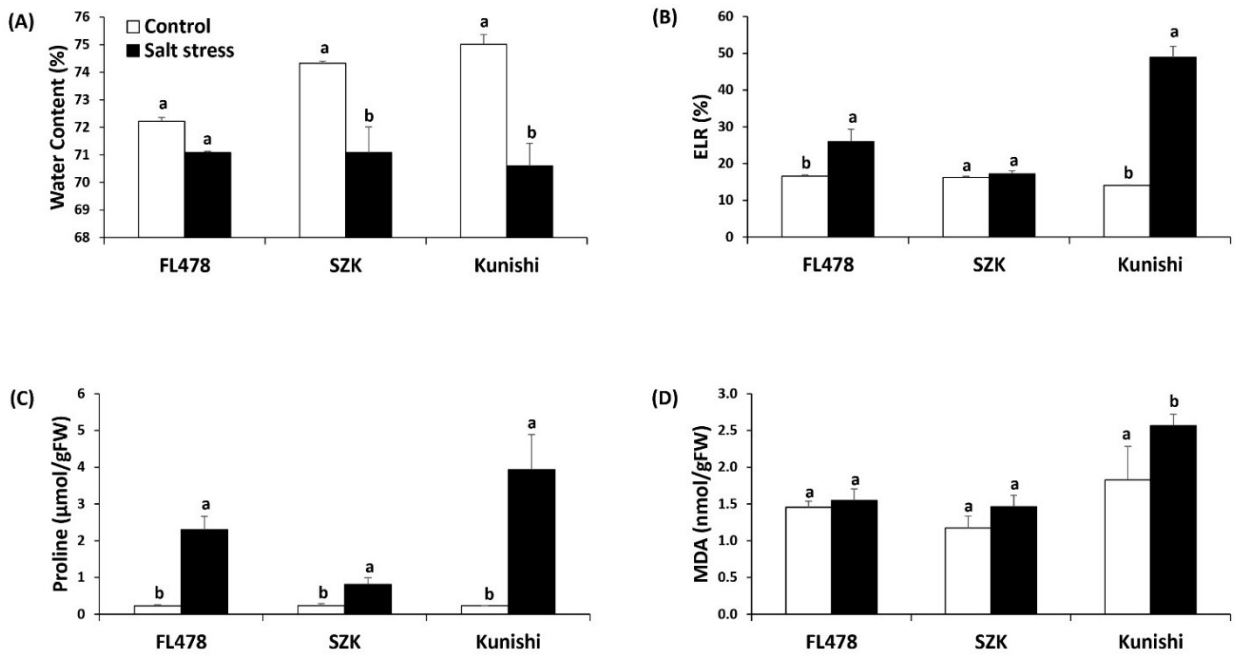

Figure 3.2 Effect of salt stress on the physiological parameters of the three varieties. (A) water content, (B) electrolyte leakage ratio, (C) proline concentration, and (D) malondialdehyde concentration. Data presented as means ± SE (n=3). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

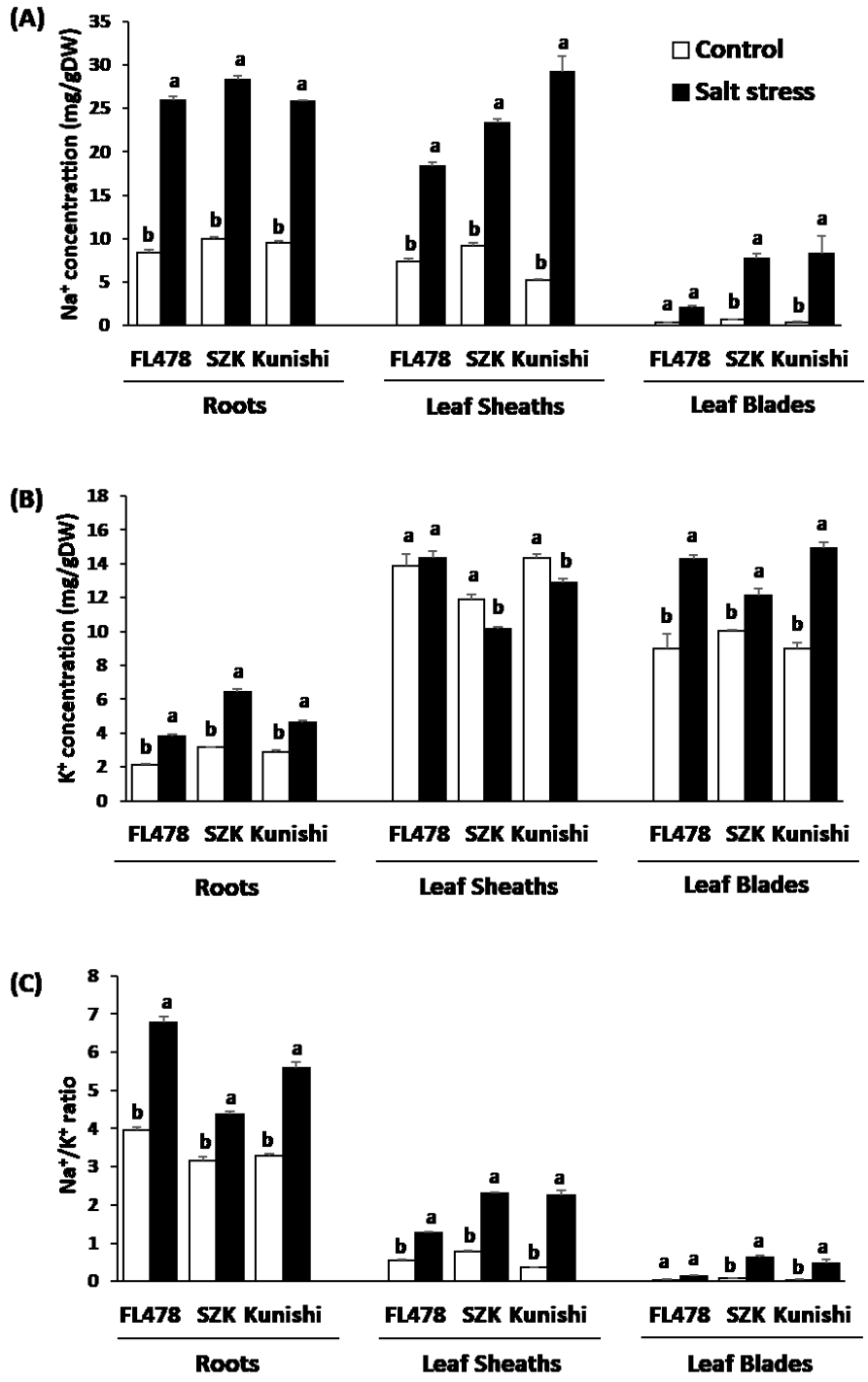

Figure 3.3 The difference in ion accumulation between three varieties under salt stress. (A) Na^+ concentration, (B) K^+ concentration, (C) Na^+/K^+ ratio. Data presented as means ± SE (n=3). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

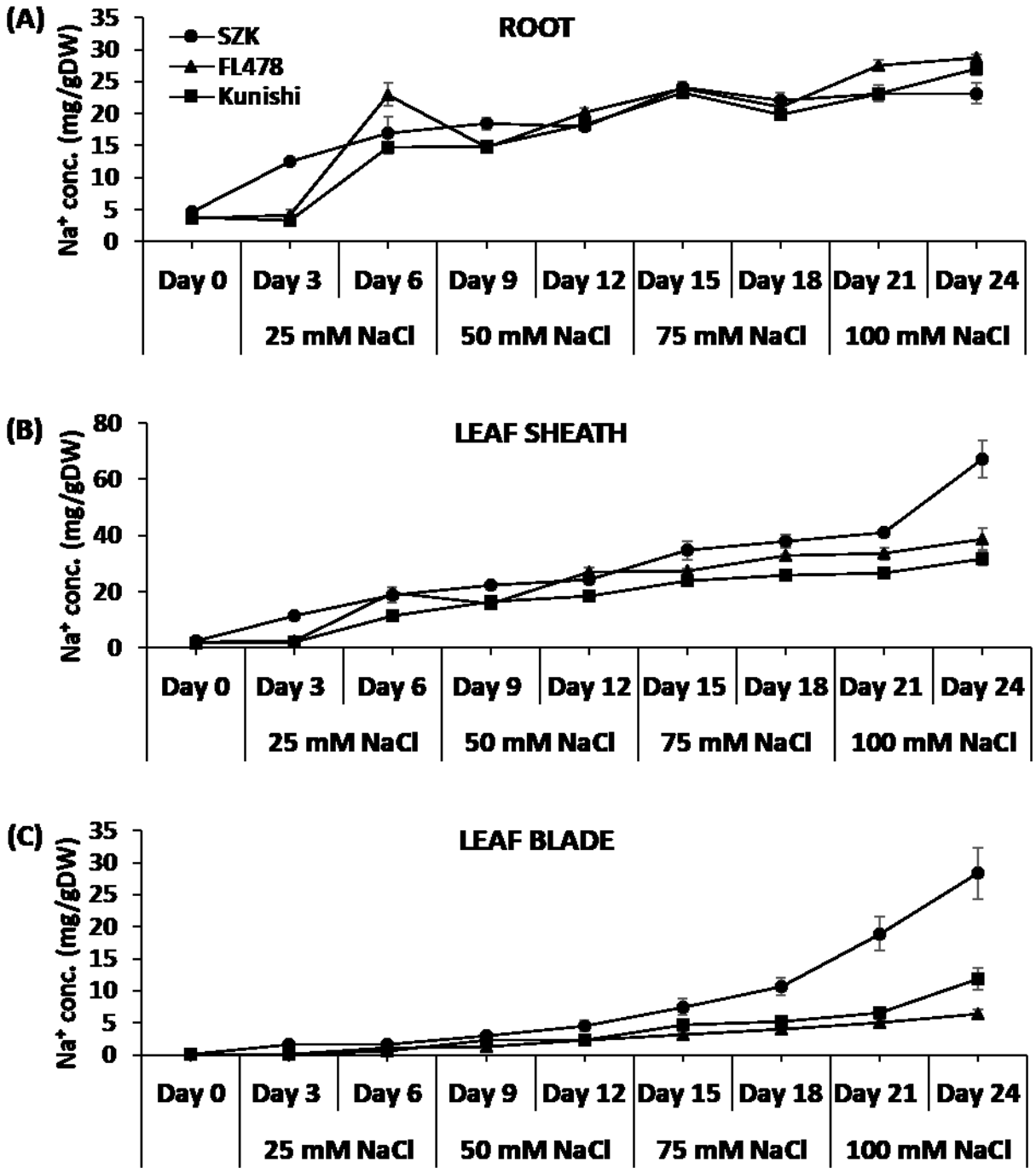

Figure 3.4 The initiation of Na^+ accumulation in the three varieties under salt stress analyzed by a time course in (A) root, (B) leaf sheath, and (C) leaf blade. Data presented as means ± SE (n=4).

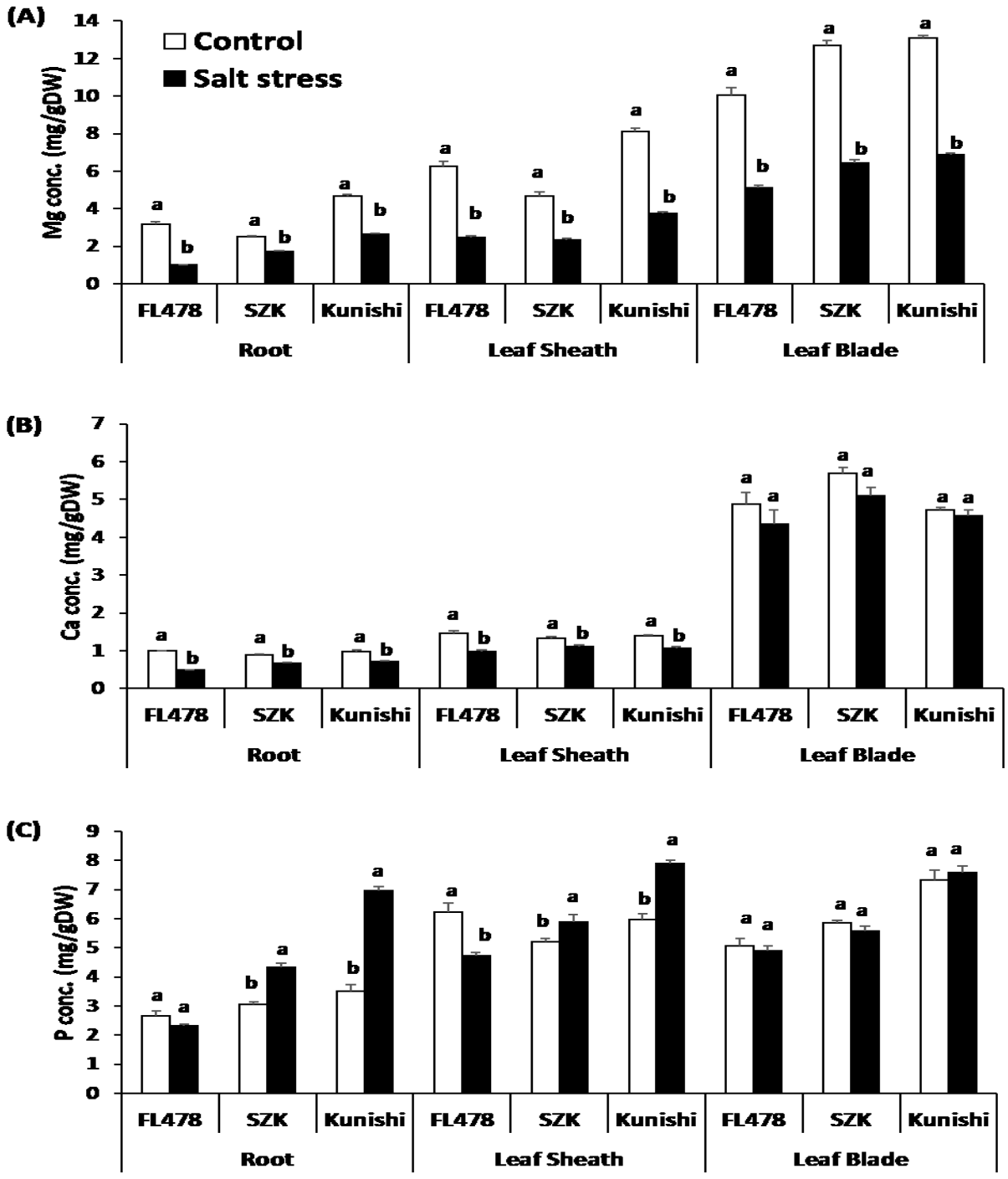

Figure 3.5 Element (macronutrient) concentrations in the roots, leaf sheaths, and leaf blades of the three varieties. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

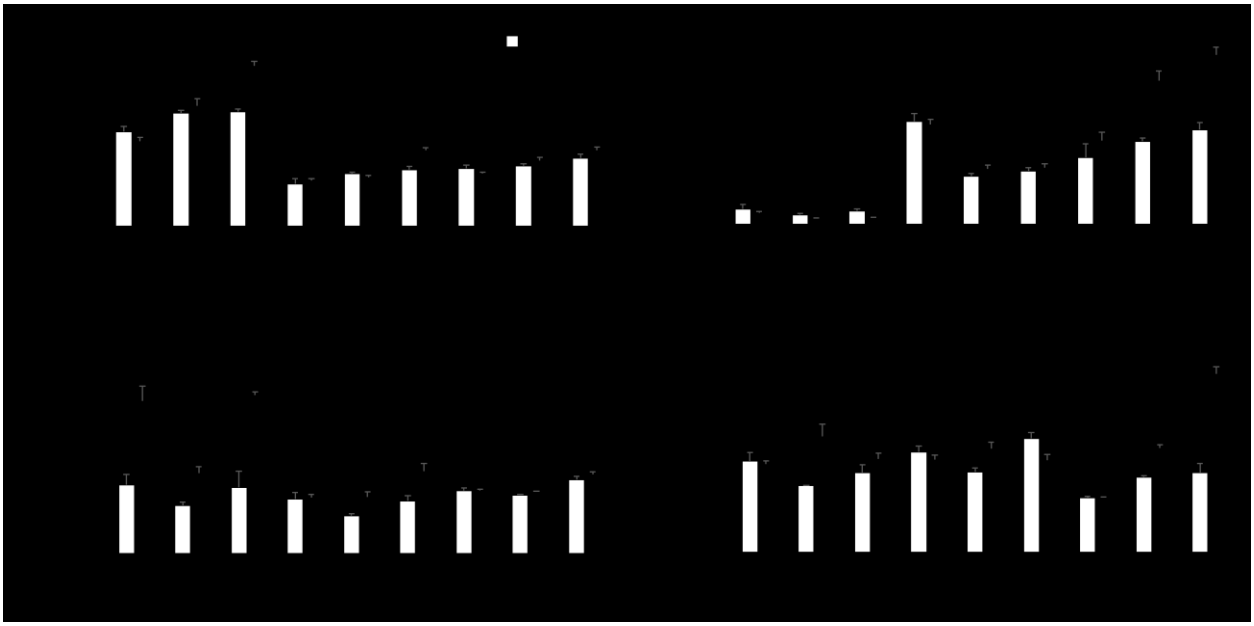

Figure 3.6 Element (micronutrient) concentrations in the roots, leaf sheaths, and leaf blades of the three varieties. Data presented as means ± SE (n=4). The same letters indicate no significant differences between the treatments in each variety ($p < 0.05$).

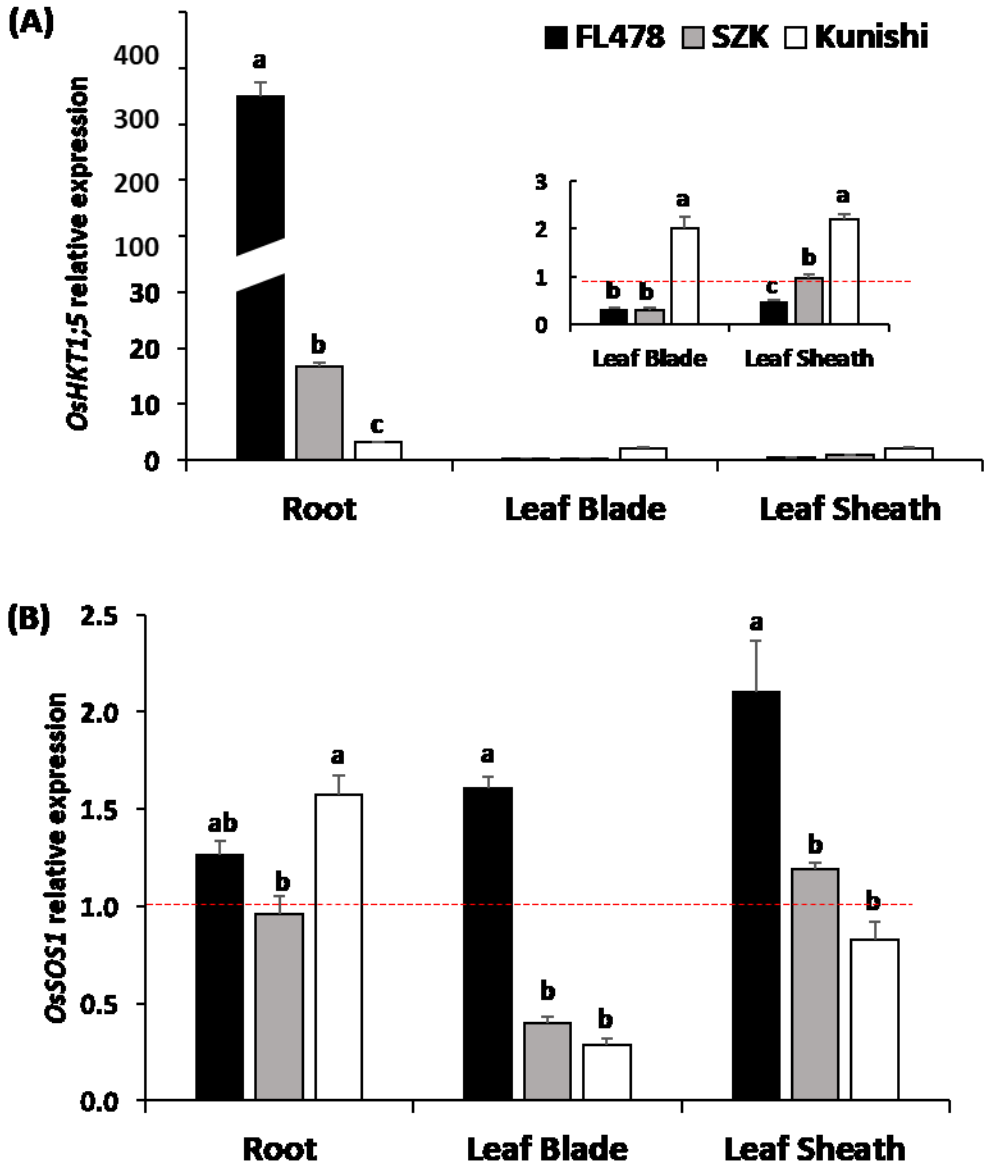

Figure 3.7 Relative expression of genes encoding Na^+ transporter in the roots, leaf sheaths, and leaf blades of the three varieties under salt stress. The number of genes was evaluated based on that of control conditions. The same letters indicate no significant differences between the varieties ($p < 0.05$).

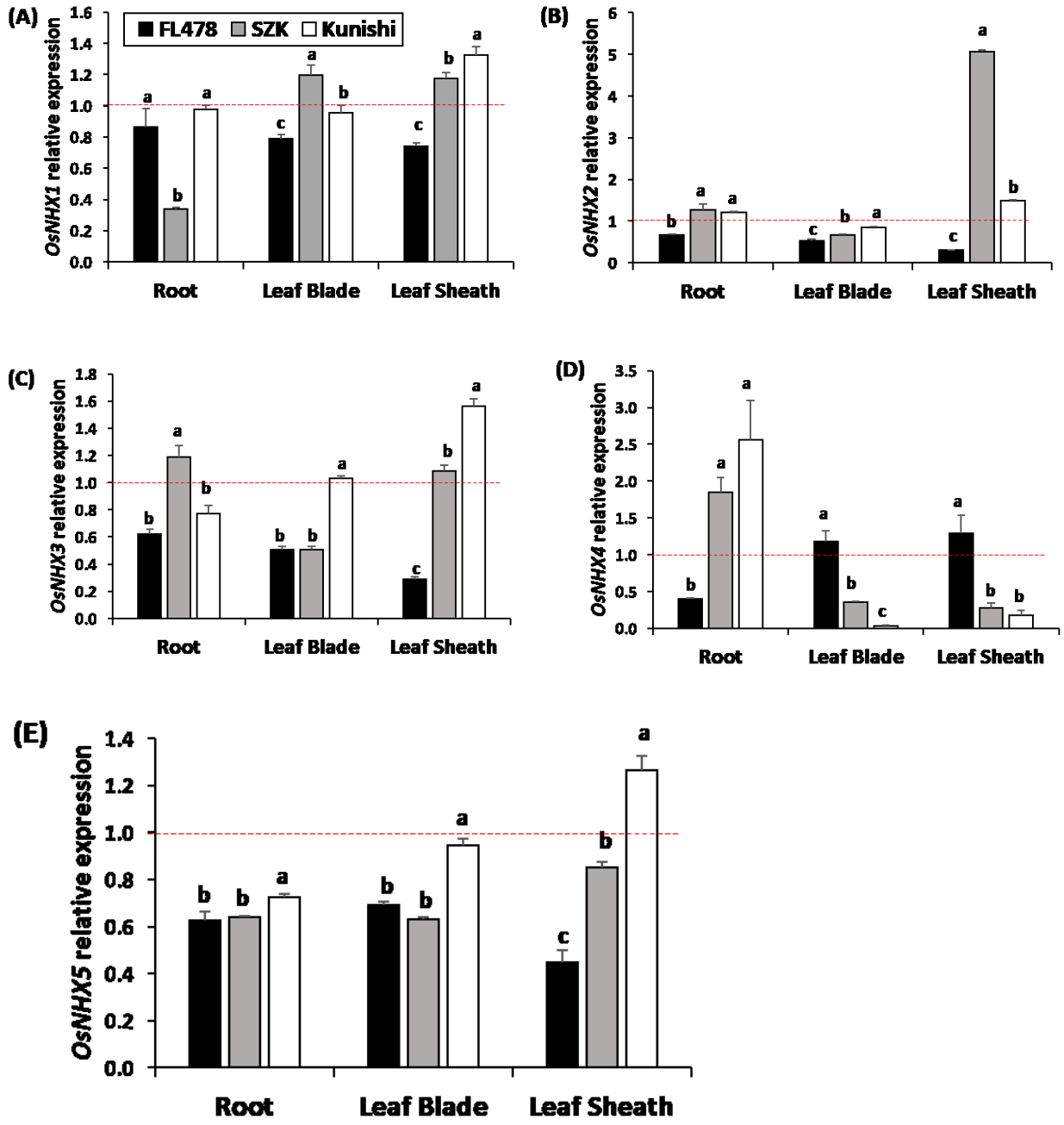

Figure 3.8 Differences in the expression level of *OsNHXs* genes among the varieties under salt stress. The number of genes was evaluated based on that of control conditions. The same letters indicate no significant differences between the varieties ($p < 0.05$).

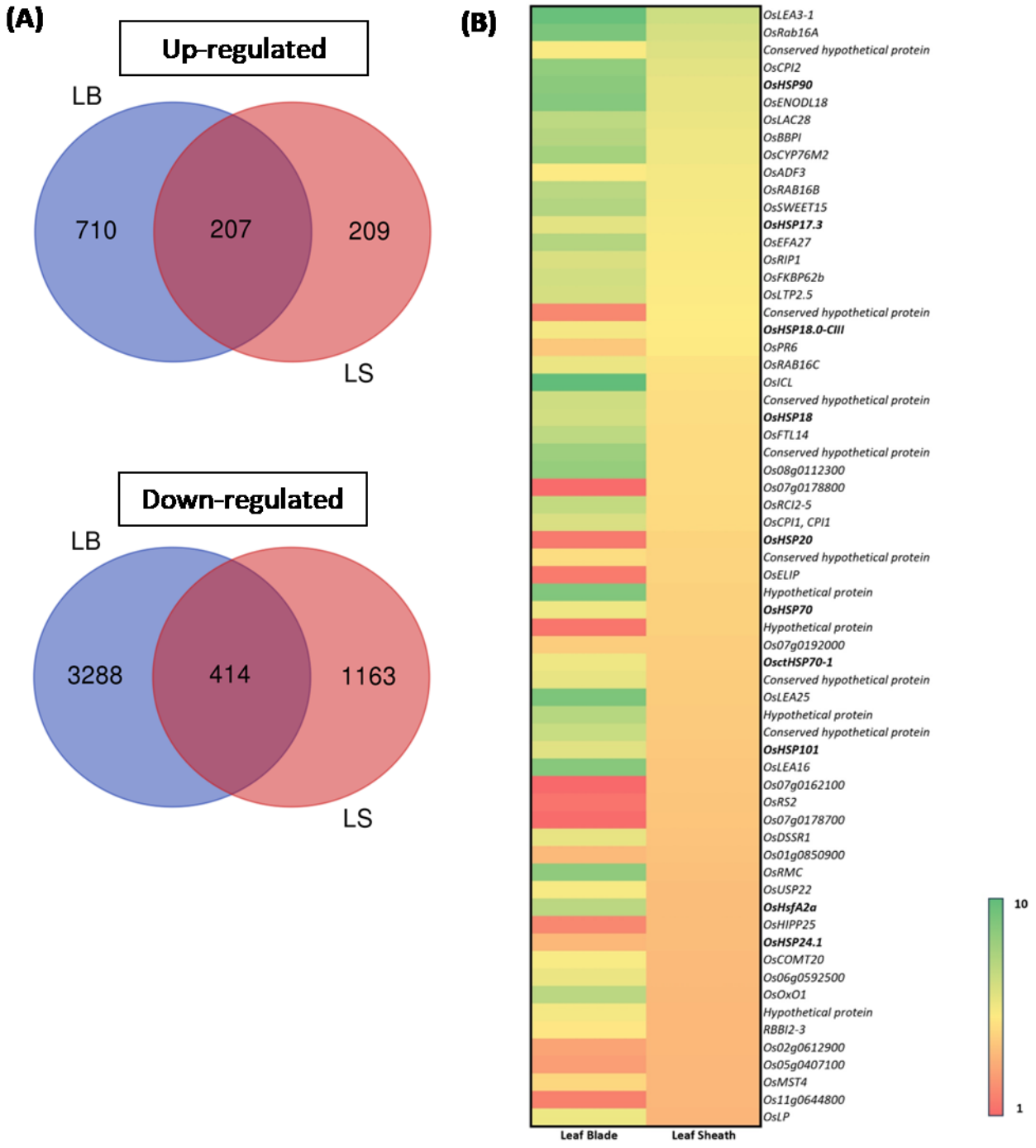

Figure 3.9 Transcriptomic analysis of SZK under salt stress. (A) Venn diagram of the up- and down-regulated genes in the leaf blade and leaf sheath of SZK under salt stress. LB, leaf blade; LS, leaf sheath. (B) The heat map shows the top 64 up-regulated genes (>2-fold) in the leaf sheath alongside the leaf blade.

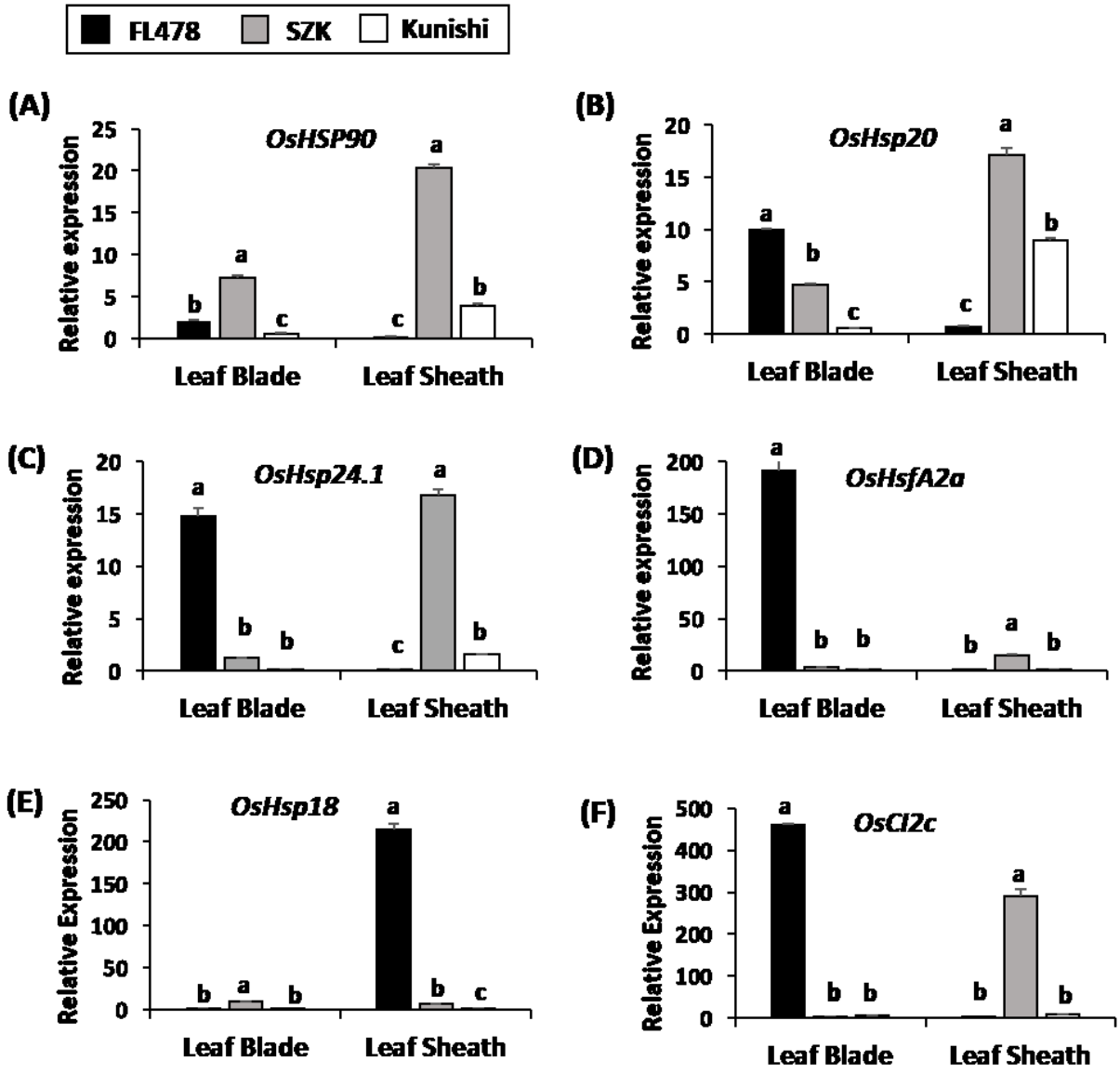

Figure 3.10 qRT-PCR confirmation of SZK-specific genes.

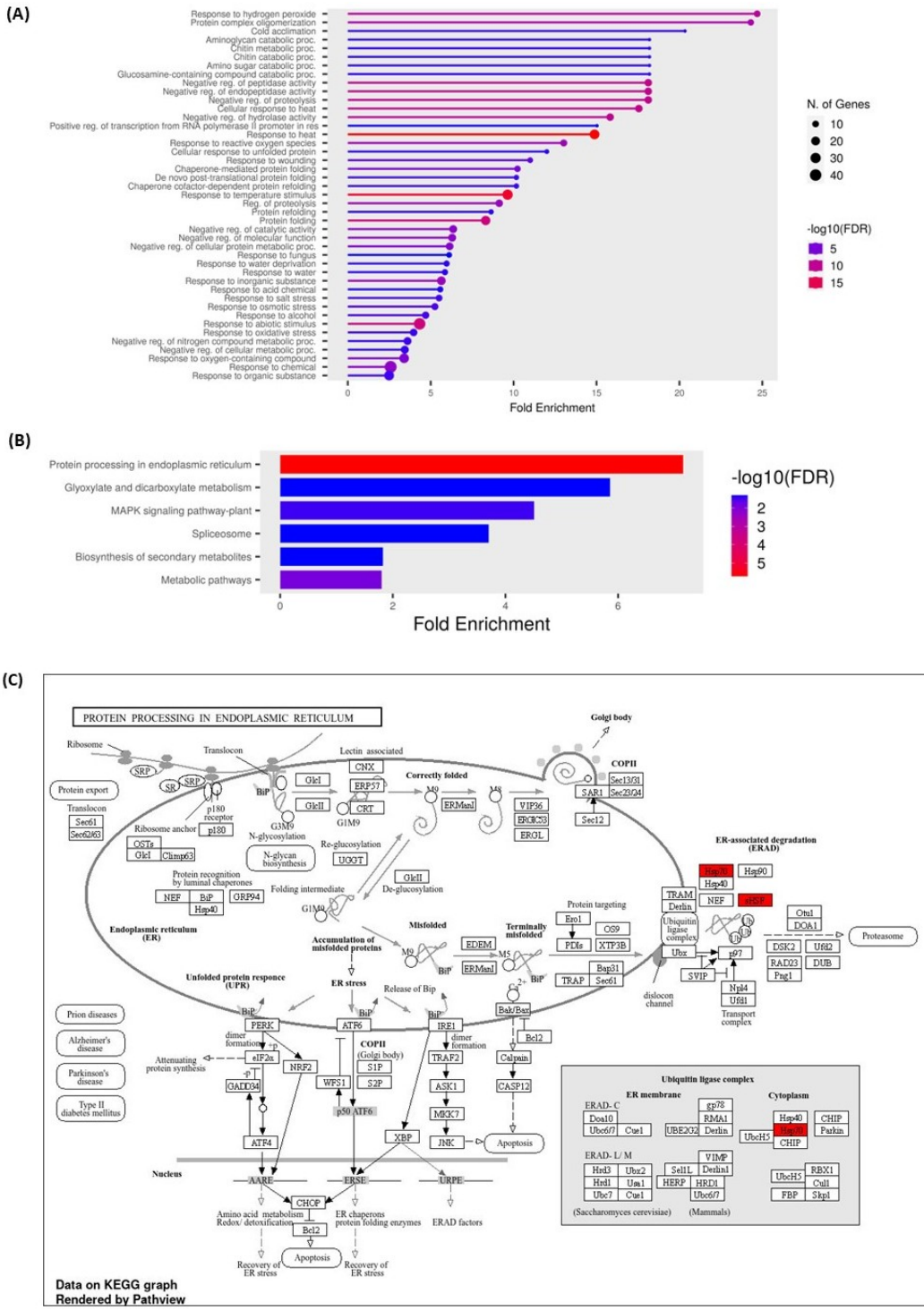

Figure 3.11 GO and KEGG analysis of the up-regulated genes in the leaf sheaths sample of SZK.

(A) GO term enrichment analysis related to biological processes. (B) KEGG pathway classification.

(C) KEGG pathway diagram.

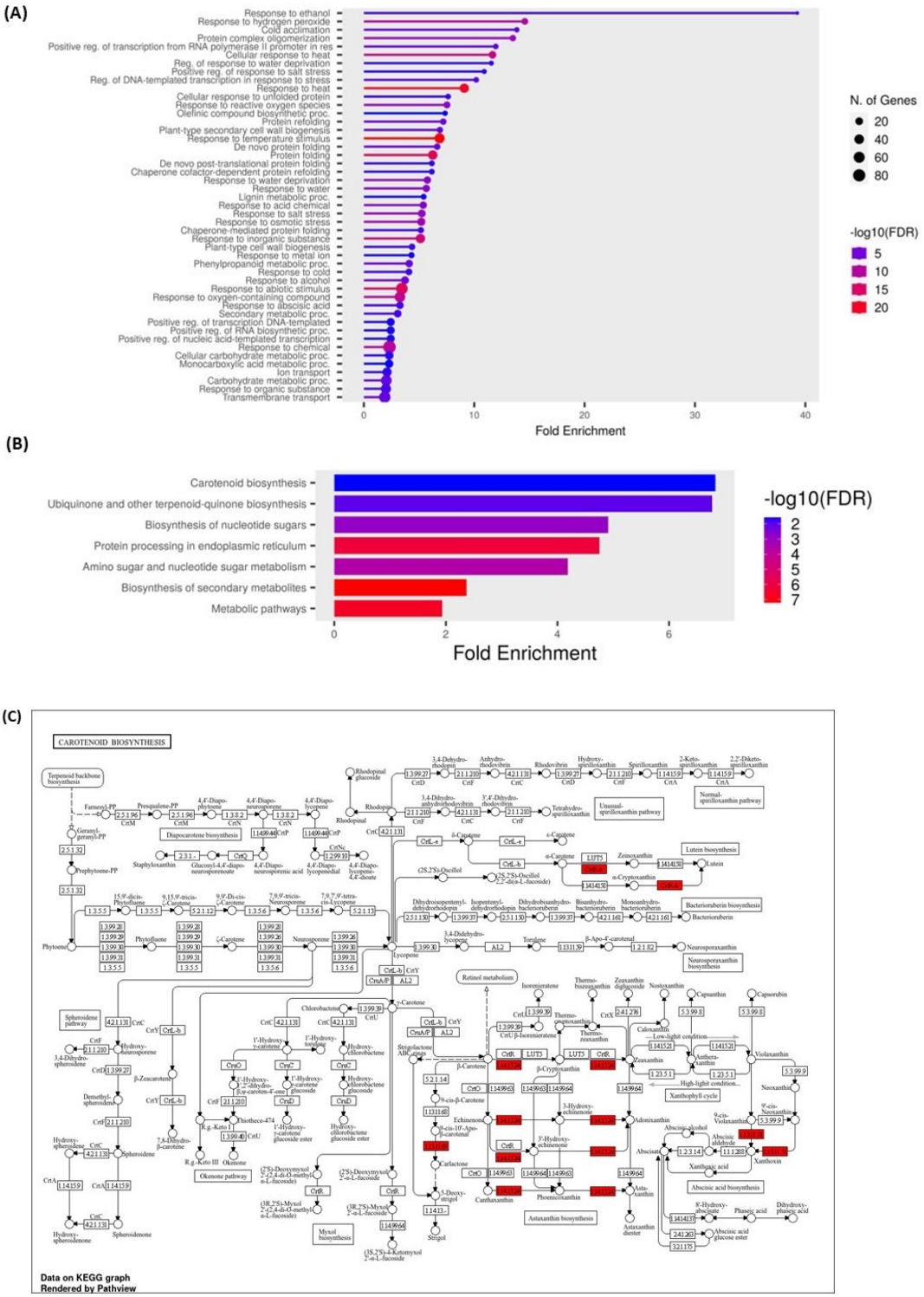

Figure 3.12 GO and KEGG analysis of the up-regulated genes in the leaf blades sample of SZK.

(A) GO term enrichment analysis related to biological processes. (B) KEGG pathway classification.

(C) KEGG pathway diagram.

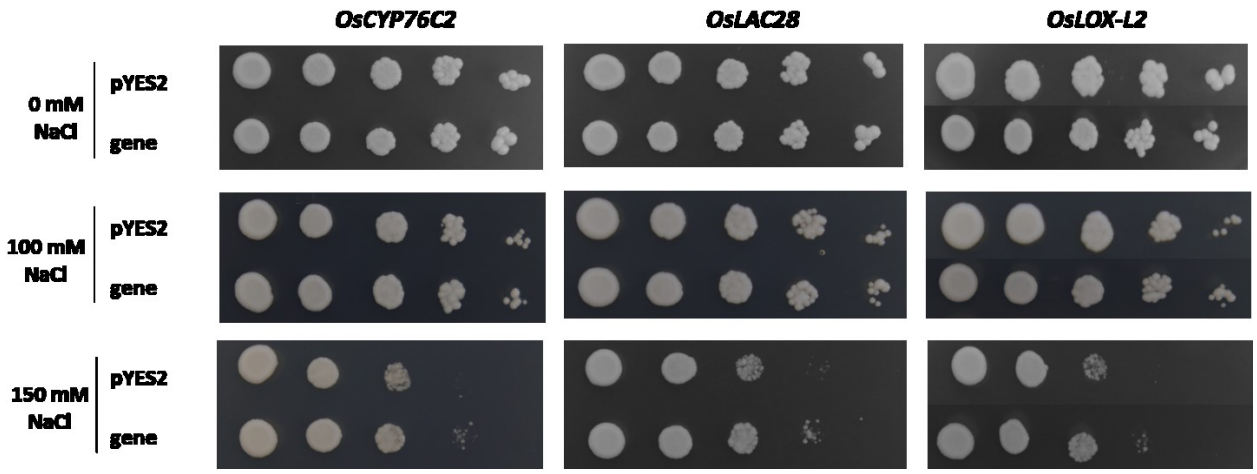

Figure 3.13 Functional analysis of *OsCYP76C2, OsLAC28,* and *OsLOX-L2* in the Na^+ sensitive yeast strain AB11c. The left figure shows the growth of AB11c cells expressing *OsCYP76M2* and empty vector (pYES2) under 0, 100, and 150 mM NaCl. The inoculated samples were serially diluted (10-fold) from left to right.

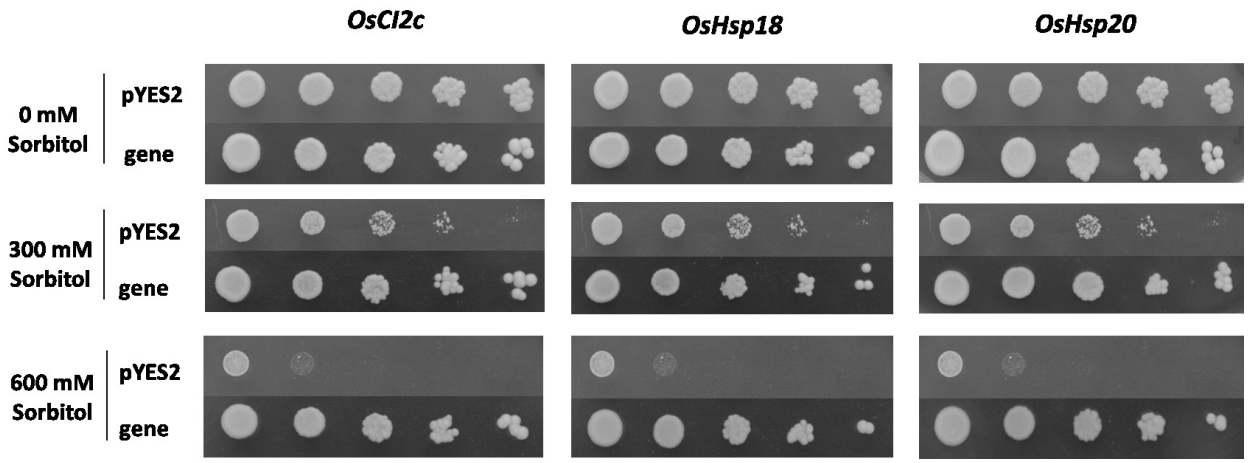

Figure 3.14 Functional analysis of *OsCI2c, OsHsp18,* and *OsHsp20* in the osmotic sensitive yeast strain BY22935. The left figure shows the growth of BY22935 cells expressing *OsCI2c* and empty vector (pYES2) under 0, 300, and 600 mM sorbitol. The inoculated samples were serially diluted (10-fold) from left to right.

3.4 Discussions

For future crop improvement, measures of salinity tolerance in landraces and new varieties or cultivars are necessary to help identify species or individuals within species that can be used to increase crop tolerance (Melino and Tester 2023). In a preliminary experiment, we identified a salt-tolerant japonica rice variety, SZK, with a unique mode of action in response to salt stress. Salt tolerance in rice is usually associated with low Na^+ transport to the shoots and Na^+/K^+ discrimination (Na^+ exclusion) (Assaha et al. 2017). However, our study demonstrated that SZK is considered a "tissue-tolerant" variety as it accumulates high Na^+ concentrations in shoots, almost the same as the salt-sensitive Kunishi while maintaining a similar level of salt tolerance to FL478. In the present study, the physiological and molecular characteristics of tissue-tolerant SZK were investigated to elucidate its adaptation to salt stress by comparing it with the salt-tolerant FL478 and salt-sensitive Kunishi.

The most significant effect of salt stress is the reduction of plant biomass. Salt tolerance can be assessed by comparing the percentage of biomass production under salt stress and control conditions over a prolonged period (Munns, 2002). From this comparative analysis, SZK was confirmed to be salt tolerant, similar to FL478, because the reduction in dry weight under salt stress was not significant in roots, leaf sheaths, and leaf blades (**Figure 3.1B**). Although SZK and FL478 were considerably tolerant of salt stress, a distinctive mechanistic difference was observed in their tolerance strategies. SZK had a physiological status similar to that of FL478 but superior to that of Kunishi. Both SZK and FL478 responded better in terms of water content (**Figure. 3.2A**), membrane integrity (**Figure 3.2B**), and degree of oxidative damage (**Figure 3.2D**). In terms of membrane integrity measured using ELR, SZK was better than FL478, as no increase in ELR under salt stress was observed. This result implied that cell injury caused by excessive salt may not occur in SZK. Synthesis of low-molecular-weight compounds or osmoprotectants such as

proline is considered a vital mechanism in plants for the maintenance of the osmotic status of the cell, facilitating water absorption to prevent water loss, and scavenging ROS from cells under prevailing stress conditions (Qureshi et al. 2013). Our study indicated that proline concentration in SZK under salt stress was the lowest (**Figure 3.2C**), suggesting that accumulation of proline may not be the mechanism by which SZK retains ion homeostasis, and this mechanism may not be associated with tissue tolerance in SZK.

While much of the emphasis in rice salt tolerance research has been placed on the exclusion of Na^+ from shoots (Lee et al. 2003; Mekawy et al. 2015; Senadheera et al. 2009; Ueda et al. 2013; Walia et al. 2007; Wangsawang et al. 2018), the present study revealed a tissue tolerance mechanism that employs Na^+ sequestration from the cytosol, resulting in a high Na^+ concentration while retaining growth under salt-stress conditions. The tissue-tolerant SZK accumulated high Na^+ in the leaf sheaths and leaf blades, similar to the sensitive Kunishi (**Figure 3.3A**), whereas FL478 had a low Na^+ concentration in the leaf sheaths and leaf blades. Furthermore, Na^+ concentration measured in time course showed that SZK accumulated Na^+ in the roots, leaf sheaths, and leaf blades from the beginning of salt stress treatments (**Figure 4**). On the last day of observation, Na^+ concentration in SZK was higher than that in Kunishi, indicating that accumulation capacity of Na^+ in SZK was superior to Kunishi. K^+ concentration in the leaf sheaths of SZK decreased significantly, leading to a high Na^+/K^+ ratio, suggesting that SZK contradicts the concept that maintaining a low tissue Na^+/K^+ ratio is key to salt tolerance. Na^+ exclusion in the root xylem epithelial region is considered the primary salt tolerance mechanism in conventional tolerant lines such as FL478, Nona Bokra, and Pokkali (Prusty et al. 2018). However, in our study, tissue-tolerant SZK showed an opposite trend in shoot Na^+ and Na^+/K^+ ratios compared to these varieties.

Among the three varieties, FL478 had a much higher expression of *OsHKT1;5* in the roots (**Figure 3.7A**), indicating superior Na^+ exclusion activity, which did not occur in SZK and Kunishi. This result explains low Na^+ concentrations in the leaf sheaths and leaf blades of FL478. At the same time, lower expression of *OsHKT1;5* in SZK and Kunishi may cause elevated Na^+ accumulation in the leaf sheaths and leaf blades, as reported by Wang et al. (2012) that the down-regulation of the *OsHKT1;5* expression may contribute to the stimulation of Na^+ accumulation in the old leaves. *OsSOS1* expression was up-regulated in the roots, leaf sheaths, and leaf blades of FL478 (**Figure 3.7B**) and is likely also involved in the mechanism to exclude Na^+ since *OsSOS1* actively extrudes absorbed Na^+ back into the extracellular space (Assaha et al. 2017; Hasegawa et al. 2000). Excluding Na^+ from the leaf sheaths and leaf blades so that a low Na^+/K^+ ratio could be achieved is the primary mechanism by which FL478 adapts to salt stress. Leaves are essential organs for carbon fixation and other primary metabolisms. Thus, protecting leaves from toxic ions is necessary for survival under salt stress. By excluding Na^+ from the leaves, FL478 can ensure proper metabolism and retain growth even under salt-stress conditions.

Unlike FL478, SZK up-regulated the expression of *OsNHX1* in the leaf sheaths and leaf blades and *OsNHX2* in the leaf sheaths (**Figure 3.8A, B**), indicating that Na^+ compartmentation in the vacuole might be active. This was confirmed by the high concentration of Na^+ in the leaf sheaths and leaf blades of SZK. Mekawy et al. (2015) reported that increased accumulation of Na^+ in a sensitive rice cultivar is likely a consequence of the activity of *OsNHX1* as the antiporter that facilitates Na^+ uptake into vacuoles in exchange for H^+ in the cytoplasm. Excess Na^+ in the cytosol can disrupt cellular processes, resulting in metabolic failure and cell death. The metabolic toxicity of Na^+ is primarily due to its ability to compete with K^+ for binding sites essential for cellular functions. K^+ activates over 50 enzymes, and Na^+ cannot substitute this role (Hasanuzzaman et al.

2018; Tester and Davenport 2003). Rather than excluding Na^+, SZK actively compartmentalizes Na^+ into vacuoles through *OsNHX1* and *OsNHX2* to sequester it from the cytosol, where most cellular processes occur. Barragán et al. (2012) reported that *NHX1* and *NHX2* have similar expression patterns and identical biochemical activities and that reverse genetics showed functional redundancy. SZK likely uses these two genes to regulate tissue tolerance.

At the cellular level, tissue tolerance is achieved by compartmentalization of Na^+ and Cl^- in the vacuole, together with the synthesis of compatible solutes and their location within the cytoplasm to balance the osmotic pressure of the ions in the vacuole (Munns et al. 2016). However, our results suggested that the proline synthesis does not follow the compartmentalization of Na^+ in SZK. Tissue tolerance is a specialized trait, generally reported to be present in salt susceptible rice varieties such as IR29 (Chakraborty et al. 2020) and in some newly identified wild rice accessions (Prusty et al. 2018). The halophytic wild relative of rice, *Oryza coarctata*, was also found to possess considerable tissue tolerance along with other important salt tolerance traits (Mangu et al. 2019; Mondal et al. 2022). Thus, SZK may have a unique tissue tolerance mechanism that has not been observed in other varieties.

Further, the molecular mechanisms underlying tissue tolerance in SZK was studied at the transcriptomic level using RNA-Seq analysis. The results showed that some *OsHSP* gene families, such as *OsHSP90, OsHSP18, OsHSP70, OsHSP101,* and *OsHSP24.1,* were up-regulated in both the leaf blades and leaf sheaths (**Figure 3.9B**). Heat shock transcription factors, such as *OsHsfA2a*, small heat shock proteins, such as *OsHSP17.3* and *OsHSP18,* and cytosolic HSP70 (*OsctHSP70*) were also up-regulated. Heat shock proteins (HSPs) and small heat shock proteins (sHSPs) play critical roles in preventing protein damage during high-temperature and salinity stress by associating with partially denatured proteins to form stable complexes and preventing

their irreversible aggregation in an ATP-independent manner (Do et al. 2023). Interestingly, expression of *OsHSP90*, *OsHsp20, OsHsp24.1,* and *OsHsfA2a* were found to be up-regulated specifically in the leaf sheaths of SZK (**Figures 3.10**), indicating that these genes may associated with tissue tolerance in SZK. Furthermore, GO enrichment analysis related to biological processes and KEGG pathway analysis showed that up-regulated DEGs in the leaf sheaths of SZK were predominantly associated with protein folding and refolding which involves HSP proteins (**Figure 3.11 A-C**). Functional analysis of *OsHsp18 and OsHsp20* was conducted to confirm these results. The growth of the BY22935 cells expressing *OsHsp18,* and *OsHsp20* was better than those expressing the empty vector (**Figure 3.12**) under osmotic stress conditions, indicating that these genes may play a role in SZK tissue tolerance.

HSPs function as a general chaperone that transiently binds to folding intermediates in vitro, prevents aggregation, and supports the refolding of intermediates to their native states (Liu et al. 2009; Raman and Suguna 2015; Schopf et al. 2017). Protein synthesis requires high concentrations of K^+ for the binding of tRNA to ribosomes (Blaha et al. 2000; Tester and Davenport 2003) and preserve ribosome integrity (Rozov et al. 2019). Under salt stress conditions, protein synthesis was disrupted because K^+ concentration in the cell is lower and cannot be substituted by Na^+. Proteins that are formed incorrectly will be degraded by the proteasome or fixed by molecular chaperone. The ability of SZK to maintain a better physiological status than the salt-sensitive variety while having a high Na^+ concentration may occur under the activity of HSPs, which prevents protein damage, allowing the plants to retain active cellular metabolisms and growth.

Functional analyses of several highly up-regulated genes (*OsCYP76M2, OsLAC28, OsLOX-L2)* and SZK-specific genes (*OsCI2c, OsHsp18, and OsHsp20*) were conducted to identify

novel genes responsible for tissue tolerance in SZK. The growth of the AB11c cells expressing *OsCYP76M2, OsLAC28,* and *OsLOX-L2* was better than those expressing the empty vector (**Figure 3.11**) under high NaCl, indicating that these three genes may play a role in SZK tissue tolerance. However, no studies have reported the function of these genes in salt tolerance in rice. CYP (cytochrome P450) belongs to the oxidoreductase class of enzymes and represents one of the largest enzyme families containing heme-thiolate as a cofactor (Pandian et al. 2020) with key roles in plant evolution and metabolic diversification (Hansen et al. 2021). Members of the CYPs family have many clans and diverse functions. CYPs are involved in plant defense against biotic and abiotic stresses. *AtCYP76C2* from Arabidopsis is associated with rapid hypersensitive cell death, a defense mechanism against bacterial canker (*Pseudomonas syringae*) infection (Godiard et al. 1998). *OsLACs* encode laccase, an enzyme that oxidizes monolignols to produce higher-order lignins that are involved in plant development and stress responses, including salt stress (Liu et al. 2017). Some studies have reported an increase in lignification under salt stress (Dissanayake et al. 2023). *OsLAC28* is highly expressed in the endosperm and was induced by Cu treatment (Liu et al. 2017). *OsLOX-L2* encodes a lipoxygenase that catalyzes the hydroperoxidation of fatty acids (Ohta et al. 1992). *OsLOX* is involved in jasmonic acid (JA) biosynthesis and is a biotic defense-related enzyme (Tong et al. 2023). Although there is no information regarding the roles of *OsCYP76C2, OsLAC28,* and *OsLOX-L2* in salt tolerance, our results suggest that these three genes may confer tissue tolerance to SZK. Further investigation of these three genes is required to understand their roles in salt tolerance, particularly tissue tolerance.

The growth of the BY22935 cells expressing *OsCI2c, OsHsp18,* and *OsHsp20* was better than those expressing the empty vector (**Figure 3.12**) under osmotic stress conditions, indicating that these three genes may play a role in SZK tissue tolerance. *CI2c* encodes a protease inhibitor

and belongs to a family of six chymotrypsin inhibitor 2 (*CI2*) genes (Losvik et al., 2017). Protease inhibitors (PIs) are small proteins that regulate plant physiological processes and defense responses and are often induced during pathogenesis and upon attack by insect herbivores (Losvik et al., 2018). PIs can inhibit insect growth and reproduction by disrupting their digestive physiology. In barley, *CI2c* plays a role in defense against aphids (Losvik et al., 2018). However, the role of *CI2c* in plant salt tolerance remains unknown. Further investigation is required to understand the role of *CI2c* in salt tolerance, particularly tissue tolerance.

3.5 Conclusions

In this study, we demonstrated that a japonica rice landrace SZK is a salt-tolerant variety comparable to the tolerant indica FL478 yet has a different coping mechanism for salt stress. FL478 and most salt-tolerant rice varieties employ the OsHKT1;5 to restrict Na^+ accumulation in shoots. Conversely, SZK accumulated a high Na^+ concentration in the shoot, which was almost the same as that in the salt-sensitive japonica Kunishi, suggesting that SZK is tissue-tolerant. SZK can maintain better growth at high Na^+ concentrations by activating *OsNHX1* and *OsNHX2* to sequester Na^+ into vacuoles so that toxic cytosolic Na^+ can be avoided. SZK also activated *OsHSPs* to prevent protein degradation. *OsCYP76C2, OsLAC28, OsLOX-L2,* and *OsCI2c* are novel target genes that confer tissue tolerance to SZK.

Chapter 4
General Discussion

Climate change and the increasing world population are two existential challenges to the sustainability of the environment and natural assets. Climate change causes increased global temperatures, a greater chance of catastrophic weather events, rising sea levels, and increased coastal land flooding (IPCC, 2023), leading to soil salinization. The rapidly growing population demands higher productivity of crops and pastures on decreasing areas of traditional agricultural land (Munns and Millar, 2023). Crop yield is severely limited by salinity, particularly for crops that are irrigated or planted in coastal lowlands that are vulnerable to seawater intrusion. NaCl is the most prevalent salt and has been the focus of much work on salinity to date. Despite being a micronutrient for plants, excess Na^+ can harm plant growth (Sahi et al., 2006). Both the quantity and the quality of crop production are negatively impacted by salinity. Salt-affected soils in arid and semi-arid regions of Asia, Africa, and South America caused considerable agronomic problems. In Asia alone, 21.5 million ha of the land area is considered salt-affected (of which 12 million ha is due to saline conditions, and the remaining 9.5 million ha is due to alkaline/sodic conditions) (Sahi et al., 2006). Rice is one of the most important crops that suffered a decline in productivity due to salinity. Rice, a staple food crop for more than half of the world's population, is grown in more than a hundred countries, with 90% of the total global production coming from Asia (Fukagawa and Ziska, 2019). Therefore, there is a great urgency in providing adequate rice production.

Some traditional cultivars and rice landraces are more tolerant to various stresses than many elite cultivars (Walia et al., 2005). These resistant genotypes are considered a good source of tolerance traits and can be used to improve plant resistance to salt stress. Exploration and identification of rice landraces or traditional cultivars with salt tolerance traits can be one potential approach to overcoming the decline in rice productivity due to high salinity. In addition, the

characterization of salt tolerance mechanisms is critical for further experiments to increase crop yield on salinized agricultural land. The objective of this study is to 1) to identify rice genotypes from the local Japanese variety that are tolerant to salt stress during the vegetative stage using FL478 and IR29 as the salt-tolerant and salt-sensitive checks, respectively, 2) to characterize the mechanisms underlying the salt-tolerance in SZK by comparing its physiological parameters and expression profiles of some essential genes for salt-tolerance with FL478 and Kunishi, and 3) to elucidate transcriptomic profile and identify genes associated with tissue-tolerance in SZK.

The screening experiment identified a salt-tolerant japonica rice variety, SZK, with a unique mode of action in response to salt stress. Salt tolerance in rice is generally associated with low Na^+ transport to the shoots, which employ the Na^+ exclusion mechanism through the function of *OsHKT1;5* and *OsSOS1*. However, our study demonstrated that SZK is considered a "tissue-tolerant" variety as it accumulates high Na^+ concentrations in shoots, almost the same as the salt-sensitive Kunishi while maintaining a similar level of salt tolerance to FL478. The physiological and molecular characteristics of tissue-tolerant SZK were investigated to elucidate its adaptation to salt stress by comparing it with the salt-tolerant FL478 and salt-sensitive Kunishi. From the comparative analysis, SZK was confirmed to be salt-tolerant, similar to FL478. Although SZK and FL478 were considerably tolerant of salt stress, a distinctive mechanistic difference was observed in their tolerance strategies. The tissue-tolerant SZK accumulated high Na^+ in the shoots, similar to the sensitive Kunishi, whereas FL478 had a lower Na^+ concentration. Furthermore, Na^+ concentration measured in time course showed that SZK accumulated Na^+ earlier than FL478 and Kunishi. At the end of cultivation, Na^+ concentration in SZK was even higher than that in Kunishi, indicating that the accumulation capacity of Na^+ in SZK was superior to Kunishi's. K^+ concentration in the leaf sheaths of SZK decreased significantly, leading to a high Na^+/K^+ ratio,

suggesting that SZK contradicts the concept that maintaining a low tissue Na^+/K^+ ratio is key to salt tolerance and showed an opposite trend in Na^+ discrimination compared to several well-known salt-tolerant cultivars such as Pokkali and Nona Bokra.

Na^+ exclusion and compartmentalization are the two mechanisms which plants cope with ion toxicity. The latter is the plant's strategy to sequester Na^+ and Cl^- in vacuoles and maintain a low concentration in the cytosol, where most cellular metabolism occurs, resulting in a situation known as tissue tolerance. Tissue tolerance is the capacity of the tissue to function while containing high levels of internal Na^+ or Cl^- (Munns et al. 2016). The main component of tissue tolerance is the compartmentalization of ions within vacuoles, which involves ion transporters in the tonoplast, particularly vacuolar Na^+/H^+ antiporters (Flowers and Colmer 2015). Rather than excluding Na^+, SZK actively compartmentalizes Na^+ into vacuoles through *OsNHX1* and *OsNHX2* to sequester it from the cytosol, where most cellular processes occur. At the cellular level, tissue tolerance is achieved by compartmentalization of Na^+ and Cl^- in the vacuole, together with the synthesis of compatible solutes and their location within the cytoplasm to balance the osmotic pressure of the ions in the vacuole (Munns et al. 2016). However, our results suggested that the proline synthesis does not follow the compartmentalization of Na^+ in SZK. These findings give additional evidence that SZK has a unique mechanism to achieve salt tolerance.

The molecular mechanisms underlying tissue tolerance in SZK were studied at the transcriptomic level using RNA-Seq analysis. The results showed that some *OsHSP* gene families, heat shock transcription factors, and cytosolic HSP were up-regulated in the shoots. The expression of *OsHSP90*, *OsHsp20, OsHsp24.1,* and *OsHsfA2a* were found to be up-regulated specifically in the leaf sheaths of SZK, indicating that these genes may associated with tissue tolerance in SZK. HSPs function as a general chaperone that transiently binds to folding intermediates in vitro,

prevents aggregation, and supports refolding intermediates to their native states (Liu et al. 2009; Raman and Suguna 2015; Schopf et al. 2017). Protein synthesis requires high concentrations of K^+ to bind tRNA to ribosomes (Blaha et al. 2000; Tester and Davenport 2003) and preserve ribosome integrity (Rozov et al. 2019). Under salt stress conditions, protein synthesis was disrupted because K^+ concentration in the cell is lower and cannot be substituted by Na^+. Proteins that are formed incorrectly will be degraded by the proteasome or fixed by molecular chaperone. The ability of SZK to maintain a better physiological status than the salt-sensitive variety while having a high Na^+ concentration may occur under the activity of HSPs, which prevents protein damage, allowing the plants to retain active cellular metabolisms and growth.

Functional analysis of several novel genes using yeast complementary assay showed that *OsCYP76C2, OsLAC28, OsLOX-L2,* and *OsCI2c* genes may play a role in SZK tissue tolerance. However, no studies have reported the function of these genes in salt tolerance in rice. Although there is no information regarding the roles of *OsCYP76C2, OsLAC28, OsLOX-L2* and *OsCI2c* in salt tolerance, our results suggest that these genes may confer tissue tolerance to SZK. Further investigation of these three genes is required to understand their roles in salt tolerance, particularly tissue tolerance.

Summary

Soil salinity is a significant abiotic stress in agricultural crop productivity worldwide. One crop that has suffered a decline in productivity due to high salinity is rice. Rice is a glycophyte known as a sensitive plant to high sodium environments. On the other hand, rice is the most widely consumed food crop, and due to population growth, the global rice demand is increasing. Therefore, improving rice resistance to salinity is essential to meet the yearly increase in rice demand.

There are apparent differences in salinity tolerance mechanisms among rice varieties. Exploration of rice landraces or traditional cultivars with salt tolerance traits can be one solution to overcome the decline in rice productivity due to high salinity. In order to find rice genotypes that have the potential for salt tolerance traits, screening rice genotypes that are tolerant to salt stress is essential. Thus, the present study was conducted to identify rice genotypes from the local Japanese variety that are tolerant to salt stress during the vegetative stage. Furthermore, characterization of the mechanism underlying the salt tolerance in the identified salt tolerance (Shuzenji-kokumai, SZK) and elucidation of its transcriptomic profile, followed by identifying genes associated with tissue tolerance in SZK, was conducted.

Fiveteen rice varieties were screened by hydroponic cultivation to identify the tolerant rice genotypes with better growth under salt stress conditions. This experiment identified a Japanese rice variety, SZK, as salt-tolerant with a unique response under salt-stress conditions. This variety can maintain high biomass while having a high sodium concentration in the shoot. These manners differ from FL478, which can exclude sodium from shoots in response to salt stress, so FL478 shows low sodium concentration in shoots. Based on those findings, this variety is considered to have a tissue tolerance ability.

Analyzing the difference in salt tolerance between the rice genotypes could give a better understanding of the salinity tolerance mechanism in rice so that improving rice resistance to

salinity could be achieved. Both physiological and molecular mechanisms behind the salt tolerance in SZK were analyzed by comparing its physiological and molecular characteristics with FL478 and Kunishi, which are salt-tolerant and salt-sensitive, respectively. Under salt stress, SZK accumulates high sodium in the shoot, almost as high as the salt-sensitive Kunishi. The electrolyte leakage ratio and malondialdehyde concentration of SZK do not change significantly under salt stress, indicating that salt stress does not affect the membrane damage of SK. The transcript levels of the genes encoding sodium transporters were analyzed to understand the mechanisms behind the differences in salt tolerance between the three genotypes. In response to salt stress, FL478 showed induction of expression of *OsHKT1;5* genes in roots that may contribute to sodium exclusion from the shoots, while SZK showed induction of expression of *OsNHX2* gene in the leaf sheaths. This result implied that SZK could compartmentalize Na^+ in the vacuole through the function of *OsNHX2* to avoid sodium toxicity in the cytosol and maintain better physiological status under salt stress. RNA-seq analysis was then performed to find transcriptomic profiles of SK under salt stress. Among the 4623 and 1998 DEGs in the leaf blade and leaf sheath, respectively.

The expression of *OsHSP90*, *OsHsp20, OsHsp24.1,* and *OsHsfA2a* were found to be up-regulated specifically in the leaf sheaths of SZK, indicating that these genes may associated with tissue tolerance in SZK. HSPs function as a general chaperone that transiently binds to folding intermediates in vitro, prevents aggregation and supports refolding intermediates to their native states (Liu et al. 2009; Raman and Suguna 2015; Schopf et al. 2017). Protein synthesis requires high concentrations of K^+ for the binding of tRNA to ribosomes (Blaha et al. 2000; Tester and Davenport 2003) and to preserve ribosome integrity (Rozov et al. 2019). Under salt stress conditions, protein synthesis was disrupted because K^+ concentration in the cell is lower and cannot be substituted by Na^+. Proteins that are formed incorrectly will be degraded by the

proteasome or fixed by molecular chaperone. The ability of SZK to maintain a better physiological status than the salt-sensitive variety while having a high Na^+ concentration may occur under the activity of HSPs, which prevents protein damage, allowing the plants to retain active cellular metabolisms and growth.

Functional analysis of several novel genes using yeast complementary assay showed that *OsCYP76C2, OsLAC28,* and *OsLOX-L2* genes may play a role in SZK tissue tolerance. However, no studies have reported the function of these genes in salt tolerance in rice. Although there is no information regarding the roles of *OsCYP76C2, OsLAC28,* and *OsLOX-L2* in salt tolerance, our results suggest that these three genes may confer tissue tolerance to SZK. Further investigation of these three genes is required to understand their roles in salt tolerance, particularly tissue tolerance.

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