DOCTORAL THESIS

Molecular Physiological Study of Saline-Alkaline Stress Tolerance in Rice

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We hereby recommend that the dissertation by Ms. SUMANA CHUAMNAKTHONG titled "Molecular Physiological Study of Saline-Alkaline Stress Tolerance in Rice" be accepted in partial fulfilment of the requirements for the degree of DOCTOR OF AGRICULTURE.

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ABBREVIATIONS

cDNA	Complementary deoxyribonucleic acid
C_T	Threshold cycle
DMA	Deoxymugineic acid
DW	Dry weight
EC	Electrical conductivity
HKT	High affinity potassium transporter
MAs	Mugineic acids
MDA	Malondialdehyde
NHX	Vacuolar Na^+/H^+ antiporter
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time-polymerase chain reaction
ROS	Reactive oxygen species
S.E.	Standard error
SOS	Salt overly sensitive
ΔC_T	Change in threshold cycle

Contents

Chapter 1

General Introduction

1.1 Saline-alkaline soils

Saline, alkaline or mixed saline-alkaline soils frequently co‐occur in the nature. More than 800 million hectares of land are severely threatened by salinization and alkalization (FAO, 2008), especially in arid and semiarid areas that have a high rate of evaporation and temperature. The differences between those soils are pH levels and main anion components. The pH of saline soils is generally below 8.5, which contains a high concentration of neutral salts (NaCl and Na_2SO_4). While the pH of alkaline or saline-alkaline soils is often greater than 8.5 due to the hydrolysis of two sodium carbonates ($NaHCO_3$ and Na_2CO_3) and the presence of those salts creates white colored deposits on the soil surface (Tang et al., 2014). In addition, saline soils have an exchangeable sodium percentage (ESP) of less than 15%, whereas alkaline or saline-alkaline soils have an exchangeable sodium percentage greater than 15% (Sparks, 2003).

1.2 Impact of saline-alkaline stress on plants

Plants grown under saline-alkaline soils must cope with physiological drought (osmotic stress), Na^+ toxicity (ionic stress), and also high pH stress. The combination of these three factors markedly inhibits plant growth and development rather than sole saline stress. Only few plant species can survive and complete their life cycle on such soils (Parida and Das, 2005). Under high Na^+ environments, the first phase of growth reduction in plants is caused by osmotic stress. The water uptake from roots is inhibited due to higher osmotic pressure generated by accumulation of soluble salts in the soil solution than that in the root cells and this makes plants difficult to acquire both water and nutrients, thus the rate of plant growth falls significantly (Verslues et al., 2006). The study by Munns and Tester (2008) has been reported that at initial response of saline stress, shoot growth is often more decreased than root growth, this is probably because a reduction in the leaf area development relative to root growth would decrease the water use by plants. Later on, ionic stress can be observed as a second phase response, which becomes gradually more severe in plant cells by an excess of Na^+ or Cl^- accumulation (Munns, 2005; Munns and Tester, 2008). The negative effects of ionic stress (Na^+ toxicity) on plant tissue are

frequently observed in older leaves rather than younger leaves and the injury symptoms may be visible as yellowing (chlorosis) or death of old leaves. At the cellular levels, high Na^+ concentrations in the cytoplasm not only cause inhibition of protein synthesis and metabolic enzymes, but also disrupt the homeostasis of other mineral elements such as K^+. Both Na^+ and K^+ are classified as monovalent cations, which have similar physicochemical properties, but their functions in plants are different. K^+, which is widely used by plants, plays important roles in maintaining cell turgor and osmotic adjustment, whereas Na^+ is considered as non-essential element for plants (except in some C_4 plant species) and it easily reaches toxic levels (Kronzucker et al., 2013; Neives-Cordones et al., 2016). Under salinity stress conditions, the competitive inhibition of K^+ uptake by Na^+ often leads to the Na^+ interfering in many K^+-dependent processes (Assaha et al., 2015). Therefore, increasing the concentration of K^+ in the cytoplasm above a certain threshold as well as maintaining a high cytosolic K^+/Na^+ ratio is required for salinity tolerance in plants.

Beside osmotic and ionic stresses, high pH of saline-alkaline soils is considered as an additional factor which causes many adverse effects on plant growth rather than saline soils. A high pH environment typically reduces availability of proton (H^+) for plant, resulting in the destruction or inhibition of transmembrane electrochemical-potential gradients in root cells, and the loss of normal physiological root functions such as ion absorption (Yang et al., 2008a, b). Several elements such as Fe, Mn, Cu, and Zn are commonly precipitated under high pH conditions, resulting in low availability of plant nutrients or ion imbalance in the root zones. There have been various reports showing that plants respond differently after exposed to either saline (NaCl and Na_2SO_4) or saline-alkaline ($NaHCO_3$ and Na_2CO_3) stress conditions (Yang et al., 2007; Gao et al., 2010; Wang et al., 2011; Wang et al., 2015). A study by Gao et al. (2010) suggested that under saline stress conditions, wheat seedlings might enhance fructan synthesis to maintain a stable ion balance, but the high pH of alkaline stress might inhibit the fructan synthesis. Furthermore, Wang et al. (2011) reported that an increase in the concentrations of organic acids such as citrate and malate was considered as an essential mechanism for rice to maintain the intracellular ion balance under saline-alkaline stress, while the accumulation of inorganic anions (Cl^-, HCO_3^-, and CO_3^{2-}) was prominent in rice seedlings grown under saline stress.

1.3 Saline-alkaline tolerance mechanisms in plants

Plants have evolved several mechanisms to withstand restrictive growth imposed by saline-alkaline stress conditions. These include Na^+ extrusion (minimizing the entry of Na^+ into the plant), Na^+ compartmentalization (minimizing the concentration of Na^+ in the cytoplasm), the accumulation of compatible solutes (small molecules that are used to maintain turgor pressure under water deficit conditions), the secretion of organic acid anions, and the secretion of H^+ to the acidification of the rhizosphere (Munns, 2002; Liu et al., 2012; Wang et al., 2015; Babgohari et al., 2013). In addition, plants grown under saline-alkaline stress often suffer from Fe deficiency, resulting in chlorosis of the young leaf blades. Although Fe is classified as a micronutrient which is required by plants with smaller amounts than macronutrients (N, P, K, Ca, Mg, and S), it is very important for chlorophyll formation as well as protein synthesis and enzymatic functions (Jone, 2012). Higher plants have developed two distinct strategies to acquire Fe from alkaline soils (Marschner et al., 1986a, b); the reduction-based Strategy I system is employed by nongraminaceous plants (tomato, soybean, and pea) and the chelation-based Strategy II system is used by graminaceous plants (rice, barley, and maize) (Schmidt, 2003; Kobayashi and Nishizawa, 2012). Strategy I plants have to reduce Fe^{3+} to Fe^{2+}, this process is driven by the activity of Fe^{3+} chelate reductases, then the reduced Fe^{2+} is transported into the plant cells via Fe^{2+} transporters. Whereas the Fe acquisition by Strategy II plants is relied on the secretion of Fe^{3+}-chelating substances rather than reduction process (Guerinot et al., 1994). To solubilize Fe in the alkaline soils, Strategy II plants have to synthesize and release mugineic acids (MAs) family phytosiderophores into the rhizospheres, then plants can uptake Fe^{3+}-MAs complex through specific transporters (Takagi et al., 1984; Araki et al., 2015; Jolley and Brown, 1989). Both biosynthesis and secretion of MAs are unique among the graminaceous plants (Mori and Nishizawa 1987; Higuchi et al., 2001; Kanazawa et al., 1994). Barley (*Hordeum vulgare* L.) is considered as the most tolerance crop plants to Fe deficiency conditions, which can synthesize and secrete several kinds of MAs such as deoxymugineic acid (DMA) and 3-epihydroxymugineic acid (Higuchi et al., 2001). Whereas other crop plants such as rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.), and maize (*Zea mays* L.) are classified as the sensitive crop

plants to low Fe availability which can produce and secrete only small amount of MAs, especially DMA (Kanazawa et al., 1994).

1.3.1 Control of Na^+ uptake and transport in plants

The regulation of Na^+ transport into plant cells is one of the most important mechanisms for both saline and saline-alkaline tolerances in plants. When Na^+ is taken up by roots, it is then transported into the xylem via symplastic and apoplastic pathways (Apse and Blumwald, 2007). Most of plant species have ability to exclude the large amount (98%) of Na^+ from roots to rhizosphere and allowing only a small amount (2%) of Na^+ to be continue transported in the xylem to the shoots (Munns, 2005). This extrusion mechanism is driven by the activity of the plasma membrane Na^+/H^+ antiporter encoded by SOS1 (salt overly sensitive 1) (Shi et al., 2002). The study conducted by Shi et al. (2002) in Arabidopsis plants showed that *AtSOS1* was mainly expressed in epidermal cells at the root tip and overexpression of this gene in the wild-type roots resulted in lower Na^+ accumulation in its shoots under high saline stress conditions (100 mM NaCl), which suggested that *AtSOS1* may be responsible for Na^+ efflux outside cells. Besides acting as a Na^+ excluder, *AtSOS1* can also play a role in loading Na^+ into the xylem, this phenomenon was observed when wild type plants were grown under mild saline stress conditions (25 mM NaCl), which resulting in the induction of Na^+ transport to the shoots. Therefore, these lines of evidence indicate that SOS1 expressed in plants may have dual functions either Na^+ influx or Na^+ efflux, and each function is regulated by the different concentrations of Na^+ and H^+ between intracellular and extracellular.

In addition to the Na^+ exclusion mechanism, saline and/or saline-alkaline tolerance in plants is also involved in the Na^+ retrieval mechanism which is governed by HKT genes. In rice, the HKT gene family is divided into two classes; Class I HKT has widely been studied due to the prominent function to preventing the large amount of Na^+ transport from roots to shoots (Rus et al., 2004), whereas Class II HKTs play an important role in Na^+ uptake from external solutions, particularly when K^+ is limiting (Horie et al., 2007). Among class I gene members, *OsHKT1;5* has been narrowed down as a determinant of saline tolerance by quantitative trait locus (QTL) analysis and attributed to the SKC1 locus (Ren et

al., 2005). *OsHKT1;5* encodes a plasma membrane-localized protein and is mainly expressed in the parenchyma cells surrounding the xylem vessels of roots (Deinlein et al., 2014). The previous study by Walia et al. (2007) showed that the salt-tolerant rice genotype Pokkali could be able to maintain a much lower shoot Na^+ concentration relative to the salt-sensitive genotype IR29 under high saline stress conditions (7 dS m^{-1}), and molecular analysis behind this tolerance reveled that the expression of *OsHKT1;5* gene was increased in the roots of Pokkali, but not in IR29. *OsHKT1;4* is another class I gene members in rice which involves in Na^+ removal from xylem to protect the leaf blades from an excess Na^+ accumulation. Suzuki et al. (2016) found that knockdown of *OsHKT1;4* in Nipponbare rice genotype resulted in overaccumulation of Na^+ in both leaf blade and leaf sheath tissues under saline stress conditions, implying that this transporter may be associated with retrieval of Na^+ from the transpiration stream for storage in the leaf sheaths. In contrast to Na^+ removal, Class II HKTs are known as a Na^+ influx transporter (Garciadeblás et al., 2003). The report of Horie et al. (2007) reveled that uptake Na^+ into the plant cells via expression of *OsHKT2;1* gene could be able to enhance growth of rice under K^+ starvation conditions.

An excessive accumulation of Na^+ in the cytosol is deleterious to several metabolic and physiological processes. Plant vacuolar Na^+/H^+ antiporters (NHXs) have been shown to play critical roles in sequestration of Na^+ into the vacuole, cellular pH regulation, and intracellular trafficking under saline and/or saline alkaline stress conditions (Yang et al., 2012; Yamaguchi et al., 2005; Sottosanto et al, 2004). Overexpression of NHX1, a Na^+, K^+/H^+ exchanger, enhances Na^+ toxicity tolerance in various plant species, including Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), tomato (*Lycopersicon esculentum*), rapeseed (*Brassica napus*), and tobacco (*Nicotiana tabacum*), showing the significance of vacuolar Na^+ sequestration in plant overall saline and/or saline-alkaline tolerances (Apse et al., 1999; Yang et al., 2012; Zhang et al., 2001a, b; Gouiaa et al., 2012).

1.3.2 Fe uptake and H^+ secretion in plants

Plants require a tiny amount of iron (Fe) to maintain their normal growth. Under aerobic conditions (the presence of free O_2 in soils), Fe has been presented in an oxidized-form and that cannot be taken up by plants (Guerinot and Yi, 1994). The problems of Fe deficiency in plants can be observed not only under

aerobic conditions, but also under alkaline and/or saline-alkaline conditions, which soils pH is greater than 7.4 (Liu et al., 2012). The oxidized form of Fe is known as ferric (Fe^{3+}), it is usually forms brown ferric hydroxide precipitation ($Fe(OH)_3$) under either aerobic or high pH conditions, whereas ferrous (Fe^{2+}) is more abundant under anaerobic conditions or at low pH.

Rice is classified as graminaceous crops which can use not only strategy II for Fe acquisition, but also a strategy I-like system that may take up Fe^{2+} from rhizosphere into the root cells via *OsIRT1* and *OsIRT2* transporters (Ishimura et al., 2006). To take up Fe^{2+}, Strategy I plants have to reduce Fe^{3+} to Fe^{2+} by the activity of ferric reductase oxidase (FROs) protein (Robinson et al., 1999). However, both expression and enzyme activity of FROs were not detectable in Fe-deficient roots of Nipponbare rice genotype, implying that acquisition of Fe^{2+} in rice is going without the process of Fe^{3+} reduction (Ishimura et al., 2006, 2007). However, it has been reported that the exudation of MAs by graminaceous plants is considered a highly efficient Fe acquisition mechanism as compared to reduction process (Ishimura et al., 2006). MAs biosynthesis in roots results primarily from *S*-adenosyl-L-methionine catalyzed by three consecutive enzymes, including nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS) (Inoue et al., 2003; Lee et al., 2012). Genes encoding NAS and NAAT enzymes have been isolated in many plant species and their activities are well correlated with tolerance to Fe deficiency (Ling et al., 1999; Takahashi et al., 2003; Mizuno et al., 2003). For example, Takahashi et al. (2003) found that overexpression of *HvNAS1* gene from barley resulted in doubling concentrations of Fe and Zn in both young leaf blades and seeds of tobacco. Likewise, overexpression of *AtNAS1* from Arabidopsis plants could be able to increase the concentration of Fe in leaf blades of tobacco (Takahashi et al., 2003; Douchkov et al., 2005). NAAT is another critical enzyme in biosynthesis of the MA family of phytosiderophores which converts nicotianamine (NA) to DMA (Mori and Nishizawa, 1987). Nozoye et al. (2004) reported that the expressions of *NAAT1* and *NAAT2* genes together with *OsNAS1-3* genes in rice plants were markedly enhanced under Fe deficiency conditions, resulting in increased secretion of MAs from roots into the rhizospheres.

Graminaceous plants take up Fe^{3+}-MA complexes via specific transporters called "Yellow Strip-Like" (YSLs). Several YSL gene members have been identified in rice and other crop plants. Both *OsYSL2* and *OsYSL15* expressions in rice are induced by Fe deficiency conditions; *OsYSL2* is preferentially expressed in aerial parts such as leaf blades phloem or developing seeds, suggesting a role in internal/long-distance transport of Fe in plants (Koike et al., 2004) whereas, *OsYSL15* expression is strongly detected in the roots (at epidermis layer and phloem), suggesting its roles in both Fe^{3+}-DMA transport from the soils and in phloem transport of Fe (Aoyama et al., 2009). Li et al. (2016) reported that the expression levels of *OsYSL2* and *OsYSL15* were markedly induced by saline-alkaline stress conditions at pH 8.5 in both the salt-sensitive and salt-tolerant rice genotypes, however, the magnitude of those genes, up-regulation was greater in the salt-tolerant rice genotype than in the sensitive one.

All the above-mentioned genes have been identified as Fe deficiency-related genes which are controlled by a basic helix-loop-helix transcription factor (IRO2). Ogo et al. (2006, 2007) confirmed that *OsIRO2* affected the expression of genes corresponding to all of the steps in MAs biosynthesis of rice plants. Thus, overexpression of *OsIRO2* could be used as an indicator for alkaline/saline-alkaline tolerance in rice. Besides Fe deficiency-responsive genes, the activity of plasma membrane H^+-ATPase is also important for saline-alkaline tolerance in rice.

1.4 Sensitivity of rice to saline-alkaline stress

Rice is considered as one of the most important crops for human diet. It is well documented that most of rice genotypes are susceptible to high salinity stress as generally rice can tolerate salinities ranging between 20-30 mM NaCl (2-3 dS m^{-1}) (Grattan et al., 2002; Walia et al., 2007). The sensitivities of rice to salinity stress depend upon several factors, including genotypes, growth stages, types and concentrations of salts together with duration and levels of stress. However, salt-affected soils commonly contain a mixture of both cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) and anions (Cl^-, SO_4^{2-}, CO_3^{2-}, and HCO_3^-) which are unsuitable for rice growth and development. The combination of alkaline salts usually generates a high pH condition at rhizospheres, resulting in the precipitation of Fe and other micro-

elements. Rice can use both Strategy I and II for iron acquisition, Fe deficiency symptoms are frequently observed under saline-alkaline stress conditions. In past decades, a few rice genotypes have been analyzed and reported for the combination of salinity and alkalinity responses, and the tolerance mechanisms behind both stresses have not been fully elucidated. Therefore, there is a need for a better understanding of the genetic variations affecting saline-alkaline tolerance in a large population of rice genotypes.

1.5 Study rationale

It is expected that a number of the world's population will continuously increase and reach nearly 10 billion by 2050 (Health Stats Population Estimates and Projections database). To meet the increased food demand in near future, increasing the productivity of crop plants is important. Rice is considered as one of the three major crops for human diets which is relatively susceptible to grow under salt-affected soils. In addition, most of salt-affected soils in the world usually contain alkaline anions such as carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-). An excess Na^+ accumulation together with high pH in the soils cause a saline-alkaline stress condition at rhizosphere which is severely damaged rice growth and development than saline soils. The problem of saline-alkaline soils is now being widely spread into agricultural regions around the world, including Thailand. Rice is not only the major staple food crop of Thai people, but also the important agricultural product for export. In the past three decades, Thailand was the world's largest rice exporter (FAO, 2018). According to the annual report 2017 from The Rice Department Ministry of Agriculture and Cooperative Thailand, the intensive rice farming is located in the Northeastern (63.1%), the Northern (21.9%) and the Central (15.0%) regions, respectively. However, the fertility of paddy soils in the Northeastern is very poor compared to other regions due to saline-alkaline stress condition. Many studies have been conducted to elucidate on the mechanisms of salinity tolerance in rice (Wang et al., 2011; Zhang et al., 2013). In contrast, only few researches have been focused on understanding how rice plants respond to saline-alkaline stress conditions and information on the genotypes of saline-alkaline tolerant rice is still limited. Therefore, the objective of this study was to characterize physiological and molecular mechanisms underlying tolerance to saline-

alkaline stress conditions in rice by comparing some growth parameters and transcriptional analysis of saline-alkaline stress-inducible genes between salt-tolerant (FL478) and salt-sensitive (IR29) rice genotypes. Further study has been conducted to identify rice genotypes, from a large population, that are tolerate to saline-alkaline stress conditions during vegetative stage and then elucidating the mechanisms of resistance to saline-alkaline stress in the selected-rice genotypes. Moreover, this study also provided the information about the differences in growth and physiology of rice in response to saline, alkaline, and saline-alkaline stress conditions.

1.6 Study objectives

1) To investigate both physiological and molecular mechanisms which are responsible for saline-alkaline tolerance in the salt-tolerant FL478 and salt-sensitive IR29 rice genotypes by comparing growth parameters, Na^+ and K^+ accumulation patterns and expression profiles of the genes that encode Na^+ and/or K^+ transport proteins together with Fe acquisition proteins under saline-alkaline stress conditions.

2) To identify rice genotypes (from large population screening) that are tolerant to high saline-alkaline stress conditions during vegetative stage by using hydroponic system.

3) To elucidate both physiological and molecular mechanisms underlying the saline-alkaline tolerance in the selected-rice genotype (Fukoku) by comparing growth parameters and expression profiles of some important genes for saline-alkaline tolerance with the sensitive rice genotype (IR29).

4) Finally, to differentiate how rice seedlings respond to each of the stress conditions (saline, alkaline, and saline-alkaline) at the physiological analysis by using both selected-(Fukoku) and sensitive rice genotype (IR29).

Chapter 2

Characterization of Na^+ exclusion mechanism in rice under saline-alkaline stress conditions

2.1 Introduction

Salinity is a global problem in crop cultivation. Millions of hectares of both irrigated and non-irrigated agricultural lands are affected by high salt accumulation (Munns, 2002). In nature, soil salinization often co-occurs with alkalization, especially in low precipitation areas. According to a report published by FAO/UNESCO, it is estimated that 831 million hectares of land is affected by saline-alkaline stress (Martinez-Beltran et al., 2013). Saline-alkaline soils are characterized by both high concentration of Na^+ and high pH, which cause more complex stress effects on plants than neutral saline soils (Tang et al., 2014).

Na^+ is the main ion found in salt-affected soils, but it is not an essential element for the crop plants. The negative effects of salt-affected soils on plant growth can be described in two distinct phases. During the first phase, water uptake by roots is inhibited due to osmotic stress, leading to cell dehydration, and significant reduction in the rate of shoot growth (Ueda et al., 2013). Ionic stress can be thought of as a second phase response, which becomes gradually more severe as excessive Na^+ accumulates through disturbing ion homeostasis in plant cells (Munns and Tester, 2008). In addition, soil pH is an important factor in the regulation of plant growth. The pH of saline-alkaline soils is often greater than 8.0, which is unsuitable for plant growth and development. Several studies have reported that the detrimental effects of saline-alkaline stress are more obvious than those of saline stress (Wang et al., 2011; Zhang et al., 2011). Thus, plants growing in saline-alkaline soils have to cope with both physiological drought and Na^+ toxicity in addition to the cellular damages induced by high pH.

Rice is one of the most important human crops in the world. It is classified as a glycophyte and its physiological traits are poor when exposed to excessive Na^+ in the soil compared to other crops (Munns, 2008). Genetic studies by Sahi et al. (2002) reported that rice genotypes differ in their sensitivity to saline stress due to additive gene effects. Some rice genotypes have been well documented to be tolerant to saline stress, including FL478, the recombinant inbred line created using the salt-tolerant Pokkali cultivar and salt-sensitive IR29 (Walia et al., 2005). To date, extensive studies have focused on the physiological and molecular biological mechanisms behind saline stress in plants (Zhang

et al., 2013; Li et al., 2017). However, few studies have been conducted to examine saline-alkaline stress.

Several genes involved in salt tolerance in rice have been studied. The genes encoding Na^+ transport proteins such as SOS and HKT are considered to be important factors in the control of Na^+ accumulation in plant cells under saline stress (Munns, 2005; Mekawy et al., 2015; Assaha et al., 2015). SOS1 (Salt Overly Sensitive 1) encodes Na^+/H^+ antiporters which are localized in plasma membranes and are responsible for the transportation of Na^+ from the cytosol to the apoplast (Martinez-Atienza et al., 2007; Ji et al., 2013). A study by Shi et al. (2000) on Arabidopsis showed that SOS1 could function in both Na^+ loading into and retrieval from the xylem; under mild saline stress at 25 mM NaCl, SOS1 may mediate active loading of Na^+ to the xylem, whereas, at high salinity (100 mM NaCl), expression of SOS1 was induced and was responsible for the retrieval of Na^+ from the xylem. HKT transporters are categorized into K^+/Na^+ uniporters or K^+-Na^+ symporters (Himabindu et al., 2016; Assaha et al., 2017; Wangsawang et al., 2018). In addition, recent functional analysis showed that OsMGT1 in rice is required to confer salt tolerance via the enhancement of the transport activity of OsHKT1;5 (Chen et al., 2017). In recent decades, a few rice genotypes have been analyzed for their combined saline and alkaline responses, and the tolerance mechanisms behind both stresses have not been well-understood. Therefore, elucidating the molecular and physiological mechanisms by which rice genotypes respond and adapt to saline-alkaline stress are crucial.

The objective of this study was to investigate differences in both physiological and molecular responses of two rice genotypes, salt-tolerant FL478 and salt-sensitive IR29, to a combination of saline stress at 50 mM Na and high alkaline stress at either pH 9 (severe), pH 8 (moderate), or pH 7 (mild). Molecular analysis revealed that the saline-alkaline tolerance of salt-tolerant FL478 is associated with expression of the *OsHKT1;5* gene and alkaline-responsive genes in the roots under saline-alkaline conditions. These findings suggest that saline-alkaline tolerance in rice plants is correlated with the expression of Na^+ transporters.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Two rice (*Oryza sativa* L.) genotypes, FL478 (salt-tolerant) and IR29 (salt-sensitive), were used in this study. FL478 is known as a salt-tolerant recombinant inbred line, IR66946-3R-178-1-1 (Walia et al., 2005), and was developed by crossing a salt-tolerant indica landrace, Pokkali, with a salt-sensitive indica genotype, IR29 (Bonilla et al., 2002; Cotsaftis et al., 2011). After incubation in tap water at 60ºC for 10 mins, seeds of each genotype were surface-sterilized with 5% (v/v) sodium hypochlorite solution for 30 mins and were then thoroughly rinsed with distilled water. Seeds were subsequently soaked in tap water for 24 h at 30ºC. The germinated seeds were transferred onto a nylons mesh floating in 2-L plastic pots containing tap water for one week. Then, the uniform seedlings were selected and grown in half-strength slightly modified Kimura B nutrient solution containing the following macronutrients (mM): 0.18 $(NH_4)_2SO_4$, 0.27 $MgSO_4 \cdot 7H_2O$, 0.09 KNO_3, 0.18 $Ca(NO_3)_2 \cdot 4H_2O$ and 0.09 KH_2PO_4 and the following micronutrients (μM): 28 $FeSO_4 \cdot 7H_2O$, which was used instead of Fe-EDTA, 9 $MnSO_4 \cdot 5H_2O$, 48 H_3BO_3, 9 $Na_2MoO_4 \cdot 2H_2O$, 0.7 $ZnSO_4 \cdot 7H_2O$ and 0.3 $CuSO_4 \cdot 5H_2O$ (Mekawy et al., 2018). This hydroponic experiment was carried out in a growth chamber maintained at 28/25°C (16 hr light period/ 8 hr dark period) under a photosynthetic photon flux density of 400/0 μmol m^{-2} s^{-1} (day/night) at relative humidity of 70%. At day 21, the 4-5 leaf stage rice seedlings were transferred to either half-strength Kimura B nutrient solution (control: pH 5.0-5.5) or to a saline-alkaline nutrient solution supplemented with 50 mM Na at pH 9 (severe), pH 8 (moderate), or pH 7 (mild) for three weeks. The components of the saline-alkaline treatments used were listed in **Table 2.1**. All treatments were performed with four replicates. The pH of the nutrient solution was measured daily using a pH meter (AS700 Type) and was regulated with either 2 N HCl or 2 N KOH throughout the growth period. The nutrient solution was renewed every four days, and water lost by evaporation was compensated for by daily addition of tap water.

2.2.2 Physiological parameters

After three weeks of saline-alkaline treatment, the fresh weight (FW) of the 42-day-old seedlings was measured following the separation of leaf blades, leaf sheaths and roots. To determine dry weight (DW), leaf blades, leaf sheaths and roots were dried at 70°C for three days prior to being weighed. The water contents in the leaf blades was calculated using the equation (FW-DW)/FW.

2.2.3 Determination of Na^+ and K^+ concentrations

The Na^+ and K^+ concentrations in leaf blades, leaf sheaths, and roots were measured using a flame photometer (ANA-135; Tokyo Photoelectric, Tokyo, Japan) as described previously (Mekawy et al., 2018). Dried samples were gently agitated in 1 N HCl overnight, and the concentrations of Na^+ and K^+ ions were estimated from the Na^+ and K^+ standard curves. Na^+ distribution was calculated as the ratio of Na^+ accumulated in each tissue to that in a whole seedling.

2.2.4 Expression analysis of the genes encoding Na^+ transport proteins

Total RNA was extracted from leaf sheaths and roots of control and saline-alkaline stressed FL478 (salt-tolerant) and IR29 (salt-sensitive) cultivars using a TRIzol reagent. After digestion with DNaseI, total RNA (0.5 µg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit, according to the manufacturer's protocol (Toyobo, Osaka, Japan). Quantitative polymerase chain reaction was performed using a TUNDERBIRD SYBR qPCR Mix and ABI StepOne System (Applied Biosystems, CA) as previously described (Ueda et al., 2013). The reaction mixture contained 7.5 µl of THUNDERBIRD SYBR qPCR Mix, 0.3 µl of 50 × ROX reference dye, 1.5 µl of forward primer, 1.5 µl of reverse primer, 1 µl of cDNA, and 3.2 µl of RNase-free water. Quantitative RT-PCR was performed using the following profile: an initial incubation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 15 s and extension at 60°C for 60 s. Relative expression levels of the gene transcripts were calculated using the comparative $2^{-\Delta\Delta CT}$ method (Livak et al., 2001) with the *Os25SrRNA* gene as an internal control (Jain et al., 2006). Data shown are the average of two technical

replicates using RNA extracted from the pooled tissues of four seedlings. The sequences of the primers used are listed in the **Table 2.2.**

2.2.5 Statistical analysis

The collected data were subjected to One-Way Analysis of Variance (ANOVA) using the SPSS statistics package, version 22 (IBM Inc., USA), and the means (n = 3, except for dry weight, length, and leaf water content, for which n = 5) were separated using Duncan's multiple range test at $p < 0.05$.

2.3 Results

2.3.1 Effect of saline-alkaline stress on physiological parameters

To compare the tolerance of FL478 (salt-tolerant) and IR29 (salt-sensitive) plants to saline-alkaline stress, three-week-old seedlings of the two rice cultivars were exposed to nutrient solutions supplemented with 50 mM Na at either pH 9 (severe), pH 8 (moderate), or pH 7 (mild) for three weeks. After one week of treatment, no differences in the growth of salt-sensitive IR29 were visually observed when it was grown under mild or moderate saline-alkaline stress (50 mM Na + pH 7 or pH 8). Upon exposure of the two rice cultivars to severe saline-alkaline stress (50 mM Na + pH 9), wilting and death of some seedlings were observed in both rice cultivars within one week, and all rice seedlings were completely dead within two weeks. In addition, under mild and moderate saline-alkaline stress (50 mM Na + pH 7 or pH 8), smaller leaf blades were observed in the salt-tolerant FL478 compared to the control **(Figure 2.1)**. The dry weight (DW) of both rice cultivars was affected by saline-alkaline stress treatments **(Figure 2.2)**. Under moderate saline-alkaline stress (50 mM Na + pH 8), the shoot DW of salt-sensitive IR29 plants drastically decreased by 69.3% in comparison with the salt-tolerant FL478 (52.8%). In addition, FL478 showed slight decreases in shoot DW (29.9%) compared to IR29 (76.0%) under mild stress (50 mM Na + pH 7). The root DW of each rice cultivar was not significantly affected by saline-alkaline stress at either pH 7 or pH 8. Additionally, the results showed that mild saline-alkaline stress resulted in decreased shoot and root lengths in FL478 and IR29. However, the root lengths of both rice cultivars increased when moderate saline-alkaline stress was applied, while their shoot length decreased under the same condition **(Figure 2.3)**.

To estimate the amount of water loss under saline-alkaline stress conditions, water content (WC) was measured using leaf tissues. The results indicated that salt-tolerant FL478 exhibited a greater potential to maintain tissue water than salt-sensitive IR29. Under mild and moderate saline-alkaline stress conditions (50 mM Na + pH 7 or pH 8), the WC of IR29 plants decreased from 75.1% in control plants to 70.5% and 67.3% at pH 7 and pH 8, respectively. However, there was no significant difference in the WC of FL478 plants between mild (75.1%) and moderate (75.3%) saline-alkaline stress conditions **(Figure 2.4)**. These results indicated that salt-tolerant FL478 maintained a better

physiological status than salt-sensitive IR29 under conditions of both mild and moderate saline-alkaline stress.

2.3.2 Effects of saline-alkaline stress on Na^+ and K^+ accumulation in different organs

In both rice cultivars, saline-alkaline stress led to increased Na^+ concentration in all organs examined **(Figure 2.5)**. Under mild and moderate (50 mM Na + pH 7 or pH 8) saline-alkaline stress conditions, salt-tolerant FL478 accumulated less Na^+ in the leaf blades than salt-sensitive IR29 **(Figure 2.5A)**. In the leaf sheaths, there was no significant differences in Na^+ concentration between the two rice cultivars when moderate saline-alkaline stress (50 mM Na + pH 8) was applied. However, under mild saline-alkaline stress (50 mM Na + pH 7), Na^+ concentration was lower in the leaf sheaths of FL478 compared to IR29 **(Figure 2.5B)**. Notably, under both mild and moderate saline-alkaline stress conditions, FL478 accumulated a higher concentration of Na^+ in the roots than IR29 **(Figure 2.5C)**. These findings suggest that saline tolerance mechanisms such as Na^+ exclusion in the leaf blades (low Na^+ accumulation) and Na^+ compartmentalization in the roots (high Na^+ accumulation) could confer saline-alkaline tolerance in FL478.

Saline-alkaline stress at either pH 7 or pH 8 significantly decreased the K^+ concentration in the leaf sheaths and roots of both rice cultivars compared to the control **(Figure 2.6B and 2.6C)**. In the leaf blades, there was no significant difference in K^+ concentration in FL478 between mild and moderate saline-alkaline stress conditions, but IR29 accumulated the least amount of K^+ in the leaf blades (21.7 mg/g DW) under moderate saline-alkaline stress compared to other treatments **(Figure 2.6A)**.

Maintaining minimal shoot Na^+/K^+ ratios is an important stress tolerance trait in some halophytes and tolerant glycophytes (Munns and Tester, 2008; Katschnig et al., 2015). This study found that, under mild and moderate saline-alkaline stress conditions, salt-tolerant FL478 maintained much lower Na^+/K^+ ratio in the leaf blades and leaf sheaths than the salt-sensitive IR29 **(Table 2.3)**.

Na^+ distribution in each tissue was estimated by calculating the ratio of Na^+ accumulation in each tissue to that of a whole seedling (Wangsawang et al., 2018). Under control condition, Na^+ distribution in the two rice genotypes was higher in the root than other parts. However, after exposure

to either mild (pH 7 + 50 mM Na) or moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions, the salt-tolerant FL478 accumulated much more Na^+ in the leaf sheaths (51.03% under pH 7 + 50 mM Na and 56.92% under pH 8 + 50 mM Na) than in the roots (39.71% under pH 7 + 50 mM Na and 27.35% under pH 8 + 50 mM Na) and the leaf blades, (9.27 % under pH 7 + 50 mM Na and 15.73% under pH 8 + 50 mM Na), respectively **(Figure 2.10A)**. In contrast, under both mild (pH 7 + 50 mM Na) and moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions, IR29 accumulated much less Na^+ in the roots than other tissues (21.77% under pH 7 + 50 mM Na and 25.13% under pH 8 + 50 mM Na). In detail, as shown in **Figure 2.10B**, under saline-alkaline treatments, IR29 showed higher Na^+ absorbed in the leaf sheaths (46.45% under pH 7 + 50 mM Na and 38.96% under pH 8 + 50 mM Na) than in the leaf blades (31.78% under pH 7 + 50 mM Na and 35.91% under pH 8 + 50 mM Na), respectively.

2.3.3 Differential expression of the genes encoding Na^+ transport proteins in response to saline-alkaline stress

To determine the mechanisms underlying differential Na^+ accumulation in the salt-tolerant FL478 and the salt-sensitive IR29, expression profiles of the genes encoding Na^+ transport proteins were analyzed. Several genes involved in salt tolerance have been identified in rice. OsHKT1;5 plays a major role in the transport of Na^+ from the xylem sap into the surrounding xylem parenchyma cells, thereby protecting the leaves from Na^+ toxicity (Ren et al., 2005). In the present study, quantitative RT-PCR analyses showed that expression of the *OsHKT1;5* gene was more induced in the roots of the salt-tolerant FL478 than in the salt-sensitive IR29 under both mild and moderate saline-alkaline stress conditions, which suggests that Na^+ transport from xylem sap to xylem parenchyma was active in the roots of FL478 but not in IR29 **(Figure 2.7A)**.

In addition, OsMGT1 is a plasma membrane-localized Mg^{2+} transporter involved in salt tolerance in rice through its enhancement of OsHKT1;5 activity (Chen et al., 2017). This study found that, under control and saline-alkaline stress conditions (mild and moderate), *OsMGT1* expression was

not detected in the roots of either rice cultivar **(data not shown)**, which suggests that saline-alkaline stress-induced *OsHKT1;5* expression in the roots was not related to the activity of the *OsMGT1* gene.

OsHKT1;4, is known as an alternative candidate for Na^+ exclusion, which is effective in the leaf sheaths, thereby protecting leaf blades from Na^+ toxicity. In response to moderate saline-alkaline stress, 0.20-fold repression of *OsHKT1;4* expression was observed in the leaf sheaths of salt-tolerant FL478, and 0.10-fold repression was measured under mild saline-alkaline stress. While, in the leaf sheaths of salt-sensitive IR29, *OsHKT1;4* expression did not change (0.01-fold) in response to mild or moderate saline-alkaline stress **(Figure 2.7B)**.

The Na^+/H^+ antiporter (*SOS1*), localized in the plasma membrane, is considered a general regulator of Na^+ export from the cytosol (Shi et al., 2002). Mild saline-alkaline stress (50 mM Na + pH 7) induced expression of the *OsSOS1* gene by 24.8-fold in FL478 roots, but its expression was repressed 0.6-fold in IR29 roots, which suggests that OsSOS1 mediated Na^+ extrusion from the cytosol may not have been active in IR29 when mild saline-alkaline stress was applied. However, when exposed to moderate saline-alkaline stress, expression of the *OsSOS1* gene was greatly induced in the roots of FL478 (44.6-fold) and slightly induced in IR29 (2.2-fold) roots. This result suggests that the salt tolerance mechanisms governed by OsSOS1 in the roots of both rice cultivars are activated in responses to moderate saline-alkaline stress **(Figure 2.7C)**.

2.3.4 Effects of alkaline stress on expression of the genes encoding Na^+ transporters

To further explore the relationship between the genes encoding Na^+ transporters and sole high pH stress, 21-day-old seedlings of two rice cultivars were grown under alkaline conditions without high saline stress at either pH 7 (mild) and moderate (pH 8) for three weeks. Upon exposure of the two rice cultivars to alkaline stress, expression of the *OsHKT1;5* gene was induced greatly in the roots of FL478 by 43.4-fold under moderate alkaline stress (pH 8 + 5 mM K) and by 12.5-fold under mild alkaline stress (pH 7 + 5 mM K) **(Figure 2.7A)**. However, expression of the *OsHKT1;5* gene was not highly inducible in the roots of IR29 under both mild and moderate alkaline stresses. In response to alkaline stress at either pH 7 (mild) or pH 8 (moderate), expression of the *OsHKT1;4* gene was repressed in the leaf sheath of

FL478 and not affected in that of IR29 **(Figure 2.7B)**. Expression patterns of the *OsSOS1* gene in the roots of both rice cultivars were also analyzed **(Figure 2.7C)**. Repression of *OsSOS1* expression was observed in the roots of salt-tolerant FL478 under both mild and moderate alkaline stress conditions by 0.2-fold and 0.3-fold, respectively. In contrast, induced expression of the *OsSOS1* gene in the roots of salt-sensitive IR29 was observed under mild alkaline stress (pH 7 + 5 mM K). Induction of *OsSOS1* expression was higher (5.8-fold) mild alkaline stress than under moderate alkaline stress (3.1-fold) in the roots of IR29.

2.3.5 Transcriptomic responses of alkaline-responsive genes in response to saline-alkaline stress

To further study the mechanisms underlying saline-alkaline stress tolerance in rice, transcription levels of alkaline-responsive genes were analyzed **(Figure 2.8)**. Several genes related to K^+ transport such as low affinity K^+ transporter 1 (AKT1) and some members of the HAK/KUP/KT transporters have also been studied in alkaline tolerance in rice (Yang et al., 2012). Mild saline-alkaline stress (pH 7 + 50 mM Na) induced expression of the *OsAKT1*, *OsHAK7* and *OsHAK10* genes highly in the roots of FL478 compared to these expressions under moderate saline-alkaline stress (pH 8 + 50 mM Na), while in the roots of IR29, expression of these genes were repressed in response to both mild and moderate saline-alkaline stress conditions **(Figure 2.8A, B, C)**. Transcripts of the *OsHAK16* gene in the roots of FL478 was highly detectable under both mild and moderate saline-alkaline stresses, whereas its expression was not detected in IR29 under all conditions tested **(data not shown)**. These results suggested that FL478 activate these K^+ transport system to acquire K^+ more efficiency under saline-alkaline stress.

Expression patterns of Fe deficiency-responsive genes were also investigated, including *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsIRT1* and *OsIRO2* because, in the saline-alkaline soils, Fe is often converted to insoluble forms (hydroxides and oxides) which are unusable for plant growth. Expression of the *OsNAS1* gene showed similar trend to that of the *OsNAS2* gene in the roots of both FL478 and IR29 **(Figure 2.8D, E)**. A significantly greater induction of those genes expression was observed under mild saline-alkaline stress (pH 7 + 50 mM Na) than moderate saline-alkaline stress (pH 8 + 50 mM Na).

In addition, expression levels of the *OsNAS1* and *OsNAS2* genes were significantly higher in the roots of FL478 than that of IR29 **(Figure 2.8D, E)**. Expression level of the *OsIRT1* and *OsIRO2* gene in FL478 roots was greater than that in IR29 roots under both mild and saline-alkaline stress conditions **(Figure 2.8F, G)**. Whereas transcripts of the *OsYSL15* genes were not detectable in the roots of two rice cultivars under stress conditions **(data not shown)**. These results imply that the saline-alkaline tolerance of FL478 might also involve with the up-regulation of Fe deficiency-related genes.

Three H^+-ATPase-encoding genes, including *Os03g0689300*, *Os12g0638700* and *Os03g0100800* have been reported to play an important role for saline-alkaline tolerance in rice by mediating proton secretion through the roots (Li et al., 2016; Yang et al., 2010). The present study found that, in comparison with normal pH condition (pH 5.0-5.5), expression levels of the three genes were not detected in the roots of salt-sensitive IR29 under both mild (pH 7 + 50 mM Na) and moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions **(data not shown)**. In contrast, in the roots of salt-tolerant FL478, expression of the plasma membrane H^+-ATPase genes showed a distinct pattern; a higher level of the *Os03g0689300* gene transcript was observed in both mild (33.0-fold) and moderate (5.1-fold) saline-alkaline stress conditions (pH 7 and pH 8 + 50 mM Na) **(Figure 2.8H)** and an increase in expression of the *Os03g0100800* gene was observed in the roots of FL478 under mild saline-alkaline stress (3.1-fold), but reduced under moderate saline-alkaline stress (0.5-fold) **(Figure 2.8I)**. These results indicated that in the roots of salt-tolerant FL478, function of the H^+-ATPase might be active through transcriptomic regulation under various saline-alkaline stress conditions, but not in the salt-sensitive rice cultivar.

Table 2.1 Chemicals used for saline-alkaline treatments

Treatments	Supplements			pH
Control (pH 5.0-5.5)	-			5.0-5.5
Mild saline-alkaline stress (pH 7 + 50 mM Na)	1 mM $NaHCO_3$	+	49 mM NaCl	7.0
Moderate saline-alkaline stress (pH 8 + 50 mM Na)	8 mM $NaHCO_3$	+	42 mM NaCl	8.0
Severe saline-alkaline stress (pH 9 + 50 mM Na)	45 mM $NaHCO_3$	+	2.5 mM Na_2CO_3	9.0
Mild alkaline stress (pH 7 + 5 mM K)	1 mM $KHCO_3$	+	4 mM KCl	7.0
Moderate alkaline stress (pH 8 + 5 mM K)	4.75 mM $KHCO_3$	+	0.25 mM KCl	8.0

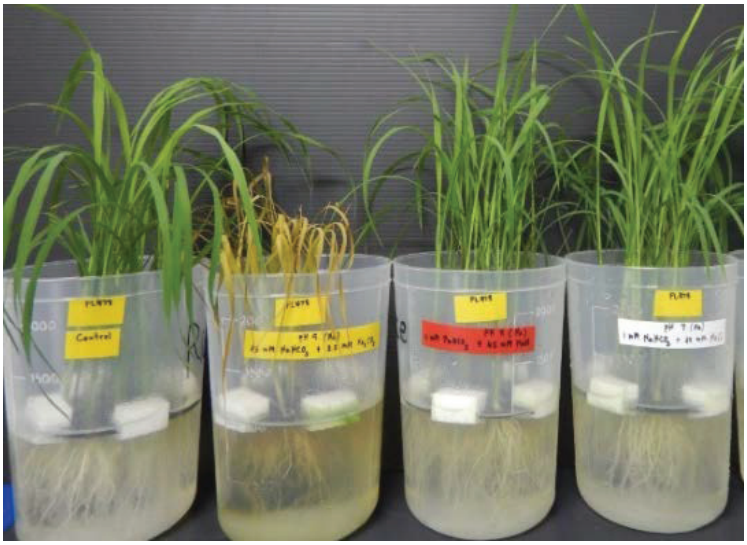
Table 2.2 Primers used for quantitative real-time RT-PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
OsSOS1	ATACTGAGTGGGGTTGTTATTGC	AAAGGTAAATTTCAAAAGGTACATGG
OsHKT1;4	GTCGAAGTTGTCAGTGCATATGG	TGAGCCTCCCAAAGAACATCAC
OsHKT1;5	TGCATTCATCACTGAGAGGAG	GGTGCAGTTTCTGCAACCTC
OsMGT1	AACACGCATCTAAAAGTTTCACC	TTCGATTATTATTGCTCCCACA
Os03g0689300	AACGATGCTCCAGCCCTAA	AATGGCGGGAAATCAAACT
Os12g0638700	CTTGCCTCT GCTGTTTACCT	GCTTCACAACCGATTCTACAT
Os03g0100800	GCTGTTGCCTATCAGGAAGT	TCAAAGAGTGGGAGAAGACC
OsNAS1	CGGTTGAGAAGGCAGAAGAG	TCGTCCGGCTGTTAGACG
OsNAS2	CGTCTGAGTGCGTGCATAGT	CACAAACACAAACCGATACCA
OsYSL15	ACTGGTACCCTGCAAACATAC	GCAATGATGCTTAGCAAGAAG
OsIRT1	CGTCTTCTTCTTCTCCACCACGAC	GCAGCTGATGATCGAGTCTGACC
OsIRO2	CTCCCATCGTTTCGGCTACCT	GCTGGGCACTCCTCGTTGATC
OsAKT1	TACGACCGCCGATACAGAA	CCAAATAAGCCACAAAGAAGG
OsHAK7	GAACTCCAACTTCCTCAAGACG	AGATCATGCCGACTTCGACGAG
OsHAK10	CGCTCTCGGCTGCTTTCCT	TAACCGCCAATCCTGACGC
OsHAK16	AGCGACTGTGTGCTAAACCC	CATAGATGCCAATCCCTGAGA
Os25SrRNA	AAGGCCGAAGAGGAGAAAGGT	CGTCCCTTAGGATCGGCTTAC

Table 2.3 Na^+/K^+ ratio in the leaf blades, leaf sheaths and roots under mild and moderate saline-alkaline stress conditions. Values are the mean of three replicates ± standard error.

Treatments	Cultivars	Leaf blades	Leaf sheaths	Roots
Control	FL478	0.01 ± 0.00	0.08 ± 0.01	0.53 ± 0.05
(pH 5.0-5.5)	IR29	0.00 ± 0.00	0.02 ± 0.00	0.19 ± 0.01
Mild saline-alkaline stress	FL478	0.23 ± 0.02	1.34 ± 0.14	3.63 ± 0.13
(50 mM Na + pH 7)	IR29	1.05 ± 0.18	3.62 ± 0.35	4.02 ± 0.21
Moderate saline-alkaline stress	FL478	0.68 ± 0.09	3.12 ± 0.57	6.76 ± 0.91
(50 mM Na + pH 8)	IR29	1.74 ± 0.33	4.77 ± 0.53	4.73 ± 0.38

(A)



(B)

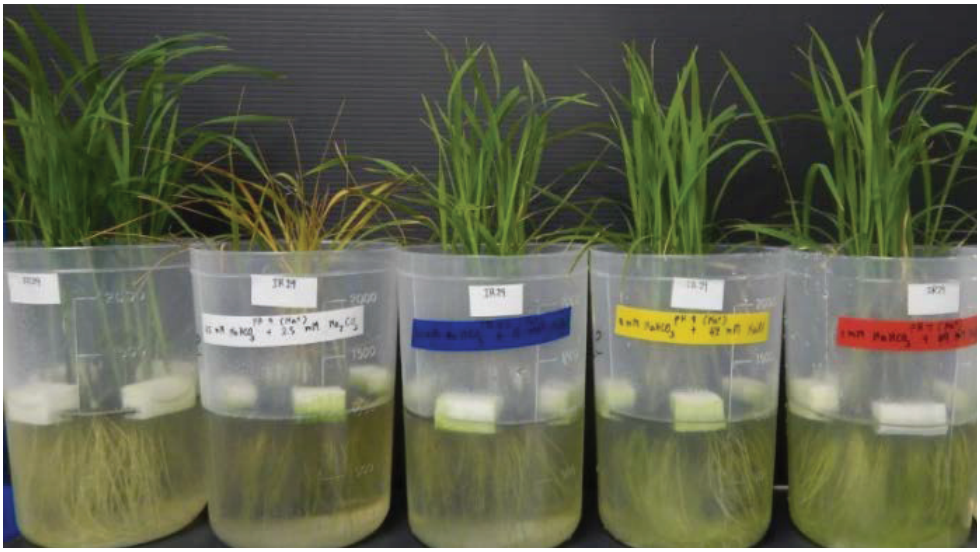


Figure 2.1 Effect of saline-alkaline stress at 50 mM Na on the growth of the rice cultivars, FL478 (A) and IR29 (B) after 7 days. (A) FL478: (L to R) Control, pH 9 (50 mM Na), pH 8 (50 mM Na), pH 7 (50 mM Na), and (B) IR29: (L to R) Control, pH 9 (50 mM Na), pH 8.5 (50 mM Na), pH 8 (50 mM Na), pH 7 (50 mM Na).

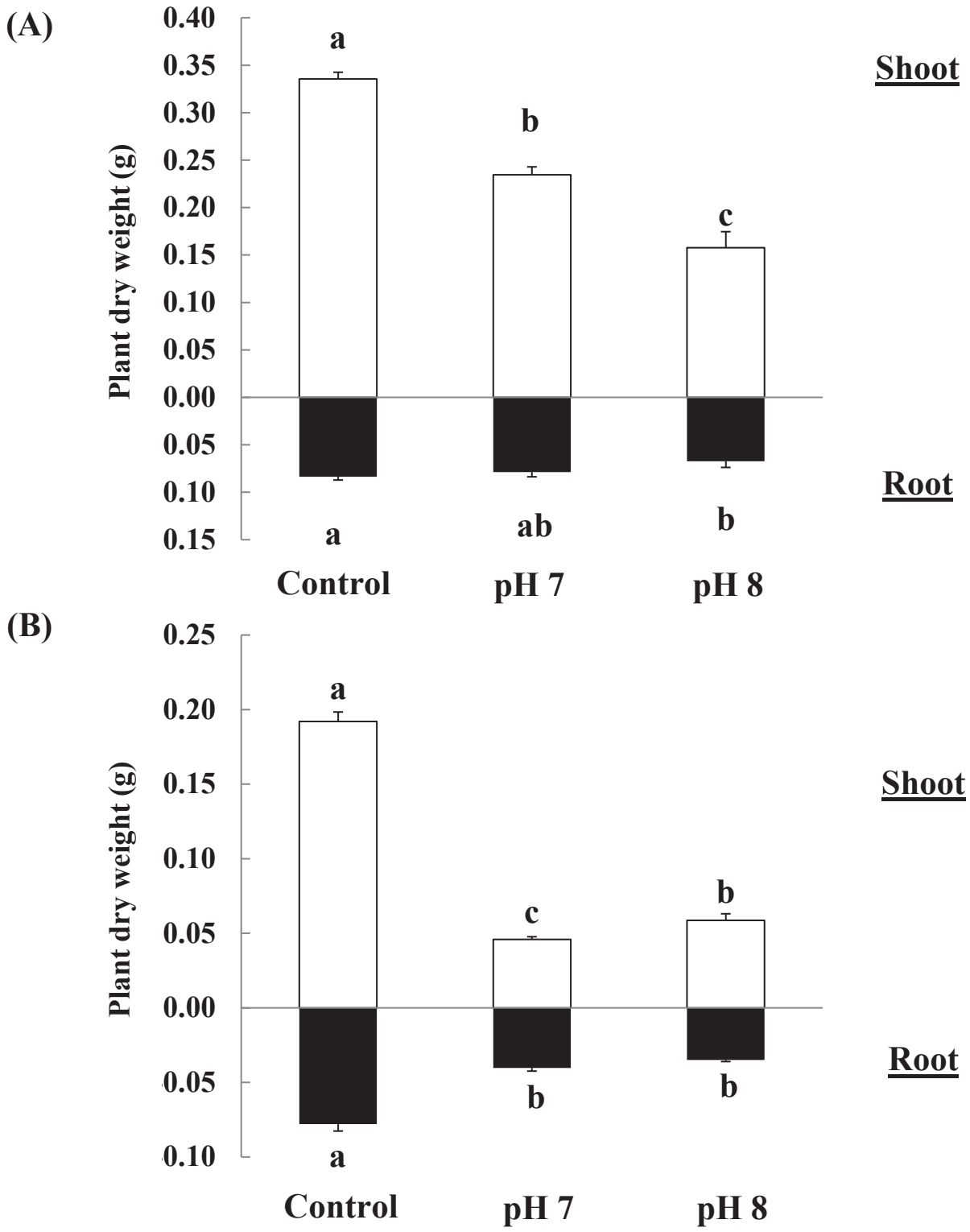


Figure 2.2 Plant dry weight (DW) of rice cultivars (A) FL478 (salt-tolerant) (B) IR29 (salt-sensitive) under control and saline-alkaline stress conditions (Severe: 50 mM Na + pH 9, Moderate: 50 mM Na + pH 8, and Mild: 50 mM Na + pH 7) for three weeks. Data represent the means of five replicates ± SE. The same letters indicate no significant difference $p < 0.05$.

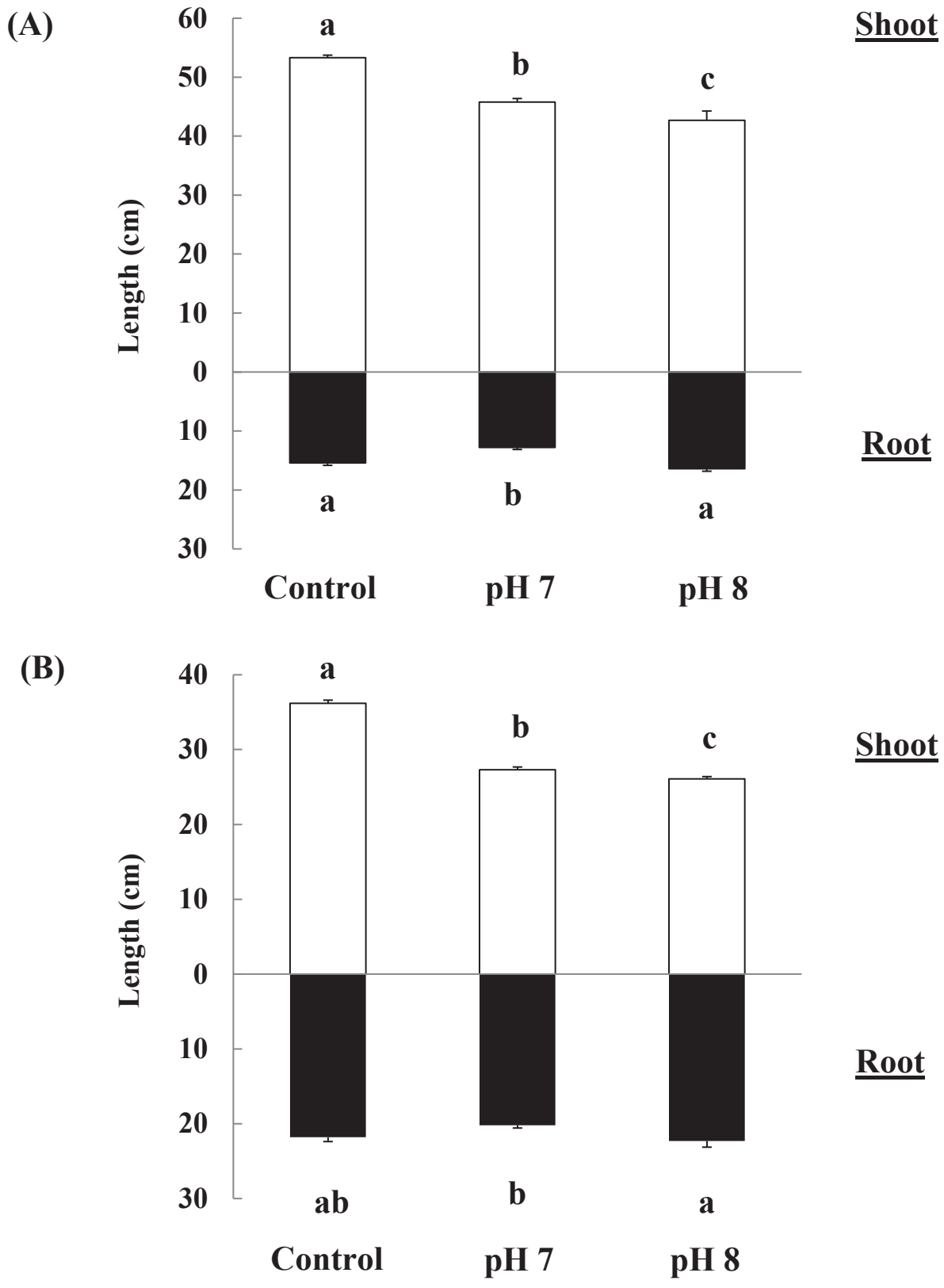


Figure 2.3 Effects of saline-alkaline stress on length of two rice cultivars (A) FL478: salt-tolerant and (B) IR29: salt-sensitive. Lengths of shoot and root were measured under control conditions and after three weeks of saline-alkaline conditions. Values are means of five replicates ± standard error.

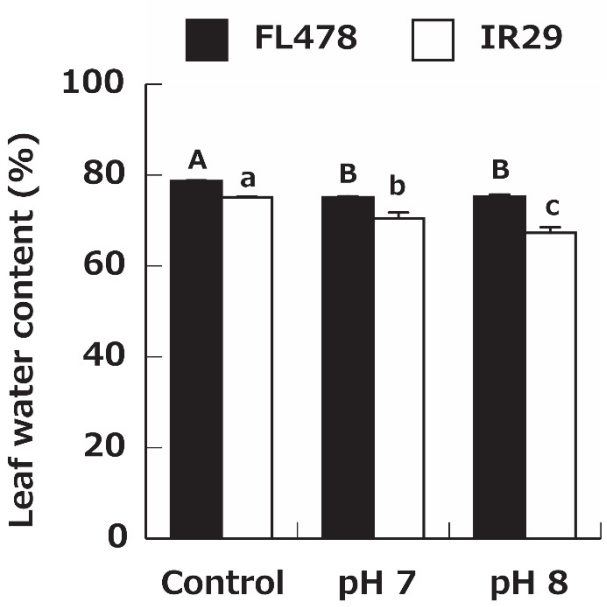


Figure 2.4 Effects of saline-alkaline stress on water content of two rice cultivars. Leaf water content was measured under control and after three weeks of saline-alkaline conditions. Values are means of five replicates ± standard error.

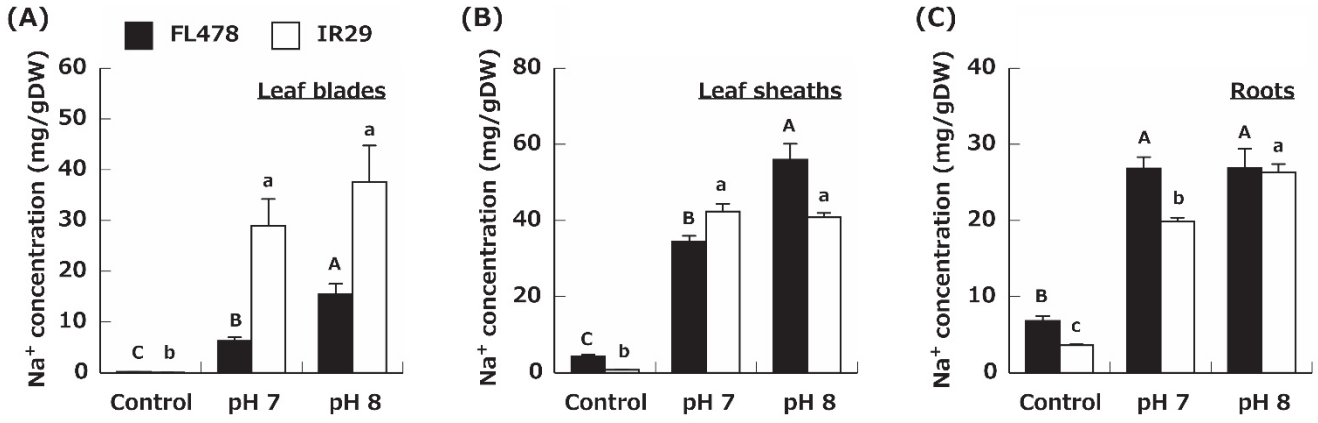


Figure 2.5 Na^+ concentrations in (A) leaf blades, (B) leaf sheaths, and (C) roots under control and saline-alkaline conditions. Values are means of three replicates ± standard error.

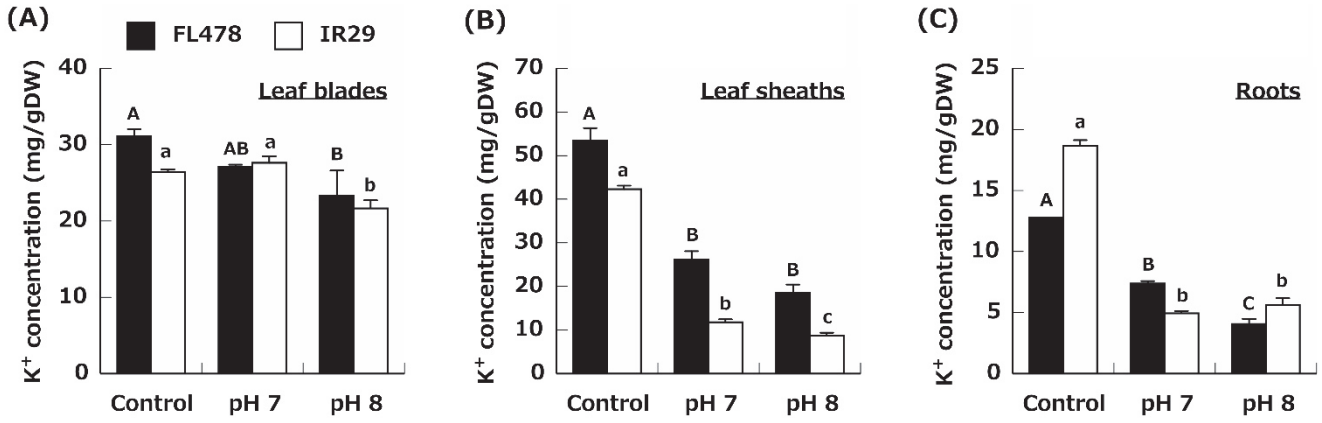


Figure 2.6 K^+ concentrations in (A) leaf blades, (B) leaf sheaths, and (C) roots under control and saline-alkaline conditions. Values are means of three replicates ± standard error.

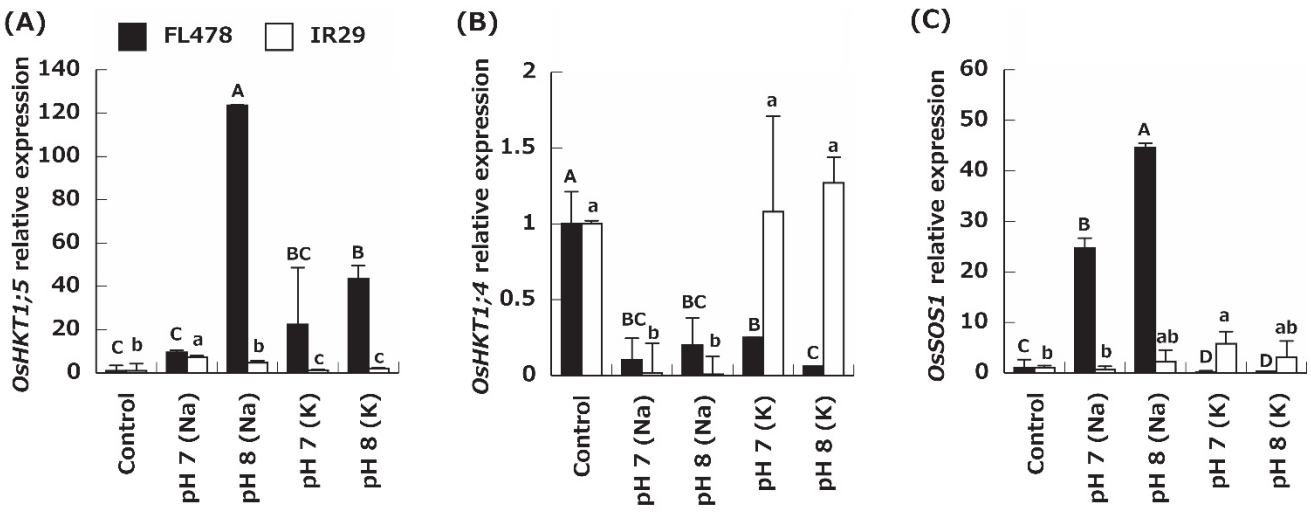


Figure 2.7 Relative expressions of genes encoding Na^+ transport proteins. (A) *OsHKT1;5* in the roots, (B) *OsHKT1;4* in the leaf sheaths, and (C) *OsSOS1* in the roots of rice seedlings of the cultivars FL478 and IR29 grown under control and saline-alkaline stress conditions for three weeks. Data represent the means of two independent experiments.

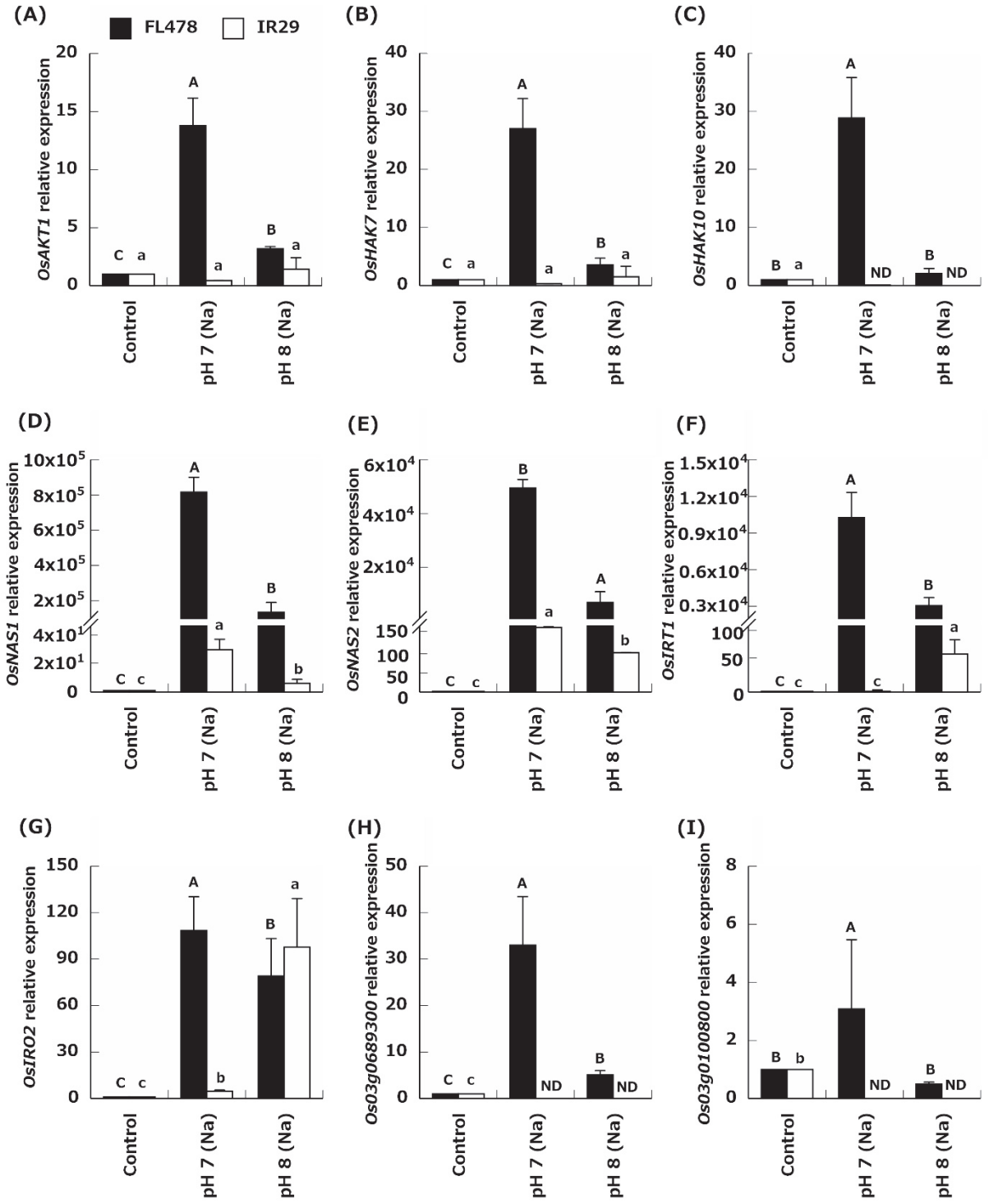


Figure 2.8 Relative expressions of alkaline-responsive genes. (A) *OsAKT1*, (B) *OsHKA7*, (C) *OsHKA10,* (D) *OsNAS1,* (E) *OsNAS2,* (F) *OsIRT1,* (G) *OsIRO2,* (H) *Os03g0689300,* and (I) *Os03g0100800* in the roots of rice seedlings of the cultivars FL478 and IR29 grown under control and saline-alkaline stress conditions for three weeks. Data represent the means of two independent experiments

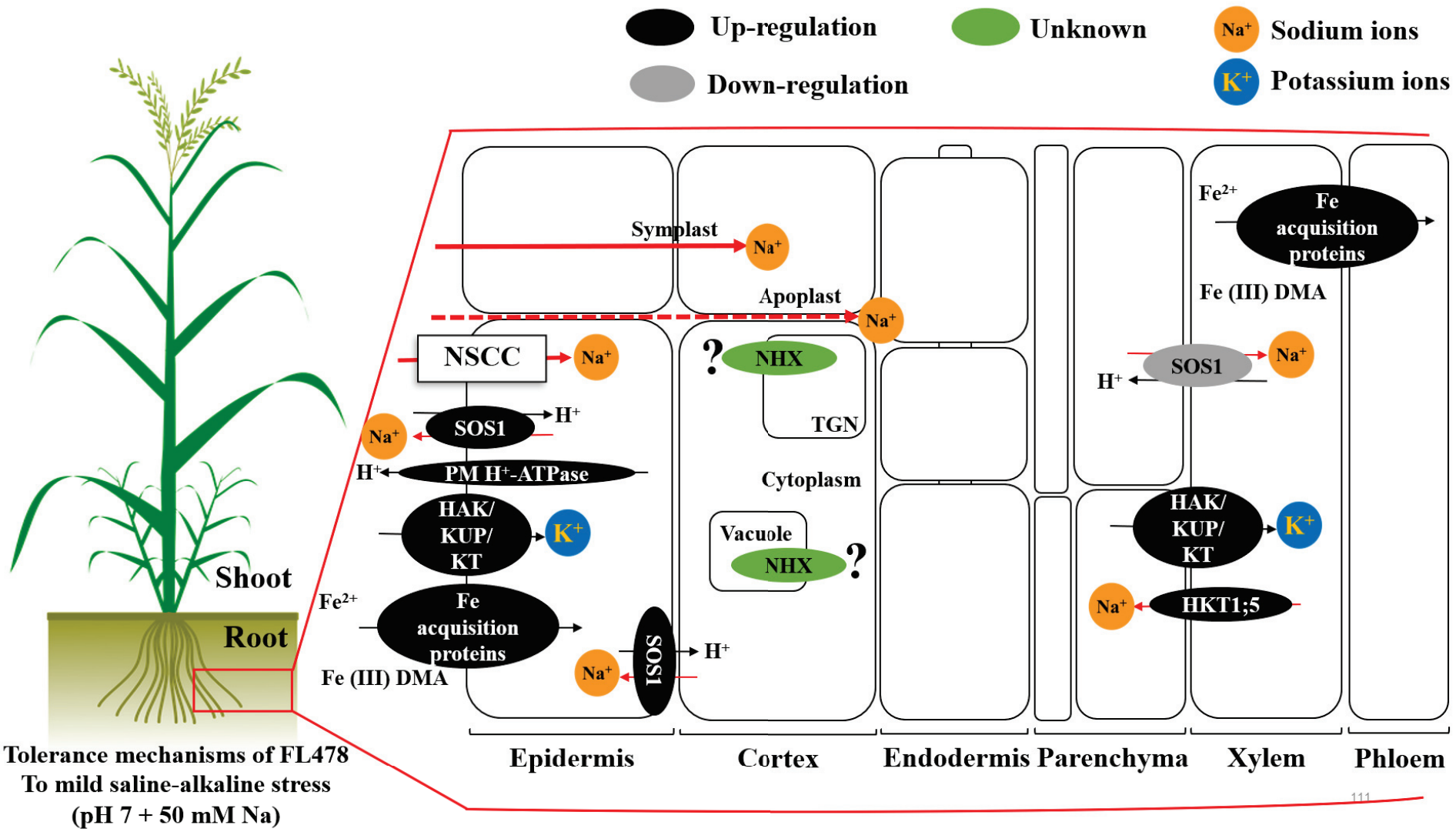


Figure 2.9 Proposed models on the mechanisms of saline-alkaline tolerance in rice.

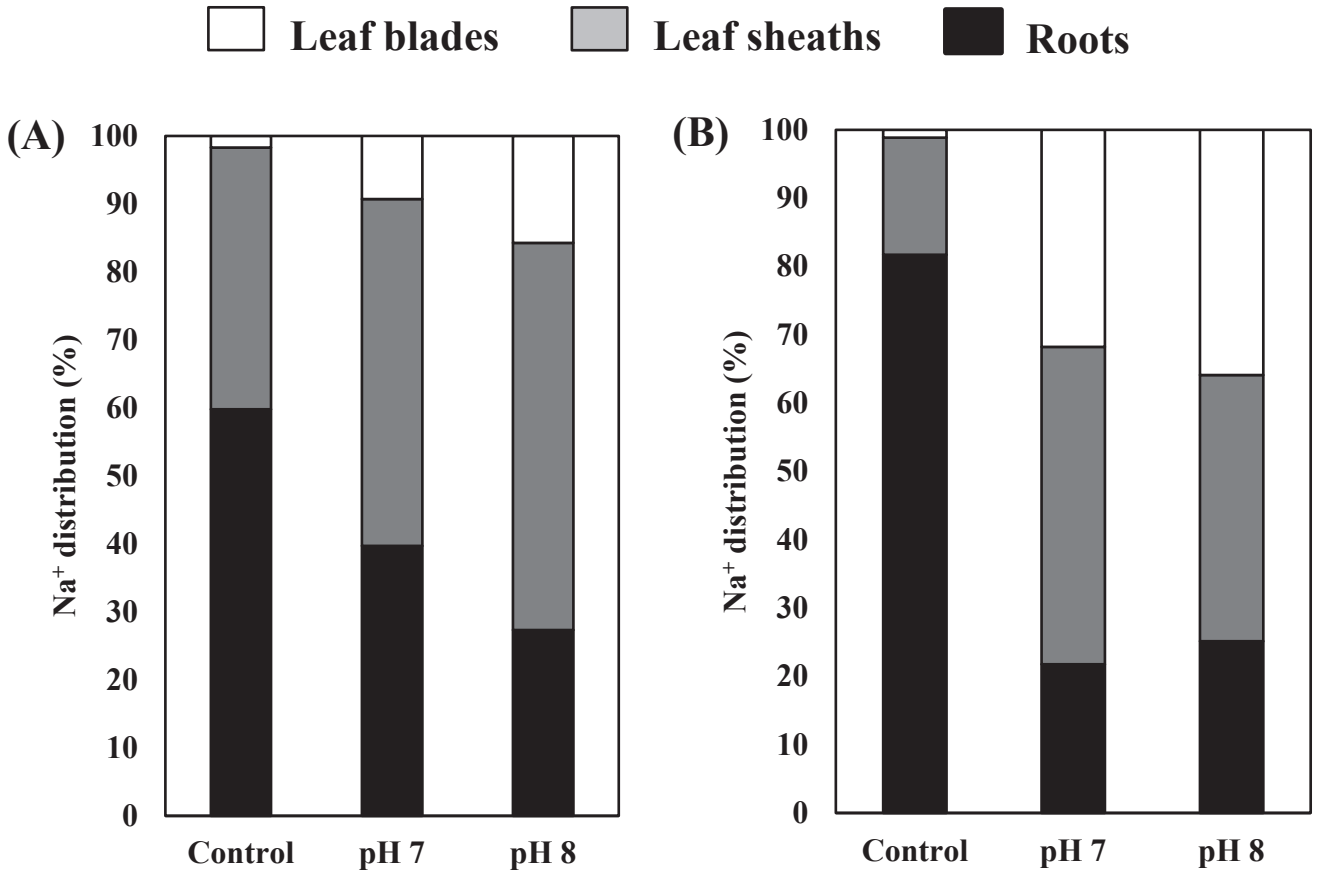


Figure 2.10 Distribution of Na^+ accumulation in the leaf blades, leaf sheaths, and roots of (A) the salt-tolerant FL478 and (B) the salt-sensitive IR29 under control, mild saline-alkaline stress (pH 7 + 50 mM Na), and moderate saline-alkaline stress (pH 8 + 50 mM Na) conditions. Na^+ distribution was evaluated by the ratio of the amount of Na^+ in each tissue to that in the whole seedlings.

2.4 Discussion

FL478 is known as a salt-tolerant rice genotype, which was developed by IRRI and was created by crossing Pokkali and IR29 genotypes. Thomson et al. (2010) reported that FL478 has seedlingstage salt tolerance up to 18 dS m^{-1}. However, the molecular physiological mechanisms of saline-alkaline tolerance in FL478 plants remain largely unknown. In the present study, the effects of severe (50 mM Na + pH 9), moderate (50 mM Na + pH 8), and mild (50 mM Na + pH 7) saline-alkaline stress conditions on salt-tolerant FL478 and a salt-sensitive, IR29 were evaluated. FL478 plants were more tolerant to mild or moderate saline-alkaline stress than IR29 plants, as shown by lower reductions in the biomass production of shoots and roots **(Figure 2.2)**. The greater dry matter production of FL478 plants may be caused by their higher leaf water content and shoot height compared to IR29 plants under both mild and moderate saline-alkaline stress conditions. Results further showed that FL478 plants had lower leaf blade and leaf sheath Na^+/K^+ ratios than IR29 in both mild and moderate saline-alkaline stress conditions. One important finding in this research is that FL478 plants can maintain lower Na^+ concentrations in the leaf blades and higher Na^+ concentrations in the roots than IR29 plants under high saline-alkaline stress at either pH 7 or pH 8; these finding suggest that FL478 is not only tolerant to saline stress but that it is also capable of growing under different pH levels of saline-alkaline environments.

In general, saline-alkaline stress is more complex than neutral-saline stress because plants suffer from both Na^+ toxicity and cellular damages induced by high pH. Several studies have reported that salinity tolerant plants often maintain a low Na^+ and high K^+ concentration in their shoots (Munns and Tester, 2008; Katschnig et al., 2015; Rahneshan et al., 2018). Thus, maintenance of a low Na^+/K^+ ratio in the shoots is considered an indicator of potential salt tolerance in rice (Yeo et al., 1982). In the present study, FL478 plants showed better growth performance than IR29 plants, by maintaining a lower Na^+/K^+ ratio in the leaf blades and leaf sheaths when grown under both mild and moderate saline-alkaline stress conditions **(Table 2.3)**. This suggests that the tolerance to saline-alkaline stress in FL478 plants is achieved by reducing Na^+ accumulation in the leaf cells.

Furthermore, under both mild (pH 7 + 50 mM Na) and moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions, the salt-tolerant FL478 accumulated Na^+ in the leaf sheaths and roots, but restricted Na^+ entry in the leaf blades. As shown in **Figure 2.10A**, under moderate saline-alkaline stress (pH 8 + 50 mM Na), FL478 accumulated 15.73% of Na^+ absorbed in the leaf blades and only 9.27% of Na^+ absorbed was found in its leaf blades when exposed to mild saline-alkaline stress (pH 7 + 50 mM Na). On the other hand, the salt-sensitive IR29 accumulated 35.91% of Na^+ absorbed in the leaf blades under moderate saline-alkaline stress (pH 8 + 50 mM Na) and 31.78% under mild saline-alkaline stress (pH 7 + 50 mM Na) **(Figure 2.10B)**, indicating that the salt-sensitive IR29 may not have an effective mechanism of Na^+ exclusion from its leaf blades under both mild and moderate saline-alkaline stress conditions.

To understand the mechanisms in restricted Na^+ transport to the leaf blades in FL478, expressions of the *OsHKT1;5* gene were analyzed **(Figure 2.7A)**. OsHKT1;5 in rice is well characterized as a key factor in salinity tolerance as its protein retrieves Na^+ from the xylem and transports it to xylem parenchyma cells. In response to mild and moderate saline-alkaline stress, FL478 markedly induced the expression of the *OsHKT1;5* gene in the roots, but IR29 induced it only slightly in the roots. This result is in agreement with the finding of Walia et al. (2007), which an increase in transcripts of the *OsHKT1;5* gene was observed in the tolerant genotype Pokkali but was reduced in the sensitive genotype IR29 (Walia et al., 2007). Therefore, differences of Na^+ accumulation observed between FL478 and IR29 can be explained by restriction of Na^+ transport to the leaf blades through OsHKT1;5 under saline-alkaline stress conditions. However, FL478 accumulated more Na^+ under moderate saline-alkaline stress than mild alkaline stress **(Figure 2.5A, 2.5B)**, although expression of the *OsHKT1;5* gene was highly induced under moderate saline-alkaline stress **(Figure 2.7A)**. Over-accumulation of Na^+ in the leaf blades and sheaths under moderate saline-alkaline stress may be due to excess amount of Na^+ absorbed by the roots whose concentration is beyond the Na^+ retrieving capacity by OsHKT1;5 in roots. Chen et al. (2017) suggested that the Mg transporter OsMGT1 is required for salt tolerance in rice as it regulates the transport activity of OsHKT1;5, but its expression was not induced by low external NaCl concentration (less than 10 mM NaCl). In the present study 50 mM Na^+ was used to create the saline-alkaline stress at either pH 7 or pH 8, and control seedlings received on

Na^+. The expression profile of the *OsMGT1* gene in the roots of both rice genotypes showed that its expression was not affected by any stress condition including the control **(data not shown)**. This finding suggests that OsMGT1 may not be able to enhance the activity of OsHKT1;5 under saline-alkaline stress conditions.

The presence of low Na^+ accumulation in the leaf cells of rice can be also driven by the *OsHKT1;4* gene, which is mainly localized in the leaf sheaths and produces a protein which functions as a Na^+ excluder by retrieving Na^+ from the xylem (Cotsaftis et al., 2012). Therefore, the expression profiles of the *OsHKT1;4* gene in both FL478 and IR29 were also investigated in this study. Under mild (50 mM Na + pH 7) and moderate (50 mM Na + pH 8) saline-alkaline conditions, expression of the *OsHKT1;4* gene was repressed in the leaf sheaths of both rice genotypes **(Figure 2.7B)**. This indicates that Na^+ retrieval in sheaths mediated by OsHKT1;4 did not contribute to restriction of Na^+ accumulation in the leaves of either genotype when saline-alkaline stress was applied.

The rice OsSOS1 transporter has been isolated based on its homology to AtSOS1 in Arabidopsis (Martinez-Atienza et al., 2007; Shi et al., 2000). Both the *OsSOS1* and *AtSOS1* genes encode a plasma membrane-localized Na^+/H^+ antiporter and, *OsSOS1* expression was upregulated by saline stress (Shi et al., 2000). One of the OsSOS1 functions in salinity tolerance is to extrude Na^+ to the outside of cells, leading to reduced Na^+ accumulation in the root cells and load Na^+ into the xylem, leading to increased Na^+ accumulation in shoots through long-distance root-shoot transport system (Shi et al., 2002). The latter function likely works in the roots of salt-tolerant FL478 under moderate saline-alkaline stress (pH 8 + 50 mM Na) because high Na^+ concentrations in both leaf blades and leaf sheaths were observed **(Figure 2.5A and 2.5B)**. On the other hand, Na^+ extrusion to the outside of roots by OsSOS1 might be not active under saline-alkaline stress conditions because plasma membrane-localized Na^+/H^+ antiporters are driven by H^+ gradient across membranes. Thus, it is possible that saline-alkaline stress at pH 8 (moderate), the mechanism of Na^+ loading by OsSOS1 is superior than the Na^+ exclusion mechanism governed by OsHKT1;5 in the roots of FL478, whereas at pH 7 (mild), OsSOS1 and OsHKT1;5 may contribute to the low Na^+ accumulation in the shoots, thereby activated in both sequestration of Na^+ to outside of the roots and retrieving Na^+ from the transpiration stream in xylem.

To gain more understanding with the relationship of Na^+ transporters and alkaline stress in both rice cultivars, expressions of the *OsSOS1* and *OsHKT1;5* genes in the roots were analyzed. The present study revealed that under both mild (pH 7 + 5 mM K) and moderate (pH 8 + 5 mM K) alkaline stress conditions (without high saline stress), the roots of salt-sensitive IR29 were able to activated transcription of the *OsSOS1* and *OsHKT1;5* genes, but in the presence of both saline and alkaline stress conditions; the expression of *OsSOS1* gene was down-regulated, this implies that reduced expression of the *OsSOS1* gene in IR29 roots was caused by Na^+ toxicity rather than high pH stress, whereas the up-regulation of the *OsHKT1;*5 expression is caused by saline-alkaline stress conditions rather than alkaline stress conditions without Na^+ toxicity. However, expression pattern of the *OsSOS1* gene in the roots of salt-tolerant FL478 showed the opposite trend with that observed in IR29 as mentioned above. The increased expression of the *OsSOS1* gene in FL478 roots was found under saline-alkaline stress conditions, but not under alkaline stress conditions without Na^+ toxicity. In addition, in response to saline-alkaline and alkaline stress at either pH 7 (mild) or pH 8 (moderate), expression of the *OsHKT1;4* gene showed no significant changes or reduction in the leaf sheaths of FL478 and IR29. Mechanisms of Na^+ exclusion mediated by OsHKT1;4 was unlikely important in tolerances to these stresses in both rice varieties.

Under high pH conditions, the availability of Fe is quite limited for plants use due to precipitation of Fe. It has been well documented that higher plants use two major Fe uptake strategies (Strategies I; Fe reduction and Strategies II; Fe chelation) to acquire more Fe under this stress condition (Guerinot et al., 1994; Kobayashi et al., 2012). In graminaceous plants including rice, Fe acquisition has been operated by only Strategy II, which can release mugineic acid family phytosiderophores to uptake Fe^{3+} from the alkaline soils (Kobayashi et al., 2012). In the current study, both mild and saline-alkaline stress conditions were markedly enhanced expression of the *OsNAS1*, *OsNAS2*, *OsYSL15* and *OsIRT1* genes in the roots of salt-tolerant FL478 rather than in the salt-sensitive IR29, except for *OsIRO2*, which exhibited a higher expression level in the roots of salt-sensitive IR29 (97.7-fold) than in FL478 roots (79.1-fold) under moderate saline-alkaline stress (pH 8 + 50 mM Na) **(Figure 2.8D, 2.8E, 2.8F, 2.8G)**. This finding suggested that, under saline-alkaline stress conditions, Fe utilization in the roots of salt-tolerant FL478 via the activities of Fe deficiency-related genes may be stronger than in

the roots of salt-sensitive IR29. However, to clearly understand the relationship between those gene expressions and Fe utilization, the concentrations of Fe in each plant tissue should be further investigated.

Root proton-secretion via the activity of plasma membrane H^+-ATPase is considered to be an adaptation of plants to alkaline stress. The present study found that, under mild and moderate saline-alkaline stress conditions (pH 7 and pH 8 + 50 mM Na), expression levels of the H^+-ATPase-encoding genes such as *Os03g0689300* and *Os03g0100800* were up-regulated in the roots of FL478 than in IR29 **(Figure 2.8H, 2.8I)**. In addition, this result is in agreement with the findings of Li et al. (2016), which suggest that under both saline-alkaline and Fe deficiency conditions, three H^+-ATPase-encoding genes (*Os03g0689300*, *Os12g0638700* and *Os03g0100800*) were highly up-regulated in the roots of saline-alkaline tolerant rice variety relative to the sensitive one. These findings indicated that the salt-tolerant FL478 might able to release more H^+ to acidify the rhizosphere and maintaining the root elongation under saline-alkaline conditions rather than the salt-sensitive IR29 rice cultivar. Acidification of the rhizosphere may be beneficial for both activation of OsSOS1 to extrude Na^+ to outside of the cells and acquirement of Fe, thereby improving rice growth under high pH conditions.

K^+ is an essential macronutrient for plant growth and development. The vital roles of K^+ in plant cells under abiotic stress conditions are related to osmoregulation. In rice, several members of the HAK/KUP/KT transporters have also been implicated in salt tolerant, for example; Na^+/K^+ homeostasis in the plant cells was found to be involve with the expression *OsHAK1*, *OsHAK5* and *OsHAK21* genes (Li et al., 2018). In response to saline-alkaline stress conditions at either pH 7 (mild) and pH 8 (moderate), expression of the *OsAKT1*, *OsHAK7*, *OsHAK10* and *OsHAK17* gene were markedly enhanced in the roots of salt-tolerant FL478 rather than in IR29, this implies that K^+ accumulation in FL478 roots under both mild and moderate saline-alkaline stress condition was strongly driven by the activities of those genes as seen in **Figure 2.8A-C**.

2.5 Conclusion

This study demonstrated that salt-tolerant FL478, was more tolerant to saline-alkaline stress at both pH 7 (mild) and pH 8 (moderate) than the salt-sensitive rice genotype IR29. FL478 plants accumulate less Na^+ in the leaf blades than IR29 plants due to higher expression of the *OsHKT1;5* gene in the roots. This allows FL478 plants to reduce Na^+ accumulation in their leaf blades under the conditions of mild (pH 7) and moderate (pH 8) saline-alkaline stress. FL478 also induced expression of the genes for K^+ acquisition, Fe acquisition, and acidification of the rhizosphere that may participate in saline-alkaline tolerance (**Figure 2.9**). In rice, the mechanisms of saline-alkaline tolerance related to the function of genes encoding Na^+ transport proteins and alkaline-responsive genes have not been fully-understood compared to saline tolerance. Further studies into this area is valuable for identifying the molecular physiological mechanisms associated with responses of rice to saline-alkaline stress.

Chapter 3

Screening rice genotypes for tolerance to saline-alkaline stress

3.1 Introduction

Climate change is enhancing the salinity problem in agricultural areas. Viswanathan et al. (2005) reported that one-fourth of the agricultural irrigated lands in the world is dramatically affected by salt contamination. Salt-affected soils can be broadly classified into two categories; (1) saline soils refer to the presence of neutral such as NaCl or Na_2SO_4, while (2) alkaline soils are associated with the presence of Na_2CO_3 or $NaHCO_3$ (Yang et al., 2007). However, mixed saline and alkaline soils are frequently generated in the nature.

Saline-alkaline soils cause much stronger negative effects on plants than saline soils due to additional effect of high pH stress (Yang et al., 2008a; Zhang et al., 2013; Zhu et al., 2016). A high pH environment not only damages plants directly, but also decreases the availability of micronutrients to plants. Deficiency of some micronutrients such as B, Cu, Fe, Mn, and Zn may affect chlorophyll formation, photosynthetic process and enzyme activities, resulting in a low productivity of plants (Suman et al., 2017). Besides a high pH stress, saline-alkaline soils also impose both osmotic and ionic stresses (Munns, 2002; Wang et al., 2012). Excessive Na^+ accumulation in plants usually inhibits the uptake of macronutrients (K^+, Ca^{2+} and Mg^{2+}), which consequently retards growth and yield (Akter and Oue, 2018). In order to survive under the combination of high Na^+ and high pH environments, plants have to use several adaptive strategies such as Na^+ exclusion mechanism (Munns and Tester, 2008), accumulation of organic osmolytes (Kumar et al., 2008), maintaining an appropriate Na^+/K^+ ratio (Li et al., 2016), and the ability to obtain both macronutrients and micronutrients from soils (Wang et al., 2011) etc. However, both physiological and molecular mechanisms behind saline-alkaline tolerance in plants have not been fully understood.

Rice is known as a sensitive plant to high Na^+ environment. Extensive studies have been conducted to elucidate the mechanisms by which rice responds and adapts to neutral salt stress (NaCl and Na_2SO_4), but not saline-alkaline stress (Na_2CO_3 and $NaHCO_3$) (Wang et al., 2011; Zhang et al., 2013). In order to produce new rice genotypes for better production under saline-alkaline stress conditions, screening of rice genotypes which have potential for saline-alkaline tolerance is important. Therefore, the purposes of this study were to (1) identify rice genotypes from a large population that

are tolerant to saline-alkaline stress during vegetative stage, (2) to elucidate both physiological and molecular mechanisms underlying the saline-alkaline tolerance in the selected-rice genotype (Fukoku) by comparing growth parameters and expression profiles of some important genes for saline-alkaline tolerance with the sensitive rice genotype (IR29), and (3) lastly, to differentiate how rice seedlings respond to each of the stress conditions (saline, alkaline, and saline-alkaline) at the physiological analysis by using both selected tolerant genotype (Fukoku) and sensitive genotype (IR29).

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

This study was conducted in a greenhouse at the Laboratory of Plant Nutritional Physiology, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, Japan (attitude/longitude, 34º 23'N/132º 26E'), during 2018-2020. The study included a series of two experiments, which are described below. All experiments were arranged in a completely randomized design with three replications.

Experiment 1: Identification of rice genotypes that tolerate to high saline-alkaline stress condition at pH 9 + 50 mM Na

In previous study, 93 rice genotypes (including both *indica* and *japonica* subspecies) were assessed their tolerance to saline-alkaline stress at pH 8.5 + 50 mM Na. Among these rice genotypes screened, 17 rice genotypes were classified as saline-alkaline tolerant **(data not shown)**. This experiment was conducted to identify rice genotypes that have a high efficiency to grow under high saline-alkaline stress by using 17 rice genotypes selected from the previous study. The uniformly-sized seeds of each genotypes were incubated in tap water at 60ºC for 10 mins, followed by surface-sterilized with 5% (v/v) sodium hypochlorite solution for 30 mins and were then thoroughly rinsed with distilled water several times. Seeds were subsequently soaked in tap water for 24 h at 30ºC. The imbibed seeds were transferred onto a nylons mesh floating in 180 L plastic containers containing tap water for one week. Then, the uniform seedlings were selected and grown in half-strength slightly modified Kimura B nutrient solution as described previously (Chuamnakthong et al., 2019). At day 28, the 6-7 leaf stage rice seedlings were transferred to either half-strength Kimura B nutrient solution (control: pH 5.0-5.5) or to a saline-alkaline nutrient solution supplemented with 50 mM Na at a pH 9.0 (45 mM $NaHCO_3$ + 2.5 mM Na_2CO_3). The pH of the nutrient solution was measured daily using a pH meter (AS700 Type) and was regulated with either 2 N HCl or 2 N KOH throughout the growth period. The nutrient solution was renewed every four days, and water lost by evaporation was compensated for by daily addition of fresh

tap water. Plants were harvested at 2 weeks after the saline-alkaline treatments. Growth conditions of the first experiment were described in **Figure 3.1**.

Experiment 2: Characterization of mechanisms involved in saline-alkaline tolerance in selected-rice genotypes

After screening under high saline-alkaline stress at pH 9.0 + 50 mM Na, only three rice genotypes (Fukoku, FL478, and Nerica18) were selected for further studies by comparing their growth parameters with the sensitive rice genotype (IR29). Rice seedlings were grown hydroponically in half-strength slightly modified Kimura B nutrient solution as described previously (Chuamnakthong et al., 2019) for 5 weeks. On day 36, uniform seedlings (in the 6-7 leaf stage) were divided into four treatments for 4 weeks; (1) control: Kimura B solution without adding any saline or alkaline salts, (2) saline treatment: Kimura B solution with 50 mM NaCl, (3) alkaline treatment: Kimura B solution with pH 8.5 + 5 mM K (4 mM $KHCO_3$ + 0.5 mM K_2CO_3) and (4) saline-alkaline treatment: Kimura B solution with pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) and all plant seedlings were harvested at the end of treatments. Growth conditions of the second experiment were described in **Figure 3.2**.

3.2.2 Physiological parameters

After measuring shoot and root lengths (cm), plants were divided into leaf blades, leaf sheaths, and roots, and their fresh weight (FW) was measure. The dry weights (DW) were recorded after drying in an oven at 70°C for 3 days. The water content in the leaf blades was calculated using the equation (FW-DW)/FW.

3.2.3 Measurement of chlorophyll concentration

To determine the total chlorophyll concentration in the leaf blades of Fukoku (the most saline-alkaline tolerant) and IR29 (saline-alkaline sensitive) plants, the third leaves from the top of those plants were harvested, weighed, and placed in tubes containing 5 mL of aqueous ethanol (95% v/v). The chlorophyll was extracted by incubating the samples into the solvent in a dark condition. The absorbance of the

supernatant was recorded using a UV-spectrophotometer (UV-1850, Hitachi, Japan) at two different wavelengths; 645 and 663 nm. Then, total chlorophyll was calculated as $8.02A_{663} + 20.21A_{645}$, and was expressed as mg chlorophyll g^{-1} fresh weight.

3.2.4 Measurement of proline concentration

The proline concentrations in the leaf blades of Fukoku and IR29 plants were determined after four weeks of saline-alkaline treatment 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.

3.2.5 Measurement of Lipid peroxidation (MDA) concentration

Malondialdehyde (MDA) concentrations in the leaf blades of Fukoku and IR29 plants were analyzed according to Hodges et al (1999). Sample (100 mg FW) was ground with liquid N_2, 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) buthyl 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});

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

$Y = [A_{440}$ (TBA (+)) – A_{600} (TBA (+))] × 0.0571

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

3.2.6 Measurement of B, Ca, Cu, Fe, K, Mg, Mn, Na, and Zn concentrations

Plant samples (leaf blades, leaf sheaths, and roots) of Fukoku and IR29 were ground into fine powder and digested with a mixture solution of nitric acid (HNO_3) and perchloric acid ($HClO_4$). Total concentration of B, Ca, Cu, Fe, K, Mg, Mn, Na and Zn were measured by using an inductively coupled plasma-optical emission spectrometer (iCAP 6000; Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.2.7 Expression analysis of the genes encoding Na^+ transport proteins

Total RNA was extracted from the roots of control and saline-alkaline stressed Fukoku (saline-alkaline tolerant) and IR29 (saline-alkaline sensitive) genotypes using a TRIzol reagent. A total amount of 0.5 µg of RNA per sample was used for first-strand cDNA synthesis using a ReverTra Ace qPCR RT Master Mix kit (TOYOBO) following the manufacturer's instructions. The gene expression level was determined by real-time RT-PCR using Thunderbird SYBR qPCR Mix (TOYOBO) and ABI StepOne System (Applied Biosystems, CA) as previously described (Ueda et al., 2013). The primers used are listed in **Table 3.4**. Normalized relative expression was calculated by the ΔΔCt method (Livak et al., 2001) with the *OsAct11* gene as an internal control (Jain et al., 2006).

3.2.8 Statistical analysis

All collected data were analyzed using the one-way analysis of variance with SPSS statistical analysis software, version 21. Differences between means (n = 4, except for proline, MDA, and microelement concentrations, for which n = 3) were determined using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

3.3 Results

3.3.1 Screening by high pH stress (pH 9.0 + 50 mM Na)

To examine the ability of saline-alkaline tolerance, 4-week-old seedlings of 21 selected-rice genotypes were exposed to a severe saline-alkaline stress condition (pH 9.0 + 50 mM Na) for 2 weeks. As shown in **Table 3.1 and 3.2**, high saline-alkaline stress resulted in severe decreases of shoot and root dry weight in all rice genotypes, but not in the root of Fukoku plants. In addition, Fukoku, FL478, and Nerica18 showed least reduction in both shoot and root dry weight under saline-alkaline stress at pH 9.0 + 50 mM Na compared to other rice genotypes, indicating that these three rice genotypes may tolerate to a severe saline-alkaline stress condition.

3.3.2 Identification of rice genotypes with high tolerance to long-term saline-alkaline stress

After screening with high saline-alkaline stress at pH 9.0 + 50 mM Na for 2 weeks, Fukoku, FL478, and Nerica18 were selected as candidate rice genotypes for further studies by comparing their growth performance with the salt sensitive rice genotype (IR29). Upon exposure of the four rice genotypes to the nutrient solution supplemented with pH 8.5 + 50 mM Na for 4 weeks, the lengths of shoot and root of all rice genotypes were decreased as compared to those under control condition **(Figure 3.3A and B)**. However, this study found that the shoot length of Fukoku seedlings was little affected by saline-alkaline stress, while a significant reduction in the shoot length was observed in other rice genotypes **(Figure 3.3A)**. In addition, the reduction of root lengths in all rice genotypes was no significant difference upon exposure to saline-alkaline stress for 4 weeks. However, in comparison to control condition, the magnitude of the reduction in root length was greater in Nerica18 (13.46%) than in Fukoku (10.49%), IR29 (10.48%), and FL478 (9.33%), respectively **(Figure 3.3B)**.

Maintaining a high biomass under abiotic stress conditions is considered as one of the most important physiological parameters to determine the tolerance of rice plants (Knowledgebank.irri). As shown in **Figure 3.4A and B**, shoot and root dry weights of the saline-alkaline tolerant Fukoku, FL478, and Nerica18 were slightly affected by saline-alkaline stress at pH 8.5 + 50 mM Na, while the saline-

alkaline sensitive IR29 showed a significant decrease in both shoot and root growth upon exposure to saline-alkaline solutions. In addition, under saline-alkaline stress, the saline-alkaline tolerant Fukoku was found to have a less decline in the dry weights of shoot (19.74%) and root (15.14%) compared to FL478 (20.72% and 18.28%) and Nerica18 (43.17% and 23.76%), respectively.

Leaf water content was measured to estimate the amount of water loss under saline-alkaline stress. In comparison to control plants, there was non-significant decreases in the leaf water content of the three saline-alkaline tolerant genotypes (Fukoku, FL478, and Nerica18) after exposure to saline-alkaline stress at pH 8.5 + 50 mM Na for 4 weeks **(Figure 3.5)**, suggesting that these rice genotypes had a greater potential to maintain the water balance in their leaf tissues under saline-alkaline stress. In contrast, the leaf water content of the saline-alkaline sensitive IR29 was dramatically decreased by 18.72% by exposure to saline-alkaline stress condition, implying that this rice genotype has a low capacity to maintain its leaf water status under a such condition.

From all above results indicate that Fukoku is the most saline-alkaline tolerant of the four genotypes. To gain a better understanding of the mechanism underlying saline-alkaline tolerance of Fukoku seedlings at vegetative stage, Fukoku (the most saline-alkaline tolerant) and IR29 (saline-alkaline sensitive) were selected as candidates rice genotypes for further analysis, including the measurements of chlorophyll, proline, malondialdehyde (MDA), Na, K, microelements (B, Ca, Cu, Fe, Fe, Mg, Mn, Mo, and Zn), and Na^+/K^+ ratio in plant tissues.

3.3.3 Effects of saline-alkaline stress on chlorophyll concentration in Fukoku and IR29 plants

As shown in **Figure 3.6**, the foliar chlorophyll concentrations of the two rice genotypes were slightly reduced by saline-alkaline stress at pH 8.5 + 50 mM Na. In detail, the highest concentration of total chlorophyll in the leaves was observed in Fukoku, while the lowest concentration was observed in IR29 under both control and saline-alkaline stress conditions. This result suggests that the ability of Fukoku to maintain a higher concentration of total chlorophyll in the leaf blades is probably one of the important mechanisms aiding to saline-alkaline tolerance.

3.3.4 Effects of saline-alkaline stress on proline accumulation in Fukoku and IR29 plants

Saline-alkaline stress at pH 8.5 + 50 mM Na significantly increased the proline concentration in the leaf blades of both rice genotypes **(Figure 3.7)**. However, under saline-alkaline stress, the proline concentration was markedly increased in the leaf blades of the most saline-alkaline tolerant Fukoku than in the saline-alkaline sensitive IR29 plants.

3.3.5 Effects of saline-alkaline stress on lipid peroxidation in Fukoku and IR29 plants

Plants suffering from abiotic stress often exhibit symptoms of oxidative stress as evidenced by enhanced accumulation of reactive oxygen species (ROS) and malondialdehyde (MDA). As shown in **Figure 3.8**, exposure to saline-alkaline stress at pH 8.5 + 50 mM Na for 4 weeks led to increase in MDA concentration in the leaves of both Fukoku and IR29 plants. However, compared with the control condition, the magnitude of the increase in MDA concentration was much greater in IR29 (98.14%) than in Fukoku (15.78%) plants under saline-alkaline stress condition, and this finding suggests that Fukoku plants may equip with greater tolerance to the oxidative damage rather than IR29 plants.

3.3.6 Effects of saline-alkaline stress on Na^+ and K^+ concentrations in Fukoku and IR29 plants

Exposure to saline-alkaline stress for 4 weeks led to a significant increase in Na^+ concentration in all tissues examined in both the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 **(Figure 3.9)**. However, under saline-alkaline stress at pH 8.5 + 50 mM Na, Fukoku plants can maintain lower Na^+ concentration in both leaf blades and roots than IR29 plants **(Figure 3.9A and C)**. In the leaf sheaths, no significant differences in Na^+ concentrations in Fukoku and IR29 plants were found when grown in saline-alkaline solution **(Figure 3.9B)**, thus leading to a significantly higher Na^+ concentrations in the shoots of IR29 than in Fukoku plants under saline-alkaline stress **(Figure 3.9C)**.

Saline-alkaline stress significantly increased the K^+ concentration in the leaf blades of IR29 plants, while the K^+ concentration in the leaf blades of Fukoku plants was relatively unchanged when grown in saline-alkaline solution **(Figure 3.10A)**. In addition, exposure to saline-alkaline stress solution led to similar reductions in leaf sheaths K^+ concentrations in the two rice genotypes, such that no

significant difference was found between two genotypes **(Figure 3.10B)**. In roots, both Fukoku and IR29 plants showed comparable root K^+ concentration when grown in control solution **(Figure 3.10D)**, however, there was a significant decrease in K^+ concentration in the roots of both rice genotypes under saline-alkaline stress; the magnitude of reduction in root K^+ concentration of IR29 plants was significantly less than that in Fukoku plants.

In order to survive under high Na^+ environment, rice plants need to maintain a low Na^+/K^+ ratio in the cytosol (Zhang et al., 2018). As shown in **Table 3.3**, the most saline-alkaline tolerant Fukoku showed lower Na^+/K^+ ratio in the leaf blades under saline-alkaline stress condition, but not in the saline-alkaline sensitive IR29, thus maintenance of lower Na^+/K^+ ratio in the leaf blades is likely one of the key factors for Fukoku to cope with saline-alkaline stress.

3.3.7 Effects of saline-alkaline stress on Ca^{2+} and Mg^{2+} concentrations in Fukoku and IR29 plants

As shown in **Figure 3.11A**, under saline-alkaline stress condition, the shoot Ca^{2+} concentrations of Fukoku and IR29 plants were much lower than those of the control plants. Additionally, the magnitude of reduction in shoot Ca^{2+} concentration of Fukoku plants was significantly less than that in IR29 plants. In roots, there was significant increases in Ca^{2+} concentration of both rice genotypes upon exposure to saline-alkaline solution; the highest concentration of Ca^{2+} was observed in Fukoku (2.16 mg/gDW), followed by IR29 (1.52 mg/gDW) **(Figure 3.11B)**. The reduction trends of Mg^{2+} concentration in the shoots of Fukoku and IR29 plants were similar to Ca^{2+} concentration **(Figure 3.11A)**, while in the roots, the concentration of Mg^{2+} was significantly increased only in Fukoku plants after exposed to saline-alkaline solution for 4 weeks **(Figure 3.11B)**.

3.3.8 Effects of saline-alkaline stress on microelements (Fe, Mn, Cu, Zn and B) concentrations in Fukoku and IR29 plants

At high pH conditions in the rhizosphere, some micronutrients (Fe, Mn, Cu, Zn, and B) become less available in the soils, therefore plants that grow in such soils usually suffer from micronutrient

deficiencies resulting in a yellowing of leaf tissues (chlorosis). Thus, maintaining a high concentration of microelements in the plant tissues is considered as one indicator to determine the saline-alkaline tolerance in plants. As shown in **Figure 3.11**, Fukoku plants accumulated much more Fe, Mn, Cu, Zn, and B in shoots and roots than IR29 plants under both control and saline-alkaline stress conditions. In detail, compared with the control plants, saline-alkaline stress at pH 8.5 + 50 mM Na resulted in a non-significant change in the concentrations of Fe, Cu, Zn, and B in the shoots of two rice genotypes **(Figure 3.11E, I, K, and M)**. However, a significant reduction in the shoot Mn concentration was observed in both rice genotypes after exposure to saline-alkaline solution for 4 weeks **(Figure 3.11G)**. In roots, the concentration of Fe and B were significantly decreased in both rice genotypes when exposed to saline-alkaline solution **(Figure 3.11F and N)**. In contrast to the trend of Fe and B accumulation, the accumulations of Mn and Zn in the roots of two rice genotypes were remarkably increased by saline-alkaline stress **(Figure 3.11H and L)**. Moreover, the difference between Fukoku (the most saline-alkaline tolerant) and IR29 (the saline-alkaline sensitive) was clearly observed in the accumulation pattern of Cu in roots, as shown in **Figure 3.11J**. Under saline-alkaline stress conditions, the concentration of Cu was increased by 4.28% in the roots of Fukoku plants, while it was drastically decreased by 40.65% in the roots of IR29 plants in comparison to those under control conditions. These findings suggest that microelements are more available in both shoot and root tissues of Fukoku plants rather than IR29 plants under either normal or saline-alkaline stress conditions.

3.3.9 Effects of saline, alkaline and saline-alkaline stresses on growth and physiological changes in rice plants

To clearly understand the physiological responses of rice to saline-alkaline stress, the growth parameters of Fukoku and IR29 rice genotypes were further examined under either saline (50 mM Na), alkaline (pH 8.5), or saline-alkaline (50 mM Na + pH 8.5) stress conditions. As shown in **Figure 3.13C and D**, a significant reduction in shoot length was observed in IR29 plants after exposure to saline (50 mM Na), alkaline (pH 8.5 + 5 mM K) and saline-alkaline (pH 8.5 + 50 mM Na) stress conditions for 4 weeks, however, the shoot length of Fukoku plants was not altered by those stress conditions. The root lengths of both rice genotypes were significantly decreased upon exposure to saline (50 mM Na) and

alkaline (pH 8.5 + 5 mM K) treatments, while a slight decrease in root length of two rice genotypes was detected under saline-alkaline (pH 8.5 + 50 mM Na) treatment.

Shoot and root dry weights of IR29 plants were dramatically decreased upon exposure to treatments either saline (50 mM Na), alkaline (pH 8.5 + 5 mM K) or saline-alkaline stress (pH 8.5 +50 mM Na) conditions **(Figure 3.14A and B)**. Among the treatments, a total dry mass production of IR29 plants was severely affected by saline-alkaline stress (pH 8.5 + 50 mM Na) rather than saline stress (50 mM Na) and alkaline stress (pH 8.5 + 5 mM K), respectively. In Fukoku plants, a significant decrease in both shoot and root dry weights was only observed in the plants grown under saline solution (50 mM Na) **(Figure 3.14A and B)**. These findings suggest that the growth of IR29 plants was sensitive to both high salinity and high pH stresses, whereas the growth of Fukoku plants was limited by only high salinity stress.

Na^+ concentrations in both shoots and roots of two rice genotypes increased sharply when challenged by saline and saline-alkaline stress conditions **(Figure 3.15A and B)**. In detail, Fukoku plants accumulated much less Na^+ in shoots and roots than IR29 plants after exposure to either saline or saline-alkaline stress conditions. In addition, there was a significant difference in Na^+ accumulation between plants grown under saline and saline-alkaline stress treatments; the Na^+ concentration of plants grown under saline-alkaline stress solution was markedly higher than that of saline-stressed plants. Shoot Na concentrations in the two rice genotypes were comparable under control and alkaline stress conditions. In contrast, compared with the control, a significant reduction in roots Na^+ concentration was observed in both rice genotypes after exposure to alkaline solution. These findings suggest that the effects of saline-alkaline stress (pH 8.5 + 50 mM Na) on the accumulation of Na^+ in both rice genotypes were stronger than that of saline (50 mM Na) and alkaline (pH 8.5 + 5 mM K) stresses, respectively.

Increased salinity generally reduces K^+ concentration in plants (Munns and Tester 2008). As shown in **Figure 3.15C and D**, saline-alkaline treatment (pH 8.5 + 50 mM Na) had a more severe reduction in K^+ concentration of shoot and root in all rice genotypes rather than saline treatment (50 mM Na). In addition, IR29 plants had a higher K^+ concentration in their shoots and roots than Fukoku plant under both control and treatments, this suggests that maintaining a relatively high K^+ concentration in plant tissues is important for the growth of IR29 plants than Fukoku plants.

3.3.10 Effects of saline-alkaline stress on expression of the genes encoding Na^+ transporters

Under 4 weeks of saline-alkaline treatment (pH 8.5 + 50 mM Na), Fukoku showed relatively higher saline-alkaline tolerance than IR29 plants due to the low Na^+ accumulation in its leaf blades and roots. To gain a better understanding of the mechanism underlying tolerance to saline-alkaline stress at a transcriptional level in both rice genotypes, the expressions of genes encoding Na^+ transport proteins (*OsSOS1* and *OsHKT1;5*) were investigated in their roots by quantitative RT-PCR.

The Na^+/H^+ antiporter salt overly sensitive1 (*SOS1*), localized in the root epidermis, offers the first route to counteract Na^+ influx by extruding Na^+ to the external soil environment (Shi et al., 2002). The results obtained from quantitative RT-PCR analysis showed that under saline-alkaline stress condition, there was a higher level of induced expression of *OsSOS1* gene in the roots of Fukoku (2.97-fold) **(Figure 3.16B)**, which might be responsible for relatively low Na^+ accumulation in its roots **(Figure 3.15D)**. In contrast, the *OsSOS1* expression in the roots of IR29 was repressed by 0.79-fold after exposure to saline-alkaline stress treatment **(Figure 3.16 A)**, which suggests OsSOS1 mediated Na^+ extrusion from the cytosol may not be active in the roots of IR29.

OsHKT1;5 is known as one of the key players for salt tolerance in rice (Chen et al., 2017). This gene encodes a plasma membrane-localized protein and plays a role in the transport of Na^+ from the xylem sap into the surrounding xylem parenchyma cells (Ren et al. 2005). In the present study, saline-alkaline treatment at pH 8.5 + 50 mM Na induced the expression of *OsHKT1;5* highly in the roots of Fukoku (22.94-fold) compared to its expression in the IR29 roots (7.65-fold) **(Figure 3.17 A and B)**. This finding implies that Na^+ transport from xylem sap to xylem parenchyma was active in the roots of both Fukoku (saline-alkaline tolerant) and IR29 (saline-alkaline sensitive) rice genotypes when exposed to saline-alkaline stress.

3.3.11 Transcriptomic responses of genes encoding Fe acquisition proteins

Under saline-alkaline conditions, plants are exposed to high soil pH, thereby suffering from Fe deficiency. Fe deficiency-responsive genes are reported to be involved in plant adaptation to saline-alkaline environments (Kobayashi et al., 2012; Kim et al., 2016). Therefore, to clearly understand the

mechanisms of Fe acquisition in rice, transcription levels of genes encoding Fe acquisition proteins (*OsIRO2*, *OsIRT1*, and *OsYSL15*) were analyzed in the roots of both Fukoku and IR29 rice genotypes.

In rice, the transcription factor OsIRO2 controls the expression of several genes involved in the process of phytosiderophore synthesis (*OsNAS1, OsNAS2, OsNAAT1*, and *OsDMAS1*) (Ogo et al., 2007). The results of present study demonstrated that the expression of *OsIRO2* was markedly induced by saline-alkaline stress (pH 8.5 + 50 mM Na) in the roots of Fukoku (15.89-fold) **(Figure 3.18B)**, but it was repressed in those of IR29 (0.24-fold) **(Figure 3.18A)**. This finding suggests that under saline-alkaline stress condition at pH 8.5 + 50 mM Na, Fukoku plants may have an efficient system of phytosiderophore synthesis than IR29 plants.

Rice is not a typical strategy II plant, as it not only synthesizes DMA in roots to chelate Fe^{3+}-DMA, but also acquires Fe^{2+} through OsIRT transporters (strategy I system). The present study found that under saline-alkaline treatment (pH 8.5 + 50 mM Na), the repression of *OsIRT1* expression was observed in the roots of both rice genotypes **(Figure 3.19A and B)**. OsYSL15 is known as one of specific transporters for Fe^{3+}-DMA uptake in rice (Aoyama et al., 2009). The results obtained from quantitative RT-PCR analysis indicated that, under both control and saline-alkaline stress conditions, *OsYSL15* transcripts are not detected in the roots of both rice genotypes **(data not shown)**, which implies that this transporter was unlikely important for Fe^{3+}-DMA acquisition in both rice genotypes.

Table 3.1 Shoot dry weight of 17 rice genotypes under control and saline-alkaline stress condition at pH 9 + 50 mM Na (45 mM $NaHCO_3$ + 2.5 mM Na_2CO_3) for 2 weeks. Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

No.	Genotypes	Shoot dry weight (g)		(B)/(A)
		(A) Control	(B) Saline-alkaline	
1	Nerica18	2.08 ± 0.36	1.57 ± 0.19	**0.75**
2	FL478	3.11 ± 0.12	2.22 ± 0.16	**0.71**
3	Fukoku	1.77 ± 0.17	1.18 ± 0.22	**0.67**
4	Milyang23	1.12 ± 0.16	0.68 ± 0.09	**0.61**
5	Dular	1.97 ± 0.11	1.21 ± 0.08	**0.61**
6	Khao Nam Jen	4.20 ± 0.40	2.53 ± 0.24	**0.60**
7	Hinode	4.05 ± 0.21	1.98 ± 0.14	**0.49**
8	Ratul	4.09 ± 0.28	1.80 ± 0.19	**0.44**
19	Vandaran	3.69 ± 0.24	1.54 ± 0.52	**0.42**
10	ARC11094	4.40 ± 0.37	1.76 ± 0.24	**0.40**
11	Aka Sho	2.26 ± 0.08	0.90 ± 0.07	**0.40**
12	Agami	1.16 ± 0.39	0.44 ± 0.17	**0.38**
13	Khau Tan Chiem	5.61 ± 0.19	2.09 ± 0.24	**0.37**
14	Basilanon	2.27 ± 0.36	0.65 ± 0.03	**0.29**
15	Khau Mac Kho	4.38 ± 0.43	1.19 ± 0.14	**0.27**
16	Kalo Dhan	3.17 ± 0.13	0.77 ± 0.01	**0.24**
17	Hong Cheuh Zai	5.23 ± 0.20	1.22 ± 0.16	**0.23**

Table 3.2 Root dry weight of 17 rice genotypes under control and saline-alkaline stress condition at pH 9 + 50 mM Na (45 mM $NaHCO_3$ + 2.5 mM Na_2CO_3) for 2 weeks. Data represent the means of quadruplicates ± SE. The same letters indicate no significant differences at $p < 0.05$.

No.	Genotypes	Root dry weight (g)		(B)/(A)
		(A) Control	(B) Saline-alkaline	
1	Fukoku	0.36 ± 0.04	0.37 ± 0.07	**1.03**
2	Nerica18	0.66 ± 0.11	0.51 ± 0.05	**0.77**
3	FL478	0.78 ± 0.06	0.52 ± 0.06	**0.67**
4	Khao Nam Jen	1.40 ± 0.13	0.92 ± 0.12	**0.66**
5	Dular	0.63 ± 0.02	0.37 ± 0.03	**0.59**
6	Milyang23	0.40 ± 0.03	0.23 ± 0.02	**0.58**
7	Hinode	1.48 ± 0.11	0.78 ± 0.03	**0.53**
8	Khau Tan Chiem	1.40 ± 0.11	0.64 ± 0.08	**0.46**
9	Agami	0.49 ± 0.16	0.20 ± 0.08	**0.41**
10	Aka Sho	0.55 ± 0.04	0.22 ± 0.01	**0.40**
11	Basilanon	0.64 ± 0.11	0.22 ± 0.01	**0.34**
12	Khau Mac Kho	1.49 ± 0.15	0.49 ± 0.04	**0.33**
13	Ratul	1.60 ± 0.08	0.49 ± 0.03	**0.31**
14	Vandaran	1.48 ± 0.50	0.45 ± 0.15	**0.30**
15	ARC11094	1.44 ± 0.18	0.43 ± 0.03	**0.30**
16	Hong Cheuh Zai	1.32 ± 0.04	0.37 ± 0.02	**0.28**
17	Kalo Dhan	1.23 ± 0.08	0.30 ± 0.01	**0.24**

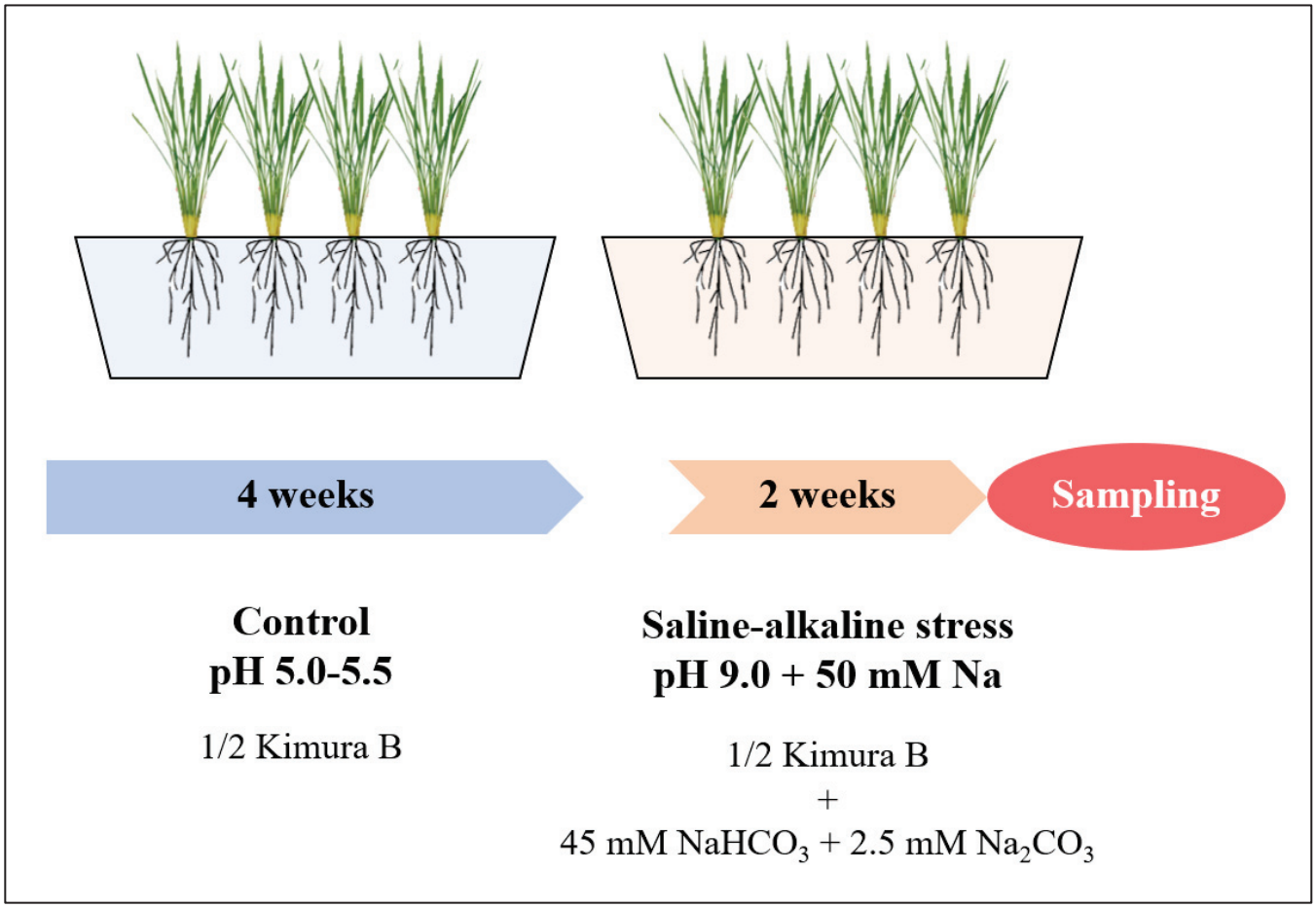


Figure 3.1 Growth conditions of the first experiment. 17 rice genotypes were grown hydroponically for 4 weeks in half-strength Kimura B nutrient solution. On day 29, uniform seedlings of each rice genotype were then subjected to saline-alkaline stress at pH 9.0 + 50 mM Na (45 mM $NaHCO_3$ + 2.5 mM Na_2CO_3) for another 2 weeks.

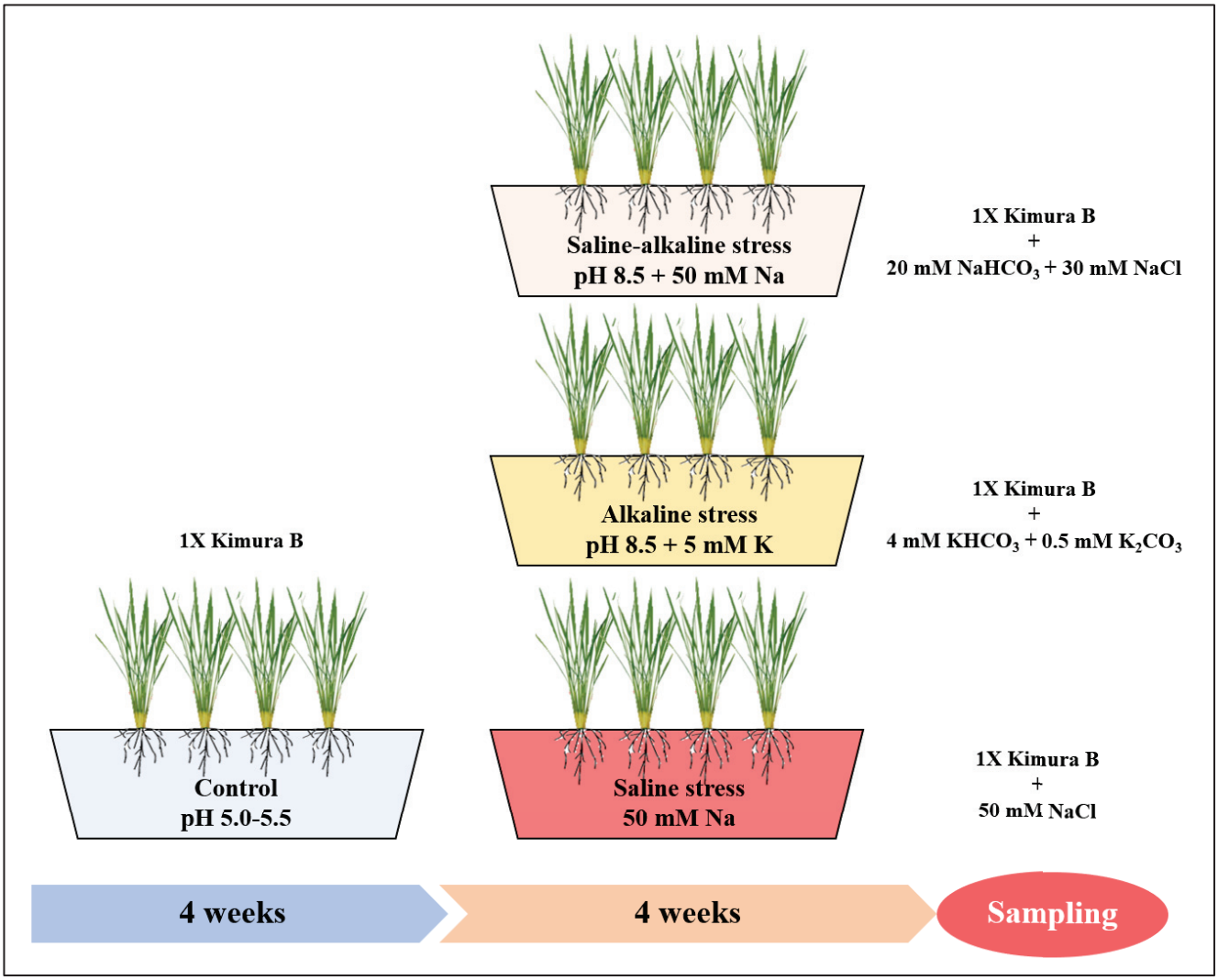


Figure 3.2 Growth conditions of the second experiment. Seedlings were established under control conditions (no saline or alkaline salts) for 5 weeks. On day 36, uniform seedlings of each rice genotypes were divided and grown under four treatments for 4 weeks; (1) control: 1X Kimura B solution without adding any saline or alkaline salts, (2) saline treatment: 1X Kimura B solution with 50 mM NaCl, (3) alkaline treatment: 1X Kimura B solution with pH 8.5 + 5 mM K (4 mM $KHCO_3$ + 0.5 mM K_2CO_3) and (4) saline-alkaline treatment: 1X Kimura B solution with pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl).

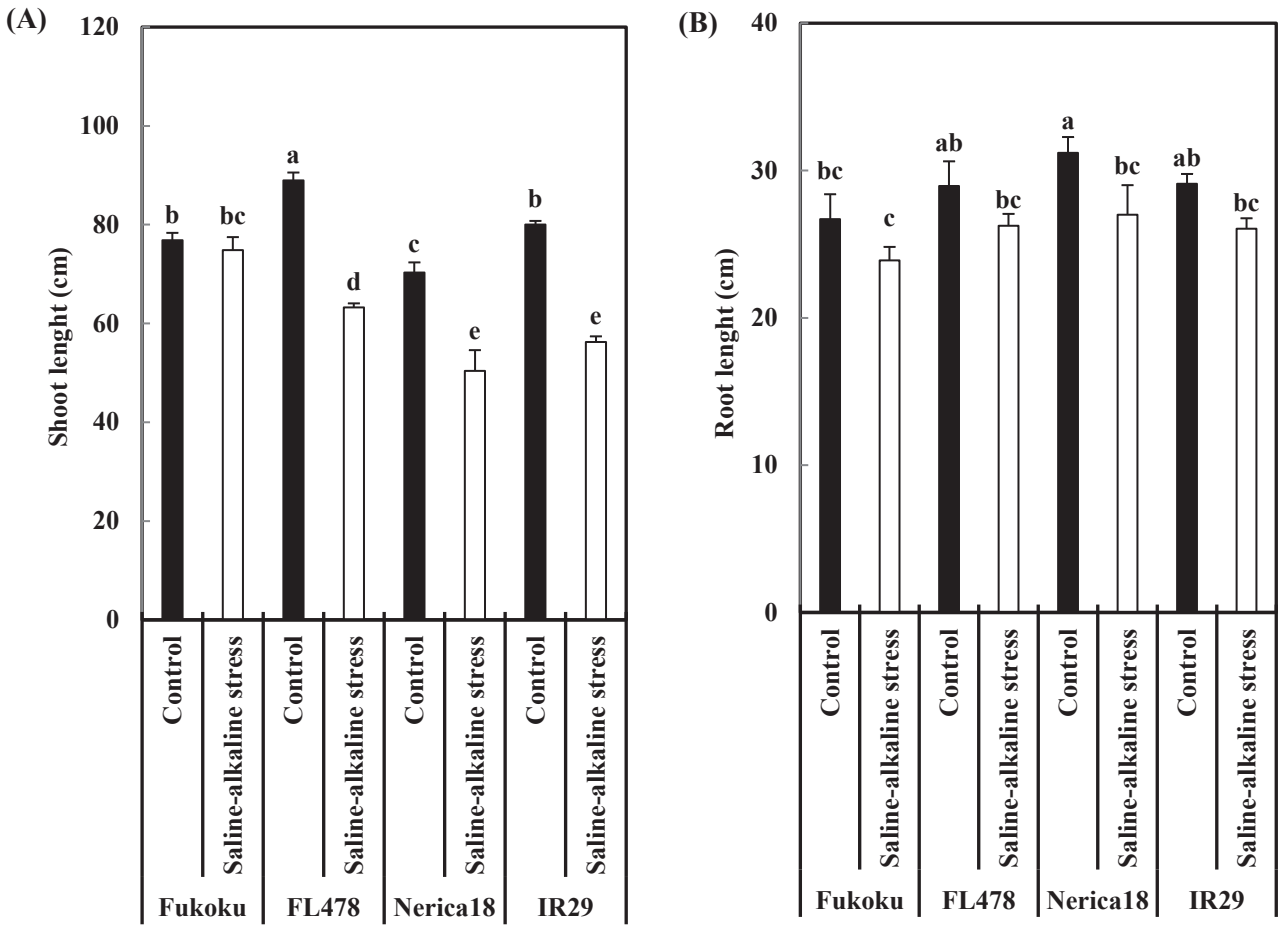


Figure 3.3 Effects of saline-alkaline stress on (A) shoot and (B) root lengths of four rice genotypes; Fukoku (saline-alkaline tolerant), FL478 (saline-alkaline tolerant), Nerica18 (saline-alkaline tolerant), and IR29 (saline-alkaline sensitive). Lengths of shoot and root were measured under control and after four weeks of saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

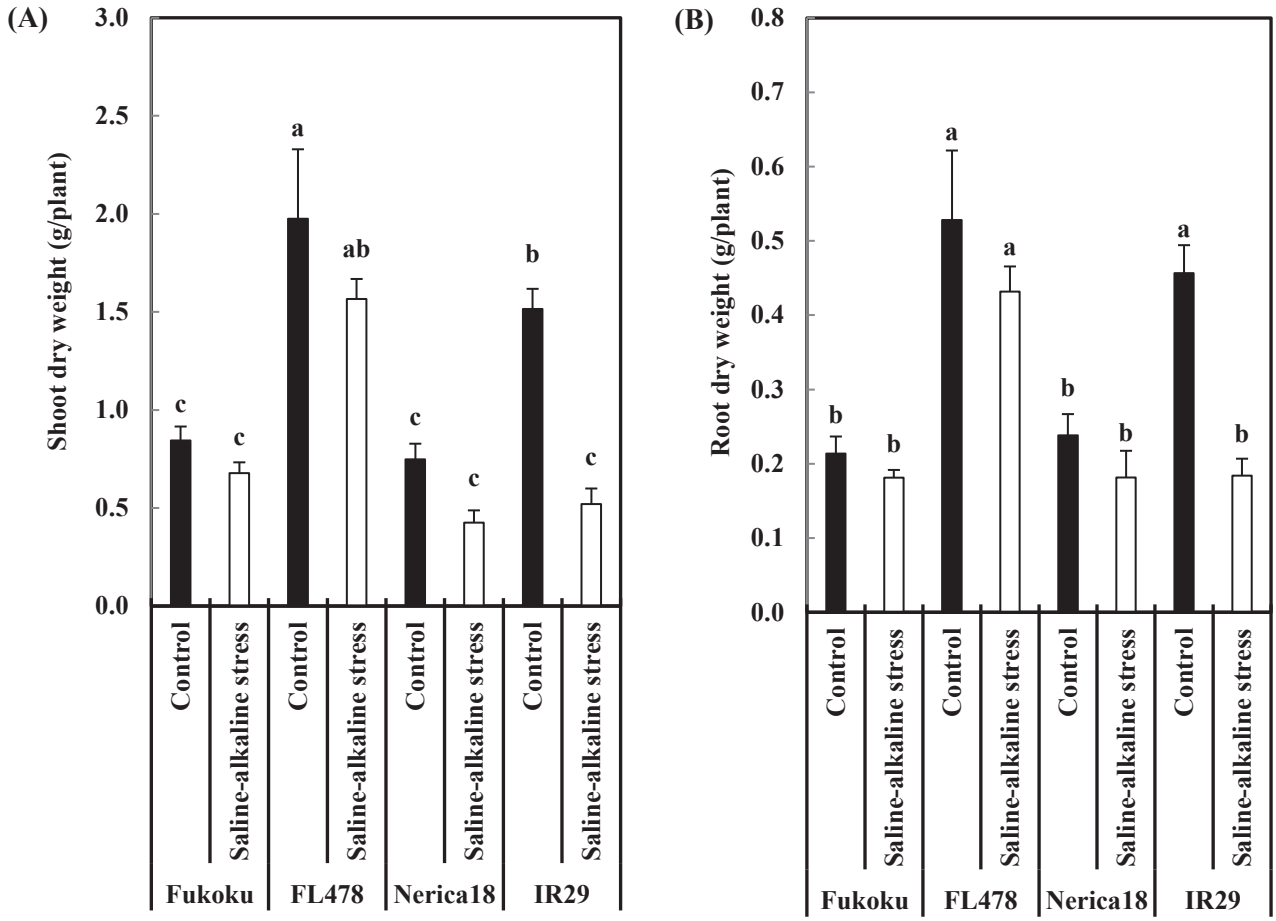


Figure 3.4 Effects of saline-alkaline stress on (A) shoot and (B) root dry weights of four rice genotypes; Fukoku (saline-alkaline tolerant), FL478 (saline-alkaline tolerant), Nerica18 (saline-alkaline tolerant), and IR29 (saline-alkaline sensitive). Dry weights of shoot and root were measured under control and after four weeks of saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

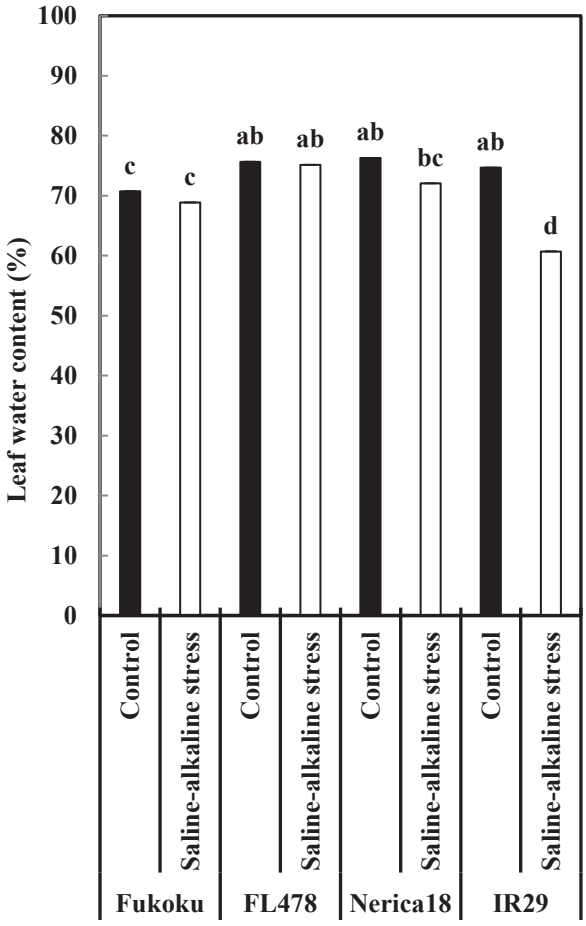


Figure 3.5 Effects of saline-alkaline stress on leaf water content of four rice genotypes. Leaf water content was measured under control and after four weeks of saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

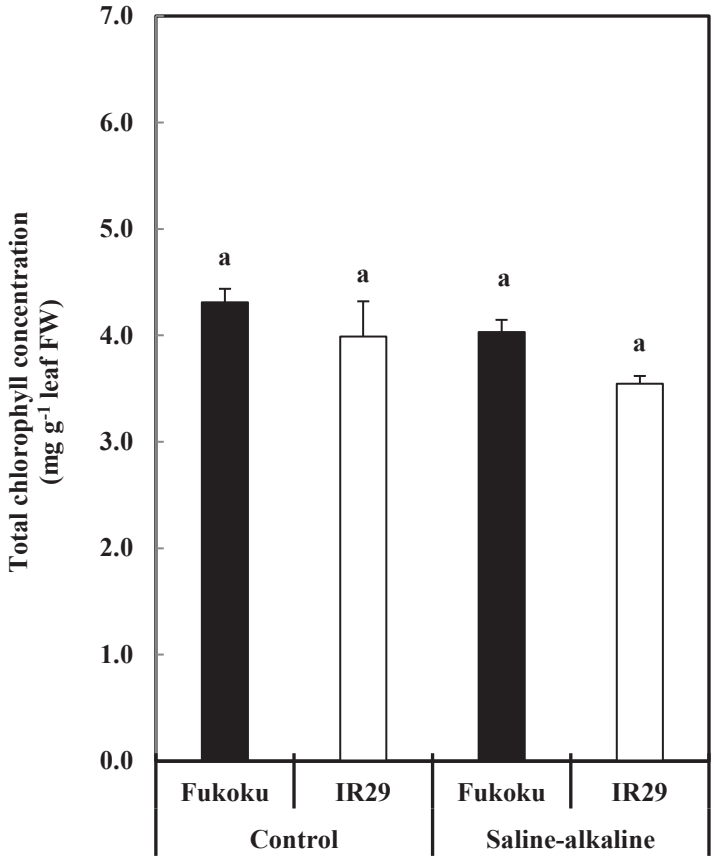


Figure 3.6 Foliar chlorophyll concentration in the leaves of the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

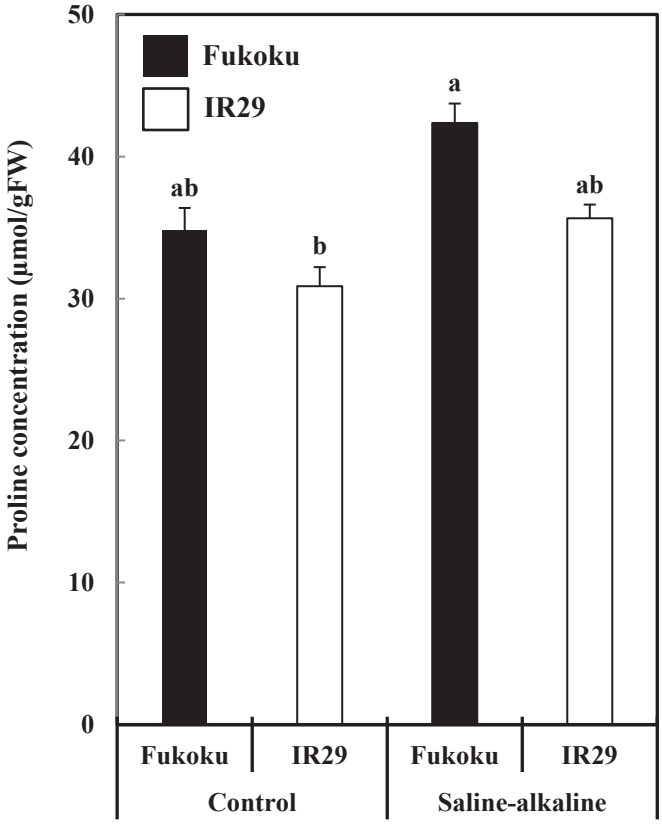


Figure 3.7 Proline concentration in the leaves of the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of three replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

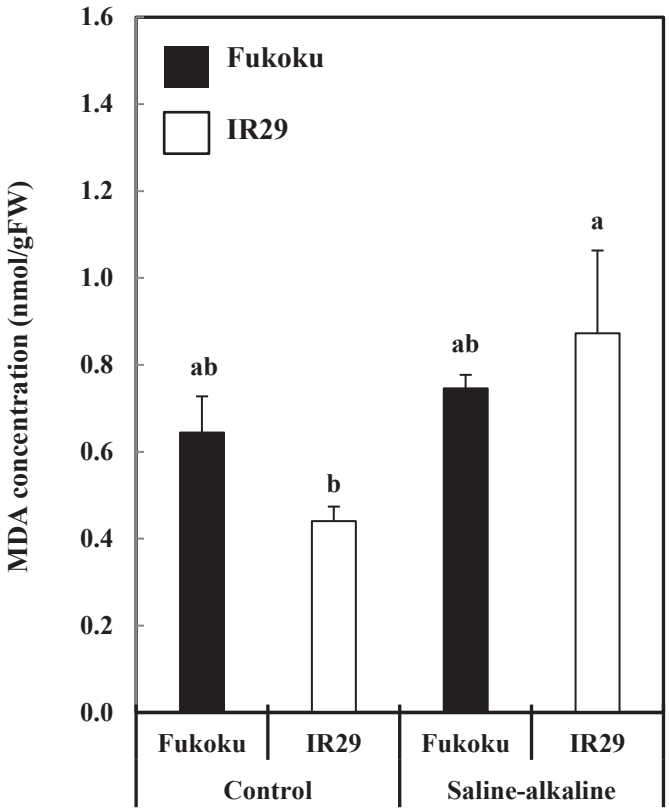


Figure 3.8 Malondialdehyde (MDA) concentration in the leaves of the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of three replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

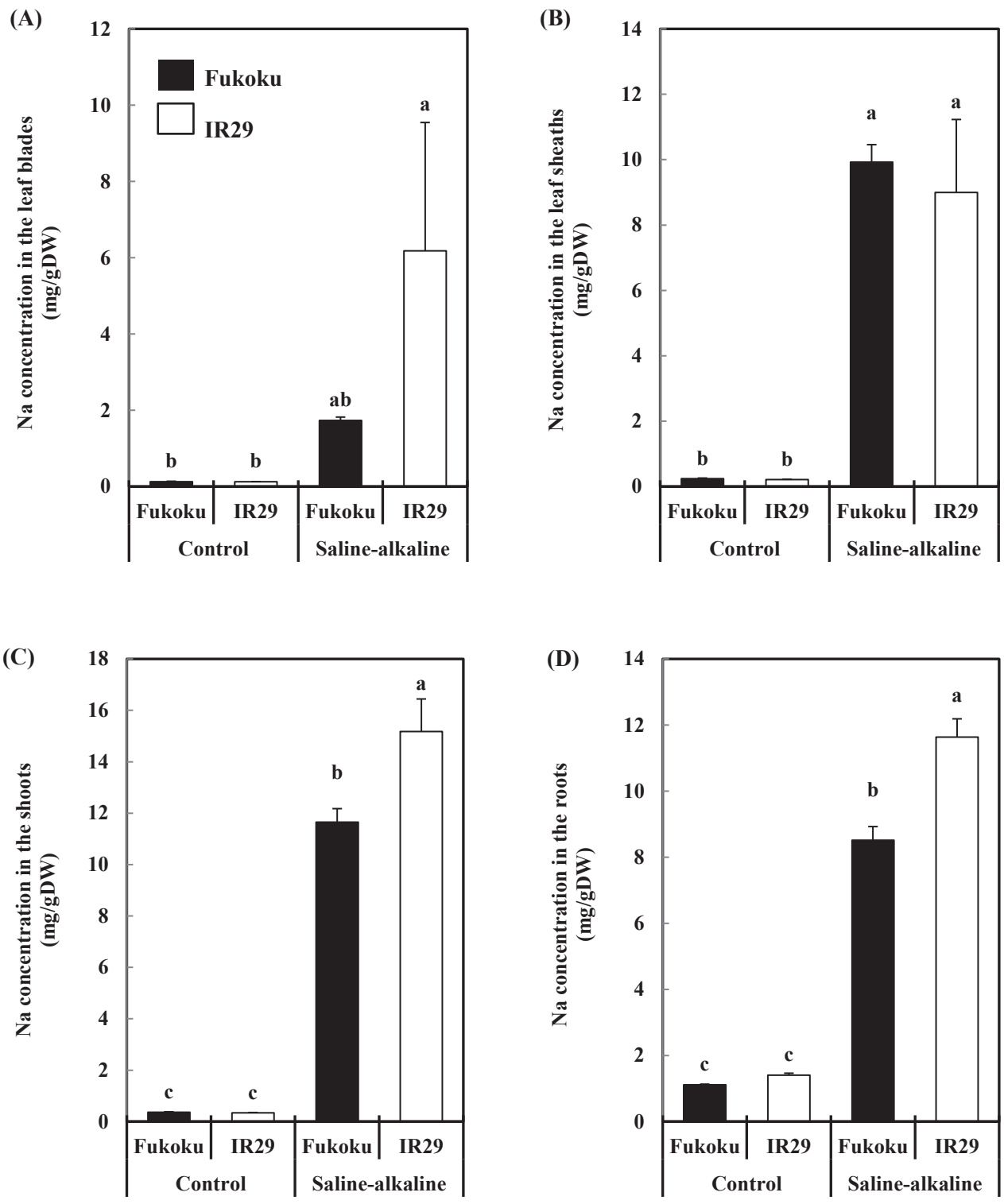


Figure 3.9 Na concentration in (A) leaf blades, (B) leaf sheaths, (C) shoots, and (D) roots under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

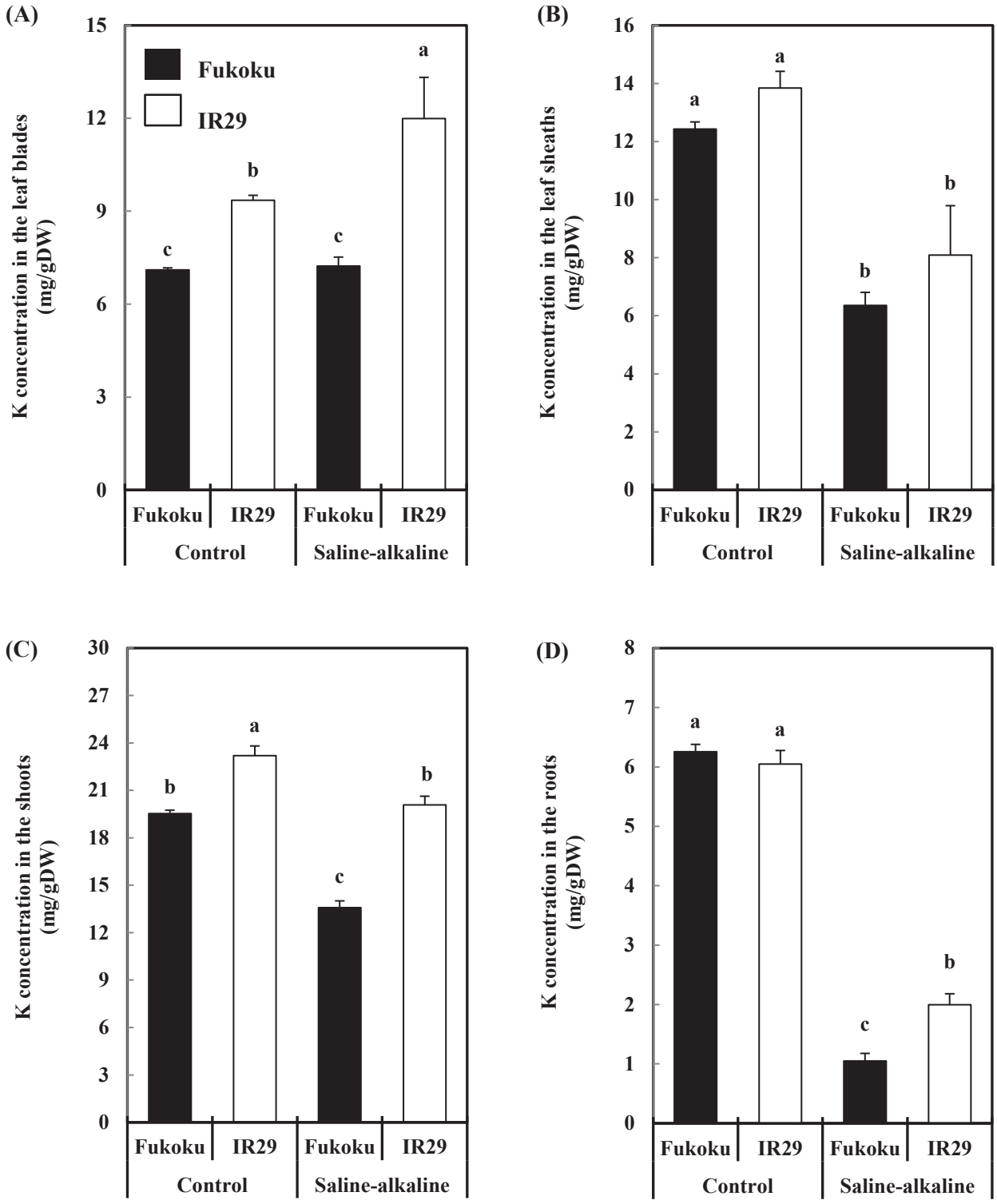
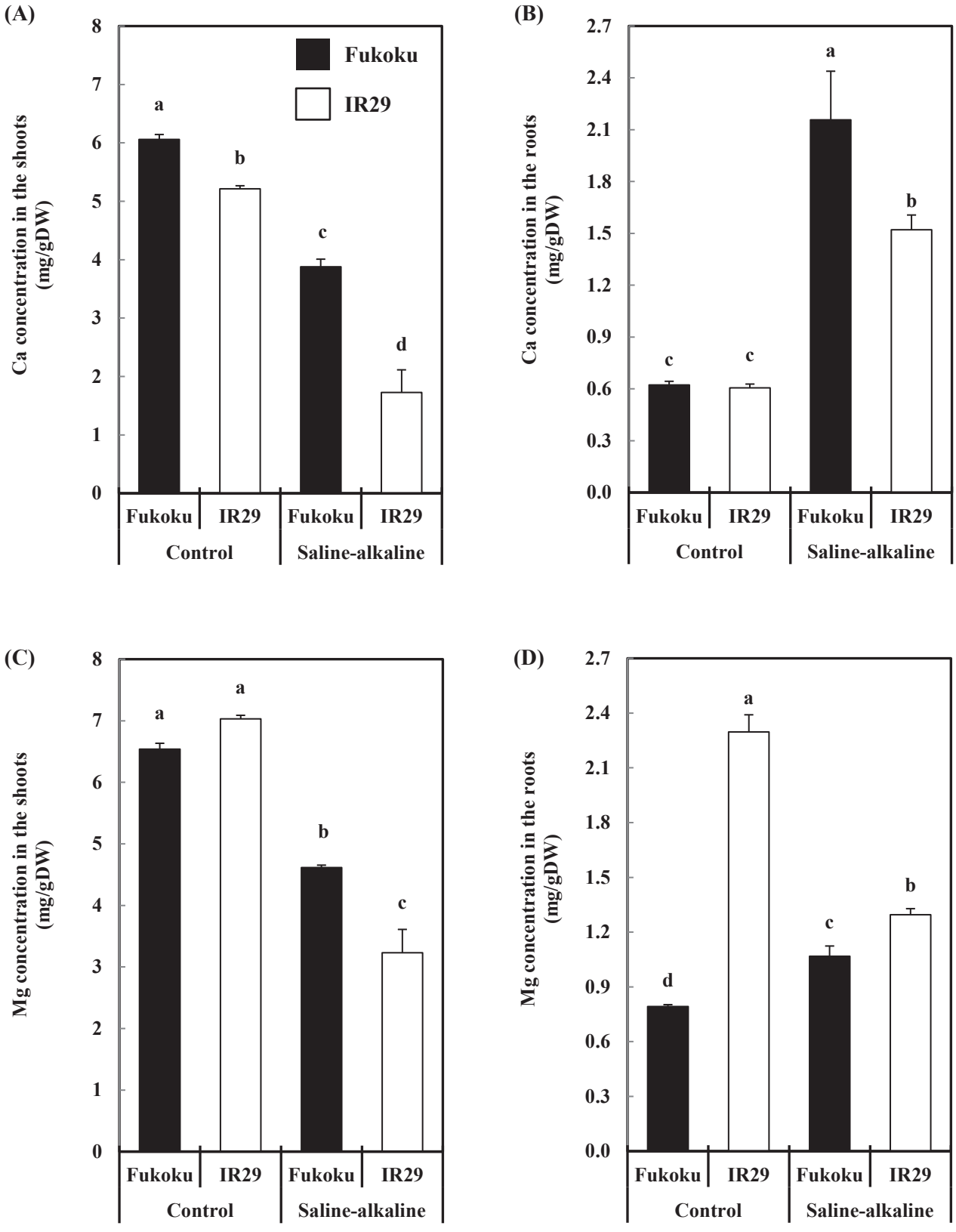
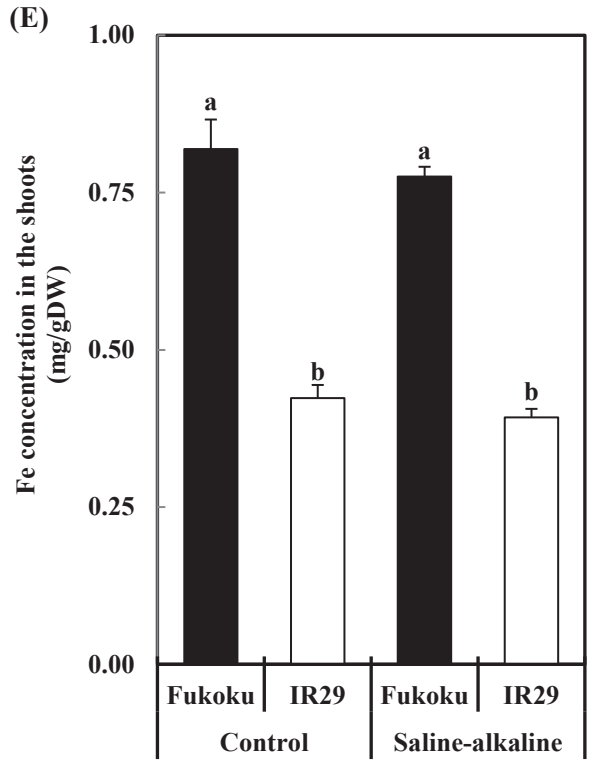


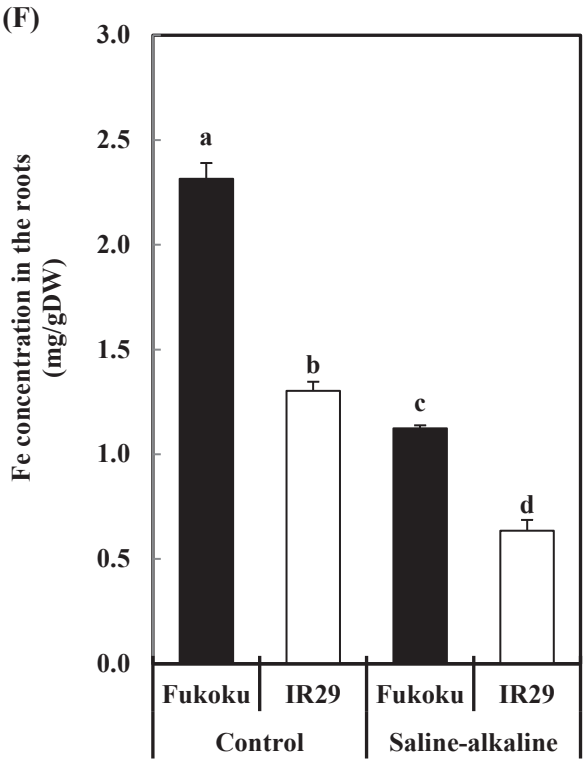
Figure 3.10 K concentration in (A) leaf blades, (B) leaf sheaths, (C) shoots, and (D) roots under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

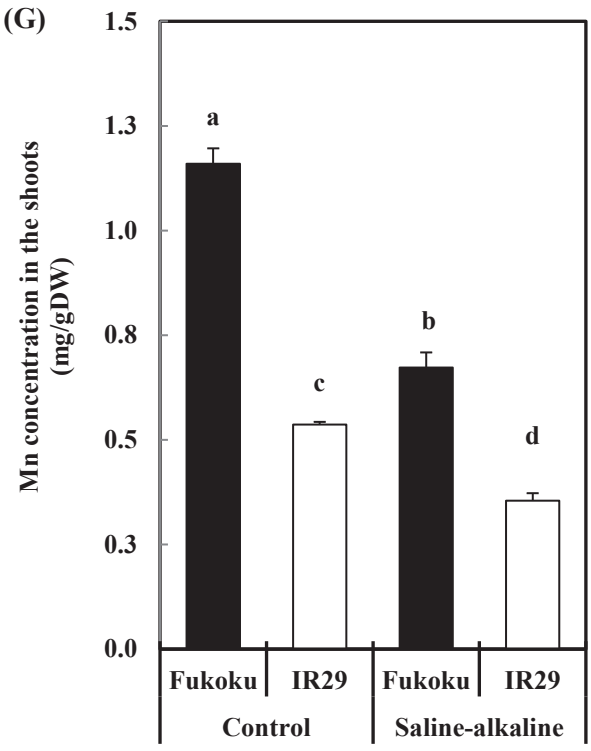
Table 3.3 Na^+/K^+ ratio in the leaf blades, leaf sheaths, and roots under saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl). Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

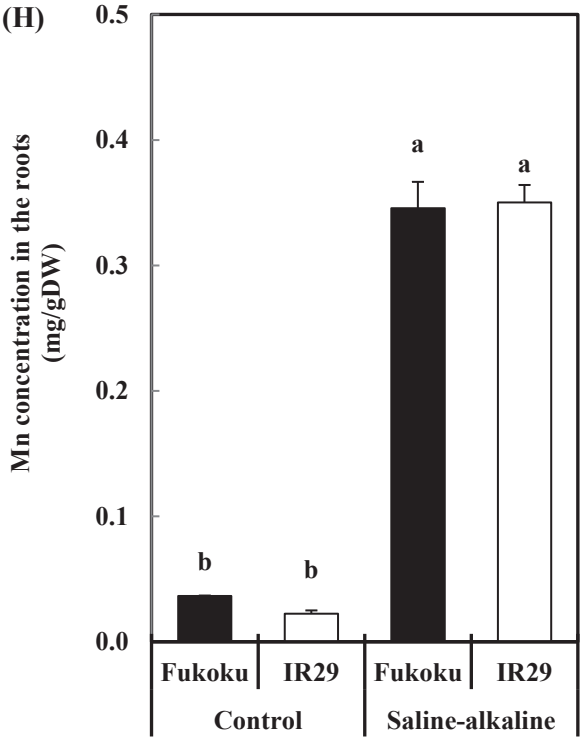
Treatments	Genotypes	Leaf blades	Leaf sheaths	Roots
Control (pH 5.0-5.5)	Fukoku	0.02±0.00 [a]	0.02±0.00 [b]	0.18±0.00 [c]
	IR29	0.01±0.00 [a]	0.02±0.00 [b]	0.23±0.01 [c]
Saline-alkaline stress (pH 8.5 + 50 mM Na)	Fukoku	0.24±0.01 [a]	1.58±0.12 [a]	8.35±0.70 [a]
	IR29	0.67±0.45 [a]	1.36±0.40 [a]	5.90±0.27 [b]

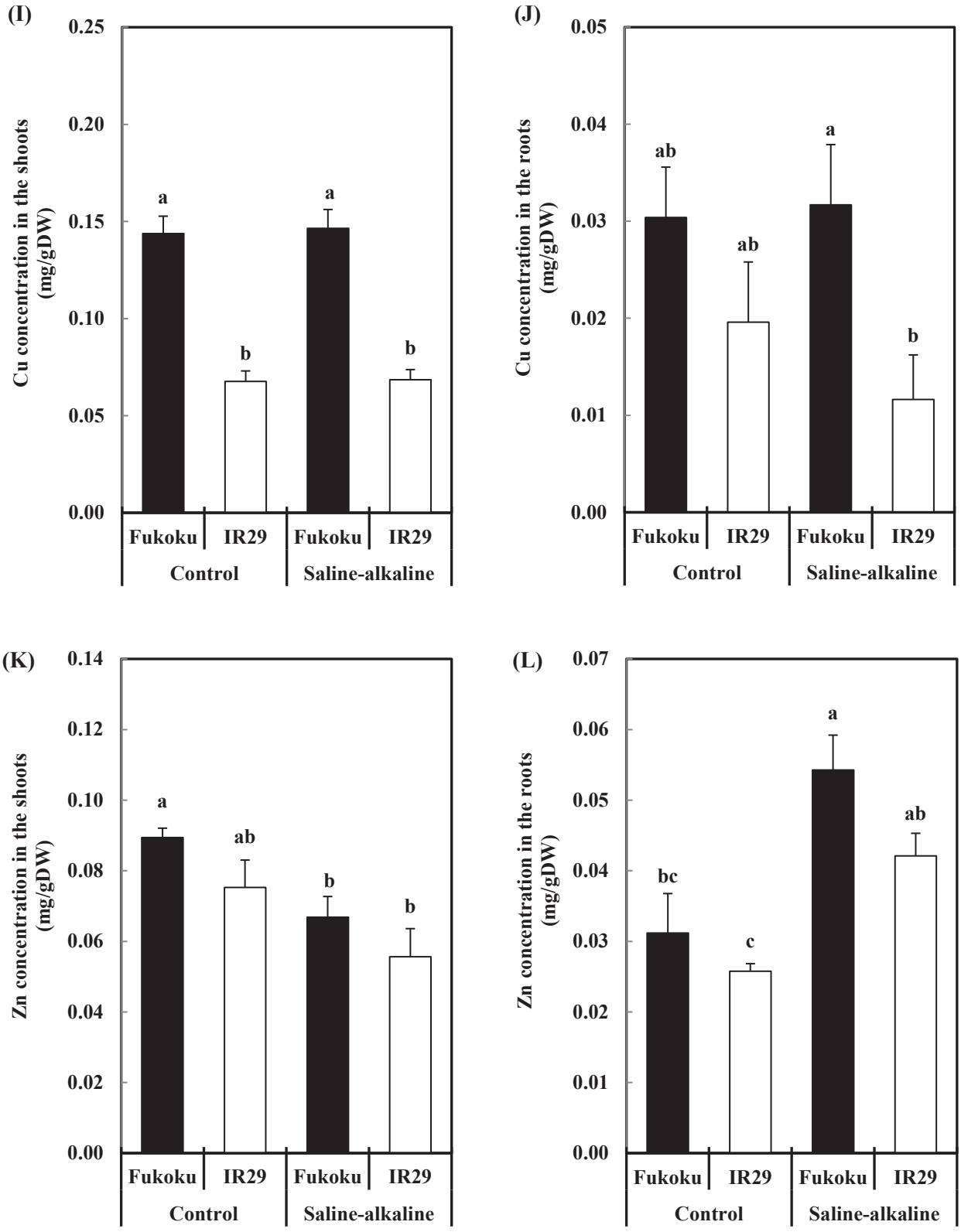

(A)
Ca concentration in the shoots (mg/gDW)
Fukoku
IR29
a
b
c
d
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(B)
Ca concentration in the roots (mg/gDW)
c
c
a
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(C)
Mg concentration in the shoots (mg/gDW)
a
a
b
c
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(D)
Mg concentration in the roots (mg/gDW)
d
a
c
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline


(E)
Fe concentration in the shoots (mg/gDW)
1.00
0.75
0.50
0.25
0.00
a
b
a
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline


(F)
Fe concentration in the roots (mg/gDW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
d
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline


(G)
Mn concentration in the shoots (mg/gDW)
1.5
1.3
1.0
0.8
0.5
0.3
0.0
a
c
b
d
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline


(H)
Mn concentration in the roots (mg/gDW)
0.5
0.4
0.3
0.2
0.1
0.0
b
b
a
a
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline


(I)
Cu concentration in the shoots (mg/gDW)
0.25
0.20
0.15
0.10
0.05
0.00
a
b
a
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(J)
Cu concentration in the roots (mg/gDW)
0.05
0.04
0.03
0.02
0.01
0.00
ab
ab
a
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(K)
Zn concentration in the shoots (mg/gDW)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
a
ab
b
b
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline
(L)
Zn concentration in the roots (mg/gDW)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
bc
c
a
ab
Fukoku
IR29
Fukoku
IR29
Control
Saline-alkaline

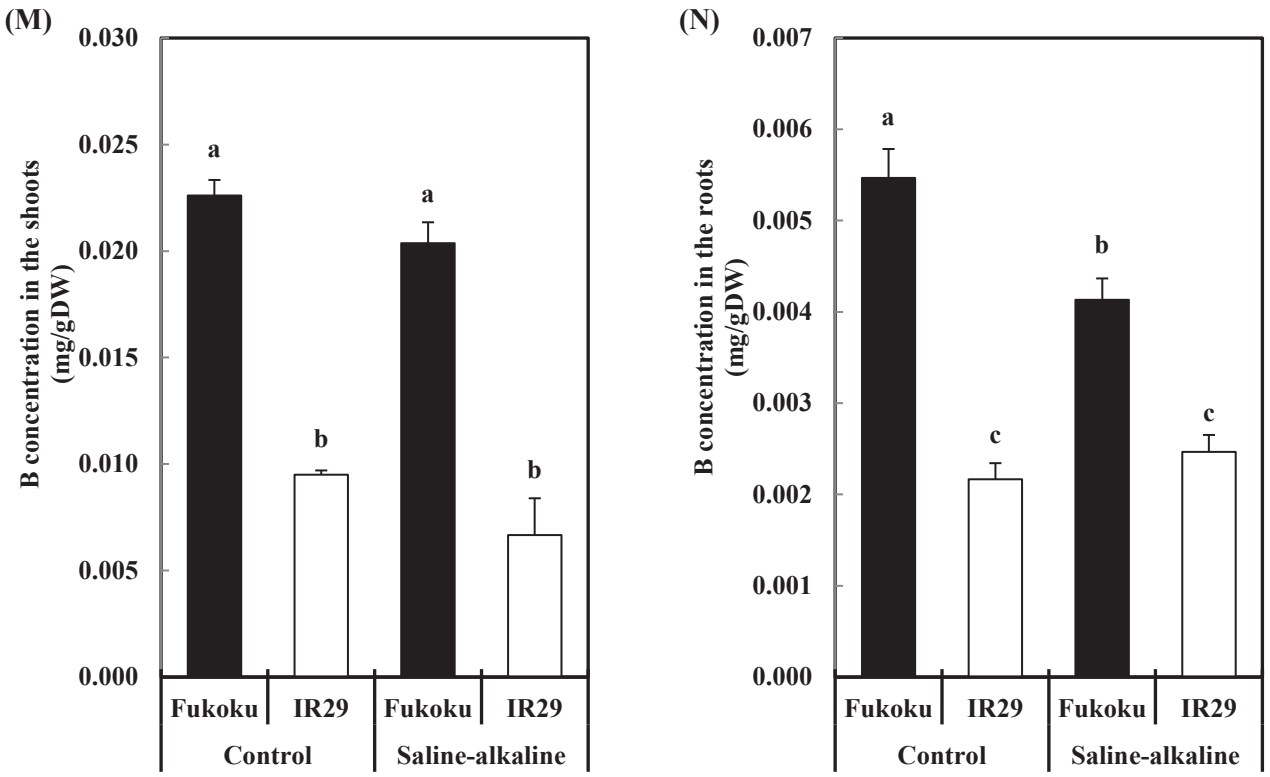


Figure 3.11 Ca, Mg, Fe, Mn, Cu, Zn and B concentrations in (A, C, E, G, I, K, and M) shoots and (B, D, F, H, J, L, and N) roots under control and saline-alkaline stress at pH 8.5 + 50 mM Na (20 mM $NaHCO_3$ + 30 mM NaCl) conditions for 4 weeks. Data represent the means of three replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

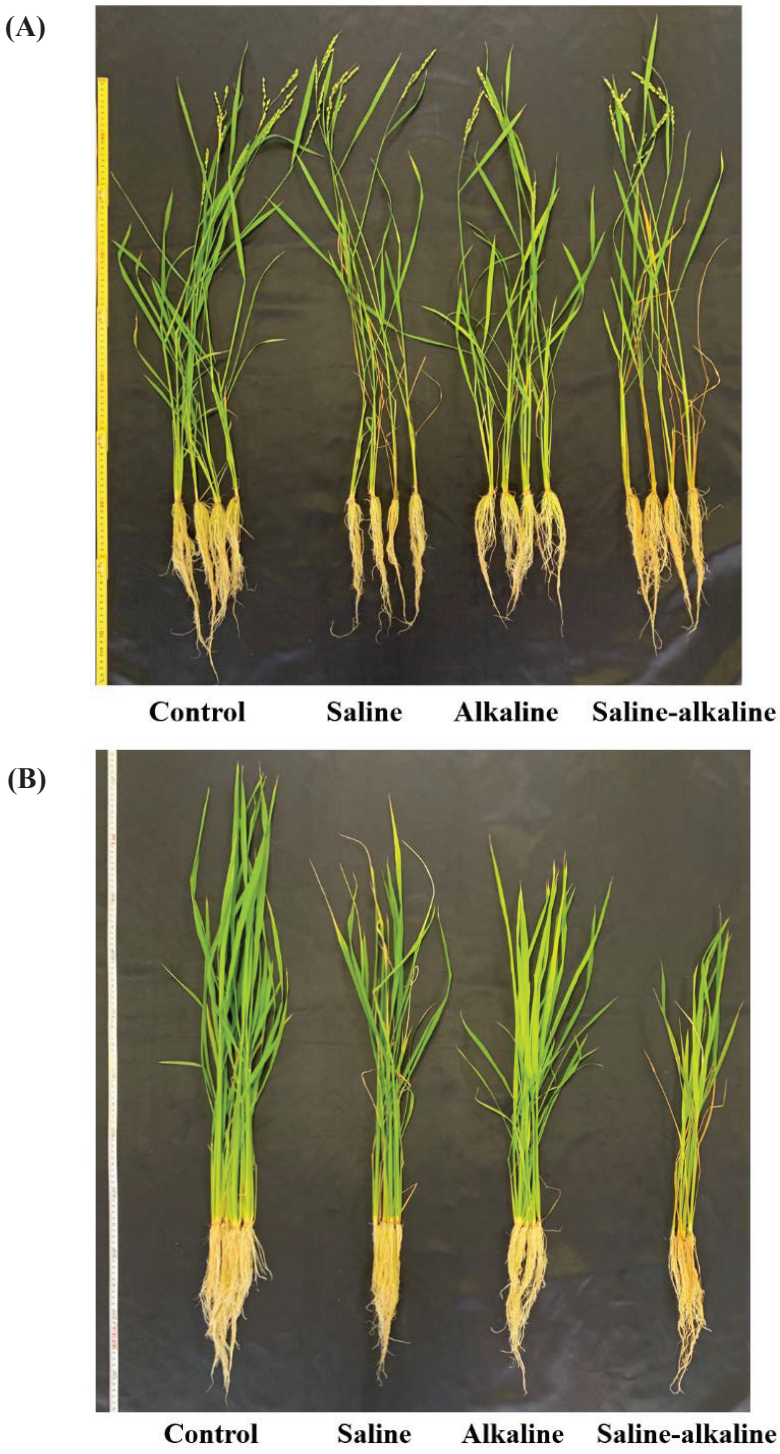


Figure 3.12 Effects of saline, alkaline, and saline-alkaline stresses on growth performance of (A) Fukoku (the most saline-alkaline tolerant) and (B) IR29 (saline-alkaline sensitive). Five-week-old rice seedlings grown in normal solution were transferred to solution supplemented with saline salts (50 mM Na), alkaline salts (5 mM K + pH 8.5), and saline-alkaline salts (50 mM Na + pH 8.5) for 4 weeks. days.

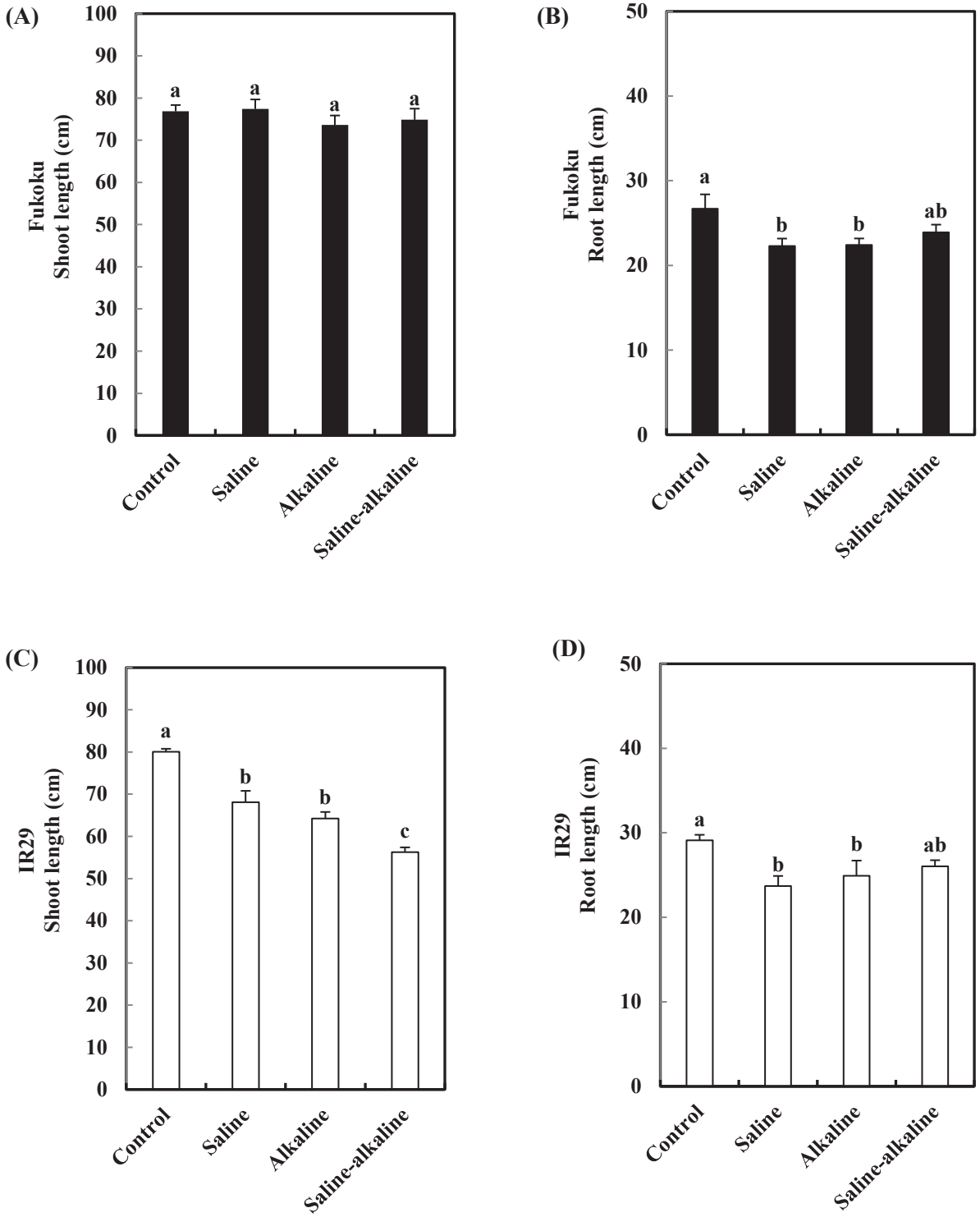


Figure 3.13 Effects of saline, alkaline, and saline-alkaline stresses on (A and C) Shoot and (B and D) Root lengths of Fukoku (the most saline-alkaline tolerant) and IR29 (the saline-alkaline sensitive). Lengths of shoot and root were measured under control and after four weeks of saline, alkaline, and saline-alkaline stress conditions. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

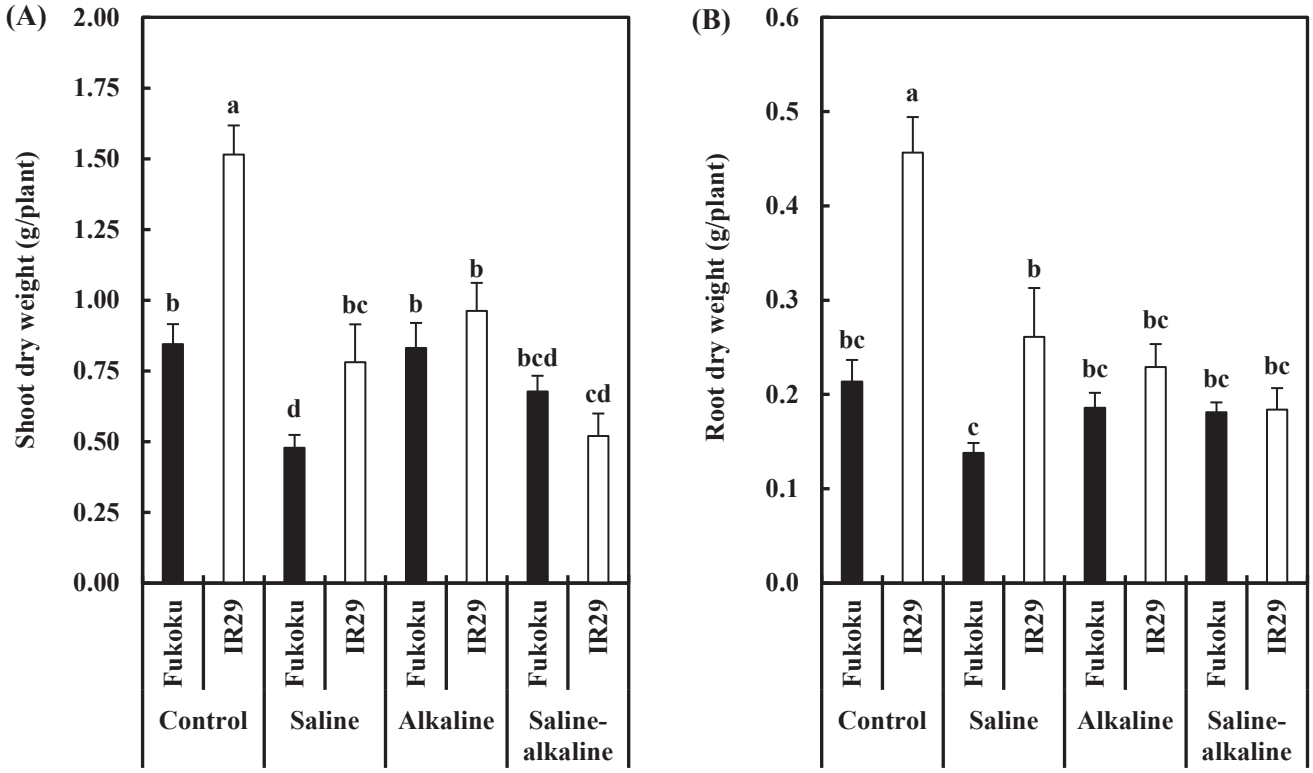


Figure 3.14 Effects of saline, alkaline, and saline-alkaline stresses on (A) Shoot and (B) Root dry weights of Fukoku (the most saline-alkaline tolerant) and IR29 (the saline-alkaline sensitive). Dry weights of shoot and root were measured under control and after four weeks of saline, alkaline, and saline-alkaline stress conditions. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

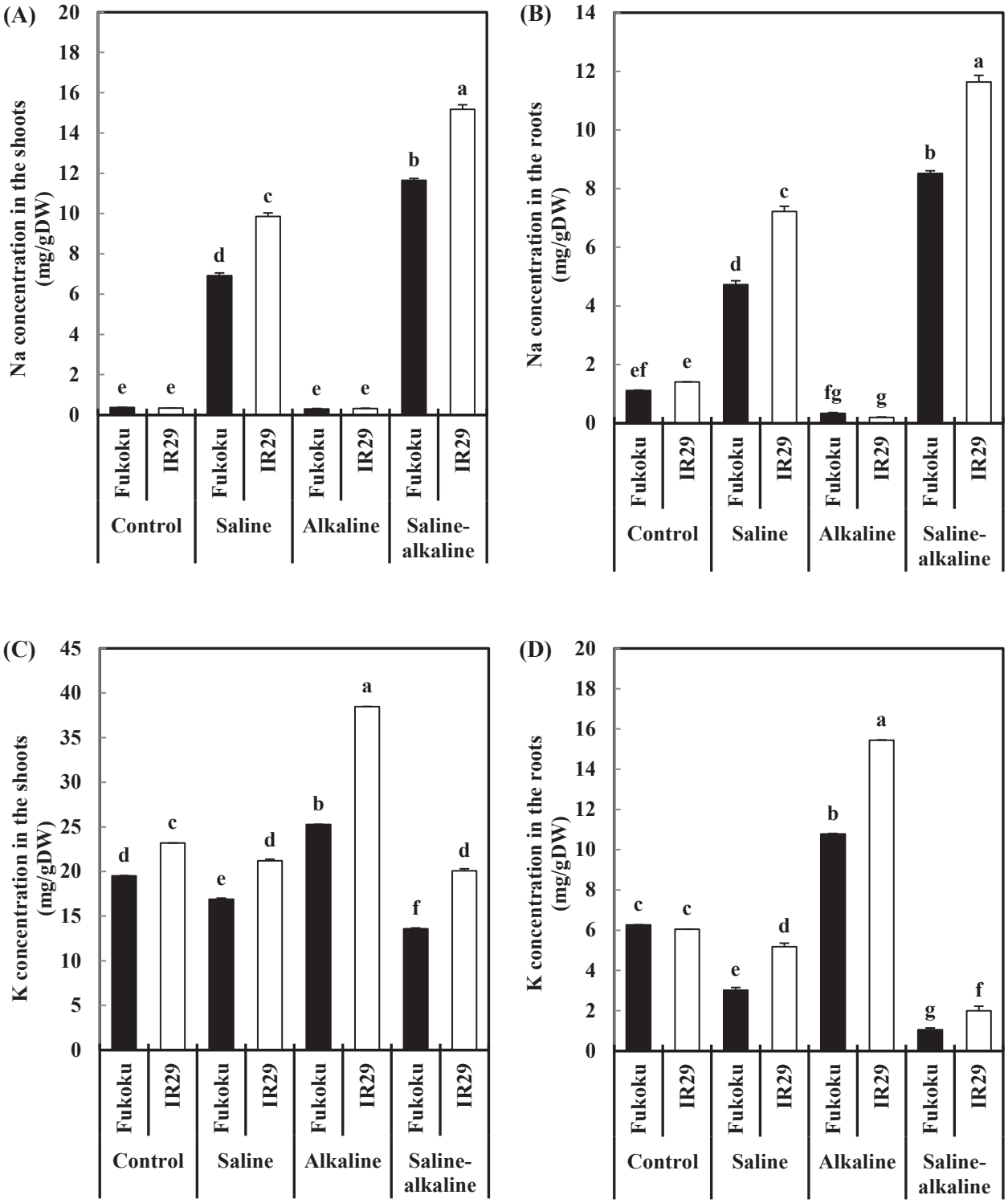


Figure 3.15 Na and K concentrations in (A and C) shoots and (B and D) roots under control, saline (50 mM Na), alkaline (pH 8.5 + 5 mM K) and saline-alkaline stress (pH 8.5 + 50 mM Na) conditions for 4 weeks. Data represent the means of four replicates ± SE. The same letters indicate no significant difference $p > 0.05$.

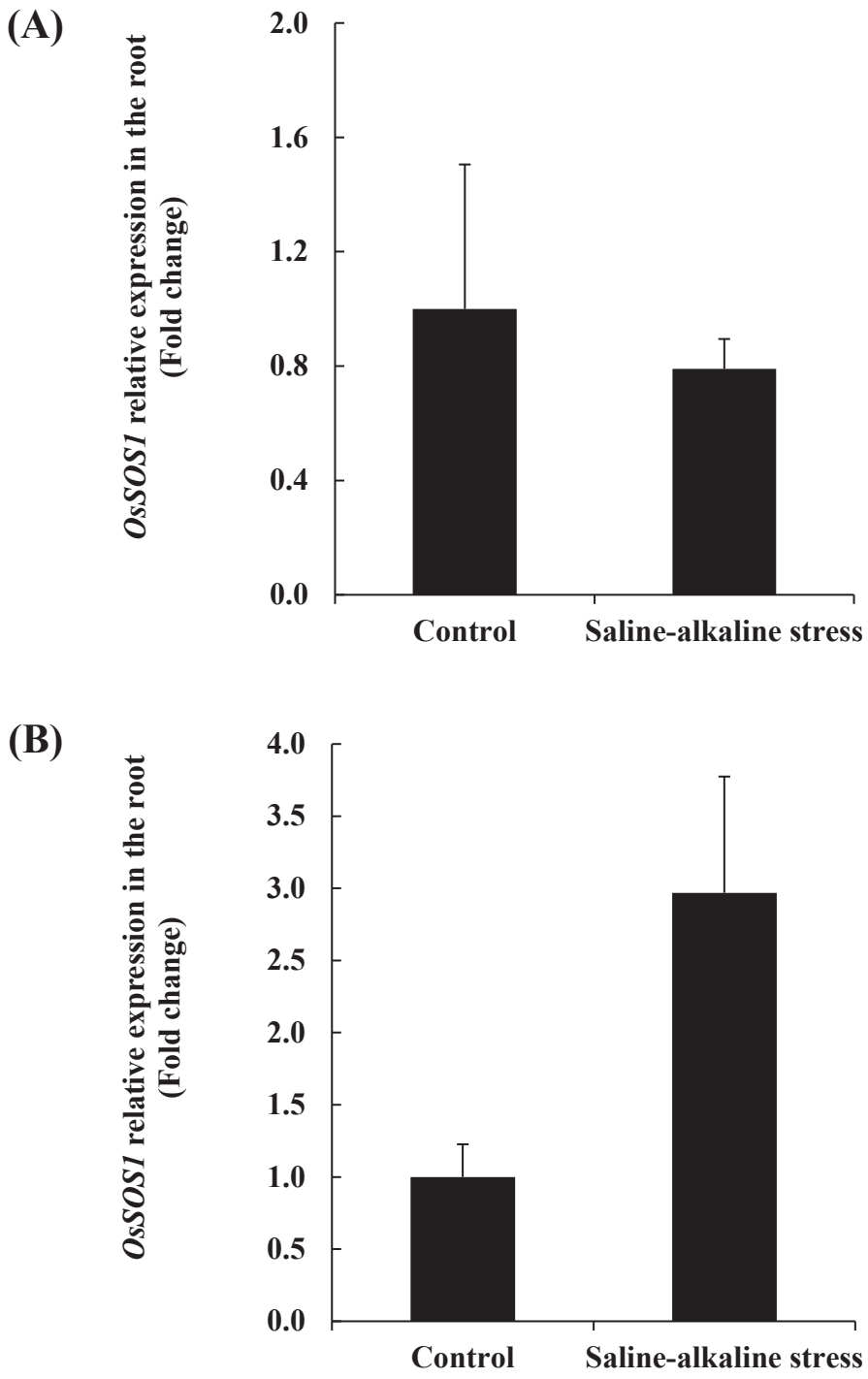


Figure 3.16 Effects of saline-alkaline stress at pH 8.5 + 50 mM Na on the expression of genes encoding the Na^+ transport proteins. The transcript level of *OsSOS1* was determined by quantitative RT-PCR in the roots of (A) IR29 and (B) Fukoku rice genotypes. The values are the mean (± SD) of duplicate samples.

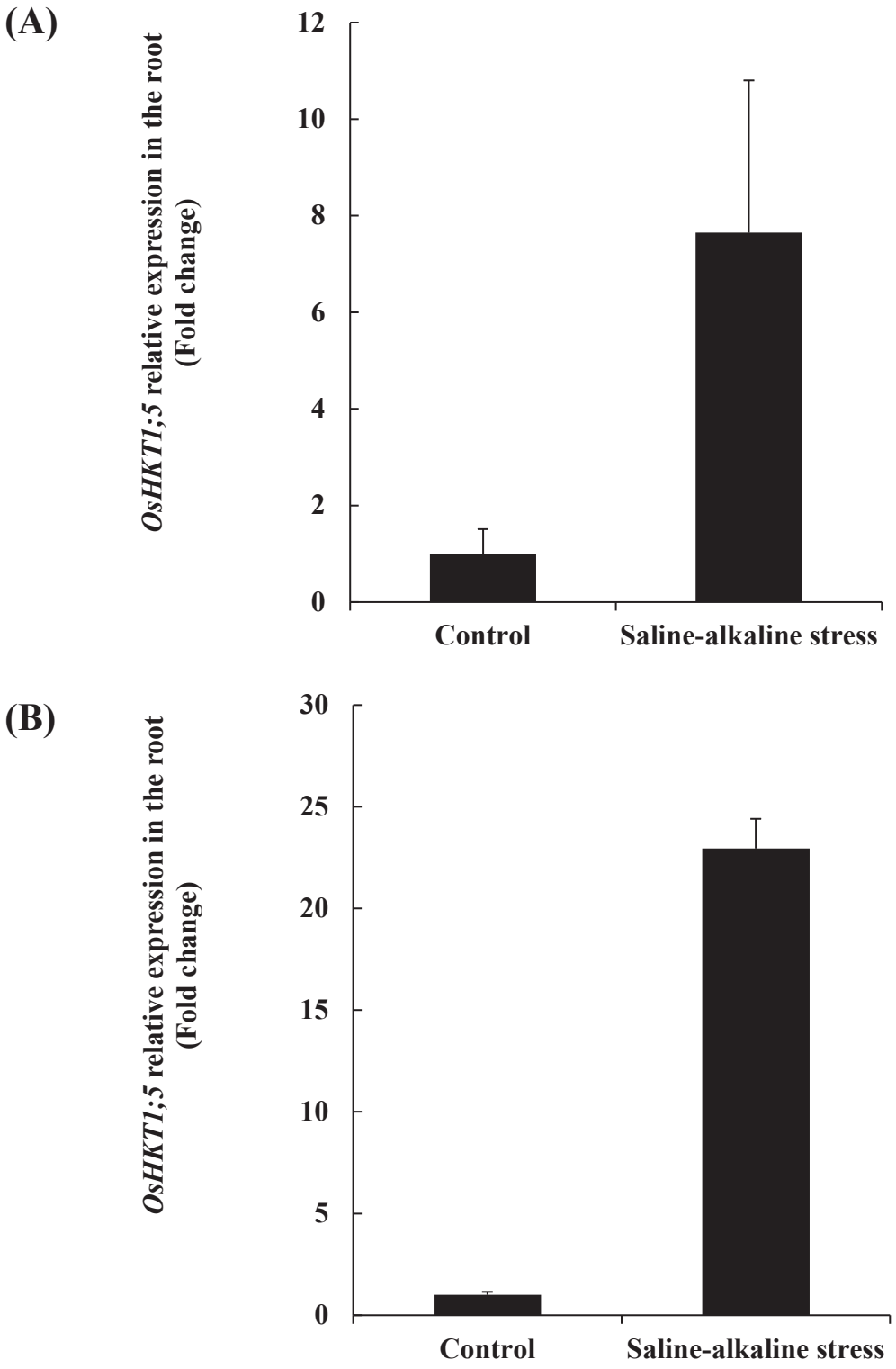


Figure 3.17 Effects of saline-alkaline stress at pH 8.5 + 50 mM Na on the expression of genes encoding the Na^+ transport proteins. The transcript level of *OsHKT1;5* was determined by quantitative RT-PCR in the roots of (A) IR29 and (B) Fukoku rice genotypes. The values are the mean (± SD) of duplicate samples.

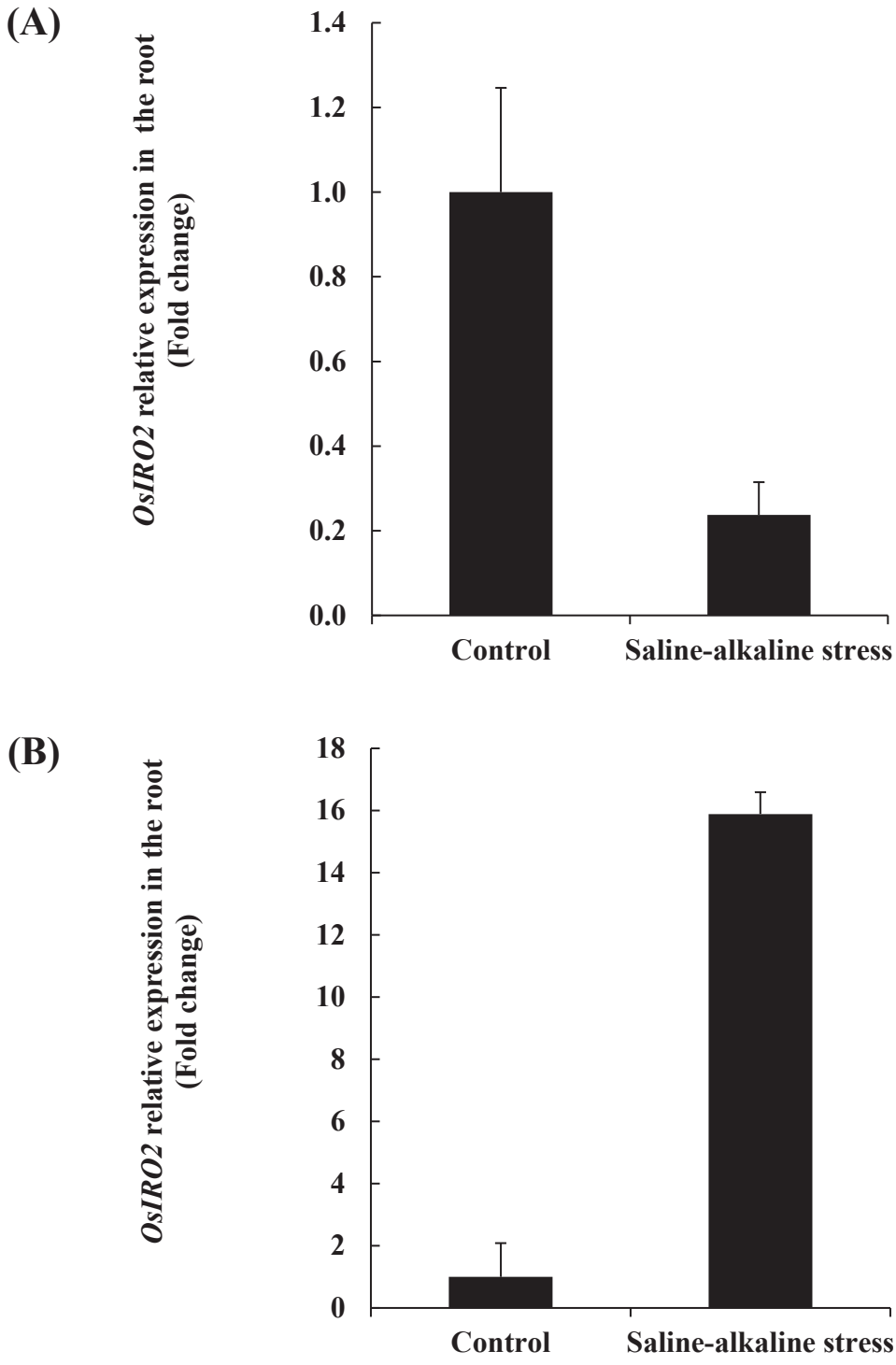


Figure 3.18 Effects of saline-alkaline stress at pH 8.5 + 50 mM Na on the expression of genes encoding Fe acquisition proteins. The transcript level of *OsIRO2* was determined by quantitative RT-PCR in the roots of (A) IR29 and (B) Fukoku rice genotypes. The values are the mean (± SD) of duplicate samples.

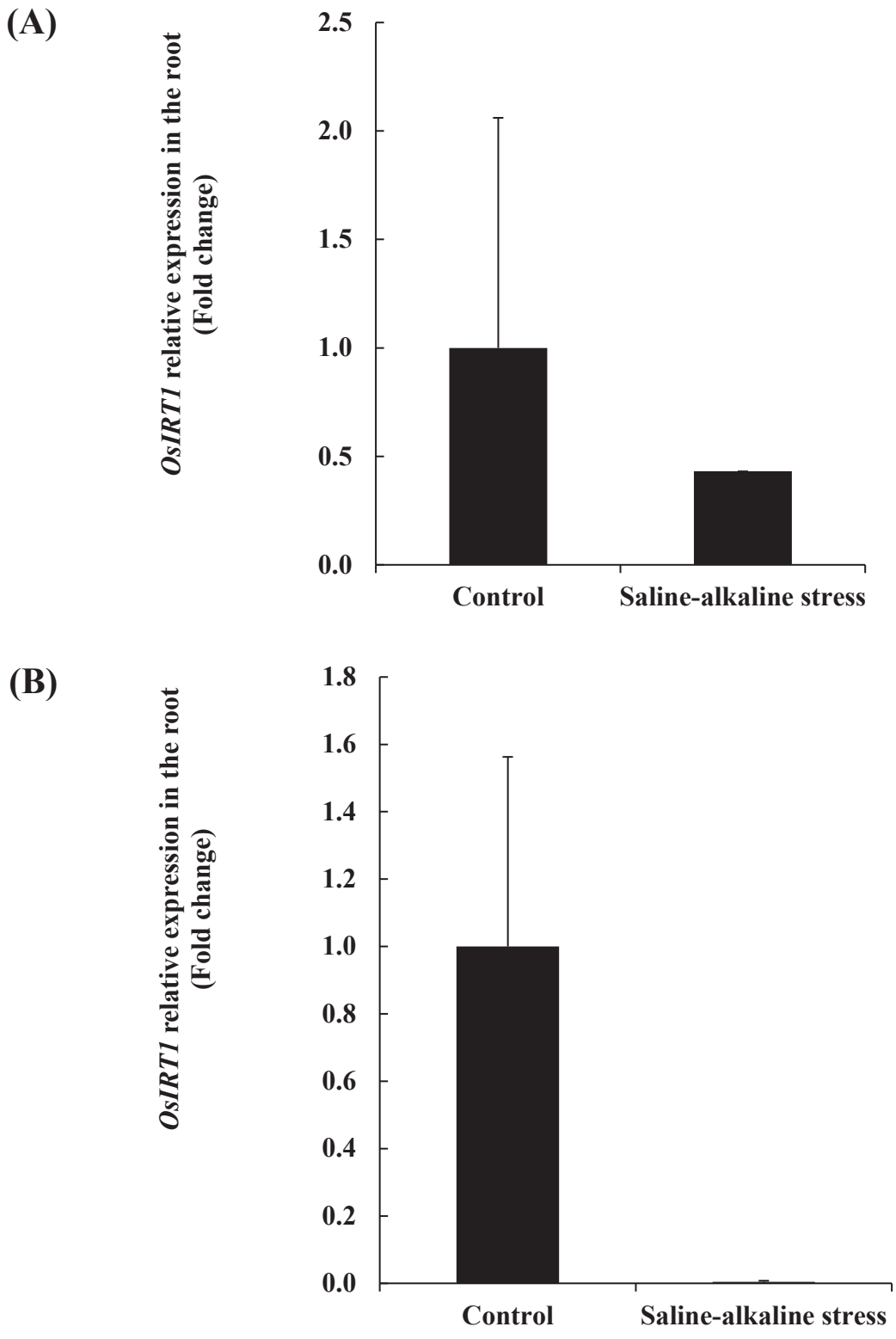


Figure 3.19 Effects of saline-alkaline stress at pH 8.5 + 50 mM Na on the expression of genes encoding Fe acquisition proteins. The transcript level of *OsIRT1* was determined by quantitative RT-PCR in the roots of (A) IR29 and (B) Fukoku rice genotypes. The values are the mean (± SD) of duplicate samples.

Table 3.4 Primers used for quantitative real-time RT-PCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
OsSOS1	ATACTGAGTGGGGTTGTTATTGC	AAAGGTAAATTTCAAAAGGTACATGG
OsHKT1;5	TGCATTCATCACTGAGAGGAG	GGTGCAGTTTCTGCAACCTC
OsIRO2	CTCCCATCGTTTCGGCTACCT	GCTGGGCACTCCTCGTTGATC
OsIRT1	CGTCTTCTTCTTCTCCACCACGAC	GCAGCTGATGATCGAGTCTGACC
OsYSL15	ACTGGTACCCTGCAAACATAC	GCAATGATGCTTAGCAAGAAG
OsAct11	CAGCCACACTGTCCCCATCTA	AGCAAGGTCGAGACGAAGGA

3.4 Discussion

Soil salinization/alkalinization is considered as one of the most vital soil problems for rice cultivation (Boivin et al., 2002). In past decade, only few studies have paid attention to the effects of saline-alkaline stress on rice and tolerant rice genotypes to saline-alkaline stress conditions are not well documented. In previous study, 93 rice genotypes (including both *indica* and *japonica* subspecies) were examined under saline-alkaline stress conditions and only 17 rice genotypes were selected for the present study. The results obtained from all experimental screenings have been confirmed that Fukoku is relatively saline-alkaline tolerant compared to other rice genotypes. To explore the physiological mechanisms underlying the greater saline-alkaline tolerance in Fukoku, several growth parameters between the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 were analyzed. This study demonstrated that Fukoku, the japonica rice that was bred from the cross between the Japanese warm region variety "Nakate-Aikoku", and the Hokkaido variety "Bozu 6" (Saito et al., 2019), was more tolerant to saline-alkaline stress at pH 8.5 + 50 mM Na than the indica rice, IR29. Fukoku plants displayed better growth performance, as it maintained greater shoot elongation **(Figure 3.3A)** and dry weight **(Figure 3.4A and B)**, and higher concentrations of total chlorophyll **(Figure 3.6)**, proline **(Figure 3.7)**, and microelements **(Figure 3.11)**, while it had a lower Na^+ concentration in both shoot and root tissues **(Figure 3.9C and D)** and a lower concentration of malondialdehyde **(Figure 3.8)**, and a lower Na^+/K^+ ratio in the leaf blades **(Table 3.3)**, in comparison to IR29 plants.

Several studies have been reported that, under high saline stress environments, maintaining a low cytosolic Na^+/K^+ ratio in the photosynthetic tissues is an essential mechanism for saline tolerance in plants (Zhu, 2003; Abdelaziz et al., 2018; Zhang et al., 2020), while a high cytosolic Na^+/K^+ ratio may have deleterious effects on plants such as cell membrane injury (Blumwald, 2000), nutritional imbalance (Peng et al., 2008) and disruption of enzyme activities (Hasegawa et al., 2000). Thus, Fukoku rice, which had a low Na^+/K^+ ratio in the leaf blades and a low concentration of Na^+ in the shoots **(Table 3.3 and Figure 3.9)**, is more tolerant and better adapt to saline-alkaline stress conditions than IR29 rice. In addition, Na^+ exclusion mechanism at the leaf sheaths via the high affinity K^+ transporter 1;4 (OsHKT1;4) has been found to play an important role in restricting Na^+ accumulation in the leaf blades

(Suzuki et al., 2016). This mechanism likely works in the leaf sheaths of Fukoku plants, but not in IR29 plants. As shown **in Figure 3.9A and B**, Fukoku accumulated much less amount of Na^+ in the leaf blades than IR29, although Na^+ concentration in the leaf sheaths was much higher in Fukoku than in IR29. This finding suggests that Fukoku may have an effective Na^+ exclusion mechanism at the leaf sheaths to prevent Na^+ accumulation in the leaf blades.

Maintaining high K^+ concentration in shoots is also crucial for plants tolerance to saline stress (Anschütz et al., 2014). In the present study, the saline-alkaline sensitive IR29 exhibited a significant higher concentration of K^+ in shoots than the saline-alkaline tolerant Fukoku when grown under control and saline-alkaline stress conditions **(Figure 3.10C)**, suggesting that IR29 plants may require a larger amount of K^+ concentration to maintain their shoot growth under both normal and saline-alkaline stress conditions, while the maintenance of higher K^+ concentrations in the shoots seemed to be less important for the growth of Fukoku plants under either control or saline-alkaline treatments. In addition, this result is in agreement with the previous findings by Li et al. (2016), who suggested that tolerance of Dongdao-4 plants (the saline-alkaline tolerant rice genotypes) to saline-alkaline stress at pH 8.5 relies on other mechanisms rather than potassium homeostasis.

Both Ca^{2+} and Mg^{2+} are classified as secondary macronutrients for plants. Ca^{2+} plays a fundamental role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi, 2004), while Mg^{2+} plays a significant role as the central atom of chlorophylls (Shaul, 2002). The previous studies reported that Ca^{2+} and Mg^{2+} accumulation in plants is usually inhibited by high salinity stress (Khan 2011; Munns and Tester, 2008). Similar results were observed in this study showing adding saline-alkaline salts (30 mM NaCl + 20 mM $NaHCO_3$) to nutrient solution caused a significant reduction in both Ca^{2+} and Mg^{2+} concentrations in the shoots of two rice genotypes **(Figure 311A and C)**. However, Wang et al. (2017) found that mixed alkaline salts ($NaHCO_3$:Na_2CO_3 = 9:1) treatment can increase the accumulation of Ca^{2+} and Mg^{2+} in the roots of salt-tolerant (Zhongmu1) and salt-sensitive (Algonqin) alfalfa plants. In the present study, both Ca^{2+} and Mg^{2+} concentrations were highly increased in the roots of Fukoku plants by saline-alkaline treatment, while in the roots of IR29 plants **(Figure 3.11B and D)**, saline-alkaline stress caused an increase in Ca^{2+} concentration, but decrease in Mg^{2+}

concentration. These observations suggest that, (1) the reduction in both Ca^{2+} and Mg^{2+} concentration in the shoots is considered as a common phenomenon for plants grown under high salinity stress condition, and (2) high pH stress that creates by alkaline salt may induce a positive effect in enhancing Ca^{2+} and Mg^{2+} accumulation in the roots of glycophyte plants (alfalfa and rice), however the mechanism behind Ca^{2+} and Mg^{2+} induction should be further investigated.

Soil pH is considered as a key factor that controls the availability of plant nutrients. At high pH, several soil micronutrients (B, Fe, Cu, Mn, and Zn) become less available to plants, resulting in an abnormality of plant growth and development. B plays primarily role in cell wall biosynthesis and structure and plasma membrane integrity (Shelp, 1993). Both Fe and Cu are involved in the formation of chlorophyll and they are also required for certain enzyme functions (Vose, 1982; Yruela, 2005). Mn is necessary in photosynthesis, nitrogen metabolism, and to form other compounds required for plant metabolism (Marschner, 1995). And, Zn is a key constituent of enzymes and required for chlorophyll biosynthesis (Hafeez et al., 2013). This study found that under both control and saline-alkaline stress conditions, Fukoku plants can maintain a higher concentration of all microelements (B, Fe, Cu, Mn, and Zn) in shoots and roots than IR29 plants **(Figure 3.11E-N)**, indicating that maintaining a high concentration of microelements is important strategy for Fukoku plants tolerant to saline-alkaline stress.

Proline is one of the most common compatible osmolytes that accumulates in a variety of plant species in response to environmental stresses (Majumder et al., 2009). Li et al. (2017) demonstrated that under salinity stress at 60 mM NaCl, the concentration of proline in the shoots was markedly increased in the saline-alkaline tolerance rice genotypes (Dongdao-4) than in the sensitive one (Jigeng-88). Similar results were observed in this study, that is, after 4 weeks of saline-alkaline treatment at pH 8.5 + 50 mM Na, the accumulation of proline in the leaves of the saline-alkaline tolerant Fukoku was higher than in the saline-alkaline sensitive IR29 **(Figure 3.7)**, this finding suggests that the saline-alkaline tolerant Fukoku had greater capacity to accumulate proline in the leaves than the saline-alkaline sensitive IR29.

In addition to the protective function as an osmolyte, proline can also act as a ROS scavenger in plants (Szabados and Savoure, 2010). The ability of proline to scavenge free radicals has been reported by Okuma et al. (2004), under salinity stress at 200 mM NaCl, an increase in endogenous proline can alleviate salt-induced damage in tobacco cells by reducing MDA accumulation. Under abiotic stress conditions, several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism (Apel and Hirt, 2004). High levels of ROS can enhance lipid peroxidation, leading to impaired membrane structure and cell death in plants (Turkan et al., 2013). MDA is known as one of the final products of lipid peroxidation, and the presence of MDA in plants is often used as a marker of oxidative damages (Yin et al., 2010). This study found that, under saline-alkaline stress at pH 8.5 + 50 mM Na, Fukoku plants accumulated lesser amounts of MDA than IR29 plants **(Figure 3.8)**, indicating that Fukoku plants may equip with greater tolerance to the oxidative damages than IR29 plants. Moreover, high proline concentration in the leaves may contribute to greater tolerance of Fukoku plants to saline-alkaline stress by promoting the role of ROS scavenger **(Figure 3.7)**, resulting in a low MDA concentration in its leaf blades **(Figure 3.8)**.

All above results indicated that two rice genotypes differing in their tolerance to saline-alkaline stress. In order to gain better understanding of saline-alkaline tolerance in rice, the growth parameters of Fukoku and IR29 rice genotypes were further examined under either saline (50 mM NaCl), alkaline (pH 8.5 + 5 mM K), or saline-alkaline (pH 8.5 + 50 mM Na) stress conditions. As shown in **Figure 3.14A and B**, shoot and root dry weights of IR29 plants were appreciably reduced when grown in the treatments either saline, alkaline, or saline- alkaline stress conditions, and the reductions of those parameters were more pronounced in saline-alkaline treatment (pH 8.5 + 50 mM Na) than saline (50 mM NaCl) and alkaline (pH 8.5 + 5 mM K) treatments, respectively. These results are in agreement with previous report in the glycophyte *Leuresthes tenuis* (Paz et al., 2012), the total dry mass production of legume was dramatically decreased by the three types of salt stress treatments (alkaline; pH 8.0, saline; pH 5.8 + 100 mM Na, and saline-alkaline; pH 8.0 + 100 mM Na), and this effect was more severe in the mixed saline-alkaline treatment, followed by saline and alkaline treatments, respectively. Those observations suggest that the toxic effect of saline-alkaline stress on glycophyte plants (rice and

legume) was generally more severe than that of a single stress factor either saline stress or alkaline stress due to the combined damage of high pH and Na^+ toxicity. In contrast with the growth of IR29 plants, the growth of Fukoku plants was better adapted to saline-alkaline stress because this rice genotype was tolerant to high pH stress condition (alkaline stress at pH 8.5), but sensitive to saline stress (50 mM Na). This finding suggests that the greater tolerance of Fukoku plants to saline-alkaline stress may be caused by high pH tolerance rather than salt stress tolerance **(Figure 3.14A and B)**.

The patterns of Na^+ and K^+ accumulation in shoots and roots of both rice genotypes were similar; an increase in Na^+ concentration and decrease in K^+ concentration was detected in plants grown under saline-alkaline stress rather than under saline stress **(Figure 3.15A and B)**. Notably, the magnitude of Na^+ induction in both shoots and roots was higher in the saline-alkaline sensitive IR29 plants than in the saline-alkaline tolerant Fukoku plants. These findings highlighted that (1) plants that were grown under mixed saline-alkaline stress may suffer from Na^+ toxicity than those of plants grown under saline stress and (2) Fukoku plants may have an effective Na^+ exclusion mechanism rather than IR29 plants. Although, the Na^+ accumulation in shoots and roots of Fukoku plants was higher under saline-alkaline treatment (pH 8.5 + 50 mM Na) than under saline treatment (50 mM NaCl), both shoots and roots dry weights of Fukoku plants were more retarded under saline treatment (50 mM NaCl); this finding suggested that (1) the trend of growth reduction in Fukoku plants might be associated with an increase in Cl^- concentration of nutrient solutions, and (2) the growth reduction of Fukoku plants under saline stress (50 mM NaCl) might be caused by Cl^- toxicity rather than high Na^+ accumulation. However, the examination of how anions (Cl^-, SO_4^{2-}, CO_3^{2-}, HCO_3^-) affects the growth of rice plants should be further investigated.

To understand the mechanisms underlying limited Na^+ transport to the leaf blades in Fukoku rice genotype under saline-alkaline stress at pH 8.5 + 50 mM Na, expression profiles of genes encoding Na^+ transport proteins (*OsSOS1* and *OsHKT1;5*) were investigated by quantitative RT-PCR analysis **(Figure 13.16-13.17)**. The Na^+/H^+ antiporter (SOS1) is involved in maintaining ion homeostasis by transporting the toxic Na^+ out of the cell under high salinity conditions (Qiu et al., 2002; Shi et al., 2002). In the present study, in response to saline-alkaline stress (pH 8.5 + 50 mM Na), the expression

of *OsSOS1* gene was considerably increased in the roots of Fukoku (2.97-fold) **(Figure 13.16B)**, but it was repressed in the roots of IR29 (0.79-fold) **(Figure 13.16A)**. This implies that after exposure to saline-alkaline stress for 4 weeks, the saline-alkaline tolerant Fukoku could restrict Na^+ accumulation in the roots via the functions of OsSOS1, and its expression would also help limit the overaccumulation of Na^+ in the shoots **(Figure 13.15A)**.

The Na^+ transporter, OsHKT1;5 functions by transporting Na^+ out of xylem vessel into xylem parenchyma (Na^+ efflux) minimizing the harmful effects to the plant due to Na^+ accumulation (Sharif Shohan et al., 2019). Under saline-alkaline stress at pH 8.5 + 50 mM Na, the expression of *OsHKT1;5* gene was up-regulated in the roots of both rice genotypes compared with the control plants **(Figure 13.16A and B)**, however the magnitude of the up-regulation was greater in Fukoku than in IR29. This finding suggests that the saline-alkaline tolerant genotype Fukoku may have better ability to restrict Na^+ accumulation by OsHKT1;5 in the shoots than the saline-alkaline sensitive rice genotype IR29.

In saline-alkaline soils, Fe occurs mainly in the form of insoluble hydroxide and oxides, limiting its bioavailability for plants (Li et al., 2016). Thus, in order to grow under saline-alkaline conditions, plants should have a greater capacity to acquire Fe. Rice is generally considered as a strategy II plant which has chelation-based strategy for acquisition of Fe^{3+}. However, several studies have been reported that rice could also directly take up Fe^{2+} via specific transporters (Ishimaru et al., 2006; Kim and Guerinot, 2007).

To take up Fe^{3+} from saline-alkaline soils, rice plants secrete mugineic acids (MAs) family phytosiderophores from their roots to solubilize rhizospheric Fe^{3+} (Takagi, 1976), then Fe^{3+}-MAs complexes are transported through the Fe^{3+}-MAs transporters (OsYSL2 and OsYSL15) (Curie et al., 2001). In the present study revealed that under both control and saline-alkaline stress conditions, the expression of *OsYSL15* gene was not detected in the roots of Fukoku and IR29 **(data not shown)**, which suggests that Fe^{3+}-MAs acquisition in both rice genotypes was not associated with the function of *OsYSL15* transporter.

OsIRT1 and OsIRT2 are known as transporters for direct uptake of Fe^{2+} from soils, which are expressed in the epidermal cells of Fe-deficient roots (Ishimura et al., 2006). In the present experiment, under saline-alkaline stress condition (pH 8.5 + 50 mM Na), *OsIRT1* expression was repressed in the roots of both rice genotypes **(Figure 3.19A and B)**, which suggests that OsIRT1 in the roots was not a major transporter for Fe^{2+} (strategy I system) uptake in both rice genotypes after exposure to saline-alkaline stress condition.

The rice transcription factor OsIRO2 has been identified as key regulators of the genes that control Fe uptake and phytosioderophore synthesis (Ogo et al., 2007; Itai et al., 2013). In the current study, saline-alkaline stress at pH 8.5 + 50 mM Na was markedly enhanced expression of *OsIRO2* in the roots of Fukoku **(Figure 3.18B)**, but it was repressed in the roots of IR29 **(Figure 3.18A)**, this finding suggests that under saline-alkaline stress condition, an increase in transcript level of *OsIRO2* gene in the roots may enhance the activity of phytosiderophore synthesis in the saline-alkaline tolerant Fukoku than the salt-sensitive IR29.

3.5 Conclusion

In conclusion, this study demonstrated that Fukoku rice genotype is more tolerant to saline-alkaline stress than other rice genotypes. When grown in saline-alkaline stress solution (pH 8.5 + 50 mM Na), Fukoku plants can maintain several growth parameters better than IR29 plants **(Figure 3.3-11)**. Maintaining a low Na^+ concentration in both shoots and roots of Fukoku plants is considered as one important strategy to decrease the toxic effects caused by high Na stress in a saline-alkaline solution **(Figure 3.9C and D)**. Beside a salt tolerance mechanism, Fukoku plants also have a greater ability to tolerate to high pH stress **(Figure 3.13A and B)**, which allow this rice genotypes to acquire micronutrients more efficiently under saline-alkaline stress condition. Molecular analysis by quantitative RT-PCR revealed that the tolerance of Fukoku rice genotype to saline-alkaline stress condition (pH 8.5 + 50 mM Na) is associated with the up-regulation of genes encoding Na^+ transport proteins (*OsSOS1* and *OsHKT1;5*) **(Figure 13.16-13.17)** and gene for Fe acquisition protein (*OsIRO2*) **(Figure 13.18)** in the roots. These findings will be useful for further examination into saline-alkaline tolerance in rice.

Chapter 4

General Discussion

This study was conducted to (1) investigate both physiological and molecular mechanisms which are responsible for saline-alkaline tolerance in two well-known rice genotypes (FL478; the salt-tolerant and IR29; salt-sensitive) by comparing growth parameters, Na^+ and K^+ accumulation patterns and expression profiles of the genes that encode Na^+ and/or K^+ transport proteins together with Fe acquisition proteins under saline-alkaline stress conditions, (2) identify rice genotypes (from a large population screening) that are tolerant to a wide range of saline-alkaline stress conditions during vegetative stage by using a hydroponic system, (3) to elucidate both physiological and molecular mechanisms underlying the saline-alkaline tolerance in the selected-rice genotype (Fukoku) by comparing growth parameters and expression profiles of some important genes for saline-alkaline tolerance with the sensitive rice genotype (IR29), and (4) finally to differentiate how rice seedlings respond to each of the stress conditions (saline, alkaline, and saline-alkaline) at the physiological analysis by using Fukoku (selected-rice genotype) and sensitive rice genotype (IR29).

4.1 Growth, physiological responses and transcriptional analysis between the salt-tolerant (FL478) and the salt-sensitive (IR29) rice genotypes under saline-alkaline stress conditions

To characterize the differences in saline-alkaline tolerance between FL478 (salt-tolerant) and IR29 (salt-sensitive) plants, 3-week-old rice seedlings of the two rice genotypes were exposed to nutrient solution supplemented with 50 mM Na at either pH 9 (severe), pH 8 (moderate), and pH 7 (mild) for 3 weeks. The results indicated that FL478 was more tolerant to saline-alkaline stress conditions at pH 7, 8, and 9 than IR29, and this was evident in its higher dry mass production, lower Na^+ accumulation in the leaf blades, and maintenance of water balance under both mild and moderate saline-alkaline stress conditions.

Plants suffering from saline-alkaline stress have to cope with high osmotic pressure (osmotic stress), Na^+ toxicity (ionic stress), and nutrient deficiency (high pH stress) (Li et al., 2016). This study found that, FL478 plants exhibited a lower Na^+/K^+ ratio in both leaf blades and leaf sheaths than IR29 plants when grown under both mild and moderate saline-alkaline stress conditions. Maintaining a balanced cytosolic Na^+/K^+ ratio has become a key salinity tolerance mechanism (Assaha et al., 2015).

Thus, a low Na^+ accumulation in both leaf blades and leaf sheaths of FL478 plants under pH 7 and pH 8 of saline-alkaline stress conditions might be associated with the Na^+ exclusion mechanism at the leaf sheaths or roots, and this mechanism may not be active in IR29 plants. Previous studies reported that salt tolerance in rice is controlled by multiple stress responsive genes (Munns and Tester, 2008; Mekawy et al., 2015; Wangsawang et al., 2018; Sriskantharajah et al., 2020). To understand the mechanisms underlying differential Na^+ accumulation in two rice genotypes, the transcript levels of some genes encoding Na^+ transport proteins in the leaf sheaths and roots of FL478 and IR29 plants were analyzed. In response to mild and moderate saline-alkaline stress conditions, salt-tolerant FL478 had highly induced expression of the *OsHKT1;5* in the roots, but IR29 induced it only slightly in the roots. OsHKT1;5 is known as a key transporter that contribute to salt tolerance by reducing Na^+ accumulation in the shoots (Ren et al., 2005; Kobayashi et al., 2017). Thus, higher transcript levels of *OsHKT1;5* in the roots may contribute to greater tolerance of FL478 plants to saline-alkaline stress than IR29 plants by mediating Na^+ unloading from the xylem. Beside an increase in transcript levels of *OsHKT1;5*, the expression of *OsSOS1* gene was also important for saline-alkaline tolerance in FL478 plants. The previous studies showed that SOS1 could function in both Na^+ loading into and retrieval from the xylem; under mild salinity stress (25 mM NaCl), SOS1 may mediate active loading of Na^+ to the xylem, while under high salinity stress (100 mM NaCl), an increase in SOS1 activity may function in Na^+ retrieval from the xylem sap (Shi et al., 2000). This study found that under saline-alkaline stress conditions, the bifunctional roles of SOS1 were active in the roots of FL478 plants than in IR29 plants, indicating that FL478 plants had greater capacity to induce several mechanisms involved in salt tolerance than IR29 plants. Saline-alkaline tolerance in FL478 was not only involved in Na^+ exclusion mechanisms, but also related to the expression of alkaline-responsive genes. The previous study demonstrated that the greater tolerance of Dongdao-4 rice genotype to saline-alkaline stress at pH 8.5 + 60 mM Na was associated with induction of expression of Fe deficiency-responsive genes such as *OsIRO2*, *OsIRT1*, *OsNAS1*, *OsNAS2*, *OsYSL15*, and *OsYSL2* in the roots (Li et al., 2016). In the present study showed that expression of the determinant genes for alkaline tolerance, such as K^+ and Fe acquisition together with plasma membrane H^+-ATPase was markedly induced in the roots of FL478 plants, but not in IR29

plants. These findings suggested that induction of the transcript levels of genes encoding Na^+ transport proteins and alkaline-responsive genes are important for saline-alkaline tolerance in FL478 plants.

4.2 Identification of rice genotypes with high tolerance to long-term saline-alkaline stress

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Rice is known as an important crop for human diets, which is very sensitive to high Na^+ environment (Munns and Tester, 2008). However, a wide genetic variation was reported in rice for salinity tolerance (Ravikiran et al., 2018), and there has been considerable effort expended in the selection for salinity resistance in rice (Akbar, 1986). It is now accepted that saline-alkaline stress causes much stronger negative effects on plants than saline stress, but tolerance of rice germplasms to saline-alkaline stress is not well documented. Thus, it is important to understand the physiological mechanism of saline-alkaline tolerance in rice and to screen for germplasms with better adapt to various types of saline-alkaline stress conditions at vegetative stage.

In the previous study, two well-known rice genotypes differing in their tolerance to salinity stress, FL478 and IR29, were used to explore physiological and molecular mechanisms underlying tolerance to saline-alkaline stress conditions at pH 9 + 50 mM Na (severe), pH 8 + 50 mM Na (moderate), and pH 7 + 50 mM Na (mild). The difference in saline-alkaline tolerance between the two rice genotypes lies in the ability to exclude Na^+ at the roots through the expression of *OsHKT1;5* and *OsSOS1* genes together with ability to enhance the transcription levels of alkaline-responsive genes. However, in order to produce new rice genotypes that are better adapt to high saline-alkaline environments, screening of rice genotypes having saline-alkaline tolerance from a large population of rice landraces is important. In addition, it is unclear whether saline-alkaline tolerance in rice plants is triggered by the effects of high Na^+ stress or high pH stress. Therefore, the present study was designed to identify the rice genotype that is tolerant to a wide range of saline-alkaline stress conditions and then study on physiological and molecular mechanisms behind the saline-alkaline tolerance. The results obtained from all experimental screenings have been confirmed that the japonica rice, Fukoku, that was developed by crossing the Japanese warm region variety “Nakate-Aikoku”, with the Japanese cold

region variety “Bozu 6” (Saito et al., 2019), was more tolerant to saline-alkaline stress at pH 8.5 + 50 mM Na than the indica rice, IR29. One important finding in the present study is that Fukoku plants can maintain a lower Na^+ concentration and a larger amount of microelements concentration in both shoots and roots than IR29 plants under saline-alkaline conditions. The previous studies revealed that, upon exposure to salinity stress, the salt-tolerant plants could maintain lower Na^+ accumulation in the shoots than salt-sensitive plants (Lutts and Guerrier, 1995; Flowers and Yeo, 1981). Beside maintaining a low Na^+ concentration in the cytosolic, accumulation of microelements at high concentration is also important for plants to survive and grow in saline-alkaline stress conditions. Although microelements are required in very small quantities for normal plant growth and development, but they are essential for certain enzyme activities, especially in a photosynthetic process (Suman et al., 2017). A high pH environment typically reduces availability of microelements for plant, resulting in growth retardation. Thus, the saline-alkaline tolerance in Fukoku plants might be associated with their ability to restrict Na^+ accumulation in leaf blades and maintaining a high concentration of microelements.

In order to further understand the mechanisms of saline-alkaline tolerance in Fukoku, expression profiles of the genes encoding Na^+ transport proteins (*OsSOS1* and *OsHKT1;5*) and the genes for Fe acquisition proteins (*OsIRO2*, *OsIRT1*, and *OsYSL15*) were analyzed by quantitative RT-PCR. In response to saline-alkaline stress at pH 8.5 + 50 mM Na, Fukoku had highly induced expression of *OsSOS1* gene in the roots, corresponding to low Na^+ accumulation in the roots. Furthermore, the expression level of *HKT1;5* gene was more strongly induced in the roots of Fukoku than IR29 rice genotypes after exposure to saline-alkaline stress condition, leading a significantly lower Na^+ accumulation in the shoots of Fukoku. On the other hand, the saline-alkaline sensitive IR29 had lower expression of both *OsSOS1* and *OsHKT1;5* genes in the roots, leading to higher Na^+ accumulation in its shoots. Further studies on the expression of genes for Fe acquisition proteins revealed that saline-alkaline tolerant in Fukoku rice genotype is also associated with the up-regulation of *OsIRO2* gene in the roots, which is responsible for regulating the genes involved in Fe homeostasis and phytosiderophore synthesis in rice (Kobayashi and Nishizawa, 2012; Ogo et al., 2006).

To test whether saline-alkaline tolerance in rice plants is triggered by the effects of high Na^+ stress or high pH stress, the growth parameters of Fukoku and IR29 rice genotypes were further examined under either saline (50 mM NaCl), alkaline (pH 8.5 + 5 mM K), or saline-alkaline (pH 8.5 + 50 mM Na) stress conditions. The results indicated that the greater tolerance of Fukoku plants to saline-alkaline stress at pH 8.5 + 50 mM Na may be caused by high pH tolerance (pH 8.5 + 5 mM K) rather than salt stress tolerance (50 mM NaCl). In contrast, IR29 plants were sensitive to both high pH and high Na^+ stress conditions, leading to a severe growth reduction under saline-alkaline treatment.

4.3 Conclusion

This study aimed at characterizing the physiological responses of the two well-known rice genotypes, FL478 (salt-tolerant) and IR29 (salt-sensitive) to various pH of saline-alkaline stress conditions and to elucidate differences in the mechanisms of saline-alkaline tolerance between them. By comparing the growth parameters, Na^+ and K^+ accumulation and the analysis of transcript levels of some key genes for saline-alkaline tolerance. It was demonstrated that, salt-tolerant, FL478, is relatively saline-alkaline tolerant compared to IR29, and this was evident in its higher dry mass production, lower Na^+ concentration in the leaf blades, and maintenance of water balance under both mild and moderate saline-alkaline stress conditions. Moreover, FL478 exhibited lower Na^+/K^+ ratios in both leaf blades and leaf sheaths under pH7 and pH 8 of saline-alkaline stress conditions. Furthermore, the ability to restrict Na^+ accumulation in leaf blades is considered as an important strategy to maintain the growth of FL478 plants under mild and moderate saline-alkaline stress conditions. In addition, this study found that, the greater tolerance to saline-alkaline stress of FL478 plants is involved in several mechanisms, including (1) Na^+ exclusion (*OsHKT1;5, OsHKT1;4* and *OsSOS1*), (2) K^+ homeostasis (*OsAKT1, OsHAK7,* and *OsHAK10*), and (3) Fe acquisition (*OsNAS1, OsNAS2, OsIRO2, OsIRT1*, and genes encoding H^+-ATPase plasma membrane).

Screening rice genotypes for saline-alkaline tolerance demonstrated that Fukoku is a relatively saline-alkaline tolerant genotype compared to other genotypes because it was able to grow in a wide range of saline-alkaline stress conditions during vegetative stage (50 mM Na + pH 8, pH 9, or pH 8.5).

When saline-alkaline stress at pH 8.5 + 50 mM Na was applied to nutrient solution, Fukoku plants can maintain several growth parameters better than IR29 plants. Maintaining a low Na^+ concentration in both shoots and roots together with accumulation of microelements at high concentration are considered as important strategies to maintain the growth of Fukoku under saline-alkaline stress conditions. At molecular level, the results obtained from quantitative RT-PCR analysis revealed that the tolerance of Fukoku rice genotype to saline-alkaline stress condition (pH 8.5 + 50 mM Na) is associated with the up-regulation of genes encoding Na^+ transport proteins (OsSOS1 and OsHKT1;5) and gene for Fe acquisition protein (OsIRO2) in the roots. Whereas, the sensitivity of IR29 might be due to the repression of *OsSOS1* and *OsIRO2* expression in the roots, thus leading to high Na^+ accumulation in the roots and low ability to acquire micronutrients, respectively.

In summary, both studies demonstrated that FL478 and Fukoku rice genotypes were more tolerant to saline-alkaline stress conditions than IR29. Under saline-alkaline stress conditions, the two rice genotypes (FL478 and Fukoku) accumulate less Na^+ concentration in the leaf blades than IR29 due to higher expression of *OsHKT1;5* gene in their roots. This allows the two rice genotypes (FL478 and Fukoku) to decrease Na^+ accumulation in their leaf blades under saline-alkaline stress conditions. The Na^+/H^+ antiporter (SOS1) is known as a general regulator of Na^+ export from the cytosol (Shi et al., 2002). Overexpression of *SOS1* gene increases the salt tolerance in Arabidopsis (Zhu, 2002; Shi et al., 2003), thereby reducing Na^+ accumulation in the roots. The present studies found that after exposure to saline-alkaline stress conditions, both FL478 and Fukoku rice genotypes were able to induce the expression of *OsSOS1* gene in the roots than IR29, but there were differences in patterns of Na^+ accumulation between the two rice genotypes. In comparison to the Na^+ accumulation in the roots of IR29 under saline-alkaline stress conditions, FL478 accumulated a higher concentration of Na^+ in its roots under both mild (pH 7 + 50 mM Na) and moderate (pH 8 + 50 mM Na) saline-alkaline stress conditions, while Fukoku showed remarkably less accumulation of Na^+ in the roots when saline-alkaline stress at pH 8.5 + 50 mM Na was applied. These findings suggest that under saline-alkaline stress condition, FL478 is not only induced the expression of *OsSOS1* gene, but it may also induce other salt tolerance mechanisms such as Na^+ compartmentalization in its roots, leading to increased Na^+

accumulation in the roots rather than IR29. Besides having a salt tolerance mechanism (OsSOS1 and OsHKT1;5), under saline-alkaline stress conditions, both FL478 and Fukoku rice genotypes also have a greater ability to induce the expression of genes for Fe acquisition proteins (especially OsIRO2) in the roots than IR29 rice genotype, which suggests that the two rice genotypes may be equipped with an efficient phytosiderophore synthesis and Fe acquisition than IR29.

Summary

Soil salinization/alkalinization is an important agricultural contaminant and has complex effects on plant metabolism. The combinations of high Na^+ and high pH stresses in saline-alkaline soils have more severe effects on plant growth and development than high Na^+ stress in saline soils. In order to overcome the toxic effects caused by saline-alkaline stress, plants have to use several adaptive strategies. However, both physiological and molecular mechanisms behind saline-alkaline tolerance in plants have not been fully elucidated. Rice is classified as a glycophyte which is relatively sensitive to salinity stress. Although rice is cultivated in many regions around the world, a significant reduction in yield is frequently observed under saline-alkaline stress environments. In past decades, a few rice genotypes have been analyzed for their combined saline and alkaline responses. Thus, the present study was conducted to investigate the molecular physiological responses of two well-known rice genotypes (FL478; salt-tolerant genotype, and IR29; salt-sensitive rice genotype) to different pH of saline-alkaline stress conditions and to elucidate differences in the mechanisms of saline-alkaline tolerance between them by comparing the several growth parameters; Na^+ and K^+ accumulation, expression profiles of the genes that encode Na^+ and/or K^+ transport proteins together with Fe acquisition proteins. In order to produce new saline-alkaline tolerant rice genotypes in the future, 17 rice genotypes were screened by hydroponic culture to identify tolerant rice genotypes with better adapt to saline-alkaline stress conditions. Then, both physiological and molecular mechanisms behind the saline-alkaline tolerance in the selected-rice genotype (Fukoku) were analyzed by comparing growth parameters and expression profiles of some important genes for saline-alkaline tolerance with the sensitive rice genotype (IR29).

1. Growth, physiology, and transcriptional analysis between the salt-tolerant and the salt-sensitive rice genotypes under saline-alkaline stress conditions

To investigate the physiological responses under saline-alkaline stress, the growth parameters of two well-known rice genotypes; FL478 (the salt-tolerant rice), and IR29 (the salt-sensitive rice) were examined under saline-alkaline stress conditions at either pH 9 + 50 mM Na (severe), pH 8 + 50 mM Na (moderate), and pH 7 + 50 mM Na (mild) for 3 weeks. The results indicated that FL478 was relatively saline-alkaline tolerant compared to IR29, and this was evident in its higher dry mass

production, lower leaf Na^+ concentration in the leaf blades, and enhanced water conservation under both mild and moderate saline-alkaline stress conditions. In addition, under pH 7 and pH 8 of saline-alkaline stress conditions, FL478 plants can maintain lower Na^+/K^+ ratios in the photosynthetic tissues (both leaf blades and leaf sheaths) than IR29 plants. Thus, the greater tolerance of FL478 plants to saline-alkaline stress is related to low Na^+ accumulation in the shoots, which might be due to a mechanism which excludes Na^+ from roots, and that this mechanism may not be operating in IR29 plants. In order to understand the mechanisms underlying differential Na^+ accumulation in two rice genotypes, some important genes encoding Na^+ and K^+ transport proteins together with Fe acquisition proteins were analyzed in the roots. In response to mild and moderate saline-alkaline stresses, FL478 plants had highly induced expression of some membrane transporter/channel genes that may contribute to low Na^+ accumulation in the shoots (*OsHKT1;5* and *OsSOS1*) and also induced expression of the genes for K^+ acquisition (*OsAKT1, OsHAK7, OsHAK10*, and *OsHAK17*), Fe acquisition (*OsNAS1, OsNAS2, OsIRT1*, and *OsIRO2*), and rhizosphere acidification (H^+-ATPase-encoding genes). Therefore, these results highlight that a higher expression of the genes encoding Na^+ and K^+ transport proteins together with Fe acquisition proteins may confer greater tolerance of FL478 plants to mild and moderate saline-alkaline stress conditions. Differences in the mechanisms of saline-alkaline tolerance between the two rice genotypes can be clearly explained by the distinct regulation of genes encoding Na^+ and K^+ transport proteins together with Fe uptake-related genes.

2. Identification of rice genotypes with high tolerance to long-term saline-alkaline stress

In previous study, 93 rice genotypes (including both *indica* and *japonica* subspecies) were assessed their tolerance to saline-alkaline stress at pH 8.5 + 50 mM Na. Among these rice genotypes screened, 17 rice genotypes were classified as saline-alkaline tolerant. Therefore, this study was conducted to identify the most saline-alkaline tolerant rice genotype by using 17 rice genotypes selected from the previous study. The results obtained from all experimental screenings have been confirmed that Fukoku is relatively saline-alkaline tolerant compared to other rice genotypes. To gain a better understanding of mechanism underlying saline-alkaline tolerance of Fukoku plants, several growth parameters

between the most saline-alkaline tolerant Fukoku and the saline-alkaline sensitive IR29 were investigated after 4 weeks of saline-alkaline stress condition at pH 8.5 + 50 mM Na. Fukoku plants displayed better growth performance, as it maintained greater shoot elongation and dry weight and higher concentrations of total chlorophyll, proline, and microelements, while it had a lower Na^+ concentration in both shoot and root tissues and a lower concentration of malondialdehyde, and a lower Na^+/K^+ ratio in the leaf blades, in comparison to IR29 plants. In addition, to test whether saline-alkaline tolerance in rice plants is driven by the effects of high Na^+ tolerance or high pH tolerance, the growth parameters of two rice genotypes were further examined under saline (50 mM Na), alkaline (pH 8.5 + 5 mM K), and saline-alkaline (pH 8.5 + 50 mM Na) stress conditions. This study found that maintaining a low Na^+ concentration in the shoots and roots of Fukoku plants is considered as one important strategy to decrease the deleterious effects caused by high Na^+ stress in both saline (50 mM Na) and saline-alkaline (50 mM Na + pH 8.5) treatments. Besides having a salt tolerance mechanism, Fukoku plants also have a greater ability to tolerate to high pH stress of alkaline treatment (pH 8.5 + 5 mM K), which allow this rice genotype to acquire micronutrients more efficiently under the combinations of high pH and high Na^+ stresses in saline-alkaline treatment. Differences in the mechanisms of saline-alkaline tolerance between Fukoku and IR29 rice genotypes could be explained by the distinct regulation of genes encoding Na^+ transport proteins and genes for Fe acquisition proteins. In response to saline-alkaline stress at pH 8.5 + 50 mM Na, Fukoku showed induction of expression of *OsSOS1*, *OsHKT1;5,* and *OsIRO2* genes that may contribute to Na^+ exclusion from the roots, restriction of Na^+ accumulation in the leaves, and Fe homeostasis, respectively. However, IR29 had lower expression of those genes in its roots after exposure to saline-alkaline stress condition (pH 8.5 + 50 mM Na) for 4 weeks. Thus, this study suggests that the rice genotype identified here will provide useful genetic traits to develop new rice genotypes having saline-alkaline tolerance.

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