Identification of Salt-tolerant Wild *Oryza*: Physiological and Molecular Investigation

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, whether in full or in part, for a degree at this or any other institution. I certify that I have complied in all other respects with the rules, requirements, procedures, and policies relating to the award of this degree at Western Sydney University.

[redacted]

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List of Abbreviations

A	net CO_2 assimilation rate
ABA	Abscisic acid
ABC	ATP-binding cassette
adh2	Alcohol dehydrogenase 2
AKT	Highly selective inward-rectifying potassium channel
ANOVA	Analysis of variance
AP2/ERF	APETALA2/Ethylene Responsive Factor
ATP	Adenosine triphosphate
bHLH	Basic helix–loop–helix
bZIP	Basic leucine zipper
CHX	Cation/H^+ exchanger
Ci	Intercellular CO_2 concentration
CM-H2DCFDA	5-(and 6-) chloromethyl-2',7'-Dichlorodihydrofluorescein diacetate, acetyl ester
CNGC	Cyclic nucleotide-gated channel
CPA	Cation-proton antiporter
CPK/CDPK	Ca2+-dependent protein kinases
DAS	Days after stress
DEGs	Differentially expressed genes
dSm^{-1}	DeciSiemens per metre
E	Transpiration rate
EC	Electrical conductivity

ECe	Electrical conductivity of saturated soil extract
EF1A	Elongation factor 1-alpha
FAO	Food and Agriculture Organization
FC	Fold change
FDR	False discovery rate
FPKM	Fragments per kilobase of exon per million fragments mapped
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLK2	Golden2-like
GLR	Glutamate receptor
GO	Gene ontology
GORK	Guard cell outward potassium channel
gs	stomata conductance
HAK	High-affinity potassium transporter
HKT	High-affinity K^+ transporter
HPS	High Pressure Sodium
HSTF	Heat stress transcription factor
INO1	Myo-Inositol 1 phosphate synthase
JA	Jasmonic acid
KC	Potassium Channel
LTP	Lipid transport protein
Mha	Million hectare
NADPH	Nicotinamide adenine dinucleotide Phosphate hydrogen
NADPME	NADP-dependent malic enzyme

NCL	Na^+/Ca^{2+} exchanger
NHX1	Tonoplast Na^+/H^+ antiporter
NSCC	Non-selective cationic channels
P	Phosporus
PCR	Polymerase chain reaction
PEPC	Phosphoenolpyruvate Carboxylase
PIP	Plasma membrane intrinsic protein
PM	Plasma membrane
PP2CA	Protein phosphatase 2A catalytic subunit alpha
PPDK	Pyruvate phosphate dikinase
qPCR	Quantitative PCR
RBCL	Ribulose-bisphosphate carboxylase
RBCS	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit
RbohD	Respiratory burst oxidase homolog D
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SOS	Salt overly sensitive
SOS1	Plasma membrane Na^+/H^+ antiporter
TEA^+	Tetraethylammonium
TF	Transcription factor
Tleaf	Leaf temperature
V-ATPase	Vacuolar-type ATPase
VHA-C	V-type proton ATPase subunit C
VpdL	Leaf vapor pressure

V-PPase	Vacuolar membrane-bound proton-pumping pyrophosphatase
WT	Wild type
WUE	Water use efficiency

Publications during the PhD candidature

First and co-first author Publications

- **Yong, M.T.**, Solis, C.A., Amatoury, S., Sellamuthu, G., Rajakani, R., Mak, M., Venkataraman, G., Shabala, L., Zhou, M.X., Ghannoum, O., Holford, P., Huda, S., Shabala, S., Chen, Z.H. Proto Kranz-like leaf traits and cellular ionic Regulation are Associated with Salinity Tolerance in a Halophytic Wild Rice. ***Stress Biology***. 2, 8 (2022). **(Chapter 4)**

- **Yong, M.T.**, Solis, C.A., Rabbi, B., Huda, S., Liu, R., Zhou, M., Shabala, L., Venkataraman, G., Shabala, S. and Chen, Z.H., 2020. Leaf mesophyll K^+ and Cl^- fluxes and reactive oxygen species production predict rice salt tolerance at reproductive stage in greenhouse and field conditions. ***Plant Growth Regul***. pp.5-020. **(Chapter 3)**

- **Yong, M.T.**, Doyle, R, Fisher, P and Mann, L 2017, 'Subsurface drip irrigation (SDI) and GYP-FLO in processing tomato production', in C Argerich and S Colvine and M Camara (eds.), ***Acta Horticulturae***, International Society for Horticultural Science, Belguim, pp. 37-45.

- Solis, C.A., **Yong, M.T.,** Venkataraman, G., Milham, P., Zhou, M., Shabala, L., Holford, P., Shabala, S. and Chen, Z.H., 2021. Sodium Sequestration Confers Salinity Tolerance in an Ancestral Wild Rice. ***Physiologia Plantarum .*** *2021; 172: 1594– 1608.* **(Chapter 3)**

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- Pornsiriwong, W., Estavillo, G.M., Chan, K.X., Tee, E.E., Ganguly, D., Crisp, P.A., Phua, S.Y., Zhao, C., Qiu, J., Park, J., **Yong, M.T**., Nisar, N., Yadav, A.K., Schwessinger, B., Rathjen, J., Cazzonelli, C. I., Wilson, P.B., Gilliham, M., Chen, Z.H. & Pogson, B.J. 2017. A chloroplast retrograde signal, 3'-phosphoadenosine 5'-phosphate, acts as a secondary messenger in abscisic acid signaling in stomatal closure and germination. ***Elife***, *6*, p.e23361.

Manuscripts in preparation

- Solis, C.A., **Yong, M.T.**, Zhou, M.X, Venkataraman, G., Shabala, L., Holford, P., Shabala, S., Chen, Z.H., Evidence of divergent evolution of tissue tolerance to Salinity in the genus *Oryza,* ***International Journal of Molecular Science***, resubmitted on January 2022 (under review).

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Poster presentation

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Abstract

Soil salinity is a major abiotic stress constraining global crop production. Salinity is already affecting more than 800 million hectares (Mha) of total global land area, where 20 Mha and 4 Mha are in Australia and India, respectively. Salinity stress is thought to be only secondary to the drought stress in hindering rice productivity in the major rice-growing regions. Rice (*Oryza sativa* L.) is highly susceptible to salinity stress. Improvement in rice salinity tolerance using existing cultivars is facing bottlenecks of narrow genetic diversity and negative linkage between key salinity tolerance traits and yield and quality traits. Wild rice preserved wide diversity of genetic pools in undisturbed natural habitats, which draws a lot of attention from the plant breeders for its outstanding performance under different environmental stresses including salinity. In this thesis, I studied the salinity tolerance of some wild *Oryza* species through parallel sets of greenhouse and field trials. Salinity treatment was applied at the late vegetative stage for a prolonged period through to the reproductive stages of different wild and cultivated *Oryza* species.

A wild range of approaches (agronomical, physiological, electrophysiological, and molecular) was employed to evaluate the performance of wild *Oryza* and rice cultivars. I first evaluated the physiological responses of three Australian commercial cultivars (*O. sativa japonica* and *O. sativa indica*) to salinity stress in a set of greenhouse and field trials (**Chapter 3**). This chapter aimed to expand our understanding of the physiological response of Australian commercial cultivars to salinity stress. The result indicated that salt-tolerant variety conferred higher K^+ retention and low recovery Cl^- efflux in mesophyll cells than the salt-sensitive cultivars. Low recovery Cl^- efflux might be the result of lower Na^+ accumulation in tolerant species. Dynamic ROS production and regulation in mesophyll cells were found over the duration and strength of salinity stress. Compared to the sensitive cultivar, the tolerant cultivar

was observed with lower ROS production at the early stage of salinity stress but increased to higher ROS at the later stage of salinity stress.

The salinity tolerance of selected wild *Oryza* species was evaluated in another set of greenhouse and field trials (**Chapters 4 & 5**). Based on the significant correlation results, it was indicated that salt-tolerant wild *Oryza* species such as *O. coarctata*, *O. alta*, *O. latifolia*, and *O. officinalis* have higher relative biomass, ROS production, chlorophyll contents, net CO_2 assimilation (*A*), and transpiration rate (*E*), but they showed lower Na^+/K^+ ratio, recovery K^+ & Cl^- efflux, and mesophyll Na^+ accumulation. In addition, the salt--tolerant wild *Oryza* species showed lower mesophyll Na^+ accumulation compared to the control and the salinity sensitive ones. Variation in environmental conditions especially in the field trial had a significant effect on plant growth, biomass, and *A*. However, these factors did not alter the overall ranking of salinity tolerance in the wild and cultivated rice species in the greenhouse and field.

The salt tolerance screening experiments identified 4 tolerant wild *Oryza* species: *O. officinalis* (CC), *O. latifolia* (CCDD), *O. alta* (CCDD), and *O. coarctata* (HHKK). *O. officinalis*, *O. latifolia*, and *O. alta* are members of the *Oryza officinalis* complex with CC genome background in common. Other genomes of *Oryza officinalis complex*- BB (*O. punctata*) and EE (*O. australiensis*), were moderately tolerant to the salinity stress. Apart from the halophytic species *O. coarctata* (HHKK), the *Oryza* species with CC genomes were overall tolerant to salinity stress. The result highlighted the potential value of *Oryza* species with C genome and the halophyte *O. coarctata* in salinity tolerance. However, further exploration with a larger species population of all genomes in *O. officinalis* complex and evaluation of salinity tolerance mechanism of species with C genome are needed to confirm the salt-tolerant related alleles in the C genome and traits in these *Oryza* species.

Oryza coarctata is the only halophyte in the *Oryza* genus. It is adapted to the daily inundation of seawater with salinity up to 40 dS m^{-1}. Salt glands formation on the leaf is

proposed to be the major feature in *O. coarctata* to adapt to extreme saline conditions. I proposed that salinity tolerance through salt exclusion from the salt glands in *O. coarctata* is accompanied with tissue tolerance of other parts of the leaf. I conducted further evaluations of salinity tolerance mechanisms of *O. coarctata* in a set of experiments. in *O. coarctata*. In **Chapter 4**, *O. coarctata* showed C4-like photosynthetic characteristics in the field and leaf morphological assessment suggested that *O. coarctata* possess Kranz-like leaf anatomy. *O. coarctata* also exhibited exceptionally higher expression of C4 pathway-related genes in the leaves compared with those in two cultivars (IR64 & Pokkali) of *O. sativa*. The result indicated that *O. coarctata* may have evolved initial intermediate traits of C3-C4 photosynthesis. Na^+ imaging analysis showed that *O. coarctata* maintains low apoplastic Na^+ content & sequestrates majority of Na^+ in the vacuole and chloroplasts of mesophyll cells, implying the activities of key membrane transporters behind this observation. The RNA-sequencing and analysis of leaf epidermis identified several specific membrane transporters such as high-affinity K^+ transporter OcHKT2;4 and cation proton exchanger OcCHX3 that may contribute to salinity tolerance *O. coarctata*. However, detailed functional characterization of those key transporters is subject to future studies.

This study provides useful insights into physiological and molecular mechanisms of salinity response in the *Oryza* genus. I also suggest the potential use of traits and genes from wild rice species, especially those with the C genome and the halophyte *O. coarctata*, in breeding for salt-tolerant rice cultivars. Outcomes of this Ph.D. project will not only improve the understanding of the complex salt tolerance mechanisms in wild and cultivated rice species but also guide future research towards a more sustainable rice production by improving tolerance of cultivated rice with the salt-tolerant traits of their wild relatives.

Chapter 1: Literature Review

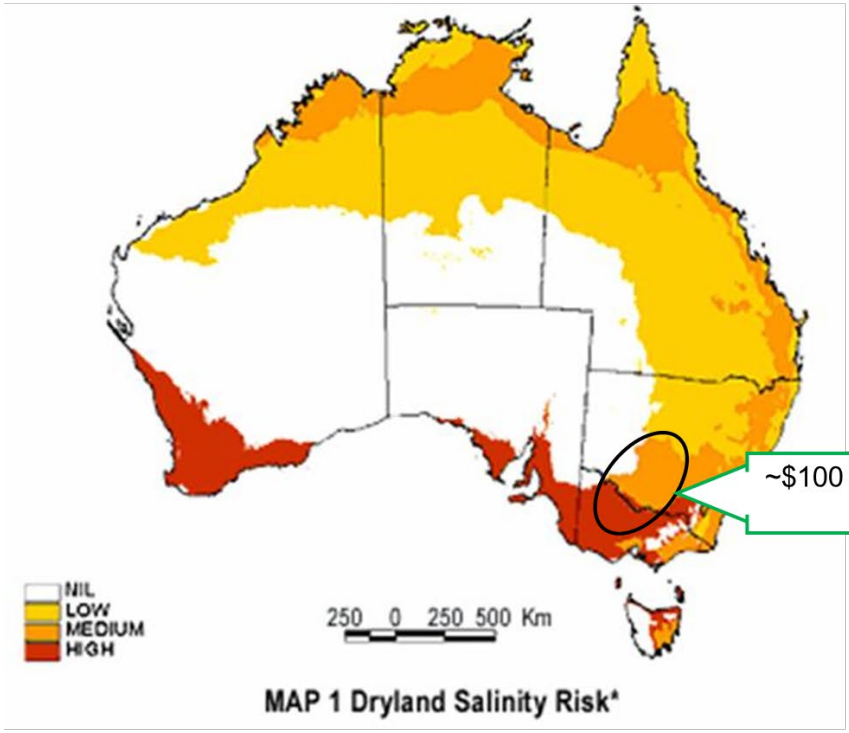
1.1 Background

Saline soil is defined when the electrical conductivity of its saturation extract (ECe) is higher than 4 dS m^{-1}. The saline soil generally contains large amounts of cations such as Na^+, Ca^{2+}, Mg^{2+} and to a lesser amount of K^+ and Fe^{2+} while the most common anions are Cl^-, SO_4^{2-}, NO_3^- and HCO_3^-. Among those, the most detrimental elements to plants are Na^+ and Cl^- as they usually occupy a major portion of available ions (Hasegawa et al., 2000). The adverse effects of Na^+ and Cl^- can affect plants through poor soil chemical and physical properties. According to the concentration of available Na^+ Cl^- and HCO_3^-, the soils can be separated into saline or saline-sodic soil types (Northcote and Srene, 1972).

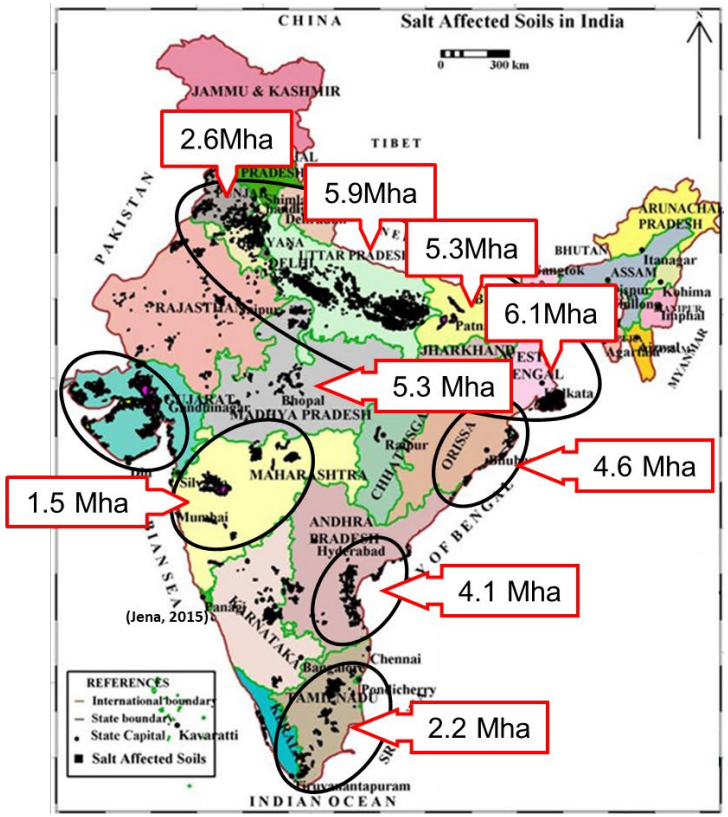
Soil salinity is one of the major abiotic stresses constraining global crop production (Zhu, 2001). Salinity is already affecting more than 800 million hectares (Mha) (6.5%) of the total land area (Ghassemi et al., 1995, Thimmappa et al., 2017). In Australia and India, 20 Mha and 4 Mha of land are affected by salinity, respectively (Thimmappa et al., 2017, Shahid et al., 2018). This estimate does not include the land area of sodic soil, which occupied another 340 and 3.7 Mha of land in Australia and India, respectively. Salinity stress is thought to be only secondary to drought stress in hindering rice productivity in the major rice-growing regions.

Most of the saline soil is formed through the natural process such as weather, salt accumulation from rainfall, and deposition of windblown salt. Formation of this type of saline soil generally takes thousands to millions of years. Some natural events such as sea water intrusion, drought, and catastrophic disasters around the coastal area such as earthquake, typhoon, and tsunami can cause soil salinization to establish more quickly, within years or even days (Jesus et al., 2015, Yoshii et al., 2013, McLeod et al., 2010, Badaruddin et al., 2015).

Secondary saline soil is usually the result of human activities. Application of fertilizers containing high salt content, poor irrigation strategies, and poor water quality are the main factors causing soil salinity in agriculture (Gu et al., 2013). These practices are unlikely to stop as they are the keys to successful crop production with a high yield. Secondary salinization was affecting around 76 Mha of land globally, where approximately 60% of these areas are irrigated, accounting for 20% of the total irrigated land (Shahid et al., 2018).



MAP 1 Dryland Salinity Risk*



Rice is a highly salinity susceptible cereal crop among glycophytes. Soil electrical conductivity (EC) between 6.9 and 11 dSm^{-1} are estimated to cause 50% yield loss (Van Genuchten and Gupta, 1993) and total yield loss of rice respectively (Munns and Tester, 2008). However, salinity sensitivity is genotype and growth stage-dependent (Zeng and Shannon, 2000). Therefore, the development of a highly productive, salt-tolerant rice cultivar is needed to cope with future demand and climate change. To fulfill the increasing food demand for the growing population, a significant yield increase (approx. 150%) in cereal crops is required by 2050 (Godfray et al., 2010). However, FAO (2017) estimated that the total area of rice cultivation is more likely to decrease slightly compared to the current total land used for rice cultivation. The main reason behind this is reported to be the human-induced soil degradation and the consequence of climate change which will result in seawater intrusion to the arable area in the future. Approximately 75% of the world rice production relies on irrigated low-land cultivation (Muthayya et al., 2014), in another word, salinity is a big issue for these low-land irrigated areas for crops cultivation. It is especially important in Eastern India as the farmers also rely on the rotation of other crops after rice cultivation (Cornish et al., 2015a, Cornish et al., 2015b).

Australia and India are countries suitable for rice cultivation. Although Australia produces ~0.1% of global rice production, Australia has the most efficient rice-producing system and the highest rice yield (Fell et al., 2020). Water usage for rice cultivation in Australia is 20 to 40% less than the world average water usage, but the yield per hectare is the highest in the world (Figure 1.2). India is the second largest rice-producing country, which contributes approx. 22% of global rice production in 2016 (FAO, 2021). Solutions such as soil amelioration are impracticable due to, firstly, difficulty in removing large amounts of salt from large areas of land; secondly, soil amelioration is always time and cost-intensive. Plant breeders have tried hard to find alternative ways to develop new varieties that can adapt to the stressful environment

through the biotechnological aspect (Lusser et al., 2012), such as traditional breeding techniques, transgenic techniques, and the recently developed novel techniques such as genome editing (Sander and Joung, 2014).

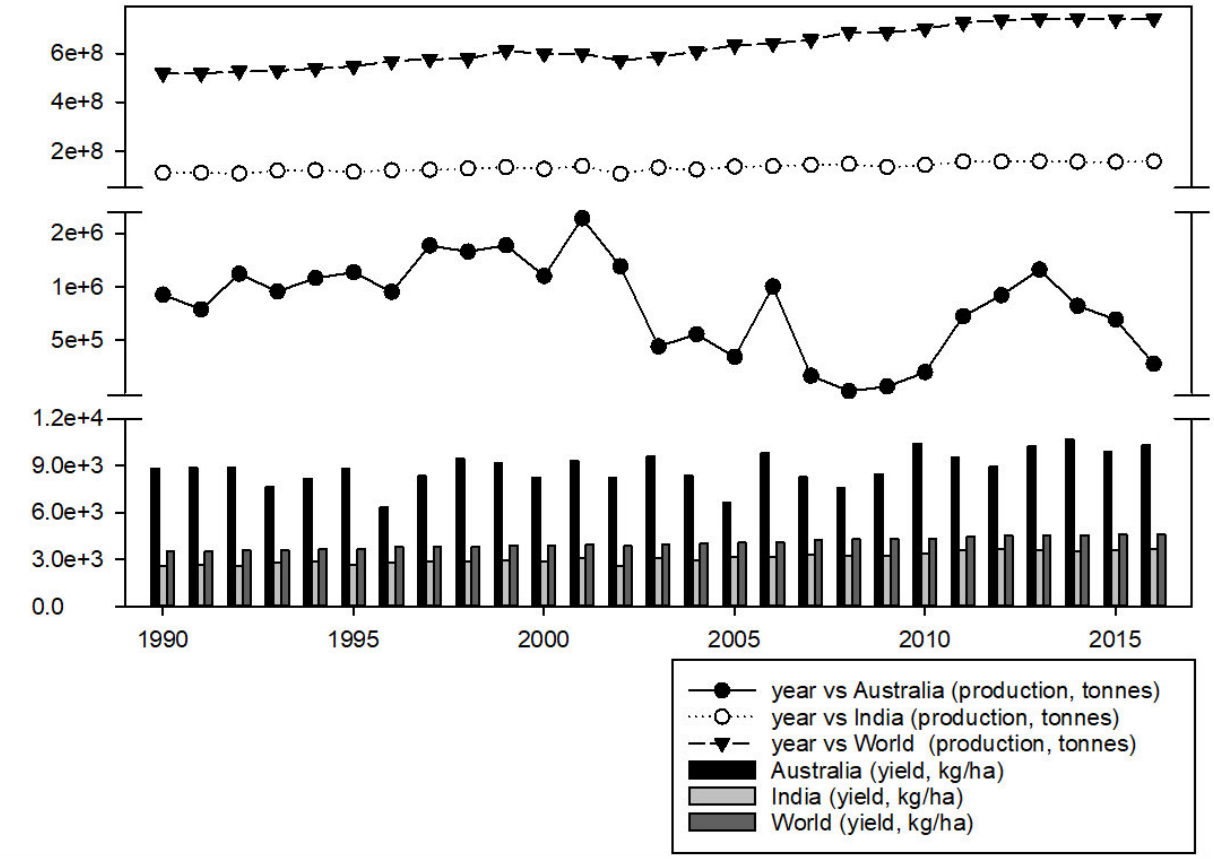


Figure 1.2. Total rice production (tons) and average yield (kg/ha) in Australia, India and the World from 1990 to 2016 (FAO, 2021).

Screening studies of salinity tolerance traits for breeding programs were commonly targeted on *Oryza sativa* subspecies *indica* and *japonica* in past decades (Negrão et al., 2011). Several salt-tolerant varieties have been developed through breeding programs, but the results are not satisfactory because there is only a limited number of major genetic traits have been identified and limited techniques exist to enhance salt-tolerant traits in new cultivars (Negrão et al., 2011). This might also indicate that the primary genes pool (*O. sativa*) does not have a sufficient number of salinity tolerance genes. Meanwhile, there are very few salinity tolerance studies of other wild rice relatives, except for *O. rufipogon* and *O. coarctata*, compared to other abiotic stresses such as drought and cold (Neelam et al., 2017, Zhang et al., 2017, Liu et al., 2003, Dai et al., 2015, Julia et al., 2016, Nguyen et al., 2003, Verma et al., 2017).

1.2 Physiological response of rice to salinity

1.2.1 Major stresses that are imposed by salinity: osmotic and ionic stress

Plant salinity tolerance is regulated by multiple genes and associated mechanisms. Bahmani et al. (2015) outlined a myriad of cellular components related to salinity tolerance from very upstream signaling and hormone regulation to cellular protection and ion homeostasis against salinity. These components work interactively to maintain cellular activities under salinity stress via three mechanisms: osmotic modulation, antioxidative regulation, and ion homeostasis. Shabala and Cuin (2008) further pointed out the indicator of the salinity tolerance from the interactive effects between these mechanisms, namely Na-induced K efflux from the cytosol and cytosolic Na:K ratio.

Rice is a glycophyte that is highly susceptible to salinity stress. Munns and Tester (2008) indicated that rice is only slightly more salt-tolerant than Arabidopsis. Sensitive rice was

reported to have a yield penalty from soil EC*e* exceeding 3 dSm^{-1} and failure to produce grain at EC*e*> 10 dSm^{-1} (Munns et al., 2006, Asch and Wopereis, 2001, Munns and Tester, 2008, Khatun and Flowers, 1995). It was reported to be relatively more salt-tolerant at seed germination (Khan et al., 1997) and vegetative stages (Zeng and Shannon, 2000) but more salt susceptible at the seedling stage (Lutts et al., 1995) and highly susceptible at the reproductive stage. Salinity significantly reduces fertility, tiller numbers, panicle number, and panicle length by lowering pollen viability and thus sterility of rice (Shereen et al., 2005, Zeng and Shannon, 2000).

Salinity affects plant growth mainly through osmotic and ionic stresses. When a plant senses salinity stress, ROS signaling is possibly one of the first defense responses in the plant (Schneider et al., 2018) to drive other defense mechanisms for salinity stress (Mittler and Blumwald, 2015). Hypertonic soil solution forces water out from the cell along the osmotic gradient (Yeo et al., 1991). It was reported that leaf and root elongation rates are reduced instantaneously at the onset of salt stress (Yeo et al., 1991), and consequently plant growth is restricted under saline conditions (Munns, 1993). Normal plant growth and function do not resume immediately when they are moved from osmotic stress to a normal conditions (Munns, 2002), indicating that plant's response to the osmotic pressure is controlled by signaling rather than water retention (Munns, 2002). On the other hand, ROS accumulation is associated with increased oxidative stress along with salinity stress due to its chemically reactive characteristic (Gupta et al., 2016).

Salinity-induced stomatal closure leads to a reduction in photosynthetic activities and return, this will accelerate continuous ROS generation in two major cellular ROS producing compartments- chloroplast and mitochondria (Gómez et al., 2019) Thus, the less photosynthetic rate reduction is reported in salinity-tolerant rice varieties and species, might not only be the

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result of active defense but also be part of the protective tool to generate energy and relieve oxidative stress. Plant transpiration was reported as the main factor that contributed to the aggregation of soluble salt toward the rhizosphere through soil water movement and led to higher osmotic pressure at the soil water to plant interface (Schröder et al., 2014). The extent of soluble salt aggregation is increasing with the initial salinity level in soil, and the transpiration rate. Van Den Akker et al. (2011) reported that soil-water Cl^- accumulation caused by transpiration was 3 to 50 times higher than Cl^- accumulation caused by evaporation under flooded irrigation in crops. However, the contribution of transpiration-driven salt accumulation around the rhizosphere of salt-stressed plant in terms of ion uptake and osmotic adjustment is unclear.

Ionic stress mainly refers to Na^+ toxicity in salinity stress. It occurs when the concentration of Na^+ and Cl^- are elevated beyond threshold levels in the cytosol. Na^+ transportation within a plant is quicker than Cl^-, and thus Na^+ toxicity initiates and becomes fatal to the plant before Cl^- accumulates to a toxic level in most plant species, especially for herbaceous plants. The occurrence of ionic stress after salinity stress is dependent on plant species. In rice, salinity-induced ionic toxicity will take hours to days to happen due to its leaky root structure (Yeo et al., 1991). The rice root system is different compared to other monocot species such as wheat and barley. The rice shoot has larger lacunae on its conical parenchyma to increase oxygen transfer efficiency from shoot to root under a hydroponic environment. Garcia et al. (1997a) reported that apoplastic ion and solute leakage in rice is 10 times higher than in wheat. The first Na^+ toxicity-induced response is net cellular K^+ efflux from the plant via membrane potential depolarization (Shabala and Cuin, 2008). As a plant macronutrient, K^+ is responsible for the activation of many important metabolic processes (Marschner, 2011). As a consequence, regular metabolisms that require the involvement of K^+ are disrupted due to K^+ deficiency. Secondly, the binding site between enzymes and K^+ has low selectivity towards Na^+

due to similar chemical properties between Na^+ and K^+. Na^+ can compete with K^+ for these binding sites when the cytosolic Na^+:K^+ ratio increases under saline conditions. NSCC is the important entrance for Na^+ uptake in plant under salinity stress (Assaha et al., 2017, Hanin et al., 2016), and the bypass flow pathway is believed to contribute significantly to Na uptake in rice. However, Kavitha et al. (2012) and Malagoli et al. (2008) reported no significant contribution of NSCC in Na^+ uptake at the early stage of salinity stress in rice. This is suggesting another dominant Na uptake pathway in rice, such as aquaporin PIP (Byrt et al., 2017).

Ionic stress affects plants in two ways: NaCl toxicity and nutrient deficiency (K, Fe, and Zn). It occurs when the concentration of Na^+ and Cl^- are elevated beyond threshold levels in the cytosol. Na^+ transportation within a plant is quicker than Cl^-, and thus Na^+ toxicity initiates and becomes fatal to the plant before Cl^- accumulates to a toxic level in most of the plant species, especially for herbaceous plants. The occurrence of ionic stress after salinity stress is species-dependent. In rice, salinity-induced ionic toxicity will take hours to days to happen due to its leaky root structure (Yeo et al., 1991). Rice's root system is different compared to other monocot species. The rice shoot has larger lacunae on its conical parenchyma to increase oxygen transfer efficiency from shoot to root under a hydroponic environment.

Potassium is responsible for the activation of more than 50 cytosolic enzymes (Shabala and Cuin, 2008). However, when the cytosolic Na^+/K^+ ratio increases significantly, Na^+ can easily compete with K^+ for the binding sites of enzymes. Na^+ is also able to bind with some K^+ responsive enzymes as Na^+ has similar physicochemical properties to the K^+ however, its binding effectiveness is only 5-10% of K^+ (Duggleby and Dennis, 1973). Furthermore, when extensive amounts of Na^+ are crossing the plasma membrane (PM) under saline conditions, PM is depolarized, by 60-80mV (Shabala et al., 2005, Shabala et al., 2006, Shabala et al., 2003). PM depolarization does not only cause K^+ uptake via inward K^+ rectifying channel to be impossible [AKT, threshold PM potential -120mV; Hirsch et al. (1998)], it also promotes K^+

efflux from cell to external via depolarization-activated outward rectifying K^+ channels [GORK, Ache et al. (2000)]. On the other hand, PM depolarization also promotes passive uptake of Cl^- under salinity treatment, which will increase the level of salinity stress (Teakle and Tyerman, 2010).

1.2.2 Molecular mechanism for salt tolerance: Compatible solute

Accumulation of compatible solute is well-known when the plant is suffered from abiotic stress. To compensate for the osmotic potential differences between cytosol and apoplast and between cytosol and vacuole under saline conditions, compatible solutes are accumulated in the cytosol to ameliorate osmotic stress (Munns and Tester, 2008). Since this mechanism mainly targets osmotic stress, it is believed to be activated in the early time of the stress and works continuously to maintain the osmotic balance between vacuole and cytosol. Why is it so important in rice in salinity stress adaptation? To date, sodium exclusion from aboveground tissue is found as the core strategy in salt-tolerant rice varieties such as Pokkali to cope with salinity stress (Gerona et al., 2019, Lutts et al., 1996, Prusty et al., 2018). However, the plant will have to increase tissue osmotic gradient to facilitate long-distance transport, which can be either achieved by reducing water content or accumulation of osmolytes (Wani et al., 2013). The former will reduce cell turgor and affect regular physiological processes such as photosynthesis (Jones, 2006).

There are 4 major types of compatible solutes based on their chemical properties: sugars (e.g. glucose), complex sugars (e.g. trehalose), sugar alcohols (e.g. mannitol), amino acid, and derivatives (e.g. proline) (Hoang et al., 2016). However, it is still unknown which and why particular compatible solutes work better for particular stress. For drought and salinity stress, proline is the most common osmo-regulator in plants. Trehalose accumulation in rice is also reported to ameliorate salinity stress by reducing sodium accumulation, reducing chlorophyll

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damage, and preserving root integrity (Garcia et al., 1997b). Starch degradation is believed to be an important metabolism in response to abiotic stress (Thalmann and Santelia, 2017). This results in the release of sugars and metabolites for osmotic regulation, and the release of energy for other metabolisms (formation and active transportation of substances) in response to stress. However, the synthesis and accumulation of compatible solutes in response to stress always come with a cost in production. It is estimated to cost at least 10 times more ATP for compatible solute synthesis and accumulation than using Na^+ as cheap osmoticum (proline: 41 ATP, mannitol: 34 ATP, glycine betaine: 50 ATP) (Chen et al., 2007a, Raven, 1985). Furthermore, the accumulation of compatible solute also increases plants' susceptibility to fungal diseases and lodging. The beneficial effect of this approach is often marginal (Babourina et al., 2000).

1.2.3 Molecular mechanism for salt tolerance: Antioxidant regulation

In a plant, reactive oxygen species (ROS) acts as an important defense and signaling system when the plant is invaded by insects and diseases or affected by abiotic stresses. However, over-production of ROS will result in oxidative damage to the plant (Munns and Tester, 2008). It will actively react with cellular molecules and metabolites, resulting in programmed cell death. Under the saline conditions, reduced available CO_2 content and photosynthetic rate due to stomatal closure results in an accumulation of ROS. The photoinhibition is prevented by two processes, which are heat scattering by the xanthophyll pigments and electron transfer to oxygen acceptor rather than water. Meanwhile, enzymes responsible for antioxidant increases to detoxify the ROS produced at the later process. It has been pointed out that superoxide and H_2O_2 content in chloroplast are three times higher in salinity-treated plants than those in the control (Munns and Tester, 2008). In rice, Kaur et al. (2016b) found that activities of NADPH oxidase and superoxide dismutase were stronger in salt-tolerant varieties than in salt-sensitive varieties. Meanwhile, salt-tolerant varieties are also found to be better in preventing the

accumulation of ROS with higher gene expression and activities of antioxidant enzymes. The result indicated the importance of synchronization between ROS generation and ROS removal in salinity tolerance. However, Munns and Tester (2008) pointed out that the expression of antioxidant enzymes in some salinity sensitive genotypes can be as high as expression in salinity tolerant genotypes. This indicated that synchronization of ROS accumulation and detoxification can be important but is not an indicator that can be used solely in measuring plant salinity tolerance.

1.2.4 Molecular mechanism for salt tolerance: Ion homeostasis

Ion homeostasis is considered one of the most important strategies for salt tolerance in plants. It is involved in the regulation of cation uptake, exclusion, and allocation to different parts of the plant with functional regulation of major ion transporters (Sun et al., 2009a). Salinity studies were focused on the regulation of Na^+ glycophytes such as cultivated rice as it was thought to be more important than K^+ regulation to maintain a low Na^+:K^+ ratio in plant homeostasis for salt tolerance. Therefore, these studies were targeted on the genes and physiological traits related to Na exclusion, compartmentation, retrieval, and other components which facilitate Na regulation through biotechnological aspects and plant breeding programs.

Studies of Na accumulation in plants do not always support a strong correlation between Na content and plant salt tolerance (Shabala and Cuin, 2008). Some studies showed higher Na^+ accumulation in salt-tolerant species compared to sensitive species. In rice, a salt-tolerant variety Pokkali accumulated higher Na^+ in the leaf than salt-sensitive variety- IR64 (Sengupta and Majumder, 2009). Similar results were found in a study on tomato salinity tolerance (Santa-Cruz et al., 1999). A study of salinity treated roots of 70 barley varieties reported that sodium content in root only became a determinate component in salinity tolerance when the K^+ fluxes were similar between the studied varieties, otherwise, K^+ efflux was highly negatively

correlated to the relative grain yield, shoot biomass, plant height, net CO_2 assimilation, survival rate. and 1000 seed weight (Chen et al., 2007b). Cuin et al. (2008) found a similar relation between K^+ flux and salinity tolerance but no obvious correlation between salinity tolerance and Na^+ content in wheat by comparing the salt-tolerant bread and durum wheat species. The salinity-tolerant species have a higher ear dry weight and ear number than salt-sensitive species under saline conditions. Both studies concluded that genotypic variation in salinity tolerance was related to the roots' ability to retain K^+ in the mature root zone when salinity stress was introduced. Shabala and Pottosin (2014) also indicated that plant salt tolerance is positively correlated to K^+ content and K^+ flux.

Under normal growth conditions, K^+ content is generally at least many folds higher than Na^+ content in the plant(Singh and Sarkar, 2014, Abdullah et al., 2001, Kumar and Khare, 2015, Wei et al., 2017, Xue et al., 2004, Lv et al., 2015, Hu et al., 2015, Dong et al., 2015, Chen et al., 2011b, Ahmad et al., 2016). However, the difference is almost leveled or even less than Na^+ content in sensitive species when they are suffering salinity stress. *O. coarctata* with 10 dSm^{-1} salinity treatment was reported to maintain a K^+:Na^+ ratio of 1.4 in mesophyll and 0.75 in the root (Flowers et al., 1990). This is normally the consequence of a sharp decrease in K^+ and an increase in Na^+. Therefore, it is not surprising that K^+ retention becomes such an important indicator of salinity tolerance after losing a high amount of the metabolically important element. In most cases, plants accumulate a higher Na^+ content in the shoot rather than the root under saline conditions including *O. coarctata.* Secondly, unique salinity tolerance mechanisms are developed to cope with this unavoidable salinity stress in *O. coarctata*. Surprisingly, the physiological studies of *O. coarctata* found that the K content in shoots treated with different salt concentrations has no significant difference between them (Flowers et al., 1990, Sengupta and Majumder, 2009). This indicated that long-distance transport of K might be an important

salinity tolerance mechanism in *O. coarctata* (Flowers et al., 1990, Sengupta and Majumder, 2009).

In rice, salt-sensitive variety was reported to have high net Na^+ permeability at early stress (Lakra et al., 2018, Nemati et al., 2011) due to higher unidirectional Na^+ influx (Kavitha et al., 2012, Malagoli et al., 2008). The salt-sensitive rice cultivar- Dongjin (Reddy et al., 2017) was also reported to behave similarly to other sensitive crops (Ishikawa and Shabala, 2019), restricting xylem Na^+ loading compared to increasing Na^+ loading in early days of stress in salt-tolerant crops (Zhu et al., 2017, Bose et al., 2014). This is suggesting firstly high Na^+ intake and lower tissue tolerance to Na^+ accumulation in aboveground part of the sensitive variety. For this reason, the sensitive variety triggers more powerful Na^+ retrieval earlier than the tolerant variety. It also highlighted the importance of Na^+ exclusion in rice at early stress stage. It may provide lag time for tolerant plant to mediate regulation of stress responsive genes for salinity adaptation.

The importance of Na^+ retrieval in salinity tolerance has been demonstrated in many studies (Horie et al., 2009, Horie et al., 2007, Flowers et al., 2015). Nevertheless, a study using reported salt toxicity in *OsHKT1;4* overexpressing plant's root led to a salt-sensitive transgenic line compared to WT (Oda et al., 2018). Similarly, Suzuki et al. (2016) found no difference in visual characteristics between Nipponbare (WT) and *OsHKT1;4* knockdown line in two weeks of 50mM NaCl stress while the knockdown line did accumulate higher Na^+ content in the shoot. These provide insight into components that are also essential together with amplification of HKT1 family in the plant for salinity tolerance improvement in high productivity but salt-sensitive rice cultivars. In the overexpressing line, the majority of Na^+ will be transferred to the lower part of plants including leaf and root tissue (Oda et al., 2018). To compensate for Na^+ toxicity, the root has to increase the content of osmolytes to alleviate Na^+ toxicity and oxidative

stress if Na^+ exclusion cannot exclude extra Na^+ from the root. On top of that, this might increase the biophysical difficulty to the long-distance transportation of water against the osmotic gradient for the regulation of normal photosynthesis. This might explain why salt-tolerant plant species do not rely heavily on Na^+ retrieval from shoot during early period of salinity stress (Zhu et al., 2017, Bose et al., 2014).

1.3 Current progress of salinity tolerance in wild *Oryza*

Crop domestication is thought to be one of the most important activities of our ancestors that initiated human civilization. These domesticated crops are highly influenced by human selection towards genetic traits contributed in higher crop production and better quality during centuries of domestication (Sengupta and Majumder, 2009). Consequently, the traits which are responsible for tolerance to biotic and abiotic stress were not particularly selected and are lost over the long-term of biased human selection. Plant breeding is now refocusing on the naturally existing traits that facilitate the tolerance to abiotic and biotic stresses for our current crops. The target is then shifted to the wild relatives and non-crop plants which reserve a wide range of tolerance genes and traits and remain undisturbed in their natural habitat (Dai et al., 2014). Due to these conditions, the genetic modification method is preferred rather than traditional plant breeding methods as it is not suitable in most cases due to incompatible fertilization between two different species, even between some wild relatives and domesticated species (Ochatt et al., 2004, Rottenberg and Zohary, 2005, Solis et al., 2020).

Salinity tolerance study of wild relatives of rice for breeding was not common due to direct breeding incompatibility between different species. *O. rufipogon* has the highest compatibility with *O. sativa* due to their close evolutionary distance (Londo et al., 2006).

However, Ganeshan et al. (2016) showed that crossing between *O. sativa* and other wild relatives is also feasible by studying salinity tolerance of *O. nivara* by crossing with cultivated varieties. The result suggested that wild rice species have salinity tolerance-related genes. Nakamura et al. (2002) compared salinity tolerance of *O. latifolia* and *O. rufipogon* species with two cultivated rice varieties- SR26B (salt-tolerant) and IR28 (salt-sensitive). They reported that *O. rufipogon* was susceptible to salinity stress, while *O. latifolia* has a higher survival rate compared to a salt-tolerant variety. *O. latifolia* accumulated higher Na^+ content compared to other tested varieties, while the photosynthesis rate was almost unchanged under 12 dSm^{-1} of salt treatment. It was reported that the net photosynthetic rate in *O. latifolia* is not affected by 300 mM NaCl of salt treatment and dropped to 40% when it was treated with 650 mM NaCl treatment (Nakamura et al., 2004). They found that the thylakoid membrane in chloroplasts of *O. latifolia* was unique compared to other species. Nishizawa et al. (2015) and *Nishizawa et al. (2016)* reported a similar tolerance mechanism between *O. officinalis* and *O. latifolia*. Both species accumulated high content of Na^+ content in lower leaf and maintained constant net photosynthetic rate under high salinity conditions. *O. officinalis* has a positive relationship between net photosynthetic rate and stomatal conductance. In contrast, the relationship between net photosynthetic rate and stomatal conductance was not related to *O. latifolia.* These studies provided strong support that there are other potential salinity tolerance-related genes preserved in wild relatives of rice. The development of salinity tolerance rice varieties should integrate the understanding of these unique tolerant mechanisms from wild relatives of rice with existing ideal yield, quality, and biotic stress resistance traits in elite commercial rice. Comparison between lines within same species or between species in broader level are needed to identify more potential mechanisms and genes related to salinity tolerance.

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1.3.1 A halophytic species in the Oryza family: Oryza coarctata

Among the 24 *Oryza* species, only two species (*O. sativa*- AA and *O. glaberrima*- AA) are extensively cultivated and domesticated. According to the cytogenetic classification of *Oryza* (Ge et al., 1999), they are grouped into 10 genome types: diploid- AA, BB, CC, EE, FF, and GG; allotetraploid- BBCC, CCDD, HHJJ, and HHKK. *O. coarctata* is designated as the HHKK genome. In the present study, the selected halophyte species- *O. coarctata*, a tetraploid (2n=48) wild relative of *O. sativa*, is a native species in South Asia. *O. coarctata* was originally classified under the *Porteressia* genus based on its morphology and embryo anatomy by Tateoka (1965). Sengupta and Majumder (2010) discussed the studies that contributed to the reposition of *P. coarctata* in the *Oryza* genus (Ge et al., 1999, Flowers et al., 1990, Latha et al., 1998). Ge et al. (1999) further suggested that *O. coarctata* is an earlier origin than *O. sativa* as the KK genome shows closer relation with DD and HH genomes based on the phylogenetic reconstruction of rice. However, Lu et al. (2009) reported that there is no homology between HH subgenomes in *O. coarctata* and *O. ridleyi* based on the Monoculm genomic regions comparison. The report suggested that *O. coarctata* has a unique genome type and should be designated as KKLL.

O. coarctata is widely distributed in mangrove ecosystems of the coastline in India, Bangladesh, and Pakistan. As a pioneer species, *O. coarctata* plays an important role in coastline protection from seawater erosion. It also can tolerate inundation of seawater twice a day with EC content up to 20-40 dSm^{-1} as none of the other salt-tolerant rice cultivars can survive under such extreme saline conditions (Sengupta and Majumder, 2009). Furthermore, Gaut (2002) estimated that the divergence between rice and other temperate region grasses was around 46 million years ago (MYA). Rice is the only one that required hydroponic cultivation compared to other cereal crops. It is also relatively more susceptible to salinity due to its root

structures compared to other cereal crops (Chen et al., 2016b). Therefore, *O. coarctata* is an ideal model plant for research on salinity tolerance in *Oryza* species.

Table 1.1 General information of members of the *Oryza* genus (Sengupta and Majumder, 2009, Singh et al., 2018).

Species	Genome	Complex	Habitat	Status	Origin
O. sativa	AA	*O. sativa*	Glyco	Domesticated	Asia
O. glaberrima	AA	*O. sativa*	Glyco	Domesticated	Senegal
O. nivara	AA	*O. sativa*	Glyco	Wild	India
O. rifipogon	AA	*O. sativa*	Glyco	Wild	Thailand
O. longistaminata	AA	*O. sativa*	Glyco	Wild	Tanzania
O. breviligulata	AA	*O. sativa*	Glyco	Wild	Africa
O. meridionalis	AA	*O. sativa*	Glyco	Wild	Australia
O. glumaepatula	AA	*O. sativa*	Glyco	Wild	America
O. punctata	BB	*O. officinalis*	Glyco	Wild	Ghana
O. officinalis	CC	*O. officinalis*	Glyco	Wild	Philippines
O. rhizomatis	CC	*O. officinalis*	Glyco	Wild	Sri Lanka
O. minuta	BBCC	*O. officinalis*	Glyco	Wild	Philippines
O. eichingeri	CC	*O. officinalis*	Glyco	Wild	Sri Lanka
O. malampuzhaensis	BBCC	*O. officinalis*	Glyco	Wild	India
O. alta	CCDD	*O. officinalis*	Glyco	Wild	Surinam
O. grandiglumis	CCDD	*O. officinalis*	Glyco	Wild	Brazil
O. latifolia	CCDD	*O. officinalis*	Glyco	Wild	Guatemala
O. australiensis	EE	*O. officinalis*	Glyco	Wild	Australia
O. longiglumis	HHJJ	*O. ridleyi*	Glyco	Wild	Indonesia
O. ridleyi	HHJJ	*O. ridleyi*	Glyco	Wild	Thailand

O. granulata	GG	*O. meyeriana*	Glyco	Wild	Sri Lanka
O. meyeriana	GG	*O. meyeriana*	Glyco	Wild	Philippines
O. brachyantha	FF	Unclassified	Glyco	Wild	Sierra Leone
O. schlechteri	KKLL	Unclassified	Glyco	Wild	Papua New Guinea
O. coarctata	KKLL	Unclassified	Halo	Wild	India

Abbreviations: Halo- halophyte; Glyco- glycophyte; IRRI- International Rice Research Institute

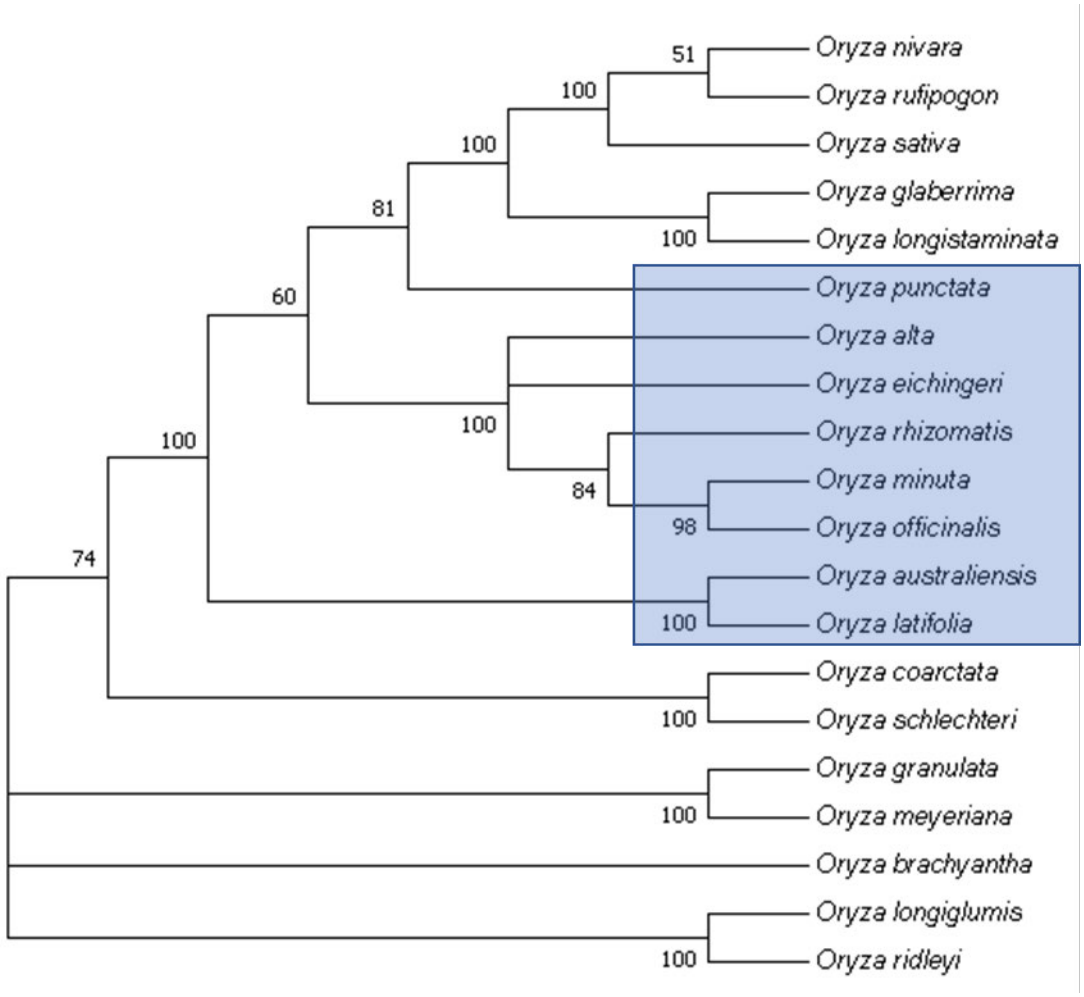

Figure 1.3. Maximum likelihood tree showing the phylogenetic relationship of *Oryza* genus with bootstrap values shown in percentage based on the Tamura 3-parameter model (Tamura, 1992). The evolutionary relationship of adh2 of wild and cultivated *Oryza* was analyzed using MEGA 7 software. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories ($^{+}$G, parameter = 0.6975)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The highlighted species are the members of the *O. officinalis* complex (Shenton et al., 2020).

1.3.2 Morphological feature and salinity tolerance studies of O. coarctata

To adapt to the coastal environment, *O. coarctata* has developed some unique morphological features compared to the cultivated rice. Its highly differentiated rhizome system deposits high content of mechanical tissue to tolerate the daily intrusion of seawater (Sengupta and Majumder, 2010). Reproductive propagation is rare for *O. coarctata* as the seed always dehisced before ripening. It normally propagates through vegetative propagation from nodes of the rhizome where the leaf buds derive. Roots of *O. coarctata* do not grow deep, which may be due to the fact that it is unnecessary to grow through the soil profile, where nutrient availability is consistent in the coastal area. *O. coarctata* branches like a runner from pseudo taproot to anchor firmly to protect itself from seawater inundation (Sengupta and Majumder, 2010).

O. coarctata's leaves are very waxy in contrast to those of the cultivated rice. The unicellular trichome salt hairs on leaves are the most important morphological advantage supporting its establishment in a highly salinized environment (Bal and Dutt, 1986, Flowers et al., 1990). These finger-shaped salt hairs on the adaxial leaf epidermis can continuously secrete salt at any conditions and crystals are formed on the leaf surface, whereas peg-shaped salt hairs on the abaxial side of the leaf epidermis swell and collapse after a certain amount of salt accumulates and regrow at low salt concentration. *O. coarctata* has a very successful estuarine adaptation strategy to maintain a low Na^+:K^+ ratio in the cytosol. Flowers et al. (1990) reported that *O. coarctata* starts to lose water content in 25% artificial seawater when the EC is above 10 dSm^{-1} though the dry matter was not affected. Meanwhile, *O. coarctata* maintained the Na^+:K^+ ratio at 0.7 in mesophyll cell, 1.3 in the root, and 7.3 in root hairs with a Na^+:K^+ ratio of 35 in the environment. Bal and Dutt (1986) reported that *O. coarctata* can exclude around 0.5 g Na^+ per kg of fresh leaves per day under such salinity level. Sengupta and Majumder (2009) reported a greater Na^+:K^+ ratio of 0.1 in root and 0.05 in shoot under salinity level of 20

to 40 dSm^{-1}. The calcium content in the root of *O. coarctata* increased significantly with increased salt treatment compared to the increment of calcium content in the root of salt-sensitive and tolerant rice cultivars. This indicated that the role of calcium in salinity tolerance in *O. coarctata* might be critical. Proteins responsible for salt tolerance in different roles such as ROS detoxification, photorespiration, and cell wall biosynthesis, were highly upregulated in salt-stressed (200 mM and 400 mM NaCl) leaves (Sengupta and Majumder, 2009).

O. coarctata cannot avoid constant contact with saline water with a minimum EC level of 20 dS m^{-1} even though it has very efficient salt excluding micro-hairs. It reveals the potential strong surviving mechanisms possessed by *O. coarctata* to fight against osmotic stress and Na^+ influx from the external solutions. Unfortunately, there are very limited numbers of studies on the physiological performance of *O. coarctata* under saline water conditions, especially regarding the study of its root and leaf cells. Furthermore, cation concentration in leaf and dry matter were the only two measurements that were repeated in these studies, which did not show a similar trend (Shabala and Cuin (2008). Two main points can be concluded from these basic physiological studies of *O. coarctata.* Firstly, *O. coarctata* is similar to most plants in that it accumulates higher Na^+ and K^+ content in shoot than root (Bal and Dutt, 1986, Flowers et al., 1990). The K^+ content is barely decreased with the increasing concentration of salt treatment (Sengupta and Majumder, 2009). This reveals that *O. coarctata* might have very strong K^+ long-distance transport and retention mechanisms. Secondly, *O. coarctata* accumulates a much higher root calcium content than in salinity-tolerant cultivated rice varieties (Pokkali). Therefore, calcium is likely to be an important factor in salinity tolerance of *O. coarctata* by preventing sodium influx and K^+ efflux from NSCC and other calcium-activated mechanisms.

Table 1.2. Summary of salinity tolerance-related gene studies in *O. coarctata.*

Genes	Protein name	Highlight	Reference
OcSrp	serine-rich protein	the transgenic plant exhibited increased tolerance to salinity stress	(Mahalakshmi et al., 2006)
OcINO1	myo-inositol 1 phosphate synthase (MIPS)	first salt-tolerant MIPS	(Dastidar et al., 2006, Sengupta et al., 2008)
OcCFR	Chloroplastic fructose-1,6-bisphosphatase	facilitates photosynthetic activity, protection from ROS damage, OcCFR remain active up to 500mM NaCl conditions while OsCFR is inactivated	(Chatterjee et al., 2013, Ghosh et al., 2001)
OcVHA	H^+-ATPase	no study on functional differences between *O. sativa* and *O. coarctata* has been done;	(Senthilkumar et al., 2005)
OcNHX1	tonoplast Na^+/H^+ antiporter	no study on functional differences between *O. sativa* and *O. coarctata* has been done;	(Kizhakkedath et al., 2015)
OceIF1	translation initiation factor 1	Gene up-regulation was observed in salinity, mannitol, and ABA treatment.	(Latha et al., 2004)

A very limited number of genes were functionally characterized in *O. coarctata* so far (Table 2). OcINO1 is the most studied gene from *O. coarctata* which is responsible for MIPS production, which is the first salt tolerance-related MIPS recognized in a monocot. Detailed physiological processes of salt-tolerant mechanisms of this MIPS were reviewed (Sengupta and Majumder, 2010). Functional study of the genes from *O. coarctata* indicated that they are better in salinity tolerance than genes from *O. sativa*. *OcVHA* and *OcNHX1* are the only two transporter-related genes reported so far, however, the functional difference has not been compared with the same transporters from *O. sativa.* Therefore, this Ph.D. study will focus on the transporter and signaling genes in wild *Oryza* species such as *O. coarctata* that are not yet being compared with orthologous genes from *O. sativa*.

1.4 Salinity tolerance related ion transporters

1.4.1 Na transporters

When salinity stress occurs, Na influx is mainly mediated through non-selective cation channels (NSCC) (Tester and Davenport, 2003). This family can be separated into 2 groups- cyclic nucleotide-gated channels (CNGC) and glutamate receptors (GLRs). This family has high selectivity for K^+ over Na^+, which K^+/Na^+ selectivity ratio of NSCCs ranged between 0.3 and 3 (Demidchik et al., 2002). Shabala et al. (2006) reported that this ion channel family is responsible for part of NaCl-induced K efflux via voltage-gated NSCC. This can be regulated by PM repolarization via H^+ ATPase (Maathuis and Sanders, 2001, Zepeda-Jazo et al., 2008, Demidchik and Tester, 2002). Some of the NSCC transcripts of Arabidopsis such as *CNGC1*, *CNGC3*, *CNGC8*, *CNGC19,* and *CNGC29* were upregulated after salinity treatment.

Jayakannan et al. (2015) suggested that salicylic acid-responsive GLRs might involve in positive Na^+ regulation under saline conditions. In contrast, NSCCs except depolarization gated members can be potentially active channels involved in Na^+ and K^+ regulation under saline conditions. In most halophytes, the influx of Na^+ via this channel can be beneficial for salinity tolerance by reducing the osmotic potential of the plant when the plant has very active Na^+ compartmentation activities in vacuoles.

Na^+ exclusion and compartmentation from cell-extracellular and vacuole have mainly relied on the NHX family. NHX family is a member of the monovalent cation-proton antiporter (CPA) superfamily. It is responsible for K^+/Na^+ and pH homeostasis for regular plant growth. Chanroj et al. (2012) reported that the NHX family is highly conserved in algae and plants via phytogenic study which indicated the importance of NHX function in plants. In Arabidopsis, all of the NHX members prefer K^+ transport over Na^+ under normal conditions. There are only 2 members who will act as Na^+ antiporters under saline conditions. These two NHX members are NHX7/SOS1 (salt overly sensitive 1) responsible for Na^+ exclusion at PM and NHX1 responsible for Na^+ compartmentation at tonoplast. Figure 2.2 showed the localization of different NHX transporter in Arabidopsis cells). Transgenic study of NHX7 and NHX1 confirmed their important role in Na regulation induced by salinity stress (Yadav et al., 2012, Zhang et al., 2001, Zhang and Blumwald, 2001, Apse et al., 1999). Surprisingly, the transgenic study of NHX2 and NHX3 also showed improved salinity tolerance of transgenic plants by the accumulation of K^+ in vacuoles (Liu et al., 2008, Rodríguez-Rosales et al., 2008). Theoretically, NHX1 activities promoted Na^+ compartmentation, as the consequence, the plant is more salinity tolerance due to the reduction of cytosolic Na^+ content. Accumulation of K^+ in vacuoles should result in a reduction in cytosolic potassium and thus less salt tolerance in the transgenic plant according to the case of NHX1. Yet, the transgenic NHX2 and NHX3 results showed opposite results (Liu et al., 2008, Rodríguez-Rosales et al., 2008) and revealed two potential mechanisms

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behind NHX activities. Enhanced K^+ dynamic between cytosol and vacuole (Walker et al., 1996) might be critical for salinity tolerance (Bassil and Blumwald, 2014). Intracellular osmotic pressure regulation plays an important role in salinity tolerance to stimulate other related activities in the cell. This can also explain why Na^+ compartmentation plays important role in salinity tolerance, when the influx pressure of extracellular Na^+ is so high to refill the cytosolic cation content is lost due to Na^+ compartmentation. The role of compartmentation is more or at least equally important than the type of cation being compartmented.

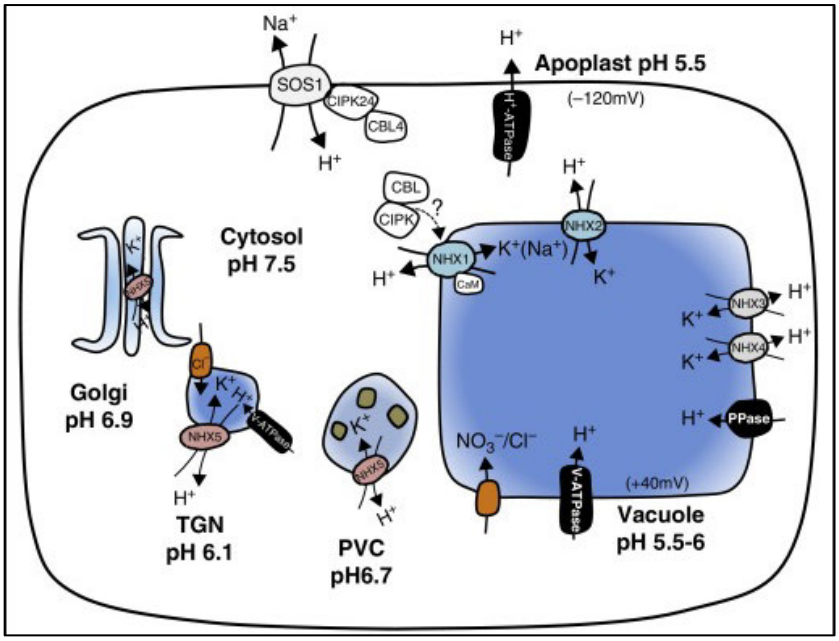

Figure 1.4. Distribution of 8 NHX members in Arabidopsis and its function. TGN- trans Golgi network, PVC- prevacuolar compartment. (Adapted from Bassil and Blumwald (2014)).

The importance of High-affinity K^+ transporter (HKT) in salinity tolerance was detailed in many reviews (Horie et al., 2009, Horie et al., 2008, Hamamoto et al., 2015). According to the phylogenic and functional comparison between HKT isoforms, the HKT family can be divided into two subfamily- HKT1 and HKT2. HKT1 family is highly selective toward Na^+ over K^+. HKT2 is normally permeable for both Na^+ and K^+.

HKT1 is confirmed as one of the most important transporters in salinity tolerance by Na retrieval from long-distance transportation. It is especially important to rice as bypass uptake of Na^+ in rice's root is higher than other plant species due to its leaky root structure. It was found at the PM of the stele cell in the root and PM of the xylem parenchyma cell in the shoot. Møller et al. (2009) reported that stellar-specific expression of *HKT1*;*1* responsible for 37-47% of Na^+ removal from the shoot and led to a significant increase in salinity tolerance. The growth of *OsHKT1;1* overexpressing line was not affected under 100 mM NaCl treatment while the growth of WT suffers a 19 to 37% reduction in biomass. It highlighted the importance of Na^+ retrieval from transpiration stream in xylem for salinity tolerance. For Na^+ retrieval from leaf, Horie et al. (2005) hypothesized a phloem sap Na^+ recirculation model based on the localization of AtHKT1;1. In this model, HKT1;1 will also indirectly promote K^+ acquisition from phloem to leaf due to membrane depolarization induced by Na^+ influx in xylem parenchyma (Horie et al., 2009). However, the later studies suggested that the phloem sap Na^+ recirculation model is unlikely (Horie et al., 2005, Davenport et al., 2007, Berthomieu et al., 2003). It is quite surprising as the removed Na has no place to go except compartmentation into the vacuole. Few studies have confirmed that Na retrieval from leaf xylem has only a very minor effect to improve salinity tolerance (Kobayashi et al., 2017, Byrt et al., 2007, Suzuki et al., 2016). Kobayashi et al. (2017) reported that OsHKT1;5 also mediated Na^+ exclusion in phloem parenchyma cells to prevent Na^+ transport to phloem sieve tubes and translocated to young leaf blades with upward sap flow.

Recent studies found that OsHKT2;4 can also transport Ca^{2+} and Mg^{2+} (Lan et al., 2010, Horie et al., 2011). It is thus likely to be an important transporter of Ca^{2+} for Ca^{2+} signaling in plants under salinity stress. OsHKT2;1 is responsible for Na^+ influx and promotes plant growth in rice and its transcripts and activities are enhanced under K^+ starvation conditions so that plants can use Na^+ as an osmolyte to substitute potassium (Horie et al., 2007). Horie et al. (2007)

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also reported that OsHKT2;1 was downregulated by protein kinase inhibitors under saline conditions. This confirmed its Na^+ influx role and does not contribute to salinity tolerance. However, Mian et al. (2011) reported that overexpression of *HvHKT2*;1 improved plant growth in the presence of salinity stress (50-100mM NaCl) with increased Na^+ uptake. It will be interesting to know the role of HKT2 members in halophytes such as *O. coarctata*, especially for those who were mainly responsible for Na^+ uptake in other species.

1.4.2 K^+ transporters

PM hyperpolarization induced K^+ uptake is mainly controlled by NSCC and inward rectifying K^+ channel AKT1/ regulatory K^+ channel KC1 complex. Hyperpolarization-activated AKT1 and KC1 belong to the Shakers family. AKT1 is an inward rectified potassium channel that is controlled by PM hyperpolarization. KC1 was found as a general modulator of shakers inward rectifying channels in Arabidopsis but not for outward rectifying channels (Jeanguenin et al., 2011, Grefen et al., 2010, Honsbein et al., 2009). Under saline conditions, PM depolarization induced by Na^+ influx causes passive K^+ uptake via AKT channel to be nearly impossible. When the osmotic gradient between root and soil solution reached equilibrium, K^+ efflux through PM is still not avoidable even the plant has very strong K^+ retention mechanisms such as high proton pump activity and reduced GORK activity. The question is how the K^+ is lost in this dynamic system replenished when the Na influx pressure is high in the external environment. If the activity of AKT is suppressed under saline conditions, K^+ replenishment through the HAK transporters may be the only way to maintain the balance. Furthermore, the plant can also replenish H^+ via HAK and generate more proton pumps to maintain PM potential. Then, what is the role of AKTs in halophytes? If the function of AKT in halophyte is always suppressed by salinity, then its presence in halophyte can be an evolutionary failure, which is

unlikely. *AKT* transcriptions in the presence of salinity stress were reduced (Fuchs et al., 2005, Kaddour et al., 2009). Garriga et al. (2017) suggested that AKT downregulation is promoted by KC1 to minimize K^+ efflux through AKT under saline conditions. Although *AKT* gene expression in halophytes was maintained in *P. tenuiflora* (Ardie et al., 2010) and upregulated in *S. salsa* (Duan et al., 2015) under saline conditions, electrophysiological properties of AKT from halophyte need to be conducted to provide convincing conclusions.

The guard cell outward rectifying K^+ channel (GORK) channel is named based on its function and place that it has been first discovered in guard cells of Arabidopsis (Blatt, 1988, Blatt and Gradmann, 1997, Ache et al., 2000). In the later studies of this channel, different names were used based on the genus of the studied plant, such as ZORK in *Zea mays* (Büchsenschütz et al., 2005) and NtGORK in *Nicotiana tabacum* (Dai et al., 2009). It has been grouped as PM depolarization activated of the Shaker K^+ channel family. The patch-clamp studies of its activities in guard cells confirmed the outward rectifying activity is voltage and external K^+ dependent, and sensitive to external pH value (Ache et al., 2000, Becker et al., 2003, Li et al., 2016). GORK transcriptions were found in every part of the plant but the expression is relatively higher at root hair, root vascular (Nguyen et al., 2017), flower (Kim et al., 2015b), and guard cells. Becker et al. (2003) reported that GORK transcription by ABA signaling is highly dependent on Ca^{2+} signaling as its transcription was highly suppressed without Ca^{2+} supplement compared to control. Corratgé-Faillie et al. (2017) found that GORK activity was enhanced with CPK33 co-expression in the oocyte, which supported the relationship between Ca^{2+} signaling and GORK. ABA-induced GORK expression in Arabidopsis *ABA insensitive 1* (*abi1*) mutant was reduced compared to WT (Becker et al., 2003). However, ABI1 was found to inhibit GORK activity. Lefoulon et al. (2016) found direct physical interaction between GORK and PP2CA. GORK activity was inactivated in GORK-PP2C interaction. Therefore, ABA signaling promoted GORK's activity by binding with ABA insensitive as ABA-

PYR/PYL/RCAR complex. Meanwhile, GORK transcription is upregulated by an uncertain mechanism downstream of the ABA signaling such as Ca^{2+} signaling. The transcription is obviously affected by the presence of ABA insensitive (Becker et al., 2003) which indicated an interaction between ABA insensitive and the mechanism responsible for GORK expression or feedback signal from ABA insensitive to stimulate more GORK transcription. However, this model does not seem to work for GORK transcription in stomata as Becker et al. (2003) found GORK expression was insensitive to ABA signalling. Surprisingly, Nguyen et al. (2017) suggested that GORK is also responsible for long-distance transport of potassium in rice as they found high gene transcription in the vascular bundle and *Osk6.2* mutant showed less shoot accumulation than root. Whether its role in long-distance K^+ transport is direct involvement, or it indirectly affects transporter located at stele is still unknown. GORK's activity under salinity stress is shown to have negative effect on plant by increasing K^+ efflux from roots. Studies showed that salinity-tolerant *Brassica* species (Chakraborty et al., 2016) and barley (Adem et al., 2014) were better in preventing salinity stress induced GORK transcription. Jayakannan et al. (2013) found that NaCl induced K efflux was reduced in plant pre-treated with Salicylic acid (SA) but there was no difference between SA treatments in *gork-1*. Among factors that reduce K efflux in SA pre-treated plants, enhanced H^+-ATPase activity is believed be the main factor. This result indicated the conversely role between SA and ABA (Planes et al., 2014) on H^+ ATPase in plant roots. By concluding the characteristic of GORK to date, it is a gate which openness is highly dependent on external issues such as PM potential, hormones signaling, H^+ flux etc. Plant will try to decrease the quantity of GORK or make the cytosol conditions less favourable for GORK activities under stress conditions.

KT/HAK/KUP transporters were first found in plants based on homology to the bacterial K^+ Uptake permease (KUP) and fungal High-affinity K^+ transporter (HAK). KT/HAK/KUP transporters comprised a big family responsible for K uptake and translocation

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in plants. Plantae is the only kingdom that preserved these transporters in all species, none are found in animal cells and are only present in certain species in other kingdoms (Feng et al., 2020b, Chen et al., 2017b). There are 13 KT/HAK/KUP in *Arabidopsis*, 27 in rice, 21 in tomato, and 57 in *Panicum virgatum.* The cluster classification by the phytogenic tree comparison was different between studies. Nieves-Cordones et al. (2016) suggested that this transporter family should be divided into five clusters based on the full sequences comparison of 46 angiosperm genomes (913 sequences). Véry et al. (2014) and Yang et al. (2009) suggested 4 clusters classification of this family by comparing coding sequences of various dicot and monocot species although there are some differences in the grouping of cluster III and cluster 1-b. Despite the difference in cluster classification, (Véry et al., 2014) and (Nieves-Cordones et al., 2016) indicated the uneven distribution of KT/HAK/KUP members between clusters among plant species. Gierth and Mäser (2007) studied the contribution of AKT1 and AtHAK5 in K acquisition by comparing the Rb^+ uptake in the mutants and the WT. They concluded that AKT1 is a low-affinity channel and HAK5 is a high-affinity transporter which total Rb^+ uptake of two transporters in Arabidopsis was estimated as 84% under low Rb^+ and 74% under high Rb^+ environment. This highlighted the importance of cluster Ia in regulating K uptake compared to the remaining 12 HAK members as AtHAK5 is the only member in cluster 1a. The growth of akt1 hak5 *Arabidopsis* double mutant was similar to WT, when it was supplied with millimolar level of K^+ (Pyo et al., 2010). This indicated that other low-affinity HAK members such as AtHAK7 (Han et al., 2016) has compensatory effect under high K conditions while both of the major K transporters were eliminated from the system. In rice, salinity studies were mainly focused on the members from KT/HAK/KUP cluster Ia. OsHAK1 (Chen et al., 2015), HAK5 (Yang et al., 2014), and HAK21 (Shen et al., 2015) were highlighted for their contribution in maintaining K^+ uptake and translocation under salinity stress. Salinity treatment of mutants indicated their role in K uptake and translocation under low K^+ conditions induced by salinity.

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s. However, expression is downregulated in low K^+ salinity treatment compared to in low K^+ conditions. The result indicated that activity and transcription of *OsHAK1* is sensitive to salinity treatment since K^+ is limited under salinity conditions. Transcription of *OsHAK21* was upregulated under saline conditions though the effect of low external K^+ was not tested. Salinity induced expression of *OsHAK21* is mainly in stele cell instead of PM of root epidermis. Moreover, *OsHAK5* expression response to salinity stress was found modest between root leaf and sheath. Overexpression lines showed significant increase in shoot K^+ content compared to WT, and thus, dry matter of shoot is 50% higher than DW of shoot in WT. Therefore, HAKs might play important role in K^+ transport under saline conditions and their roles in the salinity tolerance of wild rice species require further investigation.

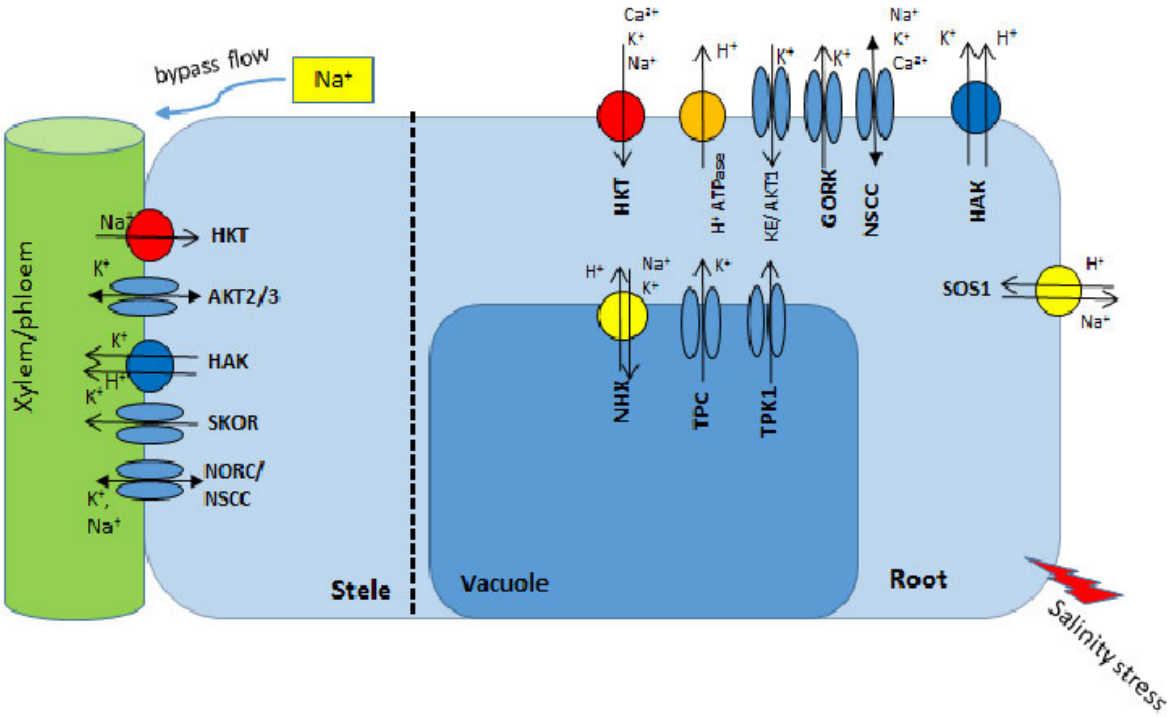

Figure 1.5. Schematic diagram of transporters and channels responsible for salinity tolerance modified from (Shabala and Cuin, 2008).

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1.5 Aims of the thesis

Soil salinization is a major threat to global crop cultivation. Soil salinization is estimated to affect the addition of 0.3 – 1.5 Mha of farmland per year. In India and Australia, major rice cultivating regions are severely threatened by soil salinization. It is especially important for rice cultivation as rice is in large demand and susceptible to salinity stress. To be able to secure rice production, the world is urged to breed salinity-tolerant rice. In the past, breeding for salinity-tolerant varieties was acquiring gene materials from salt-tolerant landrace (*O. sativa*), however, the outcomes were not satisfying. *O. sativa* might have already lost some salinity-tolerant genes in centuries of domestication. This highlights the need to assess salinity tolerance of near relative of *O. sativa*.

Does wild rice contain unique salinity tolerance traits and genes to cope with salinity stress? The overall objective of this Ph.D. project was to identify salinity-tolerant wild *Oryza* and evaluate potential tolerant mechanisms presented in salinity-tolerant wild *Oryza* species. I hypothesized that wild *Oryza* species have developed highly effective salinity tolerance mechanisms to cope with the salinity environment, especially in the halophyte wild rice *O. coarctata*.

To answer this research question, four experiments were designed and conducted to:

1. Understand the physiological responses of leaf tissue of rice cultivars to salinity treatment in both greenhouse and field trials to deepen the understanding of salinity stress responses at the reproductive stage (Chapter 3)
2. Evaluate salinity tolerance of wild *Oryza* species in both greenhouse and field trials through agronomical, physiological, and molecular approaches (Chapters 4 & 5)

3. Decipher novel mechanisms through evaluating the role of potential C3-C4 photosynthetic characteristics in *O. coarctata* for adaptation to the extreme saline environment (Chapter 4)

4. Identify salinity tolerance-related transporters in mesophyll by evaluating Na^+ distribution in mesophyll cells and salinity tolerance-related components in leaf epidermis via RNA-Sequencing (Chapter 6)

Chapter 2: Materials and Methods

2.1 Plant material and trial setup

2.1.1 Trial 1: Greenhouse salinity trial setup- three commercial Oryza sativa varieties in Australia

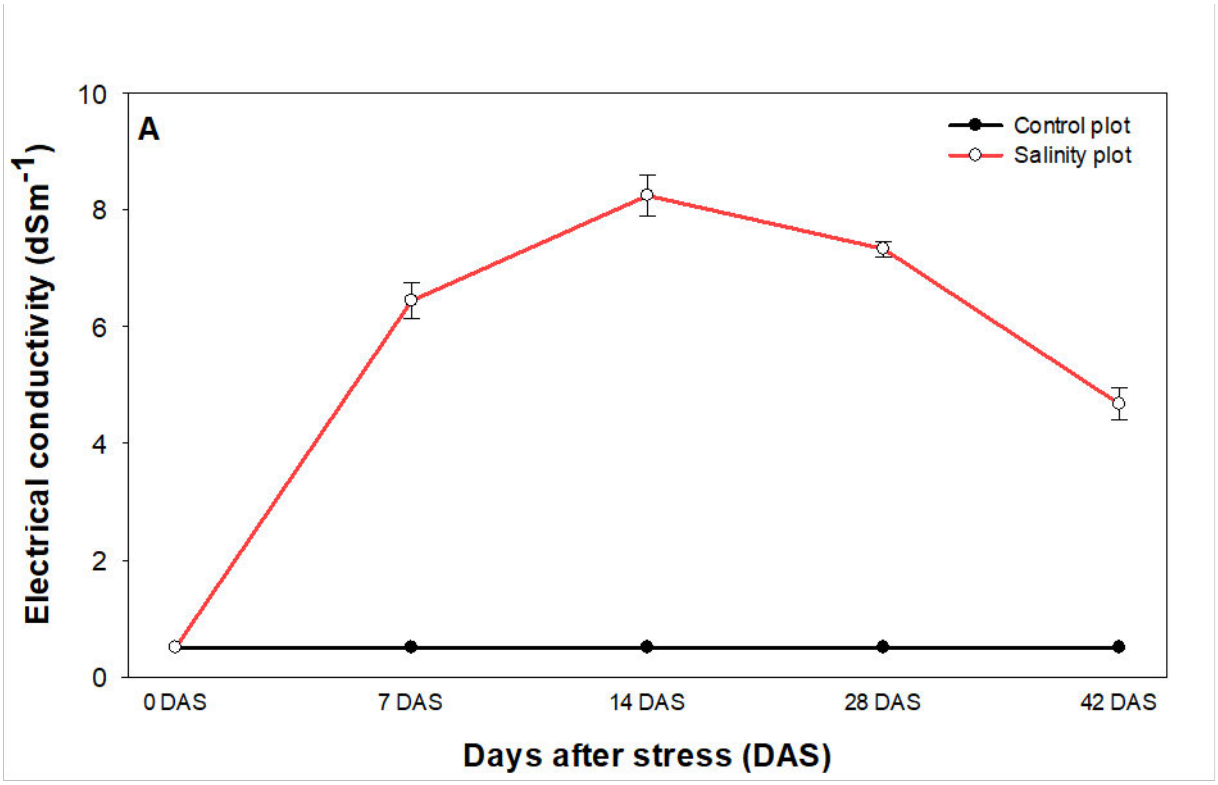
Trials 1 and 2 evaluated the salinity tolerance of three Australian cultivars- Koshihikari, Doongara, and Reiziq in the greenhouse and field, respectively. Seedlings of the three cultivars were raised in sand and then transferred to 9 L buckets filled with a loamy sandy soil (collected from the field trial site), macronutrient (Yates, NSW, Australia), and micronutrient supplements (Manutec, SA, Australia). In a greenhouse at Western Sydney University, Hawkesbury Campus (33.62º S, 150.75º E), the rice plants were grown under controlled day/night temperatures of 30ºC/24ºC and supplemental high-pressure sodium (HPS) lights with a 14h/10h day/night cycle. Relative humidity was maintained at 70%–80%. Additional fertiliser was administered before the start of the salinity application. The initial salinity value of the soil was 0.25 dSm^{-1}. Incremental salinity treatments commenced at flag leaf initiation (65 days after sowing) using NaCl with increments of 2 dSm^{-1} in EC per day until a salinity of 8 dSm^{-1} was reached. For each treatment and cultivar, 12 replicates (12 buckets with 3 plants per bucket) were used for the different measurements. Water levels were maintained up to a height of 30 mm above the soil by daily watering with tap water. The EC values during salt treatment were monitored and maintained at the desired level throughout the growing season.

2.1.2 Trial 2: Field salinity trial setup- three commercial Oryza sativa varieties in Australia

The field experiment was conducted at the Western Sydney University farm, Hawkesbury Campus (33.62º S, 150.75º E) in 2018. The weather conditions at the site over the trial period were obtained from the weather station at Richmond (33.60º S, 150.78º E), NSW (Figure 2.2_B-C). Six controlled rice paddy plots were prepared (3.5 m × 2.5 m × 0.75 m) and lined with water-proof plastic layers to retain the water and salt. Fertilizers were applied at a basal rate of 50:40:40 kg ha^{-1} N:P: K and an equal amount of N was applied at the tillering and late vegetative stages (1 week before salinity treatment). The seedlings were transplanted at a density of 40 plants m^{-2}. Salinity stress was imposed 65 days after sowing by scattering NaCl into the three treatment plots every 2 days to reach 8 dSm^{-1} as calculated based on the volume of soil and water solution using an increment of 2 dSm^{-1} of EC. Soil EC was measured (Figure 2.2_A) using a FieldScout Soil Sensor Reader with WaterScout 300 soil Moisture/EC/Temperature Sensor (Spectrum Technologies, Inc., USA).

Physiological responses and gene expression of the three rice cultivars Koshihikari, Doongara, and Reiziq (lines suitable for Australian weather conditions) to salinity stress were evaluated at the reproductive stage both in the greenhouse and field experiments. Salinity treatment was applied when >50% of plants entered the panicle initiation stage. Plant height was measured fortnightly. Biomass, tiller number, and elemental content were measured 42 days after salinity was applied (DAS). Net ion (K^+, Na^+, and Cl^-) flux and ROS production in mesophyll cells of the flag leaves were measured fortnightly after salt stress. Chlorophyll contents and gene expression in flag leaves were measured at 42 DAS.

Figure 2.1. Rice growth in the paddy at 21 days after transplanting (35 days after sowing). Two main blocks were setup up for 2 salinity treatments (Control and 8 dSm^{-1}). In each block, it was subdivided into 6 subblocks as 6 replicates.



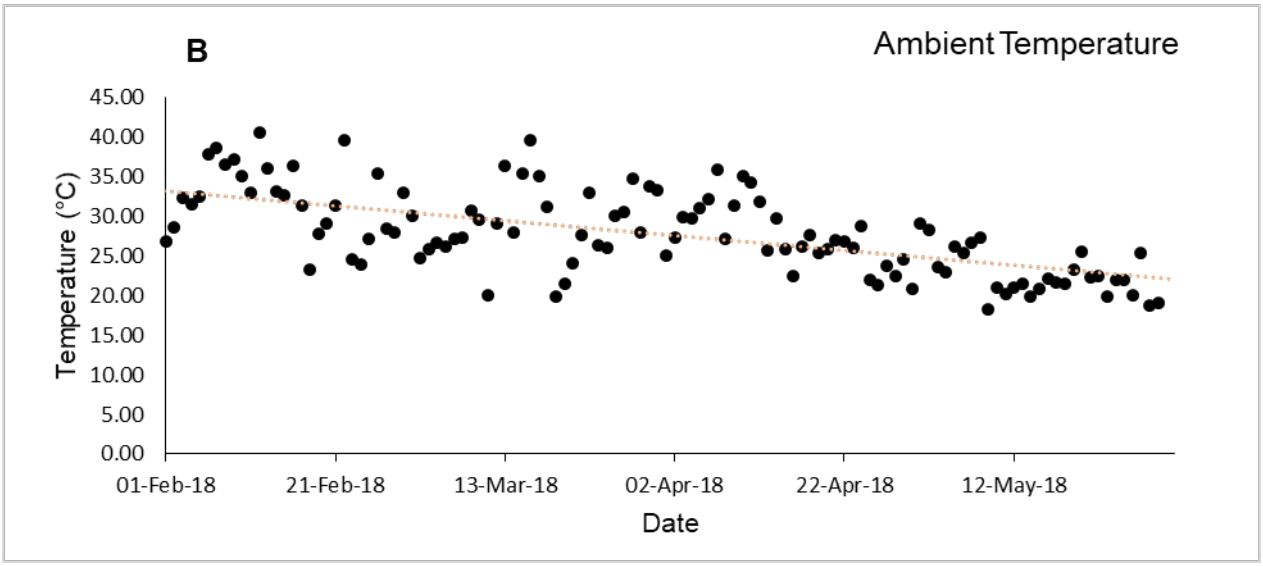


Figure 2.2. Field soil electrical conductivity (EC) and ambient temperature of the growing season. EC of control and stressed plot were measured fortnightly (A); Daily maximum temperature of the field site since transplantation of seedlings (B) Data are sourced from Bureau of Meteorology website, the weather station at Richmond (33.60º S, 150.78º E).

2.1.3 Trial 3: Greenhouse trial setup for salinity study of wild rice

Six wild rice species (*O. longiglumis* Jansen, *O. australiensis* Domin, *O. rufipogon* Griff., *O. latifolia* Desv., *O. officinalis* Wall. ex Watt and *O. coarctata* Roxb.) and two *O. sativa* subspecies indica cultivars (IR64 and Pokkali) were used in the greenhouse trial at Western Sydney University, Hawkesbury Campus (33.62º S, 150.75º E). Seedlings (14 days old) of five wild rice species and the two cultivars of *O. sativa* were raised in sand for 14 days and then transferred to 9 L buckets filled with loamy sand textured soil. The remaining wild rice, *O. coarctata* was prepared through vegetative propagation, by separating freshly emerged tillers (2 leaves tiller) from parent plants. The young tillers were recovered in Yoshida nutrient solution (Yoshida et al., 1971) for 14 days and transferred to the 9 L buckets with loamy sand soil. The final salinity treatment was maintained for six weeks before the collection of leaf samples for the measurement. The times of sowing and vegetative propagation of the wild and cultivated rice species were synchronized to ensure that the plants were in the same growth stage during the measurement. Fertilizers were applied at a basal rate of 50:40:40 kg ha^{-1} N:P:K and an additional 25 kg ha^{-1} of N fertiliser was administered at the early tillering stage and before the start of the salinity application. The greenhouse was maintained with controlled day/night temperatures of 29ºC/24ºC and supplemental HPS lights with a 14/10 h day/night cycle. Relative humidity was maintained at 70–80%.

For each treatment and rice line, 4 replicates (4 buckets with 2 plants per bucket) were used for the different measurements. Water levels were maintained up to a height of 30 mm above the soil by daily watering with tap water. The initial EC of the soil was 0.25 dSm^{-1}. Incremental salinity treatments commenced 55 days after sowing using NaCl with increments of 2 dSm^{-1} per day until a salinity of 10 dSm^{-1} was reached. The EC values were monitored and

maintained at the desired level throughout the growing season. After 42 days of salinity stress, plant height, tiller number, and biomass were measured. The 1st and 2nd fully expanded leaves were collected for measurement of electrophysiological properties of mesophyll tissue, and determination of concentrations of Na and ROS imaging in mesophyll cells, chlorophyll contents, nutrient analysis, gas exchange measurement, and quantitative real-time PCR (qPCR).

2.1.4 Trial 4: Hydroponic trial setup- O. coarctata preparation for leaf ion localization

O. coarctata seedlings were prepared through vegetative propagation, by separating freshly emerged tillers (tillers with 2 leaves) from parent plants. The young tillers were recovered in hydroponic solution with Yoshida nutrient formula in a conical flask (Yoshida et al., 1971). Each beaker was covered with aluminum foil to minimize exposure to sunlight of the roots . Koshihikari seeds were germinated in dark at 30 °C for 2 days and transferred to Yoshida solution. The solution was renewed every 3 days. After 2 weeks of growth (both *O. coarctata* & Koshihikari), an incremental salinity stress treatment was applied through the subsequent addition to the Yoshida solution of 25, 50, and 100 mM NaCl at 2-day intervals. For chapter 4, leaf of *O. coarctata* was collected after 1 month of salinity stress, Na^+ localization in leaf tissue was measured using confocal imaging. For chapter 6, leaf samples were collected for acute salinity treatment and evaluated using Microelectrode ion flux estimation (MIFE) and confocal imaging. Leaf samples were also collected on the 7th day of stress to measure Na^+ distribution using confocal microscopy and to harvest leaf epidermis for RNA- sequencing.

Figure 2.3. Leaf sample preparation for MIFE, confocal microscopy, and RNA-Seq analysis. 1- Cross-sectioned *O. coarctata* leaf for confocal imaging; 2- Isolated leaf abaxial epidermis for RNA-sequencing; 3-4- Leaf sample preparation for MIFE measurement. The epidermis of the measured site was removed, marked, and submerged in buffer solution prior to measurement.

2.1.5 Trial 5: Field trial setup for salinity evaluation of wild rice

This trial was designed to assess the salinity tolerance of wild rice evaluated in Chapter 4 under open field conditions. Additional to the seven species used in Chapter 4, *O. alta*, salt-sensitive *O. sativa* (IR29), Koshihikari, *O. brachyantha*, and *O. punctata* were selected. Unfortunately, most of the *O. longiglumis, O. officinalis*, and IR64 seedlings failed to establish in the field due to extreme heat conditions which occurred the day after the transplantation so they were not included in the field trial. Therefore, the field trial evaluated salinity tolerance of seven wild *Oryza* species (*O. brachyantha* A. Chev. and Roehr, *O. punctata* Kotschy ex Steud., *O. australiensis* Domin, *O. rufipogon* Griff., *O. latifolia* Desv., *O. alta* Swallen, and *O. coarctata* Roxb.) and three *O. sativa* cultivars (Koshihikari, IR64, and Pokkali). The field experiment was conducted at the Western Sydney University farm, Hawkesbury Campus (33.62º S, 150.75º E) in 2019. Six controlled rice paddy plots were prepared (10 m × 2.5 m × 0.75 m) and lined with water-proof plastic layers to retain the water and salt. Fertilizers were applied at a basal rate of 50:40:40 kg ha^{-1} N:P:K and an equal amount of N was applied at the tillering & late vegetative stages (1 week before salinity treatment). The seedlings were transplanted at a density of 40 plants m^{-2}. Incremental salinity treatments commenced 55 days after sowing using NaCl with increments of 2 dSm^{-1} per day until a salinity of 10 dSm^{-1} was reached. The EC values were monitored and maintained at the desired level throughout the growing season.

Physiological responses of the wild rice to salinity stress were evaluated at the late vegetative stage after 42 days of salinity stress. The measurements included plant height, tiller number, gas exchange properties, and biomass. The 1st and 2nd fully expanded leaves were collected for measurement of electrophysiological properties (net K^+, Na^+, Cl^- and Ca^{2+} flux) of mesophyll tissue, determination of concentrations of Na and ROS in mesophyll cells through confocal imaging, and measurement of gas exchange properties using LICOR-6400.

Figure 2.4 Gowth of *Oryza* species (40 days old) in the paddy before the application of salinity treatment. Six main blocks were setup for 2 salinity treatments (Control and 10 dSm^{-1}).

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2.2 Elemental content analysis

In this thesis, X-ray fluorescence spectrometry (**Chapter 3**) and flame photometry (**Chapter 4**) were employed for the elemental content analysis of plant tissue. In Chapter 3, shoot macronutrient (K, Ca, and Mg), Na, and micronutrient (Cl, Mn, Fe, Cu, Zn, and Si) concentrations were analyzed by XRF using a Niton XL3t XRF analyzer (Mak et al., 2019). Dry samples were finely ground using a Geno Grinder. ~1 g of samples was pressed into a round sample holder (2cm diameter) sealed with polyester film. The holders with the sample were loaded into the autosampler to measure the elemental content. Elemental concentrations of the shoot were calculated using standard calibration curves.

In Chapter 4, K^+ and Na^+ contents in leaves were measured using a flame photometer (Jenway PFP7, John Morris) modified from Chen et al. (2007a). The 1^{st} and 2^{nd} fully expanded leaves of each plant were harvested and oven-dried at 60 °C for 2 days. The dried leaf samples were finely ground and mixed using Retsch Mixer Miller 400. Aliquots (~50 mg) of each sample were digested in 4 ml of concentrated HNO_3 (69%) in a boiling water bath until the sample solution was clear. The solutions were then diluted to 100 mL using MilliQ water for measurement. Na and K standard curve was calibrated using six NaCl and KCl solutions at a concentration of 0, 10, 50, 100, 500, and 1000 μM, respectively.

2.3 Ion flux measurement

Transient ion flux response to acute NaCl treatment and recovery ion flux measurements from leaf mesophyll cells after 1 hr of recovery from excision were measured in this thesis. Both are useful tools that reflect salinity response and the extent of damage after prolonged salinity and drought stress (Mak et al., 2014, Wu et al., 2018a). Microelectrode preparation and

measurement protocols were according to Shabala et al. (2012). Before the measurement, cross-sectioned leaf samples (2 cm length) were clamped in Perspex measuring chambers and submerged with standard MIFE solution (0.5 mM KCl, 0.1 mM $CaCl_2$) for 1 h (the recovery phase). For each combination of species, treatment, and/or growth stage using plants from the different trials, the measurements were repeated with 5- 8 biological replicates Net ion fluxes were calculated using MIFEFLUX software based on the ion concentration gradient recorded between two positions.

In Chapters 3-5, steady-state net K^+, Na^+ Cl^- and Ca^{2+} fluxes after recovery were measured from leaf mesophyll cells of flag leaves using non-invasive MIFE. The cross-sectioned leaf sample was measured in 3 mL of MIFE solution. Five minutes of steady-state ion flux reading were taken for each sample. To narrow down the corresponding K^+ efflux channel contributed to K^+ efflux detected in recovery K^+ flux measurement, GORK's activities of variety (Koshihikari) with the highest K^+ efflux was measured. The leaf section of salt-stressed Koshihikari from greenhouse trial (Chapter 3) was submerged in 40 mM NaCl solution for 1 hour prior to measurement. Steady state ion flux of leaf section in 40 mM NaCl was measured to record the initial K^+ flux, and then 20 mM of K^+ channel blocker-Tetraethylammonium (TEA^+) was applied, and flux response was measured for another 15 minutes.

In chapter 6, transient response of K^+ Na^+ and H^+ flux of leaf (salt-tolerant- *O. coarctata* & salt-sensitive Koshihikari) in response to 100 mM NaCl treatment was measured. The leaf section (2×2 cm^2) was clamped in Perspex measuring chambers and submerged with standard MIFE solution (0.5 mM KCl, 0.1 mM $CaCl_2$) for 1 h before the measurements. Net ion flux was first measured for 5 minutes in the MIFE solution to record the initial flux. 100 mM NaCl treatment was then applied, and transient ion flux was measured for 20 minutes, then finally 50

mM of K^+ channel blocker Tetraethylammonium (TEA^+) was applied to evaluate K^+ efflux channel activities differences between tolerant and sensitive species.

2.4 Chlorophyll content

Chlorophyll contents were determined following (Lichtenthaler and Buschmann, 2001) with slight modification (**Chapter 3** & **4**). Ten milligrams of homogenized leaf samples were placed in Eppendorf tubes with 1 mL of 80% acetone and incubated in the dark overnight. Absorbance values were obtained at 470, 649, and 664 nm using a SPECTROstar^{Nano} spectrophotometer (BMG Labtech). The chlorophyll concentrations (C_a = chlorophyll a; C_b = chlorophyll b) were determined by following the equation of Lichtenthaler and Buschmann (2001). Total chlorophyll content was the sum of chlorophyll a & b contents.

$$C_a = 12.25A_{664} - 2.79A_{649}$$

$$C_b = 21.50A_{649} - 5.10A_{664}$$

2.5 Gas exchange traits measurements

The *LI-6400XT* Infra-red gas analyzer (LI-COR, Lincoln) was employed to measure gas exchange parameters from the fully expanded leaves modified from (Liu et al., 2017b) in **Chapters 4** and **5**. The parameters were Net CO_2 assimilation rate (A), stomatal conductance (g_s) and transpiration rate (E), intercellular CO2 concentration (C_i) leaf vapor pressure (VpdL), leaf temperature (Tleaf), and water use efficiency (WUE). The chamber conditions were programmed as follows: Flow rate- 500 mL min^{-1} µmol s^{-1}, Reference CO^2- 400 µmol m^{-2} s^{-1}, Chamber Temperature- 30ºC, light intensity- 1000 µmol m^{-2} s^{-1}. The chamber reference

humidity was maintained between 55-65% in the greenhouse. The reference humidity in the field was already below 45 % without the interference of the desiccator. Three biological replicates were randomly selected for measurement. In each measurement, the leaf was held in the chamber for 3-5 minutes before the reading was taken.

For the A/Ci curve measurements of *O. coarctata* (only in **Chapter 4**), the chamber conditions were set as follows: flow rate, 500 mL min^{-1}, block temperature, 30°C; light intensity, 1000 µmol m^{-2} s^{-1}, and reference CO_2, 425 µmol m^{-2} s^{-1}. Prior to the measurement, the selected leaf was clipped in the setup chamber for 20 minutes. Net CO_2 assimilation rate was recorded at each incremental ambient CO_2 rate of 425, 600, 800, 1000, 1250, 1500, 1750, and 2000 µmol m^{-2} s^{-1}. The reading at each lower ambient CO_2 concentration of 350, 250, 150, 100, and 50 µmol m^{-2} s^{-1} were taken after 20 minutes of adaptation at 425 µmol m^{-2} s^{-1} from 2000 µmol m^{-2} s^{-1}. The CO_2 compensation point was measured using the A/C_i curve according to (Laisk, 1977).

2.6 Confocal microscopy

Confocal microscopy was employed to evaluate ROS & Na accumulation in leaf tissue in **Chapters 3-6**. The production of ROS in plant cells was measured using confocal microscopy according to (Wang et al., 2017a, Wu et al., 2015c). Leaf epidermis was removed by using a scalpel or hand-peeled depending on the age and texture of the leaf. Based on the targeted tissue (mesophyll or epidermis), the leaf tissue was stained using 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) to monitor cellular oxidative stress or stained using CoroNa Green to localize Na^+ distribution in the targeted tissue. The leaf tissues (mesophyll or epidermis) of 1st or 2nd fully expanded leaf were incubated in a measuring buffer-MB (10 mM KCl, 5 mM Ca^{2+}-MES, pH 6.1) with 20 µM of CM-H2DCFDA

or 50 µM of CoroNa Green for 1 h in the dark at room temperature. The stained sample was washed three times using the measuring buffer solution after 1 hour of staining. Fluorescence images were taken to capture ROS & Na^+ in the tissue using an upright Leica laser scanning confocal microscope SP5 with 50 × objective lenses (laser power: 10%, excitation laser: 488nm (20%), emission range: 505–550, filter: TP488/543/633). 3 – 4 biological replicates were prepared for each treatment/species. ROS florescent intensity was then quantified on a cell-by-cell basis using ImageJ software (https://imagej.nih.gov/ij/).

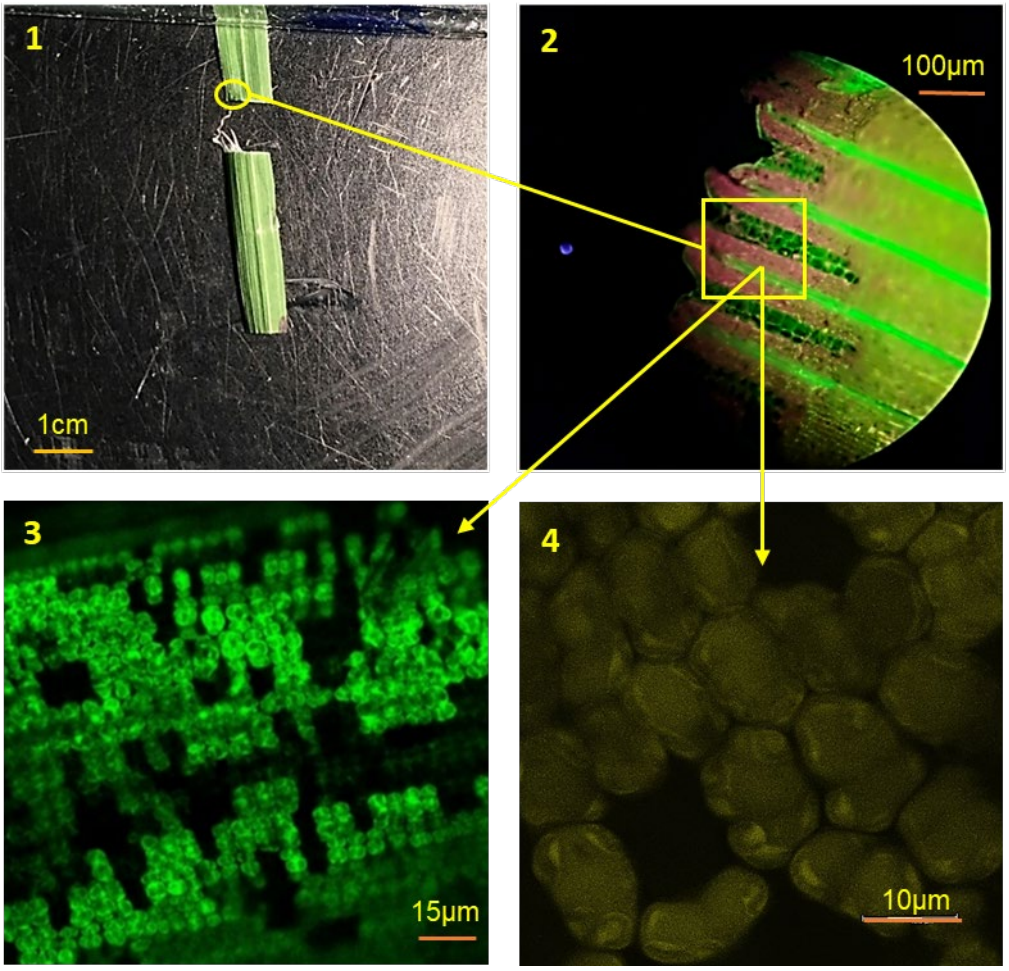

Figure 2.5. The epidermal layer was removed before staining with ROS dye- CM-H2DCFDA and Na^+ dye CoroNa Green (1-2). Examples of ROS (3) and Na^+ (4) fluorescence detected in mesophyll cells of *Oryza sativa* (3) and *Oryza coarctata* (4).

2.7 Quantitative real-time PCR

Expression of the salinity tolerance-related genes tonoplast Na^+/H^+ antiporter (*NHX1*), V-type proton ATPase subunit C (*VHA-C*), K^+ outward rectified channel (*GORK*), respiratory burst oxidase homolog D (*RbohD*), high-affinity potassium transporter 1;4 (*HKT1;4*), Na^+/H^+ antiporter (*SOS1*) and high-affinity potassium transporter (*HAK1*) and C4 pathway-related genes NADP-dependent malic enzyme (*NADPME*), phosphoenolpyruvate carboxylase (*PEPC*), pyruvate phosphate dikinase (*PPDK*), ribulose-bisphosphate carboxylase (*rbcL*) and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*) (Liu et al., 2014, Wang et al., 2016b), was quantified using qPCR. Total RNA was extracted from the first fully expanded leaf samples collected in Trials 1, 2, and 3 at 42 DAS in **Chapter 3** and **Chapter 4**. Reverse transcription was performed as per the manufacturer's instructions (Bioline, Australia). qPCR was performed using Quantinova SYBR Green Kit (QIAGEN, USA) in a Rotor-Gene 3000 quantitative PCR instrument (QIAGEN, USA). Relative gene expression was calculated using the comparative threshold cycle (C_t) $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Elongation factor 1-alpha (EF1A) were used as two internal reference genes. The experiments were conducted with three biological replicates and three technical replicates. The primer pairs are listed in Table 2.1 and Table 2.2.

Table 2.1. Primers of transporters & reference gene- *OsGAPDH* used for gene expression analysis in Trial 1 & 2 (**Chapter 3**).

Target Gene	Primer Name	Primer Sequence
HAK	OsHAK1-F	CTCATCATCATCCCCATGCT
	OsHAK1-R	CGGGAGATCAGCGAGTAGAG
HKT1;4	OsHKT1;4-F	TTTGAGCTTTCTTCTCTCGGTGA
	OsHKT1;4-R	TGAGCCTCCCAAAGAACATCAC
GORK	OsGORK-F	GATAGGGAGCTTGCAGTTGG
	OsGORK-R	TCACAGCATGAATGTCACCA
SOS	OsSOS1-F	TGCTGCTAATCTGTCCATTCC
	OsSOS1-R	TGAAACCAGTTGACGGAACA
RBOHD	OsRBOHD-F	GCCCAACTACTGGTGGTTCGT
	OsRBOHD -R	TGCTTACCATTGGAACGGCG
GAPDH	OsGAPDH F	AAGCCAGCATCCTATGATCAGATT
	OsGAPDH R	CGTAACCCAGAATACCCTTGAGTTT

T**able 2.2.** Primers of transporters, C4 pathway-related genes, and reference genes GAPDH & ELF-a, used for gene expression analysis in Trial 3 (Chapter 4).

Target Gene	Primer Name	Primer Sequence
HAK	HAK1-F	CTCATCATCATCCCCATGCT
	HAK1-R	CGGGAGATCAGCGAGTAGAG
HKT1;4	HKT1;4-F	TTTGAGCTTTCTTCTCTCGGTGA
	HKT1;4-R	TGAGCCTCCCAAAGAACATCAC
GORK	GORK-F	GATAGGGAGCTTGCAGTTGG
	GORK-R	TCACAGCATGAATGTCACCA
SOS	SOS-F	TGCTGCTAATCTGTCCATTCC
	SOS-R	TGAAACCAGTTGACGGAACA
VHA-C	VHA-C-F	CTGTCGTTCTTTTTAGCACTATGG
	VHA-C-R	GGTGACAGGATGGCCTGA
NHX	NHX-F	CTGTCGTTCTTTTTAGCACTATGG
	NHX-R	GGTGACAGGATGGCCTGA
Rubisco SS	Rubisco-SS-F	ACTCCAGCTTCGGCAACGTCAGCA
	Rubisco-SS-R	ATACGGACGAATGCATCAGGGTAC
Rubisco LS	Rubisco-LS-F	ACACTGATATCTTGGCAGCATTCCGAG
	Rubisco-LS-R	GTAGAGCGCGTAGGGCTTTGAAAC
PEPC	PEPC-F	GGAAGAAGATTTCTCCAGGAGAACCTTA
	PEPC-R	CAAGAACTGCTCGACATTGGTGTAAGTC
NADPME	NADPME-F	CTGATACAGTTTGAGGACTTCGCCAATC
	NADPME-R	GAGCAATGAGTTCTGCAATACCAGTTCC
PPDK	PPDK-F	CACGAACGACCTTACGCAGA
	PPDK-R	ACGGATCAAACGCCATCAC
	G6PDH F	AAGCCAGCATCCTATGATCAGATT

G6PDH	G6PDH_R	CGTAACCCAGAATACCCTTGAGTTT
ELF-a	ELF-a-F	CAGCAACTTGACTATGGATTGGTGGA
	ELF-a-R	CATCCAGCACAAACATCTTAATGTGGTC

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2.8 RNA- sequencing and data analysis of leaf abaxial epidermis of *O. coarctata*

2.8.1 Leaf epidermal tissue preparation

Leaf epidermal tissue preparation was modified from (Zhao et al., 2019a). The epidermal layer of control and salinity stressed leaf (1 week) on the abaxial side were peeled from a young and fully expanded leaf. The epidermal peels were immediately transferred to the opening buffer (50 mM KCl, 5 mM MES, 50 μM $CaCl_2$, 0.1% PVP-40, pH 6.1 adjusted with KOH) in the ice bath for 1 hour. The sample was then stored in liquid nitrogen until RNA extraction.

2.8.2 RNA isolation and sequencing

Epidermis RNA was extracted using a Qiagen RNeasy Plant Mini Kit. Nanodrop, Agilent Bioanalyzer 2100, and Agilent RNA 600 Pico Kit were employed to determine RNA quality. Sequencing of the control and stressed leaf epidermis (biological replicates= 3) was performed by the commercial service provider- the Next Generation Sequencing Facility (Sydney, Australia). Illumina Hi-seq 2500 platform was employed for RNA-sequencing of the leaf epidermis. RNA Library construction was conducted using the Illumina TruSeq™ RNA Sample Preparation Kit (Dai et al., 2014). Low-quality data (Q<30 and length<104 bp) was removed and adaptor sequences were at the 3' ends. RNA-Seq reads were assessed for quality control with FastQC (version 0.10.1; Babraham Bioinformatics, Cambridge, UK). Reads were mapped to a reference genome- *Oryza sativa subsp. japonica* Nipponbare is available at Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) using HISAT version 0.1.6 (Kim et al. (2015a)). The transcript abundances were measured as fragments per kilobase of exon per million fragments mapped (FPKM) by Cufflinks (Trapnell et al., 2010).

2.8.3 Differential genes analysis and GO enrichment analysis

Venn diagrams of expressed genes in the control and salt-stressed sample were generated at (http://bioinfogp.cnb.csic.es/tools/venny/). Principal component analysis of control and salinity stressed RNA was conducted using FPKM value in limma.RStudio (version 1.4.1106). The volcano plot of log_2FC was plotted using gglot2.package. Differentially expressed genes (DEG) in salt-stressed samples with $log_2FC>0.6$ and $log_2FC>1$ were identified using edgeR.package at False Discovery Rate $FDR<0.05$. Gene ontology analysis of DEG was conducted using databases from GeneOntology (http://geneontology.org/), and AgriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php).

2.9 Statistical analysis

ANOVA, Duncan's multiple range test, independent samples t-tests, Pearson's correlation, and mean value analysis were performed using SPSS Statistics Version 22-24. For all of the relative value calculations, average data in salinity treatment was divided by the average of control. All graphs and tables were prepared using SigmaPlot 12.0 and Microsoft Excel.

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Chapter 3: Leaf mesophyll K^+ and Cl^- fluxes and Reactive Oxygen Species production predict rice salt tolerance at reproductive stage in greenhouse and field conditions.

3.1 Abstracts

Rice, as a staple food for more than half of the world population, is susceptible to salinity stress suffering from significant growth and yield reductions. Extensive research on salinity tolerance in rice has been mostly carried out at the seedling stage in single experimental trials. Here, I aimed to understand the roles of ion transport and oxidative responses of leaf mesophyll in salinity tolerance of rice (*Oryza sativa* L.) at its reproductive stage using comparative investigations in both greenhouse and field trials. Two experimental trials were conducted to assess the salt tolerance of three rice cultivars at their reproductive stage in the greenhouse and field. I employed agronomic, physiological, electrophysiological, molecular and cell imaging techniques to compare the physiological response of control and salinity stressed rice plants. I found that K^+ retention and low recovery Cl^- efflux in mesophyll cells confers salt tolerance in rice. Also, dynamic ROS production and regulation of the NADPH oxidase gene, *OsRBOHD,* in mesophyll cells is crucial for the salinity tolerance of rice at the reproductive stage. There were strong correlations among recovery ion effluxes, gene expression, and growth parameters in response to salinity in both greenhouse and field conditions. This study brings together, for the first time, potential links between cellular ionic stress and oxidative stress components of salinity tolerance in rice at the reproductive stage in both greenhouse and field conditions. This study will provide guidance to examine crop salinity tolerance at reproductive stages in controlled environments and natural climatic conditions in the future.

3.2 Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops in the world; it is also a culturally irreplaceable crop in most Asian countries. By 2055, the global population is forecast to approach 11 billion and Asian countries will contribute to half of this population growth between 2019 and 2055 (Grieve et al., 2019). As a consequence, the demand for rice is projected to increase by 13% per annum for the next 10 years (FAO, 2017). This increase in rice production cannot be achieved through the expansion of rice cultivating areas due to growing urbanization, land use prioritization, industrial water demand, environmental conservation, and production of other crops and livestock. Stronger reliance on irrigation practices and sea water intrusion in some rice-growing areas are predicted to exacerbate soil salinization issues, thus reducing rice production in existing growing areas (Gonzalez-Perez and Neuerburg, 2019, Maggio et al., 2011). This calls for an urgent need to develop salinity-tolerant rice varieties and to boost rice production in saline areas.

Rice is highly susceptible to salinity stress with significant growth and yield reduction observed under low salinity levels of up to 3 dSm^{-1} at seedling and reproductive stages (Munns et al., 2006, Asch and Wopereis, 2001, Lutts et al., 1995, Khatun and Flowers, 1995, Munns and Tester, 2008). High Na^+ permeability and significant Na^+ accumulation are believed to be the main reasons for salt susceptibility at the seedling stage in sensitive rice genotypes (Lakra et al., 2018, Nemati et al., 2011) due to higher unidirectional Na^+ influx (Kavitha et al., 2012, Malagoli et al., 2008). In addition, a sensitive rice variety, at early stages of salt stress, also shows greater Na^+ unloading (Ishikawa and Shabala, 2019). These findings suggest that rice has a low tissue tolerance to Na^+ accumulation, leading to intensive Na^+ exclusion response at early stress in the sensitive varieties (Chakraborty et al., 2019, Gerona et al., 2019, Lakra et al., 2018, Nemati et al., 2011, Prusty et al., 2018). This is in stark contrast to salt-tolerant wild rice

species that have higher leaf Na^+ accumulation and leaf osmotic potential (Prusty et al., 2018, Nakamura et al., 2002, Nishizawa et al., 2015). However, an understanding of leaf mesophyll tissue salinity tolerance mechanisms at the reproductive stage of rice is still limited, especially with regard to ion fluxes and changes in levels of ROS.

After the initial salinity-induced osmotic stress to plants, ionic and oxidative stress become dominant factors (Shabala et al., 2020, Munns et al., 2020a). For tolerance to ionic stress, high cytosolic K^+/Na^+ ratios of leaf mesophyll cells are crucial for maintaining photosynthesis, which is tightly linked to membrane transporters at the plasma membrane and tonoplast (Deinlein et al., 2014, Munns and Tester, 2008, Shabala et al., 2020, Shabala and Cuin, 2008). These membrane transport systems include HKT transporters, NHX antiporters, HAK transporters, two-pore K^+ channels, and Shaker K^+ channels (Wu et al., 2018b). While a basal level of ROS is essential to maintain regular metabolism and stress tolerance, excessive accumulation of ROS leads to tissue and cell damage (Gómez et al., 2019, Mittler, 2017). The signaling process leading to ROS generation is mainly carried out by members of the NADPH oxidase (RBOHs) family and many other ROS-related protein families (Xie et al., 2011, Wang et al., 2019a, Wang et al., 2016b). ROS production by various cell types can serve as a marker to test their response to abiotic stress conditions (Noctor et al., 2016, Zhang et al., 2018b, Bonales-Alatorre et al., 2013, Wang et al., 2016b, Zhao et al., 2019b). Previously, leaf tissue-specific ion homeostasis mechanisms in rice have been mostly examined in the seedling stage and under short-term salinity stress. Therefore, it is important to assess the ionic and oxidative responses of rice at reproductive stages under both greenhouse and field experiments.

In this study, I hypothesize that the physiological differences between phenotypes will match with other typical findings. In salinity-sensitive variety, I expect higher mesophyll-specific recovery K^+ efflux and ROS accumulation but lower Na^+ and Cl^- effluxes and *RBOHD*

expression in leaf tissues of reproductive stage rice plants, contributing significantly to the salt susceptibility in both the greenhouse and field. Here, recovery ion flux measurements, cellular ROS accumulation, nutrient analysis, and qPCR techniques were employed to determine the salinity tolerance of leaf mesophyll tissue over prolonged salinity treatments at the reproductive stage of rice in both greenhouse and field conditions. However, the results find that ion (K^+ Na^+ and Cl^-) effluxes were generally higher in sensitive variety. ROS accumulation in sensitive variety was higher at an earlier period of stress but was lower at a later period of stress.

3.3 Results

3.3.1 Effect of prolonged salinity stress on physiological traits of rice cultivars

Overall, prolonged salinity treatment at the reproductive stage had a strong, negative impact on rice growth in both the greenhouse and the field (Figure 3.1-3). Significant growth reductions were observed for plant heights (Figure 3.2_A, 3.2_D), biomasses (Figure 3.2_B, 3.2_E), tiller numbers (Figure 3.2_C, 3.2_F), shoot K^+ (Figure 3.3_A, 3.3_D), and chlorophyll contents (Figure 3.4), and increases in shoot Na^+ and Cl^- contents (Figure 3.3_B-C, 3.3_E-F) were found after 42-day of salt stress. In both greenhouse and field trials, Koshihikari was the most salt-sensitive cultivar to salinity stress with the greatest reduction in most of the tested physiological parameters. Reiziq was the most salt-tolerant cultivar with the greatest relative plant heights, biomasses, photosynthetic pigment contents, shoot K^+ contents, and the lowest relative shoot Na^+ and Cl^- contents after salinity stress in both greenhouse and field conditions (Figure 3.2, 3.4).

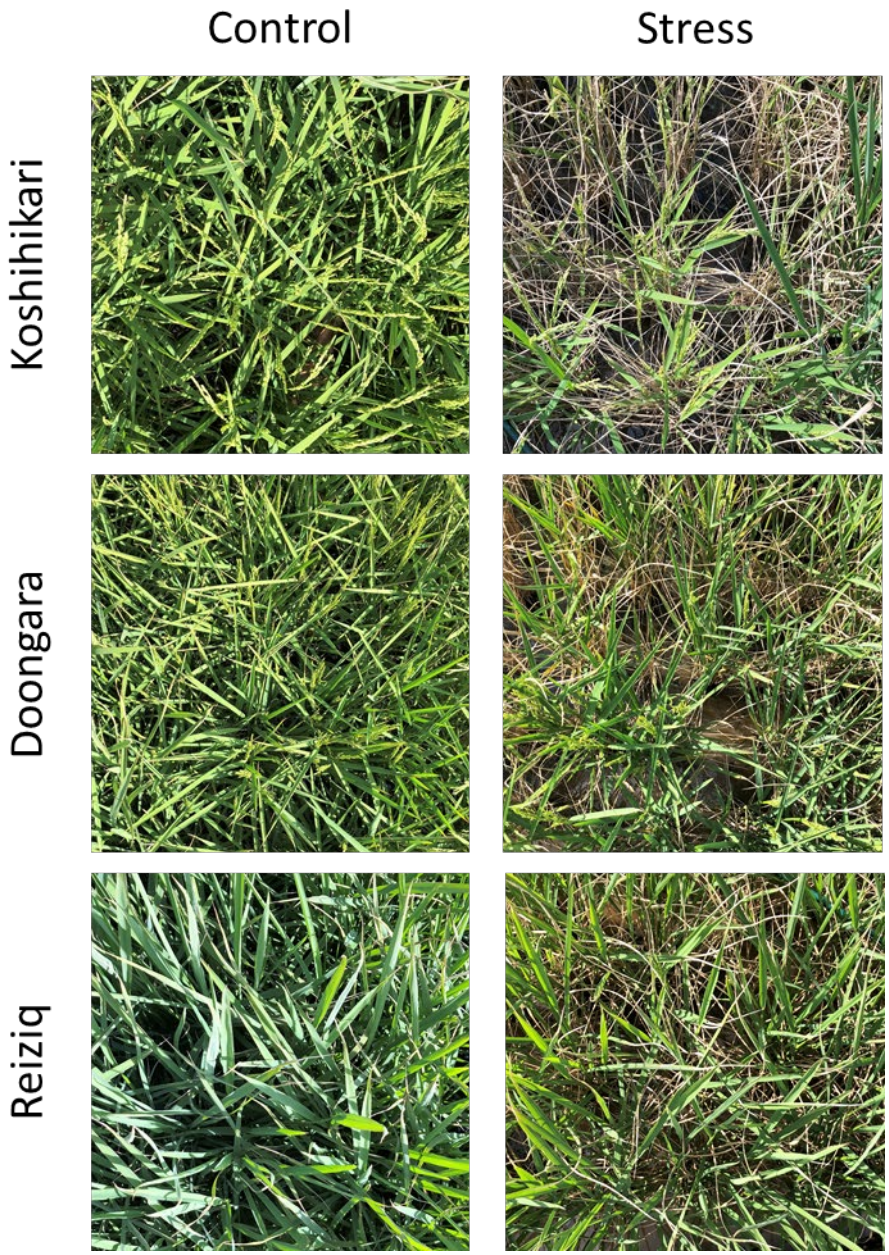


Figure 3.1. Comparison in visual appearance of three rice genotypes in response to salinity stress at 4th week of salinity stress.

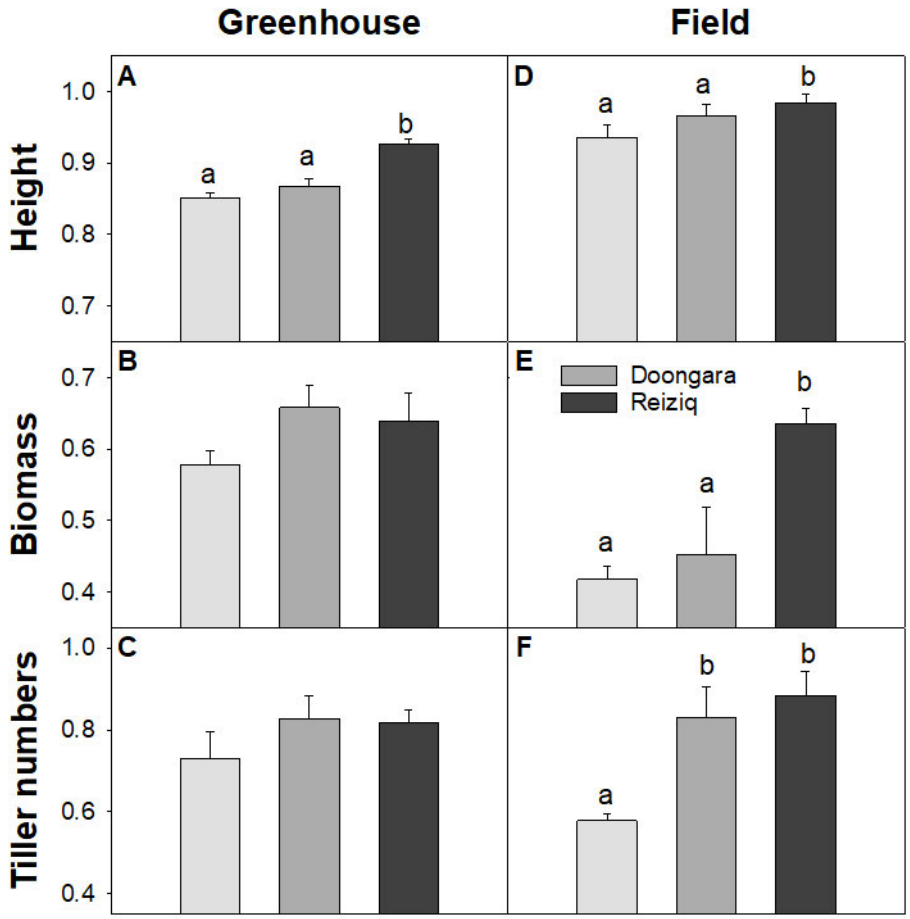


Figure 3.2. Effects of salinity on agronomical traits of three rice genotypes at their reproductive stage. Data are relative heights (A, D), relative tiller numbers (B, E), and relative biomasses (C, F) in the greenhouse (left column) and the field (right column) after prolonged salinity stress. Different lowercase letters indicate significant differences at $P<0.05$ (n=4).

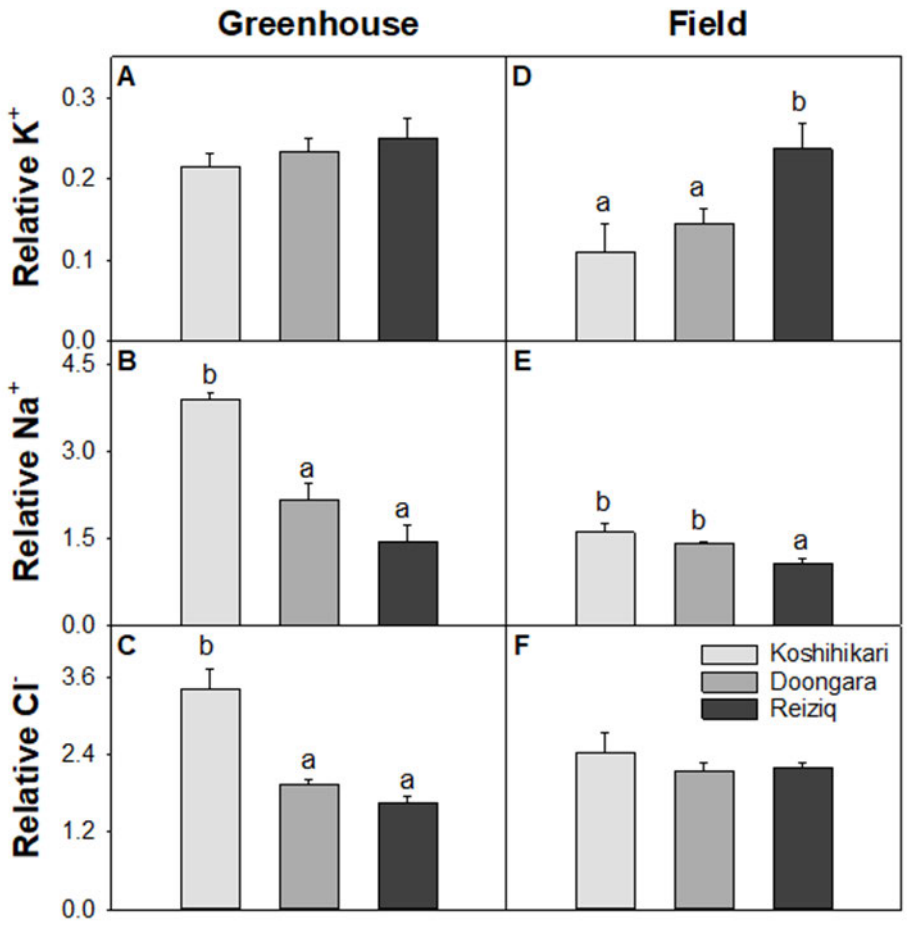


Figure 3.3. Effects of salinity on leaf ion contents of three rice genotypes at reproductive stage in both greenhouse and field conditions. Data are relative shoot K^+ (A, D), Na^+ (B, E), and Cl- (C, F) contents in the three rice varieties after prolonged salinity stress in the greenhouse (left column) and field (right column). Different lowercase letters indicate significant differences at $P<0.05$ (n=4).

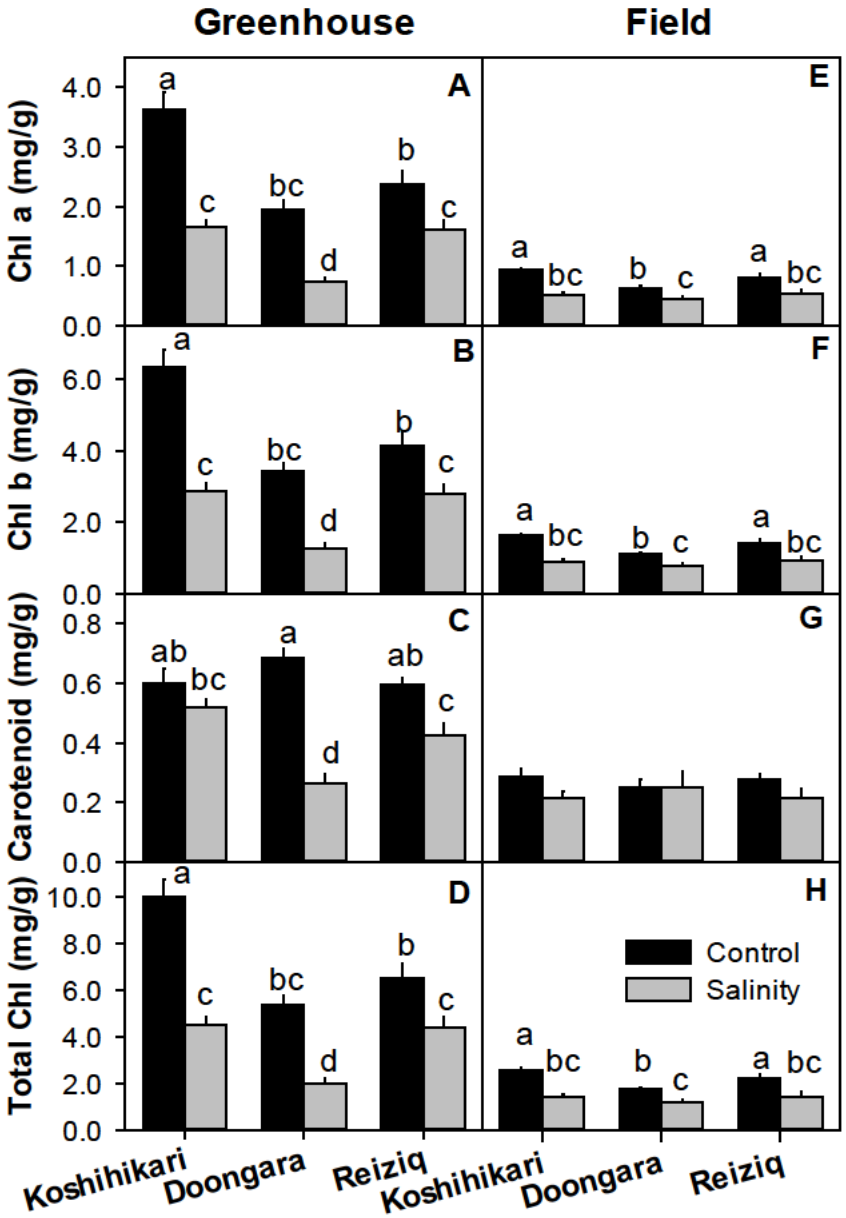


Figure 3.4. Effect of salinity stress on leaf chlorophyll content of three rice varieties at reproductive stage both greenhouse and field. Data are (A, E) chlorophyll a, (B, F) chlorophyll b, (C, G) carotenoid, and (D, H) total chlorophyll content after prolonged salinity stress in the greenhouse (left column) and field (right column). Different lowercase letters indicate significant differences at $P<0.05$ (n=4).

3.3.2 Mesophyll cells of salt-sensitive rice cultivar release more K^+, Na^+, and Cl^- after prolonged salinity stress at reproductive stage in recovery ion flux measurements

Leaf mesophyll ions fluxes after recovery were significantly different among the three genotypes, between the two experimental conditions and over the 42-days of salinity treatment (Figure 3.5). Control leaf segments of all three genotypes and time-course studies show relatively small fluxes, indicating good recovery of the mesophyll cells after 1 h of pre-incubation (Figure 3.5). The flux differences between control and salt-stressed samples increased with the prolonged stress duration in most cases (Figure 3.5). In general, Koshihikari showed the highest recovery K^+, Na^+, and Cl^- effluxes after salt treatment among the three cultivars in both trials. Net K^+ effluxes after recovery from leaf mesophyll were significantly higher in Koshihikari, especially at 42 DAS, and the effects of salinity on the effluxes were more pronounced in the greenhouse trial (Figure 3.5_A, 3.5_D). However, the difference between the three cultivars was marginal when recovery Na^+ fluxes were considered. A significant difference in recovery Na^+ efflux due to salt treatment among the cultivars was found only for Koshihikari; this cultivar showed higher recovery Na^+ effluxes than the other two cultivars particularly at 14 DAS in the field trial (Figure 3.5_B, 3.5_E). Significant differences in recovery Cl^- effluxes due to salt treatment were found in both trials; Koshihikari showed the highest recovery Cl^- effluxes and Reiziq the lowest (Figure 3.5_C, 3.5_F).

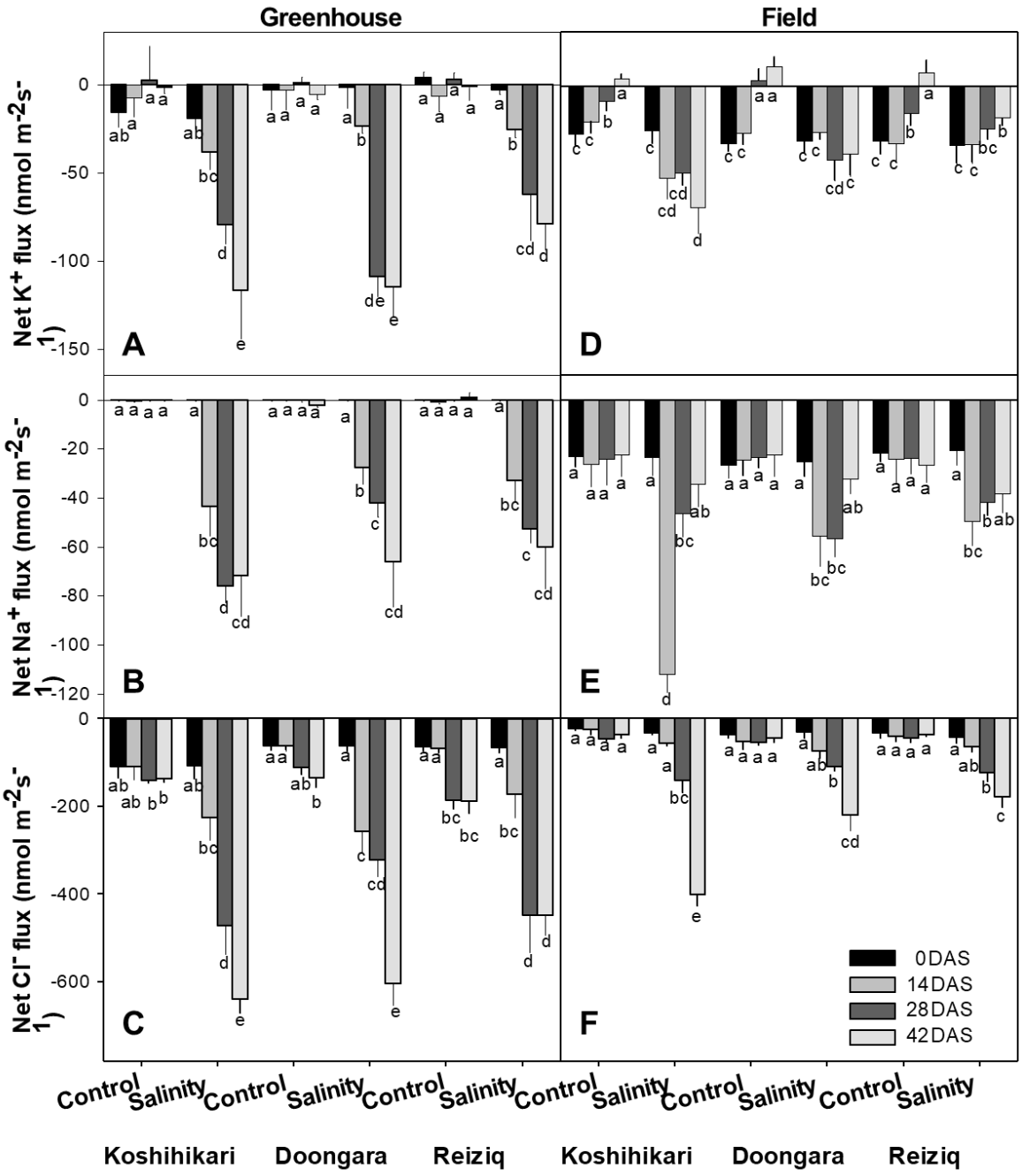


Figure 3.5. Effects of salinity on steady-state net ion fluxes of leaf mesophyll of three rice genotypes at their reproductive stage. Data are net K^+ (A, D), Na^+ (B, E) and Cl^- (C, F) fluxes of leaf mesophyll collected from control and salinity stressed plants from the greenhouse (left column) and the field (right column). The measurements were taken fortnightly until 42 DAS using a recovery ion flux protocol. Different lowercase letters indicate significant differences at $P<0.05$ (n=4-8 biological replicates).

3.3.3 Dynamic response of ROS production in rice leaf mesophyll cells to prolonged salinity stress

Salinity stress induces the accumulation of ROS in the early phase of stress that is important in stress signaling (Schneider et al., 2018). In this study, rice leaf mesophyll cell ROS production in all control samples from both greenhouse and field trials remained unchanged over the duration of six weeks of salt stress (Figure 3.6), whereas salinity stress-induced significant ROS accumulation in mesophyll cells (ANOVA of both trials, treatment effect, $P<0.01$). Consistently, leaf mesophyll samples from the field trial were less affected by salinity stress compared to those from the greenhouse trial. The ROS production in mesophyll cells peaked between 14 and 28 DAS and then reduced significantly at 42 DAS (Figure 3.6). Koshihikari showed significantly higher ROS accumulation than Doongara and Reiziq in both trials at 14 DAS (Figure 3.6_A) that reduced below control levels at 42 DAS (Figure 3.6_B, 3.6_C). Both the rate of decline in ROS and the magnitude of the reduction was greatest in Koshihikari and least in Reiziq in both trials (Figure 3.6_B-C).

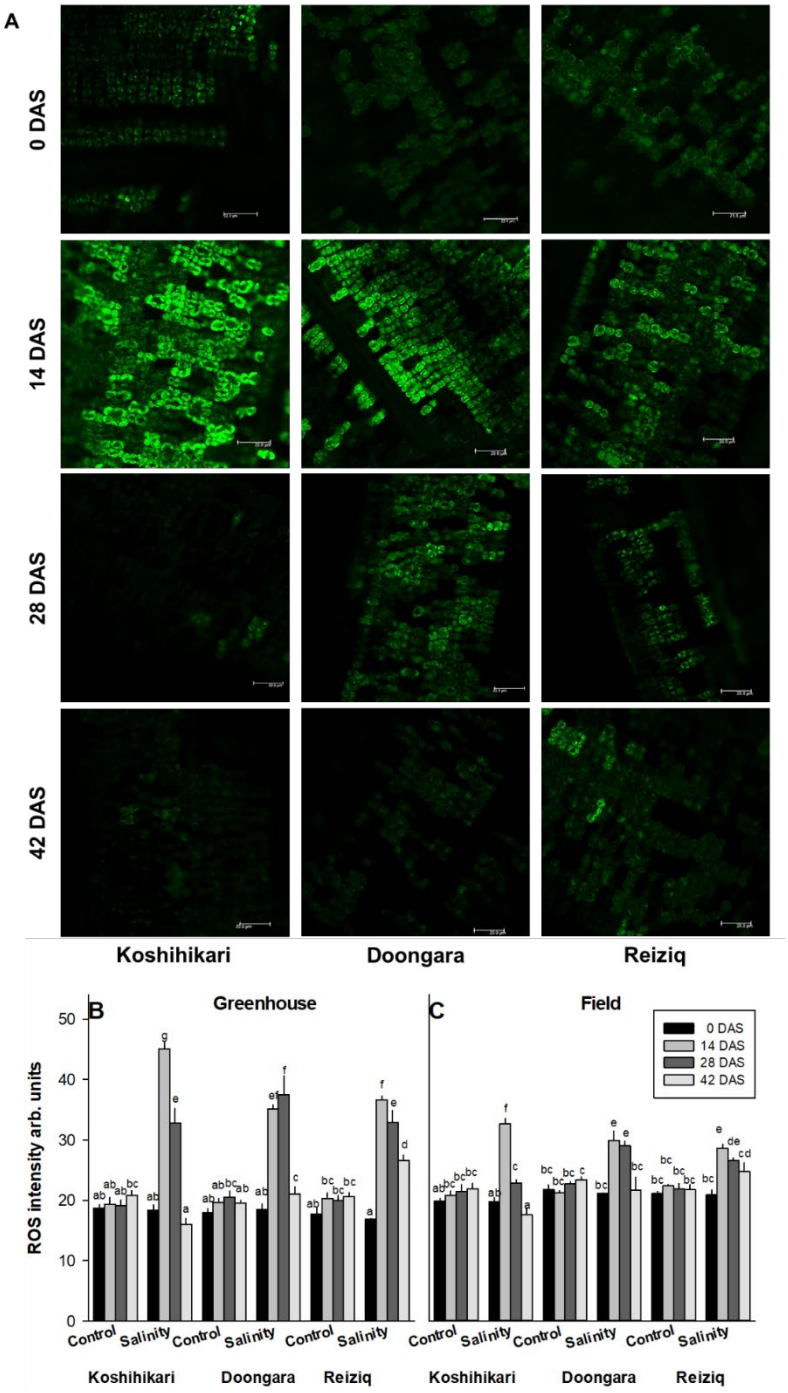


Figure 3.6. Effects of salinity on the accumulation of reactive oxygen species (ROS) in leaf mesophyll of three rice genotypes at their reproductive stage. (A) representative image of ROS in mesophyll over 42 days of salinity stress in the field. The scale bars=20µm. The mean ROS intensity from the greenhouse (B) and field (C) was compared. Different lowercase letters indicate significant differences at $P<0.05$ (n=4 biological replicates).

3.3.4 Gene expression differs in varieties and growth conditions after prolonged salinity stress

I then conducted experiments on the expression of genes (*OsHAK1*, *OsGORK*, *OsSOS1*, *OsHKT1;1*, and Os*RBOHD*) involved in controlling ion fluxes and ROS accumulation in rice (Figure 3.7). Salt-tolerant Reiziq showed significant downregulation of *OsGORK* and upregulation of *OsSOS1*, *OsHAK1*, and *OsRBOHD* in both trials (Figure 3.7). For salt-sensitive Koshihikari, *OsRBOHD* was found to be significantly upregulated in both trials with the other key genes being either expressed at low levels or not significantly responding to 42-days of salinity treatment (Figure 3.7). In Doongara, only *OsGORK* showed a significant salt-induced change in regulation and was upregulated in both trials (Figure 3.7).

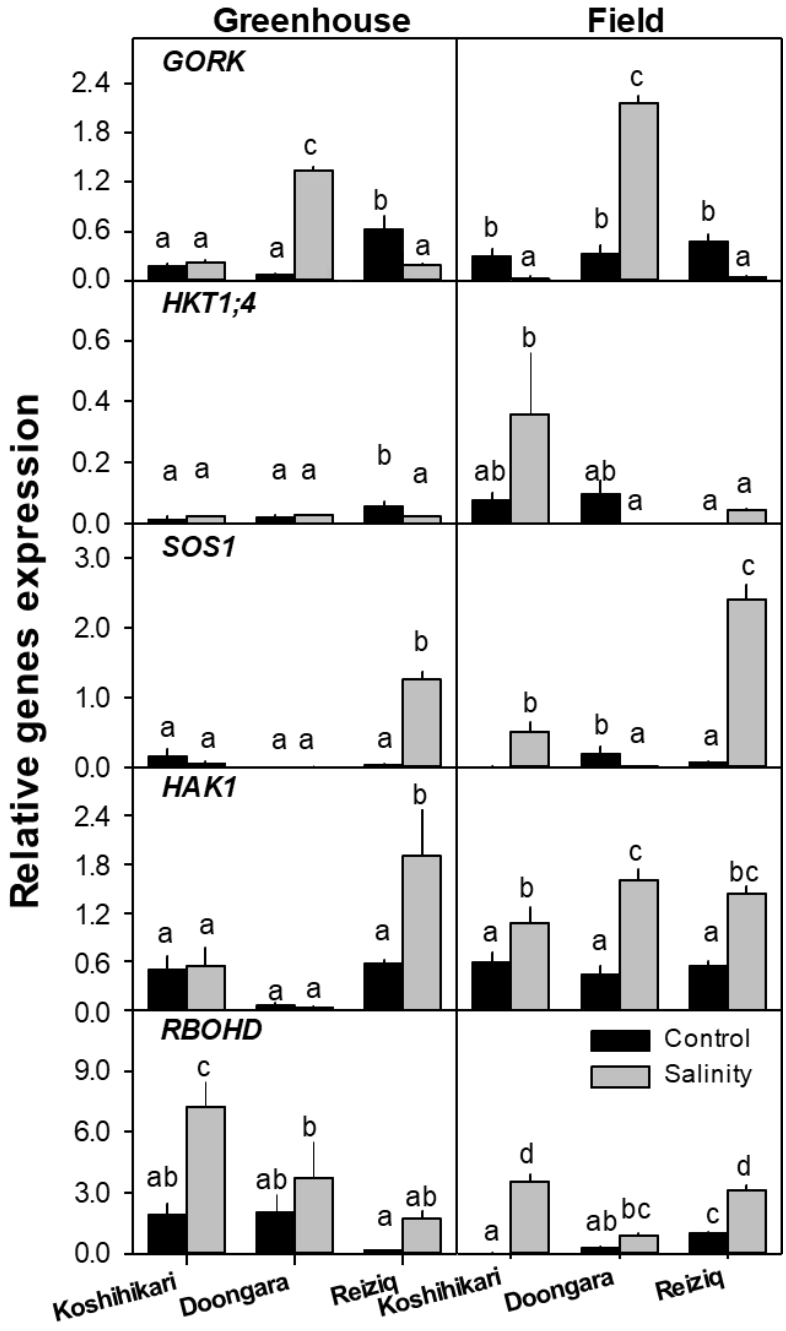


Figure 3.7. Effects of salinity on gene expression in leaf mesophyll of three rice genotypes at their reproductive stage in both the greenhouse and the field. qPCR of signature salt tolerance related genes *OsGORK, OsHKT1;1, OsSOS1, OsHAK1*, and *OsRBOHD* after 42 days of salinity stress. Different lowercase letters indicate significant differences at P<0.05 (200-300 cells from 3 biological replicates).

3.3.5 Net K^+ and Cl^- fluxes after recovery are significantly correlated with growth parameters and OsRBOHD expression

Analysis of data pertaining to each parameter collected at 42 DAS and between parameters collected at different weeks (Tables 3.3.1 and 3.3.2) revealed significant correlations among net K^+ (Figure 3.8_A, 3.8_G), Na^+ (Figure 3.8_B) and Cl^- (Figure 3.8_C, 3.7_I) flux with growth parameters (biomass and relative height) and *OsRBOHD* expression. Recovery K^+ and Cl^- fluxes were significantly positively correlated to relative plant height (Figure 3.7_G–I; 3.2) and significantly negatively correlated to *OsRBOHD* expression (Figure 3.7_D–F; Table 3.1).

Table 3.1. Pearson correlation analysis of all physiological parameters collected at 42 Days After Salinity (DAS). * and ** indicate a significant correlation at P<0.05 and P<0.01.

Parameter	K^+ flux	Na^+	Cl^- flux	rPH	rTN	rDW	r*GORK*	r*HKT1:4*	r*SOS1*	r*HAK1*	r*RBOHD*	rChl a	rChl b	rCarotenoid	rTotal chl	rROS
K^+ flux	1															
Na^+ flux	0.86**	1														
Cl^- flux	0.99**	0.79**	1													
rPH	0.64*	0.66*	0.63*	1												
rTN	0.46	0.44	0.47	0.76**	1											
rDW	0.67*	0.58*	0.68*	0.90**	0.69*	1										
r*GORK*	-0.18	-0.19	-0.12	-0.34	-0.22	-0.15	1									
r*HKT1:4*	-0.03	0.03	-0.04	-0.01	-0.09	-0.06	-0.33	1								
r*SOS1*	-0.04	-0.27	-0.04	-0.37	-0.33	-0.43	-0.34	0.08	1							
r*HAK1*	-0.13	-0.31	-0.09	-0.50	-0.46	-0.64*	0.12	0.06	0.63*	1						
r*RBOHD*	-0.78**	-0.62*	-0.80**	-0.43	-0.25	-0.50	-0.18	0.04	0.13	-0.07	1					
Chl a	0.17	0.49	0.06	0.44	0.31	0.19	-0.33	-0.34	-0.22	-0.28	-0.01	1				
Chl b	0.17	0.49	0.06	0.44	0.31	0.19	-0.33	-0.34	-0.22	-0.28	-0.01	0.98**	1			
Carotenoid	0.09	0.45	-0.02	0.35	0.17	0.11	-0.32	-0.39	-0.31	-0.36	0.08	0.87**	0.87**	1		

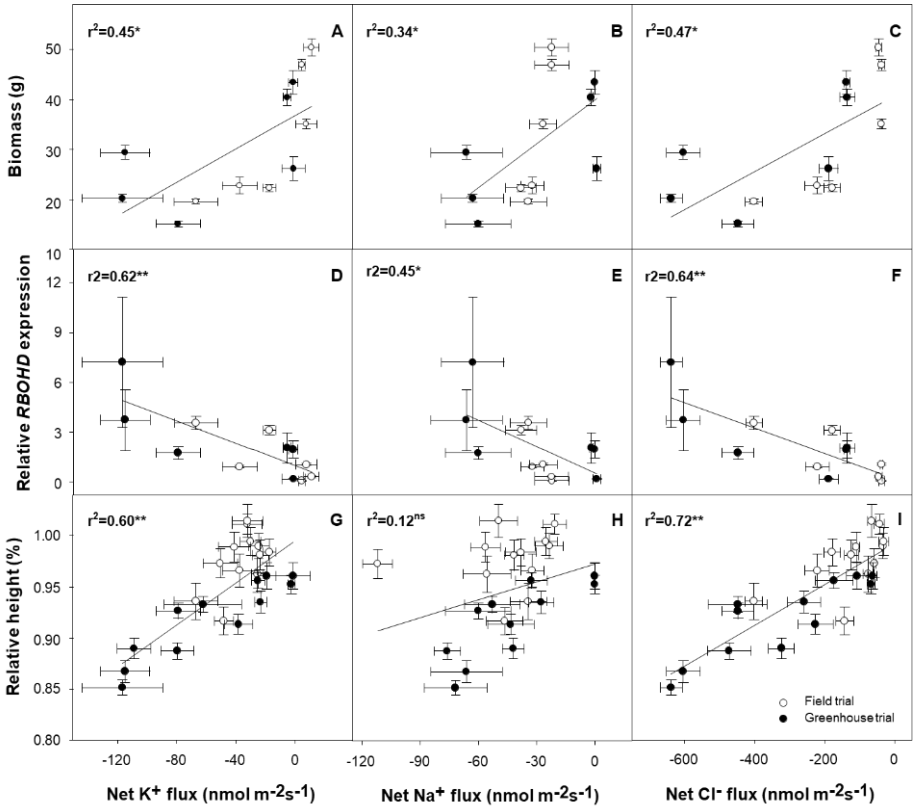
Total Chl	0.17	0.49	0.06	0.44	0.31	0.19	-0.33	-0.34	-0.22	-0.28	-0.01	0.97**	0.97**	0.87**	1	
ROS	0.29	-0.07	0.33	-0.02	-0.14	0.04	0.05	-0.27	0.57	0.50	-0.55	-0.16	-0.16	-0.30	-0.16	1

Abbreviation: K^+ flux- net K^+ flux of stress plant, Na^+ flux- net Na^+ flux of stress plant, Cl^- flux- net Cl^- flux of stress plant, rPH- relative plant height, rTN- relative tiller numbers, rDW- relative dry weight r*GORK*- relative *GORK* expression, r*HKT1;4* relative *HKT1;4* expression, r*SOS1*- relative*SOS1* expression, r*HAK1* relative *HAK1* expression, r*RBOHD*- relative *RBOHD* expression rChl a- relative chlorophyll a content, rChl b- relative chlorophyll b content, rCarotenoid- relative carotenoid content, rTotal chl, relative total

Table 3.2. Pearson correlation analysis of net ion flux and ROS production of leaf mesophyll with relative plant height from 0 to 42 days of salinity stress. * and ** indicate a significant correlation at P<0.05 and P<0.01.

Parameter	Plant height	K^+ flux	Na^+ flux	Cl^- flux	ROS
Plant height	1				
K^+ flux	0.78^{**}	1			
Na^+ flux	0.32	0.58^{**}	1		
Cl^- flux	0.85^{**}	0.85^{**}	0.43^{*}	1	
ROS	0.20	0.13	-0.52^{*}	0.31	1

Abbreviation: K^+ flux- net K^+ flux t, Na^+ flux- net Na^+ flux, Cl^- flux- net Cl^- flux


r²=0.45*
A
r²=0.34*
B
r²=0.47*
C
Biomass (g)
r2=0.62**
D
r2=0.45*
E
r2=0.64**
F
Relative RBOHD expression
r²=0.60**
G
r²=0.12ns
H
r²=0.72**
I
Relative height (%)
Field trial
Greenhouse trial
Net K+ flux (nmol m-2s-1)
Net Na+ flux (nmol m-2s-1)
Net Cl- flux (nmol m-2s-1)

stress respectively. The data points are the average value with the standard error bar. Significant statistical correlation is indicated by **($P<0.01$), *($P<0.05$), and ^{ns} (not significant).

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3.4 Discussion

3.5.1 K^+ retention is a key trait for rice salt tolerance at the reproductive stage in both greenhouse and field

In mesophyll cells, salinity-induced depolarization shifts membrane potentials towards less negative values, leading to K^+ efflux due to inactivation of AKTs and activation of GORK (Shabala and Cuin, 2008). The recovery MIFE measurements suggested that NaCl-induced, long-term mesophyll damage (Figure 3.6) in terms of K^+ outward transport activities are possibly irreversible due to the programmed cell death (Figure 3.5_A). High K^+ leakage from damaged tissue in recovery was previously reported in *Arabidopsis* seedlings given short-term salinity stress (Liu et al., 2017a) and in soybean treated with long-term drought stress (Mak et al., 2014). In addition, Liu et al. (2017a) found a strong, negative correlation between recovery K^+ flux and cytosolic K^+ content, of which the latter parameter is a well-known indicator of salinity tolerance (Shabala and Cuin, 2008).

A positive correlation between *GORK* expression and salinity tolerance was found in transiently stressed roots and leaves of *Arabidopsis* (Adem et al., 2014, Chakraborty et al., 2016, Wang et al., 2019a). Here, the K^+ outward-rectifying channel gene, *OsGORK,* was significantly downregulated in salt-tolerant Reiziq but significantly upregulated in Doongara and unchanged in Koshihikari (Figure 3.7). These changes in expression, however, did not match the K^+ efflux patterns in the rice cultivars in the two trials (Figure 3.5). Therefore, I postulate that GORK might not be the key channel responsible for K^+ efflux observed from mesophyll tissues of rice plants taken during their reproductive stage (Figure 3.5_A, 3.5_D). Similarly, a study of an *Arabidopsis gork* mutant found no difference in K^+ efflux between the mutant and the WT at the end of transient measurement in response to salinity (Azhar et al., 2017, Demidchik et al., 2010, Jayakannan et al., 2013). Other than GORK, the results suggested that weak or non-

voltage dependent K^+ efflux channels such as NSCC and NORC (Zepeda-Jazo et al., 2008) may be the channels responsible for the net K^+ efflux (Figure 3.5). However, these novel ion channels have not been assigned to any molecular identities and require future investigation.

Differences in recovery K^+ flux between rice varieties can also be affected by K^+ inward transport mechanisms such as the K^+ inward channels (OsAKTs) and high-affinity K^+ transporters (OsHAKs). For instance, in rice, three HAK members, OsHAK1, OsHAK5, and OsHAK21, are reported to have a positive role in K^+ limiting environment including salinity stress via active K^+ uptake (Chen et al., 2015, Horie et al., 2011, Shen et al., 2015). In this study, *OsHAK1* was upregulated in the salt-tolerant, Reiziq, but no change was observed in the salt-sensitive Koshihikari (Figure 3.7), indicating that OsHAK1 could be one of the key components responsible for smaller net K^+ effluxes and higher shoot K^+ contents in the salt-tolerant Reiziq at its reproductive stage. Consistent with a large-scale screening of barley seedlings using MIFE (Wu et al., 2014, Wu et al., 2015b), the results suggest a positive correlation between the magnitude of the recovery K^+ flux and salinity tolerance (Figure 3.8_A, 3.8_G) at the reproductive stage after prolonged salinity stress that is controlled by few inward and outward K^+ transporters in rice mesophyll cells.

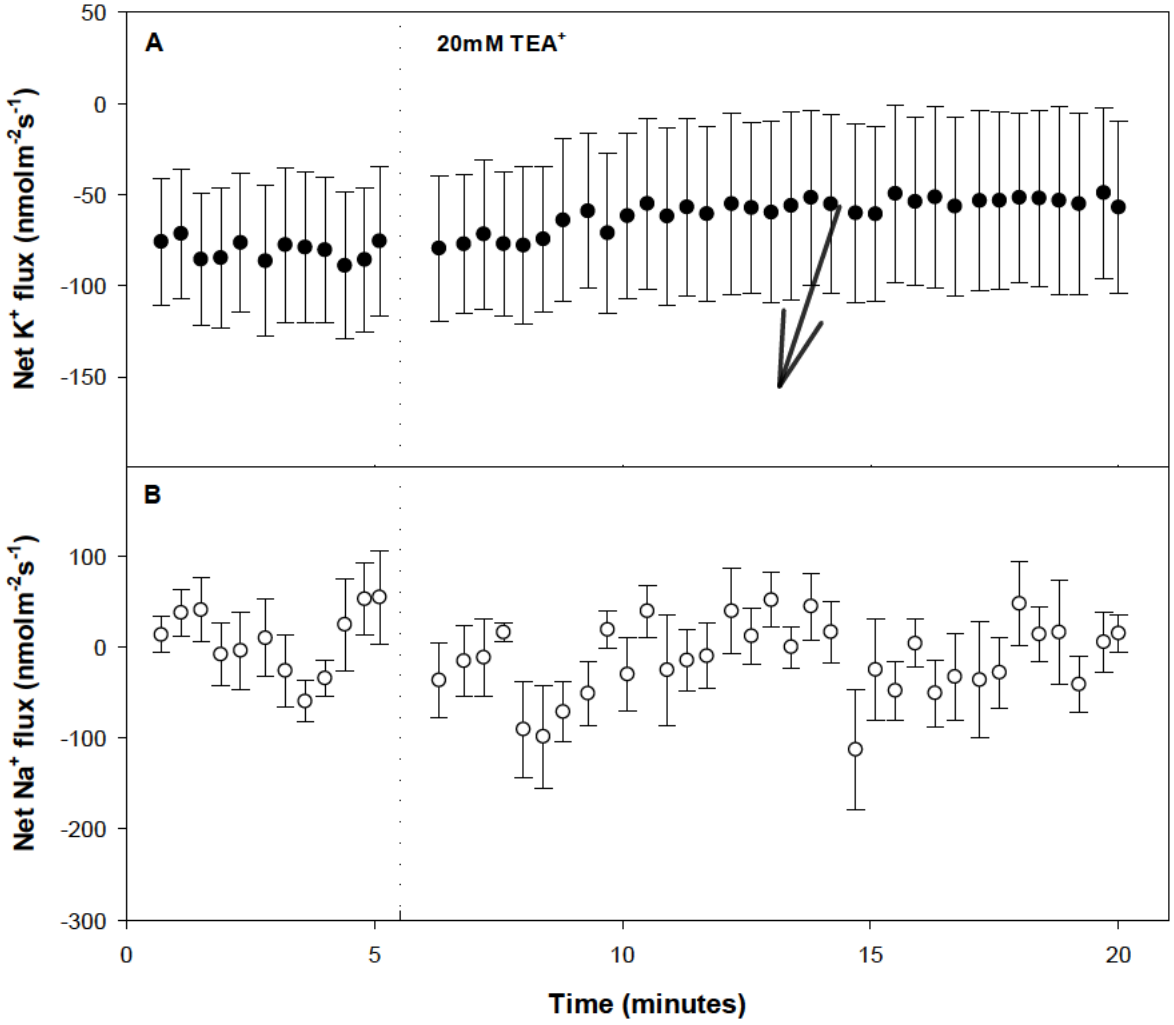

Fig 3.9. Transient net K^+ and Na^+ flux kinetics measured in the leaf mesophyll of the salinity stressed Koshihikari seedlings to K^+ channel blocker TEA^+. The result showed that GORK activities were insignificant when the leaf is adapted to external Na^+. The leaf segments were detached and floated on 40mM NaCl solution overnight before measurements. Steady flux in 40 mM NaCl solution was recorded before transient TEA^+ treatment. Values are mean ± SE (n=6).

3.5.2 Mesophyll recovery Cl^- efflux is an indicator for salt stress at the reproductive stage of rice

In contrast to active K^+ retention in cells under salinity stress, plants exclude excessive Na^+ and Cl^- from the cytosol or sequester these ions into vacuoles (Munns et al., 2020a, Shabala et al., 2020). Leaf mesophyll tissues from rice plants subjected to prolonged salt stress at the reproductive stage showed significant, positive correlations between salinity tolerance (relative biomass and height) and net Cl^- flux after an hour of recovery (Figure 3.8_B–C, 3.8_H–I), that also correlated well with shoot elemental content and general salt tolerance of the three rice cultivars (Figure 3.1-8; Tables 3.1-2). The positive correlation was less significant between salinity tolerance and net Na^+ efflux (Figure 3.8_B). The recovery and transient Na^+ efflux of short-term salt-stressed barley varieties showed higher net Na^+ efflux (Wu et al., 2018a) and recovery Na^+ flux in the root of two poplar species (Sun et al., 2009b). As both barley and poplar are more tolerant to salinity in comparison to rice, the results indicate that there are much weaker Na^+ regulatory mechanisms operating in leaf mesophyll cells of rice plants at the reproductive stage.

Chloride is a key nutrient that is involved in enzyme activation, pH regulation, turgor maintenance, and photosynthesis (Franco-Navarro et al., 2019, Teakle and Tyerman, 2010). I found that net recovery Cl^- flux was the most responsive index to the prolonged salinity stress and was highly positively correlated with salt tolerance in rice at its reproductive stage in both greenhouse and field trials (Figure 3.5, 3.8; Table 3.1-2). High Cl^- accumulation in plant leads to Cl^- toxicity (Teakle and Tyerman, 2010), and salinity tolerance was positively correlated with reduced net Cl^- uptake by roots, xylem (Läuchli et al., 2008, Teakle et al., 2007), intercellular Cl^- (Flowers and Hajibagheri, 2001) and intracellular compartmentation

(Abbaspour et al., 2013, Zhao et al., 2010). Highly positive correlations between Cl^- accumulation in leaves and economically important traits have been found in woody and leguminous plants, citrus (Brumós et al., 2009, Moya et al., 2003), grapevine (Tregeagle et al., 2010, Fort et al., 2013), soybean (Lee et al., 2009, Luo et al., 2005) and fava bean (Geilfus et al., 2015). However, Counce et al. (1999) found that rice was more sensitive to osmotic stress rather than Cl^- toxicity, and Cl^- was relatively less toxic than Na^+ in rice (Khare, 2015, Khare et al., 2015). Long-term cellular Cl^- accumulation is usually accompanied by increasing accumulation of Na^+ and reduced K^+ under saline conditions (Teakle and Tyerman, 2010, Kader and Lindberg, 2010). Therefore, I suggest that low recovery Cl^- efflux (Figure 3.5) is linked to high K^+ and low Na^+ accumulation (Figure 3.3) in leaf mesophyll with Cl^- being a 'cheap' osmolyte in salt-tolerant Reiziq to provide better tolerance to osmotic stress instead of synthesizing more energy-intensive compatible solutes (Chen et al., 2007a, Munns et al., 2020a). This strategy will provide salt-tolerant Reiziq a distinct advantage allowing more energy to be directed towards the reproductive process resulting in better yield under salinity stress as compared to the salt-sensitive rice genotypes.

3.5.3 Dynamic ROS production and regulation in leaf mesophyll cells for rice salt tolerance

ROS are vital secondary messengers for stress tolerance and signalling transduction in plants (Mittler, 2017, Zhao et al., 2019b). Salinity stress-induced ROS accumulation is commonly studied at the seedling stage with most studies involving short-term treatments of high salt (e.g. hours to days, 100–300 mM NaCl). In these cases, ROS contents were found to be negatively related to salinity tolerance (Ahammed et al., 2018, Paiva et al., 2019, Basu et al., 2017, Saibi et al., 2015, Nxele et al., 2017, Zhu et al., 2019, Zheng et al., 2015, Torun, 2019, Nath et al., 2016, Banu et al., 2015, Zhang et al., 2019). In response to transient salinity stress, dramatic

ROS accumulation and reduction in short periods (hours) of salinity stress have been extensively reported in seedlings treated with low to medium salinity: 10–80 mM NaCl for 0–24 h (Xie et al., 2014, Wang et al., 2019a, Sekmen et al., 2014, Cao et al., 2017). In comparison to studies with high salt treatments, the increase in ROS is negatively related to salinity sensitivity under low salt concentrations (Cao et al., 2017, Xie et al., 2011). ROS contents can still increase if low salt stress is continued after 24 h (Saibi et al., 2015, Zhu et al., 2019). In the study, salt-sensitive Koshihikari showed the highest ROS accumulation in mesophyll cells over prolonged salinity stress at the reproductive stage in both the greenhouse and the field (Figure 3.6). This indicated that high ROS accumulation is a sign of salinity-induced damage at an early stage before programmed cell death and was followed by a significant reduction of ROS levels in mesophyll cells. Interestingly, the work shows a significant negative relationship between ROS production and Na^+ flux over the duration of 42 days of salinity in both trials (Table 3.1), indicating a role of ROS for long-term salinity tolerance in reproductive stage rice.

In plants, the RBOH family is one of the most important ROS producing sources, responding to salinity stress *via* signaling and activation of defense systems (Xie et al., 2011, Wang et al., 2019a). This was proposed based on the ROS variation in plants in response to 24 h of low salt treatment as the early ROS accumulation was significantly delayed in *Arabidopsis rbohd* mutant and a salt-sensitive cucumber (Cao et al., 2017, Xie et al., 2011). In the study, *OsRBOHD* expression was negatively correlated with the net K^+, Na^+, and Cl^- fluxes (Figure 3.8_D–F), indicating salt-induced regulation of ROS in on ion channels of mesophyll cells that are similar to those reported in guard cells (Chen et al., 2016b, Pornsiriwong et al., 2017, Wang et al., 2013). Therefore, the results suggest that fine modulation of ROS accumulation in leaf mesophyll cells and expression of *OsRBOHD* are key components of salt tolerance in the reproductive stage of rice plants, which are consistently found in both greenhouse and field trials.

Chapter 4: Evidence of C3-C4 intermediate characteristics linked to salinity tolerance in a halophytic wild rice

4.1 Abstract

Wild rice in the *Oryza* genus possesses a wide range of genetic variation and stress tolerance that can be potentially utilized in breeding climate-resilient rice cultivars (*Oryza sativa*) for global food security. Different mechanisms (e.g. salt glands) of salinity tolerance in wild rice species such as *Oryza coarctata* have been reported, but the link of salinity tolerance to C4 has not been reported in detail in wild *Oryza* species. I performed a greenhouse trial to evaluate the salinity tolerance of six wild rice species, one rice cultivar IR64, and one landrace Pokkali at the reproductive stage. I assessed growth performance, ion homeostasis, photosynthetic capacity, and gene expression of the *Oryza* species, in response to salinity stress. Salinity stress had a minimal effect on the tolerant species (*O. latifolia, O. officinalis,* and *O. coarctata)*. The result showed that sensitive species tend to have higher Na^+ accumulation in leaf and mesophyll cells. This could lead to greater salinity-induced K^+ efflux in recovery MIFE measurement and reduced photosynthetic capacity in stressed plants. There were significant correlations between the relative biomass and with K^+ and Cl^- fluxes and ROS and Na^+ fluoresce except the halophyte *O. coarctata*. Moreover, *O. coarctata* showed C4-like photosynthetic capacity, Kranz-like leaf anatomy- (enlarged bundle sheath cell and reduced mesophyll numbers), and higher expression of C4-related genes (e.g. *NADPME*, *PPDK*, *RBCL*). It was concluded that halophytic *O. coarctata* is a clear halophytic outliner of salinity tolerance among the studied wild *Oryza* species and rice cultivars. The high net photosynthetic rate, water use efficiency, expression of C4-related genes, and unique phylogenetic relationship of *O. coarctata* with C4 grasses provide important evidence of superior salinity tolerance in *O. coarctata*, indicating a potential use for breeding salinity tolerance "C4 rice" in the future.

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4.2 Introduction

Plant salinity tolerance is a polygenetic trait that has evolved in nature involving the modification of multiple physiological metabolisms and anatomical structures to adapt to the saline environment (Solis et al., 2020, Munns et al., 2020a, Chen and Soltis, 2020). It is a multiple single-origin and difficult event that starts from modifying a random trait under saline conditions (Bromham et al., 2020), leading to different extents of diversification from the "tippy" pattern (on the tip of the phylogeny) in most of the salt-tolerant lineages, such as the majority of halophytes (Bromham et al., 2020), branched phylogeny, such as 'PACMAD' clade-C4 (Bromham and Bennett, 2014) and Quinoa family (Adolf et al., 2013), based on the evolved mechanism and characteristic of the species. These may suggest a potential loss or gain of salt-tolerant traits in different species during their evolutionary and ecological adaptation to saline conditions (Chen and Soltis, 2020, Caperta et al., 2020, Bromham et al., 2020)

The *Oryza* genus (Poales, Poaceae) has approximately 24 species (Ge et al., 2001), with the Asian rice *O. sativa* and African rice *O. glaberrima* as the staple crop of over half of the global population (Grieve et al., 2019). Cultivated rice is highly susceptible to salinity stress, and a yield penalty occurs at low salinity levels of 3 dSm^{-1} electrical conductivity (EC) (Khatun and Flowers, 1995, Lutts et al., 1995, Munns et al., 2006). In cultivated rice *O. sativa*, salt exclusion, Na^+ compartmentation, tissue tolerance, Na^+ retrieval, and osmotic regulation are reported to be the main salinity tolerance mechanisms (Kavitha et al., 2012, Lakra et al., 2018, Malagoli et al., 2008, Nemati et al., 2011, Oda et al., 2018). Attempts to increase salinity tolerance have mostly focused on traits found in salt-tolerant landraces, such as Pokkali, Nona Bokra, and FL468 with poor reproductive performance. Salinity tolerance in these lines is mainly achieved by restricting Na^+ accumulation in aboveground tissues and by maintaining

relatively higher K^+ content (Gerona et al., 2019, Lutts et al., 1996, Prusty et al., 2018). However, the development of salinity-tolerant lines using these parental lines has produced plants with poor reproductive traits (Solis et al., 2020), suggesting a close linkage between salt sensitivity and good reproductive traits and insufficient genetic background for salinity-tolerant traits in these *O. sativa* lines.

Some wild *Oryza* species are tolerant to different biotic and abiotic stresses (Stein et al., 2018). Wild rice species have variable tolerance to salinity stress, inter and intra-species and screening experiments have identified highly salt-tolerant *Oryza* species such as *O. coarctata* (KKLL) and *O. australiensis* (EE) (Prusty et al., 2018, Yichie et al., 2018). Previous findings suggested that salt-tolerant wild rice employed very different mechanisms from *O. sativa*, though their performances did not outperform remarkably compared to the salt-tolerant cultivar, Pokkali, except the only halophytic species, *O. coarctata* (Flowers et al., 1990). This suggested that the acquisition of salinity tolerance in the *Oryza* genus may be multiple independent and recent events similar to other genera with both halophytic and glycophytic species (Bromham et al., 2020). Breeding for salinity-tolerant lines using wild relatives has mostly focused on *O. rufipogon* and *O. nivara*, *Oryza* species that have the same AA genome as cultivated rice (Ganeshan et al., 2016, Wang et al., 2017d). On the contrary, the glycophytic species, *O. officinalis* (EE), *O. latifolia* (CCDD), and *O. alta* (CCDD) were found with higher leaf Na^+ accumulation and reduced photosynthetic capacity after salinity treatment (Prusty et al., 2018, Yichie et al., 2018, Nakamura et al., 2002, Nishizawa et al., 2015), indicating a significant limitation of these wild rice species for breeding high yield rice cultivate with salt tolerance.

O. coarctata is native to coastal environments that have daily fluctuations in EC between 20 and 40 dSm^{-1} and has unique morphological and anatomical features such as thick and waxy leaves that contain salt glands and a differentiated rhizome adapted for high salinity

(Sengupta and Majumder, 2010). *O. coarctata* is the only *Oryza* species that developed putative Kranz anatomy with enlarged bundle sheath cells associated with deep leaf furrow on the adaxial side of the leaf and a pair of vertically paralleled vascular veins on each leaf ridge (Chatterjee et al. 2016). In C4 plants, enlarged vascular bundle sheath reduced mesophyll cell/vascular bundle sheath cell size ratio, and reduced number of mesophyll cells between the vascular system in the leaf are fundamental leaf structures for C4 photosynthesis (Hatch, 1987). These unique C4-like features in *O. coarctata* may compensate for the increased distance between the upper mesophyll cell and the main vascular vein from increased leaf thickness, contributing to the adaptation to the extreme saline environment. The incorporation of C4 photosynthesis into new rice cultivars is suggested as a potentially cutting-edge technology towards another green revolution to meet future food demand (Von Caemmerer et al., 2012). However, the development of C4 rice mostly relies on the introduction of genes from C4 grasses (e.g. maize and sorghum) and there is no C4 *Oryza* species has been discovered (Wang et al., 2016c). A C4 *Oryza* species will be ideal to study evolution and function Kranz anatomy and C4 photosynthesis to benefit future C4 rice development.

Here, salinity tolerance of 6 wild *Oryza* species, one salt-tolerant landrace, and a salt-sensitive cultivar were evaluated in the greenhouse. I hypothesized that *O. coarctata* is a C3-C4 intermediate grass species that has salt tolerance linked to the evolution of C4-like leaf anatomy and photosynthesis. The measurements were focused on the salinity response of typical salinity tolerance indicators (e.g. biomass, Na^+/K^+ ratio), photosynthesis, ion transport properties using MIFE, confocal imaging of ROS, and Na^+ intensity in mesophyll cells, and genes expression of salinity-responsive ion transporters and C4-related proteins. The halophytic wild rice *O. coarctata* showed the highest tolerance to salinity among the wild rice and exhibited a C4-like net photosynthetic rate, high chlorophyll content, and Kranz-like leaf anatomy.

4.3 Results

4.3.1 Oryza coarctata exhibits unique growth and photosynthesis responses to salinity

Overall, salinity stress had a significant, negative effect on the lines after six weeks of NaCl treatment at 100 mM applied at the late vegetative stage and maintained at the reproductive stage with a large variation in stress tolerance exhibited among the rice lines. The relative values of the growth and physiological parameters of these lines were ranked using the homogenous groups assigned by DMRTs (Table 4.1). According to the ranking, performance under saline conditions was, from poorest to greatest, as follows: *O. longiglumis*, IR64, *O. australiensis*, *O. rufipogon, O. latifolia*, Pokkali, *O. officinalis*, and *O. coarctata.*

Table 4.1. Salinity tolerance scores of cultivated and wild rice species based on the key physiological parameters.

Salinity ranking	Biomass	Chl a	Chl b	ROS	Na^+	Na:K ratio	*A*	*E*	Average score
O. coarctata	4	4	4	2	2	3.5	1.5	1	2.7
O. officinalis	3	1	2.5	2	3	3.5	2	2	2.4
O. sativa L. Pokalli	3	3.5	3	2	2.5	4	1.5	1	2.4
O. latifolia	2.5	3	3	2	2.5	3.5	1.5	1	2.3
O. australiensis	1.5	3	2.5	1.5	2	2.5	1.5	1	1.9
O. rufipogon	2	2	2.5	2	2	2	1.5	1	1.8
O. sativa L. IR64	1	1	1.5	1.5	1.5	1	1	1	1.2
O. longiglumis	1	1.5	1	1	1	1	1	1	1.1

Significant reductions in biomass due to salinity stress were found in *O. longiglumis*, IR64, *O. australiensis* and *O. rufipogon* ($P<0.05$; salinity-sensitive lines) (Figure 4.1) but not for *O. latifolia,* Pokkali, and *O. officinalis* and *O. coarctata* (salinity-tolerant lines); *O. coarctata* even showed a non-significant increase in biomass in the salinity treatment. Tiller numbers and plant heights were not significantly affected by salinity treatment in any of the species (Figure 4.2). Similarly, significant reductions in total chlorophyll contents, CO_2 assimilation rates (A), stomata conductance (g_s), and transpiration rates (E) were found in *O. longiglumis* (except g_s and E, $P>0.05$), IR64, and *O. rufipogon* (Figure 4.1_A–C). Compared to the other rice species, *O. coarctata* showed unique photosynthetic parameters such as significantly higher A and water use efficiency (WUE) and lower intercellular CO_2 concentrations (Ci); these parameters were not affected by the salinity treatment (Figure 4.1).

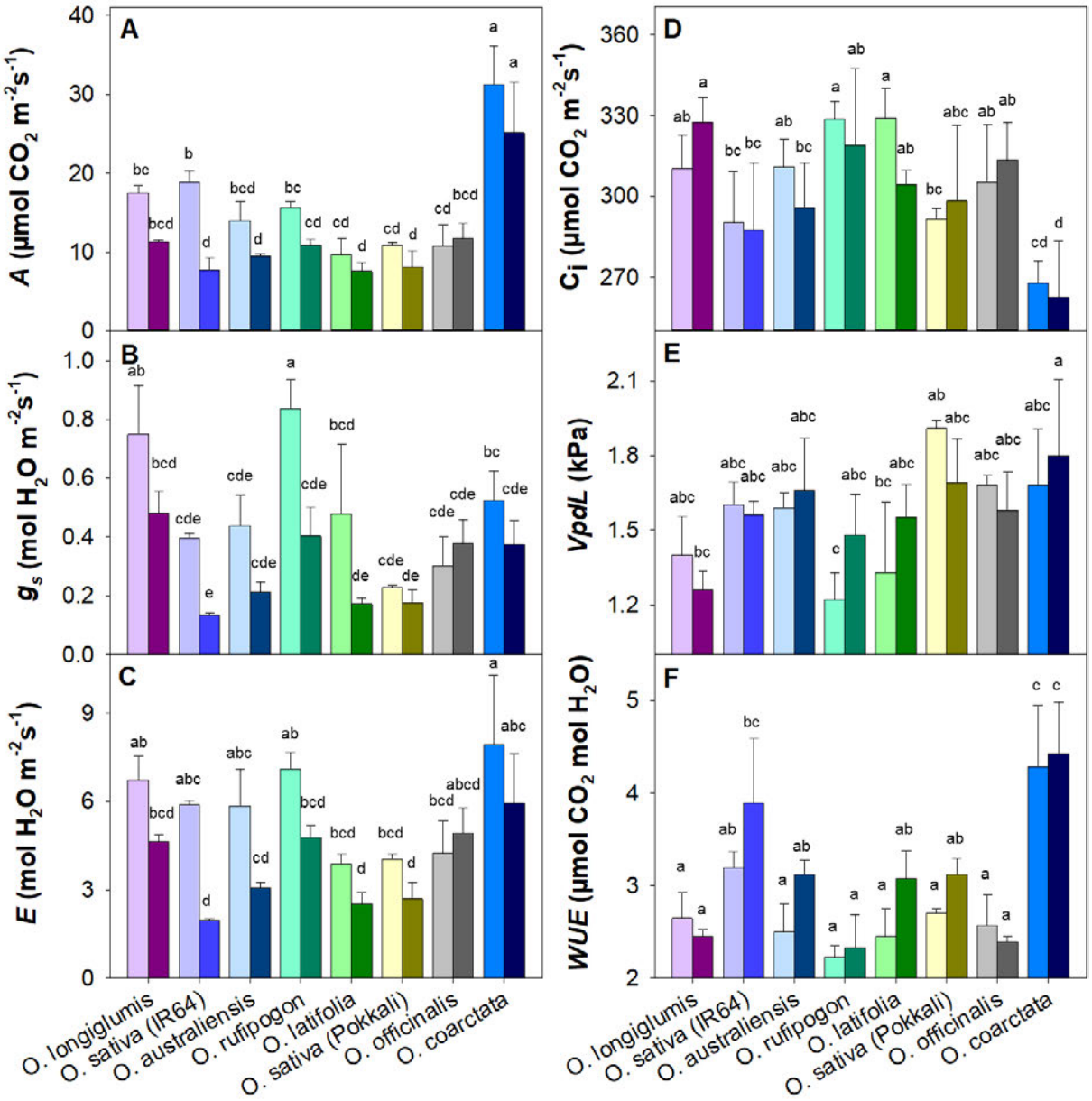

Figure 4.1. Effects of salinity on leaf gas exchange parameters of wild and cultivated rice**.** (A) net CO_2 assimilation rate- *A*, (B) stomata conductance- g_s, (C) and transpiration rate- *E*, (D) intercellular CO_2 concentration- C_i, (E) vapor pressure difference of leaf- VpdL, (F) water use efficiency- WUE after 6 weeks of salinity stress. For each species, the left bar indicates the control, and the right bar indicates the salt-stressed sample. Data are mean value ± SE (n=3).

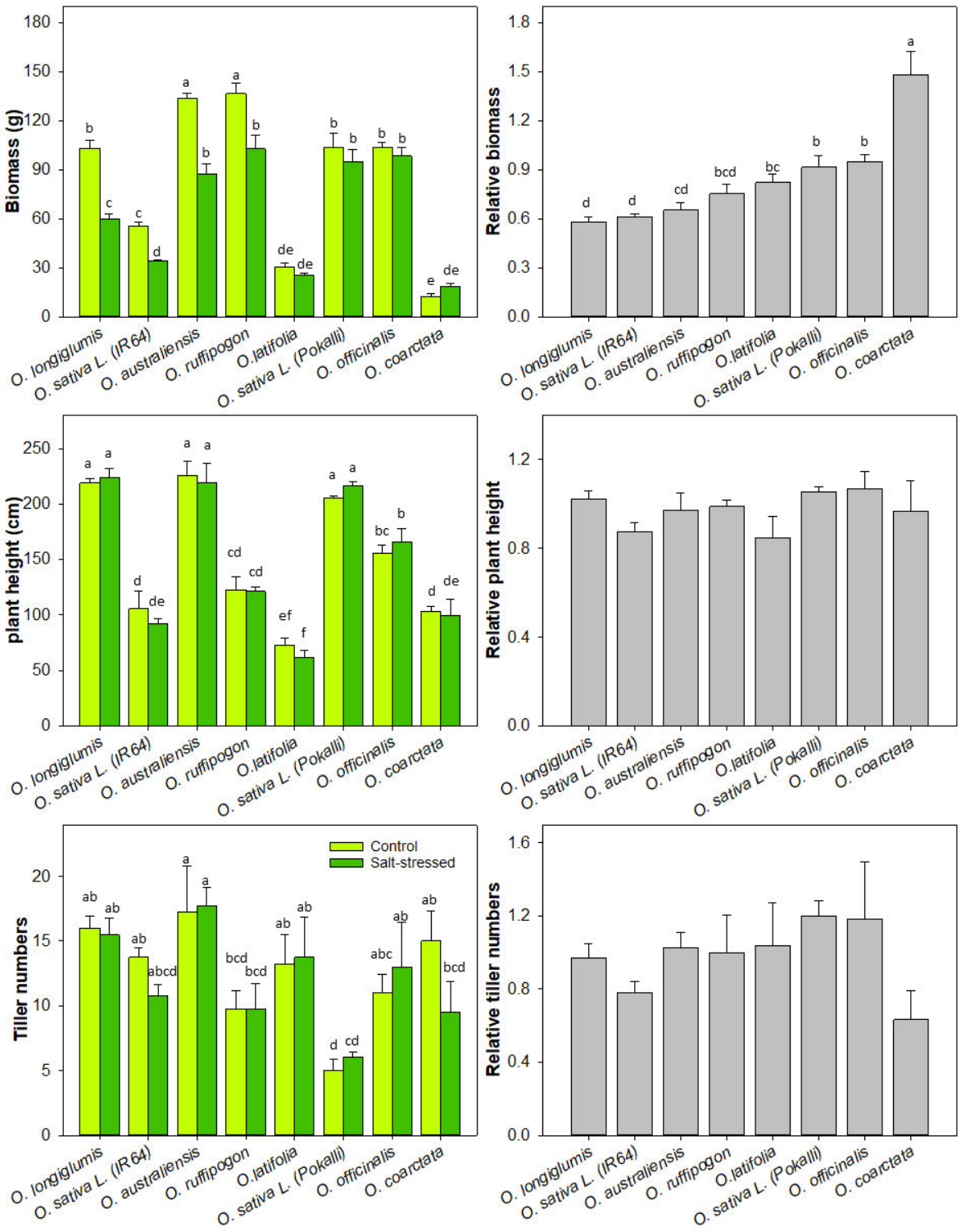

Figure 4.2. Effect of salinity on biomass, plant height, and tiller numbers of cultivated and wild rice species. The data are the mean value (n= 3 - 4 replicates) and the error bars indicate the standard errors. Different lowercase letters indicate significant differences at P<0.05 or no letter (relative plant height and relative tiller numbers) at P>0.05.

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4.3.2 Unusual patterns of ion flux and gene expression of transporters in Oryza coarctata

The effect of the salinity stress on the wild and cultivated *Oryza* species was evaluated by measuring net K^+, Na^+, Cl^- and Ca^{2+} flux of mesophyll tissues after recovery. In general, control leaves of most species had similar relatively low fluxes (Figure 4.3). Compared to the controls, the net K^+, Na^+, Cl^- and Ca^{2+} fluxes from the mesophyll of the salinity-stressed rice after recovery were mainly effluxes in all *Oryza* species (Figure 4.4). Net K^+ flux (r^2= 0.80, P<0.01) and Cl^- flux r^2= 0.59, P<0.05) were significantly positively correlated to biomass. In addition, net K^+ and Cl^- fluxes were also significantly correlated to mesophyll Na^+ and leaf Na^+/K^+ ratio (Figure 4.5_A), which are the key salinity tolerance indicators (Table 4.2). Most of the species showed no significant difference in net Na^+ flux except the most salt-sensitive *O. longiglumis*. For Ca^{2+} flux. Interestingly, the most salt-tolerant *O. coarctata* showed small Na^+ efflux, which resembled the salt-sensitive cultivar IR64 (Figure 4.3). Among the *Oryza* species, *O. coarctata* was the only species with significant salt-induced Ca^{2+} influx compared to control (Figure 4.3-4). No significant correlations between Na^+ and Ca^{2+} fluxes and biomass were observed.

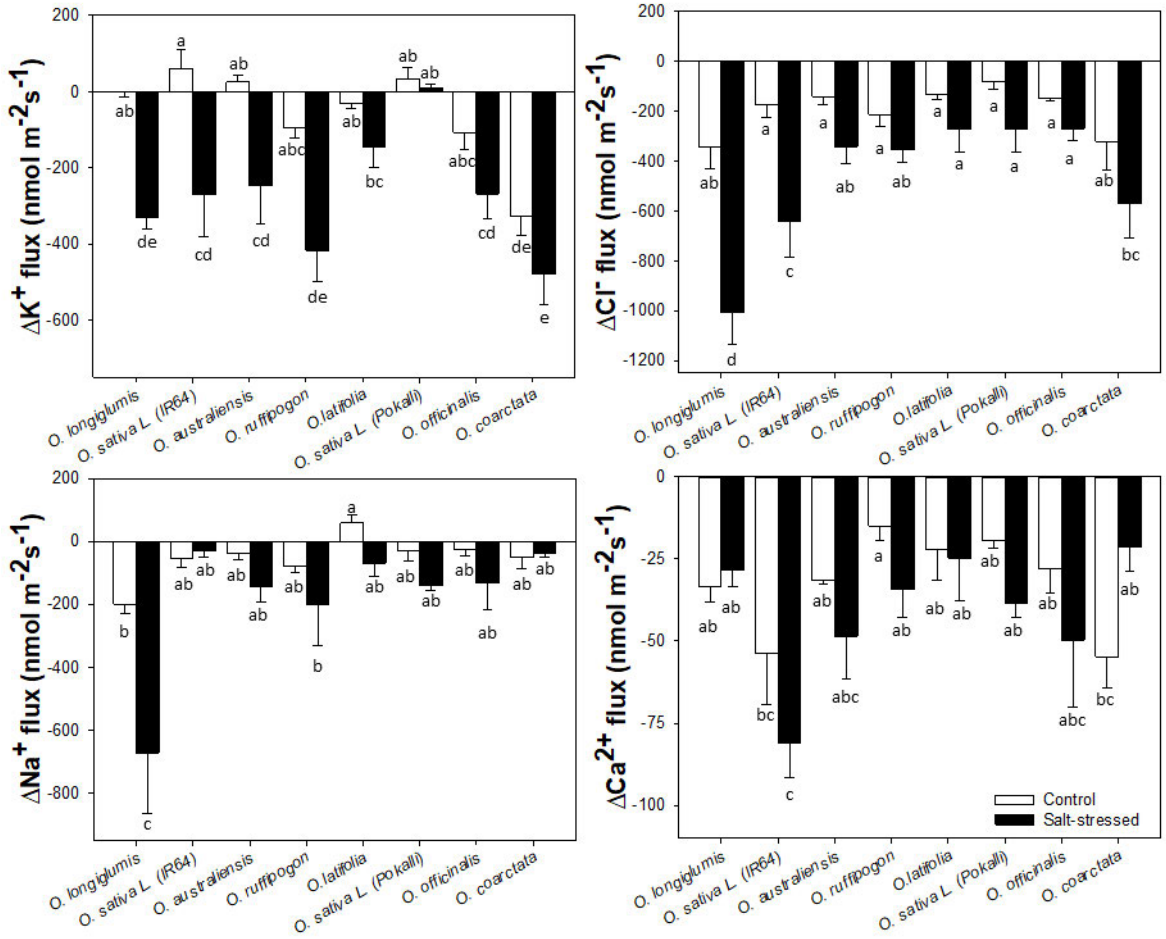

Figure 4.3. Effects of salinity on steady-state net ion fluxes of leaf mesophyll of cultivated and wild rice species. Data are net K^+ Na^+, Cl^- and Ca^{2+} fluxes of leaf mesophyll collected from control and salinity stressed plants. The data are the mean ± standard errors (n= 4 - 8 replicates). Different lowercase letters indicate a significant difference at $P<0.05$.

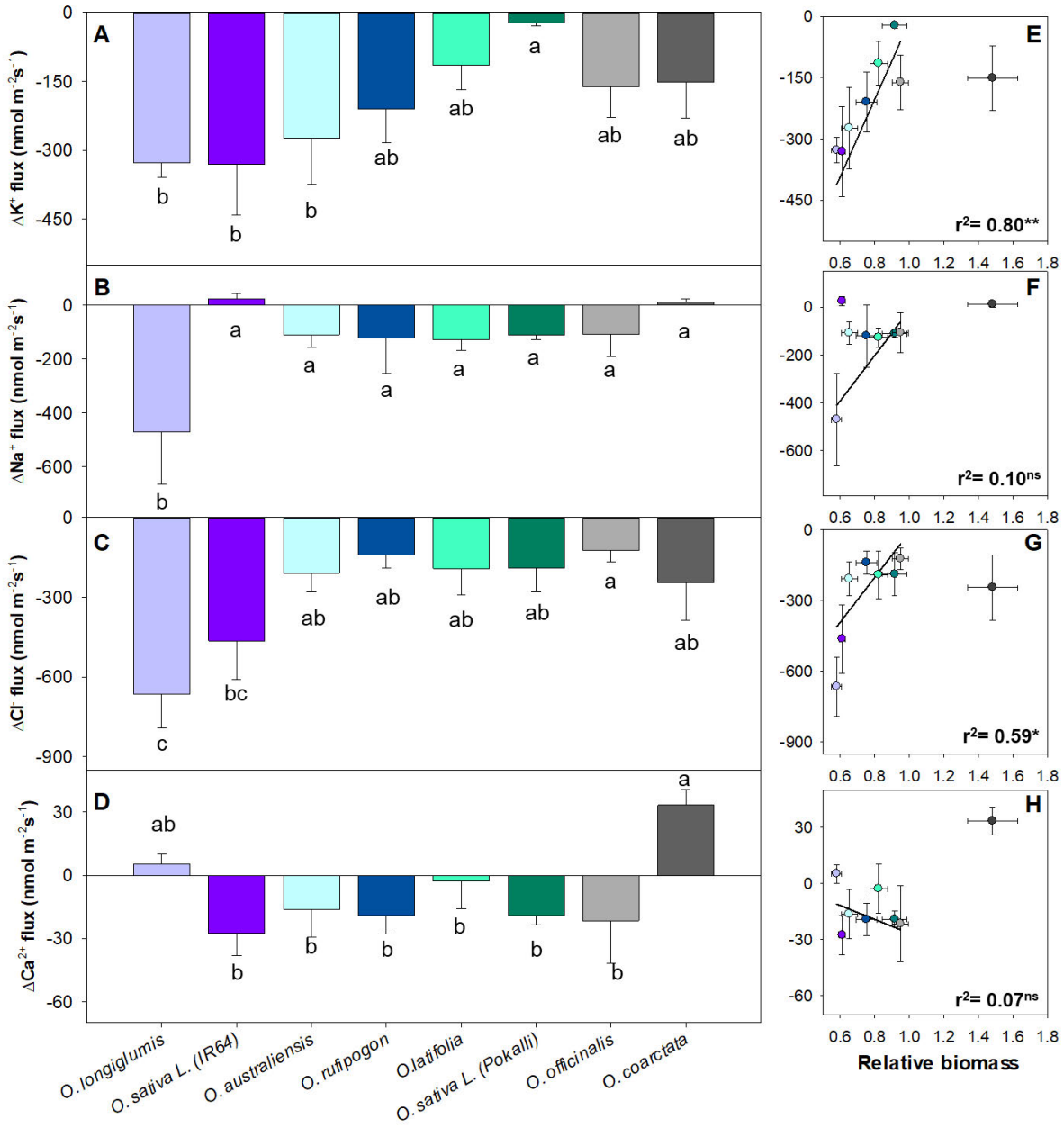

Figure 4.4. Linking salinity tolerance of wild and cultivated rice to recovery net ion fluxes of mesophyll cells. Δ flux was the flux difference between flux from salinity stressed and control samples: (A) K^+, (B) Na^+, (C) Cl^- and (D) Ca^{2+} flux. Pearson analysis of each flux: (E) K^+, (F) Na^+, (G) Cl^- and (H) Ca^{2+} with relative biomass. Data are mean value ± SE (n=3). Different lowercase letters indicate a significant difference at $P<0.05$.

I then evaluated the expression of transporters known for K^+ Na^+, and proton transport and salinity tolerance - *HAK1*, *HKT1*, *NHX1*, *SOS1*, and *VHA-C.* Among the tested genes, *NHX1* and *HAK1* were highly responsive to the salinity stress in most of the *Oryza* species with had significant fold change relative to the control (Figure 4.5_B and 4.5_F). However, the expression pattern was not unique within the salt-tolerant group or distinct between the salt-tolerant and sensitive group of the wild and cultivated rice species (Figure 4.5). Interestingly, the expression of *HKT1;4*, SOS1, and *VHA-C* in most salt-tolerant species *O. coarctata* were insignificant ($P>0.05$). This reveals the importance of these transporters for wild rice in long-term salinity stress.

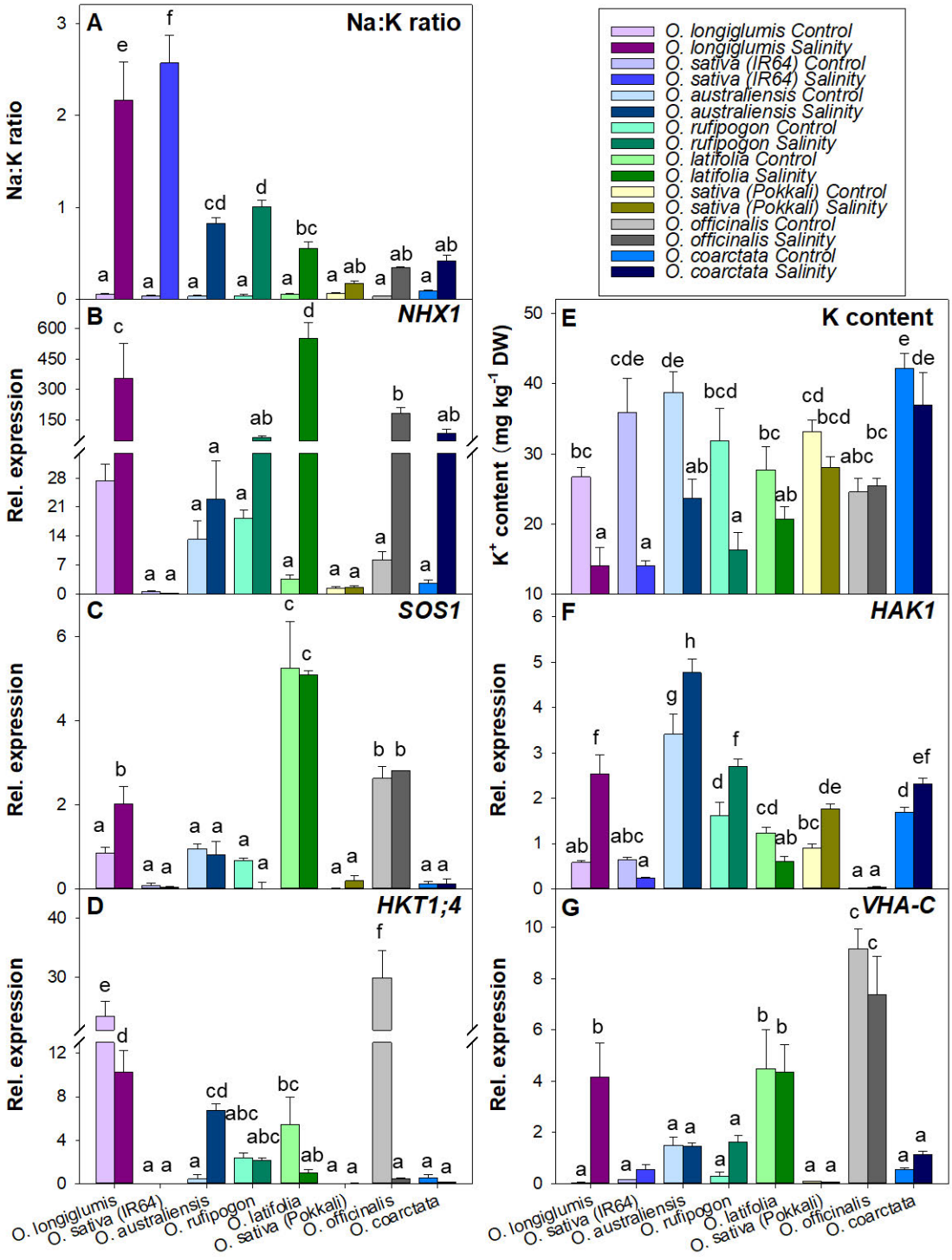

Figure 4.5. Leaf ion content and expression of salinity tolerance marker genes in wild and cultivated rice. (A) leaf Na^+/K^+ ratio and (E) leaf K^+ content. Gene expression of salinity tolerance-related transporter of wild rice and the cultivars - (B) *NHX1, (C) SOS1,* (D) *HKT1;4*, (F) *HAK1*, and (G) *VHA-C.* Data are mean value ± SE (n=4). Different lowercase letters indicate a significant difference at P<0.05.

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4.3.3 Salt-tolerant wild rice species maintained higher ROS production and lower leaf Na^+ accumulation

ROS accumulation in leaf mesophyll cells of wild and cultivated rice species was observed after 6-week salinity stress (Figure 4.6_A-C). The correlation analysis indicated that ROS accumulation was significantly correlated to the key salinity tolerance indicators: biomass (negative, r^2= 0.66, P<0.05), Na^+/K^+ ratio (negative, r^2= 0.75, P<0.05), mesophyll Na^+ intensity (negative, r^2= 0.56, P<0.05) and net Cl^- flux (positive, r^2=0.79, P<0.01). Halophytic *O. coarctata* produced higher ROS in mesophyll cells and maintained the largest relative biomass in response to salinity treatment (Figure 4.6_B).

Na^+/K^+ ratio is one of the golden standards for salinity tolerance evaluation (Liu et al., 2019). The result showed that the leaf Na^+/K^+ ratio in all *Oryza* species was significantly increased after the prolonged salinity stress and a smaller leaf Na^+/K^+ ratio was found in salt-tolerant wild rice species. Interestingly, Among the salt-tolerant species, the leaf Na^+/K^+ ratio in *O. latifolia*, *O. officinalis*, *and O. coarctata* was significantly higher compared to the tolerant landrace Pokkali (Figure 4.5_A). *O. longiglumis*, IR64, *O. rufipogon*, and *O. australiensis* had a significant reduction in leaf K^+ content, suggesting higher salt sensitivity in these species (Figure 4.5_E). The leaf Na^+ (negative; r^2=0.74, P<0.05), K^+ (positive; r^2=0.80; P<0.01) and Na^+/K^+ ratio (negative; r^2=0.74, P<0.05) were strongly correlated to the relative biomass (Table 4.2). To further confirm whether the salt-tolerant wild rice species accumulate higher Na^+ in mesophyll cells, Na^+ accumulation in mesophyll cells was evaluated using confocal imaging. The result showed a similar trend found in leaf Na^+ content where mesophyll cell Na^+ fluorescence decreased along with the increase of salt tolerance in those rice species (Figure 4.6_E-F). The Na^+ intensity was moderately correlated to the leaf Na^+ content (positive, r^2=

0.65) (Table 4.2) and highly correlative to biomass (negative, r^2=0.80, P<0.01) (Figure 4.6_E). It is worth noting that *O. coarctata* showed higher Na^+ accumulation compared to the other three most tolerant rice species (Figure 4.6_E-F).

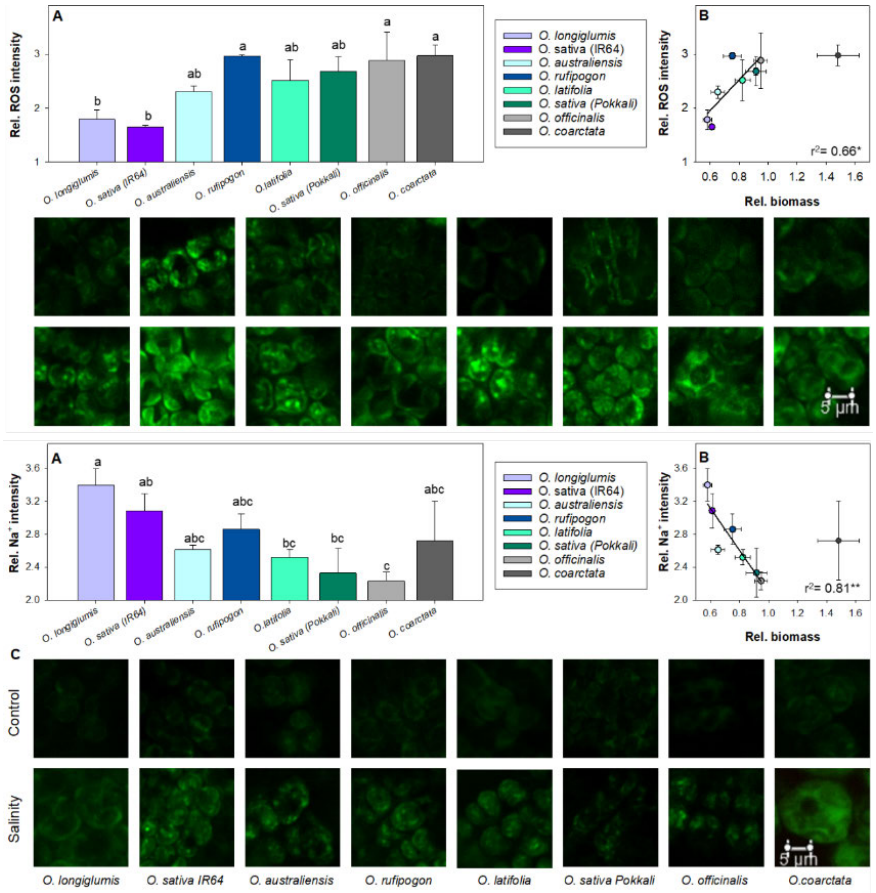

Figure 4.6. Linking salinity tolerance of wild and the cultivated rice to accumulation of reactive oxygen species (ROS) and Na^+ in leaf mesophyll cells. (A) relative ROS and Na^+ intensity in the mesophyll of salinity stressed and control plant; (B) Pearson correlation of relative ROS and Na^+ intensity

and relative biomass; (C) representative image of ROS and Na^+ in the mesophyll. The scale bars=5μm. Data are mean value ± SE (n=4 biological replicates). **(P<0.01), *(P<0.05) and ns (not significant). Different lowercase letters indicate a significant difference at P<0.05.

Table 4.2. Correlation analysis among the physiological parameters and gene expression in cultivated and wild rice species. * P<0.05 ** P<0.01.

	rDW	rPH	rTN	ΔK^+ flux	ΔNa^+	ΔCl^- flux	ΔCa^{2+}	rROSf	rNaf	rNa	rK	rNa:K
rDW	1											
rPH	0.38	1										
rTN	0.84*	0.67	1									
ΔK^+ flux	0.89**	0.24	0.80*	1								
ΔNa^+ flux	0.32	-0.36	-0.09	0.24	1							
ΔCl^- flux	0.77*	0.12	0.61	0.69	0.60	1						
ΔCa^{2+} flux	-0.27	-0.06	0.04	-0.09	-0.86*	-0.46	1					
rROSf	0.81*	0.40	0.76*	0.72	0.24	0.89**	-0.23	1				
rNaf	-0.90**	-0.25	-0.79*	-0.81*	-0.51	-0.87*	0.38	-0.75	1			
rNa	-0.86*	-0.36	-0.89**	-0.97**	-0.12	-0.73	0.00	-0.80*	0.81*	1		
rK	0.90**	0.51	0.90**	0.73	0.07	0.59	-0.05	0.65	-0.85*	-0.75	1	
rNa:K	-0.86*	-0.37	-0.91**	-0.86*	-0.21	-0.86*	0.09	-0.86*	0.91**	0.94**	-0.82*	1
r*A*	0.56	0.25	0.50	0.69	0.58	0.67	-0.60	0.53	-0.67	-0.68	0.33	-0.62
rg_s	0.49	0.19	0.55	0.73	0.29	0.37	-0.21	0.24	-0.60	-0.69	0.42	-0.57

r*E*	0.70	0.66	0.74	0.40	-0.22	0.39	0.08	0.63	-0.52	-0.47	0.83*	-0.57
r*HAK1*	0.33	0.22	0.34	0.03	-0.33	0.07	0.35	0.24	-0.21	-0.06	0.58	-0.22
r*HKT1:4*	-0.43	0.20	0.02	-0.25	-0.30	-0.02	0.20	-0.01	0.20	0.04	-0.33	-0.05
r*SOS1*	0.29	-0.29	0.24	0.24	-0.24	0.09	0.59	0.12	-0.25	-0.23	0.45	-0.29
r*NHX1*	-0.66	0.19	-0.18	-0.58	-0.82*	-0.61	0.68	-0.44	0.61	0.41	-0.36	0.37
r*VHA-C*	0.04	-0.33	0.06	0.09	-0.49	-0.18	0.81*	-0.04	0.09	-0.09	0.18	-0.05
rChl a	0.22	-0.15	0.39	0.54	0.02	0.42	-0.45	0.24	-0.37	-0.64	0.16	-0.55
rChl b	0.55	-0.29	0.41	0.87*	0.61	0.79*	-0.87*	0.51	-0.73	-0.71	0.36	-0.71

	r*A*	rg_s	r*E*	r*HAK1*	r*HKT1:*	r*SOS1*	r*NHX1*	r*VHA-C*	rrchl a	rchl b
rDW										
rPH										
rTN										
ΔK^+ flux										
ΔNa^+ flux										
ΔCl- flux										
$\Delta Ca2^+$ flux										
rROSf										
rNaf										
rNa										
rK										
rNa:K										
r*A*	1									
rg_s	0.83*	1								
r*E*	-0.05	-0.09	1							

r*HAK1*	-0.55	-0.46	0.81*	1						
r*HKT1:4*	0.13	0.08	-0.29	-0.39	1					
r*SOS1*	-0.45	-0.16	0.41	0.74	-0.38	1				
r*NHX1*	-0.56	-0.35	-0.10	0.10	0.68	0.01	1			
r*VHA-C*	-0.61	-0.30	0.25	0.62	-0.28	0.92**	0.22	1		
rChl a	0.54	0.74	-0.26	-0.41	0.40	0.14	-0.01	0.13	1	
rChl b	0.72	0.69	-0.10	-0.28	0.01	0.19	-0.57	-0.01	0.78*	1

Abbreviation: rDW- relative dry weight, rPH- relative plant height, rTN- relative tiller numbers, rROSf- relative mesophyll ROS

4.3.4 O. coarctata has Kranz-like anatomical feature, C4-like photosynthetic capacity, and highly expressed C4 photosynthesis-related genes

As the only known halophytic species in *the Oryza* genus, *O. coarctata* showed C4-like photosynthetic capacity in the greenhouse trial (Figure 4.1, Figure 4.7_D-G). To evaluate whether *O. coarctata* is a C4 or C3-C4 intermediate species, I first constructed a phylogenic tree of a C4 related gene *PEPC* of in a group of C3 and C4 plants. The phylogeny showed that *O. coarctata* is phylogenetically closer to C4 grasses such as Setaria, maize and Sorghum compared to other C3 species in the *Oryza* genus (Figure 4.7_A).

Leaf anatomy was investigated to confirm if *O. coarctata* possesses proto-Kranz or Kranz anatomy for C4 photosynthesis (Sage et al. 2014). The *O. coarctata* leaf cross-section showed a larger vascular bundle sheath cell relative to the mesophyll cell, higher numbers of chloroplasts in the vascular bundle, and lower numbers of mesophyll cells between vascular systems. In *O. coarctata*, the leaves have a very distinct adaxial surface, which is formed with a high density of ridges and furrows (Figure 4.8_B). Each ridge contains a vascular system, and salt glands are found on the surface of the furrows for salt exclusion. The number of mesophyll cells between each adjacent vascular system are significantly lower in *O. coarctata* than reported in *O. sativa* (Chatterjee et al., 2016). The chlorophyll pigment is nearly undetectable in the vascular bundle sheath cells under the confocal microscope (Figure 4.7_B-C). Under transmission electron microscopy (TEM), plasmodesmata connection between mesophyll cells and vascular bundle sheath was observed (Figure 4.7_H). The confocal results showed a strong Na^+ signal in the epidermal layer, salt glands, vascular system, sclerenchymatous thickening, and mesophyll cells (Figure 4.7_B-C). Na^+ accumulation in mesophyll cells was most likely being stored in the vacuole (Figure 4.7_C). Overall, *O. coarctata* had significantly ($P<0.01$) higher total chlorophyll content, CO_2 assimilation rate (*A*), Water use efficiency (WUE), and

significantly lower intracellular CO_2 concentration (C_i) compared to the other six *Oryza* species (Figure 4.8_D-G). The AC_i curve result indicates that *O. coarctata* was highly responsive to increasing CO_2 concentration withCO_2 compensation points at 65.5 µmol CO_2 m^{-2} s^{-1} (3-parameter exponential curve) (Figure 4.7_I) and 50.4 µmol CO_2 m^{-2} s^{-1} with a linear fitting of the first 6 points.

Further, I compared the expression of five C4 photosynthesis-related genes between *O. coarctata and O. sativa* (IR64 and Pokkali) in the control and salinity treatment (Figure 4.8). The result indicated that expression of C4 photosynthesis-related genes was overall significantly higher (up to 800-fold for *RBCL*) in both control and salinity stressed *O. coarctata* as compared to those in *O. sativa*. *PEPC* was the only gene upregulated in *O. coarctata* after the salinity stress while *PPDK, RBCL,* and *RBCS* were significantly downregulated after salinity stress (Figure 4.8_B-E).

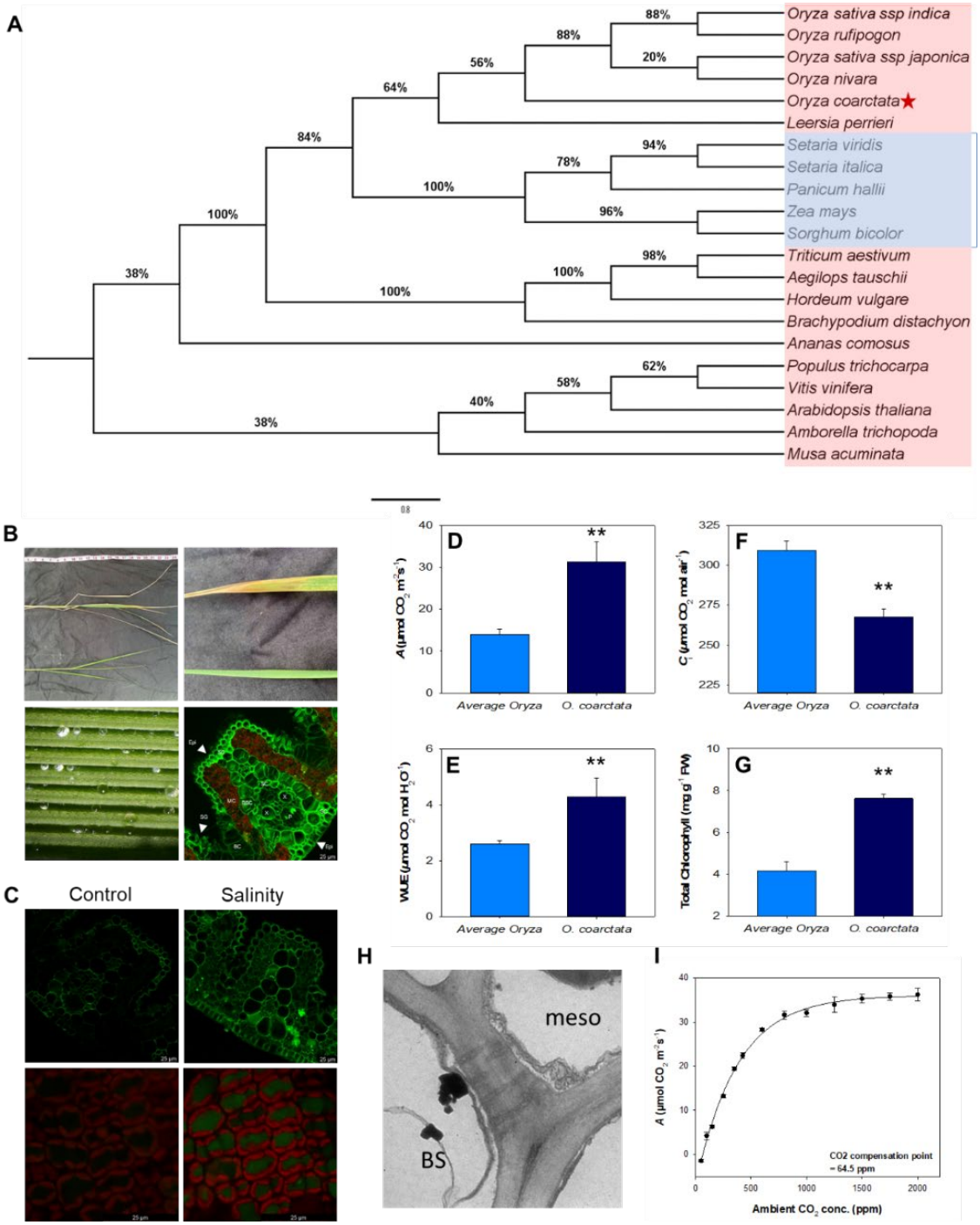

Figure 4.7. Phylogenetic, morphological, and photosynthetic evidence of C3-C4 intermediate in *Oryza coarctata*. (A) Maximum likelihood tree showing the phylogenetic relationship of PEPC a C4 key enzyme in C3 (red) and C4 (blue) species with bootstrap values shown in percentage. (B-C) Leaf morpho-anatomical traits of superior tolerance and cell-specific Na^+ accumulation in response to high salinity stress. (D-G) High leaf gas exchange

characteristics compared to other *Oryza* species used in this study. (H) Leaf ultrastructure of the presence of plasmodesmata connecting mesophyll cell and bundle sheath cells. (I) Photosynthesis dependence on intracellular CO_2 curve fitting a C4 type photosynthesis model species. The curve was fitted with a 3-parameters exponential rise to maximum [Net CO_2 assimilation=-6.68641^{+}42.9581*(1-exp(-0.0027* CO_2 concentration) with a calculated CO_2 compensation point of 65.6. Data are mean value ± SE (n=3). **(P<0.01), *(P<0.05).

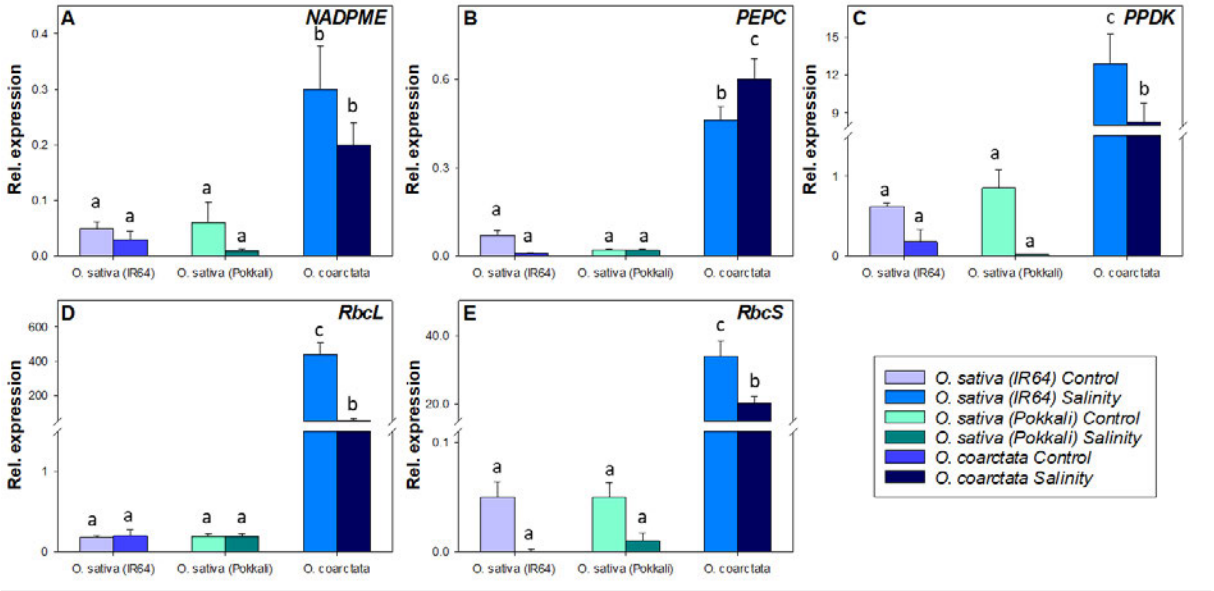

Figure 4.8. Molecular evidence of C3-C4 intermediate for *Oryza coarctata*. Effect of salinity on the C4 photosynthesis-related genes in *O. coarctata,* Pokkali, and IR64- (A) *NADPME*, (B) *PEPC, (C) PPDK,* (D) RBCL, and (E) *RBCS.* Data are mean value ± SE (n=4). Different lowercase letters indicate a significant difference at P<0.05.

4.4 Discussion

More than 10,000 years ago, ancient humans began to consume grains of *O. rufipogon* that grew in the marshes throughout Asia. Continuous domestication for desirable traits has slowly transformed *O. rufipogon* into *O. sativa*, a staple crop for billions of people worldwide (Kovach *et al.,* 2007; Molina *et al.,* 2011). However, *Oryza sativa* was estimated to only conserve 20% of the genetic diversity of wild *Oryza* after almost 8,000 years of cultivation from the lower Yangtze region, China (Zong et al. 2007; (Palmgren et al., 2015). Narrow genetic diversity is the major constraint for breeding salinity-tolerant rice lines (Waziri et al., 2016, Chen et al., 2020). Thus, investigating gene expression, ion homeostasis, Na^+ transport between vascular system, and mesophyll tissue in salt-tolerant wild rice species may be able to decipher new mechanisms contributing to salt tolerance.

4.4.1 Salt-tolerant wild rice exhibits distinct leaf tissue tolerance to Na^+ and K^+ transport compared to O. sativa

Salt-tolerant *O. sativa* generally employs the Na^+ exclusion mechanism to avoid high Na^+ accumulation in above-ground tissue due to a lack of tissue tolerant to Na^+ in this rice species (Gerona et al., 2019, Lutts et al., 1996, Prusty et al., 2018). In this study, I evaluated the performance of 6 wild *Oryza* species under salinity stress with the cultivars after prolonged stress at a moderate level of salinity of 10 dSm^{-1}. I found *O. coarctata*, *O.officinalis,* and *O. latifolia* were highly tolerant to salinity stress. However, only the halophytic *O. coarctata* showed high Na^+ accumulation in mesophyll cells in salt stress (Figure 4.5-6), suggesting that salt-tolerant wild rice *O. officinalis* and *O. latifolia* still avoid Na^+ accumulation in mesophyll cells.

In this study, one of the most interesting findings is that the expression of *SOS1* and *HKT1;4* in the halophytic *O. coarctata* was consistently low and was not affected after 6 weeks of salt treatment (Figure 4.5), suggesting that this wild rice species may not employ the typical Na^+ exclusion mechanism to salt tolerance. SOS1, HKT1;4 and NHX1 are important Na^+ transporters responsible for Na^+ exclusion, compartmentation and retrieval (Wu et al., 2018b). SOS1 (Oh et al., 2009, El Mahi et al., 2019) and HKT1;4 (Hauser and Horie, 2010, Mishra et al., 2016, Garciadeblás et al., 2003) work complementary to exclude Na^+ from mesophyll and then retrieve from intercellular space to the vascular system to reduce leaf Na^+ accumulation. regardless of salt sensitivity, all wild rice species including *O. coarctata* maintained a significantly higher level of *NHX1* expression after six weeks of salinity stress compared to the control and the two domesticated cultivars IR64 and Pokalli (Figure 4.5_B), indicating the key role of this vacuolar Na^+/H^+ exchanger in wild rice. However, the expression of *SOS1* and *HKT1;4* were high in salt-tolerant wild rice *O. officinalis,* and *O. latifolia* in the control conditions, but they mostly remained unchanged or downregulated in most species (Figure 4.5_C-D). Therefore, Na^+ accumulation (Figure 4.6) was more related to *NHX1* expression instead of transcripts of *SOS1* and *HKT1;4* at the late stage of salt stress in *O. coarctata*.

I previously reported a significant positive correlation between net K^+ flux and salinity-tolerant in *O. sativa* (Yong et al., 2020). Here, I extended this finding to the wild *Oryza* species and cultivar except for *O. coarctata* (Figure 4.4). The result showed a significant, strong, and positive correlation between net K^+ flux and biomass (Figure 4.4_E). In addition, net K^+ flux was also significantly and positively correlated with mesophyll Na^+ intensity, leaf Na^+ content, and Leaf Na^+/K^+ ratio (Table 4.2). K^+ is the macro-nutrient, responsible for coordination with more than 50 enzymes in the plant (Shabala and Cuin, 2008). Stress-induced K^+ leakage from the tissue in recovery was reported in salinity (Liu et al., 2017a), hypoxia (Wang et al., 2017b), drought (Mak et al., 2014), and high light (Babla et al., 2020). This mechanism enables the

operation of the GORK channels as a master switch of the cell metabolism, thus adjusting intracellular K^+ homeostasis to altered environmental conditions (Adem et al., 2020). Also, the K^+ efflux may be conducted by NSCCs and NORCs (Zepeda-Jazo et al., 2008). The result indicated that these ion channels may be a key factor for K^+ leakage in sensitive wild rice species. Alternatively, K^+ can be actively transported into the cell through the HAK transporter family. Its importance in salinity tolerance was reported with some key members in rice: OsHAK1, OsHAK5, and OsHAK21 (Chen et al., 2015, Feng et al., 2019, Mangano et al., 2008, Nieves-Cordones et al., 2017, Shen et al., 2015). *HAK1* expression was overall significantly upregulated in wild rice but downregulated in cultivated rice after the salinity stress (Figure 4.5_F). Here, I demonstrate the fundamental difference of K^+ transport systems between wild and cultivated rice, which requires further research work.

4.4.2 Oryza coarctata is an outlier of salt tolerance mechanisms in the Oryza species

Photosynthesis is one of the first lines of stress response and the integrated outcome at the later stage of stress. Plant gas exchange cannot be maintained without balancing ions, organic compounds, and water within a tolerable range of species-specific tissue tolerance under salinity stress (Shabala and Cuin, 2008, Munns et al., 2020b, Mishra et al., 2020). . In cultivated rice, photosynthetic activities are negatively correlated with salinity stress in terms of concentration and duration of salinity stress (Radanielson et al., 2018, Yeo et al., 1985). However, larger scale screening of inter-varieties correlation comparison study is limited or barely related in other related parameters, such as chlorophyll content- salinity tolerance (Kanawapee et al., 2012). In this study, *O. coarctata* is the only species that showed an increase of biomass after the 6-week 10 dSm^{-1} salinity stress (Figure 4.2). In comparison to other *Oryza* species, *O. coarctata* exhibited the highest *A*, WUE, and total chlorophyll content in both control and salt treatments. None of these parameters was significantly affected by salinity

stress (Figure 4.1 and Figure 4.7_D-G), indicating remarkable modification in photosynthetic traits to adapt to saline conditions in this species (Bromham and Bennett, 2014). In addition, across the six key physiological parameters that were significantly correlated to relative biomass in the studied glcophytic rice species (Figure 4.3, 4.5), *O. coarctata* is a consistent outliner that its salinity tolerance showed links neither to net K^+ and Cl^- efflux nor to ROS production and Na^+ intensity. Thus, the salinity tolerance of *O. coarctata* must be determined by other mechanisms.

I also found that confocal imaging of Na^+ localization in *O. coarctata* reveals higher Na^+ intensity in salt glands, and intercellular region of vascular tissue, epidermal tissue than that in the mesophyll tissue (Figure 4.7_B). Therefore, the majority of the Na^+ might be transferred through the whole plant to salt glands without passing through the mesophyll cells – the major cell types of photosynthesis. This was validated by experiments of 2-hours of direct treatment of 50 mM and 100 mM NaCl on the mesophyll tissue of *O. coarctata* that had epidermis removed. The directly stressed mesophyll showed obvious accumulation of Na^+ around or within the chloroplast. This was not found on leaf samples with intact epidemics from the plant after 1 month of salinity treatment in the hydroponic solution (Figure 4.7_B). Thus, *O. coarctata* can avoid Na^+ accumulation around the critical mesophyll tissue for the maintenance of high photosynthesis under salinity (Figure 4.1), which was also reported in other plant species (Kotula et al., 2020, Kotula et al., 2019).

4.4.3 Linking C3-C4 intermediate photosynthetic type to high salt tolerance O. coarctata

There is an increasing number of reports about the potential genetic engineering of C4 photosynthesis into C3 crops such as rice to improve productivity and stress tolerance (Wang et al. 2017; Ermakova et al. 2020). Evolutionarily, C3 to C4 photosynthesis transition was

closely related to ambient CO_2 concentration and temperature (Sage, 2004, Edwards et al., 2010), which may be stepwise to convert a few key anatomical structures and cellular biochemistry in leaf to the Kranz anatomy (Wang et al., 2017). Three C3-C4 intermediates were proposed as proto-Kranz, C2-$C2^+$ photosynthesis, and C4-like photosynthesis, based on the mesophyll cell numbers between veins, the size of bundle sheath cells, amount and coordination of chloroplast and mitochondria in bundle sheath cell, and Rubisco and PEPC regulation (Sage et al., 2014). In grasses, no C4 plant is reported so far in the BEP (Bambusoideae, Ehrhartoideae, Pooideae) clade and the *Oryza genus* belongs to this clade (Grass Phylogeny Working Group II, 2012). However, many members of BEP clade were also reported with C4-like leaf structure and *O. coarctata* is currently classified in this group (Christin et al., 2013). In addition, C4 plants usually have thin leaves so that the mesophyll and bundle sheath cells can coordinate closely to assure the efficiency of C4 photosynthesis (Ghannoum et al., 2005). *O. coarctata* developed a minor vein on top of the major vein that shortens the distance between mesophyll cells on the adaxial side and vascular system, but this feature was not found in other *Oryza* species (Chatterjee et al., 2016). The A/C_i curve and CO_2 compensation point indicated that photosynthetic activities of *O. coarctata* were responsive to fluctuating CO_2 concentration without true C4 photosynthesis (Schlüter et al., 2017, Ueno et al., 2007, Ku et al., 1983) but rather a proto-Kranz type (Yorimitsu et al., 2019, Vogan and Sage, 2012, Monson and Jaeger, 1991).

In C4 photosynthesis, NADP-ME, PEPC, and PPDK were responsible for oxaloacetate and phosphoenolpyruvate conversion in mesophyll cell, and CO_2 release in bundle sheath cell in C4 cycle (Hibberd and Covshoff, 2010). rbcL and RbcS are two important Rubisco in C4 responsible for the conversion of carbon into an organic form, localized in chloroplast and nucleus respectively (Berry et al., 2016). In this study, the expression of all C4 photosynthesis-related genes (*NADP-ME*, *PEPC*, *PPDK*, *rbcL*, and *RbcS*) were overall significantly higher in

O. coarctata compared to those in the two cultivars (IR64 and Pokkali). Although *O. coarctata* does not possess true C4 photosynthesis mechanisms similar to that of maize and other C4 grasses, the higher gene expression and photosynthesis rate, and water use efficiency as well as the unique cell morphology of *O. coarctata* will be useful for research work in the understanding of potential transition from C3 to C4 in grass species and realise the "C4 rice" research goal in the future.

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Chapter 5: Physiological and cellular response of wild *Oryza* species to salinity stress at reproductive stage in the field

5.1 Abstract

The result of salinity screening conducted in the greenhouse may not be repeatable in the field. This trial was designed to evaluate the salinity tolerance of wild rice species in open field conditions. Here, I aimed to confirm whether the salinity tolerance of wild *Oryza* is different and whether there are significant relations between physiological parameters observed in open-field conditions. Seven wild species and three *O. sativa* varieties were evaluated in this trial. I measured the traits (agronomic, gas exchange traits, recovery ion flux of leaf, mesophyll Na^+ accumulation, and ROS production) that were found significantly correlated to salinity tolerance in the previous chapter. Environmental effect (humidity) had some effect on the plant performance, but it did not alter the salinity ranking. The result indicated that the salinity tolerance of wild rice is highly similar compared to the outcome of the greenhouse (Chapter 4) and other studies. Again, K^+ retention and low Cl^- efflux were found positively correlated to relative fresh weight (RFW), dry biomass (RDW), and relative water content (RWC). Mesophyll's ROS & Na^+ accumulation and relative net CO_2 assimilation rate (R*A*) were moderately correlated to RDW. The result suggested that the salt-tolerant trait from C genomes was originated and inherited in C genomes rather than the common ancestor or before the branching point of B, C, E genome in *O. officinalis* complex. *O. bracyantha* is phylogenetically closely related to *O. coarctata*. It shares 2 leaf anatomical features with *O. coarctata*- numbers of mesophyll cells between 2 adjacent vascular bundle systems & high furrows and ridges density on adaxial size of the leaf. The 2 shared features in *O. bracyantha* did not contribute much to the salinity tolerance. Enlarged vascular bundle sheath and salt gland in *O. coarctata* may be the key anatomical features that contribute to salinity tolerance only with the addition

of other salinity tolerance mechanisms. This chapter provides some insight into species selection for salinity tolerance study in *Oryza*.

5.2 Introduction

Rice is one of the main staple foods for a large segment of the global population which occupied around 50 % of their daily caloric intake (Kaur et al., 2016a). India and Australia are rice-producing countries. It is cultural irreplaceable food in the second most populated nation- India. India contributed 23.5% of the global rice production in 2019 (FAO, 2021). Although rice cultivation in Australia is small (0.1%) relative to the global scale, it was one of the main short & medium grain rice exporters which occupied 5% of the global supply (Fell et al., 2020). However, half of the rice consumption in Australia comes from imports. Besides the water scarcity, saline soil is also one of the main constraints in Australia to expand rice cultivation. Salinity issue is increasing threatening agriculture production. Australia and India have a large area of land that has salinization issues (Prăvălie et al., 2021). Rengasamy (2006) advised that Australia had 16 % of agricultural land that was already affected by salinity and another 67% of the land is at risk. Soil salinization in India is expected to affect 50% of the arable land in 2050 (Kumar and Sharma, 2020). Rice is highly susceptible to salinity stress. It is most prone to salinity stress at the reproductive stage, where yield penalty was observed at electrical conductivity of 3 dSm^{-1} (Yeo et al., 1990). Land salinization is expected only to increase due to global warming, breeding of salinity tolerance rice cultivars is urged for future food security in countries with high rice consumption, such as India.

Oryza genus is divided into 5 complexes based on their genetic characteristic: *Oryza sativa* complex - AA, *O. officinalis* complex - BB, CC, BBCC, CCDD and EE, *O. ridleyi* complex -

HHJJ, O. *meyeriana* complex – GG, and unclassified – (F, K, L genomes) (Shenton et al., 2020, Kumagai et al., 2010, Singh et al., 2018). The only 2 domesticated species, Asian *O. sativa* L. and African *O. glamberrima* Steud with other 5 AA genomes wild rice species are members of *O. sativa* complex. Interestingly, we can barely find tolerant wild *Oryza* species from this complex, reported as tolerant as any highly tolerant *O. sativa* such as Pokkali and Nona Braka. Yet, most of the salinity tolerance studies of wild *Oryza* species were focused on AA - *O. rufipogon* (Scopus search: *O. rufipogon* x salinity, 65 articles) in the past decades. This species was mainly reported as a moderately tolerant species to salinity stress (Prusty et al., 2018, Yichie et al., 2018, Solis et al., 2021).

To date, limited numbers of highly salt-tolerant wild *Oryza* were reported. Excluding the only *Oryza* halophyte- *O. coarctata* (KKLL), these species were all members of the *Oryza officinalis* complex, such as EE - *O. australiensis (Yichie et al., 2018),* CC - *O. officinalis* & *O. eichingeri (Prusty et al., 2018, Nishizawa et al., 2015, Nishizawa et al., 2016)* and CCDD - *O. latifolia* & *O. alta* (Prusty et al., 2018, Nakamura et al., 2002, Nakamura et al., 2004). In *Oryza, the* establishment of salinity-tolerant accession is believed to be a multiple single events. As the consequence, it was found that only a few numbers of landrace in a species are highly tolerant to the salinity stress such as *O. coarctata*, few accessions in *O. sativa,* and accession in *O. australiensis.* In the C genome, several species (CC, CCDD, BBCC) were reported as tolerant to highly tolerant to salinity stress. Considering numbers of accession (>1) in *O. officinalis* and *O. latifolia* were reported as salinity-tolerant, salinity tolerance traits might be successfully evolved and already widely inherited in the C genome compared to others. However, this would need further evaluation with the inclusion of more accessions and species with C genome. For phylogenetically early evolved species, no tolerant species was yet reported in the *HHJJ* complex. *O. coarctata* (KKLL) was the only halophytic species reported in the unclassified

complex. The other member, *O. brachayantha* (FF) was reported as the highly salinity-sensitive species (Prusty et al., 2018).

Salinity screening of rice in the open field for salinity tolerance is challenging due to interference from the environment, such as soil heterogeneity, biological interference, and weather conditions (Shrivastava and Kumar, 2015). However, assessing the salinity tolerance of rice in field conditions is necessary to provide a realistic result compared to the greenhouse and laboratory conditions. It is advised to conduct a salinity screening trial under both controlled and open field conditions, to verify the salinity tolerance ranking result (Qin et al., 2020). Chapter 5 aims to evaluate whether the salinity ranking result in the greenhouse is consistent in the open-field conditions. This includes evaluation of the relationship between cellular responses (net ion flux and Na^+ intensity of mesophyll) and biomass. This chapter included extra lines, *O. brachayantha* (FF), *O. punctata* (BB), *O. alta* (CCDD), Koshihikari, and IR29 (AA) to increase coverage of *Oryza* species in this trial. *O. brachyantha* belongs to the unclassified complex and phylogenetically closely related to *O. coarctata*. It also shared a few common leaf anatomical features presented in *O. coarctata* (Chatterjee et al., 2016). This study also aims to evaluate if these features can improve salinity tolerance. Lastly, the performance of *O. punctata and O. australiensis* will answer if the salinity-tolerant trait is only present in the C genome or originate in the ancestor of the *O. officinalis* complex.

5.3 Result

5.3.1 Salinity response of the wild rice at reproductive stage in the open field

In this study, prolonged salinity treatment on wild *Oryza* at the late vegetative stage and maintained at the reproductive stage was evaluated in an open field experiment. Overall, the

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salinity treatment had a strong, negative impact on the growth of salinity-sensitive *Oryza* lines in the field. Visually, these sensitive lines had shown obvious salinity damage symptoms after 4 weeks of salinity treatment (Fig. 4.3.1_A). The damage symptoms included leaf necrosis from tips, yellowing, rolling, and then finally total senescence of the leaf & tiller, leading to reduced green leaf coverage. The symptoms were more severe on the sensitive lines- *O. brachyantha, O. sativa* IR29, and *O. sativa* (Koshihikari).

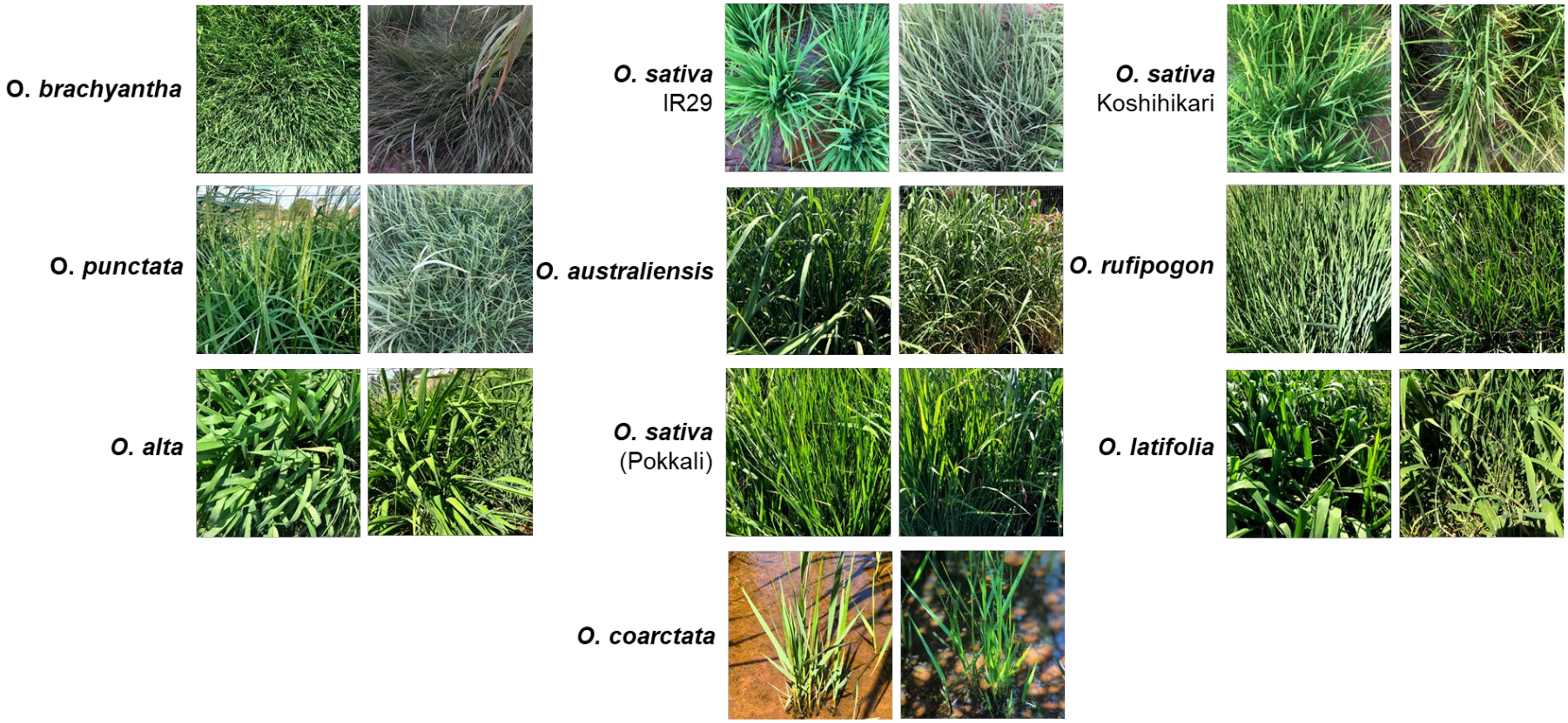

Figure 5.1. Comparison in visual appearance of wild *Oryza* and cultivated rice in response to 10 dS m^{-1} salinity treatment at 4^{th} week of salinity stress. Severe damage on stem and leaf was observed in susceptible species and *O. sativa* compared to the tolerant *Oryza* species.

The salt-sensitive lines also had a significant reduction (ANOVA species*treatment effects, $P<0.05$) in tiller numbers (Figure 5.1_C) and shoot biomass (Figure 5.2). No difference in plant height was found between control and salinity stressed plants (Figure 5.1_B, ANOVA treatment effect, $P>0.05$). *O. bracyantha* is the most susceptible species to the salinity stress, which has the highest reduction in tiller numbers, shoot fresh weight (FW), shoot dry weight (DW) and shoot water content after the salinity stress. It is followed by IR29, Koshihikari, *O. punctata,* and *O. australiensis* as the sensitive group based on the ranking of the relative value of DW, FW, mesophyll ROS production & Na^+ accumulation, K^+ & Cl^- flux, and *A* result (Table 5.1). *O. rufipogon* as moderately tolerant species, and *O. alta,* Pokkali, *O. latifolia*, and *O. coarctata* as the tolerant – highly tolerant species. The result showed that *O. coarctata* is least responsive to the salinity treatment as all the control and salinity treated *coarctata* had the same homologous group in all morphological parameters (Figure 5.2-3), except tiller numbers. Salinity-treated *O. coarctata* had significantly higher tiller numbers compared to the control (Figure 5.1-2).

Table 5.1. Salinity ranking of *Oryza* species based on the relative value of the physiological parameters.

Species	RFW	RDW	RWC	RROS	RNaF	ΔK^+	ΔCl^-	R*A*	Overall
O. bracyantha	1	1	3	1	1	1	1	1	1.2
O. sativa (Koshihikari)	2	3	4	2	3	4	3	6	3.4
O. sativa (IR29)	3	2	7	3	2	2	2	8	3.6
O. punctata	4	4	1	5	4	6	4	3	3.9
O. australiensis	5	5	2	7	5	3	6	2	4.4
O. rufipogon	6	6	6	8	6	7	5	4	6.0
O. alta	7	7	5	10	9	8	7	5	7.2
O. sativa (Pokkali)	8	8	9	6	8	5	8	7	7.4
O. latifolia	9	9	8	9	7	9	9	9	8.6
O. coarctata	10	10	10	10	10	10	10	10	10.0

Abbreviation: RFW- relative fresh weight, RDW- relative dry weight, RWC- relative water content RROSF- relative mesophyll ROS fluorescence, RNaF- relative mesophyll Na^+ fluorescence, ΔK^+- K^+ flux difference(stress/control), ΔCl^-- Cl^- flux difference (stress/control), R*A*- relative net CO_2 assimilation rate

IR29 was found with a high relative shoot water content (0.70). The relative shoot water content was even higher than the tolerant species *O. alta* (0.68). This might also directly affect the gas exchange traits and lead to greater photosynthetic performance Furthermore, two sensitive-moderate tolerant species *O. australiensis* and *O. punctata* had the lowest relative shoot water content- 0.31 and 0.26 respectively, which was lower than the highly sensitive species- *O. bracyantha*- 0.32. Two tolerant species- *O. alta* and *O. latifolia* (0.73) had significantly lower shoot water content compared to the other 2 tolerant species Pokkali (0.93) and *O. coarctata* (1.15). The result indicated variation in osmotic adjustment strategy in different species leading to variation in stress-induced water lost in the shoot.

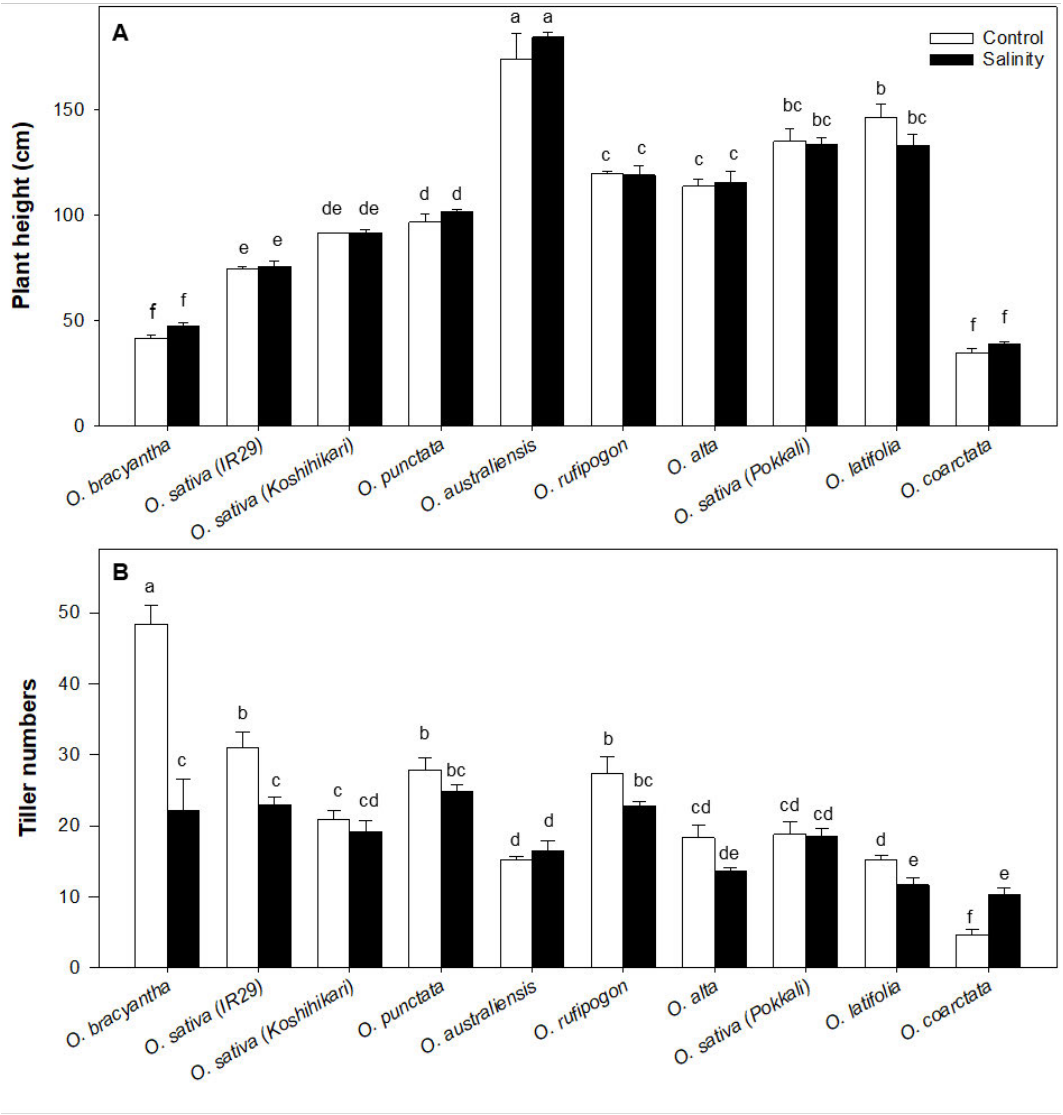

Figure 5.2. Effects of salinity on growth performance of *Oryza* lines after prolonged salinity stress in the field at their late vegetative stage. Data are plant height (A) and tiller numbers (B) after prolonged salinity stress. Data are mean ± SE (n=3). Different lowercase letters indicate significant differences at $P<0.05$.

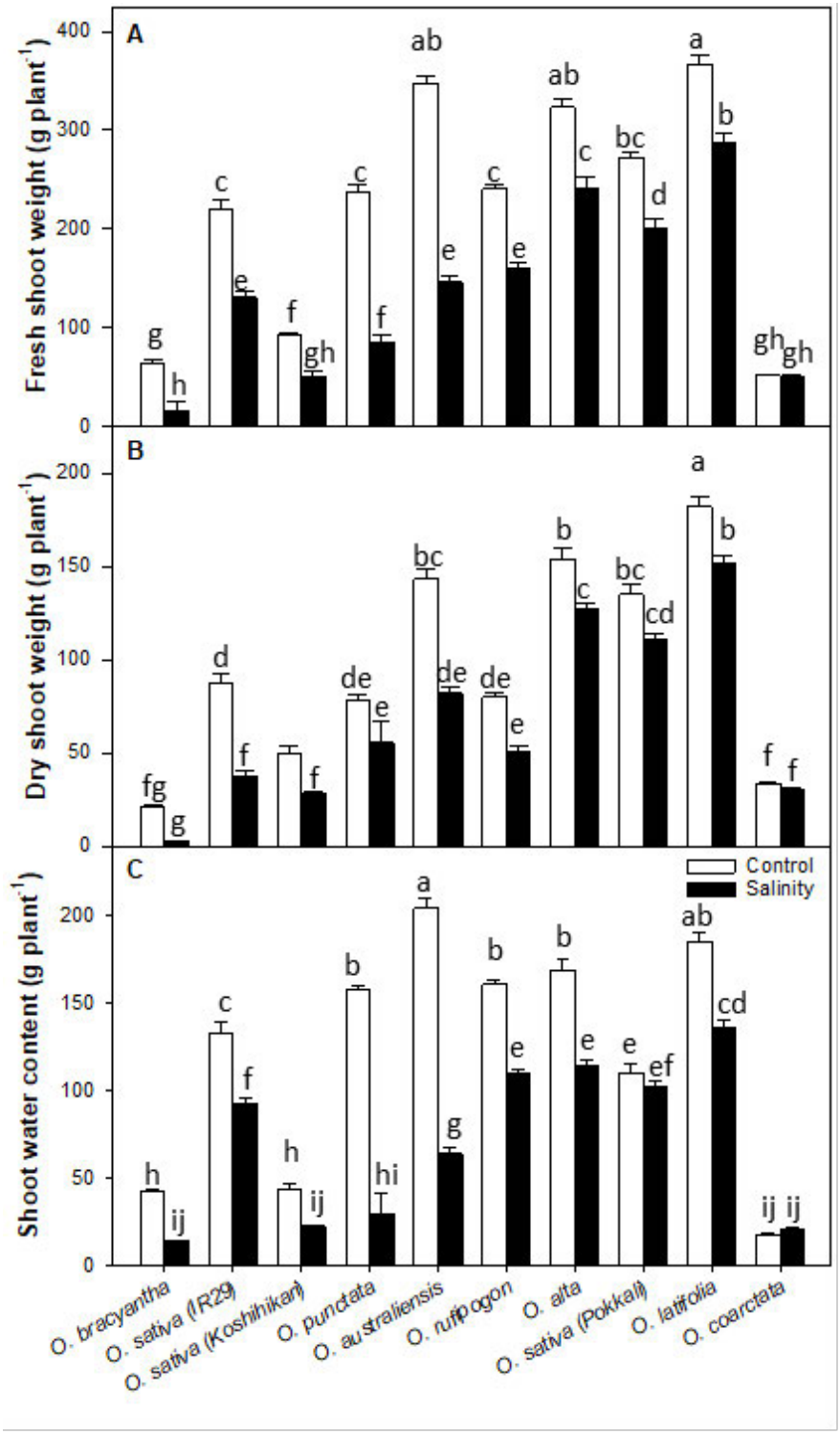

Figure 5.3. Effects of salinity on shoot biomass of *Oryza* lines after 6 weeks of salinity stress in the field at their late vegetative stage. Data are Fresh shoot weight(A), Dry shoot weight (B) and Shoot water content (C) after prolonged salinity stress. Data are mean ± SE (n=3). Different lowercase letters indicate significant differences at P<0.05.

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Table 5.2. Pearson correlation analysis among the physiological parameters in cultivated and wild rice species. * and ** indicate a significant correlation at P<0.05 and P<0.01. (Continue)

	RFW	RDW	RWC	RPH	RTN	RROSF	RNaF	ΔK^+	ΔNa^+	ΔCl^-	$\Delta Ca2^+$
RFW	1.00										
RDW	0.87**	1.00									
RWC	0.96**	0.71*	1.00								
RPH	0.22	0.39	0.13	1.00							
RTN	0.61	0.58	0.62	0.43	1.00						
RROSF	0.47	0.69*	0.23	0.28	0.00	1.00					
RNaF	-0.81**	-0.79**	-0.72*	-0.56	-0.59	-0.53	1.00				
ΔK^+	0.81**	0.91**	0.65*	0.34	0.63*	0.64*	-0.76*	1.00			
ΔNa^+	0.41	0.57	0.34	0.64*	0.64*	0.31	-0.68*	0.59	1.00		

ΔCl^-	0.87**	0.93**	0.76*	0.38	0.65*	0.58	-0.75*	0.87**	0.69*	1.00	
ΔCa^{2+}	-0.21	-0.32	-0.05	0.29	0.42	-0.75*	-0.01	-0.18	0.28	-0.21	1.00
R*A*	0.81**	0.64*	0.81**	0.33	0.54	0.19	-0.59	0.67*	0.27	0.71*	0.08
Rg_s	0.62	0.32	0.73*	-0.09	0.53	-0.20	-0.28	0.35	-0.18	0.28	0.16
RC_i	-0.63	-0.66*	-0.49	-0.29	-0.14	-0.60	0.62	-0.73*	-0.44	-0.70*	0.23
R*Tr*	0.71*	0.38	0.80**	-0.11	0.46	-0.06	-0.35	0.39	-0.18	0.34	0.01
RVpdL	0.36	0.20	0.35	-0.23	-0.27	0.51	-0.37	0.10	-0.02	0.23	-0.59
RTleaf	-0.56	-0.55	-0.47	-0.06	-0.39	-0.54	0.72	-0.57	-0.54	-0.58	0.22
RC_i_C_a	0.63	0.67	0.46	0.32	0.17	0.69	-0.62	0.68*	0.41	0.73*	-0.35
RWUE	-0.44	-0.38	-0.38	-0.54	-0.15	-0.27	0.50	-0.30	-0.32	-0.50	-0.03

	R*A*	Rg_s	RC_i	R*Tr*	RVpdL	RTleaf	RC_i_C_a	RWUE
RFW								
RDW								
RWC								
RPH								
RTN								
RROSF								
RNaF								
ΔK^+								
ΔNa^+								
ΔCl^-								
ΔCa^{2+}								
R*A*	1.00							

Rg_s	0.67*	1.00						
RC_i	-0.69*	-0.05	1.00					
RTr	0.69*	0.98**	-0.12	1.00				
RVpdL	0.04	-0.11	-0.33	0.09	1.00			
RTleaf	-0.16	0.03	0.41	-0.08	-0.65*	1.00		
R$C_i_C_a$	0.65*	-0.02	-0.91**	0.08	0.46	-0.54	1.00	
RWUE	-0.67*	-0.03	0.67*	-0.07	-0.26	0.21	-0.79**	1.00

Abbreviation: RFW- relative fresh weight, RDW- relative dry weight, RWC- relative water content, RPH- relative plant height, RTN- relative tiller numbers, RROSF- relative mesophyll ROS fluorescence, RNaF- relative mesophyll Na^+ fluorescence, ΔK^+- K^+ flux difference$_{(stress-control)}$, ΔNa^+- Na^+ flux difference$_{(stress-control)}$, ΔCl^-- Cl^- flux difference$_{(stress-control)}$, ΔCa^{2+}- Ca^{2+} flux difference$_{(stress-control)}$, RA- relative net CO_2 assimilation rate, Rg_s- relative stomata conductance, RC_i- relative intercellular CO_2 concentration, RTr- relative transpiration rate, RVpdL- relative leaf vapor pressure, RTleaf- relative leaf temperature, RCi_Ca- relative C_i/C_a, RWUE- relative water use efficiency

Leaf gas exchange was significantly affected by the salinity stress (Figure 5.2-3 ANOVA, treatment effect mostly $P<0.05$, except *Ci* & *Tleaf*). The P-values of ANOVA of these parameters in relative value were mostly significant, except *VpdL*, *Tleaf,* and *WUE*. Among, ANOVA analysis of relative *A, Ci,* and *Ci/Ca* were highly significant (ANOVA species effect, $P<0.01$).

In correlation analysis, relative *A, Ci,* and *Ci/Ca* were significantly correlated (all $P<0.05$) to relative DW, ΔK^+, and ΔCl^- which were found significantly correlated to salinity tolerance (Table 5.2). Relative *A* and *Ci/Ca* were positively correlated to relative DW, ΔK^+, and ΔCl^-. Relative *Ci* was negatively correlated with relative DW, ΔK^+, and ΔCl^-. Relative *Tr* was significantly and positively correlated with relative FW ($P<0.05$) and water content ($P<0.01$).

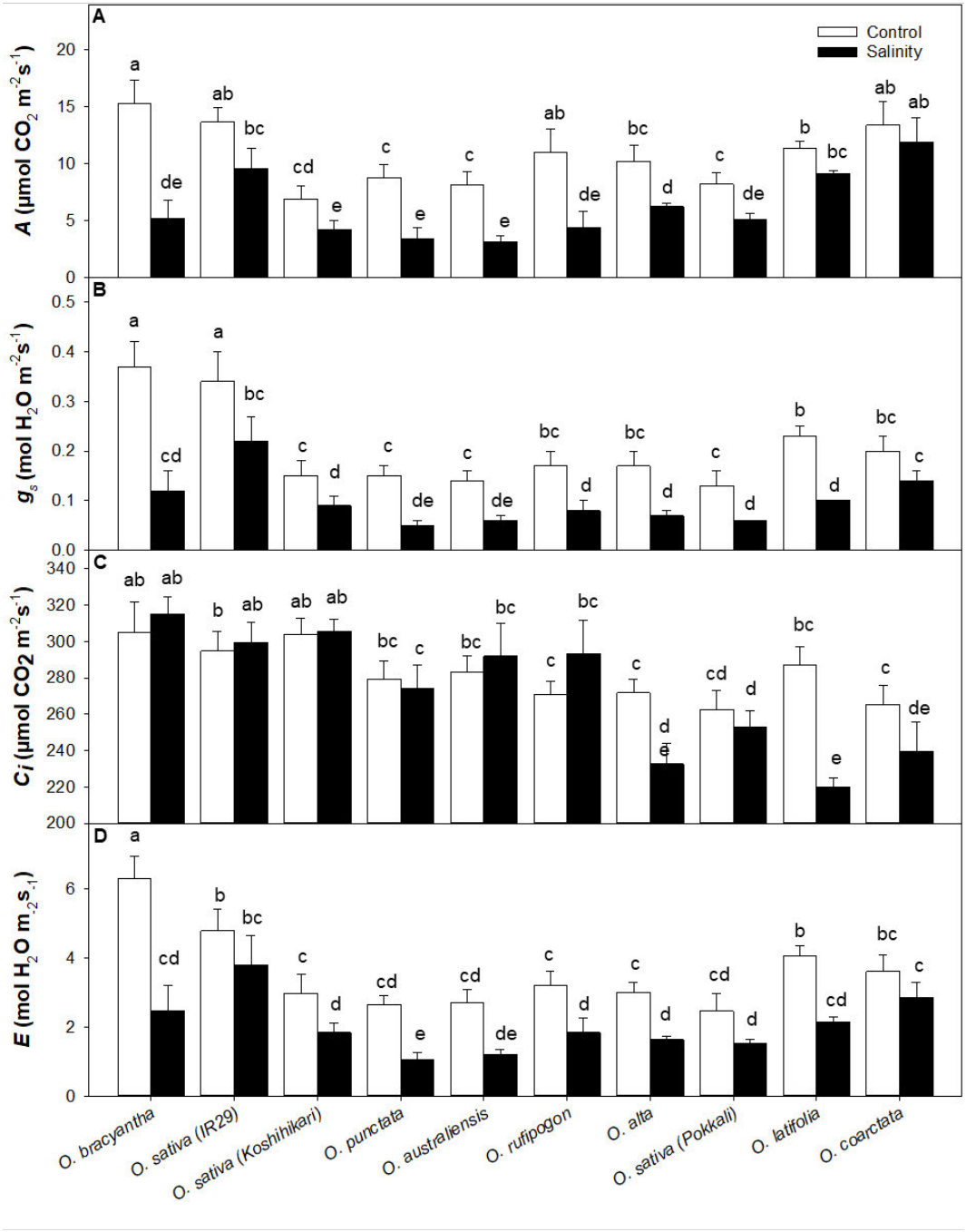

Figure 5.4. Effects of salinity on leaf gas exchange parameters of wild and cultivated rice**.** (A) net CO_2 assimilation rate- *A*, (B) stomata conductance- g_s, (C) transpiration rate- *E*, and (D) intercellular CO_2 concentration- C_i, after 6 weeks of salinity stress. For each species, the left bar indicates Control and the right bar indicates salinity stressed sample. Data are mean value ± SE (biological n=3, technical n= 5). Different lowercase letters indicate significant differences at $P<0.05$.

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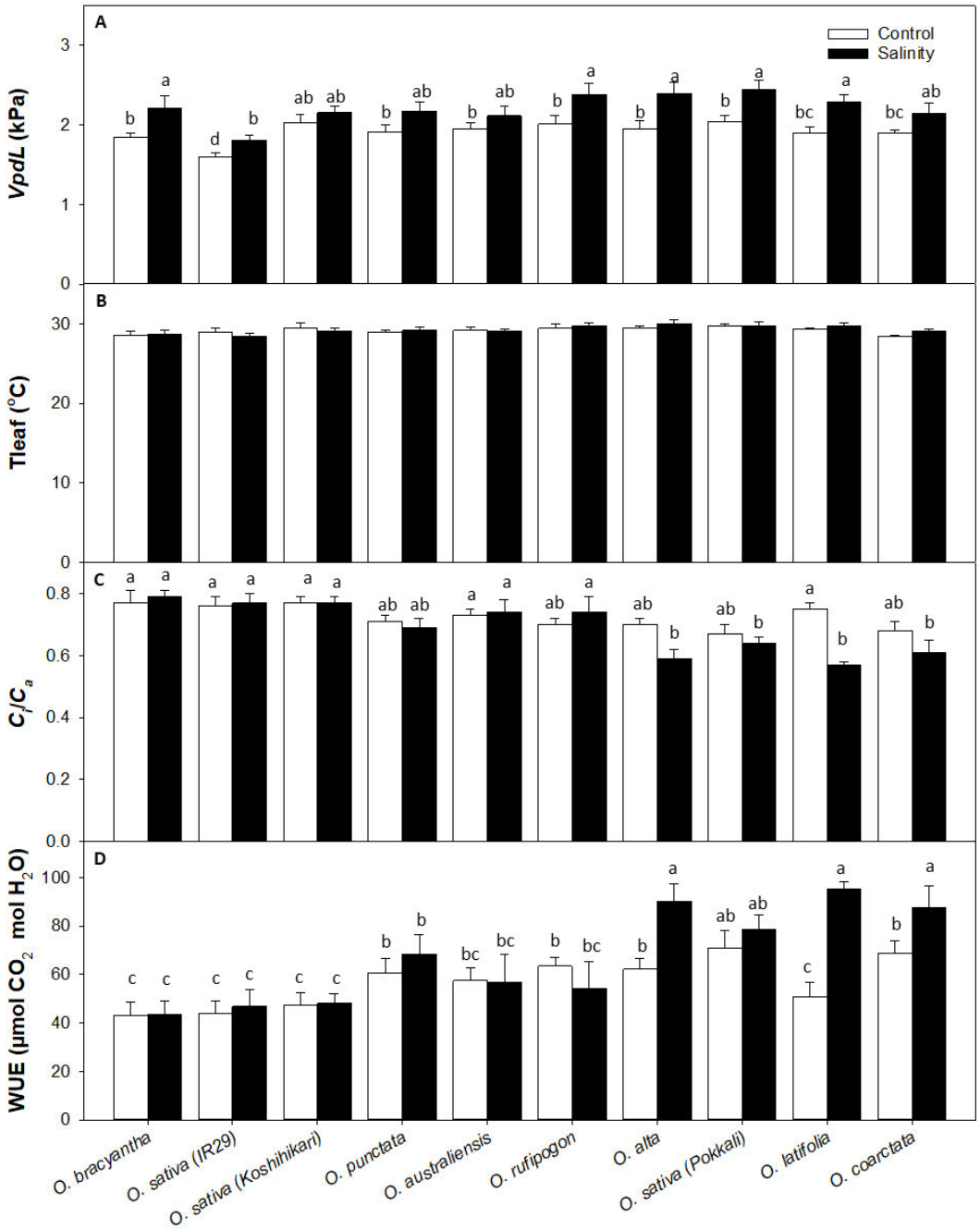

Figure 5.5. Effects of salinity on leaf gas exchange parameters of wild and cultivated rice**.** (A) Vapour pressure difference of leaf- *VpdL*, (B) ratio of intercellular CO_2 concentration to ambient CO_2 concentration.- *Ci_Ca*, (C) leaf temperature- *Tleaf,* and (D) water use efficiency- *WUE* after 6 weeks of salinity stress. For each species, the left bar indicates Control and the right bar indicates Salinity stressed sample. Data are mean values ± SE (biological n=3, technical n= 5). Different lowercase letters indicate significant differences at $P<0.05$.

5.3.2 Mesophyll cells of salt-sensitive rice cultivar release more K^+ and Cl^- after prolonged salinity stress at reproductive stage in recovery ion flux measurements

I evaluated the effect of the salinity stress on the wild and cultivated *Oryza* species by measuring net K^+, Na^+, Cl^- and Ca^{2+} flux of mesophyll tissues after recovery (Figure 5.6). Based on the DMRT's grouping, there was no significant difference between control leaf segments of most species except the net K^+ flux of *O. coarctata*. The fluxes of control were relatively small, indicating good recovery of the mesophyll cells after 1 h of pre-incubation (Figure 5.6).

Consistent with the result reported in Chapters 3 & 4, the net K^+, Na^+, Cl^- and Ca^{2+} fluxes from the mesophyll tissue of the salinity-stressed rice in the open field after recovery were mainly effluxes in all *Oryza* species compared to the control. Among the 4 fluxes results, salinity-sensitive lines with lower FW and DW tend to have higher net K^+ (Figure 5.6_A) and Cl^- (Figure 5.6_C) efflux. Correlation analysis indicated that net K^+ (Figure 5.7_A-C) and Cl^- (Figure 5.7_E-G) efflux were significantly and negatively correlated to the relative biomass-FW, DW, and water content. In which, both fluxes results had the strongest correlation with the relative DW (K^+ flux, $R^2 = 0.831$; Cl^- flux, $R^2 = 0.875$). The correlations with relative FW were moderately strong (K^+ flux, $R^2 = 0.657$; Cl^- flux, $R^2 = 0.762$). In addition, net K^+ and Cl^- fluxes were also significantly correlated to mesophyll's Na^+ content (Figure 5.7_D, 5.7_H), which were the key salinity tolerance indicators. Compared to sensitive lines (*O. brachayantha,* IR29 & Koshihikari), most of the wild lines with moderate to high salinity tolerance had relatively lower Na^+ efflux. Among the *Oryza* species, *O. coarctata* was the only species with significant salt-induced Ca^{2+} and Na^+ influx compared to the control (Figure 5.6_B, 5.6_D). There is no significant correlation between biomass and net Na^+ flux so salinity tolerance selection using net recovery Na^+ flux method modified.

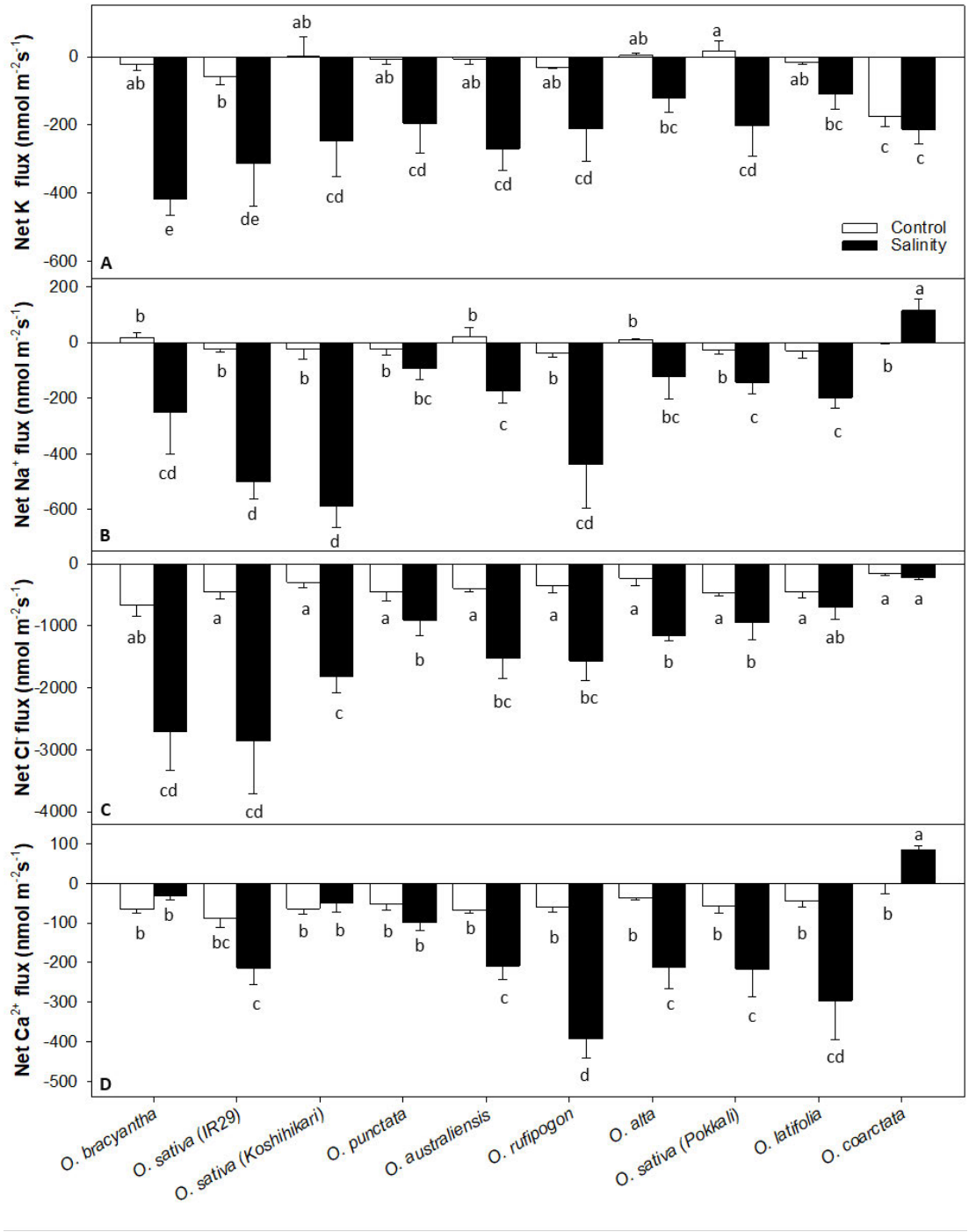

Figure 5.6. Effects of salinity on steady-state net ion fluxes of leaf mesophyll of *Oryza* lines after 6 weeks of salinity stress in the field at their late vegetative stage. Data are net K^+, Na^+, Cl^-, and Ca^{2+} fluxes of leaf mesophyll collected from control and salinity stressed plants. Different lowercase letters indicate significant differences at $P < 0.05$. Data are mean ± SE (n = 4 – 8 biological replicates).

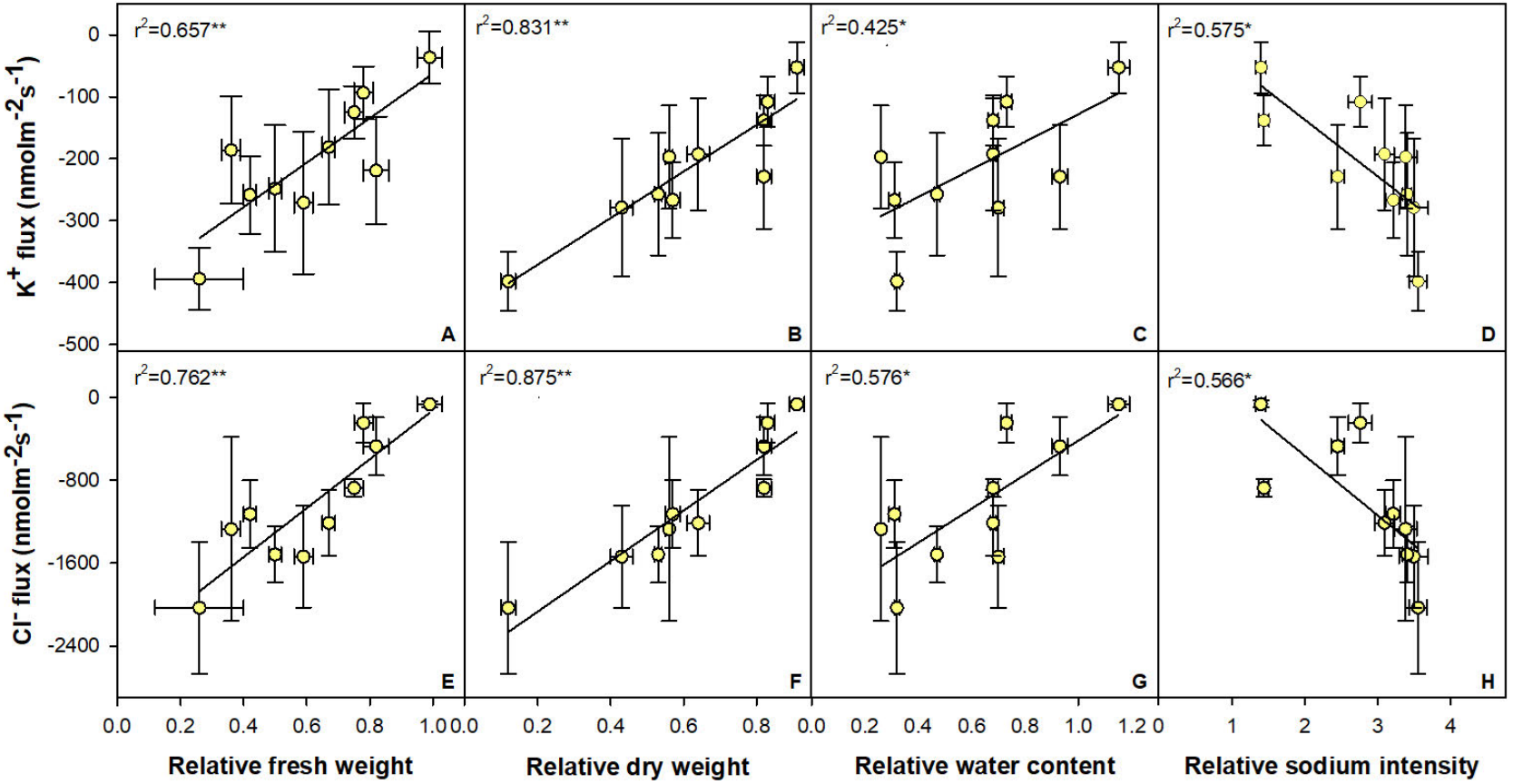

Figure 5.7. Correlation between agronomic traits and recovery ions fluxes of 10 rice species at the late vegetative stage in the field after 6 weeks of salinity stress. Correlation analysis of Δ net K^+ (A-D), and Cl^- (E-H) flux ($flux_{stress-control}$) with agronomic traits, and mesophyll Na^+ intensity respectively. Statistically, a significant correlation is indicated by *(P<0.05) and **(P<0.01).

5.3.3 Salt-tolerant wild rice species maintain higher ROS production and lower Na^+ accumulation in mesophyll cells

Consistent with the mesophyll ROS and Na^+ intensity results reported in Chapter 3, salinity-tolerant *Oryza* lines had significantly higher (Both ROS & Na^+ intensity, ANOVA, main effects, 2-ways interactive effects, all $P<0.01$) ROS production and lower Na accumulation in mesophyll cells after 6 weeks of salinity stress (Figure 5.8). However, The DMRT's result showed that few sensitive, moderately tolerant, and highly tolerant lines were grouped within the same homologous group in the ROS production result (Figure 5.8_A). Relative ROS intensity was only weakly correlated to relative DW (positive, $r^2=0.47$, $P<0.05$) and net K^+ flux (positive, $r^2=0.41$, $P<0.05$). It is possibly due to the dynamic ROS production effect mentioned in Chapter 2. Mesophyll Na^+ accumulation could be a good salinity tolerance indicator for salinity tolerance *Oryza* selection (Figure 5.8_B)., Salinity tolerance wild *Oryza* tends to have higher leaf Na^+ accumulation relative to salinity-tolerant *O. sativa*- Pokkali (Chapter 3), while uniquely maintaining low mesophyll Na^+ accumulation, probably a key factor to maintain physiological activities in photosynthetic tissues. In this Chapter, there is the same pattern of mesophyll Na^+ accumulation after 6 weeks of salinity stress, supporting the result in Chapter 3. Mesophyll Na^+ accumulation was highly significantly correlated to FW (Negative, $R^2=0.65$, $P<0.01$), DW (Negative, $R^2=0.62$, $P<0.01$) and significantly correlated to water content (Negative, $R^2=0.52$, $P<0.05$), net K^+ (Negative, $R^2=0.57$, $P<0.05$) & Cl^- (Negative, $R^2=0.57$, $P<0.05$) flux result. Overall, mesophyll Na^+ accumulation can be a good salinity screening indicator.

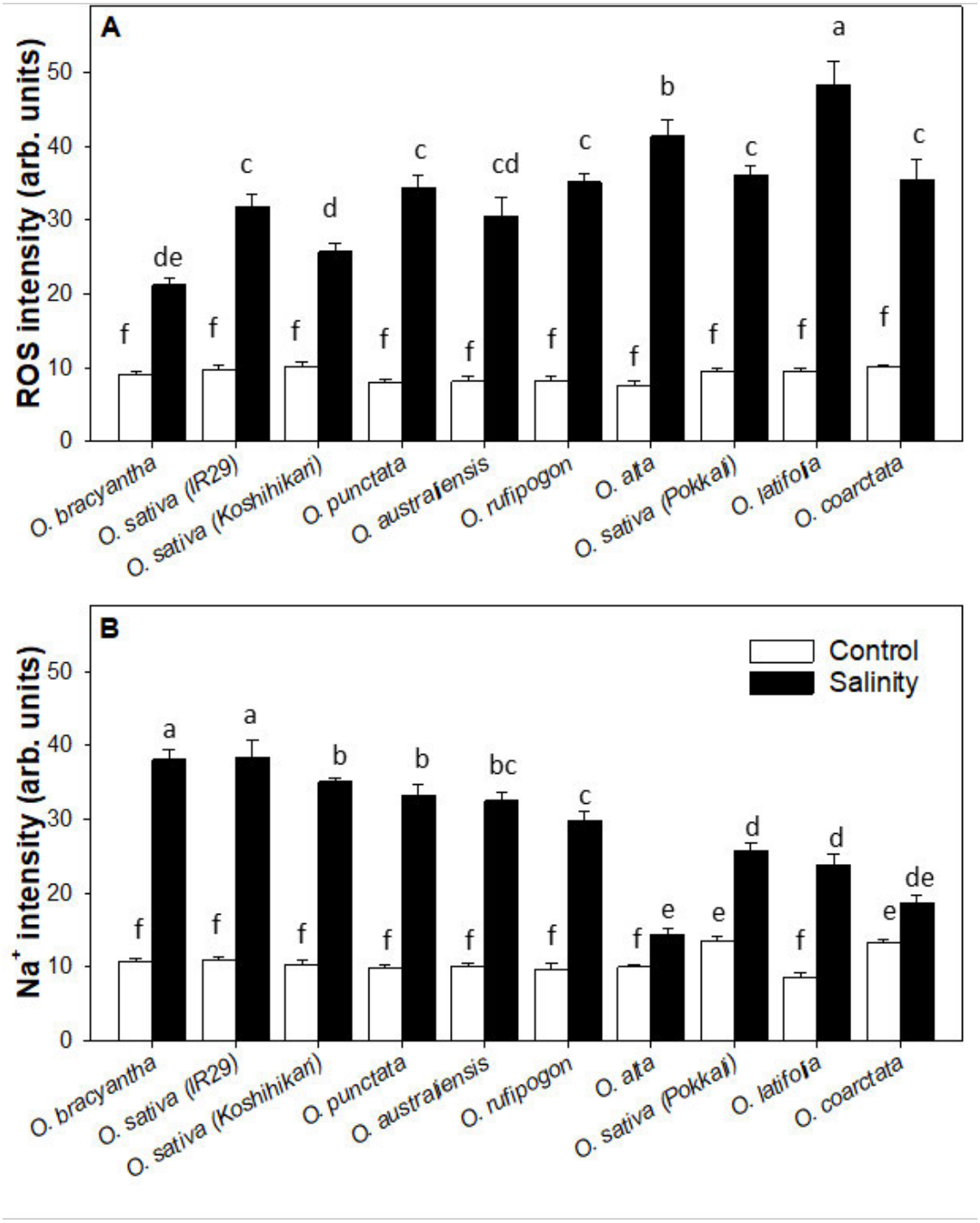

Figure 5.8. Linking salinity tolerance of wild and the cultivated rice to accumulation of reactive oxygen species (ROS) and Na^+ in leaf mesophyll cells after 6weeks of salinity stress in the field. Data are mean ROS and Na^+ intensity of the mesophyll's cell from control and salinity treated plant. Different lowercase letters indicate significant differences at $P < 0.05$. Data are mean ± SE (n = 4 – 8 biological replicates).

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5.4 Discussion

In field trials, environmental factors can introduce uncertain effects to the plant and result in consistent or even contrasting outcomes compared to the results obtained in the greenhouse (Van Straten et al., 2019). The uncertainties for salinity trials include but are not limited to the soil type, EC of irrigation water, EC of rainwater, humidity, and temperature variation (De Vos et al., 2016, Straten et al., 2016). Through this study, I confirmed that the selected wild species had similar salinity tolerance performance in the field trial. Four of the wild *Oryza* (*O. coarctata*, *O. australiensis*, *O. rufipogon,* and *O. latifolia*) were assessed in chapter 4. The remaining three species- (*O. punctata*, *O. alta,* and *O. bracyantha*) were previously assessed under controlled conditions at the early growth stage (Prusty et al., 2018). Environmental effect on the *Oryza* growth in the field was observed in gas exchange measurements. The photosynthetic traits were overall not well performed compared to their performance in the greenhouse trial (Chapter 4). The daily humidity was too low that Licor Measuring Chamber can only reach reference humidity of ~40% as compared to 55-65% in the greenhouse. This could be the reason why salinity stressed *O. coarctata* did not outperform the control in this study, where it was found to accumulate higher biomass in the other studies under similar conditions (Sengupta and Majumder, 2009, Bal and Dutt, 1986). Overall, the environmental effect did not alter the salinity tolerance ranking of the wild *Oryza* in this field study.

5.4.1 Mesophyll's healthiness is related to salinity tolerance in wild Oryza

Secondly, I confirmed that the salinity-induced damage on leaf tissue in wild *Oryza* can be highly reflected in the recovery K^+ & Cl^- efflux, and both ROS production & Na accumulation in the mesophyll cells. My results consistently showed that salt-tolerant species had lower net K^+ and Cl^- efflux in the field trial and complementary greenhouse trial (**Chapter 3 & 4**). The former was believed to be the outcome of lower potassium leakage in salt-tolerant species from

outward channels that mediates K^+ efflux such as NSCC and NORC (Zepeda-Jazo et al., 2008). GORK channel is not likely to mediate the K^+ efflux under such conditions which were previously tested in Koshihikari in Chapter 3 due to its characteristic (Azhar et al., 2017, Demidchik et al., 2010, Jayakannan et al., 2013). Potassium inward transporter such as the HAK family (Chen et al., 2015, Feng et al., 2019, Okada et al., 2018, Shen et al., 2015), can contribute to the reduction of net K^+ efflux observed in the tolerant species. Nayyeripasand et al. (2021) identified a HAK transporter HAK25 was related to the shoot length and dry biomass traits in rice using GWAS. The work also identified a plasma membrane proton pump and cation chloride cotransporter related to dry weight and fresh weight traits. Plasma membrane proton pump provided proton source for K^+ inward transport via HAK and Na^+ exclusion via *SOS1* (Queirós et al., 2009, Vitart et al., 2001). Cation chloride cotransporter (CCC) is also named as K^+/Cl^- cotransporter was reported for its role (OsCCC1) in K^+ homeostasis in rice (Chen et al., 2016a). The K^+ regulation was associated with Cl^- cotransport into the cell. This might explain why net Cl^- flux was found lower in tolerant species and strongly correlated with net K^+ flux. In addition, tolerant species can also have less Cl^- transferred from root to shoot tissue as the result of active Cl^- homeostasis under the same saline conditions (Wu and Li, 2019). As the consequence, Cl^- content in mesophyll was initially lower in tolerant species thus, leading to low Cl^- efflux. Further study on Cl^- transporter can confirm if Cl^- is also responsive to ion homeostasis in salinity stress.

ROS is an important plant growth regulator, which is constantly presented at the basal level to maintain normal plant growth (Huang et al., 2019, Mittler, 2017, Mittler et al., 2011). Under stress conditions, ROS production is elevated and act as the secondary messengers for stress tolerance and signaling in plants. Yong et al. (2020) revealed that ROS production and regulation in leaf in response to salinity stress was dynamic over duration and strength of salinity stress. This suggested the difficulty of linking ROS production with salinity tolerance.

The result showed that mesophyll ROS production was positively correlated to DW and net K^+ flux, which was commonly suggested as a negative relation between ROS production and salinity tolerance (Bose et al., 2013). Mutant study of the autophagy-related gene *ATatg2* found that it was responsible for ROS homeostasis in the plant (Yamauchi et al., 2019). Interestingly, both mutation and overexpression of autophagy-related genes (*atg*s & *NBR1*) studies found elevated antioxidant activities (Su et al., 2021, Yamauchi et al., 2019, Luo et al., 2017). In studies on the relationship between antioxidant and salinity-tolerance, only half reported positive relation, while another half reported nil to negative relations (Bose et al., 2013). This suggested the complexity of ROS homeostasis and the relation between ROS and salinity tolerance. However, this trend may change at any time based on the dynamic ROS homeostasis concept (Yong et al., 2020, Yamauchi et al., 2019).

5.4.2 O. brachyantha, one of the most diverged species from O. sativa was highly sensitive to salinity stress

O. brachyantha (FF) was another species that belongs to the unclassified complex. It shares 2 leaf anatomical features with *O. coarctata* – a low number of mesophyll cells between two adjacent vascular bundles system and a high density of furrows and ridges on the adaxial side of the leaf (Chatterjee et al., 2016). "Lower numbers of mesophyll cells between two adjacent vascular bundles system" was thought to be a replaced feature over evolution in the *Oryza* genus to increase the surface area of chloroplast to maximize photosynthetic efficiency. I assessed if these features might be important to contribute to salinity tolerance in *O. coarctata*. The result indicated that *O. bracyantha* was highly sensitive to the salinity treatment. The two features mentioned above did not contribute to salinity tolerance or cannot function without a key feature that is present in *O. coarctata,* and it is most likely be the enlarged vascular bundle sheath and salt glands in *O. coarctata*.

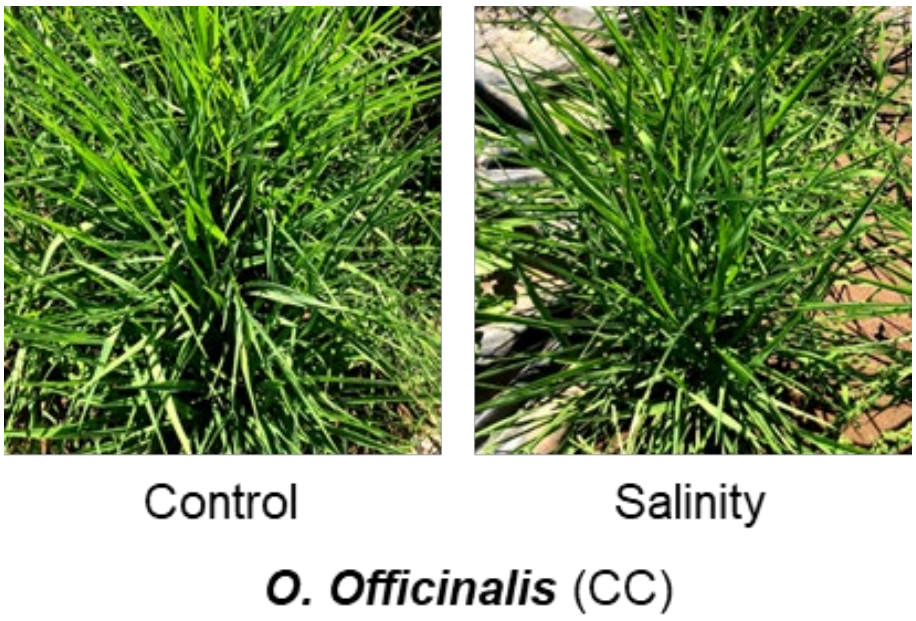

Figure 5.9. The visual appearance of *O. officinalis* with and without salinity treatment. Photos were taken after 42 days of salinity stress.

5.4.3 Salinity tolerance traits are likely inheritable only in the C genome rather than the whole O. officinalis complex

O. officinalis complex is comprised of 3 diploid members- B, C, and E genomes. They were derived from a common ancestor, which EE was believed to have originated before CC then BB genomes (Shenton et al., 2020, Ge et al., 1999). The tetraploid members- BBCC and CCDD were the results of hybridization of BB or EE genomes with CC genomes. *Oryza* with CC genomes was generally determined as tolerant to highly tolerant species to salinity stress. It is also well known for its excellent tolerance traits to various biotic and abiotic stress, such as heat (Ishimaru et al., 2010), drought (Jiang et al., 2019, Feng et al., 2012), and disease resistances (Zhang et al., 2014, Jiang et al., 2019). In the present study, I evaluated the tolerance of other 2 diploid members of *O. officinalis* to the salinity stress in the field to confirm if salinity tolerance traits were only inherited in CC genomes. Although most of the *O. officinalis* failed to establish after transplanting, the appearance of the remaining plant in the salinity plot showed almost no visual difference compared to the control plants (Figure 5.9). Thus, it may still be the highly tolerant species in the field trial. The result indicated that *O. punctata* and *O. australiensis* were between sensitive to moderately tolerant to salinity stress, while *O. officinalis*, *O. latifolia* and *O. alta* were from tolerant to highly tolerant to salinity stress. This supported the hypothesis that the salinity tolerance trait may have been inherited in the CC genomes. It also supports the origin of BB, CC, and EE genomes are multiple single events from a common ancestor as salinity tolerance trait was not presented in all EE genomes, not inherited by BB genomes and in later derived AA genomes from BB ancestor (Kim et al., 2007).

Chapter 6 Leaf defense to prolonged salt stress in *Oryza coarctata*: Na^+ allocation and transcriptomic study of leaf epidermis

6.1 Abstract

Salt secretion through salt glands is one of the main mechanisms in the halophytic wild rice species *O. coarctata*, leading to its adaptation in saline conditions. Tissue tolerance has also been proposed as a key strategy of plant salinity tolerance, but it is unclear whether a specific part of the leaf such as the epidermis and vascular bundle sheath contributes to the overall tissue tolerance. Therefore, this study evaluates Na^+ distribution in, epidermal cells, bundle sheath cells, mesophyll cells, and guard cells as well as RNA-sequencing of the leaf adaxial epidermis in the control and salt treatment. The pattern of Na^+ accumulation in those cell types would be useful to identify if Na^+ sequestration or exclusion is the main mechanism in these cells responsible for photosynthetic activities. The result showed that *O. coarctata* mesophyll cells mainly compart Na^+ into the vacuole to avoid Na^+ toxicity. However, it could not maintain low chloroplastic and cytosolic Na^+ when mesophyll was directly exposed to 100 mM NaCl solution. The result indicated that low apoplastic Na^+ may be another key feature for *O. coarctata* to adapt salinity tolerance. Moreover, RNA-sequencing of the epidermis in the control and salt treatment indicated that enrichment of upregulated differentially regulated genes (DEGs) was mainly significantly grouped in transcription regulatory activity, membrane transporters, and lipid binding. The result reveals the potential roles of several transporter protein families such as lipid transport proteins, ATP-binding cassette transporters, and aquaporins in the epidermis for salinity tolerance in *O. coarctata*. These genes may be beneficial for salinity adaptation by regulating stomata, cuticle synthesis, membrane transport of substances in *O. coarctata*. For instance, the upregulated cation transporter genes *HKT2;4* and *CHX3* might be responsible for Na^+ transport in different cell types of *O. coarctata* leaf epidermis. Several stress-related

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transcription factors (TFs) were also found differentially upregulated in the epidermis. In summary, Chapter 6 highlights the unique salt tolerance features potentially via the regulation of cation transport and gene transcription in *O. coarctata.*

6.2 Introduction

Oryza coarctata is the only tetraploid halophyte in the *Oryza* species. It is native to the mangrove ecosystem of the coastal region in India (Sengupta and Majumder, 2010, Bal and Dutt, 1986). Evolutionary analysis showed that it is grouped with *O. brachyantha* (FF), representing the earliest diverged members in *Oryza* and members of unclassified *Oryza* complex (or FF genome complex) in the *Oryza* genus (Singh et al., 2018, Kumagai et al., 2010). The similarity between the protein sequence of *O. coarctata O. sativa* was low (Garg et al., 2014) and the similarity of protein sequence between the two species was even lower as compared to the similarities between *O. sativa* and other *Oryza* species (Sakai et al., 2011, Chen et al., 2013). The lower similarity in protein sequences between species in the same genus can result in structural and functional differences in protein that may be useful for salinity tolerance in cultivated rice.

O. coarctata belongs to the recretohalophyte group, which is capable of excluding excessive Na^+ from leaf tissue to the salt glands on both the adaxial and abaxial sides of the leaf (Fan, 2020). As a recretohalophyte, salt secretion is believed to be the main adaptation mechanism in *O. coarctata* under saline conditions. To support the proper function of salt secretion, *O. coarctata* will also require other salinity tolerance related cellular regulators such as metabolites, membrane transporters (mainly Na^+ transporters), and transcription factors to improve the efficiency of salt secretion and tissue tolerance (Kizhakkedath et al., 2015,

Somasundaram et al., 2020, Mukherjee et al., 2021, Jegadeeson et al., 2019). Sengupta and Majumder (2009) reported a positive relation between *O. coarctata* growth and salinity treatment up to 400 mM NaCl. In addition, large bundle sheath cells in *O. coarctata* can also play important role in salinity tolerance via improving photosynthetic capacity, osmotic regulation, carbohydrate synthesis, nitrogen regulation and sulphur assimilation (Leegood, 2008). However, it is still technically difficult to genetically engineer the function of a whole tissue structure such as the large bundle sheath from one species to another. For example, functional and structural improvement of bundle sheath cells in rice is still the main challenge for C4 rice since the last decade (Ermakova et al., 2020). Therefore, the salt secretion mechanism alone cannot be possible to promote such growth improvement under such extreme saline conditions.

Halophytes are well known to maintain higher photosynthetic capacity under salinity stress as compared to glycophytes (Bose et al., 2017). Superior CO_2 concentrating mechanism was believed to be the main mechanism that overcoming water loss risk from stomatal opening. For instance, the chloroplastic fructose-1,6-bisphosphatase (FruP2ase) from *O. coarctata* was proposed to be one of the main components that contributed to the CO_2 concentrating mechanism. The functional evaluation indicated that FruP2ase from *O. coarctata* can remain active under high salinity treatment and promoted the growth of transgenic tobacco under salinity stress (Ghosh et al., 2001, Bose et al., 2017). L: -Myo-inositol 1-phosphate synthase (OcINO1) was also reported to promote salinity tolerance of transgenic tobacco (Patra et al., 2010, Majee et al., 2004). These components may be easier to transfer for salinity tolerance improvement in cultivated rice.

Leaf Na^+ exclusion and Na^+ sequestration are two important mechanisms of plants in response to Na^+ stress (Hanin et al., 2016). Na^+ Exclusion is mainly mediated by the SOS1

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antiporter to exclude Na^+ from mesophyll and the retrieved into xylem parenchyma cell via HKT1 transporter (Hamamoto et al., 2015). The former is controlled by the SOS signaling pathway, which is consisted of SOS3, SOS2, and SOS1 mediated by Ca^{2+} and ROS signals (Ji et al., 2013). Na^+ sequestration was mainly carried by NHX1 antiporter from cytosol to vacuole (Kronzucker and Britto, 2011). Sequestered Na^+ can be used as a 'cheap osmoticum' to increase salt concentration in the vacuole and then enhance water retention in cells when the external Na^+ is high. Moreover, transporters involved in K^+ retention is also important to maintain a high cytosolic K^+/Na^+ ratio (Shabala and Cuin, 2008), such as HAK transporter, NHX transporter, and KEA transporter (Bassil et al., 2011, Rodríguez-Rosales et al., 2008, Wang et al., 2016a, Chen et al., 2015, Shen et al., 2015, Tsujii et al., 2019). Tonoplast and plasma membrane-based proton pumps V-PPASE, V-ATPase, and H^+-ATPase are also important to provide H^+ source for Na^+ transport (Queirós et al., 2009, Zhang et al., 1999). However, the functions of these two salt tolerance mechanisms are not fully explored in the halophytic *O. coarctata.*

RNA-sequencing has become a powerful tool to study stress tolerance in plants at the whole plant, tissue, cell populations, or even at single-cell levels (Wang et al., 2018b, Chen et al., 2019, Feng et al., 2020a, Wang et al., 2009). Candidate genes detected from RNA-sequencing were usually verified with qRT-PCR to identify stress-related genes (Chandran et al., 2019). RNA-sequencing was also employed together with the quantitative trait locus (QTL) technique to identify genes that were located in the stressed related locus (Lei et al., 2020, Zhu et al., 2020). Therefore, it will be ideal to combine the physiological measurements at tissue and cellular levels with transcriptome profiling of the epidermis to better understand the Leaf defense mechanism to prolonged salt stress in *O. coarctata.*

Chapter 6 aimed to identify the most relevant Na^+ transporter in leaf mesophyll and identify potential new components in leaf epidermis via RNA-sequencing comparison between

leaf adaxial epidermis from control and salinity stressed *O. coarctata.* The results showed that cation transporter in chloroplast and vacuole and TF in leaf epidermis may have significant roles to maintain cell health, thus high photosynthesis can be maintained under high Na^+ environment *O. coarctata*.

6.3 Result

6.3.1 Effect of short-term salinity treatment on Na^+ accumulation in the leaf of O. coarctata

In the present study, the short-term (7 days) salinity effect on Na^+ accumulation and allocation in *O. coarctata*'s leaf was studied. Young seedlings of salt-sensitive *O. sativa cv*. Koshihikari and *O. coarctata* showed significant accumulation of Na^+ in the leaf after a week of salinity treatment (Figure 6.1_A). The Na^+ accumulation was confirmed in mesophyll cells in both species (Figure 6.2_B, ANOVA, treatment effect, $P< 0.05$) and was potentially also highly accumulated in the vascular system and epidermis. However, the later statement cannot be confirmed as the cells on the leaf epidermis and vascular system may have been damaged during sample preparation (sectioning the leaf). The Na^+ signal was mainly detected on the cell wall and plasma membrane of the cells of the epidermis and vascular bundle. In comparison to the salt-sensitive Koshihikari, Na^+ accumulation in the mesophyll of *O. coarctata* was relatively lower but not statistically significant (ANOVA, treatment × species, $P>0.05$).

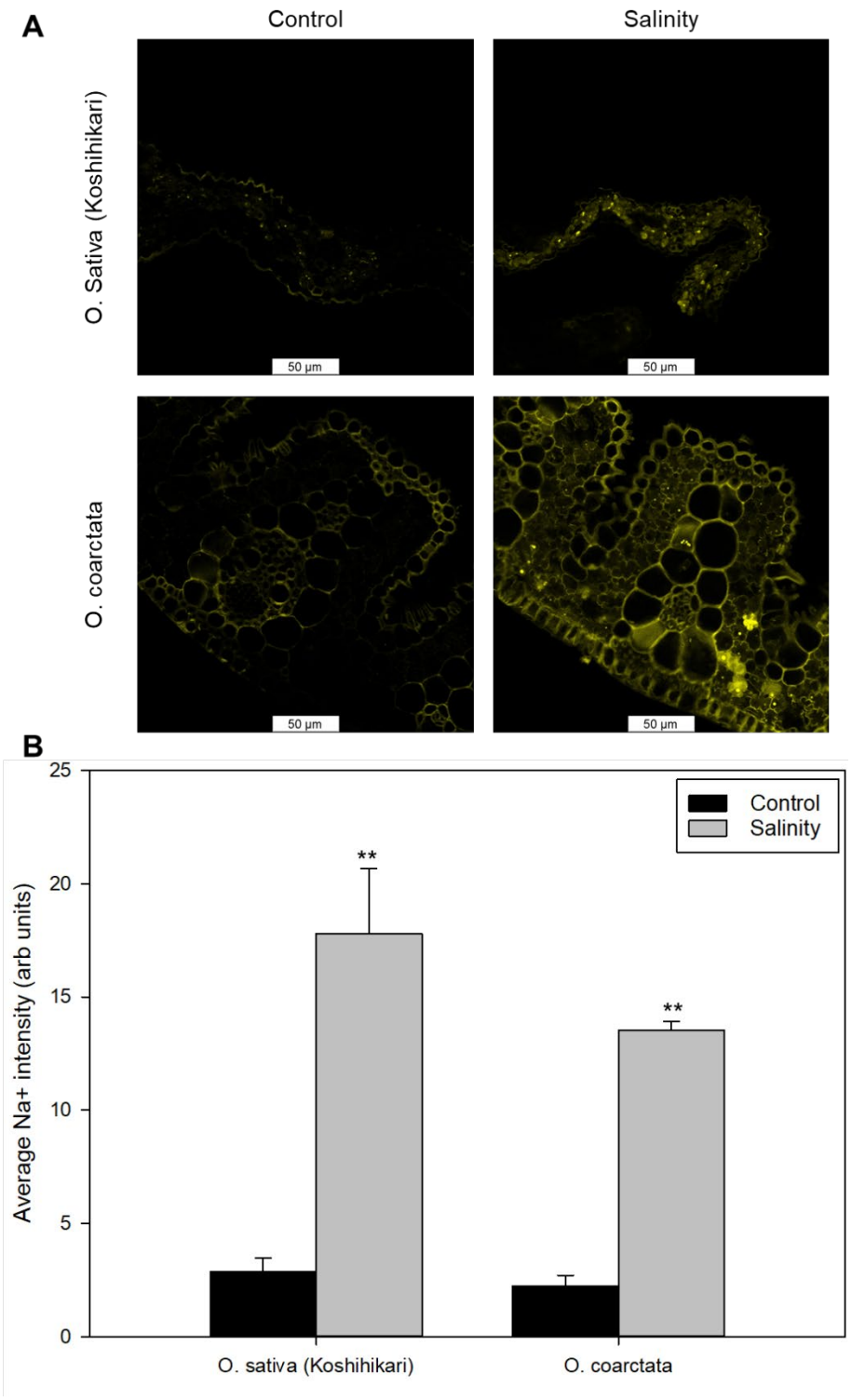

Figure 6.1. Na accumulation and distribution in the leaf of salt-sensitive cultivar-Koshihikari and *O. coarctata* after 7 days of salinity treatment. (A) Confocal imaging of Na^+ fluorescence in leaf cross-section, yellow color indicates Na^+ fluorescence; (B) Mean Na^+

fluorescence in mesophyll cell of two species. Data are mean ± SE (n = 3 replicates, 11 cells per replicate).

6.3.2 O. coarctata's mesophyll response to acute NaCl treatment and GORK activity evaluation

In Chapter 4 and Chapter 5, *O. coarctata* was reported to accumulate significantly lower Na^+ in mesophyll cells in long-term stress compared to other sensitive cultivars (IR29, IR64 & Koshihikari). Here, the Na^+ accumulation difference was not significant between the mesophyll of *O. coarctata* and Koshihikari in short-term salinity stress. This result suggested that *O. coarctata* did not avoid Na^+ accumulation in the early period of stress. To evaluate this finding, transient K^+, Na^+, and H^+ flux kinetics in responses to acute 100 mM NaCl treatment in leaf mesophyll tissue were studied (Figure 6.2). In response to acute salinity treatment, leaf mesophyll of both species showed massive net K^+ and H^+ efflux as well as net Na^+ influx. By comparing the two *Oryza* species, the result showed no difference in salinity-induced K^+ efflux from leaf mesophyll between *O. coarctata* and Koshihikari (Figure 6.2_A). Salinity-induced H^+ efflux was slightly higher in *O. coarctata* but not significant (Figure 6.2_C). Surprisingly, *O. coarctata* showed higher salinity-induced Na^+ influx in the first 5 minutes of salinity treatment compared to Koshihikari (Figure 6.2_B). However, *O. coarctata* tend to exclude Na^+ in later minutes of treatment.

K^+ efflux channel blocker TEA^+ treatment was applied after 15 minutes of salinity treatment to evaluate activities of the "master regulator" GORK in both species. The result indicated that mesophyll cells were highly responsive to TEA^+ treatment, resulting in a sudden shift of net K^+ flux from net efflux to net influx in both species (Figure 6.2). *O. coarctata* was relatively less responsive to the TEA^+ compared to Koshihikari. This suggested that *O.*

coarctata may have less NaCl-induced GORK channel activity for K^+ leakage from the cells. As the consequence, net Na^+ and H^+ efflux were unaffected in *O. coarctata*. Moreover, Koshihikari showed a significant increase in Na^+ influx and H^+ efflux in response to TEA^+ treatment.

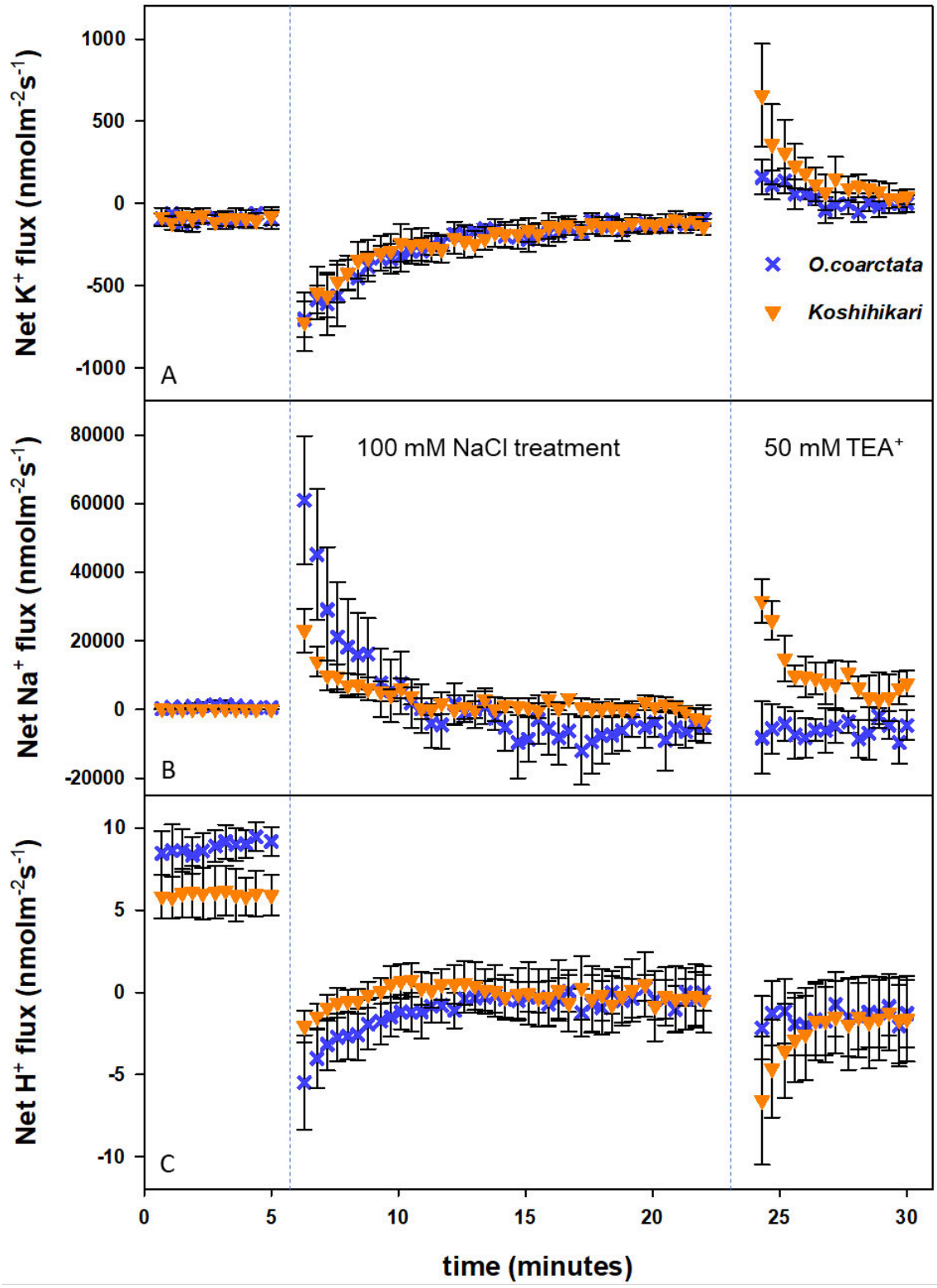

Figure 6.2. Transient net K^+ (A), Na^+ (B), and H^+ (C) flux kinetics of leaf mesophyll in response to 100 mM NaCl treatment, followed by 50 mM TEA^+ treatment 15 minutes after the salinity stress measured in *O. coarctata* and Koshihikari. Data are means ± SE (n= 5-6).

6.3.3 Effect of short-term salinity treatment on Na^+ accumulation in mesophyll and guard cell of O. coarctata

Na^+ distribution in mesophyll was further evaluated in *O. coarctata* (Figure 6.3). The *O. coarctata* samples were treated with both short-term (7 days to the whole plant) and acute salinity treatment (2 hours to leaves without epidermis). In response to 7 days of 100 mM NaCl treatment, Na^+ accumulation in the mesophyll of the whole plants was highly increased (Figure 6.4_A, $P<0.01$). Surprisingly, the Na^+ fluorescence in the chloroplast was not increased compared to the control (Figure 6.4_B, $P>0.05$). Based on the area and shape of the Na^+ fluorescence in the cell detected at the planar dimension, the Na^+ was most likely located in the vacuole (Figure 6.3). In response to 2 hours of direct salinity treatments on exposed mesophyll tissue, Na^+ fluorescence increased slightly in mesophyll treated with 50 mM NaCl treatment (Figure 6.4). However, the fluorescence was highly elevated ($P<0.01$) in mesophyll treated with 100 mM NaCl where the Na^+ fluorescence in mesophyll was even higher than the mesophyll from a plant treated with 7 days of salinity stress. The results indicated that a significant portion of Na^+ may be accumulated around or within the chloroplasts.

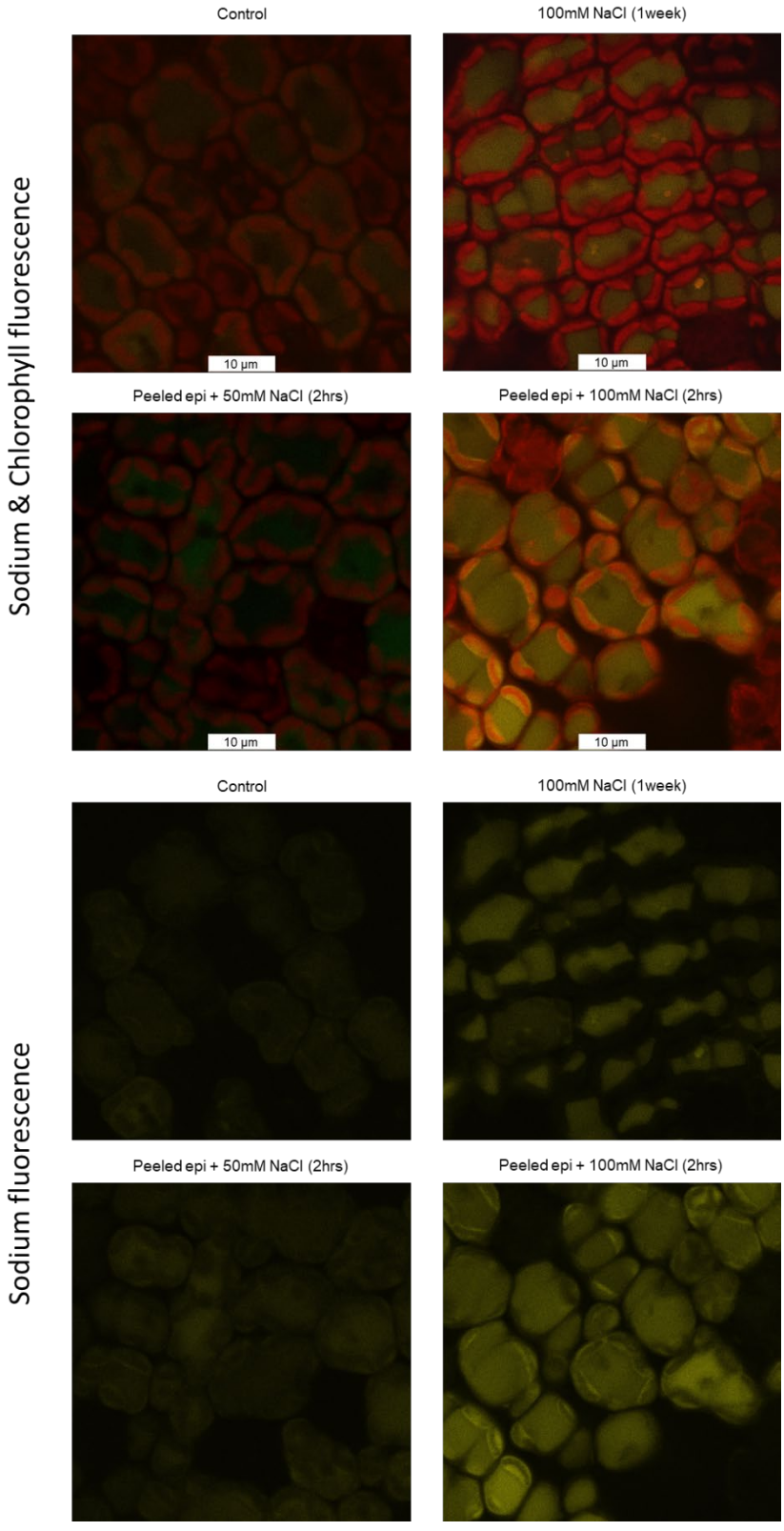

Figure 6.3. Confocal imaging of Na distribution in mesophyll cell of *O. coarctata*. The yellow color represents Na^+ signal and the red color represents the chlorophyll signal. Representative images are shown out of at least three biological replicates.

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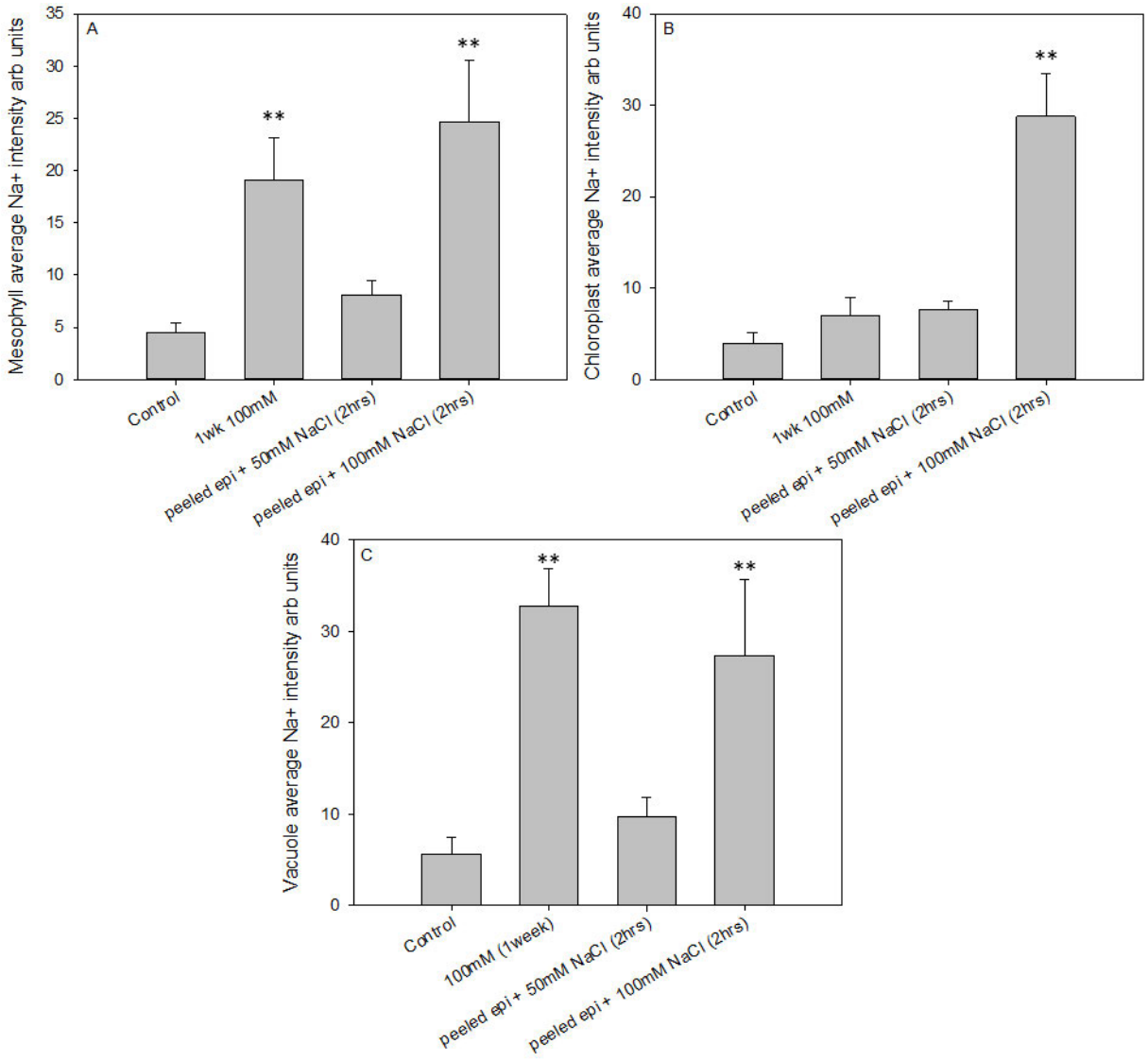

Figure 6.4. Mean Na^+ fluorescence detected in (A) mesophyll cell, (B) chloroplast and (C) vacuole. Data are mean value ± SE (n=3-5 biological replicates). ** represent significant difference at P<0.01 compared to control.

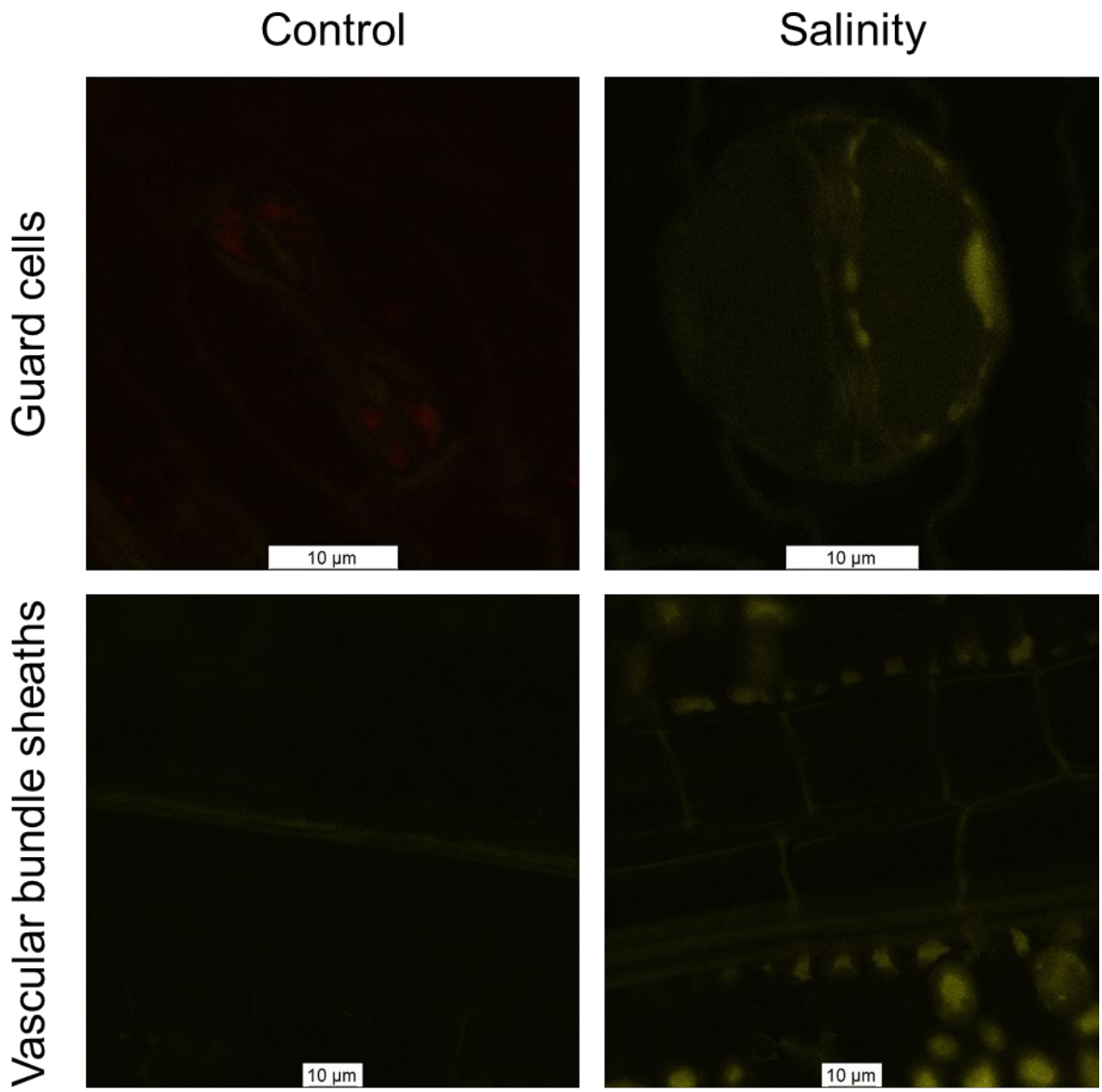

Figure 6.5. Confocal imaging of Na distribution in guard cells and vascular bundle sheath cells of *O. coarctata*. The yellow flourescence represents Na^+ signal. Representative images are shown out of at least three biological replicates.

6.3.4 RNA-sequencing of salinity stressed leaf epidermis.

The abaxial leaf epidermis of *O. coarctata* consists of three cell types - guard cells, epidermal cells, and epidermal bladder cells (different form compared to adaxial salt gland). Na^+ imaging and gas exchange results in Chapters 4 & 5 suggested that the epidermis could accumulate a high amount of Na^+, meanwhile, photosynthetic activities were slightly affected in both greenhouse and field trials. In this study, RNA sequencing of the abaxial side of *O. coarctata* was conducted to investigate epidermis-specific transcriptome response to short-term of 100 mM NaCl treatment (7 days).

In all samples, RNA-seq experiments generated 36 to 44 million read bases, and 61 - 62 % of the *O. coarctata* reads were mapped to the genome of *O. sativa* Nipponbare *(Kawahara et al., 2013)* (Table 6.1). The Venn diagram showed that RNA-seq identified overlapping 18,297 genes between control and salinity stressed plants (Figure 6.6_A). Among those, 490 and 1659 genes were only detected in control or salinity stressed plants, respectively. Quasi-Likelihood F-test found 409 differentially expressed genes (False discovery rate (FDR) < 0.05) with a fold change greater and equal than 1.5 in total (Figure 6.6_B-C). Among the differentially expressed genes (DEGs), 122 and 179 of upregulated and downregulated genes respectively, had a fold change greater and equal to 2.

Table 6.1. Summary of RNA-sequencing read mapping result of *O. coarctata*.

	Control	Salinity
Total bases (Million)	4,286.78	3,570.89
read bases (Million)	43.51	36.46
mapped reads (Million)	27,84	23,81
mapped rate (%)	61	62
Q30 (%)	77.62	75.90
GC%	44.53	44.92

Abbreviations: G, guanine C, cytosine, Q, Phred quality score, a measure to assess the quality of sequencing in next-generation sequencing

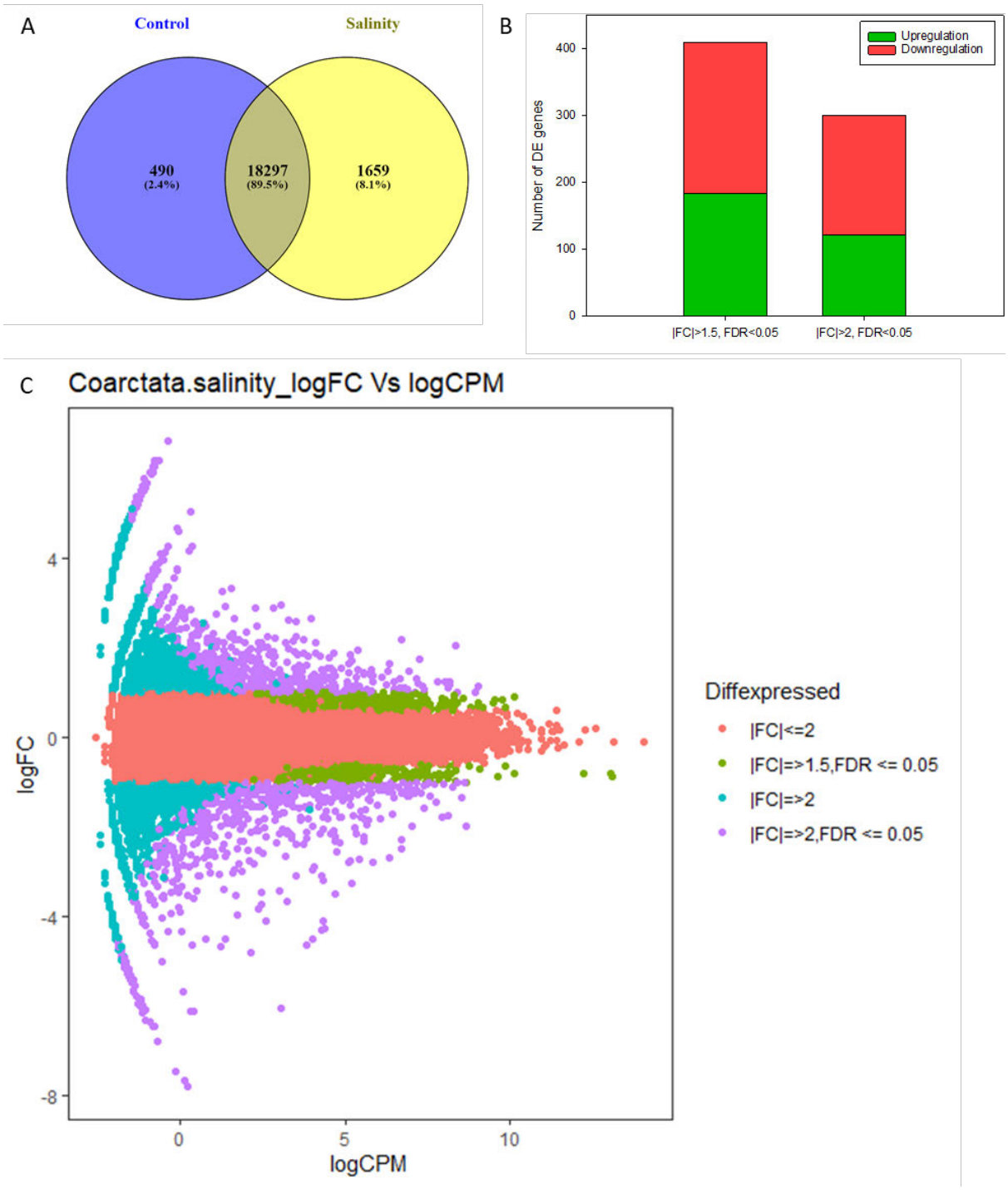

Figure 6.6. Venn diagram (A) shows the number of genes detected in leaf abaxial epidermis in response to salinity treatment. The number of DEGs was shown in the bar chart and volcano plot. Genes with a significant change in regulation ($|FC| \geq 1.5$, $FDR \leq 0.05$ & ($|FC| \geq 2$, $FDR \leq 0.05$) are illustrated as red and blue dots in the volcano plot.

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6.3.5 GO enrichment analysis of DEGs

Gene Ontology (GO) analysis of 409 DEGs (|FC| ≥ 1.5, FDR ≤ 0.05) was conducted using the databases: GeneOntology (http://geneontology.org/) and AgriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php). The GO mapping of biological process, molecular function, and cellular components showed key biological process network-regulatory activities, phosphorylation related activities, photosynthetic electron transport activities, and lipid transport & localization (Figure 6.7_A); molecular function network-transcription regulator activities, electron transport pathway, binding activities (lipid, enzyme) and kinase activity (Figure 6.7_B); and cellular components network- membrane part, light-harvesting complex, and cytoplasmic vesicle (Figure 6.7_C). To further evaluate salinity-induced regulation in the epidermis, GO analysis of differentially upregulated and downregulated genes (|FC| ≥ 1.5, FDR ≤ 0.05) was conducted separately. The result further highlighted the significant components that were mainly comprised of upregulated or downregulated genes. From the cellular component result, a significant network of upregulated genes was only found in the membrane part. Downregulated genes were significantly grouped in light-harvesting complex and cytoplasmic vesicles. They were also insignificantly grouped in other intercellular organelles except for the membrane part. This highlighted the expression difference between organelle after salinity stress.

In biological process mapping, 3 main networks were detected from all DEGs, which are transcript regulatory activities, phosphorylation-related activities, photosynthesis, and lipid transport and localization. GO network analysis showed that upregulated genes were mainly found in transcript regulatory activities lipid transport localization and membrane transport. GO network of downregulated genes was mainly found in phosphorylation-related activities and photosynthetic electron transport activities. Upregulated and downregulated genes were also

found in both transcript regulatory activities and phosphorylation-related activities (Figures 6.7-6.9).

The molecular function of the GO network result indicated that upregulated genes were only significantly grouped in transcription regulatory activity and lipid binding. Compared to the network of all DEGs, the lipid-binding category was completely comprised of upregulated genes (7 genes in both overall and upregulated mapping). Upregulated genes occupied 19 of 32 transcriptional regulatory activity-related genes by comparing both upregulated and overall networks. In downregulated genes network, binding activities (Ca, Mg, coenzyme, and ATP), kinase activities, lyase activity, and electron transporter activities were significantly highlighted.

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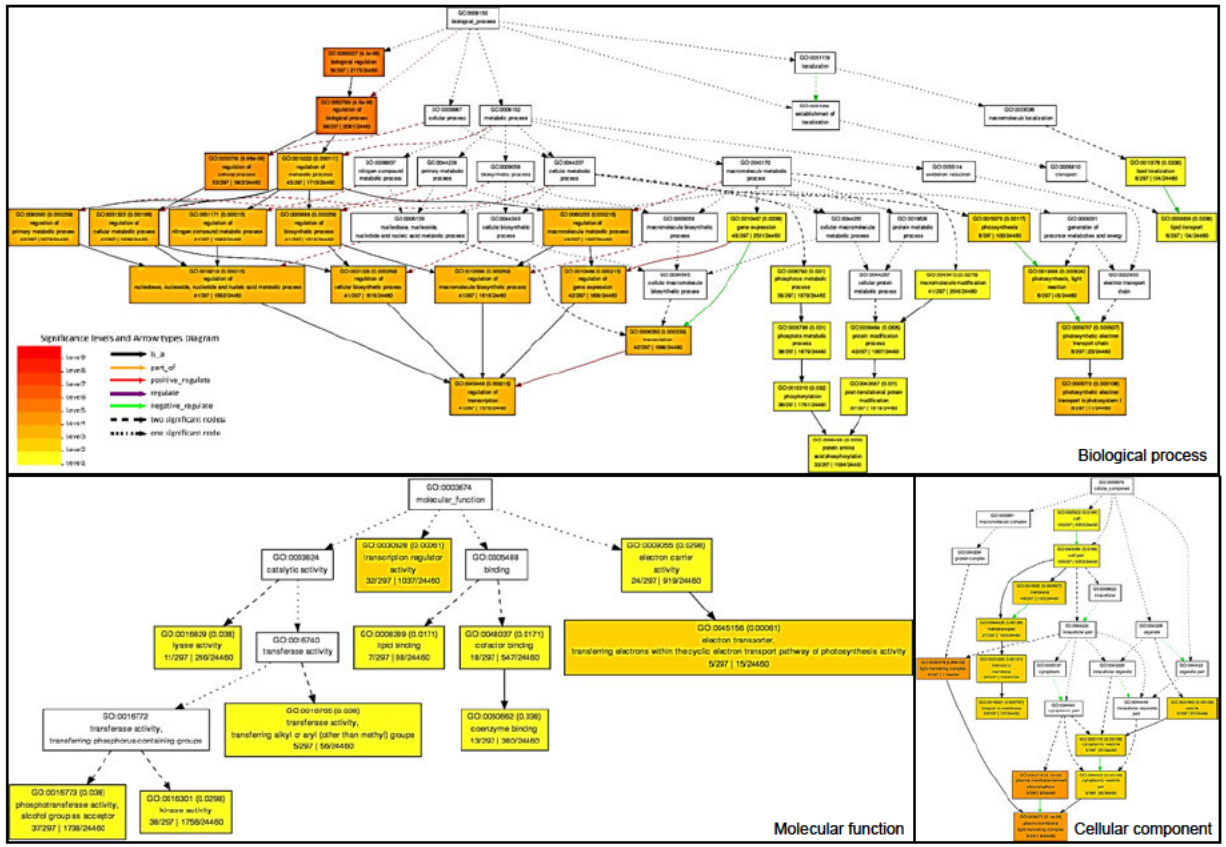

Figure 6.7. GO network analysis of DEGs (|FC| ⩾ 1.5, FDR⩽0.05) of leaf epidermis of *O. coarctata* after 7 days of salinity stress. Enriched GO categories were highlighted as indicated. The network was analyzed and generated from http://bioinfo.cau.edu.cn/agriGO/analysis.php.

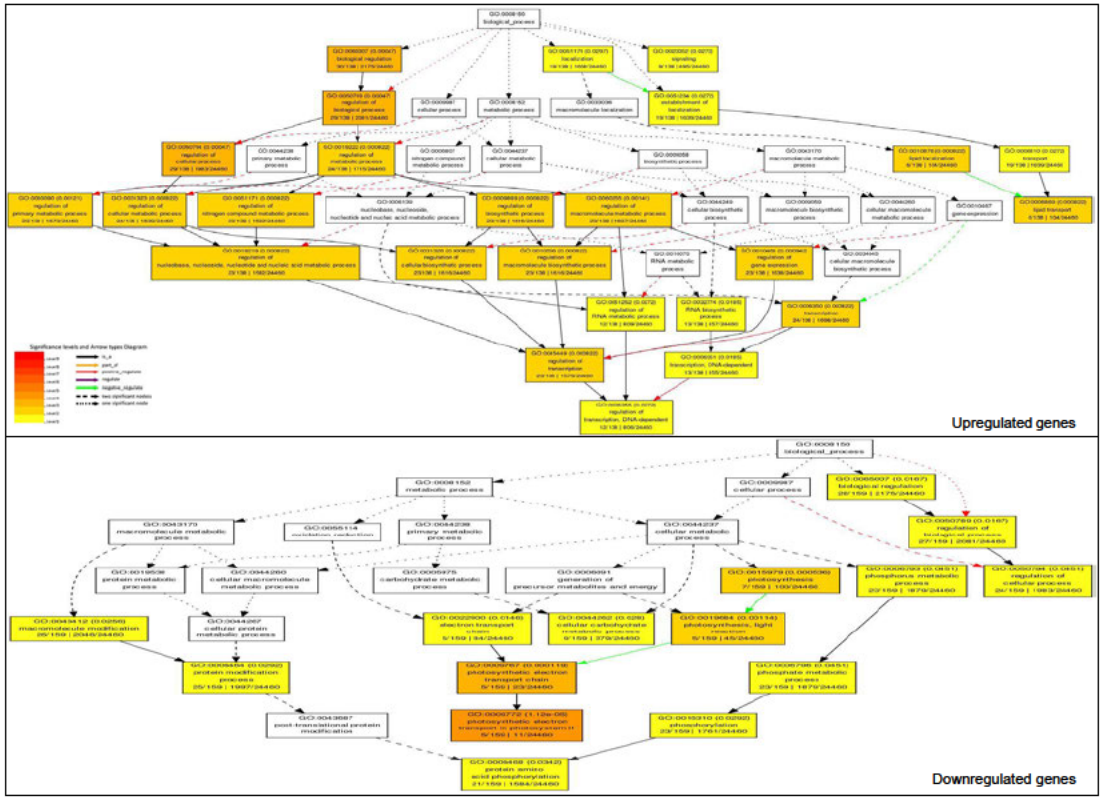

Figure 6.8. GO network- the biological process of differentially (A) upregulated and (B) downregulated expressed genes (|FC| ⩾ 1.5, FDR⩽0.05) of leaf epidermis of *O. coarctata* after 7 days of salinity stress. Enriched GO categories were highlighted as indicated. The network was analyzed and generated from http://bioinfo.cau.edu.cn/agriGO/analysis.php

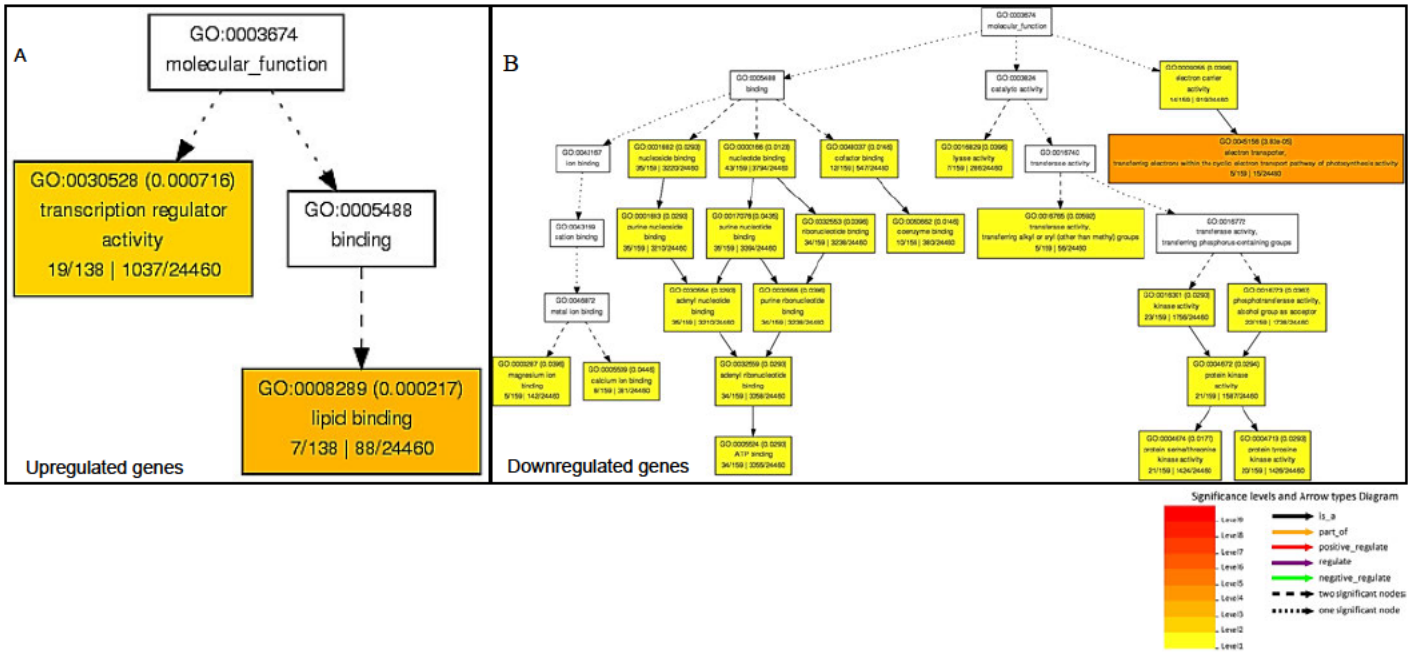

Figure 6.9. GO network- the molecular function of differentially (A) upregulated and (B) downregulated expressed genes (|FC| ⩾ 1.5, FDR⩽0.05) of leaf epidermis of *O. coarctata* after 7 days of salinity stress. Enriched GO categories were highlighted as indicated. The network was analyzed and generated from http://bioinfo.cau.edu.cn/agriGO/analysis.php.

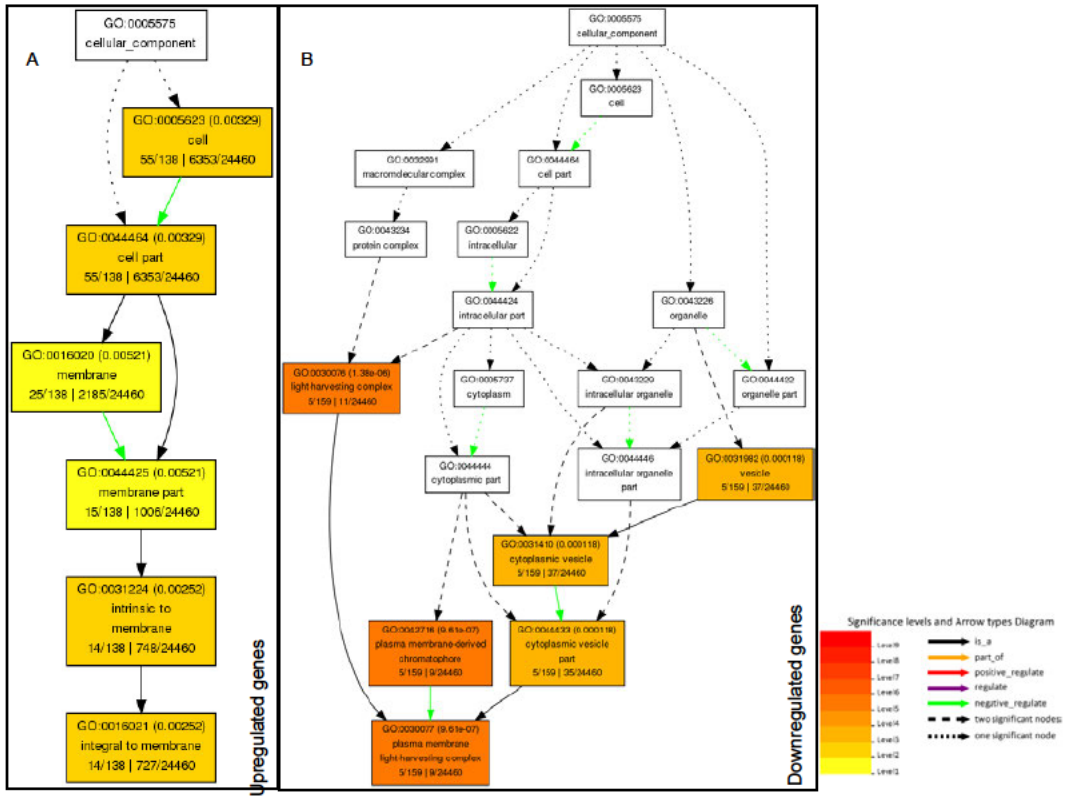

Figure 6.10. GO network- cellular component of differentially (A) upregulated and (B) downregulated expressed genes (|FC| ⩾ 1.5, FDR⩽0.05) of leaf epidermis of *O. coarctata* after 7 days of salinity stress. Enriched GO categories were highlighted as indicated. The network was analyzed and generated from http://bioinfo.cau.edu.cn/agriGO/analysis.php.

6.3.6 Membrane transporters and transcription factors were significantly upregulated in the epidermis

The significantly enriched GO categories that were potentially related to salinity stress are listed in Table 6.2. A fold change heat map of the involved genes in the enrich Go categories were generated to provide an overview of the expression pattern of each group (Figure 6.11). Similar GO categories were grouped under a subgroup such as transport (GO:0006869, GO:0010876, GO:0006810, GO:0051234, GO:0051179). Within a group, repeated genes from different categories were removed.

In the heat map, the membrane transport group had the highest numbers of differentially upregulated genes (61) compared to other groups. It included the establishment of localization to transporter genes related to transport of water- aquaporin, plasma membrane intrinsic protein (*PIP1;3*), cation transporters (*HKT2;4*), ATP- ATP-binding cassette transporter (*ABCG16*), lipid- lipid transport protein (*LTP1*), metabolites- major facilitator superfamily (*VPE1*). Noteworthy, 6 lipid transporters (*LTP4, LTP7, LTP8, LTP9, LTP10,* and *LTP20*) were significantly upregulated (Table 6.4, >2-folds) and formed a GO category- lipid transport (GO: 0006869) which is solely comprised of these transporters. However, genes related to the lipid metabolic process (GO: 0006629) were overall significantly downregulated. The expression of common salinity tolerance-related cation transporters and channels, such as *GORK*, potassium channel (*KAT1)*, *SOS1*, and *NHX1* were not differentially expressed in the epidermis. Interestingly, *HAK1,* Na^+/Ca^{2+} exchanger (*NCL2*), and *HKT1;4* were significantly downregulated ($|FC| \geq 1.5$, FDR$\leq$0.05) and Na^+ and K^+ transporters *HKT2*;*4* and cation: proton antiporter (*CHX3*) were increased >3-fold after salinity stress, which is ranked as top 30 upregulated genes (Table 6.3). Thus, these transporters may play an important role to transport Na^+ from mesophyll cells and vascular bundle cells to the epidermis and secreted via salt glands.

Moreover, differentially upregulated genes encoding transcription factors activities (GO:0003700, GO:0140110) were also identified in the GO network analysis. Several different types of transcription factors were detected with differential expression in the epidermis after the salinity stress. These transcription factors were APETALA2/Ethylene Responsive Factor (AP2/ERF), Basic helix–loop–helix (bHLH), Basic leucine zipper (bZIP), Calmodulin-binding, GRAS-domain transcription factors (GRAS), heat stress transcription factor (HSTF), Homeobox-leucine, Golden2-like (GLK2), Sigma factor and WRKY (Table 6.5). The gene expression values are listed in Table 6.5.

Table 6.2. Highlighted gene ontology terms (GO) of salinity treated leaf epidermis of *O. coarctata*.

GO name (term)	NO. of genes
ATP binding (GO:0005524)	41
calcium ion binding (GO:0005509)	9
calcium-dependent protein kinase activity (GO:0010857)	4
calcium-dependent protein serine/threonine kinase activity (GO:0009931)	4
calmodulin-dependent protein kinase activity (GO:0004683)	4
cellular lipid metabolic process (GO:0044255)	11
cellular response to stimulus (GO:0051716)	20
establishment of localization	30
Jasmonic acid biosynthetic process (GO:0009695)	5
Jasmonic acid metabolic process (GO:0009694)	5
kinase activity (GO:0016301)	26
lipid binding (GO:0008289)	9
lipid metabolic process (GO:0006629)	13
lipid localization (GO:0010876)	6
lipid transport (GO:0006869)	6
localization (GO:0051179)	29
organic acid metabolic process (GO:0006082)	17
oxidoreductase activity (GO:0016491)	23
peptidyl-serine phosphorylation (GO:0018105)	10
phosphorylation (GO:0016310)	29
protein autophosphorylation (GO:0046777)	6
protein kinase activity (GO:0004672)	21
protein phosphorylation (GO:0006468)	21

protein serine kinase activity (GO:0106310)	8
protein serine/threonine kinase activity (GO:0004674)	18
protein threonine kinase activity (GO:0106311)	37
regulation of cellular metabolic process (GO:0031323)	29
regulation of defense response (GO:0031347)	6
regulation of jasmonic acid-mediated signaling pathway (GO:2000022)	5
regulation of metabolic process (GO:0019222)	32
regulation of response to stimulus (GO:0048583)	9
regulation of response to stress (GO:0080134)	34
response to stimulus (GO:0050896)	37
response to stress (GO:0006950)	28
transcription factors activities (GO:0003700, GO:0140110)	29
transport (GO:0006810)	27
transmembrane transport (GO:0055085)	19
transporter activity (GO:0005215)	18

Figure 6.11. Heat map based on the fold change of the DEGs found in the highlighted GO terms from the transcriptome of leaf epidermis of *O. coarctata*.

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Table 6.3. Expression abundance of differentially expressed transporters in the transcriptome of leaf epidermis of *O. coarctata* after 7 days of salinity stress. The values are mean FKPMs on the control and stress columns.

Gene ID	Gene name	Control	Stress	Relative expression
Lipid transport protein				
Os11g0115350	LTPL7 - LTP family protein precursor, expressed	2.45	10.75	4.38
Os11g0116000	LTPL8 - LTP family protein precursor, expressed	2.43	7.15	2.94
Os11g0115400	Lipid transfer protein LPT IV	5.07	13.45	2.65
Os11g0116200	LTPL9 - LTP family protein precursor, expressed	2.23	5.83	2.62
Os11g0427800	LTPL10 - PLTP family protein precursor, expressed	10.16	25.62	2.52
Os12g0115100	LTPL12 - LTP family protein precursor, expressed	105.71	262.18	2.48
ATP-binding cassette				
Os07g0288700	ABC transporter OsABCG17	0.95	3.41	3.60
Os06g0731200	ABC transporter OsABCG16	0.70	1.66	2.38
Os05g0137100	ABC transporter OsABCG16	10.56	19.93	1.89
Os12g0411700	ABC transporter ABCG30	76.49	120.81	1.58
Os01g0609300	ABC transporter ABCG36	45.31	20.72	0.46
Cation transporter				
Os09g0545000	cation:proton antiporter-2 family, CHX3	0.40	1.27	3.16
Os06g0701600	Probable cation transporter HKT9	1.69	5.16	3.05
Os03g0684400	Magnesium transporter MRS2-A	5.37	8.80	1.64
Os04g0607600	Probable cation transporter HKT1;4	2.81	1.03	0.37
Os04g0401700	Potassium transporter 1;HAK1	0.76	0.25	0.33
Os02g0247800	Sodium/calcium exchanger NCL2	15.20	4.63	0.30
aquaporin				
Os02g0823100	Aquaporin PIP 1-3	7.44	15.71	2.11
Os07g0448800	Probable aquaporin PIP2-1	5.33	10.28	1.93
Sugar & metabolite				
Os12g0512100	Sugar inositol transporter	0.11	1.08	9.96
Os08g0155400	NPF6.3 nitrate transpoter	4.17	10.64	2.55
Os05g0426000	Bidirectional sugar transporter SWEET1b	29.99	69.20	2.31
Os03g0197100	Mannitol transpoter	34.55	77.06	2.23
Os06g0264500	peptide transporter PTR2	3.80	8.31	2.19
Os04g0555300	vacuolar Pi efflux transporter 1	23.09	46.58	2.02
Os06g0129400	Vacuolar phosphate efflux transporter	18.15	30.58	1.68
Os02g0229400	Tonoplast monosaccharide transporter 2	3.95	2.20	0.56
Os07g0581000	Sugar transport, DUF250 domain containing protein	43.24	22.45	0.52
Os01g0172100	Phosphoenolpyruvate/phosphate translocator PPT3;	27.27	7.12	0.26
TGA, Amino acid and others				
Os01g0226600	Malic acid transport protein	0.16	0.71	4.42
Os05g0426100	clathrin assembly protein, putative, expressed	1.51	6.59	4.36
Os04g0470700	Amino acid transporter	4.36	8.02	1.84
Os09g0484900	citrate transporter, putative, expressed	15.37	26.67	1.74
Os08g0503700	citrate transporter, putative, expressed	100.75	170.96	1.70
Os12g0207500	ATP synthase subunit beta	72.62	42.16	0.58
Os06g0633800	Amino acid transporter	80.01	42.73	0.53
Os03g0751100	OPT7 oligopeptide transporter	4.12	0.83	0.20
Os04g0571600	Protein detoxification	14.52	2.11	0.15

Table 6.4. Expression abundance of the differentially expressed transcription factor in the transcriptome of leaf epidermis of *O. coarctata* after 7 days of salinity stress. The values are mean FKPMs on the control and stress columns.

Gene ID	Gene name	Control	Stress	Relative expression
Os09g0286600	AP2/ERF domain-containing protein	0.01	0.27	21.35
Os09g0423800	AP2/ERF153	8.60	17.29	2.01
Os03g0183300	AP2/ERF domain-containing protein	8.24	15.43	1.87
Os09g0457900	AP2/ERF domain-containing protein	3.06	0.40	0.13
Os09g0487900	BHLH domain-containing protein	0.48	1.82	3.80
Os08g0506700	BHLH domain-containing protein	12.72	24.55	1.93
Os02g0673500	BHLH domain-containing protein	35.47	6.31	0.18
Os04g0301500	BHLH domain-containing protein	7.90	0.86	0.11
Os01g0542700	BZIP domain-containing protein	2.07	6.33	3.06
Os09g0456200	BZIP domain-containing protein	6.29	11.17	1.78
Os03g0778000	Calmodulin binding protein-like family protein	5.29	2.04	0.39
Os04g0555000	GRAS protein 21	4.19	8.80	2.10
Os04g0590400	GRAS protein 23	3.55	6.25	1.76
Os07g0545800	GRAS; CIGR1	32.09	15.56	0.48
Os02g0232000	Heat stress transcription factor C-2a;HSFC2A	1.69	4.92	2.90
Os03g0224700	Heat stress transcription factor A-9;HSFA9	9.68	5.41	0.56
Os05g0530400	Heat stress transcription factor A-4d;	3.25	1.29	0.40
Os04g0541700	Homeobox-leucine zipper protein HOX22	3.78	12.99	3.43
Os02g0729700	Homeobox-leucine zipper protein HOX16	16.32	31.59	1.94
Os04g0627000	Homeobox-leucine zipper protein ROC2	10.93	17.40	1.59
Os01g0239000	Probable transcription factor GLK2	2.42	5.06	2.09
Os05g0571100	Protein WHAT'S THIS FACTOR 1 homolog	19.72	9.41	0.48
Os05g0586600	Sigma factor SIG5	1.23	2.97	2.42
Os05g0183100	WRKY domain-containing protein	9.43	4.42	0.47
Os02g0181300	WRKY transcription factor WRKY71	29.90	8.73	0.29
Os09g0334500	WRKY transcription factor	12.13	2.73	0.23

Table 6.5. Top 30 significantly upregulated genes of leaf epidermis of salt-treated plants over the control ones of *O. coarctata*. These DEGs are selected from the highlighted GO categories.

ID	Description	Fold change
Os12g0138500	BTB/POX VIRUS AND ZINC FINGER (POZ) domain	24.51
Os09g0286600	ERF domain containing protein.	19.25
Os12g0512100	Sugar/inositol transporter domain containing protein.	9.91
Os09g0556700	SET domain containing protein.	9.49
Os01g0235900	Thioredoxin fold domain containing protein. Protein kinase	6.70
Os02g0106900	Protein kinase, core domain containing protein.	6.35
Os09g0240200	Zinc finger, B-box domain containing protein.	4.64
Os08g0504800	Similar to protein kinase.	4.56
Os01g0357100	Ferredoxin-nitrite reductase, Nitrate reduction (assimilation)	4.50
Os01g0226600	Similar to C4-dicarboxylate transporter/malic acid transport protein.	4.45
Os03g0228200	Conserved hypothetical protein.	4.37
Os05g0426100	clathrin assembly protein, putative, expressed	4.33
Os11g0115350	Similar to Non-specific lipid-transfer protein 2.	4.29
Os07g0162100	3-oxo-5-alpha-steroid 4-dehydrogenase	4.06
Os05g0130100	Hypothetical conserved gene.	4.06
Os09g0487900	Helix-loop-helix DNA-binding domain containing protein.	3.78
Os02g0606200	Zinc finger, B-box domain containing protein.	3.60
Os07g0288700	ABC transporter-like domain containing protein.	3.59

Os02g0765900	Similar to Ferredoxin-nitrite reductase.	3.54
Os05g0498900	Serine/threonine protein kinase domain containing protein.	3.53
Os05g0358500	Protein phosphatase 2C domain containing protein.	3.49
Os04g0541700	Homeodomain-leucine zipper protein	3.44
Os01g0770200	Aromatic L-amino acid decarboxylase (AADC),	3.42
Os03g0327800	NAC Family transcriptional activator, Abiotic stress response	3.30
Os01g0858350	Similar to cytochrome P450.	3.22
Os09g0545000	Cation/H^+ exchanger domain containing protein.	3.14
Os01g0846300	Clade A type 2C protein phosphatase	3.09
Os06g0701600	Monovalent cation transporter, HKT9 (HKT2;4)	3.05
Os01g0542700	bZIP transcription factor, bZIP-1 domain containing protein.	3.04
Os11g0116000	Similar to ipid-transfer protein 1 (LTP 1)	2.93

Table 6.6. Top 30 significantly downregulated genes of leaf epidermis of salt-treated plants over the control ones of *O. coarctata*. These DEGs are selected from the highlighted GO categories.

ID	Description	Fold change
Os05g0476200	Similar to DNA replication licensing factor MCM3.	-225.31
Os01g0821300	WRKY transcription factor, Promotion of phosphate accumulation.	-202.57
Os05g0545300	Serine/threonine protein kinase domain containing protein.	-174.71
Os06g0215900	Similar to 12-oxophytodienoic acid reductase.	-110.42
Os05g0512000	Zinc finger, RING/FYVE/PHD-type domain containing protein.	-86.80
Os06g0216200	Similar to Oxo-phytodienoic acid reductase.	-69.37
Os09g0451400	ACC oxidase, Ethylene biosynthesis	-28.14
Os12g0150200	Cytochrome P450 enzyme, Salt tolerance	-24.71
Os12g0491800	Similar to Ent-kaurene synthase 1A.	-24.71
Os06g0216300	Similar to 12-oxophytodienoic acid reductase.	-22.98
Os08g0538700	Retinoblastoma-related protein.	-22.63
Os04g0572200	Similar to OSIGBa0127A14.7 protein.	-20.05
Os11g0151400	Cytochrome P450 family protein.	-19.14
Os03g0618300	Isopenicillin N synthase family protein.	-14.18
Os02g0661100	Trehalose-6-phosphate phosphatase.	-12.56
Os09g0457900	AP2/ERF transcription factor.	-11.58
Os04g0419900	Similar to H0525E10.7 protein.	-11.38

Os05g0506000	Similar to MS5-like protein (Fragment).	-9.64
Os04g0301500	Basic helix-loop-helix transcription activator	-9.16
Os05g0545400	Serine/threonine protein kinase domain containing protein.	-8.60
Os01g0699500	Serine/threonine protein kinase domain containing protein.	-8.54
Os06g0176300	2OG-Fe(II) oxygenase domain containing protein.	-8.27
Os01g0862800	NAC TF, Early and transient regulator of abiotic stress responses	-7.85
Os01g0892800	Integrin-linked protein kinase domain containing protein.	-7.35
Os01g0627800	Similar to Cytochrome P450 monooxygenase CYP72A5 (Fragment).	-7.30
Os03g0685300	Glutamine amidotransferase class-I domain containing protein.	-7.11
Os03g0122300	Flavanone 3-hydroxylase, Positive regulation of flavonoid biosynthesis	-7.03
Os01g0699600	Mitogen-activated protein kinase kinase kinase (MAPKKK)	-6.92
Os07g0537500	Serine/threonine protein kinase-related domain containing protein.	-6.64
Os03g0741100	Basic helix-loop-helix transcription factor, Drought tolerance	-6.50

6.4 Discussion

6.4.1 The role of Na^+ transporter on the plasma membrane, tonoplast, and chloroplast of mesophyll in O. coarctata

The result showed no difference between sodium accumulation in mesophyll cells of salt-tolerant- *O. coarctata* and salt-sensitive- *O. sativa* (Koshihikari). It was further supported by the minor difference in ion flux in response to acute 100 mM NaCl treatment. However, 50 mM TEA^+ treatment after the salinity treatment found that GORK channel activity was more active in Koshihikari compared to *O. coarctata* (Figure 6.2). The results indicated that *O. coarctata* may have regulated and adapted to the salinity treatment quicker than Koshihikari and thus GORK channels were less active at 15 minutes (Adem et al., 2020). It can also be a result of tissue tolerance to Na^+ influx, leading to insensitive GORK of *O. coarctata*. The Na^+ influx was immediately increased after the GORK was blocked by TEA^+. The fluxes were significantly higher in Koshihikari. It suggested that net Na^+ influx is likely triggered by a sudden shift in net K^+ flux by promoting Na^+ influx channels such as NSCCs and PIPs or inhibiting Na^+ efflux antiporters such as SOS1 at the plasma membrane (Ji et al., 2013, Byrt et al., 2017, Demidchik and Maathuis, 2007). However, these hypotheses need to be tested in future studies to discover whether there are different functions between OcGORK and OsGORK and other Na^+ transporters in the two *Oryza* species.

Na^+ localization in mesophyll cells from 7 days stressed plant suggested that Na^+ was mainly compartmented into the vacuole. This highlighted the potential activities of tonoplast Na antiporter in *O. coarctata*- NHX1 to pump Na^+ from cytosol to vacuole (Fukuda et al., 2004). Secondly, tonoplast proton pumps V-PPase and V-ATPase are also important to provide H^+ source for Na^+ transport (Queirós et al., 2009). Comparing mesophyll cells directly exposed to

2 hours of 50 mM and 100 mM NaCl treatment, the Na^+ fluorescence in mesophyll was 3 times higher in leaf exposed to 100 mM NaCl. Based on the previous studies, the fluorescence of CoroNa can be proportionally related to the actual concentration (Park et al., 2009, Iamshanova et al., 2016, Wu et al., 2015c) so the Na^+ accumulation after 2 hours of 100 mM NaCl treatment was higher (3 times higher equivalent to one third higher relative to 50 mM NaCl treatment) than the proportion of NaCl treatment. This may indicate that SOS1 excluded a certain amount of Na^+ in response to 50 mM NaCl treatment, but the Na^+ exclusion was not promoted further in response to a higher concentration of salt at 100 mM. The results were matching with the expression result of *OcNHX1*, *OcSOS1*, and *OcVHA-C* result found in the greenhouse trial (Chapter 4), where *OcNHX1* (> 30-folds increase) & *OcVHA-C* (> 2-folds increase) in salinity stressed *O. coarctata* were significantly upregulated and *OcSOS1* expression was found no difference between control and salinity stressed sample.

Mesophyll cells exposed to 100 mM NaCl also accumulated a significantly higher amount of Na^+ around the chloroplast or within stroma based on the shape of Na^+ fluorescence. The other 2 treatments had similar Na^+ fluorescence relative to control. These results indicated that *O. coarctata* grown under 100 mM NaCl conditions can maintain low apoplastic Na^+ content to minimize the pressure of Na^+ influx into mesophyll cells. In addition, accumulated Na^+ was compartmented into a vacuole by the NHX1 transporter. Low chloroplast Na^+ might also be achieved by Na^+ transporter located on chloroplasts such as PIPs and Na^+/H^+ antiporter (NHD) (Bose et al., 2017). Efficient Na^+ transport from mesophyll cells to salt glands and Na^+ retrieval will be able to alleviate pressure from mesophyll tissue. In rice, Na^+ retrieval from leaf was mainly carried by *OsHKT1;* (Kobayashi et al., 2017). Na^+ transfer pathway from the vascular system to salt glands in the leaf of *O. coarctata* was proposed but not confirmed yet (Flowers et al., 2019, Bose et al., 2017).

Nevertheless, Flowers et al. (2015) reported that halophytes can accumulate high Na^+ content in chloroplast without compromising photosynthetic activities. Considering the salinity conditions (> 200 mM NaCl) of its native habitat, high Na^+ accumulation in chloroplast after 1 hour of direct exposure to 100 mM NaCl solution observed in this study can be a normal phenomenon in *O. coarctata.* This can be confirmed by evaluating Na^+ distribution in mesophyll from *O. coarctata* grown under higher saline conditions (> 200 mM NaCl) in the future.

6.4.2 Metabolism was downregulated in leaf epidermis O. coarctata under salinity conditions

Evolutionary divergence can be significantly higher between the epidermis of *O. coarctata* and *O. sativa.* This can lead to structural and functional variation between homolog genes from wild rice and *O. sativa* (Chen et al., 2013, Huang et al., 2012). In the present study, the transcriptome of the abaxial leaf epidermis of *O. coarctata* was sequenced under salinity conditions using the next-generation sequencing method to identify tissue-specific genes in the epidermis of *coarctata.* The sequencing result identified 50% fewer mapped reads compared to a transcriptome study of *O. coarctata* under salinity stress at whole plant basis (Garg et al., 2014). The difference indicated successful identification of tissue-specific genes relative to whole plant basis. The mapped reads are generally higher from RNA isolated from a whole-plant basis (Garg et al., 2014), followed by specific organ (Yu et al., 2017, Zhu et al., 2020), and then specific cell type (Hua et al., 2021, Han et al., 2017). Data analysis showed low numbers of DEGs in leaf epidermis in response to salinity treatment. It may be due to the length of the stress (7 days) which was also reported in the salinity response transcriptomic study of rice in Razzaque et al. (2019) and Chandran et al. (2019). Furthermore, RNA-seq analysis using edgeR was reported with a lower number of DEGs relative to other models such as DEseq2 (Wang et

al., 2019b). However, the result from edgeR had significantly less false-positive result and higher accuracy. Based on the GO enrichment analysis from Geneontology & AgriGO, significantly downregulated genes were mainly found in organelles related to phosphorylation, kinase activities, binding (ATP, calcium, and magnesium), photosystem 2 related protein and jasmonic acid regulation pathway. Some GO categories were surprising solely consisting of significantly downregulated genes such as calcium-bind protein, magnesium-binding protein, photosystem II-related protein and jasmonic acid regulation pathway (Atif et al., 2019, Rahman et al., 2000, Štefanić et al., 2013).

Jasmonic acid (JA) is a phytohormone that is initially identified to be involved in stress regulation in higher plants. Under saline conditions, JA involves mainly in the regulation of ROS production & signaling, antioxidant activities, proline content, and photosynthesis rate (Wu et al., 2015a, Kang et al., 2005, Moons et al., 1997, Walia et al., 2007, Bandurska et al., 2003). In guard cells, JA coordinates with ABA to regulate stomatal closure via ROS signaling by controlling cytosolic pH value (Gonugunta et al., 2009). Interestingly, the results in this Chapter showed downregulation of all genes (10) related to JA synthesis, metabolic, and mediated signaling pathways such as *TIFYs, OPRs, and CYP74A1.* Differentially expressed TF family, *WRKY*s which are involved in crosstalk between the salicylic acid and JA, were also downregulated. This was also in contrast to the JA regulation-related genes found in salinity transcriptomes study of *O. coarctata* (Garg et al., 2014). In this study, salinity treatment was applied for a lengthen term compared to 24 hours salinity treatment in Garg et al. (2014). Reduction in JA regulation-related genes was also reported in tolerant varieties in rice and tomato (Pedranzani et al., 2003, Wang et al., 2018a). Ismail et al. (2014) indicated that continuous Jasmonic acid pathway induction can damage the tissue from oxidative burst due to oversaturation of ROS.

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The genes under photosynthesis system II (PSII) were mainly putative and related to electron transfer. The electron transfer rate was reported to decline with an increase in non-photochemical quenching to dissipate excessive heat from light in sorghum and tolerant rice under salinity stress (Moradi and Ismail, 2007, Netondo et al., 2004). Other than that, a chloroplastic ferredoxin reductase- *NIR1* was listed in the top 30 significantly upregulated genes. It might play important role in oxidative stress detoxification in the chloroplast of guard cells (Huang et al., 2020, Higuchi-Takeuchi et al., 2011). For magnesium binding protein, 3 out of 5 putative genes were downregulated and related to lyase activities. Overexpression of a lyase activity-related gene- *Isocitrate lyase* (*OsICL*) in Arabidopsis found a positive effect of lyase activities on salinity tolerance (Yuenyong et al., 2019). Enhanced salinity tolerance might be promoted by autophagy of large compounds to adjust intercellular osmotic potential for salinity tolerance (Signorelli et al., 2019). Moreover, the four downregulated calcium-binding protein genes were under calcium-dependent protein kinase family (CDPK or CPK)- *CPK4*, *CPK24*, *CPK1*, and *CPK13*. 31 CPK proteins were identified in *O. sativa* (Ray et al., 2007). They are the Ca^{2+} sensor that detects the change in Ca^{2+} concentration in response to stimuli from stress or growth regulation, and conduct the signal through phosphorylation events (Atif et al., 2019). Among all *CPK* members, *OsCPK21, OsCPK12,* and *OsCPK7* overexpression were found to positively regulate defense response against salinity stress (Asano et al., 2012, Saijo et al., 2000, Asano et al., 2011). Wan et al. (2007) reported that salinity stress positively upregulated the expression of *OsCPK13*, *OsCPK21*, *OsCPK23,* and *OsCPK29* in salt-tolerant rice varieties. *OsCPK13* was negatively regulated in response to salinity stress (Abbasi et al., 2004). So far, no salinity study on the remaining 3 detected differentially expressed *CPK* genes in this RNA-seq experiment.

Most of the downregulated genes detected in this study were previously reported for their positive role in salinity tolerance in rice. The result suggested that these salinity tolerances

related genes may not be essential for functioning in the leaf epidermis of *O. coarctata* under long-term salinity conditions.

6.4.3 Membrane-specific proteins in the epidermis are important for salinity adaptation in the epidermis of O. coarctata

Salinity-induced upregulation of genes in leaf epidermis was significantly expressed in membrane components. The function of the genes was mainly related to transcription factors regulation (Santos et al., 2011, Golldack et al., 2011) and transportation of salinity tolerance related substances such as sugar, ion, lipid, and ATP (Sharom et al., 2011, Flowers et al., 2019, López-Pérez et al., 2009, Saha et al., 2015). Lipid transport and binding categories were solely comprised of upregulated genes, revealing the importance of lipid in salinity tolerance in *O. coarctata*. In this study, *LTP7*, *LTP8*, *LTP9*, *LTP10,* and *LTP12* were differentially upregulated after salinity stress. LTP (Lipid transport protein) transporter is responsible for phospholipids transport between membranes (Moraes et al., 2015). It was also found involved in lipid composition modification and membrane biogenesis (Wu et al., 2004, Kirubakaran et al., 2008, Yi et al., 2009). Most of the members of this LTP family were found responsive to salinity stress in various plants, including rice (Lin et al., 2017, Sui et al., 2018, Akhiyarova et al., 2020, Gangadhar et al., 2016, Wu et al., 2004, Moraes et al., 2015). To date, several roles of LTP in stress-induced defense responses were found. Cuticle formation is one of its main roles, which is important for the regulation of water transpiration from leaf epidermis (Jarzyniak and Jasiński, 2014). Xu et al. (2018) found direct interaction between NtLTP4 and a stress signaling-related MAPK, WIPK that enhances salinity tolerance from upstream regulation. It was also involved in the direct regulation of stomatal closure induced by ROS signal (Balmant et al., 2021). These indicated that LTP may involve in the regulation and synthesis of several important components in leaf epidermis that promote salinity tolerance in *O. coarctata.*

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In this Chapter, five ABC transporters were upregulated after the salinity stress. Four of them were belonging to the G group family (ABCG17, ABCG16, ABCG30, and ABCG36) and one belonged to the B group family (ABCB21). ATP-binding cassette (ABC) protein superfamily is one of the most abundant groups with over 120 members in both Arabidopsis and *O. sativa (Kang et al., 2011, Rea, 2007).* In plants, ABC transporters are involved in diverse processes such as growth regulation, plant nutrient, acid soil tolerance, disease resistance, heavy metal resistance, abiotic stress resistance, lipid catabolism, stomata regulation, phytate accumulation in seeds, and transport of the phytohormones auxin and ABA (Zhang et al., 2018a, Saha et al., 2015, Niu et al., 2013, Meng et al., 2020, Lin et al., 2013, Lee, 2016, Hinrichs et al., 2017, Chen et al., 2011a, Chen et al., 2017a, Zhao et al., 2016, Liu et al., 2021). ABC transporters might be involved in ABA signaling regulation in guard cells to regulate gas exchange and water loss from evaporation (Matsuda et al., 2016). Chen et al. (2011a) reported that a rice homolog of *HvABCG31* was involved in leaf water retention by promoting cutin deposition on the cuticle. These results indicate part of the main roles of ABC transporters in the leaf epidermis in response to salinity stress by regulating stomata closure and cuticle formation to reduce transpiration rate.

Salt accumulation was observed in the leaf epidermis (both epidermal cells and guard cells). The accumulated Na^+ can be on the way to the salt glands located on both sides of the leaf epidermis. In the RNA-seq result, signature Na^+ & Cl^- transporters' expressions were not differentially expressed in leaf epidermises, such as *CCC, NHX1,* and *SOS1* (Fukuda et al., 2004, Li et al., 2017, Martínez-Atienza et al., 2007). The result found three ion transporters that were differentially upregulated, *CHX3*, *HKT9* (*HKT2;4*), and *MRS2-A*. OsHKT2*;*4 is a class II HKT that was reported to mediate K^+ and divalent ions (Mg^{2+}, Ca^{2+}) transport. Na^+ transport was observed when the external Na^+ concentration is > 10 mM (Sassi et al., 2012, Horie et al., 2011). Expression of another member of this HKT class- *OsHKT2;1* was reported to increase

salinity sensitivity of rice by mediating Na^+ influx into the root (Horie et al., 2007). HKT2;4 may have an identical role in leaf epidermis and mediate Na^+ translocation from other leaf tissue to the epidermis. In addition, salinity-induced K^+ sensitivity reduction in *OsHKT2;4* expressing yeast was significantly lower compared to OsHKT2;2 expressing yeast (Horie et al., 2011). This indicates that *OsHKT2;4* is also contributed to K^+/Na^+ homeostasis in the epidermis under saline conditions. Functional characterization of OsCHX3 was not previously reported. It is either K^+ or Na^+ antiporter on membrane of an organelle or plasma membrane (Isayenkov et al., 2020). Aquaporin is involved in regulating photosynthesis and transpiration in plant tissue. It was reported to provide precise control of water usage and carbon assimilation under stress conditions (Pawłowicz and Masajada, 2019). *PIP2;1* and *PIP1;3* can be important in the epidermis to regulate transpiration and photosynthesis in *O. coarctata (Yooyongwech et al., 2013, Ding et al., 2019, Abdelkader et al., 2012).* These differentially upregulated transporters can be important in leaf epidermis in *O. coarctata* for salinity adaption by mediating Na^+ transport, water transport, stomata closure, and more.

Transcription factors play key roles in regulating plant response to various environmental stimuli (Singh and Sengar, 2014, Santos et al., 2011, Debbarma et al., 2019, Abiri et al., 2017). Some TF families were well studied for their stress response. In this study, several TF families were found in DE genes. More than 50 % of the DE TF were upregulated. DE *WRKY*s were overall downregulated in leaf epidermis. Based on the numbers and expression level of differentially upregulated genes, AP2/ERF, Homeobox-leucine zipper protein, bZIP, and GRAS may be important in salinity stress. Since the number of DE TF detected in this study was much lower than in other studies, these DE genes may have a key role in the regulation of plants under long-term salinity stress.

Chapter 7: General discussion and conclusions

In this Ph.D. thesis, salinity tolerance of ten *Oryza* species was explored using agronomical, physiological, and molecular approaches to expand the understanding of salinity tolerance in rice. I studied the agronomic, physiological, electrophysiological, and molecular responses of three rice cultivars (Koshihikari, Doongara, and Reiziq) differing in salt tolerance to NaCl treatment in both greenhouse and field experiments. The assessment was also conducted in the wild *Oryza* species to evaluate if the observed relation between salinity tolerance and the measured parameters in *O. sativa*, can also be validated in wild *Oryza* species. I then studied the adaptation mechanism of leaf tissue of the only halophytic *Oryza* species - *O. coarctata* and evaluated whether different leaf tissues of *O. coarctata* had a specific contribution to the overall tissue tolerance to NaCl using physiological, cell biological, and transcriptomic approaches.

7.1 Greenhouse and field environmental factors did not alter the overall salinity ranking of cultivated and wild rice species

To provide a consistent overall salinity tolerance assessment (Straten et al., 2016, Qin et al., 2020, De Vos et al., 2016) of different cultivated and wild rice accessions, two sets of complementary greenhouse and field trials were employed to validate the salinity tolerance in this Ph.D. thesis. The result indicated that plant growth and physiological properties were affected by environmental factors such as season of the year, light conditions, and humidity. Both greenhouse trials were conducted from late winter to early summer which is earlier than the complementary field trials. Better plant growth (eg. biomass) was observed in the field trial due to higher growth temperature, solar radiation, and longer daylight period in the summer. Interestingly, the impact of relative humidity on plant photosynthetic activities was observed

(see details in Chapter 5). In the open field, the relative humidity was ranged between 35 and 45%, which was significantly lower than the relative humidity in the greenhouse 55 and 65%. Although low humidity had a negative impact on photosynthetic activities in rice (Hirai et al., 2000), it protected plants from heat stress by lowering the tissue temperature during a hot day (Yan et al., 2010).

In addition, the shading effect was also found in the salinity response of slow-growing halophytic species *O. coarctata* in the field. Positive biomass accumulation was expected under the saline conditions, which was previously observed in the greenhouse (Chapter 4) and other studies (Sengupta and Majumder, 2010, Flowers et al., 1990). *O. coarctata* can normally grow to the height of approximately 100 cm in its full form. However, it has a very long "latency" period from the two-leaves stages compared to other *Oryza* species. *O. coarctata* usually chooses to emerge more tillers rather than an increase in height at this stage. As the consequence, it was shaded by the tall species which were growing next to it in the field (Figure 5.1_A). In the greenhouse, this effect was minimized by the weekly random shifting of the location of the buckets of rice plants. Considering salinity effect may only be a regular growth condition to halophytic species, *O. coarctata, it* may be more responsive to the effect of shading in the field, which leads to less biomass in both control and salinity treatment as compared to those plants in the greenhouse trial.

Overall, environmental factors such as humidity and light (shading) showed some effect on the growth of the *Oryza* species, but they did not cause a significant change in the results of salinity tolerance screening observed in Chapters 3, 4, and 5. Using *O. sativa* IR 29 (sensitive), IR64 (moderately tolerant), and Pokkali (tolerant) as reference lines, I concluded that the salinity tolerance ranking of the following species and cultivars: *O. brachyantha* (Chapter 5, highly sensitive), Koshihikari (Chapter 3 & 5, highly sensitive), Doongara (Chapter 3,

moderately sensitive), Doongara (Chapter 3, moderately sensitive), *O. longiglumis* (Chapter 4, moderately sensitive), *O. punctata* (Chapter 5, moderately tolerant), *O. australiensis* (Chapter 4 & 5, moderately tolerant), *O. rufipogon* (Chapter 4 & 5, moderately tolerant), *O. officinalis* (Chapter 4, tolerant), *O. alta* (Chapter 5, tolerant), *O. latifolia* (Chapter 4 & 5, tolerant) and *O. coarctata* (Chapter 4 & 5, highly tolerant). Therefore, the complementary greenhouse and field experiments are suitable and should be used as common practice for pre-breeding selection of salinity tolerance lines of rice and other crops.

7.2 Ion and ROS homeostasis in mesophyll cells are important traits for salinity tolerance in rice

Based on the salinity tolerance screening results, I showed that relative biomass is significantly correlated to net K^+ flux, net Cl^- flux, and relative mesophyll ROS & Na^+ intensity (Yong et al., 2020). In Chapters 3-5, I found that net K^+ and Cl^- efflux were negatively correlated with biomass accumulation. K^+ retention in the plant has been emphasized to maintain cytosolic K^+ content under the saline conditions (Wu et al., 2015b, Percey et al., 2014, Chen et al., 2005) and the K^+ efflux is mainly mediated through NSCC and NORC (Zepeda-Jazo et al., 2008, Demidchik and Tester, 2002). K^+ inward transporters such as HAK transporters might alleviate the net efflux (Shen et al., 2015, Feng et al., 2019, Chen et al., 2015). It was found that upregulation of HAK1 transporter may contribute to the significantly less K^+ efflux in the salinity-tolerant species and cultivars (Chapter 3 & 4).

Cation Chloride Cotransporter (CCC) might also indirectly contribute to K^+ retention by transporting more Cl^- in mesophyll cells as a counter ion. Cl^- is an important micronutrient but an excessive amount of Cl^- under saline conditions is harmful to plants (Wu and Li, 2019).

However, salinity-tolerant *Oryza* species had significantly lower net Cl^- flux compared with the sensitive ones. My results suggested that the negative effect of Cl^- to mesophyll cells may not be as serious as the Na^+ toxicity, at least in this case in the *Oryza* genus.

Surprisingly, Na^+ flux did not have a strong correlation with most of the salinity tolerance-related genes. This may explain why the expression of Na^+ exclusion and retrieval related genes- *SOS1* and *HKT1;4* were not significantly regulated in all tolerant species (El Mahi et al., 2019, Horie et al., 2009). Instead, leaf Na^+/K^+ ratio and mesophyll Na^+ fluorescence were found to be highly significantly correlated to biomass, net K^+ flux, and net Cl^- flux (Chapter 4 & 5). Therefore, Na^+ sequestration may contribute significantly to the maintenance of leaf Na^+/K^+ ratio and mesophyll Na^+ fluorescence because Na^+ sequestration-related genes such as *VHA-C* and *NHX1* were significantly upregulated in salt-tolerant *Oryza* species (Ishak et al., 2015, Queirós et al., 2009).

The importance of ROS regulation in response to salinity stress in the plant has been extensively studied (Mittler, 2017, Mittler et al., 2011, Zhao et al., 2019b). However, high accumulation of ROS can lead to detrimental effects such as programmed cell death (PCD), lipid peroxidation, and DNA damage. This thesis revealed that dynamic regulation of ROS production in salinity stressed leaves over 42 days of salinity treatment in rice. ROS production was found to be correlated with salinity tolerance negatively after the short-term treatment and positively after the long-term treatment (Yong et al., 2020). Therefore, elevated ROS activities after long-term salinity stress may be an important trait in salt-tolerant *Oryza* species to maintain the balance of cellular signaling transduction for salt stress adaptation.

7.3 C3-C4 intermediate and Na^+ sequestration traits contribute to salinity tolerance in *O. coarctata*

O. coarctata is the only species that showed an increase of biomass after the salinity treatment in the greenhouse trial (Chapter 4). *O. coarctata* exhibited C4-like photosynthetic traits (highest *A*, C_i, and chlorophyll content in both control and salt-stressed plants), but the AC_i curve result suggested that it does not possess C4-like photosynthesis. Moreover, leaf anatomy, AC_i curve, and C4 pathway-related gene expression all indicated that *O. coarctata* might have evolved pro-Kranz like photosynthesis or advanced CO_2 concentrating mechanism (CCM), which was also reported in other halophytes (Bose et al., 2013, Chowrasia et al., 2021, Sage et al., 2014). It exhibited the Kranz anatomy – enlarged bundle sheath cells, low numbers of mesophyll cells between two adjacent vascular veins, and high density of leaf ridges/furrows (Chowrasia et al., 2021, Rajakani et al., 2019) as well as significantly higher expression of C4 pathway-related genes- *NADP-ME*, *PEPC*, *PPDK*, *rbcL*, and *RbcS O. coarctata* compared to *O. sativa* L.- IR64 & Pokkali (Chowrasia et al., 2021). Therefore, the thesis provides strong evidence of C3-C4 intermediate characteristics that are linked to salinity tolerance in halophytic wild rice.

Na^+ fluorescence in the leaf of *O. coarctata* using confocal imaging revealed that Na^+ is significantly accumulated in the mesophyll cells, sclerenchymatous thickening, and guard cells (Chapter 4 & 6). Na^+ fluorescence in sclerenchymatous thickening and guard cells suggested that Na^+ is likely transferred to the salt gland vascular system through the epidermis layer. The result showed that Na^+ in the mesophyll cells was mainly compartmented into vacuoles to avoid Na^+ toxicity in cytosol and chloroplast (Assaha et al., 2017). The result was validated by higher gene expression of *NHX1* and *VHA-C* for Na^+ sequestration and vacuole proton pumping that are important mechanisms for salt tolerance in *O. coarctata.* Moreover, *O. coarctata* maintained high Na^+ in chloroplasts but not in the cytosol of mesophyll cells when

the leaf mesophyll tissue was directly exposed to 100 mM NaCl. It is suggested that *O. coarctata* managed to maintain low cytosol and apoplastic Na^+ content to avoid Na^+ toxicity in mesophyll cells. The chloroplasts are evolved from organisms that are tolerant to stress. Recently, interesting results have been obtained on the extremely high tolerance of isolated *O. sativa* chloroplasts to salinity (Shabala et al. unpublished). However, some halophytes were able to tolerate Na^+ accumulation in chloroplast without backstabbing photosynthetic activities (Bose et al., 2017). Furthermore, *O. coarctata* is native to the extreme saline habitat (20 - 40 dSm^{-1}). Na^+ accumulation in chloroplast and cytosol observed after 2 hours of direct exposure to 100 mM NaCl treatment might be a regular phenomenon on *O. coarctata* grown in extreme conditions, which requires further investigation in the future.

7.4 Identification of DEGs that contribute to salinity tolerance in *O. coarctata*

Leaf epidermis may be responsible for Na^+ storage and intermediate tissue of Na^+ transfer from the vascular system to salt glands in *O. coarctata*. However, the membrane transporters involved in Na^+ transport are unclear. Leaf epidermis (Figure 2.3) with cuticular wax and thick cell walls in *O. coarctata* may play important role in water retention regulation (Xue et al., 2017) and also prevent salt absorption through the leaf surface. Here, I conducted RNA-sequencing of leaf abaxial epidermis from control and salinity stressed plants to identify leaf epidermis-specific genes related to salinity stress in *O. coarctata*. The leaf epidermis RNA-sequencing showed 50% fewer mapped reads compared to RNA-sequencing of *O. coarctata* in response to salinity stress at whole plant basis (Garg et al., 2014). The difference may indicate successful identification of tissue-specific genes relative to whole plant basis. The transcriptomic analysis indicated that salinity stress significantly induces regulation of 409 DEGs ($|FC| \geqslant 1.5$, FDR $\leqslant 0.05$). 122 and 179 of DEGs were upregulated and downregulated

respectively with a |FC| ⩾ 2. (GO) enrichment analysis of upregulated DEGs identified significant clusters of genes in membrane component. GO network analysis showed that these upregulated DEGs are significantly enriched in lipid transport, membrane transport, and transcript regulatory activities.

The lipid and membrane transport include mainly LTP, ABC transporter, PIP, and cation transporters. The former three types of transporters have roles in a physiological process that are related to salinity tolerance, such as water transport, cuticle mediated regulation of transpiration, repair of salinity-induced damage on the membrane, and stomata regulation (Ding et al., 2019, Jarzyniak and Jasiński, 2014, Matsuda et al., 2016). One of the two identified cation transporters OsHKT2;4 was previously reported for its primary selectivity towards Na^+ and then K^+ under high external Na^+ conditions. It might also involve K^+ homeostasis under saline conditions as the reduction of K^+ sensitivity was lower than other HKT2 transporters (Horie, T. *et al.* 2011). Therefore, HKT2;4 may be the candidate transporter that mediates primary Na^+ transport under high external Na^+ conditions and/or K^+ transport to the epidermis, contributing to K^+/Na^+ homeostasis in *O. coarctata*. The role of another cation transporter OsCHX3 was not previously reported. Functional characterization of *Oryza* CHX can be barely found. In other plants, members of this family were reported for their positive roles under salt stress via Na^+ exclusion and K^+ accumulation in leaf and root (Qu et al., 2021, Jia et al., 2021, Jia et al., 2017, Cellier et al., 2004). It was also found to mediate Na^+ influx and increased salinity sensitivity in overexpressing lines (Jia et al., 2021). The upregulated TFs may have important roles in salinity tolerance. The TF family with more than one member upregulated in the result can be good candidates- AP2/ERF, Homeobox-leucine zipper protein, bZIP, and GRAS (Abiri et al., 2017, Wang et al., 2020).

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The downregulated genes formed significant GO terms related to physiological regulation such as JA regulation and signaling, PS II system electron transport, and Ca^{2+}-dependent signaling proteins (CPK). The importance of these components in salinity tolerance was covered in Chapter 6. Expression of these genes may not be necessary or harmful in leaf epidermis over short-term salinity stress. JA accumulation in the plant can lead to oxidative damage due to JA-induced ROS over-accumulation in tissue (Ismail et al., 2014). Reduction in electron transfer in the PS II system was found to protect the plant from excessive heat damage in plants under salinity stress (Moradi and Ismail, 2007, Netondo et al., 2004). Out of four differentially downregulated *CPK* genes, Abbasi et al. (2004) reported that *OsCPK13* expression was downregulated under salinity stress. The response of the other three members *CPK4*, *CPK24*, and *CPK1* to salinity stress was unclear. Wan et al. (2007) indicated that the extent of responses of *OsCPK*s to salinity stress can be tissue-specific. Differentially downregulated CPK might be the tissue-specific response.

The RNA-Sequencing analysis of leaf epidermis of *O. coarctata* presented in this thesis identified several tissue-specific candidate genes involved in salinity tolerance. These genes can be an important target for genetic engineering in the future.

7.5 Concluding remarks and future directions

This study identified a few *Oryza* species that are tolerant to salinity stress. *Oryza* species with C genomes were overall tolerant to salinity stress. Including the evaluation of other members of this genome, which was consistently reported as salt-tolerant species (Prusty et al., 2018, Nishizawa et al., 2015, Nakamura et al., 2002, Nakamura et al., 2004, Nishizawa et al., 2016). Future studies on this species on a larger scale can be considered to evaluate whether there is a

set of common traits reserved in C genomes. The C3-C4 intermediate photosynthesis (pro-Kranz) related traits may be helpful for Kranz structure modification in C4 rice development (Wang et al., 2017c) since *O. coarctata* is more closely related to *O. sativa* than other C4 crops.

Sodium accumulation in *O. coarctata* mesophyll cells highlighted the superior activities of several Na^+ transporters OcNHX1, OcSOS1, OcHKT1;4 and OcHKT1;5 to maintain low Na^+ content in the chloroplast and apoplastic region between mesophyll cells and high Na^+ compartmentation in the vacuole. These transporters require coordination of other components but are not limited to, Ca^{2+} signaling (SOS2, SOS3) and membrane proton pump (V-PPase and V-ATPase) (Ji et al., 2013). These candidate genes can be important for salinity-tolerant rice development in the future. Future studies can be conducted functional validation of these genes to confirm whether they are the key salinity tolerance-related genes in *O. coarctata.* The Na^+ distribution pattern can be completely different from the pattern observed in mesophyll after 7 days of salinity treatment as halophyte can tolerate higher Na^+ content in cells as found in mesophyll exposed to 2 hours of salinity stress. Future work should confirm whether the pattern can be repeated in mesophyll treated with 200 mM NaCl. Furthermore, modification of staining formula/technique would be required to evaluate Na^+ accumulation in epidermis and guard cells.

RNA-sequencing of epidermis transcriptomes reveals the potential role of several transporter proteins of the epidermis in salinity tolerance, such as lipid transport protein, ATP-binding cassette protein, and aquaporin. The upregulated cation transporter genes (*HKT2*;4, *CHX3*) might be responsible for Na^+ transfer to salt glands. Several TFs were also found differentially upregulated in the epidermis by the salinity treatment. Functional validation of these genes will be required to confirm their expression under salinity tolerance. To further explore tissue-specific defense mechanisms in the leaf, a transcriptome study of other tissues, such as mesophyll cells and bundle sheath cells will be needed. These can provide insight into

what salinity tolerance components are tissue-specific and how are each tissue coordinates to adapt salinity tolerance.

However, a comprehensive study of the most tolerant species *O. coarctata* was only carried out in this thesis in the form of C3-C4 intermediate photosynthesis and gene expression and RNA-sequencing of leaf epidermis due to limitation of the Ph.D. time frame. Future work will be conducted on proteomics and metabolomics of the leaf epidermis and functional analysis of key genes discovered in the Ph.D. thesis. Nevertheless, this study provides some new insights into the potential use of traits and genes from wild rice species in the breeding of salt-tolerant rice cultivar. The outcome of the project will not only improve the understanding of the complex salt tolerance mechanisms in wild and cultivated rice species but also guide future research towards a more sustainable rice production by improving salinity tolerance of cultivated rice with the traits of their wild relatives.

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