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Genetic basis of ionomic variations in rice and its implication for the sulfate transporter gene contributing to the sulfur accumulation

（イネにおけるイオノーム変動の遺伝的基盤とそれが示唆した硫黄蓄積に寄与する硫酸トランスポーター遺伝子）

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農学専攻／生産フロンティアコース 博士後期課程

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Chapter 1. General Introduction

1.1 Plant ionome and ionomics

It has been known for more than 2000 years that applying plant ash, lime or other forms of minerals to soils in practice can improve plant growth, but the broader understanding of the functions of certain minerals was arose in the nineteenth century and that was established as a scientific discipline by Justus von Liebig (1803-1873) (Kirkby 2012). Thereafter, following extensive studies on the mineral compositions in different plant species growing on diverse edaphic conditions defined a precise set of criteria for essential elements (Arnon and Stout 1939), and accordingly confirmed at least 14 mineral elements, except for carbon, hydrogen, and oxygen, as essential element for plant growth (Kirkby 2012). Deficiency in any one of these mineral elements decreases plant growth and crop yields. Plants normally acquire these growth-demanding mineral elements from the edaphic environment in soluble form. However, the nonessential elements can also accumulate in the soil solution and be absorbed by plants (Li et al. 2014; El Azhari et al. 2017). Comparing to essential mineral elements, nonessential elements play a more complicated role in the vital process of plants. For example, aluminum (Al) toxicity represents the most serious limitation to plant production in acid soils (Kochian et al. 2015), while Al induced improvements of the root activity contributed to a growth enhancement in *M. malabathricum* (Watanabe et al. 2005). Selenium (Se) in low concentration is beneficial to plant growth and antioxidant capacity, but in high concentration is toxic to most of plants (Kolbert et al. 2019).

Moreover, plants take up and translocate nonessential elements through sharing the same pathways as essential elements due to similar chemical properties. For instance, zinc (Zn) and cadmium (Cd) share the same zinc-iron transport protein (ZIP) influx transporter into plant roots (Spielmann et al. 2020); and plant roots can absorb arsenic (As) and Se via the phosphate transporter (Zhang et al. 2014; Cao et al. 2017). Therefore, due to the differences in elemental preferences among plant species, narrow content gap between benefit and toxicity in elements, and the strong interactions between elements, the study on combination of elements in plants arose widely interests.

With the development of high-throughput elemental analysis methods, such as inductively coupled plasma–mass spectrometry (ICP-MS) and ICP-atomic emission spectrometry (AES), it is possible to detect more elements simultaneously and rapidly, that promoting the development of elemental combination as "omics". The "metallome" were firstly proposed and defined the combination of metal and metalloid elements in cell and organism (Outten and O'Halloran 2001). Expanded on this, the new concept termed "ionome", defined as "the mineral nutrient and trace element composition of an organism, representing the iorganic component of cellular and organismal systems", was proposed by incorporating in all of non-metal and inorganic elements (Lahner et al. 2003; Salt et al. 2008). High-throughput ionomics is a branch of plant nutrition that combines tightly plant nutrition with other discipline, such as genomics, ecology, and environmental science, and provides a wider range of research interests and ideas for scientists and researchers. To date, ionomics have been performed in many studies with different research materials, including *Arabidopsis thaliana* (L.) Heynh (Lahner et al. 2003), *Saccharomyces cerevisiae* (yeast) (Yu et al. 2012), *Oryza sativa* L. (rice)

(Norton et al. 2010; Pinson et al. 2015; Yang et al. 2018), *Zea mays* L. (maize) (Baxter et al. 2014; Gu et al. 2015), *Glycine max* (L.) Merr. (soybean) (Ziegler et al. 2013), *Lotus japonica* (Chen et al. 2009), *Solanum lycopersicum* L. (cherry tomato) (Sánchez-Rodríguez et al. 2010), *Brassica napus* L. (rapeseed) (Tomatsu et al. 2007), as well as humans (Sun et al. 2012; Malinouski et al. 2014). Uncovering the genetic architectures controlling the mineral ionomic homeostasis in plants is one of the most important aspects for understanding the biochemical dynamic networks that regulate the plant ionome. Moreover, many researchers investigated the ionomic profiling in many different plant species living in diverse ecological scales to explain the different contributions of plant phylogenetic and environmental factors to plant ionomic homeostasis (Watanabe et al. 2007; Neugebauer et al. 2020; Watanabe and Azuma 2021; Zhang et al. 2021). Therefore, the ionomic variations in different plant species to the environmental change is also worth to be studied to provide a new dimension to our understanding of plant adaptation to the environment and landscape formation (Baxter and Dilkes 2012). At present, scientific interests in plant ionome mainly focus on the following fields, including: (1) mutant detection and candidate gene identification (Chen et al. 2009; Chu et al. 2015); (2) environmental evaluation and prediction (Baxter et al. 2008); (3) elemental interaction and homeostasis in plants (Watanabe et al. 2007; Zhang et al. 2022); (4) niche competition and cooperation between plant species (Pillon et al. 2019; Zhang et al. 2021); (5) metal-hyperaccumulated and biofortificated plant screening (Sterckeman et al. 2017; Nkrumah et al. 2021).

1.2 Ionome, genome, and environment

Plant ionome is a dynamic network associating with plant genome, living environment, and ecological factors as well as their interactions (Neugebauer et al. 2020). By using the inductively coupled plasma mass spectrometry (ICP-MS) to analyze the leaf ionome of many thousands of plants, Lahner et al. (2003) screened the content of 18 elements in leaves of about 6,000 *Arabidopsis* and identified 338 putative mutants with single or multiple elemental change. They found that about 90% of the mutants showed alterations in more than one element, indicating one gene could control more than one element change. Encouraged by this milestone study in development of ionomics, a set of 4,385 yeast mutant strains from the *Saccharomyces* genome deletion project was used to describe a mapping of genes on elemental homeostasis, indicating the potential of ionomics to study elemental interactions and function interpretations of uncharacterized genes (Eide et al. 2005). Chen et al. (2009) performed a similar large sample ionomic screening of 2,000 mutagenized M2 *Lotus japonica* plants in hydroponics. Their results further confirm the correlation between plant ionome and transport-related genes and suggested ionomics as a powerful functional genomics tool for determining genes. Therefore, based on the rapidly and efficiently screening of mutants, many studies have developed the identification of ionomic genes by forward genetics. For instance, the first ionomic mutant to be cloned in the forward genetic screen of Lahner et al. (2003) is the named enhanced *suberin1-1* (*esb1-1*) mutant (Baxter et al. 2009). The *esb1* mutant displays a lower leaf accumulation of Ca, Mn, and Zn, and higher levels of Na, S, K, As, Se, and Mo, due to the disruption of the normal deposition of lignin required for Casparian strip formation (Hosmani et al. 2013). The

myb36-1 mutant, also isolated by Lahner et al. (2003), showed a higher leaf Na, Mg, and Zn, and a decreased content of Ca, Mn, and Fe in leaves. Loss of function of transcription factor *MYB36* leads the Casparian strips and deposition of ectopic lignin and suberin at the endodermis (Kamiya et al. 2015). Moreover, an additional forward genetic screen following the protocol of Lahner et al. (2003), but under low Fe or P condition, identified more 36 leaf ionomic mutants with new alleles of potential candidate genes (Huang and Salt 2016).

It is notable that the plant ionome may also vary under diverse living conditions. Genetic expression and regulation in plants are stimulated by environmental signals. As a driver of elemental variation in plants, environmental signals are extremely complex, including diverse soil mineral content, climate, precipitation, temperature and other biological or abiotic factors. Plants living in different environments prefer to adjust their ionomic uptake and accumulation strategies to maximize their functional ionomic status. Thus, changes in soil conditions cause variations in plant ionome even for plants of the same species (Watanabe et al. 2015; Jiang et al. 2018). Busoms et al. (2015) concluded that local adaptation occurred between coastal and inland populations of *Arabidopsis thaliana* species living <30 km from each other and that adaptation was driven by different salinity levels. In Southwest Australia, members of the family *Proteaceae* efficiently evolved in terms of phosphorus (P) utilization mechanism to adapt to P-deficient soil (Lambers et al. 2015). Furthermore, a set of 1,972 paired leaf and soil samples from 165 European populations of *A. helleri* was collected and analyzed the ionomics by Stein et al. (2017), provide a more comprehensive and precise interpretation of intraspecies variation in soil–ionome relationships, and a basis for the

molecular–genetic dissection and ecological analysis of the observed phenotypic variation. Thus, the ionome of plants growing under different soil conditions is crucial for broadening current knowledge on environmental and ecologic adaptation.

1.3 Genome-wide association study

One of the most important functions of plant ionomics is to uncover the genetic architectures and identify the candidate genes regulating the mineral homeostasis in plants affected by environment. Genetic variation in natural genotypes provides bountiful resources for identification of quantitative trait locus (QTLs) and isolation of candidate genes. To date, based on study of map-based cloning by using biparental crosses made between accessions from the phenotypic extremes, several functional QTLs responsible for elemental uptake and accumulation in plants have been successfully identified to isolate causal genes controlling elemental variations. For instance, Rus et al. (2006) reported a *HKT1;1* gene encoding an Na transporter as the candidate gene responsible for higher leaf accumulation of Na in *A. thaliana* by screening of the survival F_2 population crossing *Tsu-1* and *Col-0* under high salty stress. Schaaf et al. (2006) proposed the *IREG2*, which encoding a membrane protein and is coregulated with *IRT1* by *FUR/FIT1* transcription factor, as a Ni transporter that regulating the loading of Ni in vacuolar in response to Fe deficiency stress. Moreover, using recombinant inbred lines (RILs) to identify ionomic QTLs has also been a great success. Using the RIL population constructed by wild *A. thaliana* accessions *Bay-0* and *Shahdara*, a major QTLs resulting variation of S content in plant was identified (Loudet et al. 2007). Combining genetic and chemical results, they have cloned the candidate

gene *APR2* to reveal a single amino acid change in an enzyme of S reduction pathway, decreasing the enzyme activity and leading to S accumulation in plants. Then, Koprivova et al. (2013) identified *ATPS1* as a complementary causal gene responsible for leaf S accumulation based on the *Bay-0* and *Shahdara* accession.

However, the number of the well-mapped mineral QTLs identified by biparental population is still limited due to the genetic variation among a few genotypes. Therefore, Genome-wide association study (GWAS), based on the large allelic diversity in natural populations, is beginning to be widely applied as a powerful complementary tool for unraveling the molecular basis for phenotypic variation. To date, GWAS of the ionomic profiles was concentratedly performed on the model plant, *A. thaliana* and rice accessions, due to the easily accessible genome information. The first comprehensive GWAS was performed on 107 phenotypic traits (18 elements) in a set of 199 (in which 93 accessions were used for elemental variation) *A. thaliana* accessions with 216,130 single nucleotide polymorphisms (SNPs) (Atwell et al. 2010). They detected significant SNPs associated with leaf Na, Mo, S, and Se in the genomic location *HKT1;1*, *MOT1*, and *SULTR1;2*, respectively. The following GWAS of ionomics were performed and identified a number of QTLs as well as causal genes controlling elemental accumulation. A set of 360 *A. thaliana* genotypes were used to perform GWAS, and finally validated with genetic complementation and gene expression analysis that the *HKT1;1*, encoding a known Na transporter as the major driver of natural leaf Na variation across the worldwide *A. thaliana* population (Baxter et al. 2010). Based on the dataset from a publicly accessible *A. thaliana* library, researchers conducted GWAS to dissect the phenotypic variance associating to genetic variance heterogeneity and screen the

genome for variance-driven loci, both identifying a known Mo transporter *MOT1* responsible for Mo accumulation in plants (Shen et al. 2012; Forsberg et al. 2015). Therefore, screening the existing big and redundant databases to dig and sort more undiscovered genetic information is also the challenge and opportunity for GWAS in the future. Furthermore, studies on transporter for heavy metal accumulation using GWAS also provide the insight on future food safety. A set of 349 *A. thaliana* accessions from 5,810 global accessions was selected to analyze the elemental profile by using ICP-MS, and performed GWAS to reveal the genetic basis underlying natural variation in Cd and As accumulation (Chao et al. 2012; Chao et al. 2014). Their identification of *HMA3* and *HAC1* for Cd and As accumulation provides an important new resource for the development of low arsenic-containing food such as rice. Yan et al. (2019) performed GWAS by using a diverse global collection of 127 rice accessions under Cd stress and isolated a major facilitator superfamily gene, *OsCd1*, is involved in root Cd accumulation. They also found that natural variation in *OsCd1* with a Val449Asp mutation is the main reason for the divergence of rice grain Cd content between *japonica* and *indica*. Recently, ionomic GWAS in rice accessions have been also largely developed (Yang et al. 2018; Cobb et al. 2021). However, due to the huge amount of genetic information and genetic variation caused by differences of geographical environment, it is still remaining a big challenge to link phenotype-genotype to provide more information of genetic architecture for controlling mineral accumulation.

1.4 Objective of the present thesis

Rice is one of the most important crops in the world, covering more than half of the daily caloric resources for more than three billion population (Khush 2005). Therefore, biofortificated and heavy metal-free rice grain is significantly of importance for human health. Ionomics is born to study this challenge with its natural advantage of speediness, efficiency, and high-throughput.

Accordingly, this thesis provides detailed information of the elemental dynamics in a set of rice accessions collected from National Agriculture and Food Research Organization (NARO), and the results of GWAS on rice ionome, to reveal the genetic architecture and underlying functional genomics of the ionome in this major crop species and provide important insights about the phenotypic variation and variety screening. On overview of the workflow is shown in Fig. 1.
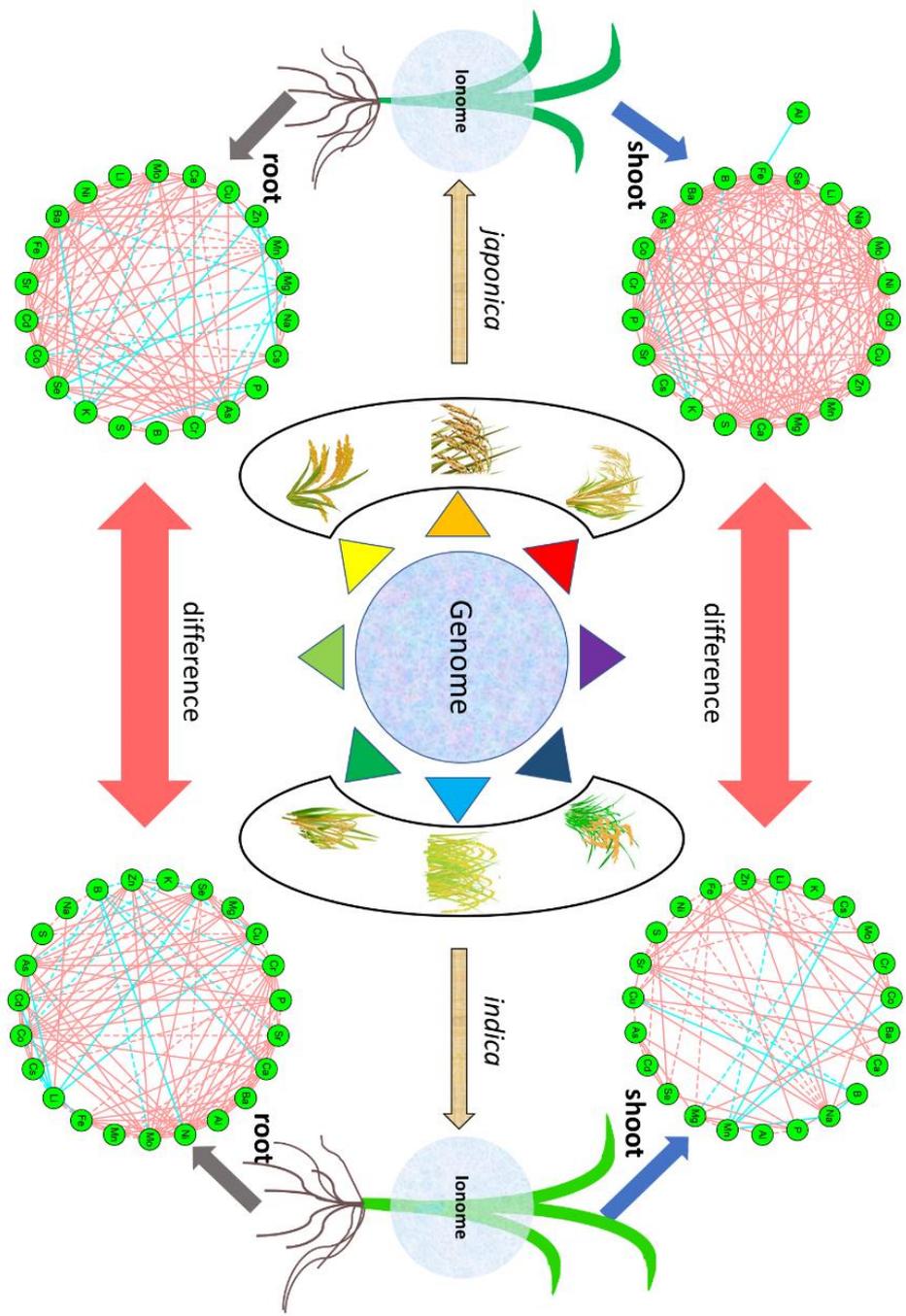
Fig. 1. The concept of the present study showed that the correlations between ionome and genome among widespread rice genotypes and subspecies.

Chapter 2. The study of ionomic profiling of rice genotypes and identification of varieties with elemental covariation effects

2.1 Introduction

At least 25 elements are required for human health, however, it is unfortunate that over 4 billion people suffer from the public health problem of 'hidden hunger' due to insufficient intake of essential elements from their daily diets, especially in developing countries (White and Brown 2010). The biofortification of staple foods has been accepted as a practical and cost-efficient way to alleviate malnutrition by increasing the concentrations of essential nutrients in crops and ultimately in humans (White and Broadley 2009; Zhang et al. 2018). Paddy rice (*Oryza sativa*), one of the most widely planted staple crops and the source of 80% of the daily caloric and micronutrients for over half of the world population, is the most suitable candidate for crop biofortification strategies (Khush 2005; Muthayya et al. 2014). Compared with medical supplementation and dietary diversification, it is easier to directly benefit the malnourished conditions of people living in poor rural regions via agronomic or genetically biofortified rice (Zhang et al. 2018; Qiao et al. 2019). Rice is adapted to diverse local edaphic and climatic conditions, resulting in the development of thousands of varieties and genotypes by selective breeding (Singh et al. 2017), however, yield and environmental adaptation are likely the most important factors for varietal breeding to enrich micronutrient content (Tan et al. 2020). For example, since the superfluous soil

Fe is one of the most significant conditions decreasing rice production in Southeast Asia and South America (Becker and Asch 2005), local farmers likely prefer rice with a lower Fe uptake or tolerance to Fe stress to ensure optimal yield. In the long-term history of rice domestication, due to the lack of attention to micronutrients, the Fe content in grains varies greatly among rice varieties. Therefore, it is important to screen for the elemental concentration status of rice varieties to meet the requirements of biofortification strategies.

Moreover, plants take up and translocate nonessential elements through sharing the same pathways as essential elements due to similar chemical properties, and elements with similar chemical properties sharing the same transportation pathways can result in competitive uptake between them. For instance, Zn and Cd share the same zinc-iron transport protein (ZIP) influx transporter into plant roots (Spielmann et al. 2020); and plant roots can absorb As and Se via the phosphate transporter (Zhang et al. 2014; Cao et al. 2017). Nonessential elements in irrigation water can accumulate in the soil and be absorbed by plants (Li et al. 2014; El Azhari et al. 2017). These nonessential elements can be enriched through the food chain from crops to humans, and the consumption of contaminated crops can pose a significant health risk. Rice is easy to accumulate toxic metal(loids) under flooded conditions due to high affinity (Zhou et al. 2018; Gu et al. 2019). Many previous studies have reported the public concern and health risks of Cd and As (Chen et al. 2018; Suriyagoda et al. 2018), and the radioactive contaminant elements cesium (Cs) and strontium (Sr) in crop grains harvested from contaminated areas, such as Fukushima in Japan and Chernobyl in Ukraine (Balonov et al. 2007;

Kubo et al. 2020). Hence, increasing the essential nutrient uptake and reducing nonessential elemental contamination in rice is a pressing issue for human health.

In the present study, thus, I cultivated 120 rice genotypes in a consistent hydroponic system to analyze the concentrations of nutrients and trace elements by inductively coupled plasma mass spectrometry (ICP-MS), and also measured concentrations of anions by capillary electrophoresis (CE) to provide a more comprehensive understanding for rice ionome.

2.2 Materials and Methods

2.2.1 Rice materials and cultivation

A total 120 rice accessions (Information in appendix 1) obtained from NARO Genebank, including *japonica*, *indica* and *aus* subspecies, was used in this study. Plump rice seeds were surface sterilized with sodium hypochlorite (NaClO with 0.5-1% available chlorine) for 10 min, thoroughly washed with running water for three times, then soaked in running water under the dark condition for 48 h to break the residual seed dormancy. After soaking, the seeds were germinated on a nylon screen floated on a polypropylene container with 8 L of 1/2 strength modified nutrient solution (as described in 2.2.2) for 7 d, and replaced to full strength nutrient solution for another 7 d. Then, the solution was replaced by a hydroponic condition with complete nutrient solution plus additional non-essential elements. In the first 7 d, half-strength of non-essential element solution (described in 2.2.2) was added in to culture solution, and the in last 7 d, added half-strength non-essential element solution was replaced by full-strength. Then the seedlings were sampled, washed with deionized water, and cut to

separate the roots from shoots. The seedling base parts contacted with solution were cut out only for lyophilization and biomass measurement, not for chemical analysis. Then, the fresh samplings were rapidly frozen in liquid nitrogen, lyophilized, weighed, and ground for further measurements.

The experiment was conducted in a greenhouse at Hokkaido University (14 h photoperiod and day/night temperature of 25–28/18–22∘C, respectively).

2.2.2 Hydroponic conditions

The modified complete nutrient solution (Maejima et al. 2014) was composed of following elements: 2.14 mM N (NH_4NO_3), 0.32 mM P ($NaH_2PO_4 \cdot 4H_2O$), 0.77 mM K (K_2SO_4:KCl=1:1), 1.2 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.82 mM Mg ($MgSO_4 \cdot 7H_2O$), 35 μM Fe (Fe-EDTA), 9.1 μM Mn ($MnSO_4 \cdot 5H_2O$), 45 μM B (H_3BO_4), 1 μM Zn ($ZnSO_4 \cdot 7H_2O$), 0.3 μM Cu ($CuSO_4 \cdot 5H_2O$), 0.5 μM Mo [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$], and 0.2 μM Co ($CoCl_2 \cdot 6H_2O$). The pH of this solution was adjusted to 5.5 (± 0.5) using 1 mM HCl or 1 mM NaOH every day.

The additional hydroponic solution including: 1 μM Al ($AlCl_3$), 0.4 μM Cr ($CrCl_2 \cdot 6H_2O$), 3 μM Cs (CsCl), 2 μM Sr ($SrCl_2 \cdot 6H_2O$), 1 μM Ba ($BaCl_2 \cdot 2H_2O$), 1 μM Se (Na_2SeO_3), 1 μM As ($NaAsO_2$), 0.2 μM Cd ($CdCl_2$), 1 μM Ni ($NiCl_2$), 1 μM Li (LiCl). The growth of rice seedlings is testified not to be affected significantly by the set concentrations of above non-essential elements in pre-experiment.

2.2.3 Ionomic analysis

Approximately 50 mg aliquot of the powdered rice sample was added with 2 ml of 61% HNO_3 (EL grade; Kanto Chemical, Tokyo, Japan) in a tube, and heated at 110 °C in a DigiPREP MS apparatus (SCP Science, Quebec, Canada) until the powder had almost disappeared. Then, 0.5 mL of H_2O_2 (semiconductor grade; Santoku Chemical, Tokyo, Japan) was added twice and continually heated, until the solution got clean. After cooling, the digested solution was filled to 10 mL with 2% HNO_3, analyzed following 23 elements by the ICP-MS (Elan, DRC-e; PerkinElmer, Waltham, MA, USA): P, potassium (K), sulfur (S), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), Zn, copper (Cu), boron (B), molybdenum (Mo), nickel (Ni), Al, barium (Ba), sodium (Na), Sr, As, Cd, cobalt (Co), chromium (Cr), Cs, Se, and lithium (Li). The compound multielement standard solution IV (Merck, Tokyo, Japan) was used for drawing standard curve.

2.2.4 Anions analysis by capillary electrophoresis (CE)

Approximately 10 mg totally powdered sample was added in a centrifugal tube (BIO-BIK, Osaka, Japan) with 1.5 mL deionized water, and shaken at room temperature for 30 min. Then, totally mixed sample was centrifuged at 120 x 100 rpm for 10 min. The supernatant was then filtered through a 45 μm membrane filter (Advantec, Tokyo, Japan) into a 1.5 mL centrifugal tube. After ten times dilution, concentrations of anions (Cl^-, SO_4^{2-} and NO_3^-) were determined by CE (Quanta 4000 CE; Waters, Milford, MA).

2.2.5 statistical analysis

All descriptive statistics of analysis of variance (ANOVA) and correlation coefficients were performed by Minitab 19 (Minitab Inc., State College, PA, USA). For correlation analysis, correlations coefficients were conducted using Pearson's correlation analysis. The visualizations of correlations were performed by R (V.3.6.3) with "corrplot" packages.

2.3 Results

2.3.1 Comparisons of element concentrations among rice subspecies

The concentrations of 26 elements (including 3 anions) in the rice shoots and roots were displayed in boxplots (Fig. 2.1 for essential elements and Fig. 2.2 for nonessential elements). Among *japonica*, *indica* and *aus*, significant differences were detected in all the elements except Ba, while the magnitudes of the element concentrations were generally similar. The comparison of element concentrations between shoots and roots showed the same pattern in each subspecies, i.e., the concentrations of macronutrients in the shoots were higher than those in the roots, whereas the concentrations of microelements [except for Mn and B] and anions in the shoots were significantly lower than those in the roots (Fig. 2.1 and 2.2). The concentrations of K in shoots and roots, as well as those of S and Ca in roots of japonica, were markedly the lowest, and the concentrations of Al, As, Cd, Co and Cr in japonica showed the highest among the three subspecies (Fig. 2.1 and 2.2).
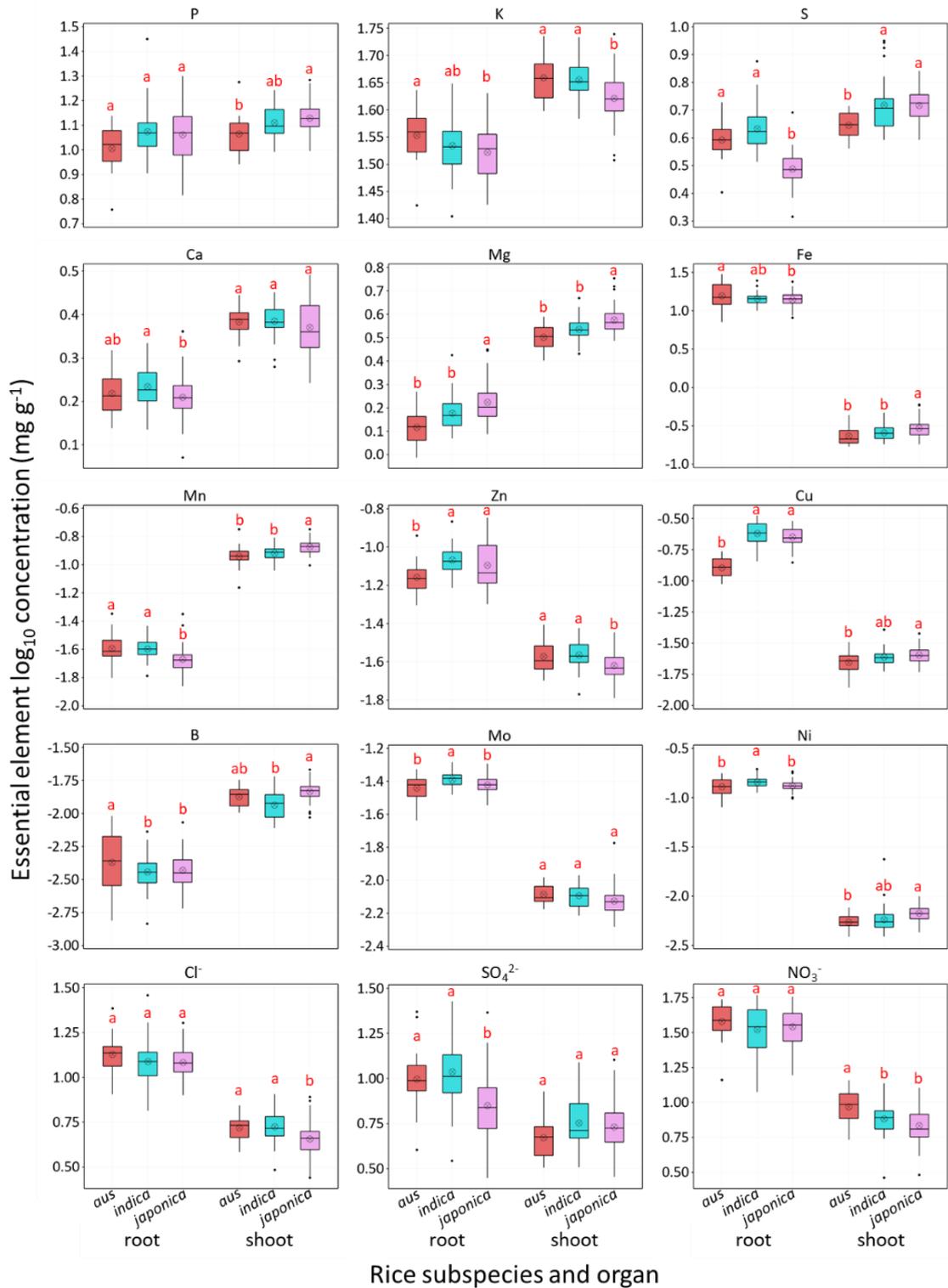
Fig. 2.1. Boxplot showing the leaf log$_{10}$ concentration of 15 essential elements (P, K, S, Ca, Mg, Fe, Mn, Zn, Cu, B, Mo, Ni, Cl$^-$, SO$_4^{2-}$ and NO$_3^-$) in shoot and root of rice

genotypes. The t-test was performed for *p*-value of pairwise comparison: different letter (a or b) represents there is significant difference between subspecies.
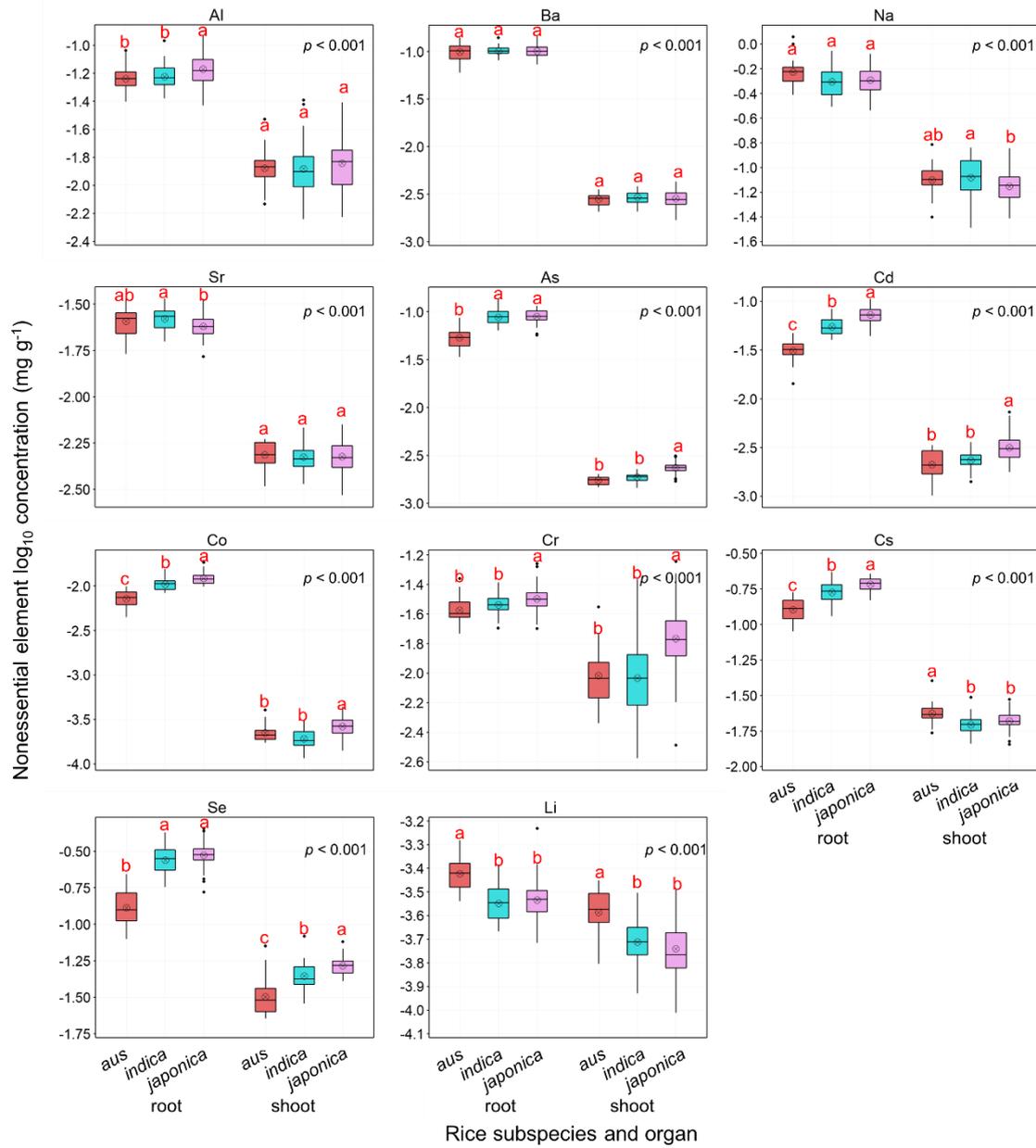
Fig. 2.2. Boxplot showing the mineral $\log_{10}$ concentration of 11 non-essential elements (Al, Na, Ba, Sr, As, Cd, Co, Cr, Cs, Se, and Li) in shoot and root of rice accessions. The t-test was performed for *p*-value of pairwise comparison: different letter (a or b) represents there is significant difference between subspecies.

2.3.2 Variations of concentrations of each element among rice genotypes

The analysis of variance (ANOVA) descriptive statistical analysis was presented in Table 2.1. Highly significant (P < 0.001) or significant (P < 0.01) differences among the rice genotypes were observed for most elements except for Fe, Ni, Cl$^-$ and Al in the shoots, and P, Ca, Zn, B, Cl$^-$, NO^{3-}, Al and Li in the roots. Correspondingly, to further quantify the linkage between element and rice genotype, variance contribution of the rice accessions to the total variance was calculated as a range from 30.21% for Al to 81.61% for Cd in the shoots and 29.09% for B to 86.03% for Mg in the roots (Table 2.1). Contributions for element less than 50% were detected in P, Ca, Fe, Zn, B, Ni, Cl$^-$, NO^{3-}, Al, Cr and Li, in which P, Ca and B were only in the roots. Among the elements with a rice variety contribution greater than 50%, only Sr and Cd were mainly contributed by rice variety in the shoots than that in the roots.

The coefficient of variation (CV), which reflected the ionomic variations in the subspecies, ranged from 0.07 to 0.68 in the shoots and 0.10 to 0.47 in the roots (Table 2.1). A similar CV trend was observed between essential and nonessential elements. The CV patterns were significantly different between the shoots and roots and similar among the *aus*, *indica* and *japonica* subspecies. For the macronutrients, the CVs in all the subspecies were less than 20% except for P in the roots. In addition, the CVs of most nonessential elements, such as As, Cd, Co, Cs and Se in the *aus* and *indica* roots varied more widely than in *japonica*.

Table 2.1. Variation in element concentrations of shoot and root among rice accessions (n = 320)

Element	Shoot					Root				
	F value[a]	Contribution (%)[b]	CV *japonica*	CV *indica*	CV *aus*	F value[a]	Contribution (%)[b]	CV *japonica*	CV *indica*	CV *aus*
P	3.31**	63.57	0.14	0.16	0.19	1.08	37.24	0.27	0.29	0.19
K	2.58**	56.71	0.09	0.07	0.09	3.60**	64.69	0.10	0.11	0.10
S	2.76**	58.33	0.13	0.23	0.10	8.10**	80.47	0.14	0.21	0.15
Ca	2.65**	57.34	0.14	0.08	0.08	1.16	37.05	0.10	0.10	0.12
Mg	4.91**	71.38	0.14	0.12	0.11	12.11**	86.03	0.20	0.19	0.17
Fe	1.29	39.48	0.30	0.21	0.28	1.53*	44.64	0.20	0.20	0.37
Mn	3.13**	61.29	0.11	0.11	0.19	3.56**	64.44	0.22	0.16	0.26
Zn	1.54*	43.90	0.17	0.16	0.19	1.32	40.24	0.30	0.17	0.20
Cu	3.74**	65.46	0.15	0.17	0.21	9.13**	82.27	0.15	0.20	0.18
B	2.86**	59.19	0.16	0.25	0.17	0.81	29.09	0.31	0.30	0.47
Mo	2.96**	59.99	0.22	0.16	0.14	3.07**	60.99	0.13	0.11	0.16
Ni	1.05	34.73	0.17	0.15	0.16	1.63*	45.36	0.12	0.13	0.19
Cl⁻	1.24	40.21	0.22	0.19	0.16	1.08	41.28	0.21	0.30	0.26
SO₄²⁻	2.13**	53.69	0.30	0.38	0.28	2.19**	59.04	0.47	0.43	0.44
NO₃⁻	1.57*	45.91	0.28	0.24	0.27	1.39	47.32	0.29	0.35	0.26
Al	0.85	30.21	0.49	0.53	0.36	1.15	37.07	0.25	0.21	0.24
Ba	2.70**	57.80	0.18	0.14	0.14	3.55**	64.35	0.14	0.11	0.19
Na	2.33**	54.19	0.29	0.34	0.30	3.03**	60.61	0.26	0.32	0.30
Sr	2.98**	60.18	0.17	0.16	0.16	2.60**	56.92	0.14	0.12	0.17
As	4.11**	67.56	0.12	0.09	0.10	4.53**	69.71	0.15	0.18	0.24
Cd	8.76**	81.61	0.30	0.19	0.32	6.77**	77.50	0.19	0.20	0.23
Co	2.99**	60.19	0.27	0.21	0.26	4.56**	69.85	0.17	0.17	0.20
Cr	1.67**	46.07	0.52	0.68	0.51	1.47*	42.76	0.20	0.14	0.22
Cs	4.21**	68.07	0.13	0.16	0.20	9.19**	82.37	0.10	0.16	0.18
Li	1.87**	48.69	0.28	0.21	0.21	1.15	36.97	0.20	0.17	0.19
Se	6.11**	75.56	0.14	0.21	0.35	8.83**	81.78	0.18	0.20	0.28

[a] ANOVA with Tukey test is conducted for F values of mineral element concentrations attributable to rice accessions. * and ** represent $p < 0.01$ and $p < 0.001$, respectively. [b] Contributions are the partition of variance for the cultivar difference (% of variance between accessions to total variance). Due to extremely small contribution of subspecies, data is not displayed.

2.3.3 Ionomic interactions among rice genotypes

Pearson's correlation analysis and visualization were performed in the interactions between pairwise individual element concentration in shoot and root of *japonica*, *indica*, and *aus* respectively. The ionomic correlation-heatmap patterns in shoot and root of subspecies in Fig. 2.3 displayed different to each other in diverse degree. According to the score plot of principle component analysis (PCA) using correlation coefficient, an extremely significant difference in correlations observed between shoot and root resulted in locating on the positive and negative axes of x, respectively (Fig. 2.4). Meanwhile, the locations of PCA score of correlations in root among subspecies were detected on the same first quadrant, yet the locations in shoot showed extremely significant different among subspecies, and were in different quadrant. It was consistent with that most of minerals in shoot of *japonica* displayed significantly positive correlation and that in shoot of *indica* as well as *aus* expressed much less correlation, while the patterns of ionomic correlations in root among subspecies were observed much more similar (Fig. 2.4).

Consistent to PCA, the elemental correlation patterns of roots were similar among subspecies, while that of shoots were largely different which the strongest was in *japonica*, the weakest was in *aus*. Although different rice subspecies and organs showed diverse strategies on interactions of element, many interactions still remained the same. Obviously, correlations between S and SO_4^{2-} were always significant positive. Ca always interacted significantly positively with Ba and Sr, as well as the significant positive correlation between P and As. The interactions of several minerals in different subspecies and organs were detected even completely opposite. For example, Mg in

root of *japonica* corelated significantly negatively with As, but positive relations showed in shoot of *japonica* and *indica*. The same situations also were detected in Mn and Co, Mn and Cs, Zn and Cs. Anions were positively correlated in root but not in shoot. Furthermore, K and Mg correlated with only a few elements in *aus* and *indica*, and P and Al only correlated with more elements in shoot of *japonica* and root of *aus*, respectively. In contrast, Ca, Cu, Zn, and Sr were detected significantly related with many elements in all plots. It indicated that these divalent cations and metals have a greater effect on ionomic interactions, rather than other elements.
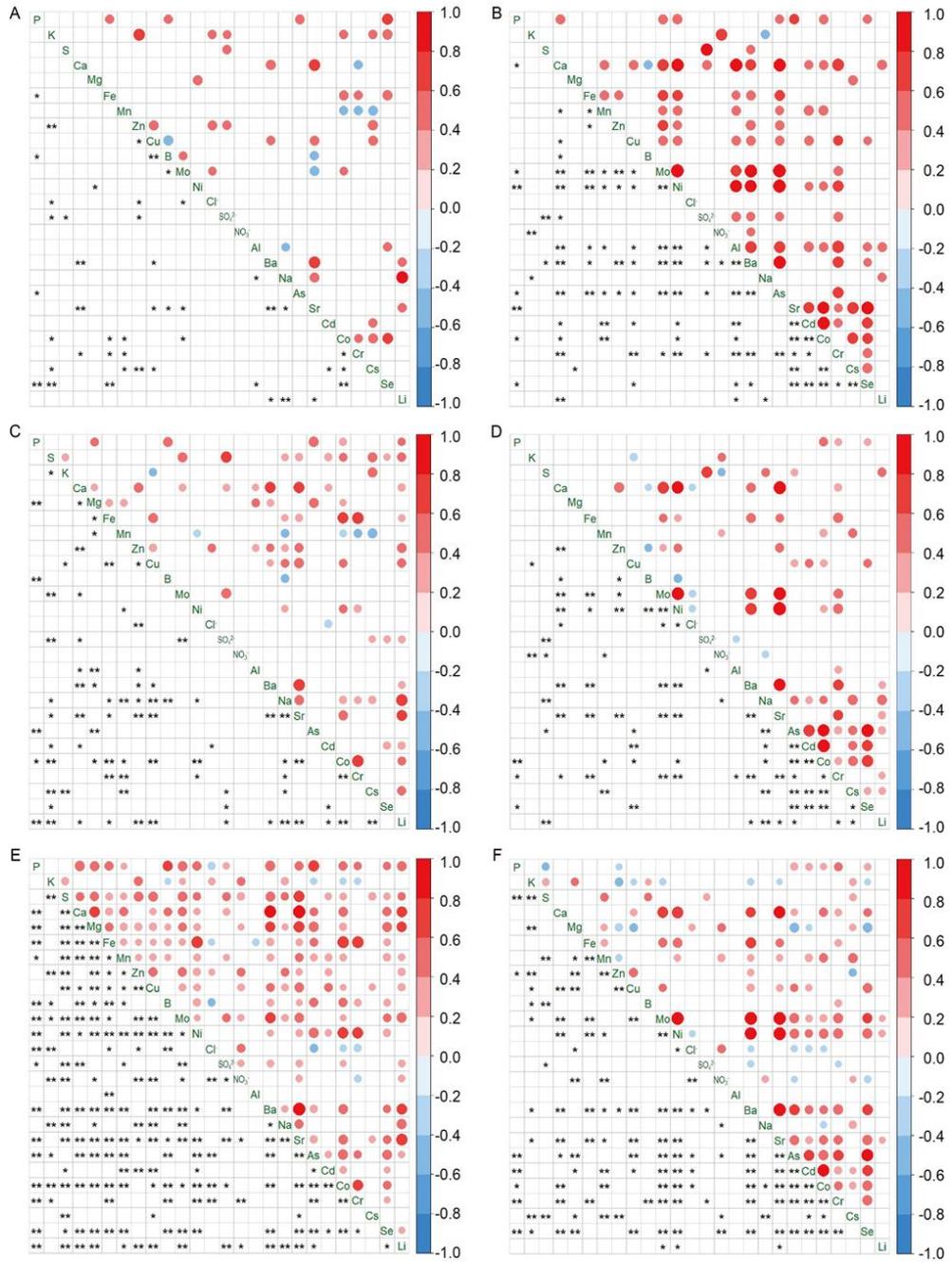
Fig. 2.3. Correlation heatmaps for (a) *aus* shoot, (b) *aus* root, (c) *indica* shoot, (d) *indica* root, (e) *japonica* shoot, and (f) *japonica* root. Only significant (P < 0.05) correlations are displayed in the Pearson's correlation analysis. The red circle indicates a positive correlation coefficient, and the blue indicates a negative one. * and ** in lower triangle matrix represent P < 0.05 and P < 0.01, respectively.

2.3.4 Identification of multielement accumulated rice varieties

To identify the potential candidate varieties with accumulation effects of high or low multielement, rice varieties with more than two elements in the top or bottom 5% concentrations in the shoots were displayed in Table 2.2. Most varieties, including JRC06 (Kaneko B), JRC12 (Oiran), JRC13 (Bouzu Mochi), WRC47 (Jaguary), WRC48 (Khau Mac Kho), WRC49 (Padi Perak), WRC50 (Rexmont), WRC51 (Urasan 1), WRC67 (Phulba) and WRC68 (Khao Nam Jen), of the *japonica* subspecies and varieties WRC26 (Jhona 2) and WRC30 (Anjana Dhan) in *aus* showed high accumulations of multi-metallic elements, including Ca, Mg, Fe, Mn, Zn, Cu and Mo. However, JRC06 (Kaneko B) accumulated the highest concentrations of Mn and B and the nonessential elements Cd, Co, As, Se and Cr, but lower concentration of K, possibly due to the negative correlations of K with B, As, Co and Cr in *japonica* shoots (Fig. 2.3). Varieties JRC12 (Oiran) and WRC67 (Phulba) accumulated the highest concentrations of essential elements, including Ca, Mn and Mg, but not toxic metals, such as Cd, As, Cr and Al, indicating that the accumulation of essential elements is antagonistic to the uptake of nonessential toxic elements in these varieties. Moreover, as an element beneficial to human health in the prevention of cancer, Se was also accumulated in the variety JRC12 (Oiran). In the *aus* subspecies, varieties WRC26 (Jhona 2) and WRC30 (Anjana Dhan) showed higher K and Cs (Table 2.2), and a positive correlation between K and Cs (Fig. 2.3). Interestingly, the *indica* variety WRC11 (Jinguoyin) showed high Zn and low Cd, and the *aus* variety WRC26 (Jhona 2) showed high Zn and low Cr, indicating a potential for high nutrient and low toxic element rice breeding.

2.3.5 Ionomic differences among rice genotypes in different origins

To identify the differentials between different parts of different subspecies, as well as the geographic factors, I compared all element concentrations in whole plant (Fig. 2.4a), shoot (Fig. 2.4b), and root (Fig. 2.4c) using PCA, and the loading plots were also displayed. As shown in Fig. 2.4a, the cumulative contribution of first two principal components (PC1 and PC2) was 81% of the total variance. A significant separation was observed between shoot and root in all rice genotypes due to the ionome of shoot mainly located on negative axis of x while that of root located on positive axis. Meanwhile, all macronutrients plus Mn and B were loading on negative axis of x to explain the ionome in shoot, and ionome in root mainly explained by microelements and anions. In Fig. 2.4b and c, the total contribution of first two PCs explained 42 % and 44%, respectively. The effect of different origins on clusters of PCA score was largely beyond that of different rice subspecies from same origins, which were not significantly separated in both shoot and root. For instance, the shoots of *japonica* from Japan were obviously separated with that of *japonica* from other origins, but were not divided with shoots of *indica* from Japan. The rice shoots from Japan were mainly explained by most nonessential and toxic elements according to the loading plot. The shoots of *aus* and *indica* were mainly located on third quadrat, and not separated. In root, rice variety of *aus* from South Asia were isolated with others to largely locate on second quadrat, whereas varieties from Japan were separated with that of *indica*, and mainly located on fourth quadrat. Heavy metals such as As and Cd also mainly attributed to rice from Japan, whereas K, Na, Li and anions mainly contributed to rice from South Asia.

Fig. 2.4. Combination of PCA score plots and loading plots for (a) all samples, (b) shoot, and (c) root. E.A, JP, S.A, SE. A represent East Asia (except Japan), Japan, South Asia, Southeast Asia, respectively.

Table 2.2. Highest and lowest multielement accumulation varieties for each element in shoot[a]

No.	Subspecies	High		Low	
		Essential element	Nonessential element	Essential element	Nonessential element
5	*japonica*	Mn + B	Cd + Co + As + Se + Cr	K	
10	*japonica*	Mn + Ca + Mg	Co + Sr + Se	Mo	
11	*japonica*	Mg + Fe + Ni + B	As + Se + Cd		
32	*japonica*			Zn + Mo + K + Cu	Ba + Sr
33	*japonica*		Al	Ca + Mo + S	Ba + Sr + Co
36	*indica*			Zn + Ca	Ba + Sr
45	*japonica*			SO_4^{2-} + Mo + Zn	Na + Li
47	*japonica*			NO_3^-	Na + Li + Sr
55	*aus*		Li	P	Na + As + Se + Cd
60	*indica*	Cl^-			Cs + Co
61	*indica*	Cl^- + Zn		NO_3^-	Cd
64	*indica*	Mg + Mo	Al		Cs
68	*indica*			P + Ni	Co + Cs
69	*indica*			Ni	Cr + Co
70	*indica*			Ni	Co + Cr
71	*indica*	SO_4^{2-} + Mo	Se		
73	*japonica*			Ni	Cs + Cr
74	*indica*			B + S	Cs
75	*aus*			S + SO_4^{2-}	Cd + Se
76	*aus*	Zn + K + Cu	Cs		Cr
77	*aus*		Li		Cd + Se
79	*aus*		Na + Li	P + Mn + Mg	Se + Cd
80	*aus*	K + Zn	Cs + Co	Mg + Mn	
82	*aus*	Mn		Ni + Cu	As
83	*aus*			Cu + Ni + Mg	Ba + Sr
88	*aus*			S + Cu + Ca	Se + Sr + As
92	*aus*	P + NO_3^- + K	Se		
97	*japonica*	Zn + Cu + Ca	Al		
98	*japonica*	P + Ca + B + Mg + Mo	Ba + Sr + As + Na		
99	*japonica*	Mo + Fe + B + P + S + Ni	Ba + Se + Cr + Sr + Co		
100	*japonica*	Fe + P + Ni	Cr + Co	Cl^- + NO_3^-	
101	*japonica*	Ca + Mg + Fe	As + Cr + Ba + Sr + Co		
102	*japonica*	Cu + NO_3^-	Cd	Ca	Al
103	*japonica*	SO_4^{2-} + Mn + S	Cs		
104	*japonica*	K + NO_3^-	Cs		
105	*indica*	Ni + Fe	Cr		
106	*indica*	S + SO_4^{2-}	Na	Mn + B	As
108	*indica*	S + SO_4^{2-}		P + Fe + B	Al
111	*indica*	Zn + Cl^-	Sr		
113	*indica*	K + S	Cs	Mn	
115	*japonica*	Cl^- + Ca + Mn	Li		
116	*japonica*	Mg + Cu	Li + Cd		

[a] The highest or lowest were defined by 5% of each element concentrations in present study, and only the varieties with multielement highest or lowest accumulated effects were displayed. Numbers represent the variety exhibited in appendix 1.

2.4 Discussions

There were significant differences in concentrations of all elements except Ba among *japonica*, *indica*, and *aus*, but generally, the ionomic profiles were displayed similar. It indicated that the ionomic variations were still within the pre-framework of phylogenetic effects of genus *Oryza*, although the genomic differentiations among subspecies have been widely confirmed (Civáň et al. 2015; Stein et al. 2018; Tanaka et al. 2020; Tanaka et al. 2020). As shown in Fig. 2.1 and 2.2, all rice subspecies follow the rule that trace elements and anions were concentrated higher in the roots than in the shoots, and it was confirmed by the separation between shoots and roots in all the rice accessions, as well as the loading of microelements on roots in PCA (Fig. 2.4a). Rice root is the main barrier to limit translocations of heavy metal(loids) and toxic element to shoots by chelation and compartmentalization (Xu et al. 2017). However, micronutrients for the goal of biofortification, such as Fe and Zn, are also indiscriminately fixed in roots (Kabir et al. 2016). Interestingly, opposite to S, the SO_4^{2-} concentration in the roots was higher than that in the shoots, primarily due to most of the inorganic S being fixed in root vacuoles or converted to organic S (cysteine) in leaf for protein synthesis (Rennenberg 1984). Thus, it would be a meaningful challenge to identify absorption and translocation mechanisms for specific microelements in roots. As a response to the element concentrations, dry biomass weights among subspecies were shown in boxplot (Appendix 2). The concentrations of essential elements in the shoots or roots of *japonica* were markedly the lowest, while that of harmful elements in

japonica showed the highest (Fig. 2.1 and 2.2). Correspondingly, the biomass weights of both shoots and roots in *japonica* were the lowest among subspecies. The results showed that improving nutrients and reducing toxic elements also showed a crucial correlation on improvement of the biomass of rice.

Significant differences among diverse rice genotypes detected in most elements in both shoots and roots showed that the phylogenetically-changed elements were more marked in the shoots than in the roots, and further indicated that the variations in elements among the rice accessions were mainly attributed to their translocation from roots to shoots, consistent with previous studies (Yang et al. 2018; Chen et al. 2019). With large contributions of genotype effects in the shoots and roots, Mg, Mn and Cu were robust to environmental perturbations (White et al. 2012). In the present study, variations of Cr concentration in the shoots among all the subspecies were the largest, which was consistent with a previous study (Chu et al. 2015). Although the transport of Cr has been demonstrated to be associated with S transporters (Schiavon et al. 2008), there was no obvious correlation were detected between these two elements in our study (Fig. 2.3), indicating that such a significant difference among the rice genotypes indicated a complex multi-influced underlying transport mechanism. Compared with S, the CV of SO_4^{2-} was higher in all the subspecies, possibly related to the different organic S conversion rates among the accessions. The CVs of macronutrients excluding P in the roots in all rice subspecies were less than that of trace elements and heavy metals, indicating that the variations in macroelements were stable among the rice accessions. Meanwhile, the lower CVs of nonessential elements in *japonica* demonstrating that microelement and heavy metal uptake in the roots showed greater

genetic diversity in *aus* and *indica* than in *japonica*, which was consistent with previous reports that *japonica* subspecies exhibits less genetic and transcriptional diversity than *indica* and *aus* (Huang et al. 2010; Campbell et al. 2020). The ionomic variation in rice accessions was mainly dependent on specific chemical element properties and genotype effects, as well as limitedly on subspecies.

One of the most important values of ionomics is in determining the interactions between elements (Baxter and Dilkes 2012; Feng et al. 2017). The occurrence of antagonism and synergism between elements on uptake and translocation in plants has been reported in many studies (Watanabe et al. 2014; Chu et al. 2015; Watanabe et al. 2016; El et al. 2018; Affholder et al. 2019). Correlation analysis showed that the rice subspecies and organs exhibited diverse strategies in establishing connections between elements, but many interactions were similar (Fig. 2.3). The significant correlations between S and SO_4^{2-} in all plots provided evidence that the correlation results in this study were reliable, although positive correlations might not always infer the same pathway (Baxter et al. 2008; Du et al. 2020). Ca was always significantly and positively correlated with Ba and Sr, and there was also positive correlation between Ba and Sr (Fig. 2.3), indicating a significant positive correlation among divalent cations, possibly explained by their sharing the non-selective cation channel transporter-protein superfamilies, such as ZIP, heavy metal ATPases and yellow stripe-like in the xylem due to their similar chemical properties and identical ionic valences (Ozaki et al. 2005; Curie et al. 2009; Pinto and Ferreira 2015). Significant positive correlations between P-As, P-Se and As-Se were detected in both shoots and roots among the subspecies. It has been reported that P and Se share the phosphate transporter *OsPT2* gene (Zhang et al.

2014). Moreover, Cao et al. (2017) reported reduced As uptake in rice via a P transporter, *OsPT4* gene knockout. In general, P application can activate the expression of *OsPT2* and *OsPT4* genes to improve As and Se uptakes in rice. These elemental interactions can provide a strategy to screen multielement accumulation rice genotypes to breed rice varieties with abundant nutrients and that are safe to consume. Moreover, combined with the results of PCA on correlation coefficient (Appendix 3), it was clear that the significantly positive correlations between minerals in the shoots of *japonica* were more obviously than those in *indica* and *aus*, while the patterns of ionomic correlations in the roots among the subspecies were similar. The elemental correlations in rice shoots mainly derive from ionomic transport, whereas in roots, it is due to element uptake, confirming that the ionomic differences were primarily caused by different transport mechanisms among the subspecies, consistent with previous studies (Chen et al. 2018; Yang et al. 2018).

In rice domestication and breeding history, farmers have preferred to plant rice varieties adapted to the local agro-climatic conditions, with higher yield and better taste, usually not considering the microelement content (Tan et al. 2020), therefore the separation of elements in PCA mainly determined by different origins rather than subspecies (Fig. 2.4bc). This finding further indicated that the genetic differences involving in ionomes in subspecies can vary with environmental changes. For example, the elemental differences in rice varieties from Japan mainly explained by most nonessential and toxic elements such as As and Cd, that can be related to the history of wide-ranging incidence of agricultural soil contamination in Japan (Arao et al. 2010). Therefore, considering the geographical and historical distributions of rice varieties

associated with the subspecies effects might be informative. Thus, due to the large genetic variation in different rice accessions, it is worthwhile to screen for rice varieties with higher nutrient concentrations, lower levels of toxic elements and healthier food values for use in biofortification strategies. The transportation of elements by the root-to-shoot process has been considered a rate-limitation factor in the shoot-to-grain system (Palmgren et al. 2008). Consequently, identification of element accumulation in shoots is positively correlated with that in grains and determines the distribution of elements in grains. Additionally, owing to the numerous correlations between elements, the elemental covariation effects in shoots should be identified to determine the nutritional values and safety of rice varieties.

Higher concentrations of essential metal or metalloid elements in rice are important in biofortification to promote the synthesis of the coenzymes or proteins required for human health (Clemens 2014). Meanwhile, consistent with the results of ionomic correlations in the shoots (Fig. 2.3), multi-metal accumulation is likely involved in the non-selective metal cation channel transporter-protein superfamilies (Curie et al. 2009; Pinto and Ferreira 2015). However, nonessential and toxic metals can be also indiscriminately accumulated in these varieties due to similar chemical properties, and potentially resulting in health risks to humans. JRC06 (Kaneko B) accumulated high concentrations of B, As, Co and Cr, while a lower concentration of K (Table 2.2). As a macronutrient, K plays a highly significant role in alleviating abiotic stress in plants (Amtmann et al. 2008; Römheld and Kirkby 2010), explaining why with low K concentration, plants' resistance to heavy metal uptake is likely suppressed, leading to a higher concentration of heavy metals (Wu et al. 2020). Both high K and Cs

concentrations in the shoots of some rice varieties can be explained by the positive correlation between K and Cs (Fig. 2.3). It shows that rice varieties with more K accumulation can also accumulate more Cs. However, Ishikawa et al. (2018) reported that the Cs concentration in rice is reduced by applying K, and Rai et al. (2017) found that the expression of K transporter *OsHAK1* in rice roots is the main route for Cs to accumulate in rice plants under a low K status. The relationship between K and Cs in the same plant is negative. The above evidences proved that K and Cs share the transport system and antagonize each other. Furthermore, the multielement accumulation in almost all the *aus* and *indica* subspecies was lower than that in the *japonica* subspecies (Table 2.2), indicating that the elemental covariations in the shoots showed subspecies differences and that the correlation of elements was stronger in *japonica* than in *indica* and *aus*, which was consistent with the results shown in Fig. 2.3. The *indica* variety WRC11 (Jinguoyin) showed high Zn and low Cd concentrations, while the *aus* variety WRC26 (Jhona 2) showed high Zn and low Cr concentrations. It has been reported that the OsHMA2 transporter in rice is associated with the co-transportation of Zn and Cd from roots to shoots, and that Zn competes with Cd by sharing the same ZIP transporter (Takahashi et al. 2012). Understanding and screening for rice varieties with significant correlations and potential for high nutritional value and safety are essential for rice breeding.

Chapter 3. Genome-wide association study on rice ionome and a related sulfate transporter gene analysis

3.1 Introduction

The uptake, translocation, and storage of mineral elements in plants are regulated by genetic, evolutional, and environmental factors as well as their reciprocal effect (Salt et al. 2008; Neugebauer et al. 2020). Therefore, the mineral accumulation and homeostasis in plant is a dynamic system controlled by complex gene networks and highly required to be explored (Lahner et al. 2003; Sasaki et al. 2016). Elements rarely have the same behavior as other elements across tissues, environments, or genotypes, and are unlikely controlled by all of the same sets of gene. For better understanding of the genetic regulation of the ionome, genes that contribute to the accumulation and distribution of each element need to be finely identified (Baxter 2009). Using high-throughput techniques in elemental analysis, large plant populations can be measured with relative ease for multiple elements simultaneously. Genetic variations in large, well-genotyped natural populations provides bountiful resources for identification of quantitative trait locus (QTL) and causal genes, and consequently develops genome-wide association study (GWAS), as a powerful tool for mapping genetic loci associated with ionomic diversity (Huang and Han 2014). It allows for simultaneous multi-elemental mapping to be rapidly performed on the same experimental populations, that make identifying a variety of genes responsible for elemental variation more efficiently (Baxter 2009).

Rice landraces have evolved from their wild progenitors and adapted to local agroclimatic conditions, resulting in a high genotypic and varietal diversity by selective breeding (Huang et al. 2010; Singh et al. 2017). It provides possible population resources with abundant natural variations for GWAS to identify the genetic basis of much of phenotypic variation in rice. A large number of traits in rice, including numerous agronomic characteristics and metabolites, have been studied using GWAS (Huang et al. 2012; Chen et al. 2014; Matsuda et al. 2015; Yano et al. 2016). As described in Chapter 2, the ionomic traits were likely not the main consider factor in farmers' select of breeding. Therefore, a large number of natural variations in element concentrations are possibly preserved and available for performing GWAS. Pinson et al. (2015) investigated that extensive heritable variation is known to exist in element concentrations in rice grain of 1,763 rice accessions from a widespread geographic and genetic origins. GWAS has been also conducted on the analysis of the concentrations of four minerals (As, Cu, Mo, and Zn) in rice grain (Norton et al. 2014), eight elements (Zn, Fe, Mn, Cu, P, Ca, K, and Mg) in brown rice (Nawaz et al. 2015), and ionome in rice plant under different field conditions (Yang et al. 2018) or hydroponics (Cobb et al. 2021). These studies show the promise of GWAS for examining ionomic traits, but the information for understanding of the complex genetic network is remain to be still limited and need more studies.

In the present study, I performed GWAS on ionome across the different accessions of rice as described in Chapter 2 to unravel the genetic basis underlying the variations of ionomics, and further validated a 3bp-base deletion in the first exon of *OsSULTR1;1*

which leading a higher S concentration to provide the complimentary information on GWAS results on rice as well as the potential candidate gene mutant.

3.2 Materials and Methods

3.2.1 GWAS analysis on rice ionome

Based on the rice ionome data as described in last section from the rice accessions (Information in appendix 1) obtained from NARO Genebank and the SNPs information from the previous studies (Tanaka et al. 2020; Tanaka et al. 2020), the mean value of each element concentration of three replications was calculated and utilized for GWAS on rice elemental profiling in both shoot and root.

For GWAS, the mixed linear model (MLM) was conducted (Yu et al. 2006). Association studies were performed using R with modified scripts from the MVP (https://rdrr.io/github/XiaoleiLiuBio/MVP/), GAPIT (Lipka et al. 2012) and GENESIS (Gogarten et al. 2019) packages. Visualization used scripts from MVP, GAPIT and the R package qqman. I removed variants with minor allele frequency (MAF) < 5% from the relevant dataset in GWAS when using the combined of JRC and WRC populations.

3.2.2 The expression analysis of *OsSULTR1;1* gene in selected rice accession with or without 3-bp base deletion

The following experiment was conducted in a greenhouse at Hokkaido University (14 h photoperiod and day/night temperature of 25–28/18–22∘C, respectively). According to the results of GWAS, rice accessions of WRC53 (*japonica*) and WRC58 (*indica*) with 3-bp deletion (hap2) on Chr03:4988355 to Chr03:4988357, as well as

WRC55 (*japonica*) and WRC59 (*indica*) with no base deletion (hap1), were selected for hydroponic experiment. The germination of seeds and cultivation of rice seedlings in hydroponics were following the steps as described in 2.2.1. Then, two-week-old rice seedlings were transplanted into nutrient solutions with two different S treatments, which were normal S and low S concentration. For normal S treatment, the set of composition of nutrient solution was described as 2.2.2, in which the S concentration was 1.25 mM. For low S treatment, all sulfate cations were replaced by corresponding chloride in the above nutrient solution. However, since 35 μM $FeSO_4 \cdot 7H_2O$ was used to make Fe-EDTA solution for Fe nutrition, the S concentration in this called low S treatment was 0.035 mM. The treatment solutions were changed with every three days during the whole rice culture period. After one-week treatments, rice plants were collected and separate into leaf, stem, and root, then immediately stored in liquid nitrogen for further analysis.

Frozen leaf and root samples were used to extract total RNA by Maxwell RSC Plant RNA kit (PROMEGA, Madison, Wisconsin). Then, cDNAs were synthesized with 3 μg of total RNA in 20 μl of reaction mixture solution by using ReverTra Ace® qPCR RT master kit (FSQ-101, Toyobo, Japan). The *OsSULTR1;1* gene expression was measure by qPCR using Thunderbird SYBR® qPCR Mix (QPS-201, Toyobo) on Roche LightCycler Nano platform with setting 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 40 s. Melt curve analysis was used to confirm the absence of non-specific amplification products. The rice actin gene was set as the internal reference gene for control. Gene-specific primers used for qPCR is listed in Table 3.2.

Table 3.1. Primers used for gene expressions

Genes	Sequence
OsActin Forward	5' TCCATCTTGGCATCTCTCAG 3'
OsActin Reverse	5' GTACCCGCATCAGGCATCTG 3'
OsSULTR1;1 Forward	5' AGGTTCTTTCTCGCGGTCAG 3'
OsSULTR1;1 Reverse	5' AAAGGATGGCATTGGGCGTA 3'

3.2.3 Statistical analysis

For a completely randomized design, three independent experiments were carried out. All descriptive statistics of analysis of variance (ANOVA) used to analyze the significant difference of data by using Minitab 19 (Minitab Inc., State College, PA, USA).

3.3 Results

3.3.1 Genetic basis of variation in the rice ionome

To analyze the natural allelic variation associating with the ionomic variation in the rice population, and identify the candidate cause genes controlling elemental homeostasis, I performed GWAS for element concentrations in both shoot and root by using a MLM model based on ionomic and sequencing data for the entire rice diversity panel of 119 accessions from NARO. Accordingly, many significant SNP peaks in GWAS results of several elements, such as S in shoot and root (Fig. 3.1 and 3.2), Fe in shoot and root (Fig. 3.3), Ni and Cs in shoot (Fig. 3.4), and Cd in shoot and Mg in root (Fig 3.5), were detected exceeding the threshold (pink line).

For S, a large number of potential SNP were detected, and the number of SNP peak in root showed more than that in shoot. A detected peak on Chr01 in shoot and root was located close to *SULTR3;6* (Os01g0719300, Chr01:29987911 to 29994075), which encodes a sulfate transporter (Fig. 3.1). A significant SNP peak on Chr03 was also detected in both shoot and root, where located near a set of *SULTR* gene family (*SULTR1;1*, *1;2*, *2;1*, and *2;2* registered RAP-ID as Os03g0195800, Os03g0196000, Os03g0195500, and Os03g0195300, respectively). Due to the further study on this region, the detailed information of this regions was displayed in Fig. 3.2. The low phytic acid (LPA) gene (also called *SULTR3;3*, Os04g0652400) was associated with the region of a significant SNP peak on Chr04 in root. On Chr06 of root, a significant SNP peak was detected close to the region of *SULTR3;4* (Os06g0143700), which encodes a *SULTR*-like phosphorus transporter. Furthermore, there were two significant SNP peaks

were detected on the front and back of Chr08, and located near the genes *SULTR5;2* (also called *MOT1;1*, Os08g0101500) and *SULTR1;3* (Os08g0406400), which encodes Mo transporter and S transporter, respectively. Finally, a SNP peak in root on the front of Chr09 was located close to *SULTR4;1* gene (Os09g0240500), encodes a S transporter. Moreover, there are many other SNP peaks on the Manhattan plot with no candidate gene in library (Fig. 3.1).

For Fe, only a limited number of significant SNP peak was detected on Manhattan plot (Fig. 3.3), that SNP08:22375122 in shoot and SNP04:21286320, SNP08:24028879, INDEL10:17498242 in root. I found the ABCB27 (ABC transporter B family member 27, Os04g0413000), encoding a gene with function in auxin transport and Fe homeostasis (Xu et al. 2014), located close to the SNP04:21286320. Moreover, yellow strip-like gene (*YSL5* and *YSL6*, Os04g0390600 and Os04g0390500) and *VIT1;2*, genes responsible for Fe transportation in rice, were also located on Chr04 near the SNP04:21286320, although the distance is approximately 2,000 kb. For other elements, abundant SNP peaks were also detected on Manhattan plots (Fig. 3.4 and Fig. 3.5), providing more possibilities for ionomic-regulation gene identification.

Fig. 3.1. The Manhattan and Q-Q plots for (a) S in shoot and (b) S in root. The pink line in Manhattan plot represented a threshold [$p<0.05$ with Bonferroni correction and -log10 (*pcorr*) > 7.8]. Red arrow represented the potential significant SNP peaks. Yellow highlighted bars indicate significant SNP peak regions of mapped potential causal gene for elemental accumulation
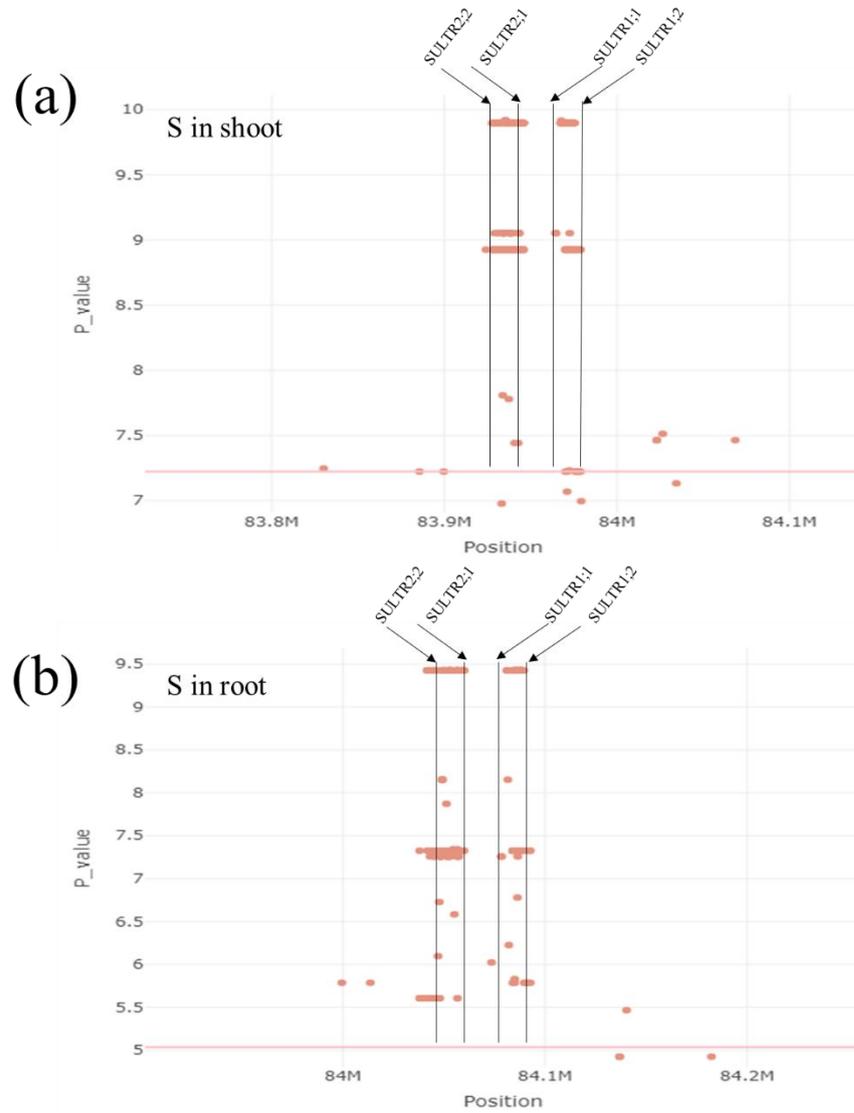
Fig. 3.2. The details of Manhattan plots for significant SNP peaks of S on Chr03 in (a) shoot and (b) root. The locations of *SULTR1*- and *SULTR2*-type transporters were displayed.
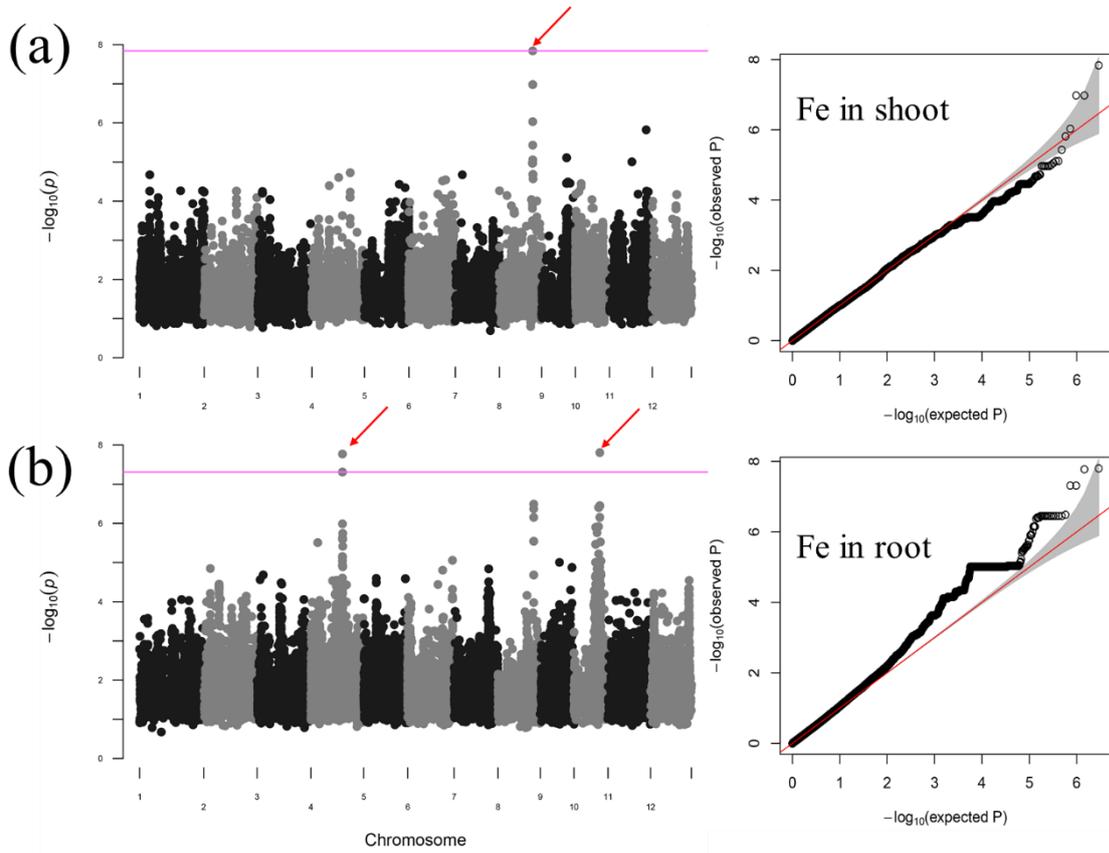
Fig. 3.3. The Manhattan and Q-Q plots for (a) Fe in shoot and (b) Fe in root. The pink line in Manhattan plot represented a threshold [$p<0.05$ with Bonferroni correction and -log10 (*pcorr*) > 7.8]. Red arrow represented the potential significant SNP peaks.
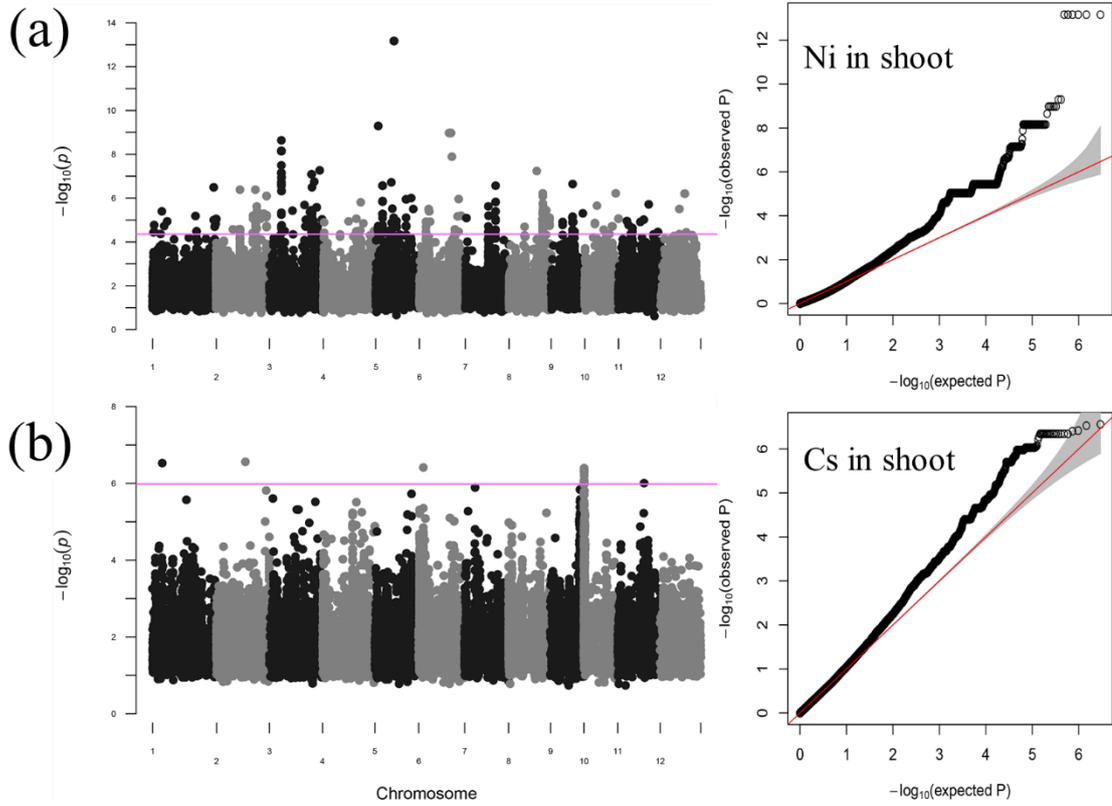
Fig. 3.4. The Manhattan and Q-Q plots for (a) Ni in shoot and (b) Cs in shoot. The pink line in Manhattan plot represented a threshold [$p<0.05$ with Bonferroni correction and -log10 (*pcorr*) > 7.8].
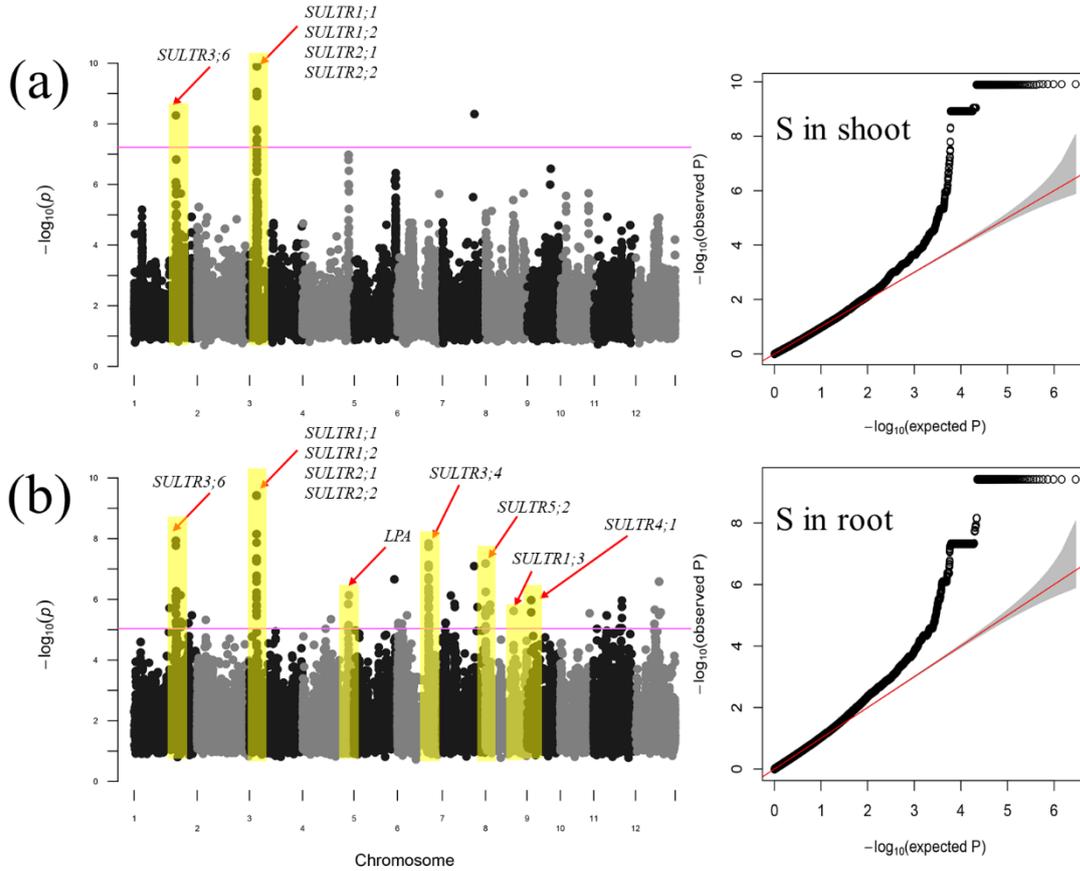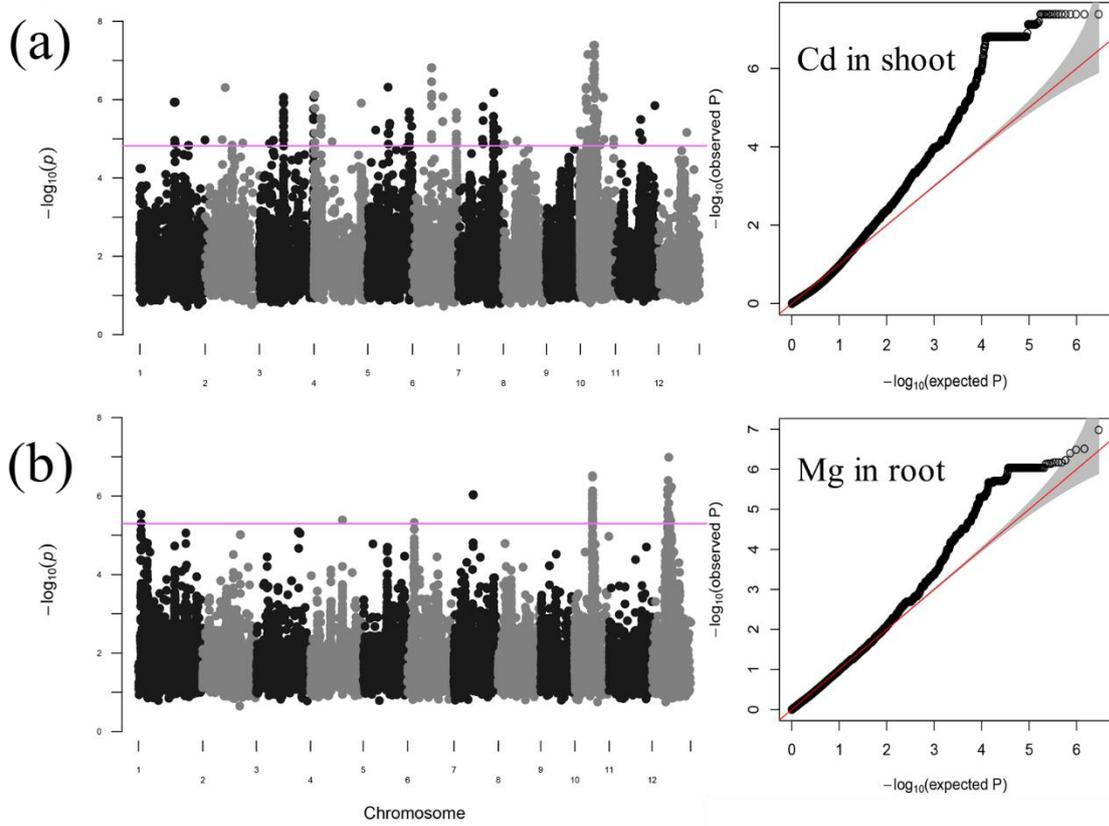
Fig. 3.5. The Manhattan and Q-Q plots for (a) Cd in shoot and (b) Mg in root. The pink line in Manhattan plot represented a threshold [$p<0.05$ with Bonferroni correction and -log10 (*pcorr*) > 7.8].

3.3.2 Haplotypic analysis of 3bp-base deletion on *OsSULTR1;1*

The GWAS on element concentrations provide a better understanding of the genetic architecture of the observed variations in the rice ionome. Based the GWAS results described above, I selected the representative and significant SNP of an S-accumulated loci for further identification of the candidate genes. I found that *OsSULTR1;1* is close to the most significant SNP (SNP03:4988716) on Chr03, which was detected at both the shoot and root locations (Fig. 3.1). The haplotype analysis showed that there were two different haplotypes of Hap1 and Hap2 in the transporter gene location (Fig 3.6a), leading the different S concentrations in both shoot and root. Especially, there are 3-bp base deletion detected on the exon of *OsSULTR1;1* (Chr03:4988355 to Chr03:4988357), with changing from ATCAT to AC, leading a significant higher S concentration in both shoot and root (Fig. 3.6bc). Therefore, I focused on this 3-bp base deletion to further verify the function results of this allelic change in *OsSULTR1;1*.

The DNA and amino acid sequence data by using Sanger sequencing chromatogram were displayed as a complementary result for confirming the DNA regions of 3-bp base deletion in rice from two haplotypes of Hap1 and Hap2 (Fig. 3.7). Accordingly, I found that Hap2, with the 3-bp base deletion at the 102th to 104th position corresponding a histidine (His) amino acid loss, showed the higher S concentration in shoot and root (Fig. 3.7).
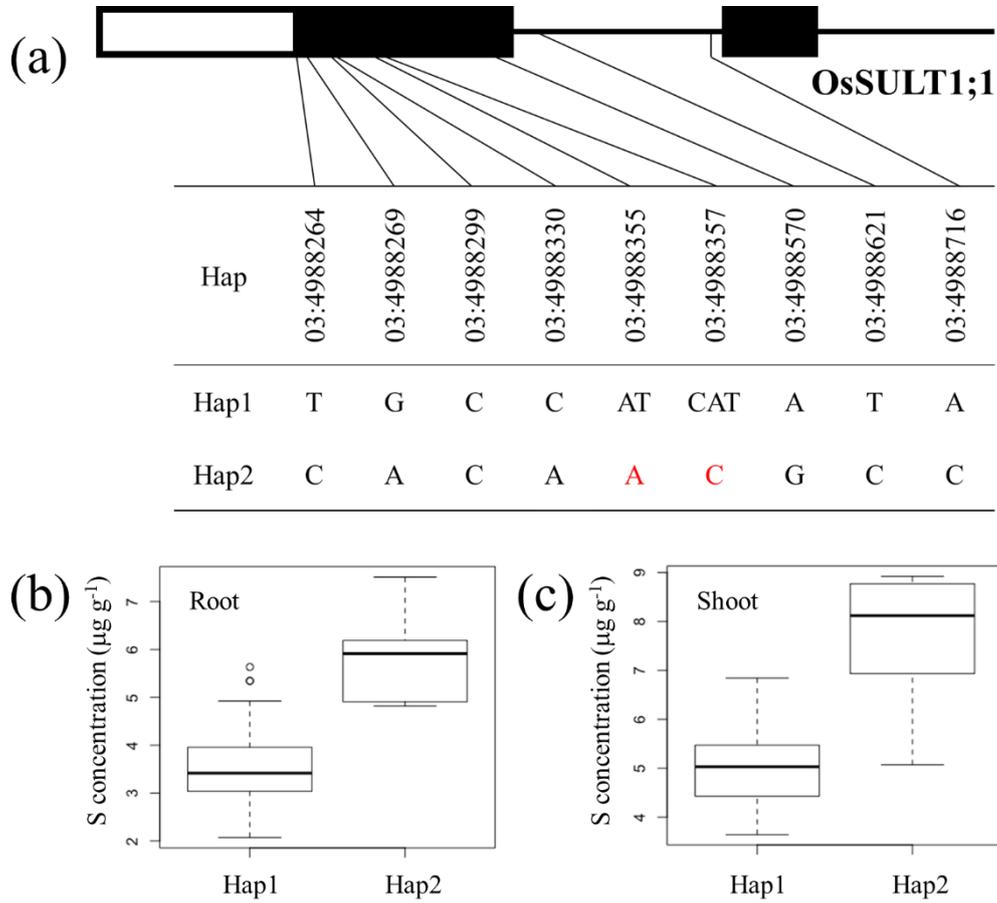
Fig. 3.6. Haplotype analysis of the S candidate gene *OsSULTR1;1*. (a) Gene model of *OsSULTR1;1* and comparison of SNP between Hap1 (n=113) and Hap2 (n=6). The black filled boxes represent the coding sequence. The lines mark the polymorphic sites identified by high-throughput sequencing. (b) Different S concentrations in root between Hap1 and Hap2. (c) Different S concentrations in shoot between Hap1 and Hap2.
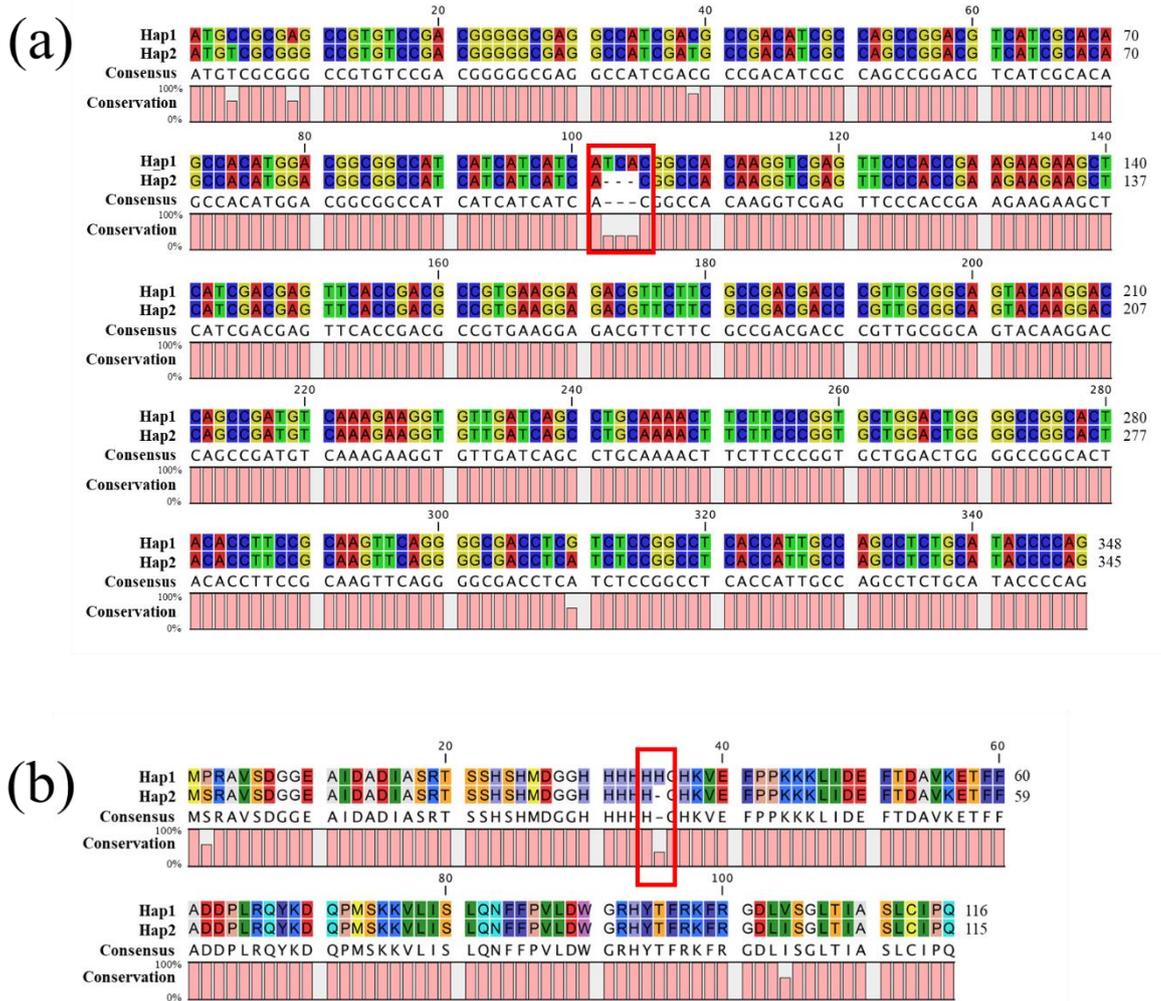
Fig. 3.7. The comparison of (a) DNA and (b) amino acid sequencing between Hap1 and Hap2. The red frame represents the location of (a) 3-bp base deletion, as well as (b) one amino acid loss, on Chr03.

3.3.3 The *OsSULTR1;1* expression analysis between haplotypes

Since the *OsSULTR1;1* gene was only detected in rice roots by using qPCR, the relative gene expression levels of *OsSULTR1;1* among different rice accessions only in root were displayed in Fig. 3.8. Results showed that the expression level of *OsSULTR1;1* gene in hap1 rice accessions (W55 and W59) were significantly up-regulated with the S deficiency in hydroponics, and that in hap2 (W53 and W58) were slightly increased under low S treatment. It indicated that this gene has a high-affinity to S and is sensitive to S deficiency. However, the gene expression level was increased significantly in W55 and W59, while slightly in W53 and W58, in which W55 and W59 are genotype with no base deletion, and W53 and W58 are that with 3-bp base deletion (Fig. 3.8). As shown that, the *OsSULTR1;1* expression level in W53 and W58 were significantly lower than that in W55 and W59 under both normal and low-S treatments, indicating that the gene expression would be decreased with this 3-bp base deletion on the exon of *OsSULTR1;1*. Therefore, gene expression levels were confirmed to be irresponsible for the higher S accumulation caused by the 3-bp base deletion.
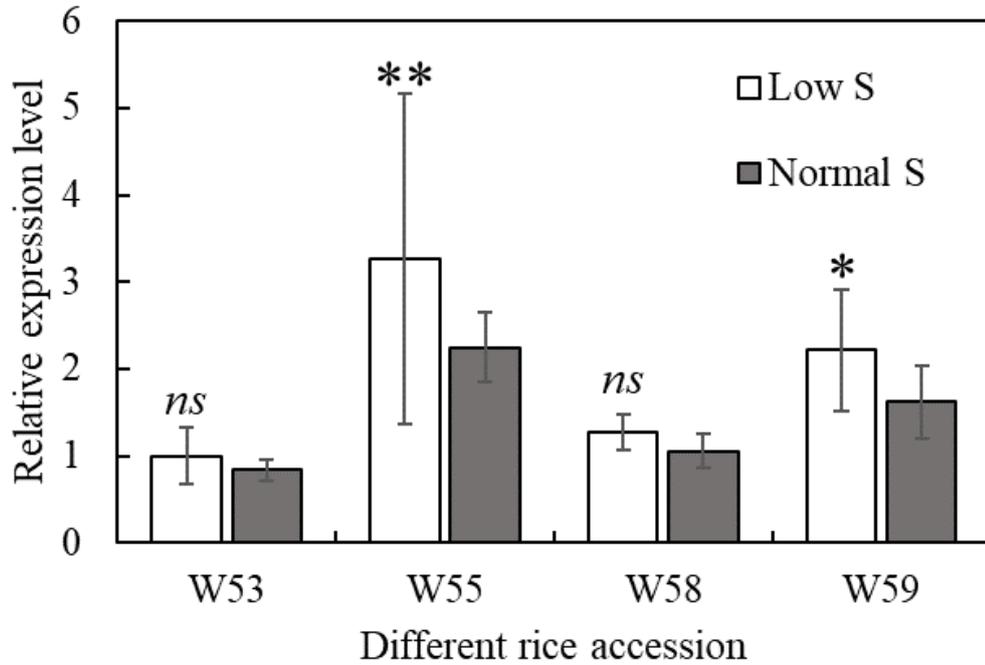
Fig. 3.8. The *OsSULT1;1* gene expression among different rice accessions under different S treatments. Rice accessions W55 and W59 represents hap1, and W53 and W58 represents hap2. The *ns*, * and ** represent no significant difference, significant difference ($p < 0.05$), and extremely significant difference ($p < 0.01$) between low S and normal S treatment, respectively.

3.4 Discussion

Plant uptake mineral elements from soils by roots, and translocate that to the organs in shoots through the xylem and phloem, to constitute the plant ionome (Jeschke and Hartung 2000). In this process, membrane transport proteins that loading and carrying the ions are proposed the main contributor for transmembrane transport (Schroeder et al. 2013). Therefore, the key targets to understand the genetic basis of ionomic variation are identification of the transporters responsible for absorption and translocation of minerals. I consequently performed GWAS, a powerful and efficient tool for analysis of natural allelic variation associated with agronomic traits using diverse populations, on the concentrations of 23 elements in shoot and root among 119 rice accessions obtained from NARO under hydroponics to dig the underlying genetic basis of rice ionome (Zhang et al. 2022). In the present study, the displayed Manhattan and quantile-quantile (Q-Q) plots should meet two criteria (Tanaka et al. 2020): The first is the separation between p-value and uniform distribution in Q-Q plot should be detected to eliminate systematic bias from potential genetic drift in GWAS results; The second is the significant peaks exceeding a threshold [$p<0.05$ with Bonferroni correction and -log10 ($pcorr$) > 7.8] should be shown in Manhattan plot. As shown in Fig. 3.1-3.4, the GWAS results on most elements were finally abandoned, and few highly reproducible and significant Manhattan and Q-Q plots were displayed.

Results showed that the GWAS results on root that meet the criteria showed less than that on shoot (Fig. 3.1-3.4), while more SNP peaks were detected on root than on shoots in the same elements (Fig. 3.1 and 3.2). Root tissue is not only the main organ through which water and mineral nutrients enter the plant organism, as the main

interface between the physical and biological environments, its membrane composition is also largely influenced by environments (Cakmak and Marschner 1988; López-Pérez et al. 2009). Furthermore, rice roots have evolved two Casparian strips in cells to adapt to flooding environment, leading a special structure for controlling the radial movement of minerals from soil to the stele (Enstone et al. 2002; Robbins et al. 2014). On the other hand, rice root is the main barrier to limit translocations of heavy metal(loids) and toxic element to shoots by chelation and compartmentalization (Xu et al. 2017). Therefore, it indicated that the ionomic dynamics network in roots is much more complicated than that in shoot, the latter mainly determined by elemental translocation from root. The linkage-mapping of phenotype-genotype may be consequently likely affected by a large number of environmental factors to locate more ionomic-genetic variation, even though the rice accessions were cultivated in a controlled consistent hydroponic system. Moreover, more genetic drift may also occur in root than that in shoot during the rice breeding history, resulting in more Q-Q plots of roots showed no separation between *p*-value and uniform distribution and were discarded. To date, only Cobb et al. (2021) systematically studied the genetic basis of ionome in rice root tissues.

In the present study, GWAS results provides some valuable genetic information for SNPs and candidate gene identification, especially for S in shoot and root that was well mapped that many significant SNP peaks associated with potential candidate genes were detected (Fig. 3.1). The understanding of the specific functions of sulfate transporters and their interactions in rice is still not clear (Mitani-Ueno et al. 2018), however, learning from the studies in *A. thaliana* indicating that *SULTR1*-type transporter gene mainly express in roots and are responsible for sulfate uptake from soil (Takahashi et al.

2000; Shibagaki et al. 2002), while *SULTR2*-types may mainly contribute to the sulfate transport from root to shoots in plant (Takahashi et al. 2000; Rausch and Wachter 2005; Maruyama-Nakashita et al. 2015). These candidate genes were all detected in both shoot and root in the present study (Fig. 3.1 and 3.2), even though the *SULTR-1* gene were identified to be expressed only in root (Kumar et al. 2011), indicating that the ionomic variations between shoot and root are strongly correlated and finally showed a genetic linkage. This outcome highlights the results of GWAS should be further validated to avoid the effects from interplays of elements and/or organs.

Then, I performed a haplotypic analysis of this region and focus on a 3-bp base deletion of *OsSULTR1;1* to further validate the natural allelic variations of causal gene (Fig. 3.5a). Results showed the S accumulations of both shoot and root in hap2 were significantly higher than that in hap1 (Fig. 3.5bc). Hap2 is rare (n=6 among 119 rice accessions) and only detected in WRC population (WRC40, WRC53, WRC58, WRC60, WRC65, and WRC97). According to Appendix 1, these rice accessions were mainly *indica* from South or Southeast Asia. Cobb et al. (2021) detected a rare rice haplotype (called hap3) being private to the *aromatic* group, leading a significant higher S accumulation. Yan et al. (2019) also found a key allelic change in *OsCd1*, encoding a Cd transporter, explains the difference in Cd accumulation between *japonica* and *indica*. Therefore, these kind of natural allele variations may be highly regionalism relating to the local geographical and climatic environment or a unique genetic characteristic in subspecies. In this study, the 3-bp base deletion constituted a histidine (Fig. 3.6) and resulted in a higher S accumulation in rice shoot and root. I thus made a hypothesis that there is no frameshift mutant of *OsSULTR1;1* gene and analyzed its expression under

low and normal S treatments. Higher expression level under low S treatment indicated that the *OsSULTR1;1* is a high-affinity gene to S deficiency in rice roots (Maruyama-Nakashita et al. 2004). However, the gene expression analysis showed that the *OsSULTR1;1* gene level in 3-bp base deletion genotypes showed significantly lower than that in no base deletion accessions (Fig. 3.7). Ren et al. (2005) proved that the differences of Na transport and salt tolerance in rice is caused by the difference in the transport activity of *OsHKT1;5*, not by the difference in the *OsHKT1;5* expression level. Similarly, in the present study, the higher S accumulation in rice hap2 may be induced by the altered transport activity of *OsSULTR1;1* caused by a 3-bp base deletion without function loss. Further studies are required to elucidate the function of 3-bp base deletion in exon of *OsSULTR1;1* on S transport.

Overall, the information about locus-element associations obtained in this study provides an important foundation for future studies on the genetic and molecular mechanisms controlling the rice ionome, especially for S transporter. Meanwhile, some limitations of linkage-mapping on the root tissues which is an organ with complex physiological features were also proposed.

Chapter 4. General Discussion

Ionomic profiles are primarily influenced by genetic and environmental factors. However, the two factors are not independent of each other, but strongly interrelated to coregulate the plant ionome. The expression of genes is largely environmentally dependent, that is, environmental deviation leads to genetic variations among plant intra- or interspecies, providing conditions for ionomic variation. Therefore, the present thesis mainly addressed two aspects of study on rice ionomics. One is the ionomic profiling of rice genotypes and ionomic responses to varietal effects among species or subspecies of rice. The differences and interplays in chemical properties between elements and other determinants for ionomic variation including geographical and historical distribution in rice breeding were the research emphasis of this issue. The other aspect is to illuminate the underlying genetic architecture of ionomic variation. To study genetic basis from phenotypic analysis is the core of reverse genetics. Here, GWAS is a powerful tool for linking phenotype-genotype to reversely discover the causal genes associated with phenotypes. A large number of natural allelic variations among the population are required as the basis to perform GWAS. In rice domestication and breeding history, farmers have preferred to plant rice varieties adapted to the local agro-climatic conditions, with higher yield and better taste, usually not considering the microelement content (Tan et al. 2020), leading a long-term gap in the study of mineral nutrition in rice, but providing an abundant natural allelic variation resource responsible for rice ionome, especially microelements. A large number of rice germplasm repositories have been established all around the world, and one of the most important

aims in maintaining genetic resources in Genebank is to select them as materials for rice improvement in breeding programs (Ghimiray and Vernooy 2017). NARO established a Genebank of rice core collections and each collection is set for diversity research, and consists of the minimum number of accessions retained genetic diversity of whole accessions. Based on this, I cultivated 120 rice (*Oryza sativa*) varieties of JRC and WRC obtained from NARO plus one Japanese common cultivar in an identical hydroponic condition and analyzed 23 elements as ionomics, then further performed GWAS on each element concentration.

Firstly, a large number of allele gene mutations occurred in diverse rice genotypes due to the historical and geographical isolation. However, since rice developed and evolved from a common wild rice ancestor, their genome is still similar. It explains the ionomic variation among rice accessions was limited by the effect of genus *Oryza* pre-framework, although the significant differences were detected in most elements. I found that the subspecies effect on elemental variation is significant, but PCA analysis showed that the influence of geographical isolation even exceeded that of subspecies effect, that is, the ionomic profile in rice genotypes living in same region clustered closer than that in those belonging to same subspecies. Meanwhile, the 3-bp base deleted haplotype contributing to higher S accumulation in our results were only rarely mainly found in rice genotypes from Southeast Asia, but one was from India. It is speculated to be caused by the exchange and flow of rice germplasm resources between regions and the rapid adaptation of rice genotype to local environmental conditions.

Secondly, numerous interactions between elements also mainly contributes to the ionomic variations, that is the occurrence of antagonism and synergism between

elements largely affects the elemental uptake and translocation in plants. For instance, Spielmann et al. (2020) reported that Zn and Cd share the same zinc-iron transport protein (ZIP) influx transporter into plant roots; It has been also reported that P and Se share the phosphate transporter *OsPT2* gene (Zhang et al. 2014). Moreover, Cao et al. (2017) reported reduced As uptake in rice via a P transporter, *OsPT4* gene knockout. In GWAS results of S, I found a significant SNP peak was detected on Chr06, close to the region of *SULTR3;4* (Os06g0143700), which encodes a *SULTR*-like phosphorus transporter involving in preferential distribution of P to the developing tissues, through xylem-to-phloem transfer mainly at the rosette basal region and leaf petiole (Ding et al. 2020). Therefore, there may be interactions between S and P at this site. Moreover, I found that the variations in the root-to-shoot ionomic transport mechanisms were the main causes of ionomic differences among the rice species and subspecies. The strong relations between root and shoot on element transport result in the *OsSULTR1;1*, which only expresses in root, being detected in the Manhattan plot of shoot.

Finally, the correlations between elements were primarily associated with the screening of varieties for elemental covariation effects that can facilitate breeding biofortified rice varieties with safe concentrations of otherwise toxic elements. The *japonica* subspecies exhibited the strongest elemental correlations and elemental covariation effects, therefore, they showed greater advantages for biofortification than the *indica* and *aus* subspecies, whereas *indica* and *aus* subspecies were likely safer in metal(loid) polluted soils. However, the Cd concentration of grains is generally lower in *japonica* than in *indica* subspecies (Yan et al. 2019). Therefore, Studies on rice biofortification and risk reduction of toxic elements should be tailored to local edaphic

conditions. Meanwhile, the discovery of more ionomic variation-controlling genes could also solve this issue with specific gene editing technology.

Overall, the results of this study provided a reference for ionomic profile in rice and further association studies to improve the nutritional status and minimize toxicity risks in rice production.

Summary

Plant ionome comprises mineral nutrients and trace elements in plants and is a multidimensional dynamic network of elements regulated by genetic and environmental factors. To adapt to a diverse range of geoclimatic environment, there is a high allelic diversity among the natural population of plants. These genotype-to-phenotype links can provide an efficient way to identify the genetic basis of ionomic traits. Rice (*Oryza sativa* L.), a staple crop for half the world's population, has a long history of domestication and breeding with widely-spreading genotypes. However, farmers have preferred planting rice varieties adapted to local agroclimatic conditions, with higher yield and better taste, usually not considering the mineral content in practice. Meanwhile, the transportation of elements by the root-to-shoot has been considered a major and common process determining the element accumulation in shoots and grains of rice. Thus, screening elemental properties in shoots and roots is worthwhile for reference to safety biofortification strategies. As a model plant with rich sequence information, an understanding of ionomic profiling and associated genetic dissection using natural genetic resources is essential to reveal the genetic basis of rice ionome. In the present study collected rice accessions with known sequence variation from the National Agriculture and Food Research Organization (NARO) GeneBank to investigate the ionomic profiles and the associated genetic basis.

1. The study of ionomic profiling of rice genotypes and identification of varieties with elemental covariation effects

First, 120 rice varieties collected from NARO GeneBank were cultivated to the seedling stage in hydroponics, and the concentrations of 23 elements and three anions in the shoots and roots of the rice were determined. Although the subspecies effects were limited by the genus *Oryza* pre-framework and its elemental chemical properties, significant differences were found in ionomic variations in most elements among the *aus*, *indica*, and *japonica* subspecies, and these differences are largely defined by geographical and historical distribution. Principal component analysis of the correlations showed that variations in the root-to-shoot ionomic transport mechanisms were the leading causes of ionomic variations among the subspecies. Furthermore, the correlations were primarily associated with screening varieties for elemental covariation effects. The *japonica* subspecies exhibited the strongest elemental correlations and elemental covariation effects. Therefore, they exhibited greater biofortification advantages than the *indica* and *aus* subspecies, whereas *indica* and *aus* subspecies were likely safer in metal (loid) polluted environments.

2. The study of genetic basis on rice ionome and association with S uptake

Based on the genome-wide association study (GWAS) of the ionomic data in rice shoot and root, many genomic regions located QTLs related to the concentration of elements were detected. Particularly, a significant single nucleotide polymorphism (SNP) peak for sulfur concentration on Chr03, and one 3-bp deletion on Chr03:4988355 to Chr03:4988357, with changing from ATCAT to AC were determined. This 3-bp

deletion is located on the first exon of a high-affinity sulfate transporter gene, *OsSULTR1;1*, which is located on Chr03:4988261 to Chr03:4988608. The haplotype analysis discovered that the sulfur concentrations in shoot and root of rice haplotype with the 3-bp deletion were significantly higher than that of rice haplotype without the 3-bp deletion. Therefore, amino acid sequencing analysis, which showed that this three-bases deletion exactly constituted one-histidine missing was performed, and it was hypothesized that there was no frameshift mutation leading to gene dysfunction. The following experiment, rice haplotypes with (hap2) or without (hap1) the 3-bp base deletion, were contrastively cultivated under normal and low sulfur treatment in hydroponics to evaluate the gene expression to sulfur deficiency. Results indicated that the expression of *OsSULTR1;1* was found only in rice roots and higher under low sulfur treatment. However, the expression in hap2 exhibited significantly lower than that in hap1, which could not explain the higher sulfur in hap2, suggesting that the sulfate ion transport activity of this transporter itself, rather than its expression level, is affected by the base deletion.

In conclusion, this study provides a potential insight that combined ionome with genetic basis, can explain the ionomic dynamics from a molecular perspective and provides a reference for cultivating safe and nutritious rice crops in practice.

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ZHANG Chengming

Appendices

Appendix 1. Rice accessions collected from NARO.

Number order	ID	Accession	Subspecies	Origin	Region
1	JRC01	GAISEN MOCHI	*japonica*	Japan (unknown)	Japan
2	JRC03	HINODE	*japonica*	Kinki	Japan
3	JRC04	SENSHOU	*japonica*	Tokyo	Japan
4	JRC05	YAMADA BAKE	*japonica*	Kagoshima	Japan
5	JRC06	KANEKO B	*japonica*	Kantou touzan	Japan
6	JRC07	IRUMA NISHIKI	*japonica*	Saitama	Japan
7	JRC08	OKKA MODOSHI	*japonica*	Japan (unknown)	Japan
8	JRC10	HIRAYAMA	*japonica*	Tokyo	Japan
9	JRC11	KAHEI	*japonica*	Kagoshima	Japan
10	JRC12	OIRAN	*japonica*	Kumamoto	Japan
11	JRC13	BOUZU MOCHI	*japonica*	Ooita	Japan
12	JRC14	MEGURO MOCHI	*japonica*	Kantou touzan	Japan
13	JRC17	AKAGE	*japonica*	Akita	Japan
14	JRC18	HASSOKUHO	*japonica*	Japan (unknown)	Japan
15	JRC19	WATARIBUNE	*japonica*	Shiga	Japan
16	JRC20	HOSOGARA	*japonica*	Aomori	Japan
17	JRC21	AKAMAI	*indica*	Kouchi	Japan
18	JRC22	MANSAKU	*japonica*	Nagano	Japan
19	JRC23	ISHIJIRO	*japonica*	Toyama	Japan
20	JRC24	JOUSHUU	*japonica*	Yamagata	Japan
21	JRC25	DANGO	*japonica*	Japan (unknown)	Japan
22	JRC26	AIKOKU	*japonica*	Fukui	Japan
23	JRC27	GINBOUZU	*japonica*	Ishikawa	Japan
24	JRC28	SHINRIKI MOCHI	*japonica*	Kumamoto	Japan
25	JRC29	SHICHIMENCHOU MOCHI	*japonica*	Japan (unknown)	Japan
26	JRC30	MORITA WASE	*japonica*	Yamagata	Japan

27	JRC31	KAMEJI	*japonica*	Shimane	Japan
28	JRC32	OMACHI	*japonica*	Okayama	Japan
29	JRC33	SHINRIKI	*japonica*	Hyougo	Japan
30	JRC34	KYOUTOASAHI	*japonica*	Kyoto	Japan
31	JRC35	KABASHIKO	*japonica*	Miyazaki	Japan
32	JRC36	SEKIYAMA	*japonica*	Aomori	Japan
33	JRC37	SHINYAMADAHO 2	*japonica*	Hyougo	Japan
34	JRC38	NAGOYA SHIRO	*japonica*	Akita	Japan
35	JRC39	SHIROINE(KEMOMI)	*japonica*	Tokushima	Japan
36	JRC40	AKAMAI	*indica*	Nagasaki	Japan
37	JRC41	AKAMAI	*indica*	Tokushima	Japan
38	JRC42	TOUBOSHI	*indica*	Kagoshima	Japan
39	JRC43	AKAMAI	*indica*	Kantou touzan	Japan
40	JRC44	KARAHOUSHI	*indica*	Kagoshima	Japan
41	JRC45	HIYADACHITOU	*japonica*	Yamagata	Japan
42	JRC46	FUKOKU	*japonica*	Hokkaido	Japan
43	JRC47	OKABO	*japonica*	Japan (unknown)	Japan
44	JRC48	HAKAMURI(YOKOYAMA)	*japonica*	Kagoshima	Japan
45	JRC49	RIKUTOU RIKUU 2	*japonica*	Japan (unknown)	Japan
46	JRC50	HIMENOMOCHI	*japonica*	Akita	Japan
47	JRC51	SHINSHUU	*japonica*	Nagano	Japan
48	JRC52	AICHIASAHI	*japonica*	Aichi	Japan
49	JRC53	RAIDEN	*japonica*	Kantou touzan	Japan
50	JRC54	HOUMANSHINDEN INE	*japonica*	Kagoshima	Japan
51	JRC55	YUMEPIRIKA	*japonica*	Hokkaido	Japan
52	WRC01	NIPPONBARE	*japonica*	Japan	Japan
53	WRC02	KASALATH	*aus*	India	South Asia
54	WRC03	BEI KHE	*indica*	Cambodia	Southeast Asia
55	WRC04	JENA 035	*aus*	Nepal	South Asia
56	WRC05	NABA	*indica*	India	South Asia
57	WRC06	PULUIK ARANG	*indica*	Indonesia	Southeast Asia
58	WRC07	DAVAO 1	*indica*	the Philippines	Southeast Asia

59	WRC09	RYOU SUISAN KOUMAI	*indica*	China	East Asia (except Japan)
60	WRC10	SHUUSOUSHU	*indica*	China	East Asia (except Japan)
61	WRC11	JINGUOYIN	*indica*	China	East Asia (except Japan)
62	WRC12	DAHONGGU	*indica*	China	East Asia (except Japan)
63	WRC13	SASU	*indica*	Bhutan	South Asia
64	WRC14	IR 58	*indica*	the Philippines	Southeast Asia
65	WRC15	CO 13	*indica*	India	South Asia
66	WRC16	VARY FUTSI	*indica*	Madagascar	Africa
67	WRC17	KEIBOBA	*indica*	China	East Asia (except Japan)
68	WRC18	QINGYU(SEIYU)	*indica*	Taiwan, China	East Asia (except Japan)
69	WRC19	DENG PAO ZHAI	*indica*	China	East Asia (except Japan)
70	WRC20	TADUKAN	*indica*	the Philippines	Southeast Asia
71	WRC21	SHWE NANG GYI	*indica*	Myanmar (Burma)	Southeast Asia
72	WRC22	CALOTOC	*indica*	Philippines	Southeast Asia
73	WRC23	LEBED	*japonica*	Philippines	Southeast Asia
74	WRC24	PINULUPOT 1	*indica*	Philippines	Southeast Asia
75	WRC25	MUHA	*aus*	India	South Asia
76	WRC26	JHONA 2	*aus*	India	South Asia
77	WRC27	NEPAL 8	*aus*	Nepal	South Asia
78	WRC28	JARJAN	*aus*	Bhutan	South Asia
79	WRC29	KALO DHAN	*aus*	Nepal	South Asia
80	WRC30	ANJANA DHAN	*aus*	Nepal	South Asia
81	WRC31	SHONI	*aus*	Bangladesh	South Asia
82	WRC32	TUPA 121-3	*aus*	Bangladesh	South Asia
83	WRC33	SURJAMUKHI	*aus*	India	South Asia
84	WRC34	ARC 7291	*aus*	India	South Asia
85	WRC35	ARC 5955	*aus*	India	South Asia
86	WRC36	RATUL	*aus*	India	South Asia
87	WRC37	ARC 7047	*aus*	India	South Asia
88	WRC38	ARC 11094	*aus*	India	South Asia
89	WRC39	BADARI DHAN	*aus*	Nepal	South Asia
90	WRC40	NEPAL 555	*aus*	India	South Asia

91	WRC41	KALUHEENATI	*aus*	Srilanka	South Asia
92	WRC42	LOCAL BASMATI	*aus*	India	South Asia
93	WRC43	DIANYU 1	*japonica*	China	East Asia (except Japan)
94	WRC44	BASILANON	*indica*	the Philippines	Southeast Asia
95	WRC45	MA SHO	*japonica*	Myanmar (Burma)	Southeast Asia
96	WRC46	KHAO NOK	*japonica*	Laos	Southeast Asia
97	WRC47	JAGUARY	*japonica*	Brazil	South America
98	WRC48	KHAU MAC KHO	*japonica*	Vietnam	Southeast Asia
99	WRC49	PADI PERAK	*japonica*	Indonesia	Southeast Asia
100	WRC50	REXMONT	*japonica*	Usa	North America
101	WRC51	URASAN 1	*japonica*	Japan	Japan
102	WRC52	KHAU TAN CHIEM	*japonica*	Vietnam	Southeast Asia
103	WRC53	TIMA	*japonica*	Bhutan	South Asia
104	WRC55	TUPA 729	*japonica*	Bangladesh	South Asia
105	WRC57	MILYANG 23	*indica*	Korea	East Asia (except Japan)
106	WRC58	NEANG MENH	*indica*	Cambodia	Southeast Asia
107	WRC59	NEANG PHTONG	*indica*	Cambodia	Southeast Asia
108	WRC60	HAKPHAYNHAY	*indica*	Laos	Southeast Asia
109	WRC61	RADIN GOI SESAT	*indica*	Malaysia	Southeast Asia
110	WRC62	KEMASIN	*indica*	Malaysia	Southeast Asia
111	WRC63	BLEIYO	*indica*	Thailand	Southeast Asia
112	WRC64	PADI KUNING	*indica*	Indonesia	Southeast Asia
113	WRC65	RAMBHOG	*indica*	Indonesia	Southeast Asia
114	WRC66	BINGALA	*indica*	Myanmar (Burma)	Southeast Asia
115	WRC67	PHULBA	*japonica*	India	South Asia
116	WRC68	KHAO NAM JEN	*japonica*	Laos	Southeast Asia
117	WRC97	CHIN GALAY	*indica*	Myanmar (Burma)	Southeast Asia
118	WRC98	DEEJIAOHUALUO	*indica*	China	East Asia (except Japan)
119	WRC99	HONG CHEUH ZAI	*indica*	China	East Asia (except Japan)
120	WRC100	VANDARAN	*indica*	Srilanka	South Asia
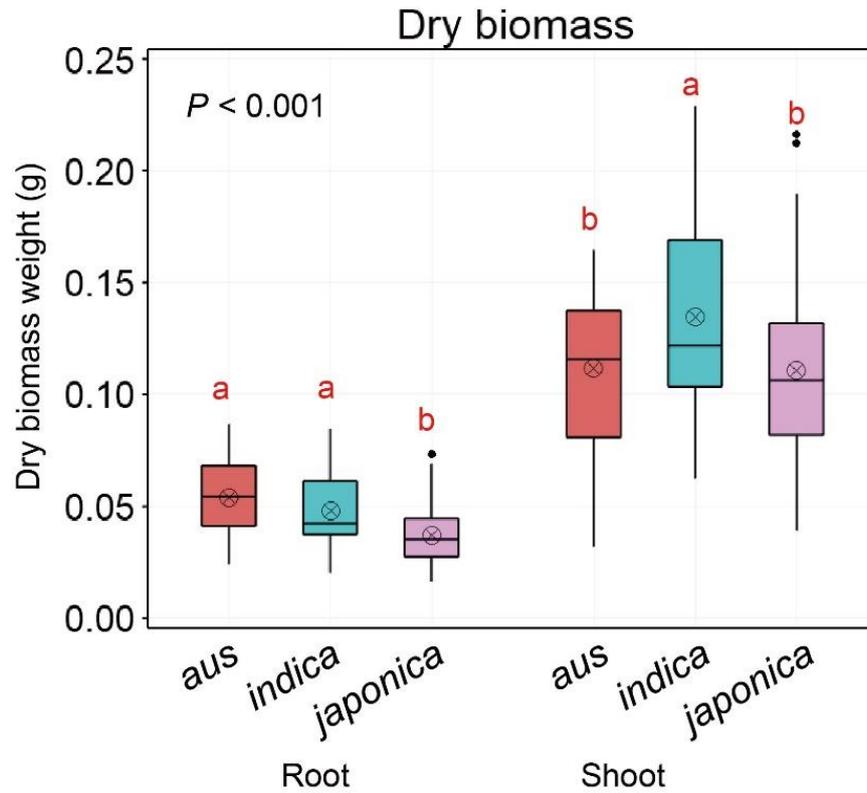
Appendix 2. Boxplot of dry biomass of rice genotypes. The ANOVA with Turkey method was performed: different letter represents significant differences between subspecies and P < 0.001 between organs.
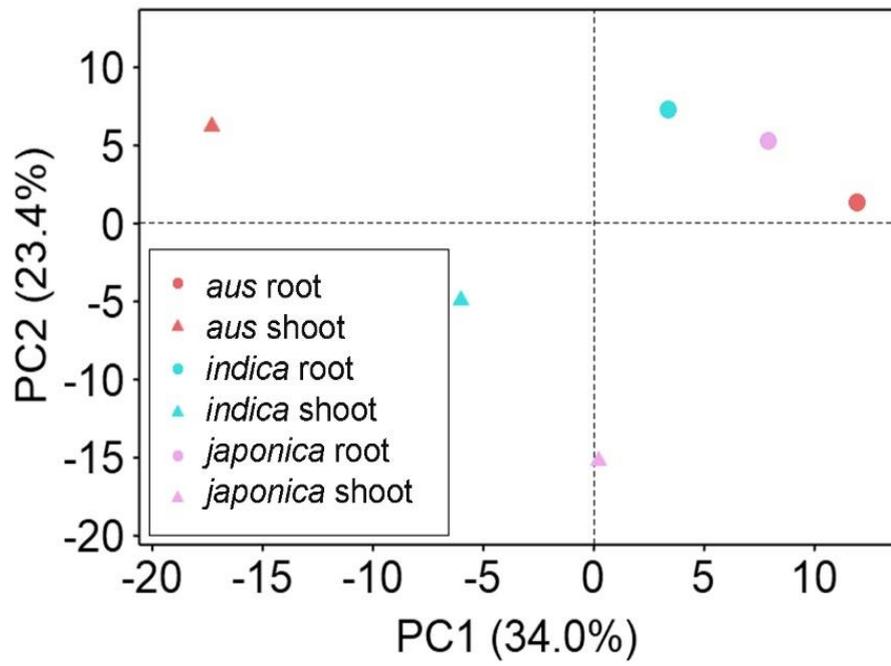
Appendix 3. Score plot of PCA using correlation coefficients.

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