Sampling Method for Identifying Salt-Tolerant Traits from the Mineral Content of Rice Seedlings

Thieu Thi Phong THU
Department of Cultivation Science, Faculty of Agronomy, Vietnam National University of Agriculture

YAMAKAWA, Takeo
Department of Agricultural Science and Technology, Faculty of Agriculture, Setsunan University

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Sampling Method for Identifying Salt–Tolerant Traits from the Mineral Content of Rice Seedlings

Thieu Thi Phong THU[1, 2]* and Takeo YAMAKAWA[2, †]

Laboratory of Plant Nutrition, Division of Molecular Biosciences, Department of Biosciences & Biotechnology,
Faculty of Agriculture, Kyushu University, 744 Motooka Nishi–ku, Fukuoka 819–0395, Japan
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This experiment evaluated a useful sampling method for studying salt tolerance in rice. Four rice varieties were studied: one salt–tolerant, two moderately salt–tolerant, and one salt–susceptible variety. Rice seeds were grown in seed bed soil for 1 week in tap water and 2 weeks in Yoshida solution. Then, in the salt treatment, seedlings were grown for 2 weeks in a 12 dS m^{-1} conductive solution, which was made by adding artificial sea water to the Yoshida solution. In the control treatment, Yoshida solution was used instead. After 2 weeks of salt stress, the seedlings were collected and dried. The shoots were then cut into four parts: the sheath part, from 0 to 1 cm including the base (0–1 sheath), the sheath part from 1 to 2 cm (1–2 sheath), the sheath part from 2 cm to the collar (2–C sheath), and the leaf blade. The mineral contents of the salt–tolerant cultivars differed significantly from those of the salt–susceptible variety in 2–C sheath and leaf blades. These plant parts were useful for identifying traits related to salt tolerance. The remaining roots and shoots 2 cm from the base were able to continue growing and produce F_1 seeds, which can be used for phenotyping the progeny. The K, Na, and Mg contents and Na/K ratio in the 2–C sheath and the Na content and Na/K ratio in leaf blades can be used as salt–tolerant traits in molecular genetic analyses.

Key words: Mineral contents, Rice, Salt stress, Sampling method

INTRODUCTION

Rice (*Oryza sativa* L.) is a stable food crop that supplies 20% of the world's daily calories, but it is susceptible to salinity. Salt stress affects many cellular and physiological processes, including photosynthesis, nutrient uptake, water absorption, plant growth, and cellular metabolism, which all lead to yield reduction (Pardo, 2010). Salt–tolerant cultivars may provide opportunities to improve the salinity tolerance of rice through breeding. The development of salt–tolerant varieties through molecular–assisted breeding is considered a key strategy for increasing rice production in coastal areas. Sahi *et al.* (2006) listed the steps used to produce salt–tolerant varieties, including evaluating the variation of genetic sources for salt tolerance in rice, identifying molecular markers associated with salt stress tolerance genes or quantitative trait loci (QTL) conferring tolerance to salt stress for their use in marker–assisted breeding programs, discovering genes that regulate salt tolerance, and developing cultivars harboring those salt–tolerance genes. Experiments to evaluate the salt tolerance of rice use the entire seedling to determine the mineral content of seedlings, so seed for the next generation cannot be produced. As a result, phenotyping salt–tolerant traits in F_1 or F_2 progeny is impossible. Besides determining salt–tolerant traits, it is important to maintain seedling growth to harvest F_1 or F_2 seeds. Therefore, this experiment evaluated a sampling method that enables the determination of useful parameters for identifying salt–tolerant traits while maintaining seedling growth after applying salt stress.

[1] Department of Cultivation Science, Faculty of Agronomy, Vietnam National University of Agriculture, Trau Quy, Ha Noi, Viet Nam

[2] Plant Nutrition Laboratory, Faculty of Agriculture, Kyushu University, 744 Motooka, Nishi–ku, Fukuoka 819–0395, Japan

[†] Present address: Department of Agricultural Science and Technology, Faculty of Agriculture, Setsunan University, 45–1 Nagaotoge–cho, Hirakata City, Osaka, 573–0101, Japan

* Corresponding Author: (Email: ttpthu@vnua.edu.vn)

MATERIALS AND METHODS

Plant materials

Four rice varieties were studied: KCR20, KCR48, KCR57, and Nipponbare. Previously, we assessed the salt–stress tolerance of these varieties based on the method of Gregorio *et al.* (1997) with the modified standard evaluation score (SES). KCR20 had SES 3.3 and was categorized as a salt–tolerant variety. KCR48 and KCR57 had SES 6.0 and were categorized as moderately salt–tolerant varieties. Nipponbare had SES 8.6 and was categorized as a salt–susceptible variety (Thu *et al.*, 2017; 2018).

Seedling preparation and growth

Rice seedlings were grown using commercial seedbed soil (Kokuryu Baido; Seisin Sangyo, Kitakyushu, Japan). Seeds of the four rice varieties were sterilized to remove fungi using 10% ethanol for 3 minutes, followed by 30 minutes of shaking in 5% NaClO. The NaClO was removed by rinsing the seeds five times with distilled water. The rice seeds were then germinated at 30°C for 24 h. One seed was planted on seedbed soil in each cell of a plastic tray, and the tray was kept in tap water. Using a hydroponic system, the seedlings were screened with salt–stress and control treatments. Yoshida (Y) solution (Yoshida *et al.*, 1976) was used for rice growth in the control and as the base solution in the salt–stress treatment. Seedlings in the two treatments were grown uniformly for 1 week in tap water and 2 weeks in Y solution. For the next 2 weeks, Y solution was used in the control treatment, while in the salt–stress treatment, the seedlings were grown in a 12 dS m^{-1} solution, which was made by adding artificial sea water (ASW) to the Y solution (ASW–Y solution). The solutions were changed twice a week. The pH was measured daily with a pH meter (pH Meter HM–10P; DKK–TOA Corporation, Tokyo, Japan) and maintained at 5.0. The electrical conductivity (EC) of the solution was measured using an EC meter (hand–held conductivity meter, Model CM–31P; DKK–TOA Corporation, Tokyo, Japan) to ensure that it was maintained at 12 dS m^{-1}. The NaCl, Na_2SO_4, $MgCl_2$, and $CaCl_2$ contents of the solution were 87.478, 5.759, 11.186, and 2.156 mM, respectively.

The hydroponic system was placed in a phytotron at a constant temperature of 30°C and humidity of 70%. The experiment used a completely randomized design with six replicates.

Sampling method and mineral content determination

After 2 weeks of salt stress, the rice plants were collected and washed from the soil. After drying at 70°C for 24 h, the shoots were cut into four parts: the leaf sheath from 0 to 1 cm including the base (0–1 sheath), the leaf sheath from 1 to 2 cm (1–2 sheath), the leaf sheath from 2 cm to the collar (2–C sheath), and the leaf blade (Figure 1). Then, the K, Na, Mg, and Ca contents of the four seedling parts were determined by atomic absorption spectrophotometry (Z5300 Polarized Zeeman Atomic Absorption Spectrophotometer; Hitachi, Tokyo, Japan) after HNO_3 digestion (Niazi, 1993).

Statistical analysis

Analysis of variance was used to test for statistical differences in the mineral contents among varieties, followed by Tukey's HSD test. The statistical analyses were performed using Statistix 8 (Analytical Software, Tallahassee, FL, USA).

RESULTS

Visible responses of the varieties to salt stress

At the end of salt stress, the different responses of the four varieties to salt stress were observed. The growth of KCR20 was nearly normal, but the leaf tips were whitish to white. The growth of KCR48 and KCR57 was severely retarded, with many whitish or rolled, brown leaves, although some leaves were elongated. The growth of Nipponbare was completely stopped; most leaves and some plants died.

Changes in the mineral contents of different plant parts with salt stress

K content

Table 1 and Figure 2 show the K contents under the control and salt–stress treatments. In the controls, the highest K content was recorded in the 2–C sheath in all four varieties. The salt–tolerant (KCR20) and moderately salt–tolerant (KCR48 and KCR57) varieties contained significantly more K than the salt–susceptible variety (Nipponbare) in all sheath parts. The leaf blade K content was significantly higher in KCR20 than in Nipponbare. Under salt stress, significant differences in

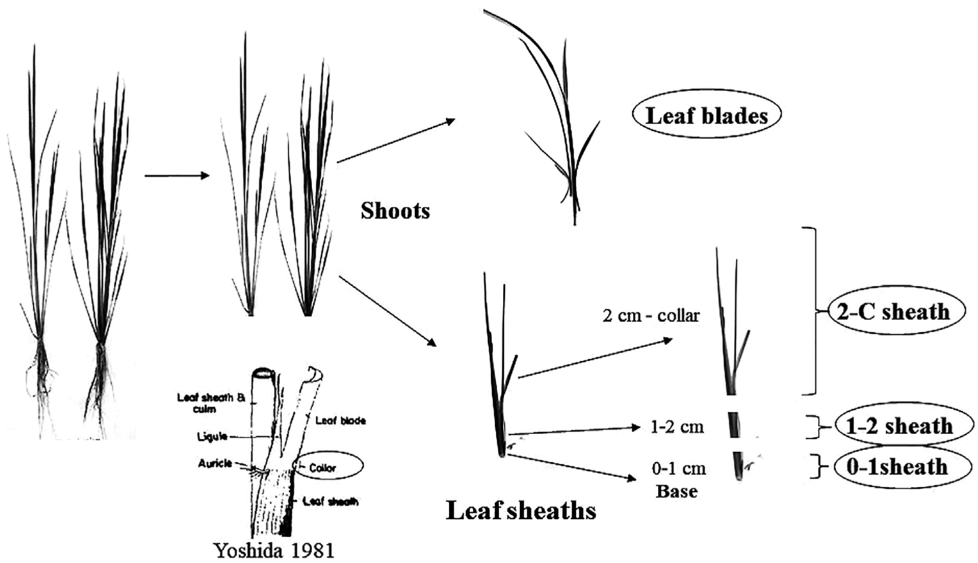

Fig. 1. Cutting method.
The shoot of two seedlings was divided into the leaf sheaths and the leaf blades by cutting at collar. Then, the leaf sheaths were separated into three parts: leaf sheath 0 cm to 1 cm from base including base (0–1 sheath), leaf sheath from 1 cm to 2 cm (1–2 sheath) and leaf sheath from 2 cm to collar (2–C sheath). The mineral contents were determined for 0–1 sheath, 1–2 sheath, 2–C sheath and leaf blades.

the K content among the four varieties were seen in all sheath parts. The K content in the 2–C sheath and leaf blade was higher than that in the 0–1 and 1–2 sheathes in all varieties. The salt–tolerant and moderately salt–tolerant varieties contained significantly more K in all sheath parts than the salt–susceptible variety (Nipponbare). The differences in K content in the leaf blades were not significant among the four varieties.

Na content and Na/K ratio

Table 1 and Figures 3 and 4 show the Na content and Na/K ratios under salt stress. All four varieties had high Na content in all sheath parts and low Na content in the leaf blades. The Na content differed significantly among varieties in the 2–C sheath and leaf blade. The Na content of the 2–C sheath and leaf blade were significantly lower in KCR20 than in the other varieties. The moderately salt–tolerant varieties had values between

Table 1. The mineral contents (mg g^{-1} DW) in different plant parts under control and salt stress treatments

Plant part	Variety	Control treatment				Salt stress treatment				
		K	Na	Mg	Ca	K	Na	Mg	Ca	Na/K
0–1 sheath	ICC20	32.82	0.96	4.52	1.71	16.23	20.08	4.82	1.27	1.24
	ICC48	36.01	0.59	6.85	2.07	19.32	21.04	7.05	1.74	1.14
	ICC57	41.76	0.88	6.90	1.61	20.22	25.77	6.56	1.52	1.27
	Nipponbare	29.12	0.93	4.61	1.65	9.72	23.83	4.96	1.21	2.45
	P–value	*<0.0135*	*>0.05*	*<0.0001*	*>0.05*	*<0.0001*	*0.0200*	*<0.0001*	*<0.0001*	*<0.0001*
1–2 sheath	ICC20	53.59	1.77	5.09	1.86	18.02	24.19	4.55	1.47	1.36
	ICC48	47.22	0.87	6.85	2.70	19.77	29.01	6.84	2.42	1.58
	ICC57	58.30	2.29	5.39	1.94	23.73	33.47	5.23	2.00	1.41
	Nipponbare	40.63	2.00	3.50	1.77	11.09	30.54	3.68	1.29	2.84
	P–value	*0.0023*	*0.0035*	*<0.0001*	*0.0024*	*<0.0001*	*0.0038*	*<0.0001*	*0.0021*	*0.0001*
2–C sheath	ICC20	77.60	0.42	4.51	1.77	29.27	20.36	5.13	1.53	0.71
	ICC48	63.26	0.55	6.01	2.82	28.87	31.44	7.53	3.40	1.10
	ICC57	66.64	0.10	4.98	2.03	36.60	30.77	6.24	2.21	0.85
	Nipponbare	52.28	0.67	3.85	2.24	18.23	35.14	6.32	2.14	1.96
	P–value	*<0.0001*	*0.0812*	*<0.0001*	*0.0005*	*<0.0001*	*0.0002*	*0.0055*	*<0.0001*	*<0.0001*
Leaf blade	ICC20	35.19	0.04	6.68	6.50	26.94	9.89	9.43	7.65	0.37
	ICC48	30.02	0.05	9.04	8.98	30.28	15.08	9.60	8.64	0.49
	ICC57	33.15	0.05	7.11	6.06	35.87	13.14	8.54	7.43	0.37
	Nipponbare	29.44	0.00	5.01	5.43	26.95	19.71	7.66	6.54	0.74
	P–value	*0.0057*	*>0.05*	*<0.0001*	*0.0002*	*<0.0001*	*0.0050*	*0.0088*	*0.0040*	*0.0009*

DW: Dry weight; 0–1 sheath: leaf sheath 0 cm to 1 cm from base including base; 1–2 sheath: leaf sheath from 1 cm to 2 cm; 2–C sheath: leaf sheath from 2 cm to collar.

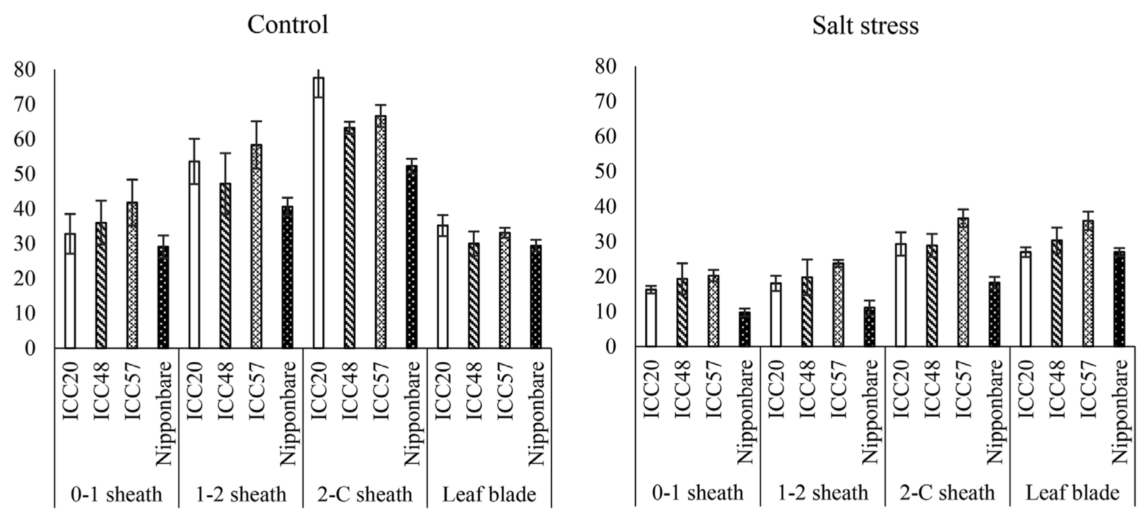

Fig. 2. The differences in K contents (mg g^{-1} DW) of different plant parts under the control and the salt stress treatments. *DW: Dry weight; 0–1 sheath: leaf sheath 0 cm to 1 cm from base including base; 1–2 sheath: leaf sheath from 1 cm to 2 cm; 2–C sheath: leaf sheath from 2 cm to collar.*

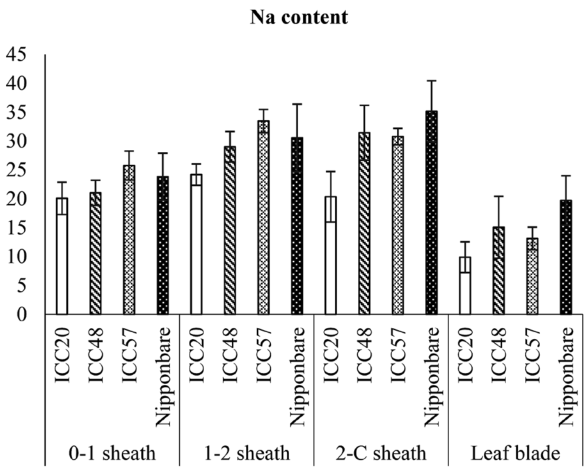

Fig. 3. The differences in Na contents (mg g^{-1} DW) of different plant parts under salt stress.
DW: Dry weight; 0–1 sheath: leaf sheath 0 cm to 1 cm from base including base; 1–2 sheath: leaf sheath from 1 cm to 2 cm; 2–C sheath: leaf sheath from 2 cm to collar.

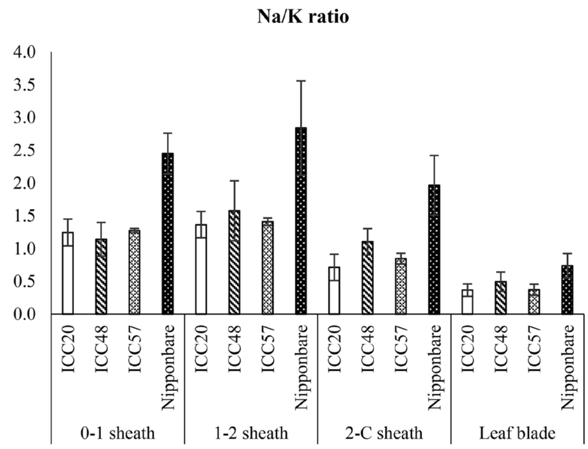

Fig. 4. The differences in Na/K ratios of different plant parts under salt stress.
DW: Dry weight; 0–1 sheath: leaf sheath 0 cm to 1 cm from base including base; 1–2 sheath: leaf sheath from 1 cm to 2 cm; 2–C sheath: leaf sheath from 2 cm to collar.

those of KCR20 and Nipponbare.

The Na/K ratios were high in all sheath parts and low in the leaf blade in all varieties. Significant differences in the Na/K ratios among the varieties were observed in every plant part. The salt–tolerant and moderately salt–tolerant varieties had significantly lower Na/K ratios than the salt–susceptible variety in all sheath parts and in the leaf blade. KCR20 had the lowest Na/K ratios in the 2–C sheath and leaf blade.

Mg content

Table 1 shows the Mg contents under the control and salt–stress treatments. All four varieties had lower Mg content in all sheath parts and higher Mg content in the leaf blade under both the control and salt–stress treatments. In the control, the Mg content did not differ significantly between the salt–tolerant and salt–susceptible varieties in any sheath part, but differed in the leaf blade. Under salt–stress, the salt–tolerant variety had the lowest Mg content in the 2–C sheath and highest value in the leaf blade compared with the other varieties.

Ca content

Table 1 shows the Ca content under the control and salt–stress treatments. All four varieties had the highest Ca in the leaf blade under both control and salt–stress treatments compared with all sheath parts. Among varieties, KCR20 had the lowest Ca in the 2–C sheath under both control and salt–stress treatment. Nipponbare had the lowest Ca in the 0–1 sheath, 1–2 sheath, and leaf blade.

DISCUSSION

The mineral contents in rice plants under control conditions

The determination of mineral contents in different plant parts (0–1 sheath, 1–2 sheath, 2–C sheath, and leaf blade) gave an overview of their distribution in rice seedlings. Under the control condition, the K content of the 2–C sheath part was always the highest of all four parts in all varieties, followed by the K content in the 1–2 and 0–1 sheathes. This suggests that K is preferentially distributed to the upper leaf sheath. K is an essential plant nutrient that improves root growth and plant vigor, helps prevent lodging, and enhances crop resistance to pests and diseases (IRRI, 2018). Consequently, K might accumulate mainly in higher parts of the leaf sheath of rice seedlings.

Mg is essential for chloroplasts as the central atom in the chlorophyll molecule and a bridging element for the aggregation of ribosome subunits necessary for protein synthesis (Beale, 1999). Mg is particularly important for photosynthesis (Gardner, 2003; Shabala and Hariadi, 2005). In this experiment, the Mg content was highest in the leaf blade in all varieties under both control and salt–stress treatments, indicating that Mg is concentrated in the seedling leaf blades. Based on the function of Mg, this result reflects the true distribution of Mg in plant.

The Ca content was higher in the leaf blade than in all sheath parts under both control and salt–stress treatments. This is probably because many Ca transporters are present in leaf cells. The Ca^{2+} transporters in leaf cells in higher plants are Ca^{2+}–pumping ATPases, Ca^{2+} channels, Ca^{2+}–H^{+} antiporters, and proton pumps (Taiz and Zeiger, 2000).

A useful sampling method to identify salt–tolerant traits by mineral contents

Many studies of salt tolerance in rice have identified salt–tolerant traits based on the mineral contents of the roots and shoots (Wang *et al.*, 2012; Zheng *et al.*, 2014; De Leon *et al.*, 2015; Rahman *et al.*, 2016; Patishtan *et al.*, 2018). In our research (Thu *et al.*, 2017; 2018), the shoots were divided into the leaf sheaths and leaf blades; we found that under salt stress, the mineral contents in each plant part of the roots, leaf sheaths, and leaf blades differed significantly between salt–tolerant and salt–susceptible varieties. Moreover, many ion transporters are expressed in the shoots, especially the shoot base. Shoot K^+ concentration 1 (*SKC1*) is a QTL that controls K^+/Na^+ homeostasis under saline conditions (Lin *et al.*, 2004). *SKC1* is expressed in parenchyma cells around xylem vessels (Ren *et al.*, 2005). *SKC1* corresponds to the *OsHKT1;5* gene (*Oryza* sativa high–affinity K^+ transporter 1;5), which encodes a Na^+–selective transporter (Kobayashi *et al.*, 2017). *OsHKT1;5* is expressed in the plasma membrane of phloem cells of the vascular bundles in the basal nodes. Notably, the transcript level of *OsHKT1;5* increased gradually during Na^+ exclusion mediated by *OsHKT1;5* in the tissues of the stem and nodes, especially node I (Kobayashi *et al.*, 2017). The expression of *OsHAK1* is up–regulated by K deficiency or salt stress in various tissues, particularly in the root and shoot apical meristem, the root epidermises and steles, and the shoot vascular bundles (Chen *et al.*, 2015). The glucuronidase (*GUS*) reporter driven by the *Oryza sativa* high–affinity K^+ 5 (*OsHAK5*) promoter was active in the root and root–shoot junction (Yang *et al.*, 2014). Thus, the mineral contents near the base might change more than those in higher sheath parts because of the existence of many transporters and enzymes under salt stress. Therefore, using sheath parts that are farther from the base might be more useful for detecting differences in salt–tolerant traits among varieties than would using sheath parts near the base.

In this study, a sampling method that cut the shoot into four parts—the 0–1 sheath, 1–2 sheath, 2–C sheath, and leaf blades—clearly reflected the mineral contents of all plant parts among varieties under salt–stress. The mineral contents in the 2–C sheath and leaf blade differed significantly among varieties. The K, Na, and Mg contents and Na/K ratio of the 2–C sheath and the Na content and Na/K ratio of the leaf blade were useful for explaining salt tolerance. The salt–tolerant varieties had higher leaf sheath K content, lower leaf sheath Na, a lower leaf sheath Na/K ratio, and lower leaf sheath Mg content than salt–susceptible varieties. The salt–tolerant varieties had lower leaf blade Na content and a lower leaf blade Na/K ratio than the salt–susceptible variety. In the 0–1 and 1–2 sheath sections, the mineral contents had large standard deviations. Moreover, the transport of water and minerals from roots to shoots at the 0–1 sheath section, which connects the root and leaf sheath, is more complex due to the change in cellular structure. Numerous studies have detected many ion transporters in this section (Chen *et al.*, 2005; Ren *et al.*, 2005; Yang *et al.*, 2014; Kobayashi *et al.*, 2017). Therefore, the 0–1 and 1–2 sheathes should not be used as samples to determine the mineral contents of rice under salt stress. In addition, the remaining 0–1 sheath, 1–2 sheath, and roots might continue to grow and produce F_1 seeds, which can be used to evaluate the phenotype of the progeny.

The results indicate that the leaf sheath from 2 cm above the base to the collar and the leaf blades are useful for identifying salinity–related traits. The remaining roots and shoots 2 cm or less from the base, including the base, might continue to grow and produce F_1 seeds, which can be used to evaluate the phenotype of the progeny.

CONCLUSION

Sampling using our cutting method is useful for studying salt tolerance in rice. The mineral contents of the 2–C sheath and leaf blades of salt–tolerant cultivars differed significantly compared with the salt–susceptible variety. Therefore, the leaf sheath section from 2 cm above the base to the collar and the leaf blades are useful for identifying salt–tolerance traits. The K, Na, and Mg contents and the Na/K ratio in the 2–C sheath, and the Na content and Na/K ratio in leaf blades should be used as salt–tolerant traits in molecular and genetic analyses. The remaining roots and shoots 2 cm from the base, including the base, might continue to grow and produce F_1 seeds, which will be used for phenotyping the progeny.

AUTHOR CONTRIBUTIONS

Thieu Thi Phong Thu designed the study, gathered and analyzed statistically the data, and wrote the first draft of the manuscript. Takeo YAMAKAWA designed the study, managed the analysis of parameters in the study and the literature search and edited the manuscript. The English in this document has been checked by at least two professional editors, both native speakers of English.

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