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Screening and Evaluation of Saline–Alkaline Tolerant Germplasm of Rice (*Oryza sativa* L.) in Soda Saline–Alkali Soil

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Abstract: The improvement and development of saline–alkali land is of great significance for promoting food production and sustainable development. It is necessary to study the mechanism of saline–alkaline tolerance and breed saline–alkaline tolerant crops to improve the utilization of saline–alkali land. For this study, we conducted a three-year pot experiment to screen the saline–alkaline tolerant germplasm of 72 rice genotypes from hundreds of elite cultivars during the whole growth period using a certain proportion of soda saline–alkali soil. The selected salt-tolerant variety was combined with a salt-sensitive variety to analyze the saline–alkaline tolerance mechanism by using the saline–alkaline soil leachate. We eliminated 36 genotypes with low seedling survival rates under salt–alkali stress, and the salt-tolerant Jiudao-66 (D68) variety had a higher survival rate than most varieties. The membership degree of Jiudao-66, according to the salt tolerance index of multiple agronomic traits, is higher than that of 34 varieties, with a higher survival rate except when compared to D36. The survival rate and these salt tolerance indexes of Jiudao-66 were significantly higher than those of Kitaake (salt-sensitive). Under the stress of leachate, the content of proline and soluble sugars in the shoots of Jiudao-66 were higher than that of Kitaake, and the total antioxidant capacity was stronger than that of Kitaake. However, the content of malondialdehyde was lower than that of Kitaake. Additionally, the Na^+/K^+ ratios in shoots and roots were not significantly differently between Kitaake and Jiudao-66. The results showed that Jiudao-66, as a salt-tolerant variety, is more tolerant to salt and alkali in a near-natural state due to its stronger tolerance of osmotic stress, and it can accumulate more proline and soluble sugars under stress. At the same time, Jiudao-66 also has a stronger antioxidant capacity. Its ion regulation ability has no obvious advantage.

Keywords: soda saline–alkali soil; rice germplasm; saline–alkaline tolerance; screening; physiological mechanism

1. Introduction

Arable land supports grain production and is a fundamental guarantee of national food security. Soil saline–alkalization is a major abiotic stressor on the world's agriculture, causing considerable damage to crop growth and resulting in serious losses in crop production [1–4]. The salinization area of land has exceeded 800 million hectares worldwide [5]. The saline–alkali land area of China is about 100 million hectares, mainly distributed in northeast, northwest, and north China, as well as coastal areas [6]. The area of saline–alkali land in the Songnen Plain in northeast China has reached 3.73 million hectares [7]. Saline–alkaline soil can be mainly classified into two types: saline soil and

alkaline soil. The main components (neutral salt containing $\text{NaCl} + \text{Na}_2\text{SO}_4$ versus alkaline salt containing $\text{NaHCO}_3 + \text{NaCO}_3$), EC (electrical conductivity) value, pH value, and other properties of these two soil types are greatly different [8]. The effects of saline soil and alkaline soil on plants are not identical. Both of them will produce ionic and osmotic stress, but alkaline soil will also form a high pH environment. High pH will affect plants and soil, so alkaline stress is more harmful to plants than salt stress [9,10]. The saline–alkali land in the Songnen Plain is made up of soda saline–alkali soil. It is one of the three typical soda saline–alkali soil distribution areas in the world [7]. Taking a large amount of saline–alkali land as a reserve arable land resource and realizing the “transformation into arable land” of the saline–alkali land by virtue of restoration and improvement is of great significance for sustainable development.

The salt and alkali tolerance mechanisms of plants mainly include ion homeostasis, osmoregulation, and antioxidant activity. Ion toxicity is the primary cause of salt damage. Osmotic stress and oxidative damage are secondary causes of salt damage. Under salt–alkali stress conditions, plants will absorb a large amount of Na^+ and inhibit the absorption of other nutrients, such as K^+ , resulting in ion imbalance and toxicity. The response mechanism of plants to ion toxicity is Na^+ exclusion and compartmentation. Plants maintain low Na^+/K^+ through the regulation of a series of genes. Under salt–alkali stress conditions, the external water potential is too low, which will cause osmotic stress to dehydrate the plant cells. At this time, plants can accumulate compatible solutes in the cytoplasm, reduce their water potential, and ensure that the volume and turgor pressure of the cells is in a suitable range to alleviate water loss. In addition, this also keeps the stomata open and the CO_2 concentration in the leaves at a high level, which can reduce the inhibition of plant photosynthesis [11]. Substances involved in plant osmoregulation include inorganic ions (such as Na^+ , K^+ , Cl^- , Ca^{2+} , etc.) and metabolites (such as sugars, organic acids, sugar alcohols, amino acids and their derivatives, etc.) [12]. Under normal conditions, the formation and elimination of reactive oxygen species (ROS) in plants is in a state of dynamic equilibrium. Saline–alkaline stress destroys this state, causing a large amount of ROS to accumulate in the plant, damaging the biomembrane system and causing a series of injuries until wilting occurs [13]. Under stress conditions, the plants scavenge excess reactive oxygen species with the help of antioxidant enzymes and antioxidants, protecting the integrity of the biomembrane structure and normal physiological functions from oxidative damage and improving the salt–alkali tolerance of the plants.

Rice (*Oryza sativa* L.) is one of the most important food crops. Half of the world’s population uses rice as a staple food, and about 60% of Chinese people use rice as a staple food [14]. Previous studies on saline–alkali tolerance in rice have been carried out for a specific period, such as the seed germination stage, seedling stage, and reproductive stage. The treatment method was to simulate saline–alkali stress in a saline–alkali solution. They treated the seeds and the hydroponic plants with a saline–alkali solution [15–20] or watered the plants in the soil with a saline–alkali solution [21–23].

In this study, we applied a certain proportion of saline–alkaline soil and normal soil mixture to implement a detailed screening in the field of saline–alkaline-tolerant germplasm of rice during the whole plant growth period. We selected 72 genotypes from hundreds of elite varieties. After screening, 36 varieties were eliminated. Finally, we obtained a salt-tolerant variety from the remaining 36 varieties. Then, we applied the saline–alkaline soil leachate to analyze the physiological mechanism of the salt-tolerant rice variety. Field screening was combined with physiological mechanism analysis to find the salt–alkali tolerance mechanism of rice in the near-natural state. This salt-tolerant variety is suitable for QTL (quantitative trait locus) mapping. Our findings will lay a foundation for identifying new salt-tolerant QTLs and cultivate new salt-tolerant rice varieties.

2. Materials and Methods

2.1. Screening of Saline–Alkaline-Tolerant Germplasm of Rice

2.1.1. Study Site

The study was conducted at the field experimental station of the Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, Anda, Heilongjiang Province (46°27' N, 125°22' E) [24] in northeast China. Anda has a temperate continental semi-arid monsoon climate. The average annual temperature is 3.3 °C, along with a frost-free period of 137 days and 2746 °C of accumulated temperature beyond 10 °C [25]. The average annual precipitation, evaporation, and sunshine hours are 438 mm, 1300 mm, and 2660 h, respectively [7,25]. Anda City is one of the most severe salinization areas, with an area of 147,600 hectares of saline–alkaline land [26]. The soil type in the area is typical soda saline–alkaline soil. The salt in the soil contains a lot of soda. The pot experiment was conducted from April to October in 2011, 2012, and 2013.

2.1.2. Rice Cultivation, Experimentation, and Data Collection

Fully developed rice seeds were sown on disks filled with nursery substrate (peat and vermiculite in a 1:1 proportion) in a greenhouse at the end of April. Three (thirty seedlings in 2011) uniform, approximately 30-day-old seedlings were transplanted into a plastic pot (30 cm diameter × 25 cm depth). Every pot was filled with a potting mixture of normal soil and saline–alkaline soil (NS:SAS volume ratios of 0, 2:1, and 1:1; Table 1) in triplicate. Fifteen days after transplantation, a complex fertilizer (N-P₂O₅-K₂O, 15-15-15, total nutrients ≥ 45%) was applied as topdressing for tillering. Then, the same topdressing was applied at the heading stage. The saline–alkaline soil was chosen and collected at 0–30 cm depth of wild “barren” lands in the serious salinization and alkalinization regions. All the pots were placed under the rain shelter of a PC (polycarbonate) sun sheet.

Table 1. Physical, chemical, and fertility characteristics of the three mixture types used in this experiment.

Mixtures	EC 1:5 $\mu\text{S}\cdot\text{cm}^{-1}$	TDS ppm	pH	Organic Matter %	Available N $\text{mg}\cdot\text{kg}^{-1}$	Available P $\text{mg}\cdot\text{kg}^{-1}$	Available K $\text{mg}\cdot\text{kg}^{-1}$	CO_3^{2-} $\text{mg}\cdot\text{kg}^{-1}$	HCO_3^- $\text{mg}\cdot\text{kg}^{-1}$	Na^+ $\text{mg}\cdot\text{kg}^{-1}$	Cl^- $\text{mg}\cdot\text{kg}^{-1}$
Normal Soil (NS)	220	96.0	8.0	2.2	45.5	35.4	164.0	—	195.0	41.0	57.0
2/3 NS	1000	445.0	8.8	1.5	66.5	32.2	218.0	192.0	512.0	181.0	114.0
1/2 NS	1498	672.0	9.0	1.5	67.2	25.7	229.0	120.0	976.0	280.0	497.0
Saline–Alkaline Soil (SAS)	2540	1150.0	9.8	1.0	58.1	12.9	272.0	840.0	2562.0	700.0	398.0

Normal Soil (NS): NS:SAS = 1:0; 2/3 NS: NS:SAS = 2:1; 1/2 NS: NS:SAS = 1:1. Saline–Alkaline Soil (SAS): NS:SAS = 0:1. The saline–alkaline soil was collected at 0–30 cm depth of wild “barren” lands. Electrical conductivity (EC), total dissolved solids (TDS), and pH were determined under soil:water = 1:5; Organic matter, available N, K, and various ions were determined by industry standards “NY/T 1121.6-2006”, “LY/T 1229-1999”, “NY/T 889-2004”, and “LY/T 1251-1999”, respectively. Available P was determined with the Olsen-P method.

2.1.3. Germplasm Survey

Seventy-two rice varieties were screened and selected half candidates of saline–alkaline tolerance, in accordance with survival rates of rice seedlings from the pot experiment in 2011 (NS:SAS = 1:0 and 2:1, in triplicate). At the mature stage in 2012 and 2013, we determined the following parameters: plant height (PHT), the number of tillers per plant (TN), effective panicle number per plant (EPN), 1000-grain weight (TGW), spikelets of the main panicle (SMP), and number of filled spikelets (NFS). For further screening, a potting mixture (1:1) was added in 2013 to the genotypes from 2011 and 2012. A typical experimental material, Nipponbare, and a salt-sensitive rice variety, Kitaake, were also tested.

2.2. Physiological Mechanism Analysis of Saline–Alkaline Tolerant Genotypes of Rice

2.2.1. Plant Material, Growth Conditions, and Stress Treatments

Two Japonica rice genotypes with different levels of saline–alkaline tolerance were used in this study: “Jiudao-66” (tolerant to SA, i.e., “D68”) and “Kitaake” (sensitive to SA). Seeds were disinfected with 3% NaClO for 30 min and rinsed with tap water six times. One day after soaking, the seeds were transferred into moist filter paper in a Petri dish and germinated for 4 days at 30 °C under bright light. The germinated seeds were then transplanted onto two multi-well plates embedded in PVC (Polyvinyl chloride) foam board floating on a plastic box containing 4 L of water for 10 days. All boxes were put in a cultivation room under the following conditions: 26–28 °C, 12 h photoperiod, 10,000 lx illuminance, and a relative humidity of approximately 70%. Then, the water was replaced with a nutrient solution containing: 1.44 mM NH_4NO_3 , 0.32 mM NaH_2PO_4 , 0.6 mM K_2SO_4 , 1.0 mM CaCl_2 , 1.6 mM MgSO_4 , 0.072 mM Fe-EDTA (Ethylene Diamine Tetraacetic Acid), 0.2 mM Na_2SiO_3 , 9.1 μM MnCl_2 , 0.154 μM ZnSO_4 , 0.156 μM CuSO_4 , 18.5 μM H_3BO_3 , and 0.526 μM H_2MoO_4 at pH 5.5 [27]. The Jiudao-66 and Kitaake seedlings were grown in this culture solution for 15 days. The water and solution were refreshed every three days.

To analyze tolerance to saline–alkaline stress, six boxes were divided into two groups as the control and saline–alkaline treatment in triplicate. Three of the boxes were kept planted in the culture solution as control. The other three boxes were replaced with 4 L saline–alkaline soil leachate for stress treatment. The leachate was extracted from 2 L saline–alkaline soil mixed with 4 L of water (stock solution). Four liters of tap water were poured into 2 L of saline–alkaline soil, stirred well, and filtered with filter paper to remove impurities. The saline–alkaline soil used for extraction was chosen and collected at 0–10 cm depth of wild “barren” lands, so it has a higher salinity. The air-dried soil was sieved using a 5 × 5 cm screen and mixed adequately. In our experiment, we used a quarter of the stock solution for stress treatment (tap water:stock solution = 3:1, volume). The process of obtaining leachate and its essential characteristics are shown Supplementary Figure S1 and Table S1. We collected samples after 24 h of control and stress treatments for various measurements.

2.2.2. Measurement of Chlorophyll Content

Leaves (0.1 g) were sampled, cut into pieces, and extracted with aqueous ethanol (95% v/v) in both Kitaake and Jiudao-66 plants to determine chlorophyll content. The absorbance (A) of the supernatant was determined at wavelengths of 645 and 663 nm. Total chlorophyll content was calculated using the following formula: $8.02 A_{663} + 20.21 A_{645}$. It was expressed using the unit mg of chlorophyll g^{-1} fresh weight.

2.2.3. Determination of Na^+ and K^+ Concentration

Shoots and roots of rice seedlings were oven-dried at 70 °C (deactivation of enzymes occurred at 105 °C during the initial stage) until they reached a constant mass and a 0.2 g dry sample was weighed separately. They were crushed and digested with 5 mL nitric acid and 2 mL perhydrol using a microwave system. Ion content was determined by inductively coupled plasma mass spectrometry (ICAP6300; Thermo Scientific, Waltham, MA, USA).

2.2.4. Determination of Proline and Soluble Sugars Content

Leaves and roots (0.1 g) were sampled for determination, and the roots were rinsed clean before weighing. Proline and soluble sugar content were determined by using commercial kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

2.2.5. Measurements of Total Antioxidant Capacity (T-AOC) and Malondialdehyde (MDA) Content

Leaves and roots (0.1 g) were sampled for measurement and the roots were rinsed clean before weighing. T-AOC and MDA content were also measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. We defined one T-AOC unit (U) as the value where 1 g of fresh plant material extract with 1 mL normal saline solution increases the absorbance (OD) value of the reaction system by 0.01 in one minute at 37 °C.

2.3. Statistical Analysis

The survival rates of seedlings from three of the same pots in 2011 were measured and averaged (means \pm SD, $n = 3$). The parameters of growth and yield components from a pot of all plants were averaged as one repetition according to the final number of survivors in 2012 and 2013. The relative parameters (salt tolerance index or STI) were the ratios of the values under stress treatments (i.e., 2:1 and 1:1 potting mixtures) to the corresponding values of controls (i.e., 1:0 potting mixture). $STI = X_{ST}/X_C$. We screened the remaining 36 varieties from 2011 using the membership degree (MD) of the relative parameters in 2012. The MD of a variety represents the mean value of the membership values (MV) of each single (relative) parameter such as PHT, TN, EPN, and etc. The formula $MV = (X - X_{min}) / (X_{max} - X_{min})$ [28] was used to calculate the MV for a specific parameter (such as PHT) of a variety, in which X was a single parameter of the variety under stress treatment (2:1 mixture). X_{max} and X_{min} were the maximum and minimum values of X in 36 rice varieties, respectively. The salt tolerance indexes were analyzed by the analysis of variance using the SPSS 17.0 (Statistical Product and Service Solutions, SPSS Statistics for Windows, Version 17.0. SPSS Inc., Chicago, IL, USA) statistical software in 2013. A general linear model (GLM) and Duncan's multiple range test (DMRT) were used for significance comparison ($p < 0.05$, $p < 0.01$). The physiological data were also analyzed through the analysis of variance using the SPSS statistical software. Significant differences were evaluated using Student's t -test ($p < 0.05$). All data represents an average of three repeat measurements and the standard deviation.

3. Results

3.1. Screening of Saline–Alkaline Tolerant Germplasm of Rice

3.1.1. Initial Screening in 2011

The seedling survival rates in the 2:1 potting mixture (NS:SAS = 2:1) are shown in Table 2. We investigated the effect of saline–alkaline soil on the survival rate of rice seedlings at 30 days after transplanting. The survival rates of seedlings under saline–alkaline stress decreased to 45.6–97.8% while they were 97.8% (two varieties), 98.9% (nine varieties), and 100% (61 varieties) in normal soil (NS:SAS = 1:0). According to these results, we selected 36 varieties for further screening in 2012.

3.1.2. Screening by Whole Growth Period in 2012

To characterize 36 varieties in the whole growth period under saline–alkaline stress, six parameters of the growth and yield components were surveyed after harvest. Then the relative parameters (salt tolerance index) of PHT, TN, EPN, TGW, SMP, and NFS were calculated (NS:SAS = 2:1). The MD values of 36 varieties according to their salt tolerance index are shown in Table 3. D36 (MD = 0.90) and D68 (MD = 0.88) were two varieties with the best overall performance under saline–alkaline stress. These two varieties could be further used as salt-tolerant varieties.

Table 2. Survival rates of 72 rice variety seedlings in 2:1 potting mixture (NS:SAS = 2:1).

Variety No.	Survival Rate %						
D10	97.78 ± 1.92	D28	88.89 ± 6.94	D25	77.78 ± 6.94	D31	58.89 ± 1.92
D70	96.67 ± 3.33	D43	88.89 ± 3.85	D20	76.67 ± 3.33	D40	58.89 ± 1.92
D36	96.67 ± 3.33	D63	88.89 ± 5.09	D45	76.67 ± 0.00	D52	58.89 ± 3.85
D30	95.56 ± 5.09	D3	87.78 ± 5.09	D60	76.67 ± 6.67	D24	56.67 ± 3.33
D57	95.56 ± 3.85	D19	87.78 ± 1.92	D61	75.56 ± 1.92	D11	56.67 ± 3.33
D68	95.56 ± 3.85	D46	87.78 ± 5.09	D62	75.56 ± 1.92	D14	56.67 ± 3.33
D1	94.44 ± 1.92	D5	86.67 ± 3.33	D15	73.33 ± 6.67	D54	54.44 ± 5.09
D47	94.44 ± 5.09	D17	86.67 ± 3.33	D33	73.33 ± 3.33	D22	53.33 ± 3.33
D71	93.33 ± 3.33	D21	86.67 ± 6.67	D41	73.33 ± 3.33	D53	53.33 ± 0.00
D8	93.33 ± 5.77	D34	86.67 ± 0.00	D26	72.22 ± 5.09	D39	52.22 ± 5.09
D49	93.33 ± 6.67	D42	86.67 ± 5.77	D44	72.22 ± 1.92	D37	51.11 ± 1.92
D64	93.33 ± 5.77	D66	85.56 ± 1.92	D18	65.56 ± 5.09	D4	50.00 ± 3.33
D23	92.22 ± 5.09	D51	85.56 ± 5.09	D48	65.56 ± 5.09	D6	50.00 ± 3.33
D35	92.22 ± 3.85	D69	84.44 ± 1.92	D32	65.56 ± 1.92	D13	50.00 ± 0.00
D2	91.11 ± 5.09	D67	84.44 ± 6.94	D29	64.44 ± 1.92	D38	48.89 ± 3.85
D27	90.00 ± 3.33	D7	82.22 ± 5.09	D56	64.44 ± 1.92	D65	48.89 ± 1.92
D50	90.00 ± 0.00	D12	81.11 ± 5.09	D59	62.22 ± 5.09	D55	46.67 ± 3.33
D72	90.00 ± 3.33	D58	81.11 ± 5.09	D9	61.11 ± 3.85	D16	45.56 ± 3.85

The data were collected after 30 days of transplanting. Values are means ± SD, $n = 3$.

Table 3. Salt tolerance index of 36 rice varieties with respect to membership degree in the 2:1 potting mixture (NS:SAS = 2:1).

Variety No.	MD						
D36	0.90	D57	0.73	D3	0.68	D66	0.56
D68	0.88	D1	0.73	D63	0.68	D5	0.53
D34	0.83	D47	0.71	D17	0.67	D42	0.51
D30	0.81	D43	0.71	D23	0.67	D51	0.50
D50	0.81	D71	0.70	D35	0.65	D12	0.47
D27	0.79	D49	0.70	D2	0.64	D69	0.47
D8	0.78	D64	0.69	D46	0.64	D58	0.45
D10	0.76	D72	0.68	D21	0.62	D67	0.45
D70	0.74	D28	0.68	D19	0.60	D7	0.32

MD: membership degree.

3.1.3. Further Verification in 2013

We compared two rice varieties (a salt-sensitive variety called “Kitaake” and a well-known variety called “Nipponbare”) with the previously studied D36 and D68 varieties. Then, we examined the growth performance and final yield composition of the four rice varieties during their growth period. In order to verify the previous results, we also added a 1:1 potting mixture as the experimental treatment. As shown in Figure 1A, no differences in the growth of four genotypes were detected when grown in a 1:0 potting mixture (NS:SAS = 1:0). Kitaake and Nipponbare were severely stressed in the 2:1 potting mixture, which caused only some seedlings to survive. Meanwhile, D36 and D68 were slightly stressed in this potting mixture, and the seedlings all survived. Kitaake and Nipponbare were more severely stressed in the 1:1 potting mixture. The seedlings were hardly able to grow. D36 and D68 were severely stressed in this potting mixture, but some of the seedlings grew well.

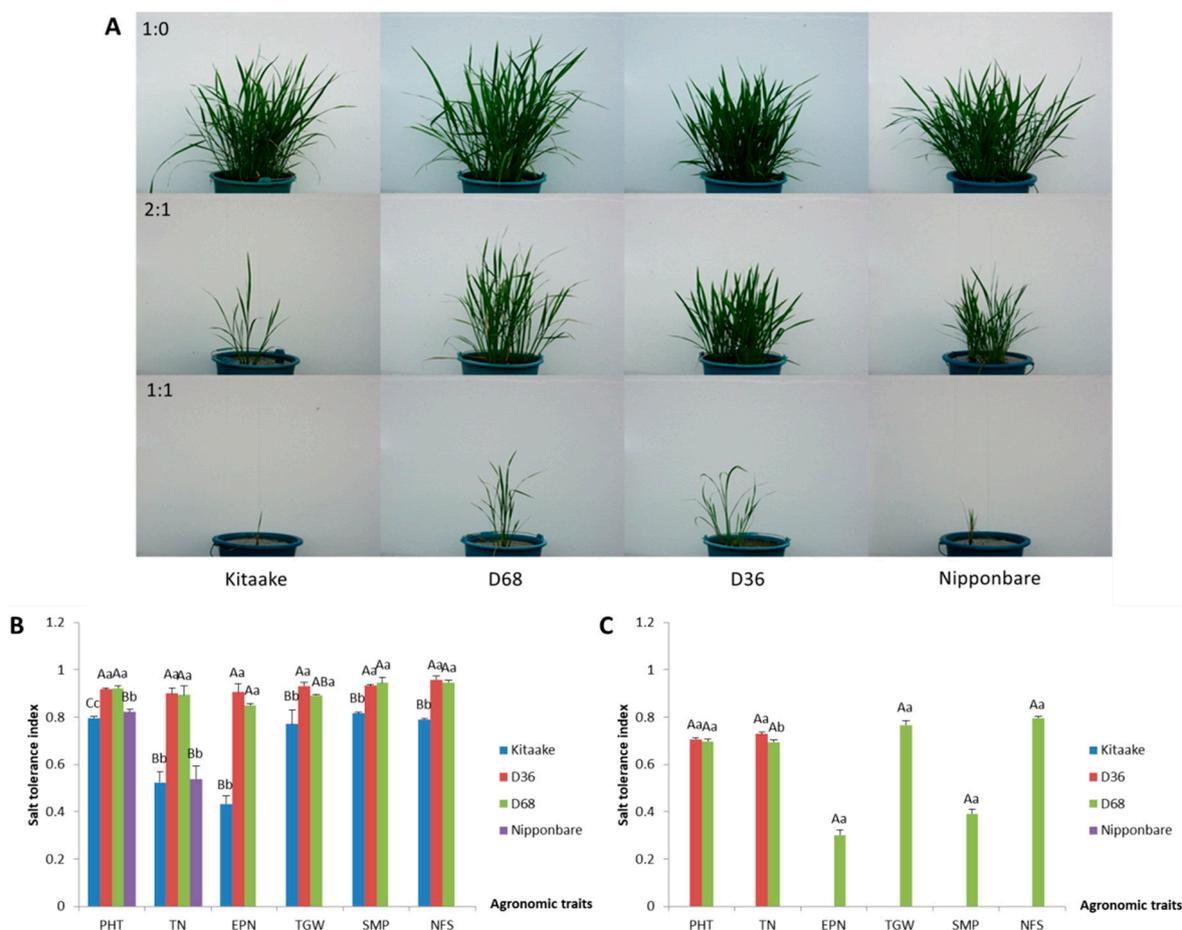


Figure 1. Pot experiment in 2013. (A) Four rice variety plants' performance in three kinds of potting mixture after 50 days from transplantation. The relative parameters (salt tolerance index) of growth and yield components of the four rice varieties in the (B) 2:1 potting mixture (C) 1:1 mixture. PHT represents plant height (cm), TN represents the number of tillers per plant, EPN represents the effective panicle number per plant, TGW represents the 1000-grain weight (g), SMP represents the spikelets of the main panicle, and NFS represents the number of filled spikelets. Values are means \pm SD, $n = 3$. Different capital letters (A, B) and lowercase letters (a, b) on columns indicate significant differences at $p < 0.01$ and $p < 0.05$ based on Duncan's test.

The salt tolerance indexes of PHT, TN, EPN, TGW, SMP, and NFS with Kitaake and Nipponbare in the 2:1 potting mixture were significantly lower than that of D36 and D68. All seedlings of Kitaake and Nipponbare in the 1:1 potting mixture were eventually dead with no gains. The salt tolerance indexes of PHT, TN, EPN, TGW, SMP, and NFS with D36 and D68 in the 1:1 potting mixture were lower than those in the 2:1 potting mixture, but harvest could be obtained. The above results indicated that the stress effect was obvious, and the salt and alkali tolerance of D36 and D68 was significantly stronger than that of Kitaake and Nipponbare.

Among the four varieties, Nipponbare was not harvested (all the plants failed to mature) due to the photoperiod, i.e., the sunshine hours in Heilongjiang Province is of the long-day characteristic with 15 h, while Nipponbare has a shorter photoperiod. Most salt tolerance indexes of D36 were higher than those of D68 (the differences were not significant), but its maturity was late. The saline-alkaline stress also delayed the maturity period (Table S2). Therefore, D36 was not harvested in the 1:1 potting mixture. D36 also had poorer rice quality than D68 (Figure S2).

Consequently, D68 and Kitaake were selected as experimental materials for the next experiment. The name of D68 was "Jiudao-66".

3.2. Evaluation of Salt and Alkali Tolerance and Physiological Mechanism Analysis of Jiudao-66

3.2.1. Effects of Saline–Alkaline Stress on Seedlings

To contrast the salt-tolerant rice variety Jiudao-66 and salt-sensitive rice variety Kitaake (pre-validation), 25-day-old rice seedlings of the two varieties were exposed to a quarter of saline–alkaline soil leachate (1/4 stock solution) for 24 h. After 24 h, the seedlings exposed to leachate were transferred to the previous culture solution for recovery. Then, we monitored the effects of saline–alkaline stress on the seedlings of the two genotypes. No differences between the two genotypes were observed when grown in the control solution. Saline–alkaline treatment for 24 h caused the seedlings to wilt. However, Jiudao-66 was better in this regard than Kitaake (Figure 2A). We measured the foliar chlorophyll content of plants undergoing control and saline–alkaline treatment. Exposure to leachate led to a reduction in foliar chlorophyll content in both Kitaake and Jiudao-66 seedlings, but the chlorophyll content in Jiudao-66 was significantly higher than that of Kitaake under stress, while there were no differences in the chlorophyll content in the two genotypes under control conditions (Figure 2B).

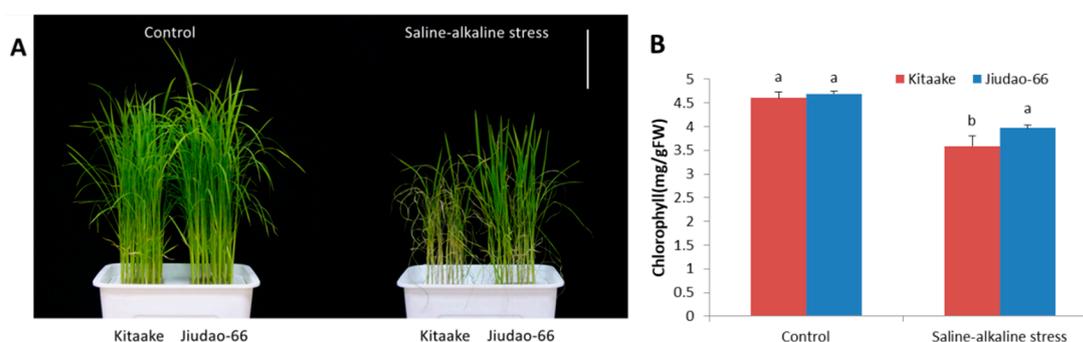


Figure 2. Effects of saline–alkaline stress on Kitaake and Jiudao-66 seedlings. (A) Seedling performance. (B) Foliar chlorophyll concentration. Twenty-five-day-old rice seedlings grown in normal culture solution were transferred to a quarter of saline–alkaline soil leachate for 24 h and exposed to normal culture solution for a recovery period of five days. Bar = 10 cm. Foliar chlorophyll content was measured at the end of the stress treatment. Values are expressed as means \pm SD, $n = 3$. Different letters (a, b) indicate significant differences between Kitaake and Jiudao-66 under the same treatment, as determined by a Student's t -test ($p < 0.05$).

3.2.2. Effect of Saline–Alkaline Stress on Na⁺ and K⁺ Concentrations

In the stress treatments, the Na⁺ concentrations in the roots and shoots of Kitaake and Jiudao-66 seedlings were significantly higher than in the control treatments, while K⁺ concentrations decreased compared to controls (Figure 3A,B,D,E). The K⁺ concentrations in the roots of the two varieties decreased markedly, while it decreased less in shoots. In the shoots, Kitaake plants showed lower K⁺ and higher Na⁺ concentrations than the Jiudao-66 plants under the control treatments. The Na⁺/K⁺ ratios of Kitaake were significantly higher than those of Jiudao-66. The K⁺ concentrations, Na⁺ concentrations, and Na⁺/K⁺ ratios in shoots of Kitaake under saline–alkaline stress were not significantly different from those of Jiudao-66 (Figure 3A–C). In the roots, Kitaake plants also showed lower K⁺ and higher Na⁺ concentrations than Jiudao-66 plants under the control treatment. The Na⁺/K⁺ ratios of Kitaake were also significantly higher than those of Jiudao-66 (Figure 3D–F). The Na⁺ concentrations in the roots of Kitaake under the saline–alkaline stress treatment were significantly lower than those of Jiudao-66 (Figure 3E). The K⁺ concentrations and Na⁺/K⁺ ratios in the roots of Kitaake under saline–alkaline stress were not significantly different from those of Jiudao-66 (Figure 3D,F). In general, the Na⁺/K⁺ ratios in the shoots and roots of Kitaake and Jiudao-66 plants were not significantly different under saline–alkaline stress.

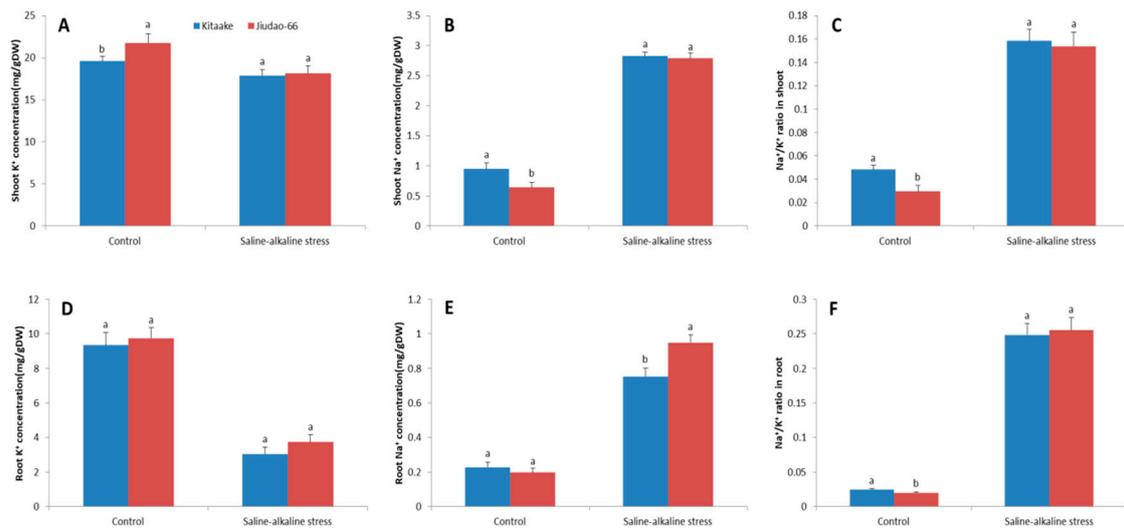


Figure 3. (A,D) Effects of saline–alkaline stress on K⁺ concentration, (B,E) Na⁺ concentration, and (C,F) Na⁺/K⁺ ratio of shoots and roots in Kitaake and Jiudao-66 seedlings grown in normal and saline–alkaline stress conditions. Treatments and statistical analyses were as described in Figure 2. Different letters (a, b) indicate significant differences at *p* < 0.05.

3.2.3. Effect of Saline–Alkaline Stress on Osmoregulation

The proline content in shoots of Jiudao-66 was higher than that in Kitaake under the control treatment. There were significant increases in shoot proline content of both Kitaake and Jiudao-66 seedlings under saline–alkaline stress. However, the increase in shoot proline content of Jiudao-66 plants was significantly higher than that in Kitaake plants under stress. The proline content in the roots of Jiudao-66 and Kitaake plants under control conditions were comparable. There was not much increase in the root proline content of Kitaake and Jiudao-66 seedlings under saline–alkaline stress. In addition, the root proline content between Jiudao-66 and Kitaake plants was not significant under stress (Figure 4A,B).

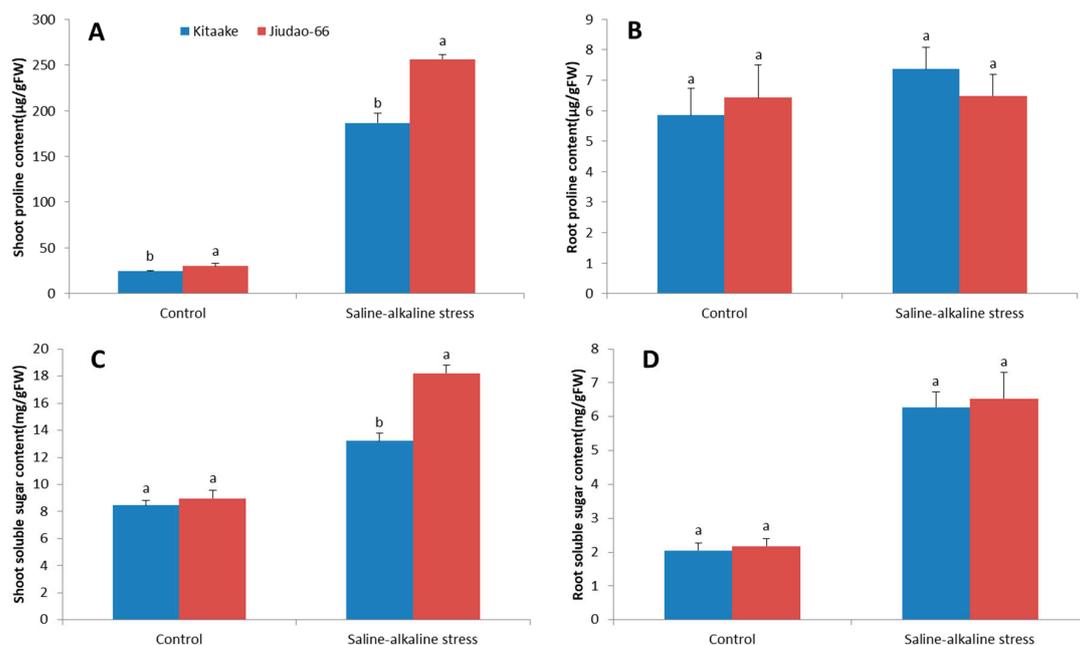


Figure 4. (A,B) Proline and (C,D) soluble sugar content of shoots and roots of Kitaake and Jiudao-66 seedlings grown in normal and saline–alkaline stress conditions. Treatments and statistical analyses were as described in Figure 2. Different letters (a, b) indicate significant differences at *p* < 0.05.

The two genotypes had a comparable soluble sugar content in their shoots and roots under the control conditions. The soluble sugar content was markedly increased in the shoots and roots of both Kitaake and Jiudao-66 seedlings under stress conditions. The increase in the shoot soluble sugar content of Jiudao-66 plants was greater than that in Kitaake plants under stress. However, no obvious differences in root soluble sugar content was found in Jiudao-66 and Kitaake plants under stress (Figure 4C,D).

3.2.4. Effect of Saline–Alkaline Stress on Oxidative Stress

Under abiotic stress, plants often exhibit symptoms of oxidative stress. The T-AOC and MDA content reflect the ability of the organism to cope with oxidative stress and the extent of damage under stress conditions. There were increases in shoot and root T-AOCs of both Kitaake and Jiudao-66 seedlings under saline–alkaline stress. The shoot and root T-AOCs of Jiudao-66 were significantly higher than those of Kitaake under control and saline–alkaline stress conditions (Figure 5A,B). There were increases in shoot and root MDA content of both Kitaake and Jiudao-66 seedlings under saline–alkaline stress. The shoot and root MDA content of Jiudao-66 was significantly lower than those of Kitaake under stress, while they had no significant differences under control conditions (Figure 5C,D).

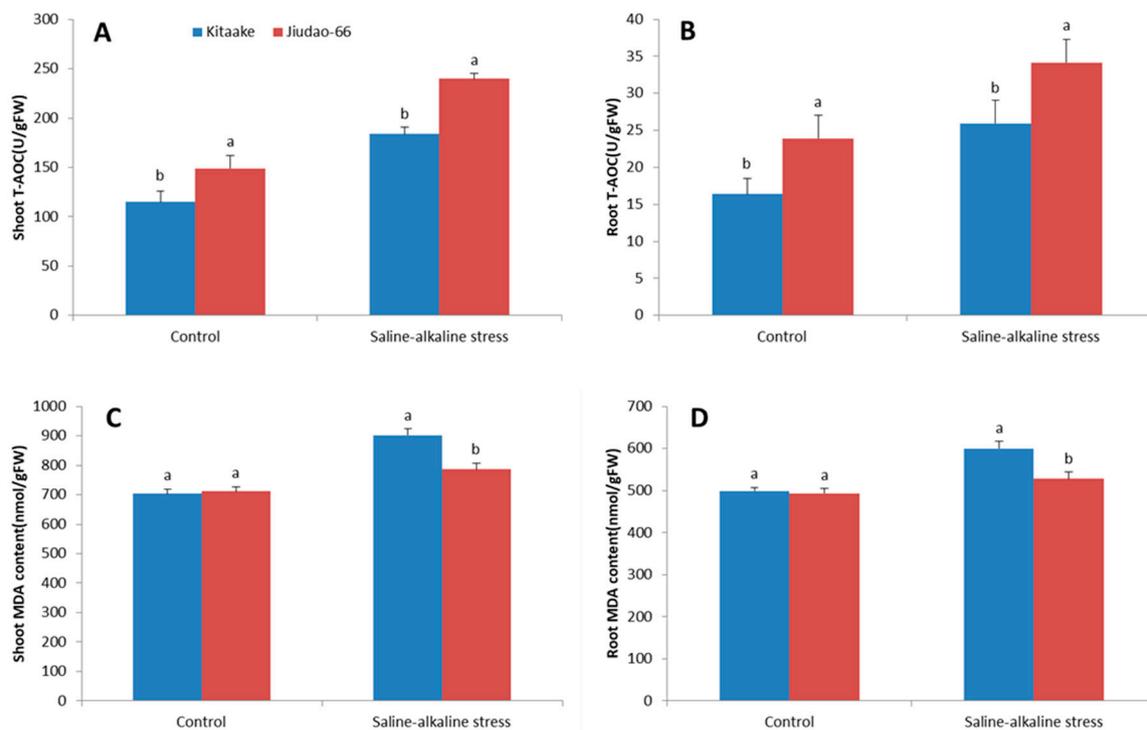


Figure 5. (A,B) Total antioxidant capacity (T-AOC) and (C,D) malondialdehyde (MDA) content of shoots and roots of Kitaake and Jiudao-66 seedlings grown in normal and saline–alkaline stress conditions. Treatments and statistical analyses were as described in Figure 2. Different letters (a, b) indicate significant differences at $p < 0.05$.

4. Discussion

The soda saline–alkali soil on the Songnen Plain has a high pH value, high EC value, and high content of carbonate (bicarbonate) (Table 1). The soil texture is very fine, the structure is poor, and the permeability is very low. Agricultural production on saline–alkali land in this area is a serious obstacle to growing. Plants are often subjected to more severe stress than in other types of saline–alkali soil.

In the past, studies on salt and alkali tolerance were often carried out by means of a salt solution (e.g., neutral salt or alkaline salt) to simulate stress conditions during screening and evaluation.

Researchers grew rice in a hydroponic culture solution with or without salt stress [27,29–33]. Previous reports have also completed phenotypic evaluation using irrigated brine or incubated rice in salt solution with perforated pots [34–37]. Takagi et al. screened 6000 ethyl methanesulfonate (EMS) mutant lines of a local elite cultivar. Young seedlings were subjected to salinity treatment in 50% GEX (Gex Co., Ltd., Osaka, Japan) artificial seawater containing 1.5% NaCl. They used a MutMap (next generation sequencing) method to accelerate breeding of a salt-tolerant rice cultivar [38]. Others findings reported implement screening and evolution during the germination period [39–41]. Importantly, Negrão et al. suggested that the salt and alkali tolerance of rice varied during different growth stages [42]. We used potting mixture with saline–alkali soil to screen the saline–alkaline tolerant germplasm of rice during the whole growth period. Seventy-two genotypes were screened with three kinds of treatment, including a control treatment. The results showed that there were differences in survival rate, related traits of growth, and yield among these rice varieties. Finally, we selected a salt-tolerant rice variety (Jiudao-66, i.e., D68) and a salt-sensitive rice variety (Kitaake) as a combination for consecutive experiments. During the screening experiments, we found that rice plants could survive after transplantation, and they would grow until harvest. Thus, the seedling stage is a key period for the resistance test. In agricultural production, the varieties with a strong salt and alkali tolerance at the seedling stage were selected to grow in normal soil first. Then, the appropriately aged seedlings were transplanted into saline–alkali soil with light salinity, so as to succeed in planting rice on saline–alkali land and developing backup farmland. After screening, we used the saline–alkaline soil leachate to evaluate salt and alkali tolerance and analyze the physiological mechanisms of the salt-tolerant rice variety. This has not been reported yet. The leachate contains plenty of tiny black soil colloid, so it looks black while the saline–alkaline soil is white or normal in color (Figure S1). The tiny colloid may also hinder the absorption of water and mineral elements by the roots. It can better represent saline–alkali land, and its properties are like those of saline–alkaline soil. The experimental results have a more important significance for crop production.

Rice plants generally tolerate salt through three main mechanisms: ion exclusion, osmotic tolerance, and tissue tolerance [43]. We determined the K^+ content, Na^+ content, proline content, soluble sugars content, T-AOC, and MDA content in shoots and roots of the plants under control and stress conditions. Ion exclusion mainly prevents excessive Na^+ from accumulating in the leaves during sodium transport processes [44]. Plants with a strong tolerance to saline stress often have a high K^+ and low Na^+ concentration in their shoots. The Na^+/K^+ ratio is often considered as an indicator of plant tolerance to saline stress [45]. However, some studies have confirmed that the Na^+/K^+ ratio of the shoots is not related to the salt and alkali tolerance of the plants [46–51]. Li et al. considered that salt-tolerant rice plants can sequester Na^+ in a vacuole, thus minimizing its toxic effect under salt stress. In the present study, a comparable Na^+/K^+ ratio was observed in Kitaake and Jiudao-66 under stress, suggesting that other mechanisms may be responsible for salt and alkali tolerance. However, we found that Kitaake plants had a higher Na^+/K^+ ratio in shoots and roots than Jiudao-66 plants under control conditions. This showed that Jiudao-66 has a stronger growth vigor, which can avoid the toxic effects of salinity [43]. Most plants accumulate some organic solutes to cope with osmotic stress. The accumulation of compatible solutes is generally considered to be a basic strategy to avoid plant salt damage. Compatible solutes accumulate as osmoprotectants in the cytosol, helping to reduce the cytoplasmic water potential [50]. Various sugars (e.g., fructose, glucose, and sucrose), polysaccharides (e.g., trehalose, raffinose, and fructans), sugar alcohols (e.g., mannitol and glycerol), amino acids, and derivatives (e.g., proline, glycine-betaine, and proline-betaine) all have this function [12,51]. In the present study, we found that Jiudao-66 plants accumulated more proline and soluble sugars in shoots than Kitaake under stress, while they have comparable amounts of proline and soluble sugars in roots under stress. The greater amount of osmoprotectants of Jiudao-66 in the shoots caused it to have more effective osmoregulation in osmotic stress and a stronger salt and alkali tolerance than Kitaake. As byproducts of photosynthesis, respiration, and photorespiration, more and more harmful reactive oxygen species (ROS) are produced under salinity stress. Excessive production

of cytotoxic ROS causes oxidative damage to different cellular components, including membrane lipids, proteins, and nucleic acids [42]. To avoid excessive accumulation of ROS, ROS scavenging mechanisms, including enzymatic and non-enzymatic (antioxidants) mechanisms, are often activated in plants [32]. T-AOC reflects the total capacity to scavenge ROS, including various related enzymes (e.g., superoxide dismutases, peroxidases, etc.) and antioxidants (e.g., ascorbic acid, carotene, etc.). The level of MDA indirectly reflects the severity of oxidative damage. In the present study, Jiudao-66 had a stronger total antioxidant capacity and lower MDA content than Kitaake, under stress. Jiudao-66 could scavenge more ROS under stress conditions, which helped to improve its salt and alkali tolerance. Furthermore, Jiudao-66 had a stronger total antioxidant capacity than Kitaake under control conditions, probably due to its high antioxidant content.

In addition, some recent studies use RNA-seq and genome-wide analysis to describe the relationship of miRNAs (target mRNAs), differentially expressed genes (DEGs), transgenic overexpression, etc. with salt tolerance, abiotic stresses in rice, and other crops. Manu Kumar et al. elaborated on the relationship of OsSta2 with salt tolerance and abiotic stress in rice [52–58]. Salt tolerant varieties can be produced using marker-assisted selection or genetic engineering by introducing salt-tolerance genes [43]. Utilizing next generation sequencing and molecular markers, an F_{6:7} RIL (Recombinant Inbred Lines) population (about 130) derived from a cross of Kitaake with Jiudao-66 was used to identify quantitative trait loci for salt and alkali tolerance in rice.

5. Conclusions

In conclusion, we obtained a salt-tolerant rice variety (Jiudao-66) through screening in the field. Then, we combined this with a salt-sensitive variety (Kitaake) to analyze the salt and alkali tolerance mechanism of the rice. Jiudao-66 is more tolerant to salt and alkali because of its stronger ability to synthesize and accumulate proline and soluble sugars and its stronger antioxidant capacity. Our findings indicate that Jiudao-66 has a greater tolerance against osmotic stress and a more effective ROS detoxifying system than Kitaake under salt–alkali stress. Our findings will lay a foundation for mapping saline–alkaline tolerant QTLs.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/8/10/205/s1>, Figure S1: The obtainment of leachate, Figure S2: The rice quality of D36 and D68, Table S1: The leachate's essential characteristics, Table S2: The maturity of D36 and D68 in the potting mixture.

Author Contributions: H.W. and S.L. conceived and designed the experiments. H.W. performed the experiments. H.W. analyzed the data. S.L. and T.T. contributed reagents, materials, and analysis tools. H.W. wrote the manuscript. S.L. and T.T. revised and approved the final article.

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