CRISPR/Cas9 Mediated Knockout of the OsbHLH024 Transcription Factor Improves Salt Stress Resistance in Rice (Oryza sativa L.)

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Abstract: Salinity stress is one of the most prominent abiotic stresses that negatively affect crop production. Transcription factors (TFs) are involved in the absorption, transport, or compartmentation of sodium (Na+) or potassium (K+) to resist salt stress. The basic helix–loop–helix (bHLH) gene family is critical for plant growth and stress responses, including salinity. Herein, we used the CRISPR/Cas9 strategy to generate the gene editing mutant to investigate the role of OsbHLH024 in rice under salt stress. The A nucleotide base deletion was identified in the osbhlh024 mutant (A91). Exposure of the A91 under salt stress resulted in a significant increase in the shoot weight, the total chlorophyll content, and the chlorophyll fluorescence. Moreover, high antioxidant activities coincided with less reactive oxygen species (ROS) and stabilized levels of MDA in the A91. This better control of oxidative stress was accompanied by fewer Na+ but more K+, and a balanced level of Ca2+, Zn2+, and Mg2+ in the shoot and root of the A91, allowing it to withstand salt stress. Furthermore, the A91 also presented a significantly up-regulated expression of the ion transporter genes (OsHKT1;3, OsHAK7, and OsSOS1) in the shoot when exposed to salt stress. These findings imply that the OsbHLH024 might play the role of a negative regulator of salt stress, which will help to understand better the molecular basis of rice production improvement under salt stress.

Keywords: antioxidants; CRISPR/Cas9; rice; OsbHLH024; salt stress; ROS

1. Introduction

Salt stress is significant abiotic stress that affects plant growth and development and renders natural production unfeasible [1]. Although rice (Oryza sativa L.) is an essential crop for feeding over half of the earth’s population, its production is limited by salt stress. Various parameters are disrupted under salinity stress at the physiological level, for instance, the stomatal closure and chlorophyll content inhibition resulted in the reduction of the photosynthesis [2]. Moreover, oxidative damage, nutrient imbalances, toxic metabolite formation, and cellular damage are often associated with plant growth inhibition and the reduction of crop production [3,4]. Such physiological parameters require particular consideration for uncovering the tolerance mechanism of salt stress in rice.

Reactive oxygen species (ROS) and ionic imbalance play a critical role in the response to salt stress [5]. ROS, including hydrogen peroxide (H2O2), superoxide radicals (O2−), hydroxyl radicals (OH), and singlet oxygen (1O2), are generated in different cellular
compartments of plants under salt stress [6]. Under aerobic conditions, ROS are produced from mitochondrial and chloroplast electron transport chains [7] and controlled through different antioxidant defense systems [8,9]. Salt stress can quickly initiate and increase ROS production, responsible for oxidative damage [10], lipid peroxidation (malondialdehyde, MDA), membrane injury [11], and eventually cell death [12]. Antioxidants are essential for scavenging ROS, and ROS mitigation occurs in plant cells [13]. Superoxide dismutase (SOD), a metalloenzyme that dismutases ROS into $H_2O_2$, is a frontline defense against ROS and peroxidases (POD), converting $H_2O_2$ to $H_2O$ [14]. The salt stress toxicity is mainly due to $Na^+$ and $Cl^-$ toxicity, which can induce remarkable disturbance of important nutrients, including N, P, $K^+$, $Ca^{2+}$, S, $Mn^{2+}$, $Mg^{2+}$, and $Zn^{2+}$ [3,15]. Therefore, the salt stress can restrict the $Ca^{2+}$ absorption, reduce cytosolic $Ca^{2+}$ levels, affect membrane stability, and influence the osmotic equilibrium and intracellular signaling [16]. Furthermore, $Zn^{2+}$ can also improve the salt stress tolerance by blocking $Na^+$ and/or $Cl^-$ absorption or translocation [17]. However, the $Zn^{2+}$ assimilation in plants is also inhibited by greater salt concentrations [18]. Development of the varieties with enhanced capacity for ROS scavenging and ionic homeostasis [19] could pave the way to improving rice tolerance towards salt stress.

Some genes are involved in salt stress regulation, including genes associated with blocking $Na^+$ entrance, translocation, and/or transport out of cells [4], and genes codifying the signaling of proteins responsible for ROS scavenging to regulate the proper nutrients balance [20]. The high-affinity potassium transporters (HKTs), KT/HAK/KUP family, Salt Overly Sensitive (SOS), as well as late embryogenesis abundant (LEA) have been reported to play a vital role in the salt stress response [21–24]. Among them, HKTs regulate the balance of $Na^+$ and $K^+$ [25], which can be divided into HKT1, a low-affinity $Na^+$ transporter, and HKT2, a permeable channel for $Na^+$ and $K^+$ [26]. Under salt stress, $OsHKT1$ excludes $Na^+$ from root xylem sap to prevent it reaching the shoots or its translocation from shoots to roots to promote efflux [27]. In rice, $OsHKT1;1$ loads $Na^+$ into parenchyma cells that might be transferred to the phloem after being transported to the leaves [28]. Moreover, $OsHKT1;1$ recovers $Na^+$ from the leaf blade and is mainly expressed in roots, vascular tissues, and leaves [28], but the exact role regarding salinity tolerance is unknown [29]. The $OsHKT1;3$ transporter is a highly selective $Na^+$ transporter found in the vascular tissue of roots and leaves [30], and it is significantly expressed when exposed to salt [31]. $OsHKT1;5$ (rice QTL, SKC1) controls $Na^+$ concentration in the xylem of leaves and is expressed in roots and shoots [32]. On the other hand, the $OsHKT2$ facilitates the uptake of $Na^+$ or $K^+$ into starved tissues in rice [33]. The KT/HAK/KUP family of $K^+$ transporters are also essential genes involved in the salt stress response, among which $OsHAK7$ is a salinity-resistant gene found in the shoots and roots of plants [34]. Furthermore, $SOS1$ functions in the SOS signaling system in plasma membranes, and its mutation, $sos1$, imparts salt sensitivity by allowing more $Na^+$ and less $K^+$ to enter the cell [35]. The late embryogenesis abundant group 3 protein in rice ($OsLEA3$) presents a protective function under extreme stress circumstances, including salt stress [21]. Thus, investigation of these genes relevant to the plant response to salt stress will help to elucidate the underlying mechanisms.

The defense mechanism could be controlled by regulatory elements, including transcription factors (TFs) [36]. TFs bind to cis-acting domains in promoters of target genes to regulate gene expression positively or negatively [37]. The regulation of TFs is one of the major mechanisms by which plants ensure growth, development, and protection from numerous environmental stresses [38]. The plant basic-helix–loop–helix (bHLH) is one of the largest TFs, compromising 25 subfamilies functioning against abiotic stress, including salt stress [39,40]. $AtbHLH092$, $AtbHLH112$ in Arabidopsis [41,42], $PebHLH-54$, $PebHLH-148$ in common bean [43], $VcbHLH1$ in grape [44], $BvbHLH93$ in sugar beet [45], $SbbHLH85$ in sweet sorghum [46], and $NtbHLH123$ in tobacco [47] have been reported to play a role in the salt stress response. Only three bHLH genes, $OsbHLH035$, $OsbHLH062$, and $OsbHLH068$, have been associated with the rice response towards salt stress [42,48,49], which belong to subfamilies V, M, and F, respectively. However, the function of the bHLH subfamily
U has not been explored yet. The rice bHLH subfamily U includes seven genes [39], and the representative OsbHLH024 (LOC_Os01g39330) gene was selected to address the function of the bHLH subfamily U response to salt stress in rice. The OsbHLH024 differentially expressed between high temperature and low temperature in thermosensitive genic male-sterile material Zheda13S [50]. In this study, to investigate the function of the OsbHLH024 gene in response to salt stress, a loss-of-function mutant was generated using the CRISPR/Cas9 approach. Interestingly, the osbhlh024 mutant (A91) exhibited a high salinity tolerance response, and accumulated less ROS, MDA, and Na⁺ but a higher K⁺ with a balanced nutritional level in the shoots and roots. Moreover, the expression of ion transporter genes of OsHKT1;3, OsHAK7, and OsSOS1 was enhanced in the A91 under salt stress. These findings suggest that the OsbHLH024 gene is a negative regulator, and its knockout enhances the salt stress tolerance in rice.

2. Results
2.1. Expression Level of OsbHLH024

Eight different tissues of rice wild type (WT) Nipponbare were sampled to investigate the expression level of OsbHLH024 gene by qRT-PCR. The results showed that OsbHLH024 was highly expressed in the flag leaves angle and roots at 7 days. While other tissues had a low expression, shoots investigated at 21 days showed very low expression levels (Figure 1).

![Figure 1. The expression level of OsbHLH024 in eight different tissues. OsUBQ5 was used as the control; three biological replicates were applied, and the values were expressed as means ± SE.](image)

2.2. Generation of A91 via CRISPR/Cas9 Method

For characterizing the function of the OsbHLH024 gene in rice, we created the A91 using the CRISPR/Cas9 genome editing method. Two single guide RNA (sgRNAs) in two targets with sequence-specific sites of OsbHLH024 were designed to induce mutagenesis. The target-1 and target-2 were separated by 312 bp and driven by OsU6a and OsU6b promoters, respectively (Figures 2a and S1). They were ligated and inserted into a CRISPR/Cas9 binary vector through two sgRNA expression cassettes [51]. Using Agrobacterium induced transformation, the final vector was transformed into the calli of WT Nipponbare. The generated transgenic plants were analyzed using PCR with site-specific (target) primers and Sanger sequencing to confirm the gene-editing type. Only one heterozygous mutant was obtained, and the homozygous mutant (A91) with one base (A) deletion in Target-1 in T₁ generation was obtained (Figures 2b and S2), which resulted in a frameshift to accumulate the stop codon (premature) with 301 of the original 455 amino acids (Figure 2c).
Figure 2. CRISPR/Cas9-induced mutation in the OsbHLH024 (LOC_Os01g39330) gene. (a) Schematic diagram of gene structure and two CRISPR/Cas9 target locations, with UTRs, exons, and introns and bHLH domain shown by blank rectangles, black rectangles, black lines, and dotted rectangles, respectively. The 20-nt target sequences are shown at the bottom of the gene structure; (b) DNA sequencing and alignments with WT identify the editing genotypes; the deletion is indicated by the red dash; (c) the expected protein structure of WT and A91. The dotted and grey showed the bHLH domain in WT and frameshift or premature stop in A91.

2.3. Phenotypic Characterization of the A91 and WT Grew in Normal Conditions

At the one-week seedling stage, the plant height was significantly different between WT (14.90 cm) and the A91 (18.16 cm) (Figure 3a,b). The difference in the root number and root length was insignificant between WT and A91 (Figure 3b). At the reproductive stage, the plant height was raised by 6.13% in the A91 compared to the WT (Figure 3c,d). Both the WT and the A91 presented five internodes, and the first, second, and third internodes of the A91 were notably longer than that of the WT, while the fourth and fifth internodes were unchanged (Figure 3e,f). The tiller biomass was increased by 14.18% in the A91 compared to the WT (Table S2). On the other hand, the tiller number, panicle size, grain shape, and leaf size remained statistically stable in the A91 and WT (Figure 3g–i and Table S2). These observations indicated that the A91 exhibits fewer effects on the rice phenotypes and could be characterized clearly by the plant height and tiller biomass at the seedling and reproductive stages under normal conditions.

2.4. The A91 Confers Salt Tolerance

The 21-day-old seedlings of the A91 and WT were exposed to 150 mM NaCl (Figure 4), and most of the WT seedlings collapsed after 12 h of salt treatment (Figure 4b). The prolongation of salt exposure resulted in visually wilted, rolled, dried, stunted, and burnt leaves after 4 days (Figure 4c). Although the stress symptoms appeared after 6 days and became clear after 7 days of salt stress exposure (Figure S3), the A91 exhibited better growth performance than the WT (Figure 4a–c). Moreover, the A91 presented a greater survival rate (7.33/20, 36.67%) than the WT (1.66/20, 8.33%) after 7 days of recovery (Figure 4d,e). While salt stress markedly decreased the growth performance of both the WT and the A91, the fresh shoot weight of the mutant was considerably higher than that of the WT after 7 days of salt exposure (Figure 4f).

The total chlorophyll content (SPAD value) and chlorophyll fluorescence were significantly greater in A91 than in WT after 4 days of salt stress exposure (Figure 4g–i). These results suggested that the knockout of the OsbHLH024 gene confers the salt tolerance in rice by improving the physiological condition of leaves, delaying the appearance of stress symptoms, and consequently promoting better shoot performance and survival rate.
Figure 3. The phenotypic comparison of the A91 and WT. (a) The height of seedlings at one-week-old growing in 1/2 MS media; (b) the comparison of the root number, the root length and the shoot length in WT and A91 (n = 9 biological replicates); (c) the morphology of plants at the reproductive stage; (d) the measurement of plant height (n = 30 biological replicates); (e) the phenotype of tiller; (f) the measurement of internode length (n = 30 biological replicates); (g) the structure of panicle; (h) 10 seeds length; (i) 10 seeds width, values are expressed as means ± SE; * and ** denote significant t-test results at p < 0.05 and p < 0.01, respectively.

2.5. OsbHLH024 Involves in the Regulation of Oxidative Stress Homeostasis in Salt Stress

Because salt stress is often accompanied by oxidative stress [3], the antioxidant enzyme (SOD and POD) activities and the levels of H$_2$O$_2$, MDA, and superoxide radicals (O$_2^{•−}$) were further investigated in shoots and roots of the A91 and WT. Although salt stress significantly increased the SOD activity in both the A91 and WT, the level of SOD activity was not significantly different between the A91 and WT (Figure 5a,e). The SOD activity regulates the production of H$_2$O$_2$, the H$_2$O$_2$ in the shoot of WT was greater than in the A91 under salt stress (Figure 5c). In addition, the salt stress coincided with a significant rise in the POD activity but at a lower level in the shoots of A91 than WT (Figure 5b). Compared to their respective controls, POD activity increased more in the shoots of WT than the A91 as a response to the H$_2$O$_2$ level (Figure 5b,c). Interestingly, no significant difference in the MDA level was recorded in the shoots of WT and A91 under control, while it was 3.8-fold higher in WT than in A91 under salt stress (Figure 5d). The MDA level in roots did not differ significantly before and after the salt exposure (Figure 5h). These physiological observations suggested the significance of salt stress in the shoots of WT compared to the A91. Moreover, the POD activity was more induced in the roots of the A91 than the WT under salt stress and indicated that the response of POD depended on the level of H$_2$O$_2$ in the roots (Figure 5f,g).
Figure 4. The growth characteristics of the A91 and WT exposed to salt stress treatments (0 and 150 mM NaCl). (a) The growth of 21-day-old seedlings before salt stress; (b) the growth of 21-day-old seedlings after 12 h stress; (c) the growth of 21-day-old seedlings after 4 days of stress; (d) 7 days recovery after 7 days of salt stress; (e) the survival rate after 7 days of recovery; (f) the fresh shoot weight after 7 days of stress; (g) total chlorophyll content; (h) SPAD value; (i) fluorescence; n = 3 biological replicates (e,f, each replicate represented 20 seedlings); 3 biological replicates (g), 9 biological replicates (h,i); data represented as means ± SE; ** indicates significant t-test results at p < 0.01; scale bar 10 cm.
Figure 5. The oxidative stress in the shoot and root of WT and A91 seedlings under salt stress. (a–d) activities of SOD, POD, H$_2$O$_2$, and MDA in shoot; (e–h) activities of SOD, POD, H$_2$O$_2$, and MDA in the root; (i) NBT staining (indicates O$_2$•$^-\$); (j) DAB staining (indicates H$_2$O$_2$); n = 3 biological replicates, data represent means ± SE; * and ** indicate significant t-test results at p < 0.05 and p < 0.01, respectively.

Furthermore, in situ histochemical DAB and NBT staining were employed to measure H$_2$O$_2$ and O$_2$•$^-$, respectively. The highly dark-brown color and more staining spots were displayed in the WT leaf than that of A91 after 4 days of salt stress (Figure 5i,j), supporting that the WT accumulated more H$_2$O$_2$ and O$_2$•$^-$ than the mutant. These results revealed the significance of oxidative stress due to salt stress in the WT compared to the A91.

2.6. *OsbHLH024* Induces Ion Homeostasis under Salt Stress

The levels of Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ in the shoots and roots of WT and A91 were studied before and after salt stress. Before salt stress, only the Zn$^{2+}$ level in the shoots was significantly higher in the A91 than that of WT (Figure 6a–f). After 7 days of salt exposure, a low level of Na$^+$ and Mg$^{2+}$ was found in the shoots of A91 seedlings compared to the WT (Figure 6a,e). By contrast, the content of K$^+$ and Zn$^{2+}$ levels were greater in the shoots of the A91 than in the WT under salt stress (Figure 6b,f). Interestingly, a lower ratio
of Na⁺/K⁺ was observed in the shoots of the A91 than in WT (Figure 6c). Ca²⁺ was not significant before and after salt stress, although there was an increasing trend observed after stress in both WT and A91 (Figure 6d). These results indicated a lower Na⁺ translocation in the shoots of the A91 compared to WT.

**Figure 6.** Measurement of nutrient elements in the shoots and roots of WT and A91 seedlings exposed to salt stress for 7 days. (a–f) Na⁺, K⁺, Na⁺/K⁺, Ca²⁺, Mg²⁺, and Zn²⁺ in shoots; (g–l) Na⁺, K⁺, Na⁺/K⁺, Ca²⁺, Mg²⁺, and Zn²⁺ in roots; n = 3 biological replicates; data represent means ± SE; * and ** indicate significant t-test results at p < 0.05 and p < 0.01, respectively.

The salt stress resulted in a higher Na⁺ content in the roots of WT than in the A91, but compared to WT, the A91 showed higher Na⁺ content in control (Figure 6g). Although the content of K⁺ in the roots was significantly decreased by salt exposure, the level of K⁺ in the A91 was higher than in WT (Figure 6h). Under salt stress, the ratio of Na⁺/K⁺ in the A91 was lower than in the WT, suggesting a superior uptake of Na⁺ in the WT than in the A91 (Figure 6i). Moreover, salt stress increased the level of Ca²⁺ and Zn²⁺ in the roots of both WT and A91 (Figure 6j,l). By contrast, the uptake of Mg²⁺ in both WT and A91 was repressed by the salt stress, which was significantly lower in A91 compared to WT (Figure 6k). These results showed that the A91 exhibited better nutritional homeostasis than WT under salt stress.

2.7. The Molecular Effect of OsbHLH024 Mutation in Ion Transporters and Cell Structure Controller Genes

As the results of the phenotypic, physiological, antioxidant activities and ion homeostasis above showed that the A91 could be tolerant to salt stress, the molecular effects of
the OsbHLH024 mutation were investigated through the expression of five ion transporters (OsHKT1;1, OsHKT1;3, OsHKT1;5, OsHAK7, OsSOS1) and one cell structure controller gene (OsLEA3), in shoots and roots under salt stress (Figure 7). The salt stress remarkably increased the expression levels of all the six genes in the shoots of both WT and A91 (Figure 7a–f). Compared to the WT, the relative expression levels of OsHKT1;3, OsHAK7, and OsSOS1 genes in the A91 were relatively higher before and after NaCl treatment (Figure 7b,d,e). On the other hand, OsHKT1;1 was expressed highly in the A91 before salt stress but significantly decreased after stress (Figure 7a). OsLEA3 was not significant before salt stress but significantly decreased after stress in the A91 (Figure 7f). The expression of OsHKT1;5 was not changed significantly between WT and the A91 (Figure 7c).

Opposite to shoots, the expression of OsHKT1;3, OsHAK7, and OsSOS1 genes were decreased in roots of the A91 when compared to WT, and OsHKT1;5 and OsLEA3 genes had similar trends, but not significant (Figure 7). The OsHKT1;1 gene had lower expression

Figure 7. Expression of genes in WT and A91 under control and salt stress for 2 days. (a–f) OsHKT1;1, OsHKT1;3, OsHKT1;5, OsHAK7, OsSOS1 and OsLEA3 in shoots; (g–l) OsHKT1;1, OsHKT1;3, OsHKT1;5, OsHAK7, OsSOS1 and OsLEA3 in roots; OsACTIN1 was used as the control; n = 3 biological replicates, data represent means ± SE; * and ** indicate significant t-test results at p < 0.05 and p < 0.01, respectively.
before stress but higher expression after salt stress in the A91 compared to WT (Figure 7g). All six genes had higher expression in shoots compared to roots under WT. These results suggested that the mutation in the OsbHLH024 gene positively affects the expression of ion transporter genes, especially in shoots, which are essential for maintaining nutrient balance.

3. Discussion

The world’s rice production, a major concern for the human diet, is hampered by excessive salinity [52]. In previous studies, the role of salinity was reported to weaken the PSII activity via promoting the compensation of the pigments in chlorophyll by the secretion of ions, which affects the electron transport chain and causes PSII inhibition [53]. Besides, the lower photosynthesis due to the salt stress is the result of either the stomata closure that leads to a drop in the intercellular CO$_2$ additional pressure or factors regarding non-stomata that involve the depletion of chlorophyll, electron transport reaction in photosynthesis, as well as fixation of carbon [54]. The rice salinity tolerance was improved by high chlorophyll content and the overexpression of OsEXPA7 and STRK1 in plants [55, 56]. By contrast, the low chlorophyll content of STRK1-RNAi transgenic plants and oshkt1;1 mutant has been associated with salinity sensitivity [28, 56]. TFs such as bHLH are essential genetic components with a potential contribution to regulate stress tolerance in plants [57]. In rice, there are several members of the bHLH TFs, among which the U subfamily has not been investigated yet, concerning its role in salt tolerance. In this study we generated the OsbHLH024 gene editing mutant by CRISPR/Cas9 method. We introduced CRISPR/Cas9-OsbHLH024 vector into rice calli many times, but achieved one mutation, and it may have some problems with the gene editing of OsbHLH024. Our findings have demonstrated that the knockout of the OsbHLH024 confers salt tolerance in rice. The mutant osbhlh024 (A91) seedlings presented some remarkable phenotypes, including the delayed stress symptoms, the higher shoot weight, an improved survival rate and chlorophyll content under salt stress (Figure 4).

The main challenge for plants exposed to salt stress is detoxifying MDA and ROS (O$_2^-$ and H$_2$O$_2$) mainly by the SOD and POD enzymes [9]. Our results clarified that antioxidant activity in the A91 contributed to better regulation of ROS and MDA to alleviate the oxidative stress under salt stress (Figure 5). In general, the ROS (H$_2$O$_2$) levels were higher in roots than shoots under salt stress because the root is the first organ to sense the environment in plant [58]. This is supported by the observation indicating the higher SOD levels in roots than shoots, especially under salt stress by at least 1.69-fold (Figure 5e). In the shoots, ROS (H$_2$O$_2$) and MDA were significantly lower in the osbhlh024 mutant than WT, which might be due to the significance of SOD activity in the OsbHLH024 loss-of-function mutant (Figure 5a). Moreover, since O$_2^-$ belongs to the short-lived ROS, it quickly generates other major ROS, such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH$^-$), perhydroxyl radicals (OH$_2^-$), and singlet oxygen ($^1$O$_2$) [59]. Hydroxyl radicals (OH$^-$) and singlet oxygen ($^1$O$_2$) are responsible for lipid peroxidation by attacking polyunsaturated fatty acids (PUFA) [60], suggesting SOD of WT might not be enough to catalyze those ROS, resulting in higher MDA levels in shoots (Figure 5d). Furthermore, less O$_2^-$ and H$_2$O$_2$ in leaves of the A91 mutant than in the WT indicated the severity of oxidative stress in WT compared to the A91 (Figure 5i, j). On the other hand, in shoots, the POD activity was significantly lower in the A91 than WT, suggesting that the level of POD activity was dependent on the level of H$_2$O$_2$ detected in the tissues responding to the salt stress. Interestingly, the greater level of POD activity in shoots of WT than in the A91 (Figure 5b) coincided with a significant MDA level, demonstrating that salt stress was more toxic in WT than in the A91. In roots, POD was significantly higher in the A91 than in the WT, suggesting that it might act as an ROS scavenger. These results indicated that the SOD and POD activity might contribute to the production and regulation of several ROS in the shoots and roots by which the A91 maintained ROS homeostasis under salt stress.
The strategy to make rice plants tolerant to salinity depends on the ability to exclude Na\(^+\) uptake from the shoots and to keep a low ratio of Na\(^+\)/K\(^+\) [61]. Previous studies have reported the effect of salt stress mediated by a high intercellular Na\(^+\) level, which can dislocate the Ca\(^{2+}\) out of membranes and cause the K\(^+\) efflux as well as leakage from cells [62]. On the other hand, the K\(^+\) loss can be mitigated by Ca\(^{2+}\) [63], suggesting the necessity of regulating the interaction of Na\(^+\), K\(^+\) and Ca\(^{2+}\) in plants exposed to salt stress. The activation of the SOS pathway can contribute directly or indirectly to salinity tolerance via the control of Ca\(^{2+}\), K\(^+\) and Na\(^+\) homeostasis in plants [64]. Several studies have shown that overexpression of OsSTLK, SAPK1 and OsSTAP1 increased K\(^+\), and decreased Na\(^+\) and Na\(^+\)/K\(^+\) ratio [65–67]. The root is the primary source where salinity-induced genes can restrict Na\(^+\) entrance into the xylem through the roots [68], and Na\(^+\) exclusion by the roots is the most efficient strategy for enhancing plant tolerance [69]. Our data indicated that a higher expression of OsHKT1;1 could mediate the salinity tolerance in the A91 through the unloading of Na\(^+\) from roots (Figure 7g). This is in agreement with the role of AtHKT1;1 in regulating the Na\(^+\) accumulation by roots in Arabidopsis, [70] and Na\(^+\) content in rice [71]. Moreover, the downregulation of OsLEA3 and OsHKT1;1 in shoots (Figure 7a,f) and OsHKT1;3, OsHAK7, and OsSOS1 in roots (Figure 7h,j,k) of the osbhlh024 mutant might not be involved in adaption to the salt stress. These genes might be regulated by other genes in specific tissues, such as those belonging to bHLH family. Therefore, the mutation in OsbHLH024 might also function in the regulation of Na\(^+\) transportation from leaves to roots. Previous research found that the Egyptian Yasmine is more salinity tolerant than Sakha 102, but that in leaf sheaths and leaves, the expression level of OsHKT1;5 was suppressed and that the OsSOS1 expression was reduced in leaves and roots, implying that these genes have dual roles [72]. In addition, HKT1 has tissue specificity known as vascular bundle or pericycle [73], mutation of HKT1 under sos2 and sos3 mutants represses hypersensitivity to salt, indicating Na\(^+\)/K\(^+\) homeostasis of plant cells is the coordination function of HKT1 and the SOS pathway [74].

However, an elevated amount of Na\(^+\) generally increased the Na\(^+\)/K\(^+\) ratio and reduced K\(^+\), resulting in sensitivity [75]. The loss-of-function osbhlh024 mutant, A91 performed lower Na\(^+\), higher K\(^+\), lower Na\(^+\)/K\(^+\) ratio accumulation in both shoots and roots (Figure 6a–c,g–i). Earlier studies investigated that OsJRL overexpression increased the expression of the OsHKT1;3 gene that conferred salinity resistance [76], the salinity sensitive osgty-2 mutant showed low expression of OsHKT1;3 [77], and loss-of-function osrr9 and osrr10 mutants performed salinity resistance with high expression of OsHKT1;3 [78]. Significantly reduced expression of OsSOS1 in the tsd2 mutant made the plant sensitive to salinity [75], while overexpression of OsTRM13 increased the OsSOS1 gene expression to positively regulate tolerance of salt stress [79]. Furthermore, overexpression of SAPK1 revealed greater expression of OsSOS1 that turned to better performance upon salinity [66]. OsHAK7 mediates K\(^+\) transport under salt stress to ensure plant resistance [80]. The OsHKT1;3, OsHAK7, and OsSOS1 genes are involved in mediating Na\(^+\)/K\(^+\) via lowering Na\(^+\) by defending the cell’s entrance, increasing K\(^+\) accumulation in rice shoots by transporting Na\(^+\) from the cytosol to the apoplast [81,82]. OsHKT1;3, OsHAK7, and OsSOS1 implicated in conferring salt tolerance exhibit increased levels in the shoots of A91 when exposed to higher salinity (Figure 7b,d,e). Zinc is another component of enzymes involved in the process of photosynthesis, such as theribulose 1,5-bisphosphate carboxylase (RuBPC), that catalyzes the fixation of carbon dioxide under photosynthesis [83]. Additionally, accumulation of higher Ca\(^{2+}\) in roots, lower Mg\(^{2+}\) and higher Zn\(^{2+}\) for these rice plants also confer the salinity tolerance in A91, while Ca\(^{2+}\) in shoots were unchanged compared to WT, Mg\(^{2+}\) and Zn\(^{2+}\) in roots were reduced (Figure 6), these results suggesting the knockout of OsbHLH024 significantly improved the tolerance to salinity.
4. Materials and Methods

4.1. CRISPR/Cas9 Vector Construction and Rice Transformation

The CRISPR-GE website (http://crispr.hzau.edu.cn/CRISPR2, accessed on: 15 January 2018) was used to design the sequences of two targets for OsbHLH024. The sequences were launched into double single guide expression cassettes of RNA (sgRNA) by overlapping PCR. Then, the PCR of the first step was performed with U-F and gR-R primers, followed by secondary PCR with appropriate primers for each site, PpS-GGL and PgS-GG2 (for Target 1), Pps-GG2/Pgs-GGR (for Target 2), having a restriction site of BsaI. Ligation of expression cassettes of two sgRNA was made into a vector named pYLCRISPR/Cas9PubiH through the Golden Gate system of ligation [51]. Primers of oligonucleotide were used to construct the recombinant vector known as pYLCRISPR/Cas9 (Table S1). The EHA105 strain of Agrobacterium tumefaciens was then injected with binary constructs. The rice (Japonica cultivar Nipponbare) was used for the transformation of tissue. The embryogenic calli were transformed using an Agrobacterium strain; after 4 weeks of rooting in the media, rice seedlings were shifted to small plastic buckets in the greenhouse, where the controlled day temperature was 30 °C and the night temperature was 26 °C.

4.2. Mutation Detection and Assay of Transgene-Free Plant Lines

The CTAB mixture was used to extract the genomic DNA from seedling leaves, and the PCR was carried out with particular primers (Table S1). The characterization of the transgene-free plants was conducted by using plants of the T1 generation. SPL-SPR and Cas9 XU-specific primers were used to analyze the genomic DNA via PCR, followed by an agarose gel electrophoresis. The CRISPR/Cas9 vector containing OsbHLH024 and T0 transgenic plants were chosen as positive controls, whereas H2O was employed as a negative control. The positive plants with SPL-SPR and Cas9 XU-specific primers were utilized to amplify the expected fragments across Target 1 and 2. The CRISPR/Cas9 positive plant DNA PCR was performed with target primers (F and R). The products of PCR were directly sequenced, using target primers (F and R) with the Sanger method. Positive and negative controls were treated as wild-type (WT) and verified transgenic plants, respectively. In summary, products of PCR were sequenced from the SunYa company.

4.3. Phenotypes Observation of the A91 Mutant

WT Nipponbare seeds and CRISPR/Cas9-mediated OsbHLH024 homozygous mutant (A91) seeds were used. Seeds were first washed 3 times with sterilized water, then surface-sterilized with 75% ethanol for 2 min, shaken with sodium hypochlorite solution (1%) for 20 min, rinsed 5 times in sterile distilled water, and kept for 3 days with tap water, germinated in 37 °C for 1 more day. After germination, seeds were put in the seedbed, and one-month-old plants were shifted to the field. At the maturity stage, we observed the plant phenotypes for statistical analysis.

4.4. Total Chlorophyll Content and Fluorescence Determination

The leaf (0.02 g) was sliced into tiny pieces and maintained in a 2 mL tube with 1 mL of acetone overnight at 25 °C. When the leaves were entirely white, samples were centrifuged (10,000 rpm) at 4 °C for 15 min. A Synergy H1 microplate reader was used to detect the absorbance at 663 nm and 645 nm. The supernatant from each sample was collected (200 µL) and used to read absorbance on a 96-plate (BioTek). Calculations were carried out using the formula [84]:

\[
\text{Chlorophyll a} = \left[\frac{12.7(A663) - 2.69(A645)}{1000 \times W}\right] \\
\text{Chlorophyll b} = \left[\frac{22.9(A645) - 4.68(A663)}{1000 \times W}\right] \\
\text{Chlorophyll (a + b) = Chlorophyll a + Chlorophyll b.}
\]
where \( A = \text{absorbance (wavelengths)}, V = \text{final volume (chlorophyll extract)}, W = \text{fresh weight (extracted tissue)} \).

A SPAD 502 plus meter was used to measure the chlorophyll content of fully inflated leaves. The chlorophyll meter, or SPAD meter, is an easy, transportable diagnostic instrument utilized to estimate the leaves’ relative chlorophyll content \[85\]. The OS-30p (product of OPTI-Sciences, Hudson, NH, USA) was used to calculate the fluorescence of chlorophyll. Dark adaption of plants was confirmed for 30 min prior to quantification at night. The value \((F_v/F_m)\) was determined using the prior technique \[86\].

4.5. Salt Stress Experiments

For the salt experiment, seeds were first cleaned 3 times with sterilized water, followed by a surface-sterilization with 75% ethanol for 2 min, then 20 min of shaking with a 1% sodium hypochlorite solution, and finally washed 5 times and kept in a 0.5% \( \text{H}_2\text{O}_2 \) solution for 2 days to break dormancy. Seeds were kept in tap water at 37°C until the occurrence of germination. The germinated seedlings were subjected to a 0.3 g/L Yoshida nutrient solution \[87\]. At this stage, seedlings were grown at 28°C for 14 h of light and 10 h of darkness for 3 days. Thereafter, the seedlings were shifted to a 0.5 g/L Yoshida nutrient solution up to 21 days. Treatments with NaCl 150 mM solution started at 21-day-old seedlings for 7 days. After that, we phenotyped the seedlings within 7 days of recovery. Solutions were changed every 3 days.

4.6. Antioxidants Measurements

After 7 days of salt treatment (150 mM NaCl), 0.2 g of shoot, and 0.1 g of root samples were harvested in liquid nitrogen. The enzymes were extracted under freezing conditions (liquid nitrogen) and homogenized in a 50 mM (potassium buffer solution) KBS (pH 7.8). Samples were centrifuged for 15 min at 13,000 rpm. The supernatant was collected in 2 mL sterilized tubes at 4°C. The activities of SOD and POD were spectrophotometrically measured from the extracted supernatant. The activity of POD was measured according to the protocol of Zhou and Leul \[88\]. The reaction solution of 210 µL included 15 µL distilled water, 5 µL enzyme extract and a 190 µL combination of 1% guaiacol and 0.4% \( \text{H}_2\text{O}_2 \) in the presence of 50 mM KBS (pH 7.0). The Synergy H1 microplate (BioTek) reader was used at 470 nm to record variations in absorbance associated with guaiacol oxidation \( (\varepsilon, \text{constant} = 25.5 \text{mM}^{-1} \text{cm}^{-1}) \) \[89\]. For the analysis of SOD, the volume of enzyme necessary to achieve a 50% reduction of the nitroblue tetrazolium (NBT) degradation was used to calculate the SOD activity of one unit. A total of 210 µL reaction solution, 20 µL enzyme extract and 190 µL mixture of NBT 75 µM, \( \text{Na}_2\text{EDTA} \) 0.1 mM, Methionine 13 mM with riboflavin 2 µM were prepared in 50 mM KBS buffer (pH 7.8). Around 4000 lux light was used to expose samples for 20 min, to achieve the reaction of SOD, and urgently covered with fuel paper to terminate the reaction, as well as blank control in the dark, and immediately measured at 560 nm wavelength by the Synergy H1 microplate reader (BioTek) \[90\].

4.7. Measurements of the Lipid Peroxidation (MDA) and \( \text{H}_2\text{O}_2 \)

Lipid peroxidation was examined based on malondialdehyde (MDA). To determine \( \text{H}_2\text{O}_2 \) content and MDA, 0.2 g shoots and 0.1 g of root samples were extracted with trichloroacetic acid (0.1% TCA) under cold conditions. A combination of 125 µL enzyme extract and a 500 µL mixture of thiobarbituric acid (0.5%) and trichloroacetic acid (20%) was used to produce the reaction under 95°C (30 min), and the reaction was stopped by cooling the samples on ice. All samples were centrifuged for 10 min, at 10,000 rpm. The Synergy H1 microplate reader (BioTek) was used to assess the reaction at 532 nm and 600 nm wavelengths (BioTek) \[91\]. To determine \( \text{H}_2\text{O}_2 \) content, 50 µL supernatant was put together with 50 µL of 10 mM KBS (pH 7.0) and 100 µL of 1 M potassium iodide (KI). The \( \text{H}_2\text{O}_2 \) was measured at a wavelength of 390 nm. The quantity of \( \text{H}_2\text{O}_2 \) in the sample was calculated using a calibration curve \[90\] (Figure S4).
4.8. Histochemical Staining

The NBT and DAB staining were implemented as previously presented [92] with modifications. To identify superoxide radicals (O$_2^-$), 0.2% NBT was produced with 50 mM KPB (pH 7.5), and to identify H$_2$O$_2$, staining with DAB (solution 1 mg/1 mL) was used. For staining, both the leaves of WT and the A91 were collected after 4 days of 150 mM stress. The samples were kept in the dark (for DAB 2 days and NBT 1 day). Six leaves from three different plants were chopped into small pieces and placed in a 5 mL tube with a staining reagent for each treatment. After the period described above, the leaves were stored in 100% ethanol for 1 day to eliminate the color due to chlorophyll. Leaves were photographed using a microscope named LEICA MZ 95.

4.9. Gene Expression Analysis

For evaluation of the expression pattern of OsbHLH024, eight different tissues of shoots (7 days), roots (7 days), shoots (21 days), roots (21 days), leaf sheath, leaf blade, flag leaf angle, and seed (14 days) after pollination were collected for RNA extraction. Tissues of shoots and roots of plants without/with 150 mM NaCl treatments were collected for RNA extraction. The RNA samples were extracted by TRIZol® reagent (Ambion, Carlsbad, CA, USA), and around 1 g (RNA) was utilized to synthesize (cDNA) by the HiScript II-RT Supermix qPCR (+gDNA wiper) kit of the Vazyme company. A Hieff qPCR SYBER green master mix kit was applied to the Roche Light Cycler® 96 (Roche, Basel, Switzerland). The relative expression levels were assessed by applying the double delta system [93], and OsActin1 was adjusted as an internal control.

4.10. Elements Analysis

The contents of Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ were measured with modification as described previously [94]. Samples (roots and shoots) were first dried for two and half days at 70 °C, followed by a digestion using 68% HNO$_3$. Three steps were performed, including 100 °C, 120 °C, and 140 °C, each step for 2 h. The 0.05 g of dried sample was digested with 2 mL of HNO$_3$, and the volume was calibrated to 50 mL by adding the distilled water. Finally, elemental analysis results were estimated from a flame photometer (FP-6410).

5. Conclusions

In conclusion, this study revealed that the OsbHLH024 negatively regulates the functions of Na$^+$ and K$^+$ transporter genes, and the osbhlh024 mutant (A91) showed a salinity tolerant phenotype. Ion homeostasis (specifically Na$^+$ and K$^+$) of osbhlh024 mutant leads to more tolerance, and antioxidants control ROS by suppressing the higher accumulation of MDA and H$_2$O$_2$. Moreover, the physiological balance of chlorophyll and photosynthesis confers the tolerance level. Therefore, OsbHLH024 confers a promising tool to improve the tolerance of rice to salt stress, which will bear insights into the OsbHLH024-mediated regulatory apparatus of salinity tolerance and provide a better perception of the importance of the OsbHLH024 gene in relation to abiotic stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11091184/s1, Figure S1. A linear form of the sgRNA vectors; Figure S2. Mutation process showing chromatograph of OsbHLH024; Figure S3: The growth characteristics of the A91 and WT after 7 days of 150 mM NaCl stress of 21-day-old seedlings; Figure S4: Standard curve for H$_2$O$_2$ determination; Table S1: Primers used in this study; Table S2: The agronomic traits of A91 and the WT in the field.

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References
1. Van Zelm, E.; Zhang, Y.; Testerink, C. Salt tolerance mechanisms of plants. *Annu. Rev. Plant Biol.* 2020, 71, 403–433. [CrossRef]
2. Munns, R.; Tester, M. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 2008, 59, 651–681. [CrossRef]
3. Hussain, S.; Zhang, J.-H.; Zhong, C.; Zhu, L.-F.; CAO, X.-C.; YU, S.-M.; BOHR, J.A.; HU, J.-J.; JIN, Q.-Y. Effects of salt stress on rice growth, development characteristics, and the regulating ways: A review. *J. Integr. Agric.* 2017, 16, 2357–2374. [CrossRef]
4. Yang, Y.; Guo, Y. Elucidating the molecular mechanisms mediating plant salt-stress responses. *New Phytol.* 2018, 217, 523–539. [CrossRef]
5. Arif, Y.; Singh, P.; Siddiqui, H.; Bajguz, A.; Hayat, S. Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance. *Plant Physiol. Biochem.* 2020, 156, 64–77. [CrossRef]
6. McCord, J.M. The evolution of free radicals and oxidative stress. *Am. J. Med.* 2000, 108, 652–659. [CrossRef]
7. Ashraf, M.; Aftab, H.; Harris, P.; KWON, S.; TUTEJA, N. Some prospective strategies for improving crop salt tolerance. *Trends Plant Sci.* 2010, 15, 78–82. [CrossRef]
8. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 2010, 48, 909–930. [CrossRef]
9. Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 2002, 7, 405–410. [CrossRef]
10. Miller, G.; Suzuki, N.; Ciﬁcti-Yilmaz, S.; Mittler, R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* 2010, 33, 453–467. [CrossRef]
11. Vaidyanathan, H.; Sivakumar, P.; Chakrabarty, R.; Thomas, G. Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa L.*)—differential response in salt-tolerant and sensitive varieties. *Plant Sci.* 2003, 165, 1411–1418. [CrossRef]
12. Demiral, T.; Türkân, I. Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environ. Exp. Bot.* 2005, 53, 247–257. [CrossRef]
13. Racchi, M.L. Antioxidant defenses in plants with attention to *Prunus* and *Citrus* spp. *Antioxidants* 2013, 2, 340–369. [CrossRef] [PubMed]
14. Gill, S.S.; Anjum, N.A.; Gill, R.; Yadav, S.; Hasanuzzaman, M.; Fujita, M.; Mishra, P.; Sabat, S.C.; Tuteja, N. Superoxide dismutase—Mentor of abiotic stress tolerance in crop plants. *Environ. Sci. Pollut. Res.* 2015, 22, 10375–10394. [CrossRef] [PubMed]
15. Razzaka, M.A.; Talukder, N.M.; Islam, M.T.; Dutta, R.K. Salinity effect on mineral nutrient distribution along roots and shoots of rice (*Oryza sativa L.*) genotypes differing in salt tolerance. *Arch. Agron. Soil Sci.* 2011, 57, 33–45. [CrossRef]
16. Rengel, Z. The role of calcium in salt toxicity. *Plant Cell Environ.* 1992, 15, 625–632. [CrossRef]
17. Weisany, W.; Sohrabi, Y.; Heidari, G.; Siosemardeh, A.; Badakhsan, H. Effects of zinc application on growth, absorption and distribution of mineral nutrients under salinity stress in soybean (*Glycine max L.*) *J. Plant Nutr.* 2014, 37, 2255–2269. [CrossRef]
18. Amanullah, I.; Inamullah, X. Dry matter partitioning and harvest index differ in rice genotypes with variable rates of phosphorus and zinc nutrition. *Rice Sci.* 2016, 23, 78–87. [CrossRef]
19. Shahid, M.A.; Sarkhosh, A.; Khan, N.; Balal, R.M.; Ali, S.; Rossi, L.; Gómez, C.; Mattson, N.; Nasim, W.; Garcia-Sanchez, F. Insights into the Physiological and Biochemical Impacts of Salt Stress on Plant Growth and Development. *Agronomy* 2020, 10, 938. [CrossRef]
20. Szymańska, K.P.; Polkovska-Kowalczyk, L.; Lichocka, M.; Maszkowska, J.; Dobrowolska, G. SNF1-related protein kinases SnRK2. 4 and SnRK2. 10 modulate ROS homeostasis in plant response to salt stress. *Int. J. Mol. Sci.* 2019, 20, 143. [CrossRef]
21. Hu, T.Z. OsLEA3, a late embryogenesis abundant protein gene from rice, confers tolerance to water deficit and salt stress to transgenic rice. *Russ. J. Plant Physiol.* 2008, 55, 530–537. [CrossRef]
22. Kumar, K.; Kumar, M.; Kim, S.-R.; Ryu, H.; Cho, Y.-G. Insights into genomics of salt stress response in rice. *Rice* 2013, 6, 1–15. [CrossRef] [PubMed]
23. Blumwald, E.; Aharon, G.S.; Apse, M.P. Sodium transport in plant cells. *Biochim. Biophys. Acta Biomembr.* 2000, 1465, 140–151. [CrossRef]
24. Basu, S.; Kumar, A.; Benazir, I.; Kumar, G. Reassessing the role of ion homeostasis for improving salinity tolerance in crop plants. *Physiol. Plant.* 2021, 171, 502–519. [CrossRef] [PubMed]
25. Hamamoto, S.; Horie, T.; Hauser, F.; Deinlein, U.; Schroeder, J.I.; Uozumi, N. HKT transporters mediate salt stress resistance in plants: From structure and function to the field. Curr. Opin. Biotechnol. 2015, 32, 113–120. [CrossRef]

26. Almeida, P.; Katschnig, D.; De Boer, A.H. HKT transporters—State of the art. Int. J. Mol. Sci. 2013, 14, 20359–20385. [CrossRef]

27. Garcia-de las, B.; Serr, M.E.; Bañuelos, M.A.; Rodríguez-Navarro, A. Sodium transport and HKT transporters: The rice model. Plant J. 2003, 34, 788–801. [CrossRef]

28. Wang, R.; Jing, W.; Xiao, L.; Jin, Y.; Shen, L.; Zhang, W. The rice high-affinity potassium transporter1;1 is involved in salt tolerance and regulated by an MYB-type transcription factor. Plant Physiol. 2015, 168, 1076–1090. [CrossRef]

29. Anwar, A.; Kim, J.-K. Transgenic breeding approaches for improving abiotic stress tolerance: Recent progress and future perspectives. Int. J. Mol. Sci. 2020, 21, 2695. [CrossRef]

30. Rosas-Santiago, P.; Lagunas-Gómez, D.; Barkla, B.J.; Vera-Estrella, R.; Lalone, S.; Jones, A.; Fronmer, W.B.; Zimmermannova, O.; Šychrová, H.; Pandoja, O. Identification of rice cornichon as a possible cargo receptor for the Golgi-localized sodium transporter OsHKT1;1. J. Exp. Bot. 2015, 66, 2733–2748. [CrossRef]

31. Mishra, P.; Mishra, V.; Singh, N.K.; Rai, V. Gene expression dynamics of HKT family genes in salt-tolerant and salt-sensitive indica rice cultivars. Indian J. Genet 2017, 77, 364–370. [CrossRef]

32. Kobayashi, N.I.; Yamaji, N.; Yamamoto, H.; Okubo, K.; Ueno, H.; Costa, A.; Taniot, K.; Matsumura, H.; Fujii-Kashino, M.; Horüchi, T. OsHKT1;5 mediates Na+ exclusion in the vasculature to protect leaf blades and reproductive tissues from salt toxicity in rice. Plant J. 2017, 91, 657–670. [CrossRef] [PubMed]

33. Horie, T.; Costa, A.; Kim, T.H.; Han, M.J.; Horie, R.; Leung, H.Y.; Miyao, A.; Hirohika, H.; An, G.; Schroeder, J.I. Rice OsHKT2;1 transporter mediates large Na+ influx component into K+-starved roots for growth. EMBO J. 2007, 26, 3003–3014. [CrossRef] [PubMed]

34. Okada, T.; Nakayama, H.; Shinmyo, A.; Yoshida, K. Expression of OsHAK genes encoding potassium ion transporters in rice. Plant Biotechnol.-Nar. 2008, 25, 241–245. [CrossRef]

35. Shi, H.; Ishitani, M.; Kim, C.; Zhu, J.-K. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+ /H+ antiporter. Proc. Natl. Acad. Sci. USA 2000, 97, 6896–6901. [CrossRef]

36. Wang, H.; Wang, H.; Shao, H.; Tang, X. Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology. Front. Plant Sci. 2016, 7, 67. [CrossRef]

37. Latchman, D.S. Transcription factors: An overview. Int. J. Exp. Pathol. 1993, 74, 417. [CrossRef]

38. Chen, W.J.; Zhu, T. Networks of transcription factors with roles in environmental stress response. Trends Plant Sci. 2004, 9, 591–596. [CrossRef]

39. Li, X.; Duan, X.; Jiang, H.; Sun, Y.; Tang, Y.; Yuan, Z.; Guo, J.; Liang, W.; Chen, L.; Yin, J.; et al. Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and Arabidopsis. Plant Physiol. 2006, 141, 1167–1184. [CrossRef]

40. Guo, J.; Sun, B.; He, H.; Zhang, Y.; Tian, H.; Wang, B. Current Understanding of bHLH Transcription Factors in Plant Abiotic Stress Tolerance. Int. J. Mol. Sci. 2021, 22, 4921. [CrossRef]

41. Jiang, Y.; Yang, B.; Deyholos, M.K. Functional characterization of the Arabidopsis bHLH92 transcription factor in abiotic stress. Mol. Genet. Genom. 2009, 282, 503–516. [CrossRef] [PubMed]

42. Chen, H.-C.; Hsieh-Feng, V.; Liao, P.-C.; Cheng, W.-H.; Liu, L.-Y.; Yang, Y.-W.; Lai, M.-H.; Chang, M.-C. The function of OsbHLH068 is partially redundant with its homolog, AtbHLH112, in the regulation of the salt stress response but has opposite functions to control flowering in Arabidopsis. Mol. Genet. Genomic. 2010, 283, 503–516. [CrossRef] [PubMed]

43. Kavas, M.; Baloglu, M.C.; Atabay, E.S.; Ziplar, U.T.; Ziplar, U.T.; Dasgan, U.Y.; Unver, T. Genome-wide characterization and expression analysis of common bean bHLH transcription factors in response to excess salt concentration. Mol. Genet. Genom. 2016, 291, 129–143. [CrossRef] [PubMed]

44. Wang, F.; Zhu, H.; Chen, D.; Li, Z.; Peng, R.; Yao, Q. A grape bHLH transcription factor gene, VvbHLH1, increases the accumulation of flavonoids and enhances salt and drought tolerance in transgenic Arabidopsis thaliana. Plant Cell Tissue Organ Cult. (PCTOC) 2016, 125, 387–398. [CrossRef]

45. Wang, Y.; Wang, S.; Tian, Y.; Wang, Q.; Chen, S.; Li, H.; Ma, C.; Li, H. Functional Characterization of a Sugar Beet BvbHLH93 Transcription Factor in Salt Stress Tolerance. Int. J. Mol. Sci. 2021, 22, 3669. [CrossRef]

46. Song, Y.; Li, S.; Sui, Y.; Zheng, H.; Han, G.; Sun, X.; Yang, W.; Wang, H.; Zhuang, K.; Kong, F. SbbHLH185, a bHLH Member, Modulates Resilience to Salt Stress By Regulating Root Hair Growth in Sweet Sorghum. Theor. Appl. Genet. 2021, 135, 201–216. [CrossRef] [PubMed]

47. Liu, D.; Li, Y.-Y.; Zhou, Z.-C.; Xiang, X.; Liu, X.; Wang, J.; Hu, Z.-R.; Xiang, S.-P.; Li, W.; Xiao, Q.-Z. Tobacco transcription factor bHLH123 improves salt tolerance by activating NADPH oxidase NrBohE expression. Plant Physiol. 2021, 186, 1706–1720. [CrossRef]

48. Chen, H.-C.; Cheng, W.-H.; Hong, C.-Y.; Chang, Y.-S.; Chang, M.-C. The transcription factor OsbHLH035 mediates seed germination and enables seedling recovery from salt stress through ABA-dependent and ABA-independent pathways, respectively. Rice 2018, 11, 50. [CrossRef]

49. Wu, H.; Ye, H.; Yao, R.; Zhang, T.; Xiong, L. OsJAZ9 acts as a transcriptional regulator in jasmonate signaling and modulates salt stress tolerance in rice. Plant Sci. 2015, 232, 1–12. [CrossRef]

50. Li, C.; Tao, R.-F.; Li, Y.; Duan, M.-H.; Xu, J.-H. Transcriptome analysis of the thermosensitive genic male-sterile line provides new insights into fertility alteration in rice (Oryza sativa). Genomics 2020, 112, 2119–2129. [CrossRef]
Plants 2022, 11, 1184

51. Ma, X.; Zhang, Q.; Zhu, Q.; Liu, W.; Chen, Y.; Qiu, R.; Wang, B.; Yang, Z.; Li, H.; Lin, Y. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol. Plant* **2015**, *8*, 1274–1284. [CrossRef] [PubMed]

52. Hussain, S.; Shaukat, M.; Ashraf, M.; Zhu, C.; Jin, Q.; Zhang, J. Salinity stress in arid and semi-arid climates: Effects and management in field crops. *Clim. Chang. Agric.* **2019**, *13*. [CrossRef]

53. Sudhir, P.; Murthy, S. Effects of salt stress on basic processes of photosynthesis. *Photosynthetica* **2004**, *42*, 481–486. [CrossRef]

54. Acosta-Motos, J.R.; Ortuño, M.F.; Bernal-Vicente, A.; Díaz-Vivancos, P.; Sanchez-Blanco, M.J.; Hernandez, J.A. Plant responses to salt stress: Adaptive mechanisms. *Agronomy* **2017**, *7*, 18. [CrossRef]

55. Jadamba, C.; Kang, K.; Paek, N.-C.; Lee, S.I.; Yoo, S.-C. Overexpression of rice expansin7 (Osexpa7) confers enhanced tolerance to salt stress in rice. *Int. J. Mol. Sci.* **2020**, *21*, 454. [CrossRef] [PubMed]

56. Zhou, Y.B.; Liu, C.; Tang, D.Y.; Yan, L.; Wang, D.; Yang, Y.Z.; Gui, J.S.; Zhao, X.Y.; Li, L.G.; Tang, X.D.; et al. The Receptor-Like Cytoplasmic Kinase STRK1 Phosphorylates and Activates CatC, Thereby Regulating $H_2O_2$ Homeostasis and Improving Salt Tolerance in Rice. *Plant Cell* **2018**, *30*, 1100–1118. [CrossRef]

57. Khan, S.A.; Li, M.Z.; Wang, S.M.; Yin, H.J. Revisiting the role of plant transcription factors in the battle against abiotic stress. *Int. J. Mol. Sci.* **2018**, *19*, 1634. [CrossRef]

58. Sun, X.; Chen, F.; Yuan, L.; Mi, G. The physiological mechanism underlying root elongation in response to nitrogen deficiency in crop plants. *Planta* **2020**, *251*, 84. [CrossRef] [PubMed]

59. Das, K.; Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* **2014**, *2*, 53. [CrossRef]

60. Hasanuzzaman, M.; Bhuyan, M.; Parvin, K.; Bhuiyan, T.F.; Anee, T.I.; Nahar, K.; Hossen, M.; Zulfiqar, F.; Alam, M.; Fujita, M. Regulation of ROS metabolism in plants under environmental stress: A review of recent experimental evidence. *Int. J. Mol. Sci.* **2020**, *21*, 8695. [CrossRef]

61. Martinez-Atienza, J.; Jiang, X.; Garciaedelas, B.; Mendoza, I.; Zhu, J.-K.; Pardo, J.M.; Quintero, F.J. Conservation of the salt overly sensitive pathway in rice. *Plant Physiol.* **2007**, *141*, 1001–1012. [CrossRef] [PubMed]

62. Cramer, G.; Läuchli, A.; Polito, V. Displacement of Ca$^{2+}$ from the plasma membrane during osmotic stress in wheat roots. *Plant, Cell Environ.* **2000**, *23*, 1653–1665. [CrossRef] [PubMed]

63. Shabala, S.; Demidchik, V.; Shabala, L.; Cuin, T.A.; Smith, S.J.; Miller, A.J.; Davies, J.M.; Newman, I.A. Extracellular Ca$^{2+}$ affects ion homeostasis and salt tolerance. *Int. J. Mol. Sci.* **2019**, *20*, 1184. [CrossRef]

64. Quintero, F.J.; Ohta, M.; Shi, H.; Zhu, J.-K.; Pardo, J.M. Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na$^{+}$ homeostasis. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 9061–9066. [CrossRef]

65. Lin, F.; Li, S.; Wang, K.; Tian, H.; Gao, J.; Zhao, Q.; Du, C. A leucine-rich repeat receptor-like kinase, OsSTLK, modulates salt tolerance in rice. *Plant Sci.* **2020**, *296*, 110465. [CrossRef]

66. Lou, D.; Yang, H.; Yu, D. The sucrose non-fermenting-1-related protein kinases SAPK1 and SAPK2 function collaboratively as positive regulators of salt stress tolerance in rice. *BMC Plant Biol.* **2015**, *15*, 257–267. [CrossRef] [PubMed]

67. Fang, C.; Li, K.; Wu, Y.; Wang, D.; Zhou, J.; Liu, X.; Li, Y.; Jin, C.; Liu, X.; Mur, L.A.J. OsTSD2-mediated cell wall modification affects ion homeostasis and salt tolerance. *Plant, Cell Environ.* **2019**, *42*, 1503–1512. [CrossRef] [PubMed]

68. He, X.; Li, L.; Xu, H.; Xi, J.; Cao, X.; Xu, H.; Rong, S.; Dong, Y.; Wang, C.; Chen, R. A rice jacalin-related mannos-binding lectin gene, OsJRL, enhances Escherichia coli viability under high salinity stress and improves salinity tolerance of rice. *Plant Biol.* **2017**, *19*, 257–267. [CrossRef] [PubMed]
77. Liu, X.; Wu, D.; Shan, T.; Xu, S.; Qin, R.; Li, H.; Negm, M.; Wu, D.; Li, J. The trihelix transcription factor OsGTγ-2 is involved in adaption to salt stress in rice. *Plant Mol. Biol.* 2020, 103, 545–560. [CrossRef]

78. Wang, W.-C.; Lin, T.-C.; Kieber, J.; Tsai, Y.-C. Response regulators 9 and 10 negatively regulate salinity tolerance in rice. *Plant Cell Physiol.* 2019, 60, 2549–2563. [CrossRef]

79. Wang, Y.; Li, D.; Gao, J.; Li, X.; Zhang, R.; Jin, X.; Hu, Z.; Zheng, B.; Persson, S.; Chen, P. The 2′-O-methyladenosine nucleoside modification gene OsTRM13 positively regulates salt stress tolerance in rice. *J. Experiment. Bot.* 2017, 68, 1479–1491. [CrossRef]

80. Banuelos, M.A.; Garcia-delblas, B.; Cubero, B.; Rodriguez-Navarro, A. Inventory and functional characterization of the HAK potassium transporters of rice. *Plant Physiol.* 2002, 130, 784–795. [CrossRef]

81. Chuamnakthong, S.; Nampei, M.; Ueda, A. Characterization of Na+ exclusion mechanism in rice under saline-alkaline stress conditions. *Plant Sci.* 2019, 287, 110171. [CrossRef] [PubMed]

82. Imran, S.; Tsuchiya, Y.; Tran, S.T.H.; Katsuhara, M. Identification and Characterization of Rice OsHKT1;3 Variants. *Plants* 2021, 10, 2006. [CrossRef] [PubMed]

83. Brown, P.H.; Cakmak, I.; Zhang, Q. Form and function of zinc plants. In *Zinc in Soils and Plants*; Springer: Berlin/Heidelberg, Germany, 1993; pp. 93–106.

84. Manjula, M.; Kumari, N.V. Effect of Drought on the Growth and Development of Mulberry. *Int. J. Appl. Res.* 2017, 12, 339–355.

85. Gholizadeh, A.; Amin, M.; Anuar, A.; Aimrun, W. Evaluation of SPAD chlorophyll meter in two different rice growth stages and its temporal variability. *Eur. J. Soil. Res.* 2009, 37, 591–598.

86. Cai, K.; Chen, X.; Han, Z.; Wu, X.; Zhang, S.; Li, Q.; Nazir, M.M.; Zhang, G.; Zeng, F. Screening of worldwide barley collection for drought tolerance: The assessment of various physiological measures as the selection criteria. *Front. Plant Sci.* 2020, 11, 1159. [CrossRef] [PubMed]

87. Coronel, V.; Akita, S.; Yoshida, S. Aluminium toxicity tolerance in rice (*Oryza sativa*) seedlings. In *Plant nutrition—Physiology and Applications*; Springer: Berlin/Heidelberg, Germany, 1990; pp. 357–363.

88. Zhou, W.; Leul, M. Uniconazole-induced tolerance of rape plants to heat stress in relation to changes in hormonal levels, enzyme activities and lipid peroxidation. *Plant Growth Regul.* 1999, 27, 99–104. [CrossRef]

89. Giannopolitis, C.N.; Ries, S.K. Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiol.* 1977, 59, 309–314. [CrossRef]

90. Lwalaba, J.W.; Zvobgo, G.; Fu, L.; Zhang, X.; Mwamba, T.M.; Muhammad, N.; Mundende, R.P.M.; Zhang, G. Alleviating effects of calcium on cobalt toxicity in two barley genotypes differing in cobalt tolerance. *Ecotoxicol. Environ. Saf.* 2017, 139, 488–495. [CrossRef]

91. Heath, R.L.; Packer, L. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* 1968, 125, 189–198. [CrossRef]

92. Alfatih, A.; Wu, J.; Jan, S.U.; Zhang, Z.S.; Xia, J.Q.; Xiang, C.B. Loss of rice PARAQUAT TOLERANCE 3 confers enhanced resistance to abiotic stresses and increases grain yield in field. *Plant Cell Environ.* 2020, 43, 2743–2754. [CrossRef]

93. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta \Delta C T}$ method. *Methods* 2001, 25, 402–408. [CrossRef] [PubMed]

94. Azhar, N.; Su, N.; Shabala, L.; Shabala, S. Exogenously applied 24-epibrassinolide (EBL) ameliorates detrimental effects of salinity by reducing K+ efflux via depolarization-activated K+ channels. *Plant Cell Physiol.* 2017, 58, 802–810. [CrossRef] [PubMed]