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Response Regulators 9 and 10 Negatively Regulate Salinity Tolerance in Rice

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Cytokinins are involved in the regulation of many plant growth and development processes, and function in response to abiotic stress. Cytokinin signaling is similar to the prokaryotic two-component signaling systems and includes the transcriptional upregulation of type-A response regulators (RRs), which in turn act to inhibit cytokinin signal response via negative feedback. Cytokinin signaling consists of several gene families and only a handful full of genes is studied. In this study, we demonstrated the function of two highly identical type-A RR genes from rice, *OsRR9* and *OsRR10*, which are induced by cytokinin and only OsRR10 repressed by salinity stress in rice. Loss-of-function mutations give rise to mutant genes, *osrr9*/*osrr10*, which have higher salinity tolerance than wild type rice seedlings. The transcriptomic analysis uncovered several ion transporter genes, which were upregulated in response to salt stress in the *osrr9*/*osrr10* mutants relative to the wild type seedlings. These include high-affinity potassium transporters, such as *OsHKT1;1*, *OsHKT1;3* and *OsHKT2;1*, which play an important role in sodium and potassium homeostasis. In addition, disruption of the genes *OsRR9* and *OsRR10* also affects the expression of multiple genes related to photosynthesis, transcription and phytohormone signaling. Taken together, these results suggest that the genes *OsRR9* and *OsRR10* function as negative regulators in response to salinity in rice.

Keywords: CRISPR/Cas9 • Cytokinin signaling • Ion transporter • *Oryza sativa* • Salt stress.

Introduction

Salinity is a major environmental factor limiting crop production. Many crops are unable to grow in saline soils with an electrical conductivity higher than 4 dS/m (4 dS/m ≈ 40 mM NaCl; Qadir et al. 2000). Rice evolved in freshwater marshes. It is a salt-sensitive species and the yield of many rice varieties can be reduced by up to 50% in response to 50 mM NaCl (Radanielson et al. 2018). Salinity influences plant growth in two phases. First, by reducing the water potential, similar to the effect of osmotic stress, whereby plants respond by translocating solutes to roots, closing stomata and reducing shoot growth. Second, a slower ionic phase follows, which results in the accumulation of toxic levels of salt in the leaves, leading to accelerated senescence and reduced uptake of various nutrients.

Sodium ions are the main toxic elements associated with salt stress. Under salt stress, glycophytes generally store low concentrations of Na^+ which prevents above-ground Na^+ from reaching toxic levels (Munns and Tester 2008, Hauser and Horie 2010, Deinlein et al. 2014). In order to maintain cellular ion homeostasis, ion pumps (e.g. symporters, antiporters and carrier proteins) are present on cell membranes. There are three major Na^+ transport systems in plants to reduce Na^+ toxicity in shoots, including Na^+ exclusion in the rhizosphere, Na^+ sequestration in vacuoles and Na^+ loading or unloading in the xylem. The excretion of Na^+ from the cytoplasm to the extracellular space requires active transport under salt stress. A previous study showed that a Na^+/H^+ antiporter, salt overly sensitive 1 (SOS1), is driven by an H^+ gradient generated by H^+-ATPases on the plasma membrane (Blumwald et al. 2000, Hasegawa et al. 2000).

Phytohormones also regulate plant responses to various abiotic stresses (Peleg and Blumwald 2011, Fahad et al. 2015, Bielach et al. 2017). Cytokinins can have both positive and negative effects on stress tolerance. In many studies, a decrease in active cytokinin concentration is observed in response to long-term stress (Albacete et al. 2008, Ghanem et al. 2008, Zwack and Rashotte 2015). However, during the intial stages of water stress, cytokinin (*trans*-zeatin riboside, ZR) levels may rapidly rise in the xylem sap, which reduce thereafter (Hansen and Dörffling 2003, Alvarez et al. 2012). Cytokinins usually increase during the rehydration period. This suggests dynamics in cytokinin levels in response to stress.

Several studies have shown that cytokinins play essential roles in stress response. Exogenous application of cytokinin caused increased sensitivity to salt stress in *Phaseolus vulgaris* (Kirkham et al. 1974). In Arabidopsis, reducing cytokinin levels either by enhanced degradation or reduced biosynthesis increased the drought and salt tolerance and enhanced the sensitivity of those plants to ABA (Nishiyama et al. 2011). On the other hand, the application of cytokinin to some species, including *Solanum melongena*, wheat and potato, resulted in an enhancement of salinity tolerance (Naqvi et al. 1982, Abdullah and Ahmad 1990, Wu et al. 2014). Disruption of *CKX2* in rice, which encodes an enzyme that degrades cytokinin, resulted in a

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higher chlorophyll content, relative water content, plant height, photosynthesis rate and yield in comparison with the wild type under salt stress (Joshi et al. 2018).

Mutants for cytokinin receptor histidine kinases, *ahk2* and *ahk3* single mutants and the *ahk2ahk3* double mutant, showed a higher tolerance to salt stress (Tran et al. 2007). A recent study indicated differences between individual cytokinin receptors, showing a mild positive effect of AHK2 (Kumar and Verslues 2015). The histidine phosphotransfer proteins (AHPs) are downstream components of cytokinin signaling pathway, and were reported to be negative regulators of drought stress in Arabidopsis (Nishiyama et al. 2013). *OsAHP1/OsAHP2* knockdown rice seedlings revealed a hypersensitive to salt stress phenotype, but were more resistant to mannitol relative to wild type (Sun et al. 2014). Type-A response regulators (type-A RRs) are primary response genes induced by cytokinin (D'Agostino et al. 2000) that negatively regulate cytokinin signaling. Thirteen type-A RRs have been identified in rice, most of which are also induced by cytokinin (Tsai et al. 2012). One clade of the type-A RRs, subfamily A-II, contains both rice (OsRR4, OsRR9 and OsRR10) and Arabidopsis (AtARR8 and AtARR9) members. AtARR8 and AtARR9 are negative regulators of cytokinin signaling (To et al. 2004) and also play roles in circadian rhythm (Salome et al. 2006, Ishida et al. 2008). OsRR9 and OsRR10, whose amino acid sequences are 99% identical to each other, are localized in the nucleus and, similar to other type-A RRs, are transcriptionally induced by cytokinin (Tsai et al. 2012).

When plants are exposed to abiotic stresses, such as low temperature, osmotic pressure, drought or salt stress, cytokinin signaling genes are differentially expressed at different time points and in different tissues. The clade II type-A RRs, including *ARR8* and *ARR9*, are significantly repressed in the shoots and roots of Arabidopsis in response to salinity stress after 24 and 6 h treatments, respectively (Kilian et al. 2007). However, other subfamily-I type-A RRs (*ARR5*, *ARR7* and *ARR15*) are repressed in shoots and induced in roots between 0.5 and 24 h of salinity stress (Kilian et al. 2007). In rice, most type-A OsRRs are induced by salinity stress, but *OsRR9*/*OsRR10* is repressed in seedlings (Jain et al. 2007). This differential expression of type-A *ARR*s suggests that there may be functional differences among type-A *RR*s. In this study, we examined the function of rice regulatory factors OsRR9 and OsRR10 in response to salinity stress. We used CRISPR/Cas9 to generate *osrr9 osrr10* double mutants. We characterized the phenotype and the transcriptome profile of this mutant and potential mechanisms of these RRs in response to salt treatment.

Results

OsRR9 and *OsRR10* are early cytokinin-response genes

We examined the expression of *OsRR9* and *OsRR10* in response to exogenous cytokinin and at different developmental stages, tissues and in response to various stress conditions. *OsRR9* and *OsRR10* encode highly similar proteins, with only three out of 201 amino acids being different, which likely arose from segmental duplication on chromosomes 11 and 12. The various microarrays used to date cannot distinguish between *OsRR9* and *OsRR10* due to their high nucleic acid sequence similarity. We designed gene-specific primers corresponding to the 5′UTR and 3′UTR to quantify *OsRR9* and *OsRR10* transcript levels.

We analyzed the levels of *OsRR9* and *OsRR10* in 10-day-old rice seedlings at various time intervals after cytokinin treatment (Fig. 1). Both *OsRR9* and *OsRR10* were significantly induced after 2 h of treatment with cytokinin 6-benzylaminopurine (BA), although their kinetics differed. *OsRR9* transcripts transiently accumulated to higher levels at 0.5 h after treatment and then declined. *OsRR10*, in contrast, was continually elevated throughout the 2-h BA treatment. These results suggest that *OsRR9* and *OsRR10* are early cytokinin-response genes, but they differ in their response kinetics.

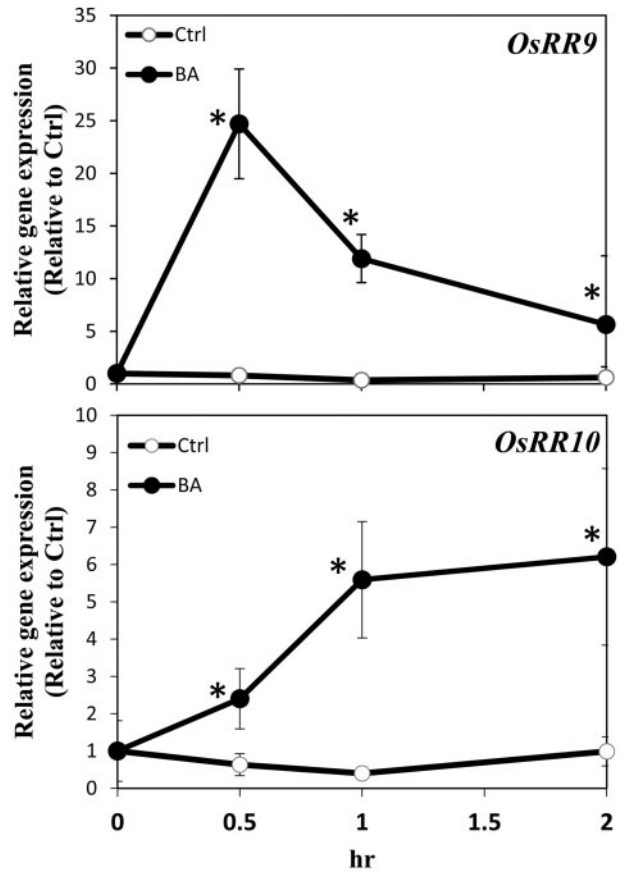


Fig. 1 Expression of *OsRR9* and *OsRR10* in response to cytokinin. 14-day-old hydroponically grown rice seedlings were treated with cytokinin (5 μM BA, close symbol) of a vehicle control (Ctrl, open symbol) by addition to the hydroponic solution and RNA extracted from the second leaves at the indicated times. *OsRR9* and *OsRR10* transcript levels were determined using qRT-PCR and expressed as expression relative to the time zero control. Values are mean ± SD ($n = 3$). Asterisks indicate statistically significant differences from the control (two-tailed Student's *t*-test, $P < 0.05$).

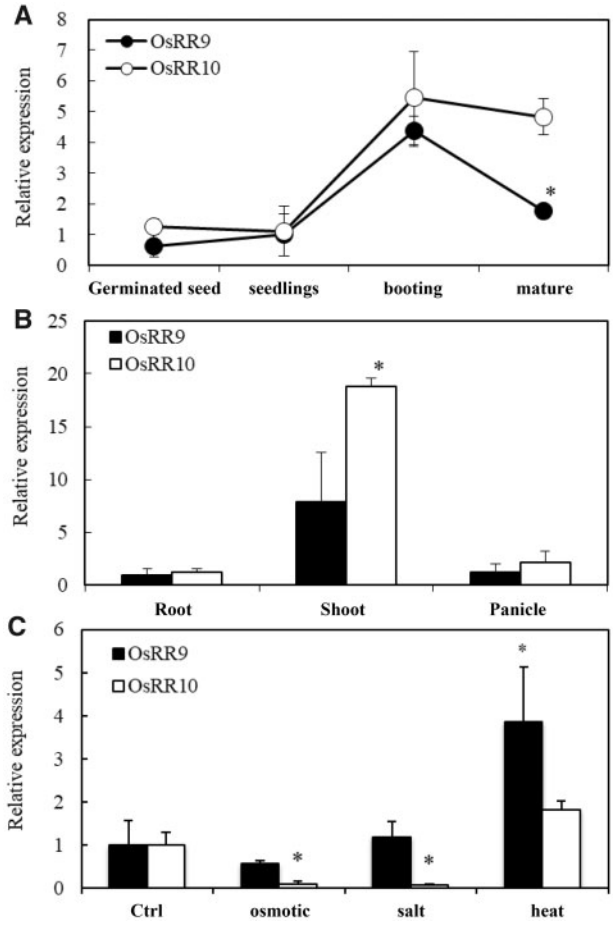


Fig. 2 Expression of rice *OsRR9* and *OsRR10* at different development stages, tissues, and in response to different stresses. (A) Expression of *OsRR9* and *OsRR10* in leaves at germinated seed (1 DAG), seedlings (14 DAG), booting (40 DAG) and mature (85 DAG) stages. (B) Expression in roots and shoots (14 DAG) and panicles (45 DAG). (C) Expression of *OsRR9* and *OsRR10* in 14 DAG seedlings treated with nothing (ctrl), 25% PEG6000 (drought), 150 mM NaCl or 47°C (heat) for 2 h. For (A–C), transcripts levels were determined by qRT-PCR and plotted relative to seedling (A), root (B) and control (C) treatment. *Ubiquitin 5* (*UBI 5*) was used as input control. Values are the mean ± SD ($n = 3$). Asterisks indicate statistically significant differences from the control (two-tailed Student's *t*-test, $P < 0.05$).

OsRR9 and OsRR10 expressions are distinct in tissues and stress responses

To gain more insight into the potential biological functions of OsRR9 and OsRR10, we examined the transcription of *OsRR9* and *OsRR10* at different developmental stages, tissues and environmental stimuli (Fig. 2). Both *OsRR9* and *OsRR10* were detected in leaves at different stages of growth, with a peak during the booting stage. On comparing different tissues, *OsRR9* and *OsRR10* were higher in the shoots relative to the roots or panicles.

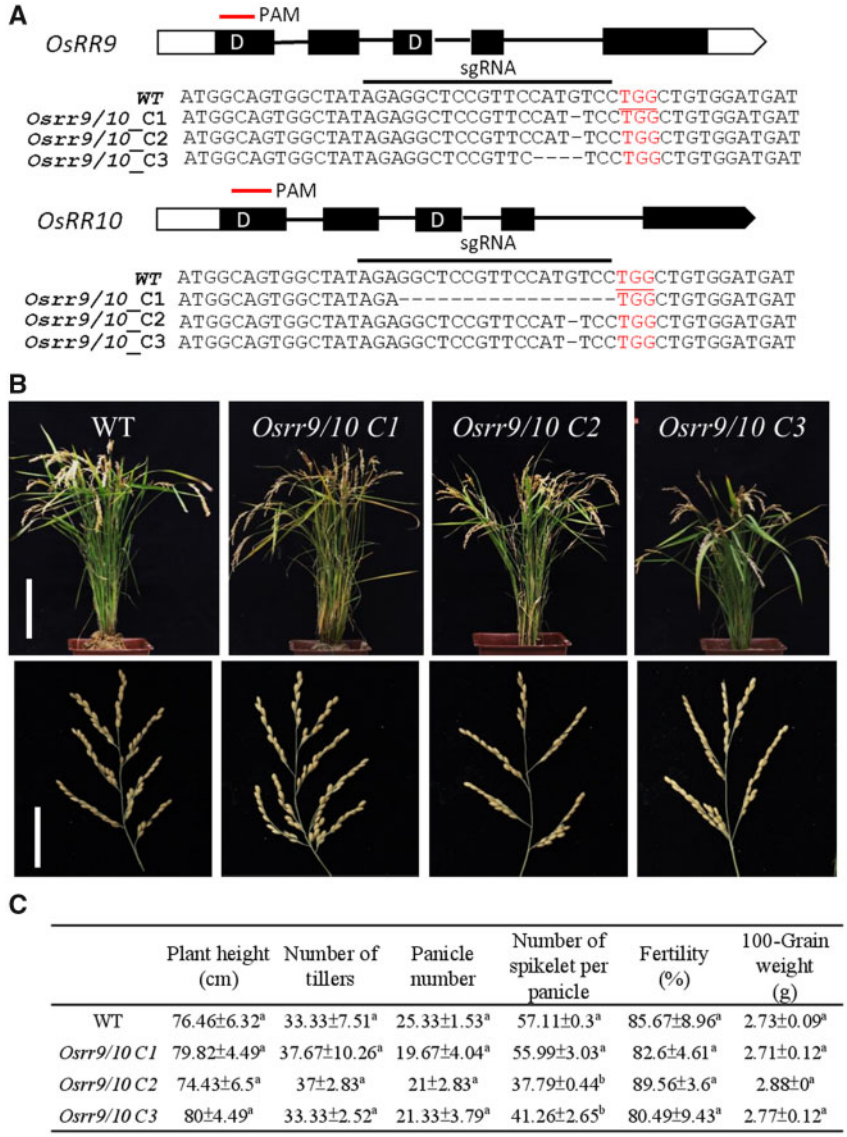
We examined the expression of *OsRR9* and *OsRR10* in response to different stresses, including osmotic stress (25% PEG6000), salinity (150 mM NaCl) and heat (47°C). Leaf tissues were collected after 2-hs treatment on 14 DAG seedlings, including 25% PEG6000 (PEG), 150 mM NaCl (salt), 47°C (heat) and control. *OsRR9* was significantly upregulated in response to the heat stress, whereas *OsRR10* was repressed after 2 h of osmotic stress and salinity stress. These results suggested that OsRR9 and OsRR10 may have functionally differentiated to mediate distinct aspects of stress responses.

Generation and verification of *osrr9* and *osrr10* gene editing mutants

To explore the roles of *OsRR9* and *OsRR10*, we used the CRISPR/Cas9 system to generate *OsRR9* and *OsRR10* double mutants. Fifteen independent transgenic T_0 lines were screened for mutations using polyacrylamide gel electrophoresis-base (PAGE)-based genotyping (Zhu et al. 2014) followed by Sanger sequencing (Supplementary Fig. S1). More than 80% of T_0 plants was identified with mutations in either *OsRR9* or *OsRR10*. The zygosity of T_0 plants included homozygote, biallele, heterozygote, chimera and wild type. To verify the T_0 genotype inheritance by T_1 plants, T_1 plants were genotyped using high resolution melting (HRM) analysis (Samarut et al. 2016) and validated with Sanger sequencing. Three of the lines had small deletions in both *OsRR9* and *OsRR10*, ranging from 1 bp up to 17 bp (Fig. 3A; Supplementary Figs. S1, S2). The CRISPR/Cas9 construct was segregated from the target genes after T_1 generation or by backcrossing with wild type. To validate the potential off-target effects of the CRISPR/Cas9 system, the genome sequence of *OsRR4*, which has the most similar sequence identity to the *OsRR9*/*OsRR10* sgRNA, was analyzed by Sanger sequencing. No new mutation was identified in the *OsRR4* genome sequence. However, the mutations in *OsRR9* and *OsRR10* are likely null alleles as they introduce a frameshift mutation early in the coding region of the respective genes (Supplementary Fig. S2). These lines were selected for subsequent analysis of their stress tolerance phenotypes.

OsRR9 and OsRR10 negatively regulate cytokinin responses

Type-A RRs are known as negative regulators of cytokinin signaling. To examine whether *OsRR9* and *OsRR10* mutations results in altering the response of other cytokinin signaling, we analyzed the transcription of type-A *OsRRs* in response to cytokinin treatment in the leaves. The transcription of *OsRR4*, which is in the same genetic clade as OsRR9 and OsRR10, is induced by 2-h BA treatment in both wild type and *osrr9*/*osrr10* which also shows comparable fold induction between different genotypes (Fig. 4). Transcription of *OsRR1*, *OsRR4* and *OsRR6* was also induced by BA treatment in *osrr9*/*osrr10* mutants and the induction levels were found to be even higher than in wild type (Fig. 4A–C). To examine the cytokinin-mediated responses in *osrr9*/*osrr10* mutants, we used a dark-induced leaf senescence assay (Fig. 4D). The last emerged leaf of three-leaf-stage wild type and *osrr9*/*osrr10* knockout rice seedlings were treated with or without cytokinin in the dark for 5 d. In the control treatment, leaf senescence was significantly reduced in *osrr9*/*osrr10* knockout rice relative to the wild type (Fig. 4). In the presence of cytokinin, leaf senescence was significantly reduced in the wild type and in *osrr9*/*osrr10* mutants. This suggests that OsRR9 and OsRR10 negatively regulate cytokinin responsiveness, similar to type-A ARRs in Arabidopsis.



C

	Plant height (cm)	Number of tillers	Panicle number	Number of spikelet per panicle	Fertility (%)	100-Grain weight (g)
WT	76.46±6.32[a]	33.33±7.51[a]	25.33±1.53[a]	57.11±0.3[a]	85.67±8.96[a]	2.73±0.09[a]
Osrr9/10 C1	79.82±4.49[a]	37.67±10.26[a]	19.67±4.04[a]	55.99±3.03[a]	82.6±4.61[a]	2.71±0.12[a]
Osrr9/10 C2	74.43±6.5[a]	37±2.83[a]	21±2.83[a]	37.79±0.44[b]	89.56±3.6[a]	2.88±0[a]
Osrr9/10 C3	80±4.49[a]	33.33±2.52[a]	21.33±3.79[a]	41.26±2.65[b]	80.49±9.43[a]	2.77±0.12[a]

Fig. 3 Generation and characterization of *osrr9 /osrr10* mutations. (A) The top shows a schematic of the target sequences for the CRISPR/Cas9 sgRNA on *OsRR9* and *OsRR10*; boxes represent exons and open and close boxes are UTRs and coding sequencing, respectively. The bottom is a sequence alignment showing the different mutations found in *OsRR9* and *OsRR10*, with the PAM target in red. (B) Mature plants (85 DAG) and panicles of *osrr9*/*osrr10* C1, C2 and C3 mutants. (C) Quantification of various aspects of growth and yield of the wild type and *osrr9*/*osrr10* mutant lines. Yield parameters are represented as mean ± SD ($n > 3$ plants). Different lowercase letters indicate statistically significant differences as indicated by Fisher's LSD test ($P < 0.05$).

osrr9/*osrr10* double mutants have an enhanced salinity tolerance phenotype

As the transcription levels of *OsRR9* and *OsRR10* altered under several abiotic stresses, we first characterized their role in salinity tolerance. To this end, three-leaf-stage rice seedlings (wild type and *osrr9*/*osrr10* mutant) were exposed to 150 mM NaCl. After 2 d of treatment, most of the first and second complete leaves of wild type plants showed a rolling phenotype (**Fig. 5A**), whereas the *osrr9*/*osrr10* double mutants maintained more flattened leaves. Based on the IRRI standard evaluation score of visual salt injury at the seedling stage (Gregorio et al. 1997), *osrr9*/*osrr10* double knockout mutants had lower injury scores than the wild type after 2 d of salt treatment (**Fig. 5B**).

In response to salinity stress, the efficiency of photosystem II (Fv/Fm) significantly decreased in wild type leaves. However, *osrr9*/*osrr10* knockout rice had higher photosystem II efficiency than wild type after 2 d of treatment (**Fig. 5C**). We also analyzed ion leakage of wild type and *osrr9*/*osrr10* mutants after salt treatment. Similar to the injury score and photosystem II efficiency, ion leakage (**Fig. 5D**) was significantly lower in *osrr9*/*osrr10* double knockout mutants relative to wild type after 2 d of salinity stress. However, the *osrr9* and *osrr10* single mutants exhibited similar ion leakage levels as the wild type (Supplementary Fig. S3). These results suggest that *OsRR9* and *OsRR10* negatively regulate salinity tolerance in rice seedlings.

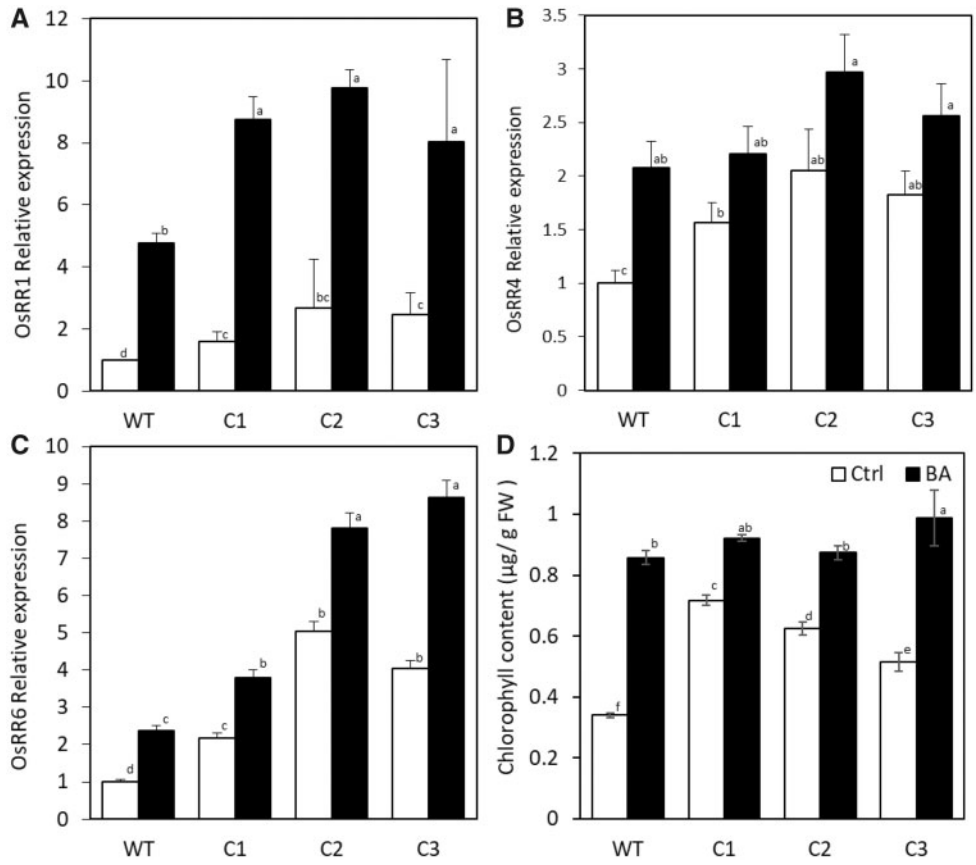


Fig. 4 Cytokinin-mediated gene expression and leaf senescence responses in *osrr9/osrr10* mutants and wild type. (A–C) The transcription levels of Type-A *OsRR*s, (A) *OsRR1*, (B) *OsRR4* and (C) *OsRR6*, in *osrr9/osrr10 C1*, *C2*, *C3* and wild type in response to exogenous BA. DMSO (Ctrl, white bars) and 5 μM BA (black bars) were applied to 14 DAG hydroponically grown rice seedlings. RNA was isolated from 14 DAG hydroponically grown rice seedlings second leaves and gene expression level of various type-A *OsRR*s as indicate were detected using qPCR, were plotted as relative expression (fold) of control. (D) The third fully expanded leaves floated on water supplemented with (black bars) or without (white bars) cytokinin for 4 d in the dark. Chlorophyll content was determined spectrophotometrically as described in the Materials and Methods section. All values are the mean ± SD ($n = 3$). Different letters at the top of each column denote statistically significant differences in genotypes and treatments base on Fisher's LSD test ($P < 0.05$).

We next determined whether the higher photosynthesis efficiency and lower ion leakage of *osrr9/osrr10* knockout mutants relative to the wild type were associated with the oxidative status of the seedlings after exposure to salt stress (**Fig. 6**). We used 3,3′-diaminobenzidine (DAB) to report the levels of H_2O_2 in wild type and mutant leaves. *osrr9/osrr10* mutants displayed significantly less DAB staining than their wild type counterparts after 24-h salt treatment (**Fig. 6A, B**). The activities of both ascorbate peroxidase (APX) and glutathione reductase (GR) were elevated in wild type after exposure to salt, but these activities were not induced in the *osrr9/osrr10* mutants (**Fig. 6C, D**). These results indicated that the *osrr9/osrr10* mutants maintained a lower oxidative status in response to salt stress relative to the wild type, while having similar APX and GR activity as the control treatment.

OsRR9 and OsRR10 mediate salt tolerance independent of osmotic adjustment

Salinity stress affects many aspects of plant growth, including osmotic and ionic effects. To elucidate whether OsRR9 and OsRR10 regulate salinity tolerance through osmotic regulation, we first determined the water and proline content of the seedlings (**Fig. 7**). *osrr9/osrr10* double knockout mutants had a comparable water content in their third leaves relative to the wild type in the absence of salt treatment (**Fig. 7A**). After 2 d of salt treatment, the wild type had a significantly reduced water content, but this still remained at higher levels in *osrr9/osrr10*. Proline levels were significantly induced in wild type leaves and roots following salt treatment (**Fig. 7B, C**). In the *osrr9/osrr10* mutant, proline levels in leaves and roots also increased in response to salt, but this induction was substantially less than that observed in the wild type. To further investigate on whether OsRR9 and OsRR10 have a role in osmotic stress response, seedlings were treated with PEG equivalent to an osmotic potential of 150 mM NaCl. No significant physiological differences, including the phenotype and relative water content, were observed between the wild type and *osrr9/osrr10* (**Fig. 7D, E**). These findings suggest that the OsRR9 and OsRR10 play a role in salinity tolerance through a mechanism distinct from osmotic adjustment.

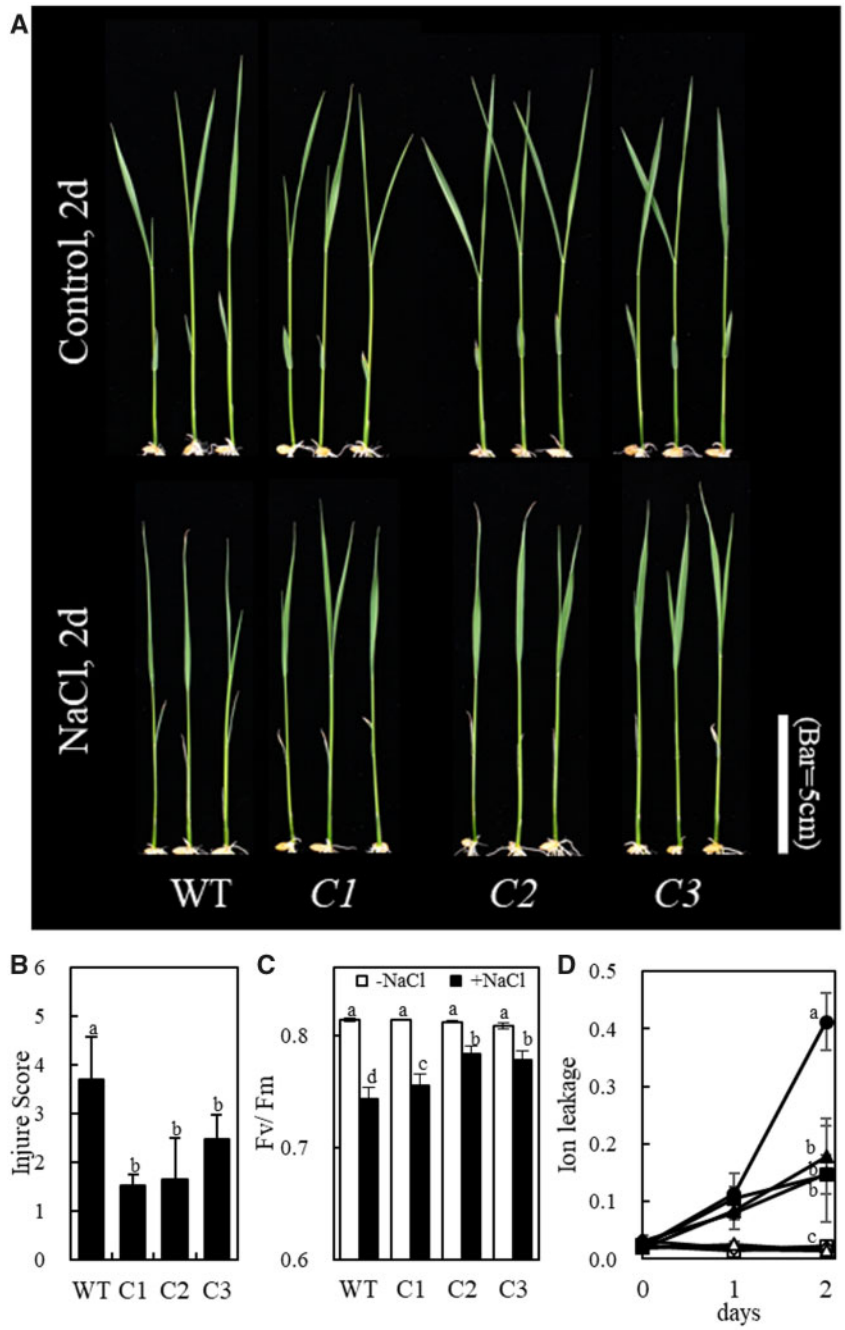


Fig. 5 Physiological responses of *osrr9*/*osrr10* mutants (C1, C2 and C3) and wild type (WT) plants under salt stress. (A) The representative photographs of 12-DAG seedlings treated with 150 mM NaCl or control medium for 2 d. (B) The injury score of various seedlings after salt treatments for 2 d. (C) Maximum quantum yield (Fv/Fm) of the third leaf of wild type and *osrr9*/*osrr10* mutants (C1, C2 and C3) were determined after 2-d NaCl treatment. (D) Time course of electrolyte leakage of the third leaf of wild type (circle), C1 (square), C2 (diamond) and C3 (triangle) after 2 d of treatment with (closed symbols) or without (open symbols) 150 mM NaCl. All values are the mean ± SD ($n = 3$). Different letters at the top of each column denote statistically significant differences in genotypes and treatments base on Fisher's LSD test ($P < 0.05$).

OsRR9 and OsRR10 mediate salt tolerance via Na^+ accumulation

As OsRR9 and OsRR10 do not appear to regulate salinity tolerance via an osmotic mechanism, we explored if they might act via modulation of ionic balance. We determined the sodium and potassium content in both shoots and roots of wild type plants and the *osrr9*/*osrr10* mutants (Fig. 8). The sodium content was reduced in the shoots and roots of the *osrr9*/*osrr10* double knockout mutants relative to the wild type after 2 d of salt treatment (Fig. 8A, B). The potassium content in *osrr9*/*osrr10* mutants and wild type was comparable in the shoots,

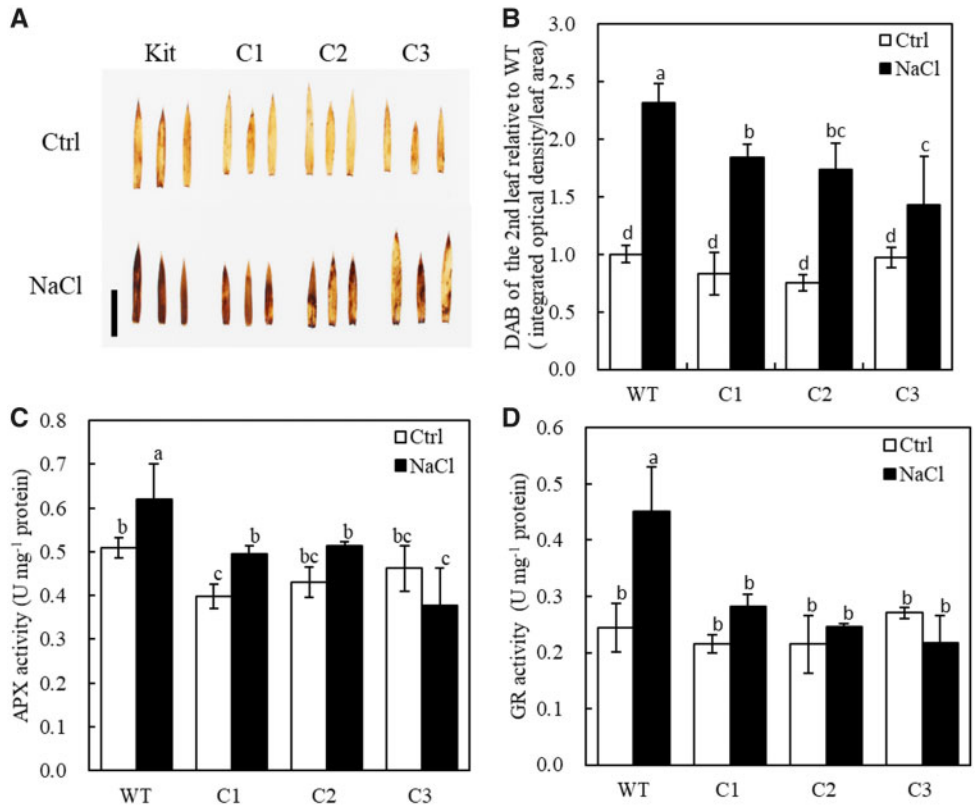


Fig. 6 Oxidative stress of wild type and *osrr9/osrr10* double mutants in response to salinity stress. Representative DAB staining images (A) and quantification (B) of the second leaves of the third-leaf stage seedlings after 150-mM NaCl treatment for 24 h ($n = 3$). (C, D) Determination of APX (C) and GR (D) activity in response to 150-mM NaCl treatment for 24 h. All values are the mean ± SD ($n = 3$). Different letters at the top of each column denote statistically significant differences in genotypes and treatments base on Fisher's LSD test ($P < 0.05$).

but significantly lower in response to salt in roots. Both shoots and roots of *osrr9/osrr10* knockout mutants maintained lower Na^+/K^+ ratios relative to the wild type in response to elevated salinity (Fig. 8E, F). These results indicate OsRR9 and OsRR10 mediate rice salinity tolerance via effects on Na^+ content.

Differentially expressed genes mediated by OsRR9 and OsRR10

To explore the potential mechanisms by which OsRR9 and OsRR10 modulate salinity tolerance, we performed a transcriptome analysis using the leaves of *osrr9/osrr10* mutants and wild type in control conditions and in response to 2-h NaCl treatment. Seedlings were grown hydroponically, and NaCl was then added to the hydroponic media at the four-leaf stage for 2 h and RNA-Seq was performed using three biological replicates. On average, 40 to 60 million 150-bp pair-end reads were obtained per sample, of which >97% could be mapped to the Kitaake genome (Li et al. 2017). For the functional interpretation of the transcriptome, we used the annotation of *Oryza sativa* Kitaake v3.1 (DOE-JGI, http://phytozome.jgi.doe.gov/). Differentially expressed genes (DEGs) were defined as those >2-fold upregulated or downregulated and with a *P*-value <0.05.

In *osrr9/osrr10* mutant leaves, 1,024 DEGs were identified as upregulated or downregulated in control conditions compared with the wild type (Supplementary Table S2). Gene ontology (GO) terms that were highly enriched for these DEGs included 'regulation of transcription', 'transcription regulator activity', 'transferase activity', 'transcription factor activity', 'photosynthesis' and 'MAPK signaling pathway', with the term 'plant hormone signal transduction' being the most significantly enriched. After 2 h of salt treatment, there were 4,200 and 3,930 DEGs in *osrr9/osrr10* mutants and wild type, respectively (Fig. 9; Supplementary Table S3). Of these, 1,765 and 1,032 genes were upregulated and downregulated in both the *osrr9/osrr10* mutant and wild type. Of these, 'oxidoreductase activity', 'signal transduction', 'sequence-specific DNA binding' and 'transmembrane transport' were significantly enriched under salt treatment.

We focused on the genes that were only upregulated or downregulated in either the *osrr9/osrr10* mutant or wild type after 2 h of salt treatment. About 740 and 656 DEGs were specifically identified in the *osrr9/osrr10* mutant after salt treatment. These *osrr9/osrr10*-specific DEGs were enriched in 61 GO terms, including 'amine metabolic process', 'cellular nitrogen compound metabolic process', 'regulation of transcription', 'transcription regulator activity', 'transporter activity' and 'symporter activity' (Supplementary Fig. S4). The GO terms identified here as being closely related to salt stress in the absence of OsRR9 and OsRR10 might prove useful for improving our understanding of the molecular mechanisms involved in plant salt tolerance.

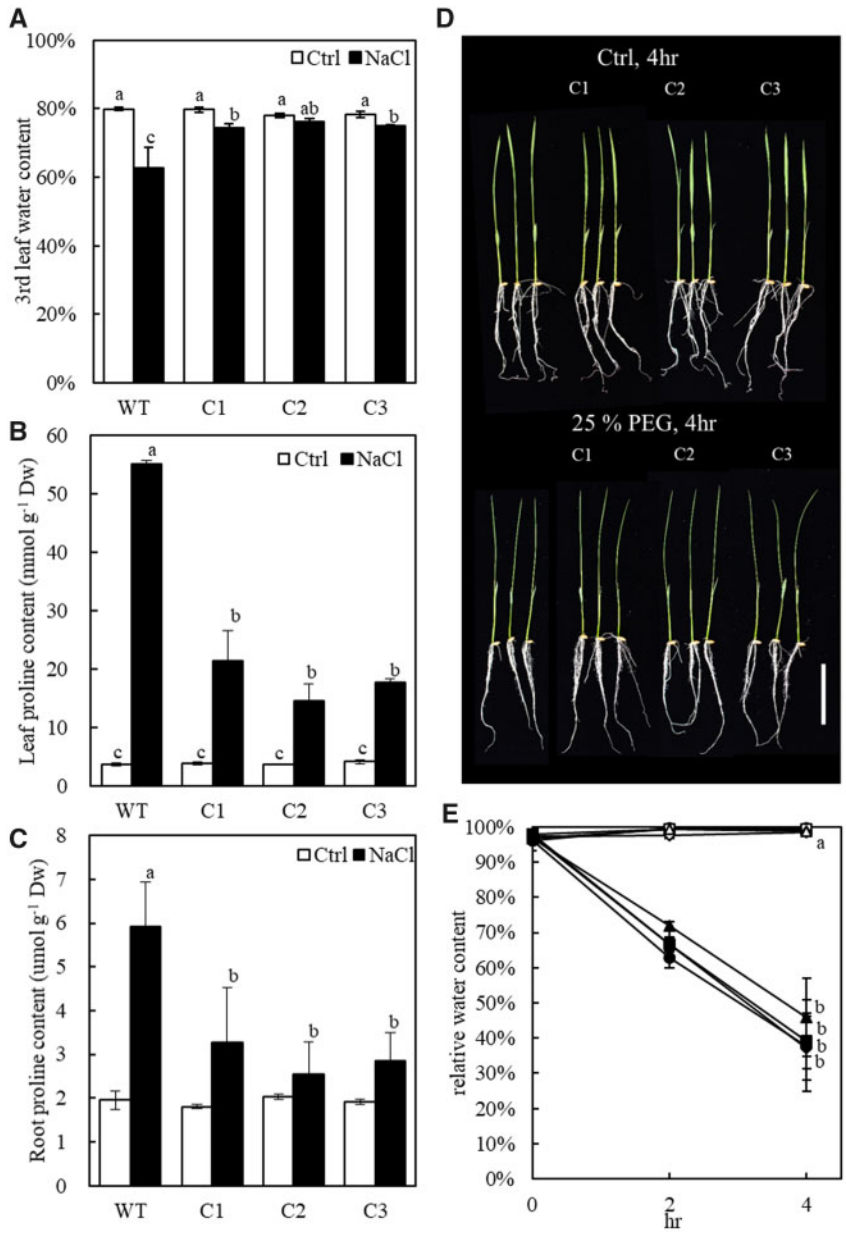


Fig. 7 Physiological responses of *osrr9/osrr10* mutant (C1, C2 and C3) and wild type plants under salt and osmotic treatments. (A–C) Quantification of the water content (A), leaf proline content (B) and root proline content (C) of 12-DAG wild type and *osrr9/osrr10* mutants. (D, E) The phenotype (D) and relative water content (E) of third-leaf stage rice seedlings (WT, circle; C1, square; C2, diamond; C3, triangle) after the treatments with 25% PEG6000 (closed symbols) or control (open symbols) for 4 h. All values are mean ± SD ($n = 3$). Different letters at the top of each column denote statistically significant differences in genotypes and treatments base on Fisher's LSD test ($P < 0.05$).

Functional classification of the leaf DEGs in *osrr9/osrr10* mutant through Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed the physiological pathways in which the *osrr9/osrr10* mutant is significantly different from the wild type in normal growth conditions, including MAPK signaling, the zeatin biosynthesis pathway, plant hormone signal transduction and carotenoid biosynthesis (Supplementary Fig. S5). After salt treatment, the categories including terpenoid backbone biosynthesis, zeatin biosynthesis, carotenoid biosynthesis and diterpenoid biosynthesis were the most significant terms between *osrr9/osrr10* mutant and wild type. These pathways are all related to phytohormones and salt stress signal transduction (Bahmani et al. 2015, Gao et al. 2008).

In cytokinin signaling, the transcription of *OsRR9* and *OsRR10* could still be detected by RNA-Seq, which may be due to the short nucleotide deletion in the coding sequence. Eight of the 13 type-A *OsRR*s were able to be detected in the wild type leaves. We validated the transcription of *OsRR9* and *OsRR10* in three *osrr9/osrr10* mutants with quantitative real-time PCR (qRT-PCR) and only *OsRR10* in *osrr9/osrr10* C1 double mutant was significantly repressed, which correlated

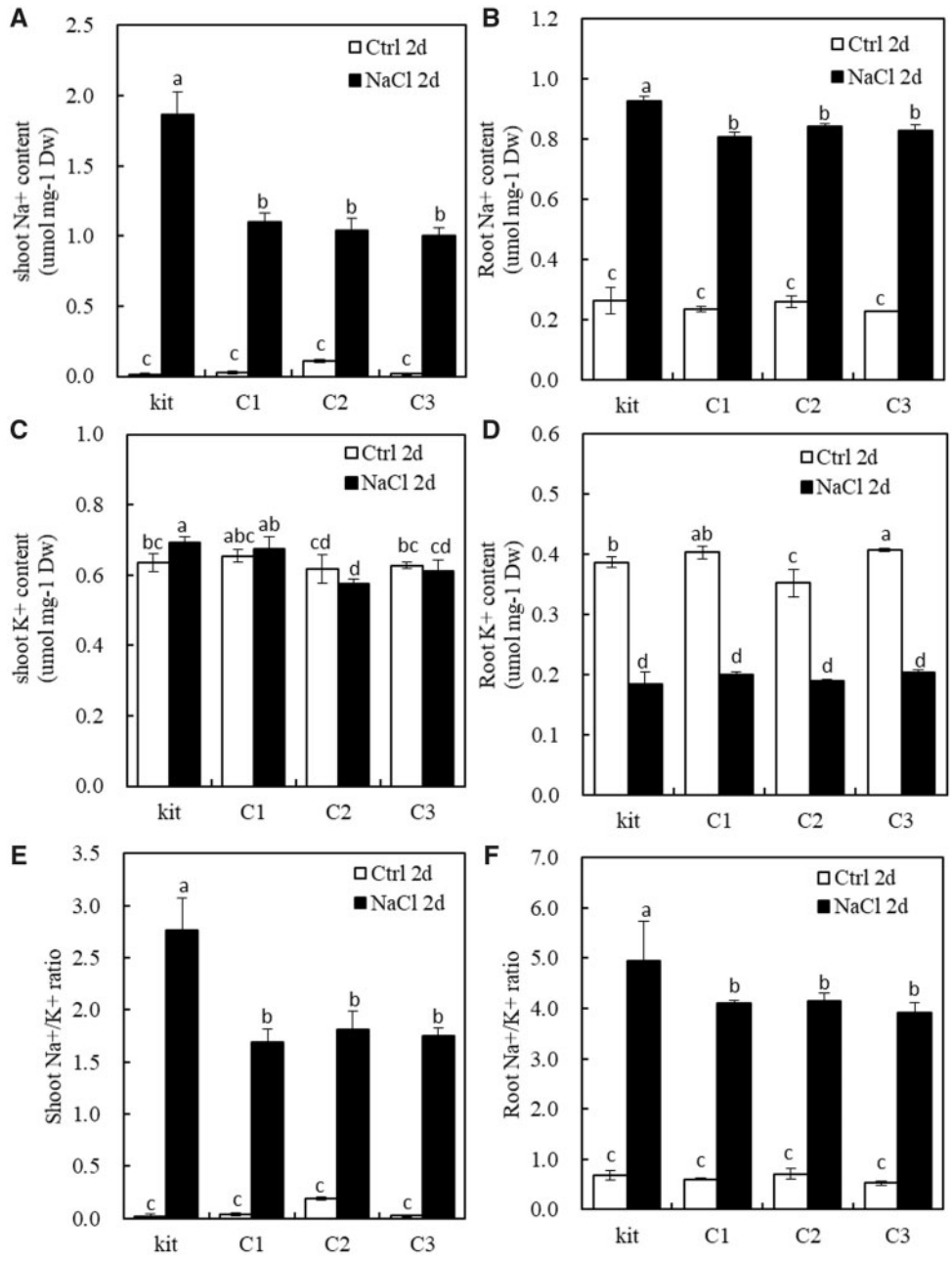


Fig. 8 *osrr9/osrr10* double mutants accumulated less Na^+ in leaves and roots in elevated salinity. Third-leaf stage seedlings were treated with 150 mM NaCl for 2 d and the sodium (A, B) and potassium content (C, D) were determined from shoots (A, C, E) or roots (B, D, F). All values are mean ± SD ($n = 3$). Different letters at the top of each column denote statistically significant differences in genotypes and treatments base on Fisher's LSD test ($P < 0.05$).

with a long deletion in the *OsRR10* coding sequence (Supplementary Fig. S6). The transcription of *AHPs* in two-component signaling in the control treatment was repressed in the *osrr9/osrr10* mutant relative to the wild type. The type-A *OsRR* transcript levels were slightly higher or similar in the *osrr9/osrr10* mutants than in the wild type plants in the control treatment, which was similar to the previous results (**Fig. 4**). In addition, the genes involved in 'plant hormone signal transduction' (Supplementary Fig. S5) related to the stress hormone, ABA and ethylene, signal transduction were upregulated in the *osrr9/osrr10* mutant in the control treatment.

DEGs between overexpression *OsRR6* lines and *osrr9/osrr10* knockout mutants

We compared the DEGs in an *OsRR6* overexpression (OsRR6-ox) line (Hirose et al. 2007) and the *osrr9/osrr10* mutant to the wild type. We identified 441 genes with 3-fold differences in

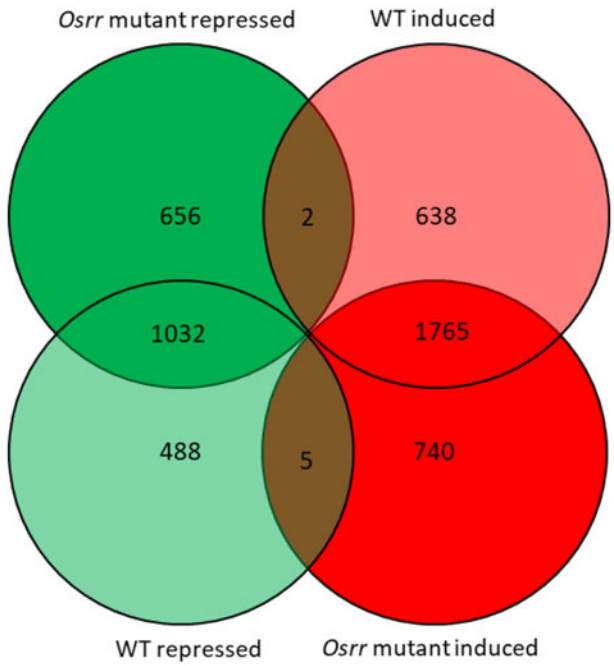


Fig. 9 Overlaps of DEGs in wild type (kit) or the *osrr9/osrr10* mutant (9/10) leaves in response to 150 mM NaCl for 2 h. See Materials and Methods section for more details.

expression level in *OsRR6ox* plants compared to the wild type, and 120 of these genes were also significantly ($P < 0.05$) different in *osrr9/osrr10* compared to the wild type (Supplementary Table S4). More than half of the DEGs (78/120) were either upregulated or downregulated in both OsRR6ox and *osrr9/osrr10*, including one of the ion transporters, *OsHK1;1*. Forty-two genes show opposite expression between OsRR6ox and *osrr9/osrr10*.

The expression of ion transporter genes is altered in *osrr9/osrr10*

Many genes involved in ion transportation or transporter activity were also significantly altered in *osrr9/osrr10* mutants relative to the wild type in response to salt treatment. These genes include sodium ion transporters, Na^+/K^+ transporters or Na^+/H^+ transporters. These genes were induced by salt treatment in the *osrr9/osrr10* mutant relative to the wild type. In addition, many *OsHKT* genes, including *OsHKT1;1*, *OsHKT1;3* and *OsHKT1;4* were expressed at lower levels in the leaves of *osrr9/osrr10* mutants in the control treatment relative to the wild type. Several ion transporter genes, K^+ uptake channel (*OsAKT1*, *OsAKT2*), vacuolar Na^+/H^+ transporter (*OsNHX1*) and K^+ transporter (*OsKAT10*) are also significantly induced in *osrr9/osrr10* mutants relative to the wild type (Supplementary Table S2). The transcription of *OsKAT5* is constitutively higher in *osrr9/osrr10* than in the wild type. Genes for 'transcription', 'transcription regulator activity' and 'transcription factor activity' were also significantly induced by salt stress in the *osrr9/osrr10* mutant, which might play a role in regulating salinity tolerance.

Validation of the DEGs with qRT-PCR

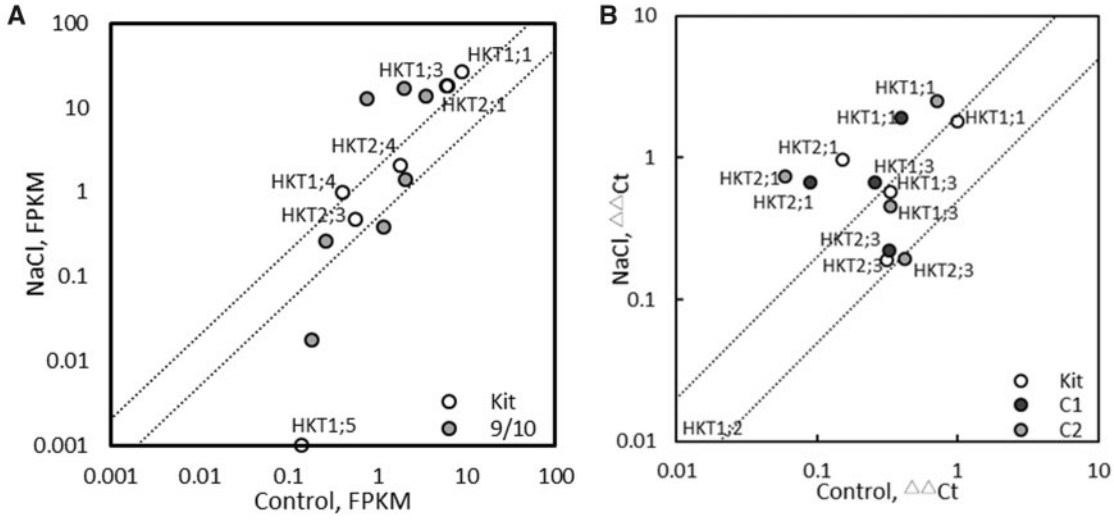
To validate the expression of ion transporters in response to salt stress, we analyzed the transcription levels of genes involving salt tolerance and cytokinin signaling. The leaves of *osrr9/osrr10* C1 and C2 mutants and wild type from two independent experiments were collected after 2 h of salt stress for the validation of DEGs with qRT-PCR (**Fig. 10**). We found that *OsHKT1;1* and *OsHKT2;1* were induced in the *osrr9/osrr10* mutants to a higher degree than in the wild type, which was similar to the RNA-seq results. In addition, genes involved in both ABA-dependent (ABI5) and independent (DREB1C) pathways were also significantly induced in both mutants and wild type. These results suggest that the *osrr9/osrr10* mutants may enhance tolerance to salinity stress by regulating sodium/potassium transporters or other phytohormone signaling pathways.

Discussion

Salinity stress is an important limiting factor in crop production. Approximately 20% of the world's agricultural land is affected by salinity (Munns and Tester 2008). To adapt and respond to various environmental stimuli, plants use several signal transduction systems, including phytohormones. Cytokinins regulate several developmental events and responses to environmental stimuli. Previous studies have found that salt stress affects the expression of many cytokinin-response genes (Argueso et al. 2009). Recent studies have shown that cytokinin degradation through cytokinin oxidase/dehydrogenase2 (OsCKX2) plays a role in determining grain yield under salinity stress (Li et al. 2013, Joshi et al. 2018). The present study investigated whether the cytokinin two-component element type-A RRs OsRR9 and OsRR10 play a role in mediating salinity stress tolerance in rice.

The type-A RRs are primary cytokinin-response genes and negative regulators in cytokinin-mediated signal transduction (Brandstatter and Kieber 1998, D'Agostino et al. 2000, Kiba et al. 2003, To et al. 2004, To et al. 2007, Lee et al. 2008). About 10 and 13 type-A RRs have been identified in Arabidopsis and rice, respectively. Several biochemical and genetic studies have proposed that type-A RR family members are functionally redundant in specific signal transduction pathways. Two of the most similar type-A OsRRs, OsRR9 and OsRR10, likely arose through gene duplication (Tsai et al. 2012). In response to cytokinin treatment, most of the type-A *OsRR*s are upregulated, with the transcription level of *OsRR9/OsRR10* being the highest (Tsai et al. 2012).

Previous microarray analysis of rice gene responses to various abiotic stresses showed that *OsRR9/OsRR10* are repressed by salinity stress (Jain et al. 2007). In our study, using gene-specific primers, we showed that only *OsRR10* was repressed by salinity stress. The protein interaction assay also indicated only OsRR10 but not OsRR9 interacts with OsRR22 (Sharan et al. 2017). In this study, we used the CRISPR/Cas9 system to generate *osrr9/osrr10* double and single knockout mutants to characterize the role of OsRR9 and OsRR10 in salt stress.



C

NaCl/Ctrl fold change		RNA-seq		qRT-PCR		
Gene	Gene KitaakeID	WT	*osrr9/osrr10*	WT	*osrr9/osrr10 C1*	*osrr9/osrr10 C2*
ABI5	OsKitaake01g407100	3.21	3.17	5.48	2.64	3.68
DREB1C	OsKitaake06g016801	49.23	79.34	37.40	62.14	14.72
OsCKX5	OsKitaake01g344600	68.55	43.07	27.05	3.02	2.04
OsKAT5	OsKitaake01g458400	0.55	0.81	6.36	1.78	1.19
OsAHP1	OsKitaake08g247800	0.56	0.66	0.80	0.66	0.84

Fig. 10 Validation of RNA-seq with qRT-PCR. Effect of salt treatment on the expression of *OsHKT* gene family in *osrr9/osrr10* and wild type. (A) Comparison plot show transcript levels as determined by RNA-seq in the control or salt treatments for the *OsHKT* gene family. (B) qRT-PCR validation of relative expression of *OsHKT* genes between wild type and *osrr9/osrr10* mutants (C1 and C2) in response to salt stress. Note that the FPKM are plotted on a $\log_{10}$ scale. Dotted lines represent variation >2-fold in expression levels between control and salt treatment. (C) Genes involved in ABA signal pathway, cytokinin metabolism, signaling and potassium transport were validated with qRT-PCR. Three independent biological repeats were performed in the analysis ($n = 3$).

Various types of mutations were detected in T_0 plants. Most of the mutations confered a 1-bp deletion at the fourth nt next to the PAM site. This is similar to several previous studies that have shown mutation types and high mutation efficiency with multiple-gene knockouts in rice using the CRISPR-Cas9 system (Zhang et al. 2014, Xu et al. 2015, Shen et al. 2017).

The plant morphology of *osrr9/osrr10* double mutants was similar to the wild type in normal growth conditions, with only a reduction in the number of spikelets per panicle (**Fig. 3**). This may indicate that OsRR9 and/or OsRR10 positively regulate rice spikelet number. It has been shown that cytokinin oxidase 2, OsCKX2, which was identified from a quantitative trait locus (QTL_*Gn1a*), regulates rice grain number (Ashikari et al. 2005). Although type-A RRs do not contain a DNA-binding motif, comparing the transcription profiles of the *osrr9/osrr10* mutants and the wild type showed that the loss of function of *OsRR9* and *OsRR10* caused many gene transcription changes (**Fig. 9**; Supplementary Table S2). A subset of cytokinin metabolising genes, including *OsIPTs* and *OsCKXs*, were altered in *osrr9/osrr10* relative to the wild type. Whether OsRR9 and OsRR10 are involved in OsCKX2-mediated grain number needs to be evaluated.

Hirose et al. (2007) also found that transgenic plants overexpressing *OsRR6* had several phenotypes including dwarf plants, small root systems and a reduction in the number of spikelets. It may seem counterintuitive that OsRR6-ox and *osrr9/osrr10* knockout lines have both reduced the spikelet number relative to the wild type. In addition, more than half of the 120 DEGs between wild type and OsRR6-ox or *osrr9/osrr10* knockout lines had a similar response direction (Supplementary Table S4). A similar study on cytokinin-deficient (*CaMV35S* > *GR* > *HvCKX2*) and cytokinin-overproducing (*CaMV35S* > *GR* > *ipt*) seedlings also showed an extensive overlapping proteomic and metabolomic profile (Cerny et al. 2013). These results indicate the importance of maintaining cytokinin homeostasis.

Genes related to photosynthesis were enriched in the *osrr9/osrr10* double mutants, which have higher electron transport rates and photon yield than the wild type under the salt stress condition. This may be associated with the delay in chlorophyll degradation in the dark which was observed in the *osrr9/osrr10* mutants. It has been shown the type-A *arr3, 4, 5, 6* mutant is hypersensitive to cytokinin in dark-induced leaf senescence (To et al. 2004). These results indicate that OsRR9 and OsRR10 negatively regulate dark-induced leaf senescence signal, which could be partially related to the feedback signal of type-A RR to two-component signal transduction.

In this study, we found the basal transcription level of ion transporters, *OsHKT1;1*, *OsHKT1;3* and *OsHKT2;1*, was

constitutively lower in *osrr9/osrr10* relative to the wild type. The basal transcription level of *HKT1* was shown to be significantly reduced in type-B *ARR10* overexpression lines (Zubo et al. 2017). After subjecting to salt stress, *osrr9/osrr10* double mutants accumulated lower sodium levels in the shoot and showed a higher transcription induced ratio of *OsHKT1;1*, *OsHKT1;3*, *OsHKT2;1* (**Fig. 10**), and were more tolerant to salt stress at the seedling stage than in wild type plants (**Fig. 5**). Plants control Na^+ content in the xylem sap and roots by regulating Na^+/H^+ ion exchange in the roots to minimize sodium ion accumulation in the shoots and leaves (Ismail and Horie 2017). High-affinity K^+ transporters (HKT) from multiple plant species have been identified that unload Na^+ from the xylem to reduce its content in the xylem sap (Munns and Tester 2008, Horie et al. 2009, Hauser and Horie 2010). HKT proteins are divided into two subfamilies. Members of the first HKT subfamily, HKT1s, preferentially transport Na^+ to reduce its accumulation in the leaves (Mäser et al. 2002, Horie et al. 2005, Ren et al. 2005). Members of the second subfamily, HKT2s, encode Na^+/K^+ symporters. At high concentrations of Na^+ (>10 mM), HKT2s selectively regulate Na^+ transport only (Horie et al. 2009). Studies on the HKT1 family in Arabidopsis indicate that the transporter AtHKT1;1 is responsible for unloading Na^+ from the xylem and enhancing plant tolerance to salt tolerance (Horie et al. 2005, Davenport et al. 2007).

Several previous studies on cytokinin signaling revealed its potential function in salinity stress. Arabidopsis cytokinin receptors, AHK2 and AHK3, have found to be induced by salt treatment, and *ahk2* and *ahk3* mutants were more tolerant to salt stress (Tran et al. 2007). A double mutant in two type-B RRs, *arr1/arr12*, also shows increased tolerance to salinity, which implies that cytokinin signaling negatively regulates the response to salinity stress in Arabidopsis (Mason et al. 2010). However, knocking down the His phosphotransfer proteins (OsAHP1 and OsAHP2) in rice revealed the hypersensitive response to salt treatment (Sun et al. 2014). Previous studies have also shown that OsRR9 and OsRR10, which belong to the A-II group together with ARR8 and ARR9 in Arabidopsis, may have opposite and mutually antagonistic functions compared to other groups of Type-A RR (To et al. 2004, Mason et al. 2010). The Arabidopsis quadruple type-A *RR* mutants accumulate higher sodium levels and have lower germination rates relative to the wild type (Munns and Tester 2008). However, only the quadruple type-A RR, *arr3, 4, 8, 9* and *arr5, 6, 8, 9* mutants but not *arr3, 4, 5, 6*, show reduced *AtHKT1;1* expression (Mason et al. 2010). This implicates the complex functioning of type-A RR and that ARR8/9 and ARR3/4 might have opposing functions in some responses (To et al. 2004). As *OsRR9/OsRR10* genes are orthologous to *ARR8/ARR9*, they may act in a similar manner to antagonize other type-A RRs in rice.

Studies in Arabidopsis have also shown that different type-A RRs respond differently to salt treatment. For example, ARR4 and ARR5 are induced by salinity stress, but ARR8 and ARR9 are not (Urao et al. 1998). In this study, several genes involved in ABA-dependent and independent or ethylene signaling pathway were identified using RNA-Seq. Some of the genes are upregulated in both wild type and mutant. However, the fold change of the upregulation is quite different, which will require additional experiments to elucidate the relationship. This suggests that the response of each type-A RR to salt stress is likely to also be regulated by a non-cytokinin signaling pathway, similar to the role of WUSCHEL in regulating type-A ARR expression (Leibfried et al. 2005).

Unlike type-B RR, which contains a DNA-binding motif, type-A RRs do not bind DNA. Recent studies on yeast-two-hybrid and biomolecular fluorescence complementation indicate that the OsRR10 directly interacts with type-B RR-OsRR22 (Sharan et al. 2017). OsRR22 was also identified from a salt-tolerant rice EMS-mutant pool as *hitomebore salt tolerant 1* (*hst1*) (Takagi et al. 2015). Twenty-one genes were at least 2-fold differentially expressed between *osrr22* and wild type in response to salt stress. One of these genes, *OsHKT1;1*, has higher induction ratio in *osrr22* (4.67 fold) than in the wild type (2.75 fold), which is similar to our results showing 8.53- and 2.88-fold induction in *osrr9/osrr10* mutant and wild type, respectively. This result is related to the induction of *AtHKT1;1* in type-B *arr1/arr12* mutants and enhanced salinity tolerance (Mason et al. 2010). OsHKT1;1 plays a role in the transport of Na^+ into the phloem and returning Na^+ from the shoot to the roots or the young leaves to the old leaves under salt stress (Wang et al. 2015). OsHKT1;1 is a key transporter for unloading of Na^+ from the xylem and cooperates with OsHKT1;4 and OsHKT1;5 to reduce the damage to rice under salinity stress (Ismail and Horie 2017). In addition, OsHKT1;1 has also been shown to be regulated by MYB-type transcription factors, and type-B RR is a transcription factor belonging to this class (Hosoda et al. 2002, Argyros et al. 2008). OsHKT1;5, which was identified from rice salt tolerance QTLs, has similar functions to AtHKT1;1. However, whereas *AtHKT1;1* is expressed in both roots and shoots, the majority of *OsHKT1;5* expression is in the root, where it functions to reduce Na^+ accumulation (Horie et al. 2005, Ren et al. 2005). In addition, OsHKT1;4 regulates the excretion of Na^+ in leaf sheath cells (Cotsaftis et al. 2012) and also regulates unloading of Na^+ in stem sap (Suzuki et al. 2016).

Type-B RRs are transcription factors that regulate many cytokinin signals, including the expression of type-A RRs. Previous studies in Arabidopsis indicate that the gene expression of type-A RRs, *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8* and *ARR9*, is inhibited in type-B *arr1, 12* double mutants (Argyros et al. 2008). The transcription level of *AtHKT1;1* is higher in the roots of *arr1, 12* double mutants, which causes less sodium accumulation in the shoots and enhances salinity tolerance. Studies have also indicated that plants lacking the cytokinin receptors AHK2, AHK3 and AHK4 have reduced sensitivity to salinity stress (Tran et al. 2007).

To the best of our knowledge, this is the first study on the function of rice cytokinin signaling type-A RRs in salinity tolerance. Using CRISPR/Cas9 technology, we generated *OsRR9/OsRR10* double knockout mutants and explored the whole-genome transcript profile in response to salt stress. Although OsRR9 and OsRR10 negatively regulate cytokinin signaling in leaf senescence and photosynthesis, it is similar to the role of

other type-A RRs in Arabidopsis. In addition, OsRR9/OsRR10 are negative regulators of salinity tolerance in rice seedlings, which is mediated by the transcription levels of ion transporters and Na^+/K^+ accumulation in the roots and shoots.

Materials and Methods

Plant growth conditions and genotypes

The rice (*O. sativa*) cultivar Kitaake was used in this study. Seeds were surface sterilized with 2.5% sodium hypochlorite with Tween-20 for 15 min, washed with distilled water and germinated on moist filter paper at 37°C for 24–48 h. The uniformly germinated seeds were placed in the hydroponic culture system with Kimura B nutrient medium (Ma et al. 2001) for 12 d in a growth chamber at 30°C/25°C (day/night) with a 12-h-light/12-h-dark cycle. Third-leaf stage rice seedlings were treated with 5 μM BA, 25% PEG 6000, 150 mM NaCl or the cognate mock treatment in the hydroponic medium as a control. Three independent biological replicates were prepared for each experiment. Visual salt stress injury was scored according to the IRRI guidelines (Gregorio et al. 1997).

RNA isolation and RNA expression analysis

For quantitative real-time PCR, tissues were collected and total RNA extracted using Trizol (ThermoFisher, Waltham, MA USA) followed by treatment with TURBO DNase (ThermoFisher) as described by the manufacturer. cDNA was synthesized from the DNase-treated RNA templates following reverse transcription using SuperScripe IV (ThermoFisher) with oligo $(dT)_{12-18}$ primers. Quantitative RT-PCR was performed using QuantiNova SYBR green PCR (Qiagen, Venlo, Netherlands) on Applied Biosystem 7500 system (ThermoFisher). The primers used for qPCR are listed in Supplementary Table S1.

For RNA-Seq analysis, three independent biological replicates were used for each treatment and genotype. The purified total RNA was quantified using a ND-1000 spectrophotometer (ThermoFisher), and the quality was confirmed using a Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA). All procedures for RNA sample library preparation were carried out according to the Illumina protocol. Agilent's SureSelect Strand-Specific RNA Library Preparation Kit was used for library construction followed by AMPure XP Beads size selection. Sequences were determined using Illumina's sequencing-by-synthesis technology. Sequencing data (FASTQ files) generation was based on Illumina's base-calling program bcl2fastq v2.2.0. Trimmomatic was used to remove poor-quality reads from the original fastq file, followed by alignment to the OsKitaake 3.1 reference database with samtools (Li et al. 2017). The aligned reads were assembled against the OsKitaake3.1 gene annotation using StringTie assembler. To determine all expressed transcripts, 12 samples were merged using the StringTie-merge option. To calculate the length of the individual genes and Fragments per Kilobase of transcript per Million mapped reads (FPKM), the merged annotation file was subsequently analyzed using the R/DEseq2 package.

DEGs were defined as having *P*-values <0.05 and FDR <0.05. The GO analysis was performed on the DEGs using EXPath 2.0. The functional orthologs of the DEGs were clustered with the KEGG pathways.

Generation and identification of CRISPR/Cas9 editing rice mutants

Approximately, 20-bp specific target sequence (AGAGGCTCCGTTCCATGT CC) with NGG at 3′ end was selected as sgRNA for *OsRR9* and *OsRR10*. The sgRNA target sequence was cloned into a *pCAB255* binary vector harboring a sgRNA cassette, a maize UBQ10-drived Cas9 and *hygromycin* selection marker (Supplementary Fig. S7). The plasmid was transformed into *Agrobacterium* strain LBA4404 and *Agrobacterium*-mediated transformation of the rice (*O. sativa* L. cv. Kitaake) embryogenic callus was performed at the Transgenic Plant Core Laboratory in Academia Sinica.

To identify the *OsRR9* and *OsRR10* mutants and the type of mutation, genomic DNA was PCR amplified. The PCR amplicons were subjected to one-step PAGE (Zhu et al. 2014), HRM analysis (Dahlem et al. 2012) or Sanger sequencing (Sanger and Coulson 1975). To remove the marker genes transformed with the CRISPR/Cas9 construct, the *osrr9*/*osrr10* double mutants were backcrossed with the wild type.

Electrolyte leakage determination

Two fully extended leaves were collected from each genotype, rinsed with deionized water and incubated with 10 ml of deionized water. After incubation for 2 h under dim light, the electrical conductivity (EC1) of the solution was measured using a conductivity meter (SUNTEX SC-170, TW). The leaves were then autoclaved at 121°C for 20 min and the solution electrical conductivity (EC2) was determined again. The electrolyte leakage was defined as follows: Electrolyte leakage (%) = $[(EC1-EC_{blank})/(EC2-EC_{blank})] \times 100$. Three independent biological replicates and each replicate of at least four plants was prepared for each experiment.

Chlorophyll content, chlorophyll fluorescence, H_2O_2 content, antioxidant enzyme activity, proline content and Na^+/K^+ content determination

Chlorophyll content was determined as described (Wintermans and De Mots 1965). The maximum quantum yield of PSII (Fv/Fm) was measured using an imaging-PAM fluorometer (Walz, Effeltrich, Germany) according to (Yuan et al. 2014). H_2O_2 in situ detection was determined using DAB as described (Orozco-Cardenas and Ryan 1999). For APX and GR assays, tissues were homogenized and assayed as previously described (Foster and Hess 1980, Nakano and Asada 1981). For proline determination, the tissues were homogenized with sulfosalicylic acid followed by centrifugation and mixing of the supernatant with ninhydrin and acetic acid (Bates et al. 1973). For analysis of sodium and potassium contents, shoots and roots were dried, weighted and digested with HNO_3. The sodium and potassium contents were quantified using an atomic absorption spectrophotometer (Model AA-680, Shimadzu, Kyoto, Japan). Three independent biological replicates and each replicate of at least four plants was prepared for each experiment.

Statistical analysis

Data are expressed as the mean ± SD. All statistical analyses were performed using R (version 3.2.0, R Core Team 2015). Significant differences between measurements ($n > 3$) for different treatments or different times were analyzed following an LSD test or two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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