

# Response Regulators 9 and 10 Negatively Regulate Salinity Tolerance in Rice

Wei-Chen Wang<sup>1</sup>, Te-Che Lin<sup>1</sup>, Joseph Kieber<sup>2</sup> and Yu-Chang Tsai <sup>1,\*</sup>

<sup>1</sup>Department of Agronomy, National Taiwan University, Roosevelt Road, Taipei, Taiwan

<sup>2</sup>Department of Biology, University of North Carolina, Chapel Hill, NC, USA

\*Corresponding author: E-mail, yuchangt@ntu.edu.tw; Fax, +886-2-23620879.

(Received April 2, 2019; Accepted July 19, 2019)

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  $\approx$  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<sup>+</sup> which prevents above-ground Na<sup>+</sup> 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<sup>+</sup> transport systems in plants to reduce Na<sup>+</sup> toxicity in shoots, including Na<sup>+</sup> exclusion in the rhizosphere, Na<sup>+</sup> sequestration in vacuoles and Na<sup>+</sup> loading or unloading in the xylem. The excretion of Na<sup>+</sup> from the cytoplasm to the extracellular space requires active transport under salt stress. A previous study showed that a Na<sup>+</sup>/H<sup>+</sup> antiporter, salt overly sensitive 1 (SOS1), is driven by an H<sup>+</sup> gradient generated by H<sup>+</sup>-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 initial 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

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 *ARRs* suggests that there may be functional differences among type-A *RRs*. 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.

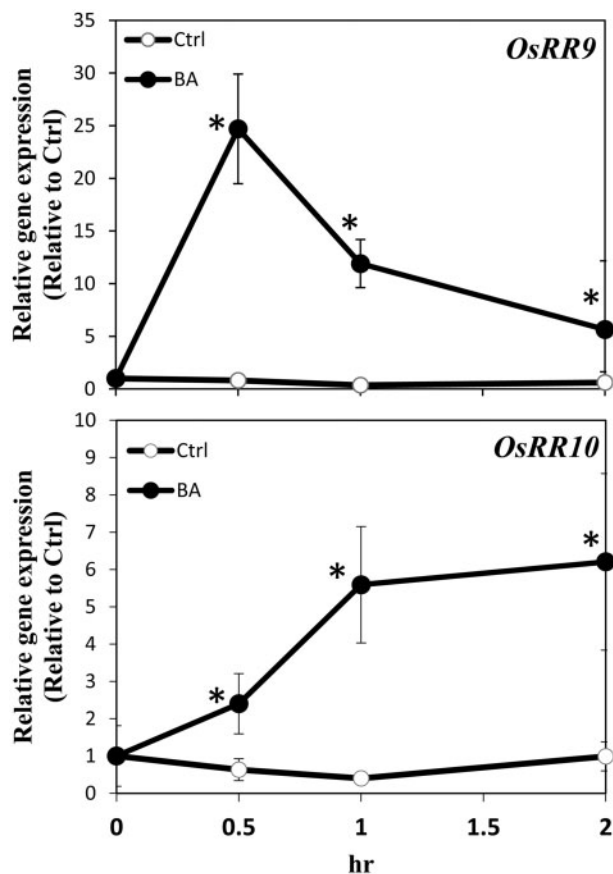
## Results

### *OsRR9* and *OsRR10* are early cytokinin-response genes

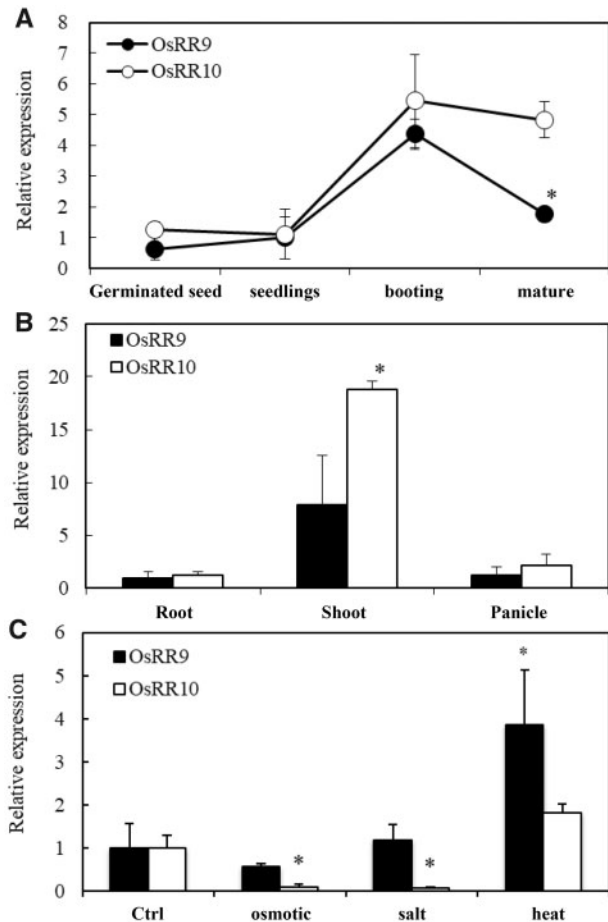
We examined the expression of *OsRR9* and *OsRR10* in response to exogenous cytokinin and at different developmental stages, tissues and in response to various stress conditions. *OsRR9* and

*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  $\mu$ M BA, close symbol) or 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  $\pm$  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 developmental 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  $\pm$  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

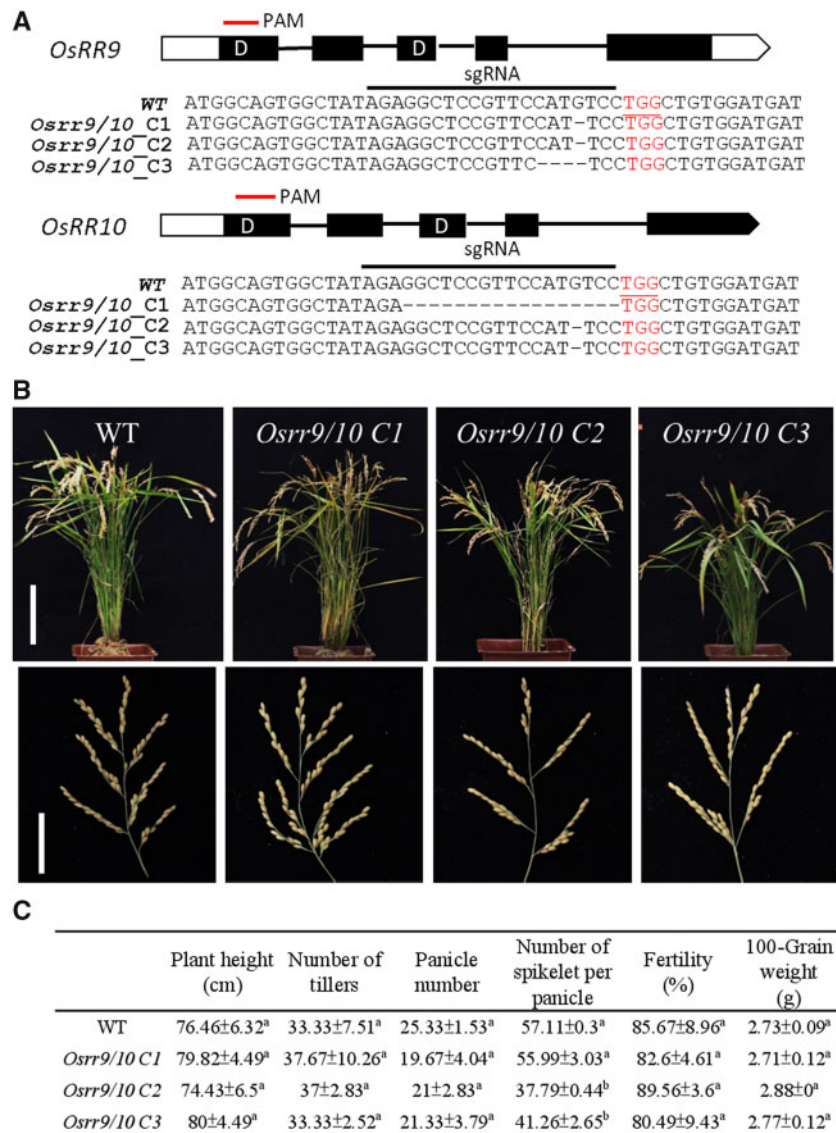
response to the heat stress, whereas *OsRR10* was repressed after 2 h of osmotic stress and salinity stress. These results suggested that *OsRR9* and *OsRR10* may have functionally differentiated to mediate distinct aspects of stress responses.

### 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.

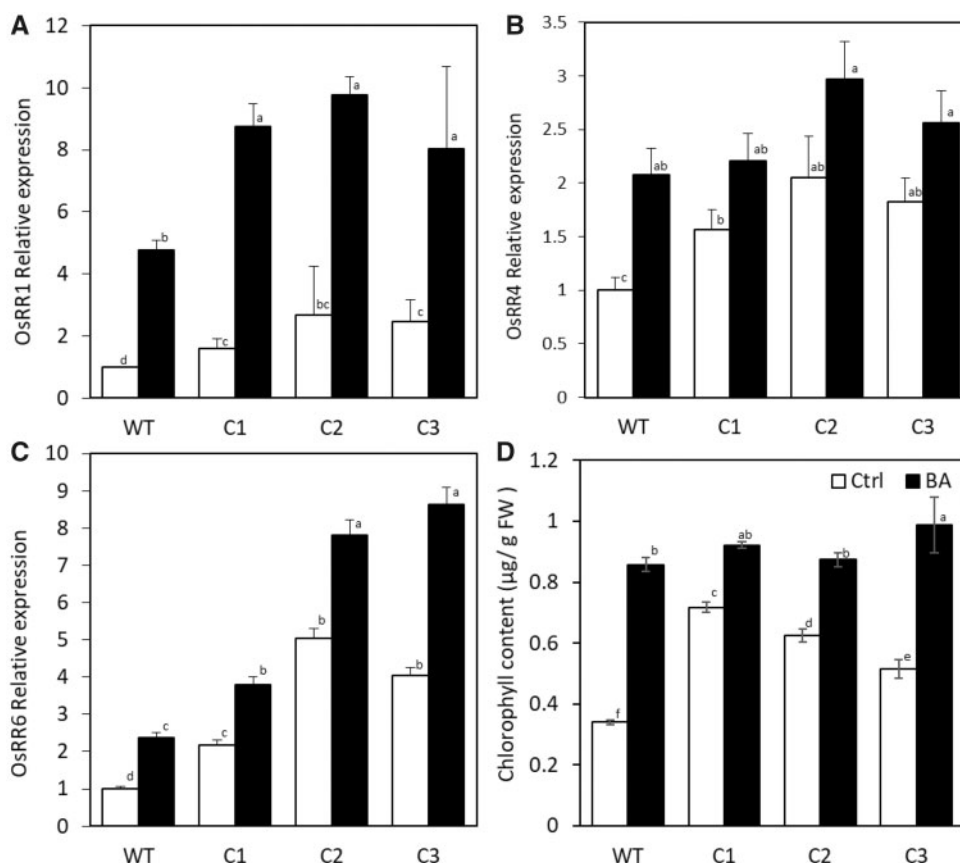


**Fig. 3** Generation and characterization of *osrr9/osrr10* mutations. (A) The top shows a schematic of the target sequences for the CRISPR/Cas9 sgRNA on *OsRR9* and *OsRR10*; boxes represent exons and open and close boxes are UTRs and coding sequencing, respectively. The bottom is a sequence alignment 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 IRR1 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.



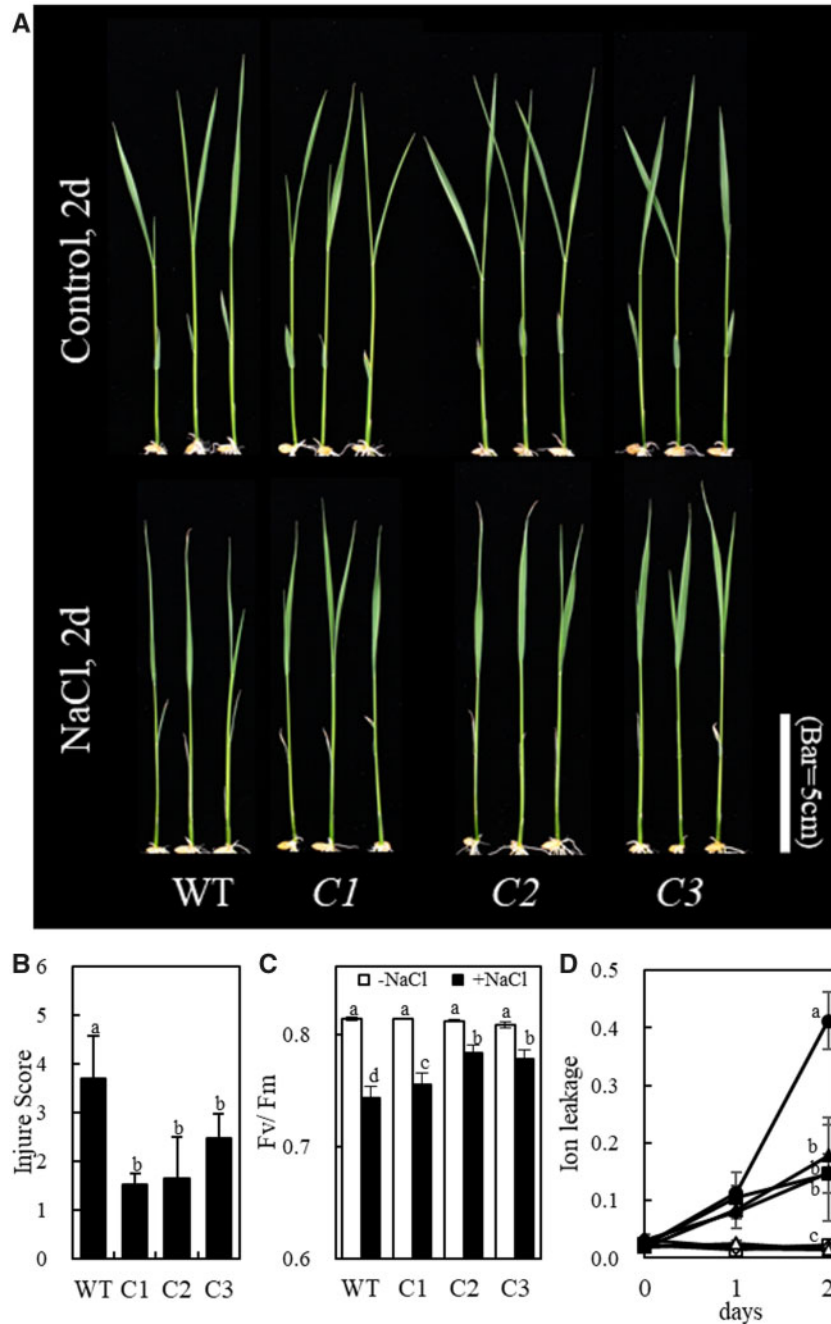
**Fig. 4** Cytokinin-mediated gene expression and leaf senescence responses in *osrr9/osrr10* mutants and wild type. (A–C) The transcription levels of Type-A OsRRs, (A) *OsRR1*, (B) *OsRR4* and (C) *OsRR6*, in *osrr9/osrr10* C1, C2, C3 and wild type in response to exogenous BA. DMSO (Ctrl, white bars) and 5  $\mu$ M 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 OsRRs 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  $\pm$  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.

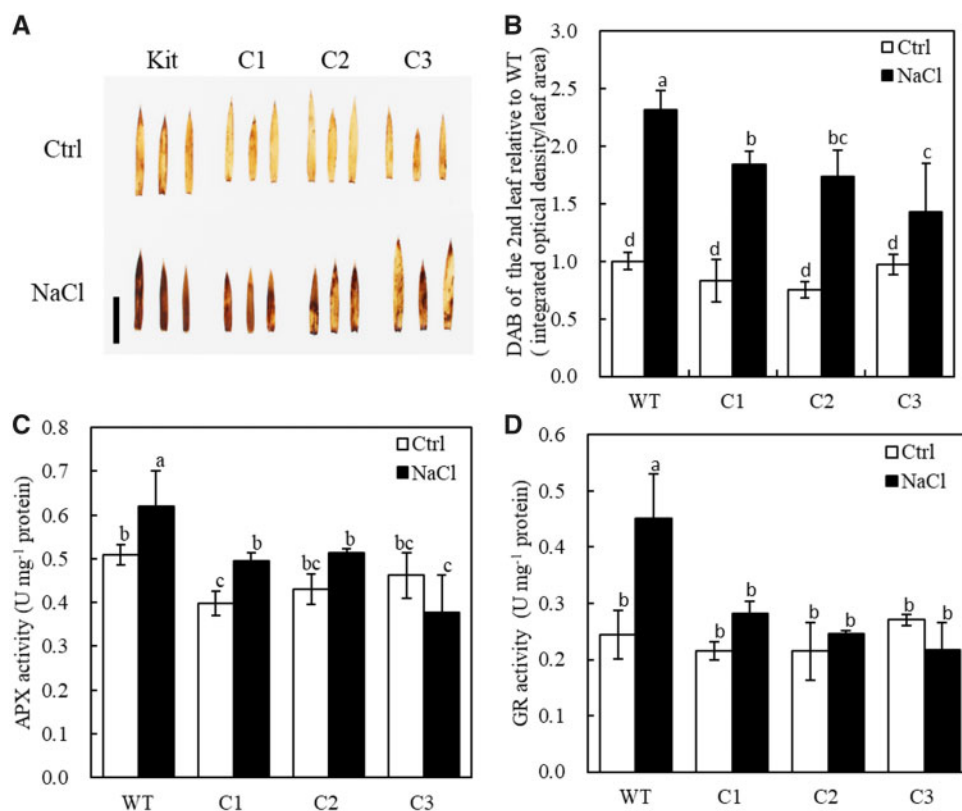


**Fig. 5** Physiological responses of *osrr9/osrr10* mutants (C1, C2 and C3) and wild type (WT) plants under salt stress. (A) The representative photographs of 12-DAG seedlings treated with 150 mM NaCl or control medium for 2 d. (B) The injury score of various seedlings after salt treatments for 2 d. (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  $\pm$  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<sup>+</sup> 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  $\pm$  SD ( $n = 3$ ). Different letters at the top of each column denote statistically significant differences in genotypes and treatments based 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  $\text{Na}^+/\text{K}^+$  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  $\text{Na}^+$  content.

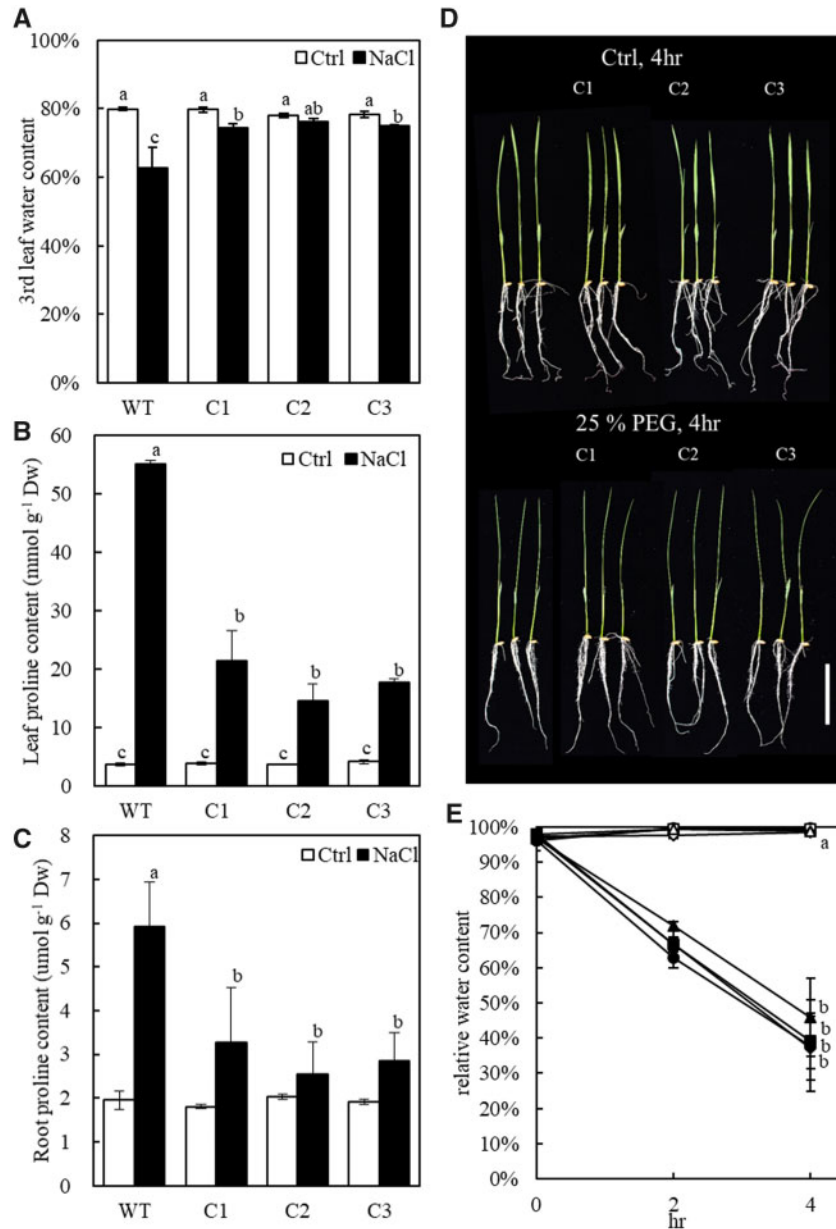
### Differentially expressed genes mediated by OsRR9 and OsRR10

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.



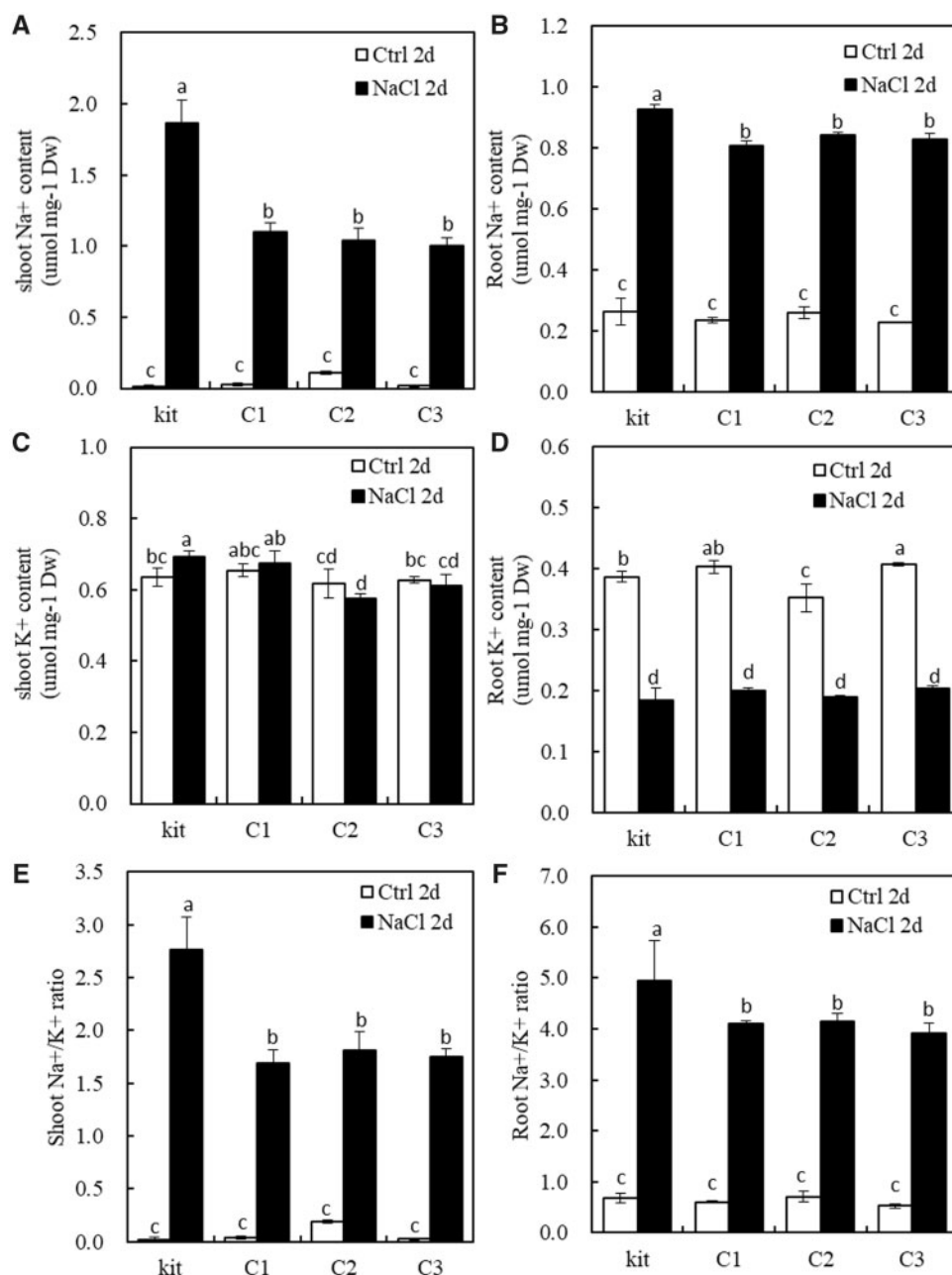
**Fig. 7** Physiological responses of *osrr9/osrr10* mutant (C1, C2 and C3) and wild type plants under salt and osmotic treatments. (A–C) Quantification of the water content (A), leaf proline content (B) and root proline content (C) of 12-DAG wild type and *osrr9/osrr10* mutants. (D, E) The phenotype (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  $\pm$  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 *OsRRs* 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





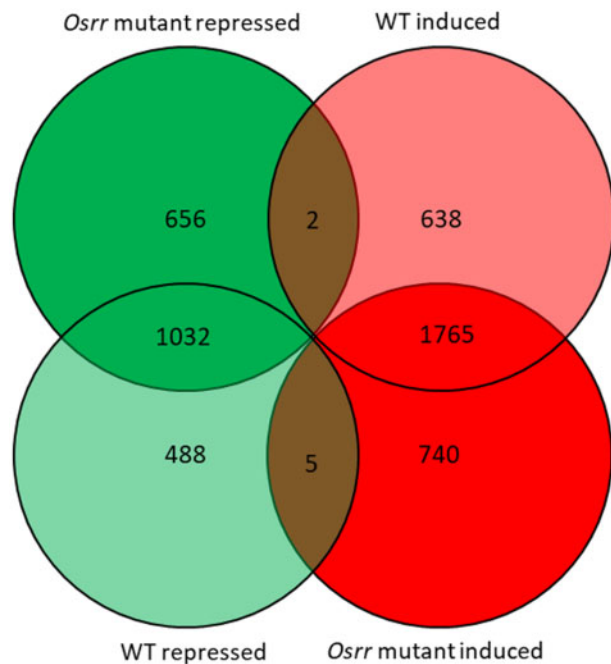
**Fig. 8** *osrr9/osrr10* double mutants accumulated less Na<sup>+</sup> in leaves and roots in elevated salinity. Third-leaf stage seedlings were treated with 150 mM NaCl for 2 d and the sodium (A, B) and potassium content (C, D) were determined from shoots (A, C, E) or roots (B, D, F). All values are mean  $\pm$  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,  $\text{Na}^+/\text{K}^+$  transporters or  $\text{Na}^+/\text{H}^+$  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,  $\text{K}^+$  uptake channel (*OsAKT1*, *OsAKT2*), vacuolar  $\text{Na}^+/\text{H}^+$  transporter (*OsNHX1*) and  $\text{K}^+$  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

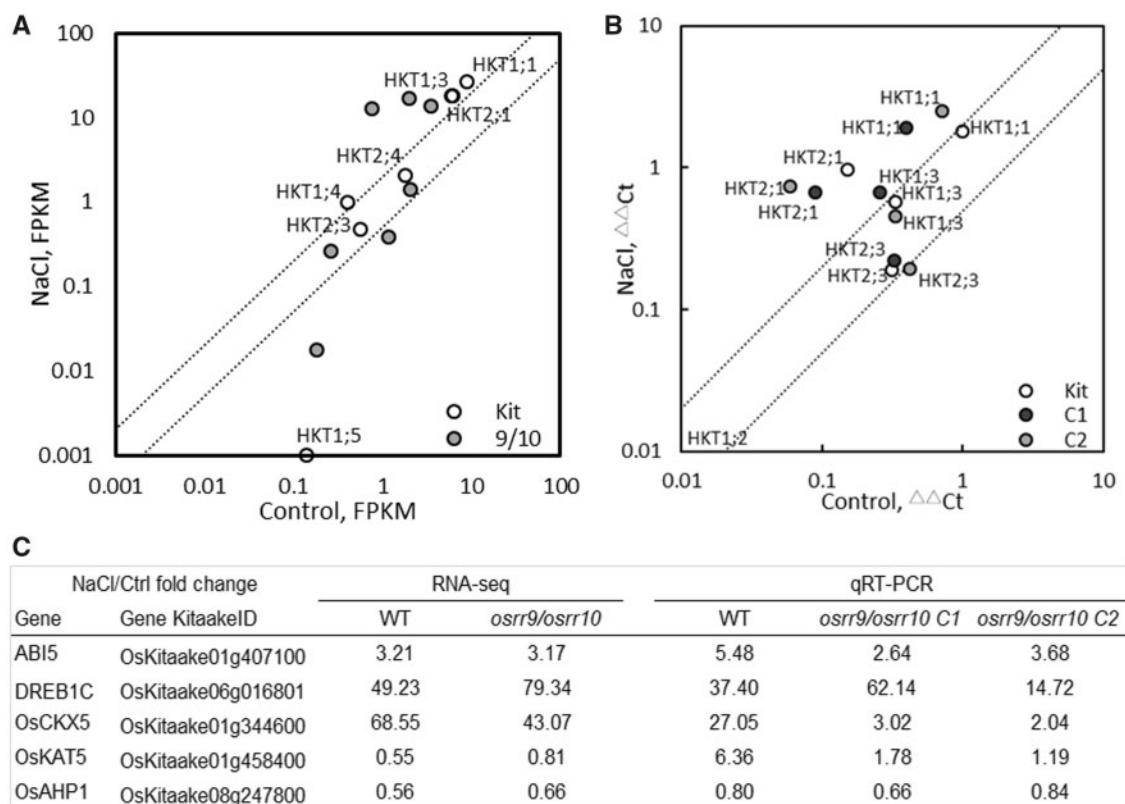
To validate the expression of ion transporters in response to salt stress, we analyzed the transcription levels of genes involving salt tolerance and cytokinin signaling. The leaves of *osrr9/osrr10* C1 and C2 mutants and wild type from two independent experiments were collected after 2 h of salt stress 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 *OsRRs* 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.



**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 conferred 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 over-expressing 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  $\text{Na}^+$  content in the xylem sap and roots by regulating  $\text{Na}^+/\text{H}^+$  ion exchange in the roots to minimize sodium ion accumulation in the shoots and leaves (Ismail and Horie 2017). High-affinity  $\text{K}^+$  transporters (HKT) from multiple plant species have been identified that unload  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+/\text{K}^+$  symporters. At high concentrations of  $\text{Na}^+$  (>10 mM), HKT2s selectively regulate  $\text{Na}^+$  transport only (Horie et al. 2009). Studies on the HKT1 family in Arabidopsis indicate that the transporter *AtHKT1;1* is responsible for unloading  $\text{Na}^+$  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  $\text{Na}^+$  into the phloem and returning  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  accumulation (Horie et al. 2005, Ren et al. 2005). In addition, *OsHKT1;4* regulates the excretion of  $\text{Na}^+$  in leaf sheath cells (Cotsaftis et al. 2012) and also regulates unloading of  $\text{Na}^+$  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  $\text{Na}^+/\text{K}^+$  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  $\mu\text{M}$  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 IRR1 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 SuperScribe IV (ThermoFisher) with oligo (dT)<sub>12–18</sub> 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 (AGAGGCTCCGTTCCATGTCC) 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-driven 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 (%) =  $[(\text{EC1} - \text{EC}_{\text{blank}})/(\text{EC2} - \text{EC}_{\text{blank}})] \times 100$ . Three independent biological replicates and each replicate of at least four plants was prepared for each experiment.

### Chlorophyll content, chlorophyll fluorescence, H<sub>2</sub>O<sub>2</sub> content, antioxidant enzyme activity, proline content and Na<sup>+</sup>/K<sup>+</sup> 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<sub>2</sub>O<sub>2</sub> 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<sub>3</sub>. 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  $\pm$  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.

### Funding

The Ministry of Science and Technology [106-2311-B-002-027-], Ministry of Science and Technology [107-2313-B-002-023-] to Y.-C.T. and the National Science Foundation [IOS-1238051] and the United States Department of Agriculture [2018-67013-27423] to J.K. The funding agencies had no role in the preparation of study design, data collection and analysis, decision to publish or drafting of the manuscript.

### Disclosures

The authors have no conflicts of interest to declare.

## References

- Abdullah, Z. and Ahmad, R. (1990) Effect of pre-and post-kinetin treatments on salt tolerance of different potato cultivars growing on saline soils. *J. Agron. Crop Sci.* 165: 94–102.
- Albacete, A., Ghanem, M.E., Martínez-Andújar, C., Acosta, M., Sánchez-Bravo, J., Martínez, V., *et al.* (2008) Hormonal changes in relation to biomass partitioning and shoot growth impairment in salinized tomato (*Solanum lycopersicum* L.) plants. *J. Exp. Bot.* 59: 4119–4131.
- Alvarez, J.M., Vidal, E.A. and Gutierrez, R.A. (2012) Integration of local and systemic signaling pathways for plant N responses. *Curr. Opin. Plant Biol.* 15: 185–191.
- Argueso, C.T., Ferreira, F.J. and Kieber, J.J. (2009) Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant Cell Environ.* 32: 1147–1160.
- Argyros, R.D., Mathews, D.E., Chiang, Y.-H., Palmer, C.M., Thibault, D.M., Etheridge, N., *et al.* (2008) Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. *Plant Cell* 20: 2102–2116.
- Ashikari, M., Sakakibara, H., Lin, S., Yamamoto, T., Takashi, T., Nishimura, A., *et al.* (2005) Cytokinin oxidase regulates rice grain production. *Science* 309: 741–745.
- Bahmani, K., Noori, S.A.S., Darbandi, A.I. and Akbari, A. (2015) Molecular mechanisms of plant salinity tolerance: a review. *Aust J Crop Sci.* 9: 321.
- Bates, L.S., Waldren, R.P. and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39: 205–207.
- Bielach, A., Hrtyan, M. and Tognetti, V.B. (2017) Plants under stress: involvement of auxin and cytokinin. *Int. J. Mol. Sci.* 18: 1427.
- Blumwald, E., Aharon, G.S. and Apse, M.P. (2000) Sodium transport in plant cells. *Biochim. Biophys. Acta* 1465: 140–151.
- Brandstatter, I. and Kieber, J.J. (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. *Plant Cell* 10: 1009–1019.
- Cerny, M., Kuklova, A., Hoehenwarter, W., Fagner, L., Novak, O., Rotkova, G., *et al.* (2013) Proteome and metabolome profiling of cytokinin action in Arabidopsis identifying both distinct and similar responses to cytokinin down- and up-regulation. *J. Exp. Bot.* 64: 4193–4206.
- Cotsaftis, O., Plett, D., Shirley, N., Tester, M. and Hrmova, M. (2012) A two-staged model of Na<sup>+</sup> exclusion in rice explained by 3D modeling of HKT transporters and alternative splicing. *PLoS One* 7: e39865.
- D'Agostino, I.B., Deruere, J. and Kieber, J.J. (2000) Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol.* 124: 1706–1717.
- Dahlem, T.J., Hoshijima, K., Jurynek, M.J., Gunther, D., Starker, C.G., Locke, A.S., *et al.* (2012) Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. *PLoS Genet.* 8: e1002861.
- Davenport, R.J., Munoz-Mayor, A., Jha, D., Essah, P.A., Rus, A. and Tester, M. (2007) The Na<sup>+</sup> transporter AtHKT1;1 controls retrieval of Na<sup>+</sup> from the xylem in Arabidopsis. *Plant. Cell Environ.* 30: 497–507.
- Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G. and Schroeder, J.I. (2014) Plant salt-tolerance mechanisms. *Trends Plant Sci.* 19: 371–379.
- Fahad, S., Hussain, S., Matloob, A., Khan, F.A., Khaliq, A., Saud, S., *et al.* (2015) Phytohormones and plant responses to salinity stress: a review. *Plant Growth Regul.* 75: 391–404.
- Foster, J.G. and Hess, J.L. (1980) Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. *Plant Physiol.* 66: 482–487.
- Gao, J.P., Chao, D.Y. and Lin, H.X. (2008) Toward understanding molecular mechanisms of abiotic stress responses in rice. *Rice* 1: 36–51.
- Ghanem, M.E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I.C., *et al.* (2008) Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). *J. Exp. Bot.* 59: 3039–3050.
- Gregorio, G.B., Senadhira, D. and Mendoza, R.D. (1997) Screening rice for salinity tolerance. In *IRRI Discussion Paper*, pp. 1–30. International Rice Research Institute, Manila, Philippines.
- Hansen, H. and Dörffling, K. (2003) Root-derived trans-zeatin riboside and abscisic acid in drought-stressed and rewatered sunflower plants: interaction in the control of leaf diffusive resistance? *Funct. Plant Biol.* 30: 365–375.
- Hasegawa, P.M., Bressan, R.A., Zhu, J.-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51: 463–499.
- Hauser, F. and Horie, T. (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K<sup>+</sup>/Na<sup>+</sup> ratio in leaves during salinity stress. *Plant Cell Environ.* 33: 552–565.
- Hirose, N., Makita, N., Kojima, M., Kamada-Nobusada, T. and Sakakibara, H. (2007) Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. *Plant Cell Physiol.* 48: 523–539.
- Horie, T., Hauser, F. and Schroeder, J.I. (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. *Trends Plant Sci.* 14: 660–668.
- Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., *et al.* (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na<sup>+</sup> unloading from xylem vessels to xylem parenchyma cells. *Plant J.* 44: 928–938.
- Hosoda, K., Imamura, A., Katoh, E., Hatta, T., Tachiki, M., Yamada, H., *et al.* (2002) Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the Arabidopsis response regulators. *Plant Cell* 14: 2015–2029.
- Ishida, K., Yamashino, T. and Mizuno, T. (2008) Expression of the cytokinin-induced type-A response regulator gene ARR9 is regulated by the circadian clock in *Arabidopsis thaliana*. *Biosci. Biotechnol. Biochem.* 72: 3025–3029.
- Ismail, A.M. and Horie, T. (2017) Genomics, physiology, and molecular breeding approaches for improving salt tolerance. *Annu. Rev. Plant Biol.* 68: 405–434.
- Jain, M., Nijhawan, A., Arora, R., Agarwal, P., Ray, S., Sharma, P., *et al.* (2007) F-box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. *Plant Physiol.* 143: 1467–1483.
- Joshi, R., Sahoo, K.K., Tripathi, A.K., Kumar, R., Gupta, B.K., Pareek, A., *et al.* (2018) Knockdown of an inflorescence meristem-specific cytokinin oxidase—OsCKX2 in rice reduces yield penalty under salinity stress condition. *Plant Cell Environ.* 41: 936–946.
- Kiba, T., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T., *et al.* (2003) The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in *Arabidopsis thaliana*. *Plant Cell Physiol.* 44: 868–874.
- Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., *et al.* (2007) The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.* 50: 347–363.
- Kirkham, M.B., Gardner, W. and Gerloff, G. (1974) Internal water status of kinetin-treated, salt-stressed plants. *Plant Physiol.* 53: 241–243.
- Kumar, M.N. and Verslues, P.E. (2015) Stress physiology functions of the Arabidopsis histidine kinase cytokinin receptors. *Physiol. Plant.* 154: 369–380.
- Lee, D.J., Kim, S., Ha, Y.M. and Kim, J. (2008) Phosphorylation of Arabidopsis response regulator 7 (ARR7) at the putative phospho-accepting site is required for ARR7 to act as a negative regulator of cytokinin signaling. *Planta* 227: 577–587.
- Leibfried, A., To, J.P., Busch, W., Stehling, S., Kehle, A., Demar, M., *et al.* (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* 438: 1172–1175.

- Li, G.T., Jain, R., Chern, M., Pham, N.T., Martin, J.A., Wei, T., et al. (2017) The sequences of 1504 mutants in the model rice variety kitaake facilitate rapid functional genomic studies. *Plant Cell* 29: 1218–1231.
- Li, S.Y., Zhao, B.R., Yuan, D.Y., Duan, M.J., Qian, Q., Tang, L., et al. (2013) Rice zinc finger protein DST enhances grain production through controlling Gnl1a/OsCKX2 expression. *Proc. Natl. Acad. Sci. USA* 110: 3167–3172.
- Ma, J.F., Goto, S., Tamai, K. and Ichii, M. (2001) Role of root hairs and lateral roots in silicon uptake by rice. *Plant Physiol.* 127: 1773–1780.
- Mäser, P., Eckelman, B., Vaidyanathan, R., Horie, T., Fairbairn, D.J., Kubo, M., et al. (2002) Altered shoot/root Na<sup>+</sup> distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na<sup>+</sup> transporter AtHKT1. *FEBS Lett.* 531: 157–161.
- Mason, M.G., Jha, D., Salt, D.E., Tester, M., Hill, K., Kieber, J.J., et al. (2010) Type-B response regulators ARR1 and ARR12 regulate expression of AtHKT1;1 and accumulation of sodium in Arabidopsis shoots. *Plant J.* 64: 753–763.
- Munns, R. and Tester, M. (2008) Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59: 651–681.
- Nakano, Y. and Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* 22: 867–880.
- Naqvi, S.S.M., Ansari, R. and Khanzada, A.N. (1982) Response of salt-stressed wheat seedlings to kinetin. *Plant Sci. Lett.* 26: 279–283.
- Nishiyama, R., Watanabe, Y., Fujita, Y., Le, D.T., Kojima, M., Werner, T., et al. (2011) Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic Acid responses, and abscisic Acid biosynthesis. *Plant Cell* 23: 2169–2183.
- Nishiyama, R., Watanabe, Y., Leyva-Gonzalez, M.A., Ha, C.V., Fujita, Y., Tanaka, M., et al. (2013) Arabidopsis AHP2, AHP3, and AHP5 histidine phosphotransfer proteins function as redundant negative regulators of drought stress response. *Proc. Natl. Acad. Sci. USA* 110: 4840–4845.
- Orozco-Cardenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA* 96: 6553–6557.
- Peleg, Z. and Blumwald, E. (2011) Hormone balance and abiotic stress tolerance in crop plants. *Curr. Opin. Plant Biol.* 14: 290–295.
- Qadir, M., Ghafoor, A. and Murtaza, G. (2000) Amelioration strategies for saline soils: a review. *Land Degrad. Dev.* 11: 501–521.
- Radanielson, A.M., Gaydon, D.S., Li, T., Angeles, O. and Roth, C.H. (2018) Modeling salinity effect on rice growth and grain yield with ORYZA v3 and APSIM-Oryza. *Eur. J. Agron.* 100: 44.
- Ren, Z.-H., Gao, J.-P., Li, L.-G., Cai, X.-L., Huang, W., Chao, D.-Y., et al. (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat. Genet.* 37: 1141.
- Salome, P.A., To, J.P., Kieber, J.J. and McClung, C.R. (2006) Arabidopsis response regulators ARR3 and ARR4 play cytokinin-independent roles in the control of circadian period. *Plant Cell* 18: 55–69.
- Samarut, E., Lissouba, A. and Drapeau, P. (2016) A simplified method for identifying early CRISPR-induced indels in zebrafish embryos using high resolution melting analysis. *BMC Genomics* 17: 547.
- Sanger, F. and Coulson, A.R. (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* 94: 441–448.
- Sharan, A., Soni, P., Nongpiur, R.C., Singla-Pareek, S.L. and Pareek, A. (2017) Mapping the 'Two-component system' network in rice. *Sci. Rep.* 7: 9287.
- Shen, L., Hua, Y.F., Fu, Y.P., Li, J., Liu, Q., Jiao, X.Z., et al. (2017) Rapid generation of genetic diversity by multiplex CRISPR/Cas9 genome editing in rice. *Sci. China Life Sci.* 60: 506–515.
- Sun, L., Zhang, Q., Wu, J., Zhang, L., Jiao, X., Zhang, S., et al. (2014) Two rice authentic histidine phosphotransfer proteins, OsAHP1 and OsAHP2, mediate cytokinin signaling and stress responses in rice. *Plant Physiol.* 165: 335–345.
- Suzuki, K., Yamaji, N., Costa, A., Okuma, E., Kobayashi, N.I., Kashiwagi, T., et al. (2016) OsHKT1;4-mediated Na<sup>+</sup> transport in stems contributes to Na<sup>+</sup> exclusion from leaf blades of rice at the reproductive growth stage upon salt stress. *BMC Plant Biol.* 16: 22.
- Takagi, H., Tamiru, M., Abe, A., Yoshida, K., Uemura, A., Yaegashi, H., et al. (2015) MutMap accelerates breeding of a salt-tolerant rice cultivar. *Nat. Biotechnol.* 33: 445–449.
- To, J.P., Deruere, J., Maxwell, B.B., Morris, V.F., Hutchison, C.E., Ferreira, F.J., et al. (2007) Cytokinin regulates type-A Arabidopsis response regulator activity and protein stability via two-component phosphorelay. *Plant Cell* 19: 3901–3914.
- To, J.P., Haberer, G., Ferreira, F.J., Deruere, J., Mason, M.G., Schaller, G.E., et al. (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *Plant Cell* 16: 658–671.
- Tran, L.S., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., et al. (2007) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 104: 20623–20628.
- Tsai, Y.-C., Weir, N.R., Hill, K., Zhang, W., Kim, H.J., Shiu, S.-H., et al. (2012) Characterization of genes involved in cytokinin signaling and metabolism from rice. *Plant Physiol.* 158: 1666–1684.
- Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Stress-responsive expression of genes for two-component response regulator-like proteins in *Arabidopsis thaliana*. *FEBS Lett.* 427: 175–178.
- Wang, R., Jing, W., Xiao, L., Jin, Y., Shen, L. and Zhang, W. (2015) The rice high-affinity potassium transporter1;1 is involved in salt tolerance and regulated by an MYB-type transcription factor. *Plant Physiol.* 168: 1076–1090.
- Wintermans, J.F.G.M. and De Mots, A. (1965) Spectrophotometric characteristics of chlorophylls a and b and their phenophytins in ethanol. *Biochim. Biophys. Acta* 109: 448–453.
- Wu, X., He, J., Chen, J., Yang, S. and Zha, D. (2014) Alleviation of exogenous 6-benzyladenine on two genotypes of eggplant (*Solanum melongena* Mill.) growth under salt stress. *Protoplasma* 251: 169–176.
- Xu, R.F., Li, H., Qin, R.Y., Li, J., Qiu, C.H., Yang, Y.C., et al. (2015) Generation of inheritable and “transgene clean” targeted genome-modified rice in later generations using the CRISPR/Cas9 system. *Sci. Rep.* 5: 11491.
- Yuan, Y.H., Shu, S., Li, S.H., He, L.Z., Li, H., Du, N.S., et al. (2014) Effects of exogenous putrescine on chlorophyll fluorescence imaging and heat dissipation capacity in cucumber (*Cucumis sativus* L.) under salt stress. *J. Plant Growth Regul.* 33: 798–808.
- Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., et al. (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12: 797–807.
- Zhu, X., Xu, Y., Yu, S., Lu, L., Ding, M., Cheng, J., et al. (2014) An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Sci. Rep.* 4: 6420.
- Zubo, Y.O., Blakley, I.C., Yamburenko, M.V., Worthen, J.M., Street, I.H., Franco-Zorrilla, J.M., et al. (2017) Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 114: E5995–E6004.
- Zwack, P.J. and Rashotte, A.M. (2015) Interactions between cytokinin signalling and abiotic stress responses. *J. Exp. Bot.* 66: 4863–4871.