ORIGINAL ARTICLE

OsRR26, a type-B response regulator, modulates salinity tolerance in rice via phytohormone-mediated ROS accumulation in roots and influencing reproductive development

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Abstract

Main Conclusion **OsRR26 is a cytokinin-responsive response regulator that promotes phytohormone-mediated ROS accumulation in rice roots, regulates seedling growth, spikelet fertility, awn development, represses NADPH oxidases, and negatively affects salinity tolerance.**

Plant two-component systems (TCS) play a pivotal role in phytohormone signaling, stress responses, and circadian rhythm. However, a significant knowledge gap exists regarding TCS in rice. In this study, we utilized a functional genomics approach to elucidate the role of OsRR26, a type-B response regulator in rice. Our results demonstrate that *OsRR26* is responsive to cytokinin, ABA, and salinity stress, serving as the ortholog of Arabidopsis ARR11. OsRR26 primarily localizes to the nucleus and plays a crucial role in seedling growth, spikelet fertility, and the suppression of awn development. Exogenous application of cytokinin led to distinct patterns of reactive oxygen species (ROS) accumulation in the roots of both WT and transgenic plants (OsRR26OE and OsRR26KD), indicating the potential involvement of OsRR26 in cytokinin-mediated ROS signaling in roots. The application of exogenous ABA resulted in varied cellular compartmentalization of ROS between the WT and transgenic lines. Stress tolerance assays of these plants revealed that OsRR26 functions as a negative regulator of salinity stress tolerance across different developmental stages in rice. Physiological and biochemical analyses unveiled that the knockdown of OsRR26 enhances salinity tolerance, characterized by improved chlorophyll retention and the accumulation of soluble sugars, K^+ content, and amino acids, particularly proline.

Abbreviations

HK	Histidine kinase
H2DCFDA	2,7-dichlorodihydrofluorescein diacetate
ROS	Reactive oxygen species
RR	Response regulator
SOD	Superoxide dismutase
TCS	Two component signaling

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Introduction

Histidine-aspartate phosphorylation (multistep phosphorelay) operating in two-component system (TCS) is a signaling mechanism that regulates various plant processes such as cytokinin and ethylene signaling, circadian rhythm, and responses to abiotic stresses (Urao et al. 2000; Grefen and Harter 2004; Nongpiur et al. 2012, 2019). His-asp phosphorelay in plants uses a hybrid-type sensory histidine kinase (HK), which contains a sensory domain, a transmitter domain, and a receiver domain. Histidine-containing phosphotransfer protein(s) (HPTs) act as mediators of signaling between the sensory HKs and response regulators (RRs) (Lohrmann and Harter 2002; Pareek et al. 2006). In

the His-asp phosphorelay, signals are perceived by the HK, which autophosphorylates, and this phosphoryl group is eventually transferred to the type-B RRs via the HPTs.

The RRs constitute the largest and most diverse group among the plant TCS family. The RRs constitute the effector molecules from which the TCS signaling in plants diverges into various other signaling cascades. They also form one of the most important feedback regulation steps for TCS phosphorelay in plants. Among these RRs, the type-B RRs are of particular interest since they contain a MYB DNA-binding domain in their C-terminal end and thus could potentially function as transcription factors. While a lot of work has been done in Arabidopsis to identify the role of response regulators in phytohormone-mediated development, not much information is available for rice.

In rice, our group has identified all the members of the two-component signaling system and reported its architecture to be similar to that in Arabidopsis (Pareek et al. 2006). The rice TCS family comprises of 11 HKs, 5 HPTs, and 36 RRs, some of which have been shown to function in various processes such as cytokinin signaling and stress responses (Choi et al. 2012; Tsai et al. 2012; Kushwaha et al. 2014; Sun et al. 2014; Ding et al. 2017; Naruse et al. 2018; Zhao et al. 2020). Transcript abundance studies have shown that the TCS genes in rice could play a key role in the abiotic stress responses (Karan et al. 2009; Singh et al. 2015; Shankar et al. 2016). Sun et al. (2014) reported that the OsHPTs, OsAHP2 and OsAHP3, are positive regulators of salinity stress tolerance in rice. In particular, reports have shown that the type-B response regulators, OsRR21, OsRR22, OsRR23, and OsRR24 have a functional overlap, playing a key role in various processes including leaf and root growth, inflorescence architecture, flower development and fertilization, trichome formation, cytokinin sensitivity, and tolerance to salinity stress (Takagi et al. 2015; Zhang et al. 2019; Worthen et al. 2019; Yamburenko et al. 2020). CRISPR/Cas9 generated mutants of 13 type-B OsRRs have been shown to vary in various traits such as plant height, tiller number, tillering angle, heading date, panicle length and yield (Li et al. 2022). OsRR22 has been shown to negatively regulate salinity tolerance in rice through interaction with OsHKT1 (Liu et al. 2023). We have reported the interaction map of the various TCS components in rice, which revealed that OsRR26, a type-B RR, interacted with all of the 3 rice pseudo-HPTs (OsPHPs) (Sharan et al. 2017). This indicated that OsRR26 could be involved in diverse processes mediated by the TCS in rice. Here, we adopted a transgenic approach to provide the functional characterization of OsRR26 in rice. Our findings reveal that *OsRR26* expression is regulated by cytokinin, ABA and osmotic stress. OsRR26 regulates the phytohormone-mediated ROS accumulation in rice root which plays a key role in its development. Furthermore, we show that OsRR26 negatively regulates salinity tolerance and plays a major role in reproductive development in rice.

Materials and methods

Plant materials and growth conditions

Oryza sativa L. (cv 1R64) was grown in the green house at the School of Life Sciences, Jawaharlal Nehru University (JNU), New Delhi, India. Mature seeds were then harvested and used for subsequent experiments.

Stress treatments

For salinity stress treatments, WT, OE and KD plants were treated with 200 mM NaCl for different time durations under the hydroponics system containing half-strength Yoshida media. Simultaneously, untreated (control) were maintained in half-strength Yoshida media. At each time point of stress treatment, untreated as well as stress-treated plants were harvested and used for recording different biochemical and physiological parameters.

Phylogenetic analyses

The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (-21962.72) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis for Fig. 1a involved 7 amino acid sequences. There were a total of 755 positions in the final dataset. The analysis for Fig. 1b involved 18 amino acid sequences. There were a total of 823 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Subcellular localization of OsRR26

OsRR26 were PCR amplified from cDNA obtained from seedlings of IR64 using RevertAid™ cDNA synthesis kit (Fermentas Life Sciences, Waltham, MA, USA) and cloned in pCAMBIA1304 for C-terminal GFP fusion. Particle bombardment of onion peel epidermal cells was carried out as previously described (Kikkert 1993). Transformed peels were kept in the dark for 18 h at 25 °C, and GFP fluorescence was viewed using a confocal laser scanning microscope (FluoView-FV1000-MPE, Olympus, Tokyo, Japan).
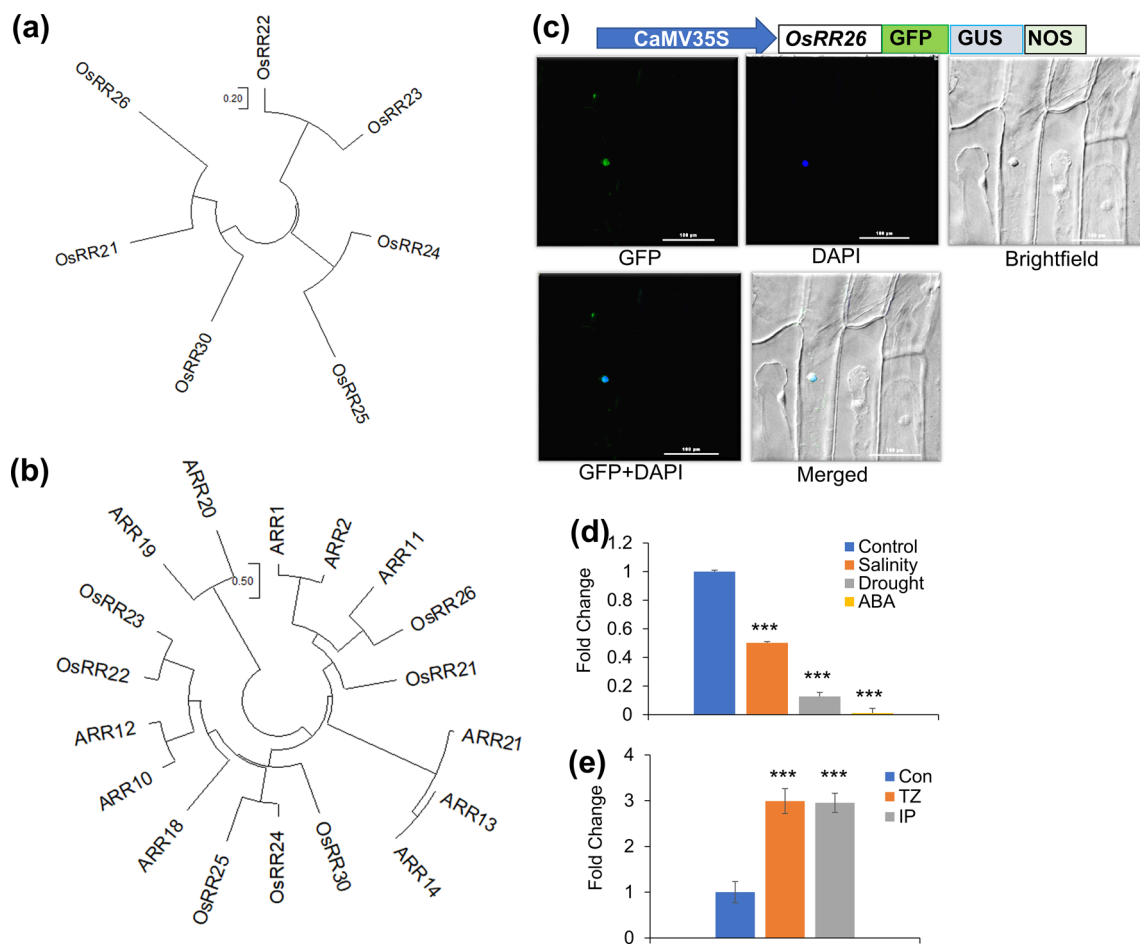
Fig. 1 *OsRR26* is a cytokinin, ABA and osmotic stress-responsive type-B Response Regulator (RR). **a** Phylogenetic tree showing the evolutionary relationship between the type-B response regulators in rice. **b** Phylogenetic tree showing the evolutionary relationship between the type-B RRs of rice and Arabidopsis. *OsRR26* is the ortholog of *ARR11*, a cytokinin-responsive, nuclear-localized, DNA binding Arabidopsis type-B RR. **c** Confocal microscopy showing the nuclear and cytoplasmic subcellular localization of GFP tagged OsRR26 in biolistically transformed onion epidermal cells. **d** qRT-PCR revealing the expression of *OsRR26* under salinity, drought, and ABA treatment in shoot tissues of 14-day-old rice seedlings. *OsRR26* expression is repressed under salinity, drought, and ABA treatment. **e** Exogenous cytokinin application of TZ and IP caused increased expression of *OsRR26* in tissues of 14-day-old rice seedlings. Mean values $\pm$SE ($n = 3$); ***$P \leq 0.001$

Total RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA was isolated from shoots of all the untreated and stressed seedlings, and mRNA was isolated as described previously (Karan et al. 2009). cDNA synthesis was done using the ReverAidTM cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's instructions. Primers designing and real-time PCR reactions were performed as previously described (Karan et al. 2009).

Rice transformation

The constructs for overexpression and knockdown of *OsRR26* in rice were transformed into *Agrobacterium tumefaciens* (LBA4404) and confirmation of the transformants

was performed using colony PCR. A detailed description of the constructs is provided in Suppl. Figs. S2 and S3. The transformed *Agrobacterium* cells were then used for the *Agrobacterium*-mediated transformation of rice as described (Sahoo et al. 2011).

Southern-blot analysis of *OsRR26* transgenic plants

The copy number of the inserted transgene was determined by Southern analysis. 25 µg of genomic DNA was digested with *Eco*R1 (NEB-High fidelity) enzyme and subjected to overnight electrophoresis on a 1% agarose gel at 30 volts. The gel was washed, followed by its depurination, denaturation, and neutralization. The DNA was then transferred onto a nylon membrane (Hybond N+ membrane,

Amersham Pharmacia Biotech, Chalfont St Giles, UK) by capillary action. The membrane was then air-dried, and DNA immobilization on the membrane was performed by UV-crosslinking in a UV-crosslinker (Amersham Pramacia Biotech). For the *OsRR26* overexpression lines, radiolabeled ATP was used for probe synthesis using the decalabel DNA labeling kit (Thermo Fischer Scientific) as per the manufacturer's instructions. The probed blots were washed and kept for IP exposure overnight. Detection was performed using a Typhoon Fla 9000 laser scanner. For the *OsRR26* knockdown lines, fall-out Southern blotting was performed, and DNA was digested by *Asc*I and *Spe*I. Blotting was done using a DIG DNA labeling kit (Roche Life Science, Basel, Switzerland) as per the manufacturer's instructions. The probe specific to the GUS spacer sequence of pFGC1008 was used. The probe was prepared using a DIG DNA labeling kit (Roche Life Science), and hybridization was performed according to the manufacturer's instructions. Detection was performed by immunological detection using anti-DIG-alkaline phosphatase-conjugated antibodies. Colour development was carried out directly on the membrane using NBT/BCIP.

Seed germination and seedling growth assay

Seeds of WT and transgenic rice were placed on cotton wool soaked with half-strength Yoshida medium containing 200 mM NaCl and the plates were kept in the dark for 48 h. The seeds were then allowed to germinate at 28 ± 2 °C in a plant growth chamber with 12-h alternate light/dark cycles for a further period of up to 14 days. Seeds germinating in half-strength Yoshida Medium were used as control. For seedling growth assay, root length, shoot length and fresh weight of WT and transgenic lines were measured for salt-stressed plants along with their control samples. For the measurements of dry weight, seedlings were kept in an oven at 60 °C for 48 h and weighed.

Leaf disc assay

Leaf discs were excised from healthy and fully expanded rice leaves of 14-d-old transgenic and WT plants and floated in a 6 ml solution of half Yoshida medium containing 200 mM NaCl for 96 h. Leaf discs floated in half-strength Yoshida medium were kept as control. These leaf discs were used for measuring chlorophyll content.

Chlorophyll estimation

For chlorophyll estimation in leaf discs, 100 mg of tissue was homogenized thoroughly in 1 ml of 80% acetone and centrifuged at $1200 \times g$ for 2–3 min. The supernatant was retained, and absorbance was recorded using a spectrophotometer at 663 and 645 nm. For seedlings, chlorophyll estimation was performed using the Soil Plant Analysis Development (SPAD, Minolta Camera Co., Osaka, Japan) chlorophyll meter as per the manufacturer's instructions.

Proline estimation

Proline content was estimated using the standard protocol developed by Bates et al. (1973). 100 mg tissue was crushed in liquid N_2 and homogenized in 10 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at $18,000 \times g$, and to the 2 ml of an upper aqueous phase, 2 ml of 0.2% ninhydrin and 2 ml of glacial acetic acid were added. After incubation at 100 °C for 1 h, the reaction was terminated by transferring to ice. Proline was extracted from the mixture with 4 ml of toluene. The O.D. of the upper aqueous phase was measured at 520 nm. Proline concentration was calculated using a standard curve for proline.

Total amino acids estimation

100 mg tissue was crushed in liquid N2 and homogenized in 10 ml of 3% sulfosalicylic acid. The homogenate was centrifuged at $18,000 \times g$, and to the 2 ml of an upper aqueous phase, 2 ml of 0.2% ninhydrin was added. After incubation at 100 °C for 10 min, the reaction was terminated by transferring to ice. Total amino acids were estimated using standard curves prepared with L-proline and L-glycine as described before (López-Hidalgo et al. 2021).

Na$^+$ estimation in leaves

To determine endogenous Na^+, 100 mg of leaf tissue (control and stressed) were taken and digested in 0.1% HNO_3. Ions were extracted in distilled H_2O by boiling for 30 min twice. The filtered extract, thus obtained, was used to measure Na^+ ions using atomic absorption spectroscopy.

Antioxidant enzyme activity assay

Superoxide dismutase (SOD) assay: SOD activity was estimated according to the protocol developed by Giannopolitis and Ries (1977). The reaction mixture was composed of crude enzyme extract (5 µg of protein) 1.3 mM riboflavin, 13 mM methionine, 63 mM NBT, 0.05 M sodium carbonate (pH 10.2) with a final volume of 3 ml made up with water.

Ascorbate peroxidase (APX) assay: APX activity was measured according to the protocol described by Nakano and Asada (1981). The reaction mixture consisted of 50 mM (pH 7.5) K_2PO_4 buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 10 mM H_2O_2 and enzyme extract with a final volume of 1 ml.

Catalase (CAT) assay: Catalase activity was measured according to the protocol described by Aebi (1984). The decrease in absorbance at 240 nm due to the decomposition of H_2O_2 was measured. The reaction mixture was composed of 50 mM K_2PO_4 buffer (pH 7.0) and leaf extract (5 μg protein). The reaction was started by the addition of H_2O_2 to a final concentration of 20 mM.

Detection of ROS

ROS production was detected using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Thermo Fisher Scientific). Stock solution of 10 mM H2DCFDA was prepared in dimethyl sulphite (DMSO) and diluted with ddH_2O to a final concentration of 10 μM for infiltration. For the assay, seeds of WT and transgenic rice were germinated and treated with 50 μM 6-BAP and ABA for seven days. Root tips from three biological replicates of each seedlings were excised and vacuum infiltrated in 10 μM H2DCFDA for 5 min and incubated for 10 min under dark conditions. Further, washing was done with ddH_2O to remove extra dye and green fluorescence was assessed by confocal laser scanning microscope at 488 nm excitation wavelength.

Total soluble sugars content

100 mg tissue was homogenized in a mortar pestle containing hot aqueous ethanol (80% v/v). The homogenate was centrifuged, 0.2 ml of supernatant was taken in separate test tubes and volume was made upto 1 ml using deionized water. 0.2% anthrone reagent was added in test tubes and samples were then heated for 8 min in boiling water and cooled rapidly. Intensity of dark green colour was measured at 620 nm. Glucose concentration was calculated using a standard curve.

Yield analysis

Yield analysis was carried out on control and stressed WT and transgenic rice plants as per protocol prescribed by the International Rice Research Institute (IRRI) (Gregorio et al. 1997).

Statistical analysis

Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigmaplot), and comparisons of each transgenic line against the WT values only were considered, where *, **, and *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Values are the mean of three independent experiments.

Results

OsRR26 is a cytokinin, ABA and osmotic stress-responsive type-B response regulator

OsRR26 is a typical of type-B response regulator containing the characteristic receiver domain and a GARP domain (Pareek et al. 2006). To gain insight about the possible role of OsRR26, we conducted a phylogenetic analysis of all the rice type-B RRs and found that the type-B RRs in rice are classified into 3 subgroups in a pairwise manner (OsRR22/OsRR23, OsRR24/OsRR25, OsRR21/OsRR26), with OsRR30 not falling under any subgroup (Fig. 1a). In Arabidopsis, the RRs are also classified into subgroups as pairs, with a few exceptions (Imamura et al. 2003). Another phylogenetic study of all the putative type-B RRs of rice and Arabidopsis revealed that OsRR26 is the ortholog of AtARR11 (Fig. 1b), a nuclear-localized, cytokinin-responsive type-B response regulator which displays both in vitro phosphorylation and DNA binding capabilities with the ARR11 GARP domain preferentially binding to GGATT suggesting a role for ARR11 as a transcription regulator (Imamura et al. 2003). The protein sequences of both the receiver domains and GARP domains of OsRR26 and AtARR11 are 88% conserved, further indicating that OsRR26 could have functional similarities to AtARR11 (Suppl. Fig. S1). For OsRR26 to function as a transcriptional regulator, it has to be localized in the nucleus. To determine its subcellular localization, we transformed an *OsRR26-GUS-GFP* construct into onion epidermal cells. Confocal microscopy using DAPI as a nuclear marker revealed that OsRR26 was localized predominantly in the nucleus, although GFP signals were also detected in the cytoplasm (Fig. 1c).

Since ARR11 is a negative regulator of salinity stress (Imamura et al. 2003), we analysed the expression of *OsRR26* in rice seedlings under various treatments and observed that *OsRR26* expression is induced by cytokinin, but repressed under drought, salinity, and ABA treatment (Fig. 1d, e). This indicated that perhaps OsRR26 could function in cytokinin signaling and also act as a negative regulator of salinity and drought stress.

## *OsRR26* causes phytohormone-mediated ROS accumulation in the rice root

To functionally characterize *OsRR26*, we generated *OsRR26* overexpression (*OsRR26*OE) and *OsRR26* knockdown (*OsRR26*KD) transgenic rice plants in the IR64 background. Schematic diagrams of the overexpression and knockdown constructs, as well as the confirmation

of cloning, have been provided in Suppl. Fig. S2 and S3. Transgene insertion was screened by PCR, and qRT-PCR was used to determine *OsRR26* expression levels (Suppl. Fig. S4a–c). The transgenic lines with the highest (for OE) and lowest (for KD) expression levels of *OsRR26* were used for confirmation of transgene insertion through Southern-blot analysis (Suppl. Fig. S4d).

As *OsRR26* expression is cytokinin and ABA-responsive, we analyzed the role of these two phytohormones in the ROS accumulation in WT, *OsRR26*OE, and *OsRR26*KD lines using H2DCFDA staining (Fig. 2). Under control condition, WT showed negligible ROS, which increased drastically in the vascular region under 6-BAP treatment. On the contrary, ROS accumulation was observed in the middle vascular region in OE under control condition which reduces under 6-BAP treatment. KD showed increased ROS in the root epidermis and cortex under 6-BAP treatment, but was dispersed throughout the root under control condition. When ABA was applied exogenously, ROS accumulated in the elongation zone in all three seedling types. Notably, the roots of WT, KD, and OE seedlings were swollen in response to ABA treatment. ROS accumulated in intracellular as well as extracellular space in WT under ABA treatment, while in OE and KD, ROS was confined to the extracellular space; however, its levels were comparatively higher in the former.

Knockdown of *OsRR26* results in elongated awns and a drastic reduction in spikelet fertility

Comparative morphological analysis between the WT, *OsRR26*OE, and *OsRR26*KD transgenic rice plants revealed that OE and KD plants showed no significant differences to the WT with regards to germination and seedling growth characteristics. The leaf architecture was slightly different in the KD lines and the KD plants were taller but the difference was not statistically significant. Representative plants of the WT, *OsRR26*OE, and *OsRR26*KD plants are shown in Fig. 3a. The WT and OE lines showed little difference in panicle development and size, while the KD lines had longer panicles with a lower number of spikelets per panicle (Fig. 3b, upper panel, c). The seeds of the KD lines had extremely long awns (Fig. 3b, lower panel). The seeds of the OE-1 and OE-5 lines were 0.41 and 0.34 mm shorter in length than that of the WT, while those of the KD-2 and KD-8 lines were 0.56 and 0.84 mm longer than the WT (Fig. 3d, e). The weight of one thousand seeds of OE lines were ~2.5 g lower than that of the WT, while that of the KD lines were ~5–7 g higher than the WT (Fig. 3f). No significant difference was observed with regards to the number of tillers and panicles of the WT, OE, and KD plants (Suppl. Fig. S5a and b). There were no significant differences between the spikelet fertility of the OE lines and the WT (Fig. 3g). However, spikelet fertility was severely hampered in the KD transgenic plants (Fig. 3g). The spikelet fertility of the OE-1, OE-5, WT, KD-2, and KD-8 lines under control conditions were 87.55, 88.89, 87.74, 1.96, and 9.62%,
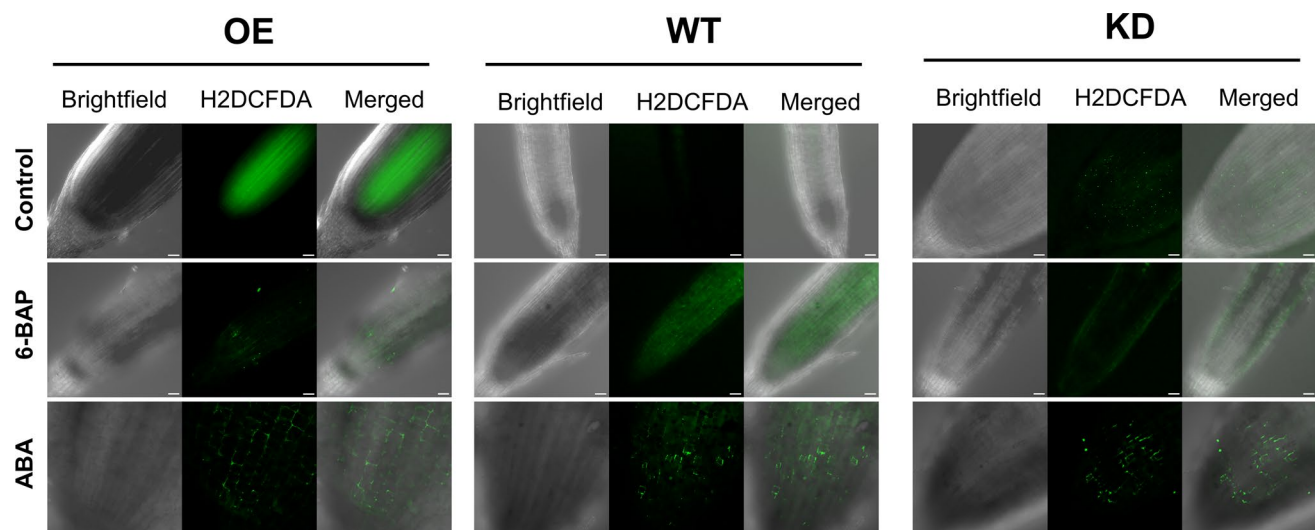
Fig. 2 In vivo ROS imaging in WT, *OsRR26*OE and *OsRR26*KD transgenic rice plants treated with cytokinin and ABA. Roots of WT, *OsRR26*OE, and *OsRR26*KD grown under control, 50 µM 6-BAP, and 50 µm ABA conditions were excised at the tip and infiltrated with 10 µM H2DCFDA fluorescent dye. For microscopy, samples were visualized under a confocal laser microscope. The intensity of green fluorescence indicates the level of ROS accumulation. ROS levels were localized at elongation zone under cytokinin treatment. Exogenous ABA treatment caused ROS accumulation in the extracellular space. Scale bars = 50 µm

respectively (Fig. 3g). As a result of the effect of *OsRR26* knockdown on spikelet fertility, the grain weight per plant was significantly reduced in the *OsRR26*KD lines (Fig. 3h).

OsRR26 regulates the expression of NADPH oxidases, *OsRBOHB* and *OsRBOHE*, and the activity of downstream superoxide dismutase (SOD)

OsRR26 interacts with OsPHP3, which inturn interacts with the histidine kinase, OsHK3 (Sharan et al. 2017). OsHK3 has been shown to regulate the rice NADPH oxidases, OsRBOHB and OsRBOHE (Wen et al. 2015). Therefore, we determined the expression of *OsRBOHB* and *OsRBOHE* in the WT, OE and KD lines to determine if OsRR26 has any overlapping functions with OsHK3 with which it shares an interacting partner. Expression levels of the *OsRBOHB* and *OsRBOHE* in the WT, OE, and KD transgenic seedlings using qRT-PCR revealed no significant changes in the transcript level of *OsRBOHB* between the WT and OE transgenic seedlings, but the transcript levels were higher in the KD transgenic lines (Fig. 4a). Transcript levels of *OsRBOHE* were reduced considerably in the OE lines than in the WT, while those of the KD transgenic lines were increased (Fig. 4b). Thus, OsRR26 negatively regulates the expression of the rice NADPH oxidases, *OsRBOHB*, and *OsRBOHE*.

Under oxidative and osmotic stress, an increase in the expression of *OsRBOHB* and *OsRBOHE* resulted in the increased activities of antioxidant enzymes, SOD, ascorbate peroxidase (APX), and catalase (CAT) (Wen et al. 2015). Therefore, we also assayed the activity of SOD, APX and CAT in control and salinity-stressed WT, *OsRR26*OE, and *OsRR26*KD rice seedlings. Salinity was chosen as a treatment based on the fact that it exerts osmotic stress, ionic stress, leads to oxidative stress, and also induces ABA response (Hernández et al. 2001; Munns and Tester 2008; Zelm et al. 2020). We observed lower SOD activity in the OE plants under control and salinity-stressed conditions compared with the WT. The SOD activity was significantly higher in the KD transgenic plants in both control and salinity-stressed seedlings (Fig. 4c). APX activity was also affected, but only in the KD lines where the activity of APX was significantly higher than that of the WT and OE transgenic seedlings in the control condition only (Fig. 4d). There were no significant differences among the APX activities in the WT, OE, and KD seedlings under salinity stress (Fig. 4d). We observed no significant difference in the catalase activity among the WT, OE, and KD transgenic seedlings under control conditions. Under salinity, no significant differences were observed between the WT and OE lines for catalase activity, while the CAT activity was significantly higher for KD lines as compared to the WT and OE lines (Fig. 4e).

OsRR26 has a negative regulatory role in osmotic stress response in rice

To verify the role of OsRR26 with regards to osmotic stress, WT, and OsRR26 transgenic plants were used for comparative osmotic stress tolerance assays at various development stages. Knockdown of OsRR26 results in increased germination percentage and enhanced percent survival of seedlings under salinity. Under non-stress conditions, the germination percentage and the number of seedlings that survived were not affected as every OE, WT, and KD seed germinated and developed into seedlings (Fig. 5a–c). Under salinity, the germination percentage of the OE-1 and OE-5 transgenic lines were 73 and 63%, respectively, while that of the WT was insignificantly higher at 77%. However, the germination percentage of the KD-2 and KD-8 lines were significantly higher at 93% each (Fig. 5b). The seedling survival was better for the KD transgenic lines (82%) than the WT (57%) and OE (<47%) lines (Fig. 5c). To further compare the tolerance of the WT, OE, and KD transgenic rice plants, the 14-day-old seedlings, which were germinated and grown under non-stress conditions, were transferred to 0.5X Yoshida medium. These seedlings were subjected to salinity stress for 72 h, after which they were subjected to a recovery phase for 4 days. We observed that the OE and WT seedlings had wilted and died, whereas the KD lines were able to recover and grow after the treatment (Suppl. Fig. S6a). Thus, the OsRR26KD lines were more tolerant to salinity at germination and early seedling stages, revealing a negative regulatory role for OsRR26 in osmotic stress tolerance.

We also measured the physiological parameters such as length, fresh weight, and dry weight of roots and shoots as well as whole seedlings of WT, OE, and KD lines under control and salinity conditions (Fig. 6). No significant differences were observed in the root length of WT and transgenic lines under control conditions. However, the root growth was inhibited in KD lines under salinity (Fig. 6a). The shoot length of three lines showed no differences under control and salinity (Fig. 6d). The fresh and dry weights of KD roots were significantly lower than WT under control and salinity stress (Fig. 6b, c). On the contrary, KD lines showed significant gain in shoot fresh and dry weights as compared to WT under control and salinity stress (Fig. 6e, f). No significant differences were observed between the OE and WT root fresh weight, whereas dry weight was significantly higher than WT under control as well as salinity (Fig. 6b, c). One of the OE lines (OE-1) showed a significantly lower shoot fresh weight under control condition as compared to the WT, while lower dry weight was observed under both control and salinity (Fig. 6e, f). The results indicated that salt treatment inhibited the growth of primary root of KD lines while the shoot length remained unaffected. The seedling fresh and dry weight was found to be high in KD lines with respect
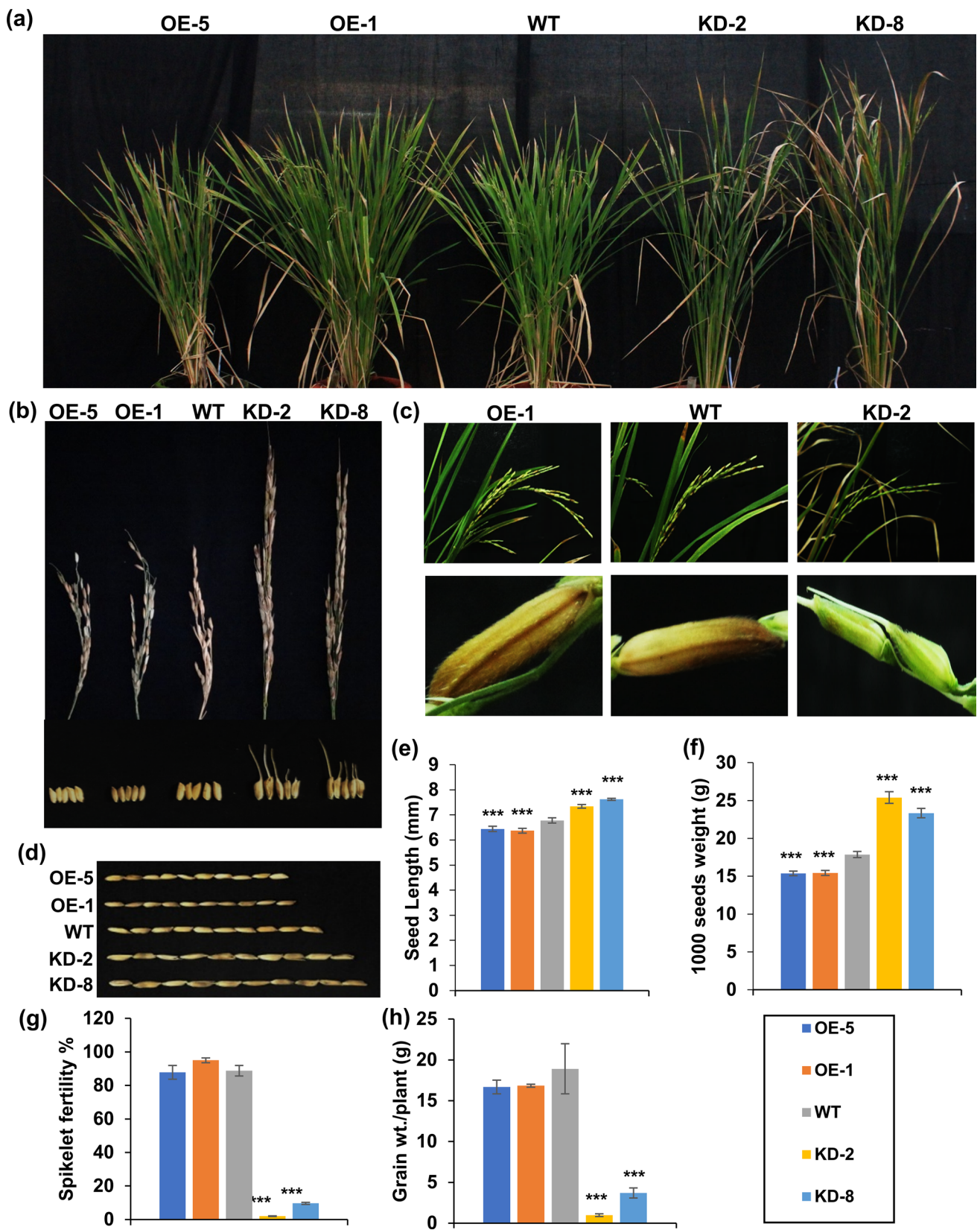
◂**Fig. 3** Knockdown of *OsRR26* has severe reproductive development implications, leading to an extreme reduction in yield. **a** Picture showing representative plants for WT, OE, and KD transgenic plants. **b, c** Morphological differences among the mature panicles and seeds of the WT, OE, and KD rice. **c** Developing panicles (upper panel) and seeds (lower panel) of the WT, OE, and KD lines at grain filling stage; abortion of grain filling was observed in the KD plants, and the grains were elongated and tapering towards the end as compared to the WT and OE seeds. The awn is highly elongated in the KD grains. **d, e** The differences in the husked (awns removed) and de-husked seed length of WT, OE, and KD rice. **f** The difference in seed weight among the WT, OE, and KD transgenic rice seeds. **g** Spikelet fertility of the WT, OE, and KD lines. **h** The grain weight per plant (grams) of the WT, OE, and KD lines. Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigmaplot), and comparisons of each transgenic line against the WT values only were considered, where *** denote significant differences at $P \leq 0.001$, $n = 9$

to WT seedlings under control and salinity (Fig. 6g, h) and thus KD lines were found to perform better under salinity.

For a preliminary assessment of the tolerance of the OE and KD transgenic plants to osmotic stress at the reproductive stage, we performed leaf disc assays where the leaves of the WT, OE, and KD plants at the heading stage were used (Suppl. Fig. S6b). WT and OE transgenic lines did not show a significant difference in the chlorophyll content (Suppl. Fig. S6c). The KD transgenic lines had significantly higher chlorophyll content after the stress treatment. However, the chlorophyll content in the KD-2 transgenic line was higher even under control condition (Suppl. Fig. S6c). Grain yield is an excellent phenotypic marker for abiotic stress tolerance. The use of yield to compare tolerance of the WT, OE, and KD lines, in this case, is however hampered by the fact that the KD lines have severely reduced spikelet fertility. Therefore, inference obtained from grain yield would not give an accurate account of the contribution of *OsRR26* towards stress tolerance. Nevertheless, a comparative yield analysis was carried out and results are provided in Suppl. Fig. S6d, e.

OsRR26 regulates various physiological parameters associated with osmotic stress tolerance in rice

To understand the basis of tolerance to salinity in the OsRR26 transgenic seedlings, we measured parameters such as chlorophyll content, total sugar content, total amino acid content, Na^+ content, K^+ content and proline levels under control and salinity stress conditions in WT, OsRR26OE and OsRR26KD plants. Fourteen days old WT, OsRR26OE, and OsRR26KD seedlings were subjected to 200 mM NaCl for 24 h after which all the measurements were made (Fig. 7a–d). We used SPAD to measure the total chlorophyll content of the control and salinity-treated OE, WT, and KD seedlings. We observed no significant differences between the SPAD values of the WT and OE seedlings under control and salinity stress conditions but the SPAD values of the KD lines were significantly higher than both WT and OE lines (Fig. 7b). Analysis of the

total soluble sugar content revealed no significant differences between the WT and transgenic seedlings in control condition (Suppl. Fig. S7a). Under salinity stress, the total soluble sugar of WT and OE seedlings decreased while that of the KD seedlings increased as compared to control conditions (Suppl. Fig. S7a). Under control condition, the total amino acid (TAA) content was significantly higher in the OE seedlings as compared to the WT and KD (Suppl. Fig. S7b). However, under salinity stress, a significant increase in the TAA content was observed in all lines as compared to the control, with the TAA content being significantly higher in the KD plants in comparison with the WT and OE (Suppl. Fig. S7b).

We observed a duration-dependent gradual increase in the Na^+ concentration in the WT, OE and KD transgenic plants subjected to salinity (Fig. 7c). However, we found no significant differences in the Na^+ accumulation in the OE and KD plants as compared to the WT under salinity stress (Fig. 7c). Analysis of K^+ levels revealed that the KD lines accumulated higher amounts of K^+ while the OE lines accumulated significantly lower amounts of K^+ as compared to the WT seedlings at 48 h of salinity stress (Fig. 7d). There were no significant differences in the proline concentration of the WT and the OE lines under control conditions, but the proline content was significantly higher in the KD lines (Fig. 7e). This same pattern was observed at 30 min of salinity stress. After 24 h of treatment, the proline levels were increased in all the genotypes. However, the overall proline levels in the OE lines were significantly lower than the WT, which was considerably lower than the KD transgenic lines. After 48 h of salinity stress, we observed no significant differences between the proline content of the WT and OE transgenic lines. However, the KD transgenic lines maintained significantly higher proline levels (Fig. 7e). Expression analysis of proline biosynthetic and degradation pathways genes, pyrroline-5-carboxylate synthase (P5CS) and proline dehydrogenase (PDH), in the WT, OE and KD lines revealed significantly higher proline biosynthesis in the KD lines under control conditions, while a spike in proline biosynthesis was observed in the OE line under salinity stress (Suppl. Fig. S7c, d). P5CS expression was significantly higher in the KD line as compared to the WT under salinity stress (Suppl. Fig. S7c). No significant difference in expression of PDH was observed among the WT, OE and KD lines under control condition (Suppl. Fig. S7d). However, under salinity stress an increase in the expression of PDH was observed in the OE lines (Suppl. Fig. S7d).

Discussion

Phylogenetic analysis revealed that the ortholog of OsRR26 in Arabidopsis is ARR11. The high degree of conservation in the sequence of the functional domains of OsRR26 and AtARR11 indicate functional conservation between these
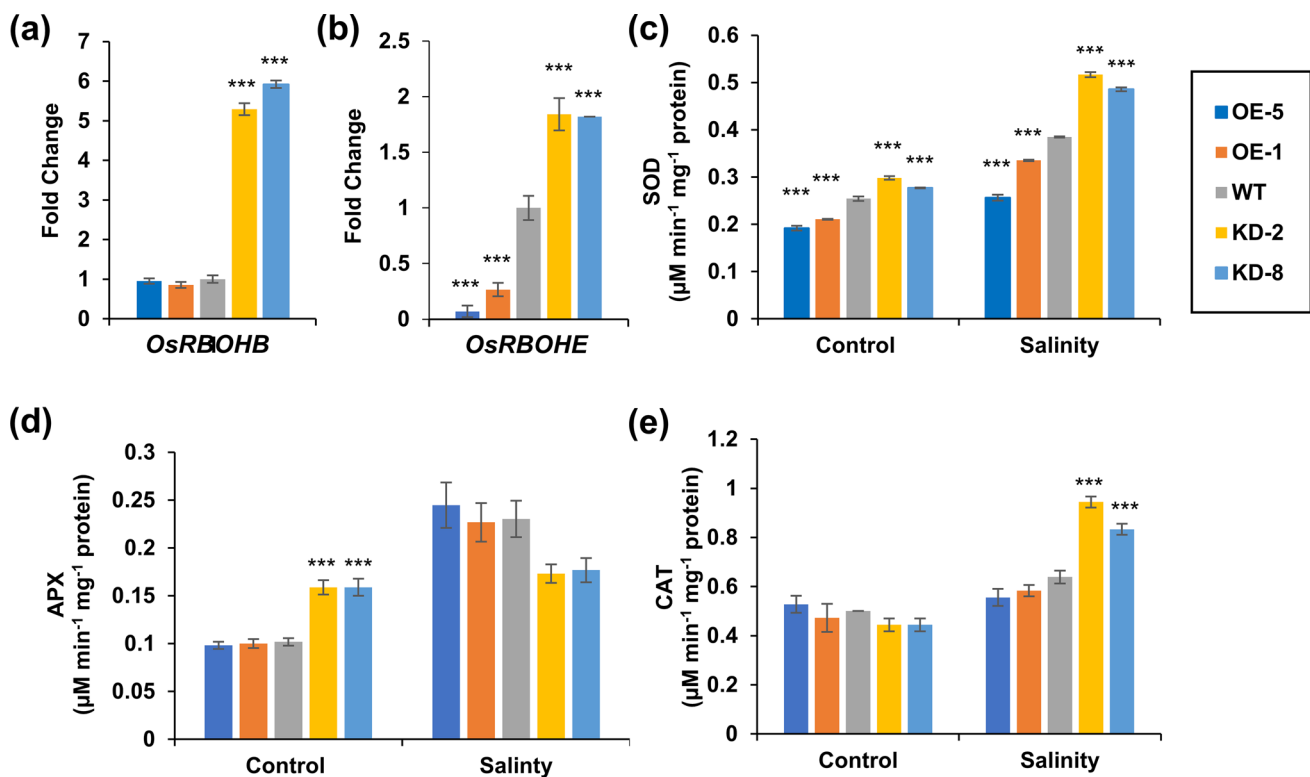
Fig. 4 *OsRR26* regulates expression of NADPH oxidases, *OsRBOHB* and *OsRBOHE*, and activity of downstream antioxidant enzymes. **a, b** qRT-PCR showing the expression of *OsRBOHB* and *OsRBOHE* in the WT, *OsRR26*OE and *OsRR26*KD lines reveals the negative regulatory role of *OsRR26* on the expression of *OsRBOHB* and *OsR-BOHE*. **c–e** Antioxidant enzyme activity assay for superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) in the WT, *OsRR26*OE and *OsRR26* KD lines under control and salinity stress (24 h, 200mM NaCl) reveals that *OsRR26* negatively regulates SOD in rice under control and salinity stress conditions. APX activity was increased only in the KD lines under control conditions

with no significant differences observed between transgenic lines and WT under salinity stress. No significant differences in CAT activity were observed between WT and transgenic lines under control conditions while CAT activity was induced to significantly higher levels in the KD lines as compared to WT and OE lines under salinity stress. Values are means of three independent experiments (±SE). Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigmaplot), and comparisons of each transgenic line against the WT values only were considered, where *** denote significant differences at $P \leq 0.001$

orthologs. ARR11 is a nuclear-localized, subfamily-I type-B RR, which functions to mediate cytokinin responses with other members of the subfamily-I in a partially redundant manner and proposed to function downstream of the cytokinin receptor HKs in a multistep phosphorelay (Mason et al. 2005; Argyros et al. 2008). The fact that OsRR26 is nuclear-localized and its expression is cytokinin responsive indicates that it also functions in cytokinin responses. Functional conservation among orthologous genes is not uncommon. Tsai et al. (2012) have shown that the rice type-B RR - OsRR22 could functionally complement the missing subfamily-I type-B RRs of Arabidopsis in the Arabidopsis *arr1-3 arr12-1* mutant, indicating functional conservation among the TCS orthologs of Arabidopsis and rice. Thus, OsRR26 and ARR11 could be functionally conserved.

As the expression of *OsRR26* is cytokinin- and ABA-responsive, we assessed its role in phytohormone-mediated ROS accumulation in the WT as well as transgenic rice

roots. The root images of H2DCFDA fluorescence of control and salinity treated WT and transgenic plants revealed that cytokinin promotes ROS induction in the elongation zone whereas ABA causes ROS accumulation at the extracellular space. Studies have identified ROS as a regulator of cellular proliferation, elongation, and differentiation based on its gradient across the root length (Tsukagoshi et al. 2010; Li et al. 2019). The cell size and shape are often controlled by apoplastic ROS by maintaining the cell wall extensibility (Schmidt et al. 2016). Under 6-BAP treatment, the ROS accumulation was observed in the elongation zone but not at the root tips. ROS functioning downstream of cytokinins could regulate the transition between cell division and differentiation (Mase and Tsukagoshi 2021). Moreover, exogenous supply of cytokinin in WT increased endogenous *OsRR26* expression which may have a possible role in inducing the ROS accumulation in middle vascular region of root. In OE transgenic plants, ROS accumulation was seen mainly in
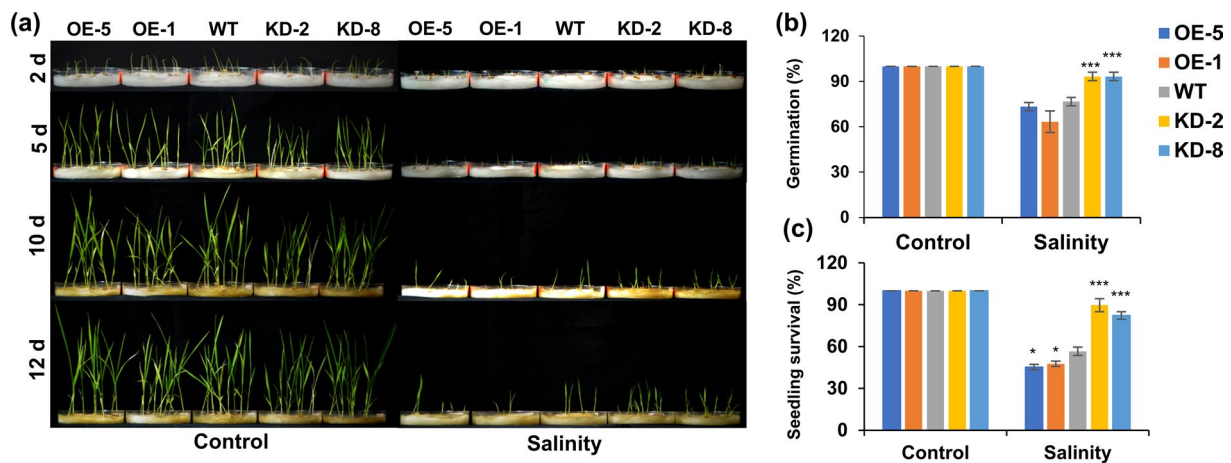
Fig. 5 *OsRR26* is a negative regulator of salinity tolerance in rice during germination and early seedling growth. **a** Representative picture of the analysis of the difference in salinity stress tolerance among the WT, *OsRR26*OE, and *OsRR26*KD transgenic rice plants at germination and seedling emergence. Seeds of the WT, *OsRR26* overexpression and knockdown transgenic rice were germinated under control and saline conditions (200 mM NaCl), and the treatment was maintained for 5 days after which recovery was performed using water. **b** Germination percentages. **c** Percent survival of seedlings germinated under salinity reveals that the *OsRR26*KD lines were significantly more toler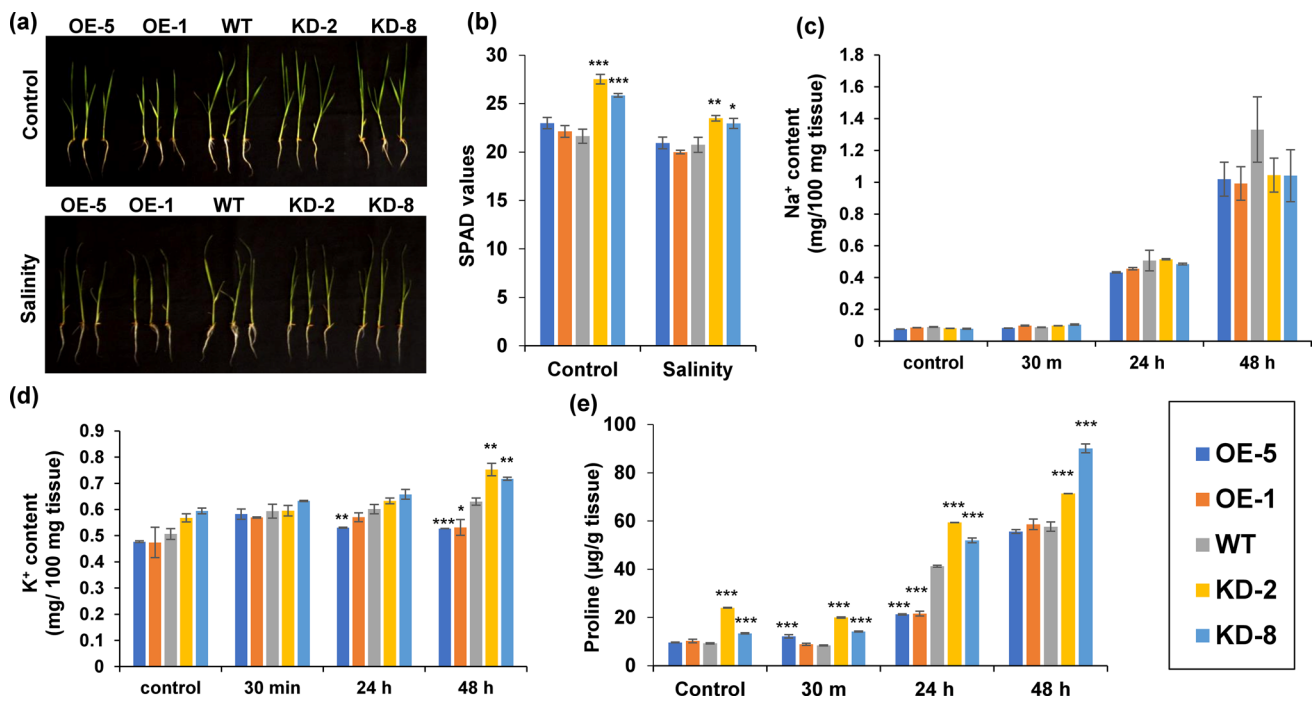ant to salinity as compared to the WT and OE lines. Values are means (±SE) of three independent experiments. Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigmaplot), and comparisons of each transgenic line against the WT values only were considered, where * and *** denote significant differences at $P \leq 0.05$ and $P \leq 0.001$, respectively

the vascular part of root. In KD, ROS was majorly observed in the root epidermal region under 6-BAP treatment. The results indicate that the expression level of *OsRR26* may induce BAP-mediated ROS accumulation in the vascular cells. Earlier report on type-B RR in Arabidopsis has shown that knockout of *arr1/arr10/arr12* affected the cytokinin-mediated specification of vascular tissues in roots (Yokoyama et al. 2007). Interestingly, exogenous treatment of cytokinin in OE seedlings reduced the ROS levels drastically. In addition to initiating the cytokinin transcriptional response, type-B RRs also commence a negative feedback loop that results in desensitization of plants to cytokinin (Zubo and Schaller 2020). Perhaps, a similar negative feedback regulation of cytokinin signaling also exists in rice, whereby the exogenous treatment of cytokinin in OE seedlings possibly provides a negative feedback loop that eventually results in reduced ROS accumulation in the roots. On the other hand, exogenous ABA treatment caused the accumulation of ROS in the extracellular space in WT, OE, and KD roots. The levels of ROS were higher in WT and OE as compared to KD seedlings. The results suggest that altering the expression levels of endogenous *OsRR26* might not affect the location of ROS accumulation but ROS levels under ABA treatment. Notably, the swelling of roots was observed in WT, KD, and OE roots under ABA treatment which is consistent with other reports which have shown that ABA causes root swelling in rice by promoting cell expansion through inducing ROS production (Chen et al. 2006; Choudhary et al. 2020; Huang et al. 2021). Additionally, abiotic stresses, including salinity induce ABA accumulation in primary root that

further promotes root swelling (Huang et al. 2021). As the ROS accumulation under salinity stress in all three lines was shown to be uniformly localized, i.e., in the extracellular space, it indicates that ABA-induced ROS accumulation in rice is not mediated by *OsRR26*.

OsRR26 is expressed in the reproductive organs and has high expression in seeds (Rice Expression Database; http://expression.ic4r.org), implicating its role in reproductive and grain development. We observed prominent differences among the WT, *OsRR26*OE, and *OsRR26*KD transgenic plants in panicle and seed development. However, the differences between the OE lines and the WT were not as prominent as those between the KD lines and WT. While the OE lines had shorter and lighter seeds, the KD lines had heavier and longer seeds and panicles as compared to the WT. Moreover, the KD lines displayed a delayed grain-filling response, elongated awns, and severely reduced spikelet fertility. Recently, it has been reported that type-B RRs in rice, OsRR21-OsRR23, function redundantly to regulate various aspects of growth and development (Worthen et al. 2019). The report revealed that the *rr21/22/23* mutant displayed a 50% reduction in spikelet fertility and this decrease was attributed to defects in fertilization caused by a lack of brush structures in the carpels (Worthen et al. 2019). Similarly, the *OsRR26*KD seeds displayed 100% germination, indicating that the reduction in spikelet fertility was probably due to fertilization defects and not defects in grain development. Thus, *OsRR26* could share a functional redundancy with the other subfamily-1 type-B RRs of rice. It should be noted that the TCS in rice including OsHKs, OsHPTs and OsRRs
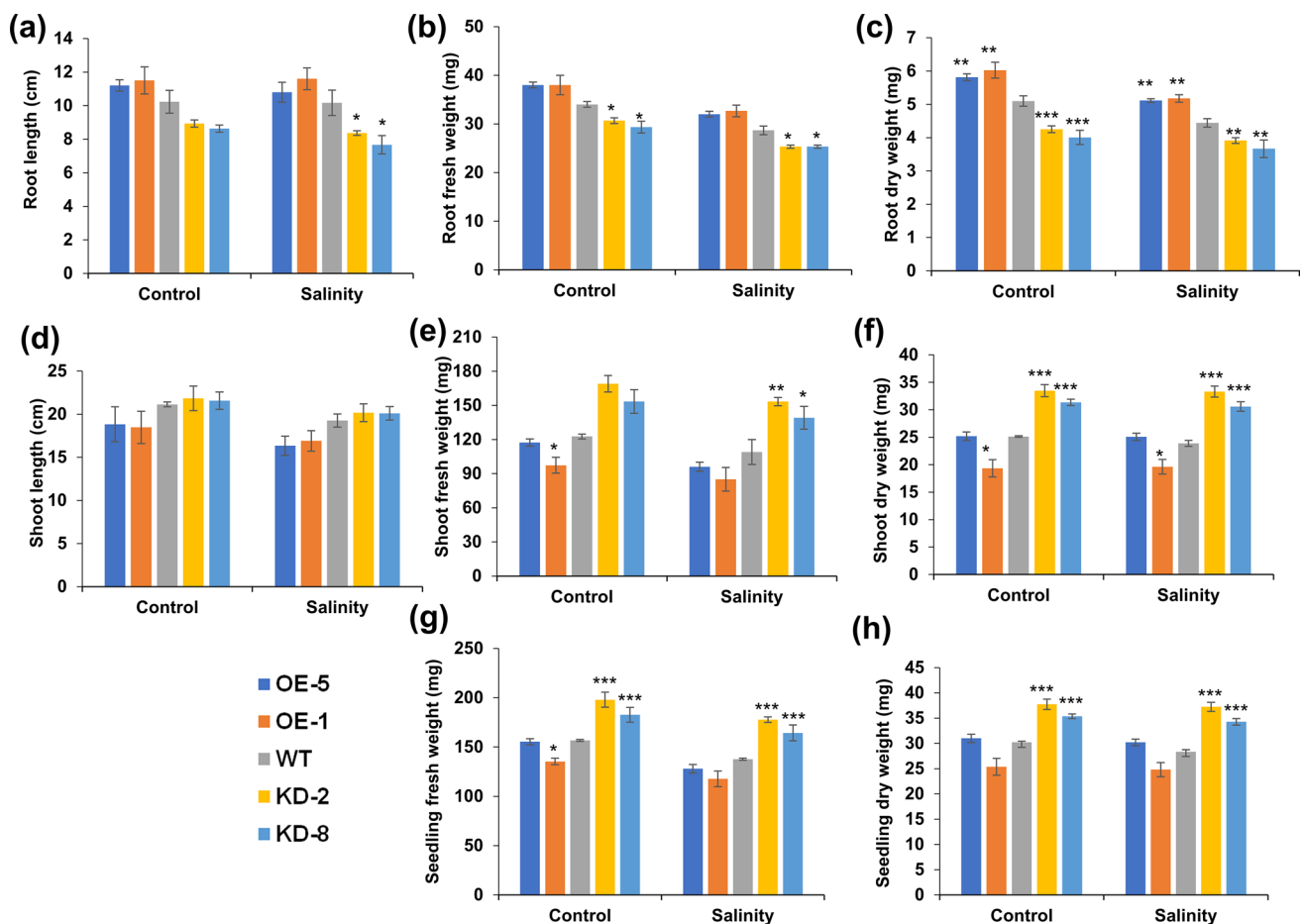
Fig. 6 Comparison of morphological features of transgenic lines with the WT under control and salinity conditions. Assessment of root length (**a**), root fresh weight (**b**), root dry weight (**c**), shoot length (**d**), shoot fresh weight (**e**), shoot dry weight (**f**), seedling fresh weight (**g**), and seedling dry weight (**h**) under control and salinity stress (200 mM NaCl). Seedling growth assay revealed that salinity inhibited root growth while the shoot and whole seedling growth was signifi-cantly better in KD lines as compared to WT and OE lines. Values are means ($\pm$ SE) of three independent experiments. Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigma-plot), and comparisons of each transgenic line against the WT values only were considered, where *, **, and *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively

have been implicated in cytokinin signaling and the role of *OsRR26* and other TCS genes in cytokinin signaling (if any) is currently being investigated in our laboratory. Thus, *OsRR26* plays an integral role in spikelet development and may function downstream of cytokinin receptors and have overlapping functions with the other cytokinin-responsive type-B RRs in rice.

The elongated awns observed in the KD lines suggested that *OsRR26* may be a negative regulator of awn development. An interesting study has shown a correlation between cytokinin signaling and awn development. Hua et al. (2015) reported that the presence of long, barbed awns in wild rice is controlled by a gene, *LONG AND BARBED AWN1* (*LABA1*), which encodes an enzyme that converts inactive cytokinin nucleotide 59-monophosphates, iPRMP, and tZRMP, to the biologically active free-base forms, iP and tZ. *LABA1* activity increases cytokinin content in the awn

primordia resulting in awn elongation (Hua et al. 2015). The connection between *LABA1* and *OsRR26* (if any) is still unknown. Considering the TCS genes in plants have been implicated in cytokinin signaling and responses, there is a possibility that *OsRR26* may regulate cytokinin-dependent awn development. We are currently trying to determine if *OsRR26* participates in cytokinin signaling and how awn development is regulated by *OsRR26*.

The type-B RRs have been shown to negatively regulate the ABA and osmotic stress response in Arabidopsis (Nguyen et al. 2016). Similarly, rice type-B RR, *OsRR22*, has been shown to negatively regulate salinity tolerance (Takagi et al. 2015; Zhang et al. 2019). A strong indication for the negative regulatory role of *OsRR26* with regards to ABA, salinity stress, and drought stress was provided by the repression of its expression under these treatments. This was per our earlier report which showed that the expression of

Fig. 7 Increased tolerance to salinity in *OsRR26*KD lines is partly contributed by better chlorophyll retention and proline accumulation as compared to the WT and OE lines. **a** Representative pictures of plants of 14-day-old WT, *OsRR26*OE and *OsRR26*KD transgenic lines under control and after 24 h salinity stress conditions. **b** SPAD values obtained from the leaves of the WT, OE, and KD transgenic seedlings reveal a higher chlorophyll concentration in the KD lines as compared to the WT and OE lines under control and salinity stress conditions. **c** Comparison of the Na^+ content of the WT, OE and KD lines subjected to salinity stress reveals no significant differences in sodium accumulation between WT and transgenic seedlings. **d** Comparison of the K^+ content of the WT, OE and KD lines subjected to salinity stress reveals significant differences in K^+ accumulation between WT and transgenic seedlings under control and 30 min of stress. At 48 h of salinity stress, a significant decrease in K^+ accumulation in OE lines was observed, while a significant increase in K^+ accumulation in the KD lines was observed compared to the WT. **e** Knockdown of *OsRR26* leads to significant increase in proline accumulation under control and salinity stress (30 m, 24 h, 48 h) conditions as compared to the WT and OE lines. A significant reduction in proline accumulation was also observed in the OE lines as compared to the WT at 24 h salinity stress. Values are means ($\pm$ SE) of three independent experiments. Statistical analysis was performed using one-way ANOVA (Holm-Sidak method, Sigmaplot), and comparisons of each transgenic line against the WT values only were considered, where *, **, and *** denote significant differences at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively

type-B RRs in rice was either repressed or unaltered under abiotic stresses (Singh et al. 2015). Using contrasting cultivars IR64 (salt-sensitive) and Pokkali (salt-tolerant), we have previously reported that germination assays, as well as tolerance assays during emergence and early seedling growth, are reliable indicators for overall salinity tolerance in rice (Lakra et al. 2018), thereby giving impetus to the observed negative regulatory role of *OsRR26* under salinity in our germination and early seedling assays. The fresh weight and dry weight of shoots and seedlings was found to be significantly higher in KD lines under salinity as compared to the WT and OE lines. Likewise, the germination percentage and seedling survival rate of *OsRR26*KD lines were significantly higher under salinity. These assays clearly showed that *OsRR26* negatively regulates salinity stress tolerance.

Investigations into the possible mechanisms to which *OsRR26* regulates salinity stress responses revealed that it functions to negatively regulate *OsRBOHB* and *OsRBOHE*.

In Arabidopsis, it has been reported that the *Atrbohd/rbohf* double mutant was less tolerant to salinity as compared to the wildtype plants, and this difference was attributed to the decreased Na^+/K^+ ratio observed in the mutants (Ma et al 2012). In our experiments, there were no differences between the Na^+ content of the WT, OE and KD plants subjected to salinity stress, indicating that *OsRR26* may not regulate Na^+ accumulation in rice. However, the increased K^+ accumulation in the KD transgenic lines after 48 h of salinity indicates that *OsRR26* may regulate K^+ influx into seedling shoots, which could contribute to decreasing the Na^+/K^+, leading to stress tolerance in the KD lines. The increased soluble sugar and amino acid content in the *OsRR26* KD seedlings as compared to the WT and OE seedlings subjected to salinity may also contribute to increased salinity tolerance of the KD lines. The accumulation of soluble sugars has been shown to regulate the osmotic adjustment, provide membrane protection and scavenge stress-induced
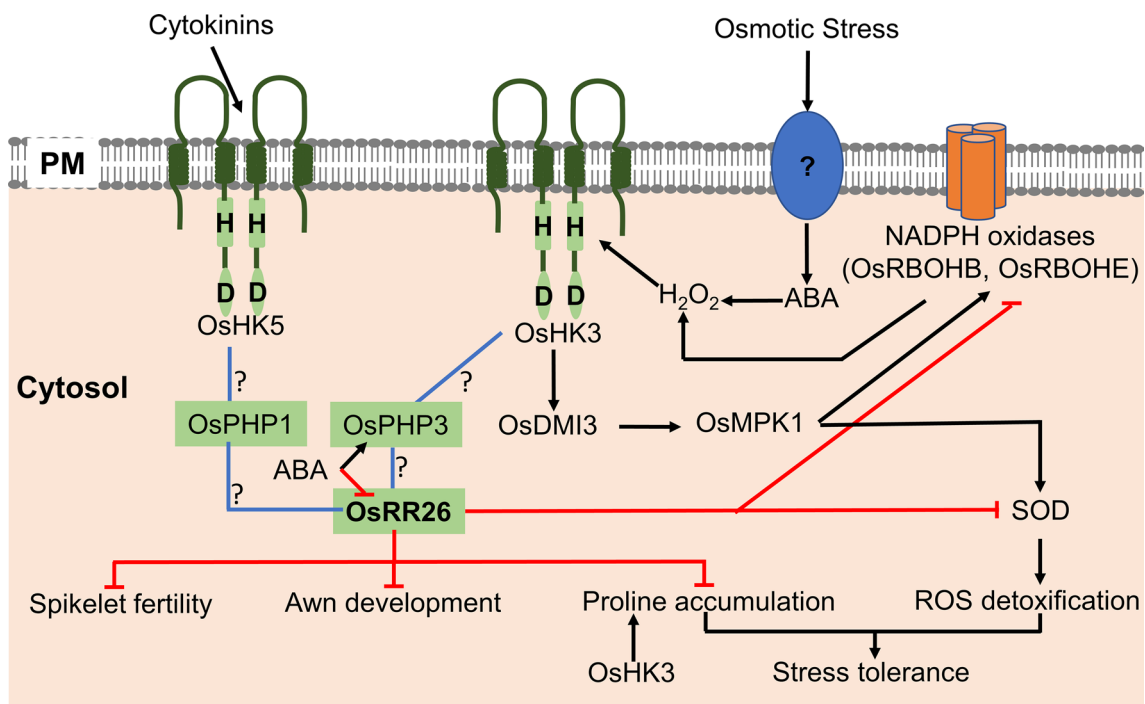
Fig. 8 A proposed pathway for the role of OsRR26 in the ABA-dependent OsHK3b mediated signaling in rice under osmotic stress. Upon exposure to osmotic stress, OsHK3b expression is induced by ABA via H_2O_2. ABA also induces the expression of OsPHP3 and inhibits OsRR26. OsHK3b signals through OsDMI3 and OsMPK1 to activate antioxidant defense system to neutralize stress generated reactive oxygen species. Expression of two NADPH oxidases *OsR-BOHB* and *OsRBOHE* are also upregulated by OsHK3b signaling. These two NADPH oxidases contribute towards the production of H_2O_2 for stress signaling. H_2O_2, in turn, upregulates OsHK3 forming a positive feedback loop. OsHK3 also induces proline biosynthesis which helps in osmoregulation. OsRR26 negatively regulates OsR-BOHB and OsRBOHE, as well as proline accumulation. OsHK3b interacts with OsPHP3 which subsequently interacts with OsRR26. This interaction of OsPHP3 with OsRR26 may lead to the deactivation of OsRR26, enabling the OsHK3b mediated MAPK activation of OsRBOHB and OsRBOHE. This pathway has been proposed based on our current findings as well as previous findings by Wen et al. (2015) and Sharan et al. (2017)

ROS under various abiotic stress conditions including salinity in plants (Murakeozy et al. 2003; Koyro et al. 2012). while the role of various amino acids in salt stress is not clearly defined. There are amino acids such as asparagine and proline whose accumulation has been shown to promote salinity tolerance in rice (Hare and Cress 1997; Verbruggen and Hermans 2008). The increased proline accumulation may be one of the contributing factors to increased tolerance in *OsRR26* knockdown plants. In Arabidopsis, NADPH oxidases have been shown to facilitate the production of H_2O_2 in response to NaCl or mannitol stress, ultimately increasing transcript levels of proline biosynthetic gene, *Δ1-Pyrroline Carboxylate Synthetase*, leading to increased proline production (Rejeb et al. 2015). Increased expression of *OsRBOHB* and *OsRBOHE* in the *OsRR26*KD lines may have resulted in increased endogenous production of H_2O_2. Whether this situation also results in increased expression of *Δ1-Pyrroline Carboxylate Synthetase* and eventual increase in proline accumulation in rice remains to be investigated. Regardless of this, the increased proline accumulation in the

*OsRR26*KD lines may be a contributing factor for increased salinity tolerance.

We show that *OsRR26* functions to negatively regulate *OsRBOHB*, *OsRBOHE*, and antioxidant enzymes in rice. Additionally, Wen et al. (2015) have shown that ABA signaling in rice involves the OsHK3 mediated MAPK activation of ROS production through OsRBOHB and OsRBOHE, which in turn activate the ROS scavenging enzymes, SOD, APX, and CAT. Thus, for the activation of OsRBOHB and OsRBOHE through OsHK3 signaling, OsRR26 needs to be repressed. Our previous report has also shown that OsRR26 interacts with all the rice OsPHPs, which in turn interact with the rice HKs including OsHK3 and OsHK5 (Sharan et al. 2017). Based on all the data available, we provide a hypothesis whereby *OsRR26* may function in canonical TCS signaling, downstream of the OsHKs and OsHPTs in rice (Fig. 8). Research currently being undertaken in our laboratory is trying to elucidate whether this hypothesis is true. Further studies in this regard could provide valuable information about the role of TCS multistep phosphorelay in cytokinin, ABA, and abiotic stress signaling in rice.

In conclusion, this study shows that OsRR26 plays a crucial role in root growth via phytohormone-mediated ROS accumulation, plant development and stress responses. This study shows how overall development and abiotic stress tolerance are intricately linked.

Declarations

Conflict of interest All authors have approved the manuscript and declare no conflict of interests.

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