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Cellulose synthase-like protein OsCSLD4 plays an important role in the response of rice to salt stress by mediating abscisic acid biosynthesis to regulate osmotic stress tolerance

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Summary

Cell wall polysaccharide biosynthesis enzymes play important roles in plant growth, development and stress responses. The functions of cell wall polysaccharide synthesis enzymes in plant growth and development have been well studied. In contrast, their roles in plant responses to environmental stress are poorly understood. Previous studies have demonstrated that the rice cell wall cellulose synthase-like D4 protein (OsCSLD4) is involved in cell wall polysaccharide synthesis and is important for rice growth and development. This study demonstrated that the OsCSLD4 function-disrupted mutant *nd1* was sensitive to salt stress, but insensitive to abscisic acid (ABA). The expression of some ABA synthesis and response genes was repressed in *nd1* under both normal and salt stress conditions. Exogenous ABA can restore *nd1*-impaired salt stress tolerance. Moreover, overexpression of *OsCSLD4* can enhance rice ABA synthesis gene expression, increase ABA content and improve rice salt tolerance, thus implying that OsCSLD4-regulated rice salt stress tolerance is mediated by ABA synthesis. Additionally, *nd1* decreased rice tolerance to osmotic stress, but not ion toxic tolerance. The results from the transcriptome analysis showed that more osmotic stress-responsive genes were impaired in *nd1* than salt stress-responsive genes, thus indicating that *OsCSLD4* is involved in rice salt stress response through an ABA-induced osmotic response pathway. Intriguingly, the disruption of OsCSLD4 function decreased grain width and weight, while overexpression of *OsCSLD4* increased grain width and weight. Taken together, this study demonstrates a novel plant salt stress adaptation mechanism by which crops can coordinate salt stress tolerance and yield.

Introduction

Plant cell walls not only determine cell shape and provide structural support, but they also act as the front line of various environmental stresses (Doblin and Pettolino, 2010; Liu *et al*., 2021a; Tenhaken, 2014; Voxeur and Höfte, 2016). Plant cell wall polysaccharides exhibit a striking variety in composition in accordance with environmental conditions, and therefore, there is a close relationship between the variety of plant cell wall polysaccharide compositions and the response of plants to environmental stresses (Corrêa-Ferreira *et al*., 2019; Doblin *et al*., 2010; Houston *et al*., 2016; Lenk *et al*., 2019; Liu *et al*., 2021b; Yang *et al*., 2021; Zhang *et al*., 2021).

Plant cell wall contains three types of polysaccharides, including cellulose, hemicellulose and pectin. Cellulose is composed of β-1,4 glucans and is synthesized by plasma membrane-localized cellulose synthase (CESA) complexes (Doblin *et al*., 2002; Suzuki *et al*., 2006). In contrast, hemicelluloses, including several types of glycans such as xyloglucans, xylans, mannans, glucomannans and β-(1,3; 1,4)-glucans (Doblin *et al*., 2009; Scheller and Ulvskov, 2010). Cellulose synthase-like (CSL) proteins and Golgi-localized β-glycan synthases play a critical role in hemicellulose biosynthesis (Richmond and Somerville, 2000; Yin *et al*., 2014). According to protein phylogeny, CSL proteins can be grouped into eight subfamilies that include CSLA, CSLB, CSLC, CSLD, CSLE, CSLF, CSLG and CSLH (Kaur *et al*., 2017; Yin *et al*., 2014). CSLA subfamily members mediate mannan backbone synthesis (Dhugga *et al*., 2004; Liepman and Wilkerson, 2005; Zou *et al*., 2018). CSLC subfamily proteins respond to the biosynthesis of the xyloglucans β-1,4-glucan backbone (Cocuron *et al*., 2007). CSLF and CSLH subfamilies are considered to be unique in monocots and participate in (1,3;1,4)-β-D-glucan biosynthesis (Burton *et al*., 2006; Doblin *et al*., 2009). CSLD share high amino acid similarity with CESA and is involved in the biosynthesis of several types of glycans (Bernal *et al*., 2007; Galway *et al*., 2011; Li *et al*., 2017; Peng *et al*., 2019). CSLDs can regulate different types of cell wall

polysaccharide synthesis with tissue and organ specificity, and they play important roles in plant growth and development (Favery *et al*., 2001; Gu *et al*., 2016; Hu *et al*., 2018; Kim *et al*., 2007; Li *et al*., 2009; Peng *et al*., 2019; Wang *et al*., 2001; Wang *et al*., 2011a; Yang *et al*., 2021; Yoshikawa *et al*., 2013). In *Arabidopsis*, AtCSLD1 and AtCSLD4 are involved in pollen tube growth (Wang *et al*., 2011a). AtCSLD2 and AtCSLD3 can affect the morphogenesis and growth of root hair (Favery *et al*., 2001; Galway *et al*., 2011; Hu *et al*., 2018; Wang *et al*., 2001). AtCSLD5 plays an important role in plant growth by regulating the synthesis of cell wall xylan and homogalacturonan (Bernal *et al*., 2007).

Cell wall polysaccharides also play important roles in plant responses to environmental stresses (Corrêa-Ferreira *et al*., 2019; Houston *et al*., 2016; Lenk *et al*., 2019; Liu *et al*., 2021b; Tenhaken, 2014; Yang *et al*., 2021). Salinity stress is one of the most widespread abiotic stresses and has a serious threat to global food security. Plants can remodel cell wall polysaccharide synthesis to cope with salt stress (Fujita *et al*., 2013; Hu *et al*., 2018; Peng *et al*., 2019; Yoshikawa *et al*., 2013). Therefore, disturbing the activity of cell wall polysaccharide synthesis enzymes seriously interferes with salt stress tolerance (Kesten *et al*., 2019; Zhang *et al*., 2016; Zhu *et al*., 2010). For example, the mutation of cellulose synthase 6 (CESA6) or its companion protein CC1 (companion of cellulose synthase 1) disturbs cellulose synthesis and decreases *Arabidopsis* salt tolerance (Endler *et al*., 2015; Kesten *et al*., 2019; Zhang *et al*., 2016). Xyloglucan is one of most important compositions of hemicelluloses. Xyloglucan endotransglucosylases/hydrolase (XTH) is a member of the xyloglucan-modifying enzyme family that exerts an important effect on cell wall remodelling (Rose *et al*., 2002). *XTH* gene *DkXTH1* and *CaXTH3* can modify transgenic *Arabidopsis* and tomato cell morphogenesis and enhances drought and salt tolerance (Cho *et al*., 2006; Choi *et al*., 2011; Han *et al*., 2017). The *Arabidopsis XTH19* is involved in the response of plants to cold stress (Takahashi *et al*., 2021). The roles of cellulose and hemicellulose synthesis involved in plant stress tolerance are complex. Except for the positive role of cellulose or hemicellulose synthetases in the response of plants to environmental stress, some play a negative role in plant stress tolerance. Disruption of AtCesA8/IRX1 enhances *Arabidopsis* tolerance to drought and osmotic stress (Chen *et al*., 2005). *Arabidopsis* xyloglucan endotransglucosylase-hydrolase30 (XTH30) can modulate xyloglucan side chains. Disruption of XTH30 function partially inhibits xyloglucan-derived oligosaccharide synthesis, sustains crystalline cellulose content and microtubule depolymerization, and enhances plant salt stress tolerance (Yan *et al*., 2019). These results indicate that cell wall polysaccharide synthesis-related enzymes play a complex and important role in the adaptation of plants to adverse environmental conditions. Although cell wall polysaccharide synthesis enzymes play important roles in the response of plants to salt stress, the detailed mechanism of these enzymes involved in plant stress responses remains obscure (Kesten and Menna, 2017; Novaković *et al*., 2018).

OsCSLD4 (rice cellulose synthase-like D4) encodes a CSLD subfamily protein. Previous studies have revealed the critical role of OsCSLD4 in rice growth and development by regulating cell wall polysaccharide synthesis (Ding *et al*., 2015; Hu *et al*., 2010; Li *et al*., 2009; Luan *et al*., 2011; Wu *et al*., 2010; Yoshikawa *et al*., 2013). However, the role of OsCSLD4 in the response of rice to environmental stresses remains unclear. This study revealed that OsCSLD4 was involved in the rice salt stress response by mediating abscisic acid (ABA) content to enhance rice osmotic stress tolerance.

Results

OsCSLD4 is highly homologous to AtCSLD5 and its gene expression is induced by salt

The results of the phylogenetic analysis revealed that OsCSLD4 was highly homologous to *Arabidopsis* AtCSLD5 and maize ZmCSLD4 (Figure 1a,b). AtCSLD5 played a vital role in the *Arabidopsis* salt stress response (Zhu *et al*., 2010). The high homology of OsCSLD4 to AtCSLD5 implies that OsCSLD4 may exert a similar function in the rice salt stress response. To elucidate the role of OsCSLD4 in the rice salt stress response, we studied the expression pattern of *OsCSLD4* under salt stress by using quantitative RT-PCR (qPCR). The results revealed that the expression of *OsCSLD4* was induced significantly by salt stress (Figure 1c). The expression of *OsCSLD4* was induced quickly within 1 h and remained high until 8 h. In contrast, the expression of other members of the CSLD subfamily in rice was not noticeably induced by salt treatment (Figure 1c). The result indicates that OsCSLD4 may play an important role in rice salt stress response.

Disruption of OsCSLD4 impairs rice salt stress tolerance

To further analyse the role of *OsCSLD4* in the rice salt stress response, we used the reported *OsCSLD4* function disruption mutant *narrow leaf and dwarf1* (*nd1*) in an indica variety Zhongxian 3037 background (Li *et al*., 2009) to study the effect of OsCSLD4 on rice salt stress tolerance. *nd1* possesses a narrow and rolled leaf and dwarf phenotype (Figure S1a; Hu *et al*., 2010; Li *et al*., 2009; Luan *et al*., 2011; Yoshikawa *et al*., 2013). When 2-week-old *nd1* and WT seedlings were treated with salt (150 mM NaCl) for 7 days with another 7-day recovery (watered without salt), the majority of *nd1* leaves were markedly wilted, while only a small number of WT leaves exhibited a wilted phenotype (Figure 2a,b). As presented in Figure 2b, *nd1* exhibited a low survival rate of only approximately 37%, while the WT survival rate was as high as approximately 68% after 7-day recovery, thus indicating that OsCSLD4 is important for rice salt stress tolerance.

Salt stress can damage and inhibit the growth of plant roots, therefore, the root growth can represent plant salt stress tolerance (Arsova *et al*., 2020; Dinneny, 2019; Li *et al*., 2021; Shao *et al*., 2021). The results from qPCR assays revealed that *OsCSLD4* was expressed in rice roots, stems and leaves (Figure S2; Ding *et al*., 2015). Different from shoots and crown roots that displayed markedly dwarf and rolled leaf or shortened phenotypes, respectively, in 10-day-old *nd1* seedlings, the growth of *nd1* primary roots was similar to that of WT (Figure S1; Figure 2c, d). To better analyse and display the effect of OsCSLD4 function on the rice salt stress response, we examined the primary root growth to analyse the role of OsCSLD4 in the rice salt stress response. When germinated WT and *nd1* seeds were transferred to 1/2 Murashige & Skoog (MS) medium with 150 mM NaCl, and then grown for 7 days, the growth of *nd1* primary roots was observed to be more severely inhibited compared to that of WT (Figure 2c,d). The length of *nd1* primary roots was approximately 3.2 cm, and in contrast, the length of WT primary roots was approximately 5.6 cm (Figure 2d). Additionally, to confirm the function of OsCSLD4 in rice salinity stress tolerance, we also analysed the salt tolerance of the *nd1* allelic mutant *nrl1* (in

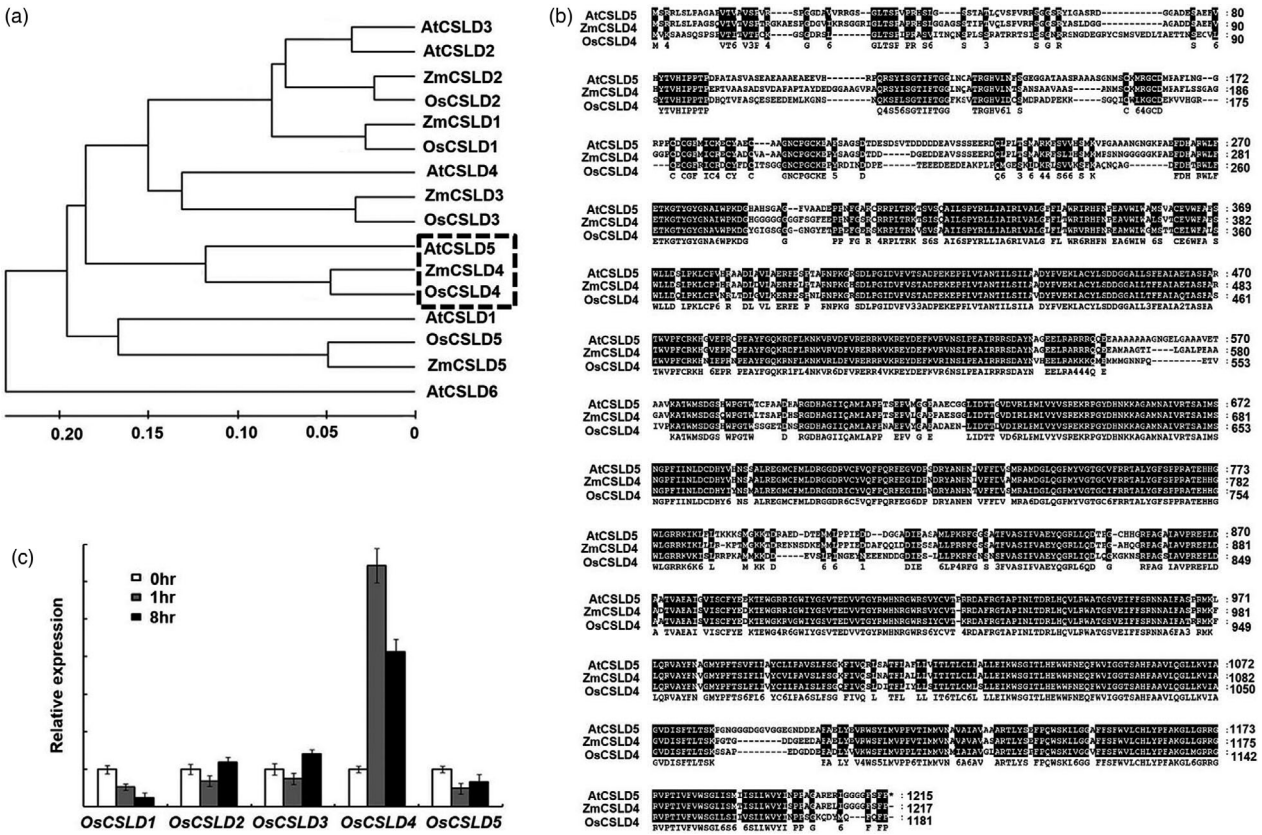

Figure 1 Phylogenetic analysis of OsCSLD4 homologous proteins and *OsCSLD4* salt-induced expression patterns. (a) Phylogenetic analysis of OsCSLD4 homologous proteins in *Arabidopsis*, maize and rice. (b) Alignment of amino acid sequences of AtCSLD5, ZmCSLD4 and OsCSLD4. White or black background represents the identity of amino acid sequences. (c) Expression level of rice *OsCSLD* subfamily genes under salt treatment (150 mM NaCl). The genes expression level under normal condition (0 h) is standardized to '1'. Figure shows the relative expression level of genes under salt treatment to normal condition. The figure presents the average from three independent experiments. Bar represents the standard error (±SE).

Zhonghua 11 background). The results revealed that *nrl1* also exhibited decreased rice salt stress tolerance (Figure S3). The above results demonstrate that OsCSLD4 is important for salinity tolerance of rice seedlings.

Disruption of OsCSLD4 increases rice seedlings' osmotic stress sensitivity

Salinity stress can disturb cellular ions and water balance in plant, ultimately resulting in ion toxicity and osmotic stress damage to plant (Amin *et al*., 2021; Dinneny, 2019; García *et al*., 2009; Huang *et al*., 2012; Zhu, 2002). To illustrate the role of OsCSLD4 in the response of rice to salinity stress, we first studied the ion toxicity tolerance of *nd1*. LiCl is easily absorbed and transported into plants, and this can result in marked ion toxicity at low concentrations. To avoid osmotic stress interference from high concentration salinity, low concentration (18 mM) LiCl was used to analyse the ion toxicity tolerance of *nd1* seedlings. The results revealed that the growth of *nd1* seedlings was similar to that of WT under LiCl treatment (Figure S4), thus indicating that there was no marked difference in ion toxicity tolerance between *nd1* and WT.

Additionally, considering that the effect of OsCSLD4 on cell wall polysaccharide composition and cell wall morphology may disturb ion absorption or transport, we measured Na^+ and K^+ content in rice roots and leaves before and after salt treatment. The results demonstrated that the Na^+ and K^+ contents in rice roots and leaves were not significantly different between WT and *nd1* before or after salt treatment (Figure S5), thus indicating that the salt-sensitive phenotype of *nd1* is not due to ion absorption or transport.

We further analysed the function of OsCSLD4 in the context of osmotic stress tolerance in rice. The results from PEG treatment experiments revealed that the growth of *nd1* roots was inhibited more markedly by 10% PEG treatment compared to that of WT (Figure 3a). The primary root length of *nd1* seedlings grown under 10% PEG for 10 days was approximately 2.6 cm, and this was only approximately 31% of that grown under normal conditions (approximately 8.8 cm). The primary root length of WT seedlings treated with 10% PEG for 10 days was approximately 4.5 cm, and this was approximately 51% of that in plants grown under normal conditions (Figure 3b), indicating that the disruption of OsCSLD4 impairs rice osmotic stress tolerance.

A previous study demonstrated that the *OsCSLD4* homolog *AtCSLD5* is involved in the *Arabidopsis* salt stress response through the ROS scavenging system (Zhu *et al*., 2010). Here, we sought to determine if OsCSLD4 plays the same role in rice. Firstly, we analysed the oxidative damage in *nd1* seedlings under salt treatment. MDA is one of the main products of cellular oxidized components, so MDA content can represent cell oxidative damage degree. The MDA content in rice seedlings

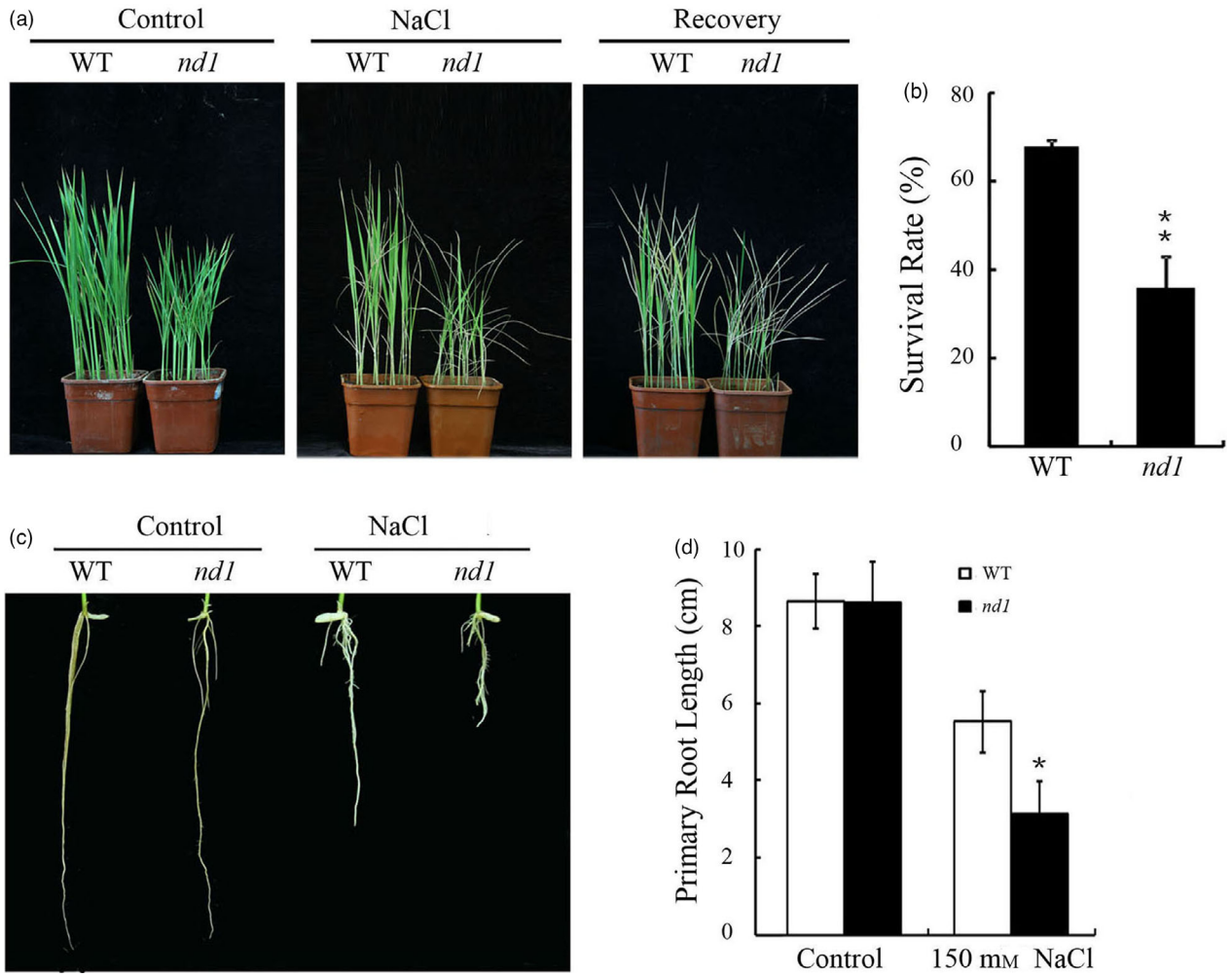

Figure 2 Disruption of OsCSLD4 decreases salt tolerance in rice seedlings. (a) Phenotype of rice seedlings treated by salt stress in soil. Two-week-old rice seedlings grown in soil were treated with 150 mM NaCl for 7 days with another 7-day recovery. (b) Survival rate of rice seedlings treated with 150 mM NaCl for 7 days with another 7-day recovery. (c) Phenotype of rice primary roots grown on 1/2 MS medium supplemented with 150 mM NaCl for 7 days. (d) The length of rice primary roots grown on 1/2 MS medium supplemented with 150 mM NaCl for 7 days. Experiments were repeated at least three times. Bars represent the SE (±), and asterisks indicate a significant difference. Significance was evaluated using the *t*-test (*$P < 0.05$ and **$P < 0.01$).

revealed that the MDA content in *nd1* seedlings was markedly higher than that in WT after long-term salt treatment (treated with 150 mM NaCl for 3 days) (Figure 3c). The higher MDA content in *nd1* seedlings indicates more serious ROS damage to *nd1* seedlings under salt stress (You and Chan, 2015). To distinguish the ROS damage in *nd1* seedlings under salt treatment, rice seedlings were treated with high salt for a short time period, and the ROS (O_2^-) and MDA contents were then analysed immediately. The results revealed that the ROS content was not significantly different in the leaves of *nd1* and WT (Figure 3d,e) under short-term salt treatment (treated with 150 mM NaCl for 1 h and 12 h respectively). Additionally, the results from H_2O_2 and MV treatment experiments demonstrated that the growth of *nd1* seedlings was similar to that of WT under oxidant treatments (Figure S6). The above results reveal that the accumulated MDA in *nd1* is not due to decreased ROS scavenging ability and is instead due to more serious damage to plant cells caused by the decreased plant salt tolerance, thus indicating that the pathway of OsCSLD4 involved in salt stress response in rice is different from that of AtCSLD5 in *Arabidopsis*.

OsCSLD4 regulates global gene responses to salt stress

To elucidate the mechanism by which OsCSLD4 is involved in the rice salt response, we studied the global impression of OsCSLD4-dependent gene expression in response to salt stress by analysing the transcriptome of WT and *nd1* that were treated with water (mock) or NaCl (150 mM NaCl). *nd1* possessed 1,318 differentially expressed genes (DEGs, marked as group A) compared to those of WT, and 554 genes were up-regulated and 764 were down-regulated, thus implying that OsCSLD4 plays an important role in rice under normal growth conditions. After treatment with 150 mM NaCl, *nd1* exhibited 888 DEGs (marked as group B) compared to those of WT, and 585 genes were up-regulated and 303 were down-regulated, thus indicating that OsCSLD4 plays an important role in regulating gene expression under salt stress (Figure 4a and Table S2). Statistical gene ontology (GO)-term enrichment analysis revealed that group A converges on cell wall organization or biogenesis, hormone synthesis or response, and transcript regulation. Group B converged in response to oxygen, hormone signal or synthesis and abiotic stress, with the

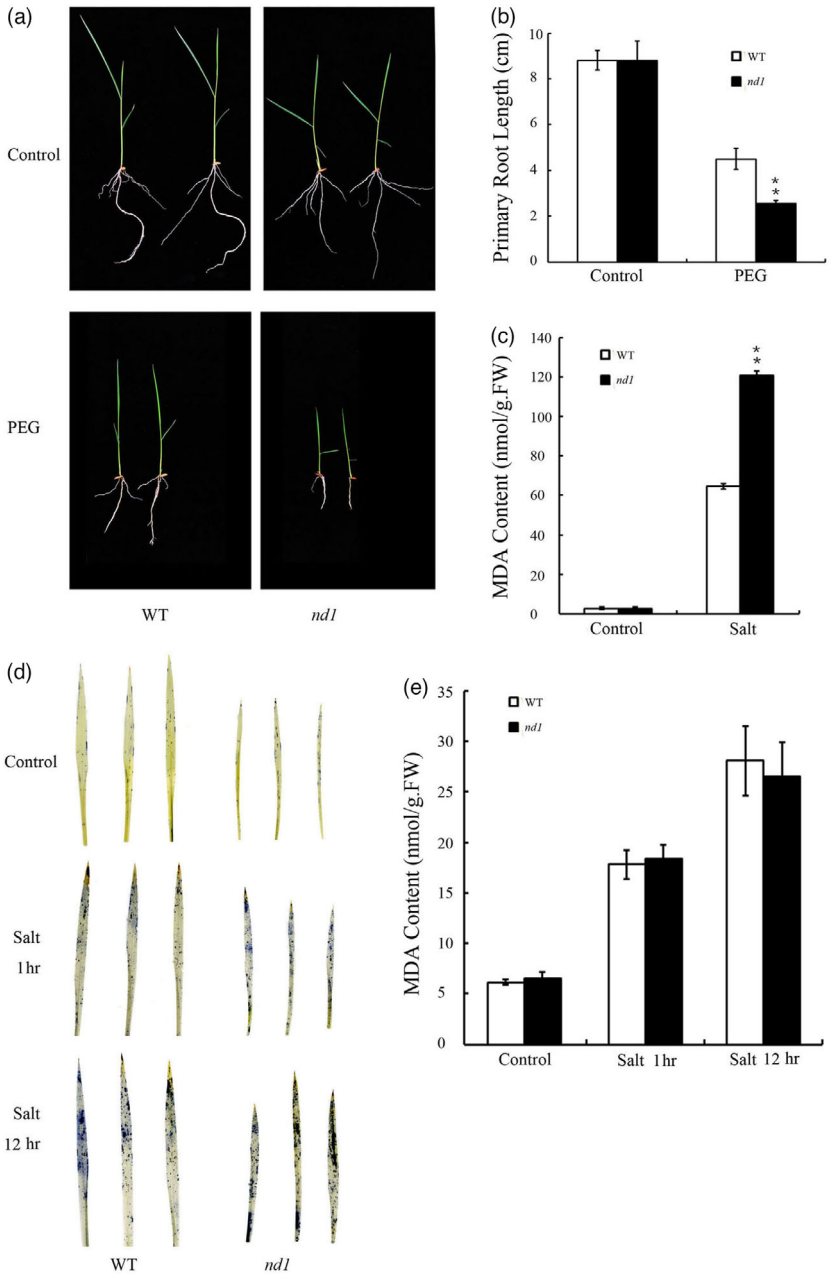

Figure 3 Disruption of OsCSLD4 decreases rice osmotic stress tolerance. (a) Phenotype of rice seedlings after PEG treatment. Control rice seedlings were grown on 1/2 MS medium for 10 days. PEG-treated rice seedlings were grown on 1/2 MS medium with 10% PEG for 10 days. (b) Primary root length of rice seedlings treated with PEG for 10 days. Control rice seedlings were grown on 1/2 MS medium for 10 days. PEG-treated rice seedlings were grown on 1/2 MS medium with 10% PEG for 10 days. (c) MDA content in rice seedlings under salt treatment. Control rice seedlings without salt treatment. Salt-treated rice seedlings were treated with 150 mM NaCl for 3 days. (d) O_2^- content in rice leaves after short-term salt treatment. (e) MDA content in rice seedlings after short-term salt treatment. Figures present the average of three repeated experiments. Bars represent the SE (±), and asterisks indicate the significant differences. Significance was evaluated using the *t*-test (*$P < 0.05$ and **$P < 0.01$).

exceptions of cell wall organization and biogenesis (Figure S7). These results reveal that OsCSLD4 is important for allowing rice to balance its development and salinity tolerance.

Moreover, the WT possessed 1068 differentially expressed genes compared to those in plants treated with water as a control (group C). In contrast, *nd1* exhibited 1641 differentially expressed genes (group D). Among these genes, 1200 DEGs exhibited different patterns compared to those in WT, and 331 DEGs between *nd1* and WT under salt stress may be responsible for the *OsCSLD4*-dependent salt stress response (Figure 4a and Table S2). We selected 107 *OsCSLD4*-dependent DEGs between *nd1* and WT for further GO enrichment. The results demonstrated that *OsCSLD4*-dependent DEGs were primarily involved in cell wall organization or biogenesis (36 genes, about 33.6% of total *OsCSLD4*-dependent DEGs), processes related to ABA (9 genes, about 8.4%), signal transduction (21 genes, about 19.6%) and stress response (36 genes, about 33.6% of total genes, Figure 4b, Table S3). We verified the relative expression in response to different treatments of some *OsCSLD4*-dependent differentially expressed genes using qPCR. For example, the cellulose synthesis genes *OsCesA4* (Tanaka *et al.*, 2003; Zhang *et al.*, 2009) and ATP-binding cassette (ABC) transporter G subfamily member 5 *OsABCG5* (Matsuda *et al.*, 2016; Shiono *et al.*, 2014), the calcium/calmodulin dependent protein kinase CRINKLY4 (Pu *et al.*, 2012), the ABA-responsive gene *RAB16C* (El-Esawi and Alayafi, 2019), the ABA-biosynthesis gene *9-cis-epoxycarotenoid dioxygenase 4 OsNCED4* (Hwang and Lee, 2018) and the transcription regulator DREB1C (Dubouzet *et al.*, 2003) all exhibited similar results in both the qPCR and transcriptome experiments (Figure 4c). These results suggest that disruption of OsCSLD4 has an obvious effect on ABA synthesis and signal pathway, and impaired salt stress tolerance may be due to the decreased transcription of stress-responsive

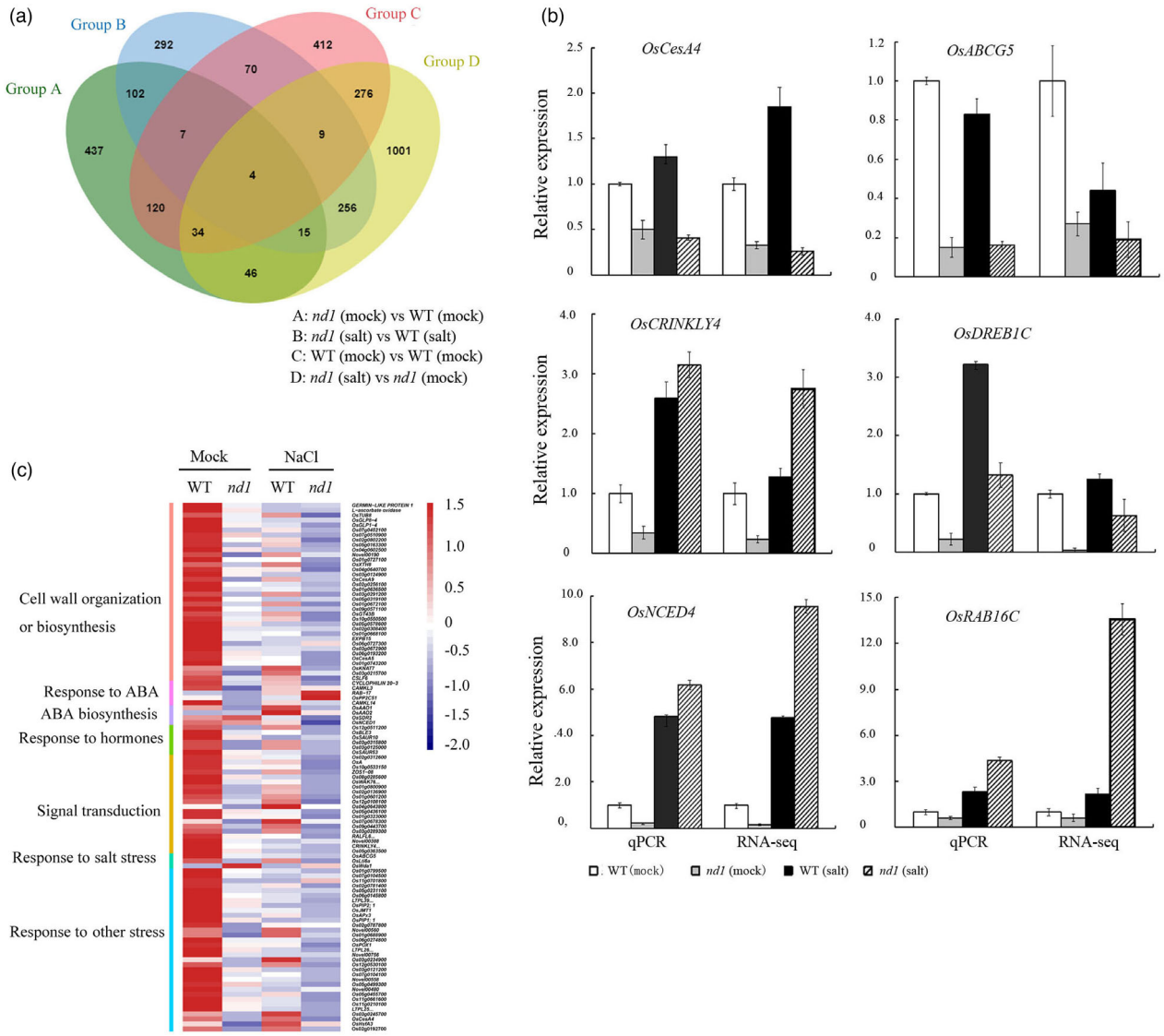

Figure 4 Global transcriptome analysis of OsCSLD4-dependent genes associated with salt stress in rice. (a) Venn diagram of DEGs between WT (Zhongxian 3037) and *nd1* under the two different experimental conditions. (b) Numbers of differentially expressed genes (DEGs) between WT and *nd1* under the two different treatment conditions ($P < 0.05$). Red bars indicate down-regulated DEGs in *nd1*; green bars indicate up-regulated DEGs in *nd1*. (c) Hierarchical clustering and expression levels of OsCSLD4-dependent DEGs that respond to NaCl treatment or are unresponsive. Coloured boxes on the right indicate GO terms that are statistically significantly enriched within each gene cluster. (d) Verification of the relative expression in response to different treatments of some OsCSLD4-independent differentially expressed genes by qRT-PCR and RNA-seq respectively. The expression level of genes in WT under normal conditions is standardized to '1'. The expression level of *OsCSLD4* is presented as the relative level to that of WT under normal condition.

genes by interfering with plant stress signal transduction, particularly ABA signal pathway.

Disruption of OsCSLD4 decreases the ABA sensitivity of rice seedlings

The phytohormone abscisic acid is known to play a key role in the response of plants to osmotic stress by regulating the expression of a large number of osmotic stress-responsive genes in plants (Chen *et al.*, 2016; Geng *et al.*, 2013; Sah and Reddy, 2016; Vishwakarma *et al.*, 2017; Wang *et al.*, 2011b; Zhu, 2002, 2016). We observed that there were a large number of DEGs between WT and *nd1* that are involved in ABA synthesis or its response in the transcriptome. To determine if and how OsCSLD4 is involved in the rice salt stress response via the ABA pathway, we first investigated the response of *nd1* to exogenous ABA treatment. The results demonstrated that the sensitivity of *nd1* roots to ABA treatment was lower than that of WT plants. The length of *nd1* primary roots was longer than that of WT under exogenous ABA treatment (Figure 5a,b), thus indicating that the disruption of OsCSLD4 may affect the sensitivity of rice to ABA.

Disruption of OsCSLD4 decreases ABA synthesis under both normal and salt conditions

Plants can regulate ABA responses by activating the action of the ABA signalling pathway or by increasing ABA content (Yoshida

and Mogami, 2014; Zhu, 2002, 2016). To illustrate the pathway by which the OsCSLD4 mutant alters rice ABA sensitivity, ABA content was measured. The results revealed that the ABA content in *nd1* seedlings under normal growing conditions was significantly lower compared to that of WT seedlings. The ABA content in the WT was approximately 169 ng/g. FW. In contrast, the ABA content in *nd1* was approximately 155 ng/g. FW (Figure 5c). The ABA content of *nd1* under salt stress was also measured. The results demonstrated that the ABA content in *nd1* under salt stress was lower compared to that in WT (Figure 5c). After a 7-day salt treatment, the ABA content in *nd1* was approximately 232 ng/g. FW. In contrast, the ABA content in the salt-treated WT was approximately 264 ng/g. FW. The above results indicated that the disruption of OsCSLD4 decreased rice ABA content.

ABA is critical for OsCSLD4 to modulate rice salt stress tolerance

To further analyse the role of ABA in OsCSLD4-regulated rice salt stress tolerance, we used low concentrations of exogenous ABA to treat *nd1* seedlings. The *nd1* seedlings that were treated with 50 nM ABA exhibited similar salt tolerance to that of WT. The length of the primary root was not significantly different compared to that of the WT under salt stress (Figure 5d,e), thus implying that ABA plays a crucial role in OsCSLD4-regulated rice salt stress response.

Disruption of OsCSLD4 impairs the expression of ABA synthesis and response genes

We analysed the expression of ABA synthesis genes in *nd1*. The results of the qPCR assay revealed that the expression of key genes involved in ABA synthesis in *nd1* seedlings was significantly lower than was that in WT seedlings (Figure 6a). For example, the transcript level of the rice ABA synthesis key gene *OsNCED4* was only approximately 20% of that in WT. The expression levels of *9-cis-epoxycarotenoid dioxygenase 1* (*OsNCED1*), *abscisic aldehyde oxidase1* (*OsAAO1*), *OsAAO2* and rice *short-chain dehydrogenase/reductase-like 2* (*OsSDR2*) in *nd1* were approximately half of those in WT (Figure 6a). The above results indicate that the decreased ABA level in *nd1* seedlings may be due to the impaired expression of ABA synthesis genes.

Abscisic acid can induce downstream stress-responsive gene expression to enhance plant stress tolerance (Yoshida *et al.*, 2014; Zhu, 2002). To analyse the role of ABA in OsCSLD4-mediated regulation of the salt stress response, we tested the expression of ABA-responsive genes in *nd1*. The results of qPCR assays revealed that the expression levels of ABA-responsive stress tolerance genes, including regulatory genes, such as *OsbZIP23* and *OsDREB1C*, and function genes, such as *OsRAB16C*, *OsRAB17*, *OsLIP9* and *OsRD22*, were markedly impaired in *nd1* (Figure 6b). For example, the expression levels of *OsRD22* and *OsRAB17* in *nd1* were approximately 19% and 10%, respectively, of those in the WT (Figure 6b).

Expression of *OsCSLD4* is induced by salt stress through an independent ABA pathway

To analyse the role of ABA in the salt-induced *OsCSLD4* expression, we studied the expression pattern of *OsCSLD4* under ABA treatment using qPCR assay. The expression of *OsCSLD4* under ABA treatment was increased to up to approximately 2.7-fold compared to that under the control conditions after 2 h of ABA treatment (Figure 7a), indicating that the expression of *OsCSLD4* was slightly induced by ABA.

Secondly, we analysed the expression of *OsCSLD4* in *nd1* without ABA or salt treatment. The results from qPCR assays revealed that the *OsCSLD4* expression level in *nd1* was approximately 70% of that in WT (Figure 7b), and the salt-induced expression of *OsCSLD4* was significantly inhibited in *nd1*, which was only approximately 30% of that in WT (Figure 7b), thus indicating that the disruption of OsCSLD4 impaired its expression under normal conditions or salt stress.

Following, to analyse the role of ABA in salt-induced *OsCSLD4* expression, we treated WT and *nd1* seedlings with ABA and salt together. The results from qPCR assays demonstrated that exogenous ABA cannot restore the salt-induced expression of *OsCSLD4* in *nd1*. The expression level of *OsCSLD4* in *nd1* was markedly lower than that in WT treated by salt alone (Figure 7b, c), but was similar to that in WT treated with ABA only (Figure 7a, c), thus indicating that salt-induced *OsCSLD4* expression was independent of ABA-induced *OsCSLD4* expression.

Overexpression of *OsCSLD4* enhances rice salt stress tolerance and grain yield

The above studies demonstrated that the loss function of OsCSLD4 markedly impaired rice salt stress tolerance. To well display the role of *OsCSLD4* in rice salt stress response, we further analysed the effect of the gain function of OsCSLD4 on rice salt tolerance. Firstly, we impaired the expression of *OsCSLD4* in the japonica variety Kitaake by RNA interference technology (Figure S8b). RNA interference plants (RIs) showed a salt stress-sensitive phenotype (Figure S8a,c), which was similar to that of the *OsCSLD4* mutants under Zhongxian 3037 and Zhonghua 11 background, indicating that OsCSLD4 has a similar role in rice salt stress response under different backgrounds. Then, we studied the effect of the gain function of OsCSLD4 on rice salt tolerance by using *OsCSLD4* overexpression plants (OEs) under Kitaake background (Figure 8 and Figure S9). We treated 2-week-old *OsCSLD4*-overexpressing rice seedlings with 150 mM NaCl for 7 days and then recovered for another 7 days. The results revealed that *OsCSLD4*-overexpressing rice seedlings exhibited higher salt tolerance after 7-day NaCl for with another 7-day recovery (Figure 8a). The survival rate of *OsCSLD4*-overexpressing plants was 70%–85%, and in contrast, the survival rate of the wild type (Kit) was only approximately 45% (Figure 8b).

The result also revealed that ABA content in *OsCSLD4* overexpression seedlings was higher than that in Kit (wild type) (Figure 8c). The ABA content in Kit was approximately 115 ng/g. FW. In contrast, the ABA content in the *OsCSLD4* overexpression lines OE1 and OE2 was approximately 195 ng/g FW and 205 ng/g FW respectively (Figure 8c). Additionally, the expression levels of ABA synthesis and responsive genes were markedly up-regulated in *OsCSLD4* overexpression plants (Figure 8d). The above results reveal that OsCSLD4 can play a positive role in prompting ABA synthesis to cope with salt stress in rice.

Intriguingly, we observed that overexpression of *OsCSLD4* not only enhanced rice salt tolerance but also increased grain size and weight. As presented in Figure S10b,d, the grain width (approximately 3.6 mm) of *OsCSLD4*-overexpressing plants was obviously larger than that of the wild type (Kit, approximately 3.3 mm). In contrast, the grain width of *nd1* (approximately 2.3 mm) was smaller than that of WT (Zhongxian 3037, approximately 2.9 mm) (Figure S10a,c). The 1000-grain weight of *nd1* was only approximately 16.8 g, while that of wild type (WT, Zhongxian 3037) was approximately 24.8 g (Figure S10e). The 1000-grain

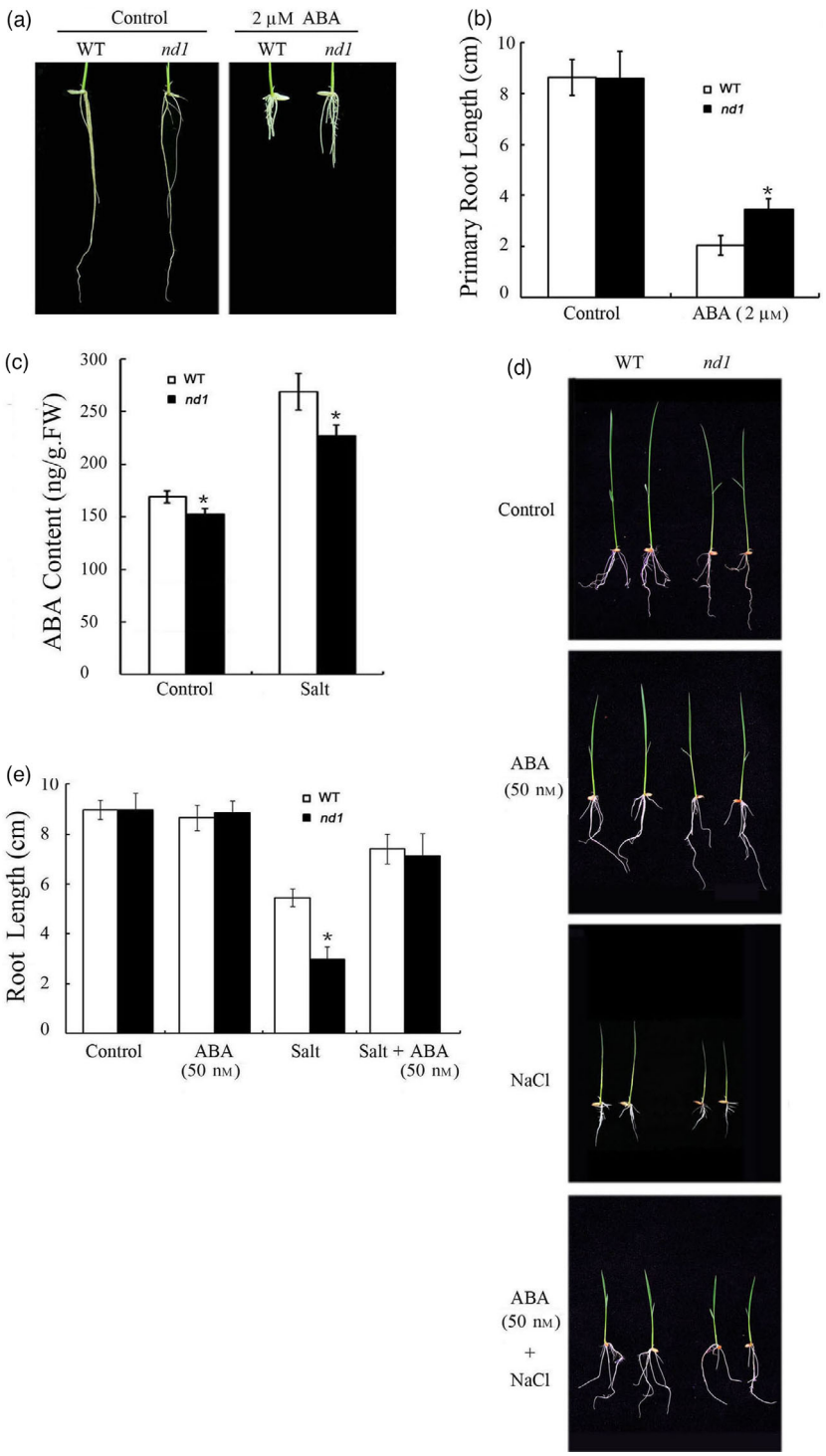

Figure 5 ABA is related to OsCSLD4 function. (a) Phenotype of rice primary roots treated with ABA for 7 days. (b) Primary root length of rice seedlings treated with ABA for 7 days. (c) ABA content in 2-week-old rice seedlings under normal and salt treatment conditions. (d) Phenotype of rice seedlings treated with NaCl and ABA together. Rice seedlings were grown in 1/2 MS medium without or with ABA and 150 mM NaCl for 7 days, respectively, or with both ABA and 150 mM NaCl. (e) Primary root length of rice seedlings treated with NaCl and ABA together. Figures present the average primary root length from three independent experiments. Bars represent the SE (±) from three repeated assays. Asterisks indicate a significant difference. Significance was evaluated using the *t*-test (*$P < 0.05$ and **$P < 0.01$).

weights of the *OsCSLD4* overexpression lines OE1 and OE2 were approximately 25.6 g and 24.9 g, respectively, while that of Kit was only about 23.3 g (Figure S10f). The above results demonstrate that OsCSLD4 plays a positive role in both salt tolerance and grain yield.

Discussion

Cell wall polysaccharide synthases play critical roles in plant stress tolerance (Hamann, 2012; Liu *et al.*, 2021a; Novaković *et al.*, 2018; Rajashekar and Lafta, 1996; Rao and Dixon, 2017; Wang *et al.*, 2019; Wang *et al.*, 2016). However, the detailed mechanism of polysaccharide synthases in plant stress responses remain obscure. Previous studies have demonstrated that the polysaccharide synthase OsCSLD4 can catalyse the biosynthesis of rice cell wall polysaccharides, and plays an important role in rice growth and development (Hu *et al.*, 2010; Li *et al.*, 2009; Luan *et al.*, 2011; Yoshikawa *et al.*, 2013). The present study further demonstrated the mechanism of OsCSLD4 involved in rice salt tolerance, which will be beneficial for people to

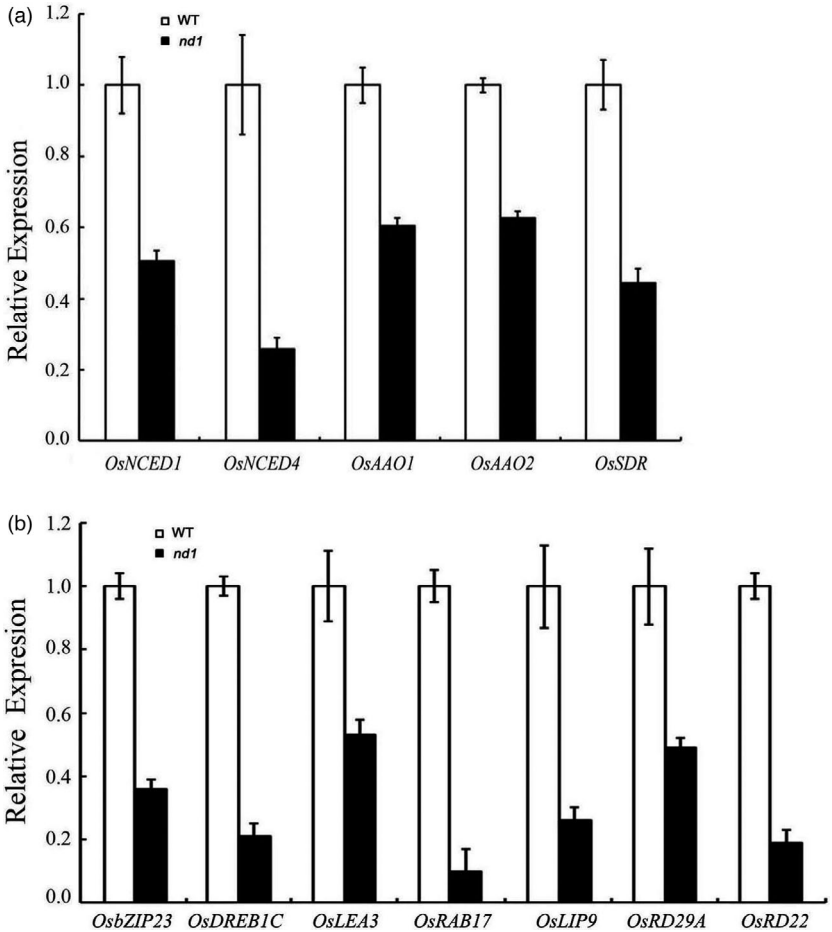

Figure 6 Relative expression levels of ABA synthesis and response-related genes in *nd1* seedlings. (a) Relative expression levels of ABA synthesis genes in 2-week-old *nd1* seedlings. (b) Relative expression levels of ABA-responsive and salt stress-related genes in 2-week-old *nd1* seedlings. The expression of *OsActin1* was used as an internal control. The figure presents the relative expression levels of genes relative to that in WT. Bars represent SE (±) from three repeated assays.

understand the roles of polysaccharide synthases in plant salt stress response.

OsCSLD4 plays a positive role in enhancing rice salinity stress tolerance

Studies revealed that cell wall polysaccharide synthesis or modification-related enzymes play critical roles in the response of plants to abiotic stresses (Corrêa-Ferreira *et al*., 2019; Hamann *et al*., 2009; Novaković *et al*., 2018; Tenhaken, 2014; Wang *et al*., 2019). For example, the mutation of cellulose synthase 6 (CESA6) or its companion protein CC1/2 (companion of cellulose synthase 1/2) disturbs cellulose synthesis and decreases *Arabidopsis* salt tolerance (Kesten *et al*., 2019; Zhang *et al*., 2016). Overexpression of hot pepper endotransglucosylase/hydrolase CaXTH3 enhances the tolerance of transgenic tomato and *Arabidopsis* to salt stress (Cho *et al*., 2006; Choi *et al*., 2011). The disruption of *Arabidopsis XTH* genes *XTH19* and *XHT23* decreased plant salt tolerance (Xu *et al*., 2020). Except for positive role in enhancing plant salt stress tolerance, some enzymes also play negative roles in plant salt stress tolerance. The overexpression of *Arabidopsis* β-1,4-galactan synthase GALACTAN SYNTHASE 1 (GALS1) decreased *Arabidopsis* salt tolerance (Yan *et al*., 2021).

Previous studies have showed that OsCSLD4 plays an important role in the synthesis of rice cell wall polysaccharides (Li *et al*., 2009, 2010). Here, this study showed that the disruption of OsCSLD4 impaired rice salt and osmotic stress tolerance (Figure 2, 3 and Figure S3), and in contrast, the overexpression of *OsCSLD4* enhanced rice salt stress tolerance (Figure 8), thus revealing the positive role of OsCSLD4 in rice salt stress tolerance. Previous study demonstrated that OsCSLD4 homolog AtCSLD5 also positively regulated *Arabidopsis* salt stress tolerance (Zhu *et al*., 2010), indicating the function of OsCSLD4 homologous proteins may be conserved in plant salt stress response.

OsCSLD4 enhances rice salt stress tolerance by regulating ABA synthesis to enhance plant osmotic stress tolerance

Studies showed that cell wall can be involved in plant salt stress response through several pathways, such as controlling ion transport, regulating osmotic homeostasis and coordinating plant hormone action (Liu *et al*., 2021a; van Zelm and Zhang, 2020; Zhao *et al*., 2021b). Plant cell wall polysaccharide synthases have important roles in plant stress response, but the pathways of cell wall polysaccharide synthases involved in salt stress response are still obscure (Liu *et al*., 2021a; Novaković *et al*., 2018; van Zelm *et al*., 2020; Voxeur and Höfte, 2016; Zhao *et al*., 2021b; Zhu *et al*., 2010).

Zhu *et al*. (2010) demonstrated that the *OsCSLD4 Arabidopsis* homolog *AtCSLD5* may be involved in the salt stress response by regulating ROS scavenging to enhance *Arabidopsis* osmotic stress tolerance under high salt stress. Here, ROS (O_2^-) content in the OsCSLD4-disrupted mutant *nd1* was similar to that in wild-type plants during short-term salt treatment (Figure 3d,e). The sensitivity of *nd1* to MV and H_2O_2 was also similar to that of the wild type (Figure S6), thus indicating that the decreased salt tolerance

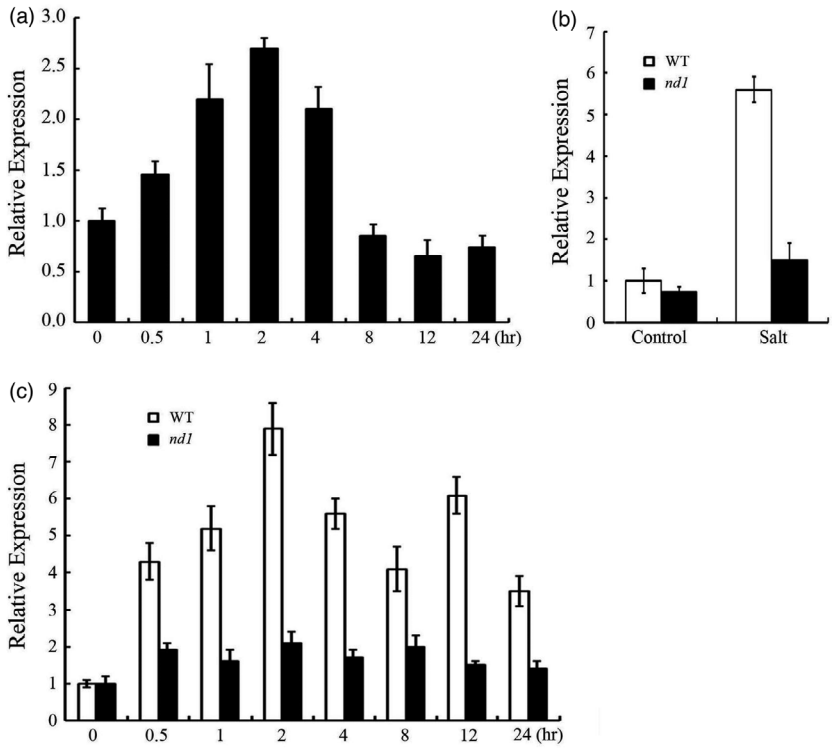

Figure 7 Salt-induced expression of *OsCSLD4* is independent of ABA. (a) Expression pattern of *OsCSLD4* under ABA treatment. The *OsCSLD4* expression level under normal condition (0 h) is standardized to '1'. The figure presents the relative expression level of *OsCSLD4* under ABA treatment compared to that under normal condition. (b) Expression level of *OsCSLD4* in *nd1* under salt treatment. The *OsCSLD4* expression level in WT under normal condition is standardized to '1'. The expression level of *OsCSLD4* was provided as the relative level to that in WT under normal conditions. (c) Expression pattern of *OsCSLD4* under treatments with salt and ABA. The expression level of *OsCSLD4* under normal condition (0 h) is standardized to '1'. The figure presents the relative expression level of *OsCSLD4* under salt and ABA treatment to that under normal conditions (0 h). The expression of *OsActin1* was used as the internal control. The figure presents the average of at least three independent experiments. Bars represent the SE (±).

of *nd1* is not due to impaired ROS scavenging ability. The Na^+ and K^+ contents in the roots and leaves of *nd1* were also similar to those of the wild type before or after salt treatment (Figure S5), thus indicating that OsCSLD4 is not involved in the absorption and transport of ions in rice. In contrast, the sensitivity of *nd1* to ABA is impaired (Figure 5a,b), and exogenous ABA can restore *nd1* salt tolerance (Figure 5d,e), thus revealing that ABA plays a critical role in OsCSLD4-mediated rice salt stress response.

ABA plays an important role in regulating the response of plants to environmental stress (Sah *et al*., 2016; Vishwakarma *et al*., 2017; Zhu, 2002). Plants can induce ABA biosynthesis to stimulate stress-responsive gene expression to adapt to adverse environmental conditions (Sah *et al*., 2016; Vishwakarma *et al*., 2017; Wang *et al*., 2011b; Zhu, 2016). Studies demonstrated that cell wall composition and integrity can affect ABA synthesis and the ABA signalling (Chen *et al*., 2005; Hernández-Blanco *et al*., 2007; Liu *et al*., 2021a; Novaković *et al*., 2018; Palmeros-Suárez *et al*., 2017). For example, osmotic stress can induce ABA synthesis in the leaf discs of beans or barley, but this process fails in their protoplasts, thus indicating that the cell wall is essential for osmotic stress-induced ABA synthesis (Lahr and Raschke, 1988; Loveys and Robinson, 1987). Additionally, the cellulose synthase AtCesA8/IRX1 allelic mutants *lew2-1* and *lew2-2* both exhibited increased expression levels of ABA synthesis genes and ABA-responsive genes, and accumulated more ABA (Chen *et al*., 2005; Hernández -Blanco *et al*., 2007), thus indicating that polysaccharide synthesis enzymes are closely related to ABA biosynthesis and ABA signalling. Here, the disruption of OsCSLD4 function decreased ABA synthesis (Figure 5c), and in contrast, overexpression of *OsCSLD4* increased ABA content (Figure 8c). The expression levels of ABA synthesis-related genes were decreased and enhanced in *nd1* and *OsCSLD4* overexpression plants, respectively, thus indicating that OsCSLD4 plays an important role in mediating ABA content by regulating the expression of ABA synthesis genes. The analysis of gene transcript level showed that amounts of ABA-responsive genes, such as *OsRAB16B* (*Os11g0454200*), *OsLEB24* (*Os01g0702500*) and *OsRAB16C*, can also be obviously induced by salt stress in *nd1* (Tables S2 and S3). Additionally, exogenous ABA can restore *nd1* salt tolerance (Figure 5d,e). These results indicate that OsCSLD4 may be involved in salinity stress response by mediating ABA content but not the activity of ABA signalling pathway in rice. Moreover, the disruption of OsCSLD4 decreased rice seedlings' osmotic stress tolerance, which indicated that OsCSLD4 is involved in rice high salt adaptation by modulating rice osmotic stress tolerance.

OsCSLD4 plays a positive role in coordinating rice salinity stress tolerance and grain growth

The cell wall-localized proteins play critical roles in coordinating plant salt stress responses and growth and development (Liu *et al*., 2021a). Several plasma membrane-localized cell wall

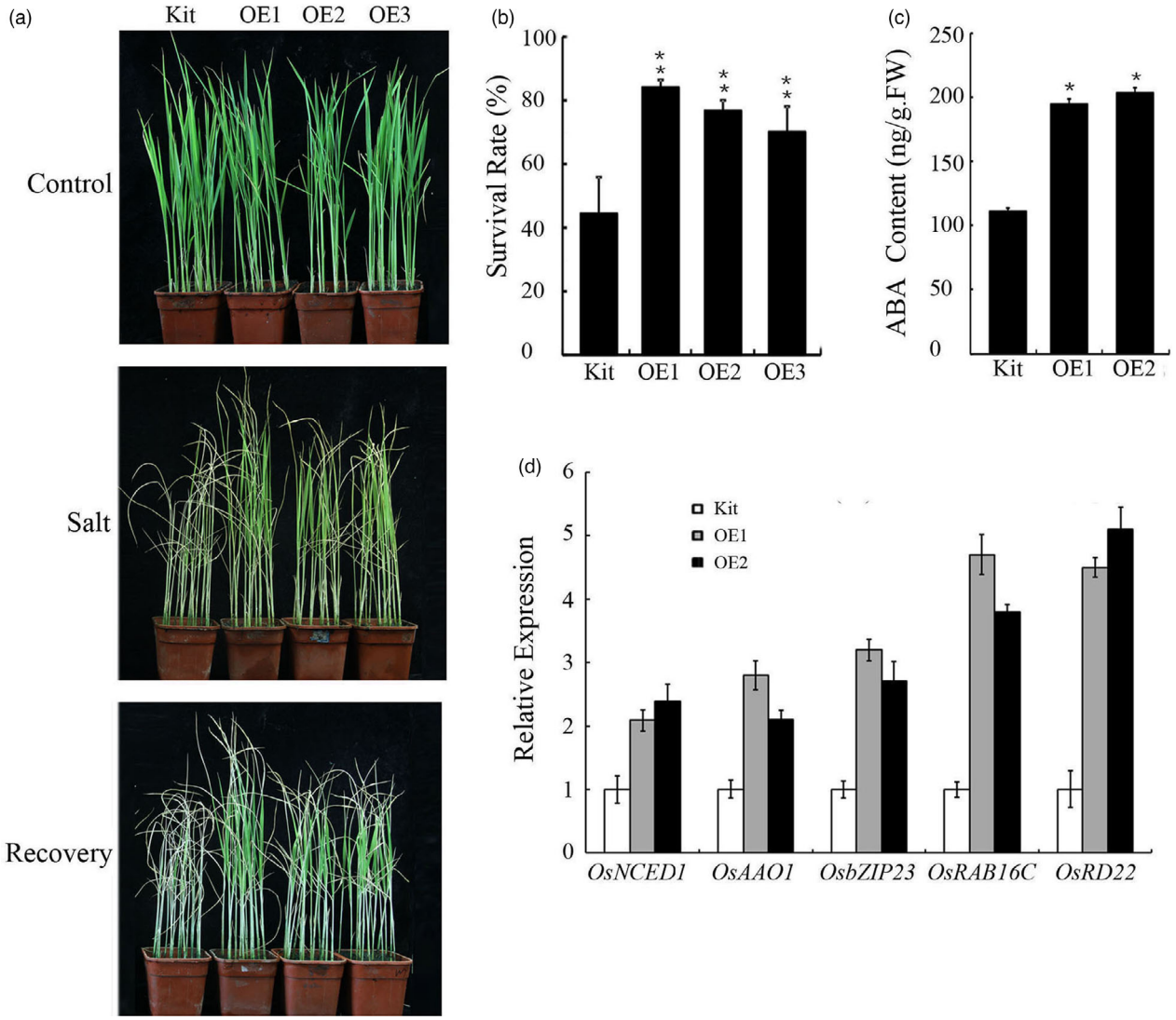

Figure 8 Overexpression of *OsCSLD4* enhances rice salt tolerance by up-regulating ABA synthesis. (a) Phenotype of *OsCSLD4* overexpression rice seedlings treated with salt stress in soil. Two-week-old rice seedlings grown in soil were treated with 150 mM NaCl for 7 days with another 7-day recovery. (b) Survival rate of *OsCSLD4* overexpression rice seedlings treated with 150 mM NaCl for 7 days with another 7-day recovery. (c) ABA content in 2-week-old *OsCSLD4* overexpression rice seedlings. (d) Expression level of ABA synthesis and responsive genes in 2-week-old *OsCSLD4* overexpression transgenic rice seedlings. The expression of *OsActin1* was used as the internal control. The gene expression levels in transgenic rice seedlings were presented as relative to the levels in the wild type (Kitaake). Kitaake: wild type. OEx: independent *OsCSLD4* overexpression lines. 'x' indicates the numbering of different *OsCSLD4* overexpression transgenic lines. The figure presents the average of at least three repeated experiments. Bars indicate the SE (±) from three repeated experiments. Asterisks represent significant difference as evaluated by *t*-test (*$P < 0.05$ and **$P < 0.01$).

integrity sensors have been identified that perceive cell wall changes and are involved in plant salt stress responses and growth and development. Wall-associated kinases (WAKs), which can be cross-linked with pectin, are a family of receptor-like Ser/Thr kinases (Decreux and Messiaen, 2005). WAKs can coordinate plant salt stress response and growth. For example, the mutant of barley WAK protein HvWAK1 obviously impaired salt stress tolerance and retarded root growth under salt stress (Kaur and Singh, 2013). The tomato WAK mutant *Slwak1* disrupted osmotic homeostasis and reduced plant shoot growth under salt stress (Meco *et al*., 2020). The rapid alkalinization factors (RALFs) are a type of small signalling peptides. RALFs play important roles in growth and development, and are involved in stress responses (Liu *et al*., 2021b; Murphy and De Smet, 2014). In *Arabidopsis*, the constitutive expression of *AtRALFL8* increased transgenic plant susceptibility to drought stress (Atkinson and Lilley, 2013). The overexpression of *AtRALF22* and *AtRALF23* can bind to leucine-rich repeat extensins (LRXs) or receptor-like kinases, such as FERONIA (FER), THESEUS1 (THE1), to control plant growth and salt stress responses by modulating the synthesis and signalling of phytohromones, such as ABA and jasmonic acid (Chen *et al*., 2016; Yu *et al*., 2012; Zhao *et al*., 2021a).

Previous studies have revealed that OsCSLD4 is involved in the synthesis of cell wall xylan, cellulose and homogalacturonan and has pleiotropic effects on the growth and development of the leaf, stem and root (Hu *et al*., 2010; Li *et al*., 2009; Luan

et al., 2011; Yoshikawa *et al*., 2013; Figure 2a and Figures S2a, S4b). The present study showed OsCSLD4 also played a positive role in rice grain growth. Although the mechanism of his synergistic regulation of rice salt stress response and growth and development needs to be further studied in detail, the results from the transcriptome analysis showed the expressions of WAKs gene *OsWAK76* (*Os08g0501700*, *WALL-ASSOCIATED KINASE GENE 76*) and RALFs gene *RALFL6* (*Os01g0358100*, *Rapid ALkalinization Factor 6*) in OsCSLD4 mutant *nd1* are obviously impaired under both normal and salt stress conditions (Table S3), indicating OsCSLD4 may coordinate rice salt stress response and growth and development by modulating the homeostasis of phytohormones *via* these cell wall-localized proteins.

Taken together, this study demonstrated that the cellulose synthase-like protein OsCSLD4 plays important roles in rice coping with salinity stress by mediating ABA synthesis to enhance osmotic stress tolerance. Considering the profound influence of OsCSLD4 on cell wall polysaccharide composition and structure, we believe that the cell wall polysaccharide composition or structure altered by OsCSLD4 is critical for rice to regulate the action of cell wall-localized proteins, which further modulate intracellular signal to regulate ABA synthesis to adapt to salt stress and adjust growth and development cases (Figure 9).

Materials and methods

Plant materials and growth conditions

The rice indica variety Zhongxian3037 and its mutant *nd1* (Li *et al*., 2009), japonica variety Zhonghua 11 and its mutant *nrl1* (Wu *et al*., 2010), japonica variety Kitaake and *OsCSLD4* overexpression transgenic plants (OE1, OE2 and OE3, in Kitaake background) were used in this study. Unless otherwise specified, wild type (WT) refers to Zhongxian3037. All rice seedlings were grown at 28 °C under a 14-h light/10-hr dark photoperiod. For rice seedlings grown on 1/2 Murashige & Skoog (MS) medium, rice seeds were first sterilized with 75% alcohol for approximately 2 min, treated with 2.5% sodium hypochlorite for 20–30 min, washed with sterile water and then planted on 1/2 MS medium with or without different exogenous reagents.

Phylogenic analysis

We used the OsCSLD4 full-length amino acid sequence to identify homologous sequences using the Blastp search program of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). All of the sequences in the CSLD subfamily from rice (*Oryza sativa*), Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) were used to construct a phylogenetic tree using MEGA 5.0 with 500 bootstrap repetitions. The accession numbers of the CSLDs used in this study are At2g33100 (AtCSLD1), At5g16910 (AtCSLD2), At3g03050 (AtCSLD3), At4g38190 (AtCSLD4), At1g02730 (AtCSLD5), At1g32180 (AtCSLD6), GRMZM2G436299 (ZmCSLD1), GRMZM5G870176 (ZmCSLD2), GRMZM2G044269 (ZmCSLD3), GRMZM2G015886 (ZmCSLD4), GRMZM2G061764 (ZmCSLD5), Os10g42750 (OsCSLD1), Os06g02180 (OsCSLD2), Os08g25710 (OsCSLD3), Os12g36890 (OsCSLD4) and Os06g22980 (OsCSLD5).

Treatments using NaCl, PEG and LiCl

To analyse the expression level of *OsCSLD*4 under salt stress, 2-week-old rice seedlings were sprayed with 150 mM NaCl for different time periods. To study the salt stress tolerance of rice seedlings in soil, 2-week-old rice seedlings grown in pots under normal conditions were transferred into trays containing 150 mM NaCl solution for 7 days. Then, the rice seedlings were removed from the NaCl solution and grown under normal conditions. The survival rate of rice seedlings was determined after another 7-day recovery period. To study the effect of OsCSLD4 on the salt tolerance of rice roots, the germinated rice seeds were then planted on 1/2 MS with or without 150 mM NaCl for another 7 days. The seedling phenotype was photographed, and the length of rice seedling primary roots was measured after 7 days of salt treatment.

To study the effect of OsCSLD4 on rice osmotic tolerance, the germinated seeds were planted on 1/2 MS with or without 10% (w/v) PEG (polyethylene glycol 6000) for 10 days. Photos of the seedling phenotype were taken, and the length of the primary roots was measured after 10-day PEG treatment.

To analyse the role of OsCSLD4 in rice ion stress tolerance, germinated rice seeds were grown on 1/2 MS or 1/2 MS supplemented with 18 mM LiCl for another 7 days. The seedling phenotype was photographed, and the length of rice seedling primary roots was measured after a 7-day LiCl treatment.

Transcriptome analysis

Two-week-old 3037 and *nd1* plants were cultivated, treated and inoculated as described above. Three biological replicates (each consisting of pooled leaves from ~20 plants from one pot) were collected after 2 d of treatment and then snap-frozen in N_2(l) for total RNA extraction. The mRNA library preparation and sequencing were outsourced (Allwegene Tech., Beijing, China). The 12 libraries were sequenced on an Illumina HiSeq 2500 platform, and paired-end 150-bp reads were generated. Reads containing greater than 10% poly-N and greater than 50% low-quality reads ($Q \leq 20$) were removed from the raw data using Trimmomatic v0.33 (Bolger *et al*., 2014). Concurrently, the Q20 and Q30 values, GC content and sequence duplication levels of the clean data were calculated. All downstream analyses were based on clean and high-quality data. RNA-Seq reads were aligned to the rice reference genome (Oryza sativa Japonica Group, Ensembl_IRGSP_1.0.34) using TopHat 2 (v2.1.0) (Trapnell *et al*., 2012). Each read was mapped with Cufflinks (v2.1.1), a program that assembled the alignments in the Sequence Alignment/Map format into transfrags (Trapnell *et al*., 2012). The assembly files were then merged with reference transcriptome annotations for further analysis (Trapnell *et al*., 2012). The differential expression of each gene was calculated by quantifying the Illumina reads based on fragments per kilobase of transcript per million fragments mapped (FPKM; Mortazavi *et al*., 2008). Gene Ontology (GO, http://www.geneontology.org/) enrichment analysis of the DEGs was performed using GOseq (v. 1.22) (Young *et al*., 2010) using Wallenius' noncentral hypergeometric distribution that can adjust for gene length bias in DEGs.

ABA treatment

To determine the expression level of *OsCSLD*4 that was induced by ABA, the leaves of 2-week-old rice seedlings were sprayed with 100 μM ABA for different time periods. To analyse the role of ABA in salt-induced *OsCSLD*4 expression, 2-week-old rice seedlings were sprayed with 100 μM ABA and 150 mM NaCl for different durations.

To analyse the sensitivity of OsCSLD4 to exogenous ABA, rice seeds were sterilized and germinated, and the germinated rice

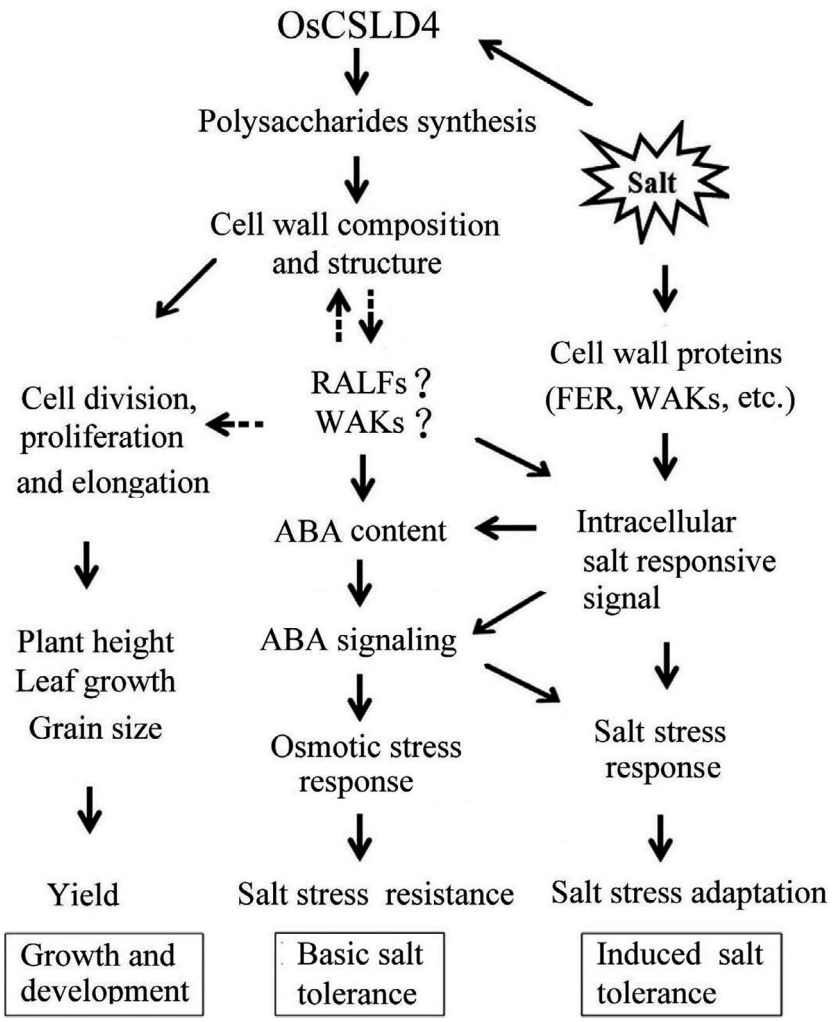

Figure 9 Proposed model for OsCSLD4 in rice salt stress response and growth and development. Rice can adapt to high salt environment by modulating the action of cell wall-localized proteins, such as FERONIA (FER) and THESEUS1 (THE1), to regulate intracellular salt response to enhance plant salt stress tolerance. Except for involving in rice growth and development by regulating cell division, proliferation and elongation, OsCSLD4 plays an important role in rice basic and induced salt tolerance by modulating the content or activity or location of cell wall-localized proteins, such as rapid the alkalinization factors (RALFs) and the wall-associated kinases (WAKs), to sustain high ABA content to enhance osmotic stress tolerance.

seeds were then planted on 1/2 MS medium or 1/2 MS medium with 2 μM ABA for another 7 days. The seedling phenotype was photographed, and the length of primary roots was measured after a 7-day ABA treatment.

To study the effect of ABA on OsCSLD4-regulated salt tolerance, the germinated rice seeds were planted on 1/2 MS medium or 1/2 MS medium with 0.05 μM ABA, 150 mM NaCl alone or with 150 mM NaCl plus 0.05 μM ABA for another 7 days. Photos of the seedling phenotype were taken, and the length of the primary roots was measured after a 7-day treatment.

Measurement of MDA content

To measure the MDA content of rice seedlings, approximately 0.2 g of leaf tissue was homogenized with 1.5 mL of 10% (w/v) trichloroacetic acid. After centrifugation, approximately 1 mL of supernatant was transferred into a clean 15-mL collection tube containing 0.25% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid. The samples were incubated at 100 °C for 30 min, cooled to ambient temperature and centrifuged at a speed of 1 *g* for 15 min. The absorbance of the supernatant was measured at 450, 532 and 600 nm. The MDA content was calculated using the following equation: 6.45× (OD532-OD600)-0.559 × OD450 (Qin *et al*., 2016).

Measurement of Na^+ and K^+ content

To test the Na^+ and K^+ content in rice shoots and roots, 2-week-old rice seedlings were grown in 1/2 MS liquid medium with or without 150 mM NaCl for 7 days. The shoots and roots of rice seedlings were collected, rinsed with deionized water and dried at 80 °C in paper bags to a constant weight. The weight of each sample was then measured. Subsequently, these samples were incinerated in a furnace at 575 °C for 9 h, dissolved in 0.1N hydrochloric acid for 4 h and diluted 50 times with 0.1N hydrochloric acid. K^+ and Na^+ concentrations were determined using atomic absorption spectrophotometry (SpectrAA-10; Springvale, Australia).

Measurement of ABA content

ABA content was measured according to the ELISA method described by Yang *et al*. (2001) with some modifications. Approximately 200 mg of frozen 2-week-old rice seedlings were ground to a fine powder with liquid nitrogen. The powder was then transferred into a covered, siliconized borosilicate tube containing 500 μL extraction buffer (90% [v/v] methanol and 200 mg/L sodium diethyldithiocarbamate trihydrate) and mixed thoroughly. The samples were centrifuged at 8000 *g* for 10 min at 4 °C after incubation at 4 °C for approximately 8 h. The supernatant was transferred to a new pre-cooled tube and

evaporated at 4 °C to dry. The residue was dissolved in 500 μL methanolic Tris-HCl buffer (50 mM, pH 7.4), and the ABA content was measured using an ELISA kit according to the manufacturer's instructions (Enzyme-linked Biotechnology, Shanghai, China; No: ml077235).

O_2^- staining

To assess the O_2^- content, 2-week-old rice seedlings were watered with or without 150 mM NaCl for 1 h or 12 h. The rice leaves were then incubated in 25 mmol HEPES buffer (pH 7.6) containing 1% nitrotetrazolium blue chloride (NBT) in the dark at 25 °C for 12 h. Next, these leaves were destained with 80% ethanol (v/v) to remove chlorophyll until clear blue deposits were observed. The detail process was described by Qin *et al*. (2016).

Quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol solution (Tiangen, Beijing, China). Next, 2 μg of total RNA was reverse transcribed into cDNA using oligo (dT) as primers and M-MLV as reverse transcriptase (Toyobo, Osaka, Japan). qPCR was performed using 2X SYBR Green mix (Takara, Cat No. 330523) with iQ5 according to the manufacturer's instructions (Bio-Rad, iQ5). The expression of *OsActin1* was used as an internal standard, and the expression levels of other genes were normalized using the comparative C_t method (Livak and Schmittgen, 2001).

Genetic transformation in rice

To generate rice plants that limit the expression of Os*CSLD4*, we used RNA interference (RNAi) technology to decrease its expression in japonica variety Kitaake. One artificial microRNA (amiRNA) sequence (*amiRNA-CSLD4*, 5′-TAAAGTACTTTTACTGAACTG-3′) targeting *OsCSLD4* transcript was selected among those proposed by the WMD3 program (web MicroRNA designer; http://wmd3.weigelworld.org/). This special *amiRNA-OsCSLD4* will be in pairing to the amiRNA exactly mimic the foldback structure of the endogenous *osa-MIR528*. The fusion *amiRNA-CSLD4* product was further cloned into vector pCAMBIA 5300 using the *Kpn* I and *BamH* I site, placing the DNA sequence of the amiRNA precursor under control of the regulatory region of the maize *Ubiquitin* promoter. The detailed process was described earlier (Warthmann *et al*., 2008). The plant expression vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404. Then, the resultant plasmid was introduced into Kitaake using *Agrobacterium*-mediated transformation. The transformed plants were selected by hygromycin. The efficiency and specificity of the RNAi lines were confirmed using real-time quantitative PCR. The T2 transgenic lines were used in this study.

To create *OsCSLD4* overexpression transgenic rice plants, the full-length ORF of *OsCSLD4* was cloned into vector pCAMBIA 1307 using *Xba* I and *BamH* I sites. The recombinant plasmid was then transferred into *Agrobacterium tumefaciens* strain LBA4404. *OsCSLD4* was introduced into the japonica variety Kitaake using Agrobacterium-mediated transformation. *OsCSLD4* overexpression transgenic lines were selected using hygromycin and confirmed by qPCR. The detailed selection process for transgenic plants was described by Qin *et al*. (2016). Homozygous T3 transgenic lines were used in this study.

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Author contributions

H.Z., Z.L. and Y.W. performed most experiments. M.X. and R.Q. performed the construction of OsCSLD4 overexpression transgenic plants. J.W. measured grain traits. R.H., L.Z. and Z.Z. designed experiments and wrote the manuscript. H.L. and H.Z. edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phenotype of the OsCSLD4-disrupted mutant *nd1*.
Figure S2 Expression level of *OsCSLD4* in rice roots, stems and leaves.
Figure S3 The *nd1* allelic mutant *nrl1* also decreases rice salt tolerance.
Figure S4 Sensitivity of the OsCSLD4 mutant *nd1* to LiCl.
Figure S5 Absorption and transportation of Na^+ and K^+ under normal or salt stress condition.
Figure S6 Sensitivity of *nd1* to MV and H_2O_2.
Figure S7 Statistical gene ontology (GO) term enrichment analysis for DEGs in group A (*nd1* mock vs. WT mock) and group B (*nd1* salt vs. WT salt).
Figure S8 Inhibited expression of *OsCSLD4* by RNA interference (RNAi) impairs rice salt tolerance in Kitaake background.
Figure S9 Expression level of *OsCSLD4* in *OsCSLD4* overexpression transgenic lines.
Figure S10 Grain width and weight of the *OsCSLD4* overexpression rice.
Table S1 Primers used in quantitative real-time PCR and vector construction.
Table S2 Differentially expressed genes (DEGs) in WT and *nd1* under different conditions.
Table S3 OsCSLD4-dependent differentially expressed genes for GO term enrichment analysis.