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ORIGINAL PAPER

Functional analysis of rice *OSCA* genes overexpressed in the arabidopsis osca1 mutant due to drought and salt stresses

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Abstract Drought and salt are two major abiotic stresses that severely impact plant growth and development, as well as crop production. A previous study showed that *OsOSCA1.4*, one of eleven rice *OSCAs* (*OsOSCAs*), complements hyperosmolality-induced $[Ca^{2+}]_{cyt}$ increases ($OICI_{cyt}$), salt stress-induced $[Ca^{2+}]_{cyt}$ increases ($SICI_{cyt}$) and the associated growth phenotype in Arabidopsis *osca1* (*reduced hyperosmolality-induced* $[Ca^{2+}]_{cyt}$ *increase 1*). In this study, Except for *OsOSCA2.3* and *OsOSCA4.1*, we generated independent transgenic lines overexpressing eight other *OsOSCAs* in the *osca1* to explore their functions in osmotic Ca^{2+} signalling, stomatal movement, leaf water loss, and root growth in response to hyperosmolality and salt stress. Similar to *OsOSCA1.4*, overexpression of *OsOSCA1.1* or *OsOSCA2.2* in *osca1* complemented $OICI_{cyt}$ and $SICI_{cyt}$, as well as stomatal closure and root growth in response to hyperosmolality and salt stress treatments, and drought-related leaf water loss. In addition, overexpression of *OsOSCA1.2*, *OsOSCA1.3* or *OsOSCA2.1* in *osca1* restored $OICI_{cyt}$ and $SICI_{cyt}$, whereas overexpression of *OsOSCA2.5* or *OsOSCA3.1* did not. Moreover, *osca1* overexpressing these five *OsOSCAs* exhibited various abiotic stress-associated growth phenotypes. However, overexpression of *OsOSCA2.4* did not have any of these effects. These results indicated that multiple members of the *OsOSCA* family have redundant functions in osmotic sensing and diverse roles in stress adaption.

Keywords $OICI_{cyt}$ · $SICI_{cyt}$ · Hyperosmolarity stress · Salt stress · Stomatal movement · Root growth

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Introduction

Throughout the whole growth period from seed germination to flowering, plants are confronted with continual abiotic stresses, including drought and salt stress, which can impede growth and development and decrease crop production (Shavrukov 2013; van Zelm et al. 2020; Zhu 2016). To adapt to the changing environment, plants must perceive and transduce extracellular stress signals into intracellular second messengers, such as phytohormones, phosphatidylinositol lipids and calcium ions, and then initiate multiple signalling cascades that activate or inhibit the expression of a specific set of genes to regulate metabolism and physiology in response to drought and salt stress (Feijó and Wudick 2018; Zhang, Y. et al. 2018). In Arabidopsis, *osca1* (*reduced hyperosmotic-induced* $[Ca^{2+}]_i$ *increase 1*) encoded a hyperosmotic gated calcium channel that functions as an osmotic sensor (Yuan et al. 2014). Furthermore, three-dimensional structural analysis of the OSCA family members OSCA1.1, OSCA1.2 and OSCA3.1 showed that these channels have high structural similarity, and belong to a class of mechanosensitive ion channels (Jojoa-Cruz et al. 2018; Liu et al. 2018; Murthy et al. 2018; Zhang, M. et al. 2018). Recently, Thor et al. (Thor et al. 2020) reported that the plasma membrane (PM)-associated cytosolic kinase BIK1 phosphorylates the N-terminal cytosolic loop of OSCA1.3 to increase Ca^{2+} transport activity upon treatment with flg22, which regulates stomatal closure for Arabidopsis immunity. These results suggest that members of the OSCA family have diverse biological functions in plants.

There are multiple members of *OSCA* family in plants, including 15 Arabidopsis *OSCAs* (Yuan et al. 2014), 11 rice *OSCAs* (*OsOSCAs*) (Li et al. 2015), 12 maize *OSCAs* (*ZmOSCAs*) (Ding et al. 2019), and 16 pear *OSCAs* (*PbrOSCAs*) (Gu et al. 2018). Moreover, 10 *OsOSCA* genes were differentially induced by various osmotic-related abiotic stresses, including 20% polyethylene glycol (PEG)-6000, 150 mM NaCl, and 100 μM abscisic acid (ABA) (Li et al. 2015), indicating that OsOSCAs have redundant functions in the perception of abiotic stress signals. Our previous study showed that OsOSCA1.4 is characterized as a calcium-permeable channel that mediates hyperosmolality-induced $[Ca^{2+}]_{cyt}$ increases ($OICI_{cyt}$) and salt stress-induced $[Ca^{2+}]_{cyt}$ increases ($SICI_{cyt}$) in HEK293 cells (Zhai et al. 2020). Overexpression of *OsOSCA1.4* in *Arabidopsis thaliana osca1* mutant rescued $OICI_{cyt}$ and $SICI_{cyt}$, root growth, and stomatal movement phenotypes in response to hyperosmolality and salt stress (Zhai et al. 2020). However, the roles of the other 10 OsOSCAs expressed under conditions of hyperosmolality and salt stress remain unknown. In this study, we generated transgenic Arabidopsis *osca1* lines individually overexpressing eight *OsOSCAs*, representing all ten OsOSCAs aside from *OsOSCA2.3* and *OsOSCA4.1*. Then, we explored their functions in osmotic Ca^{2+} signalling, stomatal movement, leaf water loss, and root growth in response to hyperosmolality and salt stress. We found that overexpression of *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1* or *OsOSCA2.2* in *osca1* led to the recovery of $OICI_{cyt}$ and $SICI_{cyt}$ levels, whereas overexpression of *OsOSCA2.4*, *OsOSCA2.5* or *OsOSCA3.1* did not. Moreover, the transgenic lines overexpressing different *OsOSCAs* showed differing abiotic stress-associated growth phenotypes related to stomatal closure, leaf water loss and root growth. These results indicate that members of the *OsOSCA* family play diverse roles in stress adaption in rice.

Materials and methods

DNA cloning and vector construction

The coding sequences (CDS) of eight *OsOSCA* genes, namely *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1*, *OsOSCA2.2*, *OsOSCA2.4*, *OsOSCA2.5,* and *OsOSCA3.1*, were separately amplified via polymerase chain reaction (PCR) and inserted into the *pE2c* (including HA-tag) plasmid (Addgene, http://www.addgene.org/). Then, the target genes on *pE2c*, *pE3c* or *pE6c* were inserted into *pMDC32* using the Gateway LR II kit (Invitrogen, USA) to generate plant expression vectors for Agrobacterium-mediated transfection. All clones were confirmed through sequencing. All primers used are listed in Supplementary Table S1.

Plant materials and abiotic stress treatments

All *Arabidopsis thaliana* lines used in this study were from the Col-0 ecotype background. *Arabidopsis thaliana* wild-type (WT) and *osca1* mutant plants

were grown in soil under a 16/8-h light (120 μmol m^{-2} s^{-1}) / dark cycle at 22 °C in environmentally controlled growth rooms. Eight *OsOSCAs* were driven by the CaMV 35S promoter and introduced into *osca1* harbouring *35S:Aequorin* through the floral dip transformation method (Clough and Bent 1998). Transformation 0-generation (T0) seeds were collected, germinated, and screened on half-strength Murashige and Skoog (MS) medium containing 50 mM Hygromycin, then T1 seeds were continually screened for 3:1 segregation to characterize the singer insertion on half-strength MS medium containing 50 mM Hygromycin. Finally, the T2 seeds were screened for no 3:1 segregation, which indicated that we obtain the homozygous and single insertion transgenic plants. T3 single-insertion homozygous transgenic Arabidopsis lines were used for $[Ca^{2+}]_{cyt}$ measurement and phenotypic observation in response to hyperosmolality and salt stress, as described previously (Zhai et al. 2020).

Total RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (semi-quantitative rt-pcr)

Total RNA was extracted from 9-day-old seedlings of the WT, *osca1* and eight different *OsOSCA*-overexpressing *osca1* transgenic plants using the EastepR Super Total RNA Extraction Kit (Promega, Madison WI, USA) according to the manufacturer protocols. Approximately 2 μg of total RNA was reverse-transcribed into first-strand cDNA using First-Strand cDNA Synthesis SuperMix (TransScript, Beijing, China). Then, semi-quantitative PCR was performed to detect the expression of *OsOSCAs* in the transgenic plants. Arabidopsis *Actin* was used as an internal control. All primers used in this study were listed in Supplementary Table S1.

Aequorin bioluminescence-based CA2+ imaging

Aequorin luminescence was measured according to the method described in a previous study (Zhai et al. 2020). Nine-day-old Arabidopsis seedlings were treated with 3.3 mL of 10 μM coelenterazine (Prolume, Pinetop, AZ, USA) in a 100 mm^2 Petri dish and incubated in the dark for 12 h. Next, the seedlings were sprayed with 600 mM sorbitol or 300 mM sodium chloride for photon emission measurement. After several hours, the seedlings were treated with a solution containing 0.9 M $CaCl_2$ and 10% (v/v) ethanol, to measure the total amount of aequorin. The bioluminescence images were captured with a Lumazone FA1300 Imaging System (Roper Scientific, USA) and analysed using ImageJ software (National Institutes of Health, Bethesda MD, USA).

Stomatal closure and leaf desiccation assays

Arabidopsis stomatal closure and leaf water loss assays were performed as described previously (Zhai et al. 2020). Rosette leaves were detached from 3 to 4 week-old plants and floated in a solution containing 10 mM KCl and 10 mM MES-Tris at pH 6.15 for 2 h in a growth chamber under constant light (120 μmol m^{-2} s^{-1}) and relative humidity 50% at 22 °C. Sorbitol (200 mM) or NaCl (100 mM) was added to the solutions, followed by incubation for 2 h. Stomatal apertures were imaged using a DP72 digital camera (Olympus Corp., Tokyo, Japan) attached to a BX51 fluorescence microscope (Olympus Corp.) and the width and length of stomatal apertures were measured using DP2-BAW software (Olympus Corp.).

For the leaf water loss assay, fully expanded rosette leaves were detached from three-week-old plants and maintained under 40–50% relative humidity at a constant temperature of 26 °C. Each sample comprised 10 individual leaves and was weighed after 3 h. Water loss was calculated with respect to the initial fresh mass.

Root growth in response to hyperosmolality and salt stress

After germination, Arabidopsis seedlings were transferred into half-strength MS medium containing 250 mM sorbitol or 125 mM NaCl for 10 days. Then, the seedlings were photographed and their root lengths were measured.

Statistical analysis

Statistical analyses were conducted using Data Processing System software (Tang and Zhang 2013). Bartlett's test was used to confirm the equal variances (P value > 0.05) among the data from different Arabidopsis lines, then one-way analysis of variance

(ANOVA) and the Tukey multiple range test were conducted to assess the significance of differences. NS indicates no significant difference; * and different letters indicate a significant difference at $P < 0.05$, ** indicates an extremely significant difference at $P < 0.01$.

Results

Generation of eight *OsOSCA*-overexpressing in *osca1* transgenic plants

In previous studies, we showed that the rice genome contains 11 *OsOSCA* genes with tissue-specific and stress-induced expression profiles (Li et al. 2015), and that overexpression of one member of the *OsOSCA* family, *OsOSCA1.4*, rescued osmotic-related growth phenotypes under hyperosmolality and salt stress in the Arabidopsis *osca1* mutant (Zhai et al. 2020). To characterise the roles of the other OsOSCAs in osmotic sensing, we overexpressed eight further *OsOSCA* genes, namely *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1*, *OsOSCA2.2*, *OsOSCA2.4*, *OsOSCA2.5,* and *OsOSCA3.1*, in Arabidopsis *osca1* to obtain transgenic lines. Then, we performed semi-quantitative RT-PCR to detect the expression of eight *OsOSCAs* in the corresponding T3 generation transgenic plants and each plant for two independent lines, and found that every *OsOSCA* gene was expressed in both two individual lines (Fig. 1). In addition, because of T1 hygromycin-resistance seedling of *osca1* harbouring *OsOSCA2.3* or *OsOSCA4.1* not acquired, we have used other eight kinds of transgenic plants for the following study.

OsOSCA1.1, OsOSCA1.2, OsOSCA1.3, OsOSCA2.1 or OsOSCA2.2, but not OsOSCA2.4, OsOSCA2.5, and OsOSCA3.1, -overexpressing in osca1 rescues both OICIcyt and SICIcyt

We previously showed that OsOSCA1.4 complements hyperosmolality-induced $[Ca^{2+}]_{cyt}$ increases ($OICI_{cyt}$) and salt stress-induced $[Ca^{2+}]_{cyt}$ increases ($SICI_{cyt}$) after overexpressed in *osca1* (Zhai et al. 2020). Therefore, we used 600 mM sorbitol and 300 mM NaCl to monitor $OICI_{cyt}$ and $SICI_{cyt}$, respectively, in these eight *OsOSCA*-overexpressing in *osca1* transgenic plants, each plant for two independent lines. We found that overexpression of *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1* or *OsOSCA2.2* recovered $OICI_{cyt}$ and $SICI_{cyt}$ phenotypes in *osca1* plants, whereas overexpression of *OsOSCA2.4*, *OsOSCA2.5* and *OsOSCA3.1* did not (Fig. 2). In addition, the seedlings were treated with a discharge solution of 0.9 M $CaCl_2$ and 10% (v/v) ethanol, and no difference in aequorin luminance intensity was observed among the WT, *osca1,* and all OsOSCA-overexpressing *osca1* lines (Supplementary Figure S1). These results suggested that overexpression of *OsOSCAs* has no effect on the total amount of aequorin in the transgenic lines, and that some OsOSCAs play important roles in osmotic sensing, while others do not.

Excluding OsOSCA1.3, OsOSCA2.4 and OsOSCA2.5, the other five OsOSCA-overexpressing in osca1 complemented hyperosmolality and salt stress-induced stomatal closure and leaf water loss phenotypes

We further analysed stomatal movement and leaf water loss in response to hyperosmolality and salt stress in WT, *osca1*, and eight *OsOSCA*-overexpressing in *osca1* plants. Similar as *OsOSCA1.4*, *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA2.1*, *OsOSCA2.2* and *OsOSCA3.1* rescued hyperosmolality and salt stress-induced stomatal closure when separately overexpressed in *osca1*, whereas, however, *OsOSCA1.3*, *OsOSCA2.4* and *OsOSCA2.5* did not (Fig. 3a, b). The leaf desiccation assay provided a similar result. The leaves of *OsOSCA1.1-*, *OsOSCA1.2-*, *OsOSCA2.1-*, *OsOSCA2.2-*, and *OsOSCA3.1-overexpressing* in *osca1* plants showed slower water losses than *osca1*, similar to the WT (Fig. 3c). On the other hand, leaves of *OsOSCA1.3-*, *OsOSCA2.4-*, and *OsOSCA2.5-overexpressing osca1* lines showed the same water losses as *osca1* plant (Fig. 3c). These results indicated that overexpression of *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA2.1*, *OsOSCA2.2*, or *OsOSCA3.1* in *osca1* rescued drought stress-induced stomatal movement and associated leaf water loss.

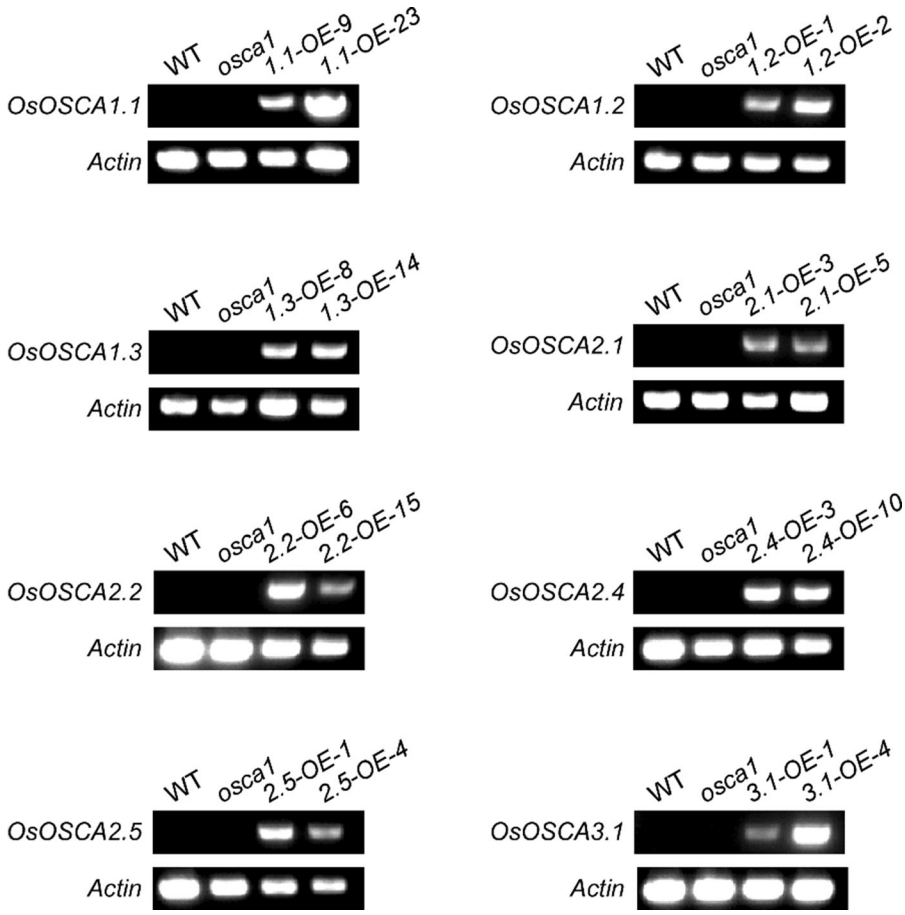


Fig. 1 Detection of eight *OsOSCA* genes overexpressed in the *osca1* mutant. Transgenic *osca1* plants overexpressing eight *OsOSCAs*, i.e. *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1*, *OsOSCA2.2*, *OsOSCA2.4*, *OsOSCA2.5* and *OsOSCA3.1*, were generated. The expression of each *OsOSCA* in the transgenic lines was detected through semi-quantitative PCR. *Actin* was used as a loading control

Of eight OsOSCAs-overexpressing in osca1, only OsOSCA1.1, OsOSCA2.2, or OsOSCA2.5 rescued the root growth responses to hyperosmolality and salt stress, and OsOSCA2.1 complemented the root growth responses to hyperosmolality stress

We continuously observed the root growth of *osca1* seedling lines overexpressed eight *OsOSCA*s in response to treatment with 150 mM sorbitol or 125 mM NaCl. Similar to *OsOSCA1.4*, overexpression of *OsOSCA1.1* was associated with recovery of root growth in response to hyperosmolality and salt stress to the level of the WT (Fig. 4). One interesting thing was that overexpression of *OsOSCA2.2* and *OsOSCA2.5* in *osca1* endued more resistant to hyperosmolarity stress in root growth than that in WT (Fig. 4). However, overexpression of *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.4*, or *OsOSCA3.1* cannot rescue root growth in response to hyperosmolarity and salt stress in *osca1* (Fig. 4). Notably, overexpression of *OsOSCA2.1* in *osca1* promoted root growth in response to hyperosmolality stress, but not salt stress (Fig. 4). These results suggested that different members of the *OsOSCA* family have diverse functions in the responses to different abiotic stresses.

In summary, eight OsOSCA genes were overexpressed in *osca1* plants to generate individual transgenic lines. We found that only overexpression of *OsOSCA1.1* or *OsOSCA2.2* complemented $OICI_{cyt}$ and $SICI_{cyt}$, as well as stomatal closure and root

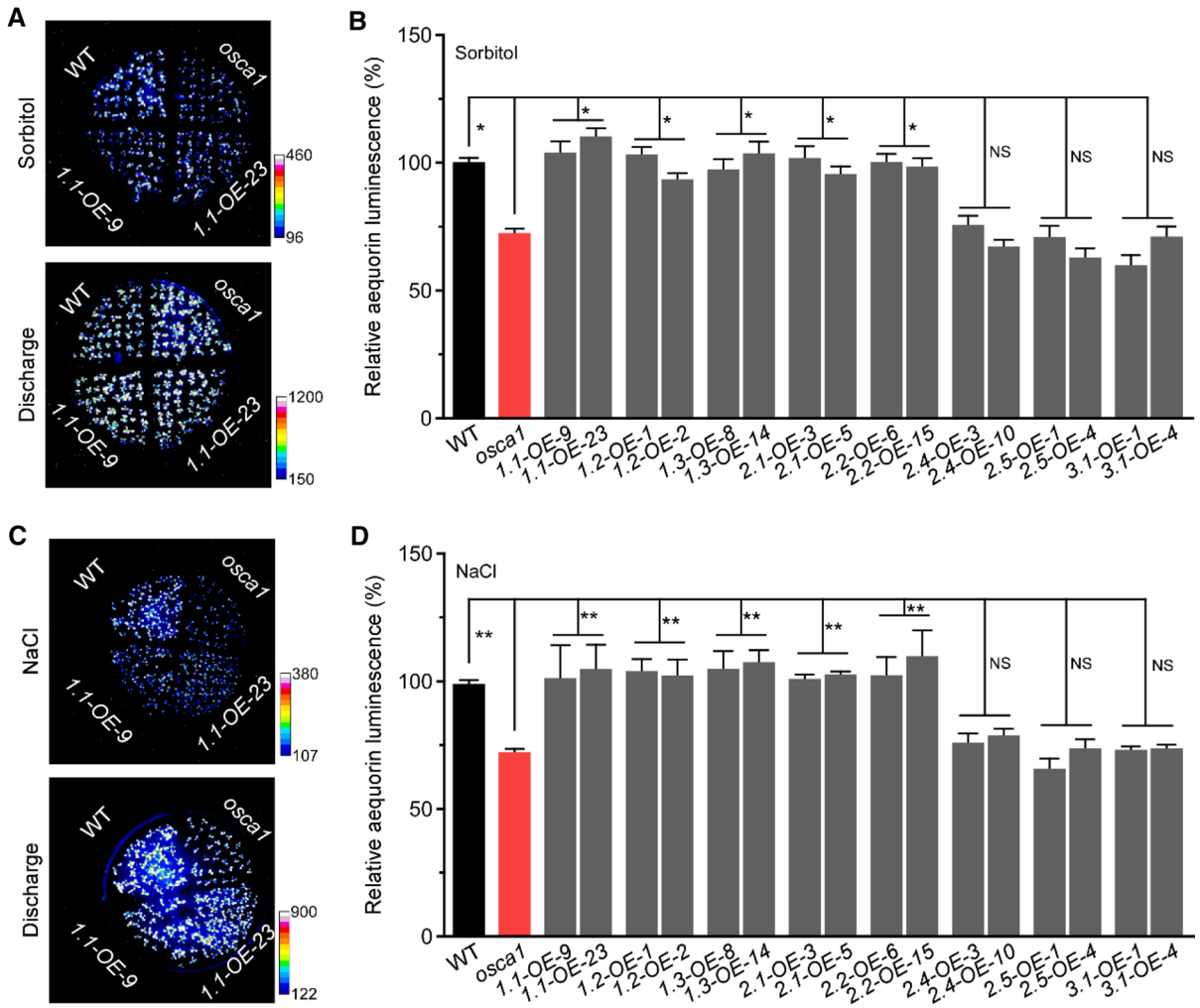
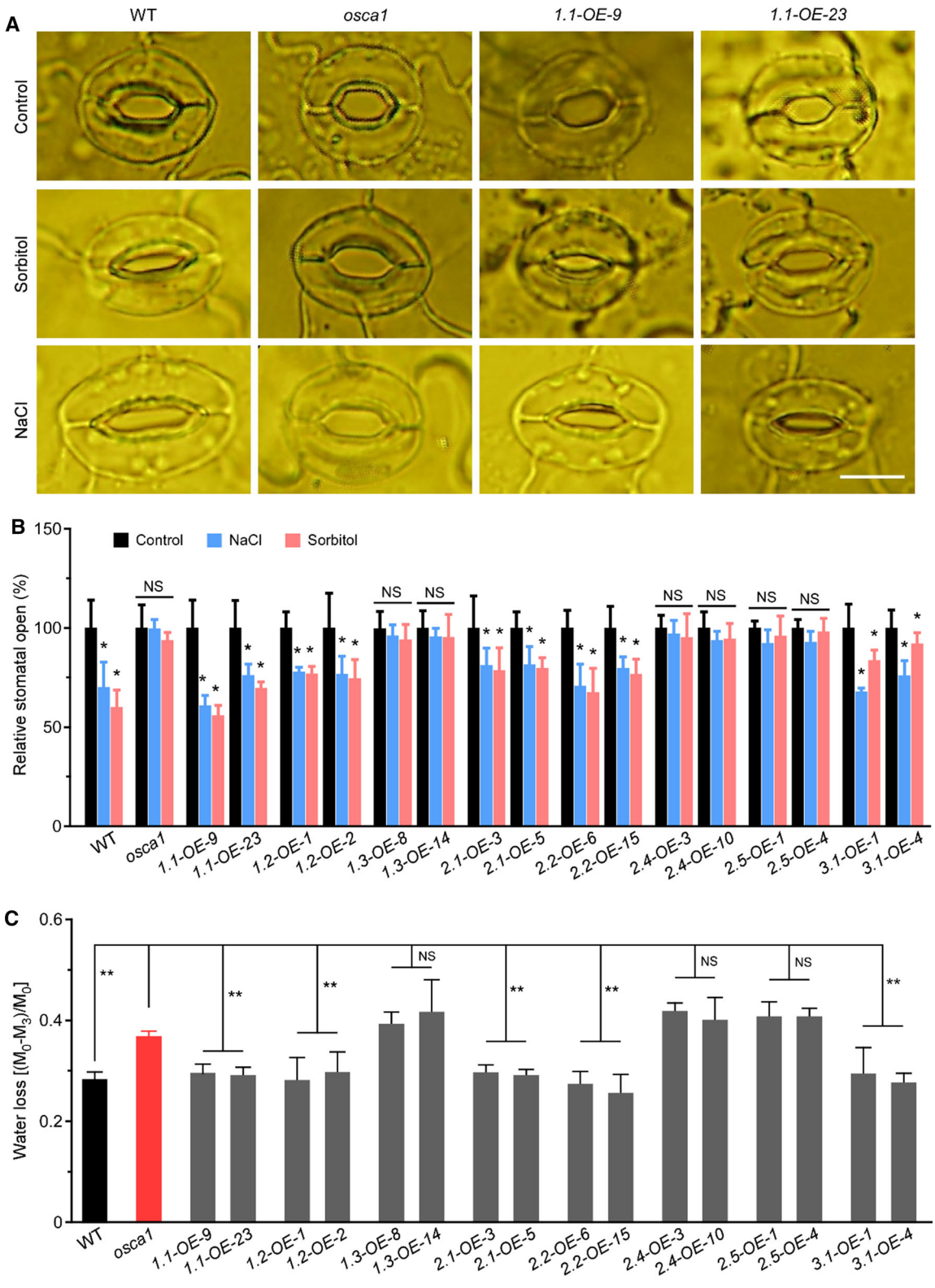


Fig. 2 Monitoring of cytosolic [Ca^{2+}] increases triggered by hyperosmolality and salt stress in eight *OsOSCA*-overexpressing *osca1* plants. **a** Hyperosmolality-induced cytosolic [Ca^{2+}] increases ($OICI_{cyt}$) in Arabidopsis seedlings of the wild type (WT), *osca1*, and *OsOSCA1.1*-overexpressing *osca1* lines *1.1-OE-9* and *1.1-OE-23*. Plants expressing aequorin were separately treated with 600 mM sorbitol, then discharged with a solution containing 0.9 M $CaCl_2$ and 10% (v/v) ethanol for aequorin measurement. $[Ca^{2+}]_i$ was analysed by imaging aequorin and shown as a pseudo-colour bar. **b** Quantification of $OICI_{cyt}$ in the seedlings of WT, *osca1*, and eight different *OsOSCA*-overexpressing *osca1* lines in the same treatment condition as (**a**). Data for three representative experiments are shown (means ± SD; $n = 120$). NS, no significant difference, and *, significant difference ($P < 0.05$) (Tukey's test). **c** Salt stress-induced cytosolic [Ca^{2+}] increases ($SICI_{cyt}$) was monitored in the seedlings of WT, *osca1*, and *OsOSCA1.1*-overexpressing *osca1* lines *1.1-OE-9* and *1.1-OE-23*. Plants were treated with 300 mM NaCl, and then with a solution containing 0.9 M $CaCl_2$ and 10% (v/v) ethanol for aequorin measurement. **d** Quantification of $SICI_{cyt}$ in WT, *osca1*, and eight different *OsOSCA*-overexpressing *osca1* lines in the same treatment condition as (**c**). Data for three representative experiments are shown (means ± SD; $n = 120$). NS, no significant difference, and **, extremely significant difference ($P < 0.01$) (Tukey's test)

growth phenotypes in response to hyperosmolality and salt stress, and drought-related leaf water loss; this is in accordance with the results of *OsOSCA1.4*-overexpressing in *osca1*, and *OsOSCA2.4* cannot. However, overexpression of the other five *OsOSCAs* in *osca1* had variable effects on $OICI_{cyt}$ and $SICI_{cyt}$, and growth phenotypes under abiotic stress conditions.


A
WT
osca1
1.1-OE-9
1.1-OE-23
Control
Sorbitol
NaCl
B
Control
NaCl
Sorbitol
Relative stomatal open (%)
150
100
50
0
NS
WT
osca1
1.1-OE-9
1.1-OE-23
1.2-OE-1
1.2-OE-2
1.3-OE-8
1.3-OE-14
2.1-OE-3
2.1-OE-5
2.2-OE-6
2.2-OE-15
2.4-OE-3
2.4-OE-10
2.5-OE-1
2.5-OE-4
3.1-OE-1
3.1-OE-4
C
Water loss [(M0-M3)/M0]
0.6
0.4
0.2
0
**
NS

◀**Fig. 3** Different members of the *OsOSCA* gene family show differing stomatal phenotypes in *OsOSCA*-overexpressing *osca1* plants. **a** Stomatal status of the 4-week-old rosette leaves from WT, *osca1*, and *OsOSCA1.1*-overexpressing *osca1* lines *1.1-OE-9* and *1.1-OE-23* in response to 200 mM sorbitol or 100 mM NaCl treatment. Scale bars indicate 100 μm. **b** Relative stomatal opening (width/length, with untreated WT set to 100%) of WT, *osca1*, and eight different *OsOSCA*-overexpressing *osca1* lines in the same treatment conditions as (**a**) (mean ± SD; $n = 120$). NS indicates no significant difference, and * indicates a significant difference ($P < 0.05$) according to Tukey's test. **c** Transpiration water loss from 4-week-old detached rosette leaves of WT, *osca1*, and eight different *OsOSCA*-overexpressing *osca1* plants in response to desiccation treatment for 3 h. The water loss was calculated as $(M_0\text{-}M_3)/M_0$, M_0 indicates the initial mass of leaves and M_3 as the leaves mass with desiccation treatment for 3 h. Data are presented as mean ± SD ($n > 45$). NS indicates no significant difference and ** indicates an extremely significant difference ($P < 0.01$) according to Tukey's test

Discussion

In this study, we systematically investigated the role of the *OsOSCA* gene family in osmotic sensing and associated growth phenotypes with overexpressing eight members of the family in the Arabidopsis *osca1* mutant. We found that overexpression of *OsOSCA1.1*, *OsOSCA1.2*, *OsOSCA1.3*, *OsOSCA2.1* or *OsOSCA2.2* rescued $OICI_{cyt}$ and $SICI_{cyt}$ in *osca1*, whereas overexpression of *OsOSCA2.4*, *OsOSCA2.5* or *OsOSCA3.1* did not. In addition, OsOSCA1.1 and OsOSCA2.2 promoted stomatal movement and root growth in response to hyperosmolality and salt stress, as well as drought-induced leaf water loss, similar to OsOSCA1.4 (Zhai et al. 2020). However, overexpression of the other six *OsOSCAs* in *osca1* led to various phenotype responses to hyperosmolality and salt stress. These results suggest that *OsOSCA* family members play the redundant roles in the responses to abiotic stresses.

The OSCA family is characterized by the presence of the conserved DUF221 domain, which is composed of four to six transmembrane (TM) helix regions (Li et al. 2015; Yuan et al. 2014). Using TMHMM Server v2.0 to predict the TM helices in OsOSCAs, we previously showed that OsOSCA2.2 contains the entire DUF221 domain, but lacks three N-terminal TMs (Li et al. 2015). However, in this study, we found that overexpression of *OsOSCA2.2* in *osca1* can rescue $OICI_{cyt}$ and $SICI_{cyt}$, as well as associated growth phenotypes, similar to *OsOSCA1.1* and *OsOSCA1.4* previously published (Zhai et al. 2020). These results indicate that the three N-terminal TMs of the OSCA family are not essential for osmotic sensing or $[Ca^{2+}]_{cyt}$ increases. In addition, we showed that nine OsOSCAs were mainly localized in the endoplasmic reticulum membrane after being transiently transformed into Arabidopsis mesophyll protoplasts, although OsOSCA1.4 was exclusively localised to the PM (Zhai et al. 2020). We found that OsOSCA1.1, OsOSCA1.2, OsOSCA1.3, OsOSCA2.1 and OsOSCA2.2 all complemented $OICI_{cyt}$ and $SICI_{cyt}$ when stably overexpressed in *osca1*, similar to OsOSCA1.4. These results suggest that either a few OsOSCAs localized to the PM, or ER-localized OsOSCAs, function in osmotic sensing. Moreover, we found that overexpression of *OsOSCA2.4*, *OsOSCA2.5* or *OsOSCA3.1* cannot rescue $OICI_{cyt}$ and $SICI_{cyt}$ in *osca1*. However, our prior study showed that the expression of these three genes are upregulated by all four different stress treatment: PEG 6000 (20%), NaCl (150 mM), drought, and ABA (100 μM) (Li et al. 2015). In addition, we showed that overexpression of *OsOSCA3.1* promotes hyperosmolality and salt stress-induced stomatal closure phenotypes, while overexpression of *OsOSCA2.5* promotes hyperosmolality and salt stress-induced root growth phenotypes. These results indicated that OsOSCA2.4, OsOSCA2.5 and OsOSCA3.1 function in different development stage, such as pollen maturation or seed dehydration period, because we previously revealed that overexpression of *OsCPK21* which is specifically expressed in late developmental stage of pollen and caryopsis facilitates the response to ABA and salt stress in rice (Chen et al. 2017; Wen et al. 2019).

Hyperosmotic stress is the first and common phase for drought and salt stress (Shavrukov 2013), which seriously threatens agriculture as it restricts the growth and yield of crops (Kumar 2013). Plant cells are capable of perceiving osmotic signals in response to drought and salt stress to trigger $[Ca^{2+}]_{cyt}$ increases. The calcium signal is then decoded by different calcium-binding proteins, such as calmodulins, calcium-dependent protein kinases, and calcineurin B-like proteins (CBLs) and their targets, CBL-interacting protein kinases (CIPKs), which activate transcription factors (TFs) that induce the expression of a particular set of genes for regulating plant growth and development and adaption to adverse environments.

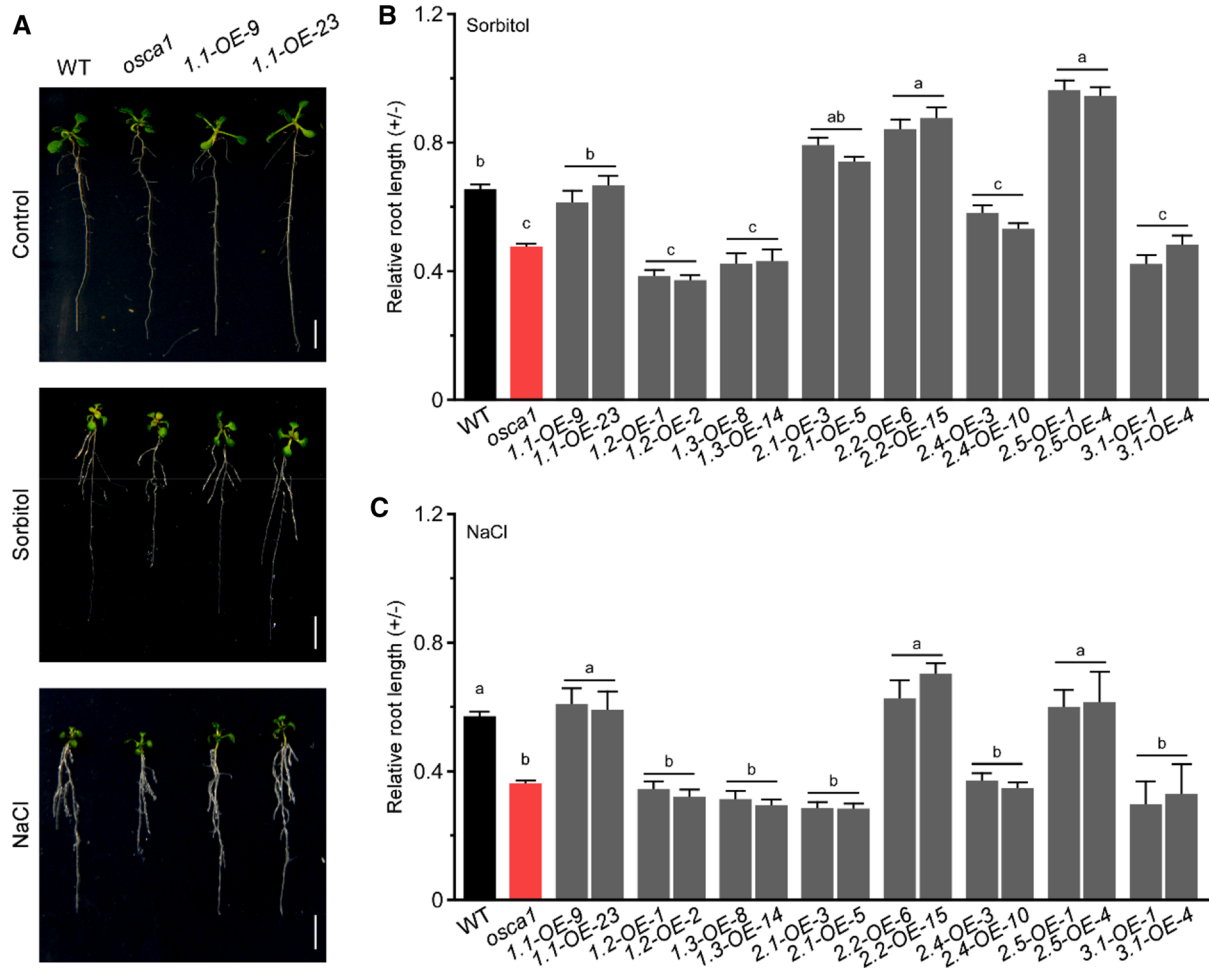


Fig. 4 *OsOSCA*-overexpressing *osca1* plants showed specific root growth phenotypes in response to hyperosmolality or salt stress. **a** Effect of hyperosmolarity or salt stress on the seedling growth of WT, *osca1*, and *OsOSCA1.1*-overexpressing *osca1* lines (*1.1-OE-9* and *1.1-OE-23*). The 3-day-old seedlings were transplanted into half-strength Murashige and Skoog medium plates in the presence or absence of 250 mM sorbitol or 125 mM NaCl, and continually grown for 10 days. Scale bars indicate 1 cm. **b** and **c**, Statistic analysis of root length of WT, *osca1*, and eight different *OsOSCA*-overexpressing *osca1* lines under the same growth condition as (**a**). Relative root length (±) indicates the ratio between treated and untreated plants. Data were collected during three independent experiments (mean ± SD; $n > 45$). The different letters are used to denote significance among the means ($P < 0.05$, Tukey's test). Bars sharing the same letter indicates no significantly different

Yuan et al. (2014) firstly showed that Arabidopsis *OSCA1.1* encodes a calcium-permeable ion channel that activates an inward current after receiving an osmotic signal exerted by hyperosmolality stress. Our previous study showed that overexpression of rice *OsOSCA1.4* in Arabidopsis *osca1* mutant complemented osmotic Ca^{2+} signalling, root growth, and stomatal movement in response to hyperosmolality and salt stress (Zhai et al. 2020). Here, we continually showed that, similar to OsOSCA1.4, OsOSCA1.1 and OsOSCA2.2 complemented $OICI_{cyt}$ and $SICI_{cyt}$, stomatal movement and root growth in response to hyperosmolality and salt stress, as well as drought-induced leaf water loss in *osca1*. These results suggested that OsOSCA1.1, OsOSCA1.4 and OsOSCA2.2 function redundantly on sensing hyperosmolarity and salt stress in rice. In addition, OsOSCA1.2, OsOSCA1.3 and OsOSCA2.1 separately enabled $OICI_{cyt}$ and $SICI_{cyt}$, but presented diverse phenotype responses to hyperosmolality and salt stress after overexpressed them in *osca1*, indicating that these three genes play the roles on sensing abiotic

stress in a different manner. A recent study showed that Arabidopsis OSCA1.3 is activated through flg22-induced BIK1 phosphorylation, which is critical for plant stomatal immunity (Thor et al. 2020). Therefore, we proposed that some members of OsOSCA family are involved in biotic stress induced $[Ca^{2+}]_{cyt}$ increases. In order to test our hypothesis and better understand the sensing stress signalling by OsOSCA in rice, we will generate OsOSCA-overexpressed lines or OsOSCA-knock-down lines with CRSPR-Cas9 techniques to study the functional genomics of the salt stress response. Rice is known to be a salt-sensitive crop (Kumar et al. 2013) and our future study possibly provide the potential molecular genetic target for engineering salt-resistant rice.

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Author contributions Y.Z., H. Z., Y-d. W. and S.H. conceived the study and analysed the data. Y.Z., Z.W., W. F., Y-x.W., C. X. and J. L. performed the experiments. Y.Z. drafted the manuscript. Y.Z. and S. H. revised the manuscript.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

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