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Aequorin-based luminescence imaging reveals differential calcium signalling responses to salt and reactive oxygen species in rice roots

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Abstract

It is well established that both salt and reactive oxygen species (ROS) stresses are able to increase the concentration of cytosolic free Ca²⁺ ([Ca²⁺]i), which is caused by the flux of calcium (Ca²⁺). However, the differences between these two processes are largely unknown. Here, we introduced recombinant aequorin into rice (Oryza sativa) and examined the change in [Ca²⁺]i in response to salt and ROS stresses. The transgenic rice harbouring aequorin showed strong luminescence in roots when treated with exogenous Ca²⁺. Considering the histological differences in roots between rice and Arabidopsis, we reappraised the discharging solution, and suggested that the percentage of ethanol should be 25%. Different concentrations of NaCl induced immediate [Ca²⁺]i spikes with the same durations and phases. In contrast, H₂O₂ induced delayed [Ca²⁺]i spikes with different peaks according to the concentrations of H₂O₂. According to the Ca²⁺ inhibitor research, we also showed that the sources of Ca²⁺ induced by NaCl and H₂O₂ are different. Furthermore, we evaluated the contribution of [Ca²⁺]i responses in the NaCl- and H₂O₂-induced gene expressions respectively, and present a Ca²⁺- and H₂O₂-mediated molecular signalling model for the initial response to NaCl in rice.

Key words: Aequorin, calcium, Oryza sativa, reactive oxygen species, rice, root, salt.

Introduction

Rice (Oryza sativa L.) is the staple food for more than half of the world’s population. Salinity is one of the most common abiotic stresses encountered by rice, which is classified as a salt-sensitive crop in early stages of development, and limits its productivity (Lutts et al., 1995; Todaka et al., 2012). To improve the rice yield under saline conditions, it is important to understand the molecular mechanisms involved in how plants respond to salt stress (Kumar et al., 2013).

Many studies have been carried out to dissect the genetic and molecular mechanisms of how plants respond to salt stress. A well-defined pathway is the Salt Overly Sensitive (SOS) signalling pathway, which comprises SOS3, SOS2 and SOS1 (Zhu, 2000), and is required to mediate the highly complex regulatory networks involved in plant response to salinity (Ji et al., 2013). Importantly, the SOS signal transduction cascade is activated by a calcium (Ca²⁺) spike, which is caused by the flux of Ca²⁺. This salt stress triggered increase of cytosolic free Ca²⁺ ([Ca²⁺]i) is considered to be the first recorded response to salt stress (Knight et al., 1997; Tracy et al., 2008). Ca²⁺ is an essential second messenger in the sophisticated network of plant signalling pathways responding to a large array of external stimuli, including salt stress (Hetherington et al., 2009).
and Brownlee, 2004; Pandey et al., 2004; Dodd et al., 2010). The Ca\(^{2+}\) channels and transporters activated by these stimuli form specific Ca\(^{2+}\) signatures, and these changes in Ca\(^{2+}\) signatures are transmitted by protein sensors that preferably bind Ca\(^{2+}\). The binding of Ca\(^{2+}\) results in conformational changes which modulate their activity or their ability to interact with other proteins, and activate the expression of downstream salt response genes through a Ca\(^{2+}\) signalling cascade (Rentel and Knight, 2004; Dodd et al., 2010; Kudla et al., 2010; Batistic and Kudla, 2012).

Furthermore, salt stress also increases the level of reactive oxygen species (ROS) predominantly represented by H\(_2\)O\(_2\) (Bienert et al., 2006; Hong et al., 2009; Miller et al., 2010). Although toxic by nature, ROS are now considered as important signalling molecules in many biological processes, including biotic and abiotic stress tolerance (Mittler et al., 2011; Schippers et al., 2012). In Arabidopsis, Respiratory Burst Oxidase Homolog F (RBOHF, an NADPH oxidase catalysing ROS production) is required for shoot sodium homeostasis during salt stress (Jiang et al., 2012). Furthermore, the RBOHF-dependent salinity-induced ROS accumulation is regulated by protein phosphorylation in a Ca\(^{2+}\)-dependent manner (Drerup et al., 2013). Interestingly, ROS have also been shown to trigger an increase of [Ca\(^{2+}\)]\(_i\) (Mori and Schroeder, 2004). Previous limited evidence implied that NaCl-gated Ca\(^{2+}\) channels and H\(_2\)O\(_2\)-gated Ca\(^{2+}\) channels may differ (Jiang et al., 2013). However, the different mechanisms between NaCl- and H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)]\(_i\), changes are yet to be explored. Also, SALT-RESPONSIVE ERF1 (SERF1) is reported to function as a central hub to regulate ROS-dependent signalling during the initial response to salt stress in rice (Schmidt et al., 2013). Therefore, it is attractive to investigate the relationship among Ca\(^{2+}\), H\(_2\)O\(_2\) and SERF1 in the early salt-stress signalling cascade.

Genetically encoded fluorescent Ca\(^{2+}\) probes are useful tools to non-invasively describe Ca\(^{2+}\) signatures in plants (Monshausen, 2012). The fluorescence resonance energy transfer (FRET)-based probes yellow cameleon YC2.1 (Miyawaki et al., 1999) and the improved YC3.6 (Nagai et al., 2004) are currently used to monitor the [Ca\(^{2+}\)]\(_i\) in the cytoplasm. With high quantum yield, FRET-based probes are suitable to measure the Ca\(^{2+}\) signatures in the cellular or subcellular resolutions (Monshausen, 2012). Aequorin, a photoprotein derived from the luminescent jellyfish Aequoria victoria, reacts specifically with Ca\(^{2+}\) and emits blue light at ~460 nm (Shimomura et al., 1963). Although the aequorin-based probe gives a low quantum yield, it is more suitable for cell population or whole plant measurement of [Ca\(^{2+}\)]\(_i\), (Monshausen, 2012). Since the transformation of recombinant aequorin in plant systems (Knight et al., 1991), it has proved to be a useful tool for non-invasive investigation of Ca\(^{2+}\)-mediated signalling in response to various stresses in whole seedlings (Zhu et al., 2013). Specific stimuli can trigger unique Ca\(^{2+}\) signatures, which are decoded subsequently by intracellular Ca\(^{2+}\) sensors, leading to the activation of downstream events (Luan, 2009). Kurusu et al. (2011) established a transgenic rice cell line expressing aequorin, and characterized the regulation mechanism of microbe-associated molecular pattern-induced [Ca\(^{2+}\)]\(_i\) transients. In spite of the progress achieved by rice cell lines, stable transgenic rice expressing aequorin is needed to investigate the changes of [Ca\(^{2+}\)]\(_i\) that appear in response to various environmental stimuli.

In this paper, we introduced recombinant aequorin, as a reporter of [Ca\(^{2+}\)]\(_i\), into rice. Transgenic rice harbouring aequorin showed strong luminescence in roots when treated with exogenous Ca\(^{2+}\). We also showed that NaCl and H\(_2\)O\(_2\) treatments induce different [Ca\(^{2+}\)]\(_i\) spikes, and may employ different Ca\(^{2+}\) channels. Furthermore, we present a Ca\(^{2+}\)- and H\(_2\)O\(_2\)-mediated molecular signalling model for the initial response to NaCl in rice.

**Materials and methods**

**Vector construction and transformation of rice**

In order to improve the aequorin expression vector for transgenic research in rice, the coding region of aequorin in pMAQ2 (Knight et al., 1991) was transferred to 35S-pCAMBIA1301 (Zhou et al., 2008) through XbaI and PstI. Plasmids were introduced into Agrobacterium tumefaciens EHA105 by electroporation. Rice transformation was performed by the Agrobacterium-mediated method, as previously described (Chen et al., 2003).

**Plant materials and growth conditions**

Rice (Oryza sativa L. cv. Nipponbare) seeds were sterilized with 75% ethanol and planted in a square plate containing half-strength Murashige and Skoog salts (MS; Gibco), and 1.5% (w/v) agar (Becton Dickinson). Seedlings were grown vertically in the growth chamber conditioned with 16 h of light at 28°C and 8 h of dark at 22°C for five days. The seedlings were then sprayed with coelenterazine for reconstitution of aequorin before subsequent experiments began.

**Root cell death detection**

A root cell death assay was performed as previously described (Qin et al., 2013). Roots of five-day-old seedlings were submerged in different concentrations of NaCl solution for 30 s and then stained with 1% Evans blue solution for 10 min, washed by distilled water for 2 h, and then photographed.

**Southern-blot analysis of transgenic rice**

Genomic DNA of transgenic rice was isolated following the instructions of a Plant Genomic DNA Kit (TIANGEN) and the purified DNA was digested with restriction enzyme EcoRI. 2 μg of digested DNA was separated on 0.8% agarose gel. After electrophoresis, the digested DNA was transferred to Hybond-N+ nylon membrane (Amersham Pharmacia) and hybridized with a \(^{32}\)P-dCTP-labelled hygromycin-resistant gene probe. The blots were washed at 65°C under stringent conditions and analysed using Typhoon-8600. The primers used to amplify the probe are listed in Supplementary Table S1.

**RT-PCR analysis**

For the examination of aequorin expression in different tissues, the shoot, shoot base and root of five-day-old transgenic rice seedlings were selected, and the PCR was conducted with 28 cycles for both aequorin and OsACTIN. For the examination of NaCl and H\(_2\)O\(_2\)-induced gene expression, the roots of five-day-old rice seedlings were selected. Ca\(^{2+}\) channel blocker pre-treatment was
Aequorin reconstitution and luminescence imaging

Seedlings were grown on half-strength MS medium for five days. Reconstitution of aequorin was performed in vivo by spraying seedlings with 10 μM coelenterazine and followed by incubation at 21°C in the dark for 12–16h. For surfactant treatment, 0.01% or 0.1% of silwet L-77 (Sigma) was added to the coelenterazine solution. For Ca

inhibitor treatments, rice roots were treated with different concentrations of GdCl

, LaCl

, neomycin and thapsigargin, respectively for 30 min before 0.25 M NaCl and 1 mM H

O

2 treatment. Treatments and aequorin luminescence imaging were performed at room temperature using a ChemiPro HT system as described previously (Jiang et al., 2013). The recording was started about 5 s prior to treatment and luminescence images were acquired for 3 min. For the analysis of time courses of increase in [Ca

], each exposure time was 30 s and the images were taken continuously for several minutes. To avoid the interference of chloroplast auto-fluorescence signal in the aequorin luminescence imaging, all the treatments were performed in the complete darkness. To record the chloroplast auto-fluorescence signal, seedlings were first exposed to strong light for 1 min. After that, the light was turned off and the chloroplast auto-fluorescence was recorded. WinView/32 and Meta Morph 7.7 were used to analyse recorded luminescence images.

Results

Production and characterization of transgenic rice expressing apoaequorin

In order to monitor [Ca

] responses in rice, we developed transgenic rice over-expressing apoaequorin under the control of cauliflower mosaic virus (CMV) 35S promoter (Fig. 1A). Three independent transgenic lines (AQ-2, AQ-3 and AQ-5) harbouring one copy of apoaequorin were selected by Southern blot analysis, and the homozygous T

 generations of these lines were used for subsequent experiments (Fig. 1B). In addition, the heterologous expression of apoaequorin had no effects on the growth and life cycle of transgenic rice (data not shown). After the reconstitution of aequorin by spraying seedlings with coelenterazine, the aequorin luminescence of these seedlings were recorded using a photo-counting camera by treating plants with exogenous Ca

 (see Materials and methods for detail). Ca

-treated seedlings showed strong and diverse luminescence in roots, and AQ-3 with the strongest luminescence was selected for further analysis (Fig. 1D). To our surprise, the aequorin-based luminescence signal was only observed in roots and we failed to detect any signal in shoots when treated with Ca

 (Fig. 1D) compared to bright-field in Fig. 1C and chloroplast auto-fluorescence in Fig. 1E). To confirm the expression of apoaequorin in the whole plant, we extracted RNA from different tissues of transgenic seedlings, and the expression of apoaequorin was examined using reverse transcription-polymerase chain reaction (RT-PCR). The results showed that apoaequorin is expressed in all the selected tissues (Supplementary Fig. S1A). It is likely that the leaf wax protects the permeation of coelenterazine (Supplementary Fig. S1B). To test this hypothesis, we added surfactant (Silwet L-77, Sigma) while spraying coelenterazine. Both luminescence signals in roots and dotted signals in shoots were observed (Supplementary Fig. S1C–H). These results showed that transgenic rice expressing apoaequorin was able to reflect the [Ca

], level in rice roots.

The optimization of discharging solution for luminescence imaging in rice

The discharging solution is used to estimate the amount of remaining aequorin in the calibration and is important to calculate the Ca

 concentration based on the luminescence intensity (Knight et al., 1996). Considering the histological differences in roots between rice and Arabidopsis (Rebouillat



Fig. 1. Transgenic rice harbouring aequorin showed strong and diverse luminescence in roots. (A) The construction of apoaequorin expression vector for transgenic research in rice. (B) Southern-blot analysis of five independent lines (AQ-1 to AQ-5) of transgenic rice. (C–E) Ca

-treated seedlings showed strong and diverse luminescence exclusively in roots. (C) Bright-field image. (D) Pseudocolour image of aequorin luminescence in roots. (E) Pseudocolour image of chloroplast auto-fluorescence. Red rectangles indicate the places of shoots, and there is no aequorin luminescence signal in shoots shown in (D). The relationship between luminescence intensity and the pseudocolour images are scaled by pseudocolour bars.
et al., 2009), we re-examined the percentage of ethanol in the discharging solution for the rice experiment. We tested a series of discharging solutions with different percentages of ethanol from 0% to 50%. Low percentages of ethanol (below 15%) had no significant difference in the luminescence imaging compared with 0% ethanol. Interestingly, the average luminescence intensity of plants increased by about one fold when treated with discharging solution containing 20% ethanol compared with that treated with a low concentration of ethanol (Supplementary Fig. S2). In spite of the leap from 15% to 20%, even higher percentages of ethanol in the discharging solution had little effect in the luminescence intensity of rice, indicating the saturation of discharged aequorin. Based on these results, we suggested that in contrast with 10% ethanol which is normally used in Arabidopsis (Yuan et al., 2014), the percentage of ethanol in the discharging solution for rice should be 25%.

**NaCl induced an immediate [Ca\(^{2+}\)] spike in rice roots**

In order to investigate the [Ca\(^{2+}\)] changes in response to salt stress in rice roots, we examined the aequorin-based luminescence under various concentrations of NaCl treatments. The intensity of [Ca\(^{2+}\)]-dependent luminescence signals relied on the strength of salt stimuli (Fig. 2A). Detailed analysis showed that 0.1 M (or less) of NaCl failed to induce visible [Ca\(^{2+}\)]-dependent luminescence signals, while 0.15 M NaCl successfully induced a visible concentration of luminescence signals. 0.25 M NaCl had a more obvious effect than 0.2 M, and was very similar to 0.5 M NaCl treatments (Fig. 2A). To further investigate the salt concentration-dependent increase of [Ca\(^{2+}\)] in rice roots, we calculated the average luminescence intensity of rice roots in response to different concentrations of NaCl treatments. As shown in Fig. 2B, a rapid increase of luminescence intensity in response to NaCl treatment occurred within a narrow range of NaCl concentrations (0.1 M to 0.25 M). NaCl treatments below or above this region had minor effect on the [Ca\(^{2+}\)] responses in rice roots (Fig. 2B).

To investigate the time courses of [Ca\(^{2+}\)] responses induced by different concentrations of NaCl in rice roots, the average luminescence intensity of continuous images with exposure time of 30 s were analysed and a comparison of basic parameters (amplitudes, durations and phases) of [Ca\(^{2+}\)] responses were made. As shown in Fig. 2C, a strong luminescence signal was detected in the first image, which collected the luminescence signal within the first 30 s. However, almost no luminescence signal was detected after the first 30 s. This indicated that salt stress immediately induced a sharp spike of [Ca\(^{2+}\)], within 30 s, which quickly declined to the basal level after the spike. The amplitudes of luminescence signals varied according to different concentrations of NaCl, while both the durations and phases were the same. Detailed analysis showed that 0.1 M NaCl had little effect on the induction of [Ca\(^{2+}\)], while 0.15 M NaCl was able to induce an obvious spike of [Ca\(^{2+}\)]. The effect of 0.2 M NaCl was not significantly different in the increase of amplitude of [Ca\(^{2+}\)] increase compared with 0.15 M. Interestingly, increasing the concentration of NaCl to 0.25 M dramatically increased the amplitude of [Ca\(^{2+}\)], by about two fold, but concentrations higher than 0.25 M NaCl had little effect on the increasing of amplitude compared with 0.25 M (Fig. 2C). These results were similar to the curves shown in Fig. 2B. It is worth noting that the spike of [Ca\(^{2+}\)] failed to decline to the basal level after the induction by 2 M NaCl treatment (Fig. 2C). This indicated a destruction of calcium transport systems, which are responsible for maintaining low [Ca\(^{2+}\)], and crucial to the living cells. As expected, Evans blue staining revealed that treatment with a high concentration of NaCl resulted in serious cell death in the rice roots (Supplementary Fig. S3).

**H\(_2\)O\(_2\) induced a delayed [Ca\(^{2+}\)] spike in rice roots**

In order to investigate the [Ca\(^{2+}\)] changes in response to ROS stress in rice roots, we examined the aequorin-based luminescence after the application of H\(_2\)O\(_2\). We collected the luminescence signals 45 times at one minute intervals after treatment with H\(_2\)O\(_2\). We found clear luminescence signals in the first and second minute. Interestingly, after the weak luminescence signals in the third minute, we failed to collect any additional signals over the remaining 42 min (Supplementary Fig. S4). This was different from that in Arabidopsis, which was reported to have a second peak 5–20 min after the application of H\(_2\)O\(_2\) (Rentel and Knight, 2004).

To investigate the H\(_2\)O\(_2\) concentration-dependent increase of [Ca\(^{2+}\)] in rice roots, we examined the aequorin-based luminescence signals and calculated the average luminescence intensity of rice roots in response to different concentrations of H\(_2\)O\(_2\) treatments. Overall, the H\(_2\)O\(_2\) response was quite similar to the NaCl response, when looking only at the concentration-dependent increase in luminescence (Fig. 3A). A concentration of 0.2 mM H\(_2\)O\(_2\) was able to induce clear luminescence signals, and the more H\(_2\)O\(_2\) was applied, the stronger the luminescence signals would be (Fig. 3A). Detailed analysis showed that there was almost a linear relationship between luminescence signals and the H\(_2\)O\(_2\) concentrations when the concentration of H\(_2\)O\(_2\) was low (below 0.5 mM). Higher concentrations of H\(_2\)O\(_2\) (more than 0.5 mM) were able to induce stronger luminescence signals but with a reduced rate of increase (Fig. 3B). Concentrations of H\(_2\)O\(_2\) higher than 5 mM had little additional effect on the luminescence changes (Supplementary Fig. S5).

Next, we investigated the time courses of increase in [Ca\(^{2+}\)] induced by different concentrations of H\(_2\)O\(_2\) in rice roots. H\(_2\)O\(_2\) did not induce an immediate spike of [Ca\(^{2+}\)], as NaCl did. Our results showed that within the first 30 s, only the highest concentration of H\(_2\)O\(_2\) (5 mM) was able to induce any luminescence signal, this is in contrast to NaCl which within 30 s was able to induce luminescence signals for all but the lowest concentration (0.1 M) (Fig. 3C compared with Fig. 2C). Furthermore, the phase of [Ca\(^{2+}\)] responses were also different among treatments with different concentrations of H\(_2\)O\(_2\). Treatments with higher concentrations of H\(_2\)O\(_2\) (more than 0.4 mM) had a peak between 30 s and 1 min, lower concentrations of H\(_2\)O\(_2\) (below 0.4 mM) would delay the peak by about 30 s. Moreover, the spike of [Ca\(^{2+}\)],
Calcium signaling in rice roots

induced by H\textsubscript{2}O\textsubscript{2} did not decline to the basal level as quickly as NaCl. The luminescence signals declined gradually to the basal level within 3 min (Fig. 3C). These results revealed that H\textsubscript{2}O\textsubscript{2} induces a delayed [Ca\textsuperscript{2+}] spike compared to NaCl.

The different effects of Ca\textsuperscript{2+} inhibitors in NaCl- and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] responses and downstream gene expression

To further investigate the source of Ca\textsuperscript{2+} in NaCl- and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] responses, we tested GdCl\textsubscript{3}, LaCl\textsubscript{3}, neomycin- and thapsigargin-treated plants on the [Ca\textsuperscript{2+}] increase in response to NaCl and H\textsubscript{2}O\textsubscript{2} respectively. Gd\textsuperscript{3+} and La\textsuperscript{3+} are agonists of Ca\textsuperscript{2+}, and they have been used as Ca\textsuperscript{2+} channel blockers to inhibit Ca\textsuperscript{2+} flux (Tracy et al., 2008). In our experiment, GdCl\textsubscript{3} and LaCl\textsubscript{3} had similar inhibitory effects in NaCl- and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}], increases respectively (Fig. 4). 1 mM of GdCl\textsubscript{3} and LaCl\textsubscript{3} almost completely inhibited the [Ca\textsuperscript{2+}], increase in response to NaCl, and inhibited about 90% of [Ca\textsuperscript{2+}] increase in response to H\textsubscript{2}O\textsubscript{2} (Fig. 4). Furthermore, significant dosage effects were observed except for LaCl\textsubscript{3} in H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] increase. Different concentrations of LaCl\textsubscript{3} treatment had similar inhibitory effects in H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] increase (Fig. 4B, C). Neomycin is an inhibitor of InsP\textsubscript{3}-stimulated Ca\textsuperscript{2+} release from internal stores (Munnik et al., 1998). 0.01 mM of neomycin treatment had no significant inhibitory effect, while 0.1 mM and 1 mM of neomycin treatment inhibited about 50% of [Ca\textsuperscript{2+}] increase in response to NaCl. In the case of H\textsubscript{2}O\textsubscript{2}, 0.01 mM and 0.1 mM of neomycin treatment inhibited about 20% of [Ca\textsuperscript{2+}] increase, while 1 mM of neomycin treatment inhibited about 50% of [Ca\textsuperscript{2+}] increase (Fig. 4B, C). Thapsigargin is an inhibitor of endoplasmic reticulum (ER) Ca\textsuperscript{2+}-ATPases, and application of thapsigargin would empty the intracellular

Fig. 2. Aequorin-based luminescence in roots under NaCl treatments. (A) Pseudocolour images of aequorin luminescence in roots treated with different concentrations of NaCl. The relationship between luminescence intensity and the pseudocolour images are scaled by a pseudocolour bar. (B) The line chart of luminescence signal intensity of every treatment. (C) The time courses of [Ca\textsuperscript{2+}] changes induced by different concentrations of NaCl in rice roots. Data for independent experiments are shown (mean±sd; n=10; *** P<0.001; NS, not significant P>0.05; Student’s t-test).
Ca\textsuperscript{2+} store in ER (Treiman et al., 1998). In our experiment, thapsigargin significantly inhibited the NaCl-induced [Ca\textsuperscript{2+}] increase, and a dosage effect for inhibition was observed. By contrast, thapsigargin had no significant effect on the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] increase (Fig. 4B, C). These results showed that the sources of Ca\textsuperscript{2+} in NaCl- and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] responses are different.

To evaluate the contribution of [Ca\textsuperscript{2+}], responses in the NaCl- and H\textsubscript{2}O\textsubscript{2}-induced gene expression, the expression levels of \textit{SERF1}, \textit{MITOGEN-ACTIVATED PROTEIN KINASE5 (MAPK5)}, \textit{DEHYDRATION-RESPONSIVE ELEMENT BINDING2A (DREB2A)} and \textit{STRESS-RESPONSIVE NAC1 (SNAC1)}, which were reported to be induced by NaCl and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] responses, are different.

After the pre-treatment of LaCl\textsubscript{3}, the induction by NaCl was seriously inhibited for all the genes examined, indicating a Ca\textsuperscript{2+}-dependent manner of these inductions (Fig. 5A). On the other hand, H\textsubscript{2}O\textsubscript{2} induced three of the four genes with \textit{DREB2A} as the exception. Interestingly, only the expression of \textit{MAPK5} showed Ca\textsuperscript{2+}-dependent induction by H\textsubscript{2}O\textsubscript{2}. The expression of \textit{SERF1} and \textit{SNAC1} were induced by H\textsubscript{2}O\textsubscript{2}, however, pre-treatment of LaCl\textsubscript{3} did not inhibit the induction of the expression by H\textsubscript{2}O\textsubscript{2}, indicating a Ca\textsuperscript{2+}-independent manner of these inductions (Fig. 5B).

**Discussion**

Since the transformation of aequorin in plants (Knight et al., 1991), it has proved to be a useful tool for non-invasive investigation of Ca\textsuperscript{2+}-mediated signalling in response to various stresses in whole seedlings (Zhu et al., 2013). Furthermore,
GAL4 transactivation of aequorin in enhancer trap lines enabled the testing of the stimulus- and cell-specific $[\text{Ca}^{2+}]_i$ signalling in specific tissues of Arabidopsis (Kiegle et al., 2000; Marti et al., 2013). In our experiment, the luminescence of aequorin was limited to rice roots, although the expression of apoaequorin was universal. It is unlikely that the leaf wax blocked the aequorin luminescence, because we detected chloroplast auto-fluorescence, which was emitted from the leaf cells (Fig. 1E). However, the leaf wax could have prevented the permeating of coelenterazine. To test this hypothesis, a surfactant was used to allow the coelenterazine to permeate through the leaf wax. After the addition of 0.01% surfactant,
both strong luminescence signals in roots and weak luminescence signals in shoots were detected (Supplementary Fig. S1C). The luminescence signals in shoots were dotted, indicating insufficient permeation of coelenterazine. Although a higher concentration of surfactant (0.1%) increased the dotted luminescence signals in shoots, it greatly decreased the luminescence signals in roots, indicating the toxic effect of the surfactant to rice roots (Supplementary Fig. S1F). These results showed that our system is able to reflect the \([\text{Ca}^{2+}]_{i}\) level only in roots, not shoots.

Fig. 5. Relative expression levels of initial response genes induced by NaCl (A) and \(\text{H}_2\text{O}_2\) (B). (A) \(\text{H}_2\text{O}+\text{H}_2\text{O}\), untreated control. \(\text{H}_2\text{O}+\text{NaCl}\), NaCl treatment without pre-treatment. \(\text{LaCl}_3+\text{H}_2\text{O}\), only \(\text{LaCl}_3\) pre-treatment, without NaCl treatment. \(\text{LaCl}_3+\text{NaCl}\), \(\text{LaCl}_3\) pre-treatment before NaCl treatment. (B) \(\text{H}_2\text{O}+\text{H}_2\text{O}\), untreated control. \(\text{H}_2\text{O}+\text{H}_2\text{O}_2\), \(\text{H}_2\text{O}_2\) treatment without pre-treatment. \(\text{LaCl}_3+\text{H}_2\text{O}\), only \(\text{LaCl}_3\) pre-treatment, without \(\text{H}_2\text{O}_2\) treatment. \(\text{LaCl}_3+\text{H}_2\text{O}_2\), \(\text{LaCl}_3\) pre-treatment before \(\text{H}_2\text{O}_2\) treatment. Data for independent experiments are shown (mean±sd; \(n=3\)).
In *Arabidopsis*, the discharging solution contains 10% ethanol, which is sufficient to permeate the exogenous Ca\(^{2+}\) and combine the remaining aequorin in the cell (Yuan et al., 2014). In our experiment, we suggested a much higher concentration of ethanol (about 25%) to discharge all the aequorin. There are several differences in root radial structure between rice and *Arabidopsis*. From outside in, the epidermis, the ground tissue consisting of four tissues (exodermis, sclerenchyma cell layer, midcortex or mesodermis, and endodermis), and the central cylinder are present in a rice root, compared with single cell layers of epidermis, cortex, endodermis and the central cylinder in *Arabidopsis* (Dolan et al., 1993; Rebouillat et al., 2009). These additional cell layers in rice roots make it much thicker than that of *Arabidopsis*, and they form barriers that inhibit the permeation of exogenous Ca\(^{2+}\) in discharging solutions. In this research, we used luminescence intensity instead of real [Ca\(^{2+}\)], in rice Knight et al. (1996) described the equation to determine the Ca\(^{2+}\) concentration based on the luminescence intensity in *Arabidopsis*. However, the parameters in the equation can be variable among different species. Thus, for the accurate quantification of [Ca\(^{2+}\)], in rice, a titration curve for analyzing the relationship between the Ca\(^{2+}\) concentration and luminescence intensity should be shown and real [Ca\(^{2+}\)] should be estimated.

We investigated the [Ca\(^{2+}\)] changes in response to various concentrations of salt stress, and found that it was sensitive within a very narrow range of NaCl concentrations (Fig. 2B). We observed that severe salt stress (more than 0.25 M NaCl) did not increase [Ca\(^{2+}\)], significantly compared with salt stress by 0.25 M NaCl. Rice is a salt-sensitive crop and continued exposure to about 0.15 M NaCl does not allow rice to complete its life cycle (Munns and Tester, 2008). In this view, there is no need for rice to evolve an energy wasting mechanism to respond to such a high concentration of NaCl. Alternatively, it is possible that severe salt stress (more than 0.25 M NaCl) does increase [Ca\(^{2+}\)], significantly. However, due to the saturation of our detection system, we failed to detect stronger luminescence signals when treated with high concentrations of NaCl. Moreover, we also observed that treatment with 0.1 M NaCl (or less) did not induce an obvious increase of [Ca\(^{2+}\)], although it is sufficient to induce the expression of many salt response genes (Schmidt et al., 2013). Our evidence suggests that the induction of these salt response genes are Ca\(^{2+}\) dependent (Fig. 5A); it is likely that low strength salt stress induces a very tiny [Ca\(^{2+}\)], response which is beyond the resolution of our detection system. It was reported that H\(_2\)O\(_2\) triggered a biphasic [Ca\(^{2+}\)] increase in *Arabidopsis* seedlings, and the second peak 5 to 20 min after stress application was located exclusively in the root (Rentel and Knight, 2004). However, in our experiment, we detected the luminescence signals only in the first 3 min and failed to detect additional signals even with the time extended to 45 min (Supplementary Fig. S4). Considering the different environments rice and *Arabidopsis* roots are adapted to, rice may have evolved a different mechanism to cope with ROS stress.

We investigated the time courses of [Ca\(^{2+}\)], changes in response to different concentrations of NaCl and H\(_2\)O\(_2\) treatments in rice roots. Treatments of NaCl at different concentrations induced immediate [Ca\(^{2+}\)], spikes within 30 s (Fig. 2C). In contrast, H\(_2\)O\(_2\) did not induce any [Ca\(^{2+}\)], increases within the first 30 s unless the concentration of H\(_2\)O\(_2\) was high (Fig. 3C). In the view that the salt sensors are still unknown in plants, we propose that the salt sensors may be on the surface of the root and are closely coupled with Ca\(^{2+}\) channels. These salt sensors can respond to different concentrations of salt directly. On the other hand, it is likely that the effect of H\(_2\)O\(_2\) on calcium signalling is less direct. The treatment of roots with H\(_2\)O\(_2\) will in the first instance cause oxidation of the apoplast, and potentially it can affect calcium fluxes once it has been taken up by the cell through aquaporins, and thus a delay in response is observed. The H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)], spikes extended for several minutes (Fig. 3C), which also indicated a complicated and indirect way of H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)], response.

GdCl\(_3\) and LaCl\(_3\) are widely used to block Ca\(^{2+}\) channels (Tracy et al., 2008). As expected, 1 mM of GdCl\(_3\) and LaCl\(_3\) almost completely inhibit the [Ca\(^{2+}\)], increase in response to NaCl. Interestingly, although 1 mM of GdCl\(_3\) and LaCl\(_3\) were able to inhibit about 90% of [Ca\(^{2+}\)], increase in response to H\(_2\)O\(_2\), it cannot go further. There were no significant differences between 0.1 mM and 1 mM of blocker treatments, indicating a saturation of Ca\(^{2+}\) channels blocked by GdCl\(_3\) and LaCl\(_3\) (Fig. 4B, C). These results suggested that H\(_2\)O\(_2\) is able to induce a [Ca\(^{2+}\)], increase via different channels, and a small portion of these channels are GdCl\(_3\)/LaCl\(_3\) insensitive. We also examined the source of Ca\(^{2+}\) in NaCl- and H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)], responses by treatment with neomycin, which is an inhibitor of InsP\(_3\)-stimulated Ca\(^{2+}\) release from internal stores (Munnik et al., 1998). Neomycin inhibited about 50% of [Ca\(^{2+}\)], increase in response to both NaCl and H\(_2\)O\(_2\). These results suggested that the [Ca\(^{2+}\)], increases caused by NaCl and H\(_2\)O\(_2\) treatments came from both internal and external stores of Ca\(^{2+}\). ER is an important internal Ca\(^{2+}\) store in the cell (Franzini-Armstrong, 1998). In our experiment, we used thapsigargin to empty the intracellular Ca\(^{2+}\) store in ER. As a result, NaCl induced [Ca\(^{2+}\)], increase was significantly inhibited, indicating a participation of ER in the NaCl-induced [Ca\(^{2+}\)], increase. Interestingly, thapsigargin had no significant effect on the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)], increase (Fig. 4B, C). This suggested that another internal Ca\(^{2+}\) store, rather than ER, may participate in the H\(_2\)O\(_2\)-induced [Ca\(^{2+}\)], increase.

Recently, the molecular processes controlling early stress perception and signalling have been explored and several genes have been identified as initial response genes in rice (Dai et al., 2005; Schmidt et al., 2013). Among these genes, *SERF1* has a critical role and functions as a central hub in the ROS-dependent signalling during the initial response to salt stress in rice (Schmidt et al., 2013). In our research, we examined NaCl- and H\(_2\)O\(_2\)-induced expression levels of *SERF1* and other initial response genes with or without LaCl\(_3\). As expected, *SERF1* and all the other initial response genes examined were induced by NaCl treatment. Furthermore, the induction of all these genes was seriously inhibited by the pre-treatment of LaCl\(_3\) (Fig. 5A). In the H\(_2\)O\(_2\) treatment experiments, expression of the initial response genes *SERF1* and *SNAC1* were induced. However, the induction of...
these genes was not inhibited by the pre-treatment of LaCl$_3$ (Fig. 5B). Considering the fact that a [Ca$^{2+}$] spike is the first response to salt stress (Knight et al., 1997), and salt-induced ROS accumulation is regulated by Ca$^{2+}$ (Drerup et al., 2013), we propose a Ca$^{2+}$ and H$_2$O$_2$ mediated molecular signalling model for the initial response to NaCl in rice (Fig. 6). Salt stress immediately induces a [Ca$^{2+}$] spike, and the increase of [Ca$^{2+}$], triggers a ROS burst probably through the activation of NADPH oxidases. The increased H$_2$O$_2$ induces the expression of SERF1 and downstream initial response genes. In addition, H$_2$O$_2$ also induces a [Ca$^{2+}$] spike, and the increase of [Ca$^{2+}$], is needed for the expression of some initial response gene(s), such as MAPK5. In our experiment, H$_2$O$_2$ did not induce the expression of DREB2A significantly as previously described (Schmidt et al., 2013). We reasoned that the initial NaCl treatment may not be long enough to induce the expression of DREB2A. In the previous report, 3 h of H$_2$O$_2$ treatment was able to induce the expression of DREB2A, while 30 min of H$_2$O$_2$ treatment had no obvious effect (Schmidt et al., 2013). Considering the fact that the 1 h of NaCl treatment is sufficient to induce the expression of DREB2A (Fig. 5A), a complex network rather than a linear structure may exist to regulate the expression of DREB2A (Kim et al., 2011).

In summary, based on the aquorin luminescence, we established a calcium reporter line in rice. Using this, we tested the different [Ca$^{2+}$] responses to NaCl and H$_2$O$_2$ in rice roots. Ca$^{2+}$ inhibitor treatments revealed that both external and internal Ca$^{2+}$ stores are involved in the [Ca$^{2+}$] increases induced by NaCl and H$_2$O$_2$. Further research suggested that the internal Ca$^{2+}$ store in ER participates in the NaCl-induced [Ca$^{2+}$] increase, while another internal Ca$^{2+}$ store, rather than ER may participate in the H$_2$O$_2$-induced [Ca$^{2+}$] increase. In addition, we dissected the early salt-stress signalling cascade and presented a Ca$^{2+}$- and H$_2$O$_2$-mediated molecular signalling model for the initial response to NaCl in rice.

### Supplementary data

Supplementary data can be found at JXB online.

- **Supplementary Fig. S1.** Leaf wax prevents the permeating of coelenterazine.
- **Supplementary Fig. S2.** The optimization of discharging solution for luminescence imaging in rice.
- **Supplementary Fig. S3.** Treatment with high concentration of NaCl resulted in serious cell death in the rice roots.
- **Supplementary Figure S4.** H$_2$O$_2$ does not induce a second peak of [Ca$^{2+}$] response.
- **Supplementary Fig. S4.** H$_2$O$_2$ does not induce a second peak of [Ca$^{2+}$] response.
- **Supplementary Fig. S5.** High concentrations of H$_2$O$_2$ have little additional effect on the luminescence changes.
- **Supplementary Table S1.** The sequences of primers used in this research.

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