Relationship between NaCl- and H$_2$O$_2$-Induced Cytosolic Ca$^{2+}$ Increases in Response to Stress in Arabidopsis

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Abstract

Salinity is among the environmental factors that affect plant growth and development and constrain agricultural productivity. Salinity stress triggers increases in cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]) via Ca$^{2+}$ influx across the plasma membrane. Salinity stress, as well as other stresses, induces the production of reactive oxygen species (ROS). It is well established that ROS also triggers increases in [Ca$^{2+}$]. However, the relationship and interaction between salinity stress-induced [Ca$^{2+}$] increases and ROS-induced [Ca$^{2+}$] increases remain poorly understood. Using an aequorin-based Ca$^{2+}$ imaging assay we have analyzed [Ca$^{2+}$] changes in response to NaCl and H$_2$O$_2$ treatments in Arabidopsis thaliana. We found that NaCl and H$_2$O$_2$ together induced larger increases in [Ca$^{2+}$] in Arabidopsis seedlings than either NaCl or H$_2$O$_2$ alone, suggesting an additive effect on [Ca$^{2+}$] increases. Following a pre-treatment with either NaCl or H$_2$O$_2$, the subsequent elevation of [Ca$^{2+}$] in response to a second treatment with either NaCl or H$_2$O$_2$ was significantly reduced. Furthermore, the NaCl pre-treatment suppressed the elevation of [Ca$^{2+}$] seen with a second NaCl treatment more than that seen with a second treatment of H$_2$O$_2$. A similar response was seen when the initial treatment was with H$_2$O$_2$; subsequent addition of H$_2$O$_2$ led to less of an increase in [Ca$^{2+}$] than did addition of NaCl. These results imply that NaCl-gated Ca$^{2+}$ channels and H$_2$O$_2$-gated Ca$^{2+}$ channels may differ, and also suggest that NaCl- and H$_2$O$_2$-evoked [Ca$^{2+}$] may reduce the potency of both NaCl and H$_2$O$_2$ in triggering [Ca$^{2+}$] increases, highlighting a feedback mechanism. Alternatively, NaCl and H$_2$O$_2$ may activate the same Ca$^{2+}$ permeable channel, which is expressed in different types of cells and/or activated via different signaling pathways.

Introduction

The presence of high salinity affects almost every aspect of plant growth and development, and causes enormous losses in agricultural production worldwide. It is estimated that about 10 million hectares of agricultural land is abandoned every year because of high salinity, and salt stress affects as much as a quarter to a third of global agricultural land, particularly land which has been irrigated [1-3]. Given the continued increase in human population occurring in the world, it is estimated that crop production must be increased 50% by 2025 to stave off large-scale food shortages [4]. Thus, it is crucial to understand how plants respond to salt stress.

Many studies have been carried out to dissect the molecular and genetic mechanisms of the plant response to salt (NaCl) stress, often using the model organism Arabidopsis thaliana [5-7]. Excess NaCl is toxic to plants, causing cellular ion imbalances and hyperosmotic stress [1-3,7]. NaCl stress also triggers a calcium signaling cascade in plants, leading to transcriptional regulation and subsequent physiological and developmental responses [1]. Although the molecular nature of initial perception of salt stress is unknown, it has been well established that salt stress triggers a transient increase in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) that lasts about 2 min [8,9]. This increase has been proposed to represent a salt sensory process in plants [3,10].

In plants, Ca$^{2+}$ as a secondary messenger is a key element to understanding a sophisticated network of signaling pathways responding to a large array of abiotic and biotic stimuli, including salt stress [11-13]. These specific Ca$^{2+}$ signatures are

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formed by the tightly regulated activities of Ca\textsuperscript{2+} channels and transporters in different tissues, organelles and membranes [13-16], and the changes in [Ca\textsuperscript{2+}] are detected by cytosolic Ca\textsuperscript{2+} sensors. More than 250 Ca\textsuperscript{2+}-binding EF-hand proteins have been identified in *Arabidopsis* [17], including the calmodulin (CaM), the calmodulin-like (CML), the Ca\textsuperscript{2+}-dependent protein kinase (CDPK), and the calcineurin B-like (CBL) protein families. These cytosolic Ca\textsuperscript{2+} sensors decode and relay the information encoded within [Ca\textsuperscript{2+}] signatures, allowing the plant to tightly bring about the appropriate adaptation to its ever-changing environment.

The salinity stress-induced increase in [Ca\textsuperscript{2+}], leads to the activation of SOS3/CBL4, which functions as the primary Ca\textsuperscript{2+} sensor of [Ca\textsuperscript{2+}] changes under salt stress [3]. Upon activation, SOS3/CBL4 interacts with the C-terminal region of a CBL-interacting protein kinase (CIPK) called SOS2/CIPK24, which in turn activates a plasma membrane Na\textsuperscript{+}/H\textsuperscript{+} antiporter SOS1 that transports sodium ions out of the cell [3]. This salt signaling pathway reinforces the concept that the salt-induced [Ca\textsuperscript{2+}] increase is an essential component for bringing about the plant response to salt stress.

Interestingly, after salt stress treatment there is an overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) [18-22]. The time constants for salt-induced increases in [Ca\textsuperscript{2+}] and ROS are about 3 sec and 400 sec, respectively, as estimated from previous studies [8,21]. It appears that the increase in [Ca\textsuperscript{2+}] occurs earlier than the ROS elevation after salt stress treatment. Considering ROS have also been shown to trigger increases in [Ca\textsuperscript{2+}] [21,26-26], it is possible that ROS-induced [Ca\textsuperscript{2+}] increases might serve as a feed forward mechanism in the salt stress signal transduction pathway. However, less is known about the relationship and interaction between the salt stress-induced [Ca\textsuperscript{2+}], increases and the [Ca\textsuperscript{2+}] increases evoked by ROS, which are produced in response to either salt stress specifically or other stresses in general [1,27].

In this study, we have systematically analyzed the relationship and interaction between salt stress-induced [Ca\textsuperscript{2+}] increases and the ROS-induced [Ca\textsuperscript{2+}] increases in *Arabidopsis*. We found the increases in [Ca\textsuperscript{2+}] induced by both stimuli were higher than those induced by either single stress, suggesting that NaCl and H\textsubscript{2}O\textsubscript{2} have an additive effect on [Ca\textsuperscript{2+}]. We have also found that NaCl-induced [Ca\textsuperscript{2+}] increases might inhibit both NaCl- and H\textsubscript{2}O\textsubscript{2}-gated channels by a feedback mechanism, but more NaCl-gated channels; a similar response was seen when the H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] increases were analyzed. These data suggest responses seen involve both feedback inhibitory mechanisms, as well as an interaction between two stimuli-mediated Ca\textsuperscript{2+} signaling pathways.

**Results**

**Dose-dependence and kinetics of NaCl- and H\textsubscript{2}O\textsubscript{2}-induced [Ca\textsuperscript{2+}] increases**

To analyze whether and how increases in [Ca\textsuperscript{2+}] induced by NaCl and H\textsubscript{2}O\textsubscript{2} interact each other in *Arabidopsis*, we attempted initially to identify optimum concentrations of NaCl and H\textsubscript{2}O\textsubscript{2}, which ideally could be applied to generate about half of the maximum amplitude of [Ca\textsuperscript{2+}], for potential up- and down-regulation. In addition, we attempted to establish the kinetics of NaCl- and H\textsubscript{2}O\textsubscript{2}-induced increases in [Ca\textsuperscript{2+}] for administering these stresses in different sequential combinations. First, to analyze NaCl-induced increases in [Ca\textsuperscript{2+}], we treated *Arabidopsis* seedlings expressing aequorin with solutions containing 0 to 600 mM NaCl. Aequorin bioluminescence images were taken every 10 sec for 500 sec, and the peak [Ca\textsuperscript{2+}] was calculated and analyzed, as NaCl induces a transient increase in [Ca\textsuperscript{2+}] [8,9]. Plants grown on the half-strength MS medium had an average basal [Ca\textsuperscript{2+}] of 80 ± 21 nM (Figure 1A and C). As expected, the [Ca\textsuperscript{2+}] increased in response to NaCl treatment (Figure 1A). The magnitudes of [Ca\textsuperscript{2+}] increases were dependent on the concentration of NaCl, higher concentration of NaCl evoked a larger increase in [Ca\textsuperscript{2+}]. The NaCl concentration needed for a half-maximal response was ~200 mM, which was chosen as an optimum concentration to subsequently analyze the interaction with H\textsubscript{2}O\textsubscript{2}-induced increases in [Ca\textsuperscript{2+}].

Then, we determined the temporal dynamics of NaCl-induced [Ca\textsuperscript{2+}] increases under the imposed experimental conditions as a control for further comparison (Figure 1B). We found that [Ca\textsuperscript{2+}] increased immediately after the application of 200 mM NaCl, reached a peak of ~1 µM at about 20 sec, and then declined gradually (Figure 1B). Note that, the peaking time might be shorter than 20 sec based on previous studies [8,9]. Nevertheless, imaging aequorin bioluminescence for less than 10 sec resulted in images with low signal-noise ratio in our system. Thus, the temporal resolution was about 10 sec, which is sufficient for the current study. At about 200 sec, the [Ca\textsuperscript{2+}] was reduced to a new resting level of under 200 nM.

Similarly, we analyzed increases in [Ca\textsuperscript{2+}] in response to H\textsubscript{2}O\textsubscript{2}. Seedlings were treated with different concentrations of H\textsubscript{2}O\textsubscript{2}, from 0 to 15 mM and [Ca\textsuperscript{2+}] was analyzed. As expected, H\textsubscript{2}O\textsubscript{2} induced increases in [Ca\textsuperscript{2+}] in a dose-dependent manner (Figure 1C). The H\textsubscript{2}O\textsubscript{2} concentration for a half-maximal response was around 4 mM with the magnitude of [Ca\textsuperscript{2+}] similar to that induced by 200 mM NaCl. We then determined the temporal dynamics of the [Ca\textsuperscript{2+}] increased induced by 4 mM H\textsubscript{2}O\textsubscript{2}. Following treatment with 4 mM H\textsubscript{2}O\textsubscript{2}, [Ca\textsuperscript{2+}] increased and reached a peak of ~1 µM at 50 sec (Figure 1D). It took another 100 sec for the [Ca\textsuperscript{2+}] to reach a new basal level of just under 200 nM. Taken together, it seems that increases in [Ca\textsuperscript{2+}] occur faster in response to NaCl than H\textsubscript{2}O\textsubscript{2}, but are reset to a resting level 150 sec after treatment.

**Additive effect of NaCl and H\textsubscript{2}O\textsubscript{2} on triggering increases in [Ca\textsuperscript{2+}]**

To investigate thoroughly the relationship and/or interaction between [Ca\textsuperscript{2+}], increased induced by NaCl and H\textsubscript{2}O\textsubscript{2}, *Arabidopsis* seedlings were treated with 200 mM NaCl or 4 mM H\textsubscript{2}O\textsubscript{2} separately, or 200 mM NaCl together with 4 mM H\textsubscript{2}O\textsubscript{2}. Note that, although salt-induced ROS production could be detected within ~2 min after salt treatment [21] the estimated half-time to the peak of ROS production is more than 5 min. Note also that, we did not detect a second peak of [Ca\textsuperscript{2+}] within 5 min after salt stress treatment (Figure S1A), suggesting that salt-induced ROS could not trigger a detectable increase in
Ca^{2+}

under the current experimental conditions. Because we measured [Ca^{2+}] changes within 5 min after salt treatment, the
effect of salt-induced ROS on [Ca^{2+}] should not interfere
apparently. The [Ca^{2+}] increases recorded after single
treatments were consistent with the results described above
(Figure 2A–C). NaCl and H_2O_2 induced similar increases in
[Ca^{2+}] (Figure 2E). When plants were treated with both stimuli
simultaneously, the peaks of [Ca^{2+}] were much larger than that
induced by each individual stimulus (Figure 2D and E),
showing an additive effect. To further analyze how salt-induced
ROS affects [Ca^{2+}] increases in response to salt stress within
500 sec, we carried out an experiment by using the NADPH
oxidase inhibitor DPI [28] and ROS scavengers ascorbic acid
and glutathione [27], and found that neither of these reagents
significantly affected [Ca^{2+}] increases induced by NaCl (Figure
S1). These results suggest that the NaCl- and H_2O_2-induced
[Ca^{2+}] increases may be largely independent events. In other
words, NaCl and H_2O_2 might activate different Ca^{2+} permeable
channels.

The crosstalk between NaCl- and H_2O_2-induced [Ca^{2+}] increases
To further characterize the potential interaction between the
two stimuli-triggered [Ca^{2+}] signals, plants were successively
treated either with the same stimulus or the other. When the
Arabidopsis seedlings were treated with 200 mM NaCl, the
level of [Ca^{2+}] increased quickly to reach a peak and
decreased to the new resting level after 150 sec (Figure 3A ),
as described in Figure 1B. A subtle increase in [Ca^{2+}] could be
detected in seedlings after washing with deionized water at 200

Figure 1. Increases in [Ca^{2+}], in response to NaCl and H_2O_2 treatments. (A and C) Increases in [Ca^{2+}] induced by several
concentrations of NaCl (A) and H_2O_2 (C) in Arabidopsis. Seedlings expressing aequorin and grown for 7 days were treated with
solutions containing several concentrations of NaCl or H_2O_2, and aequorin images were taken every 10 sec for 500 sec. Data for
four independent experiments are shown (mean ± sem; n = 64).
(B and D) Time courses of increases in [Ca^{2+}] induced by 200 mM NaCl (B) or 4 mM H_2O_2 (D). Seedlings grown for 7 days were
treated with NaCl and H_2O_2 at time zero, and aequorin images were taken every 10 sec. Representative recordings from individual
seedlings were shown. Similar results were seen in six independent experiments using 128 seedlings.
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detected in seedlings after washing with deionized water at 200
Figure 2. Increases in \([Ca^{2+}]\) in response to NaCl and H\(_2\)O\(_2\) individually or combined. (A to D) Imaging of \([Ca^{2+}]\) increases in response to the treatments of water (H\(_2\)O; A), 200 mM NaCl (B), 4 mM H\(_2\)O\(_2\) (A), and 200 mM NaCl and 4 mM H\(_2\)O\(_2\) together (D) in Arabidopsis seedlings expressing aequorin. \([Ca^{2+}]\) increases were analyzed by imaging bioluminescence and scaled by a pseudo-color bar. (E) Quantification of \([Ca^{2+}]\) increases from experiments as in (A) to (D). Data for four independent experiments are shown (mean ± sd; \(n = 64\); *** \(P < 0.001\); NS, not significant \(P > 0.05\)).

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sec (Figure 3A and B; green). Then, NaCl was added again, which caused a small increase in [Ca^{2+}]. It decayed from 300 sec to a level similar to the previous resting level (Figure 3A and B). Compared to the first NaCl treatment, which led to a large [Ca^{2+}] increase to ~1 µM, the 2nd NaCl treatment resulted in a [Ca^{2+}] increase that was only a fraction of the size of the first [Ca^{2+}] increase. This observation suggests that the NaCl-activated Ca^{2+} permeable channel (NaC) might be desensitized or adapted by unknown signaling elements upstream of NaC activation. To test whether the NaC is desensitized or adapted, we waited for 3 hr and were able to detect a normal (~1 µM) [Ca^{2+}] increase in response to NaCl, suggesting that the NaC is most likely desensitized (data not shown).

Subsequently we analyzed whether the hydrogen peroxide-activated Ca^{2+} permeable channel (HpC) was affected by the initial NaCl treatment. The second NaCl treatment was replaced by 4 mM H_{2}O_{2} at 300 sec (Figure 3C). Interestingly, the peak of [Ca^{2+}], induced by H_{2}O_{2} was clearly greater than

Figure 3. NaCl-induced [Ca^{2+}] increases inhibits NaCl-activated channels more than H_{2}O_{2}-activated channels. (A and C) Arabidopsis seedlings were subjected to a 200 mM NaCl treatment once at 0 sec, and the solution was perfused by deionized water at 200 sec. Then, a second 200 mM NaCl (A), or 4 mM H_{2}O_{2} (C) treatment was applied around 300 sec. Aequorin luminescence was recorded continuously through the treatments in the dark. (B and D) Quantification of [Ca^{2+}] increases for the 2nd NaCl (B) or 2nd H_{2}O_{2} treatment (C) from experiments as in (A) to (C), respectively. Data for four independent experiments are shown (mean ± sd; n = 64; NS, not significant P > 0.05; *** P < 0.001).

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that induced by 200 mM NaCl ($P < 0.001$). After 450 sec, the 
[Ca$^{2+}$], decreased to a new basal level under 200 µM (Figure 3C and D). The lower inhibition of HpC than NaC by the initial
NaCl treatment suggests that the initial high level of [Ca$^{2+}$],
which resulted from NaC activation (called Na[Ca$^{2+}$]
microdomain/puff) subsequently inhibited NaC more than HpC
(Figure 3).

By analogy, we used H$_2$O$_2$ as the first stimul to treat the
seedlings and then analyzed the second treatment using H$_2$O$_2$
or NaCl (Figure 4). When H$_2$O$_2$ was added to the Petri dish, at
300 sec after the first H$_2$O$_2$ treatment, the [Ca$^{2+}$] level stabilized
at $178 \pm 32$ nM, similar to previous resting levels (Figure 4A
and B). But when we used 200 mM NaCl to replace H$_2$O$_2$ at
300 sec, the peak values of [Ca$^{2+}$], were $381 \pm 23$ nM, small but
significantly higher than that seen with the second H$_2$O$_2$
treatment (Figure 4B and D). Similarly, our results suggest that
the high [Ca$^{2+}$], which resulted from the initial HpC activation
(called Na[Ca$^{2+}$], microdomain), inhibited HpC more than NaC
(Figures 4 and 5).

**Discussion**

Calcium is a universal second messenger that plays an
important role in signal transduction in animals and plants
[25,29-31]. In the past 20 years, tremendous progress has
been made in understanding the changes in [Ca$^{2+}$] that appear
in response to various abiotic and biotic stresses in plants,
including salt stress, oxidative stress, drought, high and low
temperatures, and pathogen elicitors [13,25,26,32]. It is known
that a specific stimulus can trigger unique temporal and spatial
patterns of [Ca$^{2+}$], also known as [Ca$^{2+}$] signatures [33]. The
[Ca$^{2+}$] signature encodes information from the environmental
stimulus which will be decoded subsequently by intracellular
Ca$^{2+}$ sensors, such as calmodulins (CaMs) and calceinurin B-
like proteins (CBLs), leading to the activation of downstream
events [10]. It is also known that the basal [Ca$^{2+}$], is maintained
at a concentration about 10,000-fold below the extracellular
Ca$^{2+}$ concentration [29,31,34]. In general, Ca$^{2+}$ channels in the
plasma membrane and/or endomembranes are activated in
response to environmental stimuli, leading to increases in
[Ca$^{2+}$] [30,32]. Salt stress-induced increases in [Ca$^{2+}$] have
long been proposed as being involved in the process perceiving the salt signal although the properties of the salt-
activated Ca$^{2+}$ permeable channel are poorly understood and
its molecular nature remains to be identified [1,2,7].

In addition, various abiotic and biotic stresses lead to the
production of ROS and oxidative stresses, which control many
different processes in plants [27,35-37]. It has been well
established that salt stress enhances the production of reactive
oxygen species (ROS) in plants [18-22]. Interestingly, ROS has
also been shown to activate Ca$^{2+}$ permeable channels in the
plasma membrane, which in turn lead to Ca$^{2+}$ influx into the
cell and thus increases in [Ca$^{2+}$]. [19,24,38]. Note that, the
salt stress-induced [Ca$^{2+}$] increases precede the production of H$_2$O$_2$
signaling molecule [39]. Nevertheless, little is known about the
molecular mechanisms underlying ROS perception in plant
cells, and it is possible that ROS activation of Ca$^{2+}$ permeable
channels may serve as a ROS perception process.

The decay of the increases in [Ca$^{2+}$], induced by both NaCl
and H$_2$O$_2$ seen in this study (Figure 1B and D) as well as
previous studies [8,9,15] indicates that the stimulus-activated
Ca$^{2+}$ permeable channels may be inactivated via a feedback
inhibitory mechanism, i.e. elevated [Ca$^{2+}$] inhibits these
channels, a desensitization process commonly seen for
receptor ion channels in animals [31,40]. It remains to be
addressed whether the localized increases in [Ca$^{2+}$] induced by
one stimulus, called Na[Ca$^{2+}$], microdomain [29,31], inhibit the
other stimulus-activated Ca$^{2+}$ permeable channels. It is known
that NaCl induces multiple peaks of [Ca$^{2+}$], under certain
conditions [9], possibly because the same NaCl-sensitive
channels are repetitively activated, or that NaCl might trigger
H$_2$O$_2$ production which subsequently activates another Ca$^{2+}$
channel, different from the NaCl-sensitive channel. Under our
experimental conditions, we did not observe multiple peaks of
[Ca$^{2+}$], after salt treatment (Figure S1A).

NaCl and H$_2$O$_2$ together induced larger increases in [Ca$^{2+}$],
than either NaCl or H$_2$O$_2$ alone (Figure 2), suggesting that NaCl
and H$_2$O$_2$ may activate distinct Ca$^{2+}$ permeable channels, NaC
and HpC (Figure 5). NaC and HpC are likely regulated by
feedback inhibition (Figure 5), considering their desensitization
seen in this study (Figure 1B and D) as well as previous reports
[8,9]. We demonstrated that repetitive NaCl treatments failed to
trigger repetitive [Ca$^{2+}$] increases (Figure 3A and B). This
indicates that the NaC cannot be activated repetitively within a
short period of time, i.e. NaC is possibly desensitized. We
propose that a feedback inhibition may be involved in the
desensitization (Figure 5). Upon NaCl treatment, the NaC
opens, leading to the localized increase of [Ca$^{2+}$]. Na[Ca$^{2+}$]
microdomain/puff. Na[Ca$^{2+}$] in turn signals the channel to close,
which prevents further [Ca$^{2+}$] increases and allows the basal
[Ca$^{2+}$] to be reset via Ca$^{2+}$ pumps. This feedback inhibition
avoids the excessive increase of [Ca$^{2+}$], which could be
disastrous to plant cells. The same phenomenon was also
observed with the activation of HpC (Figure 5), i.e. the Na[Ca$^{2+}$],
microdomain inhibits HpC via a feedback mechanism. Clearly,
the most significant effect is that after the initial treatment by
either NaCl or H$_2$O$_2$, [Ca$^{2+}$], increases induced by both NaCl
and H$_2$O$_2$ are reduced (Figures 3 and 4). It is most likely that
localized Na[Ca$^{2+}$], and Na[Ca$^{2+}$], merge to form a relatively
global [Ca$^{2+}$], which then feedback inhibits both NaC and HpC
(Figure 5). We observed that *Arabidopsis* was unable to
recover from 200 mM NaCl treatment 5 min after an initial
stimulation. Similar results were observed after 4 mM H$_2$O$_2$
treatment. In contrast, a previous study has shown that
*Arabidopsis* is able to recover its ability to respond almost fully
to cold shock 3 min after an initial cold shock [41]. Note that,
our work does not prove that HpC and NaC are localized in
discreet and different microdomains in the plasma membrane,
rather we have shown HpC and NaC may differ and interact via
[Ca$^{2+}$], microdomains. It is also possible that NaCl and H$_2$O$_2$
may activate the same Ca$^{2+}$ permeable channel, which is
expressed in different types of cells and/or activated via
different signaling pathways, leading to the differential changes
in [Ca$^{2+}$].

In general, when plants are exposed to one stress, their
resistance to other stresses can be enhanced. It is most likely
that stress-evoked \( [\mathrm{Ca}^{2+}] \) increases as well as stress-stimulated overproduction of ROS function as key integrators, possibly mediating stress signal perception and signal transduction. Our results demonstrate the inhibitory interaction of NaCl- and \( \mathrm{H}_2\mathrm{O}_2 \)-induced \( [\mathrm{Ca}^{2+}] \) increases, and may predict distinct \( \mathrm{Ca}^{2+} \) permeable channels activated by NaCl and \( \mathrm{H}_2\mathrm{O}_2 \), respectively (Figure 5). In the future, it is important to analyze the pharmacological properties of these putative \( \mathrm{Ca}^{2+} \) permeable channels activated by NaCl and \( \mathrm{H}_2\mathrm{O}_2 \) as described previously for MAMP-activated channels [42]. Obviously, the identification of these channels or sensors will be a hallmark in the study of plant salt resistance in the future. In addition, how NaC and HpC interact to contribute to the \( [\mathrm{Ca}^{2+}] \) signatures and other downstream events can be further analyzed when their molecular nature is identified.

Figure 4. \( \mathrm{H}_2\mathrm{O}_2 \)-induced \( [\mathrm{Ca}^{2+}] \) increases inhibits \( \mathrm{H}_2\mathrm{O}_2 \)-activated channels more than NaCl-activated channels. (A and C) Arabidopsis seedlings were subjected to a 4 mM \( \mathrm{H}_2\mathrm{O}_2 \) treatment once at 0 sec, and the solution was perfused by deionized water at 200 sec. Then, a second 4 mM \( \mathrm{H}_2\mathrm{O}_2 \) (A), or 200 mM NaCl (C) treatment was applied around 300 sec. Aequorin luminescence was recorded continuously through the treatments in the dark. (B and D) Quantification of \( [\mathrm{Ca}^{2+}] \) increases for the 2nd \( \mathrm{H}_2\mathrm{O}_2 \) (B) or 2nd NaCl treatment (C) from experiments as in (A) to (C), respectively. Data for four independent experiments are shown (mean ± sd; \( n = 64 \); NS, not significant \( P > 0.05 \); *** \( P < 0.001 \)).

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Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia-0 (Col-0) constitutively expressing intracellular aequorin (pMAQ2, a kind gift from Dr. M. Knight) under the control of the cauliflower mosaic virus 35S promoter was used [8,33]. *Arabidopsis* plants were grown in 150 mm x 15 mm round Petri dishes in half-strength Murashige and Skoog salts (MS; Gibco), supplemented with 1.5% (w/v) sucrose (Sigma), and 0.8% (w/v) agar (Becton Dickinson) adjusted to pH 6.0 with KOH in controlled environmental rooms at 21 ± 2°C. The fluency rate of white light was ~110 µmol m⁻² sec⁻¹. The photoperiods were 16 h light/8 h dark cycles. Seeds were sterilized with 2.5% PPM (Plant preservative mixture; Caisson Laboratories) and stratified at 4°C for 3 days in the dark, and then transferred to growth rooms.

**Aequorin reconstitution and measurement of [Ca²⁺]**

*Arabidopsis thaliana* plants expressing cytosolic apoaequorin were used for [Ca²⁺] measurements [33,43]. Seedlings were grown on half-strength Murashige and Skoog medium for 7 days. Reconstitution of aequorin was performed in vivo by spraying seedlings with 240 µL of 10 µM coelenterazine per
Petri dish followed by incubation at 21°C in the dark for 8 hr. Treatments and aequorin luminescence imaging were performed at room temperature using a ChemiPro HT system that includes a cryogenically cooled and back-illuminated CCD camera, liquid nitrogen autofiller, camera controller, and computer-equipped WinView/32 software (Roper Scientific) as described previously [33]. The CCD camera has a 1300 × 1340 pixel resolution and is cooled to −110°C by the cryogenic solution, a four-channel peristaltic pump (Dynamax RP-1, previously [41] with modification to the ChemiPro system. The treatment solution (100 mL) at described concentrations of NaCl or H2O2 was added into Petri dish in the dark, and luminescence was recorded continuously. For changes in bath solution, a four-channel peristaltic pump (Dynamax RP-1, Rainin) was used to perfuse Petri dish with water as indicated in the figures. Then, additional stress treatment was applied by adding 100 mL solution into Petri dish.

**Calibration of calcium measurements**

The cytosolic free cytosolic Ca2+ concentrations were calculated based on the calibration equation described previously [41] with modification to the ChemiPro system. The wild-type Arabidopsis expressing aequorin were placed individually in each well in 96-well plates containing ½ MS medium, 1.5% (w/v) sucrose, and 0.8% (w/v) agar for 10 days. Kinetic luminescence measurements were performed with an automated microplate luminescence reader (Mithras LB 940, Berthold Technologies). After automatic injection of 0.2 ml of solution into the each well bioluminescence counts were integrated every 1 sec as described previously [41]. The solutions containing a range of [NaCl] from 0 to 600 mM were used to treat the plants, and the peak values of [Ca2+] were calculated used the equation described previously [41]. Similar measurements were carried out using the ChemiPro HT system as described above to obtain L/Lmax values for each treatment, where L is luminescence and Lmax is the total remaining counts for bioluminescence. Then, we fit these data to the previously describe equation pCa = a * (−log (L/Lmax)) + b, and obtained the equation pCa = 0.9057209 * (−log (L/Lmax)) + 4.7712743. Note that, the calculated Ca2+ concentrations presented in the current study are similar to those reported previously [8,41].

**Supporting Information**

Figure S1. H2O2 levels do not affect [Ca2+], increases in response to NaCl treatment. (A) Arabidopsis seedlings were treated with water (Control), the NADPH oxidase inhibitor DPI (15 µM), and ROS scavenger ascorbic acid (5 mM) and glutathione (5 mM) two hours prior to the NaCl treatment. The seedlings were then subjected to a 200 mM NaCl treatment, and aequorin luminescence was recorded continuously through the treatments in the dark. (B) Quantification of peak [Ca2+] increases from experiments as in (A). Data for three independent experiments are shown (mean ± sd; n = 35 to 62; NS, not significant, P > 0.05).

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**Author Contributions**

Conceived and designed the experiments: ZMP ZJ. Performed the experiments: ZJ SZ RY YX AC. Analyzed the data: ZJ RY ZMP. Contributed reagents/materials/analysis tools: RY YX LA. Wrote the manuscript: ZMP ZJ.

**References**


