Calcium-sensing receptor regulates stomatal closure through hydrogen peroxide and nitric oxide in response to extracellular calcium in Arabidopsis

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Abstract

The Arabidopsis calcium-sensing receptor CAS is a crucial regulator of extracellular calcium-induced stomatal closure. Free cytosolic Ca^{2+} (Ca^{2+}_c) increases in response to a high extracellular calcium (Ca^{2+}_o) level through a CAS signalling pathway and finally leads to stomatal closure. Multidisciplinary approaches including histochemical, pharmacological, fluorescent, electrochemical, and molecular biological methods were used to discuss the relationship of hydrogen peroxide (H2O2) and nitric oxide (NO) signalling in the CAS signalling pathway in guard cells in response to Ca^{2+}_o. Here it is shown that Ca^{2+}_o could induce H2O2 and NO production from guard cells but only H2O2 from chloroplasts, leading to stomatal closure. In addition, the CASas mutant, the atrbohD/F double mutant, and the Atnoa1 mutant were all insensitive to Ca^{2+}_o-stimulated stomatal closure, as well as H2O2 and NO elevation in the case of CASas. Furthermore, it was found that the antioxidant system might function as a mediator in Ca^{2+}_o and H2O2 signalling in guard cells. The results suggest a hypothetical model whereby Ca^{2+}_o induces H2O2 and NO accumulation in guard cells through the CAS signalling pathway, which further triggers Ca^{2+}_c transients and finally stomatal closure. The possible cross-talk of Ca^{2+}_o and abscisic acid signalling as well as the antioxidant system are discussed.

Key words: ABA signalling, antioxidant system, calcium-sensing receptor, extracellular calcium signalling, guard cells, hydrogen peroxide, nitric oxide.

Introduction

Extracellular calcium (Ca^{2+}_o) has long been known to promote free cytosolic Ca^{2+} (Ca^{2+}_c) increase and stomatal closure (Schwartz, 1985; MacRobbie, 1992; McAinsh et al., 1995). The calcium-sensing receptor (CAS) was then identified and proven to be involved in this Ca^{2+}_o-induced Ca^{2+}_c increase (CICI) and stomatal closure (Han et al., 2003; Tang et al., 2007). Furthermore, CAS was reported to localize in the chloroplast thylakoid membrane (Nomura et al., 2008). However, how the guard cells convert the Ca^{2+}_o signal through CAS and finally lead to CICI was not well understood.

Hydrogen peroxide (H2O2) was shown to be a second messenger in response to biotic and abiotic perturbations (Fukao and Bailey-Serres, 2004; Laloi et al., 2004).
H$_2$O$_2$ also functions as a signalling molecule in abscisic acid (ABA)-induced stomatal movements (Pei et al., 2000). The AtrobohD and AtrobohF (Arabidopsis) respiratory burst oxidase homologues D and F) NADPH oxidases double mutant atrbohD/F abolishes the ABA-driven Ca$^{2+}$ increase and stomatal closure (Kwak et al., 2003). Meanwhile, H$_2$O$_2$ has been found to stimulate Ca$^{2+}$ transients in regulation of stomatal movement (Allen et al., 2000; Pei et al., 2000). Extracellular calmodulin (ExCaM), a Ca$^{2+}$-activated protein, induces H$_2$O$_2$ generation and the increased H$_2$O$_2$ further elevates stomatal Ca$^{2+}$ concentration (Chen et al., 2004), suggesting that Ca$^{2+}$o signalling, H$_2$O$_2$ production, and Ca$^{2+}$i transients may be orchestrated in guard cells.

Nitric oxide (NO), another key intermediate in ABA and multiple stress responses (Garcia-Mata and Lamattina, 2001, 2002; Neill et al., 2002a), operates as a downstream player of H$_2$O$_2$ signalling in guard cells (Lum et al., 2002; Bright et al., 2006). Interestingly, the Ca$^{2+}$o-driven NO accumulation in guard cells was reported in recent experiments in Arabidopsis (Li et al., 2009), suggesting the possibility of Ca$^{2+}$o-driven NO accumulation through H$_2$O$_2$ generation. Like H$_2$O$_2$, an NO-induced Ca$^{2+}$ increase and stomatal closure have also been found in guard cells of Arabidopsis and Vicia faba (Neill et al., 2002a; Garcia-Mata et al., 2003). Notably, NO was shown to contribute to Ca$^{2+}$i transients in ABA-induced stomatal closure (Desikan et al., 2002). However, the underlying mechanisms by which NO contributes to the Ca$^{2+}$o-induced Ca$^{2+}$i increase and stomatal closure are not clear.

Chloroplasts are a major source of H$_2$O$_2$ and NO production in plant cells (Asada and Takahashi, 1987; Jasid et al., 2006) and also a sensor of environmental stresses through chloroplast redox signalling (Plamenschmidt, 2003). The application of exogenous ABA resulted in H$_2$O$_2$ generation and stomatal closure, which occurs in chloroplasts earlier than within the other regions of guard cells (Zhang et al., 2001). A recent study revealed that chloroplasts play a critical role in CAS-mediated CICI and subsequent stomatal closure in Arabidopsis (Nomura et al., 2008). These results suggest that stomatal movements responding to environmental signals are probably regulated by H$_2$O$_2$ from the chloroplasts. Ca$^{2+}$o-induced stomatal closure may involve cross-talk between Ca$^{2+}$o and chloroplast-localized CAS as well as H$_2$O$_2$ in chloroplasts.

Besides what is known about guard cell signalling pathways, other findings also suggested the correlation among Ca$^{2+}$o, H$_2$O$_2$, NO, and chloroplasts. Ca$^{2+}$o can increase the activity of NADPH oxidase (NOX) and trigger H$_2$O$_2$ production in maize leaves (Sagi and Fluur, 2001; Hu et al., 2007; Potocký et al., 2007). Mitogen-activated protein kinase 6 (MPK6) and the prohibitin gene PHB3 were recently identified to function in H$_2$O$_2$-mediated NO synthesis during lateral root formation and in abiotic stress responses (Wang et al., 2010; Wang et al., 2010). Consistently, NO synthesis was shown to be up-regulated by H$_2$O$_2$ in other plant species (Lum et al., 2002; Li et al., 2009). In addition, NO also promoted Ca$^{2+}$i transients in plant cells in response to biotic and abiotic stresses (Gould et al., 2003; Lamotte et al., 2004, 2006; Vandelle et al., 2006). Similarly to H$_2$O$_2$, using NO-sensitive fluorescent probes, NO synthesis was also evident first in chloroplasts of the mesophyll and across all epidermal cell types including guard cells in response to environmental stimuli (Foissner et al., 2000; Gould et al., 2003; Arnaud et al., 2006).

In this report, the Apollo 4000 system with H$_2$O$_2$- and NO-selective electrochemical sensors was used to detect H$_2$O$_2$ and NO production in epidermis or chloroplasts. Different tools were also utilized to characterize functionally the role of H$_2$O$_2$ and NO as well as the contribution of the CAS signalling pathway in Ca$^{2+}$o-induced Ca$^{2+}$i increase and stomatal closure, including CAS antisense lines (CASs) and Arabidopsis mutants defective in H$_2$O$_2$ or NO synthesis (e.g. the H$_2$O$_2$ synthetic enzymatic mutant atrbohD/F and a mutant indirectly impaired in NO synthesis Atnoa1), a histochemical technique, and H$_2$O$_2$- and NO-specific fluorescent dyes. The transcriptional activities of Arabidopsis cytosolic antioxidant enzymes such as copper/zinc superoxide dismutase 1 (CSD1), ascorbate peroxidase 1 (APX1), and glutathione-disulphide reductase (ATGR1) in leaves were also investigated for the involvement of the antioxidant system in Ca$^{2+}$o-induced H$_2$O$_2$ generation. A functional relationship among CAS, H$_2$O$_2$, and NO as well as the antioxidant system was established in Ca$^{2+}$o-dependent guard cell signalling.

**Materials and methods**

**Plant materials and growth conditions**

Arabidopsis plants of the wild type and various mutants were grown in mixture matrix (turves:vermiculite=1:1) with a 16 h light and 8 h dark cycle under a photon flux rate of 200 μM m$^{-2}$ s$^{-1}$ at 22 °C, 70% relative humidity. Fully expanded Arabidopsis leaves of ~5 weeks old were harvested for immediate use. Seeds of CASs, atrbohD/F, and Atnoa1 mutants (background Col-0) were all obtained from Duke University. These mutants were further confirmed by reverse transcription-PCR analysis with specific primers on total RNA extracted from leaves (Supplementary Fig. S1A, C available at JXB online). Primer sequences used for PCR are shown in Supplementary Table S1. CAS antisense lines were identified by western blot analysis using anti-CAS antibody as described (Han et al., 2003) (Supplementary Fig. S1B).

**Stomatal bioassay**

Stomatal assays were performed essentially as described by Desikan et al. (2002). Abaxial epidermal strips from similar rosette leaves were floated in 10 mM MES buffer (pH 6.15) containing 50 mM KCl and 50 μM CaCl$_2$ for 2 h under light conditions to open the stomata before the addition of various compounds. Following this, 2 mM CaCl$_2$, 10 μM H$_2$O$_2$, or 60 μM sodium nitroprusside (SNP) was added to the buffer and incubated for another 2 h to assay stomatal closure. To study the effect of catalase (CAT), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide potassium salt (cPTIO), or neomycin on stomatal closure, epidermal strips were transferred to and incubated in MES buffer containing 2 mM CaCl$_2$ plus 100 U ml$^{-1}$ CAT, 100 μM cPTIO, or 100 μM neomycin for 2 h.

For time-resolved measurements of Ca$^{2+}$, H$_2$O$_2$, or SNP-induced stomatal closure, abaxial epidermal strips with open stomata were incubated in various buffers for different times as...
indicated in the figure legends. Stomatal apertures were determined as the ratio of width to length using image analysis computer software (SigmaScan Pro5) and were presented as the percentage with respect to untreated control or zero time as the standard.

**Fluorescent imaging by microscopy**

The variations of H2O2 and NO in guard cells were examined by loading 2',7'-dichlorofluorescein diacetate (H2DCFDA) and 3-aminomethyl-2',7'-dichlorofluorescein diacetate (DAF-FM DA) as described (Suhiita et al., 2004; Gonugunta et al., 2008). Abaxial epidermal strips with open stomata were transferred to MES buffer with 50 μM H2DCFDA or 10 μM DAF-FM DA for 15 min in darkness at room temperature and then rinsed with MES buffer three times before various treatments. Fluorescence was observed under a Motic AE31 fluorescence microscope (Speed Fair Co., Ltd, Hong Kong, excitation filter, 488 nm and emission, 535 nm) with a digital video camera (Motic MHG-100B, Speed Fair Co., Ltd, Hong Kong). Images were acquired from fluorescence between different samples, genotypes, and during time-courses at identical illumination intensity, camera gain, and exposure time in this experiment. The fluorescent pixel intensities of subtracting the basal signal from guard cells were analysed using Meta Morph 7.5 software. H2DCF fluorescence and chlorophyll autofluorescence in guard cells were further visualized using a laser scanning confocal microscope (LEICA TCS SP2) at an excitation wavelength of 488 nm. H2DCF fluorescence was detected from 495 nm to 535 nm, whereas chlorophyll autofluorescence was detected between 630 nm and 730 nm.

**Subcellular localization of H2O2 in guard cells**

The histochemical localization of H2O2 based on generation of electron-dense cerium perhydroxide precipitates was performed as described (Bestwick et al., 1997; Pellinen et al., 1999). Tissue fragments (2×5 mm2) from leaves incubated in MES buffer with or without 2 mM CaCl2 or 10 μM ABA were excised and kept in 50 mM MOPS buffer containing 5 mM CeCl3 at pH 7.2 under vacuum for 20 min. After being double fixed with 2.5% glutaraldehyde and 1.0% OsO4, the CeCl3-treated sections were dehydrated in an ascending ethanol series and embedded with the abaxial epidermis clamping to the surface of the embedding block. Blocks were progressively dehydrated in a graded acetone series and polymerized. Blocks of abaxial epidermis were sliced into 70–90 nm sections on a ultramicrotome, and mounted on uncoated copper grids. Sections of the guard cells were observed using transmission electron microscopy (TEM; JEM-2100HC, JEOL, Japan).

**Preparations of leaf epidermis and chloroplasts**

_Arabidopsis_ leaf epidermis was extracted as described by Pandey et al. (2002). The epidermis peels, which were pale green and ~1 mm2 in size, were ready for H2O2 or NO monitoring using a four-channel free radical Apollo 4000 analyser (World Precision Instruments, Sarasota, FL, USA). The intactness of epidermal cells in the epidermal fragments was evaluated using Evans blue dye as described (Mergemann and Sauter, 2000).

_Arabidopsis_ chloroplasts were isolated using the method of Aronsson and Jarvis (2002). The integrity of the isolated chloroplasts was estimated to be >85% (Supplementary Fig. S2 at JXB online). The chloroplast pellets were finally resuspended in HMS buffer containing 50 mM HEPES, 3 mM MgSO4, 0.3 M sorbitol, pH 7.2. The chloroplast counts were determined microscopically using a haemocytometer.

**H2O2 and NO measurement by the Apollo 4000 system**

The H2O2- and NO-selective electrochemical sensors ISO-HPO and ISO-NOP (WPI, Sarasota, FL, USA), widely used for animals, plants, microalgae, and subcellular organelles (Bouchard and Yamazaki, 2008; de Oliveira et al., 2008; D’Agostino et al., 2009; Pandolfi et al., 2010), were respectively connected to the Apollo 4000 system (World Precision Instruments) and adapted for real-time detection of H2O2 and NO from _Arabidopsis_ leaf epidermis or chloroplast. Both sensors consist of a combination of an internal H2O2- or NO-sensing pair of working and counter (reference) electrodes. Each sensor fits inside a replaceable stainless steel membrane sleeve filled with an electrolyte solution and is separated from the outside environment by an H2O2- or NO-selective membrane that covers the end of the stainless steel. The poised voltage of each sensor was set to 400 mV for H2O2 or 865 mV for NO detection. Direct current from each sensor presents the environmental H2O2 or NO concentration and the data are recorded on a PC connected to the Apollo 4000 system. The H2O2 or NO sensors were inserted into a water-jacketed chamber containing 3 ml of MES buffer with epidermal peels from 0.5 g of _Arabidopsis_ rossette leaves or 1 ml of HMS buffer. A circulating bath was used to keep the temperature constant at 30°C and the sample was mixed at a low rate using a magnetic bar controlled by a magnetic stirrer. To observe Ca2+-induced H2O2 and NO production from the epidermis, 10 mM or 50 μM CaCl2 (in MES buffer) was supplemented when the current signal became stable. For H2O2 detection from chloroplasts, a chloroplast suspension of 3×106 individuals was transferred to HMS buffer with or without 10 mM CaCl2. The H2O2 sensor was calibrated in a set of known H2O2 standards (0–10 μM range) while the NO sensor was calibrated with S-nitroso-N-acetylpenicillamine (SNAP) in 0.1 M CuCl2 (0–50 nM range of NO) as described by the manufacturer (Fig. 1A, B).

**Fluorometric detection of H2O2 and NO production from chloroplasts**

The fluorometric method used for the detection of NO from barley root mitochondria suspensions, as described recently by Gupta and Kaiser (2010), was adapted for measuring H2O2 and NO production from chloroplasts. For all experiments, chloroplasts were first incubated in HMS buffer with 50 μM H2DCFDA or 10 μM DAF-FM DA for 1 h in darkness. Chloroplasts were then rinsed twice by centrifugation at 2500 g for 4 min each, resuspended with HMS buffer, and kept in darkness until analysed. To study concentration-dependent Ca2+-induced H2O2 and NO production, H2DCF- or DAF-FM-pre-loaded chloroplasts were incubated in HMS buffer with 0–2 mM CaCl2 for 10 min. For time-related analysis, 2 mM CaCl2 was applied for the time points ranging from 0 min to 10 min. All experiments were performed at a controlled temperature of 30°C. The fluorescence of 5×106 ml–1 chloroplasts was detected using a fluorescence spectrophotometer (Cary Eclipse, Varian) with 495±5 nm and 515±5 nm as the excitation and emission wavelengths, respectively.

**Real-time quantitative PCR analysis**

Detached leaves were incubated in MES buffer containing 50 μM or 2 mM CaCl2 for 0–24 h under light at 22°C. Total RNA was extracted from leaves collected at various time points given in the figure legends using the TRIZOL Reagent (Invitrogen Inc., CA, USA). For real-time quantitative PCR, first-strand cDNA was synthesized using M-MLV reverse transcriptase (Takara Bio Inc., Japan) with an oligo d(T)18 primer. The resulting cDNAs were used as templates for subsequent PCRs which were performed on the Rotor-Gene™ 6000 real-time analyser (Corbett Research, Mortlake, Australia) in standard mode with FastStart Universal SYBR Green (ROX, Roche Ltd., Mannheim, Germany) according to the manufacturer’s protocol. All cycling conditions were as follows: 10 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by a melting curve program (55–99°C, with a 5 s hold at each temperature). The primers were designed according to known sequences of _Arabidopsis_ CSD1, APX1, and ATGRI genes (GenBank accession nos AT1G08830, AT1G07890, and AT3G24170, respectively) acquired from NCBI. The primers used for
amplification are listed in Supplementary Table S2 at JXB online, and the products were checked by melting curve analysis. Amplified products were cloned into PMD-18T vector (Takara) and subjected to sequencing analyses. The mean mRNA expression level was normalized using the \( \Delta \Delta Ct \) method described by Livak and Schmittgen (2001) with Actin2 as the internal control.

**Results**

**Ca\(^{2+}\)\(_o\) induces \( \text{H}_2\text{O}_2\) and NO production in guard cells of Arabidopsis epidermis**

To assess the effects of Ca\(^{2+}\)\(_o\) on promoting \( \text{H}_2\text{O}_2\) and NO production from Arabidopsis epidermis, electrochemical sensors ISO-HPO and ISO-NOP were used to detect \( \text{H}_2\text{O}_2\) and NO, respectively, in epidermal peels. Calibrations of both sensors were performed, and changes in the concentration of \( \text{H}_2\text{O}_2\) and NO were then calculated based on a linear relationship between \( \text{H}_2\text{O}_2\) or NO and the corresponding current over the current ranges 0–250 pA or 0–100 pA (Fig. 1A, B). The specificity of both sensors was also confirmed by further experiments showing that CAT or cPTIO could, respectively, remove the \( \text{H}_2\text{O}_2\) or NO signal, while CAT or cPTIO themselves could not affect this signal (Supplementary Fig. S3 at JXB online). To initiate the reaction, a high or low concentration of CaCl\(_2\) was added to the suspension of epidermal peels. Upon high CaCl\(_2\) (10 mM) supplementation, \( \text{H}_2\text{O}_2\) production from the epidermis monitored by a \( \text{H}_2\text{O}_2\) electrochemical sensor
rapidly increased to nearly 7 μM within 100 s and then reached a plateau (Fig. 1C). While high CaCl₂ caused a gradual increase of NO in epidermis, no significant change occurred after adding a low CaCl₂ concentration to a suspension of epidermal peels (Fig. 1D). In addition, NO synthesis seems to lag behind that of H₂O₂ when the epidermis is exposed to Ca²⁺ signal.

In order to determine whether the detected production of H₂O₂ and NO was mainly released from guard cells, the H₂O₂ and NO targets in Arabidopsis epidermal peels were investigated. The endogenous H₂O₂ and NO in 10 mM CaCl₂-pre-incubated epidermal peels were determined by using the H₂O₂ and NO fluorescent probes H₂DCFDA and DAF-FM DA. Intensive green fluorescence for both probes was observed in guard cells rather than in epidermal cells (Supplementary Fig. S4A, B at JXB online). However, the fact that no fluorescence was observed from epidermal cells was not due to death during the preparation of epidermal fragments, because >80% of epidermal cells were still alive when estimated using Evans blue dye (Supplementary Fig. S4C). These results indicated that the detected H₂O₂ and NO were mainly produced from guard cells of extracted epidermis when exposed to Ca²⁺.

**Chloroplasts are the cellular source for H₂O₂ production in Ca²⁺-exposed guard cells**

It is widely accepted that chloroplasts in guard cells of most higher plants are potential sources of H₂O₂ (Wang and Song, 2008). In the present experiments, chloroplasts of guard cells on abaxial epidermal sections were selected for study of Ca²⁺-induced H₂O₂ production using the CeCl₃ staining method. After exposure to 2 mM CaCl₂, H₂O₂ accumulation was visible using TEM as black precipitate spots on thylakoids and in stroma of chloroplasts (Fig. 2A–D), similar to the results seen for methyl viologen-induced H₂O₂ generation in spongy chloroplasts (Pellinen et al., 1999). Interestingly, intensive green fluorescence of H₂DCF from chloroplasts was also detected in 2 mM CaCl₂-exposed guard cells (Fig. 2E) compared with the control. ABA was suggested to lead to the formation of H₂O₂ in chloroplasts and stomatal closure (Zhang et al., 2001). Therefore, experiments were carried out to examine whether there is downstream signalling cross-talk between Ca²⁺ and ABA. Consistently, ABA-driven H₂O₂ accumulation in chloroplasts of guard cells was also observed (Supplementary Fig. S5 at JXB online).

It was then investigated whether Ca²⁺ could directly cause H₂O₂ generation from chloroplasts of guard cells in vitro. Purified chloroplasts were transferred to HMS buffer with or without (control) 10 mM CaCl₂, and the H₂O₂ signal was detected by the H₂O₂ sensor ISO-HPO. Here, chloroplasts in suspension buffer containing no CaCl₂ can also be detected to produce H₂O₂ (Fig. 3A). This is because transferring the stable chloroplasts to a new suspension buffer will also cause H₂O₂ generation without any stimulation until they are stable again. However, the chloroplasts incubated in HMS with 10 mM CaCl₂ responded much more strongly than the control within

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**Fig. 2.** Ca²⁺-induced H₂O₂ accumulation in guard cell chloroplasts from Arabidopsis. (A, B) TEM images of chloroplasts in guard cells with CeCl₃ staining from a leaf section incubated in 50 μM (A) or 2 mM (B) CaCl₂. (C) Further detail of A. (D) Further details of B. The black spots in the TEM images represent H₂O₂ forming electron-dense cerium perhydroxide precipitates. Examples of individual precipitates are shown by arrows. s, stroma; t, thylakoids. Bar=200 nm. (E) Confocal images of changes in H₂DCF fluorescence intensity from guard cell chloroplasts of the wild type in response to 50 μM (control) and 2 mM CaCl₂. H₂DCF, chlorophyll, and overlay fluorescence images from guard cell are shown. Bar=10 μm.
H_{2}O_{2} and NO generation are essential for Ca^{2+}-induced stomatal closure

The effects of Ca^{2+}, H_{2}O_{2}, or the NO donor SNP on stomatal closure have been demonstrated before (Pei et al., 2000; Neill et al., 2002a; Han et al., 2003). Therefore, the question was asked as to whether H_{2}O_{2} and NO generation are required for Ca^{2+}-induced stomatal closure. The fluorescent probes of H_{2}O_{2} and NO enabled determination of the kinetics of H_{2}O_{2} and NO changes in guard cells upon exposure to Ca^{2+} (Fig. 4A, B, D, E). According to Allen et al. (2001), 1–10 mM Ca^{2+} can lead to stomatal closure. To prolong the response of guard cells to Ca^{2+}, a low concentration (2 mM) of Ca^{2+} was used in this experiment. In the presence of Ca^{2+}, an alteration in H_{2}O_{2} or NO production in the wild type was visible by 15 min, reaching saturation at 25 min, and then slowly decreased, compared with the untreated control. These results were analogous to ABA-driven H_{2}O_{2} and NO formation in guard cells (Suhita et al., 2004; Gonugunta et al., 2008). Here, time-resolved measurements of Ca^{2+}, H_{2}O_{2}, and SNP-induced stomatal closure were also performed (Fig. 4C). Exposure to H_{2}O_{2} or SNP induced a striking decrease in stomatal aperture after 20 min. Comparably, with Ca^{2+} treatment, stomatal aperture decrease steeply after 30 min, which was a 15 min lag

Fig. 3. Effect of Ca^{2+} on H_{2}O_{2} generation in isolated Arabidopsis chloroplasts. (A) The upper trace represents the rapid H_{2}O_{2} production in chloroplasts incubated with 10 mM CaCl_{2}, and the lower trace represents less production of H_{2}O_{2} in chloroplasts incubated without CaCl_{2} (control). Data are averages of three samples ±SE. (B) Averaged increases in relative H_{2}DCF fluorescence in chloroplasts plotted as a function of applied CaCl_{2} for 10 min (n = 8, ±SE). Data were fitted to the Hill equation. (C) The trace shows the time-course of H_{2}O_{2} production in chloroplasts upon 2 mM CaCl_{2} supplementation. H_{2}DCF fluorescence was measured from the time at which CaCl_{2} was provided (n = 8, ±SE).

100 s (Fig. 3A), suggesting that Ca^{2+} enhanced H_{2}O_{2} production in chloroplasts. Using a fluorescence spectrophotometer, a Hill curve could be fitted to the data of CaCl_{2} dose-dependent H_{2}O_{2} production (Fig. 3B) with a Hill coefficient of ~1.1. The activation time course of 2 mM CaCl_{2} could be described by a simple sigmoid equation, with an activation time constant of 6 min (Fig. 3C). However, no pronounced increase in NO from chloroplasts was observed when exposed to 0–10 mM CaCl_{2} (Supplementary Fig. S6 at JXB online), indicating no correlation between Ca^{2+} and NO production in chloroplasts. It is well known that chloroplasts are the major source of H_{2}O_{2} in plants, and H_{2}O_{2} production in chloroplasts from guard cells or mesophyll cells in response to various stimuli has been well documented (Pellinen et al., 1999; Zhang et al., 2001), suggesting that the chloroplasts from guard cells and mesophyll cells are identical in their function to produce H_{2}O_{2}. Although total chloroplasts were used to study Ca^{2+}-induced H_{2}O_{2} production, the results may also represent Ca^{2+}-induced H_{2}O_{2} production in chloroplasts from guard cells. These data demonstrated that H_{2}O_{2}, but not NO, was produced in chloroplasts in response to a lower concentration of Ca^{2+} (~1.1 mM) within a few minutes.
behind Ca\(^{2+}\)-induced H\(_2\)O\(_2\) and NO production. Thus, H\(_2\)O\(_2\) or SNP appeared to result in a more rapid decrease in stomatal aperture than Ca\(^{2+}\), suggesting the possibility that H\(_2\)O\(_2\) and NO generation are required for stomatal closure in response to Ca\(^{2+}\).

The H\(_2\)O\(_2\) scavenger CAT and NO scavenger cPTIO were further used to study their effects on Ca\(^{2+}\)-induced H\(_2\)O\(_2\) and NO production as well as stomatal closure. Ca\(^{2+}\)-induced NO production in guard cells has been shown by using NO-specific fluorescent dyes (Garcia-Mata and Lamattina, 2007; Li et al., 2009). Here a significant increase in H\(_2\)DCF or DAF-FM fluorescence was also observed in guard cells in Ca\(^{2+}\)-treated epidermal peels \((P < 0.001)\), demonstrating the Ca\(^{2+}\)-induced H\(_2\)O\(_2\) and NO production in guard cells (Fig. 5). However, pre-treatment of guard cells with CAT or cPTIO significantly suppressed H\(_2\)O\(_2\) or NO generation and stomatal responses to Ca\(^{2+}\) in the wild-type plants (Col-0) \((P < 0.001)\) (Fig. 5; Table 1). In line with this result, the guard cells from *atrbohD/F* and *Atnoa1* were less sensitive to Ca\(^{2+}\) \((P < 0.001)\) (Table 1), indicating...
a requirement for H$_2$O$_2$ and NO synthesis for Ca$^{2+}$-induced stomatal closure. Furthermore, pre-treated epidermal peels with H$_2$O$_2$ or SNP can induce stomatal closure in the wild type ($P < 0.001$); however, both atrbohD/F and Atnoa1 stomata did close in response to SNP ($P < 0.001$) while Atnoa1 stomata were less responsive to H$_2$O$_2$ compared with the wild type and atrbohD/F ($P < 0.001$) (Table 1). Since H$_2$O$_2$ unidirectionally caused NO generation within 60 s in guard cells while NO had no effect on H$_2$O$_2$ production (Bright et al., 2006; Li et al., 2009), it is assumed that Ca$^{2+}$-induced NO generation is H$_2$O$_2$ dependent.

**Fig. 5.** CAS regulates Ca$^{2+}$-induced H$_2$O$_2$ and NO production in guard cells of Arabidopsis. (A, B) Guard cells pre-loaded with 50 µM H$_2$DCFDA in the wild type and CASas were incubated in MES buffer (control), 2 mM CaCl$_2$, or 2 mM CaCl$_2$ plus 100 U ml$^{-1}$ CAT for 20 min in darkness. H$_2$DCF fluorescence images and intensities of the guard cells were recorded in A and B, respectively. (C, D) Guard cells pre-loaded with 10 µM DAF-FM DA in the wild type and CASas were incubated in MES buffer (control), 2 mM CaCl$_2$, or 2 mM CaCl$_2$ plus 100 µM cPTIO for 20 min in darkness. DAF-FM fluorescence images and intensities of the guard cells were recorded in C and D, respectively. The data are expressed as the average ±SE ($n=100$). Bar=50 µm.

**Table 1.** The effects of CAT or cPTIO on Ca$^{2+}$-, H$_2$O$_2$-, or SNP-induced stomatal closure in various Arabidopsis genotypes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomatal aperture of each genotype$^a$</th>
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<tr>
<td></td>
<td>Col-0</td>
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<tr>
<td>None (control)</td>
<td>100.0±1.0</td>
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<tr>
<td>2 mM Ca$^{2+}$</td>
<td>58.9±0.7</td>
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<tr>
<td>2 mM Ca$^{2+}$+100 U ml$^{-1}$ CAT</td>
<td>86.2±1.1</td>
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<tr>
<td>2 mM Ca$^{2+}$+100 µM cPTIO</td>
<td>96.5±1.0</td>
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<tr>
<td>10 µM H$_2$O$_2$</td>
<td>66.0±0.7</td>
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<tr>
<td>60 µM SNP</td>
<td>67.4±0.8</td>
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$^a$ Stomatal aperture from Col-0 without any treatment is taken as 100%. Results are averages ±SE derived from analyses of stomatal apertures from 100 guard cells of at least three plants for each genotype.

$^b$ ND, not determined.

**CAS mediates H$_2$O$_2$ and NO generation during Ca$^{2+}$-induced stomatal closure**

Earlier studies have shown that stomatal movement is modulated by the Ca$^{2+}$ level through CAS while Ca$^{2+}$-induced stomatal closure is abolished in CASas lines (Han et al., 2003; Tang et al., 2007). Consistently, the results demonstrate that Ca$^{2+}$ fails to induce stomatal closure in the CASas mutants ($P <0.001$), and CASas plants pretreated with CAT or cPTIO remain impaired in Ca$^{2+}$-induced guard cell responses ($P >0.05$) (Table 1).
the stomatal bioassay showed that exogenous H₂O₂ or SNP induced stomatal closure in both Col-0 and CASas plants (P <0.001) (Table 1). Further, the involvement of CAS in Ca²⁺-induced H₂O₂ and NO production was investigated. It was found that H₂DCF or DAF-FM fluorescence intensity was impaired in CASas plants after Ca²⁺ treatment (P <0.001) (Figs 4A, B, D, E, 5). Consistently, fewer black precipitate spots of H₂O₂ were seen in CASas guard cell chloroplast (Fig. 6B) than in the wild type (Fig. 6A) in response to Ca²⁺. However, no precipitate spots of H₂O₂ were found in the cytoplasm, while some were detected on the plasma membrane of both wild-type and CASas guard cells in response to Ca²⁺ (Supplementary Fig. S7 at JXB online), which was due to the activation of plasma membrane NOX by Ca²⁺ (Sagi and Fluhr, 2001; Potocký et al., 2007). Although a small amount of NOX-driven H₂O₂ production somewhat promotes Ca²⁺-induced stomatal closure, H₂O₂ production in chloroplasts is more important for this process because chloroplasts and CAS are crucial for Ca²⁺-induced stomatal closure (Han et al., 2003; Nomura et al., 2008) and because Ca²⁺-induced H₂O₂ production was mainly seen in chloroplast (Fig. 2E). Meanwhile, H₂DCF fluorescence intensity was impaired in CASas guard cell chloroplasts after the exposure to Ca²⁺ (Fig. 6C). These results indicate that CAS affects events upstream of H₂O₂-dependent NO generation, thereby triggering stomatal closure.

Cas²⁺ induces changes in transcript levels of cytosolic antioxidant enzymes in Arabidopsis

In this study, transient H₂O₂ production induced by Ca²⁺ as well as stomatal closure induced by Ca²⁺ within 2 h have been demonstrated. Previous work showed that ABA, CaCl₂, H₂O₂, or SNP could increase the gene expression of cytosolic antioxidant enzymes such as superoxide dismutase, CAT, APX, and glutathione reductase in maize plants (Hu et al., 2005, 2007; Zhang et al., 2006; Sang et al., 2008). Based on these findings, it was decided to determine whether the cytosolic antioxidant system functions in H₂O₂ metabolism of Ca²⁺ stimulation. The expression of CSDI, which encodes a cytosolic superoxide dismutase that catalyses the conversion of O₂⁻ to H₂O₂ (Kliebenstein et al., 1998), started to increase at 4 h of 2 mM CaCl₂ treatment and reached a maximum at 20 h (Fig. 7A). APX1 and ATGRI, which encode cytosolic ascorbate peroxidase and glutathione-disulphide reductase, respectively, in the metabolism of H₂O₂ (Neill et al., 2002b), were expressed in leaves after 2 mM CaCl₂ treatment, with a decrease during the first 4–8 h, followed by an increase after 8 h (Fig. 7B, C). For the control, the expression of CSDI and APX1 remained unchanged during the 24 h period without CaCl₂ treatment, while ATGRI transcription declined gradually in MES buffer under light. Although the expression of antioxidant genes in stomatal cells is hard to detect, the cytosolic antioxidant system might function as a mediator in Ca²⁺ and H₂O₂ signalling in guard cells.

Discussion

In this study, the focus was on Ca²⁺-induced stomatal movement and the involvement of CAS, H₂O₂, NO, and the antioxidant system in the process. By combining the present data with results from other studies, a model was built describing the Ca²⁺ signal transduction pathway, which cross-talks with ABA signalling, leading to stomatal closure (Fig. 8). This model allowed the systematic understanding of the molecular mechanism of guard cells in sensing environmental cues.

Chloroplasts are the potential sites of CAS-mediated H₂O₂ generation in guard cells in response to Ca²⁺ stimulation

Chloroplasts were postulated to regulate stomatal movement as a sensor of environmental stresses (Wang and Song, 2008). Further evidence was provided for the chloroplast control of Ca²⁺-induced Ca²⁺ transients and stomatal closure (Nomura et al., 2008). H₂O₂ generation in...
chloroplasts occurred much earlier than within the other regions of guard cells after the application of exogenous ABA (Zhang et al., 2001). Consistent with this, TEM and confocal data indicate that ABA- and Ca\(^{2+}\)-induced stomatal closure were both accompanied by H\(_2\)O\(_2\) accumulation in chloroplasts (Fig. 2; Supplementary Fig. S5 at JXB online), suggesting a common route for Ca\(^{2+}\) and ABA signalling mediated by H\(_2\)O\(_2\) generation in chloroplasts. In the present experiments, it was found that a low concentration of Ca\(^{2+}\) could directly cause H\(_2\)O\(_2\) generation in vitro within a few minutes (Fig. 3). Therefore, it is possible that Ca\(^{2+}\)-induced H\(_2\)O\(_2\) accumulation in chloroplasts requires an early limited increase of Ca\(^{2+}\) which might be induced by the activation of membrane calcium channels.

CAS, a putative Ca\(^{2+}\)-binding protein localized in chloroplast thylakoid membranes (Han et al., 2003; Nomura et al., 2008), elicits a cascade of intracellular signalling events including the phospholipase C–inositol 1,4,5-triphosphate (PLC–IP\(_3\)) pathway, and mediates CICI (Tang et al., 2007). This signalling cascade is probably activated by a Ca\(^{2+}\) increase in chloroplast stroma. It was noted that a large Ca\(^{2+}\) increase in chloroplast stroma followed by Ca\(^{2+}\) oscillations was detected when tobacco leaves were transferred from light to darkness (Sai and Johnson, 2002). However, Ca\(^{2+}\) oscillations were disrupted in CASas plants (Han et al., 2003; Nomura et al., 2008; Weinl et al., 2008). These results suggested that elevation of the stroma Ca\(^{2+}\) level might be required for the CAS-mediated CICI signalling pathway. An early limited increase in Ca\(^{2+}\), regulated by the membrane calcium channel in response to Ca\(^{2+}\), may meet the need for the stromal Ca\(^{2+}\) increase required for CAS activation in chloroplasts. The observation that Ca\(^{2+}\)-induced H\(_2\)O\(_2\) accumulation in chloroplasts was impaired in CASas suggests that chloroplast H\(_2\)O\(_2\) may function downstream of CAS in the Ca\(^{2+}\) signalling pathway (Fig. 5A, B). In the studies of Han et al. (2003) and Nomura et al. (2008), Ca\(^{2+}\) induced a rapid (the first step) but impaired cytoplasmic Ca\(^{2+}\) increase in CASas, and the prolonged cytoplasmic Ca\(^{2+}\) oscillations (the second step) were significantly disrupted compared with the wild type. However, they are normal in terms of ABA-driven stomatal closure (Nomura et al., 2008). Based on these findings, it was proposed that Ca\(^{2+}\) causes biphasic Ca\(^{2+}\) transients. The first step is an early increase of Ca\(^{2+}\) that activates CAS in chloroplasts, followed by the second step for H\(_2\)O\(_2\) generation and long-term Ca\(^{2+}\) transients that also act downstream of ABA signalling through a different pathway such as cytosolic alkalinization (Suhita et al., 2004) and ABA receptors. In contrast to H\(_2\)O\(_2\), no firm evidence for Ca\(^{2+}\)-induced NO production in chloroplasts has been obtained (Supplementary Fig. S6 at JXB online). Therefore, it is possible that NO is produced around the chloroplasts through H\(_2\)O\(_2\) diffusion, considering the requirement for H\(_2\)O\(_2\) in ABA-induced NO generation (Bright et al., 2006).

**Fig. 7.** The transcription level of three cytosolic antioxidant enzymes in Arabidopsis leaves challenged with Ca\(^{2+}\). qRT-PCR analysis of time-resolved relative expression levels of CSD1, APX1, and ATGR1 in leaves incubated with MES buffer (control) or with 2 mM CaCl\(_2\) during 24 h is shown in (A), (B), and (C), respectively. The transcript levels at 0 h of incubation were set at 1. Error bars indicate the SE from three independent repeats.

**Fig. 8.** Schematic representation of the possible signalling cascade leading to the stomatal closure by Ca\(^{2+}\) and ABA. This model integrates the present data as well as results from previous studies described in the ‘Discussion’. The cascades for which the evidence is either ambiguous or still lacking are indicated by dotted arrows, while the well-established events (directly or indirectly) are represented by solid arrows.
Temporal sequence of CAS-mediated CiCl and stomatal closure

As described (Allen et al., 1999, 2000, 2001; Han et al., 2003; Weinl et al., 2008), 1–10 mM Ca\(^{2+}\) can stimulate an initial Ca\(^{2+}\) spiking within 30 s, followed by a long-term Ca\(^{2+}\) transient which was blocked in CAS\(_{a}\)s. However, 1–2 mM Ca\(^{2+}\)\(_{o}\)-induced stomatal closure was visible by ~30 min as described by Weinl et al. (2008) and in the present work (Fig. 4C), while 10 mM Ca\(^{2+}\)\(_{o}\) caused a much more rapid reduction in stomatal aperture (Allen et al., 2001). According to these reports, 2 mM or 10 mM Ca\(^{2+}\)\(_{o}\) were also used as an experimental stimulus to probe the guard cell signalling pathway in this study. The long-term Ca\(^{2+}\)\(_{i}\) transients regulated by CAS seem more likely to lead to the final stomatal closure. What happens to the guard cells is still unknown from the initial Ca\(^{2+}\)\(_{i}\) spiking at 30 s to the stomatal closure at 30 min except for long-term Ca\(^{2+}\)\(_{i}\) transients when exposed to 1–2 mM Ca\(^{2+}\)\(_{o}\), and how the guard cells maintain the long-term Ca\(^{2+}\)\(_{i}\) transients during this 30 min and perform closure that is regulated by CAS remained unclear before. H\(_{2}\)O\(_{2}\) at 100 \(\mu\)M was shown to cause one or two separate transients of Ca\(^{2+}\)\(_{i}\) leading to stomatal closure (Allen et al., 2000). Therefore, there is enough time (within 30 min) for guard cells to perform CAS-mediated H\(_{2}\)O\(_{2}\) production to maintain Ca\(^{2+}\)\(_{i}\) transients. The data indicate that Ca\(^{2+}\)\(_{o}\)-driven H\(_{2}\)O\(_{2}\) and NO generation, which were inhibited in CAS\(_{a}\)s, could be detected within 50–100 s (Fig. 1C, D) and increased steeply at ~15 min in guard cells, earlier than Ca\(^{2+}\)\(_{o}\)-induced stomatal closure (Fig. 4) but later than Ca\(^{2+}\)\(_{o}\)-driven H\(_{2}\)O\(_{2}\) production from chloroplasts at low concentration (Fig. 3). Interestingly, H\(_{2}\)O\(_{2}\) and SNP had more rapid effects than Ca\(^{2+}\)\(_{o}\) on stomatal closure (Fig. 4C), supporting the speculation that Ca\(^{2+}\)\(_{o}\) induces Ca\(^{2+}\)\(_{i}\) transients and stomatal closure through CAS regulation as well as H\(_{2}\)O\(_{2}\) and NO generation. As for the order of H\(_{2}\)O\(_{2}\) and NO generation, it was shown by Bright et al. (2006) that H\(_{2}\)O\(_{2}\) → NO seemed to be more acceptable. In the present work, the electrochemical sensors were used for H\(_{2}\)O\(_{2}\) and NO detection from the epidermis and chloroplasts. Ca\(^{2+}\)\(_{o}\)-induced H\(_{2}\)O\(_{2}\) and NO production was observed from guard cells or chloroplasts (Figs 1, 3A). Moreover, more rapid generation of H\(_{2}\)O\(_{2}\) than NO suggests that NO may act downstream of H\(_{2}\)O\(_{2}\). However, the time point when Ca\(^{2+}\)\(_{o}\) induced visible H\(_{2}\)O\(_{2}\) and NO production was not basically in line when comparing the two approaches. The exact reasons for such a discrepancy in real-time H\(_{2}\)O\(_{2}\) and NO detection from guard cells are not sure clear. This could be due to the varied sensitivity of each method, which, however, also resulted in an identical conclusion supporting the Ca\(^{2+}\)\(_{o}\) signal transduction model.

ABA was shown to promote Ca\(^{2+}\)\(_{i}\) transients, leading to stomatal closure (Allen et al., 2000). However, ABA-induced cytosolic alkalinization was visible by 10 min and ABA-induced H\(_{2}\)O\(_{2}\) and NO production were detected by ~15 min (Suhita et al., 2004; Gonugunta et al., 2008). These results regarding time are almost consistent with ABA-induced initial Ca\(^{2+}\)\(_{i}\) transients at >10 min. It is well known that cytosolic alkalinization, and H\(_{2}\)O\(_{2}\) and NO production are all required for ABA-induced stomatal closure and act upstream of long-term Ca\(^{2+}\)\(_{o}\) transients (Pei et al., 2000; Suhita et al., 2004; Bright et al., 2006; Gonugunta et al., 2008). Similar to ABA-induced H\(_{2}\)O\(_{2}\) and NO production, both of them are also needed to maintain Ca\(^{2+}\)\(_{i}\) transients in response to Ca\(^{2+}\)\(_{o}\) leading to stomatal closure.

Pharmacological analysis further supports the Ca\(^{2+}\)\(_{o}\) signalling transduction model

More evidence for the Ca\(^{2+}\)\(_{o}\) signalling model was provided by studying the effects of CAT and cPTIO on Ca\(^{2+}\)\(_{o}\)-induced H\(_{2}\)O\(_{2}\) and NO as well as stomatal closure in various Arabidopsis genotypes. The use of these two compounds in CAS\(_{a}\), atrbohD/F, and Atmoa1 plants reversed Ca\(^{2+}\)\(_{o}\)-induced stomatal closure and blocked Ca\(^{2+}\)\(_{o}\)-triggered H\(_{2}\)O\(_{2}\) and NO production in guard cells. NOX is plasma membrane located; however, a report showed that ABA induced H\(_{2}\)O\(_{2}\) synthesis not only via a plasma membrane NOX but also in the chloroplast, which occurred in chloroplasts earlier than within the other regions of guard cells (Zhang et al., 2001). In addition, a recent study revealed that chloroplasts played a critical role in CAS-mediated CiCl and subsequent stomatal closure in Arabidopsis (Nomura et al., 2008). As shown in Table 1, compared with the wild type, atrbohD/F did respond to Ca\(^{2+}\)\(_{o}\) but Ca\(^{2+}\)\(_{o}\)-induced stomatal closure was partially inhibited, while CAT, the H\(_{2}\)O\(_{2}\) scavenger, showed more efficiency in this inhibition. Furthermore, compared with the wild type and atrbohD/F, Ca\(^{2+}\)\(_{o}\) could not induce stomatal closure in CAS\(_{a}\)s, and Ca\(^{2+}\)\(_{o}\)-induced H\(_{2}\)O\(_{2}\) production was largely impaired in guard cells and chloroplasts in CAS\(_{a}\)s. The present data combined with those of other studies suggest that the CAS-mediated H\(_{2}\)O\(_{2}\) synthesis in chloroplast is more critical for Ca\(^{2+}\)\(_{o}\)-induced stomatal closure than of plasma membrane NOX. The relationship between CAS, H\(_{2}\)O\(_{2}\), and NO was further established by the observations that H\(_{2}\)O\(_{2}\) and SNP caused stomatal closure in both wild-type and CAS\(_{a}\)s plants. If CAS mediates Ca\(^{2+}\)\(_{o}\)-induced stomatal closure directly by Ca\(^{2+}\)\(_{i}\) transients and this does not require H\(_{2}\)O\(_{2}\) and NO production, it could not be understood why Ca\(^{2+}\)\(_{o}\)-induced stomatal closure was also inhibited by the two compounds as well as in atrbohD/F and Atmoa1 plants. Further evidence suggests that H\(_{2}\)O\(_{2}\) is required for NO generation because H\(_{2}\)O\(_{2}\) could not induce stomatal closure in Atmoa1 plants. A similar conclusion has been drawn in a previous study (Bright et al., 2006). The finding that H\(_{2}\)O\(_{2}\) and NO are involved in the CAS-mediated Ca\(^{2+}\)\(_{o}\) signalling in guard cells raises the questions of how the signals from CAS to H\(_{2}\)O\(_{2}\) are transducted and what are the signalling components involved in this process.

The putative Ca\(^{2+}\)\(_{o}\) signal transduction pathway cross-talks with ABA and the antioxidant system

ABA was shown to promote stomatal closure in CAS\(_{a}\)s plants (Nomura et al., 2008), suggesting that there is a signal...
converging site downstream of CAS during ABA and Ca\(^{2+}\)o signalling. In addition, ABA signalling has been known to be involved in H\(_2\)O\(_2\)-dependent NO generation (Bright et al., 2006), which were proven to act as downstream signal molecules of CAS in this study. Furthermore, findings of both Ca\(^{2+}\)o- and ABA-induced H\(_2\)O\(_2\) generation in chloroplasts indicate that H\(_2\)O\(_2\) may function as a common downstream component between ABA and CAS signalling (Fig. 6; Supplementary Fig. S3 at JXB online).

Interestingly, the antioxidant system seems to allow the plants to acclimatize to Ca\(^{2+}\)o-induced H\(_2\)O\(_2\) production. Antioxidant gene transcription was analysed after a short and long period of Ca\(^{2+}\)o stimulation (Fig. 7). When exposed to Ca\(^{2+}\)o, elevation of the CSDI expression level would promote cytosolic H\(_2\)O\(_2\) production, while biphasic responses of APX1 or ATGR1 expression could contribute to H\(_2\)O\(_2\) production at the beginning and H\(_2\)O\(_2\) scavenging after saturation. This \‘H\(_2\)O\(_2\) buffer\’ can protect the plant from oxidative damage due to a long period of Ca\(^{2+}\)o signalling. A similar phenomenon was observed in ABA-treated maize plants, suggesting that the antioxidant system could also function as the convergence point between ABA and Ca\(^{2+}\)o signalling.

In summary, by using pharmacological, biochemical, and genetic approaches, this study provided comprehensive supporting evidence for a putative signalling cascade during Ca\(^{2+}\)o-induced stomatal closure. Briefly, CAS is probably activated in chloroplast stroma by a Ca\(^{2+}\)o-induced early period of Ca\(^{2+}\) transients, which initiates an intracellular signalling cascade that involves H\(_2\)O\(_2\) and NO production, Ca\(^{2+}\) transients, and subsequent stomatal closure in Arabidopsis guard cells. Ca\(^{2+}\)o-induced H\(_2\)O\(_2\) and NO production and Ca\(^{2+}\) transients as well as antioxidant enzymes transcriptional changes were also found in ABA-induced stomatal closure, which suggest a signalling interaction between Ca\(^{2+}\)o, ABA, and the antioxidant enzymes system in stomatal movement.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Verifying the mutants using PCR and western blot.

Figure S2. The integrity of isolated chloroplasts was estimated by H\(_2\)DCF fluorescent dye.

Figure S3. The effects of CAT or cPTIO on H\(_2\)O\(_2\) or NO current detected by a H\(_2\)DCF- or NO-selective electrochemical sensor.

Figure S4. Localization of H\(_2\)O\(_2\) and NO on Arabidopsis epidermal peels in response to Ca\(^{2+}\)o.

Figure S5. ABA-induced H\(_2\)O\(_2\) accumulation in Arabidopsis guard cell chloroplasts from the wild type with CeCl\(_3\) staining viewed using TEM.

Figure S6. Effect of Ca\(^{2+}\) on NO generation in isolated Arabidopsis chloroplasts.

Figure S7. TEM images of plasma membrane and cytoplasm in Arabidopsis guard cell with CeCl\(_3\) staining in response to Ca\(^{2+}\)o.

Table S1. Primer sequences used for PCR analysis.
Table S2. Primer sequences used for RT-PCR analysis.

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