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RESEARCH PAPER

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

Wen-Hua Wang[1,*], Xiao-Qian Yi[1,*], Ai-Dong Han[2], Ting-Wu Liu[1], Juan Chen[1], Fei-Hua Wu[1], Xue-Jun Dong[3], Jun-Xian He[4], Zhen-Ming Pei[5] and Hai-Lei Zheng[1,†]

[1] Key Laboratory for Subtropical Wetland Ecosystem Research of MOE, College of the Environment and Ecology, Xiamen University, Xiamen, Fujian 361005, China
[2] Key Laboratory for Cell Biology of MOE, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, China
[3] Central Grasslands Research Extension Center, North Dakota State University, Streeter, ND 58483, USA
[4] State Key Laboratory of Agrobiotechnology and School of Life Sciences, The Chinese University of Hong Kong, Hong Kong, PR China
[5] Department of Biology, Duke University, Durham, NC 27708, USA

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: zhenghl@xmu.edu.cn

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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+}_i) 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 (H_2O_2) 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 H_2O_2 and NO production from guard cells but only H_2O_2 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 H_2O_2 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 H_2O_2 signalling in guard cells. The results suggest a hypothetical model whereby Ca^{2+}_o induces H_2O_2 and NO accumulation in guard cells through the CAS signalling pathway, which further triggers Ca^{2+}_i 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+}_i) 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+}_i 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 (H_2O_2) was shown to be a second messenger in response to biotic and abiotic perturbations (Fukao and Bailey-Serres, 2004; Laloi *et al.*, 2004).

H_2O_2 also functions as a signalling molecule in abscisic acid (ABA)-induced stomatal movements (Pei *et al.*, 2000). The AtrbohD and AtrbohF (*Arabidopsis* respiratory burst oxidase homologues D and F) NADPH oxidases double mutant *atrbohD/F* abolishes the ABA-driven $Ca^{2+}{}_i$ increase and stomatal closure (Kwak *et al.*, 2003). Meanwhile, H_2O_2 has been found to stimulate $Ca^{2+}{}_i$ transients in regulation of stomatal movement (Allen *et al.*, 2000; Pei *et al.*, 2000). Extracellular calmodulin (ExtCaM), a $Ca^{2+}{}_o$-activated protein, induces H_2O_2 generation and the increased H_2O_2 further elevates stomatal $Ca^{2+}{}_i$ concentration (Chen *et al.*, 2004), suggesting that $Ca^{2+}{}_o$ signalling, H_2O_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.*, 2002*a*), operates as a downstream player of H_2O_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_2O_2 generation. Like H_2O_2, an NO-induced $Ca^{2+}{}_i$ increase and stomatal closure have also been found in guard cells of *Arabidopsis* and *Vicia faba* (Neill *et al.*, 2002*a*; 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_2O_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 (Pfannschmidt, 2003). The application of exogenous ABA resulted in H_2O_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_2O_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_2O_2 in chloroplasts.

Besides what is known about guard cell signalling pathways, other findings also suggested the correlation among $Ca^{2+}{}_o$, H_2O_2, NO, and chloroplasts. $Ca^{2+}{}_o$ can increase the activity of NADPH oxidase (NOX) and trigger H_2O_2 production in maize leaves (Sagi and Fluhr, 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_2O_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_2O_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_2O_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_2O_2- and NO-selective electrochemical sensors was used to detect H_2O_2 and NO production in epidermis or chloroplasts. Different tools were also utilized to characterize functionally the role of H_2O_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 (*CASas*) and *Arabidopsis* mutants defective in H_2O_2 or NO synthesis (e.g. the H_2O_2 synthetic enzymatic mutant *atrbohD/F* and a mutant indirectly impaired in NO synthesis *Atnoa1*), a histochemical technique, and H_2O_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_2O_2 generation. A functional relationship among CAS, H_2O_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 *CASas*, *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_2O_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-tetramethylimidazoline-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 $Ca^{2+}{}_o$-, H_2O_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 H_2O_2 and NO in guard cells were examined by loading 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) and 3-amino,4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) as described (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008). Abaxial epidermal strips with open stomata were transferred to MES buffer with 50 μM H_2DCFDA 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 MetaMorph 7.5 software. H_2DCF 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. H_2DCF fluorescence was detected from 495 nm to 535 nm, whereas chlorophyll autofluorescence was detected between 630 nm and 730 nm.

Subcellular localization of H_2O_2 in guard cells

The histochemical localization of H_2O_2 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 mm^2) from leaves incubated in MES buffer with or without 2 mM $CaCl_2$ or 10 μM ABA were excised and kept in 50 mM MOPS buffer containing 5 mM $CeCl_3$ at pH 7.2 under vacuum for 20 min. After being double fixed with 2.5% glutaraldehyde and 1.0% OsO_4, the $CeCl_3$-treated sections were dehydrated in an ascending ethanol series and embedded with the abaxial epidermis clinging 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 mm^2 in size, were ready for H_2O_2 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 $MgSO_4$, 0.3 M sorbitol, pH 7.2. The chloroplast counts were determined microscopically using a haemocytometer.

H_2O_2 and NO measurement by the Apollo 4000 system

The H_2O_2- and NO-selective electrochemical sensors ISO-HPO and ISO-NOP (WPI, Sarasota, FL, USA), widely used for animals, plants, microalga, and subcellular organelles (Bouchard and Yamasaki, 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 H_2O_2 and NO from *Arabidopsis* leaf epidermis or chloroplast. Both sensors consist of a combination of an internal H_2O_2- 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 H_2O_2- or NO-selective membrane that covers the end of the stainless steel. The poise voltage of each sensor was set to 400 mV for H_2O_2 or 865 mV for NO detection. Direct current from each sensor presents the environmental H_2O_2 or NO concentration and the data are recorded on a PC connected to the Apollo 4000 system. The H_2O_2 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* rosette 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 $Ca^{2+}{}_o$-induced H_2O_2 and NO production from the epidermis, 10 mM or 50 μM $CaCl_2$ (in MES buffer) was supplemented when the current signal became stable. For H_2O_2 detection from chloroplasts, a chloroplast suspension of 3×10^6 individuals was transferred to HMS buffer with or without 10 mM $CaCl_2$. The H_2O_2 sensor was calibrated in a set of known H_2O_2 standards (0–10 μM range) while the NO sensor was calibrated with *S*-nitroso-*N*-acetyl-penicillamine (SNAP) in 0.1 M $CuCl_2$ (0–50 nM range of NO) as described by the manufacturer (Fig. 1A, B).

Fluorometric detection of H_2O_2 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 H_2O_2 and NO production from chloroplasts. For all experiments, chloroplasts were first incubated in HMS buffer with 50 μM H_2DCFDA 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 $Ca^{2+}{}_o$ induction of H_2O_2 and NO production, H_2DCF- or DAF-FM-pre-loaded chloroplasts were incubated in HMS buffer with 0–2 mM $CaCl_2$ for 10 min. For time-related analysis, 2 mM $CaCl_2$ 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 5×10^4 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 $CaCl_2$ 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 *ATGR1* genes (GenBank accession nos AT1G08830, AT1G07890, and AT3G24170, respectively) acquired from NCBI. The primers used for

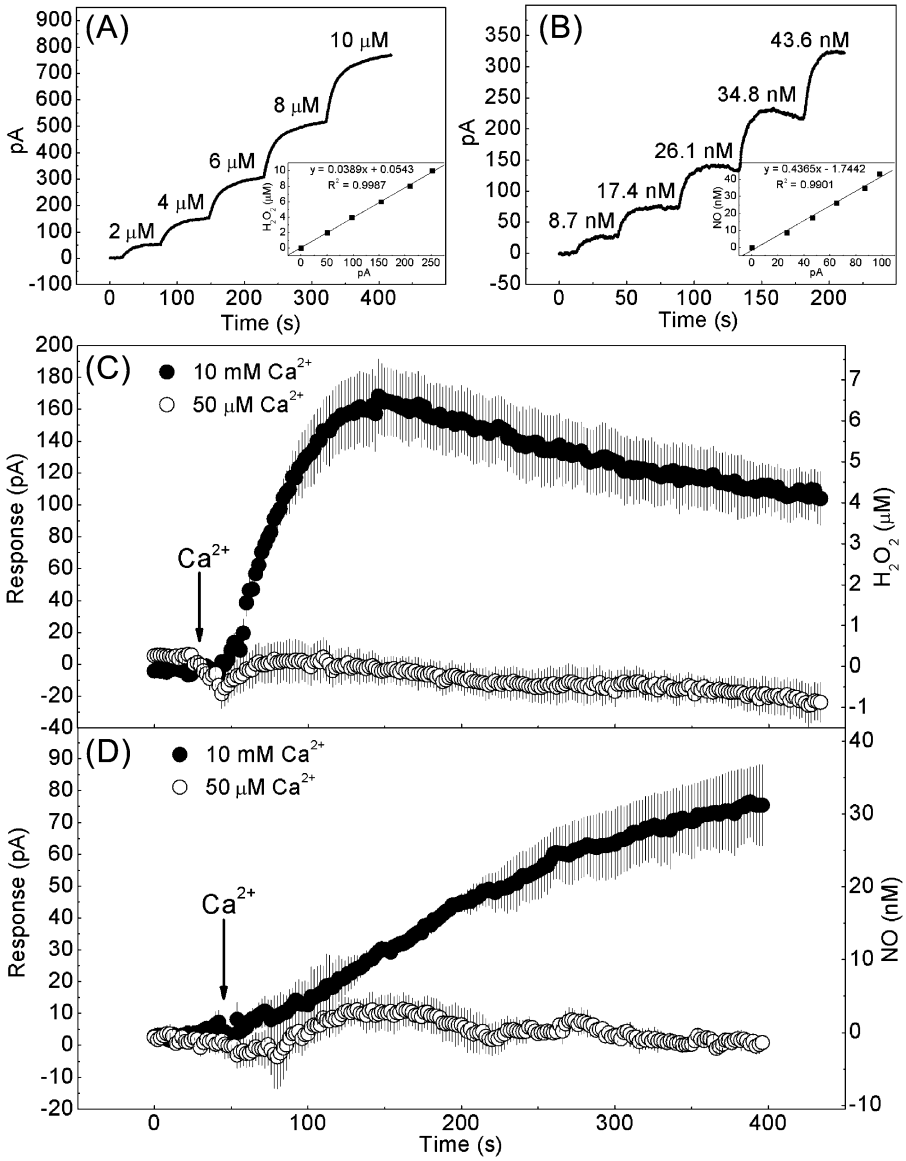

Fig. 1. Electrochemical detection of H_2O_2 and NO production from *Arabidopsis* epidermis in response to $Ca^{2+}{}_o$. (A, B) Calibration of the H_2O_2- and NO-selective electrochemical sensors ISO-HPO (A) and ISO-NOP (B). The current output jumped rapidly after each addition of H_2O_2 of known concentration or SNAP in $CuCl_2$ solution that produced a known concentration of NO. The calibration curve is shown in the inset. (C, D) Trace obtained upon supplementation with 10 mM (upper trace) or 50 μM (lower trace) $CaCl_2$ in epidermal peel suspensions using ISO-HOP (C) and ISO-NOP (D). Each trace represents the average of three samples (±SE). The arrow shows the time point at which $CaCl_2$ was applied.

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 ΔΔCt method described by Livak and Schmittgen (2001) with Actin2 as the internal control.

Results

$Ca^{2+}{}_o$ induces H_2O_2 and NO production in guard cells of Arabidopsis epidermis

To assess the effects of $Ca^{2+}{}_o$ on promoting H_2O_2 and NO production from *Arabidopsis* epidermis, electrochemical sensors ISO-HPO and ISO-NOP were used to detect H_2O_2 and NO, respectively, in epidermal peels. Calibrations of both sensors were performed, and changes in the concentration of H_2O_2 and NO were then calculated based on a linear relationship between H_2O_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 H_2O_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, H_2O_2 production from the epidermis monitored by a H_2O_2 electrochemical sensor

rapidly increased to nearly 7 μM within 100 s and then reached a plateau (Fig. 1C). While high $CaCl_2$ caused a gradual increase of NO in epidermis, no significant change occurred after adding a low $CaCl_2$ concentration to a suspension of epidermal peels (Fig. 1D). In addition, NO synthesis seems to lag behind that of H_2O_2 when the epidermis is exposed to $Ca^{2+}{}_o$ signal.

In order to determine whether the detected production of H_2O_2 and NO was mainly released from guard cells, the H_2O_2 and NO targets in *Arabidopsis* epidermal peels in response to $Ca^{2+}{}_o$ were investigated. The endogenous H_2O_2 and NO in 10 mM $CaCl_2$-pre-incubated epidermal peels were determined by using the H_2O_2 and NO fluorescent probes H_2DCFDA 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 H_2O_2 and NO were mainly produced from guard cells of extracted epidermis when exposed to $Ca^{2+}{}_o$.

Chloroplasts are the cellular source for H_2O_2 production in $Ca^{2+}{}_o$-exposed guard cells

It is widely accepted that chloroplasts in guard cells of most higher plants are potential sources of H_2O_2 (Wang and Song, 2008). In the present experiments, chloroplasts of guard cells on abaxial epidermal sections were selected for study of $Ca^{2+}{}_o$-induced H_2O_2 production using the $CeCl_3$ staining method. After exposure to 2 mM $CaCl_2$, H_2O_2 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 H_2O_2 generation in spongy chloroplasts (Pellinen *et al.*, 1999). Interestingly, intensive green fluorescence of H_2DCF from chloroplasts was also detected in 2 mM $CaCl_2$-exposed guard cells (Fig. 2E) compared with the control. ABA was suggested to lead to the formation of H_2O_2 in chloroplasts and stomatal closure (Zhang *et al.*, 2001). Therefore, experiments were carried out to examine whether there is downstream signalling cross-talk between $Ca^{2+}{}_o$ and ABA. Consistently, ABA-driven H_2O_2 accumulation in chloroplasts of guard cells was also observed (Supplementary Fig. S5 at *JXB* online).

It was then investigated whether Ca^{2+} could directly cause H_2O_2 generation from chloroplasts of guard cells *in vitro*. Purified chloroplasts were transferred to HMS buffer with or without (control) 10 mM $CaCl_2$, and the H_2O_2 signal was detected by the H_2O_2 sensor ISO-HPO. Here, chloroplasts in suspension buffer containing no $CaCl_2$ can also be detected to produce H_2O_2 (Fig. 3A). This is because transferring the stable chloroplasts to a new suspension buffer will also cause H_2O_2 generation without any stimulation until they are stable again. However, the chloroplasts incubated in HMS with 10 mM $CaCl_2$ responded much more strongly than the control within

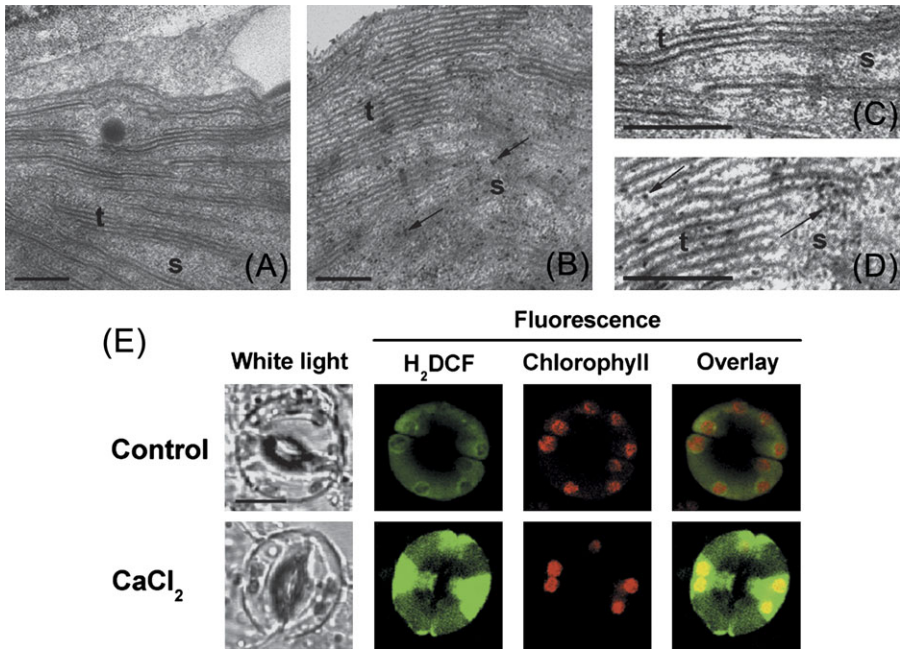

Fig. 2. $Ca^{2+}{}_o$-induced H_2O_2 accumulation in guard cell chloroplasts from *Arabidopsis*. (A, B) TEM images of chloroplasts in guard cells with $CeCl_3$ staining from a leaf section incubated in 50 μM (A) or 2 mM (B) $CaCl_2$. (C) Further detail of A. (D) Further details of B. The black spots in the TEM images represent H_2O_2 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 H_2DCF fluorescence intensity from guard cell chloroplasts of the wild type in response to 50 μM (control) and 2 mM $CaCl_2$. H_2DCF, chlorophyll, and overlay fluorescence images from guard cell are shown. Bar=10 μm.

100 s (Fig. 3A), suggesting that Ca^{2+} enhanced H_2O_2 production in chloroplasts. Using a fluorescence spectrophotometer, a Hill curve could be fitted to the data of $CaCl_2$ dose-dependent H_2O_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_2O_2 in plants, and H_2O_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_2O_2. Although total chloroplasts were used to study Ca^{2+}-induced H_2O_2 production, the results may also represent Ca^{2+}-induced H_2O_2 production in chloroplasts from guard cells. These data demonstrated that H_2O_2, but not NO, was produced in chloroplasts in response to a lower concentration of Ca^{2+} (~1.1 mM) within a few minutes.

H_2O_2 and NO generation are essential for $Ca^{2+}{}_o$-induced stomatal closure

The effects of $Ca^{2+}{}_o$, H_2O_2, or the NO donor SNP on stomatal closure have been demonstrated before (Pei *et al.*, 2000; Neill *et al.*, 2002*a*; Han *et al.*, 2003). Therefore, the question was asked as to whether H_2O_2 and NO generation are required for $Ca^{2+}{}_o$-induced stomatal closure. The fluorescent probes of H_2O_2 and NO enabled determination of the kinetics of H_2O_2 and NO changes in guard cells upon exposure to $Ca^{2+}{}_o$ (Fig. 4A, B, D, E). According to Allen *et al.* (2001), 1–10 mM $Ca^{2+}{}_o$ can lead to stomatal closure. To prolong the response of guard cells to $Ca^{2+}{}_o$, a low concentration (2 mM) of $Ca^{2+}{}_o$ was used in this experiment. In the presence of $Ca^{2+}{}_o$, an alteration in H_2O_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_2O_2 and NO formation in guard cells (Suhita *et al.*, 2004; Gonugunta *et al.*, 2008). Here, time-resolved measurements of $Ca^{2+}{}_o$-, H_2O_2-, and SNP-induced stomatal closure were also performed (Fig. 4C). Exposure to H_2O_2 or SNP induced a striking decrease in stomatal aperture after 20 min. Comparably, with $Ca^{2+}{}_o$ treatment, stomatal aperture decrease steeply after 30 min, which was a 15 min lag

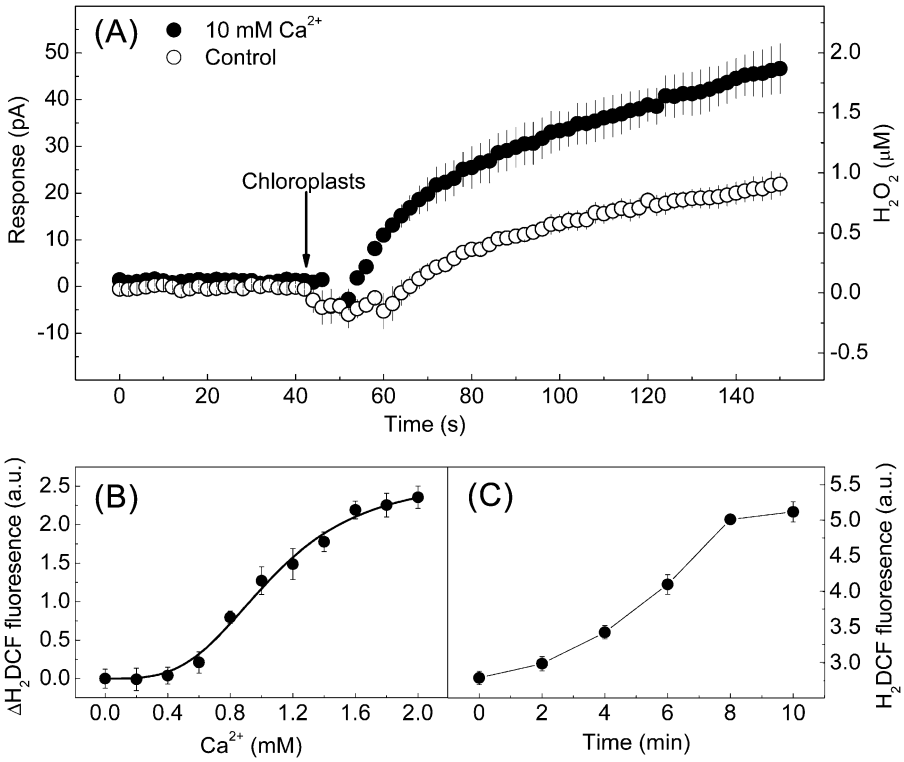

Fig. 3. Effect of Ca^{2+} on H_2O_2 generation in isolated *Arabidopsis* chloroplasts. (A) The upper trace represents the rapid H_2O_2 production in chloroplasts incubated with 10 mM $CaCl_2$, and the lower trace represents less production of H_2O_2 in chloroplasts incubated without $CaCl_2$ (control). Data are averages of three samples ±SE. (B) Averaged increases in relative H_2DCF 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_2O_2 production in chloroplasts upon 2 mM $CaCl_2$ supplementation. H_2DCF fluorescence was measured from the time at which $CaCl_2$ was provided (n=8, ±SE).

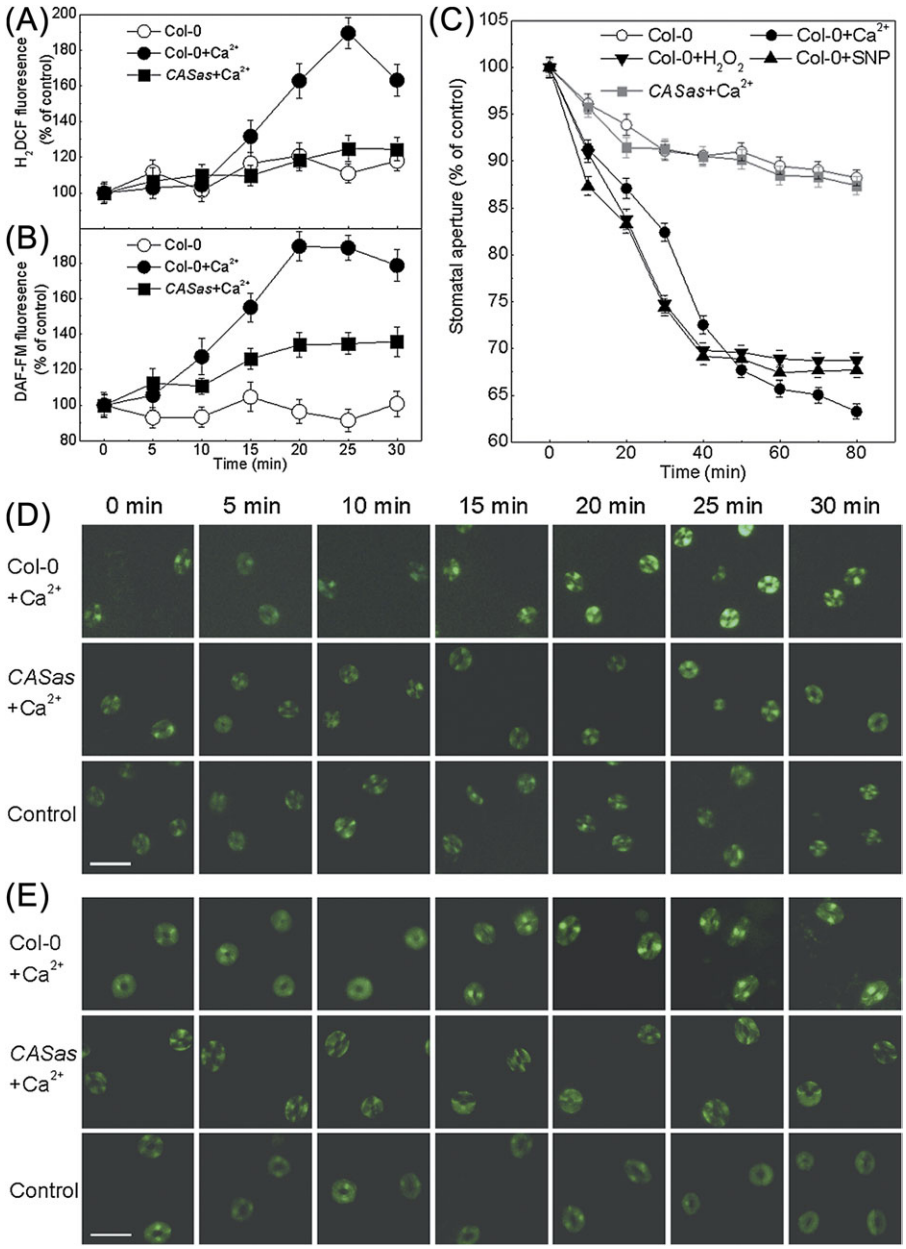

Fig. 4. H_2O_2 and NO productions occur before stomatal closure induced by Ca^{2+}_o in *Arabidopsis*. (A, B) Kinetics of the increase in H_2DCF (A) and DAF-FM (B) fluorescence intensities in guard cells of both wild type and *CASas* in response to 2 mM Ca^{2+}_o (n=50, ±SE). The relative changes in H_2O_2 and NO production were expressed using the fluorescence at time zero as the standard (100%). (D, E) Examples of H_2DCF (D) and DAF-FM (E) fluorescence images of guard cells from A and B, respectively. Bar=50 μm. (C) Kinetics of 2 mM Ca^{2+}_o-, 10 μM H_2O_2-, and 60 μM SNP-induced stomatal closure of both the wild type and *CASas* (n=100, ±SE). Stomatal apertures (width to length) were measured as the percentage using zero time as the standard (100%).

behind Ca^{2+}_o-induced H_2O_2 and NO production. Thus, H_2O_2 or SNP appeared to result in a more rapid decrease in stomatal aperture than Ca^{2+}_o, suggesting the possibility that H_2O_2 and NO generation are required for stomatal closure in response to Ca^{2+}_o.

The H_2O_2 scavenger CAT and NO scavenger cPTIO were further used to study their effects on Ca^{2+}_o-induced H_2O_2 and NO production as well as stomatal closure. Ca^{2+}_o-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_2DCF or DAF-FM fluorescence was also observed in guard cells in Ca^{2+}_o-treated epidermal peels (P <0.001), demonstrating the Ca^{2+}_o-induced H_2O_2 and NO production in guard cells (Fig. 5). However, pre-treatment of guard cells with CAT or cPTIO significantly suppressed H_2O_2 or NO generation and stomatal responses to Ca^{2+}_o 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+}_o (P <0.001) (Table 1), indicating

a requirement for H_2O_2 and NO synthesis for $Ca^{2+}{}_o$-induced stomatal closure. Furthermore, pre-treated epidermal peels with H_2O_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_2O_2 compared with the wild type and *atrbohD/F* (P <0.001) (Table 1). Since H_2O_2 unidirectionally caused NO generation within 60 s in guard cells while NO had no effect on H_2O_2 production (Bright *et al.*, 2006; Li *et al.*, 2009), it is assumed that $Ca^{2+}{}_o$-induced NO generation is H_2O_2 dependent.

CAS mediates H_2O_2 and NO generation during $Ca^{2+}{}_o$-induced stomatal closure

Earlier studies have shown that stomatal movement is modulated by the $Ca^{2+}{}_o$ level through CAS while $Ca^{2+}{}_o$-induced stomatal closure is abolished in *CASas* lines (Han *et al.*, 2003; Tang *et al.*, 2007). Consistently, the results demonstrate that $Ca^{2+}{}_o$ fails to induce stomatal closure in the *CASas* mutants (P <0.001), and *CASas* plants pre-treated with CAT or cPTIO remain impaired in $Ca^{2+}{}_o$-induced guard cell responses (P >0.05) (Table 1). However,

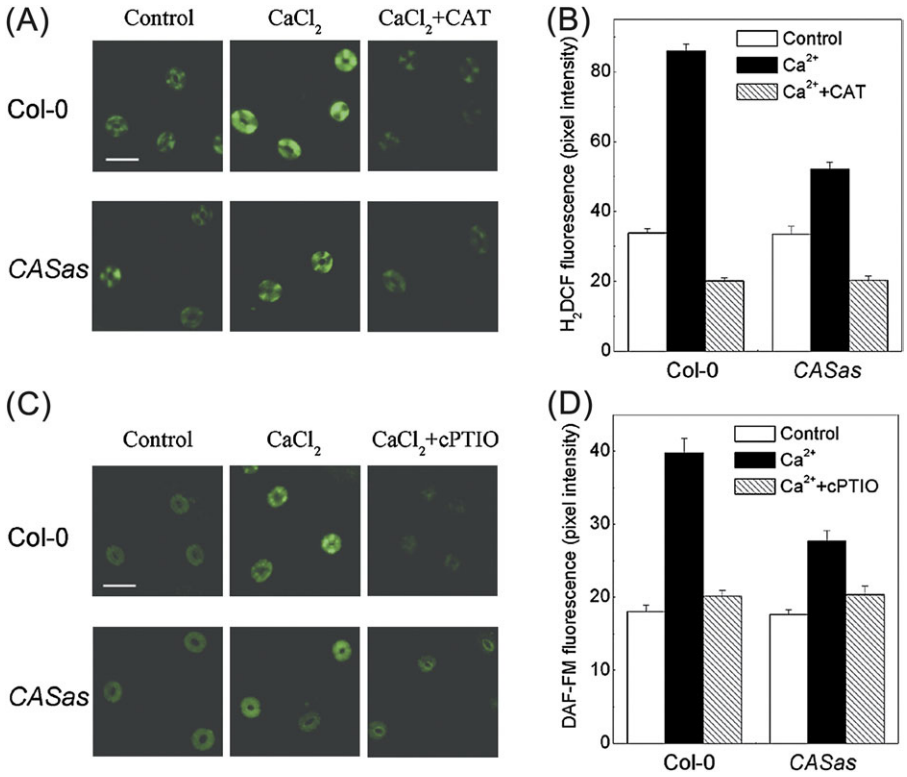

Fig. 5. CAS regulates $Ca^{2+}{}_o$-induced H_2O_2 and NO production in guard cells of *Arabidopsis*. (A, B) Guard cells pre-loaded with 50 μM H_2DCFDA in the wild type and *CASas* were incubated in MES buffer (control), 2 mM $CaCl_2$, or 2 mM $CaCl_2$ plus 100 U ml^{-1} CAT for 20 min in darkness. H_2DCF 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_2O_2-, or SNP-induced stomatal closure in various *Arabidopsis* genotypes

Treatment	Stomatal aperture of each genotype[a]			
	Col-0	***CASas***	***atrbohD/F***	***Atnoa1***
None (control)	100.0±1.0	102.3±1.2	100.7±1.2	101.3±1.1
2 mM Ca^{2+}	58.9±0.7	103.2±1.3	83.5±1.2	84.6±1.1
2 mM Ca^{2+}+100 U ml^{-1} CAT	86.2±1.1	101.1±1.4	ND[b]	ND
2 mM Ca^{2+}+100 μM cPTIO	96.5±1.0	101.7±1.2	ND	ND
10 μM H_2O_2	66.0±0.7	71.3±1.0	59.9±0.8	87.7±1.2
60 μM SNP	67.4±0.8	74.0±1.0	65.4±0.8	66.2±0.8

[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.

the stomatal bioassay showed that exogenous H_2O_2 or SNP induced stomatal closure in both Col-0 and *CASas* plants (P <0.001) (Table 1). Further, the involvement of CAS in Ca^{2+}_o-induced H_2O_2 and NO production was investigated. It was found that H_2DCF or DAF-FM fluorescence intensity was impaired in *CASas* plants after Ca^{2+}_o treatment (P <0.001) (Figs 4A, B, D, E, 5). Consistently, fewer black precipitate spots of H_2O_2 were seen in *CASas* guard cell chloroplast (Fig. 6B) than in the wild type (Fig. 6A) in response to Ca^{2+}_o. However, no precipitate spots of H_2O_2 were found in the cytoplasm, while some were detected on the plasma membrane of both wild-type and *CASas* guard cells in response to Ca^{2+}_o (Supplementary Fig. S7 at *JXB* online), which was due to the activation of plasma membrane NOX by Ca^{2+}_o (Sagi and Fluhr, 2001; Potocký *et al.*, 2007). Although a small amount of NOX-driven H_2O_2 production somewhat promotes Ca^{2+}_o-induced stomatal closure, H_2O_2 production in chloroplasts is more important for this process because chloroplasts and CAS are crucial for Ca^{2+}_o-induced stomatal closure (Han *et al.*, 2003; Nomura *et al.*, 2008) and because Ca^{2+}_o-induced H_2O_2 production was mainly seen in chloroplast (Fig. 2E). Meanwhile, H_2DCF fluorescence intensity was impaired in *CASas* guard cell chloroplasts after the exposure to Ca^{2+}_o (Fig. 6C). These results indicate that CAS affects events upstream of H_2O_2-dependent NO generation, thereby triggering stomatal closure.

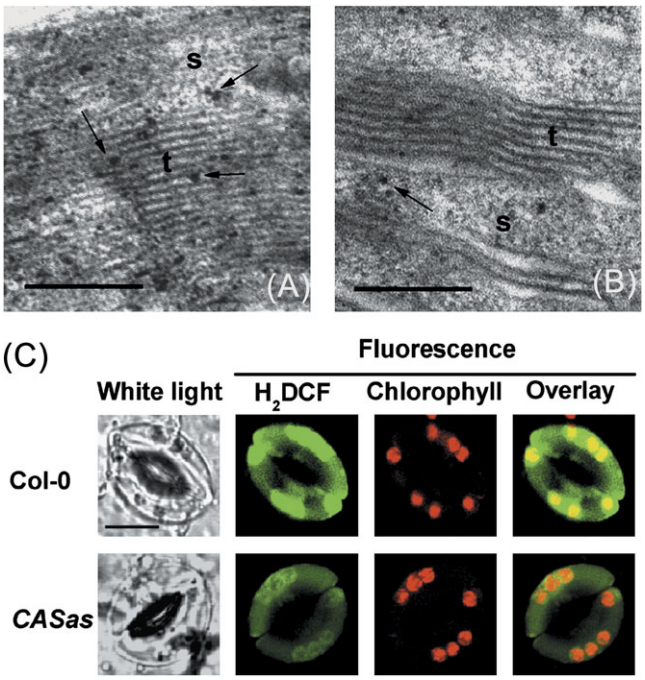

Fig. 6. Ca^{2+}_o-induced H_2O_2 accumulation in *Arabidopsis* guard cell chloroplasts is impaired in *CASas*. (A) TEM images showing the 2 mM $CaCl_2$-induced H_2O_2 accumulation in guard cell chloroplasts with $CeCl_3$ staining in the wild type. (B) Limited black precipitate spots in chloroplast of *CASas* guard cell with $CeCl_3$ staining exposed to 2 mM $CaCl_2$. The black spots in the TEM images represent H_2O_2 forming electron-dense cerium perhydroxide precipitates. Examples of individual precipitates are shown by arrows. s, stroma; t, thylakoids. Bar=200 nm. (C) Confocal images of H_2DCF fluorescence intensity from guard cell chloroplasts of the wild type and *CASas* in response to 2 mM $CaCl_2$. H_2DCF, chlorophyll, and overlay fluorescence images from guard cells are shown. The scale bar represents 10 μm.

Ca^{2+}_o induces changes in transcript levels of cytosolic antioxidant enzymes in Arabidopsis

In this study, transient H_2O_2 production induced by Ca^{2+}_o as well as stomatal closure induced by Ca^{2+}_o within 2 h have been demonstrated. Previous work showed that ABA, $CaCl_2$, H_2O_2, 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 H_2O_2 metabolism of Ca^{2+}_o stimulation. The expression of *CSD1*, which encodes a cytosolic superoxide dismutase that catalyses the conversion of O_2- to H_2O_2 (Kliebenstein *et al.*, 1998), started to increase at 4 h of 2 mM $CaCl_2$ treatment and reached a maximum at 20 h (Fig. 7A). *APX1* and *ATGR1*, which encode cytosolic ascorbate peroxidase and glutathione-disulphide reductase, respectively, in the metabolism of H_2O_2 (Neill *et al.*, 2002*b*), were expressed in leaves after 2 mM $CaCl_2$ 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 *CSD1* and *APX1* remained unchanged during the 24 h period without $CaCl_2$ treatment, while *ATGR1* 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 Ca^{2+}_o and H_2O_2 signalling in guard cells.

Discussion

In this study, the focus was on Ca^{2+}_o-induced stomatal movement and the involvement of CAS, H_2O_2, NO, and the antioxidant system in the process. By combining the present data with results from other studiess, a model was built describing the Ca^{2+}_o 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 H_2O_2 generation in guard cells in response to Ca^{2+}_o 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 Ca^{2+}_o-induced Ca^{2+}_i transients and stomatal closure (Nomura *et al.*, 2008). H_2O_2 generation in

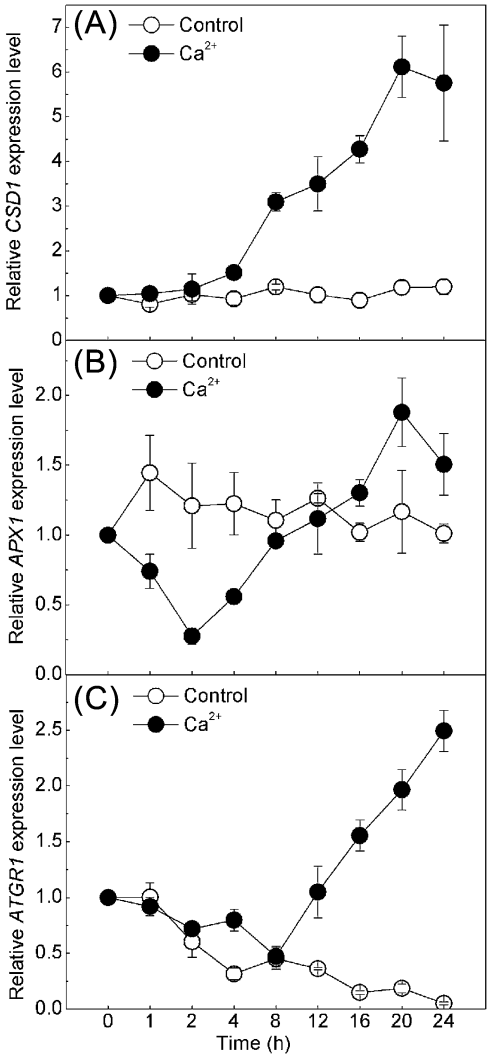

Fig. 7. The transcription level of three cytosolic antioxidant enzymes in *Arabidopsis* leaves challenged with Ca^{2+}_o. qRT-PCR analysis of time-resolved relative expression levels of *CSD1*, *APX1*, and *ATGR1* in leaves incubated with MES buffer (control) or with 2 mM $CaCl_2$ during 24 h is shown 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.

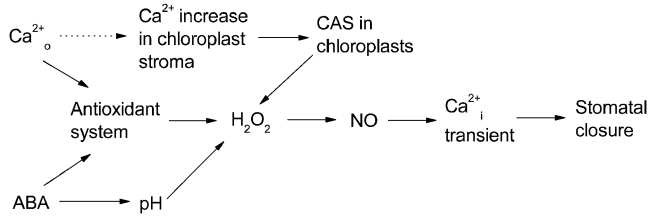

Fig. 8. Schematic representation of the possible signalling cascade leading to the stomatal closure by Ca^{2+}_o and ABA. This model integrates the present data as well as results from previous studies described in the 'Discussion'. The cascades for which the evidence is either ambiguous or still lacking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

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+}_o-induced stomatal closure were both accompanied by H_2O_2 accumulation in chloroplasts (Fig. 2; Supplementary Fig. S5 at *JXB* online), suggesting a common route for Ca^{2+}_o and ABA signalling mediated by H_2O_2 generation in chloroplasts. In the present experiments, it was found that a low concentration of Ca^{2+} could directly cause H_2O_2 generation *in vitro* within a few minutes (Fig. 3). Therefore, it is possible that Ca^{2+}_o-induced H_2O_2 accumulation in chloroplasts requires an early limited increase of Ca^{2+}_i 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+}_i transients was detected when tobacco leaves were transferred from light to darkness (Sai and Johnson, 2002). However, Ca^{2+}_o-induced Ca^{2+}_i transients 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+}_i regulated by the membrane calcium channel in response to Ca^{2+}_o may meet the need for the stromal Ca^{2+} increase required for CAS activation in chloroplasts. The observation that Ca^{2+}_o-induced H_2O_2 accumulation in chloroplast was impaired in *CASas* suggests that chloroplast H_2O_2 may function downstream of CAS in the Ca^{2+}_o signalling pathway (Fig. 5A, B). In the studies of Han *et al.* (2003) and Nomura *et al.* (2008), Ca^{2+}_o 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 is proposed that Ca^{2+}_o causes biphasic Ca^{2+}_i transients. The first step is an early increase of Ca^{2+}_i that activates CAS in chloroplasts, followed by the second step for H_2O_2 generation and long-term Ca^{2+}_i 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_2O_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_2O_2 diffusion, considering the requirement for H_2O_2 in ABA-induced NO generation (Bright *et al.*, 2006).

Temporal sequence of CAS-mediated CICI and stomatal closure

As described (Allen *et al.*, 1999, 2000, 2001; Han *et al.*, 2003; Weinl *et al.*, 2008), 1–10 mM Ca^{2+}_o can stimulate an initial Ca^{2+}_i spiking within 30 s, followed by a long-term Ca^{2+}_i transient which was blocked in *CASas*. 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_2O_2 at 100 μM 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_2O_2 production to maintain Ca^{2+}_i transients.

The data indicate that Ca^{2+}_o-driven H_2O_2 and NO generation, which were inhibited in *CASas*, 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_2O_2 production from chloroplasts at low concentration (Fig. 3). Interestingly, H_2O_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_2O_2 and NO generation. As for the order of H_2O_2 and NO generation, it was shown by Bright *et al.* (2006) that $H_2O_2 \rightarrow$NO seemed to be more acceptable. In the present work, the electrochemical sensors were used for H_2O_2 and NO detection from the epidermis and chloroplasts. Ca^{2+}_o-induced H_2O_2 and NO production was observed from guard cells or chloroplasts (Figs 1, 3A). Moreover, more rapid generation of H_2O_2 than NO suggests that NO may act downstream of H_2O_2. However, the time point when Ca^{2+}_o induced visible H_2O_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_2O_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_2O_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_2O_2 and NO production are all required for ABA-induced stomatal closure and act upstream of long-term Ca^{2+}_i transients (Pei *et al.*, 2000; Suhita *et al.*, 2004; Bright *et al*, 2006; Gonugunta *et al.*, 2008). Similar to ABA-induced H_2O_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_2O_2 and NO as well as stomatal closure in various *Arabidopsis* genotypes. The use of these two compounds in *CASas*, *atrbohD/F*, and *Atnoa1* plants reversed Ca^{2+}_o-induced stomatal closure and blocked Ca^{2+}_o-triggered H_2O_2 and NO production in guard cells. NOX is plasma membrane located; however, a report showed that ABA induced H_2O_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 CICI 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_2O_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 *CASas*, and Ca^{2+}_o-induced H_2O_2 production was largely impaired in guard cells and chloroplasts in *CASas*. The present data combined with those of other studies suggest that the CAS-mediated H_2O_2 synthesis in chloroplast is more critical for Ca^{2+}_o-induced stomatal closure than of plasma membrane NOX. The relationship between CAS, H_2O_2, and NO was further established by the observations that H_2O_2 and SNP caused stomatal closure in both wild-type and *CASas* plants. If CAS mediates Ca^{2+}_o-induced stomatal closure directly by Ca^{2+}_i transients and this does not require H_2O_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 *Atnoa1* plants. Further evidence suggests that H_2O_2 is required for NO generation because H_2O_2 could not induce stomatal closure in *Atnoa1* plants. A similar conclusion has been drawn in a previous study (Bright *et al.*, 2006). The finding that H_2O_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_2O_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 *CASas* 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_2O_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_2O_2 generation in chloroplasts indicate that H_2O_2 may function as a common downstream component between ABA and CAS signalling (Fig. 6; Supplementary Fig. S5 at *JXB* online).

Interestingly, the antioxidant system seems to allow the plants to acclimatize to Ca^{2+}_o-induced H_2O_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 *CSD1* expression level would promote cytosolic H_2O_2 production, while biphasic responses of *APX1* or *ATGR1* expression could contribute to H_2O_2 production at the beginning and H_2O_2 scavenging after saturation. This 'H_2O_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_2O_2 and NO production, Ca^{2+}_i transients, and subsequent stomatal closure in *Arabidopsis* guard cells. Ca^{2+}_o-induced H_2O_2 and NO production and Ca^{2+}_i 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_2DCF fluorescent dye.

Figure S3. The effects of CAT or cPTIO on H_2O_2 or NO current detected by a H_2O_2- or NO-selective electrochemical sensor.

Figure S4. Localization of H_2O_2 and NO on *Arabidopsis* epidermal peels in response to Ca^{2+}_o.

Figure S5. ABA-induced H_2O_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 imgages 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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