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Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in *Arabidopsis* guard cells

Rayhanur Jannat^a, Misugi Uraji^a, Miho Morofuji^a, Mohammad Muzahidul Islam^a, Rachel E. Bloom^c, Yoshimasa Nakamura^a, C. Robertson McClung^d, Julian I. Schroeder^c, Izumi C. Mori^{b,*}, and Yoshiyuki Murata^a

^aGraduate School of Natural Science and Technology, Okayama University, 1-1-1 Tsushima-naka, Okayama, 700-8530, Japan

^bInstitute of Plant Science and Resources, Okayama University, Kurashiki, 710-8530, Japan

^cCenter for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093, USA

^dDepartment of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA

Abstract

Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), are among the important second messengers in abscisic acid (ABA) signaling in guard cells. In this study, to investigate specific roles of H₂O₂ in ABA signaling in guard cells, we examined the effects of mutations in the guard cell-expressed catalase (CAT) genes, *CAT1* and *CAT3*, and of the CAT inhibitor 3-aminotriazole (AT) on stomatal movement. The *cat3* and *cat1 cat3* mutations significantly reduced CAT activities, leading to higher basal level of H₂O₂ in guard cells, when assessed by 2', 7'-dichlorodihydrofluorescein, whereas they did not affect stomatal aperture size under non-stressed condition. In addition, AT-treatment at the concentrations that abolish CAT activities, showed trivial affect on stomatal aperture size, while basal H₂O₂ level increased extensively. In contrast, *cat* mutations and AT-treatment potentiated ABA-induced stomatal closure. Inducible ROS production triggered by ABA was observed in these mutants and wild type as well as in AT-treated guard cells. These results suggest that the ABA-inducible cytosolic H₂O₂ elevation functions in ABA-induced stomatal closure, while constitutive raise of H₂O₂ did not caused stomatal closure.

Keywords

Abscisic acid; Catalase; Guard cells; Hydrogen peroxide

Introduction

Guard cells, which form stomatal pores in the epidermis of aerial parts of higher plants, respond to numerous biotic and abiotic stimuli (Schroeder et al., 2001; Hetherington and Woodward, 2003). The phytohormone, abscisic acid (ABA), which is synthesized in

*Corresponding author: Tel.: +81 86 434 1215; Fax: +81 86 434 1249 imori@rib.okayama-u.ac.jp (I.C. Mori).

response to drought stress, is known to induce stomatal closure to reduce transpirational water loss (Irving et al., 1992; Lee et al., 1999; Pei et al., 2000; Murata et al., 2001; Schroeder et al., 2001; Munemasa et al., 2007;2011; Islam et al., 2009; 2010a; b).

Hydrogen peroxide (H_2O_2) is one of the major reactive oxygen species (ROS) and plays an important role as a second messenger in ABA-induced stomatal closure (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001; Kwak et al., 2003; Bright et al., 2006; Miao et al., 2006). Genetic and pharmacological studies have demonstrated that the ROS production is mediated by NAD(P)H oxidases in ABA signaling in *Arabidopsis* guard cells (Pei et al., 2000; Kwak et al., 2003). The treatment with exogenous catalase (CAT) reduced H_2O_2 accumulation and inhibited ABA-induced stomatal closure (Zhang et al., 2001; Munemasa et al., 2007), consistent with the function of plasma membrane NAD(P)H oxidases in mediating stomatal closing (Kwak et al., 2003; Suhita et al., 2004). Direct application of ROS to guard cells is known to inhibit inward K^+ channels and also to activate hyperpolarization-activated Ca^{2+} -permeable I_{Ca} channels and stomatal closure (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001). Therefore, accumulation of H_2O_2 is thought to function in ABA-induced stomatal closure.

However, the details of the active chemical species among ROS that functions in ABA signaling are not fully understood. Reactive oxygen species can be inter-transformable by several reactions, such as Fenton reaction, Harber-Weiss reaction, and SOD-catalyzed disproportionation. This may have hampered speciation of active chemical species among ROS in ABA signaling. Furthermore, spacio-temporal speciation of ROS function is not understood. In this study we focused on the specific H_2O_2 -degrading enzyme CAT to dissect ROS species in ABA signaling.

Reactive oxygen species levels are controlled by antioxidant enzymes, such as catalases, peroxidases and superoxide dismutase in plants cooperating with ROS generating systems (Willekens et al., 1997; Corpas et al., 2001; Mittler, 2002; Apel and Hirt, 2004; Nyathi and Baker, 2006; Palma et al., 2009). The involvement of ascorbate peroxidase (APX) in stomatal function has been demonstrated but it was suggested that it was not involved in drought- or ABA-induced stomatal closure (Davletova et al., 2005). Catalase allows plant cells to remove H_2O_2 energy-efficiently because CAT decomposes H_2O_2 without consuming cellular reducing equivalents. Recently, it has been reported that Ca^{2+} -dependent peroxisomal H_2O_2 catabolism is modulated by CAT in *Arabidopsis* guard cells (Costa et al., 2010).

The *Arabidopsis* genome contains three CAT genes, *CAT1*, *CAT2* and *CAT3*, which are differentially expressed and can form up to six different isozymes (Frugoli et al., 1996). Catalase are highly specific to H_2O_2 over other chemical species of ROS, such as superoxide and hydroxyl radical. In this study, we examined the roles of constitutively accumulated H_2O_2 and ABA-inducible H_2O_2 in stomatal closure by examining *cat* mutants.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana wild type (Wassilewskija ecotype) and *cat3-1*, *cat1 cat3* mutant lines were used. *cat3-1* mutant possesses a T-DNA insertion in *CAT3* locus of the Wassilewskija (Ws) accession and isolated by a genomic PCR screening of the pooled DNA from random T-DNA inserted populations provided by Ohio State University. The *cat1 cat3* double mutant was identified in a population of Ws that had been subjected to fast neutron bombardment. (J. Dangl, personal communication). To define the limits of the deletion, we developed PCR primers to amplify genes flanking the linked *CAT1* (At1G20630) and *CAT3* (At1G20620) loci. In addition to *CAT1* and *CAT3*, the two genes immediately downstream of *CAT1*, At1G20650 (encoding a RWP-RK family protein) and At1G20660 (encoding a protein serine threonine kinase) were missing, but the third gene, At1G20670 (encoding a putative bromo-domain containing protein), was retained. Similarly, the next gene upstream of *CAT3*, At1G20619 (Encoding CYCLIN B2;3), was also retained (Fig. 1B). We then designed primers to amplify across the deletion and determined the DNA sequence of the amplified fragment. The deletion eliminates 20,625 base pair (bp), from position 7141093, 2052 bp upstream of *CAT3*, to position 7161718, 1182 bp upstream (5') of At1G220660 and 2574 bp downstream of At1G20670 (nucleotide numbers are based on the Col-0 reference genome).

Plants were grown in soil in a plant growth chamber (LPH-350SP; Nihonika Co., Osaka, Japan) at 22°C under a 16-h-light/8-h-dark photoperiod at a photosynthetic photon flux density of 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and watered twice a week with Hyponex solution (0.1%).

Isolation of total RNA from whole leaf, guard cell protoplasts and mesophyll cell protoplasts

Total RNA from whole leaf was carried out with Trizole Reagent (Invitrogen) according to the producer's manual. Guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) were isolated by the method described by Kwak et al. (2002). Total RNA from GCP and MCP was isolated as same as from whole leaf.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as follows: Single strand complementary RNA was synthesized with MMLV reverse transcriptase according to the manufacturer's manual from total RNA isolated GCP, MCP and whole rosette leaves of 4-6-week-old *Arabidopsis* plants. Polymerase chain reaction was carried out using gene-specific primer pairs as listed in supplemental Table 1 with a 30-cycle reaction steps: 94°C for 30 s, 53°C for 30 s and 72°C for 60 s. BIOTAQ DNA polymerase (Bioline, Bio-21040) was used.

Catalase activity measurements in whole leaves

For measurement of CAT activity leaves were homogenized in 5 volumes of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.5 mM EDTA, and

phenylmethylsulfonyl fluoride with a mortar and a pestle. The homogenate was centrifuged at $15,000\times g$ for 10 min at 4°C . The supernatant was used for CAT activity measurement.

Catalase activity measurements in guard cells

For measurement of CAT activity in guard cells, live guard cells in epidermis were prepared as follows: Excised rosette leaves from 5-week-old plants were blended for 40 s with a Waring Commercial Blender and epidermis fragments were collected. The epidermis fragments were exposed to pH 4.5 for 90 min in 10 mM citrate buffer. This mild acid treatment was reported as a highly effective method of destroying all cells apart from the guard cells in epidermal preparation, in which guard cells apparently remained fully functional (Squire and Mandfield, 1972). The epidermis fragments were rinsed in distilled deionized water for 5 min. For ABA treatment, the epidermis fragment were then treated with 10 μM ABA or 0.02% ethanol (solvent control) for 30 min. The peels were collected and ground with a mortar and a pestle in liquid nitrogen to burst the guard cells. The homogenate was then mixed with 5 volumes of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.5 mM EDTA, and phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $15,000\times g$ for 10 min at 4°C . The supernatant was used as the crude enzyme sample for CAT activity measurement.

Catalase activity was assayed according to the method of Aebi (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 20 mM H_2O_2 and 50 μL crude enzyme sample. The reaction was started by the addition of H_2O_2 . The activity was calculated from the decline in absorbance at 240 nm for 60 s. The extinction coefficient was $39.4 \text{ M}^{-1}\text{cm}^{-1}$. Protein contents were measured as described by Bradford (1976) using bovine serum albumin as the standard.

Stomatal aperture measurements

Stomatal aperture was measured as described previously (Murata et al., 2001; Munemasa et al., 2007; Saito et al., 2008; Islam et al., 2009; 2010a; b). Excised rosette leaves from 4-6-week-old plants were floated on the medium containing 5 mM KCl, 50 μM CaCl_2 and 10 mM MES-Tris (pH 6.15) for 2 h in the light ($80 \mu\text{mol m}^{-2} \text{ s}^{-1}$) to fully open stomata, followed by the addition of 10 μM ABA or solvent control (0.1% ethanol). Leaves incubated for 2 h were blended for 30 s with a Waring Commercial Blender and epidermal peels were collected. Width of stomatal aperture was immediately measured with a microscope after blending. Twenty stomatal apertures were measured on each preparation from a leaf. Number of biological repeats is designated in figure legends. For time course experiments, stomatal apertures were measured at each indicated time after ABA application.

Detection of ROS

Reactive oxygen species production was examined with 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) as previously described (Pei et al., 2000; Murata et al., 2001; Munemasa et al., 2007; 2011; Saito et al., 2008; Islam et al., 2009; 2010a). Epidermal tissues were isolated from 4-6-week-old plants by blending with a Waring Commercial Blender and incubated for 2 h in the medium containing 5 mM KCl, 50 μM CaCl_2 , and 10 mM MES-Tris (pH 6.15) in the light. To load the dye to guard cells, epidermal tissue was

incubated in the 50 μM $\text{H}_2\text{DCF-DA}$ -containing medium for 30 min. The excess dye was removed by washing with distilled deionized water on a piece of nylon mesh. Then, the dye-loaded tissues were treated either 10 μM ABA or 0.1% ethanol for 20 min. Fluorescent images of guard cells were captured in 8-bit gray scale with Adobe Photoshop program (Adobe Systems Inc.). The fluorescence intensity of the guard cell pair was determined using Image J software (NIH, Bethesda, MD, USA), the fluorescence intensity increase was expressed as percentage of the control.

Statistical Analysis

Significance of differences between mean values was assessed by Student's t-test or one-way ANOVA with Dunnett's test. Differences at $p < 0.05$ were considered significant.

Results

Catalase gene expression and identification of *cat3* and *cat1 cat3* mutants

Reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) as the template showed that only *CAT3* was expressed in GCP (Fig. 1), while *CAT1* and *CAT2* were expressed in MCP ($n=2$). The expression of *CAT1* was observed in GCP in only one of three experiments (Supplementary Fig. S1). Therefore we looked for *cat1* and *cat3* single mutants. Unfortunately, a *cat1* single mutant was not available in the same ecotype background (Ws). Since *CAT3* is reproducibly expressed in GCP in our study, we feel that demonstrating the *cat1 cat3* double disruption mutant phenotype is satisfactory to discuss the role of H_2O_2 by altering the accumulation pattern in the cell. No apparent difference in general morphology was found among plants under the growth condition used in this study (data not shown), while RT-PCR analyses showed no transcripts in the mutants (Fig. 1C).

Catalase activities and ROS accumulation in the catalase mutants

To elucidate roles of endogenous H_2O_2 in ABA signaling of guard cells, we assessed stomata of *CAT* mutants, *cat3-1* and *cat1 cat3*. First, we examined *CAT* activities of the *cat3-1* and *cat1 cat3* mutants. The *cat3-1* mutation ($p < 0.0005$) and the *cat1 cat3* mutation ($p < 0.001$) significantly reduced *CAT* activities in guard cell-enriched epidermis (see Materials and Methods) (Fig. 1D). These results indicate disruption of *CAT* in the guard cell of mutants.

Hydrogen peroxide accumulation was examined using the fluorescence dye, $\text{H}_2\text{DCF-DA}$ in the absence of ABA. Fluorescence intensity of guard cells of the mutants was brighter than that of wild type, indicating higher constitutive H_2O_2 accumulation level under non-stressed condition (Fig. 1E). This result was in a good agreement with lack of *CAT* activities in the mutant guard cells.

It has been reported that exogenously added H_2O_2 induces stomatal closure and endogenous ROS/ H_2O_2 elevation is involved in ABA-induced stomatal closure. Thus, we examined stomatal aperture width of the mutants in the absence of ABA. Interestingly, the

constitutively elevated H₂O₂ level in mutants' guard cells did not result in reduced aperture size (Fig. 1F).

Effects of the catalase mutation on ABA-induced stomatal closure and ABA-induced ROS accumulation

Abscisic acid-induced stomatal closure is associated with ROS production (Pei et al., 2000; Zhang et al., 2001). Furthermore, molecular genetic evidences for the contribution of ROS in guard cell ABA signaling have been unambiguously presented (Murata et al., 2001; Mustilli et al., 2002; Kwak et al., 2003; Sirichandra et al., 2009). Thus, we examined ABA-induced stomatal closure and H₂O₂ production in the *cat3-1* and *cat1 cat3* mutants and wild type plants (Fig. 2). Application of ABA induced stomatal closure in a time dependent manner in all lines. At 180 min after ABA treatment, stomatal apertures were decreased by 31.0 % in wild type, 36.0 % in the *cat3-1* mutant, and 38.4 % in the *cat1 cat3* mutant (Fig. 2A). A statistic analysis suggested significant differences in stomatal apertures between these mutants and wild type ($p < 0.03$ for *cat3-1*; $p < 0.02$ for *cat1 cat3*).

The H₂O₂ level in guard cells of the *cat3-1* ($p < 0.05$) and *cat1 cat3* ($p < 0.0002$) plants were higher than that in wild-type guard cells in the absence of ABA treatment (Fig. 2B), as essentially the same as seen in Fig. 1E. Consistent with previous reports (Pei et al., 2000; Murata et al., 2001; Bright et al., 2006; Munemasa et al., 2007; 2011; Islam et al., 2010a), ABA induced H₂O₂ production in wild type guard cells ($p < 0.002$) (Fig. 2B). In mutant guard cells, the H₂O₂ level was also elevated by ABA treatment (*cat3-1*, $p < 0.006$; *cat1 cat3*, $p < 0.008$) (Fig. 2B). Compared with wild type, the accumulation of H₂O₂ in guard cells was significantly higher in *cat3-1* mutant guard cells ($p < 0.02$) and *cat1 cat3* mutant guard cells ($p < 0.02$) (Fig. 2B).

To test the possibility that H₂O₂ accumulation by ABA in guard cell is mediated with inhibition of CAT activity with ABA in guard cells, CAT activity in guard cells-enriched epidermis after ABA treatment was examined. Essentially no difference in CAT activity in guard cells was observed with and without of ABA treatment for 30 min (Supplementary Fig. S2). Ten μ M ABA was added to extracts from guard cell-enriched epidermis to examine direct effect of ABA on CAT activity. No significant difference in the activity was observed compared to the solvent control (data not shown).

These results suggest that stomatal closure is associated with inducible ROS production, rather than constitutive elevation of ROS level, and that downregulation of CAT is not involved in ABA-induced ROS elevation.

Effects of the catalase inhibitor, 3-aminotriazole (AT), on ABA-induced stomatal closure

To further examine the involvement of CATs in ABA-induced stomatal closure, we used the CAT inhibitor, AT. Application of AT reduced CAT activity in wild type plants to a negligible level in whole leaves ($p < 0.0003$) (Fig. 3A). The treatment with AT showed a very minor but statistically significant effect on stomatal aperture in the absence of ABA (Fig. 3B). Since *cat* mutants did not show any apparent effects on stomatal aperture in the absence of ABA (Fig. 1F), the effect of AT may be attributed to a sudden decrease of CAT

activity by AT application, which is potentially associated with an artificial induction of a rapid H₂O₂ accumulation. According to the gene expression profiling results in Fig. 1A and Supplementary Fig. S1, *cat1 cat3* mutant guard cell lack CAT activity. The observed minor, but significant effect of AT on stomatal aperture in the absence of ABA (Fig. 3B) in the mutants might be attributed to H₂O₂ supply from peripheral cells, such as mesophyll cells, which possess CAT2, to a minor adverse effect of AT, or to trace *CAT2* expression, undetectable with RT-PCR, activity in guard cell.

In wild type, ABA-induced stomatal closure was enhanced by AT ($p < 0.005$) and reached a similar level to *cat* mutants (Fig. 3B). The effect of AT was not significant in *cat3-1* and *cat1 cat3* mutants, suggesting the apparent effect of AT was due to the inhibition of CAT, rather than to other non-specific effects.

Treatment with AT significantly increased ROS accumulation in ABA-untreated ($p < 0.0003$) and -treated ($p < 0.0002$) wild type guard cells in accordance with *cat* mutants (Fig. 3C).

Taken together with previous findings, these data suggest that ABA-induced production of ROS is not simple ROS elevation, but instead represents temporal-spatial specific ROS signature that plays a role in guard cell ABA signaling (Fig. 4 and Discussion). This study examining disruption mutants or inhibition of the H₂O₂ specific degrading enzyme, CAT, suggests that among ROS at least intracellular H₂O₂ have some roles in ABA signaling.

Discussion

Abscisic acid induces stomatal closure along with ROS accumulation in guard cells, which is an essential ABA signaling component in guard cells (Pei et al., 2000; Kwak et al., 2003). However, temporal and chemical speciation of ROS in guard cell ABA signaling has not been examined. In the present study, in order to assess the role of H₂O₂, we employed reverse genetic and a pharmacological approaches using *Arabidopsis thaliana*. We showed that *CAT3* is the predominantly expressed *CAT* in guard cells, while expression of *CAT1* can be observed occasionally. Catalase mutants, *cat3-1* and *cat1 cat3* demonstrated no corresponding transcript and reduced CAT activity. The CAT inhibitor, 3-aminotriazole (AT), also reduced CAT activity. Decreasing CAT activity by mutations and the inhibitor resulted in the constitutive H₂O₂ accumulation in *Arabidopsis* guard cells. However, the constitutive ROS accumulation did not affect stomatal aperture in the absence of ABA. The CAT mutations and the CAT inhibitor enhanced ABA-induced stomatal closure and also promoted ABA-induced ROS production. In addition, ABA induced stomatal closure equivalently in wild type and both mutants in the presence of AT at the concentration, which completely inhibited CAT activities. The genetic and pharmacological approaches in this study suggest that inducible ROS production, but not constitutive ROS accumulation, are closely involved in stomatal closure. This may imply that temporal-spatial specific, presumably also chemical species specific, ROS production signatures play a critical role in the signal discrimination in plant cell signaling (Fig. 4).

Arabidopsis glutathione peroxidase (GPX) isoenzymes, AtGPX1, -2, -3, -5 and -6, function to reduce H₂O₂ and hydroperoxide using thioredoxin but not glutathione as an electron donor (Iqbal et al., 2006; Miao et al., 2006). Glutathione peroxidase function in detoxifying organic hydroperoxides as well as H₂O₂, and may functionally differ from CAT in guard cells. AtGPX3 play dual roles in H₂O₂ homeostasis via the 2C-type protein phosphatase ABA INSENSITIVE2 (ABI2), acting as a general scavenger and specifically relaying the H₂O₂ signal as an oxidative signal transducer in ABA signaling (Miao et al., 2006). Interestingly, plant GPX is shown not to use glutathione as the substrate, instead it uses NADPH (Iqbal et al., 2006). On the other hand, involvement of glutathione in ABA signaling is suggested by a glutathione synthesis-deficient mutant (Jahan et al., 2008). *ASCORBATE PEROXIDASE 1 (APX1)* is involved in light-dark cycle transition response of stomata, but not in ABA response (Pnueli et al. 2003). *ASCORBATE PEROXIDASE 1* localizes in cytosol and mainly decomposes cytosolic super oxide. This difference in substrate preference may be one of the reasons for the phenotypic difference between CAT3 and APX1. Taken together, it is reasonable that APX1 has a distinct function in guard cell redox signaling from CAT.

Calcium-permeable channels activated by ROS is shown to function in the ABA signaling network in *Arabidopsis* guard cells (Pei et al., 2000; Murata et al., 2001; Mori et al., 2006). Increases in cytosolic calcium are triggered by ROS production during ABA signaling (Kwak et al., 2003), suggesting a tight link of ROS generation and Ca²⁺ oscillation. Similar to H₂O₂, constitutive Ca²⁺ elevations (Ca²⁺ plateau) in guard cells does not induce stomatal closure contrasting with Ca²⁺ oscillations (Young et al., 2006; Siegel et al., 2009). From these facts one can reasonably speculate that spatio-temporal specific ROS elevation pattern function together in a harmonized way with Ca²⁺ oscillation in mediating ABA-induced stomatal closing.

It was shown that NAD(P)H oxidase generates superoxide anion extracellularly (Kwak et al. 2003). Extracellular application of membrane impermeable CAT inhibits ABA-induced stomatal closure (Zhang et al., 2001; Munemasa et al., 2007). Our results, which showed partial enhancement of stomatal closure in CAT disruption or inhibition, suggests, at least, partial involvement of intracellular H₂O₂ in the process. Taken together these observations, we propose a model for ROS function in ABA signaling in guard cell (Fig. 4). Furthermore, the present study provides evidence that transient ROS production rather than constitutive ROS accumulation modulated by *CAT1* and *CAT3* are important for stomatal closure in *Arabidopsis* guard cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

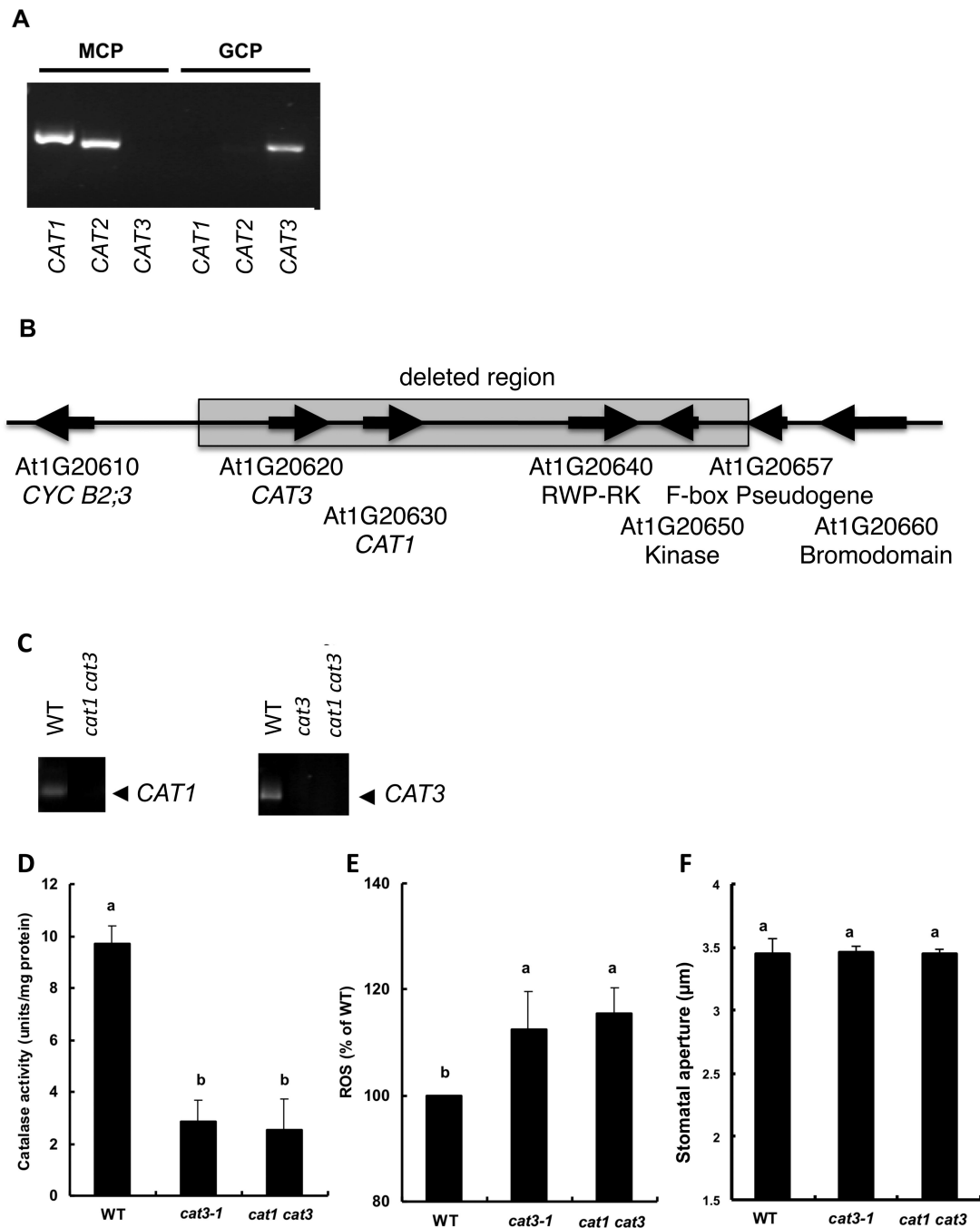
ABA	abscisic acid
APX	ascorbate peroxidase
AT	3-aminotriazole
CAT	catalase
GCP	guard cell protoplast
GPX	glutathione peroxidase
H₂DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
I_{Ca} channel	plasma membrane Ca ²⁺ -permeable channel
MCP	mesophyll cell protoplast
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction

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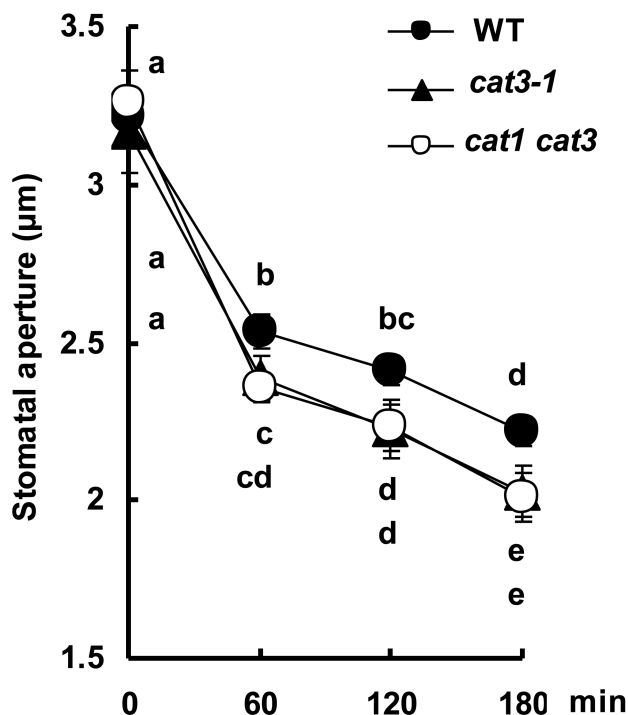
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**Fig. 1.**

Gene expression, catalase activities, ROS accumulation and stomatal aperture of wild type and *cat* mutants. A) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *CATALASE* mRNAs; *CAT1*, *CAT2* and *CAT3* in guard cell protoplasts (GCP) and mesophyll cell protoplasts (MCP) of wild-type *Arabidopsis* (WS). B) Schematic representation of the deletion of the *CAT3* and *CAT1* loci in the fast neutron mutagenized *cat1 cat3* mutant. The upper boxes indicate genomic organization of the reference sequence of the corresponding bacterial artificial chromosome clone sequence of the *Arabidopsis* (BAC clone#: F2D10).

The bottom boxes indicate the genomic organization of the fast neutron mutagenized *cat1 cat3* double deletion mutant. The grey box indicates the 20,625 bp deleted region in the mutant. Each arrow indicates open reading frame. In the mutant, two genes (RWP-RK and Kinase) are disrupted together with the tandem *CAT1* and *CAT3* genes. C) Reverse transcriptase-polymerase chain reaction analyses of *CAT* genes in *cat3-1* and *cat1cat3* mutants. Total RNA extracted from whole leaf was used for the template. D) Catalase activities of extracts from the low pH-treated epidermal fragments of wild type and, *cat3-1* and *cat1 cat3* mutants. E) Reactive oxygen species (ROS) production of guard cells in wild type, *cat3-1* and *cat1 cat3* mutants. The vertical scale represents the percentage of DCF fluorescent levels when fluorescent intensities of *cat* mutants were normalized to wild type value taken as 100% for each experiment. More than 50 guard cells were measured per experiment. F) Stomatal aperture of wild type and, *cat3-1* and *cat1 cat3* mutants in the absence of ABA. Each data was obtained from 60 stomatal aperture measurements. Figures having same letters do not differ significantly at the 5% level (one-way ANOVA with Dunnett's *post-hoc* test). Averages from three independent experiments are shown. Error bars represent standard deviation.

A



B

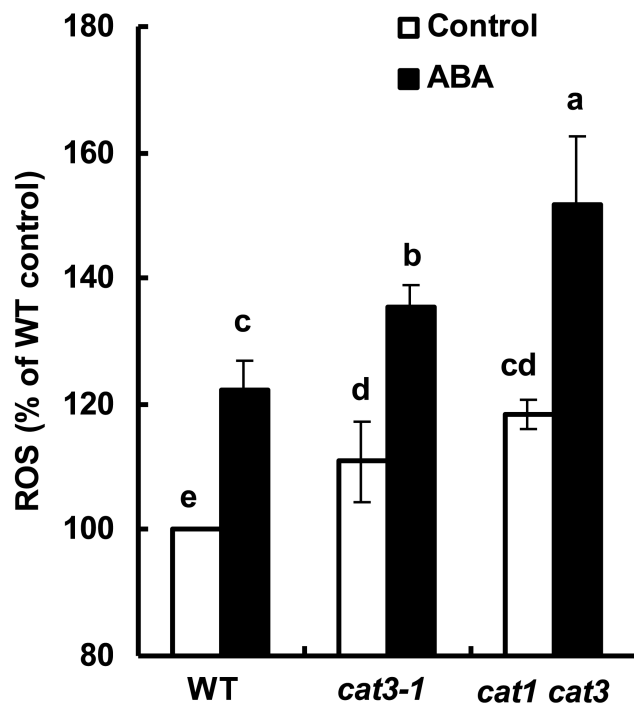


Fig. 2. Abscisic acid-induced stomatal closure and ROS accumulation of wild type and *cat* mutants. A) The time-course of stomatal closure in wild type (filled circle), *cat3-1* (filled triangle) and *cat1 cat3* (open circle) treated with 10 µM ABA. Each data was obtained from 60 stomatal aperture measurements. B) Reactive oxygen species production treated with 0.1% ethanol (as solvent control) and 10 µM ABA in guard cells of wild type and, *cat3-1* and *cat1 cat3* mutants. The vertical scale represents the percentage of DCF fluorescent levels when fluorescent intensity of wild type control value taken as 100%, and other values were expressed in relation to wild type control for each experiment. Each data was obtained from more than 50 guard cells. Figures having same letters do not differ significantly at the 5% level (one-way ANOVA with Dunnett's *post-hoc* test). Averages from three independent experiments are shown. Error bars represent standard deviation.

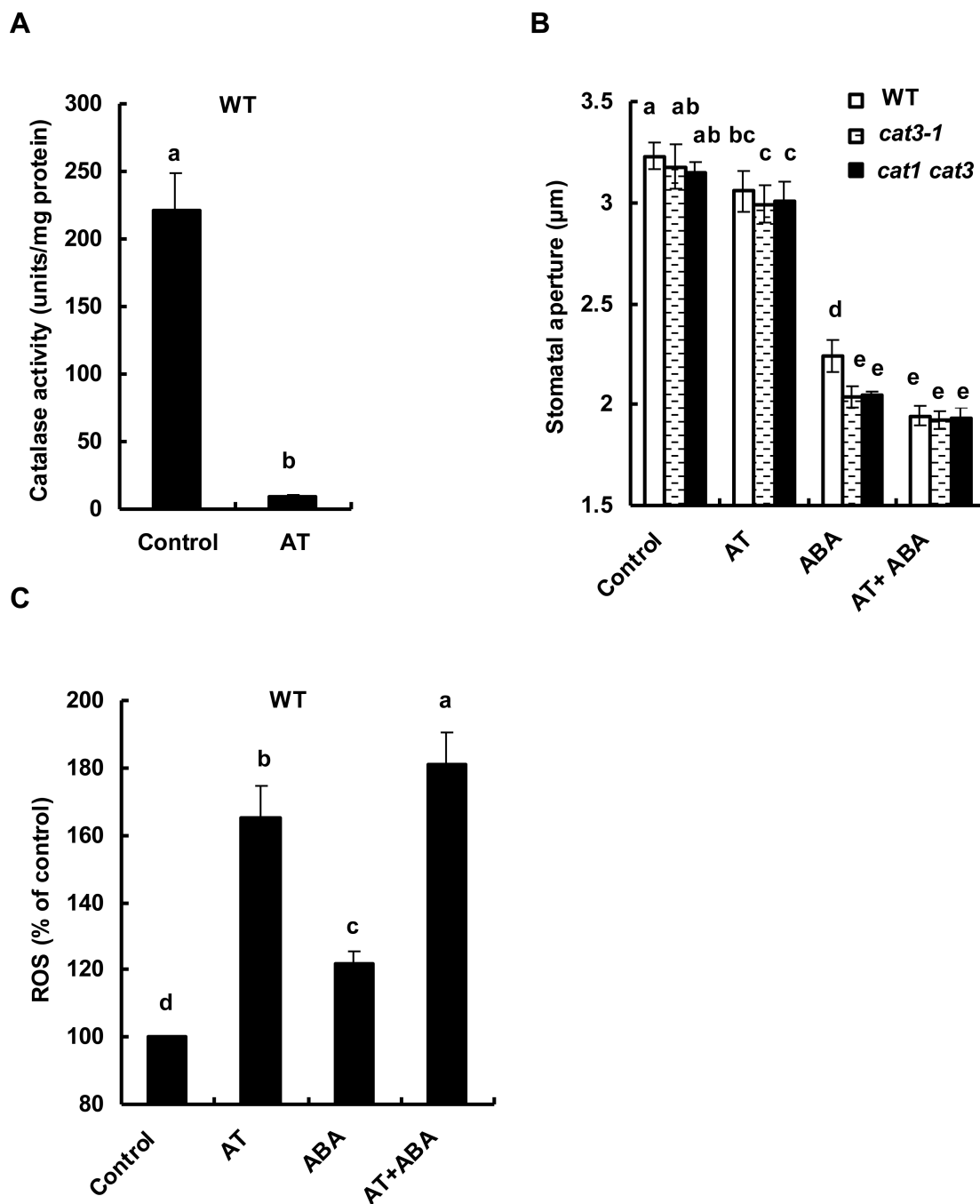


Fig. 3. Catalase activities, ABA-induced stomatal closure and ROS production in plants pre-treated with CAT inhibitor 3-aminotriazole (AT). A) Catalase activities of extracts from the whole leaves of wild type treated with 20 mM AT. Leaves were incubated for 2 h with 20 mM AT. B) Abscisic acid-induced stomatal closure in guard cells in the presence of AT. Rosette leaves of wild type (white bar), *cat3-1* (dotted bar) and *cat1 cat3* (black bar) were treated with 20 mM AT for 30 min, followed by 10 μM ABA treatment for 2 h. Each data was obtained from 60 stomatal aperture measurements. C) Abscisic acid-induced ROS

production of guard cells in wild type in the presence of AT. Guard cells were treated with 20 mM AT for 30 min, followed by 10 μ M ABA. The vertical scale represents the percentage of DCF fluorescent levels when fluorescent intensity of wild type control value taken as 100% and other values were expressed in relation to wild type control for each experiment. Each data was obtained from more than 50 guard cells. Figures having same letters do not differ significantly at the 5% level (one-way ANOVA with Dunnett's *post-hoc* test). Averages from three independent experiments are shown. Error bars represent standard deviation.

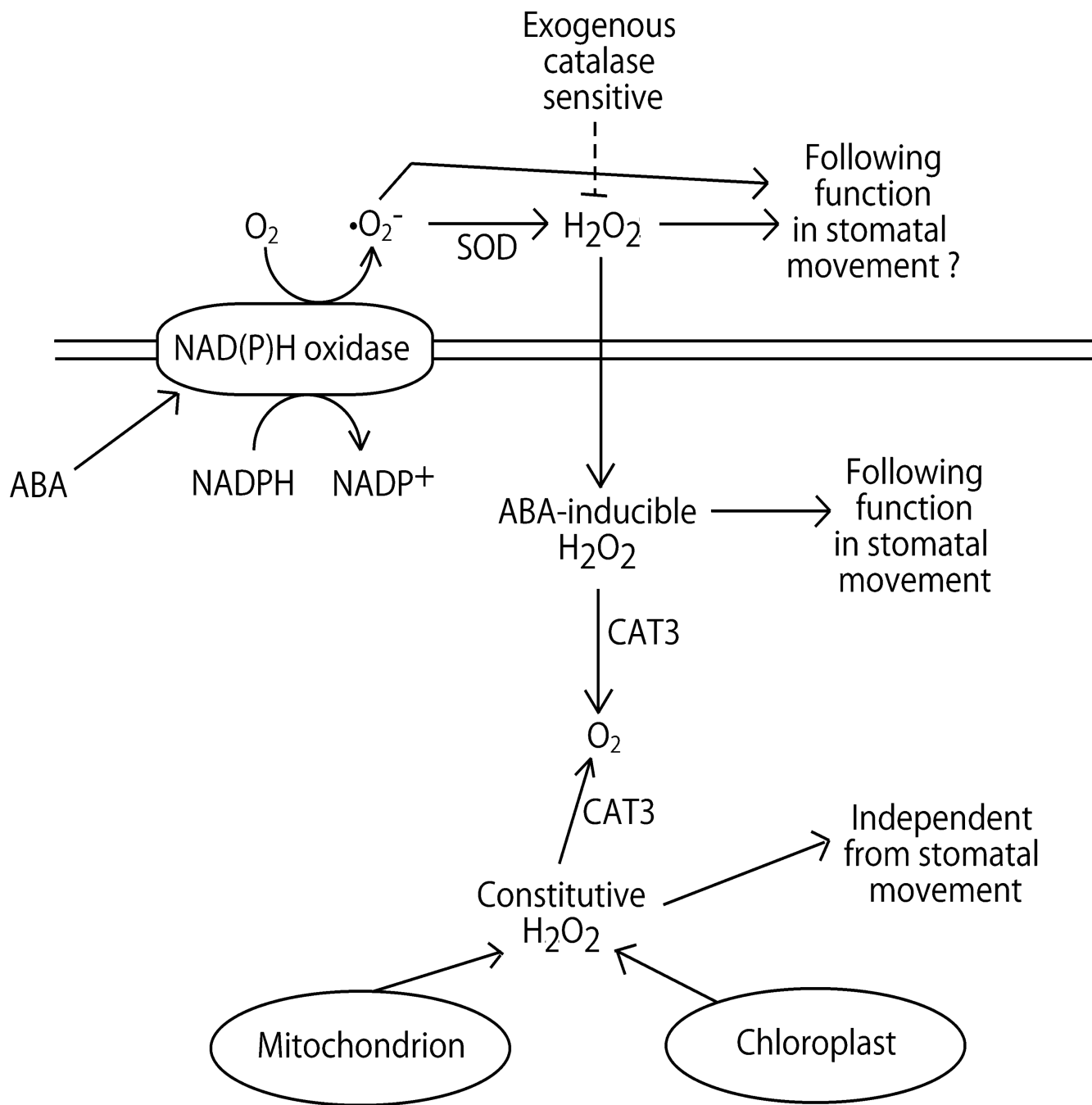


Fig. 4. A proposed model showing the roles of constitutive- and ABA-inducible cytosolic H_2O_2 accumulation in ABA signaling in guard cells. Arrows indicate positive regulation, and open blocks indicate negative regulation. In the absence of ABA, constitutive H_2O_2 is generated from mitochondria and chloroplast, which is independent from stomatal movement. In a wild type guard cell, CAT3 decomposes the constitutive H_2O_2 to O_2 . In the presence of ABA, ABA activates NAD(P)H oxidases in the plasma membrane that produces superoxide anion ($\cdot\text{O}_2^-$) by consuming the reduction

potential of NADPH in the cell. Superoxide dismutases in the apoplastic space convert $\bullet\text{O}^-_2$ to H_2O_2 , which is sensitive to exogenously added CATs. The extracellularly converted H_2O_2 migrates into cytosolic space across the plasma membrane, raises cytosolic H_2O_2 concentration (ABA-inducible H_2O_2) and functions for following stomatal movement signaling in the cell. The ABA-inducible H_2O_2 are also decomposed into O_2 by CAT3 for downregulating the signal. Thus, disruption of CAT3 causes a slight increase of ABA sensitivity of stomata. Note that this model does not necessarily deny functions of extracellular $\bullet\text{O}^-_2$ and H_2O_2 in stomatal movements. At present, at least two targets of H_2O_2 are postulated, i.e. ABI2 and I_{Ca} channels (see text). However, differential roles of extracellular and cytosolic ABA-inducible H_2O_2 to the downstream signal components have not been clarified, yet.