BIOSCIENCE, BIOTECHNOLOGY, AND BIOCHEMISTRY
2020, VOL. 84, NO. 7, 1418–1426
https://doi.org/10.1080/09168451.2020.1743168

Interaction of intracellular hydrogen peroxide accumulation with nitric oxide production in abscisic acid signaling in guard cells

Rayhanur Jannat[a*], Takanori Senba[a], Daichi Muroyama[a], Misugi Uraji[a**], Mohammad Anowar Hossain[a], Mohammad Muzahidul Islam[a], Yoshimasa Nakamura[a], Shintaro Munemasa[a], Izumi C. Mori[b] and Yoshiyuki Murata[a]

[a]Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan; [b]Institute of Plant Science and Resources, Okayama University, Kurashiki, Japan

ABSTRACT

Reactive oxygen species and nitric oxide (NO•) concomitantly play essential roles in guard cell signaling. Studies using catalase mutants have revealed that the inducible and constitutive elevations of intracellular hydrogen peroxide (H_2O_2) have different roles: only the inducible H_2O_2 production transduces the abscisic acid (ABA) signal leading stomatal closure. However, the involvement of inducible or constitutive NO• productions, if exists, in this process remains unknown. We studied H_2O_2 and NO• mobilization in guard cells of catalase mutants. Constitutive H_2O_2 level was higher in the mutants than that in wild type, but constitutive NO• level was not different among lines. Induced NO• and H_2O_2 levels elicited by ABA showed a high correlation with each other in all lines. Furthermore, NO• levels increased by exogenous H_2O_2 also showed a high correlation with stomatal aperture size. Our results demonstrate that ABA-induced intracellular H_2O_2 accumulation triggers NO• production leading stomatal closure.

Abbreviations: ABA: abscisic acid; CAT: catalase; cGMP: cyclic guanosine monophosphate; DAF-2DA: 4,5-diaminofluorescein-2 diacetate; H_2DCF-DA: 2′,7′-dichlorodihydrofluorescein diacetate; MeJA: methyljasmonate; NOS: nitric oxide synthetase; NR: nitrate reductase; POX: peroxidase; ROS: reactive oxygen species; SNAP: *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP: sodium nitroprusside; NOX: NADP(H) oxidase.

ARTICLE HISTORY
Received 20 January 2020
Accepted 10 March 2020

KEYWORDS
Abscisic acid; catalase; hydrogen peroxide; nitric oxide; stomatal closure

Reactive oxygen species (ROS) play indispensable roles as intracellular and intercellular signaling messengers in plants, besides their adverse effects [1]. In regard to stomatal movement, ROS production in guard cells participates in many signaling processes such as abscisic acid (ABA)-, carbon dioxide (CO_2)-, methyljasmonate (MeJA)-, microbe-associated molecular patterns (MAMPs)- and salicylic acid (SA)-induced closure [2–6], while the involvement of ROS in guard cell signaling is argued to be conditional [7].

In addition to ROS, nitric oxide (NO•) is widely accepted to play a crucial role in guard cell signaling [8–10]. Induction of ROS production and NO• production occurred coincidentally in guard cells upon exposure to ABA, MeJA and an MAMP [11–13]. Accumulated pharmacological and molecular biological evidences suggest that ROS generation and NO• generation in guard cell signaling are associated in the signaling process [14–16]. Among ROS, hydrogen peroxide (H_2O_2) plays major roles in spatiotemporal-specific signaling [17], although the cooperative action of H_2O_2 and NO• remains to be further elucidated.

NO• is supposed to transduce the signal to the downstream components by nitration of tyrosine residues and cyclic guanosine monophosphate (cGMP) mediated via subsequent $ONOO^-$ formation as well as direct *S*-nitrosylation of cysteine residues [18,19]. It was reported that NO• cooperatively acts with H_2O_2 to positively transduce ABA signal in stomatal closure by generation of NO_2-cGMP via the spontaneous formation of peroxynitrite ($ONOO^-$) [18]. The generation of $ONOO^-$ crucially depends on the interaction of superoxide anion radical ($O_2•^-$) and NO• [20]. The environmental stimulus-induced production of $O_2•^-$ in guard cells is supposed to occur extracellularly by catalysis of NADPH oxidase (NOX) or cell wall-associated peroxidase (cell wall POX), while NO• generation by nitrate reductase takes place in cytosol [11,21,22]. Therefore, it remains controversial where $O_2•^-$ and H_2O_2 encounter to spontaneously generate $ONOO^-$ by the current ROS and NO• generation model.

The crosstalk of ROS, NO• and Ca^{2+} has often been discussed [23]. However, the chemical species, which participates in this signal crosstalk among ROS, i.e. $O_2•^-$, hydroxyl radical (OH•) and H_2O_2,

CONTACT Izumi C. Mori imori@okayama-u.ac.jp
*Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Dhaka, Bangladesh
**Graduate School of Science, Technology and Innovation, Kobe University, 1-5-6 Minatojimaminamimachi, Chuo-ku, Kobe, 650-0047, Japan

has not been well elucidated. Catalase catalyzes the disproportionation reaction to produce an oxygen molecule (O_2) and a water molecule (H_2O) from two H_2O_2, and hence, contributes to balancing the ROS level in a cell. Loss-of-function mutations in *CATALASE* genes render the elevation of basal level of H_2O_2 in Arabidopsis guard cells [24–26]. Their studies using catalase mutants introduced the concept that H_2O_2 in guard cells is comprised of at least two distinct components, constitutive H_2O_2 and stimulus-inducible H_2O_2, and only the inducible component conveys the environmental signals eventually inducing stomatal closure. They also showed that the enhanced inducible H_2O_2 elevation in *CATALASE* mutants leads to the increase of frequency of guard cells exhibiting calcium oscillation [25].

Some second messengers have a particular temporal pattern to properly transduce a specific message. Allen et al. [27,28] demonstrated that simple rise of cytosolic free calcium concentration, denoted as *calcium plateau*, does not induce stomatal closure, but calcium oscillation patterns with a defined range of frequency (*calcium signature*) induce and maintain the closure. It was shown that the ROS production is associated with Ca^{2+}-permeable channel activation [29,30]. Furthermore, the association of inducible ROS generation and calcium oscillation induction was reported [25]. Therefore, it can be conceivable that an inducible ROS generation in guard cells modulates calcium signature.

Meantime, the spatial and temporal pattern of NO•, and the role of constitutive and inducible NO• accumulation in guard cells, if occurs, remain obscure. In this study, we investigated NO• mobilization in catalase mutant guard cells using a fluorescent indicator to explore the relation of H_2O_2 production and NO• production. Our results suggest that the level of NO• is associated with that of H_2O_2 in guard cells. We discuss a mode of interaction of ROS and NO• in a cell.

Materials and methods

Plants

Arabidopsis thaliana, wild types Columbia (Col) and Wassilewskija (WS), and *CATALASE* mutants *cat2-1, cat3-1* and *cat1;cat3* were grown in soil in an environment-controlled growth chamber (LH-100RD, Nihonika Co., Osaka, Japan) at 22°C under a light regime of 16 h light/8 h dark at a photosynthetic photon flux density of 80 μmol m^{-2} s^{-1}. Plants were watered daily and fertilized once a week with diluted (1:1000) Hyponex solution (Hyponex Japan Co., Osaka, Japan), as described previously [25]. *cat2-1* (Salk_076998) and *cat3-1* (screened from random T-DNA insertion population provided from Ohio University) were T-DNA insertion mutants in Col and WS backgrounds, respectively [25,26]. *cat1;cat3* was a deletion mutant in the chromosome 1 lacking 20,625 base pairs, coding four genes including adjacent *CAT1* and *CAT3* generated by fast neutron bombardment in WS background [26]. Homozygosity, lack of the messenger RNA of *CATALASEs* and compromised catalase activity in the mutant leaves had been confirmed in the previous studies [25,26]. Experiments with genetically modified plants were approved by Okayama University (No. G-1249).

Chemicals

The NO• donor, sodium nitroprusside (SNP) was a product of Sigma-Aldrich, Inc. (St. Louis, MO, U.S. A.). Other chemicals were analytical grade purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Nakalai Tesque Inc. (Kyoto, Japan).

Measurement of stomatal aperture

Aperture width of stomata was measured by the method described in Munemasa et al. [31]. In brief, excised rosette leaves from 4 to 5 week-old plants were floated on the 10 mM MES-Tris solution containing 5 mM KCl and 50 μM $CaCl_2$ (pH 6.15) for 2 h under illumination at 80 μmol m^{-2} s^{-1} and subsequently treated with the solution supplemented with ABA, SNP, H_2O_2 or the solvent control (0.1% ethanol or water as indicated in figure legends) for 2 h. Epidermal fragments were prepared by blending a leaf with a Waring commercial blender (51BL32, Waring, Stamford, CT, US.A.) until clear epidermal fragments were released, ~30 s. Width of stomatal aperture in abaxial epidermis was measured under a microscope.

Measurement of NO and ROS production in guard cells

Levels of ROS and NO• in guard cells were examined using fluorescence indicators, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), which detected intracellular H_2O_2 indirectly via compounds I and II of POX formed in the presence of H_2O_2, and 4,5-diaminofluorescein-2 diacetate (DAF-2DA), which was sensitive to NO•, using a fluorescence microscope (Biozero BZ-8000, Keyence corporation, Osaka, Japan) with a filter set, OP-66835 BZ filter GFP (excitation wavelength, 480/30 nm; absorption wavelength, 510 nm; dichroic mirror wavelength, 505 nm) according to Ye et al. [13]. In brief, epidermal fragment obtained by blending of rosette leaves from 4 to 5 week-old plants were incubated in the10 mM MES-Tris solution in the light (80 μmol m^{-2} s^{-1}) for 3 h, followed by 30 min of fluorescent dye loading (either 50 μM H_2

DCF-DA or 5 μM DCF-2DA in the MES-Tris solution). After the dye loading, excess dye was removed by rinsing with the 10 mM MES-Tris solution on a nylon net (30 μm opening) and resuspended in the same solution. Following ABA, SNP, or H_2O_2 treatment for 30 min; fluorescence images were taken using VH Analyzer (Keyence corporation, Osaka, Japan) the intensity of fluorescence in guard cells was analyzed using Adobe Photoshop (Adobe Inc. San Jose, CA, U.S.A.) without an image processing. Change in fluorescence intensity was expressed as % of each control.

Statistics

The significance of differences in mean values between data sets was assessed by two-tailed Student's *t*-test or Dunnett's test as indicated.

Results

Enhanced production of NO• in guard cells in catalase mutants

Through the analysis of *CATALASE* mutants, previous studies have shown that an induced accumulation of H_2O_2 in guard cells participates in the ABA signaling inducing stomatal closure [25,26]. To obtain clues to grasp the temporal and spatial mobilization manner of NO• accumulation and the relation between H_2O_2 accumulation and NO• accumulation in guard cells; we investigated stomatal movement and NO• production in catalase mutants. We examined ABA-induced stomatal closure in the mutants to confirm the results of previous studies (Figure 1(a)). Stomatal aperture widths of *CATALASE* mutants (*cat3-1, cat1;cat3* and *cat2-1*) were comparable to their corresponding wild-type plants without exogenously applied ABA. Exogenous ABA treatment induced stomatal closure in each wild type as expected. In *cat3-1* and *cat2-1*, ABA-induced stomatal closure was significantly augmented in agreement with previous reports [25,26]. Visibly, stomatal closure of *cat1;cat3* double mutant was also augmented to compared with that of wild type, while it did not show a significance ($P = 0.098$). We also reexamined ABA-induced cytosolic H_2O_2 accumulation in the catalase mutants (Figure 1(b)). The H_2O_2 accumulation level in guard cells was constitutively elevated in *cat3-1, cat1;cat3* and *cat2-1* in the absence of exogenous ABA and was additionally increased by the application of exogenous ABA in agreement with previous studies [25,26]. These results confirmed the reported characteristics of catalase mutants that ABA-induced stomatal closure and H_2O_2 accumulation were enhanced.

A slight increase in DAF fluorescence, which represents NO• production, was observed in the *CATALASE* mutants ranging 8% to 14% increases from the corresponding wild types in the absence of exogenous ABA (Figure 1(c), see also Figure 2(b) $P = 0.011$ in *cat1;cat3*), although this increase was infrequently showed a statistical significance. In accordance with earlier reports, exposure to exogenous ABA resulted in an increase in NO• accumulation in guard cells in wild-type plants (Figure 1(c)). In *CATALASE* mutants, ABA induced the elevations of NO• accumulation in guard cells higher than in wild-type guard cells (Figure 1(c)). The increases of NO• levels by ABA treatment in *cat3-1* and *cat1;cat3* were 17.6% and 26.0%, that were higher than that in the wild type (WS, 14.9%) ($P = 0.026$ and 0.018 by two-tailed unpaired Student's t-test). In *cat2-1* NO• production induced by ABA was also significantly higher than that in the wild type (Col), 20.5% against 15.8% ($P = 0.029$ by two-tailed unpaired Student's t-test).

Correlation of H_2O_2 and NO• accumulations was examined using fluorescence levels of DCF and DAF (Figure 1(d)). The accumulations of H_2O_2 and NO• in guard cells in the presence of exogenous ABA showed a high linear correlation ($r = 0.98$, $P = 0.003$). Taken together with the earlier report [11], these results suggest that the NO• production follows the H_2O_2 production in guard cells, and H_2O_2 accumulation level defines NO• accumulation level. This indicates that enhanced production of H_2O_2 induced by ABA promoted NO• production. It should be noticed that formation of DCF from H_2DCF can be mediated not only by the oxidation with H_2O_2-mediated formation of compound I and II of POX but also by the direct oxidation by OH•, $ONOO^-$, and NO_2• [32,33]; while the oxidation of H_2 DCF by O_2• $^-$ is minimum [33]. This broad specificity of H_2DCF is distinct from the high specificity of DAF to NO•. Therefore, we cannot exclude the participation of augmented productions of OH•, $ONOO^-$, and NO_2• in the increase of the DCF fluorescence in this experiment.

Constitutive accumulation of H2O2 in catalase mutant guard cells results in no apparent NO• elevation

Effects of exogenous H_2O_2 exposure on NO• accumulation in catalase mutant guard cells were examined to look further into the relation between these two second messengers. Prior to the DAF fluorescence analysis, H_2O_2-induced stomatal aperture phenotype in catalase mutants was examined. Stomatal aperture widths in the absence of exogenous H_2O_2 were comparable between mutants and the corresponding wild types (Figure 2(a)), while the H_2O_2 levels were increased in the mutants. Addition of 100 μM H_2O_2 resulted in remarkable closure in all plants examined, and the degree of closure was scarcely promoted in *cat3-1, cat1cat3* and cat*2-1* (18 – 25%), while it was not significant at $P < 0.05$ level (Figure 2(a)). This implies that the introduction of a rapid increase of H_2O_2

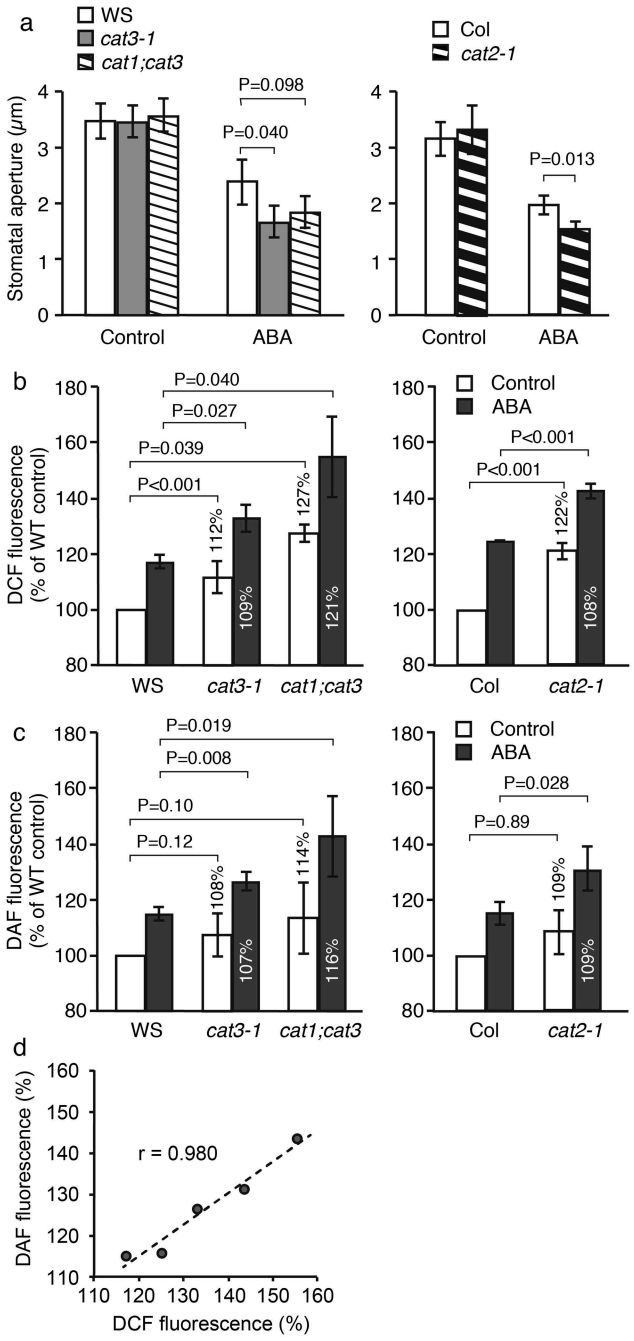

Figure 1. ABA-induced stomatal closure, H_2O_2 production, and NO• production in catalase knockout mutants.

Ten μM ABA was applied for 2 h. (a) Stomatal closure in WS, *cat3-1*, *cat1;cat3*, Col and *cat2-1*. Each datum was obtained from 4 replicate. Each replicate consisted of measurement of 20 stomata. (b) H_2O_2 production represented by dichlorofluoresein (DCF) fluorescence normalized to wild-type controls in percentage. Each datum was obtained from 4 replicate. Each replicate consisted of measurement of 50 guard cells. Percent increase from wild type data is shown. (c) NO• production represented by diaminofluorescein (DAF) fluorescence normalized to wild-type controls (WT) in percentage. Each datum was obtained from 4 replicate. Each replicate consisted of measurement of 50 guard cells. Error bars represent standard deviation. *P* values obtained from Student's *t*-test between two data sets were shown. (d) Relationship of DCF and DAF fluorescence. Plots are from fluorescence intensities in the presence of ABA, which are normalize to that of wild-type guard cells in the absence of ABA. *r* represents Pearson's correlation coefficient. Percentage above or in bars indicates the means of data to compare with the corresponding wild type treated with 10 μM ABA. 0.1% ethanol was used for the solvent dissolving ABA. 0.1% DMSO was used for the solvent dissolving H_2DCF-DA and DAF-2 DA.

has a message to induce stomatal closure and catalase activity to the sudden massive H_2O_2 application has only a modest contribution in H_2O_2 decomposition under this experimental procedure.

Exogenously added H_2O_2 to the excised leaf increased the DAF fluorescence in *CATALASE* mutant guard cells as well as the corresponding wild-type guard cells (Figure 2(b)). Statistical difference in DAF fluorescence between wild type and the *CATALASE* mutant was not observed (P = 0.11 for *cat3-1*, 0.36 for *cat1cat3* and 0.30 for *cat2-1*). This marginal difference in the effect of H_2O_2 to the NO• generation resembles the effect of H_2O_2 to the stomatal aperture (Figure 2(a)). A high negative correlation between NO• levels and aperture widths was observed: the more NO• was accumulated in guard cells, the

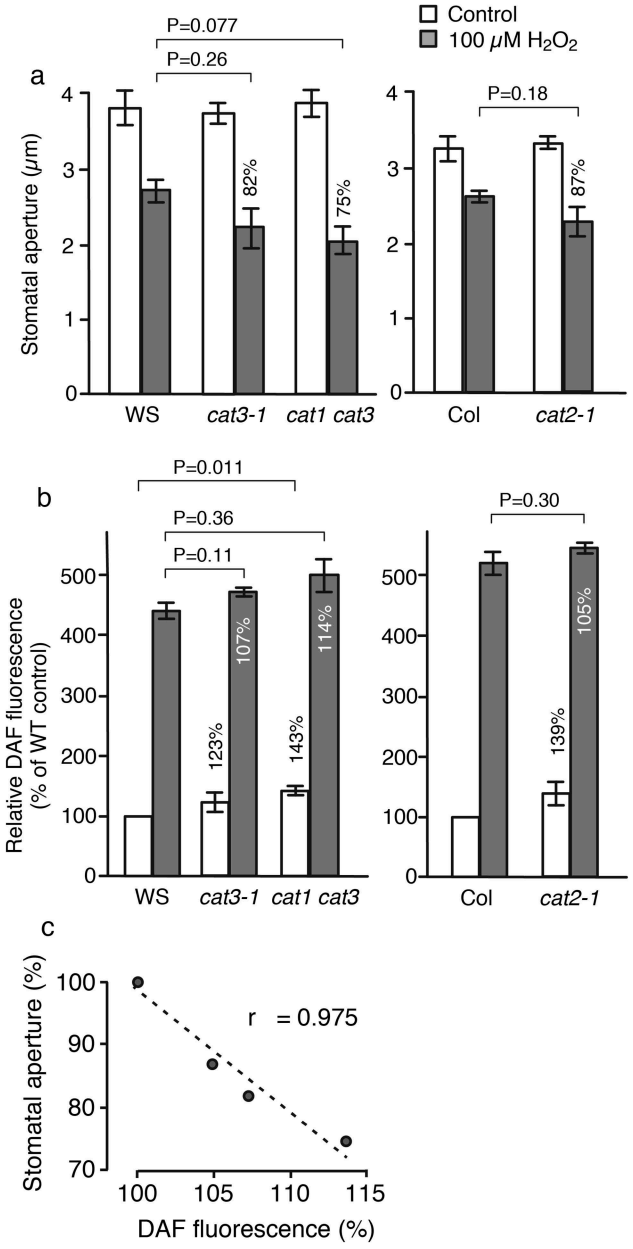

Figure 2. H_2O_2-induced stomatal closure and NO• production in catalase knockout mutants.

(a) Stomatal closure in WS, *cat3-1*, *cat1;cat3*, Col and *cat2-1*. Each datum was obtained from 3 replicate. Each replicate consisted of measurement of 20 stomata. (b) NO• production examined with DAF fluorescence. Each datum was obtained from 3 replicate. Each replicate consisted of measurement of 50 guard cells. Error bars indicate standard deviation. *P* values obtained from Student's t-test between two data sets were shown. (c) Relationship of DAF fluorescence and stomatal aperture. Data are normalize to that of wild-type guard cells in the absence of H_2O_2 and expressed as %. *r* represents Pearson's correlation coefficient. Percentage above or in bars indicates the means of data to compare with the corresponding wild type treated with 100 μM SNP. H_2O_2 stock solution was prepared in water. 0.1% DMSO was used for the solvent dissolving DAF-2 DA.

narrower stomatal aperture close (r = – 0.975, P = 0.037, Figure 2(c)), yet binary comparison of the catalase mutants with the wild type did not show a significance (Figure 2(a,b)). This relation might not be a linear but a downward convex, which is likely due to saturation of the closure.

The gain of the constitutive accumulation of H_2O_2 in catalase mutant's guard cells (Figure 1(b,c) and 2(b)) does not result in apparent increase in NO• level. Taking into account the critical role of NO• in ABA-induced stomatal closure, the inducible NO• accumulation, which is link to the inducible H_2O_2 accumulation, is associated with induction of stomatal closure.

NO• donor-induced stomatal closure in catalase mutants

Reportedly, an exogenous application of a NO• donor, such as sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP), induces stomatal closure [9]. Here, we examined the effect of SNP on stomatal aperture of catalase mutants; so that the impact of a sudden increase of NO• on guard cells, in which H_2O_2 level is augmented, is to be examined.

We examined the effects of several concentrations of SNP on stomatal apertures of wild-type plants (Figure 3(a)). In both WS and Col, no significant effect of SNP was

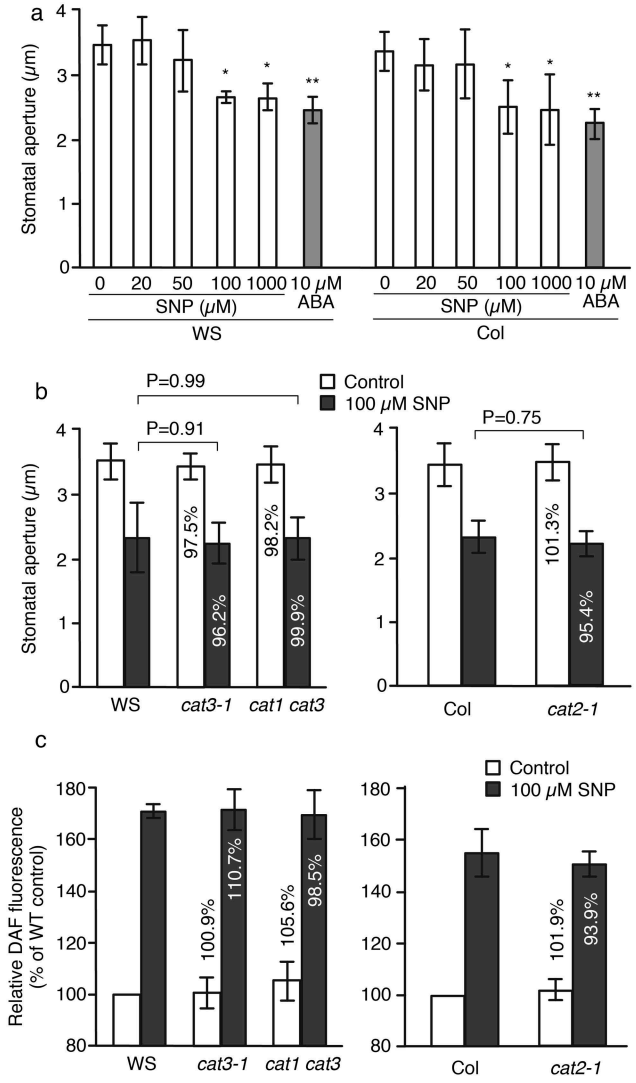

Figure 3. SNP-induced stomatal closure and NO• production in catalase knockout mutants. SNP was applied for 2 h.

Error bars indicate standard deviation. Each datum was from three independent experiments. (a) Effect of different concentrations of SNP and 10 μM ABA on stomatal aperture of wild-type plants. Asterisks indicate significance from the control at $P < 0.05$ (*) or <0.01 (**) by Dunnett's test. (b) SNP-induced stomatal closure in *cat3-1*, *cat1;cat3* and *cat2-1*. *P* values obtained from Student's t-test between two data sets were shown. (c) NO accumulation levels examined with diaminofluorescein (DAF) fluorescence in SNP-fed guard cells. The fluorescence level is normalized to wild-type controls (WT). Each replicate consisted of measurement of 50 guard cells. Percentage above or in bars indicates the means of data to compare with the corresponding wild type treated with 100 μM H_2O_2. Water and 0.1% DMSO was used for the solvent dissolving SNP and DAF-2 DA, respectively.

noticed up to 50 μM. At 100 and 1000 μM SNP reduced stomatal aperture width at a similar level to 10 μM ABA. Therefore, we used 100 μM SNP for following SNP-exposure experiments. On the course of the experiment, we did not notice any visible adverse effect of SNP to the leaves and guard cell structure at microscopic level with 40-fold magnification objective lens. The intactness of the guard cell plasma membrane was validated by that there was no apparent leakage of fluorescent dye from the cell.

Exogenous application of 100 μM SNP induced stomatal closure by approximately 35% in wild types (Figure 3(b)). The aperture width closed at almost same level of the wild type in all *CATALASE* mutants (Figure 3(b)). At the same time, NO• was highly accumulated by SNP application in *CATALASE* mutant guard cells at the same level as that in wild-type guard cells (Figure 3(c)). This suggests that an addition of NO• is sufficient to induce stomatal closure and the level of constitutive H_2O_2 accumulation in guard cells does not apparently affect NO•-induced stomatal closure level as well as NO• accumulation. Taking account of the difference between the effect of H_2O_2 application and NO• application on stomatal movements, we conclude that NO• production follows and is controlled by H_2O_2 production, while the former does not affect the latter.

Discussion

Relation of H2O2 production and NO• production in guard cell signaling

In this study, we examined the relation between H_2O_2 production and NO• production in guard cells by analyzing NO• production, H_2O_2 production, and stomatal movement in loss-of-function mutants of *CATALASE* genes taking advantage of its exclusive specificity for H_2

O_2 over other ROS. Our results showed a very high correlation of H_2O_2 accumulation and NO• accumulation levels in response to ABA (Figure 1). A high correlation was also observed between stomatal aperture and NO• accumulation (Figure 2). To the contrary, the application of NO• donor resulted in no apparent difference in stomatal phenotype between wild types and *CATALASE* mutants (Figure 3). Collectively, this suggests that a rise in H_2O_2 precedes a NO• production and finely modulate the NO• generation to eventually regulate stomatal aperture. Bright et al. [11] has argued that NO• generation is dependent on the preceding H_2O_2 production. Our results using *CATALASE* mutants support their finding and furthermore emphasize a more dominant role of intracellular H_2O_2 than the other ROS for NO• generation being inferred by the phenotype of *CATALASE* mutants, which have an exclusively degrade H_2O_2 to O_2 and H_2O; and high correlation between intracellular H_2O_2 production and NO• production. Even though the specificity of catalase to H_2O_2 is exclusive, simultaneous intensification of other ROS species could occur by equilibration of chemical reaction of ROS at local proximity in guard cells of catalase mutants.

Previously, we have shown that a constitutive elevation of H_2O_2 does not affect stomatal aperture width, but an inducible elevation of H_2O_2 is involved in induction of stomatal closure [24–26]. Like the H_2O_2 production, we assumed that only inducible NO• production is able to induce stomatal closure. In this study, we observed only a marginal elevation of constitutive NO• levels in *CATALASE* mutants (Figures 1 and 2). Therefore, we interpret these results as indicating that the inducible H_2O_2 production is capable of inducing NO• generation, but the constitutive H_2O_2 production participates only a subtle in the NO• generation, if any.

NO• is reported to be produced by nitrate reductase (NR) and nitric oxide synthetase (NOS) in the plant system [16]. In the ABA response of guard cells, H_2O_2 might activate the NO• production systems. It was reported that H_2O_2-activated MAP kinase 6 regulated the activity of a nitrate reductase, NIR2, in Arabidopsis [34]. This system might be involved in this process. Further studies on the regulation of NO• production systems by intracellular H_2O_2 are required to uncover the mechanism.

Two major O_2•$^-$ generation mechanisms in guard cell signaling have been advocated: NOX and cell wall POX. NOX generate O_2•$^-$ from O_2 using NADPH as the electron donor. Cell wall POX, which has the compound I heme, generates an organic radical when it turns to the compound II heme form. The generated organic radical subsequently produce O_2•$^-$ from adjacent O_2 [35]. ABA and MeJA signals involve NOX-mediated ROS generation [22]. It has been established that intracellular H_2O_2 production elicited by NOX-mediated O_2•$^-$ participates in successive NO• production [36]. Reportedly, SA and chitosan signaling also involve NO• generation [37]. SA and chitosan signals involve ROS generation by cell wall POX [22]. The relation of H_2O_2 and NO• in the cell wall POX-mediated guard cell signaling remains to be unveiled. We do not deny the involvement of spontaneous formation of NO_2^- by disproportionation of NO• and subsequent NO_2• radical formation by POX.

It has not been elucidated that the rapid changes in H_2O_2 level in guard cell raised by light fluctuations or sudden nutritional perturbations render stomata closing. In this study, we used stable level of light intensity that is relatively low (80 μmol m^{-2} s^{-1}) compared to the maximum photosynthetic activity, while it is sufficient to induce stomatal opening. If the light intensity increases, the status of ROS in guard cells would change. Under a saturating level of light irradiation, photorespiration occurs for compromising ROS production. We do not need to consider such bulky flux of ROS from organelles in this experimental design.

Potential downstream signaling mechanisms for the co-production of H2O2 and NO•

Nitration of cGMP and tyrosine residues are the well-documented signaling mechanisms acting downstream of the NO• production as well as *S*-nitrosylation [38]. The action of NO• is reported to be different depending on the downstream mechanisms [38]. *S*-nitrosoglutathione (GSNO) is preferably used as the donor to pharmacologically induce *S*-nitrosylation [39]. SNP and SNAP are frequently used as the donors to directly generate NO•, which can induce *S*-nitrosylation and nitrations [9]. Contrary to rich reports for the induction of stomatal closure by SNP and SNAP [37], the exogenous application of GSNO inhibits the activity of OST1 protein kinase, which a positive regulator of ABA signaling [39]. In whole Arabidopsis plants, nitration of tyrosine residues of the ABA receptors, PYR/PYL/RCAR proteins, might lead to the proteasome-dependent degradation [40]. These results suggest that the *S*-nitrosylation and nitration may have bidirectional regulation in guard cell signaling allowing the regulation of Ca^{2+} signaling to generate a particular Ca^{2+} signature pattern [27,28]. Our results support the positive regulation of ABA signaling to lead stomatal closure. Taken together with previous reports, the intracellular H_2O_2 accumulation-induced NO• production may lead to nitration of the target molecules rather than the *S*-nitrosylation. The nitration after NO• production can occur through the formation of $ONOO^-$. Therefore, ABA signal in guard cells regulate the stomatal movement through the formation of $ONOO^-$, which might be triggered by H_2O_2 production and NO• production, positively regulates the stomata-closing signal.

Yamauchi et al. [41] reported that Arabidopsis autophagy-defective mutants, in which high accumulations of constitutive H_2O_2 occurred by aggregation of

peroxisomes and reduced catalase activity in guard cell, defected light-induced stomatal opening. In this case, the enormous constitutive H_2O_2 might overflow to reach the site of NOS/NR activation and subsequently generate $ONOO^-$. *GUARD CELL HYDROGEN PEROXIDE-RESISTANT1* might be involved in the process to activate Ca^{2+} channel [42].

fEarlier reports suggested the role of $ONOO^-$ in guard cell signaling as referred above. The formation of $ONOO^-$ is strictly dependent on the reaction of NO• and $O_2•^-$, but not H_2O_2 [20]. The NOX- and cell wall POX-mediated $O_2•^-$ production takes place in apoplastic space. Our results emphasized the involvement of intracellular H_2O_2 production and associated NO• production. Therefore, $O_2•^-$ should be produced in the cytosol concomitantly with NO• to eventually generate $ONOO^-$. It remains unresolved how $ONOO^-$ is generated in guard cells following the NO• production.

Conclusion

The constitutive H_2O_2 production in catalase mutant guard cells does not induce NO• production and stomatal closure. NO• production showed very high association with the inducible H_2O_2 production and stomatal closure. In agreement with earlier studies, NO• production was suggested to follow the preceding intracellular H_2O_2 accumulation. We proposed a novel hypothetical mechanism for $ONOO^-$ generation following H_2O_2 and NO• production in guard cells.

Author contributions

RJ, SM, ICM, YM: Conceptualization. RJ, TS, DM, MU, MAH, MMI: Investigation. RJ, ST, ICM: Formal analysis and Visualization. ICM, RJ, YN, SM, YM: Writing.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Nissan Science Foundation, the Ohara Foundation for Agricultural Research, and JSPS KAKENHI No. [24114709] to ICM.

ORCID

Shintaro Munemasa http://orcid.org/0000-0003-1204-1822
Izumi C. Mori http://orcid.org/0000-0002-2235-0779
Yoshiyuki Murata http://orcid.org/0000-0002-7864-1903

References

[1] Waszczak C, Carmody M, Kangasjarvi J. Reactive oxygen species in plant signaling. Annu Rev Plant Biol. 2018;69:209–236.
[2] Chater C, Peng K, Movahedi M, et al. Elevated CO_2 -induced responses in stomata require ABA and ABA signaling. Curr Biol. 2015;25:2709–2716.
[3] Khokon MAR, Hossain MA, Munemasa S, et al. Yeast elicitor-induced stomatal closure and peroxidase-mediated ROS production in Arabidopsis. Plant Cell Physiol. 2010;51:1915–1921.
[4] Khokon MAR, Okuma E, Hossain MA, et al. Involvement of extracellular oxidation burst in salicylic acid-induced stomatal closure in Arabidopsis. Plant Cell Environ. 2011;34:434–443.
[5] Kwak JM, Mori IC, Pei ZM, et al. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. Embo J. 2003;22:2623–2633.
[6] Suhita D, Raghavendra AS, Kwak JM, et al. Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol. 2004;134:1536–1545.
[7] Guzel Deger A, Scherzer S, Nuhkat M, et al. Guard cell SLAC1-type anion channels mediate flagellin-induced stomatal closure. New Phytol. 2015;208:162–173.
[8] Desikan R, Griffiths R, Hancok J, et al. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proc Natl Acad Sci USA. 2002;9:16314–16318.
[9] Garcia-Mata C, Gay R, Sokolovski S, et al. Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proc Natl Acad Sci USA. 2003;100:11116–11121.
[10] Guo FQ, Okamoto M, Crawford NM. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science. 2003;302:100–103.
[11] Bright J, Desikan R, Hancock J, et al. ABA-induced NO generation and stomatal closure in Arabidopsis dependent on H_2O_2 synthesis. Plant J. 2006;45:113–122.
[12] Srivavstava N, Gonugunta VK, Puli MR, et al. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*. Planta. 2009;229:757–765.
[13] Ye W, Muroyama D, Munemasa S, et al. Endogenous abscisic acid is involved in methyl jasmonate-induced reactive oxygen species and nitric oxide production but not in cytosolic alkalization in Arabidopsis guard cells. J Plant Physiol. 2013;170:1212–1215.
[14] Agurla S, Raghavendra AS. Convergence and divergence of signaling events in guard cells during stomatal closure by plant hormones or microbial elicitors. Front Plant Sci. 2016;7:1332.
[15] Desikan R, Cheung MK, Bright J, et al. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J Exp Bot. 2004 Jan;55(395):205–212.
[16] Neill S, Barros R, Bright J, et al. Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot. 2008;59:165–176.
[17] Mori IC, Schroeder JI. Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol. 2004;135:702–708.

[18] Joudoi T, Shichiri Y, Kamizono N, et al. Nitrated cyclic GMP modulates guard cell signaling in *Arabidopsis*. Plant Cell. 2013;25:558–571.
[19] Zhang J, Huang D, Wang C, et al. Recent progress in protein S-nitrosylation in phytohormone signaling. Plant Cell Physiol. 2019;60:494–502.
[20] Souza JM, Peluffo G, Radi R. Protein tyrosine nitration – functional alteration or just a biomarker? Free Radic Biol Med. 2008;45:357–366.
[21] Corpas FJ, Barroso JB. Peroxynitril ($ONOO^-$) is endogenously produced in arabidopsis peroxisomes and is overproduced under cadmium stress. Ann Bot. 2014;113:87–96.
[22] Mori IC, Murata Y, Uraji M. Integration of ROS and hormone signaling. In: Del Rio LA, Puppo A, editors. Reactive oxygen species in plant signaling. Berlin, Heidelberg: Springer-Verlag; 2009. p. 43–54.
[23] Niu L, Liao W. Hydrogen peroxide signaling in plant development and abiotic responses: crosstalk with nitric oxide and calcium. Front Plant Sci. 2016;7:230.
[24] Jannat R, Uraji M, Hossain MA, et al. Catalase negatively regulate methyl jasmonate signaling in guard cells. J Plant Physiol. 2012;169:1012–1016.
[25] Jannat R, Uraji M, Morofuji M, et al. The roles of *CATALASE2* in abscisic acid signaling in *Arabidopsis* guard cells. Biosci Biotechnol Biochem. 2011;75:2034–2036.
[26] Jannat R, Uraji M, Morofuji M, et al. Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in *Arabidopsis* guard cells. J Plant Physiol. 2011;168:1919–1926.
[27] Allen GJ, Chu SP, Harrington CL, et al. A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature. 2001;411:1053–1057.
[28] Allen GJ, Chu SP, Schumacher K, et al. Alternation of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. Science. 2000;289:2338–2342.
[29] Köhler B, Hills A, Blatt MR. Control of guard cell ion channels by hydrogen peroxide and abscisic acid indicates their actin through alternate signaling pathways. Plant Physiol. 2003;131:385–388.
[30] Pei ZM, Murata Y, Bennig G, et al. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature. 2000;406:731–734.
[31] Munemasa S, Hirao Y, Tanami K, et al. Ethylene inhibits methyl jasmonate-induced stomatal closure by modulating guard cell slow-type anion channel activity *via* the OPEN STOMATA1/SnRK2.6 kinase-independent pathway in Arabidopsis. Plant Cell Physiol. 2019;60:2263–2271.
[32] Kalyanaraman B, Darley-Usmar V, Davies KJ, et al. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. Free Radic Biol Med. 2012;52:1–6.
[33] Volk APD, Moreland JG. ROS-containing endosomal compartments: implications for signaling. Methods Enzymol. 2014;535:201–224.
[34] Wang P, Du Y, Li Y, et al. Hydrogen peroxide-mediated activation of MAP kinase 6 modulates nitric oxide biosynthesis and signal transduction in *Arabidopsis*. Plant Cell. 2010;22:2981–2998.
[35] Shigeto J, Tsutsumi Y. Diverse functions and reactions of class III peroxidases. New Phytol. 2016;209:1395–1402.
[36] Murata Y, Mori IC, Munemasa S. Diverse stomatal signaling and the signal integration mechanism. Ann Rev Plant Biol. 2015;66: 369–392.
[37] Gayatri G, Agurla S, Raghavendra AS. Nitric oxide in guard cells as an important secondary messenger during stomatal closure. Front Plant Sci. 2013;4:425.
[38] Laxalt AM, Garcia-Mata C, Lamattina L. The dual role of nitric oxide in guard cells: promoting and attenuating the ABA and phospholipid-derived signals leading to the stomatal closure. Front Plant Sci. 2016;7:476.
[39] Wang P, Du Y, Hou YJ, et al. Nitric oxide negatively regulates abscisic acid signaling in guard cells by S-nitrosylation of OST1. Proc Natl Acad Sci USA. 2015;112:613–618.
[40] Castillo MC, Lozano-Juste J, Gonzalez-guzman M, et al. Inactivation of PYR/PYL/RCAR ABA receptors by tyrosine nitration may enable rapid inhibition of ABA signaling by nitric oxide in plants. Sci Signal. 2015;8:ra89.
[41] Yamauchi S, Mano S, Oikawa K, et al. Autophagy controls reactive oxygen species homeostasis in guard cells that is essential for stomatal opening. Proc Natl Acad Sci USA. 2019;116:19187–19192.
[42] Hua D, Wang C, He J, et al. A plasma membrane receptor kinase, GHR1, mediates abscisic acid- and hydrogen peroxide-regulated stomatal movement in *Arabidopsis*. Plant Cell. 2012;24:2546–2561.