

Hydrogen Peroxide Is Involved in Abscisic Acid-Induced Stomatal Closure in *Vicia faba*¹

Xiao Zhang, Lin Zhang, Facai Dong, Junfeng Gao, David W. Galbraith, and Chun-Peng Song^{2*}

Department of Biology, Henan University, Kaifeng 475001, People's Republic of China (X.Z., L.Z., F.D., C.-P.S.); Department of Basic Courses, Northwestern Agricultural University, Yangling 712100, People's Republic of China (X.Z., J.G.); and Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721 (D.W.G.)

One of the most important functions of the plant hormone abscisic acid (ABA) is to induce stomatal closure by reducing the turgor of guard cells under water deficit. Under environmental stresses, hydrogen peroxide (H₂O₂), an active oxygen species, is widely generated in many biological systems. Here, using an epidermal strip bioassay and laser-scanning confocal microscopy, we provide evidence that H₂O₂ may function as an intermediate in ABA signaling in *Vicia faba* guard cells. H₂O₂ inhibited induced closure of stomata, and this effect was reversed by ascorbic acid at concentrations lower than 10⁻⁵ M. Further, ABA-induced stomatal closure also was abolished partly by addition of exogenous catalase (CAT) and diphenylene iodonium (DPI), which are an H₂O₂ scavenger and an NADPH oxidase inhibitor, respectively. Time course experiments of single-cell assays based on the fluorescent probe dichlorofluorescein showed that the generation of H₂O₂ was dependent on ABA concentration and an increase in the fluorescence intensity of the chloroplast occurred significantly earlier than within the other regions of guard cells. The ABA-induced change in fluorescence intensity in guard cells was abolished by the application of CAT and DPI. In addition, ABA microinjected into guard cells markedly induced H₂O₂ production, which preceded stomatal closure. These effects were abolished by CAT or DPI micro-injection. Our results suggest that guard cells treated with ABA may close the stomata via a pathway with H₂O₂ production involved, and H₂O₂ may be an intermediate in ABA signaling.

The plant hormone abscisic acid (ABA) regulates many important plant developmental processes, and induces tolerance to different stresses including drought, salinity, and low temperature (Giraudat et al., 1994). ABA production is increased in tissues during these stresses, and this causes a variety of physiological effects, including stomata closure in leaves. By opening and closing stomata, the guard cells control transpiration to regulate water loss or retention. Despite the recognitions of the central role played by ABA in regulating stomatal function, the signal transduction events leading to alterations to the stomatal aperture remain incompletely understood (Schroeder et al., 2001). Previous evidence showed that an elevation of cytosolic Ca²⁺, an increase in pH, and a reduction in K⁺, Cl⁻, and organic solute content in both guard cells surrounding the stomatal pore, are downstream elements of ABA-induced stomatal closure (MacRobbie, 1998; Assmann and Shimazaki, 1999), although their spatio-temporal relationships are merely understood. In addition, cADP-Rib, phospholipase C, and phospho-

lipase D have been identified as signaling molecules in the ABA response, and exerting their effects by regulating cytosolic Ca²⁺ concentration ([Ca²⁺]_i) and inward K⁺ channels (Wu et al., 1997; Leckie et al., 1998; Jacob et al., 1999; Staxen et al., 1999). Furthermore, Ca²⁺ channels and anion channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and ABA (Pei et al., 2000; Allen et al., 2000; Hamilton et al., 2000; Li et al., 2000), and an increase in [Ca²⁺]_i resulting from the activation of Ca²⁺ channels leading to Ca²⁺ influx is known to inactivate inward-rectifying K⁺ channels, biasing the plasma membrane for solute efflux, which drives stomatal closure (Blatt and Grabov, 1997).

It is well known that utilization of molecular oxygen may be proceeded by a series of single electron transfers, which generates reactive oxygen species (ROS), such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and other free radicals that react with, and thereby damage DNA, proteins, and lipids (Bowler et al., 1992; Scandalios, 1993). Earlier studies have indicated that the production of ROS is indirectly increased by stresses such as drought and chilling (Bowler et al., 1992; Fryer, 1992). It is interesting that H₂O₂ generated from an oxidative burst in pathogen-infected cells is thought to be a second messenger, which can both orchestrate the plant hypersensitive disease resistance by initialing a series of reactions (Levine et al., 1994) and mediate systemic signaling in the establishment of plant immunity

¹ This work was supported by the National Natural Science Foundation of China (grant no. 3990407021 to C.-P.S.) and by the National Key Basic Research Special Funds (grant no. G1999011700 to C.-P.S.).

² Present address: Department of Plant Sciences, Forbes Building 303, University of Arizona, Tucson, AZ 85721.

* Corresponding author; e-mail songcp@ag.arizona.edu; fax 520-621-7186.

(Neuenschwander et al., 1995; Allan and Fluhr, 1997; Alvarez et al., 1998). It has been found that O₂⁻ and other activated oxygen species are involved in the regulation of stomatal movement (Purohit et al., 1994). The oxidative stress resulting from exposure to methyl viologen (which generates O₂⁻) or H₂O₂ has a remarkable effect on stomatal aperture (Price, 1990), and exogenous H₂O₂ can also induce [Ca²⁺]_i increases in guard cells (McAinsh et al., 1996; Pei et al., 2000), comprising one or two separate transient increases, which are necessary for stomatal closure (Allen et al., 2000). Using recombinant aequorin in transgenic tobacco (*Nicotiana plumbaginifolia*), Price et al. (1994) have demonstrated that H₂O₂ stimulates a transient increase in [Ca²⁺]_i in whole tobacco seedlings.

Although the role of H₂O₂ as an intermediate in ABA signaling in guard cells has been clearly examined in Arabidopsis plants (Pei et al., 2000), it was not known whether H₂O₂ acts as a second messenger for the induction of stomatal closure in response to ABA in other plants, and where is the source of H₂O₂ generation in guard cells. To further confirm the role of H₂O₂ in the ABA signaling in other type plants, we therefore investigated the changes in stomatal behavior in response to H₂O₂ and ABA at the cellular level, and the relationships between ABA and H₂O₂ in ABA signaling cascade. Using *Vicia faba* plants, we provide evidence that H₂O₂ generation is an early event in ABA-induced stomatal closure.

RESULTS

ABA- and H₂O₂-Induced Changes in Stomatal Behavior

ABA, which is widely accepted as a stress signal, induces a reduction in stomatal aperture in a concentration-dependent manner (Schroeder et al., 2001). To gain insights into the mechanism of ABA-induced stomatal pore changes, we analyzed whether H₂O₂ might be involved in ABA effects on stomatal movements, in a manner similar to its effects in elicitor-induced defense responses. *V. faba* epidermal tissues were treated with 1 μM ABA in the presence of either 100 units mL⁻¹ catalase (CAT) or 10 μM diphenylene iodonium (DPI), which either remove H₂O₂ or reduce the generation of H₂O₂, respectively (Levine et al., 1994; Alvarez et al., 1998; Lee et al., 1999). Both reagents reversed the ABA-induced stomatal closure (Fig. 1), suggesting that ABA promotes stomatal closure via a pathway involving H₂O₂. Treatments of the epidermis with CAT or DPI alone did not cause any changes of stomatal aperture (Fig. 1), which is the same as the results reported previously (Lee et al., 1999). It is possible that under noninducing conditions by ABA, the amount of H₂O₂ or the activity of NADPH oxidase is low in guard cells. It is important to keep the low level of H₂O₂ in the cells under optimal conditions, because the oxidative stress, resulting from ROS, is

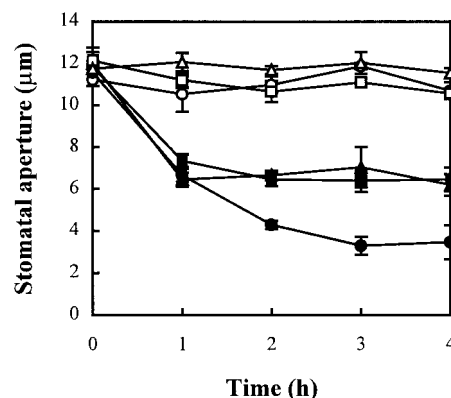


Figure 1. The effect of CAT and DPI on the ABA-induced stomatal closing. Isolated epidermis of *V. faba* was incubated in CO₂-free MES [2-(*N*-morpholino)-ethanesulfonic acid]-KCl for 3 h under conditions promoting stomatal opening and then transferred to fresh CO₂-free MES-KCl containing no ABA (○), 1 μM ABA (●), 1 μM ABA + 100 units mL⁻¹ CAT (■), 1 μM ABA + 10 μM DPI (▲), 100 units mL⁻¹ CAT (□), or 10 μM DPI (△) only for another 4 h. Stomatal apertures were determined at 1-h intervals during the 4-h incubation. Values are the means of 120 measurements ±SE.

harmful to cell (Scandalios, 1993). These dangerous cascades are prevented by efficient operation of the cell's antioxidant defense (Noctor and Foyer, 1998). Therefore, the indirect evidence suggests a role for H₂O₂ as a common and critical intermediate for the signaling in ABA-induced stomatal closure.

Previous studies have suggested that oxidative stress resulting from exposure to methyl viologen or H₂O₂ has a marked effect on stomatal aperture (Price, 1990; McAinsh et al., 1996; Allen et al., 2000; Pei et al., 2000). Exogenous application of H₂O₂ promoted stomatal closure (Fig. 2) in a dose-dependent manner. The effect of H₂O₂ on promotion of stomatal closure was significant ($P < 0.05$) at a concentration of H₂O₂ ≥ 10⁻⁵ M. The maximum promotion of stomatal closure was observed at 2 h after treatment with 10⁻³ M H₂O₂, under which conditions the stomatal apertures were 5.54 ± 0.54 μm, or 46% of the control value. However, in washout experiments, the effects of H₂O₂ (≤10⁻⁵ M) on stomatal aperture were completely reversible (Fig. 2). There was no significant ($P < 0.05$) difference between the apertures of stomata treated with H₂O₂ for 2 h followed by a 2-h washout and those that were incubated under the same conditions for 4 h in the absence of H₂O₂.

In plant cells, the most important reducing substrate for H₂O₂ removal is ascorbic acid (Noctor and Foyer, 1998). Ascorbate peroxidase uses two molecules of ascorbic acid to reduce H₂O₂ to water, with the concomitant generation of two molecules of monodehydroascorbate. The relatively high concentration of ascorbic acid in the cell ensures stable maintenance of the cellular redox state. When ascorbic acid was applied together with 10⁻⁵ M H₂O₂, the effects of H₂O₂-induced stomatal closure were partly abolished in a concentration-dependent manner (Fig.

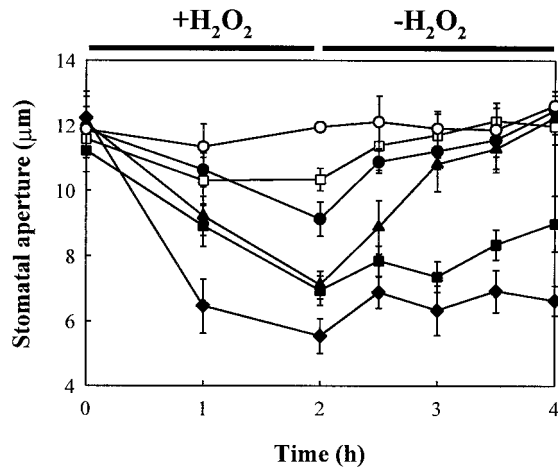


Figure 2. Promotion of stomatal closure by H_2O_2 and its reversibility. Isolated epidermis of *V. faba* was incubated in CO_2 -free MES-KCl for 3 h under conditions promoting stomatal opening and then transferred to fresh CO_2 -free MES-KCl containing H_2O_2 (0 M, \circ ; 10^{-7} M, \square ; 10^{-6} M, \bullet ; 10^{-5} M, \blacktriangle ; 10^{-4} M, \blacksquare ; or 10^{-3} M, \blacklozenge) for 2 h under opening conditions, and stomatal apertures were determined at 1-h intervals during the 2-h H_2O_2 application (+ H_2O_2), and at 30-min intervals during the 2-h H_2O_2 removal in “washout experiment” ($-\text{H}_2\text{O}_2$). Values are the means of 120 measurements \pm SE.

3). Ascorbic acid alone induced a slight opening of stomata over untreated controls. Therefore, it appears that the intercellular redox state of cell reacts rapidly to the accumulation of H_2O_2 induced by ABA.

ABA Induces H_2O_2 Production in Guard Cells

Having established that exogenous H_2O_2 is involved in the regulation of stomatal changes induced by ABA in the above epidermal strips bioassay ex-

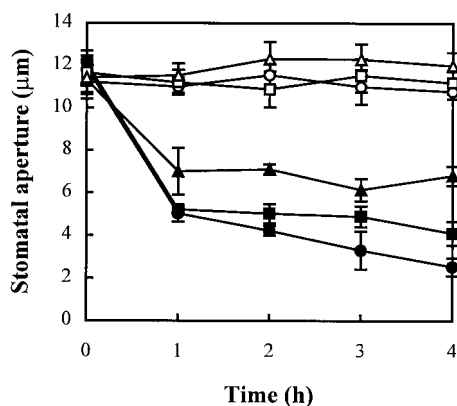


Figure 3. Effects of ascorbic acid on the promotion of stomatal closure by H_2O_2 . Isolated epidermis of *V. faba* was incubated in CO_2 -free MES-KCl for 3 h under conditions promoting stomatal opening and then transferred to fresh CO_2 -free MES-KCl containing no (\circ), 10^{-5} M H_2O_2 (\bullet), 10^{-5} M H_2O_2 + 1 mM ascorbic acid (\blacksquare), 10^{-5} M H_2O_2 + 10 mM ascorbic acid (\blacktriangle), 1 mM (\square), or 10 mM (\triangle) ascorbic acid only for another 4 h. Stomatal apertures were determined at 1-h intervals during the 4-h incubation. Values are the means of 120 measurements \pm SE.

periments, we then examined whether external ABA might increase the level of H_2O_2 in guard cells. In this study, we used the oxidatively sensitive fluorophore dichlorofluorescein (H_2DCF) to measure fast changes in intracellular H_2O_2 level directly. The nonpolar diacetate ester ($\text{H}_2\text{DCF-DA}$) of H_2DCF enters the cell (Allan and Fluhr, 1997) and is hydrolyzed into the more polar, nonfluorescent compound H_2DCF , which therefore is trapped. Subsequent oxidation of H_2DCF by H_2O_2 , catalyzed by peroxidases, yields the highly fluorescent DCF (Cathcart et al., 1983). $\text{H}_2\text{DCF-DA}$ loads readily into guard cells, and its optical properties make it amenable to analysis using laser scanning confocal microscopy.

As shown in Figure 4, exogenous application of ABA enhanced the relative fluorescence intensity of DCF in guard cells, which represents as pixel intensity averaged over the entire cells, and the effects were dose dependent. A single cell assay also illustrated that 1 μM ABA significantly induced increases in DCF fluorescence intensity in guard cells, and the H_2O_2 elevation was observable throughout the entire surface of *V. faba* guard cells treated with ABA for 5 min (Fig. 5, A–F).

To determine which cellular compartments underwent increases in fluorescence intensity, subcellular regions were delineated using bright-field analysis as previously described (Allan and Fluhr, 1997). Chloroplast regions can be easily defined in bright field under microscope, whereas the cytosol was the region devoid of visible organelles, possibly including cytoplasmic and vacuolar regions. Time course quantitative analysis was performed in the chloroplast (area a), and the cytosol (areas b–d) as shown in Figure 5G. The earliest increase in ABA-induced H_2O_2 was in the region of chloroplast. This result is similar to the early observation that the earliest increase in DCF fluorescence induced by cryptogein was in the chloroplastic regions in guard cells (Allan and Fluhr, 1997).

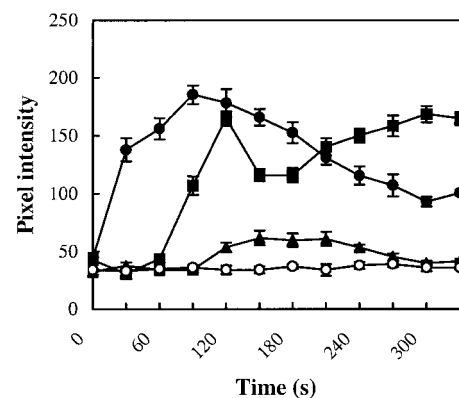


Figure 4. Effects of ABA on the DCF fluorescence in guard cells. (\circ), 0 μM ; (\blacktriangle), 0.1 μM ; (\blacksquare), 1 μM ; and (\bullet), 10 μM ABA. Each time point represents the mean of 10 measurements of pixel intensity of the whole cell determined in three independent experiments (\pm SE).

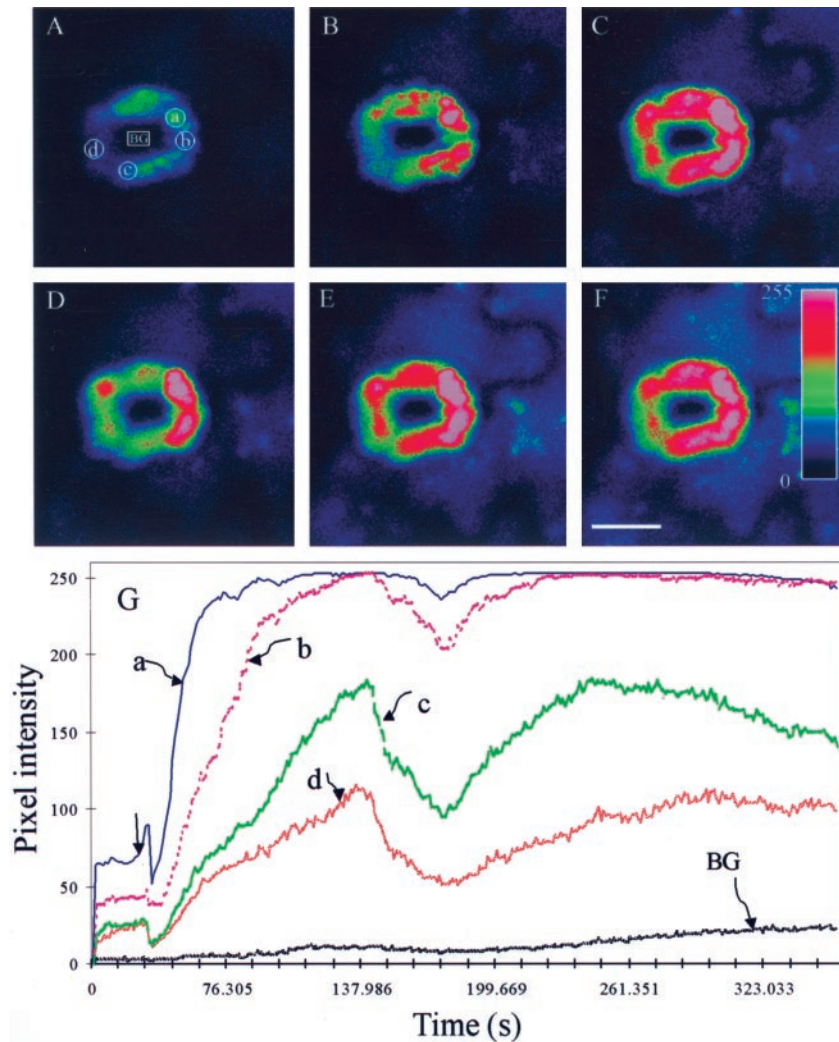


Figure 5. Exogenous ABA-induced production of H₂O₂ in guard cells. A, A pair of guard cells loaded with DCFH-DA before the addition of 1 μM ABA. B through F, The same cells shown in A at 60, 120, 180, 240, and 300 s after the addition of 1 μM ABA, respectively. G, Time course changes in the pixel intensity of selected cytosolic regions as represented by the boxes in A. BG represents background fluorescence selected region of stomatal pore. The figures show representative fluorescence image and time course from three independent experiments. The pseudocolor key is shown in the bar (F), which was applied to pixel intensity values (0–255) for all of the six fluorescence images. Scale bar represents 10 μm for all of the images. Arrow in G indicates the addition of ABA.

It was noticed that the ABA-induced increase in H₂O₂ production in the presence of 10 μM ABA in guard cells for different periods exhibited a biphasic pattern of changes, whereby about 2-min incubation increased and 2.5-min incubation decreased the DCF fluorescence intensity compared with their respective control values (Fig. 4). The mean period of the biphasic pattern in the treatment of 1 μM ABA displayed slightly changes over 10 μM ABA treatment, which shows 1-min incubation increase and 1-min decrease in H₂O₂ generation. This was also the case when the H₂O₂ production in selected different cytosolic regions was measured in the presence of 1 μM ABA (Fig. 5G). It is well known that high doses of H₂O₂ are cytotoxic (Bowler et al., 1992; Scandalios, 1993). A state of moderately increased levels of interacellular H₂O₂ is referred to as oxidative stress. The biphasic pattern of H₂O₂ production in guard cells, acting as cell signaling messenger for H₂O₂, might allow all cells to tightly control their level within a very narrow range. The homeostatic modulation of oxidant levels is a highly efficient mechanism that appeared in evolution. This observation might be consistent

with the early results that the cytosolic Ca²⁺ oscillations of differing amplitudes and frequencies induced by H₂O₂ (Allen et al., 2000).

The Effects of CAT and DPI on H₂O₂ Generation Induced by ABA

H₂O₂ is extremely sensitive to CAT, and flavin-dependent enzymes, including the mammalian NADPH oxidase, are strongly inhibited by DPI (Alvarez et al., 1998; Potikha et al., 1999). As shown in Figure 5, A through F, the increase of fluorescence in guard cells treated with ABA was accompanied by an increase of fluorescence in adjacent epidermal cells. We had expected that epidermal cells treated with ABA might also produce H₂O₂. However, the following experiments argue against this as conclusion, and suggest that the H₂O₂ in the epidermal cells came from the guard cells. Addition of CAT before ABA treatment abrogated the increases in fluorescence in guard cells and their adjacent epidermal cells, and the epidermal cells were more sensitive to exogenous CAT than guard cells. A possible explanation for this

phenomenon is that ABA-induced H_2O_2 was dissipated from guard cells to their peripheries, which was then blocked by the exogenously added CAT remaining in the apoplast. In a parallel experiment, although $10 \mu M$ DPI partly abolished ABA-induced fluorescence, the fluorescence in the chloroplasts was much more enhanced than that before ABA treatment (Fig. 6, C–F). This implies that the reduction of fluorescence intensity is due to the generation of H_2O_2 through flavin-dependent enzymes (including NADPH oxidase) blocked by DPI. However, the production of H_2O_2 from chloroplasts was not inhibited by DPI. These results further indicated that NADPH oxidase was activated by exogenous ABA treatment, and the oxidative environment of the chloroplasts was enhanced by ABA.

The Effects of Intracellular ABA on the Generation of H_2O_2 by Guard Cells and on the Stomatal Aperture

There has been no conclusive identification of an ABA receptor in plant cells. Previous work, including stomata closure induced by the ABA microinjected into guard cells, indicates an internally localized ABA reception site (Allan et al., 1994; Schwartz et al., 1994). An external reception site has also been suggested (Anderson et al., 1994), and these two possibilities are not mutually exclusive, i.e. there could be ABA reception sites both inside and outside the cell

(MacRobbie, 1995). As shown in the above experiments, externally added ABA induced production of H_2O_2 , which promoted stomatal closure. This raised the question as to whether intracellular ABA might have the same effects. To answer this question, we microinjected or comicroinjected reagents into guard cells, and examined DCF fluorescence in one of the paired guard cells at different times after microinjection. We found that ABA microinjection induced H_2O_2 production in the injected guard cell, and with increase of treatment time, the injected cells exhibited more rapid increase in DCF fluorescence than their uninjected counterparts (Fig. 7, E–H). CAT ($100 \text{ units mL}^{-1}$) completely abolished ABA-induced fluorescence increases in *V. faba* guard cells following microinjection (Fig. 7, I–L), which is the same as the results from the microinjection of buffer without ABA and CAT (Fig. 7, A–D). However, following comicroinjection of ABA and DPI, the cells showed DCF fluorescence increases only in the regions where chloroplasts were located (Fig. 7, M–P), which is similar to the observations that cells treated using externally added ABA in the presence of DPI (Fig. 6, C and F). It looks like the location of increase in DCF fluorescence might also be near the regions of the nucleus from the image (Fig. 7, M–P). Moreover, it is clearly observed that there are several green particles corresponding to its fluorescence images under bright-light microscope. This at least indicated that

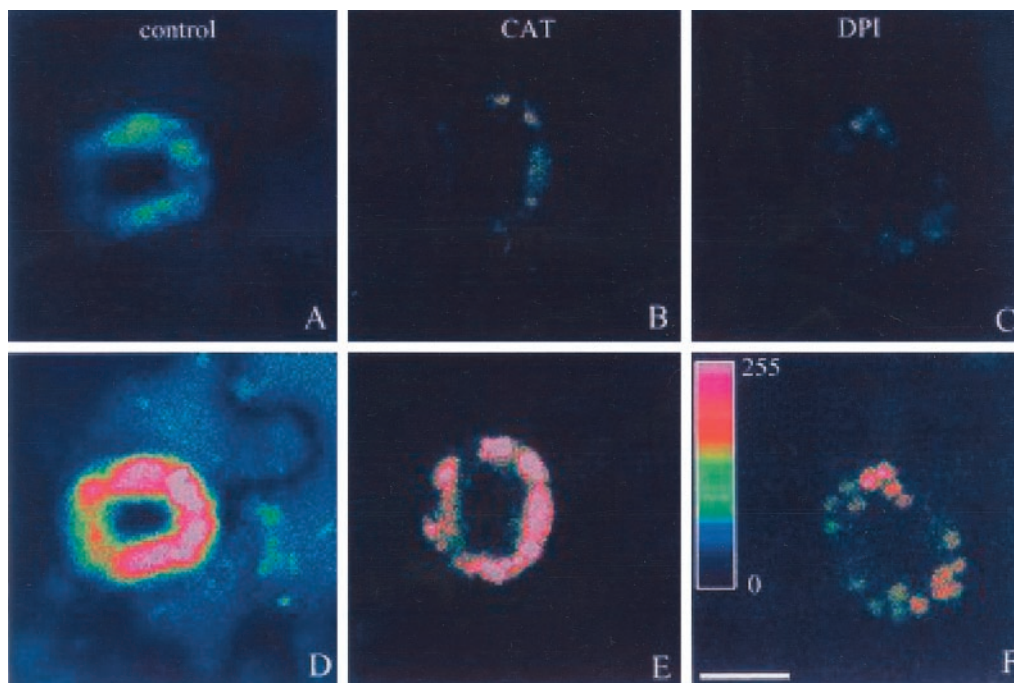


Figure 6. Effects of CAT and DPI on ABA-induced H_2O_2 in guard cells. A through C, Guard cells loaded with DCFH-DA before the addition of $1 \mu M$ ABA, in which cells (B and C) in the presence of CAT ($100 \text{ units mL}^{-1}$) and DPI ($10 \mu M$), respectively. D–F, Cells shown in A through C, respectively, 300 s after the addition of $1 \mu M$ ABA. The pseudocolor key in F is shown within the bar, which was applied to pixel intensity values (0–255) for all of the six fluorescence images. Scale bar represents $10 \mu m$ for all of the images.

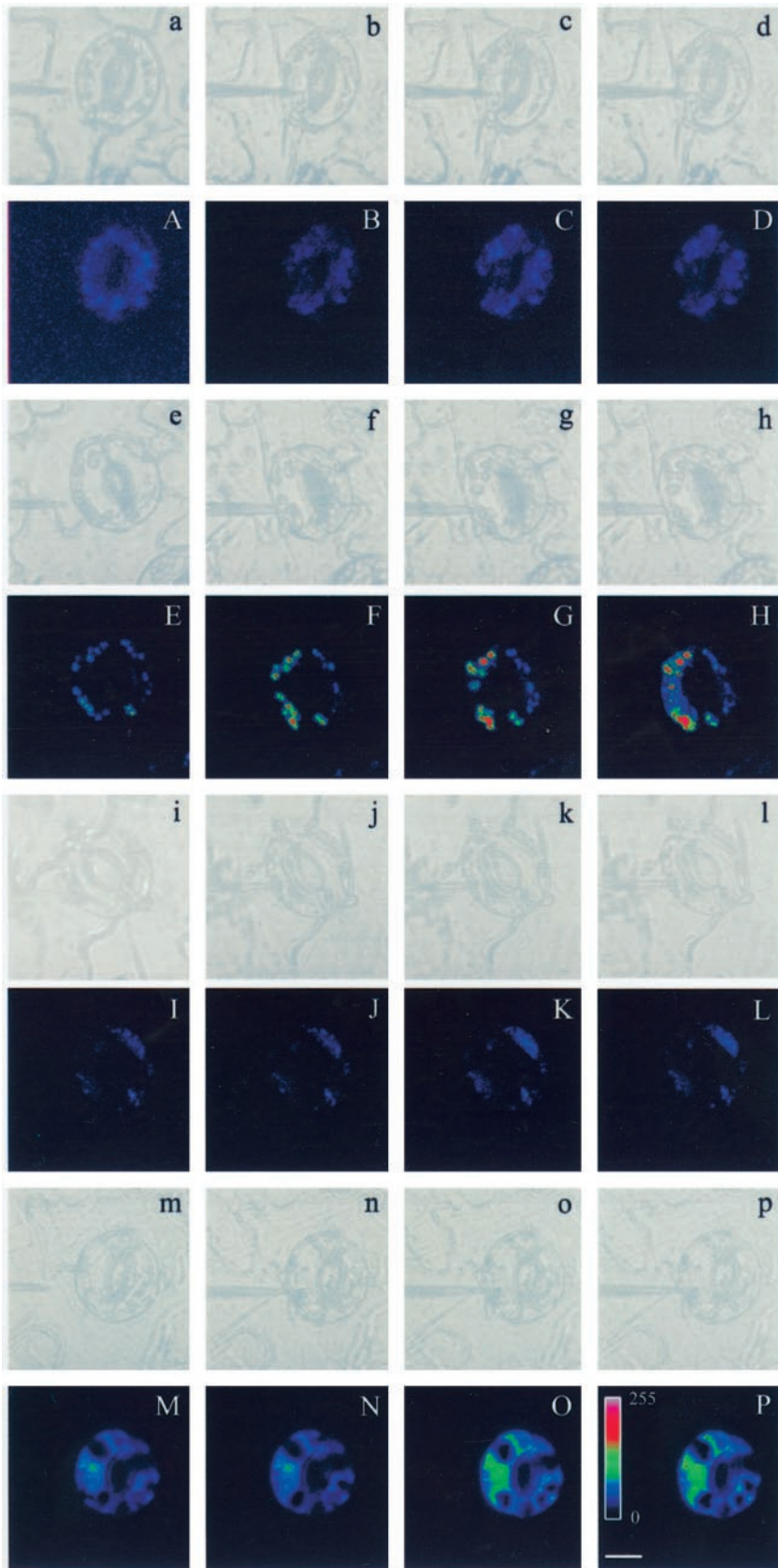


Figure 7. Laser scanning confocal imaging of (co) microinjection reagents into guard cells. A, E, I, and M, Guard cell loaded with H₂DCF-DA before microinjection. B through D, F through H, J through L, and M through P, Cells shown in A, E, I, and M 15, 60, and 120 s, respectively, after microinjection buffer (A–D), ABA (1 μM; E–H), ABA (1 μM) and CAT (100 units mL⁻¹; I–L), and ABA (1 μM) and DPI (10 μM; M–P). a through p, Bright-light images corresponding to fluorescence images (A–P, respectively). The pseudocolor key in L is shown within the bar, which was applied to pixel intensity values (0–255) for all of the fluorescence images. Scale bar represents 10 μm for all of the images.

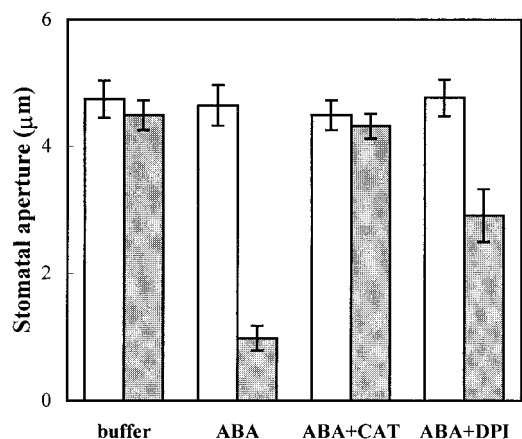


Figure 8. Effects of reagents or buffer microinjection on stomatal aperture in *V. faba*. One guard cell in a pair was injected. In each case, white bars (□) represent the half-apertures of the uninjected guard cells in the pairs and gray bars (▨) represent the half-aperture of the injected guard cells in the pairs. The stomatal aperture measurements were performed at 45 min after microinjection of ABA into guard cells. Values shown are the means \pm SE ($n = 10$ for each treatment).

oxidized dye accumulating either in the regions of chloroplasts or in the region of the nucleus.

In considering the source of H_2O_2 in the cellular regions, care must be taken to prevent DCF sequestration into cellular compartments. Previous studies have shown that such problems cannot arise with H_2DCF -DA-based assays for H_2O_2 production, due to relatively high levels of peroxidase activities in cytosol of guard cells (Allan and Fluhr, 1997), resulting in H_2DCF -DA deacetylated to essentially non-permeate H_2DCF , which is nonfluorescent but became oxidized fluorescent, and also non-permeating DCF (Cathcart et al., 1983). On the other hand, our results indicate that CAT (100 units mL^{-1}) completely abolished ABA-induced fluorescence increases, including chloroplasts, in *V. faba* guard cells following microinjection (Fig. 7, I–L). CAT sensitivity rules out the possibility that DCF is sequestered into chloroplast because the exogenously injected CAT remains in the cytoplasm.

It is important that 45 min after microinjection of ABA into guard cells (see "Materials and Methods"), the stomatal half-aperture displayed a significant ($P < 0.05$) reduction, from $4.643 \pm 0.320 \mu m$ to $0.978 \pm 0.192 \mu m$. Comicro-injection of ABA and DPI resulted in a reduction in stomatal half-aperture from $4.769 \pm 0.287 \mu m$ to $2.910 \pm 0.417 \mu m$, whereas comicro-injection of ABA and CAT resulted in no significant ($P < 0.05$) change in the half-aperture of the stomata, which is similar to the results following microinjection with buffer alone (Fig. 8). These findings suggest that H_2O_2 is a possible intermediate of the signal transduction pathway of ABA, and/or an ABA reception site is localized internally.

DISCUSSION

Under stress conditions, ABA and H_2O_2 are commonly generated in many biological systems (Assmann and Shimazaki, 1999; Potikha et al., 1999). It has been widely confirmed that ABA regulates stomatal movement as a stress signal (Assmann and Shimazaki, 1999), yet there remain considerable gaps in our knowledge regarding a detailed description of the events and underlying signal transduction mechanisms involved in stomatal closing (MacRobbie, 1998). ROS appear to play a crucial role in physiological and pathological processes of plants. H_2O_2 , in particular, has been previously implicated as a second messenger in the regulation of the plant hypersensitive response (Mehdy, 1994; Low and Merida, 1996) and plays an important intermediary role in the ABA signal transduction pathway leading to the induction of the *Cat1* gene (Guan et al., 2000).

Here, we provide new evidence that H_2O_2 is involved in ABA-induced stomatal movement in *V. faba*, which is consistent with recent findings in Arabidopsis plants (Pei et al., 2000). The following results support this conclusion: (a) exogenously added H_2O_2 induced stomatal closure, (b) scavenging of H_2O_2 and inhibiting H_2O_2 generation reversed the H_2O_2 - or ABA-induced stomatal closure, and (c) H_2O_2 generation coincided with stomatal closure. In addition, our previous work using voltage clamp method has demonstrated that the stomatal closure induced by externally applied H_2O_2 is partially due to the inhibition of K^+ uptake and the activation of K^+ release through K^+ channels on the plasma membrane of guard cells (An et al., 2000). McAinsh et al. (1996) have also provided evidence that oxidative stress induces stomatal closure, and an increase in cytosolic free Ca^{2+} concentration in guard cells of *Commelina communis*. The direct recording of Ca^{2+} currents have resulted recently in a breakthrough (Hamilton et al., 2000; Pei et al., 2000), leading to the discovery of H_2O_2 -activated Ca^{2+} channels as an important part of the mechanism for ABA-induced stomatal closure (Pei et al., 2000). It is interesting that H_2O_2 may play different roles during different biological processes. In plant defense responses, H_2O_2 functions as a signal molecule. On the other hand, their production during the environmental stresses is thought to be a byproduct of stress metabolism and is thought to induce cellular damage.

In the washout experiments, at concentrations lower than 10^{-5} M, the effects of H_2O_2 on stomatal behavior were reversible, whereas at concentrations higher than 10^{-5} M, the effects were irreversible (Fig. 2), which is the same as that seen in previous work (McAinsh et al., 1996). This implies that at low concentrations the effects of H_2O_2 may be due to the activation of a signaling cascade, whereas at high concentrations the effects of H_2O_2 may be due to the membrane integrity changes. The fact that H_2O_2 production preceded stomatal closure in guard cells challenged with externally or internally added ABA

(Figs. 5 and 7, E–H), and that the ABA-induced stomatal closure was reversible by washout experiments (data not shown), indicates that ABA-stimulated H₂O₂ does not damage guard cells, and it can induce stomatal closing that can be reversed by other stimuli.

In higher plants, ROS can be generated by several different pathways (Allan and Fluhr, 1997; Bolwell et al., 1998). These pathways may include a cell wall-localized peroxidase (Bolwell et al., 1995), amine oxidases (Allan and Fluhr, 1997), non-flavin NADPH oxidases (Van Gestelen et al., 1997), and NADPH oxidases, which resemble the flavin-containing NADPH oxidases activated by Rac in leukocytes (Xing et al., 1997).

It is important that the highly energetic reactions of photosynthesis and an abundant oxygen supply make the chloroplast a particularly rich potential source of ROS. This involves O₂ competing for electrons from photosystem I, thereby leading to the generation of ROS through the Mehler reaction (Foyer, 1997). It has been suggested that O₂ might serve as an alternative electron acceptor when NADPH availability is limited, which would result in increased O₂⁻ production (Polle, 1997). It should be noted that guard cells contain chloroplasts, which can both produce NADPH and ATP (Zeiger et al., 1981), and release O₂ (Wu and Assmann, 1993) by the action of functional light reaction. However, the amount of Rubisco is very low in guard cell chloroplasts as compared with that in leaf cell chloroplasts, and the CO₂ assimilation catalyzed by Rubisco through photosynthesis only provides 2% of sugar required for stomatal opening (Reckmann et al., 1990). This indicates that it may be possible for guard cells to accumulate high chemical energy products such as NADPH and ATP. The question of whether Rubisco activity actually contributes significantly to osmotic buildup associated with stomatal opening is still an open question (MacRobbie 1997). Therefore, until now, the exact function of chloroplast in guard cells remains unclear.

We have investigated the subcellular source and possible molecular events involved in H₂O₂ generation in guard cells challenged with ABA. Time course experiments (Fig. 5) showed that chloroplasts might be the main regions of H₂O₂ production. The inhibition of both ABA- or H₂O₂-induced stomatal closing (Figs. 1 and 3) and ABA-induced H₂O₂ elevation of guard cells (Fig. 6, B and E) by externally applied CAT showed that H₂O₂ might also act externally to the guard cell plasma membrane (Lee et al., 1999) because CAT is not likely to cross the plasma membrane. This is consistent with the high permeability of the membrane to H₂O₂ (Yamasaki et al., 1997). To assess ABA-induced H₂O₂ levels in vivo, we co-injected reagents into guard cells, finding that internally applied CAT and DPI completely or partly inhibited ABA-induced H₂O₂ production and half-

stomatal closure (Fig. 7, I–L and M–P). This further indicated that NADPH oxidase located at plasma membrane, as well as light reaction in chloroplasts, contributes to H₂O₂ production, and that there might be internal ABA reception sites in guard cells. In fact, various environmental perturbations (e.g. intense light, drought, temperature stress, etc.) can induce excessive ROS, which may overwhelm the defense system and necessitate additional defense (Scandalios, 1993; Foyer et al., 1994). Therefore, guard cell generated H₂O₂ and O₂⁻ under different environmental conditions, which can regulate stomata closure.

In summary, the accumulated evidence suggest that H₂O₂ can be generated in guard cells, thereby providing new intermediates for ABA signaling. These results not only suggest the mechanism of stomatal closure under stresses such as drought and high-intensity light, during which an oxidative burst has been found in non-stomatal tissues (Legendre et al., 1993; Auh and Murphy, 1995), but also provide a possible explanation for the function of chloroplasts in guard cells. At present, the spatio-temporal relationships between H₂O₂ and other intracellular second messengers (Ca²⁺, IP₃, and pH) remain unknown.

MATERIALS AND METHODS

Chemicals

Dichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide to produce a 50-mM stock solution, which was aliquoted. ABA (±) and DPI was from Sigma (St. Louis). CAT (bovine liver) was from Calbiochem (La Jolla, CA). Unless stated otherwise, the remaining chemicals were of analytical grade from Chinese companies.

Plant Material

Broad bean (*Vicia faba*) was grown in a greenhouse with a humidity of about 70%, a photon flux density of 0.20 to 0.30 mmol m⁻² s⁻¹, and an ambient temperature (day/night 25°C ± 2°C/20°C ± 2°C). Immediately prior to each experiment, the epidermis was peeled carefully from the abaxial surface of the youngest, fully expanded leaves of 4-week-old plants, and cut into 5-mm lengths.

Epidermal Strip Bioassay

Stomatal bioassay experiments were performed as described (McAinsh et al., 1996) with slight modifications. To study the promotion of stomatal closure by ABA and H₂O₂, freshly prepared abaxial epidermis was first incubated in CO₂-free 50 mM KCl/10 mM MES-Tris, pH 6.15 (MES-KCl) for 3 h under conditions promoting stomatal opening (at 22°C–25°C, under a photon flux density of 0.20–0.30 mmol m⁻² s⁻¹) to open the stomata. The epidermis was then transferred to CO₂-free MES-KC in the presence of ABA (0.0, 0.1, 1.0, and 10 μM) or H₂O₂ (0, 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶,

and 10^{-7} M) with and without CAT, DPI, or ascorbic acid, for another 2 h, or as indicated. In washout experiments, strips were subsequently transferred to fresh CO₂-free MES-KCl for 2 h, and stomatal apertures were determined via confocal microscopy at 30-min intervals. In all cases, the strips were subsequently examined under the microscope to determine the aperture of the stomatal pores.

Dye Loading

The epidermal strips, previously incubated for 3 h under conditions promoting stomatal opening, were placed into loading buffer with 10 or 50 mM Tris-KCl (pH 7.2) containing 50 μ M of H₂DCF-DA. Before further experiments, peels were pre-incubated in the dark for 10 to 15 min.

Micro-injection of Stomatal Guard Cells

The peels loaded with H₂DCF-DA were floated on fresh buffer (Tris-KCl at 10 or 50 mM, pH 7.2) to wash off excess dye in the apoplast, and were then affixed to a polyacrylate plastic dish with 0.5 mL of H₂DCF-DA-free loading buffer. We then performed microinjection into guard cells under a TE300 (objective 40 \times 0.60 Plan Fluor) inverted microscope (Nikon, Tokyo) with a micromanipulator system (188NE, Narishige Scientific Instruments, Tokyo) according to a method with slight modification of methods of Ma et al. (1999) and Perona et al. (1999). Micropipettes (tip diameter 0.5 μ m) for injection were made from borosilicate glass capillaries (GD-1, Narishige Scientific Instruments) using a micropipette puller (PC-10, Narishige Scientific Instruments). Micropipette tips were front filled with injection reagents by applying a negative pressure.

To examine the effects of microinjection on stomatal behavior, stomata with apertures of 8 to 10 μ m were chosen for microinjection of different reagents (Gilroy et al., 1991; Schwartz et al., 1994). Micropipettes contained buffer (Tris-KCl, pH 7.2) as well as the treatment reagents. The microinjection tip reached no more than 3 μ m into the cytoplasm. Five minutes after microinjection, the micropipette tips were slowly removed and the cells allowed to recover for approximately 10 min. Guard cells that failed to recover normally and that had any visible morphological changes, such as turgid losses and differences in organelle shape and distribution between injected guard cells and noninjected counterparts (Gilroy et al., 1991), were discarded. Epidermal strips with injected stomata that recovered (or nearly recovered) to the same aperture as those on the remaining part of the epidermal strip, and for which both the injected and uninjected cells of a single stoma exhibited the same increase in turgor, were maintained under white light at 0.20 mmol m⁻² s⁻¹ for another 30 min, and the stomatal apertures were recorded and measured. Fluorescein diacetate staining was used to further confirm cellular viability after each experiment. Criteria for cells damaged by injected were those described by Gilroy et al. (1991).

Laser Scanning Confocal Microscopy

Examinations of peel fluorescence was performed using a MicroRadiance Laser scanning confocal microscope (Bio-Rad, Hercules, CA), with the following settings: ex = 488 nm, em = 525 nm, power 3%, zoom 4, mild scanning, frame 512 \times 512, and Timecourse and Photoshop software. To enable the comparison of changes in signal intensity, confocal images were taken under identical exposure conditions (in manual setup) for all samples. ABA or other reagents were added directly to the buffer or microinjected into guard cells during the time course. The experiments were repeated at least three times in each treatment, and the selected confocal image represented the same results from about 10 time course experiments.

ACKNOWLEDGMENTS

We thank Dr. Michael Deyholos and Shuhua Yuan (University of Arizona, Tucson) for critical reading of this manuscript.

Received February 9, 2001; returned for revision April 11, 2001; accepted May 15, 2001.

LITERATURE CITED

- Allan AC, Fluhr R (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell* 9: 1559–1572
- Allan AC, Fricker MD, Ward JCL, Beale MH, Trewavas AJ (1994) Two transduction pathways mediate rapid effects of abscisic acid in *Commelina* guard cells. *Plant Cell* 6: 1319–1328
- Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF et al. (2000) Alternation of stimulus-specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. *Science* 289: 2338–2342
- Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92: 773–784
- An GY, Song CP, Zhang X, Jing YC, Yang DM, Huang MJ, Wu CH, Zhou PA (2000) Effect of hydrogen peroxide on stomatal movement and K⁺ channel on plasma membrane in *Vicia faba* guard cell. *Acta Phytophysiol Sin* 26: 458–463
- Anderson BE, Ward JM, Schroeder JI (1994) Evidence for an extracellular reception site for abscisic acid in *Commelina* guard cells. *Plant Physiol* 104: 1177–1183
- Assmann SM, Shimazaki KI (1999) The multisensory guard cell, stomatal responses to blue light and abscisic acid. *Plant Physiol* 119: 809–815
- Auh CK, Murphy TM (1995) Plasma membrane redox enzyme is involved in the synthesis of O₂⁻ and H₂O₂ by *Phytophthora* elicitor-stimulated rose cell. *Plant Physiol* 107: 1241–1247
- Blatt MR, Grabov A (1997) Signaling gates in abscisic acid-mediated control of guard cell ion channels. *Physiol Plant* 100: 481–490

- Bolwell GP, Butt VS, Davies DR, Zimmerlin A** (1995) The origin of the oxidative burst in plants. *Free Radic Res* **23**: 517–532
- Bolwell GP, Davies DR, Gerrish C, Auh C-K, Murphy TM** (1998) Comparative biochemistry of the oxidative burst produced by rose and French bean cells reveals two distinct mechanisms. *Plant Physiol* **116**: 1379–1385
- Bowler C, van Montagu M, Inze D** (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* **43**: 83–116
- Cathcart R, Schwiers E, Ames BN** (1983) Detection of picomol levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem* **134**: 111–116
- Foyer CH** (1997) Oxygen metabolism and electron transport in photosynthesis. In JG Scandalios, ed, *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 587–621
- Foyer CH, Descourvieres P, Kunert KJ** (1994) Protein against oxygen radicals: an important defense mechanism studied in transgenic plants. *Plant Cell Environ* **17**: 507–523
- Fryer MJ** (1992) The antioxidant effect of thylakoid vitamin E (α-tocopherol). *Plant Cell Environ* **15**: 381–392
- Gilroy S, Fricker MD, Read ND, Trewavas AJ** (1991) Role of calcium on signal transduction of *Commelina* guard cells. *Plant Cell* **3**: 333–344
- Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J** (1994) Current advances in abscisic acid action and signaling. *Plant Mol Biol* **26**: 1557–1577
- Guan L, Zhao J, Scandalios JG** (2000) *Cis*-elements and *trans*-factors that regulate expression of the maize *Cat1* antioxidant gene in response to ABA and osmotic stress: H₂O₂ is the likely intermediary signaling molecule for the response. *Plant J* **22**: 87–95
- Hamilton D, Hills A, Kohler B, Blatt MR** (2000) Ca²⁺ channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proc Natl Acad Sci USA* **97**: 4967–4972
- Jacob T, Ritchie S, Assmann SM, Gilroy S** (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proc Natl Acad Sci USA* **96**: 12192–12197
- Leckie CP, McAinsh MR, Allen GJ, Sanders D, Hetherington AM** (1998) Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. *Proc Natl Acad Sci USA* **95**: 15837–15842
- Lee S, Choi H, Suh S, Doo I-S, Oh K-Y, Choi EJ, Taylor SAT, Low PS, Lee Y** (1999) Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reaction oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol* **121**: 147–152
- Legendre L, Rueter S, Heinstein PF, Low PS** (1993) Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine-max*) cells. *Plant Physiol* **102**: 233–240
- Levine A, Tenhaken R, Dixon RA, Lamb C** (1994) H₂O₂ from the oxidative burst orchestrates the plant hypersensitive response. *Cell* **79**: 583–593
- Li J, Wang XQ, Watson MB, Assmann SM** (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* **287**: 300–303
- Low PS, Merida JR** (1996) The oxidative burst in plant defense-function and signal transduction. *Plant Physiol* **96**: 533–542
- Ma LG, Xu XD, Cui SJ, Sun DY** (1999) The presence of a heterotrimeric G protein and its role in signal transduction of extracellular calmodulin in pollen germination and tube growth. *Plant Cell* **11**: 1351–1363
- MacRobbie EAC** (1995) ABA-induced ion efflux in stomatal guard cells: multiple actions of ABA inside and outside the cell. *Plant J* **7**: 565–576
- MacRobbie EAC** (1997) Signaling in guard cells and regulation of ion channel activity. *J Exp Bot* **48**: 515–528
- MacRobbie EAC** (1998) Signal transduction and ion channels in guard cells. *Phil Trans R Soc Lond B* **353**: 1475–1488
- McAinsh MR, Clayton H, Mansfield TA, Hetherington AM** (1996) Changes in stomatal behavior and cytosolic free calcium in response to oxidative stress. *Plant Physiol* **111**: 1031–1042
- Mehdy MC** (1994) Active oxygen species in plant defense against pathogens. *Plant Physiol* **105**: 467–472
- Neuenschwander U, Vernooij B, Friedrich L, Uknes S, Kessmann H, Ryals J** (1995) Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? *Plant J* **8**: 227–233
- Noctor G, Foyer CH** (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 249–279
- Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JL** (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* **406**: 731–734
- Perona R, Dolfi F, Feramisco J, Lacal JC** (1999) Microinjection of macromolecules into mammalian cells in culture. In JC Lacal, R Perona, J Feramisco, eds, *Microinjection*. Birkhauser Verlag, Basel, pp 17–30
- Polle A** (1997) Defense against photoxidative damage in plants. In JG Scandalios, ed, *Oxidative Stress and the Molecular Biology of Antioxidant Defense*. Cold Spring Harbor Laboratory Press, Cold Spring, NY, pp 623–666
- Potikha TS, Collins CC, Johnson DI, Delmer DP, Levine A** (1999) The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. *Plant Physiol* **119**: 849–858
- Price AH** (1990) A possible role for calcium in oxidative plant stress. *Free Radic Res* **10**: 345–349
- Price AH, Taylor A, Ripley SJ, Griffiths A, Trewavas AJ, Knight MR** (1994) Oxidative signals in tobacco increase cytosolic calcium. *Plant Cell* **6**: 1301–1310
- Purohit S, Kumar GP, Laloraya M, Laloraya MM** (1994) Involvement of superoxide radical in signal transduction regulating stomatal movements. *Biochem Biophys Res Commun* **205**: 30–37
- Reckmann U, Scheibe R, Raschke K** (1990) Rubisco activity in guard cells compared with the solute requirement for stomatal opening. *Plant Physiol* **92**: 246–253

- Scandalios JG** (1993) Oxygen stress and superoxide dismutases. *Plant Physiol* **101**: 7–12
- Schroeder JI, Kwak JM, Allen GJ** (2001) Guard cell abscisic acid signaling and engineering drought hardiness in plants. *Nature* **410**: 327–330
- Schwartz A, Wu W-H, Tucker EB, Assmann SM** (1994) Inhibition of inward K⁺ channels and stomatal response by abscisic acid: an intracellular locus of phytohormone action. *Proc Natl Acad Sci USA* **91**: 4019–4023
- Staxen I, Pical C, Montgomery L, Gray J, Hetherington AM, McAinsh MR** (1999) Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proc Natl Acad Sci USA* **96**: 1779–1784
- Van Gestelen P, Asard H, Caubergs RJ** (1997) Solubilization and separation of a plant plasma membrane NADPH-O₂ synthase from other NAD(P) H oxidoreductases. *Plant Physiol* **115**: 543–550
- Wu W, Assmann SM** (1993) Photosynthesis by guard cell chloroplasts of *Vicia faba* L.: effects of factors associated with stomatal movements. *Plant Cell Physiol* **34**: 1015–1022
- Wu Y, Kuzma J, Marechal E, Graeff R, Lee HC, Foster R, Chua NH** (1997) Abscisic acid signaling through cyclic ADP-Ribose in plants. *Science* **278**: 2054–2055
- Xing T, Higgins VJ, Blumwald E** (1997) Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. *Plant Cell* **9**: 249–259
- Yamasaki H, Sakihama Y, Ikehara N** (1997) Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H₂O₂. *Plant Physiol* **115**: 1405–1412
- Zeiger E, Armond P, Melis A** (1981) Fluorescence properties of guard cell chloroplasts: evidence for linear electron transport and light harvesting pigments of photosystems I and II. *Planta* **67**: 17–20