

# MAPK kinase and CDP kinase modulate hydrogen peroxide levels during dark-induced stomatal closure in guard cells of *Vicia faba*

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**ABSTRACT.** We used 2'-amino-3'-methoxyflavone (PD98059) (an inhibitor of mitogen-activated protein kinase kinase, MEK) and Trifluoperazine (TFP) (a specific inhibitor of calcium-dependent protein kinase, CDPK) to investigate the role of MEK/CDPK and its effects on H<sub>2</sub>O<sub>2</sub> levels of guard cells in the dark-induced stomatal closure in *Vicia faba*. We provide evidence that both PD98059 and TFP reduced H<sub>2</sub>O<sub>2</sub> levels in guard cells and promoted stomatal opening significantly in the dark, implying that MEK/CDPK mediated dark-induced stomatal closure by influencing H<sub>2</sub>O<sub>2</sub> levels of guard cells. In addition, like ascorbic acid (ASA), an important reducing substrate for H<sub>2</sub>O<sub>2</sub> removal, but unlike diphenylene iodonium (DPI), an inhibitor of the H<sub>2</sub>O<sub>2</sub>-generating enzyme NADPH oxidase, PD98059 and TFP not only reduced exogenous H<sub>2</sub>O<sub>2</sub> levels in guard cells in light, but also eliminated the H<sub>2</sub>O<sub>2</sub> that had been generated during a dark period and promoted stomatal opening. The results suggest MEK and CDPK are probably involved in restraining the H<sub>2</sub>O<sub>2</sub> scavenging enzyme and elevating H<sub>2</sub>O<sub>2</sub> levels in guard cells during dark-induced stomatal closure. Of course, the probability of MEK and CDPK acting as the target downstream of H<sub>2</sub>O<sub>2</sub> in the signaling transduction chain is not excluded.

**Keywords:** Dark; Hydrogen peroxide; MAPK kinase and CDP kinase; Stomatal closure; *Vicia faba*.

**Abbreviation:** ASA, ascorbic acid; CDPK, calcium-dependent protein kinase; DCF, dichlorofluorescein; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; H<sub>2</sub>DCF-DA, 2, 7-dichlorofluorescein diacetate; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; PD98059, 2'-amino-3'-methoxyflavone; ROS, reactive oxygen species; TFP, Trifluoperazine.

## INTRODUCTION

Stomata are the main routes for leaf gas exchange, controlling CO<sub>2</sub> uptake and transpiration. Stomatal movements are regulated by both internal and external factors. The opening of stomata is stimulated by low CO<sub>2</sub> concentrations, a range of natural and synthetic cytokinins, and blue light and other photosynthetically active wavelengths. Stomatal closure occurs in response to environmental cues like low air humidity and high temperature (Jewer and Incoll, 1980; Assmann, 1993; Willmer and Fricker, 1996; Liang et al., 2002; Hung et al., 2005). Light and dark are the most important environmental factors affecting stomatal movement (Zeiger, 1983; Cousson et al., 1995), which is also regulated by the redox active molecule hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Neill et al., 2002; Laloi et al., 2004).

Previous evidence showed that H<sub>2</sub>O<sub>2</sub> functions as an endogenous signalling molecule mediating responses to various stresses and stimuli (Finkel, 2000; Neill et al., 2002). There is now compelling evidence that H<sub>2</sub>O<sub>2</sub> is involved in abscisic acid (ABA)-induced stomatal closure (Pei et al., 2000; Zhang et al., 2001a, b; Meihard et al., 2002). Recent research provides exciting evidence that H<sub>2</sub>O<sub>2</sub> is a key signalling molecule mediating dark-induced stomatal closure (Desikan et al., 2004; She et al., 2004).

A growing body of evidence has shown that numerous protein kinases with close sequence similarity to mammalian mitogen-activated protein kinases (MAPKs) have been identified in plants (Hirt, 1997; Mizoguchi et al., 1997; Zhang and Klessig, 2001; Ichimura et al., 2002). Increasing evidence has shown that MAPKs play an important role in plant signal transduction related to biotic and abiotic stresses. Activation of MAPKs has been observed in plants exposed to pathogens (He et al., 1999), cold (Jonak et al., 1996), salinity (Mikolajczyk et al., 2000), drought (Jonak et al., 1996), and wounding (Usami

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et al., 1995; He et al., 1999). Plant MAPKs also can be activated by ABA (Knetsch et al., 1996; Burnett et al., 2000; Heimovaara-Dijkstra et al., 2000). Most physiological responses to activation of protein kinases in stimulus factors like light, hormones, stress, and pathogen attack regulated stomatal aperture (Sopory and Munshi, 1998). Some evidence suggests that the guard cell-specific protein kinase, ABA-activated protein kinase (AAPK), is essential for ABA-induced stomatal closing (Li et al., 2000). Burnett et al. (2000) reported that ABA activation of additional types of protein kinases had been found in pea epidermal peels. Jiang et al. (2003) reported that MEK is an important regulator of stomatal movement, which is believed to mediate the  $H_2O_2$  generation induced by ABA in guard cells of *Vicia faba*. In addition, plant cells contain a group of kinases, designated as calcium-dependent protein kinases (CDPKs), which are dependent only on calcium and do not require CaM for activation (Harmon et al., 1987). Wang and Wu (1999) reported that CDPKs might be involved in the ABA-mediated signal transduction cascades of stomatal movement.

Recently, elegant work from Gomi et al. (2005) showed that the involvement of a specific MAPK has been suggested in jasmonic acid signaling and MAPK-silenced plants showed misregulation of stomatal aperture. In addition, a potential crosstalk between a CDPK and a MAPK signaling pathway mediated by ethylene during stress responses in guard cells has also been reported (Ludwig et al., 2005), as have the effects of CDPKs CPK6 and CPK3 on stomatal aperture and ion channel activity in guard cells (Mori et al., 2006). Up to now, the involvement of MAPKs and CDPKs in darkness-mediated stomatal closure has not been addressed, and the effect of MEK and CDPK on  $H_2O_2$  levels in the process needs to be demonstrated. In the present study, we seek for evidence by means of stomatal bioassay and laser-scanning confocal microscopy that MEK and CDPK mediate dark-induced stomatal closure based on 2', 7'-dichlorodihydrofluorescein diacetate ( $H_2DCF$ -DA). The effect of MEK and CDPK on  $H_2O_2$  levels in dark-induced stomatal closure in *Vicia faba* is also studied.

## MATERIALS AND METHODS

### Chemicals

Molecular probes 2', 7'-dichlorodihydrofluorescein diacetate ( $H_2DCF$ -DA, from Biotium, Hayward, California) was dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM stock solution. Diphenylene iodonium (DPI), Trifluoperazine (TFP), DMSO and 2-(N-morpholino) ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (St. Louis, MO). 2'-amino-3'-methoxyflavone (PD98059) were purchased from Calbiochem (an affiliate of Merck KGaA, Darmstadt, Germany). Unless stated otherwise, the remaining chemicals were of the highest analytical grade available and were sourced from various Chinese suppliers.

$H_2DCF$ -DA, PD98059 and DPI were dissolved in DMSO. The final concentration of the solvent was 0.5% (v/v), which did not induce any significant change in guard cell viability or stomatal aperture.

### Plant materials

Broad bean (*Vicia faba* L.) was grown in controlled-environment plant growth chamber with a humidity of 80%, a photo flux density of  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  PAR generated by cool white fluorescent tubes (Philips, New York, NY), and an ambient temperature  $25 \pm 2^\circ\text{C}$  with a 14-h light and 10-h dark cycle. The epidermis was peeled carefully from the abaxial surface of the youngest, fully expanded leaves of 4-week-old seedlings and cut into pieces about 5 mm wide and 5 mm long.

### Stomatal bioassay

Stomatal opening and closing were monitored using the method of McAinsh et al. (1996) with slight modifications. To study the role of MEK and CDPK in dark-induced stomatal movement, freshly prepared abaxial epidermis was first incubated in  $\text{CO}_2$ -free MES/KCl (10 mM MES/KOH, 50 mM KCl, 100  $\mu\text{M}$   $\text{CaCl}_2$ , pH 6.15) buffer, which included various treating reagents (PD98059, TFP, ASA or DPI), in the dark for 3 h at  $25^\circ\text{C}$ . Final stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer.

To study the effects of PD98059 and TFP on stomatal closure caused by exogenous  $H_2O_2$ , epidermal strips were incubated in MES/KCl buffer with  $H_2O_2$  alone, or in buffer containing PD98059, TFP, or other compounds for 3 h under light ( $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) conditions at  $25^\circ\text{C}$ , and then the apertures were recorded. To study the effects of MEK and CDPK on stomata that had closed in the dark, strips were incubated in MES/KCl buffer for 3 h in dark at  $25^\circ\text{C}$ , and then were treated with fresh buffer alone, or buffer containing PD98059, TFP, or other compounds for another 3 h in dark at  $25^\circ\text{C}$ . Final stomatal apertures were recorded.

To avoid any potential rhythmic effects on stomatal aperture, experiments were always started at the same time each day. In each treatment, we scored 30 randomly selected apertures per replicate, and treatments were repeated thrice. The data presented are the means of 90 measurements  $\pm$  s.e.

### Dye loading of $H_2DCF$ -DA

$H_2O_2$  measurement was performed as the method of Allan and Fluhr (1997) with some modifications. To study the effects of MEK and CDPK on  $H_2O_2$  levels in guard cells caused by darkness and exogenous  $H_2O_2$ , the epidermal strips were treated for 3 h as described for stomatal bioassay and were immediately placed into loading Tris-KCl buffer (Tris 10 mM and KCl 50 mM, pH 7.2) containing 50  $\mu\text{M}$  of  $H_2DCF$ -DA for 10 min in darkness at  $25 \pm 2^\circ\text{C}$ . To study the effects of MEK and CDPK on  $H_2O_2$  levels generated in guard cells held in the

dark, strips were incubated in MES/KCl buffer for 3 h in darkness at 25°C, and then in fresh MES/KCl containing PD98059, TFP, or other reagents for another 3 h. After these steps, H<sub>2</sub>DCF-DA was loaded in Tris-KCl buffer.

### Laser-scanning confocal microscopy

After excess dye was washed off with fresh Tris-KCl loading buffer in darkness, the epidermal strips were immediately examined by TCS SP5 laser-scanning confocal microscopy (Leica Lasertechnik GmbH, Heidelberg, Germany) with the following settings: excitation 488 nm, emission 530 nm, power 10%, PMT 959, zoom about 4, normal scanning speed, frame 512×512 pixel. Images acquired from the confocal microscope were analyzed using Leica image software, Time-Course, and Photoshop.

In the time-course plot experiments for the changes in DCF fluorescence intensity, epidermal strips were incubated in MES/KCl buffer for 3 h in darkness at 25°C, and then H<sub>2</sub>DCF-DA was loaded for 10 min. After these steps, PD98059, TFP, or other reagents were added directly to Tris-KCl buffer. The change in intensity of dichlorofluorescein (DCF) fluorescence was recorded at about 800 s, and guard cell images were taken at 0, 100, 300, 500, 700 s.

To enable the comparison of changes in signal intensity, confocal images were taken under identical conditions (in manual setup) for all samples, and in each treatment we measured three epidermal strips, and the treatment was repeated at least thrice. The selected confocal images represented the same results from three replications.

### Monitor of the fluorescence spectrum of H<sub>2</sub>DCF-DA *in vitro*

The fluorescence spectrum of H<sub>2</sub>DCF-DA was measured on a Hitachi F-2500 fluorescence spectrophotometer (Ltd., Tokyo, Japan), using Sarstedt REF67.754 cuvettes. Apparatus settings: excitation at 488 nm, emission at 530 nm, PMT voltage 950 V. To study the effects of MEK and CDPK inhibitors on the fluorescence spectrum of H<sub>2</sub>DCF-DA *in vitro* at 25±2°C, the treatments were as follows: Tris-KCl buffer including 50 μM of H<sub>2</sub>DCF-DA only, H<sub>2</sub>DCF-DA + 10 μM H<sub>2</sub>O<sub>2</sub>, H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM PD98059, H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM TFP, H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 100 μM ASA, and H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM DPI. The fluorescence intensity of the above treatments was determined respectively. Each treatment was performed at least thrice. The data from three replications were the same.

## RESULTS

### Effects of PD98059 and TFP on dark-induced stomatal closure

PD98059 is a potent and selective cell permeable inhibitor of MAPK kinase (MEK) and thus an invaluable aid in elucidating the role of MEK in a variety of biological

systems (Alessi et al., 1995). Previous studies suggested that PD98059 abolished the ABA-induced stomatal closure in *Pisum sativum*, implying that ABA effects in pea epidermal peels require MAPK activation (Burnett et al., 2000), and MEK specifically mediates the ABA-induced H<sub>2</sub>O<sub>2</sub> generation in guard cells in *Vicia faba* (Jiang et al., 2003). Additionally, Wang and Wu (1999) reported that CDPK might also be involved in ABA-mediated signal transduction cascades in regulation of stomatal movement. Previous research demonstrates that trifluoperazine (TFP) inhibits plant CDPKs (Polya and Micucci, 1985; Zhou and Zhang, 2004). Recent studies have shown that H<sub>2</sub>O<sub>2</sub> is an essential signaling molecule involving in dark-induced stomatal closure (Desikan et al., 2004; She et al., 2004). Therefore, we expected that PD98059 and TFP could affect dark-induced H<sub>2</sub>O<sub>2</sub> levels and stomatal closure in *Vicia faba*.

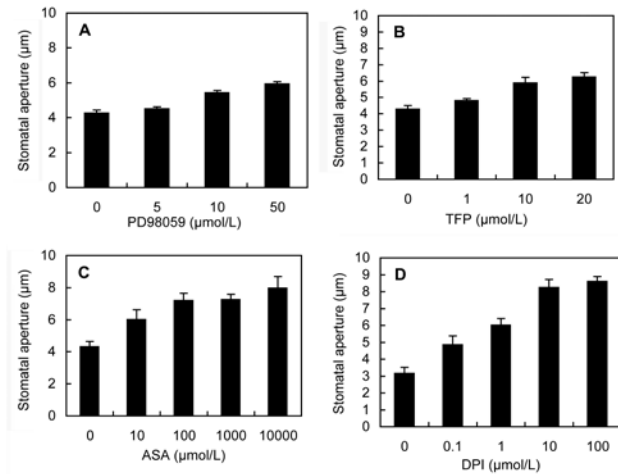
After examining the effects of PD98059 or TFP on dark-induced stomatal closure, we found that both PD98059 and TFP at a concentration of 10 μM significantly reversed darkness-induced stomatal closure ( $P < 0.01$ ) (Figure 1A and B). So we suppose that darkness induces stomatal closure via a pathway involving MEK and CDPK.

Dark-induced stomatal closure is related to endogenous H<sub>2</sub>O<sub>2</sub> (Desikan et al., 2004; She et al., 2004). To investigate the relation between the mediating of MEK/CDPK in dark-induced stomatal closure and the change of H<sub>2</sub>O<sub>2</sub> levels in guard cells, epidermal strips were treated with ASA and DPI. The former is the most important reducing substrate for H<sub>2</sub>O<sub>2</sub> removal (Noctor and Foyer, 1998), and the latter is an inhibitor of the H<sub>2</sub>O<sub>2</sub>-generating enzyme, NADPH oxidase (Lee et al., 1999). The results show that both ASA and DPI induced stomatal opening in darkness in a dose-dependent manner (Figure 1C, D). The effects of ASA and DPI on stomatal aperture were significant ( $P < 0.01$ ) at 100 and 10 μM, respectively. These results suggest that, probably like ASA and DPI, MEK/CDPK modulates H<sub>2</sub>O<sub>2</sub> levels during dark-induced stomatal closure in guard cells.

### Both PD98059 and TFP affect the dark-induced H<sub>2</sub>O<sub>2</sub> levels of guard cells

Having established that both MEK and CDPK mediate dark-induced stomatal closure (Figure 1A, B), we used H<sub>2</sub>DCF-DA, a specific probe for intracellular H<sub>2</sub>O<sub>2</sub> (Allan and Fluhr, 1997), to measure H<sub>2</sub>O<sub>2</sub> levels directly in guard cells. Upon entering the cell, the nonpolar H<sub>2</sub>DCF-DA is hydrolyzed to the oxidatively sensitive, more polar, nonfluorescent compound fluorophore dichlorofluorescein (H<sub>2</sub>DCF). H<sub>2</sub>DCF is rapidly oxidized to the highly fluorescent DCF by intracellular H<sub>2</sub>O<sub>2</sub> (Allan and Fluhr, 1997). H<sub>2</sub>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 2B, darkness could induce an intense DCF fluorescence in guard cells over the light treatment (Figure 2A), which is consistent with previous

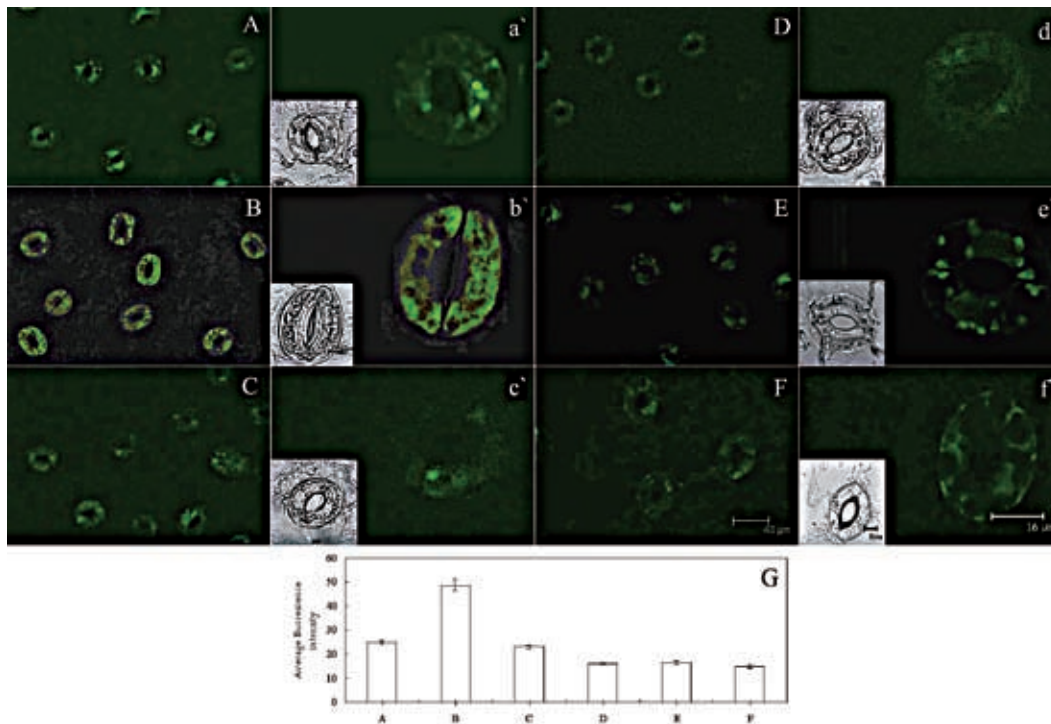


**Figure 1.** Effects of protein kinase inhibitors on dark-induced stomatal closure. Isolated epidermal strips were incubated at 25 °C in CO<sub>2</sub>-free MES[2-(N-morpholino) ethanesulfonic acid]/KCl containing different concentrations of (A) PD98059 (0, 5, 10, 50 µM), (B) TFP (0, 1, 10, 20 µM), (C) ASA (0, 10, 100, 1000, 10000 µM) and (D) DPI (0, 0.1, 1, 10, 100 µM) for 3 h in darkness. Stomatal apertures were determined. Values are the means of 90 measurements±s.e. forming three independent experiments.

reports (Desikan et al., 2004; She et al., 2004). However, darkness-induced DCF fluorescence in guard cells was largely prevented by PD98059 and TFP (Figure 2C, D). Similarly, ASA and DPI also substantially suppressed dark-induced DCF fluorescence (Figure 2E, F). Taking these results from Figures 1 and 2 together, we suggest that MEK and CDPK may be the upstream signal molecule mediating H<sub>2</sub>O<sub>2</sub> levels in the dark-induced stomatal closure of *Vicia faba*, and they can elevate the levels of endogenous H<sub>2</sub>O<sub>2</sub> and promote stomatal closure in darkness.

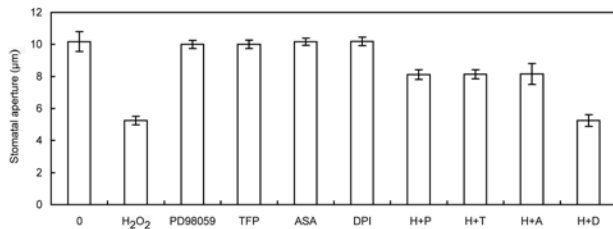
### Effects of PD98059/TFP on stomatal closure and DCF fluorescence in guard cells induced by exogenous H<sub>2</sub>O<sub>2</sub>

Having established that MEK and CDPK mediate dark-induced stomatal closure and modulate the H<sub>2</sub>O<sub>2</sub> levels of guard cells in *Vicia faba*, we wanted further insight into how MEK and CDPK affect H<sub>2</sub>O<sub>2</sub> levels in guard cells. Epidermal strips were incubated in MES/KCl with H<sub>2</sub>O<sub>2</sub> alone or H<sub>2</sub>O<sub>2</sub> with PD98059, TFP and other compounds for 3 h in light. As shown in Figure 3, exogenous application of H<sub>2</sub>O<sub>2</sub> promoted stomatal closure in light, which is consistent with the previous data (Zhang et al., 2001b; Desikan et al., 2004; She et al., 2004). PD98059,



**Figure 2.** Effects of protein kinase inhibitors on the dark-induced H<sub>2</sub>O<sub>2</sub> levels of guard cells. Guard cells of *V. faba*: (A) treated with MES/KCl only in light for 3 h; (B) in darkness alone for 3 h; (C) in darkness with 10 µM PD98059; (D) in darkness with 10 µM TFP; (E) in darkness with 100 µM ASA; (F) in darkness with 10 µM DPI, for 3 h. Above treated strips were immediately loaded with H<sub>2</sub>DCF-DA in Tris-KCl buffer for 10 min in darkness. Then excess dye was removed, and strips were examined using laser-scanning confocal microscopy; (G) shows the average fluorescent intensity of guard cells in images from (A) to (F). Data are the means±s.e. Guard cells shown in image (a') to (f') represent guard cells shown in images (A) to (F). The insets show the bright-field images corresponding to the fluorescence images (a') through (f'). The length of scale bar in image (F) and (f') represents 40 µm and 16 µm for image (A) to (F) and (a') to (f'), respectively. The bar in inset of image (f') represents 8 µm for all the insets. Each experiment was performed at least thrice, and the selected confocal image represented the same results from about nine time measurements.

TFP, ASA or DPI did not cause any changes in stomatal aperture in light. However, similar to ASA (an important reducing substrate for  $H_2O_2$  removal), but not to DPI (an inhibitor of the  $H_2O_2$ -generating enzyme, NADPH oxidase), PD98059 and TFP prevented the stomatal



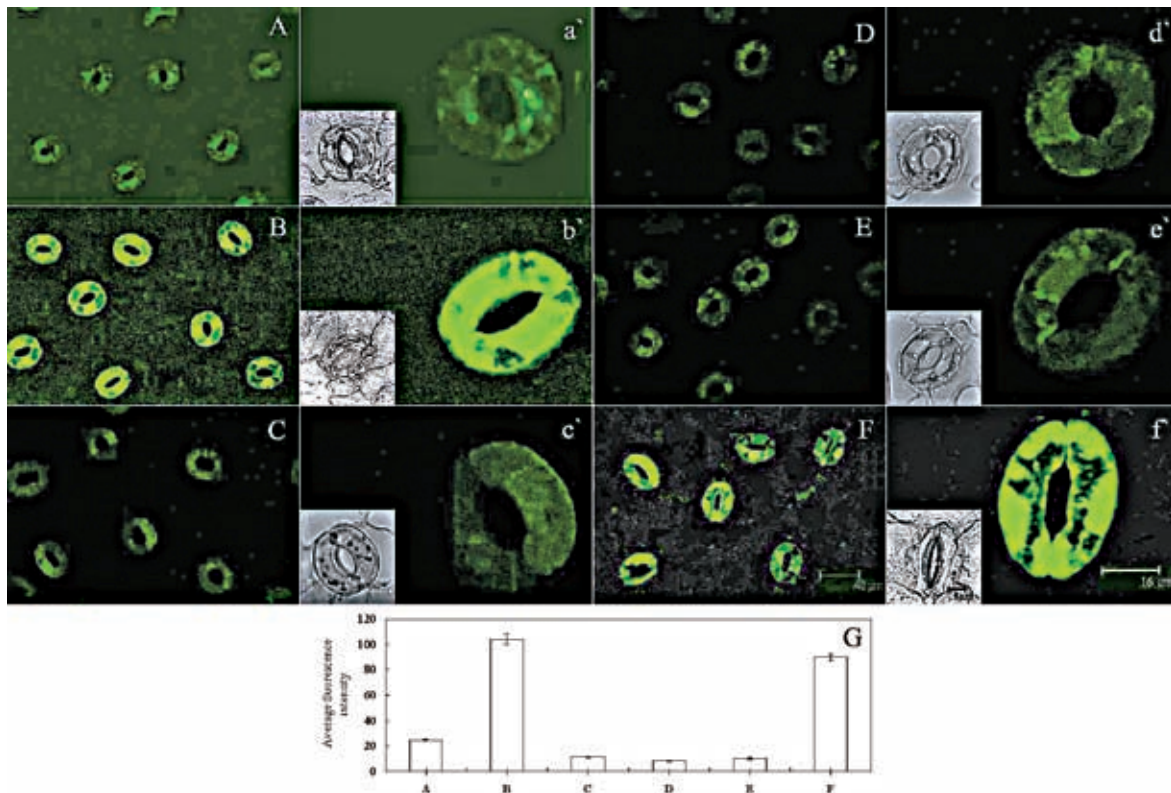
**Figure 3.** Stomatal closure induced by exogenous  $H_2O_2$  can be prevented by PD98059 and TFP. Isolated epidermal strips of *V. faba* were incubated at  $25^\circ C$  in  $CO_2$ -free MES/KCl alone, or MES/KCl containing  $10 \mu M H_2O_2$ ,  $10 \mu M$  PD98059,  $10 \mu M$  TFP,  $100 \mu M$  ASA,  $10 \mu M$  DPI,  $10 \mu M H_2O_2 + 10 \mu M$  PD98059 (H+P),  $10 \mu M H_2O_2 + 10 \mu M$  TFP (H+T),  $10 \mu M H_2O_2 + 100 \mu M$  ASA (H+A),  $10 \mu M H_2O_2 + 10 \mu M$  DPI (H+D) for 3 h under light ( $300 \mu mol m^{-2} s^{-1}$ ). Stomatal apertures were determined after 3 h incubation. Values are the means of 90 measurements  $\pm$  s.e. of three independent experiments.

closure induced by exogenous  $H_2O_2$  in light. The effects were significant ( $P < 0.01$ ; Figure 3).

To further clarify whether or not MEK/CDPK can affect exogenous  $H_2O_2$ -induced DCF fluorescence, the epidermal strips were treated with  $H_2O_2$  in the presence of PD98059, TFP, ASA or DPI for 3 h in light, and then  $H_2O_2$  levels were measured. As shown in Figure 4, a striking DCF fluorescence in guard cells was observed after treatment with  $10 \mu M$  exogenous  $H_2O_2$  in light (Figure 4B) compared with the control (Figure 4A). However,  $H_2O_2$ -induced DCF fluorescence in guard cells was abolished by PD98059, TFP (Figure 4C, D) and ASA (an important reducing substrate for  $H_2O_2$  removal) (Figure 4E), but not by DPI (an inhibitor of the  $H_2O_2$ -generating enzyme, NADPH oxidase) (Figure 4F). From these results we know that, like ASA, both PD98059 and TFP not only prevented stomatal closure by exogenous  $H_2O_2$ , but also reduced exogenous  $H_2O_2$  levels in guard cells in light.

### The closed stomata caused by dark can be reopened by PD98059 and TFP

To confirm the effects of MEK and CDPK on  $H_2O_2$  guard cell levels in response to darkness, epidermal strips were incubated in MES/KCl for 3 h in darkness and then



**Figure 4.** Exogenous  $H_2O_2$ -induced DCF fluorescence in guard cells is reduced by protein kinase inhibitors. Guard cells shown in image (A) were treated in light for 3 h with buffer only, and those in image (B) were treated with  $10 \mu M H_2O_2$ ; (C)  $10 \mu M H_2O_2 + 10 \mu M$  PD98059; (D)  $10 \mu M H_2O_2 + 10 \mu M$  TFP; (E)  $10 \mu M H_2O_2 + 100 \mu M$  ASA; (F)  $10 \mu M H_2O_2 + 10 \mu M$  DPI, in light for 3 h. Above treated strips were loaded with  $H_2DCF$ -DA for 10 min in darkness. Then excess dye was removed, and the strips were examined by laser-scanning confocal microscopy. (G) shows the average fluorescent intensity of guard cells in images from (A) to (F). Data are the means  $\pm$  s.e. Other explanations are the same as in Figure 2.

treated with fresh buffer alone or buffer containing various reagents for another 3 h in dark. As shown in Figure 5, like ASA, the most important reducing substrate for  $H_2O_2$  removal, both PD98059 and TFP promoted the reopening of stomata that had closed in the dark, but DPI (an inhibitor of the  $H_2O_2$ -generating enzyme, NADPH oxidase) did not (Figure 5). The results suggest that  $H_2O_2$  is necessary to maintaining stomatal closure in darkness, once the stomata are closed, continued  $H_2O_2$  production in dark is neither required (at least by a DPI-sensitive enzyme) nor significant.

### PD98059 and TFP reduce levels of $H_2O_2$ generated by darkness

The effects of PD98059 and TFP on the levels of  $H_2O_2$  generated in guard cells held in the dark were also measured. After an incubation of 3 h in darkness, epidermal strips were loaded with  $H_2DCF$ -DA, washed, and examined by laser-scanning confocal microscopy. During the examination of DCF fluorescence, PD98059, TFP, ASA, or DPI was added to the buffer. As shown in Figure 6A, the fluorescence intensity of controls showed no change within 800 s. PD98059 and TFP reduced the DCF fluorescence intensity compared with the control (Figure 6B and C), as did ASA (Figure 6D). However, DPI did not reduce it (Figure 6E).

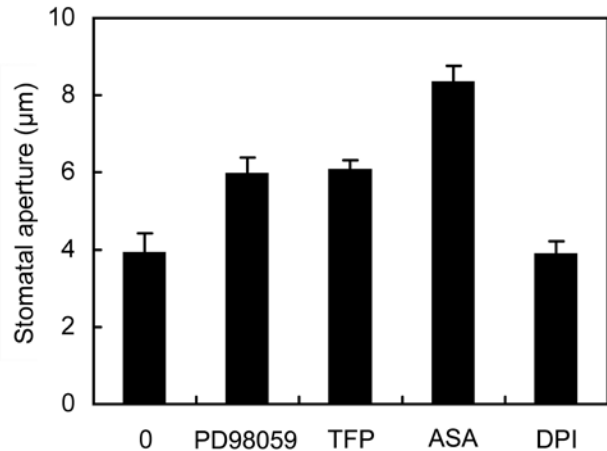
Images of guard cells treated with PD98059, TFP, ASA, and DPI at 0 and 3 h were also obtained. As shown in Figure 7, the DCF fluorescence of guard cells treated with PD98059, TFP, and ASA for 3 h in dark (Figure 7C, D, E) was less than that of the control (Figure 7B) and 0 h treatment (Figure 7A). However, the DCF fluorescence of guard cells treated with DPI for 3 h (Figure 7F) was unchanged. The results suggest that both PD98059 and TFP reduce endogenous  $H_2O_2$  levels that have been generated by darkness in guard cells.

### The effects of PD98059/TFP on the fluorescence spectrum of $H_2DCF$ -DA *in vitro*

As shown in Figure 8, *in vitro*, the fluorescence intensity of  $H_2DCF$ -DA only was 103, but in the presence of  $H_2O_2$ , the value increased. The inhibitors of MEK/CDPK, PD98059, and TFP had no effect on the fluorescence intensity of  $H_2DCF$ -DA, but ASA significantly reduced it ( $P < 0.01$ ). In addition, the fluorescence intensity of  $H_2DCF$ -DA was also reduced by DPI to some extent.

## DISCUSSION

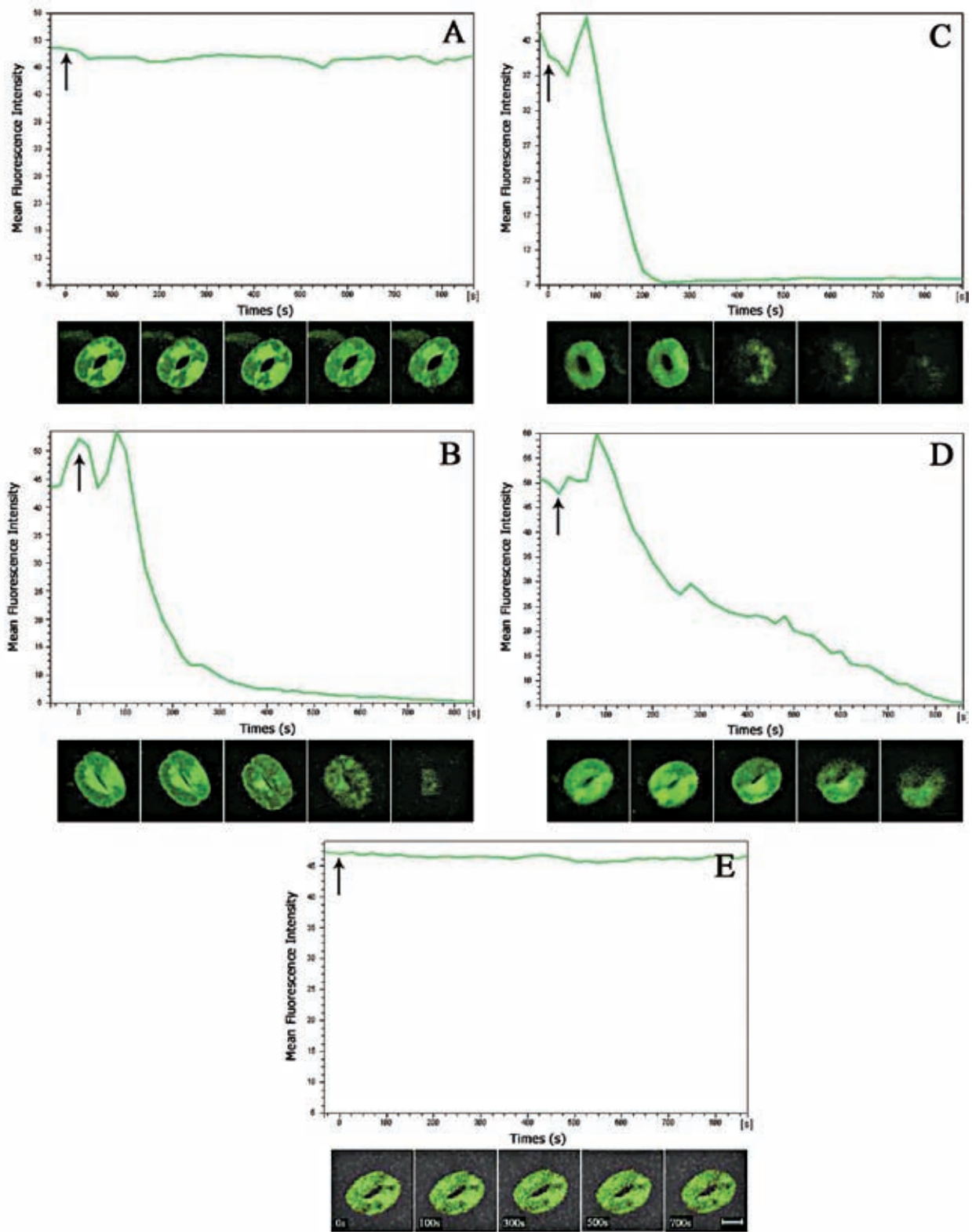
Protein phosphorylation/dephosphorylation is now known to play a very important role in response to exogenous factors like light, hormones, stress, and pathogen attack in plants (Stone and Walker, 1995; Sopory and Munshi, 1998; Schenk and Snaar-Jagalska, 1999). MAPKs are serine/threonine protein kinases that phosphorylate a range of substrates to activate various cellular responses, including gene expression and membrane transport, in dif-



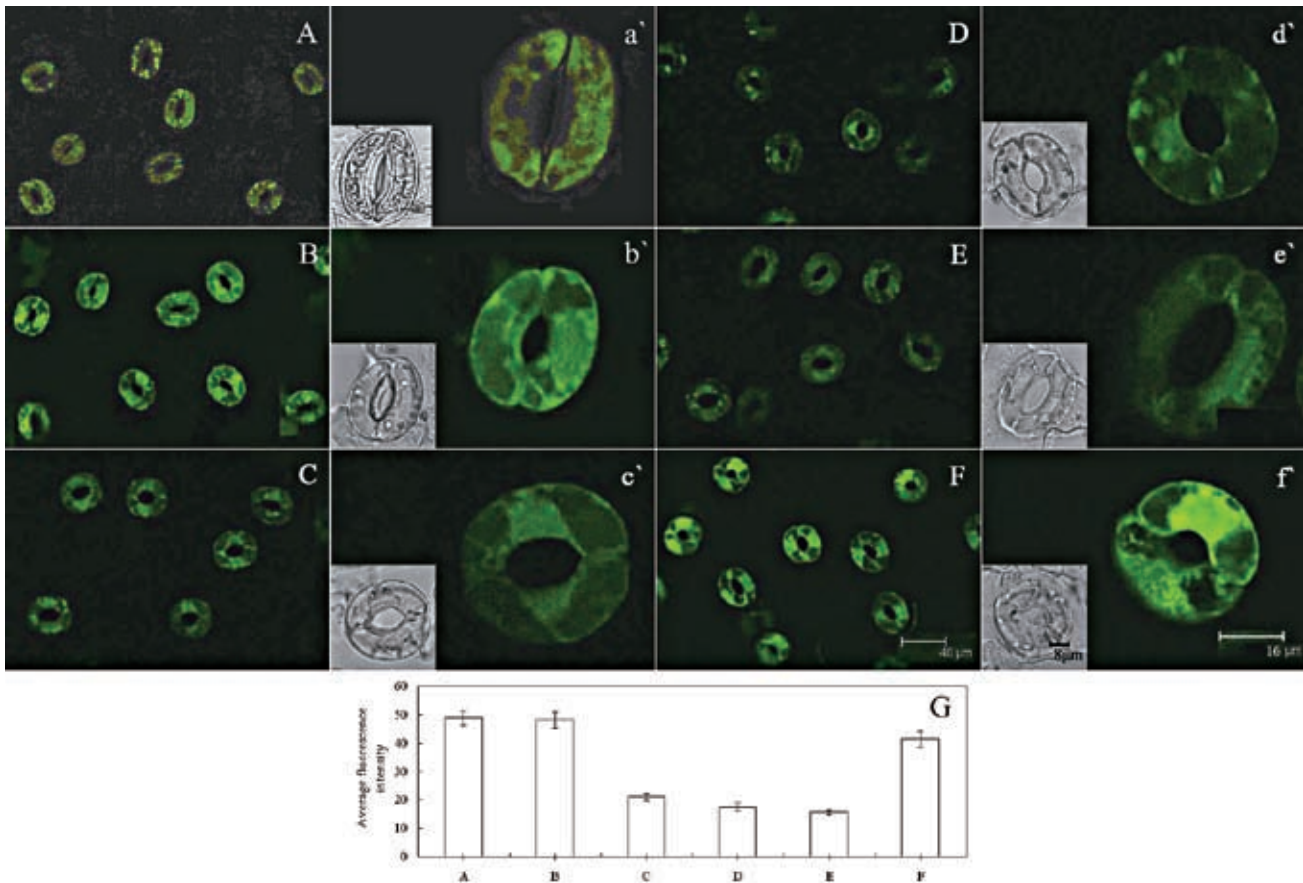
**Figure 5.** The closed stomata during dark exposure can be reopened by protein kinase inhibitors. Strips of *V. faba* incubated at 25°C in  $CO_2$ -free MES/KCl for 3 h in darkness were treated with fresh buffer alone or buffer containing 10 µM PD98059, 10 µM TFP, 100 ASA, or 10 µM DPI for another 3 h in dark, and then stomatal apertures were determined. Values are the means of 90 measurements  $\pm$  s.e. of three independent experiments.

ferent cells (Cohen, 1997; Hirt, 1997). MAPKs are themselves activated via dual phosphorylation on threonine and tyrosine residues by MAPK kinases (MEKs), which in turn are activated via phosphorylation by MAPKK kinases (MAPKKKs). MAPK-based signalling cascades are ubiquitous components of all eukaryotic cells (Hirt, 1997; Mizoguchi et al., 1997). A large number of these enzymes have already been identified in plants, and it seems very likely that they transduce responses to many external signals and plant hormones (Hirt, 1997; Mizoguchi et al., 1997). CDPKs are another class of serine/threonine protein kinases unique to plants and some protists. A large family of CDPKs has been identified recently in higher plants (Roberts and Harmon, 1992). CDPKs are dependent only on calcium and do not require CaM for activation (Harmon et al., 1987). Among the cells in epidermis CDPKs are only expressed in the stomatal guard cells (Hong et al., 1996). Previous research shows that both MAPKs and CDPKs might be involved in the ABA-mediated signal transduction cascades that regulate stomata movement (Wang and Wu, 1999; Burnett et al., 2000; Jiang et al., 2003). A crosstalk between the MAPK and CDPK pathways was mediated by ethylene and involved the generation of reactive oxygen species in guard cells (Ludwig et al., 2005). This is the first study, to our knowledge, to show the role of MEK and CDPK in dark-induced stomatal closure in *Vicia faba*.

Previous studies have suggested that PD98059 and TFP are inhibitors of MEK and CDPK activity, respectively (Polya and Micucci, 1985; Alessi et al., 1995; Burnett et al., 2000; Jiang et al., 2003; Zhou and Zhang, 2004). A report from Lu et al. (2002) showed that the inhibition of MAPKK activity by PD98059 could interfere with the ability of ABA to trigger postgermination growth arrest



**Figure 6.** Time-course plots of changes in intensity of DCF fluorescence. Epidermal strips of *V. faba*, incubated in  $\text{CO}_2$ -free MES/KCl for 3 h in the dark were loaded with  $\text{H}_2\text{DCF-DA}$  for 10 min in the dark, washed, and examined by laser-scanning confocal microscopy. During image acquisition, Tris-KCl buffer only (A, control), 10  $\mu\text{M}$  PD98059 (B), 10  $\mu\text{M}$  TFP (C), 100  $\mu\text{M}$  ASA (D), and 10  $\mu\text{M}$  DPI (E) were added directly to the buffer. (A-E) Time-course plots of changes in the DCF fluorescence intensity of guard cells; higher intensity stands for higher  $\text{H}_2\text{O}_2$  concentration. Arrow in image (A) to (E) indicates the addition of reagents. Fluorescence images of stomata were taken at 0, 100, 300, 500 and 700 s after the addition of reagents. The scale bar in the stoma of image (E) is 20  $\mu\text{m}$  for all the images.

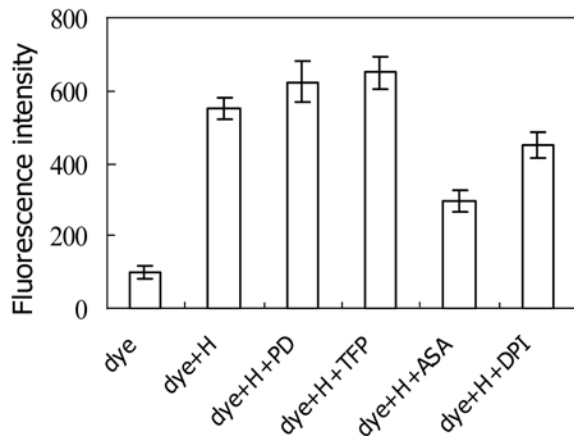


**Figure 7.** Protein kinase inhibitors reduce the  $\text{H}_2\text{O}_2$  levels generated by darkness. Epidermal strips of *V. faba*, were incubated in  $\text{CO}_2$ -free MES/KCl buffer for 3 h in darkness, and then in fresh MES/KCl containing PD98059, TFP, or other reagents for another 3 h. After this step,  $\text{H}_2\text{DCF-DA}$  was loaded in Tris-KCl buffer for 10 min in darkness and washed. (In Figure 7A at 0 h in the dark, strips in MES/KCl for 3 h in darkness, and then  $\text{H}_2\text{DCF-DA}$  was loaded). At 0 h in dark (A) and 3 h, the strips were examined by laser-scanning confocal microscopy. Image of guard cells held in dark for another 3 h in the presence of buffer only (B), and buffer and (C) 10  $\mu\text{M}$  PD98059, (D) 10  $\mu\text{M}$  TFP, (E) 100  $\mu\text{M}$  ASA, (F) 10  $\mu\text{M}$  DPI. (G) shows the average fluorescence intensity of guard cells in images from (A) to (F). Data are the means $\pm$ s.e. Other explanations are the same as in Figure 2.

by in-gel kinase assays. Desikan et al. (2001) provided evidence that PD98059 could inhibit the activity of a MAPK-like enzymew AtMPK4 in cell suspension cultures of *Arabidopsis* var. Landsberg *erecta*. Jiang et al. (2003) reported that PD98059 reversed ABA-induced stomatal closure and  $\text{H}_2\text{O}_2$  generation. In addition, CDPK exhibits a  $\text{Ca}^{2+}$ -induced electrophoretic mobility shift, and its  $\text{Ca}^{2+}$ -dependent catalytic activity can be inhibited by TFP in *Vicia faba* (Li et al., 1998) and in *Arabidopsis thaliana* (Hong et al., 1996). Wang and Wu (1999) reported that addition of TFP significantly reversed the inhibitory effect of ABA on stomatal opening, suggesting CDPKs are involved in ABA-regulated stomatal closure. The results of the present study showed that PD98059 and TFP stopped darkness-induced stomatal closure (Figure 1). From these results we presume that dark-induced stomatal closure occurs via a pathway involving MEK and CDPK and that the two protein kinases are key signaling components of this closure. Previous research shows that OST1, one of the AAPK, acts upstream of  $\text{H}_2\text{O}_2$  produc-

tion in ABA-regulated stomatal movement in *Arabidopsis* (Mustilli et al., 2002), Jiang et al. (2003) reported MAPK could specifically regulate and amplify the ABA-induced  $\text{H}_2\text{O}_2$  generation in guard cells of *Vicia faba*. Using laser-scanning confocal microscopy, we found that like ASA, an important reducing substrate for  $\text{H}_2\text{O}_2$  removal, and DPI, an inhibitor of the  $\text{H}_2\text{O}_2$ -generating enzyme NADPH oxidase (Figure 2E, F), both PD98059 and TFP prevented darkness-induced  $\text{H}_2\text{O}_2$  levels (Figure 2C, D), suggesting that MEK and CDPK may be an upstream signal molecule regulating  $\text{H}_2\text{O}_2$  levels in darkness-induced stomatal closure in *Vicia faba*.

Given that MEK and CDPK mediated in the dark-induced change of  $\text{H}_2\text{O}_2$  levels in *Vicia faba* guard cells, we wanted to further explore how MEK and CDPK affected the levels of  $\text{H}_2\text{O}_2$  in dark-induced stomatal closure. We found that, like ASA, but unlike DPI (Figure 4E, F; Figure 7E, F), both PD98059 and TFP not only eliminated the  $\text{H}_2\text{O}_2$ -induced DCF fluorescence of guard cells in light (Figure 4C, D), but also removed



**Figure 8.** Monitoring of the fluorescence spectrum of H<sub>2</sub>DCF-DA *in vitro*. The treatments, to seek for the effects of MEK and CDPK inhibitors on the fluorescence spectrum of H<sub>2</sub>DCF-DA *in vitro*, were as follows: Tris-KCl buffer including 50 μM of H<sub>2</sub>DCF-DA only (dye), H<sub>2</sub>DCF-DA + 10 μM H<sub>2</sub>O<sub>2</sub> (dye + H<sub>2</sub>O<sub>2</sub>), H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM PD98059 (dye + H<sub>2</sub>O<sub>2</sub> + PD), H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM TFP (dye + H<sub>2</sub>O<sub>2</sub> + TFP), H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 100 μM ASA (dye + H<sub>2</sub>O<sub>2</sub> + ASA), and H<sub>2</sub>DCF-DA + H<sub>2</sub>O<sub>2</sub> + 10 μM DPI (dye + H<sub>2</sub>O<sub>2</sub> + DPI). The fluorescence spectrum of H<sub>2</sub>DCF-DA was measured on a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). Apparatus settings: excitation at 488 nm, emission at 530 nm, PMT voltage 950 V. The fluorescence intensity of the above treatments was determined. Each treatment was performed at least three. The data from three replications were the same.

H<sub>2</sub>O<sub>2</sub> generated by the dark (Figure 7C, D). The DCF fluorescence intensity changes in the time-course plots showed that the DCF fluorescence caused by darkness was eliminated by PD98059/TFP and ASA (Figure 6B, C, D), but not by DPI (Figure 6E). *In vitro*, as shown in Figure 8, the fluorescence intensity of H<sub>2</sub>DCF-DA was not reduced by the inhibitors of MEK/CDPK PD98059 and TFP, however, the intensity could be reduced by ASA. In addition, DPI also reduced the fluorescence intensity of H<sub>2</sub>DCF-DA to some extent, but DPI had no effect on the H<sub>2</sub>O<sub>2</sub>-induced DCF fluorescence of guard cells (Figure 4F), and the reason should be studied in the future. These results were consistent with the observation that PD98059/TFP reversed exogenous H<sub>2</sub>O<sub>2</sub>-induced stomatal closure (Figure 3) and promoted the reopening of stomata that had closed in the dark (Figure 5).

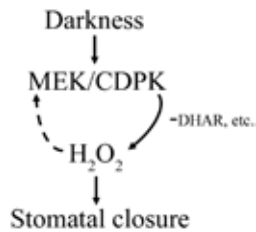
ASA is the major antioxidant that scavenges H<sub>2</sub>O<sub>2</sub>, and the balance between H<sub>2</sub>O<sub>2</sub> production and the ASA redox state establishes whether the H<sub>2</sub>O<sub>2</sub> concentration rises to a level that can trigger stomatal closure. Dehydroascorbate reductase (DHAR) catalyzes the reduction of dehydroascorbate (oxidized ascorbate) to ASA and thus contributes to the regulation of the ASA redox state (Chen and Gallie, 2004). Previous research provided evidence that stomatal pores in many species open in the morning but close in the afternoon to limit water loss (Assmann, 1993;

Assmann and Wang, 2001). The level of H<sub>2</sub>O<sub>2</sub> increases during the afternoon while the Asc redox state in guard cells decreases. Plants with an increased guard cell Asc redox state were generated by increasing DHAR expression, and these exhibited a reduction in the level of guard cell H<sub>2</sub>O<sub>2</sub> (Chen and Gallie, 2004). Here, our data imply that, in the darkness, MEK and CDPK are probably involved in restraining the H<sub>2</sub>O<sub>2</sub> scavenging enzyme DHAR to elevate the H<sub>2</sub>O<sub>2</sub> levels of guard cells in the darkness. When PD98059/TFP inhibits MEK/CDPK, DHAR expression increases. H<sub>2</sub>O<sub>2</sub> scavenging is no longer restrained, and H<sub>2</sub>O<sub>2</sub> levels decline. Of course, further experiments should be performed to confirm the effects of PD98059/TFP on antioxidant activities.

By stomatal bioassay both PD98059 and TFP not only prevented stomatal closure by exogenous H<sub>2</sub>O<sub>2</sub> in light (Figure 3), but also promoted reopening of stoma induced to close in the dark (Figure 5), implying that MEK/CDPK may be an upstream or downstream signal molecule of H<sub>2</sub>O<sub>2</sub> in guard cells. Using confocal microscopy we provide evidence that both PD98059 and TFP not only abolished H<sub>2</sub>O<sub>2</sub>-induced DCF fluorescence of guard cells in light (Figure 4 C, D), but also removed H<sub>2</sub>O<sub>2</sub> that had been generated by darkness (Figure 6B, C; Figure 7C, D). MEK and CDPK are suggested to be involved in restraining the H<sub>2</sub>O<sub>2</sub> scavenging enzyme to elevate H<sub>2</sub>O<sub>2</sub> levels. Of course, the probability of MEK and CDPK acting as the target downstream of H<sub>2</sub>O<sub>2</sub> in the signaling transduction chain is not excluded. It is well known that H<sub>2</sub>O<sub>2</sub> induces the activation of a MAPK in *Arabidopsis* (Desikan et al., 1999; Grant et al., 2000; Desikan et al., 2001). Mizoguchi et al. (1998) have shown that H<sub>2</sub>O<sub>2</sub>-induced MAPK cascade induces specific stress-responsive gene expression. H<sub>2</sub>O<sub>2</sub> also activates AtMPK6 (MEK-like) in *Arabidopsis* leaf protoplasts (Kovtun et al., 2000). Therefore, H<sub>2</sub>O<sub>2</sub> may have an autocatalytic function to facilitate its own generation, e.g. once an H<sub>2</sub>O<sub>2</sub>-generation system was triggered, a small amount of H<sub>2</sub>O<sub>2</sub> could accelerate MEK or CDPK activation due to the formation of an H<sub>2</sub>O<sub>2</sub> feedback-loop. This crosstalk of H<sub>2</sub>O<sub>2</sub> and MEK or CDPK may lead to the formation of a self-amplification loop. Once the inhibition of H<sub>2</sub>O<sub>2</sub>-induced closure by PD98059/TFP was achieved by reducing the activities of MEK/CDPK, the signal transduction pathway in guard cells was completely blocked.

In summary, the roles and functions of MAPK cascades and CDPKs in plants have been widely reported, but the whole picture is still fragmented. Furthermore, the role of MAPK cascades and CDPKs in stomatal responses to environmental stresses is not fully understood. Here, we suggest that MEK/CDPK mediates dark-induced stomatal closure by influencing H<sub>2</sub>O<sub>2</sub> levels of guard cells in *Vicia faba*. Furthermore, our data show that MEK/CDPK modulation of H<sub>2</sub>O<sub>2</sub> levels in guard cells is probably related to its restraint of the H<sub>2</sub>O<sub>2</sub> scavenging enzyme system during darkness-induced stomatal closure. These results will no doubt help us to gain further insight into the roles

of MEK and CDPK and the relationship between MEK/CDPK and the change to  $H_2O_2$  levels in guard cell signal transduction. However, little is known about the complex molecular network operating during the guard cell stomatal movement triggered by darkness, and whether or not MEK/CDPK acts as the target downstream of  $H_2O_2$  in guard cells in the darkness in *Vicia faba*. These problems should be further studied in future. Schematic representation of the relationship among darkness, MEK/CDPK, and  $H_2O_2$  signalling in stomatal guard cells was shown as follows by our results.



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## LITERATURE CITED

- Alessi, D.R., A. Cuenda, P. Cohen, D.T. Dudley, and A.R. Saltiel. 1995. PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **17**: 27489-27494.
- Allan, A.C. and R. Fluhr. 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *Plant Cell.* **9**: 1559-1572.
- Assmann, S.M. 1993. Signal transduction in stomatal guard cells. *Annu. Rev. Cell Biol.* **9**: 345-375.
- Assmann, S.M. and X.Q. Wang. 2001. From milliseconds to millions of years: Guard cells and environmental responses. *Curr. Opin. Plant Biol.* **4**: 421-428.
- Burnett, E.C., R. Desikan, R.C. Moser, and S.J. Neill. 2000. ABA activation of an MBP kinase in *Pisum sativum* epidermal peels correlates with stomatal responses to ABA. *J. Exp. Bot.* **51**: 197-205.
- Chen, Z. and D.R. Gallie. 2004. The ascorbic acid redox state controls guard cell signaling and stomatal movement. *Plant Cell.* **16**: 1143-1162.
- Cohen, P. 1997. The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* **7**: 353-361.
- Cousson, A., V. Cotellet, and A. Vavasour. 1995. Induction of stomatal closure by vanadate or a light/dark transition involves  $Ca^{2+}$ -Calmodulin-dependent protein phosphorylations. *Plant Physiol.* **109**: 491-497.
- Desikan, R., A. Clarke, J.T. Hancock, and S.J. Neill. 1999.  $H_2O_2$  activates a MAP kinase-like enzyme in *Arabidopsis thaliana* suspension cultures. *J. Exp. Bot.* **50**: 1863-1866.
- Desikan, R., J.T. Hancock, K. Ichimura, K. Shinozaki, and S.J. Neill. 2001. Harpin induces activation of *Arabidopsis* mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiol.* **126**: 1579-1587.
- Desikan, R., M.K. Cheung, A. Clarke, S. Golding, M. Sagi, R. Fluhr, C. Rock, J. Hancock, and S.J. Neill. 2004. Hydrogen peroxide is a common signal for darkness- and ABA-induced stomatal closure in *Pisum sativum*. *Func. Plant Biol.* **31**: 913-920.
- Finkel, T. 2000. Redox-dependent signal transduction. *FEBS Lett.* **476**: 52-54.
- Gomi, K., D. Ogawa, S. Katou, H. Kamada, N. Nakajima, H. Saji, T. Soyano, M. Sasabe, Y. Machida, I. Mitsuhashi, Y. Ohashi, and S. Seo. 2005. A mitogen-activated protein kinase NtMPK4 activated by SIPKK is required for jasmonic acid signaling and involved in ozone tolerance via stomatal movement in Tobacco. *Plant Cell Physiol.* **46**(12): 1902-1914.
- Grant, J.J., B.W. Yun, and G.J. Loake. 2000. Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J.* **24**: 569-582.
- Harmon, A.C., C. Putnam-Evans, and M.J. Cormier. 1987. A calcium-dependent but calmodulin-independent protein kinase from soybean. *Plant Physiol.* **83**: 830-837.
- He, C., S.H. Fong, D. Yang, and G.L. Wang. 1999. BWMK1, a novel MAP kinase induced by fungal infection and mechanical wounding in rice. *Mol. Plant-Microbe Interact.* **12**: 1064-1073.
- Heimovaara-Dijkstra, S., C. Testerink, and M. Wang. 2000. Mitogen-activated protein kinase and abscisic acid signal transduction. In H. Hirt (ed.), *MAP Kinases in Plant Signal Transduction*, Heidelberg, Germany: Springer-Verlag, pp. 131-144.
- Hirt, H. 1997. Multiple roles of MAP kinases in plant signal transduction. *Trends Plant Sci.* **2**: 11-15.
- Hong, Y., M. Takano, C.M. Liu, A. Gash, M.L. Chye, and N.H. Chua. 1996. Expression of three members of the calcium-dependent protein kinase gene family in *Arabidopsis thaliana*. *Plant Mole. Biol.* **30**: 1259-1275.
- Hung, S.H., C.W. Yu, and C.H. Lin. 2005. Hydrogen peroxide functions as a stress signal in plants. *Bot. Bull. Acad. Sin.* **46**: 1-10.
- Ichimura, K., K. Shinozaki, G. Tena, J. Sheen, Y. Henry, A. Champion, M. Kreis, S. Zhang, H. Hirt, C. Wilson, E. Heberle-Bors, B.E. Ellis, P.C. Morris, R.W. Innes, J.R. Ecker, D. Scheel, D.F. Klessig, Y. Machida, J. Mundy, Y. Ohashi, and J.C. Walker (MAPK Group). 2002. Mitogen-activated protein kinase cascades in plants, a new nomenclature. *Trends Plant Sci.* **7**: 301-308.
- Jewer, P.C. and L.D. Incoll. 1980. Promotion of stomatal opening in the grass *anthephora pubescens* nees by arrange of natural and synthetic cytokinins. *Planta* **150**: 218-221.
- Jiang, J., G.Y. An, P.C. Wang, P.T. Wang, J.F. Han, Y.B. Jia, and C.P. Song. 2003. MAP kinase specifically mediates the

- ABA-induced H<sub>2</sub>O<sub>2</sub> generation in guard cells of *Vicia faba* L. *Chin Sci Bull.* **48(18)**: 1919-1926.
- Jonak, C., S. Kiegerl, W. Ligterink, P.J. Baker, N.S. Huskisson, and H. Hirt. 1996. Stress signaling in plants, A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc. Natl. Acad. Sci. USA* **93**: 11274-11279.
- Knetsch, M.L., M. Wang, B.E. Snaar-Jagalska, and S. Heimovaara-Dijkstra. 1996. Abscisic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. *Plant Cell* **8**: 1061-1067.
- Kovtun, Y., W.L. Chiu, G. Tena, and J. Sheen. 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* **97**: 2940-2945.
- Laloi, C., K. Apel, and A. Danon. 2004. Reactive oxygen signaling, the latest news. *Curr. Opin. Plant Biol.* **7**: 323-328.
- Lee, S., H. Choi, S. Suh, I.S. Doo, K.Y. Oh, E.J. Choi, S.A.T. Taylor, P.S. Low, and Y. Lee. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. *Plant Physiol.* **121**: 147-152.
- Li, J., Y.R.J. Lee, and S.M. Assmann. 1998. Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiol.* **166**: 785-795.
- Li, J.X., X.Q. Wang, M.B. Watson, and S.M. Assmann. 2000. Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* **287**: 300-303.
- Liang, Z.S., F.S. Zhang, M.A. Shao, and J.H. Zhang. 2002. The relations of stomatal conductance, water consumption, growth rate to leaf water potential during soil drying and rewatering cycle of wheat (*Triticum aestivum*). *Bot. Bull. Acad. Sin.* **43**: 187-192.
- Lu, C., M.H. Han, A. Guevara-Garcia, and N.V. Fedoroff. 2002. Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. *Proc. Natl. Acad. Sci. USA* **99**: 15812-15817.
- Ludwig, A.A., H. Saitoh, G. Felix, G. Freymark, O. Miersch, C. Wasternack, T. Boller, J.D.G. Jones, and T. Romeis. 2005. Ethylene-mediated cross-talk between calcium-dependent and mitogen-activated protein kinase signaling controls stress responses in plants. *Proc. Natl. Acad. Sci. USA* **102(30)**: 10736-10741.
- McAinsh, M.R., H. Clayton, T.A. Mansfield, and A.M. Hetherington. 1996. Changes in stomatal behavior and guard cell cytosolic free calcium in response to oxidative stress. *Plant Physiol.* **111**: 1031-1042.
- Meihsard, M., P.L. Rodriguez, and E. Grill. 2002. The sensitivity of ABI2 to Hydrogen peroxide links the abscisic acid-response regulator to redox signalling. *Planta* **214**: 775-782.
- Mikolajczyk, M., O.S. Awotunde, G. Muszynska, D.F. Klessig, and G. Dobrowolska. 2000. Osmotic stress induces rapid activation of a salicylic acid-induced protein kinase and a homolog of protein kinase ASK1 in tobacco cells. *Plant Cell* **12**: 165-178.
- Mizoguchi, T., K. Ichimura, and K. Shinozaki. 1997. Environmental stress response in plants, the role of mitogen-activated protein kinase. *Trends Biotechnol.* **15**: 15-19.
- Mizoguchi, T., K. Ichimura, K. Irie, P. Morris, J. Giraudat, K. Matsumoto, and K. Shinosaki. 1998. Identification of a possible MAP kinase cascade in *Arabidopsis thaliana* based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants. *FEBS Lett.* **437**: 56-60.
- Mori, I.C., Y. Murata, Y. Yang, S. Munemasa, Y.F. Wang, S. Andreoli, H. Tiriack, J.M. Alonso, J.F. Harper, J.R. Ecker, J.M. Kwak, and J.I. Schroeder. 2006. CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca<sup>2+</sup>- permeable channels and stomatal closure. *PLOS Biol.* **4**: 1749-1862.
- Mustilli, A.C., S. Merlot, A. Vavasseur, F. Fenzi, and J. Giraudat. 2002. *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**: 3089-3099.
- Neill, S., R. Desikan, and J.T. Hancock. 2002. Hydrogen peroxide signalling. *Curr. Opin. Plant Biol.* **5**: 388-395.
- Noctor, G. and C.H. Foyer. 1998. Ascorbate and glutathione, keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**: 249-279.
- Pei, Z.M., Y. Murata, G. Benning, S. Thomine, B. Klüsener, G.J. Allen, E. Grill, and J.I. Schroeder. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* **406**: 731-734.
- Polya, G.M. and V. Micucci. 1985. Interaction of wheat germ Ca<sup>2+</sup>-dependent protein kinases with calmodulin antagonists and polyamines. *Plant Physiol.* **79**: 968-972.
- Roberts, D.M. and A.C. Harmon. 1992. Calcium-modulated proteins, targets of intracellular calcium signals in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**: 375-414.
- Schenk, P.W. and B.E. Snaar-Jagalska. 1999. Signal perception and transduction, the role of protein kinases. *Biochim. Biophys. Acta.* **1449(1)**: 1-24.
- She, X.P., X.G. Song, and J.M. He. 2004. The role and relationship of nitric oxide and Hydrogen peroxide in Light/Dark-regulated stomatal movement in *Vicia faba*. *Acta Bot. Sin.* **46(11)**: 1292-1300.
- Sopory, S.K. and M. Munshi. 1998. Protein kinases and phosphatases and their role in cellular signaling in plants. *Critical Rev. Plant Sci.* **17(3)**: 245-318.
- Stone, J.M. and J.C. Walker. 1995. Plant protein kinase families and signal transduction. *Plant Physiol.* **108**: 451-457.
- Usami, S., H. Banno, Y. Ito, R. Nishihama, and Y. Machida. 1995. Cutting activates a 46-kilodalton protein kinase in plants. *Proc. Natl. Acad. Sci. USA* **92**: 8660-8664.
- Wang, X.Q. and W.H. Wu. 1999. Involvement of calcium-dependent protein kinases in ABA-regulation of stomatal movement. *Acta Bot. Sin.* **41(5)**: 556-559.
- Willmer, C.M. and M.D. Fricker. 1996. Stomata. Second Edi-

- tion. Chapman and Hall, London, United Kingdom.
- Zeiger, E. 1983. The biology of stomatal guard cells. *Annu. Rev. Plant Physiol.* **34**: 441-475.
- Zhang, S. and D.F. Klessig. 2001. MAPK cascades in plant defense signaling. *Trends Plant Sci.* **6**: 520-527.
- Zhang, X., L. Zhang, F.C. Dong, J.F. Gao, D.W. Galbraith, and C.P. Song. 2001b. Hydrogen peroxide is involved in abscisic acid induced stomatal closure in *Vicia faba*. *Plant Physiol.* **126**: 1438-1448.
- Zhang, X., Y.C. Miao, G.Y. An, Y. Zhou, Z.P. Shanguan, J.F. Gao, and C.P. Song. 2001a.  $K^+$  channels inhibited by hydrogen peroxide mediate abscisic acid signalling in *Vicia* guard cells. *Cell Res.* **11**: 195-202.
- Zhou, X.Y. and H. Zhang. 2004. Roles of calcium-dependent protein kinases in ABA regulation of stomatal in poplar. *Sci. Tech. Engng.* **4(2)**: 80-84.

## 黑暗引起的蠶豆氣孔關閉與 MEK 和 CDPK 調節保衛細胞中的過氧化氫的水準有關

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本實驗應用促細胞分裂原蛋白激酶激酶 (MEK) 的抑制劑 2'-氨基-3'-甲氧基黃酮 (PD98059) 和鈣依賴的蛋白激酶 (CDPK) 的特效抑制劑三氟啦嗪 (TFP) 探究了 MEK 和 CDPK 在黑暗引起的蠶豆氣孔關閉中的作用和二者對該過程中保衛細胞中過氧化氫 ( $H_2O_2$ ) 水準的影響。我們的結果證實 PD98059 和 TFP 在黑暗條件下都能降低保衛細胞  $H_2O_2$  水準並能促進氣孔開放，暗示了 MEK 和 CDPK 介導的黑暗引起的氣孔關閉是通過影響保衛細胞中  $H_2O_2$  的水準來實現的。另外，和  $H_2O_2$  的產生有關的 NADPH 氧化酶的抑制劑二苯基碘 (DPI) 的作用效果不同，而和  $H_2O_2$  的清除劑抗壞血酸 (ASA) 的作用效果類似，PD98059 和 TFP 不僅能夠降低光下保衛細胞的外加的  $H_2O_2$  水準，而且還能清除由黑暗條件誘導的已經產生的  $H_2O_2$ ，並能引起氣孔開放。這些結果暗示 MEK 和 CDPK 介導的黑暗引起的氣孔關閉可能通過過制保衛細胞的與  $H_2O_2$  的清除體系相關的酶來實現的，當然也不排除 MEK/CDPK 在保衛細胞信號傳導鏈中作為  $H_2O_2$  的下游信號影響氣孔運動的可能性。

**關鍵詞：**黑暗；過氧化氫；MEK；CDPK；氣孔關閉；蠶豆。