Ethylene mediates UV-B-induced stomatal closure via peroxidase-dependent hydrogen peroxide synthesis in Vicia faba L.

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Abstract

Ultraviolet B (UV-B) radiation is an important environmental signal for plant growth and development, but its signal transduction mechanism is unclear. UV-B is known to induce stomatal closure via hydrogen peroxide (H2O2), and to affect ethylene biosynthesis. As ethylene is also known to induce stomatal closure via H2O2 generation, the possibility of UV-B-induced stomatal closure via ethylene-mediated H2O2 generation was investigated in Vicia faba by epidermal strip bioassay, laser-scanning confocal microscopy, and assays of ethylene production. It was found that H2O2 production in guard cells and subsequent stomatal closure induced by UV-B radiation were inhibited by interfering with ethylene biosynthesis as well as ethylene signalling, suggesting that ethylene is epistatic to UV-B radiation in stomatal movement. Ethylene production preceded H2O2 production upon UV-B radiation, while exogenous ethylene induced H2O2 production in guard cells and subsequent stomatal closure, further supporting the conclusion. Inhibitors for peroxidase but not for NADPH oxidase abolished H2O2 production upon UV-B radiation in guard cells, suggesting that peroxidase is the source of UV-B-induced H2O2 production. Taken together, our results strongly support the idea that ethylene mediates UV-B-induced stomatal closure via peroxidase-dependent H2O2 generation.

Key words: Ethylene, hydrogen peroxide, peroxidase, stomatal closure, UV-B radiation.

Introduction

Stomata play a major role in controlling gaseous exchange, especially of photosynthetic carbon dioxide (CO2) uptake, and in water release by transpiration in response to changes in the surrounding environment. The regulation of stomatal aperture is thus extremely important for the survival of plants. Ozone depletion in the stratosphere has resulted in increased ultraviolet B (UV-B; 280–315 nm) radiation at the earth’s surface since the 1980s (UNEP, 2002) and present projections estimate a return to pre-1980 levels by 2050–2075 (UNEP, 2008). In addition, stratospheric ozone recovery may possibly be delayed due to a number of uncertainties (UNEP, 2008). Therefore, it is still essential to investigate the effects of elevated UV-B radiation on various aspects of plant growth (Solomon, 2008). On exposure to elevated UV-B radiation, the stomata of plants exhibit complex responses. Several groups focusing on plant performance and photosynthesis under supplemental UV-B radiation observed UV-B-induced decreases in stomatal conductance and/or aperture (Musil and Wand, 1993; Noguès et al., 1999; Jansen and Noort, 2000). However, in some species, such as some Ericaceae and Vicia faba, UV-B has been reported to induce either stomatal opening or

Abbreviations: ABA, abscisic acid; ACC, 1-amino- cyclopropane-1-carboxylic acid; ACS, ACC synthase; AOA, aminooxyacetic acid; ASC, ascorbic acid; AVG, aminooxyvinyl glycine; CAT, catalase; DMSO, dimethyl sulphoxide; DPI, diphenylene iodonium chloride; H2DCFDA, dichlorofluorescein diacetate; H2O2, hydrogen peroxide; 1-MCP, 1-methylcyclopropene; MES, 2-(N-morpholino) ethanesulphonic acid; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; UV-B, ultraviolet B.

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stomatal closure (Musil and Wand, 1993; Eisinger et al., 2000), perhaps depending on the metabolic state of guard cells (Jansen and Noort, 2000). Although the response of stomata to UV-B radiation is well known, the underlying mechanism is poorly understood.

Studies have shown that, in plants, hydrogen peroxide (H₂O₂) generation from multiple sources can be induced by UV-B radiation (Mackerness et al., 2001; Neill et al., 2002). Because H₂O₂ is relatively stable and diffusible through membranes, it can function as a signalling molecule mediating a range of responses to environmental stresses, including UV-B radiation (Surplus et al., 1998; Mackerness et al., 2001; Neill et al., 2002). UV-B-induced stomatal closure is also mediated by H₂O₂ (He et al., 2005). However, the mechanism by which UV-B radiation induces H₂O₂ generation in guard cells is still not known.

The plant hormone ethylene influences many aspects of plant growth and development, as well as mediating a diverse array of abiotic stresses (Wang et al., 2002; Guo and Ecker, 2004). In plants, ethylene is synthesized from Met via S-adenosyl-L-Met and 1-aminoacyclopropene-1-carboxylic acid (ACC; Adams and Yang, 1979). The conversion from S-adenosyl-L-Met to ACC, catalysed by ACC synthase (ACS), is generally the rate-limiting step of ethylene biosynthesis. Expression of ACS and consequent ethylene production are induced under UV-B radiation (Garty et al., 2004; An et al., 2006). Such UV-B-induced production of ethylene is important for some UV-B-induced damage (Nara and Takeuchi, 2002) and gene expression (Wang et al., 2002; Guo and Ecker, 2004). Stomatal movement is also regulated by ethylene, but its effect in this process seems rather contradictory (Acharya and Assmann, 2009; Wilkinson and Davies, 2010). In some species, it is reported that ethylene induces stomatal opening and/or inhibits abscisic acid (ABA)-induced stomatal closure, or stomatal sensitivity to stresses/cues in which ABA is the normal signal for the given stomatal response (Madhavan et al., 1983; Levitt et al., 1987; Merritt et al., 2001; Tanaka et al., 2005, 2006; Mills et al., 2009; Wilkinson and Davies, 2009; Benlloch-González et al., 2010), whereas in others it induces closure (Pallas and Kays, 1982; Tissera and Ayres, 1986; Gunderson and Taylor, 1991; Young et al., 2004).

Pharmacological drugs that specifically inhibit H₂O₂ production, ethylene biosynthesis or ethylene perception were widely used as powerful tools to study the role of ethylene or H₂O₂ in different cellular processes including stomatal movement. For example, aminoethoxyvinyl gly cine (AVG) and aminooxyacetic acid (AOA) are two inhibitors of ACS, a rate-limiting enzyme of ethylene biosynthesis (Wang et al., 2002), while 1-methylycyclopropene (1-MCP) and silver both interfere with the function of ethylene receptor(s), and therefore have been used to block various effects of ethylene (Beyer, 1976; Sisler and Serek, 1997; Rodriguez et al., 1999). Tiron is a scavenger of O₂, application of which led to H₂O₂ reduction (Wojtaszek, 1997; Mackerness et al., 2001). Salicylhydroxamic acid (SHAM) inhibits peroxidase function to reduce H₂O₂ production whereas diphenylene iodonium chloride (DPI) inhibits that of NADPH oxidase not only in mammals but also in plants (Mackerness et al., 2001; Desikan et al., 2006).

Recent data indicated that H₂O₂-induced stomatal closure requires the ethylene receptor ETR1 (Desikan et al., 2005) and ethylene-induced stomatal closure via NADPH oxidase AtrbohF-mediated H₂O₂ synthesis (Desikan et al., 2006), implying a link between H₂O₂ and ethylene signal transduction during stomatal movement. But, to our knowledge, it is still not known whether ethylene is involved in UV-B-induced stomatal closure and, if so, whether ethylene mediates the UV-B effect via H₂O₂ production and what is the enzymatic pathway of H₂O₂ generation. It is shown here that ethylene mediates UV-B-induced stomatal closure via peroxidase-dependent H₂O₂ synthesis in broad bean (Vicia faba L.) using physiological analyses combined with pharmacological treatments. Our results support the idea that ethylene biosynthesis and signalling serve as a missing link in the H₂O₂ production in guard cells and stomatal closure induced by UV-B radiation.

Materials and methods

Chemicals

Molecular probe 2’,7’-dichlorofluorescein diacetate (H₂DCF-DA, from Biotium, Hayward, CA, US) was dissolved in dimethyl sulphoxide (DMSO, from Amresco, Solon, OH, US) to produce a 10 mM stock solution. Catalase (CAT, from bovine liver), ascorbate acid (ASC), 2-(N-morpholino)ethanesulphonic acid (MES), AVG, AOA, 1-MCP, DPI, SHAM, tiron, ethephon, and ACC were obtained from Sigma-Aldrich (St Louis, MO, US). Unless stated otherwise, the remaining chemicals were of the highest analytical grade available from various Chinese suppliers.

Plant materials

Broad bean (Vicia faba L.) was grown in growth chamber [day/night 25±2/20±2 °C; 300 μmol m⁻² s⁻¹ PAR generated by cool white fluorescent tubes (Philips, New York, NY); 14 h photoperiod; relative humidity 70%]. Immediately before 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 strips 5 mm wide and 10 mm long.

UV-B radiation

UV-B irradiation was provided by filtered 40 W Q-Panel UV313 lamps (Largo, Goteborg, Sweden; maximum output at 313 nm) in the controlled growth chamber. The lamps were suspended above and perpendicular to the Petri plates and filtered with 0.13 mm thick cellulose diacetate (transmission down to 290 nm) for UV-B radiation or 0.13 nm polyester plastic films (absorbs all radiation below 320 nm) as a control. Cellulose diacetate filters were personalized for 8 h and changed weekly to ensure uniformity of UV-B radiation. The desired radiation (0.8 W m⁻²) was obtained by adjusting the distance between the lamps and the Petri plates. The spectral irradiation from the lamps was determined with an Optronics Model 742 (Optronics Laboratories, Orlando, FL, USA) spectroradiometer and weighted with the generalized plant response action spectrum normalized to 300 nm.

Stomatal bioassay

Stomatal bioassay was performed on epidermal strips as described by He et al. (2005). Freshly prepared abaxial epidermal strips were floated under light conditions (PAR 300 μmol m⁻² s⁻¹) for 2 h
with their cuticles facing up on CO2-free MES–KCl buffer (10 mM MES–KOH, 50 mM KCl, pH 6.15). Once the stomata were fully open, epidermal strips were treated with various compounds or inhibitors for a further 4 h under light conditions with or without supplementary UV-B irradiation. Control treatments involved the addition of buffer or appropriate solvents used with inhibitors. Stomatal apertures were measured with a light microscope with a calibrated micrometer scale.

To avoid any potential rhythmic effects on stomatal aperture, experiments were always started at the same time of the day. In each treatment, 30 randomly selected apertures per replicate were scored and treatments were repeated three times. The data presented are the means of 90 measurements ±SEs.

**Treatment with 1-MCP**

For 1-MCP treatment, 8.69 mg 1-MCP was dissolved with 132 μl distilled water in a tube, and 4-week-old plants were laid with the tube in a closed chamber (9.0 l) for 12 h (overnight). The final concentration of 1-MCP in the gas phase was expected to be about 500 μl l⁻¹ (Tamaoki et al., 2003). After the treatment, the epidermis was carefully peeled from the abaxial surface of the youngest, fully expanded leaves of the treated plants and considered as the 1-MCP-treated epidermal strips to be used for experiments.

**Ethylene measurement**

After the previously described treatments, ethylene production in the epidermal strips was determined by enclosing the strips in a 2 ml vial, containing filter paper moistened by the corresponding MES–KCl buffer, for 3 h under light conditions. A 1 ml aliquot of the gaseous phase of the vial was applied to a gas chromato-

**Measurement of endogenous H2O2 by confocal laser-scanning microscopy**

H2O2 measurement were performed with fluorescent indicator dye H2DCFDA as described previously (Allan and Fluhr, 1997) with slight modifications. After the previously described treatments, the epidermal strips were immediately placed into TRIS–KCl buffer (10 mM TRIS and 50 mM KCl, pH 7.2) containing H2DCFDA at 50 μM for 10 min, in the dark at 25 °C. Excess dye was removed with fresh TRIS–KCl buffer in the dark, and an examination of the peels was immediately performed by TCS SP2 confocal laser-scanning microscopy (Leica Lasertechnik GmbH, Heidelberg) with the following settings: excitation 488 nm, emission 530 nm, normal scanning speed, frame 512×512. Images acquired from the confocal microscope were processed with Photoshop software and analysed with LEICA IMAGE software to measure the average fluorescence intensities in the guard cells following various treatments. In each experiment, three epidermal strips were measured, each of which originated from a different plant. Each experiment was repeated three times. The selected confocal image represented the same results from approximately nine time measurements.

**Statistical analysis**

Statistical analyses were performed by using a one-way ANOVA followed by the least significant difference test.

**Results**

**UV-B-induced stomatal closure depends on ethylene biosynthesis**

To examine the effects of UV-B on stomata, a system using isolated V. faba epidermal strips in which stomatal apertures could be measured was employed. When epidermal strips were floated on CO2-free MES–KCl buffer under light with supplementary UV-B radiation, time-course observations demonstrated that 3 h radiation of UV-B significantly induced stomatal closure (P <0.01) while the maximum effect was observed at 4 h (Fig. 1A).

Next, the effects of AVG, AOA, 1-MCP, and silver on UV-B-induced stomatal closure were examined. After epidermal strips were treated with AVG, AOA, 1-MCP or silver, the UV-B-induced stomatal closure was significantly inhibited (P <0.01), while the four inhibitors had no effect on stomatal apertures under the light controls (P >0.05) (Fig. 1B). Furthermore, application of the ethylene precursor ACC compensated for the effects of AVG and AOA, but not for the effects of 1-MCP and silver (Fig. 1B), which concurred with the inhibitory effects of AVG and AOA on the activity of ACS, or 1-MCP and silver on the ethylene signalling.

Having established that ethylene is involved in UV-B-induced stomatal closure in the above epidermal strip bioassays, it was subsequently examined whether ethylene production in the epidermal strips was increased by UV-B radiation. As shown in Fig. 1C, a low and constant level of ethylene was observed in the control samples, but exposure to UV-B for 1–4 h induced a rapid and striking increase in ethylene production that reached a maximum at 1 h, gradually decreased after 2 h, and kept constant but still much higher than that in control samples after 3 h. Clearly, the enhanced ethylene production induced by UV-B preceded stomatal closure. Furthermore, the UV-B-induced ethylene production was largely prevented by AVG and AOA, but not significantly inhibited by 1-MCP and silver (P >0.01) (Fig. 1D), which is consistent with the results of the stomatal bioassays described above.

The effects of exogenous ethylene on stomatal movement was then investigated. As shown in Fig. 1E, both ethephon and ACC, the ethylene releasing compound and the immediate precursor of ethylene, respectively, induced stomatal closure significantly. Such effects were largely suppressed by the addition of 1-MCP or silver (P <0.01), further confirming that the effect of ethylene on stomatal closure depends on ethylene signalling.

Taken together, the above results showed convincingly that UV-B-induced stomatal closure depends on ethylene.

**Ethylene mediates UV-B-induced stomatal closure via H2O2 generation**

First, it is proposed that if H2O2 acts downstream of ethylene, then the deficiency of ethylene could be bypassed by H2O2, but the deficiency of H2O2 could not be compensated by ethylene, and vice versa. Indeed, the inhibitory
Fig. 1. Ethylene mediates UV-B-induced stomatal closure. (A) Time-course of stomatal closure induced by UV-B. Epidermal strips were floated on buffer under light alone (white) or with supplementary UV-B radiation (0.8 W m\(^{-2}\)) (black) for indicated time. Data are the means of 90 measurements ±SEs. (B) Effect of AVG, AOA, 1-MCP, silver or ACC on UV-B-induced stomatal closure. Epidermal strips from V. faba not treated or treated with 500 pl l\(^{-1}\) 1-MCP (MCP) in a closed chamber for 12 h were exposed to light alone (white), or in the presence of supplementary UV-B radiation (0.8 W m\(^{-2}\)) without (black) or with (grey) 100 µM ACC, for 4 h on buffer alone (con) or containing 50 µM AVG, 50 µM AOA or 0.1 µM silver nitrate (Ag), respectively. Data are the means of 90 measurements ±SEs. (C) Time course of ethylene production from epidermal strips under UV-B radiation. After the epidermal strips were floated on buffer under light alone (white) or with supplementary UV-B radiation (black) for the indicated times, the epidermal strips were immediately transferred to a vial and sealed for 3 h, then the ethylene production was measured by gas chromatography. Data are the means of five measurements ±SEs. (D) Effects of AVG, AOA, 1-MCP, and silver on ethylene production from epidermal strips under UV-B radiation for 1 h. Treatments and ethylene measurements are the same as in (B) and (C), respectively. (E) Effects of 1-MCP and silver on ethephon- or ACC-induced stomatal closure. Epidermal strips from V. faba were floated on buffer alone (white), or containing 0.1 µM silver nitrate (Ag) (black) without (con) or with 300 µM ethephon or 100 µM ACC; or epidermal strips from V. faba treated with 500 pl l\(^{-1}\) 1-MCP (grey) were floated on buffer alone (con) or containing 300 µM ethephon or 100 µM ACC, for 4 h in light. Data are the means of 90 measurements ±SEs. Means with different letters in (A) to (E) are significantly different at \(P < 0.05\).
effects of AVG, AOA, 1-MCP, and silver on UV-B-induced stomatal closure were significantly reversed by H$_2$O$_2$ ($P<0.01$) (Fig. 2A), but the inhibitory effect of CAT (a H$_2$O$_2$ scavenger) or ASC (an important reducing substrate for H$_2$O$_2$ removal) on UV-B-induced stomatal closure could not be compensated by either ethephon or ACC ($P>0.05$) (Fig. 2B). Similarly, exogenous ethephon- or ACC-induced stomatal closure was completely inhibited by ASC and CAT (Fig. 3A), but exogenous H$_2$O$_2$-induced stomatal closure was not affected by either ethylene biosynthesis inhibitors AVG and AOA, or the ethylene perception inhibitors 1-MCP and silver (Fig. 3B). These results suggest that H$_2$O$_2$ acts downstream of ethylene during UV-B-induced stomatal closure. To confirm this conclusion further, H$_2$O$_2$ synthesis in guard cells was next monitored using the H$_2$O$_2$-sensitive fluorescent dye H$_2$DCFDA by confocal microscopy (Allan and Fluhr, 1997; He et al., 2005). As shown in Fig. 2C, UV-B had a cumulative effect over time on H$_2$O$_2$ production in guard cells. The relative fluorescence intensity of DCF in guard cells under UV-B radiation was slightly enhanced at 1 h, substantially enhanced at 2 h, and reached a maximum at 4 h (Fig. 2C). Clearly, under UV-B radiation, the significant rise in H$_2$O$_2$ level (Fig. 2C) was after the peak of ethylene evolution (Fig. 1C), but preceded stomatal closure (Fig. 1A), also suggesting that H$_2$O$_2$ works downstream of ethylene. Furthermore, the UV-B-induced H$_2$O$_2$ production was reduced significantly by AVG, AOA, 1-MCP, and silver ($P<0.01$) (Fig. 2C). In addition, treatment with exogenous ethylene, supplied via either ethephon or ACC, also increased H$_2$O$_2$ generation in guard cells, which was inhibited not only by ASC and CAT, but also by 1-MCP or silver (Fig. 3C). Together, the correlation between stomatal movement and change in H$_2$O$_2$ level indicates that ethylene mediates UV-B-induced stomatal closure via H$_2$O$_2$ generation.

Involvement of peroxidase-dependent H$_2$O$_2$ generation in UV-B-induced stomatal closure

In order to evaluate the potential role of NADPH oxidase and peroxidase as H$_2$O$_2$-generators in guard cells in response to UV-B exposure, epidermal strips were exposed to UV-B radiation for 4 h in the presence of tiron, DPI or SHAM, then the stomatal aperture and H$_2$O$_2$ level were examined. As shown in Fig. 4, Both UV-B-induced stomatal closure (Fig. 4A) and H$_2$O$_2$ generation (Fig. 4B) were almost completely inhibited by tiron, indicating that the first reactive oxygen species (ROS) generated by exposure to UV-B is O$_2^-$. Taken together with ASC and CAT data (Fig. 2B; He et al., 2005), it is indicated that UV-B-induced stomatal closure is regulated by H$_2$O$_2$ derived from O$_2^-$. Furthermore, both UV-B-induced stomatal closure (Fig. 4A) and H$_2$O$_2$ generation (Fig. 4B) were significantly reduced by SHAM ($P<0.01$), but not by DPI ($P>0.05$), suggesting that UV-B-induced H$_2$O$_2$ in guard cells was derived from the activity of peroxidase, but not of NADPH oxidase. Combined with the above conclusion that ethylene mediated H$_2$O$_2$ production during UV-B-induced stomatal closure, it was deduced that ethylene mediated UV-B-induced stomatal closure via peroxidase-dependent H$_2$O$_2$ generation. This result seemed to disagree with a previous report by Desikan et al. (2006) in which the NADPH oxidase AtrbohF was involved in H$_2$O$_2$ synthesis during stomatal closure induced by exogenous ethylene in Arabidopsis. To assess this discrepancy further, the origin of exogenous ethylene-induced H$_2$O$_2$ in guard cells of V. faba was clarified. Figure 3A and C indicated, respectively, that both ethephon- and ACC-induced stomatal closure and H$_2$O$_2$ generation were significantly inhibited not only by DPI, but also by SHAM, as well as tiron ($P<0.01$), suggesting that both NADPH oxidase- and peroxidase-mediated H$_2$O$_2$ are involved in exogenous ethylene-induced stomatal closure in V. faba.

Discussion

UV-B induces stomatal closure through acceleration of ethylene biosynthesis

Stomata are the main routes for leaf gas exchange, controlling CO$_2$ uptake and transpiration. Stomatal movements are regulated by many abiotic and biotic factors, including UV-B radiation and the plant hormone ethylene (Jansen and Noort, 2000; Acharya and Assmann, 2009; Wilkinson and Davies, 2010). Previous studies have proved that UV-B radiation induces the enhanced production of ethylene from plant tissues (Garty et al., 2004; An et al., 2006), which acts as a second messenger to mediate plant responses to UV-B radiation, such as leaf damage (Nara and Takeuchi, 2002) and gene expression (Wang et al., 2002; Guo and Ecker, 2004). However, whether ethylene was involved in the UV-B effect on stomata remained unknown. The data presented here show clearly that ethylene generation and its signalling are essential for UV-B-induced stomatal closure in V. faba epidermal strips. The following results supported this conclusion: (i) ethylene production in epidermal strips coincided with stomatal closure (Fig. 1A–D), (ii) inhibiting ethylene generation or perception reversed the UV-B-induced stomatal closure (Fig. 1B), and (iii) exogenous ethylene induced stomatal closure in the absence of UV-B radiation (Fig. 1E).

Desikan et al. (2006) reported that ethylene induced stomatal closure in intact Arabidopsis leaves, but not in epidermal peels. They therefore proposed that ethylene-induced stomatal closure requires some cell-to-cell communication between guard cells and mesophyll cells, which is not consistent with the data reported here. However, it must be noted that they used Arabidopsis, whereas the studies described here used V. faba. Stomata in different species have different sensitivities or responses to ethylene (Madhavan et al., 1983; Giulivo, 1986). Furthermore, endogenous ethylene is an effective regulator of inhibiting stomatal opening in epidermal strips prepared from either healthy or rusted regions of infected leaves of V. faba (Tissera and Ayres, 1986). Li et al. (2007) also observed that ethephon induced stomatal closure in V. faba epidermal peels. Taken together with the data described here, it is possible that the sensitivities of guard cells to ethylene differ between Arabidopsis and V. faba.
Fig. 2. Ethylene acts upstream of H$_2$O$_2$ during UV-B-induced stomatal closure. (A) H$_2$O$_2$ rescued the inhibitory effect of AOA, AVG, 1-MCP, and silver on UV-B-induced stomatal closure. Epidermal strips from V. faba not treated or treated with 500 pl l$^{-1}$ 1-MCP (MCP) were floated on buffer alone (white), or containing 100 µM H$_2$O$_2$ (black) without (con) or with 50 µM AVG, 50 µM AOA or 0.1 µM silver nitrate (Ag), for 4 h in light with supplementary UV-B radiation (0.8 W m$^{-2}$). Data are the means of 90 measurements ± SEs. Means with different letters are significantly different at $P < 0.05$. (B) Ethephon and ACC did not rescue the inhibitory effect of ASC and CAT on UV-B-induced stomatal closure. Epidermal strips were floated on buffer alone (white), or containing 300 µM ethephon (black) or 100 µM ACC (grey) without (con) or with 100 µM ASC or 100 units ml$^{-1}$ CAT, for 4 h in the light with supplementary UV-B radiation (0.8 W m$^{-2}$). Data are the means of 90 measurements ± SEs. Means with different letters are significantly different at $P < 0.05$. (C) Endogenous H$_2$O$_2$ production in guard cells in response to UV-B radiation without or with AOA, AVG, 1-MCP, and silver. After the following treatments, the epidermal strips were immediately loaded with H$_2$DCFDA for 10 min in the dark, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (a) Image of an epidermal strip treated with light alone for 4 h. (b–e) Images of epidermal strips treated by UV-B radiation (0.8 W m$^{-2}$) for 1 h, 2 h, 3 h, and 4 h, respectively. (f–h) Images of epidermal strips treated with UV-B radiation for 4 h in the presence of 50 µM AVG, 50 µM AOA or 0.1 µM silver nitrate, respectively. (i) Image of epidermal strips from 1-MCP-treated V. faba treated with UV-B radiation for 4 h. (j) The figure shows the average fluorescent intensity of guard cells in images from (a) to (i), data are the means ± SEs (n=200 guard cells) and means with asterisks are significantly different at $P < 0.01$ when compared to (e). Scale bar (40 µm) in image (i) is for all images.
In the literature on the effects of ethylene on stomatal movements in different species, varied reports have revealed that, in the absence of ABA, ethylene can induce stomatal closure (Pallas and Kays, 1982; Tissera and Ayres, 1986; Gunderson and Taylor, 1991; Young et al., 2004; Desikan et al., 2006; Li et al., 2007), but it has the opposite effect in the presence of ABA such that it inhibits ABA-induced closure (Tanaka et al., 2005, 2006; Desikan et al., 2006; Mills et al., 2009; Wilkinson and Davies, 2009; Benlloch-Gonzalez et al., 2010). In the present study, although ABA levels in guard cells were not directly examined, the application of fluridone, an inhibitor of ABA biosynthesis, did not affect the UV-B-induced stomatal closure (data not shown), suggesting that an ABA-independent signalling pathway may well be involved in UV-B-induced stomatal closure in *V. faba* epidermal strips. However, the UV-B-induced stomatal closure was only partially reversed by either the inhibitors of ethylene biosynthesis, AVG and AOA, or the inhibitors of ethylene perception, 1-MCP and silver (Fig. 1B), suggesting that an ethylene-independent signalling pathway may still exist in UV-B-induced stomatal closure.

**Ethylene mediates UV-B-induced stomatal closure via peroxidase-dependent H$_2$O$_2$ generation**

Our previous work has shown that H$_2$O$_2$ acts as a second messenger mediating UV-B-induced stomatal closure (He et al., 2005), but by what mechanisms UV-B induces H$_2$O$_2$ generation in guard cells was still not known. The data presented here (Figs 2, 3) further indicate that the UV-B-induced H$_2$O$_2$ generation in *V. faba* guard cells is mediated by ethylene, which again emphasizes H$_2$O$_2$ as a key component of the ethylene-induced stomatal closure (Desikan et al., 2006). On the other hand, Desikan et al. (2005, 2006) have showed that one of ethylene receptors, ETR1, is a convergent point of ethylene and H$_2$O$_2$ signalling in guard cells, but H$_2$O$_2$ signalling via ETR1 is mechanistically different to that of ethylene. The present data showed that 1-MCP and silver, two inhibitors of ethylene receptor(s), not only inhibited exogenous ethylene-induced H$_2$O$_2$ generation (Fig. 3C) and stomatal closure (Fig. 1E), but also significantly reduced the effects of UV-B-induced endogenous ethylene on H$_2$O$_2$ production (Fig. 2C) and stomatal closure (Fig. 1B). These results indicated that UV-B-induced ethylene signalling also occurs via
that NADPH oxidase-mediated H$_2$O$_2$ synthesis is present in the guard cells of Arabidopsis and Pisum sativum, and likely to mediate the ABA-, darkness-, methyl jasmonate-, ozone-, and ethylene-induced stomatal closure (Kwak et al., 2003; Desikan et al., 2004, 2006; Suhita et al., 2004; Joo et al., 2005; Sagi and Fluhr, 2006). With regard to the enzymatic sources of the UV-B-induced H$_2$O$_2$ in plants, Mackerness et al. (2001) showed that UV-B exposure is likely to induce at least NADPH oxidase- and peroxidase-mediated H$_2$O$_2$ synthesis in the leaves of Arabidopsis. In the present study, by using tiron, a scavenger of O$_2^\cdot$, DPI, an inhibitor of NADPH oxidase, and SHAM, an inhibitor of peroxidase, it is clearly shown that UV-B-induced H$_2$O$_2$ generation in V. faba guard cells depends on peroxidase, but not NADPH oxidase (Fig. 4), despite these two enzymes of H$_2$O$_2$ production both being induced by exogenous ethylene (Fig. 3). These results not only confirmed the studies of Desikan et al. (2006), but also indicated that multiple sources of H$_2$O$_2$ can be induced by ethylene. Combined with the studies of Mackerness et al. (2001), the results also showed that UV-B radiation activated different enzymatic sources of H$_2$O$_2$ between mesophyll and epidermal tissues. As UV-B-induced H$_2$O$_2$ generation is mediated by ethylene (see above), it is proposed that the peroxidase source of H$_2$O$_2$ is activated by UV-B-induced ethylene, which is not exactly the same as exogenous ethylene-induced enzymatic sources of H$_2$O$_2$ in V. faba guard cells (Fig. 3). With regard to this difference, the following two possibilities are proposed. It is possible that the spatial and temporal location of UV-B-induced ethylene in guard cells is quite different from that supplied by exogenous ethylene, which perhaps results in the differently localized enzymes of H$_2$O$_2$ generation being activated (Wojtaszek, 1997). Another possible reason may be that, besides the ethylene signalling pathway, UV-B radiation also induces other signalling pathways or some components in guard cells, which block ethylene to activate NADPH oxidase or inhibit the activity of NADPH oxidase. However, these possibilities should be examined in future.

In summary, the data presented here show that UV-B radiation causes stomatal closure in V. faba epidermal strips via ethylene-induced H$_2$O$_2$ synthesis, mediated by peroxidases. These data will no doubt help us to gain further insight into the physiological roles of ethylene and H$_2$O$_2$ in UV-B signal transduction networks. However, little is known about the complex molecular network operating during the guard cell movement triggered by UV-B, ethylene and H$_2$O$_2$, and these should be further studied.

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