Carbon monoxide-induced stomatal closure involves generation of hydrogen peroxide in *Vicia faba* guard cells

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Carbon monoxide-induced stomatal closure involves generation of hydrogen peroxide in *Vicia faba* guard cells

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Abstract. Recently, carbon monoxide (CO) was implicated as another important physiological messenger or bioactive molecule in animals. Previous researches indicate that heme oxygenase-1 (HO-1, EC 1.14.99.3) catalyzes the oxidative conversion of heme to CO and biliverdin IXa with the concomitant release of iron. However, little is known about the physiological roles of CO in plant. In the present paper, the regulatory role of CO during stomatal movement in *Vicia faba* was surveyed. Results indicated that, like hydrogen peroxide (H$_2$O$_2$), CO donor Hematin induced stomatal closure in dose- and time-dependent manners. These responses were also proven by the addition of gaseous CO aqueous solution with different concentrations, showing the first times that CO and H$_2$O$_2$ exhibit the similar regulation role in the stomatal movement. Moreover, our data showed that ascorbic acid (ASA, an important reducing substrate for H$_2$O$_2$ removal) and diphenylene iodonium (DPI, an inhibitor of the H$_2$O$_2$-generating enzyme NADPH oxidase) not only reversed stomatal closure by CO, but also suppressed the H$_2$O$_2$ fluorescence induced by CO, implying that CO induced-stomatal closure probably involves H$_2$O$_2$ signal. Additionally, the CO/NO scavenger hemoglobin (Hb) and CO specific
synthetic inhibitor ZnPPIX, ASA and DPI reversed the darkness-induced stomatal closure and H$_2$O$_2$ fluorescence. These results show that, maybe like H$_2$O$_2$, the levels of CO in guard cells of *Vicia faba* is higher in dark than that in light, HO-1 and NADPH oxidase are the enzyme systems responsible for generating endogenous CO and H$_2$O$_2$ in darkness respectively, and that CO being from HO-1 mediated darkness-induced H$_2$O$_2$ synthesis in guard cells stomatal closure of *Vicia faba*.

**Keywords:** Carbon monoxide, guard cells, hydrogen peroxide, signaling, stomata.

Exogenous carbon monoxide (CO) has long been recognized as a toxic gas with lethal effects on humans following continuous exposure in air containing ≥4 mL/L CO (equivalent to approximately 180µmol/L CO gas or more). Until 1990s, with the further studies on low concentrations of endogenous nitric oxide (NO) as the most important gaseous messenger molecule in human bodies, increasing attention has also been drawn to another gaseous molecule CO, since a number of studies have shown that CO was similar to NO in physical and chemical properties (Verma et al. 1993; Longo et al. 1999). Recent studies in mammals have demonstrated that CO may also exert its biological effect by its synergism with NO, and cGMP was the common second messenger (Coceani, 2000; Ryter et al. 2002; Dulak and Józkowicz, 2003; Otterbein et al. 2003; Watts et al. 2003). Heme oxygenase (HO, EC 1.14.99.3) is the enzyme systems responsible for generating carbon monoxide in animals (Ryter et al. 2002), and it catalyzes the oxidative conversion of heme to CO and biliverdin IXa.
In animals, there are three forms of HOs. HO-1 is inducible, while constitutively expressed HO-2 and HO-3 display very low activity. In plants, several laboratories have reported the direct emission of CO by living green plants when exposed to sunlight or during seedling growth (Wilks, 1959; Siegel et al. 1962; Tarr et al. 1995). In 2002, Muramoto et al. (2002) first found that the Arabidopsis HY1 gene encoding plastid heme oxygenase (AtHO1) exhibited the ability to catalyze the formation of CO from heme molecules with the concomitant production of biliverdin (BV) in vitro, and was responsible for phytochrome chromophore synthesis. Some studies showed that there has be HO-1 activity in plants (Terry et al. 2002, Balestrasse et al. 2005), and Zn protoporphyrin IX (ZnPPIX) could inhibit irreversibly HO-1 activity (Noriega et al. 2004). Genes encoding HO-1 have been isolated from red algae and cryptophytes (Ortiz de Montellano and Wilks 2001). But little was still known about the exact mechanism of biosynthesis and even biological roles of CO in plants.

H$_{2}$O$_{2}$ is a form of reactive oxygen species having wide-ranging effects in many biological systems (Finkel and Holbrook, 2000). Exposure of plants to various abiotic and biotic stresses induces the generation of H$_{2}$O$_{2}$ (Dat et al. 2000). Although the effects of H$_{2}$O$_{2}$ on plant physiology and development have been the subject of investigation for several years, it is only relatively recently that its role as signalling molecules has been characterized more fully. The roles of H$_{2}$O$_{2}$ in plant defence responses to pathogens have been studied in some detail. Exposure of plant cells to avirulent pathogens or pathogen-derived elicitors induces both a rapid and a more
prolonged 'burst' of $\text{H}_2\text{O}_2$. Generation of NO occurs within the same time frame as $\text{H}_2\text{O}_2$ release, and a critical balance between the two reactive species regulates cellular outcomes such as programmed cell death (Delledonne et al. 2001). Involvement of $\text{H}_2\text{O}_2$ in developmental processes such as seed germination, gravitropism and root development indicates that the signalling molecule is key regulators of plant responses to a range of endogenous signals and stimuli such as auxin and ABA (Neill et al. 2002, 2003). The effects of $\text{H}_2\text{O}_2$ on guard cells were first reported by McAinsh et al. (1996). Exposure of *Vicia faba* guard cells to exogenous $\text{H}_2\text{O}_2$ induced elevations of cytosolic calcium and stomatal closure. The discovery that ABA induces $\text{H}_2\text{O}_2$ synthesis in Arabidopsis guard cells was a significant finding that highlighted further complexities in ABA signalling in Arabidopsis and *Vicia faba* guard cells (Pei et al. 2000; Zhang et al. 2001). There is now compelling evidence that $\text{H}_2\text{O}_2$ is involved in UV-B-, elicitor-, cytokinin-, auxin- and ABA-induced stomatal movement (Lee et al. 1999, Allen et al. 2000, Pei et al. 2000, Zhang et al. 2001, He et al. 2005, Song et al. 2006). Recent researches provided exciting evidence that darkness-induced stomatal closure requires $\text{H}_2\text{O}_2$ accumulation (Desikan et al. 2004, She et al. 2004), and ABA-induced NO generation and stomatal closure are dependent on $\text{H}_2\text{O}_2$ synthesis in Arabidopsis (Bright et al. 2006).

Recently, CO has been studied intensively to elucidate the role of enigmatic molecule in plant. Previous studies discovered that CO is a novel antioxidant against oxidative stress in wheat seedling leaves (huang et al. 2006; Sa et al. 2007), and CO is able to attenuate the seed germination inhibition produced by salinity stress (Xu et al.
2006a) and also stimulated seed germination (Dekker and Hargrove, 2002).

Interestingly, CO induces adventitious rooting of hypocotyl cuttings from mung bean seedling (Xu et al. 2006b), and promotes lateral root formation in rape seedling (Cao et al. 2007). Similarly, H$_2$O$_2$ also participates in adventitious root organogenesis of cucumber and mung bean hypocotyls (Li et al. 2006) and involves the development of lateral roots in soybean (Su et al. 2006). More recently, the report from Shen`s lab suggest that CO produced by HO-1 is involved in ABA-induced stomatal closure, and NO and cGMP may function as downstream intermediates in the CO signaling responsible for stomatal closure (Cao et al. 2007). This is the first report focusing on CO involvement in the signaling pathway of guard cells. In addition, Yannarelli et al. (2006) discovered that HO in soybean plants was up-regulated as a mechanism of cell protection against ultraviolet-B irradiation-induced oxidative damage, and this process was related to the formation of H$_2$O$_2$. Up to today, the role of CO in light/darkness-regulated stomatal movement, and the relation of H$_2$O$_2$ and CO in this process are still unknown.

In this work, we discovered that CO donor Hematin induced stomatal closure in 

*Vicia faba*, as did gaseous CO aqueous solution. Furthermore, through pharmacological and surgical approaches combined with laser scanning confocal microscope (LSCM) method we found that H$_2$O$_2$ might mediate the stomatal closure induced by CO. In addition, our data showed that endogenous CO also mediated darkness-induced H$_2$O$_2$ synthesis in guard cells stomatal closure of *Vicia faba*. 
Results

Exogenous CO induces stomatal closure in a dose- and time-dependent manner

As shown in Fig. 1C, H\textsubscript{2}O\textsubscript{2} induced stomatal closure in dose- and time-dependent manners in *Vicia faba*, a similar response to that obtained with H\textsubscript{2}O\textsubscript{2} (Zhang et al. 2001; She et al. 2004), as so Hematin, used as CO donor in our experiments (Lamar et al. 1996; Longo et al. 1999) (Fig. 1A). In comparison with control, CO donor Hematin at concentrations used in our experiment significantly induced stomatal closure (p<0.01 or 0.05), with an optimum biological response concentration and time at 400 µmol/L and 3 h, respectively (p<0.01). The optimum biological response concentration and time of H\textsubscript{2}O\textsubscript{2} are 100 µmol/L and 3 h, respectively (p<0.01). In our system, although H\textsubscript{2}O\textsubscript{2} used at 1000 and 10000 µmol/L also significantly induced stomata to close (Fig. 1C), it had obviously harmful role to epidermal cells and guard cells (results not shown).

In further experiments, application of gaseous CO aqueous solution with different concentrations from 0.1% to 100.0% of saturation also proved the above results. Among these treatments, the effect of CO gas aqueous solution on stomatal aperture with 100.0% saturation was the most obvious at 2 h (p<0.01, Figs. 1 B). It was well known that CO’s solubility in water at 25°C, 1 atm is 0.03 g/L, approximately equivalent to 1.0 mmol/L. Meanwhile, the half-life of CO loss from the above solutions under our experimental conditions was about 50 min. The difference of effective concentrations and time between Hematin and CO aqueous solutions might be derived from the rapid CO loss from above aqueous solution.
Meanwhile, the similar actions between gaseous CO aqueous solution and Hematin on the stomatal behavior implied that CO, nor other compounds derived from Hematin, was responsible for the stomata to close.

**Fig. 2**

**H$_2$O$_2$ is involved in CO-regulated stomatal closure**

The data from Fig. 1 shown the similar effects of CO and H$_2$O$_2$ on the stomatal behavior attract us to detect whether they have some relation in stomatal movement of *Vicia faba*. The data in Figure 2 indicate that H$_2$O$_2$ synthesis is probably required for CO-induced stomatal closure. Hematin at 400 µmol/L significantly induced stomatal to close in light for 3h (P<0.01). However, in the present of ASA and DPI the stomatal closure induced by Hematin was reversed (P<0.01). Furthermore, 400µmol/L Hematin-induced stomatal closure was blocked by co-incubation with hemoglobin (Hb), which is chosen as CO/NO scavenger (Lamar et al. 1996), at 50µmol/L (Fig. 2), indicating that CO or NO induced by CO, nor other compounds derived from Hematin, was responsible for the above effect, which is consistent with the previous results from Fig. 1A, B. The results presume that CO induces H$_2$O$_2$ synthesis, and then results in stomatal closure.

**Fig. 3**

**CO induced H$_2$O$_2$ generation in *Vicia faba* guard cells**

Having been established that CO induces stomatal closure (Fig. 1A and B) and ASA and DPI can reverse the stomatal closure induced by Hematin (Fig. 2), it attracts us to seek for further whether CO-induced stomatal closure being accompanied by
increase of H\textsubscript{2}O\textsubscript{2} levels in *Vicia faba* guard cells. Epidermal strips were loaded with H\textsubscript{2}DCF-DA, a specific probe for intracellular H\textsubscript{2}O\textsubscript{2} (Allan and Fluhr, 1997), to measure H\textsubscript{2}O\textsubscript{2} levels directly in guard cells. The nonpolar H\textsubscript{2}DCF-DA, entering the cell, is hydrolyzed to the oxidative sensitive, more polar, nonfluorescent compound fluorophore dichlorofluorescein (H\textsubscript{2}DCF). H\textsubscript{2}DCF is rapidly oxidized to the highly fluorescent DCF by intracellular H\textsubscript{2}O\textsubscript{2} (Allan and Fluhr 1997). H\textsubscript{2}DCF-DA loads readily into guard cells, and its optical properties make it amenable to analysis using laser-scanning confocal microscopy.

Our further studies showed that the apparent green fluorescence of H\textsubscript{2}O\textsubscript{2} was detected at 3 h treatment with 400 µmol/L Hematin (Fig. 3B) in comparison with the controls (Fig. 3A), and the scavenger of CO/NO Hb at 50µmol/L abolished effectively CO-induced H\textsubscript{2}DCF-DA fluorescence (Fig. 3C). Interestingly, when ASA, which is the most important reducing substrate for H\textsubscript{2}O\textsubscript{2} removal (Noctor and Foyer 1998), at 100µmol/L was further added to the buffer with Hematin for 3 h, the CO-induced H\textsubscript{2}DCF-DA fluorescence was suppressed completely (Fig. 3D). By incubating together with DPI, an inhibitor of the H\textsubscript{2}O\textsubscript{2}-generating enzyme NADPH oxidase (Cross and Jones, 1986; Lee et al. 1999), at 10µmol/L and 400µmol/L Hematin, the CO-induced H\textsubscript{2}DCF-DA fluorescence was also prevented (Fig. 3E). Therefore, we presume that *Vicia faba* guard cells generate H\textsubscript{2}O\textsubscript{2} in response to CO via the NADPH oxidase pathway.

**Fig. 4** Darkness-induced stomatal closure involved CO and H\textsubscript{2}O\textsubscript{2} in *Vicia faba*
Previous research suggested that darkness-induced stomatal closure is related to the production of endogenous \( \text{H}_2\text{O}_2 \) (Desikan et al. 2004, She et al. 2004), and the levels of \( \text{H}_2\text{O}_2 \) are higher in darkness than in light (She et al. 2004). Here, both the ASA and DPI induced stomatal opening in darkness and the effects were in dose-dependent manner (Fig. 4A, B). The effects of ASA and DPI on stomatal aperture were highly significant (\( P<0.01 \)) at 100 and 10\( \mu \text{mol/L} \), respectively. The data indicate that \( \text{H}_2\text{O}_2 \) synthesis is necessary to stomatal closure by darkness, which is the same as the previous researches (Desikan et al. 2004; She et al. 2004).

In order to investigate the possibility of endogenous CO signal being derived from HO-1 involving darkness-induced stomatal closure in *Vicia faba*, the effects of the CO/NO scavenger Hb, and ZnPPIX, which is a potent inhibitor of heme oxygenase-1 (HO-1) (Lamar et al. 1996), were assessed. As illustrated in Fig. 4, Hb and ZnPPIX, at the concentration of 50\( \mu \text{mol/L} \) and 200\( \mu \text{mol/L} \) respectively, significantly reversed darkness-induced stomatal closure (Fig. 4C, D; \( P<0.01 \)), inferring that CO mediates darkness-induced stomatal closure in *Vicia faba*. ZnPPIX inhibited CO production from an endogenous source, implying that there might be existing HO-1 enzyme activity in *Vicia faba* guard cells, which was also related to stomatal closure by darkness. In addition, the accordant effects of ASA/DPI and Hb/ZnPPIX on the stomatal closure by darkness imply that \( \text{H}_2\text{O}_2 \) and CO probably have some link in dark-induced stomatal closure.

*Fig. 5*  
CO is involved in darkness-induced \( \text{H}_2\text{O}_2 \) synthesis
The fact, both H$_2$O$_2$ and CO mediate darkness-induced stomatal closure in *Vicia faba*, attracts us to gain the relation of H$_2$O$_2$ and CO during darkness-regulated stomatal movement. To address this question, the epidermal strips were treated with the CO/NO scavenger Hb and CO specific synthetic inhibitor ZnPPIX respectively in darkness for 3 h, and then H$_2$O$_2$ levels were measured. As shown in Fig. 5A, dark could induce an intense DCF fluorescence in guard cells, which is consistent with the previous reports (Desikan et al. 2004; She et al. 2004). However, darkness-induced DCF fluorescence in guard cells was greatly prevented by Hb and ZnPPIX (Fig. 5B, C). Similarly, treatment with ASA and DPI also substantially suppressed dark-induced DCF fluorescence (Fig. 5D, E). These results provide evidence that CO is involved in darkness-induced H$_2$O$_2$ synthesis in guard cells of *Vicia faba*.

Discussion

Guard cells are unique, highly specialised autonomous cells that surround stomatal pores. They swell to open and shrink to close stomates and thereby regulate gas exchange. Stomatal closure is induced by many abiotic and biotic factors, such as osmotic stress, darkness, high CO$_2$ concentrations, decreased humidity, abscisic acid (ABA) and some mechanical stresses (Kearns and Assmann, 1993; Schroeder et al. 2001). Stomatal opening can be induced by light, low CO$_2$ levels and humidity etc (Mansfield et al. 1990). H$_2$O$_2$ has been proved being involved in stomatal movement (Lee et al. 1999; Allen et al. 2000; Pei et al. 2000; Zhang et al. 2001; Desikan et al. 2004; She et al. 2004; He et al. 2005). CO, a by-product released during the
degradation of heme by HO-1 in animals, is regarded as an important physiological messenger or bioactive molecule involved in many biological events, and playing a major role in mediating the cytoprotection against oxidant-induced lung injury (Otterbein et al. 2003). The other laboratories have also reported CO may exert its biological effect in mammals (Coceani, 2000; Ryter et al., 2002; Dulak and Józkowicz, 2003; Watts et al. 2003) and in plant (Hargrove, 2002; Dekker and Hargrove, 2002; Huang et al. 2006; Xu et al. 2006a; Xu et al. 2006b; Sa et al. 2007).

Recently, an important research shows firstly that CO involves in the signaling pathway required for ABA-induced stomatal movement in *Vicia faba* (Cao et al. 2007). However, little was still known whether CO involves in light/darkness-regulated stomatal movement, and its relationship with H2O2 in this process. In the present study, we discovered that CO donor Hematin was able to mimic the effect of H2O2 on inducing stomatal closure in a dose-dependent manner in *Vicia faba*, as did the gaseous CO aqueous solution. Additionally, CO-induced stomatal closure in *Vicia faba* was also in a time-dependent manner (Fig. 1). In animals, it is well known that CO is produced by the action of heme oxygenase-1 (HO-1) enzymes, by which heme from Hb is degraded to CO and biliverdin. HO-1 was also thought of as potential sources of CO in plant cells (Muramoto et al. 2002). In addition, previous research suggested that darkness-induced stomatal closure is related to the production of endogenous H2O2 (Desikan et al. 2004; She et al. 2004), and the levels of H2O2 in guard cell of *Vicia faba* are higher in dark than in light (She et al. 2004). The data present here showed that the scavenger of CO/NO Hb, which
had no obvious effect on stomatal aperture in light (Fig. 3), reversed stomatal closure by darkness (Fig. 4C). Interestingly, as the effect of Hb, the potent inhibitor of HO-1 ZnPPIX dramatically arrested the darkness-induced stomatal closure (Fig. 4D), without obvious effect on stomatal aperture in light (Data not shown). Hematin is the substrate of HO-1, it was inferred that there might be existing HO-1 enzyme activity in *Vicia faba* guard cells, which was also related to stomatal closure by darkness. Endogenous CO signal might be derived from HO-1 in *Vicia faba* guard cells, which is consistent with the previous research of CO inducing adventitious rooting in mung bean hypocotyls cuttings (Xu et al. 2006b). We also presume from these results that, probably like H$_2$O$_2$, the levels of CO in guard cells of *Vicia faba* is low in light, but that is high in darkness.

It is becoming increasingly evident that signalling mechanisms in plants often do not operate as isolated pathways but extensive cross-talk occurs between signal transduction pathways (Knight and Knight 2001). The cross talking of signalling responses to certain stresses may have developed in such a way and provided a mechanism: multiple, intertwined pathways are in place to ensure a contingency so that, in the event of dysfunction, a plant cell may still operate appropriately. Guard cells, a unique signal transduction research tool, provide an elegant system to dissect an intricate network of signalling pathways. Here, our results showed both CO and H$_2$O$_2$ induced stomatal closure in dose- and time-dependent manners in light (Fig. 1), H$_2$O$_2$’s scavenger and generating enzyme NADPH oxidase inhibitor ASA/DPI reversed stomatal closure induced by Hematin (Fig. 2), and both ASA/DPI and
Hb/ZnPPIX induced stomatal opening in darkness (Fig. 4). From these data we presume that there is some link between H$_2$O$_2$ and CO in light/dark-regulated stomatal movement in *Vicia faba*. Through pharmacological and surgical approaches combined with laser scanning confocal microscope (LSCM) method, we discovered that after being treated with CO for 3 h, DCF fluorescence in guard cells was significantly increasing. However, like CO/NO scavenger Hb, ASA and DPI efficaciously suppressed the DCF fluorescence by CO (Fig. 3), indicating that H$_2$O$_2$ signal system is possibly involved in the CO-regulated stomatal movement in *Vicia faba*. Additionally, like ASA and DPI, both Hb and the specific HO-1 inhibitor ZnPPIX reduced H$_2$O$_2$ levels in guard cells caused by dark (Fig. 5), implying that CO mediated darkness-induced H$_2$O$_2$ synthesis in guard cells of *Vicia faba*. Of course, cross-talk between signalling pathways is a common phenomenon, for example, there is a interrelationship between H$_2$O$_2$ and NO in light/darkness-regulated stomatal movement (She et al. 2004), and in UV-B-induced stomatal closure (He et al. 2005). So, the probability of H$_2$O$_2$ modulating CO's levels is not ruled out in our experiment.

Taken together, the results presented in this paper directly illustrate for the first time that stomatal closure induced by CO probably is mediated by H$_2$O$_2$ signalling pathway in *Vicia faba*. The data from our experiment also provide evidence that endogenous CO being from HO-1 involves in darkness-induced stomatal closure and is related to the H$_2$O$_2$ synthesis. In addition, The facts, CO/NO being involved in ABA-induced stomatal closure, and the reversibility of darkness-induced stomatal closure by Hb, implies that CO maybe also mediates NO synthesis induced by
darkness in guard cells of *Vicia faba*. The problem should been studied in the future.

Combined with the above statements, we further deduced that ABA, CO, H$_2$O$_2$ and NO signals could contribute to more integrated communication system among guard cells signaling network pathway.

**Materials and methods**

**Chemicals**

Molecular probes 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA, from Biotium, Hayward, California) was dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM stock solution. Hematin (used as CO donor), Diphenylene iodonium (DPI), Zinc protoporphyrin IX (ZnPPIX), dimethyl sulfoxide (DMSO) and 2-(N-morpholino) ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (St. Louis, MO). Unless stated otherwise, the remaining chemicals were of the highest analytical grade available from various suppliers of the Chinese companies.

DPI was also 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 µmolm$^{-2}$s$^{-1}$ PAR generated by cool white fluorescent tubes (Philips, New York, NY), and an ambient temperature 25±2°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 width and 5 mm lengths.

**Preparation of the gaseous CO aqueous solution**

The CO gas was prepared by heating concentrated sulfuric acid (H$_2$SO$_4$), when
H$_2$SO$_4$ was ratherish bubbling up, formic acid (HCOOH) was added at a speed of 3-5
s per drop according to the reaction:

$$
H_2SO_4 (L) + HCOOH (L) \rightarrow CO (g) + H_2SO_4;H_2O (L)
$$

Theoretically, this mixture could produce CO that is >95% pure. In the present study,
CO aqueous solution was further obtained by bubbling the CO gas gently through a
glass tube into 50 mL of a CO$_2$-free MES/KCl (10 mM MES/KOH, 50 mM KCl,
100µM CaCl$_2$, pH 6.15) buffer in an open bottle for least 15min, time long enough to
make the solution saturated with CO because the saturation of CO is only 23 mL/L
water at 1 atm, 20°C. The saturation solution was then diluted with MES/KCl buffer
to the concentrations required (0.1, 1.0, 10.0 and 100.0% of saturation). We further
determined the CO content in these solutions according to the method described by
Chalmers (1991) and Xu et al. (2006b). Under the present experimental conditions,
the concentrations of CO gas in 100.0% saturation CO aqueous solution was about
0.03 g/L, approximately 1.0 mmol/L. Meanwhile, the half-life of CO loss from these
solutions at 25°C was approximately 50min.

**Stomatal bioassay**
Stomatal opening and closing were monitored as the method of McAinsh et al. (1996) with slight modifications. To study the effects of CO or its gas aqueous solution on *Vicia faba* stomatal aperture, freshly prepared abaxial epidermis was incubated in CO$_2$-free MES/KCl (10 mM MES/KOH, 50 mM KCl, 100µM CaCl$_2$, pH 6.15) buffer, which included various treating reagents (Hematin or CO gas, H$_2$O$_2$), under light (300 µmol m$^{-2}$ s$^{-1}$) conditions at 25°C for 1, 2, 3 h. Final stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. To study the effects of ASA and DPI on stomatal closure caused by Hematin, isolated epidermal strips of *V. faba* were incubated at 25°C in CO$_2$-free MES/KCl alone, or containing Hematin, ASA, DPI and other compounds for 3 h under light (300 µmol m$^{-2}$ s$^{-1}$) conditions at 25°C, and then the apertures were recorded. To study the effects of CO and H$_2$O$_2$ on darkness-induced stomatal closure, strips were incubated in MES/KCl buffer alone, or containing different concentrations of Hb, ZnPPIX, ASA and DPI for 3 h in darkness conditions at 25°C. Final stomatal apertures were recorded.

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

**Dye loading of H$_2$DCF-DA**

H$_2$O$_2$ measurement was performed as the method of Allan and Fluhr (1997) with
some modifications. To study the effects of Hematin on H$_2$O$_2$ levels in guard cells in light, the epidermal strips were treated for 3 h as described for stomatal bioassay, and were immediately placed into loading Tris–KCl buffer (Tris 10mM and KCl 50mM, pH 7.2) containing 50 µM of H$_2$DCF-DA for 10 min in darkness at 25±2°C. To study the effects of CO on darkness-induced H$_2$O$_2$ generation in guard cells, strips were incubated in MES/KCl buffer alone, or containing Hb, ZnPPIX, ASA and DPI for 3 h in darkness at 25°C, and then H$_2$DCF-DA was loaded in Tris–KCl buffer.

Laser-scanning confocal microscopy

After washed off excess dye 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 and Photoshop7.0. 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 three times. The selected confocal images represented the same results from three replications.

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Fig. 1 The carbon monoxide (CO) donor Hematin, its gas and hydrogen peroxide (H₂O₂) induce stomatal closure in light. Isolated epidermal strips of *V. faba* were incubated in CO₂-free MES [2-(N-morpholino)-ethanesulfonic acid]/KCl containing different concentrations of Hematin (0, 100, 200, 300, 400 µM) (A), CO aqueous solution (0, 0.1, 1, 10, 100%) (B) and H₂O₂ (0, 10, 100, 1000, 10000 µM) (C) for 1, 2, 3 h under light (300 µmol m⁻² s⁻¹) at 25°C. Stomatal apertures were determined. Values shown are the means of 90 measurements±standard error of three independent experiments.
**Fig. 2** Stomatal closure induced by Hematin can be prevented by ASA and DPI. Isolated epidermal strips of *V. faba* were incubated at 25°C in CO₂-free MES/KCl alone, or containing 400 µM Hematin, 10 µM DPI, 100 µM ASA, 50 µM Hb, 400 µM Hematin +10 µM DPI (H+D), 400 µM Hematin +100 µM ASA (H+A), 400 µM Hematin +50 µM Hb (H+Hb) for 3 h under light (300 µmol m⁻² s⁻¹). Stomatal apertures were determined after 3 h incubation. Values are the means of 90 measurements±s.e of three independent experiments.
**Fig. 3** Hematin-induced H$_2$O$_2$ generation in guard cells of *Vicia faba*. Guard cells shown in image (A) treated with MES/KCl buffer only, (B) with MES/KCl in the present of 400 µM Hematin, (C) 400 µM Hematin and 50 µM Hb, (D) 400 µM Hematin and 100 µM ASA, (E) 400 µM Hematin and 10 µM DPI respectively in light for 3 h. Above treated epidermal strips were loaded with H$_2$DCF-DA in Tris-KCl buffer for 10 min in darkness, then excess dye removed, examined by using laser-scanning confocal microscopy. (F) shows the average fluorescent intensity of guard cells in images from (A) to (E), data are the means±s.e. The guard cells shown in image (a’) to (e’) are the representative of guard cells shown in images (A) to (E). The insets show the bright-field images corresponding to the fluorescence images (a’) through (e’). The length of scale bar in image (E) and (e’) represents 40 µm and 16 µm for image (A) to (E) and (a’) to (e’), respectively. The bar in inset of image (e’) represents 8 µm for all the insets. Each experiment was repeated at least three times, and the selected confocal image represented the same results from about nine time measurements.
Fig. 4 Effects of carbon monoxide (CO) and hydrogen peroxide (H₂O₂) inhibitors and scavengers on dark-induced stomatal closure. Isolated epidermal strips were incubated at 25°C in CO₂-free MES[2-(N-morpholino) ethanesulfonic acid]/KCl, or containing different concentrations of (A) ASA (0, 10, 100, 1000, 10000 µM) (B) DPI (0, 0.1, 1, 10, 100 µM), (C) Hb (0, 0.5, 5, 50, 500 µM), and (D) ZnPPIX (0, 100, 200, 300, 400 µM) for 3h in darkness conditions. Stomatal apertures were determined. Values are the means of 90 measurements±s.e. form three independent experiments.
**Fig. 5** CO mediates in darkness-induced H$_2$O$_2$ synthesis in *Vicia faba* guard cells. Guard cells shown in image (A) were treated with buffer only, and those in image (B) were treated with 50 µM Hb, (C) 200 µM ZnPPIX, (D) 100 µM ASA, (E) 10 µM DPI, respectively, in dark for 3 h. Above treated strips were loaded with H$_2$DCF-DA for 10 min in darkness, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (F) shows the average fluorescent intensity of guard cells in images from (A) to (E), data are the means±s.e. Other explanations are the same as in Fig. 3.