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Differential Role of Ethylene and Hydrogen Peroxide in Dark-induced Stomatal Closure

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Abstract: Regulation of stomatal aperture is crucial in terrestrial plants for controlling water loss and gaseous exchange with environment. While much is known of signaling for stomatal opening induced by blue light and the role of hormones, little is known about the regulation of stomatal closing in darkness. The present study was aimed to verify their role in stomatal regulation in darkness. Epidermal peelings from the leaves of *Commelina benghalensis* were incubated in a defined medium in darkness for 1 h followed by a 1 h incubation in different test solutions [H_2O_2, propyl gallate, ethrel (ethylene), $AgNO_3$, sodium orthovanadate, tetraethyl ammonium chloride, $CaCl_2$, $LaCl_3$, separately and in combination] before stomatal apertures were measured under the microscope. In the dark stomata remained closed under treatments with ethylene and propyl gallate but opened widely in the presence of H_2O_2 and $AgNO_3$. The opening effect was largely unaffected by supplementing the treatment with Na-vanadate (PM H^+ ATPase inhibitor) and tetraethyl ammonium chloride (K^+-channel inhibitor) except that opening was significantly inhibited by the latter in presence of H_2O_2. On the other hand, H_2O_2 could not override the closing effect of ethylene at any concentrations while a marginal opening of stomata was found when Ag NO_3 treatment was given together with propyl gallate. $CaCl_2$ treatment opened stomata in the darkness while $LaCl_3$ maintained stomata closed. A combination of $LaCl_3$ and propyl gallate strongly promoted stomatal opening. A probable action of ethylene in closing stomata of *Commelina benghalensis* in dark has been proposed.

Key words: Stomatal regulation, ethylene, H^+ ATPase, H_2O_2, Ca^{2+}

INTRODUCTION

Stomata are microscopic pores on the surface of plants which are surrounded by a pair of guard cells, thus becoming under the control of movement of such cells. These are of crucial importance for the control of water loss and gas exchange simultaneously and as such they are major regulators of global carbon and water cycles. Stomatal opening depends on the increased turgidity of guard cells which is brought about by accumulation of solutes such as K^+, Cl^-, malate^2-and sucrose (Talbott and Zeiger, 1996). Such accumulation is triggered by light via different overlapping ways. These include an accumulation of K^+ in response to hyperpolarization caused by a light activated plasma membrane located H^+-ATPase (Zeiger, 1983). Stomatal closing, on the other hand, occurs when such solutes diffuse out due to possible membrane depolarization (Schroeder *et al*., 2001) thereby leading to turgor loss. The hormone abscisic acid (ABA) has been implicated for stomatal closure by activating specific signalling cascades involving H_2O_2 and Ca^{2+} as intermediary components (Schroeder *et al*., 2001; Fan *et al*., 2004; Zhang *et al*., 2004). Ethylene has also been demonstrated to induce stomatal closure acting through H_2O_2 (Desikan *et al*., 2006) though its role is conflicting. Typically, these experiments on stomatal regulation by ABA or ethylene treatment were carried out in light to demonstrate their counteracting ability. No reports are there so far demonstrating the effect of such agents on stomata in darkness.

In our earlier study on epidermal peeling of *Commelina benghalensis* leaves we demonstrated the effect of ethylene and H_2O_2 on stomatal behaviour during

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light-induced opening (Laha *et al*., 2009). Interestingly ethylene and Ca^{2+} was found to be associated with opening while H_2O_2 induced closure. In the present investigation the role of ethylene, Ca^{2+} and H_2O_2 and their interaction during stomatal closure in darkness was verified using again the epidermal strips from leaves of *C. benghalensis*.

MATERIALS AND METHODS

Twigs of *C. benghalensis* were collected from the Departmental garden and washed with water before use. Mature leaves from the apical part of the plants were used for peeling epidermal strips from the lower surface of leaves.

Epidermal peels of about 5×10 mm were floated on a media (50 mM KCl and 10 mM MES buffer adjusted to pH 6.15 with KOH) kept in Petri dishes in darkness at a constant temperature (25±1°C). After 1 h strips were transferred to the same media now added with test compounds in specified concentrations while sets were still in dark. Stomatal peelings were treated with H_2O_2 (1 mM, unless otherwise specified), propyl gallate (0.1 mM, unless otherwise specified), ethrel (commercial preparation of ethylene, 0.1 mM, unless otherwise specified), $AgNO_3$ (1 mM, unless otherwise specified), sodium orthovanadate (1 mM), tetraethyl ammonium chloride (1 mM), $CaCl_2$ (0.1 mM), $LaCl_3$ (1 mM) or combinations of such treatments. After 1 h dark incubation strips were examined under microscope. Stomatal apertures were measured using an ocular micrometer standardized against stage micrometer and the area of the aperture was calculated from length and breadth. Data presented were average of at least ten measurements taken from different microscopic fields.

RESULTS

Figure 1 shows the photo plate of microscopic views of stomata on epidermal peelings of *C. benghalensis* leaves incubated in darkness in the specified medium added with H_2O_2 (1 mM) or $AgNO_3$ (1 mM) along with a control set (distilled water). While the stomatal aperture was totally closed under control condition (Fig. 1a), it was widely opened in case of both the treatments (Fig. 1b-c), more prominently with $AgNO_3$. Effect of propyl gallate (0.1 mM), H_2O_2 (1 mM), $AgNO_3$ (1 mM) and ethylene (ethrel, 0.1 mM) on stomatal aperture of *Commelina* species kept in darkness has been depicted as bar graph in Fig. 2. Again treatment with H_2O_2 and $AgNO_3$ opened stomata while stomata remained almost closed in propyl gallate and fully closed in ethylene.

Table 1 includes data on the effect of combined treatment of H_2O_2 (1 mM) and $AgNO_3$ (1 mM) with either Na-vanadate (1 mM) (inhibitor of plasmalemma H^+ ATPase) or tetraethyl ammonium chloride (1 mM) (a potassium channel inhibitor) along with individual treatments for comparison. Individual treatments with H_2O_2 and $AgNO_3$ opened stomata but apertures remained at control values in Na-vanadate or tetraethyl ammonium chloride. However, stomatal opening caused by $AgNO_3$ was slightly inhibited by both Na-vanadate and tetraethyl ammonium chloride. In contrast, stomatal opening induced by H_2O_2 could not be decreased by Na-vanadate but significantly inhibited by tetraethyl ammonium chloride treatment.

Table 2 describes the effects of combination of ethylene (0.05, 0.1 mM) with H_2O_2 (0.5, 1 mM) of different concentrations and combinations of $AgNO_3$ (0.1, 1 mM) with propyl gallate (0.05, 0.1 mM). Stomata remained closed in any combination of ethylene with H_2O_2. On the other hand, a marginal opening of stomata was found in case of any combination of $AgNO_3$ and propyl gallate.

Figure 3 depicts the changes in the stomatal aperture during treatment with $CaCl_2$ (0.1 mM), $LaCl_3$

Table 1: Effect of different treatments and their combinations on stomatal opening (area of aperture, μm²) of *Commelina benghalensis* incubated in darkness

Treatment	Aperture area (μm²)
Control	24.97±3.41
H_2O_2 (1 mM)	113.55±2.79
Ag^+ (1 mM)	180.40±4.65
Na-Vanadate (1 mM)	26.65±5.08
TEAC (1 mM)	16.82±3.44
Ag^+ (1 mM)+Na-Vanadate (1 mM)	153.15±2.38
Ag^+ (1 mM)+TEAC (1mM)	140.18±4.56
H_2O_2 (1 mM)+Na-Vanadate (1 mM)	123.01±2.83
H_2O_2 (1 mM)+TEAC (1 mM)	68.60±3.11

Treatments were: H_2O_2 (1 mM), $AgNO_3$ (Ag^+, 1 mM), Na-Vanadate (1 mM) and tetraethyl ammonium chloride (TEAC, 1 mM) and combinations of these as shown below. Data presented were average of ten replications (Mean±SE)

Table 2: Effect of different combination of treatments on stomatal opening (area of aperture, μm²) of *Commelina benghalensis* incubated in darkness.

Treatment	Aperture area (μm²)
Ethylene (0.1 mM)+H_2O_2 (1 mM)	16.12±3.150
Ethylene (0.1 mM)+H_2O_2 (0.5 mM)	16.83±2.750
Ethylene (0.05 mM)+H_2O_2 (1 mM)	17.00±4.190
Ag^+ (1mM)+PG (0.1 mM)	64.01±3.720
Ag^+ (1mM)+PG (0.05 mM)	40.74±4.560
Ag^+ (0.1 mM)+PG (0.1 mM)	64.01±4.710

Treatments were either combination of ethylene (0.05 mM or 0.1 mM) and H_2O_2 (0.5 mM or 1 mM) or combination of $AgNO_3$ (Ag^+, 0.1 mM or 1 mM) and propyl gallate (PG, 0.05 mM or 0.1 mM). Data presented were average of ten replications (Mean±SE)

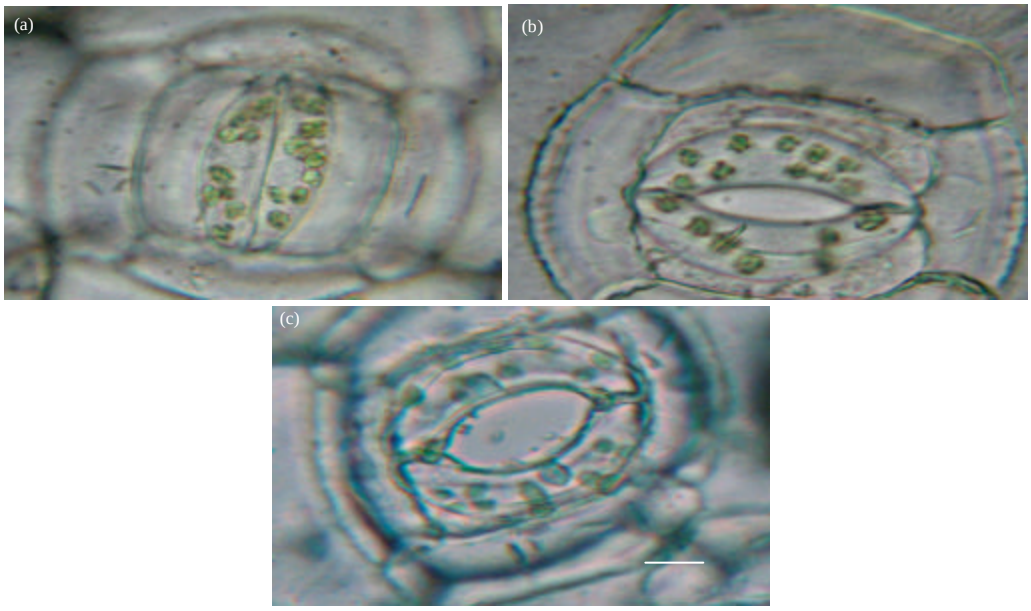

Fig. 1(a-c): Photographic plates showing microscopic view of representative stomata of *Commelina benghalensis* incubated in darkness under treatment with either (b) 1 mM H_2O_2 or (c) 1 mM $AgNO_3$ (a) Along with control distilled water. Scale bar shown in the figure measures 10 μm

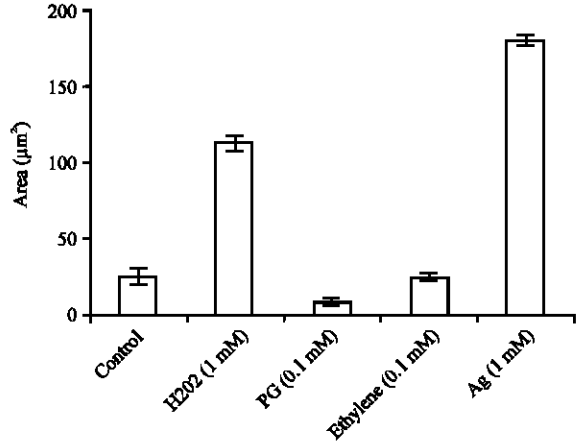

Fig. 2: Effect of treatments with 1mM H_2O_2, 0.1 mM Propyl Gallate (PG), 0.1 mM ethylene and 1 mM $AgNO_3$ along with control (distilled water) on stomatal opening (area of aperture, μm^2) of *Commelina benghalensis* incubated in darkness. SE shown as vertical bars

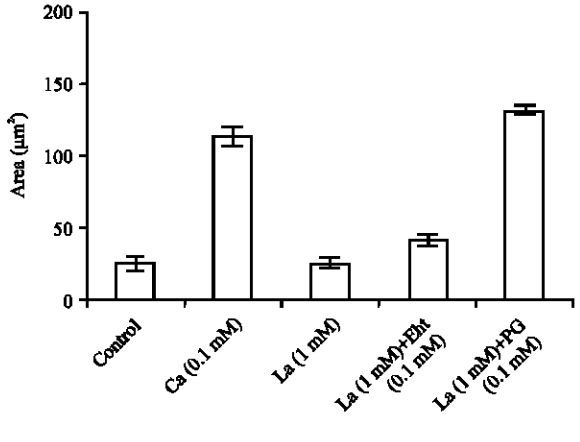

Fig. 3: Effect of treatments with $CaCl_2$ (Ca), 0.1 mM, $LaCl_3$ (La), 1 mM and combinations of $LaCl_3$ (La), 1 mM with Propyl Gallate (PG), 0.1 mM and with Ethylene (Eth), 0.1 mM on stomatal opening (area of aperture, μm^2) of *Commelina benghalensis* incubated in darkness. SE shown as vertical bars

(1 mM) (a plasma membrane located calcium channel blocker) and combinations of $LaCl_3$ with ethylene (0.1 mM) and with propyl gallate (0.1 mM) in darkness. $CaCl_2$ treatment alone caused significant opening of stomata while stomata remained unaffected by $LaCl_3$ treatment. Combining $LaCl_3$ had only a small promoting effect on opening but when combined with propyl gallate, $LaCl_3$ opened stomata widely.

DISCUSSION

Stomatal opening induced by light is known to depend on osmotic accumulation of K^+ ions and other solutes like chloride, malate anions and sugars. There is a well-defined promoting role of blue light that acts via induction of plasma membrane H^+ ATPase in facilitating K^+ uptake (Roelfsema and Hedrich, 2005). However, the regulation of such turgor development in guard cells is controversial, particularly in respect of the exact role of ethylene and H_2O_2 and Ca^{2+} as signalling component. in our earlier work on stomatal regulation in leaf epidermal peelings of *C. benghalensis* in light (Laha *et al*., 2009) we showed that though H_2O_2 treatment closed stomata, ethylene and Ca^{2+} played a positive role. In the present investigation with a similar test system we attempted to clarify the roles of ethylene and H_2O_2 in stomatal regulation in darkness. H_2O_2 was found to open stomata in darkness and treatment with propyl gallate, a potent reactive oxygen species scavenger, resulted in closed stomata (Fig. 1 and 2). This is in contrast to the finding of Desikan *et al.* (2004) who demonstrated inhibition of dark-induced stomatal closure by H_2O_2 scavenging enzyme catalase or diphenylene iodonium, an inhibitor of reactive oxygen species generating enzyme NADPH oxidase. Also, ABA has been implicated in dark-induced stomatal closure, because closure is impaired in an ABA-deficient mutant (Lee *et al*., 2006). The action of ABA occurs via signalling components such as Ca^{2+} and H_2O_2 (Pei *et al*., 2000; Kwak *et al*., 2003). H_2O_2 is reported to be generated through NADPH oxidase (Kwak *et al*., 2003) or copper amine oxidase (An *et al*., 2008). A recent finding showed that elevation of intracellular H_2O_2 induced by ABA, not constitutive increase in H_2O_2 level, functions in ABA-induced stomatal closure (Jannat *et al*., 2011). Exogenous treatment with H_2O_2 also caused stomatal closure in *Arabidopsis thaliana* and treatment with fusicoccin suppresses such closure by removing H_2O_2 (She *et al*., 2010).

In contrast to the role of ABA, involvement of ethylene in stomatal regulation is much less clearly defined and there are contradictory reports. On the one hand it is found responsible for stomatal closing (Desikan *et al*., 2006), on the other hand ethylene inhibits ABA-induced stomatal closure (Tanaka *et al*., 2005, 2006). In the former case ethylene-induced stomatal closure was seemingly mediated by H_2O_2 produced by NADPH oxidase (Desikan *et al*., 2006). In one recent report, ethylene was found to inhibit darkness-induced stomatal closure by scavenging Nitric Oxide (NO) (Song *et al*., 2011). Surprisingly, in epidermal peelings of *C. benghalensis* leaves ethylene treatment in the dark was without effect but $AgNO_3$ (1 mM), an inhibitor of ethylene perception, increased stomatal aperture markedly (Fig. 1, 2), apparently indicating a possible role of ethylene in dark-induced closing of stomata. This is in contrast to its probable role in light where treatment opened stomata in the same species (Laha *et al*., 2009).

Opening of stomata, either in darkness or light must involve osmoregulation (Talbot and Zeiger, 1998). Likely candidates involved in such regulation are plasma membrane H^+ ATPase and K^+ channels along with other transporters (Pandey *et al*., 2007). Thus possible involvement of plasma membrane H^+ ATPase and K^+ channels in case of stomata opening induced by Ag^+ and H_2O_2 in darkness in the present system was tested by combination treatments of these agents with the inhibitors, Na-orthovanadate (an inhibitor of plasma membrane H^+ ATPase) and tetraethyl ammonium chloride (an inhibitor of K^+ channels) (Table 1). It appeared that opening of stomata in darkness resulting from inhibition of ethylene action by $AgNO_3$ was not fully dependent on the action of plasma membrane H^+ ATPase and K^+ channels, as the inhibitors could marginally inhibit stomatal opening. However, H_2O_2-induced stomatal opening was only sensitive to tetraethyl ammonium chloride indicating a role of K^+ channels in such opening in darkness. In an early work on dark opening of stomata under CO_2 free air, area of stomatal aperture was correlated with the K^+ content of the guard cells (Rogers *et al*., 1980). It may be assumed that in case of H_2O_2-induced stomatal opening K^+ uptake occurred via K^+-channels, although it is not clear how H_2O_2 can drive such K^+ uptake.

Ethylene and H_2O_2 have counteracting roles in stomatal regulation instead of a collinear action at least in case of *C. benghalensis*. This was also found in case of light incubation of the epidermal peels of the same species by Laha *et al.* (2009). However, in case of dark experiments ethylene could override the opening effect of H_2O_2 (Table 2) apparently through different pathway. On the other hand, in absence of ethylene action ($AgNO_3$ treatment) reactive oxygen species scavenger (propyl gallate treatment) could not close stomata completely. This may be either due to residual amount of H_2O_2 that acted towards opening or resulted from complex interaction of signalling pathways.

Calcium is well known for its role in signalling for a number of plant processes and cytosolic Ca^{2+} plays a pivotal role in stomatal regulation (Allen *et al*., 2001). Level of cytosolic Ca^{2+} may be controlled either by the entry of apoplastic Ca^{2+} through plasma membrane located Ca^{2+} channels or release from endosomal stores. Increased cytosolic Ca^{2+} level, as downstream effect, may have

impact on anion channels and plasma membrane H^+ ATPase. As such Ca^{2+} has been reported to be involved in stomatal closing as an intermediate in the cascade induced by ABA or H_2O_2. On the contrary, our earlier finding in *C. benghalensis* leaves was that Ca^{2+} is involved in stomatal opening in light as La^{3+} (inhibits the entry of apoplastic Ca^{2+} in the cytosol) inhibited opening and also Ca^{2+} treatment prevented H_2O_2-or Ag^+-induced stomatal closing (Laha *et al*., 2009). In the present investigation with the same species, Ca^{2+} treatment was again found to trigger stomatal opening even in darkness. Although the sensitivity of stomata to extracellular Ca^{2+} may vary (Roelfsema and Hedrich, 2005), no report is there so far about the opening effect of Ca^{2+} except one report on *C. benghalensis* leaves showing Ca^{2+} requirement for blue light-induced stomatal opening (Parvathi and Raghavendra, 1997) and our earlier report (Laha *et al*., 2009). $LaCl_3$-treatment showed no effect when given individually or marginal opening when supplemented with ethylene. Ethylene-induced stomatal opening in light in *C. benghalensis* was explained by possible release of endosomal Ca^{2+} release (Laha *et al*., 2009), but in darkness ethylene is associated with stomatal closing through some different mechanism. Interestingly, a combined treatment of $LaCl_3$ with propyl gallate resulted in wide opening of stomata, which was also observed in light in the same species (Laha *et al*., 2009), but in the latter case individual treatment with propyl gallate caused stomatal opening. It is suggested that a complex interplay of Ca^{2+} influx from apoplast and Ca^{2+} release from endosomal stores through coordinated regulation of respective channels or transporters maintaining Ca^{2+} homeostasis (Roelfsema and Hedrich, 2005) and accordingly stomatal aperture is regulated by a defined window of guard cell cytosolic Ca^{2+} oscillation parameters (Allen *et al*., 2001).

Apparently ethylene operates in darkness to maintain the stomatal closure and probably it acts through maintaining a critical cytosolic Ca^{2+} balance by controlling Ca^{2+} influx from apoplast and Ca^{2+} release from endosomal stores. H_2O_2 induces stomatal opening in darkness probably by controlling K^+ channel activity which may lie downstream of Ca^{2+} signalling. Further in-depth research and analysis may provide support to this contention, which is apparently in contrast to other findings.

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