H$_2$O$_2$, Ca$^{2+}$, and K$^+$ in subsidiary cells of maize leaves are involved in regulatory signaling of stomatal movement

**CURRENT STATUS:** POSTED

Li Zhang  
State Key Laboratory of Soil and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources

Yaqin Yao  
College of Life Sciences, Northwest A&F University

Suiqi Zhang  
University of the Chinese Academy of Sciences  

Corresponding Author  

sqzhang@ms.iswc.ac.cn

**DOI:**  
10.21203/rs.2.15366/v1

**SUBJECT AREAS**  
Plant Physiology and Morphology

**KEYWORDS**  
subsidiary cells, stomatal movement, signaling substance, inhibitor
Abstract

Background: The stomata of maize (Zea mays) contain a pair of guard cells and a pair of subsidiary cells. To determine whether H$_2$O$_2$, Ca$^{2+}$, and K$^+$ in subsidiary cells were involved in stomatal movement, we treated four-week-old maize (Zhengdan 958) leaves with H$_2$O$_2$, diphenylene iodonium (DPI), CaCl$_2$, and LaCl$_3$. Changes in content and distribution of H$_2$O$_2$, Ca$^{2+}$, and K$^+$ during stomatal movement were observed.

Results: When exogenous H$_2$O$_2$ was applied, Ca$^{2+}$ increased and K$^+$ decreased in guard cells, while both ions increased in subsidiary cells, leading to stomatal closure. After DPI treatment, Ca$^{2+}$ decreased and K$^+$ increased in guard cells, but both Ca$^{2+}$ and K$^+$ decreased in subsidiary cells, resulting in open stomata. Exogenous CaCl$_2$ increased H$_2$O$_2$ and reduced K$^+$ in guard cells, while significantly increasing them in subsidiary cells and causing stomatal closure. After LaCl$_3$ treatment, decreased and K$^+$ increased in guard cells, whereas both H$_2$O$_2$ and K$^+$ decreased in subsidiary cells and stomata became open.

Conclusions: These results indicated that H$_2$O$_2$ and Ca$^{2+}$ were correlated positively with each other and with K$^+$ in subsidiary cells during stomatal movement. Both H$_2$O$_2$ and Ca$^{2+}$ in subsidiary cells promote an inflow of K$^+$, indirectly regulating stomatal closure.

Background

Stomata control the outward transport of water molecules and the gaseous exchange between plants and the external environment during photosynthesis and respiration [1, 2]. In the stomata of maize (Zea mays), a pair of guard cells surrounds a pair of subsidiary cells, which are thought to be donors and receptors of large amounts of water and ions [3] and assist in stomatal movement [4]. When guard cells perceive changes in the environment, they immediately produce signal molecules that regulate stomatal switches and allow the plants to adapt [5]. Besides external factors [6, 7, 8], these switches are controlled also by intracellular signaling substances and ions [2, 9]. Due to the absence of functional plasmodesmata in mature guard cells, the change in ion content between guard cells and subsidiary cells requires ion transport through channels and transporters [10].

Hydrogen peroxide accumulation is involved in the regulation of stomatal movement [11]. Treatment
with exogenous H₂O₂ induced stomatal closure in bean epidermis [12] and in rice plants [13]. Stomatal closure resulted also from the accumulation of H₂O₂ in subsidiary cells of maize leaves epidermis following a drought period [14]. Inhibition and scavenging of H₂O₂ have been studied using the NADPH oxidase inhibitor diphenylene iodonium (DPI) [15–17]. Calcium ions have also been found to regulate stomatal movement [18], with Ca²⁺ content in guard cells increasing following application of exogenous H₂O₂, and leading to stomatal closure [19]. Ca²⁺ channel inhibitors can change the stomatal closure induced by H₂O₂ [15]. K⁺ accumulates in guard cells following entry through its channel, resulting in a lower osmotic potential, water absorption, cell expansion, and finally stomatal opening [20]. On the contrary, when K⁺ is transported out of guard cells, the osmotic potential in guard cells increases and stomatal aperture decreases [21]. Subsidiary cells are storage cells for K⁺ and Cl⁻ ions [22]. During stomatal opening, depolarization in subsidiary cells depends on inactivation of the outward rectifying potassium channel protein to release K⁺, whereas depolarization in guard cells depends on the activation of the outward rectifying potassium channel protein to let K⁺ in [23]. Hence, stomatal aperture changes may vary with the changes in H₂O₂, K⁺, and Ca²⁺ content in guard and subsidiary cells.

Whereas the interaction between H₂O₂, Ca²⁺, and K⁺ in guard cells during stomatal movement is well known, it remains unclear what their mutual relationship and role is in subsidiary cells. Some reports of H₂O₂ and K⁺ in subsidiary cells [14, 24] have prompted the present effort to determine how H₂O₂, Ca²⁺, and K⁺ in these cells affected stomatal opening and closing, and how they regulated each other. To this end, this study applied exogenous H₂O₂, Ca²⁺, and their inhibitors, and compared their content in subsidiary and guard cells. The results provide a new theoretical basis explaining the mechanism of stomatal movement in grasses.

Results

*Effect of exogenous H₂O₂, Ca²⁺, and their inhibitors on stomatal aperture*
To verify that \( \text{H}_2\text{O}_2 \), \( \text{K}^+ \), and \( \text{Ca}^{2+} \) in subsidiary cells were involved in stomatal movement, the lower epidermis of maize leaves was treated with exogenous \( \text{H}_2\text{O}_2 \), \( \text{Ca}^{2+} \), and their inhibitors prior to scanning electron microscopy observation. Results showed that the treatments promoted or inhibited stomatal opening to varying degrees (Fig. 1). Both \( \text{H}_2\text{O}_2 \) and \( \text{Ca}^{2+} \) inhibited stomatal opening. In particular, exogenous \( \text{H}_2\text{O}_2 \) resulted in stomatal aperture reaching only 8% of that observed under light conditions, whereas exogenous \( \text{Ca}^{2+} \) reduced stomatal opening by 42% compared with the light control (Fig. 2). DPI and \( \text{LaCl}_3 \) inhibited stomatal closure by 72% and 26%, respectively, when switching from light to dark (Fig. 2). These results showed that \( \text{H}_2\text{O}_2 \), \( \text{Ca}^{2+} \), and their inhibitors could regulate stomatal opening and closing.

*Effect of exogenous \( \text{H}_2\text{O}_2 \), \( \text{Ca}^{2+} \), and their inhibitors on \( \text{H}_2\text{O}_2 \) distribution in guard and subsidiary cells during stomatal movement*

Under light conditions (i.e., when stomata were open), \( \text{H}_2\text{O}_2 \) was low and distributed at both ends of guard cells, but it was negligible in subsidiary cells (Fig. 3a). Under dark conditions (i.e., when stomata were closed), \( \text{H}_2\text{O}_2 \) content augmented in both guard and subsidiary cells, with the latter exhibiting a significantly higher increase (Fig. 3b). A low amount of exogenously added \( \text{H}_2\text{O}_2 \) under light conditions significantly increased \( \text{H}_2\text{O}_2 \) in guard and subsidiary cells (Fig. 3c), mimicking the control situation in the dark (Fig. 3b) and promoting stomatal closure (Fig. 1c). DPI was added after 3 h of light and samples were transferred to the dark for 3 h, which significantly reduced \( \text{H}_2\text{O}_2 \) content in guard and subsidiary cells (Fig. 3d). Guard cells treated with exogenous \( \text{H}_2\text{O}_2 \) under light conditions exhibited 78.5% higher level of \( \text{H}_2\text{O}_2 \) than light control (Fig. 4a). In samples exposed to 3 h of light and then treated with DPI for 3 h in the dark, \( \text{H}_2\text{O}_2 \) amounted to only 18.8% of that in dark control, and 39% of that in guard cells of light control (Fig. 4a). Subsidiary cells treated with exogenous \( \text{H}_2\text{O}_2 \) under light conditions displayed a 86.7% level of \( \text{H}_2\text{O}_2 \) in dark control, but only 10.4% of the latter when treated with DPI (Fig. 4b). The difference between treatment and control was significant,
indicating that DPI effectively inhibited the production of $\text{H}_2\text{O}_2$.

$\text{H}_2\text{O}_2$ content in guard cells was more than 1.7 times as high following $\text{CaCl}_2$ treatment than in light control (Fig. 3e, Fig. 4a). Seemingly, $\text{H}_2\text{O}_2$ content in subsidiary cells increased with the addition of $\text{CaCl}_2$ (Fig. 4b). This finding indicated a positive correlation between $\text{Ca}^{2+}$ and $\text{H}_2\text{O}_2$ in both subsidiary and guard cells. To further test this relationship in stomatal cells, $\text{LaCl}_3$ was added for 3 h under dark conditions after 3 h in the light. Results showed a significant reduction in $\text{H}_2\text{O}_2$ content in guard cells (50.9%) and subsidiary cells (72.8%) compared with dark control (Fig. 3f, Fig. 4). This finding confirmed that $\text{H}_2\text{O}_2$ in subsidiary cells correlated positively with changes in $\text{Ca}^{2+}$.

**Effect of exogenous $\text{H}_2\text{O}_2$, $\text{Ca}^{2+}$ and their inhibitors on $\text{K}^+$ distribution in guard and subsidiary cells during stomatal movement**

The amount of $\text{K}^+$ in guard cells regulates cell turgor, which is very important for stomatal opening and closing. As a $\text{K}^+$ reservoir, subsidiary cells play an important role in stomatal movement. Under light conditions, $\text{K}^+$ could be found mainly in guard cells (Fig. 5a), which absorbed water and expanded to open stomata (Fig. 2). Under dark conditions, $\text{K}^+$ flowed out from guard cells and into subsidiary cells (Fig. 5b), which caused the former to lose water and stomata to close (Fig. 2). Under light conditions, $\text{K}^+$ content in subsidiary cells was only 26.8% of that in guard cells, whereas in guard cells it was only 19.5% of that in subsidiary cells under dark conditions (Fig. 6). When the epidermis of maize leaves epidermis was treated with $\text{H}_2\text{O}_2$ under light conditions, $\text{K}^+$ was distributed mainly in subsidiary cells and less in guard cells (Fig. 5c); with the former accounting for 1.7 times as much $\text{K}^+$ as the latter. However, upon DPI treatment for 3 h in the dark following 3 h in the light, $\text{K}^+$ was found mainly in guard cells and less in subsidiary cells (Fig. 5d); with the latter accounting for only 36.2% as
much K⁺ as the former (Fig. 6). Even in the presence of light, K⁺ content in guard cells decreased with addition of exogenous H₂O₂, while it increased in subsidiary cells, reflecting the situation under dark conditions (Fig. 5b, c). H₂O₂ inhibition following DPI addition was similar under light settings (Fig. 5a, d). Therefore, H₂O₂ content correlated negatively with K⁺ in guard cells, but positively in subsidiary cells.

K⁺ content in guard cells treated with CaCl₂ under light conditions was 71% lower compared to light control, whereas in subsidiary cells it increased by 4.3 times over the control (Fig. 5e, Fig. 6). In addition, when samples were treated with LaCl₃ for 3 h in the dark after 3 h in the light, K⁺ was detected mainly in guard cells, and less in subsidiary cells (Fig. 5f), where it was only half as much as in the former (Fig. 6). These results indicated that Ca²⁺ content correlated positively with K⁺ content in subsidiary cells, but negatively in guard cells.

Effect of exogenous H₂O₂, Ca²⁺ and their inhibitors on Ca²⁺ distribution in guard and subsidiary cells during stomatal movement

Ca²⁺ content in guard cells was 52.4% higher in the dark than under light conditions (Fig. 7a, b, Fig. 8). The change in Ca²⁺ content was similar to that observed for H₂O₂ in subsidiary cells, where it was almost undetectable in the light (Fig. 7a), but increased significantly in the dark (Fig. 7b). When exogenous Ca²⁺ was applied under light conditions, Ca²⁺ content increased significantly in both cell types (Fig. 7c), becoming about twice as high as in the dark control (Fig. 8). Incubation with LaCl₃ for 3 h in the dark after 3 h in the light resulted in only a small amount of Ca²⁺ was being left in guard cells and almost none in subsidiary cells, which was similar to the light control (Fig. 7d). The difference in fluorescence intensity was only 4% following LaCl₃ treatment (Fig. 8), which indicated that exogenous Ca²⁺ and LaCl₃ augmented and reduced, respectively, Ca²⁺ content in stomata.

H₂O₂ treatment under light conditions promoted a significant increase in Ca²⁺ content in both cell
types compared with light controls (Fig. 7e, Fig. 8). The amount of Ca\textsuperscript{2+} in guard cells treated with DPI for 3 h in the dark after 3 h in the light was essentially analogous to that under light control conditions (Fig. 7a, f) with only a 1.9% difference in fluorescence intensity between them (Fig. 8a). In contrast, in subsidiary cells there was no difference at all compared to the light control (Fig. 7a, f, Fig. 8b). These findings indicated that DPI had an inhibitory effect on the increase of Ca\textsuperscript{2+} in both guard and subsidiary cells. Taken together with the changes in H\textsubscript{2}O\textsubscript{2}, the results were point to a positive correlation between Ca\textsuperscript{2+} and H\textsubscript{2}O\textsubscript{2}.

Discussion

Changes in H\textsubscript{2}O\textsubscript{2} content in guard and subsidiary cells

H\textsubscript{2}O\textsubscript{2} has a dual function in plants, acting both as a signaling substance to regulate growth and development when its level is low [25], or as an inhibitor when its concentration is high [26]. Figure 3 shows that external factors could increase H\textsubscript{2}O\textsubscript{2} content in plants [27], whereas DPI could inhibit the production of H\textsubscript{2}O\textsubscript{2} [16, 17]. In the latter case, abscisic acid-induced stomatal closure is inhibited [28]. H\textsubscript{2}O\textsubscript{2} content in guard cells increased compared with light control after treatment with H\textsubscript{2}O\textsubscript{2}, and decreased compared with dark control after treatment with DPI (Fig. 4a). In contrast, H\textsubscript{2}O\textsubscript{2} content in subsidiary cells upon H\textsubscript{2}O\textsubscript{2} treatment was higher than in dark control, but only 16.5% of dark control treated with DPI (Fig. 4b). This indicated that exogenous H\textsubscript{2}O\textsubscript{2} or DPI could effectively promote or reduce H\textsubscript{2}O\textsubscript{2} content in stomatal cells [12, 17]. At the same time, it also indicated that during stomatal switching, H\textsubscript{2}O\textsubscript{2} in guard and subsidiary cells arose mainly from the action of NADPH oxidase [16, 29].

Generally, variations in H\textsubscript{2}O\textsubscript{2} content in guard cells are believed to affect the intracellular concentration of K\textsuperscript{+} and thus regulate stomatal switching [29, 30]. Ca\textsuperscript{2+} is an important link in the regulation of K\textsuperscript{+} by H\textsubscript{2}O\textsubscript{2} in guard cells [31]. External environmental factors affect H\textsubscript{2}O\textsubscript{2} content in
guard cells, which impacts on the activity of intracellular Ca\textsuperscript{2+} channels [19] to produce a signal transmission system, and cause inward or outward flow of K\textsuperscript{+} to regulate stomatal movement [28]. According to the present results and previous studies, Ca\textsuperscript{2+} seems to promote the production of H\textsubscript{2}O\textsubscript{2} in guard and subsidiary cells. Thus, even in the presence of light, exogenous Ca\textsuperscript{2+} treatment augments H\textsubscript{2}O\textsubscript{2} content in guard cells by up to 212.3% of that in the light control, while also increasing significantly that in subsidiary cells (Fig. 3e, Fig. 4). When going form light to dark conditions, the content of H\textsubscript{2}O\textsubscript{2} in guard and subsidiary cells was significantly reduced by LaCl\textsubscript{3} compared with the dark control (Fig. 3f). Importantly, it also indicated that Ca\textsuperscript{2+} in guard and subsidiary cells promoted the production of H\textsubscript{2}O\textsubscript{2}, confirming a positive correlation between these two signaling molecules [32].

**Changes in K\textsuperscript{+} content in guard and subsidiary cells**

The flow of K\textsuperscript{+} in guard cells occurs in the opposite direction to that in subsidiary cells [33]. For example, when stomata are closed, K\textsuperscript{+} flows out from guard cells and into subsidiary cells (Fig. 5b). Ca\textsuperscript{2+} and H\textsuperscript{+} control K\textsuperscript{+} channels to regulate stomatal movement [31]. The mutual regulatory relationship between K\textsuperscript{+} and H\textsubscript{2}O\textsubscript{2} was further confirmed via H\textsubscript{2}O\textsubscript{2} and DPI treatments. Specifically, treatment with H\textsubscript{2}O\textsubscript{2} under light conditions and DPI under light-to-dark transition, led to K\textsuperscript{+} becoming concentrated in subsidiary cells and guard cells, respectively (Fig. 5c, d). This indicated that H\textsubscript{2}O\textsubscript{2} activates K\textsuperscript{+} outflow in guard cells [30] while promotes its inflow in subsidiary cells. When Ca\textsuperscript{2+} content in guard cells increases to a micromolar concentration, it significantly reduces the activity of inward K\textsuperscript{+} channels but increases the activity of outward K\textsuperscript{+} channels [31, 34]. The calcium-dependent protein kinase GORK is an outward-rectifying K\textsuperscript{+}-channel, playing an important role in stomatal closure [35]. In the presence of light, K\textsuperscript{+} content in guard cells was reduced whereas
that in subsidiary cells was increased following treatment of maize leaf epidermis with \( \text{CaCl}_2 \) (Fig. 5e).

This suggested that \( \text{Ca}^{2+} \) promoted \( \text{K}^+ \) release in guard cells and absorption in subsidiary cells. \( \text{LaCl}_3 \) addition for 3 h in the dark after 3 h in the light resulted in more \( \text{K}^+ \) in guard cells and less in subsidiary cells (Fig. 5f), indicating that \( \text{Ca}^{2+} \) promotes \( \text{K}^+ \) influx in subsidiary cells. Increased \( \text{Ca}^{2+} \) concentration in the cytoplasm activates the plasma membrane binding anion channel, resulting in membrane depolarization and inhibition of the inward \( \text{K}^+ \) channel [31]. Guard cells plasma membrane depolarized to close stomata, whereas transient hyperpolarization occurs in subsidiary [3]. Hence, the increase in \( \text{Ca}^{2+} \) concentration in subsidiary cells may result in hyperpolarization of the plasma membrane and activation of the introverted \( \text{K}^+ \) channel.

## Change in \( \text{Ca}^{2+} \) content in guard and subsidiary cells

The activation of hyperpolarization-dependent \( \text{Ca}^{2+} \) channels on the membrane of guard cells under dark conditions promoted an increase in intracellular \( \text{Ca}^{2+} \) [36]. Figure 7a, b reveal that the changes in \( \text{Ca}^{2+} \) content in guard cells are consistent with the above results. However, no one had previously studied changes in \( \text{Ca}^{2+} \) content in subsidiary cells of maize leaf epidermis stomata under dark and light conditions. In this experiment, \( \text{Ca}^{2+} \) content in subsidiary cells also increased significantly with the closure of stomata. It has been reported that during maize stomatal closure, hyperpolarization and cytoplasmic acidification of subsidiary cells differed from those of guard cells or were actually opposite to them [3]. Changes in \( \text{H}_2\text{O}_2 \) and \( \text{Ca}^{2+} \) content in subsidiary cells were akin to those in guard cells, but plasma membrane polarization and cytoplasmic acidification were the opposite, indicating that the two signaling molecules employed different transmission modes between subsidiary and guard cells.

\( \text{H}_2\text{O}_2 \), have an important effect on the activation of \( \text{Ca}^{2+} \) channels in cytoplasmic membrane [19].
After addition of H$_2$O$_2$ and DPI, Ca$^{2+}$ content in guard and subsidiary cells increased and decreased in a corresponding manner (Fig. 7e, f). Indeed, patch-clamp experiments had shown that Ca$^{2+}$ channels were sensitive to H$_2$O$_2$, confirming the hypothesis that reactive oxygen species and Ca$^{2+}$ acted synergistically [37]. This finding indicates that H$_2$O$_2$ can promote an increase of Ca$^{2+}$ in both guard and subsidiary cells.

Conclusions

The present study shows that the interaction between H$_2$O$_2$, Ca$^{2+}$, and K$^+$ in guard cells of maize during stomatal movement is consistent with existing reports. As shown in Figure 9, the interaction in subsidiary cells occurs as follows: H$_2$O$_2$ promotes an increase in Ca$^{2+}$ and the inflow of K$^+$; and Ca$^{2+}$ promotes the production of H$_2$O$_2$ and the inflow of K$^+$. Contrary to guard cells, H$_2$O$_2$ and Ca$^{2+}$ in subsidiary cells promote K$^+$ influx and indirectly regulate stomatal closure. Hence, subsidiary cells control the turgor of guard cells by establishing ion concentrations in the cytoplasm. This provides a further understanding of the movement rule and mechanism of stomata under different ecological environment conditions. It is expected to optimize and control stomatal movement, which is of great significance for inducing plant resistance to adverse effects.

Methods

Plant material and growth conditions

Experiment were carried out at Northwest A&F University, Yangling, Shaanxi Province, China (108°4′28″ E, 34°16′56″N, and 500 m a.s.l) using potted plants grown in a chamber. Topsoil from a field was collected, ground, and mixed 2:1 with vermiculite for later use. The original source of Zhengdan 958 is female parent: Zheng 58 × male parent: Chang 7–2, which is approved by China country with deposition number 20000009. Seeds of similar size and plumpness were subjected to accelerated germination at room temperature for 2–3 days after medical alcohol disinfection. Four seedlings with consistent growth were selected and planted in pots of 25 cm in diameter and 16 cm in height. Once the trifoliate stage was reached, two seedlings with vigorous growth were reserved and allowed to grow to the six-leaf stage for subsequent experiments.
Cytochemical localization of H$_2$O$_2$, K$^+$, and Ca$^{2+}$ in the epidermis of maize leaves

Cytochemical localization of H$_2$O$_2$

Cytochemical localization of H$_2$O$_2$ was performed as described by Zhang et al. [11] with some modifications. Freshly torn lower epidermis of fully unfolded maize leaves at the six-leaf stage was suspended in MES-KCl buffer (pH 6.15, 10 mM MES, 50 mM KCl, 100 µM CaCl$_2$). The epidermis was incubated for 15 min in the dark at 25 °C with 1 mL MES-KCl (pH 7.2) solution containing 1 µl of 50 mM 2′,7′-dichloro hydrogenated fluorescein diacetate ester fluorescent dye and then washed using MES-KCl buffer. A fluorescence microscope (OlympusBX51, Olympus Corporation, Japan) was used to observe and photograph the samples; H$_2$O$_2$ in guard and subsidiary cells was analyzed using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Cytochemical localization of K$^+$

The K$^+$ staining methods proposed by Raschke and Fellows [22] and [38] were combined and improved by replacing Na$_3$Co(NO$_2$)$_6$ with NaPbCo(NO$_2$)$_6$ [39]. The epidermis was incubated in MES-KCl buffer, rinsed with 20 mM CaCl$_2$, immediately transferred to 15% NaPbCo(NO$_2$)$_6$ fixing solution acidified with acetic acid, for incubated 15 min, rinsed three times for 15 min with 50% alcohol, stained with 5% (NH$_4$)$_2$S color solution for 1 min, rinsed with distilled water, and finally observed and photographed under a fluorescence microscope. The black-yellow crystals were CoS formed by the action of reaction between K-NaPbCo(NO$_2$)$_6$ and (NH$_4$)$_2$S. The above steps were carried out at 4 °C or on ice with a pre-cooled reagent.

Cytochemical localization of Ca$^{2+}$

Cytochemical localization of Ca$^{2+}$ was performed as described by Qu et al. [40]. The epidermis was transferred to MES-KCl buffer containing 4 µM Fluo-3 AM (Sigma), incubated in the dark for 2 h at 4°C to prevent esterase hydrolysis in cell walls, incubated in the dark for 2 h at 25 °C to allow the fluorescent dye to fully mark intracellular Ca$^{2+}$ following esterase hydrolysis, and washed with MES-
KCl buffer. A fluorescence microscope and Image-Pro Plus software were used to observe and analyze the content and distribution of Ca\(^{2+}\) in guard and subsidiary cells.

**Pharmacological treatment of epidermis of maize leaves**

For H\(_2\)O\(_2\) and DPI treatment, epidermis exposed to light for 3 h and then treated with 10 μM H\(_2\)O\(_2\) [41] for 3 h under light or exposed to light for 3 h and then treated with 10 μM DPI [42] for 3 h under darkness. For CaCl\(_2\) and LaCl\(_3\) treatment, epidermis exposed to light for 3 h and then treated with 10 mM CaCl\(_2\) [43] for 3 h under light or exposed to light for 3 h and then treated with 5 mM LaCl\(_3\) [44] for 3 h under darkness. In all cases, changes in H\(_2\)O\(_2\), K\(^+\), and Ca\(^{2+}\) content and distribution in guard and subsidiary cells were observed as described in the previous sections. The transitions from light to dark involved in the above treatment are carried out at room temperature in a black paper sleeve or exposed to fluorescent lamp.

### 2.3 Determination of stomatal aperture in maize leaf epidermis

Stomatal aperture was determined according to Yao et al. [14]. Maize leaves at the six-leaf stage were cut into pieces of 5 mm\(^2\) and rapidly fixed with 4% (v/v) glutaraldehyde solution. The pieces were completely submerged by vacuum and stored at 4 °C overnight. They were then rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.2), dehydrated in an alcohol gradient, ethanol was replaced by isoamyl acetate, dried at the CO\(_2\) critical point of CO\(_2\), gold-plated, and finally observed and recorded under a scanning electron microscope (Hitachi S–3400N, Hitachi Koki Co., Ltd., Japan). Image-Pro Plus software was used to analyze and quantify fluorescence intensity of guard and subsidiary cells, as well as stomatal aperture.

**Declarations**

**Abbreviations**

Diphenylene iodonium: DPI

**Acknowledgements**

Not applicable.
Authors’ contributions
SQZ and YQY designed the conception and the study. LZ designed the pot experiments, made the electron microscope samples, stained epidermis of maize leaves after pharmacological treatment, observed the stomatal and anatomy structure, and analyzed data. LZ and SQZ wrote the manuscript, and all authors proof read the manuscript.

Funding
This work was supported by the National Science and Technology Supporting Programs (2015BAD22B01), the 111 Project of the Chinese Education Ministry (B12007). The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
All data generated or analyzed during this study are included in this manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1 State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Yangling, Shaanxi 712100, China;
2 University of Chinese Academy of Sciences, Beijing 100049, China;
3 State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Northwest A&F University, Yangling Shaanxi 712100, China;
4 College of Life Sciences, Northwest A&F University, Yangling Shaanxi 712100, China

References
1. Cardoso AA, Randall JM, McAdam SAM. Hydraulics regulates stomatal responses to changes in leaf water status in Athyrium filix-femina. Plant Physiol. 2018;179:533-43.

2. Granot D, Kelly G. Evolution of guard-cell theories: The story of sugars. Trends Plant Sci. 2019;1788:1-12.

3. Mumm P, Wolf T, Fromm J, Roelfsema MRG, Marten I. Cell type-specific regulation of ion channels within the maize stomatal complex. Plant Cell Physiol. 2011;52:1365-75.

4. Higaki T, Hashimoto-Sugimoto M, Akita K, Iba K, Hasezawa S. Dynamics and environmental responses of PATROL1 in arabidopsis subsidiary cells. Plant Cell Physiol. 2014;55:773-80.

5. Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, et al. CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature. 2008;452:483-6.

6. Kim TH, Böhmer M, Hu H, Nishimura N, Schroeder JI. Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. Annu Rev Plant Biol. 2010;61:561-91.

7. Zhang W, Jeon BW, Assmann SM. Heterotrimeric G-protein regulation of ROS signalling and calcium currents in arabidopsis guard cells. J Exp Bot. 2011;62:2371-9.

8. Murata Y, Mori IC, Munemasa S. Diverse stomatal signaling and the signal integration mechanism. Annu Rev Plant Biol. 2015;66:369-92.

9. Chen YL, Huang RF, Xiao YM, Lu P, Chen J, Wang XC. Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and H2O2. Plant Physiol. 2004;136:4096-103.

10. Wille AC, Lucas WJ. Ultrastructural and histochemical studies on guard cells. Planta. 1984;160:129-42.

11. Zhang H, Pan CZ, Gu SH, Ma QM, Zhang YQ, Li X, et al. Stomatal movements are
involved in elevated CO2-mitigated high temperature stress in tomato. Physiol Plantarum. 2018;165:569-83. 12 Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP. Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in Vicia faba, Plant Physiol. 2001;126:1438-48.

12. Liu JP, Zhang CP, Wei CP, Liu X, Wang MG, Yu FF, et al. The RING finger ubiquitin E3 ligase OsHTAS enhances heat tolerance by promoting H2O2-induced stomatal closure in rice. Plant Physiol. 2016;170:429-43.

13. Yao YQ, Liu XP, Li ZZ, Ma XF, Rennenberg H, Wang X, et al. Drought-induced H2O2 accumulation in subsidiary cells is involved in regulatory signaling of stomatal closure in maize leaves. Planta. 2013;238:217-27.

14. Suhita D, Raghavendra AS, Kwak JM, Vavasseur A. Cytoplasmic alkalization precedes reactive oxygen species production during Methyl Jasmonate- and abscisic acid-induced stomatal closure. Plant Physiol. 2004;134:1536-45.

15. Singh R, Parihar P, Singh S, Mishra RK, Singh VP, Prasad SM. Reactive oxygen species signaling and stomatal movement: current updates and future perspectives. Redox Biol. 2017;11:213-8.

16. Watkins JM, Chapman JM, Muday GK. Abscisic acid-induced reactive oxygen species are modulated by flavonols to control stomata aperture. Plant Physiol. 2017;175:1807-25.

17. Minguet-Parramona C, Wang Y, Hills A, Vialet-Chabrand S, Griffiths H, Rogers S, et al. An optimal frequency in Ca2+ oscillations for stomatal closure is an emergent property of ion transport in guard cells. Plant Physiol. 2015;170:33-42.

18. Wang Y, Chen ZH, Zhang B, Hills A, Blatt MR. PYR/PYL/RCAR abscisic acid receptors regulate K+ and Cl- channels through reactive oxygen species-mediated activation of Ca2+ channels at the plasma membrane of intact Arabidopsis guard cells. Plant
19. Ward JM, Mäser P, Schroeder JL. Plant ion channels: gene families, physiology, and functional genomics analyses. Annu Rev Physiol. 2009;71:59-82.

20. Isner JC, Begum A, Nuehse T, Hetherington AM, Maathuis FJM. KIN7 kinase regulates the vacuolar TPK1 K+ channel during stomatal closure. Curr Biol. 2018;28:1-7.

21. Raschke K, Fellows MP. Stomatal movement in Zea mays: shuttle of potassium and chloride between guard cells and subsidiary cells. Planta. 1971;101:296-316.

22. Kai B, Marten I, Becker D, Philippar K, Ache P, Hedrich R. Differential expression of K+ channels between guard cells and subsidiary cells within the maize stomatal complex. Planta (Berlin). 2005;222:968-76.

23. Wolf T, Heidelmann T, Marten I. ABA regulation of K(+) -permeable channels in maize subsidiary cells. Plant & Cell Physiology. 2006;47:1372-80.

24. Singh R, Singh S, Parihar P, Mishra RK, Tripathi DK, Singh VP, et al. Reactive oxygen species (ROS): beneficial companions of plants’ developmental processes. Front Plant Sci. 2016;7:1299.

25. Song XG, She XP. The generation and the role of hydrogen peroxide in plant. Journal of Lianyungang Teachers College. 2010;4:99-103.

26. Song XJ, Matsuoka M. Bar the windows: an optimized strategy to survive drought and salt adversities. Gene Dev. 2009;23:1709-13.

27. Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, et al. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature. 2000;406:731-4.

28. Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, et al. NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. Embo J. 2003;22:2623-33.
29. Hedrich R. Ion channels in plants. Physiol Rev. 2012;92:1777-811.

30. Osakabe Y, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP. ABA control of plant macroelement membrane transport systems in response to water deficit and high salinity. New Phytol. 2014;202:35-49.

31. Romeis T, Herde M. From local to global: CDPKs in systemic defense signaling upon microbial and herbivore attack. Curr Opin Plant Biol. 2014;20:1-10.

32. Majore I, Wilhelm B, Marten I. Identification of K+ channels in the plasma membrane of maize subsidiary cells. Plant Cell Physiol. 2002;43:844-52.

33. Zou JJ, Li XD, Ratnasekera D, Wang C, Liu WX, Song LF, et al. Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE8 and CATALASE3 function in abscisic acid-mediated signaling and H2O2, homeostasis in stomatal guard cells under drought stress. Plant Cell. 2015;27:1445-60.

34. Van Kleeff PJM, Gao J, Mol S, Zwart N, Zhang H, Li KW, et al. The Arabidopsis GORK K+-channel is phosphorylated by calcium-dependent protein kinase 21 (CPK21), which in turn is activated by 14-3-3 proteins. Plant Physiol and Bioch. 2018;125:219-31.

35. Mittler R, Blumwald E. The roles of ROS and ABA in systemic acquired acclimation. Plant Cell Online. 2015;27:64-70.

36. Gilroyet S, Suzuki N, Miller G, Choi WG, Toyota M, Devireddy AR, et al. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci. 2014;19:623-30.

37. Macallum AB. On the distribution of potassium in animal and vegetable cells. J Physiol - London. 1905;32: 95-198.

38. Zhang L, Wang QY, Li S, Dong H, Yao YQ. In situ detection technology of potassium in submicroscopic structures of plants. Plant Physiology Journal. 2015;51:1524-8.
39. Qu HY, Jiang XT, Shi ZB, Liu LM, Zhang SL. Fast loading ester fluorescent Ca2+ and pH indicators into pollen of pyrus pyrifolia. J Plant Res. 2012;125:185-195.

40. McAinsh MR, Brownlee C, Hetherington AM. Visualizing changes in cytosolic-free Ca2+ during the response of stomatal guard cells to abscisic acid. Plant Cell. 1992;4:1113-22.

41. Zhang X, Miao YC, An GY, Zhou Y, Shangguan ZP, Gao JF, et al. K+ channels inhibited by hydrogen peroxide mediate abscisic acid signaling in vicia guard cells. Cell Res. 2001;11:195-202.

42. Zhang N, Gao H, Qi Z. Effect of calcium on alleviation of decreased photosynthetic ability in salt-stressed maize leaves. Acta Phytoecologica Sinica. 2005;29:324-30.

43. Cao RR, Xing BY, Dang XL, Yao YQ, Liu LC, Dong JE. Effects of Ca2+ on salicylic-acid induced biosynthesis of salvianolic acid B in young seedlings of Salvia miltiorrhiza Bunge. Chin J Biotechnol. 2013;29:1836-46.

Figures
Figure 1

State of the stomatal apparatus from the epidermis of maize leaves following different treatments: a epidermis exposed to light for 6 h (light control); b epidermis exposed to light for 3 h and then darkness for 3 h (dark control); c epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; d epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; e epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; f epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness.
Figure 2

Stomatal aperture under different treatments: LL epidermis exposed to light for 6 h (light control); LD epidermis exposed to darkness for 3 h after (dark control); LL+H2O2 epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; LD+DPI epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; LL+CaCl2 epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; LD+LaCl3 epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness. Data represent the means of 15 replicates and the bars depict the SD.
Figure 3

Distribution of H2O2 in stomata under different treatments: a epidermis exposed to light for 3 h (light control); b epidermis exposed to darkness for 3 h (dark control); c epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light, d epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; e epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; f epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness.
Figure 4

Fluorescence intensity of H2O2 in guard and subsidiary cells under different treatments: LL epidermis exposed to light for 6 h (light control); LD epidermis exposed to darkness for 3 h after (dark control); LL+H2O2 epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; LD+DPI epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; LL+CaCl2 epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; LD+LaCl3 epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness. Data represent the means of 15 replicates and the bars depict the SD.
Figure 5

Distribution of K+ in stomata under different treatments: a epidermis exposed to light for 3 h (light control); b epidermis exposed to darkness for 3 h (dark control); c epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light conditions, d epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness conditions; e epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light conditions; f epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness conditions.
Concentration of K+ in guard and subsidiary cells under different treatments: LL epidermis exposed to light for 6 h (light control); LD epidermis exposed to darkness for 3 h after (dark control); LL+H2O2 epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; LD+DPI epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; LL+CaCl2 epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; LD+LaCl3 epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness. Data represent the means of 15 replicates and the bars depict the SD.
Figure 7

Distribution of Ca2+ in stomata under different treatments: a epidermis exposed to light for 3 h (light control); b epidermis exposed to darkness for 3 h (dark control); c epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; d epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness; e epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; f epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness.
Fluorescence intensity of Ca2+ in guard and subsidiary cells under different treatments: LL epidermis exposed to light for 6 h (light control); LD epidermis exposed to darkness for 3 h after (dark control); LL+H2O2 epidermis exposed to light for 3 h and then treated with H2O2 for 3 h under light; LD+DPI epidermis exposed to light for 3 h and then treated with DPI for 3 h under darkness; LL+CaCl2 epidermis exposed to light for 3 h and then treated with CaCl2 for 3 h under light; LD+LaCl3 epidermis exposed to light for 3 h and then treated with LaCl3 for 3 h under darkness. Data represent the means of 15 replicates and the bars depict the SD.
Figure 9

Pattern diagram of H2O2, Ca2+, and K+ content in guard and subsidiary cells during stomatal opening and closing. In guard cells, H2O2 and Ca2+ are positively correlated and mutually regulated, promoting the outward flow of K+. In subsidiary cells, H2O2 and Ca2+ are also positively correlated and mutually regulated, but they both promote the inward flow of K+. Reductions in H2O2 or Ca2+ contents induced by external stimuli in guard and subsidiary cells activate intracellular outward Ca2+-channels and inhibit inward Ca2+-channels, lead to hyperpolarization of guard cells’ and depolarization of subsidiary cells’ plasma membrane, activate inward K+-channels in guard cells and outward K+-channels in subsidiary cells, inhibit outward K+-channels of guard cells and inward K+-channels in subsidiary cells, and eventually result in stomatal opening. The stomatal closure process follows a reverse event.