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Sphingosine-1-phosphate (S1P) mediates darkness-induced stomatal closure through raising cytosol pH and hydrogen peroxide (H_2O_2) levels in guard cells in *Vicia faba*

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The role and signaling of sphingosine-1-phosphate (S1P) during darkness-induced stomatal closure were examined in *Vicia faba*. Darkness substantially raised S1P and hydrogen peroxide (H_2O_2) levels and closed stomata. These darkness effects were significantly suppressed by DL-*threo*-dihydrosphingosine (DL-*threo*-DHS) and *N*,*N*-dimethylsphingosine (DMS), two inhibitors of long-chain base kinases. Exogenous S1P led to stomatal closure and H_2O_2 production, and the effects of S1P were largely prevented by the H_2O_2 modulators ascorbic acid, catalase, and diphenyleneiodonium. These results indicated that S1P mediated darkness-induced stomatal closure by triggering H_2O_2 production. In addition, DL-*threo*-DHS and DMS significantly suppressed both darkness-induced cytosolic alkalization in guard cells and stomatal closure. Exogenous S1P caused cytosolic alkalization and stomatal closure, which could be largely abolished by butyric acid. These results demonstrated that S1P synthesis was necessary for cytosolic alkalization during stomatal closure caused by darkness. Furthermore, together with the data described above, inhibition of darkness-induced H_2O_2 production by butyric acid revealed that S1P synthesis-induced cytosolic alkalization was a prerequisite for H_2O_2 production during stomatal closure caused by darkness, a conclusion supported by the facts that the pH increase caused by exogenous S1P had a shorter lag and peaked faster than H_2O_2 levels and that butyric acid prevented exogenous S1P-induced H_2O_2 production. Altogether, our data suggested that darkness induced S1P synthesis, causing cytosolic alkalization and subsequent H_2O_2 production, finally leading to stomatal closure.

sphingosine-1-phosphate, cytosol pH, hydrogen peroxide, darkness, stomatal closure

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Sphingolipids are ubiquitous membrane lipids that play important roles in membrane structure and organization in many eukaryotes. Sphingolipids comprise a fatty acid *N*-linked to a long-chain base (LCB), usually with the addition of a polar head group to generate the mature lipid [1]. In mammals, the predominant LCB is sphingosine (Sph), whereas LCBs in plants are predominantly isomers of 4,8-sphingadienine and 4-hydroxy-8-sphingenine, such as phytosphingosine (Phyto-Sph) and dihydrosphingosine (DHS) [1−4]. In eukaryotes, phosphorylated LCBs (LCBPs) are synthesized from LCBs by long-chain base kinases (LCBKs) [5,6], whereas LCBs themselves are produced via the combinatorial activities of three LCB modification enzymes, such as LCB C-4 hydroxylase, LCB Δ8-desaturase, and LCB Δ4-desaturase [7,8].

In animals, sphingosine-1-phosphate (S1P), a Δ4-desaturated LCBP, has well-defined roles in cell signaling. It

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regulates many biological processes by binding to a family of receptors that are part of the specific cell surface G-protein-coupled receptors (GPCRs) and also serves as an intracellular second messenger in eukaryotes to regulate Ca^{2+} homeostasis, cell growth, and survival [9–13]. In higher plants, S1P has been shown to be a new calcium-mobilizing molecule and involved in abscisic acid (ABA)-induced stomatal closure in *Commelina communis* [14] and *Arabidopsis thaliana* [5]. Worrall *et al.* [15] have reported that the stomata of an *A. thaliana* LCBK mutant are less sensitive than wild type to ABA, whereas the stomata of LCBK-overexpression plants are more sensitive. Because S1P is often found at trace levels in plants [16–18] and phytosphingosine-1-phosphate (Phyto-S1P), a predominant Δ4-saturated LCBP, is also capable of promoting stomatal closure in *A. thaliana* [19], the role of S1P in guard-cell signaling is doubted. Further, *A. thaliana* insertion mutants with disrupted in Δ4-desaturase genes lack any detectable Δ4-unsaturated LCBP, such as S1P, and display no perturbation to ABA-induced stomatal closure [8], suggesting that S1P is not involved in stomatal closure caused by ABA in *A. thaliana*. However, Δ4-unsaturated sphingolipids are abundant in some plant species, such as *Glycine max* and *Lycopersicon esculentum* [16,17], whereas research has been limited to the role of S1P in ABA signaling in *A. thaliana* and *C. communis* guard cells [5,8,14,15]. It remains unknown whether S1P mediates stomatal closure in response to other internal or external factors, including darkness. Furthermore, whether S1P regulates stomatal movement in plants other than *A. thaliana* and *C. communis*, needs to be investigated.

Intracellular pH changes have long been proposed to function in various physiological processes in plants, such as plant defense responses [20], coleoptile or root hair growth, nodulation, elicitation [20–24], and response to hormones such as gibberellic acid, methyl jasmonate (MJ), and ABA [25–27]. Cytosolic alkalization in guard cells is a common phenomenon in ABA- and MJ-induced stomatal closure in *A. thaliana*, *Pisum* sativum, and *Paphiopedilum tonsum* [27–30]. ABA elevates the level of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in accordance with cytosolic alkalization in *Paphiopedilum tonsum* guard cells [28]. Cytosolic alkalization and cytosolic Ca^{2+} oscillation coordinately function in ABA and MJ signaling in *A. thaliana* guard cells [31]. Cytosolic alkalization also activates outward K^+ currents and inactivates inward K^+ currents to promote a net efflux of K^+ in *Vicia faba* guard cells [32]. In addition, cytosolic acidification in guard cells has been shown to mediate stomatal opening by kinetin (KT), indole-3-acetic acid (IAA), and fusicoccin (FC) [28] and to activate inward K^+ currents [33]. These results suggest that changes in cytosol pH are closely involved in modulating stomatal movement.

Hydrogen peroxide (H_2O_2) is a reactive oxygen species having wide-ranging effects in many biological systems [34]. H_2O_2 functions as an endogenous signal molecule mediating plant responses to various stresses and stimulus [35,36]. Exogenous H_2O_2 was reported to elevate the level of $[Ca^{2+}]_{cyt}$ in guard cells and induce stomatal closure in *V. faba* [37]. The ability of guard cells to generate H_2O_2 has been demonstrated in *Nicotiana tabacum*, *L. esculentum*, and *C. communis* [38,39]. The finding that ABA promotes H_2O_2 synthesis in *A. thaliana* and *V. faba* guard cells was a significant advance, which further highlighted the fact that endogenous H_2O_2 acts as an intermediate signal in ABA signaling in guard cells [40,41]. In addition, H_2O_2 has also been shown to mediate MJ-, salicylic acid (SA)-, cytokinin-, auxin-, ethylene-, elicitor-, high CO_2 concentrations-, CO-, and UV-B-regulated stomatal movement [27,38,39,42–47]. Further studies also provided exciting evidence that darkness-induced stomatal closure requires H_2O_2 accumulation in guard cells [48,49].

However, whether S1P and cytosolic alkalization are involved in darkness-induced stomatal closure in *V. faba* remains unclear. In addition, the interactions of S1P, cytosolic pH, and H_2O_2 during stomatal responses to darkness must also be elucidated. In this study, by means of stomatal bioassays and high-performance liquid chromatography (HPLC), we provided evidence that S1P synthesis is involved in darkness-induced stomatal closure in *V. faba*. Furthermore, our study showed that the role of S1P in darkness-induced stomatal closure is associated with cytosolic alkalization and H_2O_2 production in guard cells and that a rise in cytosolic pH is a prerequisite for H_2O_2 production via stomatal bioassays and laser scanning confocal microscopy using the molecular probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM).

1 Materials and methods

1.1 Chemicals

The molecular probe H_2DCF-DA was obtained from Biotium (Hayward, CA, USA). S1P was purchased from Avanti Polar Lipids (Alabaster, AL, USA). BCECF-AM, 2-(*N*-morpholino) ethanesulfonic acid (MES), DL-*threo*-dihydrosphingosine (DL-*threo*-DHS), catalase (CAT, from bovine liver), diphenyleneiodonium (DPI), butyric acid, dimethyl sulfoxide (DMSO), and naphthalene-2,3-dicarboxaldehyde (NDA) were obtained from Sigma-Aldrich (St Louis, MO, USA). *N*,*N*-dimethylsphingosine (DMS) was purchased from Merck KGaA (Darmstadt, Germany). Unless stated otherwise, the remaining chemicals were of the highest analytical grade available from various Chinese suppliers.

1.2 Plant materials

Broad bean (*Vicia faba* L.) was grown in a controlled-

environment plant growth chamber with a humidity of 80%, a 14∶10 h light:dark cycle with a photon flux density of 300 μmol m^{-2} s^{-1} PAR generated by cool white fluorescent tubes (Philips, New York, NY, USA), and an ambient temperature of (25±2)°C. The epidermis was carefully peeled from the abaxial surfaces of the youngest, fully-expanded leaves of 4-week-old seedlings and cut into pieces of about 5 mm×5 mm. All treatments described below were at (25±2)°C in either light (300 μmol m^{-2} s^{-1}) or darkness.

1.3 Stomatal bioassays

Stomatal bioassays were performed as described by McAinsh *et al.* [37] with slight modifications. All incubations were for 3 h. To study the effects of DL-*threo*-DHS, DMS, ASA, CAT, and DPI on darkness-induced stomatal closure, freshly prepared epidermal strips were incubated in light in MES/KCl buffer (10 mmol L^{-1} MES/KOH, 50 mmol L^{-1} KCl, 100 μmol L^{-1} $CaCl_2$, pH 6.15) alone or incubated in darkness in MES/KCl buffer alone or containing 5, 10, 15, 20 μmol L^{-1} DL-*threo*-DHS, 0.1, 1, 5, 10 μmol L^{-1} DMS, 100 μmol L^{-1} ASA, 100 units mL^{-1} CAT, or 10 μmol L^{-1} DPI. Final stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. To study the effect of S1P on stomatal aperture, the epidermal strips were incubated in MES/KCl buffer containing 2, 4, 6, 8 μmol L^{-1} S1P in light, then were treated with MES/KCl buffer in light for another 3 h before the stomatal apertures were recorded. To study the effects of ASA, CAT, DPI, and butyric acid on stomatal closure caused by S1P, isolated epidermal strips were incubated in light in MES/KCl buffer alone or containing 6 μmol L^{-1} S1P, 100 μmol L^{-1} ASA, 100 units mL^{-1} CAT, 10 μmol L^{-1} DPI, 0.5, 1, 1.5, 2 mmol L^{-1} butyric acid, 6 μmol L^{-1} S1P+100 μmol L^{-1} ASA, 6 μmol L^{-1} S1P+100 units mL^{-1} CAT, 6 μmol L^{-1} S1P+10 μmol L^{-1} DPI, or 6 μmol L^{-1} S1P+0.5, 1, 1.5 or 2 mmol L^{-1} butyric acid, and then the apertures were recorded.

To avoid circadian rhythm 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 at least three times. The data presented are the mean±SE of 90 measurements.

1.4 Measurement of H_2O_2 and cytosolic pH

H_2O_2 was measured by using H_2DCF-DA, as previously described [38] with minor changes. The changes in cytosolic pH were monitored with BCECF-AM, as described earlier by Irving *et al.* [28] with minor modifications.

To study the effect of butyric acid on darkness-induced H_2O_2 generation in guard cells, the epidermal strips were incubated for 3 h in MES/KCl buffer alone in light or in MES/KCl buffer alone or containing 0.5 mmol L^{-1} butyric acid in darkness, and then were immediately loaded with 50 μmol L^{-1} H_2DCF-DA in Tris-KCl buffer (10 mmol L^{-1} Tris, 50 mmol L^{-1} KCl, pH 7.2) for 10 min in darkness. To study the effects of (i) DL-*threo*-DHS, DMS, ASA, CAT, and DPI on darkness-induced H_2O_2 generation, (ii) ASA, CAT, DPI, and butyric acid on H_2O_2 levels in guard cells treated with S1P, (iii) DL-*threo*-DHS and DMS on darkness-induced alteration in cytosolic pH, and (iv) butyric acid on cytosol pH in guard cells treated with S1P, the epidermal strips were treated as described for the stomatal bioassays. In these treatments, the concentrations of DL-*threo*-DHS, DMS and butyric acid were 15, 5 and 0.5 mmol L^{-1} respectively, the concentrations of the remaining agents were the same as those used in the stomatal bioassays. After treatment described above, the strips were loaded with 50 μmol L^{-1} H_2DCF-DA or 20 μmol L^{-1} BCECF-AM for 10 min in Tris-KCl buffer in darkness. To facilitate the loading of BCECF-AM, the Tris-KCl loading buffer included 0.05% Pluronic F-127. After these steps, excess dye was washed off with fresh Tris-KCl loading buffer in darkness, and 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%, zoom about 4, normal scanning speed, and frame 512×512 pixels. Images thus acquired were analyzed with Leica image software and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA).

In the experiments involving time-course monitoring of H_2O_2 levels and cytosolic pH in guard cells in response to S1P, epidermal strips were incubated in MES/KCl buffer in light for 3 h to allow stomatal opening, and then were immediately loaded with 50 μmol L^{-1} H_2DCF-DA in Tris-KCl buffer or 20 μmol L^{-1} BCECF-AM in Tris-KCl buffer containing 0.05% Pluronic F-127 in darkness for 10 min. After washing off excess dye, S1P (final concentration of 6 μmol L^{-1}) was added directly to Tris-KCl buffer, and then changes in H_2DCF-DA or BCECF-AM fluorescence were recorded within 30 min by laser-scanning confocal microscopy.

To compare changes in signal intensity, confocal images were taken under identical conditions (in manual setup) for all samples. In each treatment, three epidermal strips were measured, and the treatment was repeated at least three times. The confocal images depicted here represent similar results from all three replications.

1.5 S1P determination

After exposure to light for 3 h, fully-expanded leaves of 4-week-old seedlings were sprayed with MES/KCl buffer alone or containing 15 μmol L^{-1} DL-*threo*-DHS or 5 μmol

L^{-1} DMS, and then the seedlings were grown in light or in darkness for another 3 h. The leaves were cut and used for lipid extraction and S1P analysis.

Lipid extraction was performed as described by Ng *et al.* [14]. Relying on derivatization with NDA, S1P was quantified by high-performance liquid chromatography (HPLC) according to the methods of He *et al.* [50] with some modifications. The HPLC system consisted of a Shimadzu CBM-20A Controller, LC-20AT pump (Shimadzu, Kyoto, Japan), Rheodyne 7725i manual sampler (IDEX, Oak Harbor, WA, USA), and C_{18} reversed-phase column (00G-4252-E0; 4.6 mm i.d.×250 mm) (Phenomenex, Torrance, CA, USA). The mobile phase composition for the isocratic eluent system was 5 mmol L^{-1} potassium phosphate buffer (pH 6.5) for mobile phase A, 100% acetonitrile for mobile phase B, and 100% methanol for mobile phase C. The isocratic eluent composition of A∶B∶C (30∶55∶15) and a flow rate of 1 mL min^{-1} were accurately controlled during HPLC analysis. The NDA derivatives were detected selectively using a Shimadzu RF-10AXL fluorescence detector with an excitation wavelength of 252 nm and an emission wavelength of 483 nm. Quantification of the S1P peak was calculated using the Shimadzu LC Solution software according to a calibration curve derived from commercial purified S1P. In each treatment, we determined two values per replicate, and treatments were repeated three times. The data presented are the mean±SE of six measurements.

1.6 Solvent effects and statistical analyses

The control sets were added with an equal volume of solvents used for their stocks. DMSO was the solvent used for BCECF-AM and H_2DCF-DA. S1P stock solutions were prepared in methanol and DL-*threo*-DHS and DMS stocks in ethanol. The final concentrations of DMSO, methanol, and ethanol were 0.5%, 0.06%, and 0.01% (v/v), respectively, which did not induce any significant changes in guard cell viability or stomatal aperture. The NDA stock solution was prepared in 100% ethanol. Other chemicals were dissolved in double-distilled water. MES/KCl buffer was prepared in freshly double-distilled water to avoid the effect of CO_2 on stomatal aperture. The statistical significance of treatments was checked using one-way ANOVA followed by Duncan's multiple range test. The data were considered statistically significant when *P*-values were below 0.05.

2 Results

2.1 S1P synthesis mediates darkness-induced stomatal closure by triggering H_2O_2 production in guard cells

Previous studies demonstrated that darkness-induced stomatal closure was related to an increase in H_2O_2 levels in guard cells [48,49]. To explore whether stomatal closure in darkness also involved S1P and whether S1P and H_2O_2 were related in this process, the effects of DL-*threo*-DHS and DMS, two inhibitors of LCBKs in mammalian cells [51,52], on darkness-induced stomatal closure, H_2O_2 production in guard cells, and S1P content in leaves were measured. Similar to H_2O_2 modulator ASA [53], CAT [39] and DPI [54], DL-*threo*-DHS at concentrations ⩾5 μmol L^{-1} and DMS at concentrations ⩾1 μmol L^{-1} significantly inhibited darkness-induced stomatal closure; their optimum concentrations were 15 and 5 μmol L^{-1}, respectively (Figure 1A). Compared with the light treatment (Figure 1B and I), darkness induced an intense H_2DCF-DA fluorescence in guard cells (Figure 1C and I), consistent with a previous report [49]. However, 15 μmol L^{-1} DL-*threo*-DHS and 5 μmol L^{-1} DMS abolished darkness-induced H_2O_2 production (Figure 1D, E and I), as did ASA, CAT, and DPI (Figure 1F, G, H and I). Additionally, darkness significantly induced S1P synthesis in leaves, which was inhibited by 15 μmol L^{-1} DL-*threo*-DHS and 5 μmol L^{-1} DMS (Figure 1J). Together with the fact that DL-*threo*-DHS and DMS distinctly suppressed darkness-induced stomatal closure and H_2O_2 production (Figure 1A, D, E and I), the results suggested that S1P synthesis mediates darkness-induced stomatal closure by triggering H_2O_2 production.

To further prove that S1P synthesis mediated darkness-induced stomatal closure by triggering H_2O_2 production, the effects of exogenous S1P on stomatal aperture and H_2O_2 production in guard cells were measured. Treatment with ⩾4 μmol L^{-1} S1P promoted stomatal closure; the optimum concentration was 6 μmol L^{-1} (Figure 2A). A washout experiment indicated that the effect of S1P on stomatal aperture was not due to irreversible cytotoxicity (Figure 2A). Importantly, S1P-induced stomatal closure and H_2O_2 production were significantly abolished by ASA, CAT, or DPI (Figure 2B and C). The results confirmed the conclusion that S1P synthesis mediated darkness-induced stomatal closure by triggering H_2O_2 production.

2.2 S1P synthesis induces guard cell cytosolic alkalization during darkness-induced stomatal closure

To further study the role of S1P in darkness-induced stomatal closure, the relationship between S1P synthesis and changes in cytosol pH during the process was investigated. First, the effects of DL-*threo*-DHS and DMS on cytosolic pH were determined. Compared with light treatment (Figure 3A and E), darkness significantly raised cytosolic pH in guard cells (Figure 3B and E). However, application of 15 μmol L^{-1} DL-*threo*-DHS and 5 μmol L^{-1} DMS largely suppressed the darkness-induced rise in guard cell cytosol pH (Figure 3C–E). Together with the fact that DL-*threo*-DHS and DMS also suppressed darkness-induced S1P synthesis (Figure 1J) and stomatal closure (Figure 1A), we concluded that S1P synthesis was involved in darkness-induced sto-

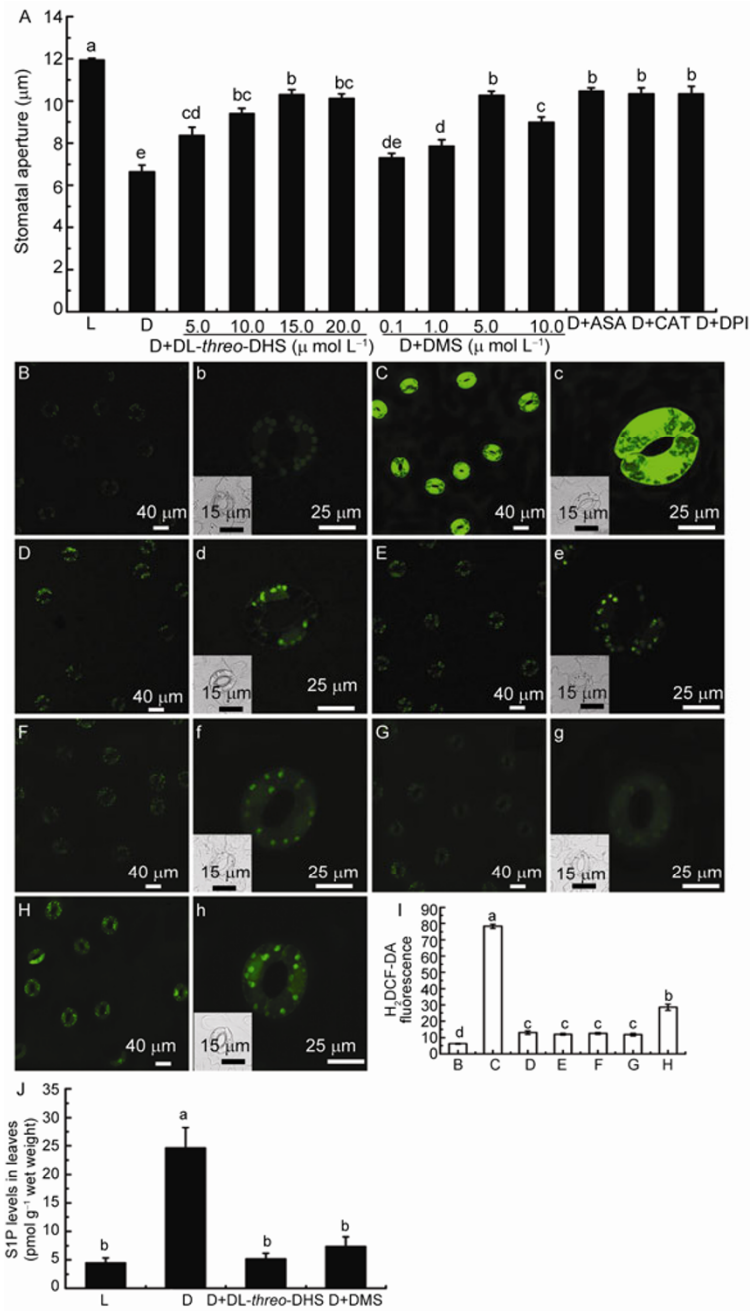

Figure 1 DL-*threo*-DHS and DMS inhibit darkness-induced stomatal closure, H_2O_2 production in guard cells, and S1P synthesis in leaves. A, DL-*threo*-DHS and DMS inhibit darkness-induced stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer alone in light (300 μmol m^{-2} s^{-1}) (L), or in MES/KCl buffer alone (D) or containing different concentrations of DL-*threo*-DHS (D+DL-*threo*-DHS) or DMS (D+DMS), 100 μmol L^{-1} ASA (D+ASA), 100 units mL^{-1} CAT (D+CAT), or 10 μmol L^{-1} DPI (D+DPI) in darkness at (25±2)°C for 3 h. Data are mean±SE of 30 stomata in each of three independent experiments (n=90). Means denoted by different letters differed significantly at P<0.05 according to Duncan's multiple range test. B–I, DL-*threo*-DHS and DMS reduce darkness-induced H_2O_2 production in guard cells. Guard cells were treated for 3 h as follows: (B) with MES/KCl buffer alone in light; (C) MES/KCl buffer alone, (D) containing 15 μmol L^{-1} DL-*threo*-DHS, (E) 5 μmol L^{-1} DMS, (F) 100 μmol L^{-1} ASA, (G) 100 units mL^{-1} CAT, or (H) 10 μmol L^{-1} DPI in darkness. Epidermal strips were immediately loaded with 50 μmol L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (I) Average fluorescent intensity of guard cells in images (B–H); data are mean±SE of three independent experiments (n=9). The means in (I) denoted by different letters differed significantly at P<0.05 according to Duncan's multiple range test. The guard cells shown in images (b–h) are representative of those in images (B–H), respectively, and the insets show the corresponding bright-field images. Scale bars in (H), (h), and (h) inset represent 40, 25, and 15 μm, respectively, in all corresponding images. J, DL-*threo*-DHS and DMS suppress darkness-induced S1P synthesis in leaves. After exposure to light for 3 h at (25±2)°C, fully expanded leaves of 4-week-old seedlings were sprayed with MES/KCl buffer without or with 15 μmol L^{-1} DL-*threo*-DHS or 5 μmol L^{-1} DMS, and then the seedlings were grown in light or darkness for another 3 h. The leaves were cut and used for lipid extraction and analysis. The levels of S1P were quantified by HPLC after NDA derivatization. The derivatives were detected with an excitation wavelength of 252 nm and an emission wavelength of 483 nm. Data are mean±SE of three independent experiments (n=6). Means denoted by different letters differed significantly at P<0.05 according to Duncan's multiple range test.

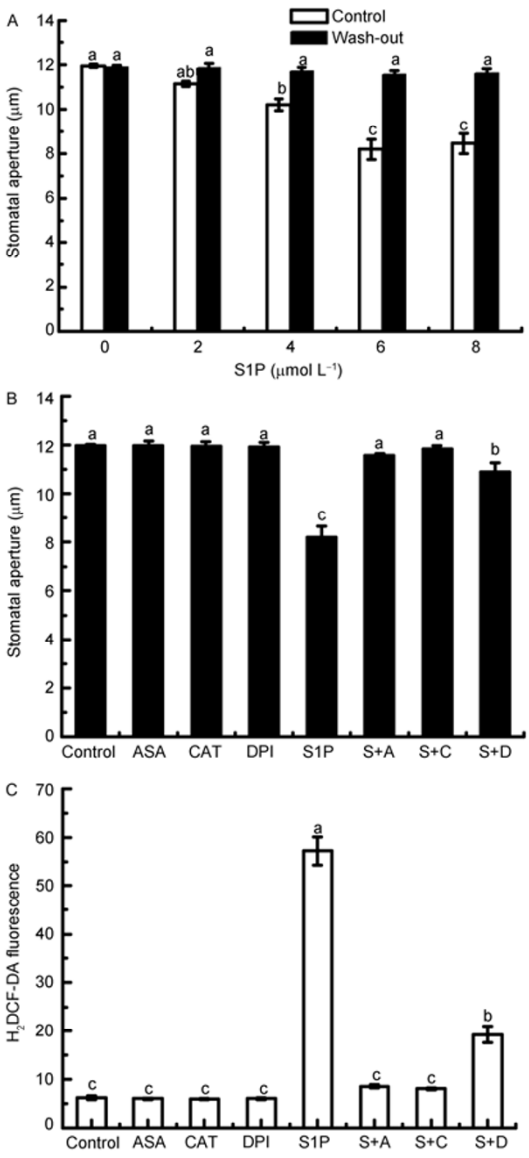

Figure 2 ASA, CAT, and DPI prevent exogenous S1P-induced stomatal closure and H_2O_2 production in guard cells. A, S1P induces stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer containing different concentrations of S1P in light (300 μmol m^{-2} s^{-1}) at (25±2)°C for 3 h, then stomatal apertures were recorded (white columns). After incubation in MES/KCl buffer containing different concentrations of S1P in light for 3 h, the strips were treated with MES/KCl buffer in light for another 3 h, then apertures were measured (black columns). See Figure 1A for further experimental details. B, ASA, CAT and DPI inhibit S1P-induced stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer alone (Control) or containing 100 μmol L^{-1} ASA, 100 units mL^{-1} CAT, 10 μmol L^{-1} DPI, 6 μmol L^{-1} S1P, 6 μmol L^{-1} S1P+100 μmol L^{-1} ASA (S+A), 6 μmol L^{-1} S1P+100 units mL^{-1} CAT (S+C), or 6 μmol L^{-1} S1P+10 μmol L^{-1} DPI (S+D) in light for 3 h, and stomatal apertures were measured. See Figure 1A for further experimental details. C, ASA, CAT and DPI suppress S1P-induced H_2O_2 production. Treatments were as in Figure 2B. After treatments, the epidermal strips were immediately loaded with 50 μmol L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness for 10 min, then excess dye was washed off and the strips were examined by laser-scanning confocal microscopy. Data show the average fluorescent intensity±SE of three independent experiments (n=9). Means denoted by different letters differed significantly at P<0.05 according to Duncan's multiple range test.

matal closure by inducing guard cell cytosolic alkalization.

To further validate that S1P was associated with cytosolic alkalization in guard cells during darkness-induced stomatal closure, the effects of butyric acid, which is frequently used to modulate guard cell cytosolic pH [29], on exogenous S1P-induced stomatal closure and cytosolic pH of guard cells were examined. Treatment with exogenous S1P significantly induced stomatal closure, but this effect was largely suppressed by ⩾0.5 mmol L^{-1} butyric acid (Figure 3F). Compared with the control, butyric acid had no obvious effect on cytosolic pH of guard cells (P>0.05). S1P significantly induced cytosolic alkalization (Figure 3G), but the effect of S1P on cytosolic pH was reversed by 0.5 mmol L^{-1} butyric acid (Figure 3G). These results reinforced the conclusion that S1P synthesis was involved in darkness-induced stomatal closure by inducing guard cell cytosolic alkalization.

2.3 S1P synthesis-induced cytosolic alkalization in guard cells is a prerequisite for H_2O_2 production during darkness-induced stomatal closure

Having established that S1P synthesis mediated darkness-induced stomatal closure by triggering cytosolic alkalization and H_2O_2 production in guard cells, we further studied whether the S1P synthesis-induced rise in cytosolic pH affected H_2O_2 production. As shown in Figure 4A, darkness significantly promoted H_2O_2 production relative to the light treatment, consistent with previous results [49]. However, the effect of darkness on H_2O_2 production was largely prevented by butyric acid (Figure 4A). Combined with the facts that both cytosolic alkalization and H_2O_2 production caused by S1P synthesis were implicated in darkness-induced stomatal closure (Figures 1–3), our data showed that S1P synthesis-induced cytosolic alkalization in guard cells was a prerequisite for H_2O_2 production during darkness-induced stomatal closure.

To further examine whether S1P synthesis-induced cytosolic alkalization was necessary for H_2O_2 production in guard cells during stomatal closure in darkness, we determined the changes in kinetics of cytosolic pH and H_2O_2 levels in guard cells treated with exogenous S1P. As shown in Figure 4C and D, there were no visible changes in cytosolic pH or H_2O_2 level in control samples within 30 min. However, the increase in cytosolic pH in guard cells upon exogenous S1P was remarkable within 6 min and peaked at 15 min (Figure 4C). In contrast, H_2O_2 level in guard cells treated with S1P started to increase sharply after 9 min and reached its maximum at 21 min (Figure 4D). The results showed that the rise in cytosolic pH was either associated with or necessary for H_2O_2 production. In addition, our data showed that exogenous S1P-induced H_2O_2 production was largely prevented by butyric acid (Figure 4B). Taken together, the results supported the conclusion that S1P synthesis-induced cytosolic alkalization was necessary for

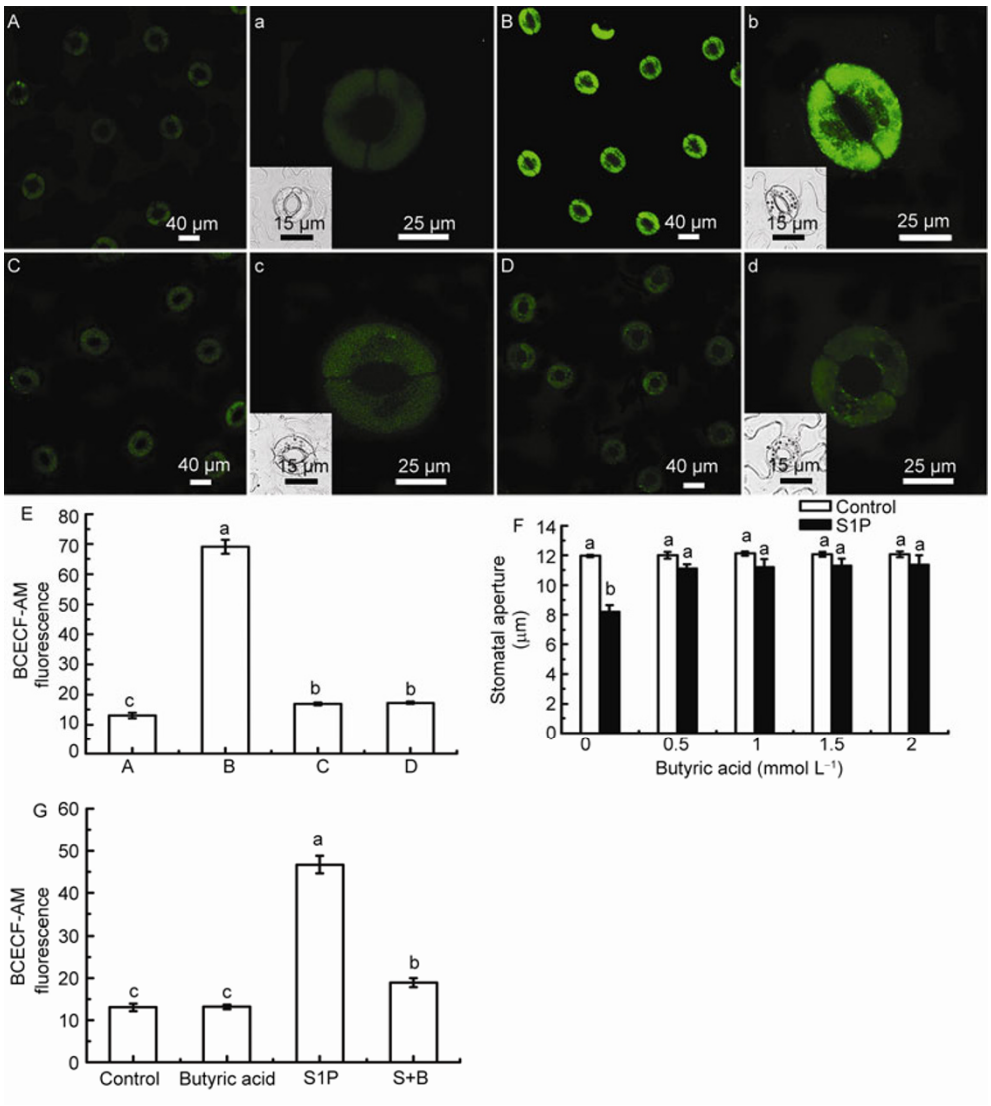

Figure 3 S1P is involved in darkness-induced stomatal closure by raising guard cell cytosolic pH. A–E, DL-*threo*-DHS and DMS restrict darkness-induced rise in cytosolic pH. Guard cells were treated at (25±2)°C for 3 h as follows: (A) with MES/KCl buffer alone in light (300 μmol m^{-2} s^{-1}); (B) MES/KCl buffer alone, (C) containing 15 μmol L^{-1} DL-*threo*-DHS, or (D) 5 μmol L^{-1} DMS in darkness. Epidermal strips were immediately loaded with 20 μmol L^{-1} BCECF-AM in Tris-KCl buffer containing 0.05% Pluronic F-127 in darkness for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. (E) Average fluorescent intensity of guard cells in images (A–D); data are mean±SE of three independent experiments (n=9). The means in (E) denoted by different letters differed significantly at P<0.05 according to Duncan's multiple range test. The guard cells in (a–d) are the representative of those in (A–D). Insets in (a–d) show corresponding bright-field images. Scale bars in (D), (d), and (d) inset represent 40, 25, and 15 μm, respectively, in all corresponding images. F, Butyric acid prevents S1P-induced stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer alone or containing different concentrations of butyric acid (white columns), 6 μmol L^{-1} S1P, or 6 μmol L^{-1} S1P+different concentrations of butyric acid (black columns) in light for 3 h. See Figure 1A for further experimental details. G, Butyric acid suppresses S1P-induced rise in cytosolic pH in guard cells. Isolated epidermal strips were incubated in MES/KCl buffer alone (Control) or containing 0.5 mmol L^{-1} butyric acid, 6 μmol L^{-1} S1P, or 6 μmol L^{-1} S1P+0.5 mmol L^{-1} butyric acid (S+B) in light for 3 h. Epidermal strips were immediately loaded with 20 μmol L^{-1} BCECF-AM in Tris-KCl buffer containing 0.05% Pluronic F-127 in darkness for 10 min, then excess dye was removed and the strips were examined by laser-scanning confocal microscopy. See Figure 2C for further experimental details.

H_2O_2 production in guard cells during stomatal closure caused by darkness.

3 Discussion

Sphingolipids are major components of membrane lipids. In animal cells, the sphingolipid metabolite S1P has been shown to be involved in the mediation of numerous cellular responses, including proliferation, survival, cytoskeletal organization, motility, differentiation, neurite retraction, and rounding [55,56]. Presently, S1P and LCBKs (referred to as SphKs) are known to participate in ABA-regulated stomatal movement [5,14,15]. However, *A. thaliana* mutants dis-

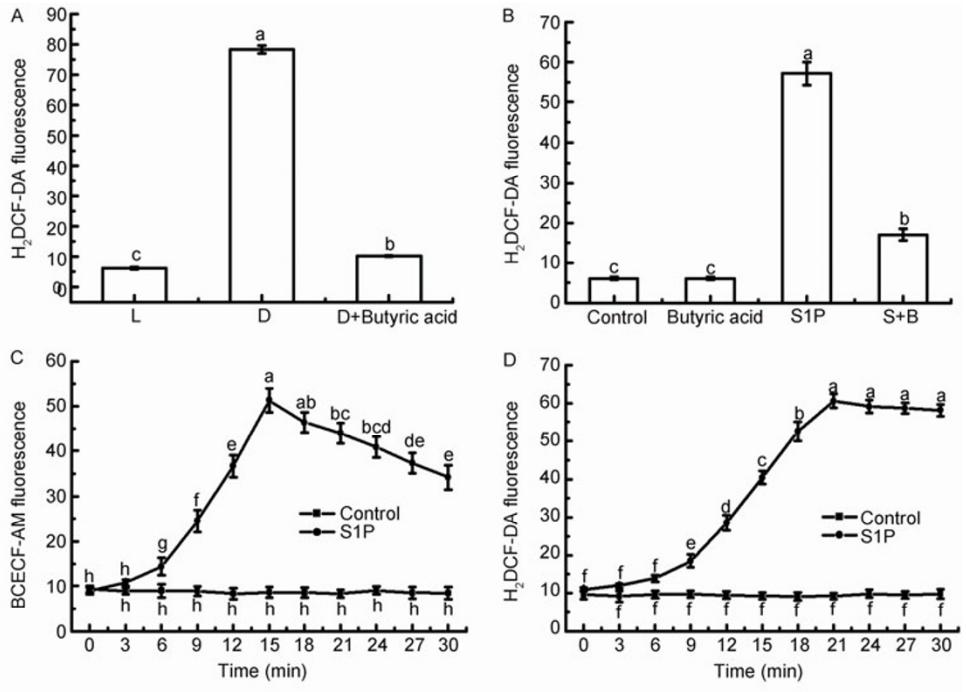

Figure 4 S1P-induced cytosolic alkalization in guard cells is necessary for H_2O_2 production. A, Butyric acid suppresses darkness-induced H_2O_2 production. Isolated epidermal strips were incubated in light (300 μmol m^{-2} s^{-1}) at (25±2)°C for 3 h in MES/KCl buffer alone (L) or in darkness in MES/KCl buffer alone (D) or containing 0.5 mmol L^{-1} butyric acid (D+butyric acid). See Figure 2C for further experimental details. B, Butyric acid reduces S1P-induced H_2O_2 levels in guard cells. Isolated epidermal strips were incubated in light in MES/KCl buffer alone (Control) or containing 0.5 mmol L^{-1} butyric acid, 6 μmol L^{-1} S1P, or 6 μmol L^{-1} S1P+0.5 mmol L^{-1} butyric acid (S+B) for 3 h. See Figure 2C for further experimental details. C and D, Kinetics change in cytosolic pH (C) and H_2O_2 level (D) in guard cells treated with S1P. Isolated epidermal strips were incubated in MES/KCl buffer in light for 3 h to induce stomatal opening, and then were loaded with 20 μmol L^{-1} BCECF-AM in Tris-KCl buffer containing 0.05% Pluronic F-127 or 50 μmol L^{-1} H_2DCF-DA in Tris-KCl buffer in darkness for 10 min. After washing off excess dye, S1P was added directly to Tris-KCl buffer, and changes in BCECF-AM and H_2DCF-DA fluorescence were recorded within 30 min by laser-scanning confocal microscopy. See Figure 2C for further experimental details.

rupted in Δ4-desaturase neither contain S1P nor display any perturbation to ABA-induced stomatal closure, indicating that S1P is not involved in the stomatal closure by ABA [8]. Therefore, the effects of S1P on stomatal movement remain unclear. In this study on darkness-induced S1P synthesis and stomatal closure, we showed that exogenous S1P affected stomatal aperture similarly to darkness treatment and that application of DL-*threo*-DHS and DMS, two LCBK inhibitors, prevented darkness-induced stomatal closure and S1P synthesis. Our data therefore indicated that S1P mediated darkness-induced stomatal closure in *V. faba*. Considering that LCBKs also phosphorylate phytosphingosine and that Phyto-S1P can promote stomatal closure in *A. thaliana* [19], our results did not preclude the possibility that other LCBPs, including Phyto-S1P, are also involved in darkness-caused stomatal closure in *V. faba*. Furthermore, whether S1P regulates ABA-induced stomatal closure in *V. faba* must also be investigated.

In animals, S1P has been shown to function as both an intracellular messenger and an extracellular ligand for G-protein-coupled receptors of the S1P receptor family, thus regulating diverse biological processes [13]. In plants, the role of S1P in cell signaling was only recently reported [5,14,57]. S1P could induce Ca^{2+} mobilization, inhibit inwardly-rectifying plasma membrane K^+ channels, and stimulate slow anion channels, thus regulating stomatal movement [5,14]. The action of S1P on ion channels was impaired in guard cells of *A. thaliana* knockout lines of the sole prototypical heterotrimeric G-protein a-subunit gene, *GPA1*, suggesting that heterotrimeric G-proteins are downstream targets for S1P in plants, as in mammals [5]. Surprisingly, more recent research showed that GCR1, a putative GPCR in *A. thaliana*, could negatively control S1P regulation of stomatal apertures [57]. Nevertheless, whether H_2O_2 participates in S1P-induced stomatal closure is still unknown. The results that S1P and H_2O_2 mediate ABA-induced stomatal closure [5,14,40,41,58] and S1P also reduces stomatal aperture [5,14], this study promoted us to investigate the effect of S1P on H_2O_2 production in guard cells during darkness-induced stomatal closure. Our results indicated that exogenous S1P induced H_2O_2 production, and that darkness caused S1P synthesis and H_2O_2 production. In addition, DL-*threo*-DHS and DMS abolished the effects of darkness on S1P synthesis and H_2O_2 production. The results demonstrated that S1P synthesis was required for darkness-induced H_2O_2 production in guard cells of *V. faba*. Additionally, the inhibition of S1P-induced H_2O_2 production by DPI suggested that NADPH oxidase might be re-

sponsible for H_2O_2 production induced by S1P in *V. faba* guard cells.

The pH is an important physiological signaling component, including in stomatal movements [28,59,60]. Effectors that decrease the cytosolic pH (auxin, fusicoccin) open stomata [28], while those that raise the cytosolic pH (ABA and MJ) result in stomatal closure [27,32]. Cytosolic alkalization has been reported to precede the production of reactive oxygen species during ABA- and MJ-induced stomatal closure [27,61]. However, whether S1P affects guard cell cytosolic pH to regulate stomatal movement is unknown. The present study showed that DL-*threo*-DHS and DMS significantly prevented darkness-induced S1P synthesis, cytosolic pH increase, and stomatal closure. Exogenous S1P markedly induced guard cell cytosolic alkalization and stomatal closure. Furthermore, the effects of S1P on cytosolic pH and stomatal aperture were evidently restricted by butyric acid. The data clearly revealed that the role of S1P was related to guard cell cytosolic alkalization during darkness-induced stomatal closure. This is the first report that darkness-induced S1P synthesis regulates stomatal movement by influencing cytosolic pH in guard cells.

Although cytosolic alkalization in guard cells has been shown to occur early in ABA- and MJ-induced H_2O_2 production and stomatal closure [27,61], it is unknown whether cytosolic alkalization is a causal factor for H_2O_2 production during darkness-induced stomatal closure. To answer this question, the effect of butyric acid on darkness-induced H_2O_2 production in guard cells was determined. The results showed that butyric acid significantly suppressed darkness-induced H_2O_2 production in guard cells. Combined with the facts that darkness induced S1P synthesis, cytosolic alkalization, H_2O_2 production, and stomatal closure, the inhibitory effect of butyric acid on darkness-induced H_2O_2 production revealed that S1P-induced cytosolic alkalization is a causal factor for H_2O_2 production in guard cells during stomatal closure in darkness. This conclusion was further supported by the observation that the exogenous S1P-induced increase in cytosolic pH had a shorter lag and peaked faster than H_2O_2 level, and butyric acid significantly inhibited exogenous S1P-induced H_2O_2 production in guard cells.

Altogether, our results showed that S1P synthesis mediated darkness-induced stomatal closure by inducing cytosolic alkalization and H_2O_2 production in guard cells; guard cell cytosolic alkalization is a prerequisite for H_2O_2 production. However, how darkness regulates S1P synthesis to raise cytosolic pH remains unknown. Furthermore, the mechanism by which cytosolic alkalization affects H_2O_2 production must be further investigated.

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