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The role of phytosphingosine-1-phosphate (Phyto-S1P) and its relationships with cytosolic pH and hydrogen peroxide (H_2O_2) during stomatal closure by darkness in broad bean

Y. Ma *, J. Niu

College of Life Sciences, Shanxi Normal University, Linfen 041004, People's Republic of China

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ABSTRACT

We examined the role of phytosphingosine-1-phosphate (Phyto-S1P) and its relationships with cytosolic pH and hydrogen peroxide (H_2O_2) during stomatal closure by darkness using broad bean as experimental material. Darkness caused cytoplasmic alkalization, induced H_2O_2 generation, and led to stomatal closure. DL-*threo*-dihydrosphingosine (DL-*threo*-DHS) and *N,N*-dimethylsphingosine (DMS) inhibited these effects of darkness. Exogenous Phyto-S1P promoted cytoplasmic alkalization, H_2O_2 synthesis, and caused stomatal closure. Butyric acid largely suppressed cytoplasmic alkalization and prevented stomatal closure by Phyto-S1P. The H_2O_2 modulators ascorbic acid (ASA), catalase (CAT), and diphenyleneiodonium (DPI) obviously restricted Phyto-S1P-promoted H_2O_2 synthesis and stomatal closure. These results suggest that Phyto-S1P was involved in darkness-induced stomatal closure by causing cytoplasmic alkalization and H_2O_2 synthesis. Furthermore, darkness-induced H_2O_2 synthesis was inhibited by butyric acid, suggesting that cytoplasmic alkalization caused by Phyto-S1P was probably necessary for H_2O_2 synthesis. The rise in cytosolic pH promoted by Phyto-S1P became noticeable at 6 min and reached a maximum at 18 min, while H_2O_2 levels sharply increased at 8 min and peaked at 22 min, and butyric acid prevented Phyto-S1P-promoted H_2O_2 synthesis, which confirmed the conclusion that Phyto-S1P-induced cytosolic alkalization acts upstream of the production of H_2O_2 during stomatal closure by darkness. We suggest that Phyto-S1P is involved in stomatal closure by darkness through cytoplasmic alkalization and H_2O_2 synthesis, and Phyto-S1P-promoted cytoplasmic alkalization acts upstream of H_2O_2 synthesis in the process.

1. Introduction

Many studies have demonstrated sphingolipid metabolites as potent messengers that mediate many cellular responses. In particular, long-chain base phosphorylates (LCBP), such as sphingosine-1-phosphate (S1P; d18:1$^{\Delta 4}$-P), play crucial signaling roles in modulating growth and survival in animals (Spiegel and Milstien, 2003). S1P has been demonstrated to play important signaling functions in mediating abscisic acid (ABA)-induced stomatal closure through G proteins, regulating pollen tube growth, and adaptation to stress tolerance in plants (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2008; Zhang et al., 2012; Wu et al., 2014). But Michaelson et al. (2009) reported that S1P does not mediate ABA responses. However, some evidence suggests that S1P mediates stomatal closure by darkness (Ma et al., 2012). Phytosphingosine-1-phosphate (Phyto-S1P; t18:1-P) was shown to be a prominent LCBP in plants and produced by sphingosine kinase (SPHK). Such evidence showed that Phyto-S1P functions in many physiological responses, including ABA guard-cell signaling, growth, and stress tolerance in plants (Coursol et al., 2005; Worrall et al., 2008; Guo et al., 2011, 2012; Dutilleul et al., 2012; Wu et al., 2014), e.g., Phyto-S1P induces stomatal closure and acts upstream of G proteins during ABA guard-cell signaling in *Arabidopsis* (Coursol et al., 2005). Phyto-S1P regulates *Arabidopsis* pollen tube growth through Ca^{2+} influx mediated by G proteins (Wu et al., 2014). However, it is largely unknown whether Phyto-S1P plays an important role in stomatal movement by darkness.

Most evidence suggests the involvement of intracellular pH during many plant physiological responses including stomatal movement by ABA, methyl jasmonate (MJ), or cytokinins (Irving et al., 1992; Gehring et al., 1997; Feijó et al., 1999; Felle, 2005;), e.g., cytosolic alkalization mediates the inductive effects of stomatal closure by ABA, MJ, and darkness (Irving et al., 1992; Suhita et al., 2004), whereas

Abbreviations: ABA, Abscisic acid; ASA, Ascorbic acid; BCECF-AM, 2′,7′-Bis(2-carboxyethyl)-5(6)- carboxyfluorescein acetoxymethyl ester;; CAT, Catalase; DL-*threo*-DHS, DL-*threo*-dihydrosphingosine; DMS, *N,N*-dimethylsphingosine; DMSO, Dimethyl sulfoxide; DPI, Diphenyleneiodonium; FC, Fusicoccin; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; H_2O_2, Hydrogen peroxide; IAA, Indole-3-acetic acid; KT, Kinetin; LCB, Long-chain base; LCBK, Long-chain base kinase; LCBP, Phosphorylated long-chain bases; MES, 2-(*N*-morpholino) ethanesulfonic acid; MJ, Methyl jasmonate; NO, Nitric oxide; PA, Phosphatidic acid; Phyto-S1P, Phytosphingosine-1-phosphate; PLDα1, Phospholipase Ds α1; SA, Salicylic acid; SPHK, Sphingosine kinase; S1P, Sphingosine-1-phosphate.

* Corresponding author.
E-mail address: mayinli1978@163.com (Y. Ma).

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cytosolic acidification participates in stomatal opening by indole-3-acetic acid, fusicoccin (FC), or kinetin (Irving et al., 1992). Additionally, changes in cytosolic pH modulate ion mobilization to cause stomatal movement (Blatt and Armstrong, 1993; Grabov and Blatt, 1997). Cytosolic pH and cytosolic Ca^{2+} oscillation have been reported coordinately to modulate ABA- or MJ-promoted stomatal movement (Islam et al., 2010). Recent data showed that darkness could induce cytosolic alkalization and cause stomatal closure (Ma et al., 2012, 2013).

It is well recognized that H_2O_2 mediates many physiological responses in plants including stomatal movement by ABA, darkness, ethylene, or UV-B (Neill et al., 2002; Desikan et al., 2004; She et al., 2004; He et al., 2005; Desikan et al., 2006). However, whether Phyto-S1P mediates darkness-promoted stomatal closure in broad bean remains unclear, and whether Phyto-S1P has any relationship with cytosolic pH and H_2O_2 in the process is also unknown. In this study, we observed that Phyto-S1P mediated darkness-promoted stomatal closure by inducing cytoplasmic alkalization and H_2O_2 synthesis and that Phyto-S1P-promoted cytoplasmic alkalization was necessary for the synthesis of H_2O_2.

2. Materials and methods

2.1. Plant materials

Broad bean (*Vicia faba* L.) was grown in controlled-environment chamber with a humidity of 80%, a 14 h: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 abaxial epidermis was peeled off carefully from the youngest, fully expanded leaves of 4-week-old seedlings and cut into strips about 5 × 5 mm. All treatments described below were at 25 ± 2 °C in either light (300 μmol m^{-2} s^{-1}) or darkness.

2.2. Stomatal bioassay

Stomatal bioassay was performed as described by McAinsh et al. (1996) with slight modifications. Freshly prepared epidermal strips were treated with MES-KCl buffer (10 mmol/L MES/KOH, 50 mM KCl, 100 μM $CaCl_2$, pH 6.15) alone or containing various compounds or inhibitors in light (300 μmol m^{-2} s^{-1}) or darkness for 3 h at 25 ± 2 °C, and final stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. Each experiment was repeated at least three times. The data presented are the means of 90 measurements ± s.e.

2.3. Endogenous H_2O_2 or cytosolic pH detection

H_2O_2 was detected with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) according to the method of Allan and Fluhr (1997), with some modifications. Cytosolic pH was detected with 2′,7′-Bis(2-carboxyethyl)- 5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) according to the description by Irving et al. (1992), with some changes. The treated epidermal strips were loaded in Tris-KCl buffer (Tris 10 mM and KCl 50 mM, pH 7.2) containing 50 μM H_2DCF-DA for 10 min or 20 μM BCECF-AM and 0.05% Pluronic F-127 at 25 ± 2 °C for 10 min in darkness. Then the epidermal strips were washed with Tris-KCl buffer and immediately examined by TCS SP5 laser-scanning confocal microscopy (LSCM) (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 pixel. In the experiments to detect the changes in H_2O_2 levels or cytosolic pH in guard cells with time after Phyto-S1P treatments, epidermal strips were treated in light for 3 h in order to make stomata open, and then were loaded with 50 μM H_2DCF-DA for 10 min or 20 μM BCECF-AM and 0.05% Pluronic F-127 for 10 min in darkness. The treated epidermal strips were washed with Tris-KCl buffer, and Phyto-S1P was added directly to Tris-KCl buffer, then the changes of H_2DCF-DA and BCECF-AM fluorescence were recorded within 30 min by TCS SP5 LSCM. The selected confocal images represented the same results from three replications for the approximately nine time measurements. The BCECF-AM or H_2DCF-DA fluorescent intensity presented are the mean ± s.e. of three duplicates. We analyzed the acquired images with Leica image software and processed with Photoshop 7.0 (Adobe, San Jose, CA, USA).

2.4. Solvent effects and statistical analysis

BCECF-AM and H_2DCF-DA were dissolved using dimethyl sulfoxide (DMSO). DL-*threo*-DHS, DMS stock solutions were prepared in ethanol and Phyto-S1P in chloroform. The final concentrations of DMSO, ethanol and chloroform, were 0.5%, 0.01%, and 0.06% (*v*/v), respectively, which didn't cause any significant effects in guard cell viability and stomatal aperture. Additionally, double-distilled water was used for dissolving other chemicals. The statistical significance of treatments were 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.

3. Results

3.1. Phyto-S1P participates in stomatal closure by darkness through causing cytosolic alkalization

Cytosolic pH changes are closely related to stomatal movement (Irving et al., 1992). Recent data showed the involvement of cytosolic alkalization in darkness-promoted stomatal closure (Ma et al., 2012, 2013). To find out whether Phyto-S1P participates in darkness-promoted stomatal closure, and whether Phyto-S1P has any effect on cytosolic pH in the process, we examined the effects of DL-*threo*-DHS, DMS (Buehrer and Bell, 1992; Kohama et al., 1998), and butyric acid (Gonugunta et al., 2008), on darkness-promoted stomatal movement and the change in cytosolic pH. Addition of 15 μM DL-*threo*-DHS, 5 μM DMS or 0.5 mM butyric acid inhibited stomatal closure by darkness (Fig. 1A). We determined the pH of guard cells by using the fluorescence probe BCECF-AM. The results showed that darkness largely caused an intense BCECF fluorescence (Fig. 1C, G) over the light treatment (Fig. 1B, G). DL-*threo*-DHS or DMS weakened darkness-caused BCECF fluorescence (Fig. 1D, E, G), as did butyric acid (Fig. 1F, G). These data indicated that Phyto-S1P probably participates in stomatal closure by darkness through causing cytosolic alkalization. Next, the responses of stomatal aperture as well as cytosolic pH to exogenous Phyto-S1P were examined. Addition of 4~8 μM Phyto-S1P significantly decreased stomatal aperture, and 6 μM Phyto-S1P was chosen to be the following experimental concentration (Fig. 2A). Additionally, the data from the wash-out experiments showed the reversible effects of Phyto-S1P on stomatal aperture (Fig. 2A). Butyric acid alone did not cause stomatal closure and cytosolic alkalization, but it significantly restricted Phyto-S1P-promoted stomatal closure and cytosolic alkalization (Fig. 2B, C). These results confirmed the conclusion that Phyto-S1P participated in stomatal closure by darkness through causing cytosolic alkalization.

3.2. Phyto-S1P mediates stomatal closure by darkness via inducing H_2O_2 synthesis

H_2O_2 synthesis has been shown to mediate darkness-induced stomatal closure (Desikan et al., 2004; She et al., 2004). We therefore explored further the relationship between Phyto-S1P and H_2O_2 during stomatal closure by darkness. Compared with the results of light treatment (Fig. 3A, E), darkness caused a marked rise in H_2O_2 levels (Fig. 3B, E), which confirmed previous findings (She et al., 2004). However, the effects of darkness on H_2O_2 synthesis were largely suppressed by DL-*threo*-DHS or DMS (Fig. 3C, D, E). Additionally, Phyto-S1P at the concentration of 6 μM obviously caused stomatal closure and induced