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SPHINGOSINE-1-PHOSPHATE MEDIATES STOMATAL CLOSURE CAUSED BY DARKNESS VIA INDUCING NITRIC OXIDE SYNTHESIS IN GUARD CELLS OF *VICIA FABA* L.

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Abstract

The relationship between sphingosine-1-phosphate (S1P) and the change of nitric oxide (NO) levels in guard cells of *Vicia faba* L. during darkness-induced stomatal closure was investigated. Results show that darkness closed stomata while increasing nitric oxide (NO) levels in guard cells. DL-*threo*-dihydrosphingosine (DL-*threo*-DHS) and *N*,*N*-dimethylsphingosine (DMS), two inhibitors of long-chain base kinases, not only suppressed darkness-induced stomatal closure but also inhibited NO synthesis caused by darkness, as did cPTIO (a specific NO scavenger) and *L*-NAME (an inhibitor of NO synthase). Additionally, exogenous S1P triggered stomatal closure and NO synthesis, but S1P-induced stomatal closure and NO synthesis were distinctly suppressed by cPTIO and *L*-NAME. Altogether, present data suggested that S1P mediated stomatal closure caused by darkness via inducing nitric oxide synthesis in guard cells of *Vicia faba*.

Introduction

Stomata are vital for plant life, as they control CO_2 uptake and water loss. The stomatal guard cells are able to sense and integrate multiple internal and external stimuli (Schroeder *et al.* 2001). Stomatal movements are known to be modulated by many factors, such as CO_2, humidity, abscisic acid (ABA) and indoleacetic acid (IAA) (Irving *et al.* 1992, Schroeder *et al.* 2001). Sphingolipids are a class of complex lipids. The metabolic precursor of sphingolipid long chain base (LCB) can be phosphorylated by specific LCB kinases (LCBKs), and phosphorylated LCB (LCB-P) has been implicated in salt and oxidative stress responses (Zhang *et al.* 2012). S1P, a Δ4-desaturated LCB-P, has been known to regulate many biological processes in animals (Spiegel and Milstien 2002). However, its biological function in plants remains unclear. S1P has been shown to mediate ABA-induced stomatal closure (Ng *et al.* 2001, Coursol *et al.* 2003, Worrall *et al.* 2008), whereas in others it is reported to have no effect on stomatal closure (Michaelson *et al.* 2009). Recently, our study indicated that S1P mediated darkness-induced stomatal closure in *Vicia faba* (Ma *et al.* 2012).

Nitric oxide (NO) is an unique signalling molecule with multiple biological functions (García-Mata and Lamattina 2001, Desikan *et al.* 2002). Nitric oxide synthase (NOS) and nitrate reductase (NR) have been known to be potential source of NO in plant cells (Neill *et al.* 2002, Desikan *et al.* 2002). There is now compelling evidence that NO mediates diverse plant physiological processes, including ABA-, UV-B- and darkness-induced stomatal movement (Beligni and Lamattina 2000, Neill *et al.* 2002, Zhao *et al.* 2004; She *et al.* 2004, He *et al.* 2005). Previous studies have shown that S1P, cytosolic alkalization and H_2O_2 production are involved in darkness-induced stomatal closure (Desikan *et al.* 2004, She *et al.* 2004, Ma *et al.* 2012, 2013), and the relationships among them have been proved (Ma *et al.* 2012). However, it is unknown whether S1P is related to NO in guard cells in darkness. This report provides some evidence for the relationship between S1P and NO in guard cells of *Vicia faba* in darkness by means of epidermal strip bioassay and using laser-scanning confocal microscopy.

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Materials and Methods

Broad bean (*Vicia faba* L.) was grown in the same conditions as described by Ma *et al.* (2013). The epidermis strips were prepared as described by Ma *et al.* (2013).

Stomatal bioassay was performed as described by McAinsh *et al.* (1996) with slight modifications. In brief, freshly prepared epidermal strips were treated with MES-KCl buffer (10 mmol/l MES/KOH, 50 mmol/l KCl, 100 µmol/l $CaCl_2$, pH 6.15) alone or containing various compounds or inhibitors in light (300 µmol/m^2·s) or darkness at 25 ± 2°C for 3 hrs and then stomatal apertures were recorded with a light microscope and an eyepiece graticule previously calibrated with a stage micrometer. Experiments were started at the same time of the day to avoid any potential rhythmic effects on stomatal aperture.

NO in guard cells was monitored by using fluorescent indicator dye DAF-2 DA, as previously described (Kojima *et al.* 1998), with minor changes. After treatments, the epidermal strips were loaded in tris-KCl buffer (tris 10 mmol/l and KCl 50 mmol/l, pH 7.2) containing 10 µmol/l DAF-2 DA for 30 min in darkness at 25 ± 2°C. Then excess dye was washed off with tris-KCl buffer in darkness, the epidermal strips were immediately examined by TCS SP5 laser-scanning confocal microscopy, as previously described (Ma *et al.* 2012). Each experiment was repeated at least three times. The selected confocal images represented the same results from three times repeat.

DMSO was the solvent used for DAF-2 DA. S1P, DL-*threo*-DHS, DMS and other chemicals stock solutions were prepared as previously described (Ma *et al.* 2012). The statistical significance of treatments was checked using one-way ANOVA followed by DMRT ($p < 0.05$).

Results and Discussion

The results showed that DL-*threo*-DHS (15 µmol/l) and DMS (5 µmol/l), two inhibitors of long-chain base kinases (Buehrer and Bell 1992, Kohama *et al.* 1998), NO specific scavenger cPTIO (200 µmol/l, García-Mata and Lamattina 2001) and mammalian NOS inhibitor *L*-NAME (25 µmol/l, Wendehenne *et al.* 2001, Neill *et al.* 2003) all largely suppressed darkness-induced stomatal closure (Fig. 1), which is consistent with the previous results (She *et al.* 2004, Ma *et al.* 2012). These data suggested that both S1P and NO mediate darkness-induced stomatal closure.

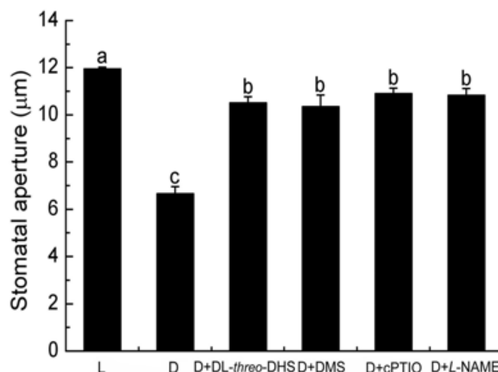

Fig. 1. DL-*threo*-DHS, DMS, cPTIO and *L*-NAME inhibit darkness-induced stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer alone in light (L), or incubated in MES/KCl buffer alone (D), or containing 15 µmol/l DL-*threo*-DHS (D + DL-*threo*-DHS), 5 µmol/l DMS (D + DMS), 200 µmol/l cPTIO (D + cPTIO) or 25 µmol/l *L*-NAME (D + *L*- NAME) in darkness, respectively. Values of stomatal apertures are the means of 90 measurements ± standard error of three independent experiments. Means denoted by different letters differ significantly at $p < 0.05$ according to DMRT.

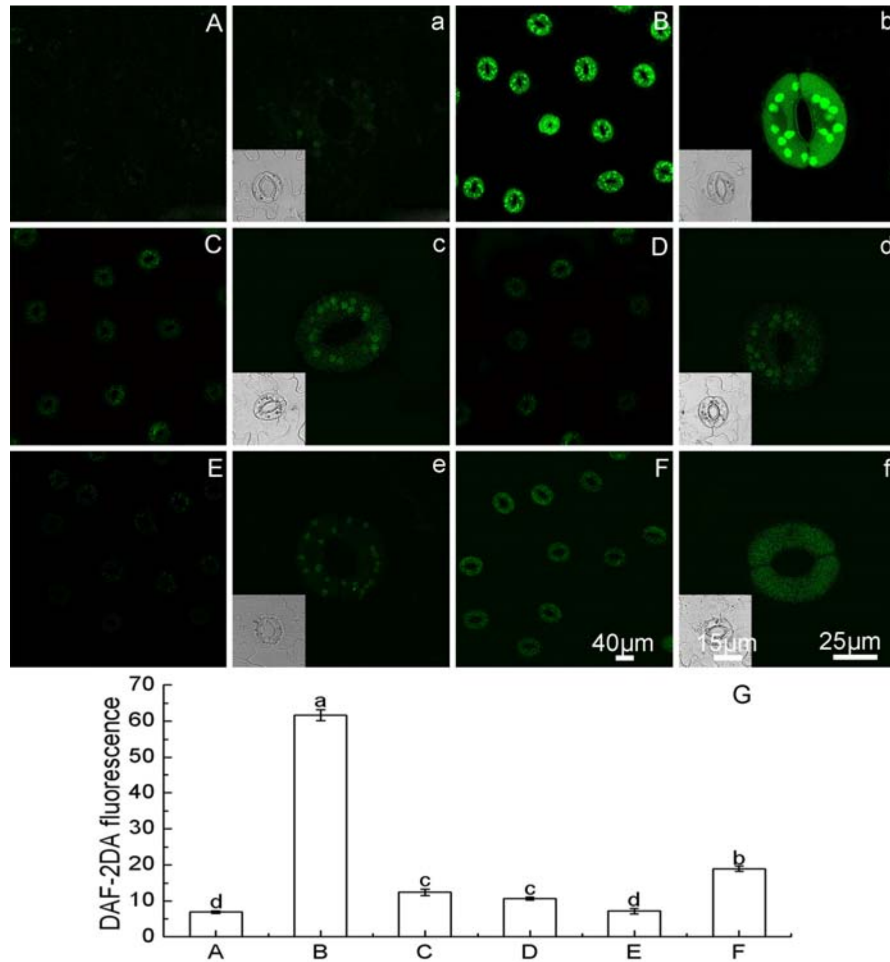

Fig. 2. DL-*threo*-DHS, DMS, cPTIO and *L*-NAME suppress darkness-induced NO synthesis in guard cells. (A) Guard cells were treated with MES/KCl buffer in light, or (B) treated with MES/KCl buffer, or containing (C) 15 µmol/l DL-*threo*-DHS, (D) 5 µmol/L DMS, (E) 200 µmol/l cPTIO or (F) 25 µmol/l *L*-NAME in darkness. Then the treated strips were immediately loaded with 10 µmol/l DAF-2 DA, and were examined by laser-scanning confocal microscopy. The guard cells shown in images (a-f) are representative of those in images (A-F), respectively, and the insets show the corresponding bright-field images. Scale bars in (F), (f), and (f) inset represent 40, 25, and 15 µm, and are for images from (A-F), (a - f) and (a inset - f inset, respectively. (G) The average fluorescent intensity of guard cells in images from (A) to (F), data are the means ± standard error (n = 9). The means denoted by different letters in (G) differ significantly at $p < 0.05$ according to Duncan's multiple range tests.

Results obtained by present authors further showed the effects of DL-*threo*-DHS, DMS, cPTIO and *L*-NAME on NO levels in darkness. Epidermal strips were loaded with NO-specific fluorophore (DAF-2 DA), which allows the detection of NO presence in both animal and plant cells (Foissner *et al.* 2000, Kojima *et al.* 1998). As shown in Fig. 2, compared with the light treatment (Fig. 2A and G), darkness induced an intense NO fluorescence in guard cells (Fig. 2B and G), consistent with a previous report (She *et al.* 2004). However, DL-*threo*-DHS and DMS suppressed darkness-induced NO fluorescence (Fig. 2C, D and G), like cPTIO and *L*-NAME (Fig. 2E, F and G). Linked with the finding that DL-*threo*-DHS and DMS inhibit darkness-induced S1P synthesis

(Ma *et al.* 2012) and the results that DL-*threo*-DHS, DMS, cPTIO and *L*-NAME all inhibited darkness-induced stomatal closure (Fig. 1), these data not only confirmed that S1P and NO are essential for stomatal closure by darkness, but also provided evidence that S1P mediates the process via inducing NO synthesis.

Authors next studied the effects of cPTIO and *L*-NAME on exgenous S1P-induced stomatal closure and NO synthesis. Six μmol/l S1P obviously closed stomata, cPTIO and *L*-NAME largely prevented the inductive effect of S1P on stomatal closure (Fig. 3). Additionally, S1P effectively induced NO synthesis, cPTIO and *L*-NAME largely obolished S1P-induced NO synthesis (Fig. 4). These data clearly showed that S1P indeed can close stomata via inducing NO synthesis in guard cells.

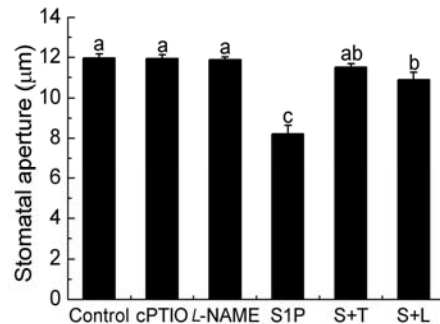

Fig. 3. cPTIO and *L*-NAME inhibit exogenous S1P-induced stomatal closure. Isolated epidermal strips were incubated in MES/KCl buffer (control), or containing 200 μmol/l cPTIO, 25 μmol/l *L*-NAME, 6 μmol/l S1P, 6 μmol/l S1P + 200 μmol/l cPTIO (S + T) or 6 μmol/l S1P + 25 μmol/l *L*-NAME (S + L) in light, respectively. Other explanations are the same as in Fig. 1.

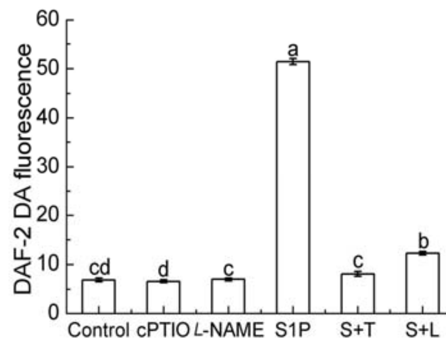

Fig. 4. cPTIO and *L*-NAME suppress exogenous S1P-induced NO synthesis in guard cells. Treatments were the same as in Fig. 3. The treated strips were loaded with 10 μmol/l DAF-2 DA, and were examined by laser-scanning confocal microscopy. Data show the average fluorescent intensity ± standard error of three independent experiments (n = 9). Means denoted by different letters differ significantly at $p < 0.05$ according to DMRT.

In animals, S1P is a well-established messenger molecule. It has been reported to participate in many signalling pathways (Spiegel and Milstien 2002, Chalfant and Spiegel 2005). In plants, S1P is also shown to be a new calcium-mobilizing molecule and to mediate ABA-induced stomatal closure (Ng *et al.* 2001, Coursol *et al.* 2003). Coursol *et al.* (2003) reported that SPHK, one of LCBKs, mediates ABA-led stomatal closure, and S1P acts upstream of the sole prototypical putative heterotrimeric G protein a-subunit encoded by the GPA1 gene, as in mammals. And the effect of S1P on stomatal apertures is also shown to be negatively regulated by GCR1, one putative

GPCR in Arabidopsis (Pandey and Assmann 2004). However, Michaelson *et al.* (2009) reported that S1P does not mediate ABA-induced stomatal closure in *Arabidopsis*. Previous results of the authors showed that S1P mediates darkness-induced stomatal closure through raising cytosol pH and H_2O_2 levels in guard cells of *Vicia faba* (Ma *et al.* 2012), which is consistent with the results of Ng *et al.* (2001) and Coursol *et al.* (2003). The discrepancy between our results and the data of Michaelson *et al.* (2009) may reflect the difference between ABA and darkness signal transduction mechanism in guard cells, or be due to the different plant species used.

NO has been proved to mediate various plant physiological processes, including stomatal closure by darkness (She *et al.* 2004). However, up to data, the relationship between S1P and NO is unknown. Present results clearly showed that DL-*threo*-DHS and DMS, cPTIO and *L*-NAME all significantly prevented darkness-induced stomatal closure in *Vicia faba*, which is similar to the previous results (Ma *et al.* 2012, She *et al.* 2004). In addition, darkness-induced NO synthesis could be supressed by DL-*threo*-DHS and DMS, as did cPTIO and *L*-NAME. Together with the facts that DL-*threo*-DHS and DMS suppressed darkness-induced S1P synthesis (Ma *et al.* 2012), these data showed that S1P mediates darkness-induced stomatal closure via inducing NO synthesis. Authors' further results indicated that exogenous S1P induced NO synthesis and stomatal closure, and cPTIO and *L*-NAME largely reversed S1P-induced NO synthesis and stomatal closure, which unequivocally consolidated the conclusion that S1P mediated stomatal closure caused by darkness via inducing NO synthesis in guard cells of *Vicia faba*. Additionally, the inhibition of darkness- and S1P-induced NO synthesis by *L*-NAME suggested that NOS-like enzyme might be responsible for NO synthesis induced by S1P in *Vicia faba* guard cells in darkness.

Present study has provided evidence that S1P mediates stomatal closure caused by darkness via inducing NO synthesis in guard cells of *Vicia faba*. The findings expand the plant biological function of S1P, and also help to further elucidate darkness signal transduction mechanisms in plant guard cells. However, whether or not the intracellular Ca^{2+} release and the protein phosphorylation act in S1P-induced NO synthesis need to be studied.

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