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Abscisic acid (ABA) inhibits light-induced stomatal opening through calcium- and nitric oxide-mediated signaling pathways ☆

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Abstract

Nitric oxide (NO) is an important signaling component of ABA-induced stomatal closure. However, only fragmentary data are available about NO effect on the inhibition of stomatal opening. Here, we present results supporting that, in *Vicia faba* guard cells, there is a critical Ca^{2+}-dependent NO increase required for the ABA-mediated inhibition of stomatal opening. Light-induced stomatal opening was inhibited by exogenous NO in *V. faba* epidermal strips. Furthermore, ABA-mediated inhibition of stomatal opening was blocked by the specific NO scavenger cPTIO, supporting the involvement of endogenous NO in this process. Since the raise in Ca^{2+} concentration is a pre-requisite in ABA-mediated inhibition of stomatal opening, it was interesting to establish how does Ca^{2+}, NO and ABA interact in the inhibition of light-induced stomatal opening. The permeable Ca^{2+} specific buffer BAPTA-AM blocked both ABA- and Ca^{2+}- but not NO-mediated inhibition of stomatal opening. The NO synthase (NOS) specific inhibitor L-NAME prevented Ca^{2+}-mediated inhibition of stomatal opening, indicating that a NOS-like activity was required for Ca^{2+} signaling. Furthermore, experiments using the NO specific fluorescent probe DAF-2DA indicated that Ca^{2+} induces an increase of endogenous NO. These results indicate that, in addition to the roles in ABA-triggered stomatal closure, both NO and Ca^{2+} are active components of signaling events acting in ABA inhibition of light-induced stomatal opening. Results also support that Ca^{2+} induces the NO production through the activation of a NOS-like activity.

Keywords: ABA; Calcium; Light; Nitric oxide; Nitric oxide synthase; Stomatal opening

Stomata are epidermal pores through which plants regulate gas exchange (i.e., CO_2 uptake and water loss during transpiration). Regulation of stomatal pore size is driven by volume variations of a pair of specialized cells known as guard cells. A rise in turgor pressure increases guard cell volume, and hence stomatal pore size. Reciprocally, a reduction of turgor pressure reduces guard cell volume reducing the size of the stomatal pore [1–3].

Gas exchange regulation is crucial for plant growth and developmental processes, therefore, guard cells must be able to sense and integrate multiple internal (chemical) and external (environmental) stimuli [4], among them light is one of the dominant environmental signals controlling stomatal movements [3]. Light induces the extrusion of H^+ by the activation of plasma membrane H^+-ATPases [5]. This proton efflux hyperpolarizes the plasma membrane, activating voltage gated K^+ channels, that pump K^+ into the cell, increasing guard cell turgor and though stomatal pore size [4]. Probably the best studied responses to internal stimuli are those generated by the phytohor-

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mone abscisic acid (ABA).[1] Under water stress conditions ABA accumulates in guard cells inducing stomatal closure and inhibiting light-induced stomatal opening [5,6]. However, while the mechanisms commanding ABA-induced stomatal closure have been widely described, those involved in ABA-inhibition of stomatal opening are still partially understood. The coordination of the responses triggered by the multiple stimuli sensed by guard cells is commanded by a network of intricate signal transduction pathways. This network has many signaling components, and a small number of highly interconnected components which are central for the functioning of the network [7]. Recently, Ca^{2+} has been proposed as one of these highly interconnected components [7]. The increase of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) not only is a common signal for most of the stimuli sensed by guard cells, but is also a common component in the signaling pathways leading to both inhibition of stomatal opening and stomatal closure induction [8].

Besides Ca^{2+}, the complexity of guard cell signaling network requires the participation of almost every second messenger reported in plants [4,9,10]. Six years ago, nitric oxide (NO) was presented as a novel component involved in this signaling network [11]. Since that report, both NO source and NO role has become a hot topic in guard cell biology [12,13]. As a result we now know that, in guard cells, NO can be synthesized both by nitrate reductase (NR) [14] and nitric oxide synthase (NOS)-like activity [15]. NO was reported to be a key component of ABA-dependent induction of stomatal closure [15,16] through the regulation of inward rectifying potassium channels (K^+_{in}) [17]. Interestingly, NO was also reported to be involved in dark-induced stomatal closure [11,18], and this NO action was prevented by a NOS inhibitor [18]. However, there is only one very recent paper, showing the participation of NO in the ABA-promoted inhibition of blue light-induced stomatal opening [19]. In the present work, we report evidence on the signaling pathways operating during the NO inhibition of light-induced stomatal opening in *Vicia faba* guard cells, and on the participation of NO in ABA-mediated inhibition of stomatal opening. Data indicate that Ca^{2+} and NO are key players in the ABA inhibition of light-induced stomatal opening.

Materials and methods

Plant material and chemicals

Vicia faba plants were germinated and grown in soil in a greenhouse under sunlight with supplemental light (200 $\mu E\ m^{-2}\ s^{-1}$) when needed, with a 16 h photoperiod and an average of 20 °C ± 5 temperature. The following reagents have been used for the chemical treatments: 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl) ester, BAPTA-AM (Calbiochem); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, carboxy-PTIO (Molecular Probes); 4,5-diaminofluorescein diacetate, DAF-2DA (Molecular Probes); ^{N}G-NO_2-L-Arg-OMe, L-NAME (Sigma); *S*-nitroso-*N*-acetyl-penicyllamine, SNAP (Molecular Probes).

Preparation of epidermal strips

Young fully expanded leaves were harvested from 4- to 6-week-old plants. Abaxial epidermis was striped with stainless steel forceps and immediately floated in 10 mM MES pH 6.1, 10 mM KCl (opening buffer). Two to 4 mm^2 strips sections were cut and put in 24 well plates containing 400 μl of opening buffer per well, for future treatments.

Dark treatments

Epidermal strips were pre-incubated in opening buffer for 2 h and 30 min in the dark at 25 °C. After pre-incubation period, treatments were performed in the same opening buffer in the dark. The different reagents corresponding to each treatment were added under green light to avoid light-induced stomatal opening [20]. After 30 min of incubation, some of the strips were mounted in a microscope slide to quantify stomatal aperture (T0), and the rest were put under light (200 $\mu E\ m^{-2}\ s^{-1}$) at 25 °C for different periods.

Stomatal opening measurements

Epidermal strips were analyzed with a Nikon E200 (Nikon, Japan) microscope with a 40× objective. For quantifying, microscope fields were digitalized with a Nikon Coolpix 990 (Nikon, Japan) digital camera. The width of the stomatal aperture was measured using the image analysis software Matrox Inspector 2.2 (Matrox Electronic System, Dorval, Canada). The pore width from 20 to 30 stomata per treatment per experiment was measured and pooled together for statistical analysis. Data are expressed as μm and are means ± SE.

Fluorescence microscopy

Nitric oxide (NO) was visualized using the specific NO dye DAF-2 DA (Molecular Probes, Oregon, USA). Epidermal strips pre-incubated in opening buffer for 2 h and 30 min in the dark were loaded with 15 μM DAF-2 DA for 30 min before washing with fresh buffer for another 20 min. Strips were then incubated for 30 min with the different compounds in the dark. Images were visualized immediately before the dark/light shift using a Nikon E200 epifluorescent microscope (excitation 495 nm; emission 515–555 nm). Images were acquired with a Nikon coolpix 990 digital camera (Nikon, Japan), and analyzed using ImageJ software (ImageJ, US National Institutes of Health, MD, USA). Fluorescence was quantified as pixel intensity of a fixed area for all guard cells. Fluorescence values are presented as relative units (RU) with respect to control treatments and are expressed as means ± standard error. Fifteen to thirty guard cells were observed per experiment in each treatment at least in four independent replicates.

Statistical analysis

Data analyses were performed using Sigmaplot for Windows (SPSS Science, Chicago, IL, USA), and the different statistical analyses were performed using Statistica v. 6.0 analysis software (Stat Soft Inc., Tulsa-OK, USA).

Stomatal closure induction (Suplemental data)

Epidermal strips were pre-incubated in opening buffer for 2 h in white light at 25 °C. After pre-incubation period, treatments were performed in the same opening buffer. Stomatal aperture measurements were performed after incubating the epidermal strips for 90 min in white light.

[1] *Abbreviations used:* ABA, abscisic acid; BAPTA-AM, 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl) ester; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; DAF-2DA, 4,5-diaminofluorescein diacetate; L-NAME, ^{N}G-NO_2-L-Arg-OMe; NO, nitric oxide; NR, nitrate reductase; NOS, nitric oxide synthase; SNAP, *S*-nitroso-*N*-acetyl-penicyllamine.

Results

NO blocks light-induced stomatal opening

Two previous publications have studied the effect of NO on the inhibition of stomatal opening, one of them showed that in Arabidopsis plants, ABA-dependent inhibition of stomatal opening was not affected in the NR double mutant *nia1/nia2*, while the second one showed that, in Arabidopsis mutant *Atnos1*, ABA was unable to inhibit stomatal opening [21]. These results lead us to investigate if NO has any effect in light-induced stomatal opening in *V. faba* epidermal strips, and try to understand the mechanism involved in this response. With that aim, *V. faba* epidermal strips were incubated for 3 h in the dark, in order to set stomata in a closed state. Then, strips were treated with 150 μM of the NO donor *S*-nitroso-*N*-penicyllamine (SNAP) or kept in opening buffer (control) and maintained in the dark for another 30 min. Stomatal aperture was measured before the dark/light shift and after different light periods. An additional set of strips was treated with 20 μM ABA as a positive control. Fig. 1A shows that stomatal aperture values of SNAP-treated epidermal strips remained constant through all the assayed times, showing statistical differences with the control treatments 30 min after the dark/light shift. Furthermore, it can be observed that, as previously reported [22], ABA-treated epidermal strips showed no significant increases of stomatal aperture values after the dark/light shift (Fig. 1A). Further experiments showed that when the epidermal strips were treated with different concentrations of SNAP, ranging from 0 to 200 μM, stomatal opening was inhibited in a dose dependent manner after 90 min of white light exposure (Fig. 1B). In addition, when epidermal strips were treated with 200 μM of the NO donor *S*-nitrosoglutathione (GSNO), the mean stomatal aperture was as low as that obtained with 200 μM SNAP (Fig. 1B, inset), indicating that the inhibition of stomatal opening is due to the NO released by the donor and not to any by-product of the donor molecule. Since SNAP was reported to release, in solution, 2 nM/NO/min/μM [23], it can be assumed that NO concentrations as low as 20 nM are able to inhibit light-induced stomatal opening. These results demonstrate that exogenous NO inhibits light-induced stomatal opening.

ABA induces NO production through a NOS-like activity and is required for inhibition of stomatal opening

The occurrence of an ABA and NO interaction has been shown in different plants and physiological processes [15,16,18,24–26]. In order to study the occurrence of an ABA-NO interaction in the inhibition of stomatal opening, pre-darkened epidermal strips were incubated with SNAP or ABA in presence or absence of the specific NO scavenger carboxy PTIO (cPTIO). As expected, cPTIO completely blocked SNAP-mediated inhibition of stomatal opening (Fig. 2A). Interestingly, cPTIO also blocked ABA-mediated inhibition of stomatal opening, showing that, endogenous NO is required by ABA for the regulation of this process.

The source of the endogenous production of NO is still uncertain for many of the physiological processes in which

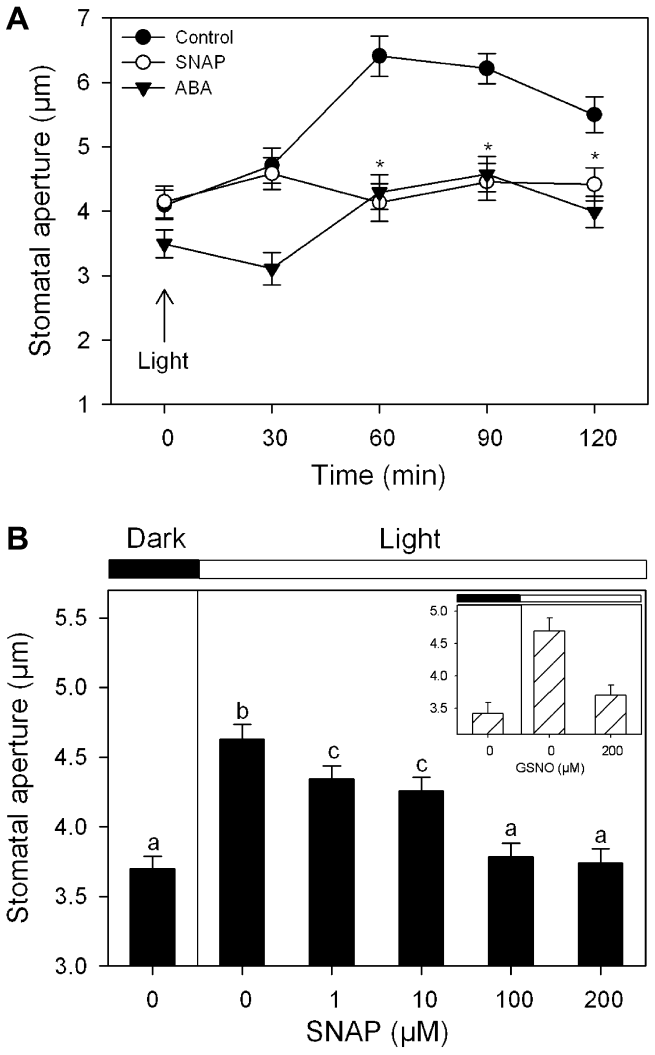

Fig. 1. NO and ABA inhibition of light-induced stomatal opening. Stomatal aperture measurements from *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark with opening buffer (10 mM MES, pH 6.1; 10 mM KCl). (A) After pre-incubation, strips were then treated with 20 μM ABA (▼), 150 μM SNAP (○) or kept in opening buffer (control, ●) for 30 min in darkness and subsequently incubated for different light periods (time). Stomatal aperture was measured as pore width and expressed in μm. Asterisks denote statistical differences between SNAP and control values (Fisher LSD post hoc test, $p < 0.05$). (B) After pre-incubation, strips were then treated with different SNAP concentrations (SNAP) for 30 min in darkness and subsequently incubated in white light for 90 min. (inset) Stomatal aperture measurements from *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark with opening buffer, then treated with or without 200 μM GSNO for 30 min and subsequently incubated in white light for 90 min. Stomatal aperture was measured as pore width and expressed in μm. Different letters denote statistical differences between treatments (Fisher LSD post hot test, $p < 0.05$). Values are expressed as means ± SE and represent the mean of 20–30 stomata per experiments from at least three independent experiments ($n = 60$–150).

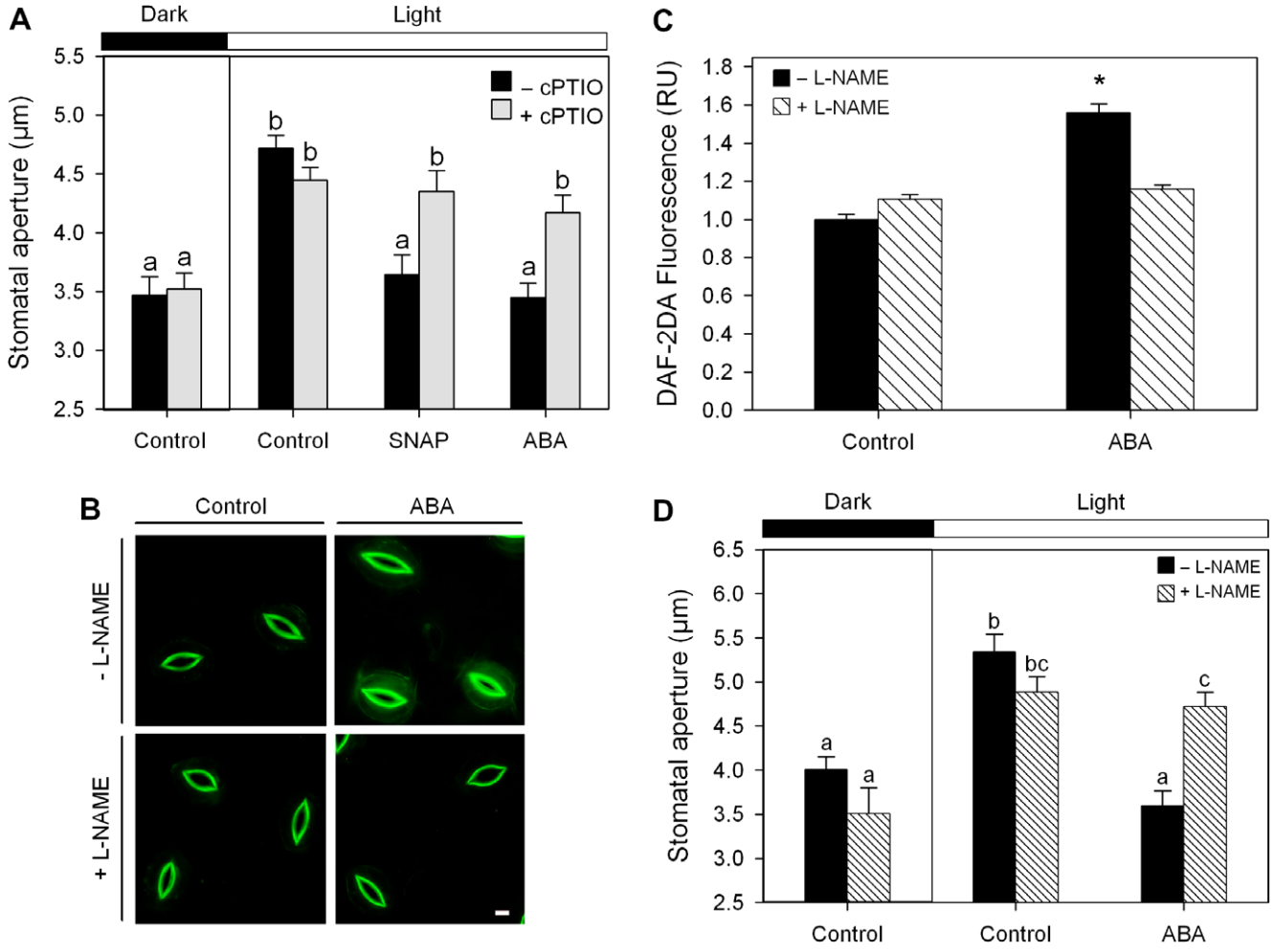

Fig. 2. Endogenous NO is required for ABA-dependent inhibition of stomatal opening. (A) Stomatal aperture measurement from *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark, in opening buffer (10 mM MES, pH 6.1; 10 mM KCl), then treated with or without 200 μM cPTIO for 30 min in the same opening buffer. Strips were then treated for 30 min without (control), with 150 μM SNAP or with 20 μM ABA. (B) Images of one representative experiment of *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark in opening buffer. Strips then were loaded with 15 μM DAF-2DA (see Materials and methods) in opening buffer and treated with opening buffer (control) or with 20 μM ABA (ABA) in presence or absence of 1 mM L-NAME for 30 min in the dark. Pictures were taken immediately after the dark/light switch. Scale bar, 20 μm. (C) Green fluorescent pixel intensity of four independent experiments. Fluorescent values are expressed as relative units (RU) with respect to the control treatment in absence of L-NAME. Values are the mean of 108 guard cells and are expressed as means ± SE. Asterisks denote statistical differences between the conditions with or without L-NAME. (D) Epidermal strips pre-incubated 2 h and 30 in the dark, in opening buffer, then treated with or without 1 mM L-NAME for 30 min in the same opening buffer. Strips were then treated for 30 min without (control) or with 20 μM ABA. Stomatal aperture (A and D) was measured immediately before (dark) or 90 min after the dark/light shift, and is expressed in μm. Values are expressed as means ± SE and represent the mean of 20–30 stomata per experiment from at least three independent experiments ($n = 60$–150). Different letters denote statistical difference (Fisher LSD post hoc test, $p < 0.05$).

NO was reported to participate in plants. One of the reasons might be that many potential sources of NO have been reported in plants [27]. In *A. thaliana* guard cells, nitrate reductase (NR) was shown to be responsible for ABA-induced NO production triggering stomatal closure. However, ABA inhibition of stomatal opening was not affected in the NR double mutant *nia1/nia2* [15]. Regarding NO synthase (NOS), even though the certainty of AtNOS1 as a plant protein sharing NOS activity is controversial [28–30], NOS activity was demonstrated in many and diverse plant systems [27]. Since ABA was shown to be unable to inhibit stomatal opening in the *Atnos1* mutant, imaging approaches using the NO specific fluorescent probe 2,4-aminofluorescein-diacetate (DAF-2DA) were conducted in order to see if ABA induces NO production in *V. faba* through a NOS-like activity. With that aim, we studied the effect of the specific NOS inhibitor L-NAME on ABA-mediated NO production in *V. faba* epidermal strips, immediately after the dark/light shift. Fig. 2B depicts pictures of one representative experiment showing that ABA-induced increase of endogenous NO levels was blocked in presence of 1 mM L-NAME. Fig. 2C summarizes the quantification of the pixel intensity of guard cells from four independent experiments and shows a 1.6-fold increase of DAF-2DA fluorescence in ABA treatment ($n = 108$) with respect to the control treatment (Fisher

LSD $P < 0.05$). The increase of DAF-2DA fluorescence was not evident when ABA was added in the presence of L-NAME ($n = 108$). The involvement of a NOS-like activity in ABA-mediated inhibition of stomatal opening was supported by stomatal aperture experiments. Fig. 2D shows that ABA was unable to inhibit light-induced stomata opening in pre-darkened epidermal strips incubated with 1 mM L-NAME. This set of experiments not only indicate that endogenous NO is required for this particular physiological process, but also that a NOS-like activity seems to be responsible for the NO production involved in ABA-mediated inhibition of stomatal opening.

Cytosolic Ca^{2+} increase mediates the ABA-induced NO production

ABA-mediated inhibition of stomatal opening was reported to occur via an increase in $[Ca^{2+}]_{cyt}$, which down regulates inward rectifying K^+ channels and H^+-ATPases activity [2,31]. Therefore, we studied the possibility of a cross-talk between Ca^{2+} and NO in the signaling pathways regulated by ABA during the inhibition of stomatal opening. Technical difficulties impede us to perform electrophysiological approaches without exposing the guard cells to light, therefore, stomatal aperture experiments using the non fluorescent permeable Ca^{2+} buffer 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM) [32] were done in order to study putative interaction between NO and Ca^{2+}. Fig. 3A shows stomatal aperture values of pre-darkened epidermal strips treated with ABA or not (control) in presence or absence of 250 μM BAPTA-AM. As expected, 250 μM BAPTA-AM prevented the inhibition of stomatal aperture in ABA-treated strips. However, BAPTA-AM has no effect on NO-dependent inhibition of stomatal opening (Fig. 3A).

To investigate if Ca^{2+} has any effect on endogenous NO production, imaging experiments similar to those shown in Fig. 2, were conducted by treating DAF-2DA loaded epidermal strips with ABA in presence of BAPTA-AM. Fig. 3B shows that DAF-2DA fluorescence level in ABA treatment was approximately 1.6-fold higher than that of the control treatment without ABA ($n = 188$). Interestingly, when ABA was added together with BAPTA-AM, there was no appreciable difference in green fluorescence with respect to the control treatment ($n = 188$). These last results strongly support the occurrence of an interaction between NO and Ca^{2+} in ABA-mediated inhibition of stomatal opening.

Ca^{2+} inhibits stomatal opening through a NOS-like-dependent NO production

Previous reports have shown that, in epidermal fragments of *Commelina communis* and *Arabidopsis thaliana*, exogenous application of Ca^{2+} inhibits light-induced stomatal opening [33,34]. Therefore, we tested if exogenous

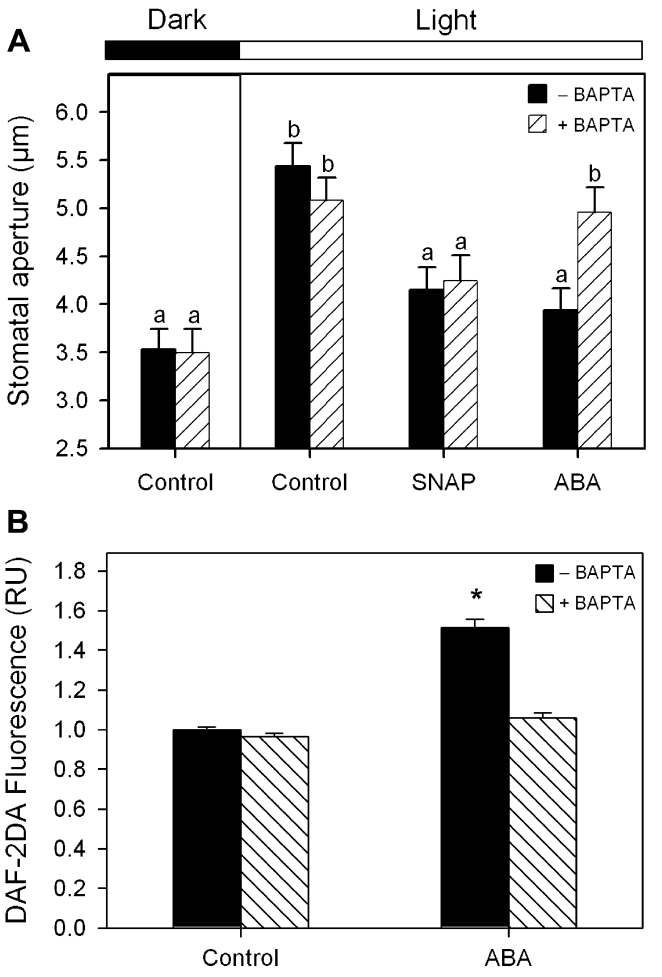

Fig. 3. Ca^{2+} is required for ABA dependent NO production in the inhibition of stomatal opening. Stomatal aperture measurement from *Vicia faba* epidermal strips pre-incubated 2 h and 30 in in the dark, in opening buffer (10 mM MES, pH 6.1; 10 mM KCl), then treated with or without 250 μM BAPTA-AM for 30 min in the same opening buffer. Strips were then treated for 30 min without (control), 150 μM SNAP or 20 μM ABA. (A) Stomatal aperture was measured immediately before (dark) or 90 min after the dark/light shift, and is expressed in μm. Values are expressed as means ± SE and represent the mean of 20–30 stomata per experiment from at least three independent experiments ($n = 60$–150). Different letters denote statistical difference (Fisher LSD post hoc test, $p < 0.05$). (B) Green fluorescence pixel intensity expressed as relative units (RU) with respect to control treatment in absence of BAPTA-AM. Values are the quantification of guard cells from four independent treatments ($n = 188$) and are expressed as means ± SE. Asterisk denotes statistical differences with respect to control treatment (Fisher LSD post hoc test, $p > 0.05$).

application of $CaCl_2$ could also be able to inhibit stomatal opening in *V. faba* epidermal strips. Fig. 4A shows that $CaCl_2$ treatments ranging from 1 to 1000 μM were able to inhibit light-induced stomatal opening. Interestingly, when epidermal strips were treated with ABA plus different $[Ca^{2+}]$ in presence of cPTIO, ABA was ineffective to inhibit stomatal opening (Fig. 4B). Since DAF-2 fluorescence was proved to be unaffected by the presence of divalent cations [35], we tested if in our system exogenous addition of $CaCl_2$ has any effect on endogenous NO concentration.

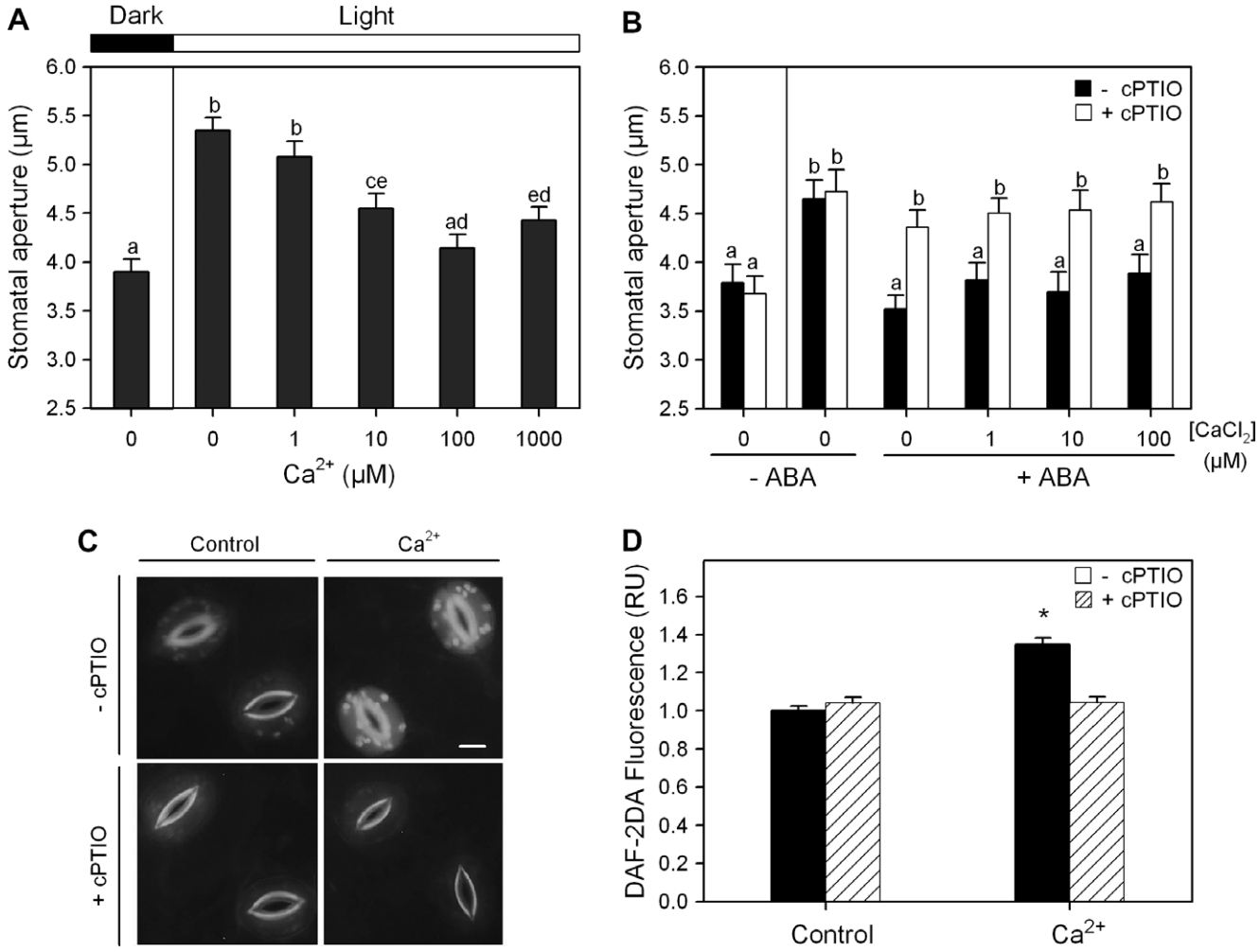

Fig. 4. NO and Ca^{2+} interaction in ABA-mediated inhibition of light-induced stomatal opening. Stomatal aperture measurements from *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark with opening buffer (10 mM MES, pH 6.1; 10 mM KCl), then treated in the same opening buffer with (A) different concentrations of $CaCl_2$ ([Ca^{2+}]); or (B) different concentrations of $CaCl_2$ ([Ca^{2+}]) without (−ABA) or with 20 μM ABA (+ABA) for 30 min in absence (black bars) or presence (white bars) of 200 μM cPTIO. Subsequently, strips were incubated in white light. Stomatal aperture was measured as pore width immediately before (dark) or 90 min after the dark/light shift, and is expressed in μm. Different letters denote statistical differences between treatments (Fisher LSD post hoc test, $p < 0.05$). Values are expressed as means ± SE and represent the mean of 20–30 stomata per experiments from at least three independent experiments ($n = 60$–150). (C) Images of one representative experiment where epidermal strips were loaded with DAF-2DA (see Materials and methods) and treated with opening buffer (control) or with 1 mM $CaCl_2$ (Ca^{2+}) in presence or absence of 200 μM cPTIO for 30 min in the dark. Pictures were taken immediately before the dark/light shift. Scale bar, 20 μm (D) strips pre-incubated 2 h and 30 min in the dark were loaded with DAF-2DA (see Materials and methods) in opening buffer and then treated without (control) or with 20 μM ABA (ABA) in presence or absence of 200 μM cPTIO for 30 min in the dark. Green fluorescence pixel intensity is expressed as relative units (RU) with respect to control treatment in absence of cPTIO. Values are the quantification of guard cells from four independent treatments ($n = 76$) and are expressed as means ± SE. Asterisk denotes statistical differences with respect to control treatment (Fisher LSD post hoc test, $p > 0.05$).

Fig. 4C depicts photographs of one representative experiment and shows that 1 mM $CaCl_2$ increased DAF-2DA fluorescence with respect to control treatment. Quantification of guard cells from four independent experiments ($n = 76$) showed that Ca^{2+} treatments raised DAF-2DA fluorescence up to 1.35-fold with respect to control treatment ($P < 0.05$) (Fig. 4D). In addition, Fig. 4B also shows that cPTIO prevented the $CaCl_2$-mediated inhibition of stomatal opening, indicating that NO is downstream from Ca^{2+}. Moreover, Fig. 4D shows that, as expected, cPTIO was able to decrease the endogenous NO generated by Ca^{2+} treatment.

In order to further investigate the interaction observed between NO and Ca^{2+}, pre-darkened epidermal strips were treated with $CaCl_2$ in presence or absence of L-NAME. Fig. 5A shows that, Ca^{2+}-mediated inhibition of stomatal opening is blocked by 1 mM L-NAME, suggesting that Ca^{2+} might be regulating the activity of a NOS-like activity. In order to see if Ca^{2+} is affecting NOS like-dependent NO production in this particular system, epidermal strips were loaded with DAF-2DA and then treated with $CaCl_2$ in presence or absence of L-NAME. As described before (Fig. 4D), Fig. 5B shows that the addition of 1 mM $CaCl_2$ induces a 1.35-fold rise of endogenous NO production

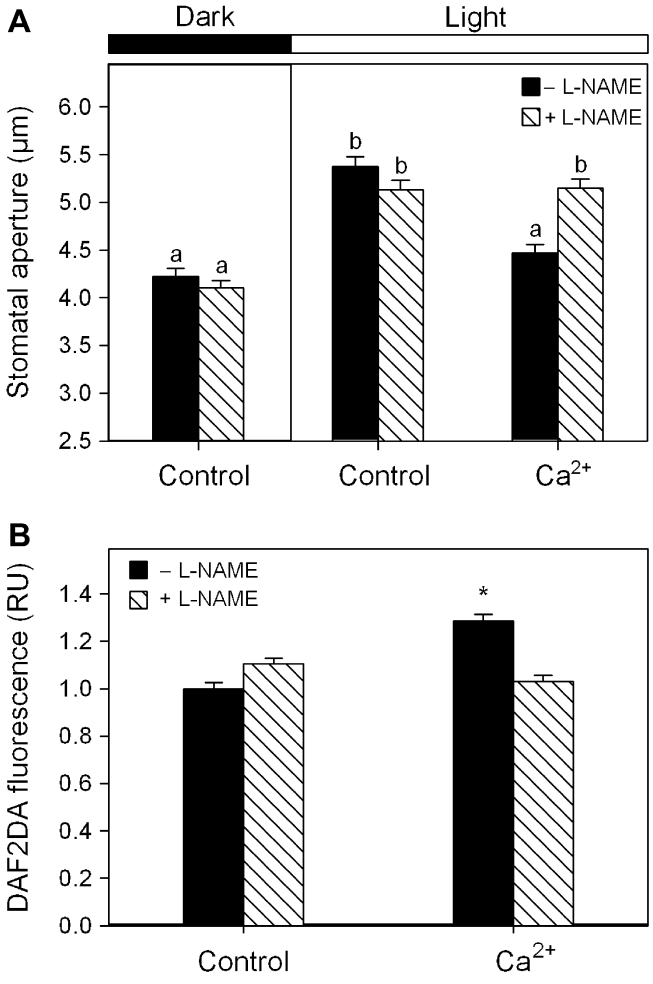

Fig. 5. The NOS inhibitor L-NAME prevents both Ca^{2+}-dependent inhibition of stomatal opening and Ca^{2+}-dependent NO production. Stomatal aperture measurements from *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark in opening buffer (10 mM MES, pH 6.1; 10 mM KCl). (A) Epidermal strips were treated with or without 1 mM L-NAME for 30 min in the same opening buffer and then treated for 30 min without (control) or with 1 mM $CaCl_2$ (Ca^{2+}). Stomatal aperture was measured as pore width immediately before (dark) or 90 min after the dark/light shift, and is expressed in μm. Values are expressed as means ± SE and represent the mean of 20–30 stomata per experiments from at least three independent experiments ($n = 60$–150). (B) *Vicia faba* epidermal strips pre-incubated 2 h and 30 min in the dark were loaded with DAF-2DA (see Materials and methods) in opening buffer and were treated with (Ca^{2+}) or without (control) of 1 mM $CaCl_2$ in presence or absence of 1 mM L-NAME for 30 min in the dark. Green fluorescence pixel intensity is expressed as relative units (RU) with respect to control treatment in absence of L-NAME. Values are the quantification of 120 guard cells of four independent treatments and are expressed as means ± SE. Asterisk denotes statistical differences between treatments (Fisher LSD post hoc test, $p > 0.05$).

($n = 120$), but also shows that the Ca^{2+}-dependent increase of the endogenous NO level was prevented when $CaCl_2$ was added together with 1 mM L-NAME (Fisher LSD, $p < 0.05$). These results further support that: (i) Ca^{2+} might be increasing NO production through the regulation of NOS-like activity and (ii) Ca^{2+} increases DAF fluorescence through the increase of NO production and not because it increases DAF sensitivity.

Discussion

Nitric oxide is an extensive signal molecule that has an active role in a myriad of physiological processes both in plants and animals [36–38]. Among them, NO has been recently reported to have a key function in the fine-tuned regulation of stomatal closure [16,18,19,39]. Stomatal movement has been frequently assumed as a model system to study signal transduction pathways, since it integrates environmental and hormonal stimuli, and in particular those regulated by ABA [40]. As a result, we now have a quite accurate panorama of the scenes and players leading to stomatal closure, however, the mechanisms leading to the inhibition of light-induced stomatal opening are still not as well understood. Zhang et al. [19] have very rencently reported that both H_2O_2 and NO inhibits blue light-induced activation of H^+-ATPase by inhibiting the signaling components upstream of H^+-ATPase. Thus, both radicals were able to inhibit blue light-dependent stomatal opening [19]. In the present work we show that: (i) NO inhibits light-induced stomatal opening in *V. faba* epidermal strips; (ii) a NOS-dependent NO production is required by ABA for the inhibition of stomatal opening, and (iii) the occurrence of a NO/Ca^{2+} interaction is critical for the inhibition of stomatal opening processes.

Two previous reports presented evidence on an ABA-NO interaction in guard cells during the transition dark/light. One of them showed that ABA-induced stomatal closure is affected in the NR double mutant *nia1/nia2*, while there were no differences between the mutant and the wild-type, regarding ABA-mediated inhibition of light-induced stomatal opening [15]. The other report shows that ABA failed to inhibit light-induced stomatal opening in the *Arabidopsis* mutant *Atnos1* [21]. More recently, Zhang et al. [19] reported a role for NO in ABA-induced inhibition of stomatal opening. In the present work, it is shown that the NO scavenger cPTIO blocks ABA inhibition of stomatal opening in *V. faba,* further supporting that NO is required by ABA for this response. Desikan et al. [14] using pharmacological and genetical approaches presented strong evidences that ruled out the participation of NR as source of NO during the ABA-mediated inhibition of light-induced stomatal opening. Here, we show that the inhibitor of NO synthase (NOS) L-NAME blocks the ABA-induced NO production, suggesting that a NOS-like activity operates during the ABA inhibition of stomatal opening. This result was further confirmed by experiments showing that L-NAME blocks ABA-dependent inhibition of stomatal opening.

Both ABA and NO have been described to regulate cytoplasmic Ca^{2+} concentrations in different plant systems, including the regulation of stomatal movements [17,41]. In the present work, using the specific Ca^{2+} buffer BAPTA-AM we showed that the Ca^{2+} buffer blocks ABA- but not NO-dependent inhibition of stomatal opening. This result opened up the question about the characteristics of the Ca^{2+}–NO interaction in ABA-mediated inhibition of

light-induced stomatal opening. This question was addressed by treating DAF-2DA loaded epidermal strips with ABA and the Ca^{2+} buffer BAPTA-AM. Results presented in Fig. 3B shows that, when added together with BAPTA-AM, ABA was not able to induce endogenous NO production, suggesting that there is an interaction between NO and Ca^{2+}.

Stomatal opening was reported to be inhibited both by Ca^{2+} and ABA [31,34,42,43]. In the present work we show that in *V. faba* epidermal strips, exogenous application of Ca^{2+} inhibited light-induced stomatal opening. In mammals, the activity of at least two of the NOS isoforms, eNOS and nNOS, is Ca^{2+}-dependent [38]. In plants, even though the existence of a NOS ortholog is still under discussion [30], the NO production that was measured from the AtNOA1 activity was also reported to be Ca^{2+} dependent [28,29]. In agreement, Lum et al. [44] also showed that NO generation required Ca^{2+} in *Phaseolus aureus*. In the present report we show that, in *V. faba* epidermal strips, there is a Ca^{2+}-dependent production of NO, which is blocked by the NOS inhibitor L-NAME, suggesting that Ca^{2+} might be activating a NOS-dependent NO production. Nevertheless, results reported with the inhibitor L-NAME just means that there is an enzyme activity that is inhibited by L-NAME, whose identity is not yet know in plants.

All together, data presented in this work support that Ca^{2+}-mediated responses during ABA inhibition of stomatal opening are NO-dependent. Several lines of evidence indicate that: (i) in guard cells endogenous NO levels are significantly increased by exogenous addition of Ca^{2+} and ABA, (ii) the specific inhibitor of NOS-like activity L-NAME prevents both ABA- and Ca^{2+}-dependent inhibition of stomatal opening and (iii) accordingly, L-NAME prevents both ABA and Ca^{2+} to induce NO increases.

If we further consider that (iv) data presented in Supplemental Fig S1 shows the lack of effect of cPTIO on Ca^{2+}-dependent induction of stomatal closure, (v) our previous data showing that under white light NO evokes $[Ca^{2+}]_{cyt}$-increases through the regulation of K_{in} channels generated by Ca^{2+} released from intracellular Ca^{2+} stores [17] and (vi) the results of this work indicating that Ca^{2+} is required for NO production but not for NO-induced inhibition of stomatal opening, it results that Ca^{2+} appears to be upstream of NO production for the inhibition of stomatal opening by ABA but downstream during ABA-induced stomatal closure. It has long been known that Ca^{2+} can be both upstream and downstream of NO production in both animals and plants [45,46]. Therefore, it can be concluded that there are different targets and/or circuitry for NO in the two distinct processes regulating ABA induction of stomatal closure and ABA inhibition of stomatal opening.

The differences between signaling pathways operating in stomatal opening inhibition and induction of stomatal closure processes are not novel for guard cell. As was previously stated [15] it have been shown that *Arabidopsis* double mutant *nia1*/*nia2* is ABA-insensitive during stomatal closure induction but not during the inhibition of stomatal opening. Other studies have also reported differences between stomatal opening and closure events involving differential contribution of signaling components such as Ca^{2+}, K^+, protein phosphatases and MAP kinases among others [43,47–49]. The evidences stated above further support the hypothesis that stomatal closure and stomatal opening processes are commanded by the same signaling components but through different mechanisms and that they are not simply "two sides of the same coin".

Overall, the results of the present work complement those recently presented by Zhang and colleagues [19], contributing to a better understanding of the mechanisms commanding stomatal opening inhibition, and support NO as a central player in controlling guard cell responses to ABA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.niox.2007.08.001.

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