

# Nitric Oxide Is a Novel Component of Abscisic Acid Signaling in Stomatal Guard Cells

Steven J. Neill\*, Radhika Desikan, Andrew Clarke, and John T. Hancock

Centre for Research in Plant Science, University of the West of England, Bristol, Coldharbour Lane, Bristol BS16 1QY, United Kingdom

Stomatal closure in response to the hormone abscisic acid (ABA) is mediated by a complex signaling network involving both calcium-dependent and calcium-independent pathways (Assmann and Shimazaki, 1999; Webb et al., 2001), activated by several signaling intermediates (Schroeder et al., 2001) that include hydrogen peroxide (Miao et al., 2000; Pei et al., 2000; Zhang et al., 2001) and lipids such as sphingosine-1-phosphate (Ng et al., 2001). Here, we provide evidence that nitric oxide (NO) is also a signaling component of ABA-induced stomatal closure. Our data show that NO synthesis is required for ABA-induced closure and that ABA enhances NO synthesis in guard cells. Exogenous NO induces stomatal closure, and ABA and NO-induced closure require the synthesis and action of cGMP and cyclic ADP Rib (cADPR).

## ABA-INDUCED STOMATAL CLOSURE REQUIRES NO SYNTHESIS

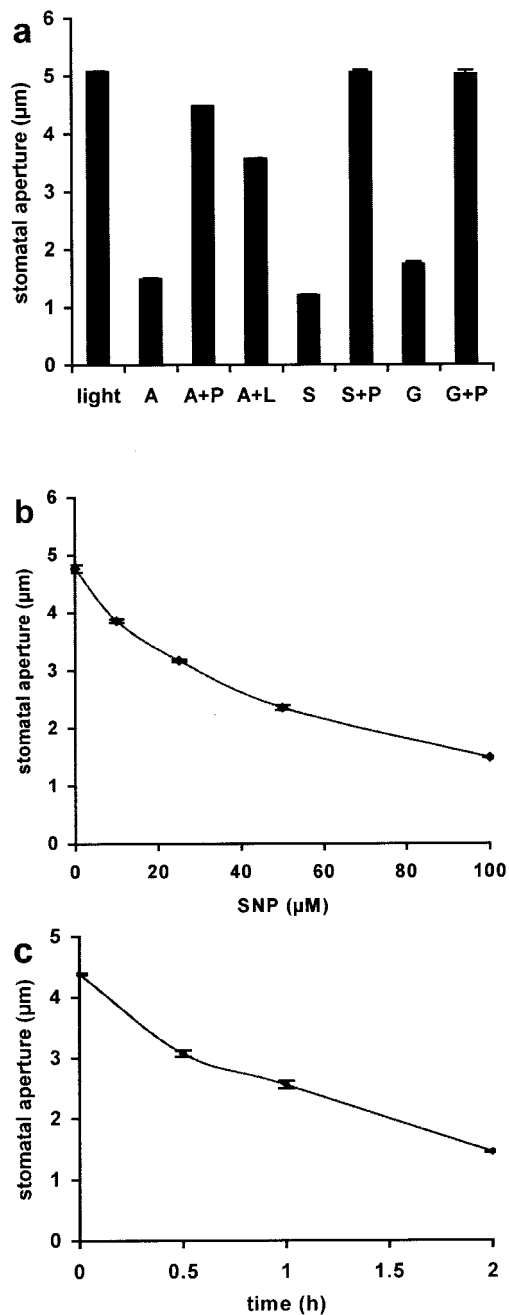
NO is a key signaling molecule in plants, mediating responses to various abiotic and biotic stresses (Delledonne et al., 1998; Durner et al., 1998; Clarke et al., 2000; Beligni and Lamattina, 2001). The recent reports that treatment with a fungal elicitor induced the rapid synthesis of NO in tobacco (*Nicotiana tabacum*) epidermal cells (Foissner et al., 2000) prompted us to determine any involvement of NO in ABA-regulated stomatal movements. Epidermal peels from pea (*Pisum sativum* L. *Argenteum*) were incubated in ABA in the presence of 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO), a specific NO scavenger previously shown to block NO effects (Delledonne et al., 1998; Clarke et al., 2000), or  $N^G$ -nitro-L-Arg-methyl ester (L-NAME), an inhibitor of NO synthase (NOS) in mammalian cells that also inhibits plant NOS (Barroso et al., 1999). Pretreatment with either L-NAME or PTIO largely suppressed stomatal responses to ABA (Fig. 1a), indicating the requirement for NO synthesis and action during ABA-induced stomatal closure. Exogenous NO also induced stomatal closure. Both sodium nitroprusside (SNP) and S-nitrosoglutathione (GSNO), two chemically different NO donors previously

shown to induce defense responses in plants (Delledonne et al., 1998; Durner et al., 1998; Clarke et al., 2000; A.-H.-Mackerness et al., 2001) induced stomatal closure, which was readily inhibited by pretreatment with PTIO (Fig. 1a). SNP effects were determined in more detail; the dose response and kinetics of SNP-induced stomatal closure are shown in Figure 1, b and c. At the concentrations tested, SNP did not reduce the viability of guard cells, and in wash-out experiments the stomata reopened fully, indicating that the effects of SNP were fully reversible (not shown).

The effects of ABA on NO synthesis were determined using the cell-permeable fluorescent NO probe diaminofluorescein diacetate (DAF-2 DA), recently used to visualize NO synthesis in tobacco (Foissner et al., 2000), and *Taxus brevifolia* and *Kalanchoe daigremontiana* (Pedroso et al., 2000). Autofluorescence was observed associated with the inner walls of the guard cells in control samples, with low-level, diffuse fluorescence also apparent in a small number of the guard cells (Fig. 2a). Exposure to 10  $\mu$ M ABA induced a rapid and striking increase in the fluorescence of guard cells that was evident after 5 min and substantial after 30 min (Fig. 2b). Fluorescence was apparent in the cytosol and particularly intense in chloroplasts. Average fluorescence intensity increased by 52% in epidermal cells and by 120% in guard cells ( $n = 21$ ). After 30 min, 35% of the guard cells fluoresced brightly ( $n = 250$ ) compared with 8% for control cells ( $n = 247$ ), and within 60 min, 80% ( $n = 362$ ) were fluorescing (17% for control,  $n = 216$ ). ABA-induced DAF-2 DA fluorescence in guard cells was largely prevented by PTIO (14% cells fluorescing,  $n = 105$ ; Fig. 2c). Pretreatment with L-NAME also substantially suppressed ABA-induced DAF-2 DA fluorescence (11% of cells fluorescing,  $n = 54$ ; Fig. 2d), suggesting that pea guard cells possess a NOS-like enzyme. Interestingly, NOS enzyme activity and a partial NOS cDNA clone have been isolated from pea leaves (Barroso et al., 1999; Corpas et al., 2001). It has been reported recently that DAF-2 DA fluorescence is amplified in the presence of  $Ca^{2+}$ , although still absolutely dependent on the presence of NO (Broillet et al., 2001). Because the stimulation by ABA of both the uptake and intracellular release of  $Ca^{2+}$  is well known, we repeated the experiments in the presence of 2 mM EGTA-AM, the membrane-permeable form of the  $Ca^{2+}$  chelator

\* Corresponding author; e-mail steven.neill@uwe.ac.uk; fax 00-44-117-3442904.

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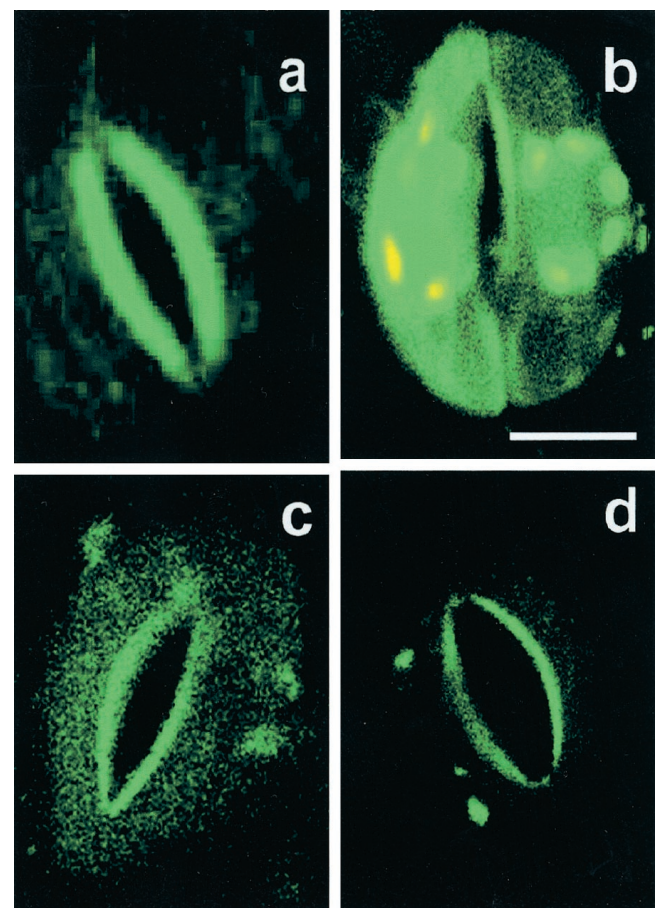
**Figure 1.** Effects of ABA and NO on stomatal closure in pea. a, Epidermal peels, prepared from *Argenteum* pea (Burnett et al., 2000), were incubated in the light in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (0.01 M MES-KOH, 0.05 M KCl, pH 6.15) to induce stomatal opening and then: incubated for 2 h in buffer alone (light), 10 µM ABA (A), ABA + 200 µM PTIO (A+P), ABA + 25 µM L-NAME (A+L), 100 µM SNP (S), SNP + 200 µM PTIO (S+P), 500 µM GSNO (G), and GSNO + 200 µM PTIO (G+P). b, Dose response for SNP, after incubation for 2 h. c, Kinetics of SNP-induced stomatal closure (100 µM SNP). Bars = SE ( $n = 180$ ).

EGTA (Wu et al., 1997). This treatment had no effect on ABA-induced DAF-2 DA fluorescence (60 min, 87% of guard cells fluorescing,  $n = 326$ ). NO synthesis by epidermal peels was also estimated using the hemo-

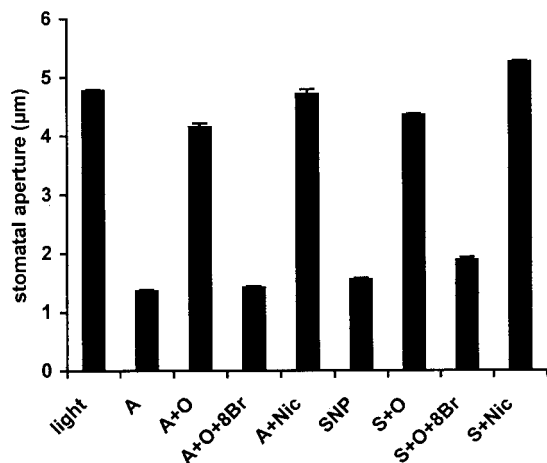
globin assay (Clarke et al., 2000). Peels were incubated for 60 min and NO release over this period subsequently determined. Constitutive NO release was estimated as  $93 \pm 7 \text{ nmol g}^{-1}$  ( $n = 7$ ). This increased significantly ( $t$  test,  $P < 0.05$ ) to  $125 \pm 7 \text{ nmol g}^{-1}$  ( $n = 7$ ), an increase of 35%, following treatment with 10 µM ABA. This increase was prevented by co-incubation with 25 µM L-NAME ( $99 \pm 11 \text{ nmol g}^{-1}$  [ $n = 5$ ]).

#### ABA AND NO SIGNALING DURING STOMATAL CLOSURE

NO signaling commonly involves the second messenger cGMP, generated via the enzyme guanylate



**Figure 2.** ABA induces NO synthesis in pea guard cells. Epidermal peels were floated in MES buffer in the light for 1 h and then loaded with DAF-2 DA (Calbiochem, Nottingham, UK; 10 µM in MES, 10 min in the dark, 20 min wash in MES). Following treatments, peels were observed with a laser confocal scanning microscope (Nikon PCM2000, Nikon Europe B.V. Badhoevedorp, The Netherlands; excitation 495 nm, emission 515–560 nm). Acquired images were processed using Adobe Photoshop (Adobe Systems, Mountain View, CA) and relative pixel intensities determined using EZ2000 version 2.1 software (Coord, Amsterdam). Images are shown after a 30-min treatment. PTIO and L-NAME treatments reduced both the intensity and the number of guard cells visibly fluorescing; figure shows those cells in which fluorescence was still visible. a, Control (buffer only). b, 10 µM ABA. c, ABA + 200 µM PTIO. d, ABA + 25 µM L-NAME. Scale bar = 7 µm.



**Figure 3.** ABA- and NO-induced stomatal closure requires cGMP and cADPR. Epidermal peels were incubated in the light to induce stomatal opening and then incubated for 2 h in buffer alone (light), 10  $\mu\text{M}$  ABA (A), ABA + 2  $\mu\text{M}$  ODQ (A+O), ABA + ODQ + 50  $\mu\text{M}$  8-Br-cGMP (A+O+8Br), ABA + 5 mM nicotinamide (A+Nic), SNP (100  $\mu\text{M}$ ), SNP + 2  $\mu\text{M}$  ODQ (S+O), SNP + ODQ + 100  $\mu\text{M}$  8-Br-cGMP (S+O+8Br), and SNP + 5 mM nicotinamide (S+Nic). Bars = SE ( $n = 180$ ).

cyclase (Wendehenne et al., 2001), and previous work has provided data consistent with cGMP involvement in plant NO signaling (Durner et al., 1998; Clarke et al., 2000). Consequently, we pretreated epidermal peels with 1*H*-(1,2,4)-oxadiazole-[4,3-*a*]quinoxalin-1-one (ODQ), an inhibitor of NO-sensitive guanylate cyclase (Durner et al., 1998; Clarke et al., 2000). ODQ by itself had no effect on stomatal aperture (not shown), but it was a potent inhibitor of both ABA- and SNP-induced stomatal closure (Fig. 3). Furthermore, treatment with 8-bromo-cGMP (8-Br-cGMP), a cell-permeable analog of cGMP known to be active in plant cells (Durner et al., 1998; Clarke et al., 2000), reversed the inhibitory effects of ODQ on ABA- and NO-induced stomatal closure (Fig. 3). Treatment with 8-Br-cGMP alone had no effect (not shown). These data indicate that cGMP is required, but not sufficient, for ABA- and NO-induced stomatal closure. One downstream signaling response to NO and cGMP is intracellular generation of cADPR, a  $\text{Ca}^{2+}$ -mobilizing molecule (Wendehenne et al., 2001). cADPR involvement in ABA responses has already been demonstrated (Wu et al., 1997; Leckie et al., 1998; MacRobbie, 2000). Consequently, we determined the effects of nicotinamide, an antagonist of cADPR production (Leckie et al., 1999; MacRobbie, 2000), on ABA- and NO-induced stomatal closure (Fig. 3). Nicotinamide inhibited the effects of both ABA and NO, suggesting that inhibition of ABA responses by nicotinamide is, at least partly, due to inhibition of cADPR synthesis following NO generation.

In summary, the results presented here demonstrate that NO is a novel component of ABA signaling in stomatal guard cells. They show that guard

cells generate NO in response to ABA via NOS-like activity, and that such NO production is required for full stomatal closure in response to ABA; that exogenous NO induces stomatal closure; and that cGMP and cADPR are both required for NO- and ABA-induced stomatal closure. Cyclic nucleotide-gated ion channels have recently been cloned and characterized in Arabidopsis (Kohler et al., 1999; Leng et al., 1999). Modulation of the activity of such channels by cGMP may be one mechanism by which NO effects stomatal closure. It will clearly be important to quantify accurately NO production in guard cells and other cell types in a range of species and to determine whether other ABA responses similarly involve NO, particularly as wilting can result in elevated NO production (Lesham and Haramaty, 1996). Very recently, Mata and Lamattina (2001) have reported that NO induces stomatal closure in fava bean (*Vicia faba*), *Salpichroa organifolia*, and *Tradescantia* spp., although a requirement for NO in ABA-induced stomatal closure was not determined. However, our preliminary data indicate that ABA-induced stomatal closure in Arabidopsis also requires NO, as in pea (not shown). These data are important because they point the way to molecular and genetic analyses, which will include studies of the ABA-insensitive and ABA-deficient *abi* and *aba* mutants. Moreover, the involvement of NO signaling during stomatal responses to ABA provides a new opportunity to manipulate plant water relations in order to increase agricultural productivity.

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