Nitrated Cyclic GMP Modulates Guard Cell Signaling in Arabidopsis

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Nitric oxide (NO) is a ubiquitous signaling molecule involved in diverse physiological processes, including plant senescence and stomatal closure. The NO and cyclic GMP (cGMP) cascade is the main NO signaling pathway in animals, but whether this pathway operates in plant cells, and the mechanisms of its action, remain unclear. Here, we assessed the possibility that the nitrated cGMP derivative 8-nitro-cGMP functions in guard cell signaling. Mass spectrometry and immunocytochemical analyses showed that absicic acid and NO induced the synthesis of 8-nitro-cGMP in guard cells in the presence of reactive oxygen species. 8-Nitro-cGMP triggered stomatal closure, but 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP), a membrane-permeating analog of cGMP, did not. However, in the dark, 8-bromo-cGMP induced stomatal opening but 8-nitro-cGMP did not. Thus, cGMP and its nitrated derivative play different roles in the signaling pathways that lead to stomatal opening and closure. Moreover, inhibitor and genetic studies showed that calcium, cyclic adenosine-5′-diphosphate-ribose, and SLOW ANION CHANNEL1 act downstream of 8-nitro-cGMP. This study therefore demonstrates that 8-nitro-cGMP acts as a guard cell signaling molecule and that a NO/8-nitro-cGMP signaling cascade operates in guard cells.

INTRODUCTION

Stomata are regulated pores on the surface of aerial plant organs; the opening and closing of these pores controls the diffusion of gasses into and out of air spaces in the plant tissues. Stomata are formed by pairs of guard cells, which sense and rapidly respond to environmental signals such as light, humidity, carbon dioxide, and pathogens, and also respond to hormones including abscisic acid (ABA), auxin, and ethylene (Schroeder et al., 2001a; Melotto et al., 2006; Shimazaki et al., 2007; Shope et al., 2008). Guard cells provide a convenient model system for studying signal transduction (Schroeder et al., 2001b; Assmann and Wang, 2001). Numerous signaling components act in the induction of stomatal closure, including the important signaling compound nitric oxide (NO) (Besson-Bard et al., 2008; Neill et al., 2008). NO is a reactive free radical that can diffuse across biological membranes; it is a second messenger in animals and also regulates various processes in plants, including root growth, cell wall lignification, senescence, chlorophyll biosynthesis, and stomatal closure (Lamattina et al., 2003).

In animals, NO enhances the activity of a soluble guanylate cyclase, which stimulates the production of cyclic GMP (cGMP), a crucial signaling molecule involved in smooth muscle relaxation, in inhibition of platelet aggregation, and in sensing systems, including vision and olfaction (McDonald and Murad, 1995). In plants, NO-induced increases in cGMP levels have been reported in plants such as spruce (Picea abies), tobacco (Nicotiana tabacum), soybean (Glycine max), and Arabidopsis thaliana (Pfeiffer et al., 1994; Durner et al., 1998; Suita et al., 2009; Dubovskaya et al., 2011). In plants, inhibitors of animal guanylate cyclase suppressed the expression of genes encoding Phe ammonia-lyase and PATHOGEN-RELATED1 protein (Durner et al., 1998). These inhibitors also prevented NO-induced plant cell death (Durner et al., 1998) and NO-induced stomatal closure (Neill et al., 2002). The cell-permeating analog of cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP), induced expression of genes encoding PATHOGEN-RELATED1 protein, Phe ammonia-lyase, cation transporters, and chalcone synthase (Bowler et al., 1994; Durner et al., 1998; Suita et al., 2006; Maathuis, 2006; Suita et al., 2009). Recent work identified a novel plant NO-sensitive guanylate cyclase (Mulauzdi et al., 2011). Thus, a NO/cGMP signaling cascade resembling that in animal cells also appears to operate in plants.

The presence of cGMP has been demonstrated in guard cells, a finding that agrees with the idea of a specific role for cGMP in stomatal function (Pharmawati et al., 2001). In addition, NO was shown to induce stomatal closure (Garcia-Mata and Lamattina, 2001) and to inhibit stomatal opening (Yan et al., 2007; Zhang et al., 2007). Inhibitors of animal guanylate cyclase also prevented NO-induced stomatal closure in plants (Neill et al., 2002). These results suggested that the NO/cGMP signaling cascade functions in stomatal closure. However, 8-bromo-cGMP did not induce stomatal closure in pea (Pisum sativum; Neill et al., 2002).
By contrast, it did induce stomatal opening in Commelina communis, Tradescantia albiflora, fava bean (Vicia faba), and Arabidopsis (Cousson and Vavasseur, 1998; Pharmawati et al., 1998, 2001; Cousson, 2003). Thus, the issue of whether the NO/cGMP cascade operates in guard cell signaling is still controversial.

Work in animals has provided evidence that 8-nitro-cGMP is present and physiologically relevant in vivo. Sawa et al. (2007) used mammalian cells to demonstrate that NO-dependent guanine nitration of cGMP does occur and that the nitrated derivative cGMP, 8-nitro-cGMP, is produced under physiological and pathophysiological conditions. This nitrated derivative activated cGMP-dependent protein kinases and induced vasodilatation in a manner similar to that of native cGMP. In addition, 8-nitro-cGMP possesses unique electrophilic properties, as it triggered the production of reactive oxygen species (ROS) and modified thiol residues of proteins. This novel 8-nitro-cGMP-mediated posttranslational modification, named S-guanylation, was studied using the redox sensor protein Keap1. S-Guanylated Keap1 induced the expression of heme oxygenase 1, which elicits cytoprotection during Salmonella infection (Zaki et al., 2009). This finding raised the question of whether nitrated cGMP functions as a guard cell signaling molecule in plants.

Here, we examined the possibility that nitrated cGMP is present in plants and participates in guard cell signaling. We found that ABA, NO, and ROS induced 8-nitro-cGMP synthesis in guard cells and that 8-nitro-cGMP triggered stomatal closure.

The effects of cGMP and 8-nitro-cGMP differed considerably; cGMP caused stomata to open in the dark, whereas 8-nitro-cGMP caused stomata to close in the light. Thus, a NO/nitrated cGMP signaling cascade that is distinct from the classical NO/cGMP cascade operates in guard cells.

RESULTS

Nitrated cGMP Acts as a Signaling Molecule in Guard Cells

To test whether guanylate cyclase is involved in the signaling pathways that lead to stomatal closure or opening, we treated Arabidopsis epidermal cells with two guanylate cyclase inhibitors, 1-\(H\)-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ) and 6-anilino-5,8-quinolinequinone (LY83583). Although these reagents alone had no effects on stomatal aperture (see Supplemental Figure 1 online), they inhibited ABA- and 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC5; NO donor)-induced stomatal closure (Figures 1A and 1C; see Supplemental Figure 2 online), which suggests that guanylate cyclase is involved in ABA- and NO-induced stomatal closure. We applied cGMP (the product of guanylate cyclase) and 8-bromo-cGMP (a membrane-permeating analog of cGMP) to epidermal strips, but these molecules did not induce stomatal closure (Figure 1B), consistent with previous observations in pea and Arabidopsis (Neill et al., 2002; Dubovskaya et al., 2011). However, cGMP or 8-bromo-cGMP

Figure 1. Guanylate Cyclase Inhibitors Prevented ABA- and NO-Induced Stomatal Closure.

Change in stomatal aperture (\(\mu m\)) in response to various treatments. The stomatal aperture in untreated cells measured 3.44 ± 0.05 \(\mu m\), 3.45 ± 0.03 \(\mu m\), and 3.48 ± 0.03 \(\mu m\) for (A), (B), and (C), respectively. Error bars represent se (\(n = 100\)). The double asterisks indicate a significant difference (\(P < 0.01\)) compared with values for untreated cells.

(A) Effect of guanylate cyclase inhibitors (ODQ and LY83583) on ABA-induced stomatal closure.

(B) Effect of cGMP on ABA-induced stomatal closure.

(C) Effect of cGMP and ODQ on NOC5-induced stomatal closure.
applied in combination with ABA and ODQ did induce stomatal closure (Figure 1B). This effect was abolished by the addition of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide (cPTIO). Furthermore, when NOC5 was applied with cGMP and ODQ, stomata closed (Figure 1C). These data indicate that cGMP is required, but not sufficient, for stomatal closure and that its effect occurs only in the presence of NO.

Next, we examined a T-DNA knockout mutant of NO-dependent guanylate cyclase, nogc1. In this mutant, ABA and NOC5 did not induce stomatal closure (Figure 2A), indicating that NOGC1 is involved in ABA- and NO-induced stomatal closure. We also applied cGMP and 8-bromo-cGMP to epidermal strips from the nogc1 mutant, but these molecules did not induce stomatal closure (see Supplemental Figure 3 online), consistent with the observation in Figure 1B. When ABA and NOC5 were applied with cGMP (Figure 2B), the stomata closed, confirming that NO and cGMP are required for stomatal closure.

Mass Spectrometric Identification of 8-Nitro-cGMP
To assess the possibility that cGMP nitration occurs in plant cells, we first performed mass spectrometry (MS) by liquid chromatography–ion trap–time-of-flight. MS analysis of 8-nitro-cGMP showed the deprotonated molecule ion mass-to-charge ratio (m/z) 389.03 [M-H]⁻ (calculated C₁₀H₁₀N₆O₉P₂, 389.0247) with a retention time of 10.01 min (Figure 3A). The plant cell extracts also showed a deprotonated molecule ion m/z 389.03 at the same retention time. The precursor ion (m/z 389.03) of 8-nitro-cGMP was fragmented by collision-induced dissociation to produce product ions at m/z 178.00, 195.03, 273.04, and 291.04 (Figure 3B). The plant cell extracts showed an identical precursor ion at m/z 389.03 and the same product ions, confirming that 8-nitro-cGMP synthesis did occur in the plant cells (Figure 3C).

Detection of 8-Nitro-cGMP by Immunocytochemistry
To further examine 8-nitro-cGMP formation in guard cells, we conducted immunocytochemical analyses using a monoclonal antibody against 8-nitro-cGMP and a fluorescent secondary antibody (Figures 4 and 5). Weak fluorescent background signals were detected in untreated guard cells. Epidermal strips treated with 8-nitro-cGMP showed strong fluorescence, but strips treated with 8-bromo-cGMP or cGMP did not differ from untreated strips (see Supplemental Figure 4 online). These results confirmed that the antibody specifically recognized 8-nitro-cGMP and that 8-nitro-cGMP permeated the guard cell membrane. We detected strong fluorescence in guard cells treated with ABA, NOC5, and S-nitroso-N-acetyl-DL-penicillamine (SNAP; NO donor) but not in epidermal pavement cells (Figure 4), showing that most of the 8-nitro-cGMP in epidermal tissues was found in the guard cells. A NO synthase inhibitor, N-nitro-L-Arg methyl ester (L-NAME), reduced the ABA-induced immunofluorescence, and the NO scavenger cPTIO decreased the immunofluorescence induced by ABA and NOC5 (Figure 4). These results indicated that 8-nitro-cGMP forms in a NO-dependent manner.

Addition of ODQ, a guanylate cyclase inhibitor, also caused a decrease in ABA- and NOCS5-induced immunofluorescence (Figure 5). When cGMP was applied to epidermal strips together with NOC5 and ODQ, guard cells showed strong fluorescence that resembled the fluorescence observed in NOC5-treated guard cells. These results indicate that cGMP and NO were required for 8-nitro-cGMP formation in guard cells, which is consistent with our findings from the stomatal aperture assays (Figure 1).

Figure 2. ABA- and NO-Induced Stomatal Closure Requires NO-Dependent Guanylate Cyclase.
(A) Effect of ABA, NOC5, and 8-nitro-cGMP (NcGMP) on stomatal aperture in the nogc1 mutant. The stomatal aperture in untreated cells measured 3.17 ± 0.05 μm.
(B) Effect of cGMP on ABA- and NOC5-induced stomatal closure in the nogc1 mutant. Gray bars, treated with 30 μM ABA; black bars, treated with 100 μM NOC5. −cGMP, treated with ABA or NOC5 without cGMP; +cGMP, treated with ABA or NOC5 with 100 μM cGMP. The stomatal aperture in untreated cells measured 3.38 ± 0.10 μm for the ABA series and 3.42 ± 0.16 μm for the NOC5 series. Error bars represent ±SE (n = 80). The double asterisks indicate a significant difference (P < 0.01) compared with values for untreated cells.
Quantitative Analyses of 8-Nitro-cGMP by Liquid Chromatography–Tandem MS

Quantitative analyses of 8-nitro-cGMP were performed by liquid chromatography–tandem MS (LC-MS/MS) in multiple reaction monitoring (MRM) mode. The intensities of MS fragments specific to 8-nitro-cGMP in epidermal cells treated with 30 μM ABA or 100 μM NOC 5 were approximately six- to sevenfold higher than in untreated epidermal cells (Figure 6A). 8-Nitro-cGMP synthesis was stimulated even at 1 μM ABA. By contrast, ABA-induced 8-nitro-cGMP synthesis was impaired in the mutant of the early ABA signaling component protein phosphatase 2C ABA-INSENSITIVE1 (ABI1, abi1-1; see Supplemental Figure 5 online). These results indicate that ABA and NO induced 8-nitro-
cGMP. We then examined the time course of 8-nitro-cGMP synthesis (Figure 6B). ABA did not induce 8-nitro-cGMP within 3 min, but within 5 min, ABA induced an increase of ~260% in 8-nitro-cGMP concentrations, which peaked at 15 min.

8-Nitro-cGMP Synthesis Depends on ROS

We used 2,7-dichlorofluorescin diacetate (H₂DCF-DA) and diaminofluorescein–2 diacetate (DAF-2 DA) to monitor ROS and NO generation in guard cells treated with ABA. ABA induced a 250% increase in ROS within 3 min, and ROS concentrations peaked at 3 to 5 min and then gradually decreased (Figure 7A). ABA also increased NO production in guard cells; there was a 200% increase in NO within 3 min after ABA treatment, and
NO continuously increased to 370% in 15 min (Figure 7A). The generation of NO and ROS occurred simultaneously and preceded 8-nitro-cGMP synthesis (Figure 6B).

To test whether ABA-induced ROS are involved in 8-nitro-cGMP formation, we used DTT (a reducing agent), 1,2-dihydroxy-3,5-benzenedisulfonic acid (Tiron; a superoxide scavenger), and catalase (a hydrogen peroxide [H$_2$O$_2$] scavenger). These reagents suppressed ROS generation induced by ABA (Figure 7B) and reduced 8-nitro-cGMP formation induced by ABA (Figures 8A and 8B). In the abi1-1 mutant, where ABA-induced ROS generation did not occur (see Supplemental Figure 6 online), ABA did not induce 8-nitro-cGMP synthesis (see Supplemental Figure 5 online). Furthermore, when epidermal tissues were treated with H$_2$O$_2$, 8-nitro-cGMP increased fivefold (Figure 8C). These results showed that ROS were required for ABA-induced 8-nitro-cGMP formation.

### Roles of cGMP and 8-Nitro-cGMP in Guard Cell Signaling

To examine the responses to the different cGMP derivatives, we applied each compound exogenously to epidermal strips and...
examined the change in stomatal aperture. In the light, 8-nitro-cGMP induced stomatal closure in a dose-dependent manner (Figure 9A). Under identical conditions, 8-bromo-cGMP did not induce stomatal closure. These results indicate that the NO signaling molecule responsible for the closure of stomata is not cGMP but is rather 8-nitro-cGMP. When epidermal strips with closed stomata were exposed to 1 to 100 μM 8-bromo-cGMP in the dark, stomata opened (Figure 9B), which agrees with previously reported results (Cousson, 2003). At the same concentrations, 8-nitro-cGMP was ineffective, which indicates that the signaling molecule responsible for stomatal opening was not 8-nitro-cGMP but was instead cGMP.

Figure 6. Quantitative Analyses of 8-Nitro-cGMP by LC-MS/MS.

(A) Levels of 8-nitro-cGMP in epidermal tissues treated with ABA and NOC5. Epidermal tissues were treated with ABA (1, 30 μM) or NOC5 (100 μM) for 30 min. The double asterisks indicate a significant difference (P < 0.01) compared with the control value.

(B) Time course of 8-nitro-cGMP synthesis in epidermal tissues treated with ABA. Epidermal tissues were treated with ABA (30 μM) for the indicated period. Error bars represent SE (n = 3). fr wt, fresh weight.

Figure 7. ROS and NO Generation in Guard Cells Treated with ABA.

(A) Time course of ROS and NO generation in guard cells treated with ABA. Epidermal tissues were treated with ABA (30 μM) for the indicated period. Circles: Fluorescence intensity from guard cells loaded with H2DCF-DA, which detects ROS. Triangles: Fluorescence intensity from guard cells loaded with DAF-2 DA, which detects NO. Fluorescence intensity per guard cell was expressed as the ratio of treated cells to untreated cells. Error bars represent SE (n = 30).

(B) Effects of DTT, Tiron, and catalase on ABA-induced ROS generation in guard cells. Epidermal tissues were treated with ABA (30 μM) in the absence or presence of DTT (2 mM), Tiron (100 μM), or catalase (CAT; 1000 units) for 5 min. Fluorescence intensity per guard cell was expressed as the ratio of treated cells to untreated cells. Error bars represent SE (n = 30). The double asterisks indicate significant differences (P < 0.01) compared with ABA-treated cells.
Calcium acts as a signaling component in ROS and NO signaling pathways in guard cells. To determine whether Ca\(^{2+}\) mediates 8-nitro-cGMP signaling in guard cells, we used the cell-permeating Ca\(^{2+}\)-chelator bis(o-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester (BAPTA-AM). This reagent inhibited stomatal closure induced by NOC5 and 8-nitro-cGMP (see Supplemental Figure 7 online; Figure 10A) and cyclic adenosine-5’-diphosphate-ribose (cADPR), a second messenger modulating intracellular calcium levels (Guse, 1999), has been shown to serve as a downstream messenger of NO in pea and fava bean (Neill et al., 2002; Garcia-Mata et al., 2003). To determine whether cADPR mediates 8-nitro-cGMP signaling in guard cells, we used two antagonists of cADPR production, nicotinamide and 8-bromo-cADPR. Although the two inhibitors themselves had no effect on stomatal aperture (see Supplemental Figure 1 online), both inhibited NO- and 8-nitro-cGMP–dependent stomatal closure (see Supplemental Figure 8 online; Figure 10B).

Figure 8. 8-Nitro-cGMP Synthesis Is Dependent on ROS.

(A) Fluorescence micrographs of epidermal tissues showing fluorescent signals from antibody against 8-nitro-cGMP. Epidermal tissues were treated with ABA (30 \(\mu\)M) in the absence or presence of DTT (2 mM), Tiron (100 \(\mu\)M), or catalase (CAT; 1000 units) for 30 min. Bars = 20 \(\mu\)m.

(B) Fluorescence intensity per guard cell expressed as the ratio of treated cells to untreated cells. Error bars represent se (n = 3). The asterisk indicates a significant difference (P < 0.05) compared with the control value.

(C) \(\text{H}_2\text{O}_2\)-induced 8-nitro-cGMP synthesis. Epidermal tissues were treated with 100 \(\mu\)M \(\text{H}_2\text{O}_2\) for 30 min. 8-Nitro-cGMP levels in epidermal tissues were determined by means of LC-MS/MS. Error bars represent se (n = 3). The double asterisk indicates a significant difference (P < 0.01) compared with the untreated control. fr wt, fresh weight.

Components of the 8-Nitro-cGMP Signaling Pathway in Guard Cells

Figure 9. Stomatal Response to 8-Nitro-cGMP and 8-Bromo-cGMP.

(A) Stomatal closure in the light. The stomatal aperture in untreated cells measured 3.87 ± 0.05 \(\mu\)m (for the 8-Br-cGMP series) and 3.74 ± 0.05 \(\mu\)m (for the 8-nitro-cGMP series).

(B) Stomatal opening in the dark. The stomatal aperture in untreated cells measured 2.60 ± 0.06 \(\mu\)m (for the Br-cGMP series) and 2.55 ± 0.04 \(\mu\)m (for the 8-nitro-cGMP series). Error bars represent se (n = 100). The double asterisks indicate a significant difference (P < 0.01) compared with values for untreated cells.
These results suggested that Ca\(^{2+}\) acts through ADP-ribosyl cyclase in 8-nitro-cGMP signaling.

SLOW ANION CHANNEL1 (SLAC1) is an essential protein functioning downstream of ABA, ROS, NO, and Ca\(^{2+}\) in mediating stomatal closure (Negi et al., 2008; Vahisalu et al., 2008; Geiger et al., 2010). In the slac1-2 mutant, stomatal closure induced by 8-nitro-cGMP or ABA was impaired, indicating that SLAC1 acts downstream of 8-nitro-cGMP (Figure 10C). ABI1 acts upstream of 8-nitro-cGMP as described above. Nonetheless, there are reports showing that it functions at other steps of ABA guard cell signaling. For example, it acts downstream of NO (Desikan et al., 2002) and cADPR (Wu et al., 2003), which act as signaling molecules in 8-nitro-cGMP signaling. To test whether ABI1 acts in 8-nitro-cGMP signaling, we examined the effects of abi1-1 on stomatal closure. We found that NOC5 and 8-nitro-cGMP did not trigger stomatal closure in abi1-1 mutant plants (see Supplemental Figure 9 online). In the nogc1 mutant, ABA and NO did not induce stomatal closure, but 8-nitro-cGMP could induce stomatal closure (Figure 2A), indicating that NOGC1 acts upstream of 8-nitro-cGMP.

**DISCUSSION**

**8-Nitro-cGMP Is a Novel Signaling Molecule in Plants**

Neill et al. (2002) previously demonstrated that NO is a downstream component of ABA guard cell signaling and that ABA- and NO-induced stomatal closure was inhibited by ODQ, a guanylate cyclase inhibitor, which suggests that an NO/cGMP signaling cascade operates in guard cells. The reaction product of guanylate cyclase is cGMP, which would be expected to induce stomatal closing, but its membrane-permeating analog, 8-bromo-cGMP, did not induce this response. However, when 8-bromo-cGMP was applied together with ABA and ODQ, stomata closed. Based on these results, Neill et al. (2002, 2008) suggested that cGMP is required but not sufficient for stomatal closure and that other ABA-induced factors might be required. We obtained similar results in Arabidopsis. Two guanylate cyclase inhibitors, ODQ and LY83583, prevented ABA- and NO-induced stomatal closure. The T-DNA knockout mutant of NO-dependent guanylate cyclase was impaired in ABA- and NO-induced stomatal closing. When cGMP was exogenously applied together with ODQ and ABA, stomata closed, which suggests that cGMP-induced stomatal closure depends on an ABA-induced factor (or factors). Our experiments using nogc1 mutants confirm these results: cGMP-induced stomatal closure occurred in the presence of ABA. NO is thought to be an ABA-induced factor because NO scavengers eliminated the effect of ABA and because cGMP-induced stomatal closure requires the presence of an NO donor. Thus, ABA-induced NO interacted with cGMP to elicit stomatal closure.

Sawa et al. (2007) reported that NO-dependent nitration of cGMP, producing 8-nitro-cGMP, occurs in mammalian cells under physiological and pathological conditions. This nitrated derivative seems to act as an NO signaling molecule in mammalian cells. We hypothesized that NO-dependent guanine nitrination
of cGMP may also occur in plants and that the resulting 8-nitro-cGMP acts as a signaling molecule in guard cells. To test this hypothesis, we performed MS and immunocytochemical analyses with monoclonal antibodies against 8-nitro-GMP. These analyses demonstrated that NO and cGMP induced the synthesis of 8-nitro-cGMP in guard cells. Furthermore, 8-nitro-cGMP induced stomatal closure in a dose-dependent manner. We concluded that NO induces the production of 8-nitro-GMP as a signaling molecule that regulates stomatal aperture. A NO/8-nitro-cGMP signaling cascade therefore operates in guard cells.

A role for cGMP in kinetin- and natriuretic peptide–induced stomatal opening has been described in T. albi flora (Pharmawati et al., 1998). In C. communis and Arabidopsis, cGMP mediated auxin-induced stomatal opening (Cousson and Vavasseur, 1998; Cousson, 2003). We also tested the possibility that 8-nitro-cGMP functions in stomatal opening in Arabidopsis. 8-Bromo-cGMP caused stomata to open, as previously reported (Cousson, 2003), but 8-nitro-cGMP did not. We conclude that cGMP and its nitrated derivative play different roles in guard cell signaling: cGMP promotes stomatal opening in the dark, whereas 8-nitro-cGMP promotes stomatal closure in the light.

ROSs Are Essential for 8-Nitro-cGMP Synthesis

ROS and NO are key signaling molecules that mediate many developmental and physiological processes. Pathogens elicited rapid NO and H2O2 production, which triggers plant defense signaling, including expression of defense-related genes, hypersensitive responses, and stomatal closure (Delledonne et al., 1998, 2001; Durner et al., 1998; Lee et al., 1999; de Pinto et al., 2002; Melotto et al., 2006). In addition, NO and H2O2 are important signaling molecules involved in other processes such as root development, root gravitropism, and seed germination (Joo et al., 2001; Pagnussat et al., 2002; Bailly, 2004; Bethke et al., 2004; Hu et al., 2005; Li et al., 2007). ROS and NO have been identified as essential components of the signaling network inducing stomatal closure in response to ABA, jasmonate, darkness, UV, and high CO2 concentrations (Zhang et al., 2001; Neill et al., 2002; Desikan et al., 2004; She et al., 2004; He et al., 2005; Liu et al., 2005; Kolla and Raghavendra, 2007; Kolla et al., 2007). In response to stressors, guard cells generate ROS and NO simultaneously; this indicates a close functional relationship between ROS and NO in guard cell signaling (Bright et al., 2006). Several research groups have suggested that ROS is an upstream component in the NO signaling network involved in stomatal closure in response to UV-B, ABA, chitosan, and extracellular calmodulin (He et al., 2005; Bright et al., 2006; Li et al., 2009; Srivastava et al., 2009). In ABA inhibition of stomatal opening, ROS also acts upstream of NO (Yan et al., 2007).

In this article, we showed that ROS and NO were required for ABA-induced 8-nitro-cGMP formation, which indicates that guanine nitration of cGMP occurred in the presence of both NO and ROS. NO and ROS act synergistically to mediate stomatal closure through guanine nitration of cGMP. In other words, the NO and ROS signaling pathways converge at guanine nitration of cGMP.

The Role of ABI1 in 8-Nitro-cGMP Signaling

ABI1 acts at a very early step of ABA signaling. ABA binding to the ABA receptor (RCAR/PYR/PYL) leads to inactivation of ABI1,
which triggers ABA signaling (Ma et al., 2009; Park et al., 2009). This led to our expectation that ABI1 functions upstream of 8-nitro-cGMP. In this article, we showed that ABA did not induce 8-nitro-cGMP synthesis and ROS generation in ab1-1 mutants, indicating that ABI1 acts upstream of 8-nitro-cGMP. We also found that NO and 8-nitro-cGMP did not elicit stomatal closure in ab1-1 mutants. Two possible explanations may account for these results. First, ABI1 may act downstream of 8-nitro-cGMP. ABI1 regulates SLAC1 (Geiger et al., 2009; Brandt et al., 2012) and KAT1 potassium channels (Sato et al., 2009), which play central roles in guard cell signaling. 8-Nitro-cGMP directly or indirectly regulates ABI1 activity, leading to stomatal closure. In the second possible explanation, ABI1 functions in parallel with 8-nitro-cGMP. ABI1 activates or induces the synthesis of a component that functions downstream of 8-nitro-cGMP. This component is not activated or not synthesized in the ab1-1 mutant, which thus does not respond to 8-nitro-cGMP. The role of ABI1 in 8-nitro-cGMP signaling will require further study.

NO/8-Nitro-cGMP Signaling Cascade

On the basis of the results presented here, and in view of the data published by other groups, we propose a model of NO signaling in guard cells (Figure 11). Biotic and abiotic stressors, such as pathogen attack, ABA, UV, jasmonate, and high CO2 concentrations, induce increased NO synthesis (Delledonne et al., 1998; Durner et al., 1998; Neill et al., 2002; He et al., 2005; Liu et al., 2005; Kolla and Raghavan, 2007), which causes higher cGMP levels through NOGC1. The same stressors also induce the formation of ROSs (Delledonne et al., 2001; Zhang et al., 2001; Desikan et al., 2004; She et al., 2004; Suhita et al., 2004; He et al., 2005; Kolla et al., 2007), which react with NO or nitrite to form reactive nitrogen oxide species (RNS) (van der Vliet et al., 1997). RNS may react with cGMP to produce 8-nitro-cGMP (Yermilov et al., 1995; Sawa et al., 2007). 8-Nitro-cGMP triggers cytoplasmic [Ca2+] elevation and then activates slow anion channel1, which induces stomatal closure. In the dark, other plant hormones, such as cytokinin, auxin, and natriuretic peptides, induce decreases in NO and ROS (She and Song, 2006; Song et al., 2006) and activate membrane-bound (NO-independent) guanylate cyclase to form cGMP (Pharmawati et al., 2001). In this case, cGMP nitration does not occur; thus, the native cGMP induces stomatal opening. Guanine nitration of cGMP is therefore considered to be a critical factor for switching the guard cell signaling pathway from stomatal opening to stomatal closure. We conclude that 8-nitro-cGMP is an important signaling molecule that mediates a dynamic signaling cascade involving NO/cGMP- and ROS-mediated pathways in guard cells.

METHODS

Plant Materials

Seeds of the Arabidopsis thaliana mutant slac1-2 were kindly provided by Koh Iba (Kyushu University, Japan). Seeds of the Arabidopsis ecotype Columbia-0 and mutant lines nosc1 (CS820406) and ab1-1 were obtained from the Arabidopsis Biological Research Center. Seedlings were grown in soil in a growth chamber for 1 to 2 months at 20 to 25°C under an 11-h-light/13-h-dark cycle (50 μmol m⁻² s⁻¹).

Stomatal Aperture Assay

Epidermal strips were peeled from the abaxial surface of young but fully expanded leaves. To study stomatal closing, strips were kept in opening medium (10 mM MES-KOH, pH 6.15, 50 mM KCl, and 0.1 mM CaCl2) for 3 h in the light (50 μmol m⁻² s⁻¹) before transfer to opening medium containing one or more of various reagents (30 μM ABA, 100 μM cPTIO, 100 μM NOCS, 2 μM ODQ, 2 μM LY83583, 100 μM cGMP, 1 to 100 μM 8-bromo-cGMP, 1 to 100 μM 8-nitro-cGMP, 10 μM 8-bromo-cADPR, 250 μM BAPTA-AM, and 5 mM nicotinamide). 8-Nitro-cGMP was synthesized as described (Sawa et al., 2007). NOCS and cPTIO were from Dojindo. ODQ was from Sigma-Aldrich. LY83583 was from Merck. Other reagents were from Wako Pure Chemical Industries. The strips were incubated in this medium for 2 h in the light. To investigate stomatal opening, epidermal strips were floated in opening medium for 2 h in the dark and were then incubated in opening medium containing 8-bromo-cGMP or 8-nitro-cGMP for 3 h in the dark. During treatments, epidermal strips were kept at 23°C. After treatment, stomata were photographed with a charge-coupled device camera (DS-Fi1; Nikon) mounted on a microscope (Eclipse E600; Nikon). Stomatal apertures (inner diameters of the stomatal pores) were measured using a digital microanalyzer (Japan Polaroid Digital Products).

Identification of 8-Nitro-cGMP by MS

About 5 g leaves treated with 30 μM ABA for 2 h were ground in liquid nitrogen with a mortar and pestle and resuspended in extraction medium (2% acetic acid/80% ethanol). The mixture was incubated for 30 min at 4°C and centrifuged at 15,000 g for 20 min. The supernatants were removed and the pellets were rehomogenized two times with extraction medium. The supernatants were combined and applied to OASIS WAX-SPE columns (Waters) preconditioned with 1 mL methanol and 1 mL water. The columns were rinsed with 2 mL 2% (v/v) acetic acid/water and 2 mL methanol and then eluted with 2 mL 5% (v/v) NH4OH/methanol. The eluates were dried under vacuum and resuspended in 0.6 mL 1% (v/v) formic acid/water (Sigma-Aldrich) and then separated by HPLC fractionation on a Mightysil RP-18 column (250 × 4.6-mm i.d.; Kanto Chemical) with a binary solvent system comprising 0.1% (v/v) formic acid/water (A) and 0.1% (v/v) formic acid/methanol (B) at 40°C with flow rate of 1.0 mL/min. The linear solvent gradient program applied was: 0 to 3 min B, 5%; 3 to 9 min B, 5 to 100%; and 9 to 20 min B, 100%. The injection volume was 20 μL each time. All 8-nitro-cGMP fractions (at 11.2 to 12.0 min) were concentrated under vacuum and reconstituted with 200 μL 0.1% formic acid.

Detection and identification of 8-nitro-cGMP were performed with a liquid chromatography–ion trap–time-of-flight mass spectrometer (Shimadzu). The fractionated samples were eluted from reverse-phase HPLC on a CAPCELL PAK C18 column (150 × 3.0-mm i.d.; Shiseido Fine Chemicals), with a binary solvent system comprising 0.1% (v/v) formic acid/water (A) and 0.1% (v/v) formic acid/methanol (B) at room temperature (~20°C) with a flow rate of 0.20 mL/min. The linear solvent gradient program applied was as follows: 0 to 1 min B, 5% solvent B; 1 to 6 min, 5 to 100% solvent B; and 6 to 20 min, hold at 100% solvent B. The injector volume selected was 10 μL. The MS was operated with a probe voltage of
eluates were dried and then dissolved in 0.1 mL water, followed by and separated on OASIS WAX-SPE columns as described above. The supernatants were removed and the pellets were rehomogenized 

Kaisha). Epidermal tissues were collected on 40-

6.15, containing 2 mM DTT using a homogenizer (AM-5; Nihonseiki Kaisha). Epidermal tissues were collected on 40-

m nylon mesh and kept in opening medium for 3 h in the light before incubation in opening medium containing ABA, NOC5, and hydrogen peroxide for the indicated times. The treated epidermal tissues (100 to 200 mg) were ground with a microtube and pestle and resuspended in the extraction medium. The mixture was incubated for 30 min at 4°C and centrifuged at 15,000g for 20 min. The supernatants were removed and the pellets were rehomogenized two times with the extraction medium. The supernatants were combined and separated on OASIS WAX-SPE columns as described above. The eluates were dried and then dissolved in 0.1 mL water, followed by analysis of 8-nitro-cGMP contents by LC-MS/MS in MRM mode. \( ^{15} \text{N} \)-labeled 8-nitro-cGMP (8-15NO2-cGMP) was synthesized as described (Fujii et al., 2010) and was used as an internal standard. LC-MS/MS was performed with a 3200 QTRAP LC-MS/MS system (AB SCIEX) after reverse-phase HPLC on a Mightysil RP-18 column (150 \( \times \) 2.0-mm i.d.) with a binary solvent system comprising 0.1% (v/v) formic acid/water (A) and methanol (B). The linear solvent gradient program applied was as follows: 0 to 7 min, 0 to 100% solvent B; 7 to 20 min, hold at 100% solvent B. The total flow rate was 0.20 mL/min, and the injection volume was 10 \( \mu \)L. Ionization was achieved using electrospray in the negative mode with the clustering potential, and collision energy were set to 350°C, 50 V, and 2000V, respectively. Quanti

cation was used for data acquisition and processing. The data are average values from at least three independent experiments.

**Quantitative Analyses of 8-Nitro-cGMP by LC-MS/MS**

Leaves were homogenized (30 s \( \times \) 3) in ice-cold 10 mM MES-KOH, pH 6.15, containing 2 mM DTT using a homogenizer (AM-5; Nihonseiki Kaisha). Epidermal tissues were collected on 40-

m nylon mesh and kept in opening medium for 3 h in the light before incubation in opening medium containing ABA, NOC5, and hydrogen peroxide for the indicated times. The treated epidermal tissues (100 to 200 mg) were ground with a microtube and pestle and resuspended in the extraction medium. The mixture was incubated for 30 min at 4°C and centrifuged at 15,000g for 20 min. The supernatants were removed and the pellets were rehomogenized two times with the extraction medium. The supernatants were combined and separated on OASIS WAX-SPE columns as described above. The eluates were dried and then dissolved in 0.1 mL water, followed by analysis of 8-nitro-cGMP contents by LC-MS/MS in MRM mode. \( ^{15} \text{N} \)-labeled 8-nitro-cGMP (8-15NO2-cGMP) was synthesized as described (Fujii et al., 2010) and was used as an internal standard. LC-MS/MS was performed with a 3200 QTRAP LC-MS/MS system (AB SCIEX) after reverse-phase HPLC on a Mightysil RP-18 column (150 \( \times \) 2.0-mm i.d.) with a binary solvent system comprising 0.1% (v/v) formic acid/water (A) and methanol (B). The linear solvent gradient program applied was as follows: 0 to 7 min, 0 to 100% solvent B; 7 to 20 min, hold at 100% solvent B. The total flow rate was 0.20 mL/min, and the injection volume was 10 \( \mu \)L. Ionization was achieved using electrospray in the negative mode with the clustering potential, and collision energy were set to 350°C, 50 V, and 2000V, respectively. Quanti

cation was used for data acquisition and processing. The data are average values from at least three independent experiments.

**Immunocytochemical Analyses**

Epidermal tissues were incubated in opening medium for 3 h and were then transferred to opening medium containing one or more of various reagents (30 \( \mu \)M ABA, 25 \( \mu \)M l-NAME, 100 \( \mu \)M cGMP, 100 \( \mu \)M NOCS, 200 \( \mu \)M SNAP, 100 \( \mu \)M cPTIO, 2 \( \mu \)M ODO, 100 \( \mu \)M Tiron, 2 mM DTT, and 1000 units of catalase), l-NAME, NOC5, cPTIO, and SNAP were from Dojindo. Other reagents were from Wako Pure Chemical Industries. After 1 h, the tissues were fixed with Zamboni solution (4% paraformaldehyde, 10 mM picric acid, and 0.1 M phosphate buffer, pH 7.4) at 4°C for 4 h. After the epidermal strips were washed three times with PBS, fixed strips were placed on silanized glass slides (Dako). Preparations were digested enzymatically (20 mM MES-KOH, pH 5.5, 0.7% cellulysin, and 0.2 M manninitol) at 25°C for 15 min, washed with PBS, and treated with 0.5% Triton X-100 at room temperature for 15 min. After the preparations were washed again with PBS, they were blocked with 3% BSA in PBS at 4°C overnight and then washed with PBS. The slides were then incubated with antibodies against 8-nitro-cGMP (1G6; Sawat et al. 2007) in Can Get Signal immunostain solution A (Toyobo) for 2 h at room temperature. The slides were washed in PBST (PBS and 0.05% Tween 20) four times for 5 min each. The fluorescent secondary antibody (2 \( \mu \)g/mL Alexa 488-conjugated goat anti-mouse IgG [Invitrogen] dissolved in Can Get Signal immunostain solution A) was added, and the samples were incubated for 60 min at room temperature. After the slides were washed, they were mounted with Vectashield (Vector Laboratories) and observed with a fluorescence microscope (Eclipse E600; Nikon) equipped with a charge-coupled device camera (DS-Fi1; Nikon). Fluorescence intensities were measured using EZ-C1 FreeViewer version 3.70 software (Nikon). At least 100 guard cells were evaluated. The data are average values from at least three independent experiments.

**ROS Detection in Guard Cells**

Epidermal tissues were incubated in opening medium for 3 h and were then transferred to opening medium containing 30 \( \mu \)M ABA and incubated for 3 to 15 min in the light. The treated epidermal tissues were collected on 40-

m nylon mesh, floated in opening medium containing 10 \( \mu \)M H2DCF-DA (Wako Pure Chemical Industry) for 20 min in the dark, and then collected on 40-

m nylon mesh. The dye-loaded epidermal tissues were washed with opening medium and floated in opening medium in the dark for 10 min. Control experiments (with only 0.01% DMSO added) showed no stimulation of ROS production. Fluorescence intensities were measured as described above, and data shown were obtained by subtracting the control values (without ABA) from the values for treated samples. At least 30 guard cells were evaluated. The data are average values from at least three independent experiments.

**NO Detection in Guard Cells**

Epidermal tissues were incubated in opening medium for 3 h, transferred to opening medium containing 10 \( \mu \)M DAF-2 DA (Sekisui Medical), and incubated for 20 min in the dark. The tissues were washed with opening medium and floated in opening medium in the dark for 10 min. The dye-loaded epidermal tissues were transferred to opening medium containing 30 \( \mu \)M ABA and incubated for 3 to 15 min in the light. Fluorescence intensities were measured for at least 30 guard cells, and the data shown were obtained by subtracting the control values (without ABA) from the values for the treated samples. The data are average values from at least three independent experiments.

**Statistical Analysis**

All data are presented as means \( \pm \) se. The statistical significance of differences between two groups was determined by Student’s t test.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: NOGC1 (At1g62580), ABI1 (At1g26080), and SLAC1 (At1g12480).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1. Effect of Inhibitors on Stomatal Aperture.**

**Supplemental Figure 2. Inhibitors of Guanylate Cyclase Reduced NOC5-Induced Stomatal Closure.**

**Supplemental Figure 3. Effect of cGMP and 8-Bromo-cGMP on Stomatal Aperture in the nobc1 Mutant.**

**Supplemental Figure 4. Fluorescence Micrographs of Epidermal Strips Showing Fluorescent Signals from Anti-8-Nitro-cGMP.**

**Supplemental Figure 5. ABA-Induced 8-Nitro-cGMP Synthesis in the abi1-1 Mutant.**
**Supplemental Figure 6.** ABA-Induced ROS Generation Did Not Occur in the \(abi1-1\) Mutant Guard Cells.

**Supplemental Figure 7.** Effect of BAPTA-AM on NOC5-Induced Stomatal Closure.

**Supplemental Figure 8.** Effect of Antagonist of Cyclic ADPR on NOC5-Induced Stomatal Closure.

**Supplemental Figure 9.** Effect of 8-Nitro-cGMP on Stomatal Aperture in the \(abi1-1\) Mutant.

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**AUTHOR CONTRIBUTIONS**


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