A Signaling Pathway Linking Nitric Oxide Production to Heterotrimeric G Protein and Hydrogen Peroxide Regulates Extracellular Calmodulin Induction of Stomatal Closure in Arabidopsis[1][W]

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Extracellular calmodulin (ExtCaM) regulates stomatal movement by eliciting a cascade of intracellular signaling events including heterotrimeric G protein, hydrogen peroxide (H_2O_2), and Ca^{2+}. However, the ExtCaM-mediated guard cell signaling pathway remains poorly understood. In this report, we show that Arabidopsis (*Arabidopsis thaliana*) NITRIC OXIDE ASSOCIATED1 (AtNOA1)-dependent nitric oxide (NO) accumulation plays a crucial role in ExtCaM-induced stomatal closure. ExtCaM triggered a significant increase in NO levels associated with stomatal closure in the wild type, but both effects were abolished in the *Atnoa1* mutant. Furthermore, we found that ExtCaM-mediated NO generation is regulated by GPA1, the Gα-subunit of heterotrimeric G protein. The ExtCaM-dependent NO accumulation was nullified in *gpa1* knockout mutants but enhanced by overexpression of a constitutively active form of *GPA1* (cGα). In addition, cGα *Atnoa1* and *gpa1-2 Atnoa1* double mutants exhibited a similar response as did *Atnoa1*. The defect in *gpa1* was rescued by overexpression of *AtNOA1*. Finally, we demonstrated that G protein activation of NO production depends on H_2O_2. Reduced H_2O_2 levels in guard cells blocked the stomatal response of cGα lines, whereas exogenously applied H_2O_2 rescued the defect in ExtCaM-mediated stomatal closure in *gpa1* mutants. Moreover, the *atrbohD/F* mutant, which lacks the NADPH oxidase activity in guard cells, had impaired NO generation in response to ExtCaM, and H_2O_2-induced stomatal closure and NO accumulation were greatly impaired in *Atnoa1*. These findings have established a signaling pathway leading to ExtCaM-induced stomatal closure, which involves GPA1-dependent activation of H_2O_2 production and subsequent AtNOA1-dependent NO accumulation.

Plant guard cells control opening and closure of the stomata in response to phytohormones (e.g. abscisic acid [ABA]) and various environmental signals such as light and temperature, thereby regulating gas exchange for photosynthesis and water status via transpiration (Schroeder et al., 2001). Cytosolic calcium ($[Ca^{2+}]_i$) has been shown to be a key second messenger that changes in response to multiple stimuli in guard cells (McAinsh et al., 1995; Grabov and Blatt, 1998; Wood et al., 2000). A large proportion of Ca^{2+} is localized in extracellular space. It has been shown that external Ca^{2+} concentration ($[Ca^{2+}]_o$) promotes stomatal closure and induces oscillation in $[Ca^{2+}]_i$ in guard cells (MacRobbie, 1992; McAinsh et al., 1995; Allen et al., 2001). However, how the guard cells perceive $[Ca^{2+}]_o$ concentration and convert $[Ca^{2+}]_o$ changes into $[Ca^{2+}]_i$ changes was not understood until a calcium-sensing receptor (CAS) in the plasma membrane of guard cells in Arabidopsis (*Arabidopsis thaliana*) was identified (Han et al., 2003). The external Ca^{2+} ($Ca^{2+}{}_o$)-induced $[Ca^{2+}]_i$ increase is abolished in CAS antisense lines (Han et al., 2003). Both $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ show diurnal oscillation that is determined by stomatal conductance, whereas the amplitude of $[Ca^{2+}]_i$ oscillation is reduced in CAS antisense lines (Tang et al., 2007). The reduced amplitude of $[Ca^{2+}]_i$ diurnal oscillation in response to $Ca^{2+}{}_o$ treatment suggests the potential existence of other $[Ca^{2+}]_o$ sensor(s) that may transmit $[Ca^{2+}]_o$ information into the $[Ca^{2+}]_i$ response in coordination with CAS. Extracellular calmodulin (ExtCaM) could be such an additional $[Ca^{2+}]_o$ sensor.

Calmodulin is a well-known Ca^{2+} sensor that is activated upon binding of Ca^{2+}. It has been shown that calmodulin exists not only intracellularly but also extracellularly in many plant species (Biro et al., 1984; Sun et al., 1994, 1995; Cui et al., 2005). ExtCaM has been implicated in several important biological

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functions, such as the promotion of cell proliferation, pollen germination, and tube growth (Sun et al., 1994, 1995; Ma and Sun, 1997; Ma et al., 1999; Cui et al., 2005; Shang et al., 2005). ExtCaM is found in the cell wall of guard cells in *Vicia faba* and in the epidermis of Arabidopsis by immunogold labeling/electron microscopy and western-blot analyses, respectively, and the endogenous CaM in the extracellular space has been shown to regulate stomatal movements (Chen et al., 2003; Xiao et al., 2004). Under natural conditions, once the activity of ExtCaM has been inhibited by its membrane-impermeable antagonist W7-agrose or CaM antibody, stomatal opening under light is enhanced and stomatal closure in darkness is inhibited in *V. faba* and Arabidopsis (Chen et al., 2003; Xiao et al., 2004). $[Ca^{2+}]_i$ and cytosolic hydrogen peroxide (H_2O_2) changes, two events involved in ExtCaM-regulated stomatal movement (Chen et al., 2004), are likely regulated by light/darkness (Chen and Gallie, 2004; Tang et al., 2007), suggesting that ExtCaM plays an important physiological role in the regulation of stomatal diurnal rhythm. Calmodulin-binding proteins have been found in the protoplast of suspension-cultured Arabidopsis cells, supporting the idea that ExtCaM functions as a peptide-signaling molecule (Cui et al., 2005). Furthermore, ExtCaM triggers $[Ca^{2+}]_i$ elevation in guard cells of *V. faba* and Arabidopsis and in lily (*Lilium daviddi*) pollen (Chen et al., 2004; Xiao et al., 2004; Shang et al., 2005). These observations support the notion that ExtCaM could be a potential $[Ca^{2+}]_o$ sensor for external calcium, and this external calcium sensing could subsequently regulate the $[Ca^{2+}]_i$ level through a signaling cascade.

It is interesting that ExtCaM and ABA induce some parallel changes in second messengers in guard cell signaling. Our previous studies show that ExtCaM induces $[Ca^{2+}]_i$ increase and H_2O_2 generation through the Gα-subunit (GPA1) of a heterotrimeric G protein, and increased H_2O_2 further elevates $[Ca^{2+}]_i$ (Chen et al., 2004). G protein, Ca^{2+}, and H_2O_2 are well-known second messengers in ABA-induced guard cell signaling (McAinsh et al., 1995; Grabov and Blatt, 1998; Pei et al., 2000; Wang et al., 2001; Zhang et al., 2001; Liu et al., 2007). However, the signaling cascade triggered by ExtCaM in guard cells is poorly understood. New ABA signaling components in guard cells could provide a clue in the study of the molecular mechanism of ExtCaM guard cell signaling.

Recently, nitric oxide (NO) has been shown to serve as an important signal molecule involved in many aspects of developmental processes, including floral transition, root growth, root gravitropism, adventitious root formation, xylogenesis, seed germination, and orientation of pollen tube growth (Beligni and Lamattina, 2000; Pagnussat et al., 2002; He et al., 2004; Prado et al., 2004; Gabaldón et al., 2005; Stohr and Stremlau, 2006). Increasing evidence points to a role for NO as an essential component in ABA signaling in guard cells (Garcia-Mata and Lamattina, 2001, 2002; Neill et al., 2002). It has been shown that nitrate reductase (NR) reduces nitrite to NO, and the *nia1, nia2* NR-deficient mutant in Arabidopsis showed reduced ABA induction of stomatal closure (Desikan et al., 2002; Bright et al., 2006). Although animal nitric oxide synthase (NOS) activity has been detected in plants and inhibitors of mammalian NOS impair NO production in plants (Barroso et al., 1999; Corpas et al., 2001), the gene(s) encoding NOS in plants is still not clear. *AtNOS1* in Arabidopsis was initially reported to encode a protein containing NOS activity (Guo et al., 2003). However, recent studies have raised critical questions regarding the nature of AtNOS1 and suggested that *AtNOS1* appears not to encode a NOS (Crawford et al., 2006; Zemojtel et al., 2006). However, the originally described *Atnos1* mutant is deficient in NO accumulation (Crawford et al., 2006). Consequently, AtNOS1 was renamed AtNOA1 (for NITRIC OXIDE ASSOCIATED1; Crawford et al., 2006). Therefore, the *Atnoa1* mutant provides a useful tool for dissecting the function of NO in plants. At present, the molecules that regulate NO generation in ABA-mediated guard cell signaling are not clear. Evidence suggests that H_2O_2, a second messenger important for the regulation of many developmental processes and stomatal movement (Pei et al., 2000; Zhang et al., 2001; Coelho et al., 2002; Demidchik et al., 2003; Kwak et al., 2003), regulates NO generation in guard cells (Lum et al., 2002; He et al., 2005; Bright et al., 2006).

Given the parallel signaling events induced by ABA and ExtCaM, we investigated whether NO is involved in the regulation of ExtCaM-induced stomatal closure in Arabidopsis and whether it is linked to G protein and H_2O_2, two key regulators of both ExtCaM and ABA regulation of stomatal movements. Using Arabidopsis mutants (e.g. *GPA1* null mutants, the NO-producing mutant *Atnoa1*, and the guard cell H_2O_2 synthetic enzymatic mutant *atrbohD/F*) combined with pharmacological analysis, we present compelling evidence to establish a linear functional relationship between Gα, H_2O_2, and NO in ExtCaM guard cell signaling.

RESULTS

AtNOA1-Dependent NO Accumulation Is Required for ExtCaM-Induced Stomatal Closure

To investigate a potential role for NO in ExtCaM-induced stomatal closure, we examined the effect of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide potassium salt (cPTIO) on ExtCaM action. Both ExtCaM and the NO donor sodium nitroprusside (SNP) induced stomatal closure (Fig. 1A), as demonstrated previously (Garcia-Mata and Lamattina, 2001; Chen et al., 2004). cPTIO greatly reduced ExtCaM-induced stomatal closure compared with ExtCaM-treated stomata ($P < 0.001$; Fig. 1A). We then monitored changes in NO levels in response to applied ExtCaM using the NO-specific

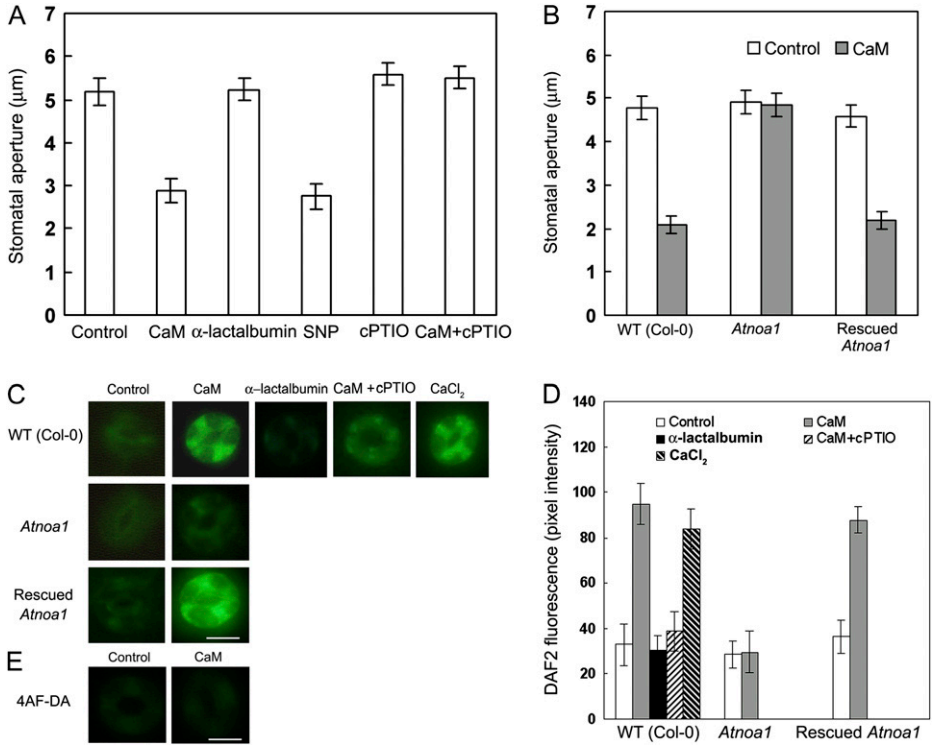

Figure 1. AtNOA1-dependent NO production regulates ExtCaM-induced stomatal closure. A, Open stomata in the wild type (WT) were incubated in MES buffer (control), 10^{-8} M CaM, 10^{-8} M α-lactalbumin, 60 μM SNP, 200 μM cPTIO, or 10^{-8} M CaM plus 200 μM cPTIO. B, Open stomata in the wild type, *Atnoa1*, and rescued *Atnoa1* were incubated in MES buffer (control) or 10^{-8} M CaM. Stomatal apertures were measured 2 h after treatment. C and D, ExtCaM induces a NO increase in guard cells in the wild type and rescued *Atnoa1* but fails in the *Atnoa1* mutant. Open stomata in each genotype were incubated in 10 μM DAF2-DA buffer for 15 min and then incubated in MES buffer (control), 10^{-8} M α-lactalbumin, 1 mM $CaCl_2$, 10^{-8} M CaM, or 10^{-8} M CaM plus 200 μM cPTIO for 30 min in darkness. Fluorescence images and intensities in guard cells were recorded and are shown in C and D, respectively. E, Open stomata in the wild type were incubated in 10 μM 4AF-DA, a negative control of DAF2-DA, for 15 min and then incubated in MES buffer or 10^{-8} M CaM for 30 min in darkness, and fluorescence images in guard cells were recorded. Bars = 10 μm.

fluorescent dye diaminofluorescein diacetate (DAF2-DA; Garcia-Mata and Lamattina, 2002; Guo et al., 2003). ExtCaM treatment induced a dramatic increase in DAF2-DA staining in guard cells compared with the untreated control ($P < 0.001$), and cPTIO significantly reduced ExtCaM-induced NO generation in guard cells ($P < 0.001$; Fig. 1, C and D). The same concentration of α-lactalbumin, which contained an EF-hand like structure (Iyer and Qasba, 1999), had no effect on stomatal movement or NO levels in guard cells (Fig. 1, A, C, and D), indicating that induction of ExtCaM on stomatal closure and NO generation is CaM specific and is not induced by any other member of the EF-hand family of proteins. Application of calcium also caused NO synthesis, as did ExtCaM, supporting the possibility that ExtCaM transmits extracellular calcium changes into intracellular response (Fig. 1, C and D). Guard cells in wild-type (ecotype Columbia [Col-0]) epidermis were loaded with 4-aminofluorescein diacetate (4AF-DA), a negative control of DAF2-DA, and fluorescence intensity was examined when treated with ExtCaM. Fluorescence of 4AF in guard cells did not increase upon ExtCaM treatment (Fig. 1E). These results support an essential role for NO in regulating ExtCaM-induced stomatal closure.

We next determined whether the *Atnoa1* mutant, which is defective in NO accumulation (Guo et al., 2003), exhibited different ExtCaM-induced guard cell responses. NO levels in guard cells and stomatal apertures in the *Atnoa1* mutant and rescued *Atnoa1* line were examined following ExtCaM treatment. ExtCaM-induced stomatal closure was impaired significantly in *Atnoa1* compared with the ExtCaM-treated wild type ($P < 0.001$; Fig. 1B), consistent with the blocking effect of the NO scavenger cPTIO on ExtCaM-induced stomatal closure ($P < 0.001$). These results were supported by NO changes: ExtCaM-induced NO accumulation was almost completely abolished in the *Atnoa1* mutant ($P > 0.05$; Fig. 1, C and D). Both stomatal closure and NO accumulation upon ExtCaM treatment in the rescued *Atnoa1* line were similar to that of the wild type ($P > 0.05$; Fig. 1, B–D). These data indicate that AtNOA1-mediated NO accumulation plays an essential role in ExtCaM-induced stomatal closure.

G Protein α Subunit Modulates AtNOA1-Dependent NO Production in ExtCaM-Induced Stomatal Closure

The GPA1 G protein α subunit (Gα) has been shown to modulate ABA regulation of stomatal opening (Wang et al., 2001) and closure (Liu et al., 2007) and ExtCaM induction of stomatal closure (Chen et al., 2004). To investigate whether Gα also regulates ExtCaM induction of NO generation, we examined NO levels in guard cells of the *gpa1* null mutants and of transgenic lines expressing a constitutively active form of *GPA1* (cGα; Okamoto et al., 2001; Wang et al., 2001). NO levels in the guard cells of two independent cGα lines were higher than that of the wild type (ecotype Wassilewskija [Ws]) in response to ExtCaM ($P < 0.001$), but ExtCaM failed to induce NO accumulation in *gpa1-1* and *gpa1-2* mutants ($P > 0.05$; Fig. 2, A and B; data from one line of cGα are shown). These results clearly indicate that Gα is required for ExtCaM induction of NO generation. These data are consistent with the faster response of cGα stomata to ExtCaM and the impaired stomatal closure of *gpa1* with ExtCaM treatment, as reported previously (Chen et al., 2004). Furthermore, the NO donor SNP induced stomatal closure in *gpa1-1* and *gpa1-2* mutants ($P < 0.001$; Fig. 2C), further supporting that NO acts downstream of Gα during ExtCaM-induced stomatal closure.

To further test the role of Gα in the regulation of NO generation in ExtCaM guard cell signaling, we performed crosses between the cGα1 line (Okamoto et al., 2001) and the *Atnoa1* mutant and between *gpa1-2* and *Atnoa1* and examined stomatal responses and NO levels in guard cells in the double mutants. As shown in Figure 3, cGα1 *Atnoa1* stomata did not close when treated with ExtCaM ($P > 0.05$; Fig. 3A) and ExtCaM failed to arouse NO production in guard cells of cGα1 *Atnoa1* ($P > 0.05$; Fig. 3, B and C). The NO donor SNP rescued the defects of cGα1 *Atnoa1* in stomatal closure and NO generation, suggesting that the defects of cGα1 *Atnoa1* are due to a lack of NO production (Fig. 3).

At the same time, ExtCaM-induced stomatal response and NO level in the *gpa1-2 Atnoa1* double mutant were similar to those in both *gpa1-2* and *Atnoa1* mutants ($P > 0.05$; Fig. 3), indicating that GPA1 and AtNOA1 act in the same pathway. Furthermore, we introduced the *35S*: *AtNOA1* gene into the *gpa1-2* mutant. NO level and stomatal response following ExtCaM treatment were examined in three independent transgenic lines, and one set of representative data is shown. Stomatal closure and NO generation in guard cells in *gpa1-2 AtNOA1* were similar to those of the wild type when treated with ExtCaM ($P > 0.05$; Fig. 3). Taken together, our results clearly demonstrate that the GPA1 G protein acts upstream of NO production to activate ExtCaM-induced stomatal closure.

Essential Role of H_2O_2 Generation in Gα-Induced NO Production in ExtCaM Guard Cell Signaling

Pharmacological evidence supports a role for H_2O_2 in the mediation of ExtCaM-induced stomatal closure

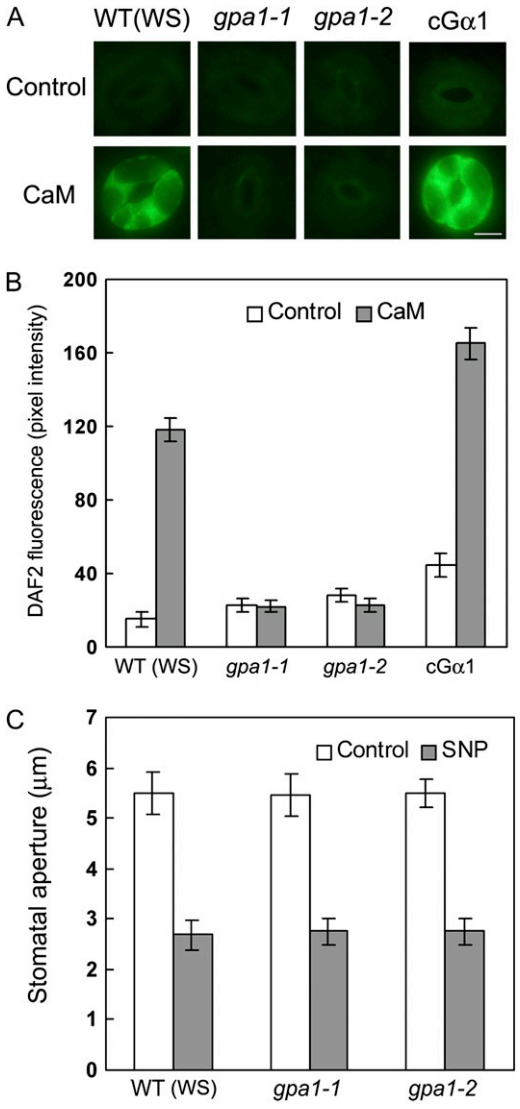

Figure 2. GPA1 modulates NO synthesis in ExtCaM guard cell signaling. A and B, Guard cells preloaded with 10 μM DAF2-DA in the wild type (WT/WS), *gpa1* mutants, and the cGα line were incubated in MES buffer (control) or 10^{-8} M CaM for 30 min in darkness. Fluorescence images and intensities in guard cells were recorded and are shown in A and B, respectively. Bar = 10 μm. C, Open stomata in the wild type, *gpa1-1*, and *gpa1-2* were incubated in MES buffer (control) or 60 μM SNP. Stomatal apertures were measured 2 h after treatment. WS, Ecotype Ws.

in *V. faba* (Chen et al., 2004). In this study, we vigorously tested the role of H_2O_2 in Arabidopsis using both pharmacological and genetic approaches. Stomata of *atrbohD/F* failed to close upon ExtCaM treatment, resembling the response of the wild type (Col-0) pretreated with catalase (CAT), a H_2O_2 scavenger ($P > 0.05$; Fig. 4A). Furthermore, we measured H_2O_2 changes using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCFDA). As shown in Figure 4, ExtCaM induced a dramatic increase in H_2O_2 level in wild-type guard cells ($P < 0.001$) but not in *atrbohD/F* guard cells,

while H_2O_2 level in the control guard cells did not change significantly during the recording period (Fig. 4, B and C). These results demonstrate that ExtCaM-induced stomatal closure depends on its activation of H_2O_2 production in guard cells.

We next sought to determine whether Gα and H_2O_2 act in the same signaling pathway triggered by ExtCaM. We examined whether *gpa1* and cGα affect

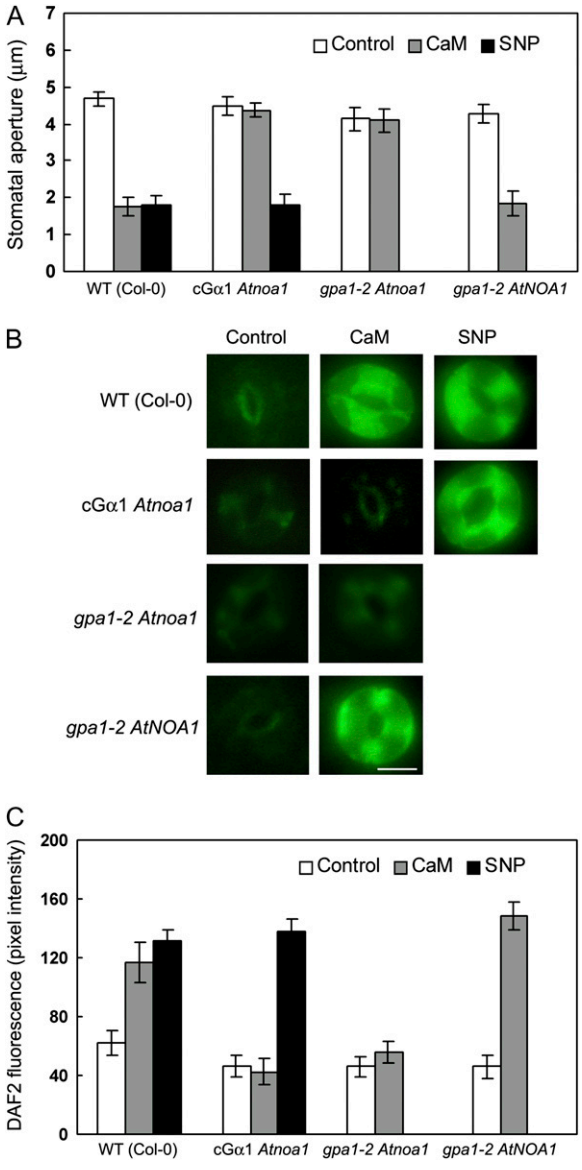

Figure 3. The effect of GPA1 depends on AtNOA1-mediated NO production. A, Open stomata in the wild type (WT), cGα1 *Atnoa1* and *gpa1-2 Atnoa1* double mutants, and *gpa1-2 AtNOA1* lines were incubated in MES buffer (control), 10^{-8} M CaM, or 60 μM SNP. Stomatal apertures were measured 2 h after treatment. B and C, Guard cells preloaded with 10 μM DAF2-DA in the wild type, cGα1 *Atnoa1* and *gpa1-2 Atnoa1* double mutants, and *gpa1-2 AtNOA1* lines were incubated in MES buffer (control), 10^{-8} M CaM, or 60 μM SNP for 30 min in darkness. Fluorescence images and intensities in guard cells were recorded and are shown in B and C, respectively. Bar = 10 μm.

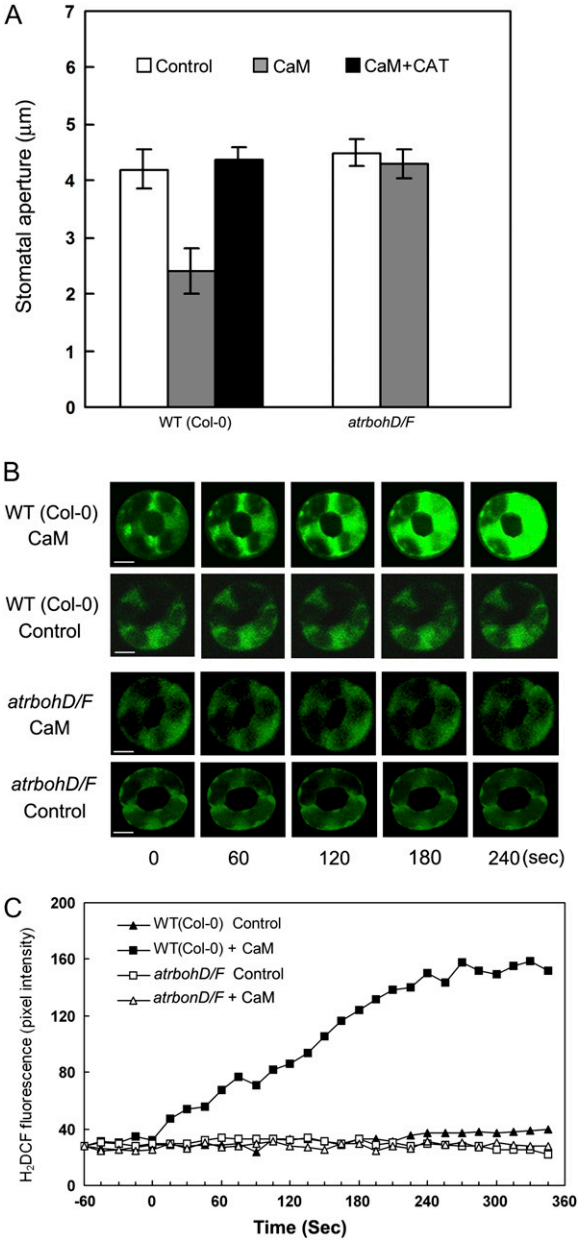

Figure 4. Impaired H_2O_2 synthesis blocks ExtCaM-induced stomatal closure. A, Open stomata in the wild type (WT) and *atrbohD/F* were incubated in MES buffer (control), 10^{-8} M CaM, or 10^{-8} M CaM plus 100 units mL^{-1} CAT. Stomatal apertures were measured 2 h after treatment. B and C, Fluorescence images (B) and a quantitative curve of fluorescence intensity (C) show that ExtCaM induces an H_2O_2 increase in the wild type but fails in *atrbohD/F*. H_2O_2 changes in guard cells preloaded with 50 μM H_2DCFDA were measured with a confocal laser scanning microscope, and 10^{-8} M CaM or MES buffer (control) was added at 0 s, as indicated in C. Both fluorescence intensities and images of the guard cells were recorded every 15 s. The experiments were repeated at least three times with seven to 10 cells each time, and one time set of data is presented. Bars = 10 μm.

H_2O_2 generation in response to an ExtCaM stimulus. As shown in Figure 5, both diphenylene iodonium (DPI), an inhibitor of NADPH oxidases, and CAT blocked

the stomatal response to ExtCaM in cGα lines as well as in the wild type (Ws; $P > 0.05$; Fig. 5A; data from one cGα line are shown). cGα lines have been reported to show faster stomatal closure in response to ExtCaM, although they exhibit the same final stomatal apertures as ExtCaM-treated wild-type controls (Chen et al., 2004). Therefore, the large final stomatal aperture in cGα lines under the treatment of ExtCaM together with DPI or CAT demonstrates that the effect of higher Gα activity depends on the generation of H_2O_2. Furthermore, exogenously applied H_2O_2 induced stomatal closure in *gpa1* mutants ($P < 0.001$; Fig. 5B), indicating that action of H_2O_2 does not depend on the activity of Gα. These results suggest that H_2O_2 acts downstream of Gα.

We next investigated whether H_2O_2 is essential for NO generation by examining the NO level in the *atrohD/F* double mutant upon treatment with ExtCaM. ExtCaM failed to induce NO generation in guard cells of *atrbohD/F* or the wild type (Col-0) incubated with CAT ($P > 0.05$; Fig. 6, A and B). The NO donor SNP induced stomatal closure of *atrbohD/F* ($P < 0.001$; Fig. 6C), supporting that NO acts downstream of H_2O_2. These results indicate that H_2O_2 is essential for NO generation induced by ExtCaM. Based on these results and our previous finding that Gα is required for ExtCaM-induced H_2O_2 generation in guard cells (Chen et al., 2004), we speculate that Gα-mediated NO production depends on H_2O_2 generation induced by ExtCaM.

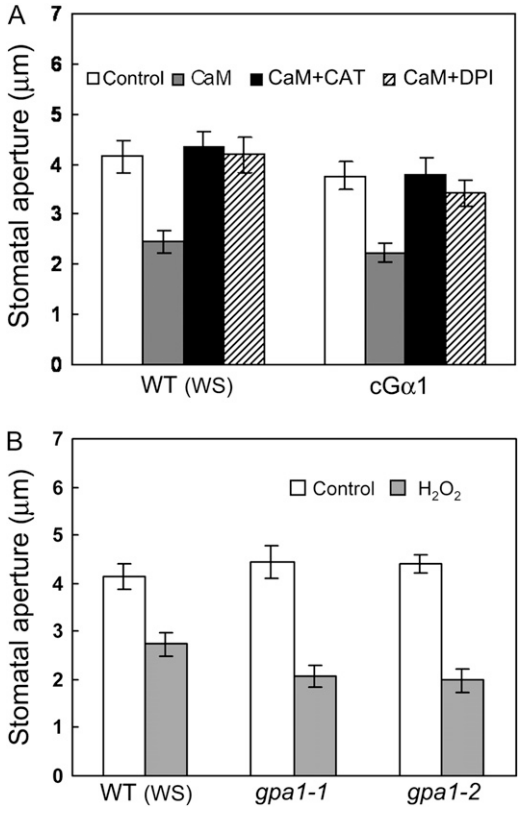

Figure 5. H_2O_2 is required for action of cGα in ExtCaM guard cell signaling. A, Open stomata in the wild type (WT/WS) and cGα lines were incubated in MES buffer (control), 10^{-8} M CaM, 10^{-8} M CaM plus 10 μM DPI, or 10^{-8} M CaM plus 100 units mL^{-1} CAT. B, Open stomata in the wild type, *gpa1-1*, and *gpa1-2* were incubated in MES buffer (control) or 5×10^{-5} M H_2O_2. Stomatal apertures were measured 2 h after treatment. WS, Ecotype Ws.

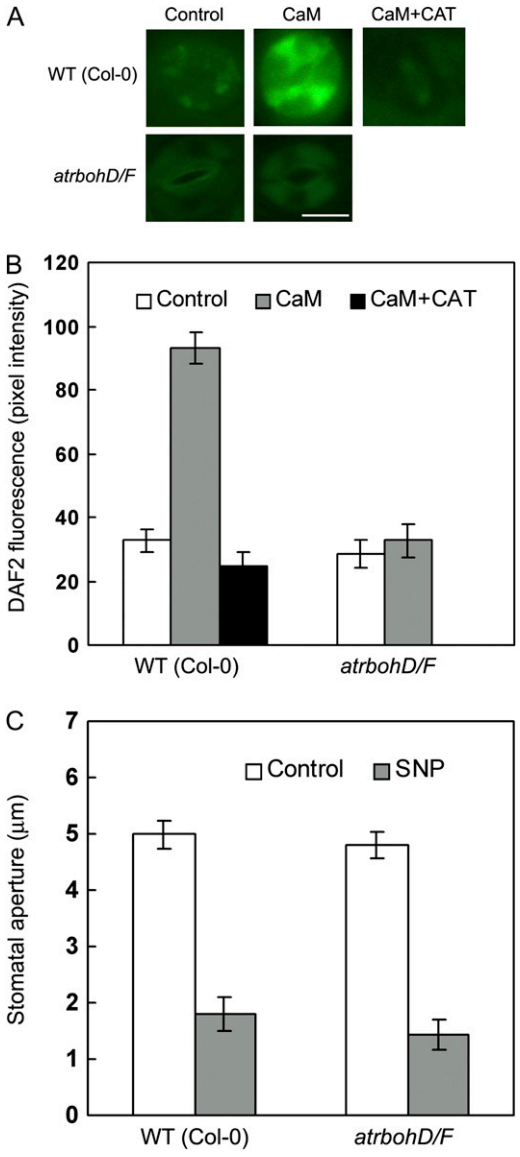

Figure 6. H_2O_2 generation is required for NO production in ExtCaM guard cell signaling. A and B, ExtCaM induces NO synthesis in the wild type (WT) but fails in *atrbohD/F* and the wild type incubated with CAT. Guard cells preloaded with 10 μM DAF2-DA in the wild type and *atrbohD/F* were incubated in MES buffer (control), 10^{-8} M CaM, or 10^{-8} M CaM plus 100 units mL^{-1} CAT for 30 min in darkness. Fluorescence images and intensities in the guard cells were recorded and are shown in A and B, respectively. Bar = 10 μm. C, Open stomata in the wild type and *atrbohD/F* were incubated in MES buffer (control) or 60 μM SNP. Stomatal apertures were measured 2 h after treatment.

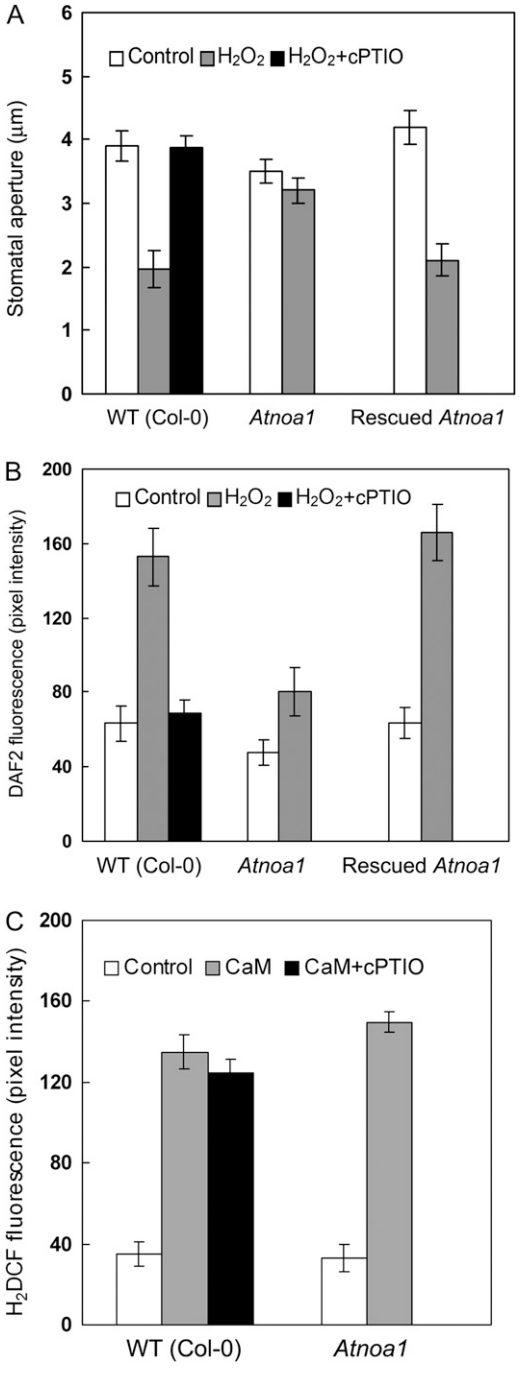

Figure 7. H_2O_2-induced stomatal closure depends on AtNOA1-mediated NO production. A, Open stomata in the wild type (WT), rescued *Atnoa1*, and *Atnoa1* were incubated in MES buffer (control), 5×10^{-5} M H_2O_2, or 5×10^{-5} M H_2O_2 plus 200 μM cPTIO. Stomatal apertures were measured 2 h after treatment. B, Guard cells preloaded with 10 μM DAF2-DA in the wild type, rescued *Atnoa1*, and *Atnoa1* were incubated in MES buffer (control), 5×10^{-5} M H_2O_2, or 5×10^{-5} M H_2O_2 plus 200 μM cPTIO for 30 min in darkness, and fluorescence intensities in guard cells were recorded. ExtCaM-induced H_2O_2 generation does not depend on AtNOA1-mediated NO production. C, Guard cells preloaded with 50 μM H_2DAFDA in the wild type and *Atnoa1* were incubated in MES buffer (control), 10^{-8} M CaM, or 10^{-8} M CaM plus 200 μM cPTIO for 30 min in darkness, and fluorescence intensities in guard cells were recorded.

AtNOA1-Dependent NO Accumulation Is Crucial for H_2O_2-Induced Stomatal Closure

Since interruption of H_2O_2 generation blocked ExtCaM-induced NO generation, we further investigated the relationship between H_2O_2 and NO. H_2O_2 induced stomatal closure in the wild type (Col-0) control and the rescued *Atnoa1* line but not in the wild type incubated with cPTIO ($P > 0.05$); meanwhile, H_2O_2 failed to induce stomatal closure in the *Atnoa1* mutant ($P > 0.05$; Fig. 7A). H_2O_2 induced NO generation in guard cells of the wild type and the rescued *Atnoa1* line, and cPTIO reduced NO generation that was induced by H_2O_2 ($P < 0.001$); H_2O_2 also stimulated NO production in *Atnoa1*, whereas the level was greatly lower than that in the H_2O_2-treated wild type ($P < 0.001$; Fig. 7B). However, ExtCaM induced H_2O_2 generation both in *Atnoa1* and in the wild type incubated with cPTIO ($P > 0.05$; Fig. 7C). These results suggest that H_2O_2-induced stomatal closure mainly depends on AtNOA1-mediated NO generation.

DISCUSSION

In this report, we have provided convincing evidence for a new signaling pathway controlling stomatal movement. Our combined genetic, pharmacological, and biochemical analyses show that ExtCaM stimulates AtNOA1-dependent production of NO, which acts as a second messenger to activate stomatal closure. Furthermore, we found that ExtCaM-triggered NO production is mediated by the activation of Gα of the heterotrimeric G protein in Arabidopsis. We also showed that the modulation of NO production by Gα requires NADPH oxidase-dependent H_2O_2 generation. Therefore, together with previous findings (Chen et al., 2004; Joo et al., 2005; Bright et al., 2006), our results support an ExtCaM-activated guard cell signaling pathway that includes a cascade of ExtCaM, heterotrimeric G protein, NADPH oxidase, and AtNOA1-dependent NO generation.

NO Plays an Important Role in ExtCaM Guard Cell Signaling

The role of NO in the regulation of stomatal closure has been well documented. The NO donor SNP was reported to induce stomatal closure in *V. faba*, *Salpichroa organifolia*, and *Tradescantia* species and to reduce transpiration in wheat (*Triticum aestivum*; Garcia-Mata and Lamattina, 2001). A requirement of NO in ABA-mediated stomatal closure has also been found in pea (*Pisum sativum*) and Arabidopsis (Desikan et al., 2002; Neill et al., 2002; Bright et al., 2006). Our results in this report provide convincing evidence that NO plays an important role in the regulation of ExtCaM-induced

stomatal closure. The NO scavenger cPTIO reversed ExtCaM-induced stomatal closure (Fig. 1A), indicating that NO is required for ExtCaM-induced stomatal closure. The apertures of open stomata measured in this study are consistent with those in reports by Guo et al. (2003) and Liu et al. (2007) but somewhat larger than those reported by Desikan et al. (2002) and Bright et al. (2006). This discrepancy may be due to the different concentrations of KCl in the buffers used in these studies. The concentration of KCl in the MES/KCl buffer is 5 mM in the methods described by Desikan et al. (2002) and Bright et al. (2006) and 50 mM in the methods described by Liu et al. (2007). DAF2-DA is a NO-specific fluorescent dye. Using DAF2-DA dye, our results indicated that ExtCaM induced NO production in Arabidopsis (Fig. 1, C and D), further supporting the essential role of NO in ExtCaM signaling.

In animal systems, it has been reported that extracellular calcium induces NO production (Miles et al., 1998). In this study, we have checked NO levels after treatment with extracellular calcium, and the results indicated that NO levels in the guard cells of the wild type increased when treated with extracellular calcium (Fig. 1, C and D), suggesting that ExtCaM may act as an extracellular calcium sensor. We propose that G protein, H_2O_2, and NO play essential roles in transducing the extracellular calcium signal.

NR has been demonstrated to be an enzyme that mediates NO synthesis in plants. NR-mediated NO generation has also been suggested to regulate stomatal closure induced by ABA (Desikan et al., 2002; Bright et al., 2006). Although it has been shown that the NOS inhibitor nitro-L-Arg methyl ester suppresses stomatal closure and NO generation in guard cells of pea in response to ABA (Neill et al., 2002), the gene(s) encoding NOS in plants is not clear. AtNOA1 was initially isolated as a NOS in Arabidopsis (Guo et al., 2003), whereas the AtNOA1 protein showed no NOS activity in recent studies (Crawford et al., 2006; Zemojtel et al., 2006). Interestingly, AtNOA1 contains a GTP-binding domain and has been shown to be a member of the circularly permuted GTPase family for RNA/ribosome binding and to be involved in ribosome assembly (Moreau et al., 2008). Although it is not clear how AtNOA1 affects NO accumulation indirectly, the *Atnoa1* mutant shows impaired stomatal closure and less NO accumulation in response to ABA and salt stress (Guo et al., 2003; Zhao et al., 2007). In this study, we showed that ExtCaM-induced NO accumulation requires AtNOA1: ExtCaM failed to induce stomatal closure and NO generation in the guard cells in *Atnoa1*, consistent with the blocking effect of cPTIO on ExtCaM-induced stomatal closure and NO production (Fig. 1). Thus, our pharmacological and genetic results suggest that NO is an essential molecule in ExtCaM-induced stomatal closure and that NO accumulation induced by ExtCaM depends on AtNOA1.

AtNOA1 was found to be targeted to mitochondria in Arabidopsis root (Guo and Crawford, 2005). However, recent evidence shows that AtNOA1 localizes in chloroplast (Flores-Pérez et al., 2008; Gas et al., 2009). Organelle localization of AtNOA1 suggests that activation of this protein requires signal transduction from extracellular stimuli to intracellular organelle. In this study, we show that AtNOA1-dependent NO accumulation in response to ExtCaM requires the activation of G protein and the accumulation of H_2O_2.

Modulation of NO Generation by G Protein in ExtCaM-Induced Stomatal Closure

Involvement of heterotrimeric G proteins in guard cell signaling has been supported by genetic evidence from null mutants for GPA1 and the G protein-coupled receptors GCR1 and GCR2 (Wang et al., 2001; Pandey and Assmann, 2004; Liu et al., 2007). Guard cells in *gpa1* mutants were insensitive to ABA inhibition of inward K^+ currents and pH independent of ABA activation of anion channels (Wang et al., 2001) and induction of stomatal closure (Liu et al., 2007). It is speculated that G protein regulates ion channels directly in guard cells, whereas modulation of radical oxygen species (ROS) by G protein has been found both in ExtCaM-induced stomatal closure and in ozone stress responses (Chen et al., 2004; Joo et al., 2005). In addition, sphingosine-1-phosphate has been found to be a signaling molecule involved in ABA regulation of guard cell turgor (Ng et al., 2001), and the Gα subunit acts downstream of sphingosine-1-phosphate during this process (Coursol et al., 2003). Those reports indicate that Gα may play a central mediating role in guard cell signaling. ExtCaM and ABA likely act independently to regulate the G protein pathway, although this has not been experimentally demonstrated. However, Gα regulation of NO generation in guard cells has not been reported. In mammalian cells, heterotrimeric G protein regulates endothelial NOS activity by increasing cellular levels of endothelial NOS (Andreeva et al., 2006). If such G protein-dependent regulation of NO generation exists in Arabidopsis, NO

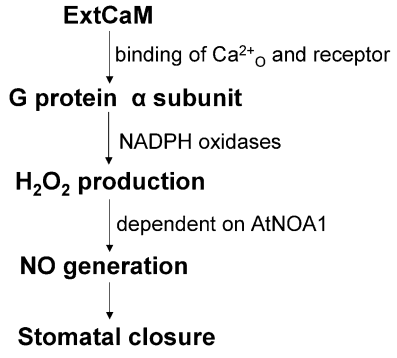

Figure 8. Model showing the possible signaling pathway for ExtCaM-induced stomatal closure. ExtCaM activates G protein to induce H_2O_2 generation by NADPH oxidases, which increases NO production depending on AtNOA1; NO subsequently arouses stomatal closure.

production will be decreased in *gpa1* mutants and increased in cGα lines. Our results were consistent with this speculation. *gpa1* guard cells generated less NO and cGα lines generated higher NO levels when treated with ExtCaM (Fig. 2, A and B). Furthermore, the similarity of cGα *Atnoa1* and *gpa1-2 Atnoa1* double mutants to the *Atnoa1* mutant in response to ExtCaM, the rescue of *gpa1* mutants by *AtNOA1* in stomatal closure and NO generation in response to ExtCaM (Fig. 3), and the enhancement of stomatal closure by SNP in *gpa1* mutants (Fig. 2C) suggest that Gα action depends on AtNOA1-mediated NO generation. These results suggest that Gα acts upstream of NO generation in ExtCaM-mediated guard cell signaling.

Gα Regulation of NO Production in ExtCaM-Induced Stomatal Closure Depends on H_2O_2 Generation

ROS has been shown to be an important second messenger in ABA-induced stomatal movement in both Arabidopsis and *V. faba* (Pei et al., 2000; Zhang et al., 2001). Pharmacological results show that H_2O_2 is also involved in ExtCaM-induced stomatal closure and acts downstream of Gα in *V. faba* (Chen et al., 2004). In this report, we confirmed the essential role of ROS in the ExtCaM-mediated guard cell response using the *atrbohD/F* double mutant in Arabidopsis. The *atrbohD/F* mutant showed impaired ROS increases and stomatal closure when treated with ExtCaM (Fig. 4), providing genetic evidence for the involvement of ROS in ExtCaM guard cell signaling. Furthermore, ExtCaM-triggered stomatal closure in cGα lines was blocked by CAT or DPI, and the defect in stomatal closure in *gpa1* was rescued by H_2O_2 (Fig. 5). These results indicate that H_2O_2 acts downstream of Gα in ExtCaM guard cell signaling.

Reports from several groups imply that H_2O_2 induces NO generation in guard cells of *Phaseolus aureus*, *V. faba*, and Arabidopsis (Lum et al., 2002; He et al., 2005; Bright et al., 2006). It has been shown that antioxidant ascorbate and CAT partially reverse SNP-induced H_2DCF fluorescence and stomatal closure in *V. faba* (He et al., 2005). However, Bright et al. (2006) reported that SNP-induced stomatal closure is not affected by CAT in Arabidopsis, implying that guard cell responses differ between Arabidopsis and *V. faba*. In this study, we showed that ExtCaM-induced H_2O_2 synthesis is essential for NO production, since ExtCaM-induced NO production was greatly impaired in the guard cells of *atrbohD/F* and CAT blocked the ExtCaM-induced NO production in the guard cells of the wild type (Fig. 6, A and B). Nitro-L-Arg methyl ester inhibits H_2O_2-mediated NO generation in guard cells of *P. aureus* and *V. faba*, implying that NOS activity is related to H_2O_2-mediated NO generation (Lum et al., 2002; He et al., 2005). However, using Arabidopsis *Atnoa1* and *nia1, nia2* mutants, Bright et al. (2006) reported that it is NR, not AtNOA1, that is responsible for the stomatal closure and NO generation in guard cells in response to H_2O_2, although Desikan et al. (2002) showed that stomatal closure in the *nia1, nia2* double mutant was induced by H_2O_2 as in the wild type. Our data indicate that H_2O_2-induced stomatal closure was greatly impaired by pretreatment with cPTIO. Meanwhile, H_2O_2-induced stomatal closure and NO generation were significantly reduced in *Atnoa1* (Fig. 7, A and B). These results indicate that H_2O_2-induced stomatal closure and NO production depend mainly on AtNOA1, which contradicts the findings of Bright et al. (2006) but is somewhat supported by Desikan et al. (2002). The discrepancy in the AtNOA1 or NR source of H_2O_2 induction of NO generation may be due to the nitrogen nutrition conditions in the soil, because NR is a nitrate-dependent enzyme (Raven, 2003).

In this study, we present pharmacological, biochemical, and genetic evidence to support the essential role played by AtNOA1-dependent NO generation in ExtCaM-induced stomatal closure. We also show that a signaling cascade of heterotrimeric G protein, NADPH oxidase-dependent H_2O_2 generation, and AtNOA1-dependent NO production regulates ExtCaM-mediated guard cell responses. Both ExtCaM and H_2O_2 raise the $[Ca^{2+}]_i$ level by promoting $Ca^{2+}{}_o$ influx through Ca^{2+} channels in the plasma membrane (Pei et al., 2000; Xiao et al., 2004; Shang et al., 2005). In addition, ExtCaM receptor-like binding protein has been found in the plasma membrane (Cui et al., 2005). Based on these observations, we propose a working model for ExtCaM action: ExtCaM is activated by $Ca^{2+}{}_o$ binding; active ExtCaM converts $Ca^{2+}{}_o$ status to intracellular events through its receptor-like binding protein in the plasma membrane. ExtCaM could elevate the $[Ca^{2+}]_i$ level by at least two means: (1) by promoting $Ca^{2+}{}_o$ influx directly, or (2) by stimulating Ca^{2+} channels in the plasma membrane via increased H_2O_2. $[Ca^{2+}]_i$ increases induced by ExtCaM might trigger Ca^{2+}-dependent NO accumulation, and subsequently NO further releases Ca^{2+} from intracellular Ca^{2+} stores (Sokolovski et al., 2005). ExtCaM could act as another $Ca^{2+}{}_o$ sensor together with CAS to regulate $[Ca^{2+}]_i$ oscillation under natural conditions. A similar pathway appears to be responsible for ABA regulation of stomatal closure: ABA activates G protein (Liu et al., 2007) to arouse H_2O_2 generation by NADPH oxidases (Kwak et al., 2003), which subsequently induces NO levels by raising the $[Ca^{2+}]_i$ level, and increased NO may cause $[Ca^{2+}]_i$ elevation (Desikan et al., 2004). Therefore, a signaling cascade involving G protein, H_2O_2, NO, and calcium may integrate both ABA and ExtCaM to regulate stomatal movement (Fig. 8).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of the wild type and various mutants of Arabidopsis (*Arabidopsis thaliana*) were sown in potting mix and grown in a greenhouse under a 16-h-light/8-h-dark cycle, a photon flux density of 0.30 mmol m^{-2} s^{-1}, and a day/night temperature cycle of 18°C/22°C for 3 to 4 weeks. Seeds of cGα lines

(background Ws) overexpressing a constitutively active form of *GPA1* and *gpa1-1* and *gpa1-2* mutants (background Ws) were obtained from Dr. L.G. Ma (National Institute of Biological Sciences). cGα plants were grown in the presence of 70 nM dexamethasone (Sigma) according to the method described by Okamoto et al. (2001). *atrbohD/F* double mutant seeds (background Col-0) were obtained from Dr. M.A. Torres (University of North Carolina at Chapel Hill). Seeds of *Atnoa1* mutant and the rescued *Atnoa1* line (background Col-0) and the construct *35S:AtNOA1* were obtained from Dr. N.M. Crawford (University of California at San Diego). The cGα1 *Atnoa1* and *gpa1-2 Atnoa1* double mutants and the *gpa1-2* mutant for transformation were back-crossed three times with Col-0 to remove the Ws background of cGα1 or *gpa1-2*. Genotypes of all mutants were confirmed by PCR analysis. Recombinant AtCaM2 protein was obtained from Dr. S.J. Cui (Hebei Normal University; Cui et al., 2005).

Transformation of Arabidopsis and PCR Analysis of Mutants

AtNOA1 cDNA was cut from pBIN-JIT (Guo et al., 2003) and ligated into binary vector pCAMBIA-1300 with hygromycin selection. Transformation of pCAMBIA-1300 harboring *35S:AtNOA1* into the *gpa1-2* mutant was performed according to the flower-dip method (Clough and Bent, 1998). Hygromycin-resistant seedlings were transferred to soil for examination of the NO level and stomatal response to ExtCaM. Data from three independent transgenic lines were obtained, and representative results are shown. Primers for PCR analysis of the mutants are shown in the Supplemental Materials and Methods S1.

Stomatal Bioassay

Stomatal assays were performed essentially as described by Chen et al. (2004). Abaxial epidermal strips were peeled gently and incubated in MES buffer (10 mM MES-Tris, 50 mM KCl, and 0.1 mM $CaCl_2$, pH 6.1) for 90 min under light to open the stomata. To study the effects of CaM, α-lactalbumin, SNP, and H_2O_2 on stomatal closure, the epidermal strips with open stomata were transferred to and incubated in MES buffer containing 10^{-8} M CaM, 10^{-8} M α-lactalbumin, 60 μM SNP, or 5×10^{-5} M H_2O_2 for 2 h. To investigate the effect of cPTIO, CAT, or DPI on CaM-induced stomatal closure, the strips with open stomata were transferred to and incubated in MES buffer containing10^{-8} M CaM plus 200 μM cPTIO, 100 units mL^{-1} CAT, or 10 μM DPI for 2 h. To investigate the effect of cPTIO on the effect of H_2O_2-induced stomatal closure, the strips with open stomata were transferred to and incubated in MES buffer containing 5×10^{-5} M H_2O_2 plus 200 μM cPTIO for 2 h. Stomatal apertures were measured with a microscope. Fifty stomata were randomly selected for three independent repeats. The data are presented as means $\pm$ SE (n = 150).

Fluorescence Microscopy

NO was visualized using the specific fluorescent NO dye DAF2-DA (Sigma). Abaxial epidermal strips with open stomata were loaded with 10 μM DAF2-DA for 15 min, followed by a wash step. The strips were subsequently incubated in MES buffer alone, 10^{-8} M CaM, 10^{-8} M α-lactalbumin, 1 mM $CaCl_2$, or 5×10^{-5} M H_2O_2 in MES buffer (or coincubated with other reagents as indicated in the figure legends) for 30 min. All images were acquired with a fluorescence microscope (Nikon; ELLIPE TE2000-U) with the following settings: excitation = 488 nm, emission = 515 nm; the fluorescence intensities were analyzed using MetaMorph. Data are presented as mean pixel intensities. Fifty guard cells were recorded for each treatment for three independent repeats.

Confocal Laser Scanning Microscopy

H_2O_2 measurement in guard cells was carried out using the method described previously (Chen et al., 2004). Abaxial epidermal strips with open stomata were incubated in 50 μM H_2DCFDA (Molecular Probes; D399) for 15 min at room temperature and then washed three times. The strips were then incubated in MES buffer alone or 10^{-8} M CaM in MES buffer (or coincubated with 200 μM cPTIO) for 30 min. The H_2O_2 fluorescence in guard cells was measured using a confocal laser scanning microscope (Bio-Rad; CLSM 1024) with the following settings: excitation = 488 nm, emission = 535 nm. For real-time recording of H_2DCF fluorescence intensity, images were recorded every 15 s. When the fluorescence stabilized around 60 s after scanning, CaM solution was added directly to the buffer in which the strips were placed, and we treated this agent addition point as zero time. Fluorescence changes were recorded, and relative fluorescence intensity was calculated by subtracting the basal signal at different time points as indicated in the figure legends. The experiments were repeated at least three times with seven to 10 cells each time, and one set of data is presented to illustrate the changes in fluorescence intensity.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At2g26300 (AtGPA1), At3g47450 (AtNOA1), At5g47910 (AtrbohD), and At1g64060 (AtrbohF).

Supplemental Data

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

Supplemental Materials and Methods S1. Primers for PCR analysis of mutants.

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