

Cross-talk between calcium-calmodulin and nitric oxide in abscisic acid signaling in leaves of maize plants

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Using pharmacological and biochemical approaches, the signaling pathways between hydrogen peroxide (H₂O₂), calcium (Ca²⁺)-calmodulin (CaM), and nitric oxide (NO) in abscisic acid (ABA)-induced antioxidant defense were investigated in leaves of maize (*Zea mays* L.) plants. Treatments with ABA, H₂O₂, and CaCl₂ induced increases in the generation of NO in maize mesophyll cells and the activity of nitric oxide synthase (NOS) in the cytosolic and microsomal fractions of maize leaves. However, such increases were blocked by the pretreatments with Ca²⁺ inhibitors and CaM antagonists. Meanwhile, pretreatments with two NOS inhibitors also suppressed the Ca²⁺-induced increase in the production of NO. On the other hand, treatments with ABA and the NO donor sodium nitroprusside (SNP) also led to increases in the concentration of cytosolic Ca²⁺ in protoplasts of mesophyll cells and in the expression of calmodulin 1 (*CaM1*) gene and the contents of CaM in leaves of maize plants, and the increases induced by ABA were reduced by the pretreatments with a NO scavenger and a NOS inhibitor. Moreover, SNP-induced increases in the expression of the antioxidant genes superoxide dismutase 4 (*SOD4*), cytosolic ascorbate peroxidase (*cAPX*), and glutathione reductase 1 (*GRI*) and the activities of the chloroplastic and cytosolic antioxidant enzymes were arrested by the pretreatments with Ca²⁺ inhibitors and CaM antagonists. Our results suggest that Ca²⁺-CaM functions both upstream and downstream of NO production, which is mainly from NOS, in ABA- and H₂O₂-induced antioxidant defense in leaves of maize plants.

Keywords: abscisic acid, antioxidant defense, calmodulin, cytosolic calcium, nitric oxide, nitric oxide synthase, *Zea mays*
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Introduction

Abscisic acid (ABA), as a stress signal, plays critical roles in the regulation of plant water balance and osmotic stress tolerance under drought, cold, and salt stress conditions [1, 2]. Accumulating evidence indicates that ABA-enhanced water stress tolerance is, at least in part, due to the induction of antioxidant defense systems [3-6]. Previous studies have shown that NADPH oxidase, hydrogen peroxide (H₂O₂), nitric oxide (NO), calcium (Ca²⁺)-calmodulin (CaM), and mitogen-activated protein kinase (MAPK) are required for ABA-induced up-regulation in the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) and glutathione reductase (GR) in leaves of maize plants [7-11]. It has been shown that ABA-induced H₂O₂ production

mediates NO generation, which, in turn, activates MAPK and results in the up-regulation in the expression and the activities of antioxidant enzymes in ABA signaling [11]. Ca²⁺-CaM has also been shown to be necessary for ABA-induced antioxidant defense and function both upstream and downstream of H₂O₂ production in leaves of maize plants [9]. However, whether Ca²⁺-CaM is involved in NO-induced antioxidant defense in plants and, if so, what the relationship between Ca²⁺-CaM and NO in the ABA signaling is remains to be determined.

An increasing body of evidence indicates that NO, which was first identified as a unique diffusible molecular messenger in animals, plays important roles in various physiological processes in plants, including defense responses and programmed cell death (PCD), hormone responses, abiotic stress, root and xylem development, germination, iron homeostasis, and flowering [12-18]. In mammals, NO is formed exclusively by the enzyme nitric oxide synthase (NOS). NOS catalyzes the conversion of L-arginine into L-citrulline and NO in the presence of molecular oxygen [12, 14, 17, 18]. Three isoforms of NOS have been identified in mammalian species: neuronal NOS (nNOS), endothelial

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NOS (eNOS), and inducible (iNOS) [12, 15, 18]. nNOS and eNOS are classified as constitutive NOSs because they are rapidly activated by Ca²⁺ and show limited transcriptional regulation. By contrast, iNOS constitutively binds Ca²⁺ and CaM and is regulated primarily at the transcriptional level. In plants, there are several potential sources of NO, including NOS-like enzyme, nitrate reductase (NR), and non-enzymatic sources [12, 14, 15, 17]. Although no plant NOS similar to the mammalian one has been identified [19-21], several lines of evidence have demonstrated the presence of NOS-like activity in plant cells [12, 14, 17, 18,

22-24]. The NOS activity in pea leaf peroxisomes has been biochemically characterized, which is strictly dependent on L-arginine, NADPH, tetrahydrobiopterin (BH₄), and CaM, and requires Ca²⁺ [22]. Previous studies showed that pretreatment with N^G-nitro-L-Arg methyl ester (L-NAME), a well-known competitive inhibitor of mammalian NOS, reduced ABA-induced increases in the production of NO in pea guard cells [25] and maize mesophyll cells [11], suggesting that NOS is involved in ABA signaling. However, more evidence is required for the involvement of NOS in ABA-induced antioxidant defense in plants.

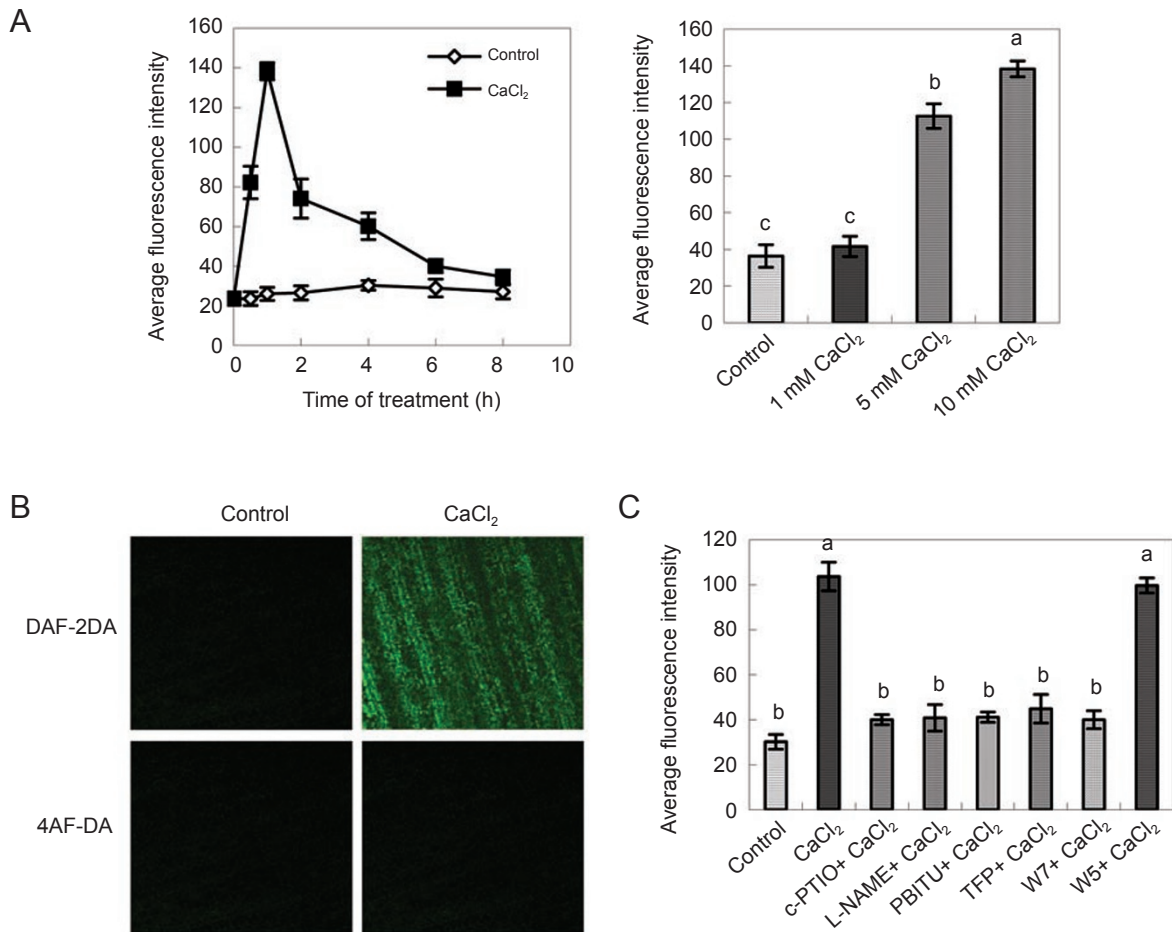


Figure 1 Nitric oxide production in response to CaCl₂ treatment in maize leaves. **(A)** Time course and dose dependence for CaCl₂-induced NO production in mesophyll cells of maize leaves. The detached plants were treated with 10 mM CaCl₂ for various times (left), or different concentrations of CaCl₂ (1, 5 and 10 mM) for 1 h (right), and then the leaf segments were loaded with DAF-2DA and detected by CLSM. **(B)** DAF-2DA fluorescence accumulation in CaCl₂-treated mesophyll cells of maize leaves. The detached plants were treated with distilled water (control), 10 mM CaCl₂ for 1 h, and the leaf segments were loaded with DAF-2DA (top) or 4AF-DA (bottom) and detected by CLSM. **(C)** Effects of pretreatments with NO scavenger, NOS inhibitors and CaM antagonists on CaCl₂-induced NO production. The detached plants were pretreated with distilled water, 200 μM c-PTIO, 200 μM L-NAME, 5 mM PBITU, 300 μM TFP, 200 μM W7, and 200 μM W5 for 2 h, and then exposed to 10 mM CaCl₂ treatment for 1 h. Detached plants treated with distilled water served as control. In **(A and C)**, values are the means ± SE of three different experiments. Means denoted by the same letter did not significantly differ at *P* < 0.05 according to Duncan's multiple range test. In **(B)**, experiments were repeated at least three times with similar results.

In the present study, using pharmacological and biochemical approaches, the signaling pathways between H_2O_2 , Ca^{2+}/CaM , and NO in ABA-induced up-regulation in the expression of several antioxidant genes such as superoxide dismutase 4 (*SOD4*), encoding a cytosolic isoform of SOD, cytosolic ascorbate peroxidase (*cAPX*), encoding a cytosolic isoform of APX, and glutathione reductase 1 (*GRI*), encoding a plastidial isoform of GR, and of the total activities of the chloroplastic and cytosolic antioxidant enzymes SOD, APX, and GR in leaves of maize plants were investigated. Our results showed that Ca^{2+} -CaM is required for ABA- and H_2O_2 -induced increase in the production of NO and the activity of NOS in leaves of maize plants, and the increases in the production of NO are mainly from NOS. Our data also suggest that there exists cross-talk between Ca^{2+} -CaM and NO in ABA and H_2O_2 signaling.

Results

Involvement of CaM in exogenous Ca^{2+} -induced NO generation and NOS activity

To investigate the effect of $CaCl_2$ treatment on the production of NO in leaves of maize plants, the leaf segments from treated and untreated leaves were loaded with the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA), and confocal laser scanning microscopy (CLSM) was used to monitor changes in NO-induced fluorescence in mesophyll cells of maize leaves. Treatment with 10 mM $CaCl_2$ led to a rapid increase in DAF-2DA fluorescence intensity (Figure 1A). The DAF-2DA fluorescence was visible as early as 0.5 h, maximized at 1 h, and then decreased after 2 h of $CaCl_2$ treatment. The $CaCl_2$ -induced fluorescence intensity was dose-dependent in the concentration range of 1-10 mM $CaCl_2$ (Figure 1A). By contrast, no increase in fluorescence intensity was observed in $CaCl_2$ -treated leaves when the leaf segments were loaded with the negative probe 4-amino fluorescein diacetate (4AF-DA), which lacks one of the amino groups that constitutes the NO specificity domain of the DAF-2DA molecule (Figure 1B). Pretreatments with the NO scavenger c-PTIO and the NOS inhibitors L-NAME and PBITU substantially reduced the DAF-2DA fluorescence intensity induced by $CaCl_2$ treatment (Figure 1C). These results clearly indicate that Ca^{2+} can induce an increased generation of NO in mesophyll cells of maize leaves.

The fact that NOS inhibitors inhibit the generation of NO induced by $CaCl_2$ treatment (Figure 1C) suggests that NO production is associated with the activity of NOS in leaves of maize plants. To further determine the relationship between NO generation and NOS activity, the cytosolic and microsomal fractions of maize leaves were isolated and the activity of NOS was determined. Under the control

conditions, the activity of NOS in the microsomal fraction was higher than that in the cytosolic fraction (Figure 2A). Treatment with 10 mM $CaCl_2$ resulted in a rapid increase in the activity of NOS in the cytosolic and microsomal fractions of maize leaves. After 1 h of $CaCl_2$ treatment, the activity of NOS in the cytosolic and microsomal fractions reached the maximum values, and increased by 92.7% and 148%, respectively, compared with the control values. Pretreatments with the NOS inhibitors L-NAME and PBITU completely blocked the increase in the activity of NOS in the cytosolic and microsomal fractions induced by $CaCl_2$ treatment (Figure 2B), but these pretreatments alone did not affect the activity of NOS in the control leaves (data not shown).

In order to investigate whether Ca^{2+} -induced increases in the generation of NO and the activity of NOS are related to the action of CaM, the CaM antagonists trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride (W7) were used. Pretreatments with these antagonists substantially prevented the increases in the generation of NO (Figure 1C) and the activity of NOS in the cytosolic and microsomal fractions (Figure 2B) induced by $CaCl_2$ treatment. However, pretreatment with W5, an inactive structural analog of W7, had very little effect on $CaCl_2$ -induced increases in the production of NO and the activity of NOS in leaves of maize plants exposed to $CaCl_2$ treatment. These results suggest that CaM is necessary for the generation of NO and the activity of NOS induced by exogenous Ca^{2+} in leaves of maize plants.

Ca^{2+} -CaM is required for ABA- and H_2O_2 -induced NO generation and NOS activity

Previous studies showed that both ABA and H_2O_2 induce NO generation in maize mesophyll cells [11], and also induce increases in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in the protoplasts of mesophyll cells and in the expression of *CaM1* gene and the content of CaM in leaves of maize plants [9]. To investigate whether Ca^{2+} -CaM is involved in ABA- and H_2O_2 -induced generation of NO, the Ca^{2+} chelator ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), the Ca^{2+} channel blockers $LaCl_3$ and verapamil, and the CaM antagonists TFP and W7 were used. Pretreatments with these inhibitors significantly reduced the increase in the production of NO induced by ABA or H_2O_2 in mesophyll cells of maize leaves (Figure 3A), but these pretreatments alone did not affect the production of NO in the untreated leaves (data not shown). Moreover, pretreatment with W5 had little effect on the ABA- and H_2O_2 -induced NO production in mesophyll cells of maize leaves (Figure 3A). These results suggest that Ca^{2+} -CaM is required for ABA- and H_2O_2 -induced NO production in leaves of maize plants.

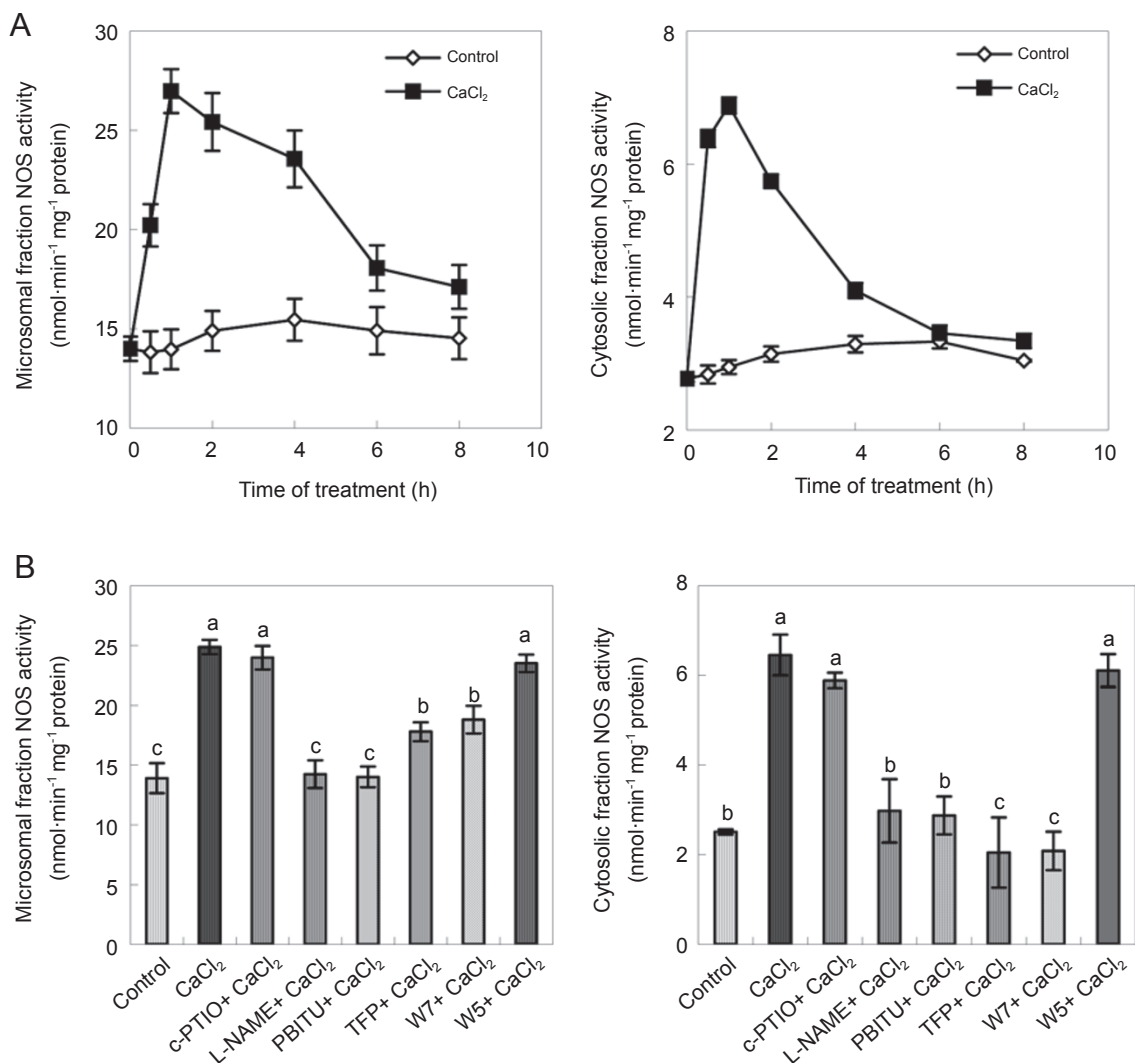


Figure 2 CaCl_2 -induced changes in the activity of NOS in cytosolic and microsomal fractions of maize leaves (A) and the effects of pretreatments with NO scavenger, NOS inhibitors, and CaM antagonists on the activity of NOS (B) in leaves of maize plants exposed to CaCl_2 treatment. (A) The detached plants were treated with distilled water (control) and 10 mM CaCl_2 for various times. (B) The detached plants were pretreated with distilled water, 200 μM c-PTIO, 200 μM L-NAME, 5 mM PBITU, 300 μM TFP, 200 μM W7, and 200 μM W5 for 2 h, and then exposed to 10 mM CaCl_2 or distilled water treatment for 1 h. Values are means \pm SE of three different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

Treatments with ABA and H_2O_2 also led to an increase in the activity of NOS in the cytosolic and microsomal fractions of maize leaves (Figure 3B), and such an increase was blocked by the pretreatments with EGTA, LaCl_3 and verapamil, and TFP and W7. However, pretreatment with W5 had little effect on the increase in the activity of NOS induced by ABA or H_2O_2 treatment. These results suggest that the ABA- and H_2O_2 -induced NOS activity is related to Ca^{2+} -CaM.

NO induces increases in $[\text{Ca}^{2+}]_i$ and CaM

To investigate whether NO also affects the levels of Ca^{2+} -CaM, the NO donor SNP was used. Treatment with SNP led to a rapid increase in Ca^{2+} -sensitive fluorescence of the protoplasts, which were isolated from untreated maize leaves and loaded with the Fluo-3/AM, a Ca^{2+} -sensitive fluorescent probe [31], and observed by CLSM (Figure 4A). The fluorescence intensity of the protoplasts reached a maximum value at 6 min, which was 1.9-fold higher than that in the control, and then remained high during the 30 min of SNP treatment. By contrast, treatment with sodium ferricyanide $[\text{Fe}(\text{III})\text{CN}]$, which shares many structural

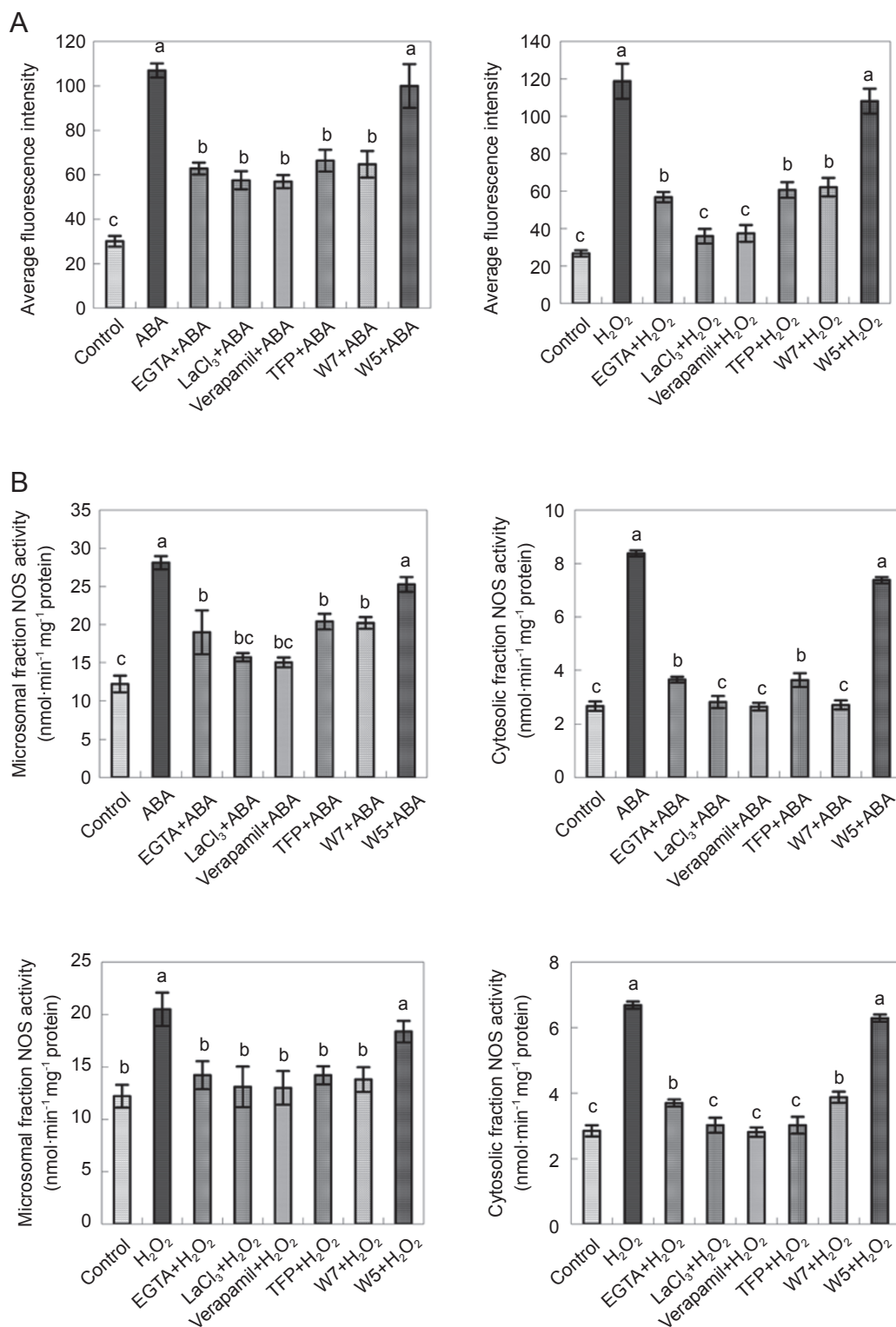


Figure 3 Effects of pretreatments with Ca²⁺ inhibitors and CaM antagonists on ABA- or H₂O₂-induced NO production in mesophyll cells of maize leaves (**A**) and NOS activity in cytosolic and microsomal fractions of maize leaves (**B**). The detached plants were pretreated with distilled water, 10 mM EGTA, 5 mM LaCl₃, 100 μM verapamil, 300 μM TFP, 200 μM W7 and 200 μM W5 for 2 h, and then exposed to 100 μM ABA or 10 mM H₂O₂ or distilled water treatment for 2 h. Values are means ± SE of three different experiments. Means denoted by the same letter did not significantly differ at *P* < 0.05 according to Duncan's multiple range test.

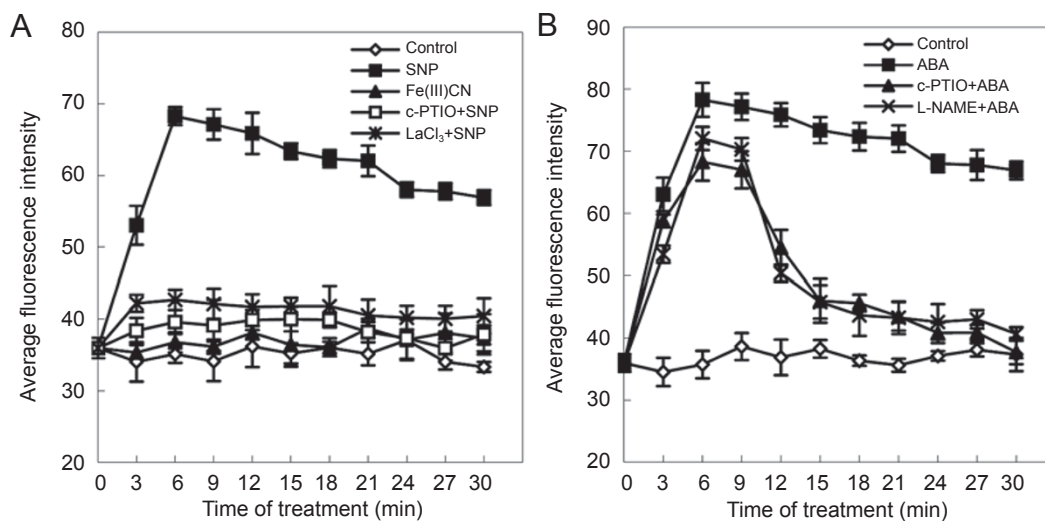


Figure 4 Changes of cytosolic calcium concentration ($[Ca^{2+}]_i$) in maize mesophyll protoplasts exposed to SNP (**A**) or ABA (**B**) treatment. The protoplasts loaded with Fluo-3/AM were pretreated with distilled water, 20 μ M c-PTIO, 20 μ M L-NAME and 500 μ M LaCl₃ for 3 min, respectively, and then exposed to 10 μ M SNP or 10 μ M Fe(III)CN or 10 μ M ABA or distilled water for various times, and detected by CLSM. The fluorescence intensity was measured by fluorescence microscopy and calculated as the average value of those obtained by scanning over 60 protoplasts from different experiments. All experiments were repeated at least five times with similar results.

features with SNP but lacks a nitroso group and thus the ability to generate NO [36], did not induce the increase in the Ca²⁺-sensitive fluorescence of the protoplasts. Moreover, pretreatments with c-PTIO and LaCl₃ substantially reduced the increase in fluorescence intensity induced by SNP treatment (Figure 4A). These results clearly indicate that NO itself can induce the increase in the concentration of $[Ca^{2+}]_i$. Meanwhile, SNP treatment also caused clear increases in the transcript level of *CaMI*, which encodes a maize CaM isoform [9, 37], analyzed by semi-quantitative RT-PCR, and in the content of CaM in leaves of maize plants (Figure 5A and 5B).

To investigate whether ABA-induced increases in the levels of Ca²⁺-CaM are related to ABA-induced NO generation, the detached plants were pretreated with the NO scavenger c-PTIO and the NOS inhibitor L-NAME, and then exposed to ABA treatment. Experimental results showed that pretreatments with c-PTIO and L-NAME had very little effect on the ABA-induced increase in the concentration of $[Ca^{2+}]_i$ within the first 9 min of ABA treatment (Figure 4B) and in the expression of *CaMI* within the first 1 h of ABA treatment (Figure 5A) and in the content of CaM within the first 1.5 h of ABA treatment (Figure 5B), but substantially blocked the increases in the concentration of $[Ca^{2+}]_i$ after 12 min of ABA treatment and in the expression of *CaMI* after 1.5 h of ABA treatment and in the content of CaM after 2 h of ABA treatment, suggesting that the initial increase in Ca²⁺-CaM induced by ABA is not dependent on ABA-induced NO production in leaves of maize plants

exposed to ABA treatment.

Ca²⁺-CaM is involved in NO-induced antioxidant defense

It has been shown that NO can induce increases in the expression of antioxidant genes such as *CAT1*, *cAPX*, *SOD4*, and *GRI* and the total activities of these antioxidant enzymes [11]. In order to investigate whether NO-induced antioxidant defense is related to the increases in Ca²⁺-CaM, the detached plants were pretreated with the Ca²⁺ chelator EGTA, the Ca²⁺ channel blocker LaCl₃, and the CaM antagonists TFP and W7, respectively, and then exposed to SNP treatment. Pretreatments with these Ca²⁺ inhibitors and CaM antagonists almost fully prevented the SNP-induced increases in the expression of the antioxidant genes *SOD4*, *cAPX*, and *GRI* (Figure 6A) and the activities of chloroplastic and cytosolic SOD, APX, and GR (Figure 6B), but pretreatment with W5 had very little effect on SNP-induced increases in the expression and activities of these antioxidant enzymes in leaves of maize plants. Meanwhile, treatment with Fe(III)CN did not induce the increases in the expression and activities of these antioxidant enzymes. These results clearly suggest that Ca²⁺-CaM is required for the NO-induced antioxidant defense.

Discussion

Previous studies showed that ABA-induced NO production is dependent on H₂O₂ generation in *Arabidopsis* guard cells [38] and in maize mesophyll cells [11]. However,

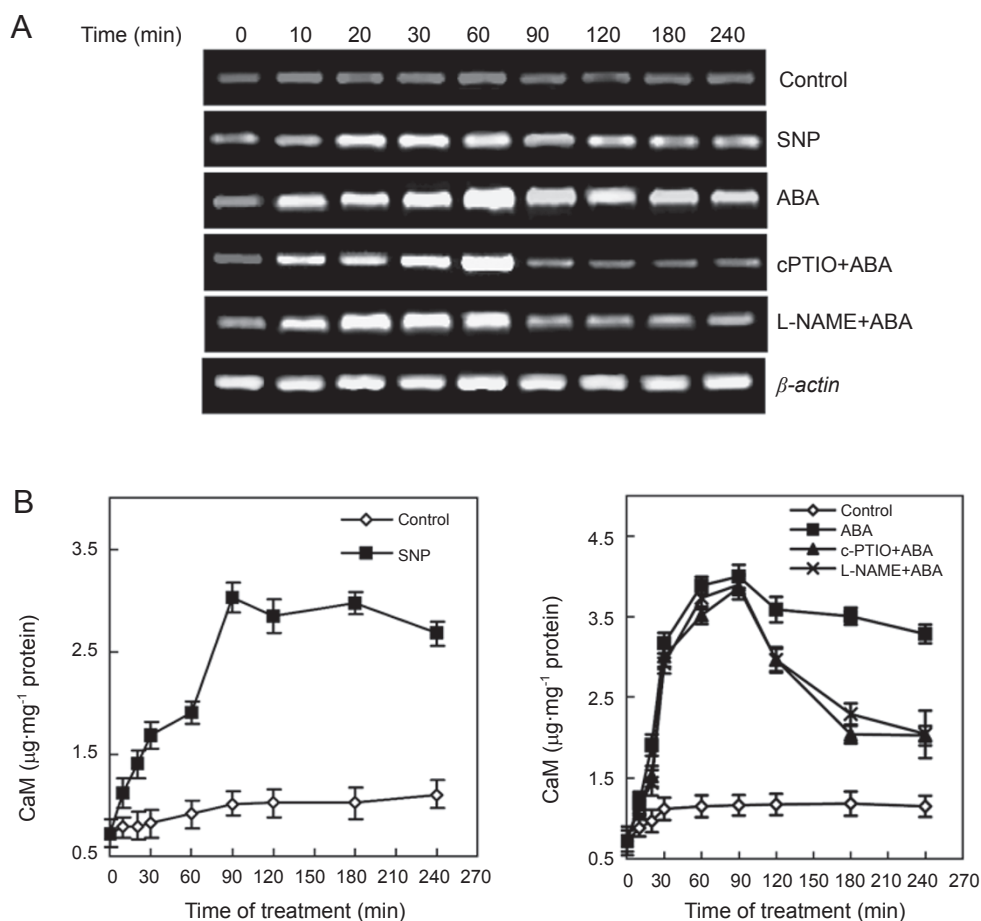
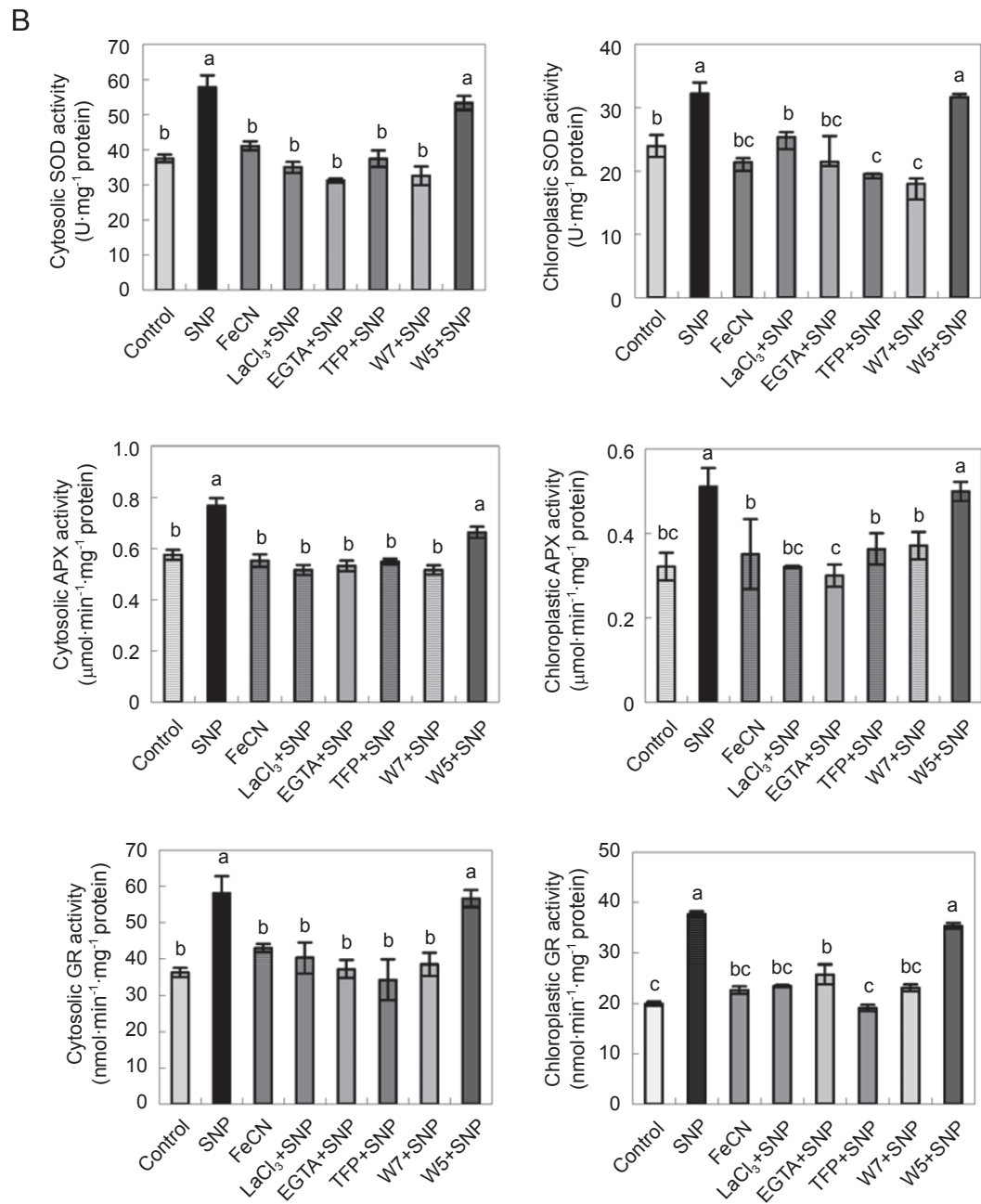
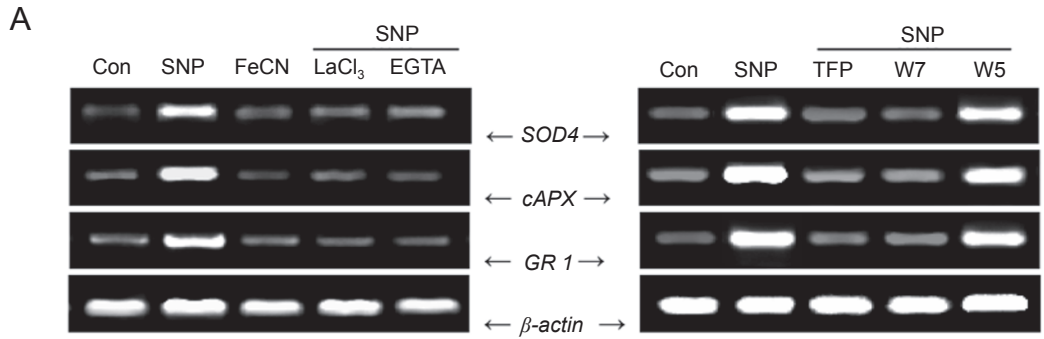


Figure 5 SNP- and ABA-induced changes in the expression of the calmodulin 1 (*CaM1*) gene and the content of CaM in leaves of maize plants. **(A)** Transcript levels of *CaM1* analyzed by RT-PCR. **(B)** Time-course of changes in the content of CaM. The detached plants were pretreated with distilled water, 200 μM c-PTIO, and 200 μM L-NAME for 2 h, and then exposed to 100 μM SNP or 100 μM ABA for various times. Detached plants treated with distilled water served as controls. In **(A)**, experiments were repeated at least three times with similar results. In **(B)**, the values are the mean \pm SE of three different experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

how H_2O_2 regulates NO production in ABA signaling is not clear. It has been shown that ABA-induced H_2O_2 can induce increases in the concentration of $[\text{Ca}^{2+}]_i$ and the expression of *CaM1* and the content of CaM in leaves of maize plants [9], and that Ca^{2+} is required for H_2O_2 -induced NO production in guard cells of mung bean [39]. The Ca^{2+} -dependent NO production was also observed in tobacco cells challenged by the elicitor cryptogein [40] and in the endopolygalacturonase 1 (BcPG1)-elicited grapevine cells [41]. Here we provide evidence that both Ca^{2+} and CaM are involved in ABA- and H_2O_2 -induced NO production in leaves of maize plants. Our data showed that ABA- and H_2O_2 -induced increase in the generation of NO was substantially blocked by pretreatments with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blockers LaCl_3 and verapamil, and the CaM antagonists TFP and W7 (Figure 3A). However,

pretreatment with W5, an inactive structural analog of W7, had very little effect on the ABA- and H_2O_2 -induced NO production in mesophyll cells of maize leaves. Moreover, exogenous Ca^{2+} also induced an increase in the production of NO (Figure 1A), and the increase was prevented by pretreatments with the CaM antagonists TFP and W7 (Figure 1C). Similarly, pretreatment with W5 did not affect the exogenous Ca^{2+} -induced NO production. Although the absolute specificities of these inhibitors used in this study can always be questioned, it has been shown that these inhibitors in the concentration range used in the present study should make it feasible to study the role of Ca^{2+} and CaM in plant cells [7, 9, 42, 43]. Our results clearly suggest that Ca^{2+} /CaM is required for the ABA- and H_2O_2 -induced NO production in maize mesophyll cells.

In animals, NO production is catalyzed primarily by



NOS. This enzyme catalyzes the oxygen- and NADPH-dependent oxidation of L-arginine to L-citrulline and NO in a complex reaction requiring FAD, FMN, BH₄, Ca²⁺, and CaM [12, 14, 17, 18]. In plants, pharmacological, biochemical and physicochemical, and immunological evidence indicates the presence of NOS-like activity similar, to a certain extent, to mammalian NOS [12, 14, 17, 18, 22-24], although no plant NOS similar to the mammalian one has been characterized [19-21]. It has been shown that NOS is localized in the cytoplasm, chloroplasts, mitochondria, peroxisomes, and nucleus in plant cells [14, 22, 24, 44]. The occurrence of NOS activity in pea leaf peroxisomes was demonstrated by biochemical methods and electron paramagnetic resonance (EPR) spectroscopy [22]. The enzyme was strictly dependent on L-arginine, NADPH, BH₄, and CaM, and required Ca²⁺. Previous studies showed that pretreatment with L-NAME, a well-known competitive inhibitor of mammalian NOS, reduced ABA-induced increase in the production of NO in pea guard cells [25] and maize mesophyll cells [11], suggesting that NOS is involved in ABA signaling. In this study, the activity of NOS in the cytosolic and microsomal fractions of maize leaves was determined. Our data showed that, under the control conditions, the activity of NOS in the microsomal fraction was higher than that in the cytosolic fraction. Treatment with ABA or H₂O₂ or Ca²⁺ induced increase in the activity of NOS in the cytosolic and microsomal fractions, and the increase was blocked by the pretreatments with the Ca²⁺ chelator EGTA, the Ca²⁺ channel blockers LaCl₃ and verapamil, and the CaM antagonists TFP and W7 (Figures 2 and 3). Furthermore, pretreatments with the NOS inhibitors L-NAME and PBITU inhibited the activity of NOS induced by exogenous Ca²⁺ (Figure 2B), and also blocked the production of NO (Figure 1C). Our results clearly suggest that ABA- and H₂O₂-induced increase in the production of NO is mainly from NOS, which is Ca²⁺- and CaM-dependent. Our data also suggest that ABA and H₂O₂ could induce the activity of NOS, which is primarily located in the microsomal fraction, in different subcellular compartments. In previous studies, however, it was reported that NR was the main source of NO production in response to ABA and H₂O₂ in *Arabidopsis* guard cells [38, 45]. The results described above suggest that there are different

sources of NO in response to ABA and H₂O₂ in guard cells and mesophyll cells. However, we do not exclude that NR could also participate in the ABA- and H₂O₂-induced NO production in the mesophyll cells of maize leaves.

NO has also been shown to induce an increase in the concentration of [Ca²⁺]_i in plant cells. NO may mobilize Ca²⁺ from internal stores through the activation of ryanodine receptor (RYR)-like channels [15, 17, 40, 41, 46-48] and plasma membrane Ca²⁺-permeable channels [40, 41, 47]. In the present study, our data showed that NO induced not only an increase in [Ca²⁺]_i in the protoplasts of maize leaves, which was sensitive to the plasma membrane Ca²⁺ channel blocker LaCl₃ (Figure 4A), but also increases in the expression of *CaM1* (Figure 5A) and the content of CaM in leaves of maize plants (Figure 5B). Furthermore, ABA-induced NO production is also involved in the regulation of [Ca²⁺]_i and CaM because pretreatments with the NO scavenger c-PTIO and the NOS inhibitor L-NAME substantially reduced the ABA-induced increases in the concentration of [Ca²⁺]_i (Figure 4B) and the expression of *CaM1* (Figure 5A) and the content of CaM (Figure 5B) after the peak values. However, pretreatments with c-PTIO and L-NAME had very little effect on the ABA-induced increases in [Ca²⁺]_i and *CaM1* and CaM before the peak values, suggesting that NO is not required for the initial increases in [Ca²⁺]_i and CaM induced by ABA. Taken together, our results suggest that Ca²⁺-CaM functions both upstream and downstream of NO production in ABA and H₂O₂ signaling.

In a recent study, our data suggest that ABA-induced H₂O₂ production mediates NO generation, which in turn activates a MAPK and results in the up-regulation in expression and activities of antioxidant enzymes in ABA signaling in leaves of maize plants [11]. Moreover, Ca²⁺-CaM has also been shown to be involved in ABA-induced antioxidant defense [9]. However, whether Ca²⁺-CaM is also involved in the NO-induced antioxidant defense is not yet clear. In this study, we provide pharmacological evidence for the involvement of Ca²⁺-CaM in the NO-induced antioxidant defense. Our data showed that pretreatments with the Ca²⁺ chelator EGTA, the Ca²⁺ channel blocker LaCl₃, and the CaM antagonists TFP and W7 substantially arrested the NO donor SNP-induced increases in the expression of the antioxidant genes *SOD4*, *cAPX*, and *GRI* (Figure 6A) and

Figure 6 Effects of pretreatments with Ca²⁺ inhibitors and CaM antagonists on SNP-induced increases in the expression and activities of chloroplastic and cytosolic antioxidant enzymes in leaves of maize plants. **(A)** Transcript levels of the antioxidant genes *SOD4*, *cAPX*, and *GRI* analyzed by RT-PCR. **(B)** The activities of the chloroplastic and cytosolic antioxidant enzymes SOD, APX, and GR. The detached plants were pretreated with distilled water, 10 mM EGTA, 5 mM LaCl₃, 100 μM verapamil, 300 μM TFP, 200 μM W7, and 200 μM W5 for 2 h, respectively, and then exposed to 100 μM SNP or 100 μM Fe(III)CN treatment for 8 h **(A)** or 12 h **(B)**. Detached plants treated with distilled water under the same conditions served as controls. In **(A)**, experiments were repeated at least three times with similar results. In **(B)**, the values are means ± SE of three different experiments. Means denoted by the same letter did not significantly differ at *P* < 0.05 according to Duncan's multiple range test.

the activities of chloroplastic and cytosolic SOD, APX, and GR (Figure 6B), but pretreatment with W5 did not affect the SNP-induced increases in the expression and activities of these antioxidant enzymes. These results clearly suggest that Ca²⁺/CaM is required for NO-induced antioxidant defense in leaves of maize plants. It seems that there are at least two pathways in NO-induced antioxidant defense: one is NO-MAPK-antioxidant; another is NO-Ca²⁺/CaM-antioxidant. However, Ca²⁺/CaM seems unlikely to directly regulate the activities of antioxidant enzymes in ABA signaling [9]. A Ca²⁺-CaM kinase pathway associated with NO sensing may be involved in the regulation of ROS-scavenging systems. To date, however, no information is available for the Ca²⁺-CaM kinase in plant cells.

Materials and Methods

Plant material and treatments

Seeds of maize (*Zea mays* L., cv. Nongda108; from Nanjing Agricultural University, China) were sown in trays of sand in a light chamber at a temperature of 22–28 °C, with a photosynthetic active radiation (PAR) of 200 μmol m⁻² s⁻¹ and a photoperiod of 14/10 h (day/night), and watered daily. When the second leaf was fully expanded, the plants were collected and used for all investigations.

The plants were excised at the base of the stem and placed in the distilled water for 1 h to eliminate the wound stress. After the treatment, the cut ends of the stems were placed in beakers wrapped with aluminum foil containing 100 μM ABA, 10 mM H₂O₂, 100 μM sodium nitroprusside (SNP), and 10 mM CaCl₂ solutions for various times at 25 °C with a continuous light intensity of 200 μmol m⁻² s⁻¹. In order to study the effects of inhibitors and scavengers, the detached plants were pretreated with 10 mM EGTA, 5 mM LaCl₃, 100 μM verapamil, 300 μM TFP, 300 μM W7 and 300 μM *N*-(6-aminoethyl)-1-naphthalene sulfonamide hydrochloride (W5) for 2 h, respectively, and then exposed to 100 μM ABA or 10 mM H₂O₂ or 100 μM SNP or 10 mM CaCl₂ treatment for various times up to 12 h under the same conditions as described above. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls. After treatments of detached maize plants, the second leaves were sampled and immediately frozen under liquid N₂.

NO detection by CLSM

NO measurement was performed with the specific NO dye DAF-2DA, using the method as described [22] with slight modifications. Leaf segments of approximately 0.5 cm² were incubated in loading buffer (0.1 mM CaCl₂, 10 mM KCl, 10 mM MES-Tris, pH 5.6) and DAF-2DA at a final concentration of 10 μM for 30 min in the dark at 25 °C, followed by washing twice in the same loading buffer for 15 min each. All images were visualized using CLSM (excitation 495 nm, emission 515 nm). Images acquired were analyzed using Leica IMAGE software. All experiments were repeated three times with similar results. Data are presented as average fluorescence intensity.

NOS activity assay

Crude enzyme extracts were prepared as described by Ninnemann

and Maier [26], with some modifications. The frozen leaf tissues were homogenized in 5 mL of homogenization buffer (50 mM triethanolamine hydrochloride, pH 7.5, containing 0.5 mM EDTA, 1 mM leupeptin, 1 mM pepstatin, 7 mM GSH, 0.2 mM PMSF). After centrifuging at 9 000 × *g* for 30 min (4 °C), the supernatant was collected and recentrifuged at 100 000 × *g* for 1 h (4 °C). The supernatant was used as cytosolic fraction. The pellet was resuspended in 1 mL of homogenization buffer and used as microsomal fraction. NOS activity was analyzed by hemoglobin assay as previously described [27, 28]. Protein content was determined according to the method of Bradford [29] with BSA as standard.

Preparation of protoplasts

Maize mesophyll protoplasts were isolated using the method described by Shang *et al.* [30]. The cell wall was digested in enzyme solution, which contained 1.5% cellulose R10 (Yakult Honsha, Tokyo, Japan) and 0.4% macerozyme R10 (Yakult Honsha, Tokyo, Japan). The isolated protoplasts were washed twice with washing and incubation solution comprising 0.5 M mannitol, 4 mM MES, pH 5.7, and 20 mM KCl.

Measurement of [Ca²⁺]_i

For measurement of [Ca²⁺]_i, the maize mesophyll protoplasts were used to load the Ca²⁺-sensitive fluorescent dye Fluo-3/AM ester (Molecular Probes, Eugene, OR, USA) and make the CLSM observations according to the method described by Zhang *et al.* [31]. Fluo-3/AM was added from a stock solution of 1 mM Fluo-3/AM in DMSO, after incubation at 25 °C for 1 h in the dark. The incubation solution contained 20 μM Fluo-3/AM ester, 0.5 M mannitol, 4 mM MES (pH 5.7), and 20 mM KCl. Fluorescent probes were excited with a 488 nm laser, and emission fluorescent was filtered by a 515 nm filter to eliminate the autofluorescence of chlorophyll. Pictures were taken by scanning three times each 30 s, and then the fluorescence intensities of these pictures were measured by fluorescence microscopy after establishing a stable baseline.

Determination of CaM content

For isolation of CaM, the maize leaves were ground in liquid N₂, and then homogenized in buffer solution [50 mM Tris-HCl (pH 8.0), 1 mM EGTA, 0.5 mM PMSF, 20 mM NaHSO₄, and 0.15 M NaCl] at 1:1 weight/volume (w/v). The homogenates were disintegrated by sonication for a total of 2 min, treated in a water bath at 90–95 °C for 3 min and then cooled, and then centrifuged at 10 000 × *g* for 30 min. The supernatants were used for measurement of protein content and CaM concentration. Protein content was determined according to the method of Bradford [29] with BSA as standard. CaM concentration was determined by enzyme-linked immunosorbent assay (ELISA) according to Sun *et al.* [32].

Isolation of chloroplasts

Chloroplasts were isolated from leaves using the method as described by Munné-Bosch and Alegre [33]. Briefly, after grinding 5 g leaves in isolation buffer [0.33 M sorbitol, 2 mM EDTA, 50 mM Hepes-KOH (pH 7.5), 0.1% (w/v) BSA, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM DTT], the homogenate was filtered through four layers of cheesecloth and centrifuged at 2 °C and 200 × *g* for 1 min. The pellet was resuspended in isolation buffer and then centrifuged at 2 °C and 2 500 × *g* for 5 min. Chloroplasts were purified by resuspending the pellets in isolation buffer, laying on 12.5 mL of 25%

(v/v) of Percoll (in isolation buffer), and centrifuging at 2 °C and 15 800 × g for 20 min. The intact chloroplasts in lower layer were resuspended in isolation buffer without BSA and MnCl₂, centrifuged at 2 °C and 2 500 × g for 5 min. The chloroplast pellets were lysed in lyses buffer [50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 1 mM MgCl₂] and used immediately for analyses. To detect APX activity, an independent organelle-isolation procedure was used (20 mM sodium ascorbate added to the extraction medium), and all other solutions also contained 2 mM ascorbic acid to prevent the possible inactivation of APX.

Preparation of cytosolic fraction

Cytosolic fraction was isolated according to the method as described by Yang and Komatsu [34]. Leaf segments (0.5 g) were homogenized in a mortar and pestle in 5 mL homogenization buffer containing 50 mM phosphate buffer (pH 7.5), 0.25 M sucrose, 10 mM EGTA, 1 mM DTT, and 1 mM PMSF. The homogenates were centrifuged at 10 000 × g for 10 min. The supernatants were centrifuged at 100 000 × g for 1 h, and the cytosolic fraction was obtained by collecting the supernatant.

Isolation of total RNA and RT-PCR

Total RNA was isolated from leaves by using RNeasy mini kit (Qiagen) according to the instruction supplied by the manufacturer. Approximately 3 µg of total RNA was reverse transcribed using oligo(dT) primer and SuperScriptTM Reverse Transcriptase (Invitrogen). cDNA was amplified by PCR using the following primers: *SOD4*, forward TGC TCG CCT ACA AAT CCA and reverse TAG CGT TCT CAA ATG CTT AA; *cAPX*, forward TCG GCA CCA TGA AGA ACC C and reverse TCC TCG TCC GCT GCG TAT T; *GRI*, forward GAA GGT CGT GGA AAG ATA and reverse TTG GCA ACG AAG ACA TCA; *CaMI*, forward AAG CAT TCA GCC TCT TCG and reverse CAT CAT AAC CTT GAC AAA CTC C; *β-actin*, forward AAA TGA CGC AGA TTA TGT TTG A and reverse GCT CGT AGT GAG GGA GTA CC. To standardize the results, the relative abundance of *β-actin* was also determined and used as the internal standard.

The cycle number of the PCR reactions was adjusted for each gene to obtain barely visible bands in agarose gels. Aliquots of the PCR reactions were loaded on agarose gels and stained with ethidium bromide.

Enzyme assays

The activities of antioxidant enzymes were determined as previously described [35]. Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitro blue tetrazolium as monitored at 560 nm. APX activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. GR activity was measured by following the change in absorbance at 340 nm as oxidized glutathione (GSSG)-dependent oxidation of NADPH.

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