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Ca^{2+} and CaM are Involved in NO- and H_2O_2-Induced Adventitious Root Development in Marigold

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Abstract Our previous results have demonstrated that both nitric oxide (NO) and hydrogen peroxide (H_2O_2) are involved in the promotion of adventitious root development in marigold (*Tagetes erecta* L.). However, not much is known about the intricate molecular network of adventitious root development triggered by NO and H_2O_2. In this study, the involvement of calcium (Ca^{2+}) and calmodulin (CaM) in NO- and H_2O_2-induced adventitious rooting in marigold was investigated. Exogenous Ca^{2+} was capable of promoting adventitious rooting, with a maximal biological response at 50 μM $CaCl_2$. Ca^{2+} chelators and CaM antagonists prevented NO- and H_2O_2-induced adventitious rooting, indicating that both endogenous Ca^{2+} and CaM may play crucial roles in the adventitious rooting induced by NO and H_2O_2. NO and H_2O_2 treatments increased the endogenous content of Ca^{2+} and CaM, suggesting that NO and H_2O_2 enhanced adventitious rooting by stimulating the endogenous Ca^{2+} and CaM levels. Moreover, treatment with Ca^{2+} enhanced the endogenous levels of NO and H_2O_2. Additionally, Ca^{2+} might be involved as an upstream signaling molecule for CaM during NO- and H_2O_2-induced rooting. Altogether, the results suggest that both Ca^{2+} and CaM are two downstream signaling molecules in adventitious rooting induced by NO and H_2O_2.

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Introduction

Adventitious roots that arise from any part other than the primary root system are the most common type of root regeneration (Smart and others 2003; Ramírez-Carvajal and others 2009). Adventitious root development is a complex process regulated by both environmental and endogenous factors such as temperature, light conditions, hormones, enzymes, metabolic constituents, and genotype (Pagnussat and others 2003; Liao and others 2010). Recently, there has been an increasing interest in understanding auxins and adventitious root response signaling pathways. It has been demonstrated that polyamines, ethylene, nitric oxide (NO; Pagnussat and others 2003), cyclic guanosine monophosphate (cGMP; Pagnussat and others 2003), mitogen-activated protein kinase cascade (MAPK; Pagnussat and others 2004), calcium (Ca^{2+}), Ca^{2+}-dependent protein kinase (CDPK; Lanteri and others 2006), phospholipase D-derived phosphatidic acid (Lanteri and others 2008), carbon monoxide (CO; Xuan and others 2008), and hydrogen peroxide (H_2O_2; Li and others 2009) are involved in auxin-induced adventitious root development. So far, however, the intricate signaling network that participates in adventitious root development has not been demonstrated.

Several lines of evidence suggest that both NO and H_2O_2 function as signaling molecules in plants. It is now clear that NO and H_2O_2 are involved in the plant response to various environmental stresses such as salinity (Zhang and others 2007), drought (Wodala and others 2008;

Lozano-Juste and León 2010), heat stress (Uchida and others 2002), ozone (Rao and Davis 2001), UV radiation (He and others 2005), disease (Delledonne and others 2001), and mechanical wounding (Orozco-Cárdenas and Ryan 2002). In addition, both NO and H_2O_2 regulate various developmental processes such as seed germination, programmed cell death, peroxisome biogenesis, flowering, and cell wall lignification (Neill and others 2002). Recently, some findings point to the existence of NO and H_2O_2 signaling pathways as a regulator of root hair formation (Rentel and others 2004; Lombardo and others 2006). NO and H_2O_2 are also likely to play important roles in the development of lateral roots (Correa-Aragunde and others 2004; Su and others 2006) and adventitious roots (Pagnussat and others 2003; Li and others 2009; Liao and others 2009).

Ca^{2+} is well established as a ubiquitous intracellular second messenger, regulating many different responses of plants to environmental signals (Besson-Bard and others 2008; Dodd and others 2010). Variations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) have been reported in response to a wide range of environmental, developmental, and growth stimuli, including heat shock (Gong and others 1998), cold shock (Knight and others 1991), UV light stress (Frohnmeyer and others 1999), pathogens (Blume and others 2000), oxidative stress (Price and others 1994), salinity, anoxia (Bush 1996), and abscisic acid (ABA; Schroeder and others 2001). Changes in $[Ca^{2+}]_{cyt}$ may proceed as single calcium transients, oscillations, or repeated spikes that have unique temporal and spatial arrangements determining the specificity of the physiological response (Lecourieux and others 2006). Current reports show that there are at least two main Ca^{2+} channels in plants: cyclic adenosine diphosphoribose (cADPR)/ryanodine-sensitive Ca^{2+} channels and inositol 1,4,5-trisphosphate (IP_3)-regulated Ca^{2+} channels (Lanteri and others 2006). For instance, an increase in $[Ca^{2+}]_{cyt}$ triggered by NO was through the activation of cADPR/ryanodine-sensitive Ca^{2+} channels in fava bean (*Vicia faba*) and tobacco (*Nicotiana tabacum*) cells (García-Mata and others 2003; Lamotte and others 2004). However, understanding of the biochemical and pharmacological properties of the two channels is not yet complete. Calmodulin (CaM) is a small, broadly distributed Ca^{2+}-binding protein that participates in signaling pathways that regulate many crucial processes such as innate immunity (Ma and others 2008), basal defense against necrotrophic pathogens (Takabatake and others 2007), pollen tube growth (Maih and others 1994), cell wall regeneration and cell division (Sun and others 1995), and heat tolerance (Gong and others 1998). CaM regulates the activity of an array of target proteins giving rise to a cascade of downstream effects, including altered protein phosphorylation and gene expression patterns (Lecourieux and others 2006). The local intracellular availability of CaM and its distribution appear to play important roles in regulating its biological activity. To date, the mechanism mediating CaM-related plant responses to growth stimuli remains unresolved.

It has been suggested in previous studies that there is a connection among NO, H_2O_2, Ca^{2+}, and CaM in plants. To date, the relationships between Ca^{2+} and NO have become more apparent (Courtois and others 2008). A Ca^{2+} chelator and channel blocker prevented pathogen-associated molecular pattern (PAMP)-induced NO generation, suggesting that there is a connection between $[Ca^{2+}]_{cyt}$ elevation and NO production (Ali and others 2007). Variations of $[Ca^{2+}]_{cyt}$ might be involved intimately in mediating NO synthesis (NOS) in plant cells and NO appears to be involved in the regulation of $[Ca^{2+}]_{cyt}$ (García-Mata and others 2003). NO treatment was shown to elevate $[Ca^{2+}]_{cyt}$ through the mobilization of intracellular pools of Ca^{2+}, potentially via cGMP- or cADPR-gated Ca^{2+}-permeable channels (Wendehenne and others 2004). Thus, NO may be an excellent candidate as a signaling molecule in every Ca^{2+}-modulated cell response (Lamotte and others 2004; Lanteri and others 2006). According to Ali and others (2007), the application of a CaM antagonist prevented Ca^{2+} channel- and NOS-mediated induction of NO, suggesting a link among Ca^{2+}, CaM, and NO in the plant pathogen response signaling cascade. Clearly, close interaction also exists between H_2O_2 and Ca^{2+} in response to biotic and abiotic stresses in plants. H_2O_2 treatment could activate Ca^{2+} channels to elevate $[Ca^{2+}]_{cyt}$ levels in guard cells, which are necessary for stomatal closure (Pei and others 2000). In addition, changes in $[Ca^{2+}]_{cyt}$ might regulate the generation of H_2O_2. Yang and Poovaiah (2002) reported that increases in $[Ca^{2+}]_{cyt}$ activated the Ca^{2+} sensor CaM and subsequently passed the signal to a downstream target "catalase" ("CAT"). This finally downregulated H_2O_2 levels by stimulating the activity of CAT. It has been also demonstrated that H_2O_2 production from the Ca^{2+}-dependent activation of a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase could trigger a Ca^{2+} influx (Pei and others 2000). More recently, the crosstalk between Ca^{2+}–CaM and H_2O_2 was reported to play a central role in ABA-induced antioxidant defense (Hu and others 2007). At present, however, the exact crosstalk among NO, H_2O_2, Ca^{2+}, and CaM remains to be elucidated.

Previous results from our laboratory have shown that both NO and H_2O_2 play crucial roles in adventitious rooting of marigold (*Tagetes erecta* L.; Liao and others 2009). Further investigations are needed to conclusively confirm the presence of downstream messengers in the signaling pathways triggered by NO and H_2O_2 to promote adventitious root development. In view of the evidence

described above, we further investigated the possible roles of Ca^{2+} and CaM in NO- and H_2O_2-induced adventitious root development in marigold.

Materials and Methods

Plant Material

Seeds of marigold (PanAmerican Seed, USA) were washed in distilled water and surface-sterilized in 5% (w/v) sodium hypochlorite for 10 min. After three washes in sterile distilled water, the seeds were germinated in Petri dishes on filter paper soaked in distilled water and maintained at 25 ± 1°C for 5 days with a 14-h photoperiod (photosynthetically active radiation = 200 μmol s^{-1} m^{-2}). Seedlings with their primary roots removed were used as explants and were maintained under the same temperature and photoperiod conditions described above for another 5 days in the presence of different media indicated below. The number and fresh weight of adventitious roots per explant were then measured.

Explant Treatments

After the primary roots were removed, marigold seedling explants were placed in Petri dishes containing filter paper soaked in distilled water (control), in different concentrations of calcium chloride ($CaCl_2$, Chinese supplier, Beijing, China), as indicated in Fig. 1, in 50 μM sodium nitroprusside (SNP, a donor of NO, Merck, Darmstadt, Germany), or in 200 μM hydrogen peroxide (H_2O_2, Sigma, St. Louis, MO, USA) and kept at 25 ± 1°C. The following chemicals were added alone or together with SNP, H_2O_2, or $CaCl_2$: 100 μM ethylene glycol-*bis*(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, Chinese supplier, Beijing, China), 100 μM 1,2-*bis*(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM, Sigma), 100 μM *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7, Sigma), 100 μM *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5, Sigma), 100 μM chlorpromazine hydrochloride (CPZ, Sigma), 100 μM trifluoperazine dihydrochloride (TFP, Merck), 200 μM 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, Sigma), or 100 μM catalase (CAT, Sigma). The concentration of these chemicals was selected based on the results of a preliminary experiment.

Measurement of Cytosolic Free Ca^{2+} Concentration

The fluorescent calcium indicator fluo-3-acetoxymethyl ester (Fluo-3/AM; Biotium, USA) was used in this study to determine $[Ca^{2+}]_{cyt}$ in hypocotyl cells (Zhang and others 1998). Fluo-3/AM was loaded into hypocotyls (10–20 mm long) at 4°C in the dark at a concentration of 10 μM. After 2 h of incubation, the hypocotyls were washed with standard medium (1 mM KNO, 0.2 mM KH_2PO_4, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KI, 0.1 mM $CuSO_4$, 0.5 M sorbitol, 0.8 M Glc, 10 mg l^{-1} myoinositol, 5 mM MES, pH adjusted to 5.5 with Tris) at 20°C for 30 min and placed under room temperature for 1 h. The Fluo-3 fluorescence of the hypocotyls was observed under a laser scanning confocal microscope (LSCM, MRC 1024, equipped with argon-krypton laser light, Bio-Rad Spectroscopy Group, Cambridge, MA, USA). The wavelength of excitation light was 488 nm and the emission signals at 515 nm were collected. After that, the kinetics of fluorescence intensity were measured with Laserpix 4.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

CaM Extraction and Analysis

Two grams of hypocotyls from marigold explants were ground in liquid N_2, then homogenized in buffer [50 mM Tris-HCl (pH 8.0), 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 mM NaCl, 20 mM $NaHSO_3$] at 1:2 (w/v). After being kept at 90°C for 2 min, the mixture was centrifuged at 4°C with 10,000×*g* for 30 min. The supernatant was analyzed for the soluble CaM and its concentration was determined by ELISA according to Sun and others (1995). The binding reaction was determined by absorbance at 475 nm in an ELISA analyzer (Shanghai Lorderan Scientific Instruments Co., Ltd, China). The antibody used for ELISA was a Promega product (Promega, Madison, WI, USA).

Determination of Endogenous NO and H_2O_2 Generation

NO and H_2O_2 contents were determined according to the procedures of Liao and others (2011). For NO content determination, 0.5 g of hypocotyls was frozen in liquid nitrogen and then ground in a mortar and pestle in 3 ml of 50 mM ice-cold acetic acid buffer (pH 3.6) containing 4% (w/v) zinc diacetate. The homogenates were centrifuged (10,000×*g* for 15 min at 4°C) and the supernatants were collected. The pellets were washed using 1.0 ml of extraction buffer and centrifuged as before. For each sample, the two supernatants were combined and 0.1 g of charcoal (Shanghai Chemical Reagent Co, Ltd.) was added. After vortex mixing and filtration, the filtrate was leached and collected. A mixture of 1.0 ml of filtrate and 1.0 ml of Greiss reagent was incubated for 30 min at room temperature to convert nitrite into a purple azo-dye. The absorbance was then determined at 540 nm.

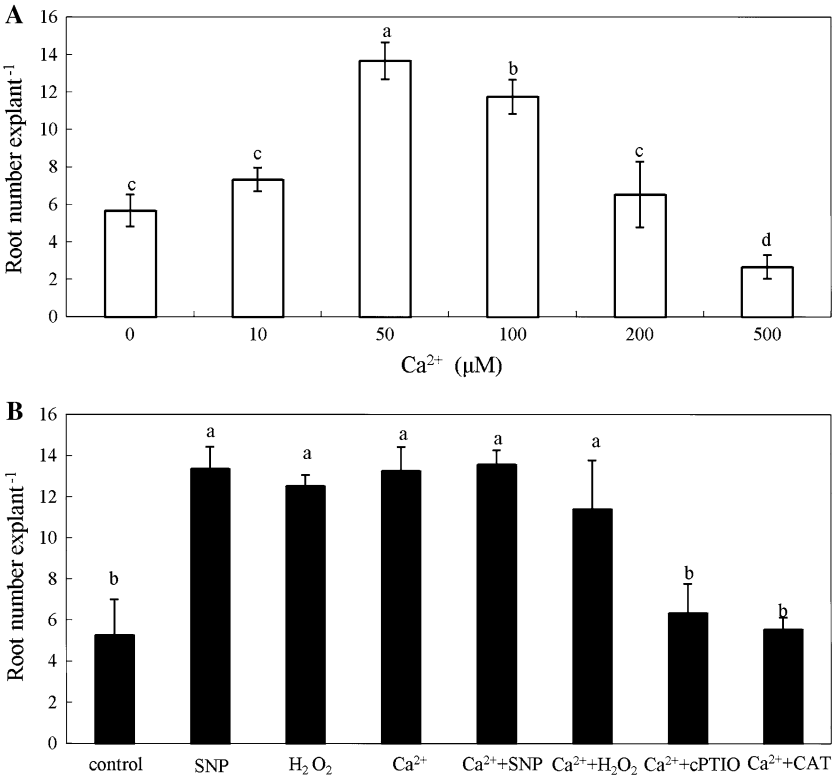

Fig. 1 Effects of exogenous Ca^{2+} on adventitious root development in marigold explants. The primary root system was removed from the hypocotyls of 5-day-old germinated marigold seedlings. **a** Hypocotyl explants were incubated for 5 days with different concentrations of $CaCl_2$, as indicated. **b** Hypocotyl explants were incubated for 5 days with 50 μM SNP, 200 μM H_2O_2, 50 μM $CaCl_2$, 200 μM cPTIO, or 100 μM CAT alone or in combination, as indicated. The number of adventitious roots per explant was expressed as mean ± SE ($n = 20$ explants from each of four independent experiments). Bars with different lower-case letters were significantly different (*t*-test; $p < 0.05$)

To determine H_2O_2 levels, 1.5 g of hypocotyls was frozen in liquid nitrogen, ground to a powder, and extracted in 3 ml of ice-cold acetone. The homogenate was centrifuged (10,000×*g* for 10 min at 4°C) and 0.5 ml of the supernatant was mixed with a 1.5 ml of a mixture of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4; 1:3 v/v). Distilled water (2.5 ml) was then added and the mixture was centrifuged (1,000×*g* for 1 min). The aqueous phase was collected. The reaction mixture contained 0.5 ml of buffer (0.2 M phosphate buffer solution, pH 7.8), 0.5 ml of water phase sample, and 20 ml of CAT solution (0.5 U; to set controls) or 0.5 U of inactive CAT (sample). After 10 min of incubation at 37°C, 0.5 ml of 200 mM Ti-4-(2-pyridylazo) resorcinol (Ti-PAR) was added. The reaction mixtures were incubated at 45°C for 20 min, then sample absorbance was measured at 508 nm against the controls.

Statistical Analysis

Where indicated, the results are expressed as mean values (±SE) from four independent experiments ($n = 20$). For statistical analysis, Student's *t*-test was used to determine the significance of the results between different treatments. Significance was established at the $p < 0.05$ level. All statistical analyses were performed using the Statistical Package for Social Sciences for Windows ver. 13.00 (SPSS, Inc., Chicago, IL, USA).

Results

Effects of Exogenous Ca^{2+} on Adventitious Root Development

In an attempt to elucidate the role of Ca^{2+} in adventitious root development, we performed a dose-response experiment with $CaCl_2$ (Fig. 1a). Compared with the control (0 μM), 50 μM and 100 μM $CaCl_2$ significantly increased the root number. The treatment of hypocotyls with 50 μM $CaCl_2$ resulted in the maximum root number being 2.4-fold of the control. At the highest $CaCl_2$ concentration tested (500 μM), many primordia failed to emerge, consequently diminishing the number of adventitious roots (Fig. 1a).

When compared with the control, treatment with 50 μM SNP, 200 μM H_2O_2, or 50 μM $CaCl_2$ alone significantly enhanced the number of adventitious roots (Fig. 1b). However, there was no significant difference in root number among SNP, H_2O_2, Ca^{2+}, Ca^{2+}+SNP, and Ca^{2+}+H_2O_2 treatments (Fig. 1b). Figure 1b also shows that pretreatment with 200 μM cPTIO or 100 μM CAT

significantly reversed partly adventitious root development induced by Ca^{2+}.

Effects of Ca^{2+} Chelators or CaM Antagonists on NO-Induced or H_2O_2-Induced Adventitious Root Development

Taking into consideration that Ca^{2+} is among the downstream components in the NO and H_2O_2 signaling cascades leading to adventitious rooting, we tested the Ca^{2+} requirement for NO- and H_2O_2-mediated adventitious root development. The results presented in Fig. 2 indicated that the membrane-impermeable Ca^{2+} chelator EGTA significantly reduced the root number and root fresh weight of explants treated with SNP and H_2O_2. Similarly, the membrane-permeable Ca^{2+} chelator BAPTA/AM was able to prevent the stimulating effect of NO and H_2O_2 on adventitious rooting. Moreover, BAPTA/AM alone was able to cause a significant reduction of root development compared with the control (Fig. 2).

Because CaM is thought to be a mediator protein of the Ca^{2+} signal, the effects of CaM antagonists (W-7, CPZ, and TFP) on NO- and H_2O_2-induced adventitious rooting were also analyzed. The CaM antagonist W-7 conferred decreased root number and root fresh weight of explants treated with SNP and H_2O_2 (Fig. 3). However, W-5, a biologically inactive structural analog of W-7, had no effect on adventitious rooting induced by NO and H_2O_2 (Fig. 3a, b). As shown in Fig. 3, the CaM antagonists CPZ and TFP were also able to prevent the promotion of adventitious rooting by SNP and H_2O_2.

Effects of NO, H_2O_2, and Their Antagonists or Chelators on $[Ca^{2+}]_{cyt}$ and CaM Levels

It seemed possible that the promotion of adventitious root development by NO and H_2O_2 involved, in part, increases in internal Ca^{2+} and CaM in the explants. Therefore, $[Ca^{2+}]_{cyt}$ and CaM levels of hypocotyls were measured in explants treated with SNP and H_2O_2 for 210 and 240 min, respectively. As shown in Fig. 4a, $[Ca^{2+}]_{cyt}$ in the control treatment (distilled water) remained constant throughout the 210 min of the experiment. A significant increase in $[Ca^{2+}]_{cyt}$ was observed in the explants treated with SNP and H_2O_2 (Fig. 4a). The initiation of this increase in $[Ca^{2+}]_{cyt}$ occurred within 90 min of SNP and H_2O_2 treatments. After 90 min of treatment, the $[Ca^{2+}]_{cyt}$ reached a

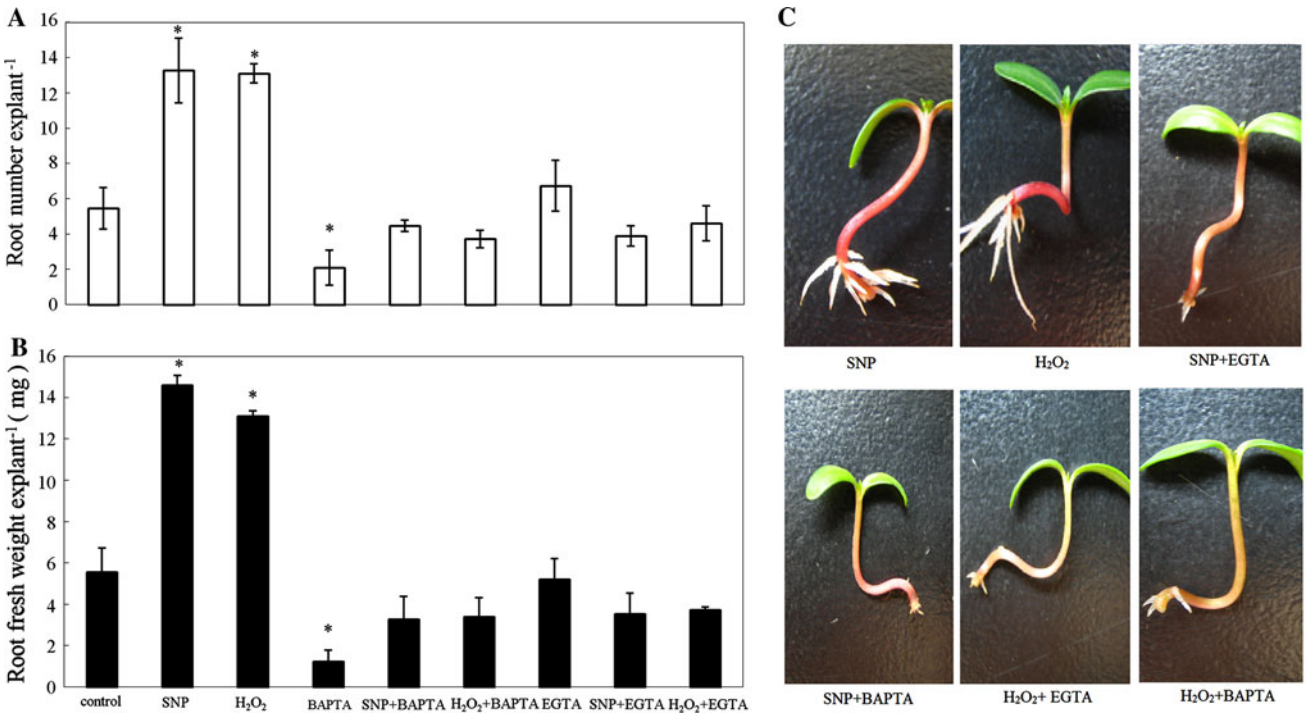

Fig. 2 Effects of Ca^{2+} chelators on SNP-induced or H_2O_2-induced adventitious root development in marigold explants. The primary root system was removed from the hypocotyl of 5-day-old germinated marigold seedlings. Hypocotyl explants were incubated for 5 days with 50 μM SNP, 200 μM H_2O_2, 100 μM EGTA, or 100 μM BAPTA/AM alone or in combination, as indicated. The number (**a**) and fresh weight (**b**) of adventitious roots per explant were expressed as mean ± SE ($n = 20$ explants from each of four independent experiments). Bars with asterisk are significantly different from the control (*t*-test, $p < 0.05$). Photographs (**c**) show hypocotyl explants after 5 days of the treatments indicated

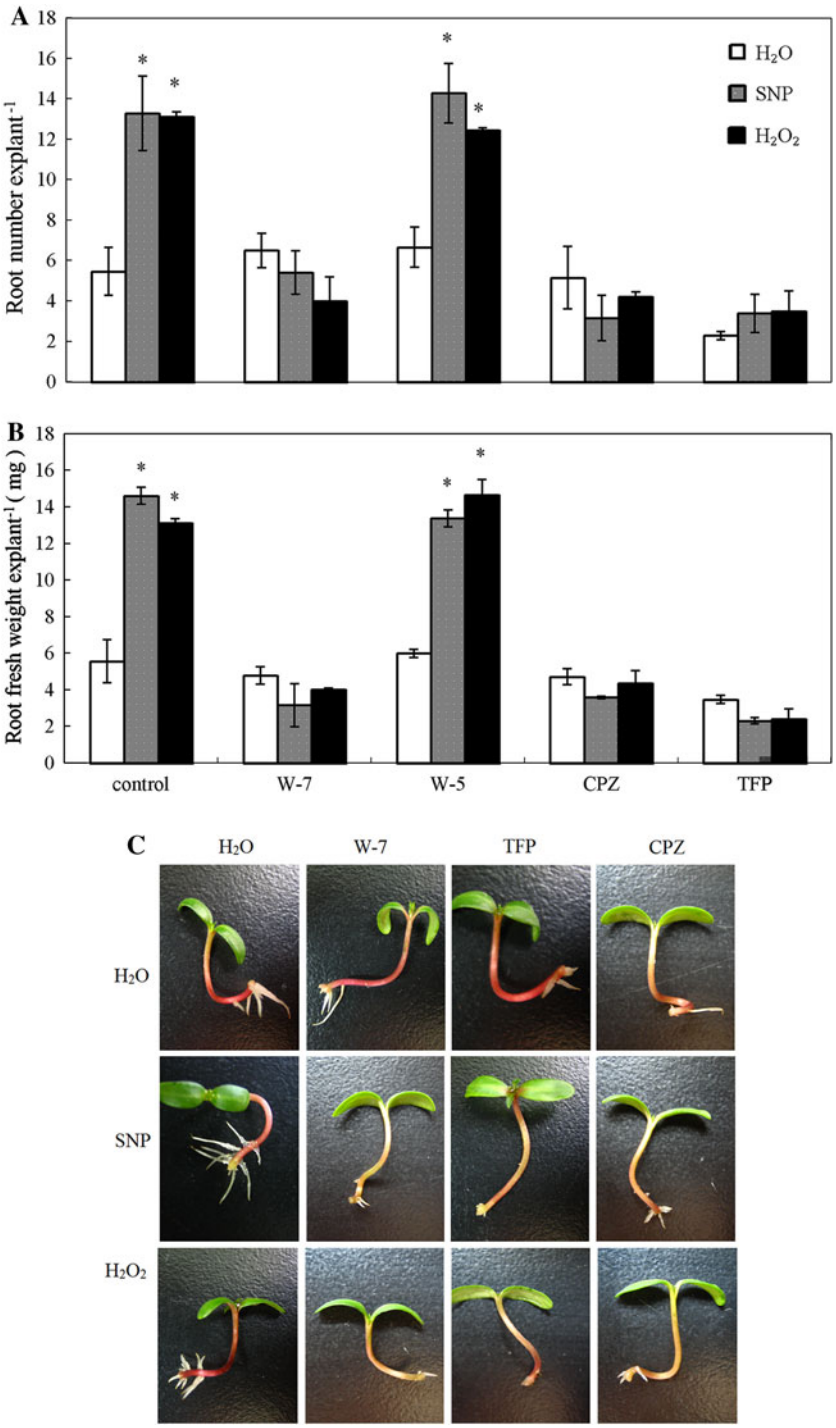

Fig. 3 Effects of CaM antagonists on SNP-induced or H_2O_2-induced adventitious root development in marigold explants. The primary root system was removed from the hypocotyls of 5-day-old germinated marigold seedlings. Hypocotyl explants were incubated for 5 days with 50 μM SNP, 200 μM H_2O_2, 100 μM W-7, 100 μM W-5, 100 μM CPZ, or 100 μM TFP alone or in combination, as indicated. The number (**a**) and fresh weight (**b**) of adventitious roots per explant were expressed as mean ± SE ($n = 20$ explants from each of four independent experiments). Bars with *asterisk* are significantly different from the control (*t*-test, $p < 0.05$). Photographs (**c**) show hypocotyl explants after 5 days of the treatments indicated

maximum 3-fold increase and then remained at high, constant levels after 120 min of treatment (Fig. 4a).

The CaM levels in explants treated with distilled water (control), SNP, and H_2O_2 were similarly low at 0 min. The concentration of CaM in explants treated with distilled water increased and reached a maximum 7.2-fold increase after 120 min (Fig. 4b). Treatment with SNP and H_2O_2 promoted the increase. The accumulation of CaM reached a maximum 15.3-fold and 15.2-fold increase after 120 min of SNP and H_2O_2 treatments, respectively, and then CaM levels decreased to the control levels after 240 min of treatment (Fig. 4b).

To further investigate the possible roles of Ca^{2+} and CaM in NO- and H_2O_2-induced adventitious rooting, the effects of the CaM antagonist W-7 and the Ca^{2+} chelator BAPTA/AM on $[Ca^{2+}]_{cyt}$ and CaM levels induced by SNP or H_2O_2 in marigold hypocotyl explants were determined, respectively. As shown in Fig. 5a, the Ca^{2+} chelator

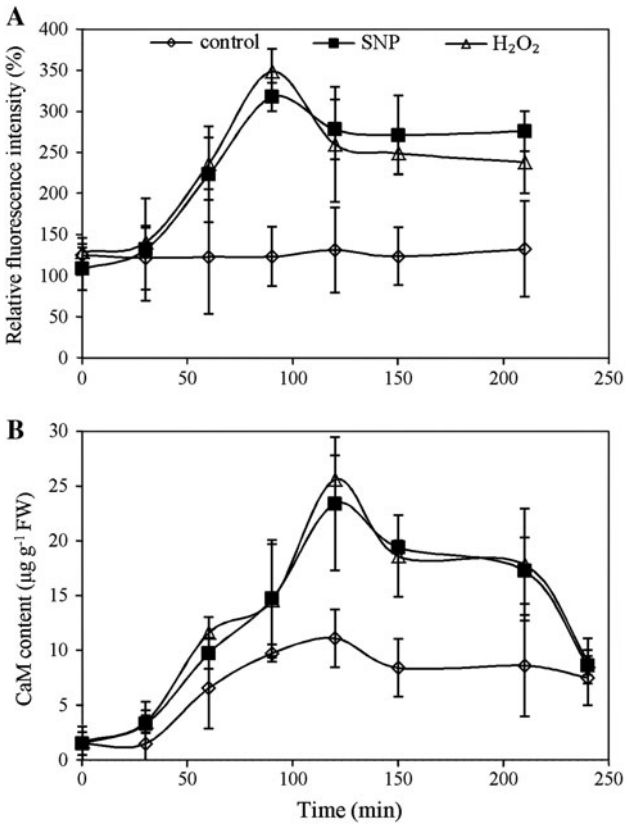

Fig. 4 Effects of NO and H_2O_2 on $[Ca^{2+}]_{cyt}$ and CaM level in marigold explants. The primary root system was removed from the hypocotyls of 5-day-old germinated marigold seedlings. $[Ca^{2+}]_{cyt}$ (**a**) and CaM levels (**b**) were determined in explants treated with distilled water (control), 50 μM SNP, or 200 μM H_2O_2. Fluo-3/AM was used as the Ca^{2+}-sensitive fluorescent probe. Changes of $[Ca^{2+}]_{cyt}$ were monitored by LSCM and expressed as relative fluorescence intensity. CaM was extracted from treated samples and quantified by ELISA. Values (mean ± SE) are the averages of four independent experiments

BAPTA/AM significantly inhibited NO- and H_2O_2-induced $[Ca^{2+}]_{cyt}$. When applied alone, BAPTA/AM depressed $[Ca^{2+}]_{cyt}$ (Fig. 5a). However, the CaM antagonist W-7 had no obvious inhibitory effect on the sustained $[Ca^{2+}]_{cyt}$ increase induced by NO and H_2O_2. When the Ca^{2+} chelator BAPTA/AM and CaM antagonist W-7 were administered to SNP- and H_2O_2-treated explants, it resulted in a significant reduction of endogenous CaM content. In addition, $[Ca^{2+}]_{cyt}$ in explants treated with BAPTA/AM or W-7 alone was significantly lower than that in the control explants (Fig. 5b).

Effects of Exogenous Ca^{2+} on Endogenous NO and H_2O_2 Production

To investigate the crosstalk between Ca^{2+}/CaM and NO/H_2O_2, the time-dependent production of NO and H_2O_2 induced by exogenous Ca^{2+} was investigated in hypocotyl explants. Figure 6a shows that in comparison with the control, treatment with 50 μM Ca^{2+} triggered a transient increase in NO production. This transient increase in NO accumulation culminated at about 6 h after Ca^{2+} treatment, then quickly attenuated to even below the control level (from 12 to 18 h after treatment), and a second wave of NO burst was detected 36 h after treatment (Fig. 6a). Figure 6b shows the time course of H_2O_2 levels affected by Ca^{2+} treatment. H_2O_2 levels of the explants treated with Ca^{2+} increased rapidly after treatment and reached the highest levels at about 8 h, then H_2O_2 levels decreased. Compared with the control, the endogenous H_2O_2 levels in Ca^{2+} treatment were higher during the treatment process (Fig. 6b).

Discussion

Recently, we demonstrated that both NO and H_2O_2 are messengers involved in adventitious root development in marigold explants (Liao and others 2009). In this study we present evidence that Ca^{2+} and CaM signaling cascades are activated during adventitious rooting induced by NO and H_2O_2. Thus, the results presented in this work are significant for describing the presence of downstream signals in the NO and H_2O_2 signaling pathways during adventitious root development.

Dose-response experiments confirmed that the effect of exogenous Ca^{2+} on adventitious rooting was dose-dependent, with a maximal biological response at 50 μM $CaCl_2$ (Fig. 1a). To date, the Ca^{2+} signal in adventitious root development is still poorly known. Falasca and others (2004) reported that application of exogenous Ca^{2+} to thin cell layers of *Arabidopsis thaliana* induced de novo adventitious root formation in a dose-dependent manner, with a maximal biological response at 0.6 mM $CaCl_2$. The exclusion of Ca^{2+} from the expression auxin-free medium reduced the percentage of adventitious rooting of poplar (*Populus tremula* L. × *P. tremuloides* L. cv. Muhs 1) cuttings by about 42%, suggesting the role of Ca^{2+} in the late phases of the adventitious rooting process (Bellamine and others 1998). In addition, exogenous Ca^{2+}, NO, and H_2O_2 applied to marigold explants exhibited similar behavior in inducing adventitious root development (Fig. 1b). Similar conclusions were reported in recent studies on the roles of NO and H_2O_2 in adventitious rooting. For instance, both NO and H_2O_2 have been shown to be involved in adventitious root development in *Arabidopsis*, cucumber (*Cucumis sativus*), mung bean (*Vigna radiata*), marigold, and chrysanthemum (*Dendranthema morifolium*) (Pagnussat and others 2003; Li and others 2009; Liao and others 2009, 2010). In a previous study, we demonstrated that NO may act synergistically with H_2O_2 to

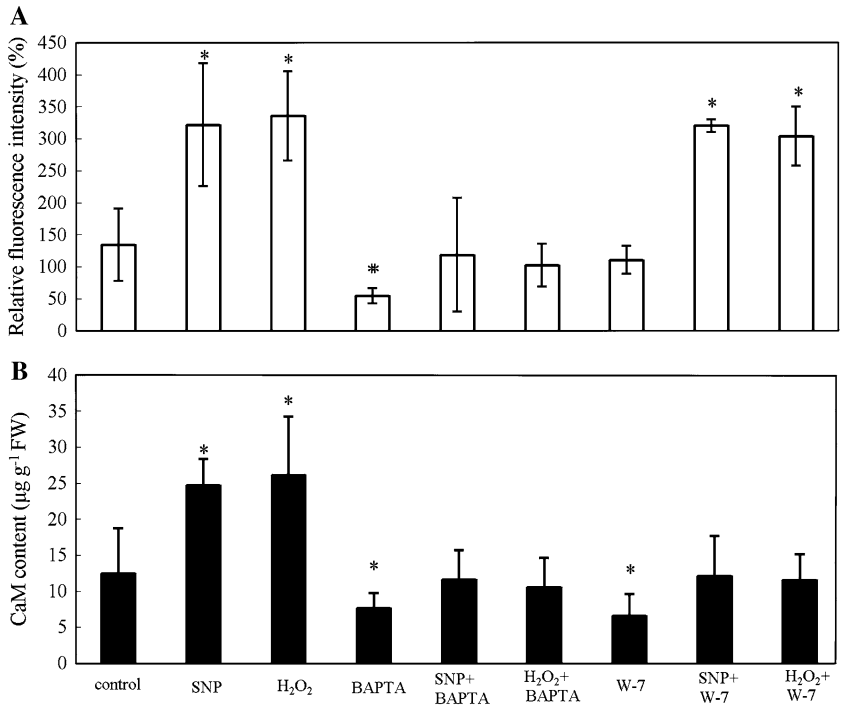

Fig. 5 Effects of Ca^{2+} chelators or CaM antagonists on $[Ca^{2+}]_{cyt}$ and CaM levels induced by SNP or H_2O_2 in marigold explants. The primary root system was removed from the hypocotyls of 5-day-old germinated marigold seedlings. Hypocotyl explants of marigold were incubated with 50 μM SNP, 200 μM H_2O_2, 100 μM BAPTA/AM, SNP + BAPTA/AM, H_2O_2 + BAPTA/AM, 100 μM W-7, SNP + W-7, or H_2O_2 + W-7 as indicated. $[Ca^{2+}]_{cyt}$ (**a**) was determined after 90 min of treatment and CaM level (**b**) was determined after 120 min of treatment. Fluo-3/AM was used as the Ca^{2+}-sensitive fluorescent probe. Changes of $[Ca^{2+}]_{cyt}$ were monitored by LSCM and expressed as relative fluorescence intensity. CaM was extracted from treated samples and quantified by ELISA. Values (mean ± SE) are the averages of four independent experiments. Bars with asterisk are significantly different from the control (*t*-test, $p < 0.05$)

enhance adventitious root development in marigold (Liao and others 2009). However, explants treated with $CaCl_2$ + SNP or $CaCl_2$ + H_2O_2 did not produce more adventitious roots than explants treated with SNP, H_2O_2, and $CaCl_2$ alone (Fig. 1b), showing that there was no synergistic effect between Ca^{2+} and NO/H_2O_2 on mediating rooting. Furthermore, pretreatment with the NO-specific scavenger cPTIO or the H_2O_2 scavenger CAT suppressed Ca^{2+}-induced adventitious root development (Fig. 1b), and exogenous Ca^{2+} may increase NO and H_2O_2 levels in hypocotyls (Fig. 6). Therefore, it may be hypothesized that NO and H_2O_2 may be involved in Ca^{2+}-induced adventitious rooting.

In our experiment, the involvement of endogenous Ca^{2+} in adventitious rooting induced by NO and H_2O_2 was further confirmed by the use of EGTA and BAPTA/AM. The results show that these compounds significantly blocked NO- and H_2O_2-induced adventitious rooting. When BAPTA/AM was administered alone, the development of adventitious roots was significantly inhibited (Fig. 2). Bellamine and others (1998) also found that both the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker lanthanum chloride ($LaCl_3$) inhibited adventitious rooting in poplar cuttings. Additionally, the promotive effects of NO and H_2O_2 on rooting were also largely depressed by the simultaneous presence of the CaM antagonists W-7, CPZ, or TFP (Fig. 3). These remarkable results further demonstrated that endogenous Ca^{2+} and CaM are essential for NO- and H_2O_2-induced adventitious rooting. Ca^{2+} and CDPK activities have previously been shown to mediate the auxin response leading to adventitious root development (Lanteri and others 2006). This is the first report to show that Ca^{2+} and CaM are involved in the H_2O_2-induced adventitious root developmental process. As we know, CaM is thought to be involved in many physiological processes in plants. The effects of CaM antagonists such as CPZ, TFP, and W-7 have suggested a role for the Ca^{2+}–CaM system in the responses to hormones (Schroeder and others 2001), light (Frohnmeyer and others 1999), abiotic stress (Gong and others 1998), and microbial elicitors (Blume and others 2000). To our knowledge, this is the first report to show that endogenous CaM may be involved in NO and H_2O_2 signaling pathways that promote adventitious rooting. One point we would like to make is that the CaM antagonists W-7, CPZ, and TFP bind to and act on proteins that, like CaMs and CAM-like (CML), contain

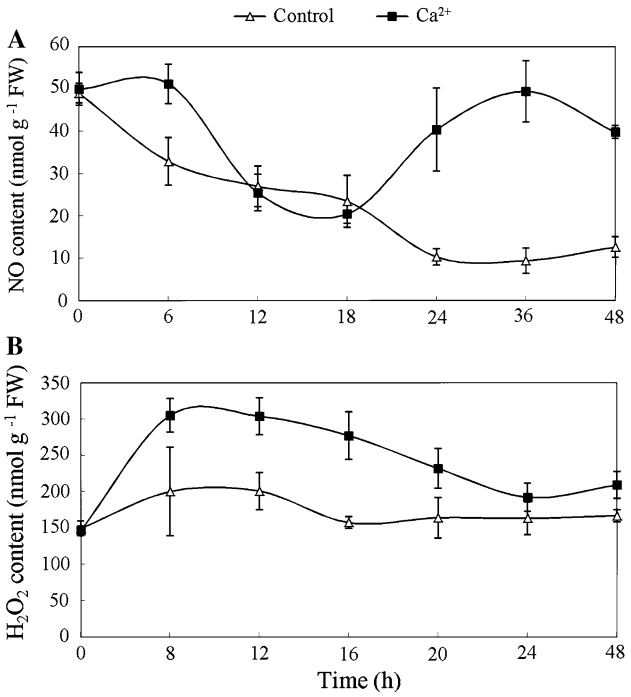

Fig. 6 Effect of Ca^{2+} on endogenous NO and H_2O_2 contents in marigold explants. The primary root system was removed from the hypocotyls of 5-day-old germinated marigold seedlings. Endogenous NO levels (**a**) and H_2O_2 levels (**b**) of hypocotyls were determined over 48 h in explants treated with distilled water (control) or 50 μM $CaCl_2$. Values (mean ± SE) are the averages of four independent experiments

paired helix-loop-helix Ca^{2+}-binding EF hands (Bouché and others 2005). Thus, these inhibitors are not specific for inhibiting CaMs, and they could also impair the function of calcineurin B-like proteins and CDPK. In our additional experiment, both NO and H_2O_2 significantly enhanced endogenous CaM levels in marigold explants (Fig. 4b). Furthermore, the Ca^{2+} chelator BAPTA/AM and the CaM antagonist W-7 significantly depressed CaM levels in explants treated with NO and H_2O_2 (Fig. 5b). These results suggest that the Ca^{2+} chelator and the CaM antagonist act to inhibit adventitious rooting through their antagonism of CaM. Recently, MAPK and CDPK activities were shown to be involved in NO- and auxin-induced adventitious rooting (Pagnussat and others 2004; Lanteri and others 2006). This study was the first to prove that CaM plays important roles in adventitious root development, and this article is the first to show correlations with endogenous CaM level and NO and H_2O_2 response of adventitious rooting. Thus, Ca^{2+}, CDPK, MAPK, and CaM have been shown to be involved in adventitious rooting. However, our understanding of the role and the order of action of CaM as a new and critical mediator in adventitious root development should include more precise experiments in further detail.

In this study, a possible link between Ca^{2+}/CaM and NO/H_2O_2 during adventitious rooting was examined by monitoring endogenous Ca^{2+} and CaM contents in NO- and H_2O_2-treated explants. Exogenous NO and H_2O_2 increased $[Ca^{2+}]_{cyt}$ and CaM levels in marigold hypocotyls (Fig. 4), suggesting that NO and H_2O_2 may enhance the development of adventitious roots partially through activation of the endogenous production of Ca^{2+} and CaM. These results further confirm the pivotal roles of endogenous Ca^{2+} and CaM in adventitious rooting induced by NO and H_2O_2. Our previous results showed that the synergistic effects of NO and H_2O_2 on adventitious root development might be dependent on their mutual amplification (Liao and others 2009). NO has been reported to raise $[Ca^{2+}]_{cyt}$ in fava bean and tobacco guard cells by promoting Ca^{2+} release from intracellular stores (García-Mata and others 2003; Lamotte and others 2004). Interestingly, Lamotte and others (2006) found that the NO-evoked Ca^{2+} influx occurred concomitantly with a Ca^{2+}-independent plasma membrane depolarization. These authors suggested that NO may promote the opening of voltage-gated Ca^{2+} channels subsequent to membrane depolarization. Thus, it was proposed that Ca^{2+} might participate downstream of NO in plant signal transduction pathways. Crosstalk between H_2O_2 and Ca^{2+} in plant cells has been well reported. It has also been observed that H_2O_2 activated Ca^{2+} channels and then induced an increase in $[Ca^{2+}]_{cyt}$ (Pei and others 2000; Rentel and Knight 2004). Our data show that both NO and H_2O_2 treatments induced an increase in $[Ca^{2+}]_{cyt}$ and CaM levels, indicating that both Ca^{2+} and CaM might be downstream signal molecules in the NO and H_2O_2 signaling cascades. This study further showed that exogenous Ca^{2+} could enhance endogenous levels of NO and H_2O_2 in marigold explants (Fig. 6). As noted above, NO and H_2O_2 were also shown to increase $[Ca^{2+}]_{cyt}$ and CaM levels during adventitious rooting. Coincidently, it has been demonstrated that concomitant changes in $[Ca^{2+}]_{cyt}$ and NO levels are apparent during the transduction of biotic and abiotic signals (García-Mata and others 2003; Vandelle and others 2006). Therefore, our preliminary results indicate that there is a complex interplay that exists between Ca^{2+}/CaM and NO/H_2O_2 in adventitious root development. The results may have important functional implications, expanding and enriching the possibilities for modulating transduction processes during the development of adventitious roots.

The possible interrelationship between Ca^{2+} and CaM during NO- and H_2O_2-induced adventitious rooting also was further investigated in this study. A Ca^{2+} chelator prevented CaM accumulation in SNP- and H_2O_2-treated explants (Fig. 5b), suggesting that the CaM accumulation induced by NO and H_2O_2 is dependent on Ca^{2+}. However, application of the CaM antagonist did not prevent NO- and

H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevation (Fig. 5a), excluding the involvement of CaM as an upstream signal molecule for Ca^{2+} during adventitious rooting. These results indicate that Ca^{2+} may act as an upstream regulator for CaM in the NO and H_2O_2 signal transduction pathways in adventitious rooting.

Our previous results demonstrated that NO treatment induced an increase in endogenous H_2O_2 levels in marigold explants (Liao and others 2009). Recently, NO levels in the root of wild-type *A. thaliana* plants have also been shown to increase (8-fold) after H_2O_2 treatment (Wang and others 2010). Thus, H_2O_2 may be involved as an upstream signaling molecule for NO. More recent evidence has suggested that both NO and H_2O_2 are involved in indole-3-butyric acid (IBA)-induced rooting in marigold (Liao and others 2011). This combination of findings provides some support for a schematic model of the suggested pathway

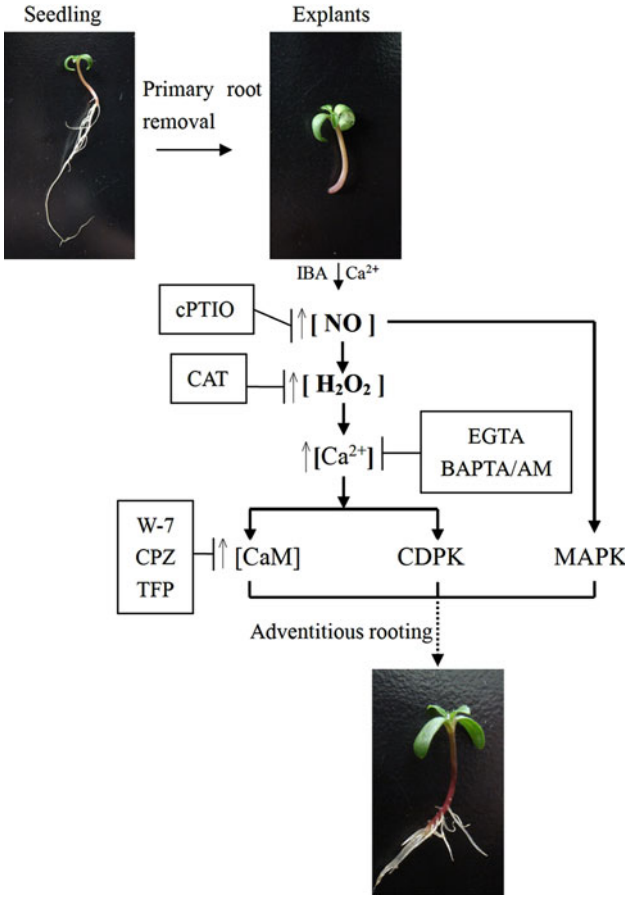

Fig. 7 Schematic model of the signaling networks involving IBA, NO, H_2O_2, and Ca^{2+} during adventitious root development in marigold. The auxin IBA (Liao and others 2011) and Ca^{2+} trigger a transient NO and H_2O_2 accumulation. In turn, NO and H_2O_2 induces an increase in $[Ca^{2+}]_{cyt}$ and CaM levels. In parallel, certain CDPKs and MAPKs become activated (Pagnussat and others 2004; Lanteri and others 2006). ⊢, inhibition. All the inhibitors assayed in this study are *boxed*

involving NO, H_2O_2, Ca^{2+}, and CaM in adventitious rooting (Fig. 7). Based on our studies, both Ca^{2+} and IBA treatment enhances the endogenous levels of NO and H_2O_2 in hypocotyl explants, and then NO and H_2O_2 trigger $[Ca^{2+}]_{cyt}$ elevation. CDPK (Lanteri and others 2006) and CaM may be downstream messengers in the signaling pathway (Fig. 7). Additionally, NO also has been demonstrated to activate a MAPK cascade in a cGMP-independent pathway (Pagnussat and others 2004).

Altogether, we revealed some components of NO and H_2O_2 signaling during adventitious rooting in this study. However, the network responsible for adventitious root development seems to be very complex. Therefore, considerably more work will need to be done to determine adventitious rooting signaling.

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