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The interaction between H_2O_2 and NO, Ca^{2+}, cGMP, and MAPKs during adventitious rooting in mung bean seedlings

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Abstract Hydrogen peroxide (H_2O_2), an active oxygen species, is widely generated in many biological systems and mediates various physiological and biochemical processes in plants. In the present study, we present a signaling network involving H_2O_2, nitric oxide (NO), calcium (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and the mitogen-activated protein kinase (MAPK) cascade during adventitious rooting in mung bean seedlings. Both exogenous H_2O_2 and the NO donor sodium nitroprussiate were capable of promoting the formation and development of adventitious roots. H_2O_2 and NO signaling pathways were elicited in parallel in auxin-induced adventitious rooting. Cytosolic Ca^{2+} was required for adventitious rooting, and Ca^{2+} served as a downstream component of H_2O_2, as well as cGMP or MAPK, signaling cascades. cGMP and MAPK cascades function downstream of H_2O_2 signaling and depend on auxin responses in adventitious root signaling processes.

Keywords Hydrogen peroxide · Nitric oxide · Calcium · Cyclic guanosine monophosphate · Mitogen-activated protein kinase · Adventitious roots · Mung bean · *Vigna radiata* L.

Introduction

For many years, H_2O_2, a form of reactive oxygen, was mainly viewed as a toxic cellular metabolite. However, it is now clear that it functions as a signaling molecule that mediates responses to various stimuli in plant cells (Neill et al. 2002). Hydrogen peroxide is continually generated from various sources during normal metabolism in plant cells. It mediates various physiological and biochemical processes, including systemic acquired resistance and the hypersensitive response (Melillo et al. 2006), senescence (Hung et al. 2006), programmed cell death (Levine et al. 1994), stomatal closure (Pei et al. 2000; Bright et al. 2006), root gravitropism (Joo et al. 2001), lateral root development (Su et al. 2006), cell wall development (Potikha et al. 1999), and pollen–stigma interactions and development (McInnis et al. 2006). Recently we demonstrated that H_2O_2 is a messenger involved in auxin-induced adventitious rooting in cucumber (Li et al. 2007) and mung bean (Li et al. 2009).

Adventitious roots are post-embryonic roots that arise from the stem and leaves, as well as from non-pericyclic tissues in existing roots. Adventitious root formation is one of the most important means of vegetative plant propagation. The formation of adventitious roots involves a process of redifferentiation in which predetermined cells switch from their morphogenetic path to act as mother cells for the root primordia (Aeschbacher et al. 1994). Many environmental and endogenous factors regulate adventitious rooting. Some endogenous factors have been identified, such as calcium (Ca^{2+}) (Bellamine et al. 1998), sugar (Takahashi et al. 2003), phenolics (Rout 2006), ethylene (Liu et al. 1990), polyamines (Nag et al. 2001), nitric oxide (NO) (Pagnussat et al. 2003, 2004), carbon monoxide (Xu et al. 2006), cyclic guanosine monophosphate (cGMP), and mitogen-activated protein kinase (MAPKs) (Pagnussat et al. 2003, 2004), phytohormones (De Klerk et al. 1999), and peroxidase (Syros et al. 2004). Some of these molecules may function in signaling and mediate auxin-induced adventitious rooting and auxin-response gene expression. To date, the intricate

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signaling network that participates in adventitious root formation remains poorly understood.

In recent years, significant progress in the elucidation of auxin and adventitious root response signaling pathways has been made, *e.g.*, IAA induces adventitious rooting via a pathway that involves NO. cGMP and MAPK cascades are downstream signals in the NO signaling pathway during adventitious rooting (Pagnussat et al. 2003, 2004). Although a variety of components participating in auxin transport and signal transduction have been identified, the molecular and biochemical mechanisms and intermediates underlying the signal transduction cascades in auxin-promoted root formation remain poorly understood.

In a previous study, we demonstrated that H_2O_2 might function as a signaling molecule in auxin-induced adventitious root formation in cucumber and mung bean seedlings (Li et al. 2007, 2009). Further investigations are needed to conclusively confirm the presence of downstream H_2O_2 signaling in adventitious rooting. The main aim of the present study was to elucidate the interactions between H_2O_2 and other signaling molecules such as NO, Ca^{2+}, cGMP, and the MAPK cascade in the formation and development of adventitious roots in mung bean.

Materials and Methods

Plant material. Seeds of mung bean, *Vigna radiata* (L.) R. Wilczek, were washed in distilled water and immersed in 70% ethanol for 1 min. After five washes in sterile distilled water, seeds were germinated in Petri dishes on perlite soaked in distilled water and maintained at 25± 1°C for 2 d in the dark, followed by 4 d in a 14-h photoperiod (PAR of 100 μmol m^{-2} s^{-1}) provided by white fluorescent lamps. 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 d in the presence of different test solutions.

Explant treatments. After the primary roots were removed, seedling explants, 10 per beaker, were inserted into the holes of filter paper in 50-ml beakers containing 20 ml of distilled water (control) or 20 ml of test solution. The test solutions utilized are described in the caption of Fig. 1. The beakers were kept in the same conditions as during germination. The chemicals used in the treatments were AR or biochemical standard reagents, *i.e.*, indole-3-butyric acid (IBA), hydrogen peroxide (H_2O_2) (Chinese supplier, Beijing), sodium nitroprusside (SNP), diphenylene iodonium (DPI), N^{ω}-nitro-L-arginine (L-NNA), ruthenium red, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 6-anilino-5,8-quinolinedione (LY83583), 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059), ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 8-bromoguanosine 3,5-cyclic monophosphate (8-Br-cGMP) (Sigma-Aldrich Co., St. Louis, MO).

Data collection and statistical analyses. The number and fresh weight of adventitious roots were quantified after a 5-d treatment. Data presented are the means ± SE of at least three independent experiments, with 10 explants per treatment. Data were analyzed using ANOVA, and comparisons between the mean values were evaluated by the least significant difference test at $P<0.05$ (indicated with the letters above the bars in the figures). Statistical analyses were performed using SPSS/PC Ver. 14.0 software (SPSS Inc., Chicago, IL).

Results

Adventitious roots grow through the epidermis of the hypocotyls within 3 d after excision of the primary roots and incubation with water (control).

H_2O_2 involvement in adventitious rooting in the NO-independent signaling pathway. The results obtained in preliminary experiments show that exogenous H_2O_2 caused a significant and dose-dependent increase in adventitious root number and fresh weight, similar to the promoting effect observed for treatment with IBA (Fig. 1: 1–3) (Li et al. 2009). When compared with the control, treatment with 1–100 mM H_2O_2 for 8 h significantly enhanced the number of adventitious roots per explant, with optimal concentrations of H_2O_2 ranging between 50 and 100 mM (data not shown). We confirmed that treatment with 300 μM NO donor SNP significantly increased the number of adventitious roots per explant (Fig. 1: 4, data not shown), as reported by She and Huang (2004).

Treatment of seedlings with 10 μM DPI, a specific inhibitor of H_2O_2 production (Desikan et al. 2004), completely inhibited adventitious rooting. When DPI was used in combination with SNP, SNP could not mitigate the inhibitory effect of DPI on rooting (data not shown). However, treatment with cPTIO (a specific NO scavenger) or L-NNA (a NO synthase inhibitor) (Hu et al. 2005; Bright et al. 2006) did not inhibit adventitious rooting (Fig. 1: 5, 8; Fig. 2). When either cPTIO or L-NNA was used in combination with H_2O_2 or IBA, cPTIO or L-NNA had no inhibitory effect on H_2O_2- or IBA-induced increases in the number (and fresh weight; data not shown) of adventitious roots (Fig. 1: 6, 7, 9, 10; Fig. 2). These results indicate that H_2O_2 is involved in the NO-independent signaling pathway or acts as a downstream component in the NO signaling

Figure 1. Effects of different treatments on the formation and growth of adventitious roots in 5-d-old mung bean seedling explants. Explants were incubated in the medium for 5 d. H_2O_2 was used at 10 mM and IBA 10 μM. *Bar*=1 cm. *1*, H_2O; *2*, 10 mM H_2O_2; *3*, 10 μM IBA; *4*, 300 μM SNP; *5*, 200 μM cPTIO; *6*, 200 μM cPTIO + H_2O_2; *7*, 200 μM cPTIO + 10 μM IBA; *8*, 10 mM L-NNA; *9*, 10 mM L-NNA + 10 mM H_2O_2; *10*, 10 mM L-NNA + IBA; *11*, 20 μM LY83583; *12*, 20 μM LY83583 + H_2O_2; *13*, 20 μM LY83583 + IBA; *14*, 20 μM LY83583 + 5 μM 8-Br-cGMP; *15*, 20 μM LY83583 + H_2O_2 + 5 μM 8-Br-cGMP; *16*, 20 μM LY83583 + IBA + 5 μM 8-Br-cGMP; *17*, 20 μM PD98059; *18*, 20 μM PD98059+H_2O_2; *19*, 20 μM PD98059 + IBA; *20*, 2 mM EGTA; *21*, 50 μM $CaCl_2$; *22*, 1 mM EGTA+50 μM $CaCl_2$; *23*, 1 mM EGTA + H_2O_2; *24*, 1 mM EGTA + IBA; *25*, 1 mM EGTA + H_2O_2 + 50 μM $CaCl_2$; *26*, 1 mM EGTA + IBA + 50 μM $CaCl_2$; *27*, 10 μM ruthenium red; *28*, 20 μM PD98059 + 50 μM $CaCl_2$; *29*, 20 μM LY83583 + 50 μM $CaCl_2$; *30*, 20 μM LY83583 + 5 μM 8-Br-cGMP + 50 μM $CaCl_2$; *31*, 5 μM DPI; *32*, 5 μM DPI + 50 μM $CaCl_2$; *33*, 5 μM DPI + H_2O_2.

pathway suggested by Bright et al. (2006), during adventitious rooting.

cGMP functions as a downstream component of H_2O_2 signaling involved in adventitious rooting. Treatment with 3 μM LY83583, the guanylate cyclase inhibitor (Alessi et al. 1995), neither inhibited the formation and growth of adventitious roots nor suppressed the promoting effects of H_2O_2 or IBA on adventitious rooting (data not shown). However, treatment with 5 μM LY83583 markedly inhibited the formation and growth of adventitious roots (Fig. 1: 11–16; Fig. 3). When 5 μM LY83583 was used together with H_2O_2, IBA, or 8-Br-cGMP, the cell-permeable cGMP derivative (Hu et al. 2005), the inhibitory effects of LY83583 on rooting were wholly or partially reversed. The results suggested that cGMP is involved in adventitious rooting, and it is worthy of study that H_2O_2 and IBA may induce the increase of cGMP in cells.

MAPK is a downstream mediator of H_2O_2- and IBA-induced adventitious root formation. PD98059 is a MAPK kinase (MAPKK) inhibitor (Alessi et al. 1995). PD98059

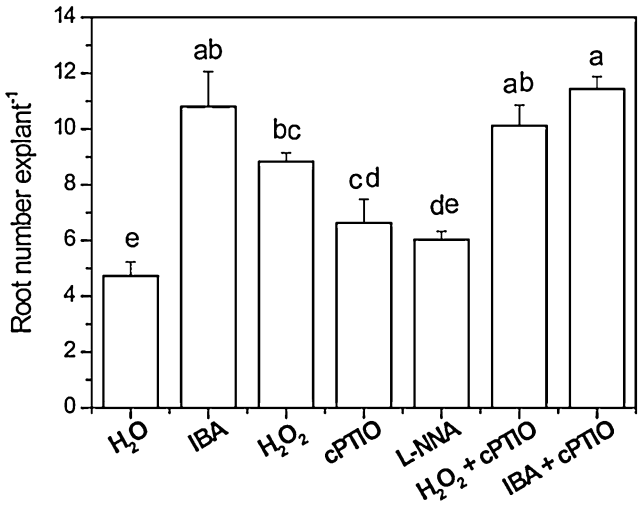

Figure 2. Effects of treatment with cPTIO and L-NNA on H_2O_2- or IBA-induced formation of adventitious mung bean roots. Explants were incubated in the medium indicated for 5 d. H_2O_2 was used at 10 mM, IBA 10 μM, L-NNA 10 mM, and cPTIO 200 μM.

(50 μM) treatment strongly inhibited the formation and growth of adventitious roots in cucumber via inhibition of the MAPKK cascade (Pagnussat et al. 2004). In our experiments, treatment with 10 μM PD98059 inhibited neither adventitious root formation nor H_2O_2- or IBA-induced adventitious rooting (data not shown). However, treatment with 20 μM PD98059 strongly inhibited adventitious rooting (Fig. 1: 17; Fig. 4). H_2O_2- or IBA-induced adventitious rooting was also suppressed by the addition of 20 μM PD98059 (Fig. 1: 18, 19). These results indicate that the MAPK cascade mediated adventitious rooting as a downstream component of H_2O_2 and IBA signaling.

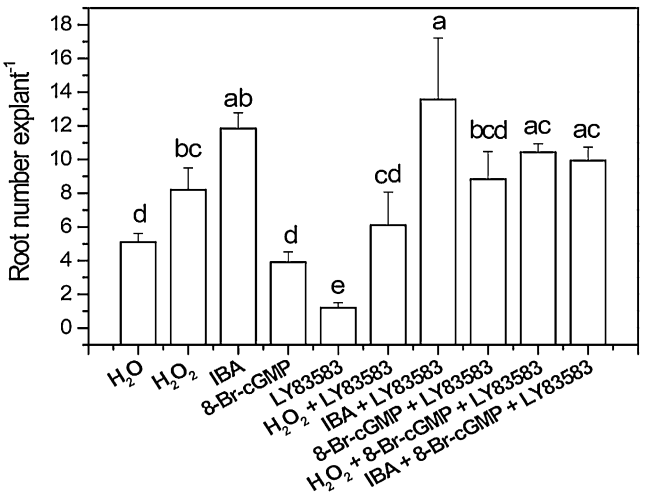

Figure 3. The effects of treatment with LY83583 and 8-Br-cGMP on H_2O_2- or IBA-induced formation of mung bean adventitious roots. Explants were incubated in the medium indicated for 5 d. H_2O_2 was used at 10 mM, IBA 10 μM, LY83583 5 μM, and 8-Br-cGMP 5 μM.

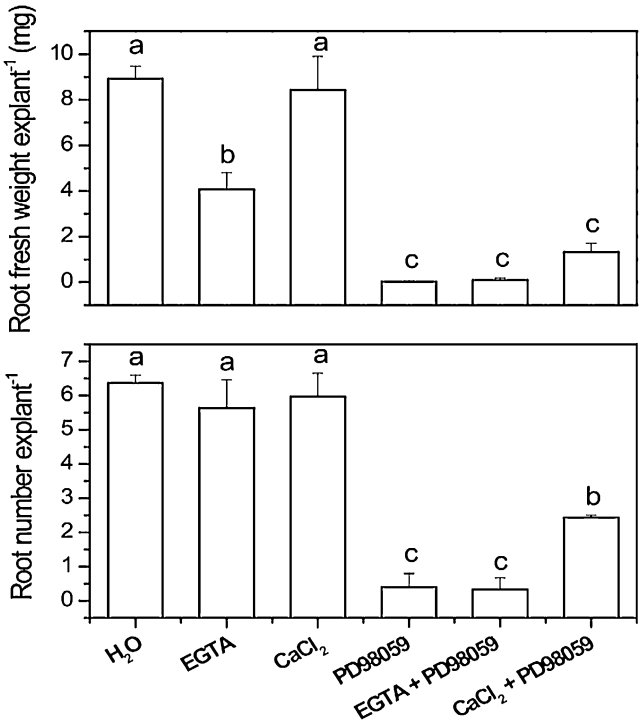

Figure 4. The effects of treatment with EGTA, $CaCl_2$, and PD98059 on formation and growth of mung bean adventitious roots. Explants were incubated in the medium indicated for 5 d. EGTA was used at 1 mM, $CaCl_2$ 50 μM, and PD98059 20 μM.

Cytosolic Ca^{2+} functions as a downstream component in H_2O_2 signaling involved in adventitious rooting. Ruthenium red is an inhibitor of the endomembrane Ca^{2+} permeable channel (Toyota et al. 2008), and EGTA is a chelator of extracellular Ca^{2+} (Pei et al. 2000). Treatment with 10 μM ruthenium red completely inhibited adventitious rooting (Fig. 1: 27). When 10 μM ruthenium red was used together with either 10 mM H_2O_2 or 10 μM IBA, the inhibitory effect of ruthenium red on adventitious rooting remained unchanged (data not shown). When 10 μM ruthenium red was used together with $CaCl_2$, the inhibitory effect of ruthenium red on adventitious rooting was not abrogated by $CaCl_2$ (data not shown). Furthermore, 1 mM EGTA treatment had no effect on the formation of adventitious roots but markedly inhibited their growth (Figs. 4, 5). Treatment with 2 mM EGTA significantly inhibited the formation and growth of adventitious roots (Fig. 1: 20). In addition, treatment with 50 μM $CaCl_2$ had no effect on either adventitious rooting or H_2O_2- or IBA-induced adventitious rooting (Fig. 1: 21–26; Figs. 4, 5). These results indicate that the formation of adventitious roots depended mainly on the cytosolic Ca^{2+} concentration, rather than the extracellular Ca^{2+} concentration. Furthermore, when DPI was used in combination with $CaCl_2$, $CaCl_2$ partially reversed the inhibitory effect of DPI on adventitious rooting (Fig. 1: 31, 32; Fig. 6). H_2O_2 can

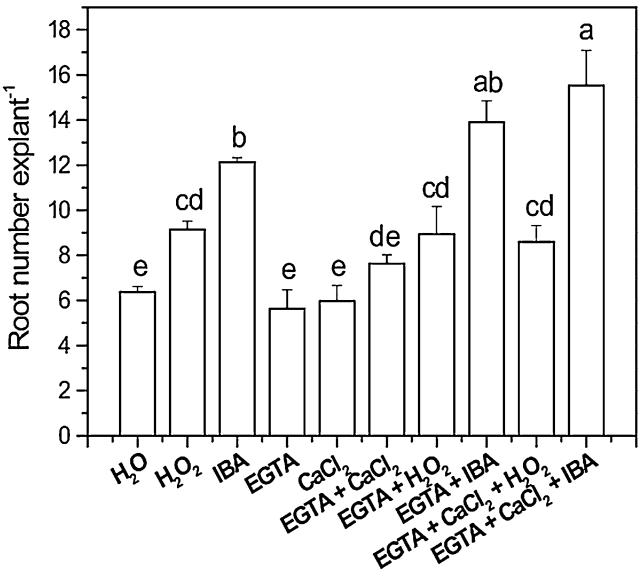

Figure 5. The effects of treatment with EGTA and $CaCl_2$ on H_2O_2 or IBA-induced formation of mung bean adventitious roots. Explants were incubated in the medium indicated for 5 d. H_2O_2 was used at 10 mM, IBA 10 μM, EGTA 1 mM, and $CaCl_2$ 50 μM.

partially reverse the inhibitory effect of DPI on adventitious rooting when DPI was used in combination with H_2O_2 (Fig. 1: 31, 33; Fig. 6). As DPI suppresses adventitious rooting by inhibiting the H_2O_2 production, and Ca^{2+} can partially reverse this inhibition, we concluded that Ca^{2+} is a downstream component of H_2O_2 signaling.

Ca^{2+} also mediates the cGMP or MAPK signaling pathways during adventitious rooting. Neither treatment with 1 mM EGTA nor $CaCl_2$ alone (Figs. 4, 5) had an effect on adventitious rooting. Similarly, treatment with EGTA plus 8-Br-cGMP and EGTA plus $CaCl_2$ and 8-Br-cGMP failed to affect adventitious rooting. In contrast, treatment with LY83583 alone significantly inhibited adventitious rooting (Fig. 1: 11; Fig. 7), and when LY83583 was used in combination with either $CaCl_2$ or $CaCl_2$ plus 8-Br-cGMP, the inhibitory effects of LY83583 were partially reversed (Fig. 1: 29, 30; Fig. 7). Furthermore, when PD98059 was used in combination with $CaCl_2$, the inhibitory effect of PD98059 on adventitious rooting was partially reversed (Fig. 1: 28; Fig. 4). These results indicate that Ca^{2+} is also

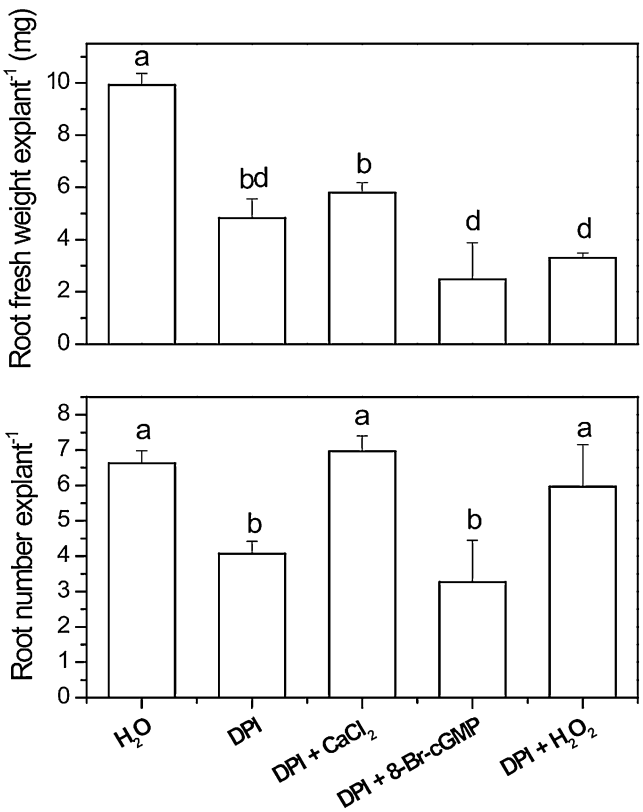

Figure 6. The effects of treatment with DPI, $CaCl_2$, and 8-Br-cGMP on H_2O_2-induced formation and growth of mung bean adventitious roots. Explants were incubated in the medium indicated for 5 d. H_2O_2 was used at 10 mM, DPI 5 μM, 8-Br-cGMP 5 μM, and $CaCl_2$ 50 μM.

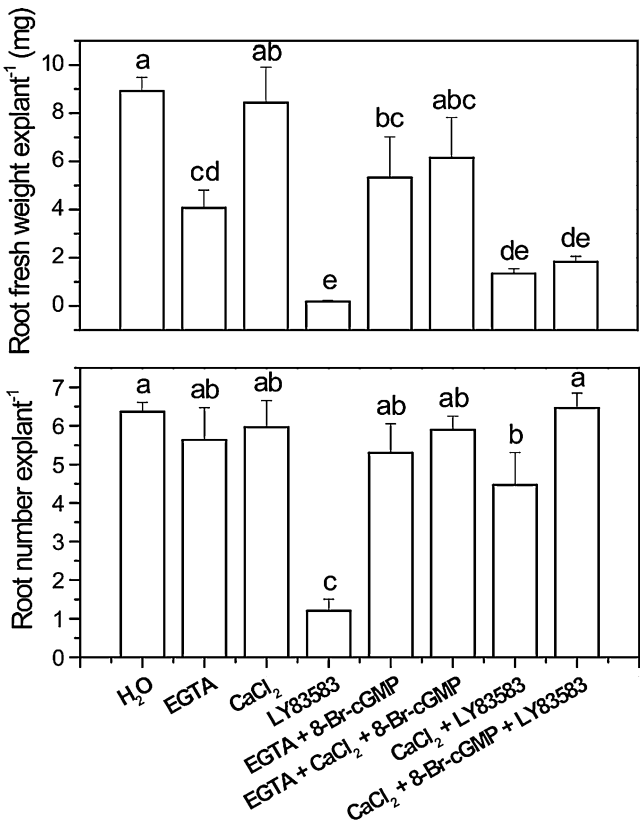

Figure 7. The effects of treatment with EGTA, $CaCl_2$, LY83583, and 8-Br-cGMP on formation and growth of mung bean adventitious roots. Explants were incubated in the medium indicated for 5 d. EGTA was used at 1 mM, $CaCl_2$ 50 μM, LY83583 20 μM, and 8-Br-cGMP 5 μM.

involved in the cGMP or MAPK signaling pathways during adventitious rooting.

Discussion

Based on the above results, we conclude that (1) both H_2O_2 and NO act as essential components of the signaling network that induces adventitious root formation after excision of primary roots; (2) H_2O_2 and NO are involved in two parallel downstream signaling pathways in auxin-induced adventitious root formation; and (3) H_2O_2 is also a downstream component of NO signaling during adventitious rooting. Similar conclusions were reported in a number of studies on the roles of NO and H_2O_2 in plant development and defense responses. For instance, NO serves to modulate H_2O_2 production and to downregulate its effects on the expression of defense-related genes (Orozco-Cardenas and Ryan 2002). H_2O_2 and NO generation occur in parallel or in short succession to each other and function synergistically and independently (Clarke et al. 2000). H_2O_2 and NO are essential components of the complex signaling network responsible for stomatal closure (Desikan et al. 2004).

cGMP is involved in plant development processes and responses to both biotic and abiotic stresses, such as stomatal closure (Neill et al. 2002), adventitious root development (Pagnussat et al. 2003), and *Arabidopsis* cell death (Clarke et al. 2000). cGMP regulates its targets, the cyclic nucleotide-gated channels involved in Na^+ and K^+ transport during cation uptake in roots, and influences salt tolerance in *Arabidopsis* (Guo et al. 2008). cGMP is involved in Ca^{2+} accumulation and ion flux that can produce a localized signal capable of regulating the pollen tip growth (Frietsch et al. 2007). In this study, LY83583 strongly inhibited the formation and growth of adventitious roots, and this inhibitory effect can be partially reversed by H_2O_2 or 8-Br-cGMP, suggesting cGMP functions as a downstream signal involved in H_2O_2-promoting adventitious rooting. Furthermore, the inhibitory effects of LY83583 were partially reversed by $CaCl_2$, suggesting that Ca^{2+} is also involved in the cGMP signaling pathway during adventitious rooting.

Cross-talk between H_2O_2 and Ca^{2+} occurs in plant cells. Ca^{2+}, or Ca^{2+} fluxes, induce the generation of H_2O_2, and H_2O_2 activity requires Ca^{2+} (Chen and Li 2001; Agarwal et al. 2005). H_2O_2 activates Ca^{2+} channels in guard cells, and the increase in cytosolic Ca^{2+} concentration in response to H_2O_2 has been observed and is necessary for stomatal closure (Pei et al. 2000). H_2O_2 treatment induces an increase in cytosolic Ca^{2+} concentration (Rentel and Knight 2004). Cross-talk between Ca^{2+}–CaM and H_2O_2 plays a pivotal role in ABA signaling (Hu et al. 2007). Our study demonstrated that Ca^{2+} is involved in adventitious rooting. Chelation of extracellular Ca^{2+} by EGTA was shown to have no clear inhibitory effect on adventitious rooting. However, ruthenium red completely inhibited adventitious rooting, suggesting that cytosolic Ca^{2+} fluxes are required for adventitious rooting and that Ca^{2+} serves as a downstream component in the H_2O_2 signaling pathway.

H_2O_2 is known to activate MAPK cascades in various tissues (Kovtun et al. 2000; Desikan et al. 2004), and H_2O_2 and NO may converge on MAPK signaling pathways involved in regulating stomatal closure (Desikan et al. 2004). A MAPK signaling cascade is activated during the adventitious rooting process induced by IAA (Morris 2001; Pagnussat et al. 2004). In the present study, PD98059 treatment strongly inhibited the formation of adventitious roots and completely suppressed the adventitious root-promoting effects of H_2O_2 or IBA, indicating that the MAPK cascade functions as a downstream component in signaling of H_2O_2 promotion of adventitious rooting.

Altogether, adventitious rooting is regulated by a complex set of cellular messengers, among which MAPK and cGMP are activated by upstream components that involve H_2O_2, NO, and Ca^{2+}. H_2O_2 and NO signaling represent two parallel pathways in this process. Activation of both pathways seems to be required for the development of adventitious roots since if one pathway is blocked, no adventitious roots develop. Ca^{2+} signaling plays a key role in adventitious root formation and functions as a downstream component in both the H_2O_2/NO and MAPK/cGMP pathways, although the network responsible for root development remains to be elucidated.

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