Hydrogen Peroxide Is a Second Messenger in the Salicylic Acid-Triggered Adventitious Rooting Process in Mung Bean Seedlings

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Abstract

In plants, salicylic acid (SA) is a signaling molecule that regulates disease resistance responses, such as systemic acquired resistance (SAR) and hypertensive response (HR). SA has been implicated as participating in various biotic and abiotic stresses. This study was conducted to investigate the role of SA in adventitious root formation (ARF) in mung bean (*Phaseolus radiatus* L) hypocotyl cuttings. We observed that hypocotyl treatment with SA could significantly promote the adventitious root formation, and its effects were dose and time dependent. Explants treated with SA displayed a 130% increase in adventitious root number compared with control seedlings. The role of SA in mung bean hypocotyl ARF as well as its interaction with hydrogen peroxide (H_2O_2) were also elucidated. Pretreatment of mung bean explants with N, N'-dimethylthiourea (DMTU), a scavenger for H_2O_2, resulted in a significant reduction of SA-induced ARF. Diphenyleneiodonium (DPI), a specific inhibitor of membrane-linked NADPH oxidase, also inhibited the effect of adventitious rooting triggered by SA treatment. The determination of the endogenous H_2O_2 level indicated that the seedlings treated with SA could induce H_2O_2 accumulation compared with the control treatment. Our results revealed a distinctive role of SA in the promotion of adventitious rooting via the process of H_2O_2 accumulation. This conclusion was further supported by antioxidant enzyme activity assays. Based on these results, we conclude that the accumulation of free H_2O_2 might be a downstream event in response to SA-triggered adventitious root formation in mung bean seedlings.

Introduction

Roots function as the interface between plants and the terrestrial environment. In higher plants, the root system is composed of primary roots, lateral roots and adventitious roots. Primary roots are initiated during embryogenesis and elongate after germination. Compared with primary roots, lateral roots and adventitious roots are 'post-embryonic' roots, as they initiate from non-pericycle tissues. Lateral roots initiate from primary roots or axes, and adventitious roots initiate from stem and leaf-derived cells. Together, these plant roots form the root system, the architecture of which can be altered in response to environmental changes and stimuli [1].

The appearance of adventitious roots may date the evolution of endogenous initiation combined with reverse auxin transport because these roots appear to have evolved repeatedly over time, and it is suggested they may have been required for the establishment of vascular continuity [2]. Adventitious root formation (ARF) is part of the normal development of plants and occurs naturally. ARF is useful for facilitating the uptake of water and nutrients from the soil, the anchorage of plants to substrates and the formation of food storage reserves.

ARF is a complex process that is influenced by multiple exogenous and endogenous factors. Plant hormones are among the internal factors that play a major role in regulating ARF. The hormone auxin has been shown to be a key regulator of AR formation [3-8]. Furthermore, ethylene [9-11] and cytokinin [12,13] are also believed to be crucial for ARF. In addition, other molecules such as polyamines [14], peroxidase (POD) [15], H_2O_2 [16,17], Ca^{2+} [18,19], nitric oxide (NO) [20-22], cyclic guanosine monophosphate (cGMP) [21,22] and mitogen-activated protein kinase (MAPKs) [23], as well as light [24], play a pivotal role in the adventitious rooting process. Although a variety of components participate in the regulation

of the adventitious rooting process, the molecular and biochemical mechanisms underlying the signal transduction involved in this process remain poorly understood.

The plant hormone SA is an endogenous growth regulator with a phenolic nature that plays a critical role in many diverse physiological processes. A well-known effect of SA is increasing the temperature in thermogenic plants [25] and flowering [26,27]. Previous studies also support the notion that SA is involved in modulating the plant response to many abiotic and biotic stresses, such as disease resistance [28,29] photosynthesis [30,31], low temperature resistance[32,33], drought resistance [34,35], salt resistance [36,37] and fruit maturity [38], among others. SA is also known to play a possible role in activating the defense response of plants to pathogen attack. SA mediates the oxidative burst that leads to programmed cell death (PCD) in the hypersensitive response, and it has been suggested that SA could act as a signal in the development of the SAR [39,40].

Nevertheless, defining the biological functions of SA in the context of the adventitious rooting process has been controversial. Still and Kling advocated that supplying SA could slight stimulate adventitious root initiation [41,42]. And in combination with indoleacetic acid (IAA), SA act synergistically effect [42]. This study revealed a distinctive role for SA in IAA-induced adventitious root formation. In 1989, Riov found that SA could effectively stimulate rooting of mung bean cuttings. However, only two concentration of SA has been examined (0.1 mM, 0.2 mM) [5]. Using SA deficient mutants, Gutierrez suggested that SA is possibly a positive regulator of adventitious rooting in *Arabidopsis* [43]. On the other side, Kang found that SA could increase the amount of scopolamine in adventitious root cultures of *Scopolia parviflora*, and without any negative effects on growth [44]. In contrast, another study yielded completely different results. Li reported that SA inhibited adventitious root formation and decreased the weights of roots in mung bean hypocotyls cuttings [45]. And De Klerk indicated that SA inhibited IAA-induced adventitious root formation in apple microcuttings during the initial post-treatment days. These findings may be the result of SA-enhanced IAA decarboxylation [46]. To date, the confirmatory role of SA in ARF is still ambiguous.

The main object of this study was to identify the role of SA in ARF in mung bean hypocotyls. Through pharmacological and physiological approaches, we demonstrate that SA is an important factor that induces adventitious root organogenesis and formation in mung bean hypocotyls. We also examine the interaction between SA with another important second signaling molecule, H_2O_2. Furthermore, we provide evidence that SA can elevate H_2O_2 levels through the regulation of the antioxidant enzymes in mung bean seedlings. Based on these results, a simple transduction pathway model is proposed, wherein H_2O_2 acts as a downstream messenger in the SA-mediated signaling pathway that induces ARF.

Materials and Methods

Plant Material

Mung bean (*Phaseolus radiatus* L) seeds were washed in distilled water and immersed in 0.1% $HgCl_2$ for 5 minutes. After five washes in distilled water, the seeds were soaked in distilled water for 12 h at 27°C and were then maintained at 27°C for 5 d, with a 14-h photoperiod (PAR of 200 µmol m^{-2} s^{-1}).

Explant Treatments

The explants consisted of a terminal bud, two primary leaves and 4 cm of the hypocotyls. After the primary roots were removed, the explants were put into 100-ml beakers containing 50 ml of distilled water (control) or 50 ml of the test solution for 24 h under the same conditions. After being washed three times, the seedlings were moved into distilled water, used as explants and maintained under the same temperature and photoperiod conditions for another five days. The distilled water was replaced daily.

Application of plant hormones and inhibitors

All chemicals were purchased from Sigma-Aldrich (St Louis, USA). SA was dissolved in ethanol for a final stock concentration of 1 M. The H_2O_2 scavenger DMTU was dissolved in distilled water to make a stock solution of 10 M. The NADPH oxidase inhibitor DPI was dissolved in dimethylsulfoxide (DMSO) to make a stock solution of 1 mM. For the DMTU and DPI treatments, after the removal of the primary roots, mung bean seedlings were pretreated in beakers for 4 h in the presence of DMTU or DPI and were then moved into test solutions for another 24 h. In addition, all of the explants treated with the inhibitors tested in this study appeared healthy.

Statistical analyses

The numbers of adventitious roots were determined after five days of treatment. The number of adventitious roots of more than 1 mm long was recorded. The data presented are the means of at least three independent experiments with 30 explants per treatment. The data were analyzed using an ANOVA. The comparisons between the mean values were conducted using the least significant difference (LSD) test, with the significance set at $P < 0.05$, and the standard error (S.E.) was calculated. The statistical analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL).

Root primordium observation

Root primordium observation was accomplished using Feulgen-staining, as described by Kordan [47]. After being treated for 48 h, the hypocotyls cut from the explants were fixed in absolute ethanol:glacial acetic acid (3:1) for 48 h and were then stored in 70% ethanol until they were required for use. The hypocotyls were rehydrated in an ethanol series (70-50-30-10%), hydrolyzed for 10 min in 10% HCl at 60°C and then placed in Schiff's reagent (24 h) followed by a thorough washing with tap water until the wash water was no longer

pink. The Feulgen-stained hypocotyls were placed in a 10% aqueous glycerine solution in uncovered plastic Petri dishes, and the glycerine allowed for concentration via the gradual evaporation of the water on a warming plate at 40°C. The stained hypocotyls were examined as whole mounts in the concentrated glycerine in Petri dishes using bright field transmission optical microscopy.

Visualization of H_2O_2 with the DAB method

H_2O_2 was detected with the DAB method [48]. Briefly, the hypocotyls cut from the explants were treated with 1 mg/ml solution of DAB, pH 3.8, for 8 h under light at 25°C. The treated hypocotyls were then placed in boiling 95% ethanol for 10 min to decolorize the hypocotyls, except for the deep brown polymerization product produced by the reaction of DAB with H_2O_2. After cooling, the hypocotyls were extracted with fresh ethanol and preserved at 4°C in ethanol and photographed.

Cytochemical detection of H_2O_2

H_2O_2 was visualized at the subcellular level using $CeCl_3$ for localization [49]. Electron-dense $CeCl_3$ deposits are formed in presence of H_2O_2 and are visible via transmission electron microcopy. Tissue samples (2×2 mm^2) were excised from the hypocotyls of the cuttings and then vacuum infiltrated with freshly prepared 5 mM $CeCl_3$ in 50 mM 3-(N-morpholino) - propane-sulfonic acid at pH 7.2 for 30 min. The tissue samples were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (CAB), pH 7.2, for 1 h at room temperature and kept overnight at 4°C. After fixation, the tissue samples were washed twice for 10 min in CAB and postfixed for 45 min in 1% (v/v) osmium tetroxide in CAB. The tissue samples were then washed twice for 10 min in CAB and dehydrated in a graded acetone series (30, 50, 70, 80, 90 and 100% [v/v]), progressively embedded in rising concentrations of acetone-resin mixtures, and incubated over two 24 h replacements of pure epoxy resin before polymerization at 60°C for 48 h. Ultrathin sections (50 to 100 nm) were obtained with a Reichert Ultracut E Microtome, using a diamond knife mounted on nickel grids (200 mesh), and examined without staining with a transmission electron microscope (H7650, HITACHI, Japan) at an accelerating voltage of 80 kV.

Endogenous H_2O_2 assay

H_2O_2 content was measured by monitoring the A_{415} of the titanium-peroxide complex, following the method described by Brennan and Frenkel [50], with slight modifications. Briefly, 0.5 g (FW) of hypocotyl material was frozen in liquid nitrogen immediately after completion of the treatment period and ground with liquid nitrogen, and the fine powdered material was mixed with 5 ml of cooled acetone in an ice bath. The mixture was centrifuged at 10,000 g for 15 min (4°C). Next, 1 ml of supernatant was obtained, and this was followed by the addition of 0.2 ml of a titanium reagent (20% w/v) and 0.4 ml of an ammonium solution to precipitate the titanium-hydroperoxide complex. The reaction mixture was centrifuged at 10,000 g for 10 min. The precipitate was dissolved in 5 ml of 2 M H_2SO_4. The supernatant's absorbance was measured at

415 nm against blanks using a LabTech UV1600 spectrophotometer (LabTech Inc. USA). A standard response curve was prepared with known concentrations of H_2O_2 using the same method as described above. The H_2O_2 content was calculated through comparison with a standard graph generated with known H_2O_2 concentrations.

Determination of hypocotyl O_2^- production

O_2^- production was measured, as described by Able [51], by monitoring the reduction of XTT in the presence of O_2^-. Mung bean hypocotyls (1 g) were homogenized with 5 ml of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at 15,000 g for 10 min. The reaction mixture (1 ml) contained 50 mM Tris–HCl buffer (pH 7.5), 50 µg of plasma membrane proteins, and 0.5 mM XTT. The reduction of XTT was evaluated at 470 nm for 5 min. Corrections were made for the background absorbance in the presence of 50 units of SOD. The O_2^- production rate was calculated using an extinction coefficient of $2.16×10^4$ M^{-1} cm^{-1}.

Isolation of the plasma membrane and determination of NADPH oxidase activity in the plasma membrane

Plasma membrane vesicles were isolated from mung bean hypocotyls using two-phase partitioning, according to a procedure described previously [52]. The membrane vesicles were resuspended in a 50 mM Tris-HCl buffer, pH 7.5 and used immediately for NADPH oxidase activity assays. The membrane vesicles (10 µg) were incubated in the reaction buffer (50 mM Tris-HCl buffer, pH 7.5, and 0.5 mM XTT). The reaction was initiated with the addition of NADPH. After 10 min at 25°C, the reaction solution was used for the spectrophotometric analysis of XTT formazan production at A_{470}. The NADPH activity was expressed as $\triangle A_{470}$ per milligram of protein per minute. $\triangle A_{470}$ represents the difference in XTT formazan absorbance at 470 nm in the presence and absence of 100 units of SOD.

Enzyme activity assays

For the determination of enzyme activity, plant material was frozen in liquid N_2 and stored at -80°C until the plants were ground and enzymes were extracted. Frozen root segments (0.3 g) were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM ASC in the case of the APX assay. The homogenate was centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was immediately used for the following described enzyme assays. Protein content was determined, according to the Bradford method, with BSA as a standard.

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Giannopolitis and Ries [53]. One unit of SOD activity was defined as the amount of enzyme that was required to produce 50% inhibition of the reduction of nitro blue tetrazolium as monitored at 560 nm.

CAT activity was assayed by measuring the rate of decomposition of H_2O_2 at 240 nm, as described by Aebi [54].

GR activity was determined, as described by Connell and Mullet [55]. The activity of the enzyme was assessed in
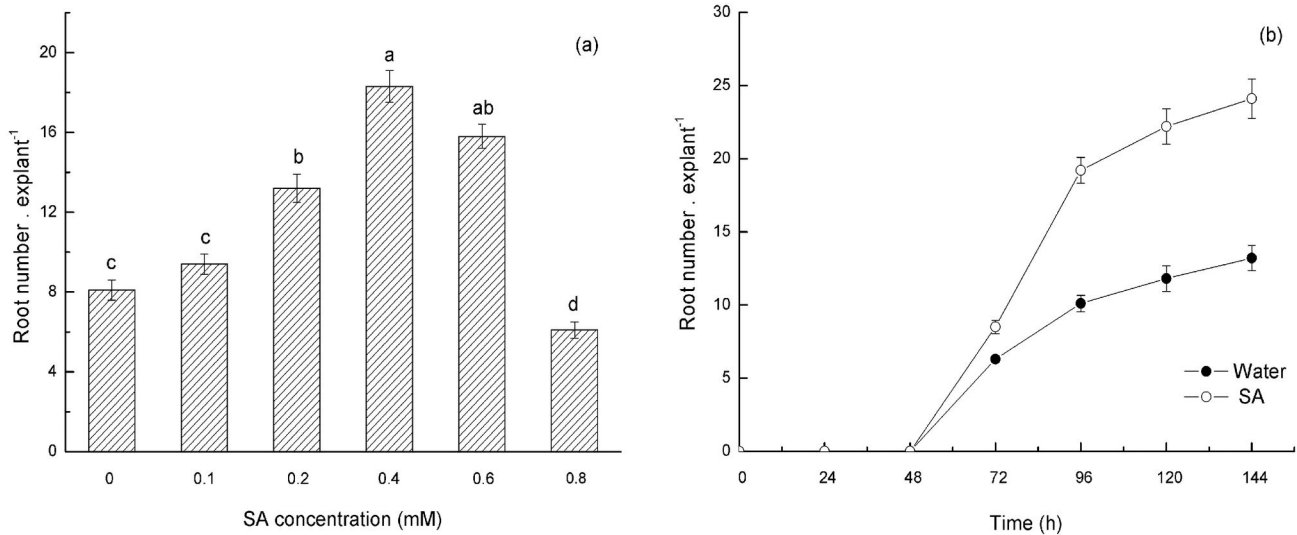
Figure 1. SA-induced ARF increases in mung bean hypocotyl cuttings. (a) Hypocotyls were incubated with 0, 0.1, 0.2, 0.4, 0.6 and 0.8 mM SA for 24 h and washed three times, and then the cuttings were transferred into distilled water. The cuttings were continuously grown for 5 days in this distilled water at 25±2°C, with a 14-h photoperiod (PAR of 200 μmol m^{-2} s^{-1}). The distilled water was replaced every day. The root numbers were determined at 5 d after treatment. In addition, adventitious roots of more than 1 mm long were quantified. The values represent the means of 30 explants, and the different letters above the bars indicate significant differences among the treatments at a P<0.05 level, according to the LSD test. (b) Time course of adventitious root formation induced by application of SA or water in mung bean hypocotyls. Hypocotyls were treated with 0.4 mM SA (the optimal concentration) or CK (water) for 24 h and were then transferred into distilled water and continuously grown for 7 days at 25±2°C, with a 14-h photoperiod (PAR of 200 μmol m^{-2} s^{-1}). The root numbers were determined in 24-h intervals. The distilled water was replaced every day, and the number of adventitious roots of more than 1 mm long was recorded. The values represent the means of 30 explants, and the error bars represent the SE (P<0.05) .

doi: 10.1371/journal.pone.0084580.g001

NADPH-containing HEPES buffer by monitoring the decrease in absorbance at 340 nm as NADPH is oxidized.

APX activity was determined by following the decrease in 290 nm for 3 min in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ASC, 0.1 mM H_2O_2, and 200μl of enzyme extract. The reaction was started by the addition of the enzyme extract. The reaction was corrected for the low non-enzymatic oxidation of ASC by H_2O_2 [56].

Results

SA induces adventitious rooting in mung bean hypocotyls in a dose- and time-dependent manner

To investigate the effect of SA on ARF in mung bean hypocotyls, 5-d-old seedlings with their primary roots removed were used as explants and incubated in a control treatment (water) and different concentrations of SA (0.1, 0.2, 0.4, 0.6 and 0.8 mM) at the same temperature and with the same photoperiod conditions for 24 h. After 5 days, the root number was analyzed and quantified. The results indicated that exogenous SA could promote adventitious root formation, and its effects were dose and time dependent (Figure 1). The treatment of the seedling explants with a low concentration of SA (0.2 mM) slightly promoted ARF, and the root number of

treatment explants exposed to 0.4 mM SA was 2.3-fold compared with the explants in the control treatment. An inhibitory effect on ARF was observed when the SA concentration was 0.8 mM. These results indicate that exogenous SA at particular concentrations promotes adventitious root formation in mung bean hypocotyl cuttings. Therefore, an SA concentration of 0.4 mM was used in the further experiments.

Effect of SA on root primordia formation

An anatomical study was performed to observe the primordium formation during the first stages of adventitious rooting. We analyzed (0-1 cm) sections of mung bean hypocotyls treated with water (control) or SA (0.4 mM SA). A photograph of a representative root primordium observation is presented in Figure 2b. At 48 h after the removal of the primary root system, adventitious root primordium formation was detected in explants treated with SA and water. In the water-treated plants, few root primordia could be observed, whereas in the SA-treated explants, we observed a greater number of root primordia than in the water-treated hypocotyls. The number of root primordia in the SA-treated explants was more than 2-fold higher than in the control seedlings. These results suggested that SA promotes AR formation in mung bean hypocotyls, possibly due to the induction of the dedifferentiation
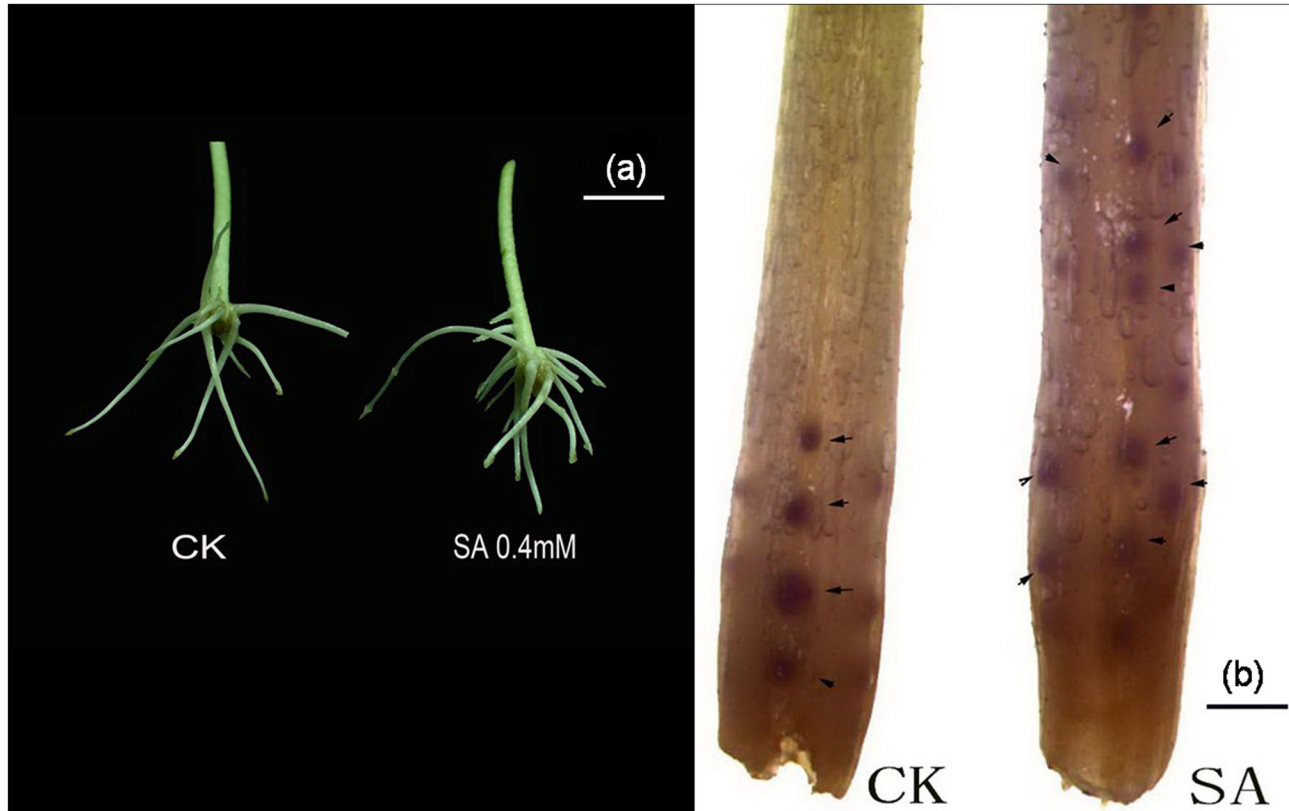
Figure 2. (a) Photograph showing explants after 5 days of treatment with CK (water) and SA (0.4 mM) Bar=1 cm. (b) Photograph depicting adventitious root primordial formation in mung bean seedlings after 48 h of treatment with H_2O or 0.4 mM SA. Bar=1 mm.

doi: 10.1371/journal.pone.0084580.g002

of cells and the consequent reestablishment of a new apical meristem.

Interaction between SA and H_2O_2 in ARF in mung bean hypocotyls

The above data indicate a SA-dependent effect on the formation of adventitious roots in mung bean hypocotyls. Previous work has shown that H_2O_2 can stimulate ARF in mung bean hypocotyls, and H_2O_2 might act as a downstream signaling molecule in IAA-induced ARF [57-59]. Whether SA influences H_2O_2-induced ARF remains unknown. To investigate whether H_2O_2 acts as a messenger in the SA-induced ARF transduction pathway, we first investigated the influence of SA on H_2O_2-induced ARF in mung bean hypocotyls. Several concentrations of SA with low concentrations of H_2O_2 (10 mM, suboptimal concentration) were tested together. We found that a combination of SA and H_2O_2 plays a synergetic role in regulating adventitious rooting in mung bean hypocotyls. At low concentrations, supplying SA with H_2O_2 can promote their respective effects on ARF in mung bean hypocotyls, whereas at a higher concentration, SA and H_2O_2 suppressed ARF (Figure 3).

DMTU and DPI prevent SA-induced adventitious root formation

To further elucidate the role of H_2O_2 in SA-triggered ARF in mung bean hypocotyls, the role of N, N'-dimethylthiourea (DMTU; a cell-permeable scavenger for H_2O_2) [60-64] in mung bean hypocotyls was investigated. We determined that H_2O_2 depletion treatment provoked a 2-fold reduction in the root number in comparison with the control explants. We believe that the inhibitory effect of DMTU on ARF can mainly be attributed to the scavenging of H_2O_2. However, pretreating hypocotyls with DMTU for 4 h, followed by a transfer into 0.4 mM SA for another 24 h, result indicated that DMTU can partially ameliorate the effect of SA on ARF (Figure 4a). The inhibition of the promotional effect of SA on ARF in mung bean hypocotyls that were pretreated with DMTU indicates that H_2O_2 may play a pivotal role in the SA-induced adventitious rooting process.

The above experiments identified that endogenous H_2O_2 might be involved in SA-induced ARF. The production of intracellular H_2O_2 was mainly attributable to NADPH oxidase, which converts O_2 to superoxide anions (O_2^-) and then to H_2O_2 [65-67]. Diphenyleneiodonium (DPI) is a specific inhibitor of membrane-linked NADPH oxidase that consequently inhibits
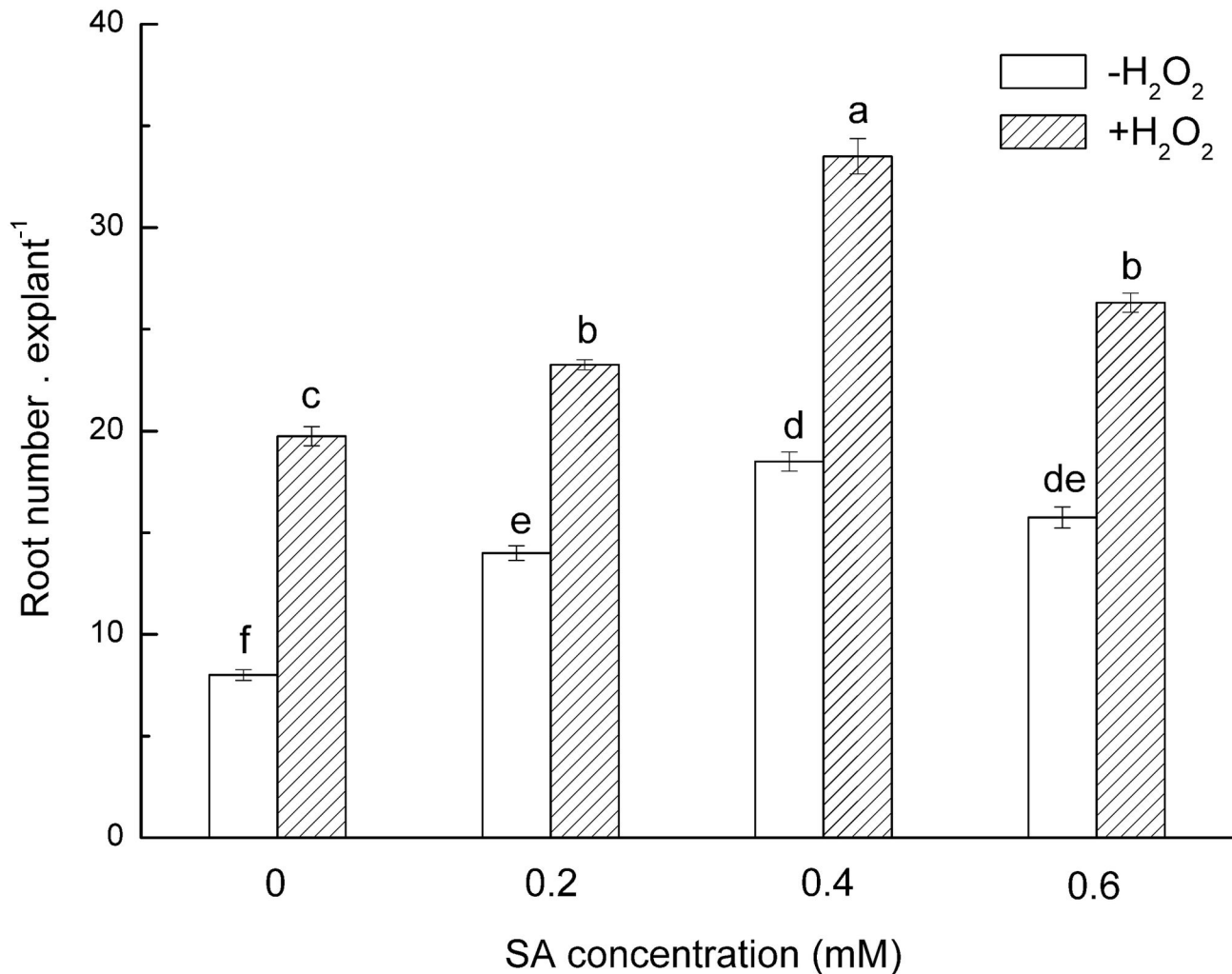
Figure 3. Interaction between SA in combination with H$_2$O$_2$ on ARF in mung bean hypocotyls. Hypocotyls were treated with different test solutions for 24 h, and then the cuttings were transferred to distilled water and continuously grown for 5 days at 25±2°C, with a 14-h photoperiod (PAR of 200 µmol m^{-2} s^{-1}). The distilled water was replaced every day, and the number of adventitious roots of more than 1 mm long was recorded. The number of roots was determined after 5 d of treatment. The values represent the means of 30 explants, and the error bars represent the SE (P<0.05). H$_2$O$_2$:10 mM H$_2$O$_2$.

doi: 10.1371/journal.pone.0084580.g003

NADPH oxidase activity (O$_2^-$ synthase) [60,68-71]. The effect of DPI treatment on ARF in mung bean hypocotyls was also investigated. As shown in Figure 4b, pretreatment with DPI alone could depress ARF. We also found that pretreatment with DPI could significantly depress the SA induction of ARF. These results imply that H$_2$O$_2$ formation might be required for SA-induced ARF. The observed changes in the H$_2$O$_2$ content after SA treatment further support this conclusion.

SA enhances H$_2$O$_2$ levels in mung bean hypocotyls

To further confirm the interaction between SA and H$_2$O$_2$ on ARF in mung bean hypocotyl cuttings, we measured the time course of endogenous H$_2$O$_2$ levels in the hypocotyls of mung beans incubated with water and SA. In this study, SA

treatments enhanced H$_2$O$_2$ levels in the hypocotyls of mung bean seedlings in a time-dependent manner. The treatment of hypocotyls with 0.4 mM SA significantly enhanced in vivo H$_2$O$_2$ contents compared with hypocotyls treated with water. Here, a rapid increase in the endogenous H$_2$O$_2$ level was detected during the first 12 h of hypocotyl incubation with SA, with a peak reached at 12 h; however, there was only a slight increase in the H$_2$O$_2$ level in the water-treated hypocotyls (Figure 5a). The H$_2$O$_2$ content at 3 h and 6 h was increased by 48% and 61%, respectively, compared with the control seedlings. After 12 h of treatment, the production of H$_2$O$_2$ reached maximum levels and increased by 33% compared with the control value. The results obtained in this study confirm that SA-mediated ARF has a close association with H$_2$O$_2$
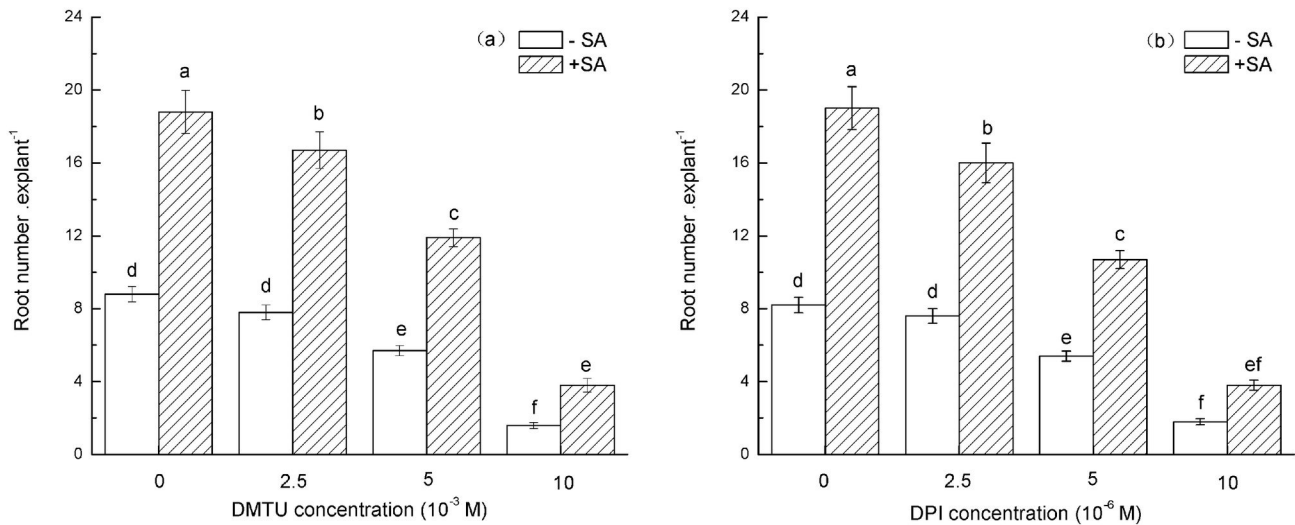
Figure 4. Impact of pre-treatment with DMTU or DPI on SA-induced ARF in mung bean hypocotyl cuttings. The primary roots were removed from seedlings of 5-day-old germinated mung beans, incubated in DMTU or DPI for 4 h, moved into 0.4 mM SA for 24 h, washed three times and cultivated in distilled water for another five days. The number of adventitious roots was quantified and is expressed as the mean from three independent experiments with 30 explants for each treatment. The different letters above the bars indicate significant differences among the treatments (P<0.05), according to the LSD test.
doi: 10.1371/journal.pone.0084580.g004

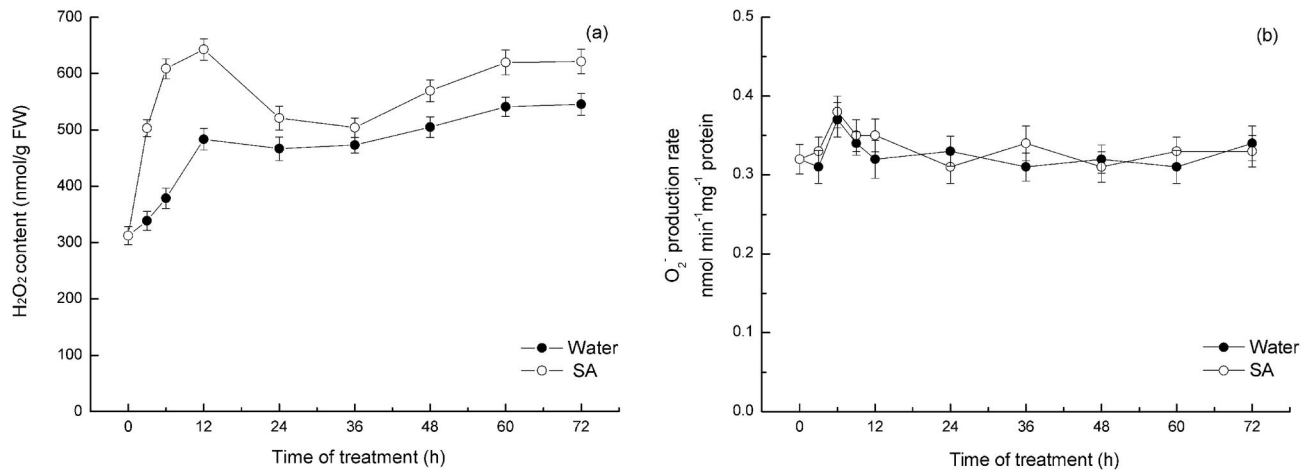**Figure 5. Time course of H_2O_2 accumulation (a) and O_2^{2-} production (b) in the hypocotyls of mung beans treated with water or SA.** Explants were incubated with SA or water for 24 h, and the H_2O_2 levels were monitored at the indicated time points. The mean values shown are the averages of three different experiments. The error bars represent the SE (n=5). The asterisks indicate that the mean values are significantly different compared with the control values (P<0.05). FW, fresh weight.
doi: 10.1371/journal.pone.0084580.g005

accumulation. However, the SA treatment did not affect the production of O_2^- throughout the treatment period (Figure 5b).

In this study, diaminobenzidine (DAB) uptake method has been adapted of *in-situ* detection of H_2O_2 [48]. The development of the DAB-H_2O_2 reaction product in hypocotyls in response to cutting was shown in Figure 6a. The color was more intense in SA incubated cuttings than water treated seeding, so did the stained area. Histochemical and cytochemical have been used for the detection of H_2O_2 generated in mung bean hypocotyls. H_2O_2 was visualized at the subcellular level using $CeCl_3$ for localization [49]. The greatest accumulation of H_2O_2 was observed in the cell wall and intercellular space in SA treated cutting after 12 h incubation, however, there is barely deposits of $CeCl_3$ in water-treated seedling (Figure 6b).
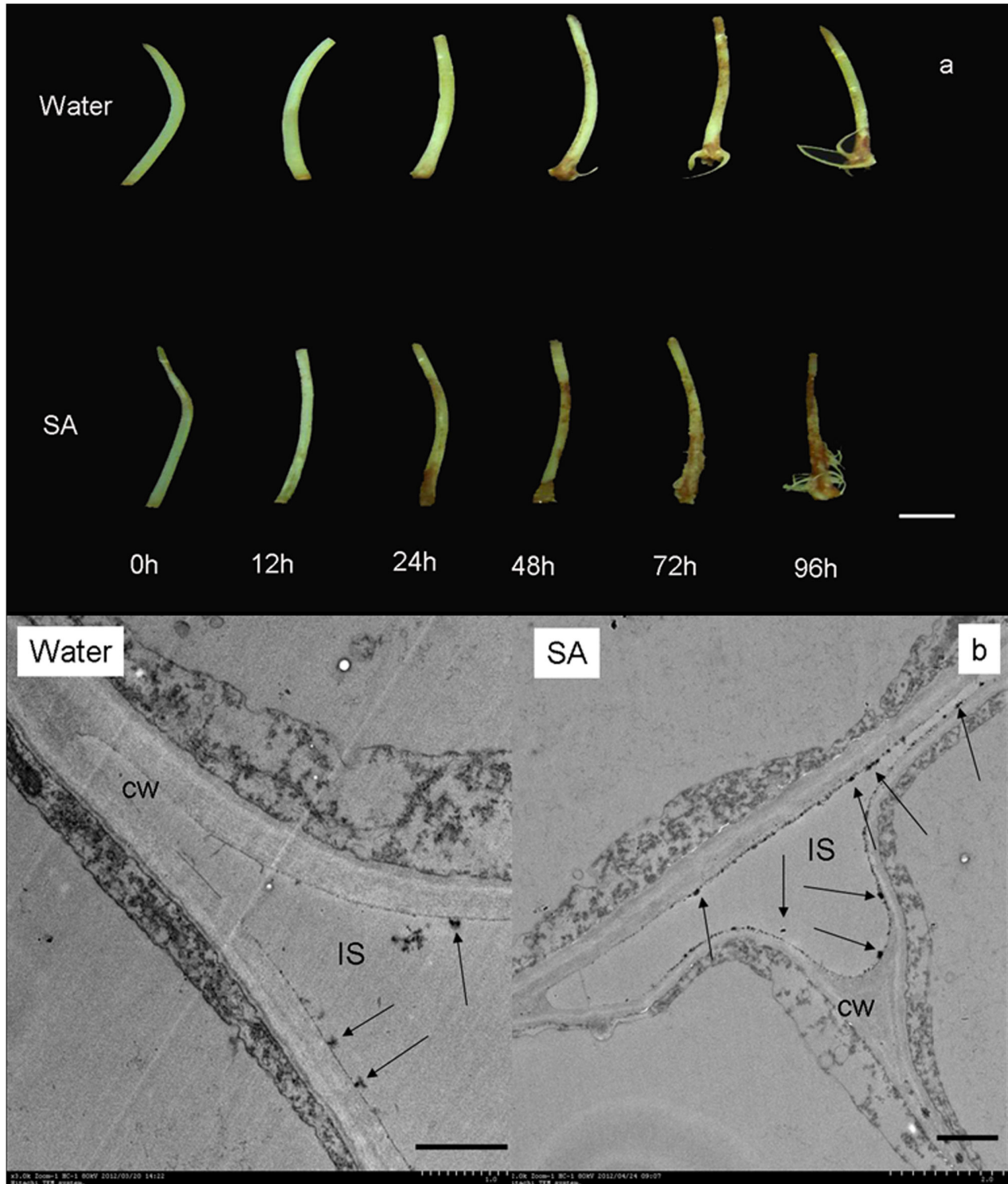
Figure 6. Histochemical and cytochemical detection of H$_2$O$_2$ accumulation induced by SA in mung bean hypocotyl cuttings. a Mung bean hypocotyl cuttings were incubated in SA for 24 h, and H$_2$O$_2$ levels were monitored at the indicated time points. All experiments were repeated at least three times with similar results. Bar=1 cm.
b Mung bean hypocotyl cuttings were incubated in SA for 12 h. Hypocotyls treated with distilled water under the same conditions served as controls. All experiments were repeated at least three times with similar results. Abbreviations: CW, cell wall; IS, intercellular space. Bar=1 μm.

Figure 7. Time course of changes in the activities of the antioxidant enzymes SOD (a), CAT (b), GR(c) and APX (d) in the hypocotyls of mung beans treated with water or SA. Explants were incubated with S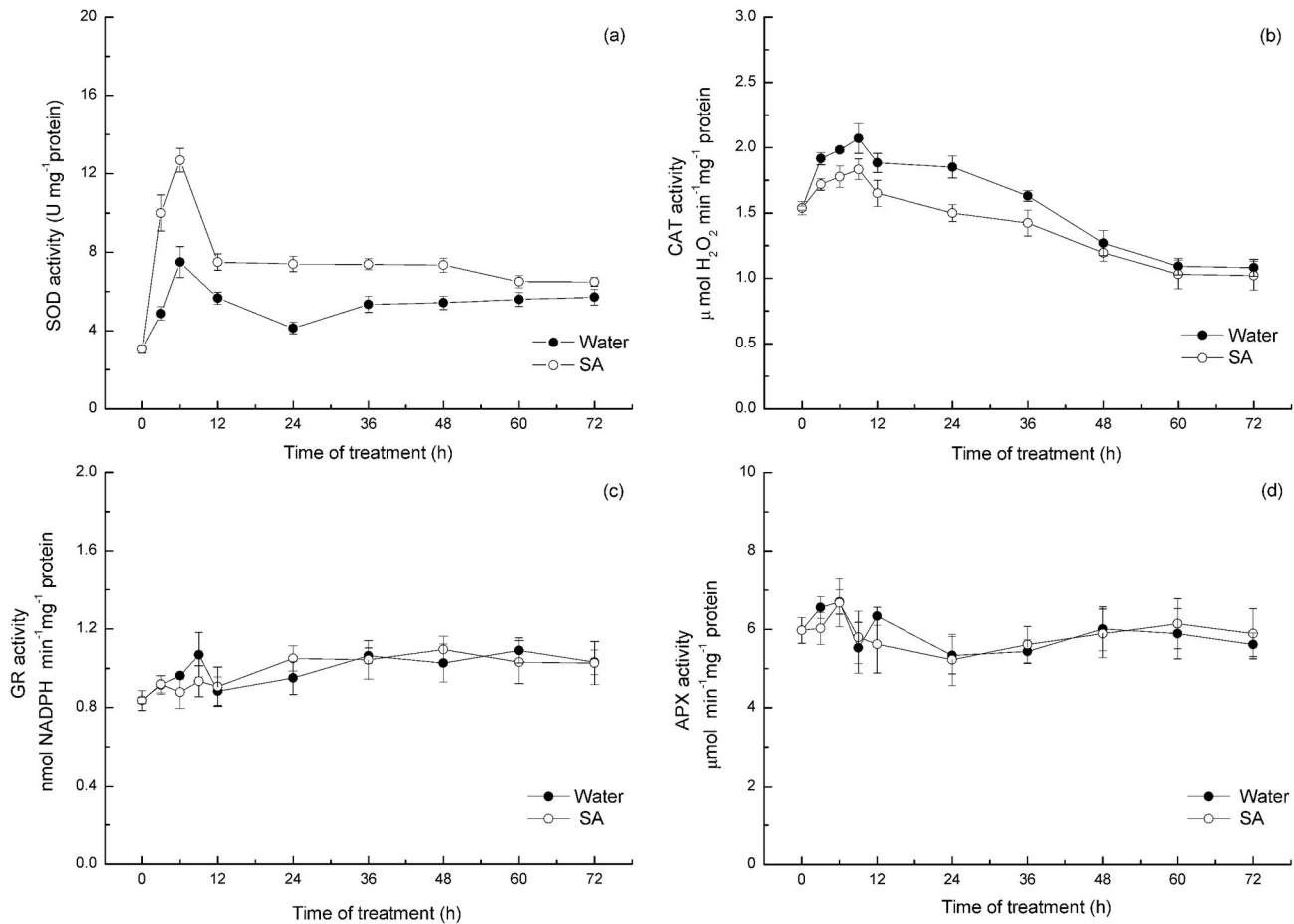A or water for 24 h, and the enzyme actives were monitored at the indicated time point. The mean values shown are the averages of three different experiments. The error bars represent the SE (n=5). Asterisks indicate that the mean values are significantly different compared with the control values (P<0.05). FW, fresh weight.

doi: 10.1371/journal.pone.0084580.g007

Influence of SA on NADPH oxidase activity and antioxidant enzymes

NADPH oxidase is a protein that transfers electrons from NADPH to an electron acceptor, which leads to the formation of reactive oxygen species. To study a possible link between SA and NADPH oxidase, the activity of NADPH oxidase was measured. Plasma membrane (PM) vesicles were isolated from roots, and the NADPH oxidase activity was determined by measuring O_2^- production. The results indicate that SA treatment did not affect NADPH activity (date not shown)

Altered ROS levels can result either from increased production or decreased scavenging. The activities of the antioxidant enzymes in the cuttings treated with water or SA were investigated to determine the source of H_2O_2 formation. As shown in Figure 7a, a significant enhancement in the activity of SOD occurred within the first 6 h of incubation with SA. The SA-induced SOD activity was enhanced by 105% compared with the control value after 3 h of incubation. After 6

h of incubation, the activity of SOD was increased by 69% compared with the control seedlings. It can be presumed that the accumulation of H_2O_2 observed with the SA treatment could be a result of SOD activity enhancement. Accordingly, the pretreatment with SA significantly repressed the activities of CAT in a time-dependent manner (Figure 7b). The CAT activities at 3 h and 6 h were decreased by 12% and 15%, respectively, compared with the control seedlings. After 12 h of treatment, the CAT activity was decreased by 17% compared with the control value. However, there was no significant change in APX and GR activities between the water and SA treatments (Figure 7c, 7d).

Discussion

AR at the base of plant cuttings is an innate de novo organogenesis process that allows the massive vegetative propagation of many economically and ecologically important

species [72]. It is necessary to understand the physiological and biochemical process of adventitious rooting. The discovery of signal molecules involved in the intricate network that triggers ARF remains a major goal for a large number of biotechnological procedures. Although a variety of plant components induce ARF and signal transduction has been identified, the molecular mechanism underlying meristem initiation is still undefined and remains to be proven.

SA is believed to be a key signaling molecular in SAR against pathogens and to play an important role in mediating plant responses to a variety of abiotic stresses. However, the effects of SA in root development are less known. A previous study demonstrated that SA might participate in root formation and development in plants. Some researchers observed that an aqueous solution of SA sprayed on the shoots of soybeans could significantly increase the growth of shoots and roots. In addition, SA induces increases in root growth of up to 100% in the field [73]. Singh [74] observed that SA stimulated root formation in the young shoots of ornamental plants. Salicylic acid and its derivatives are more closely related to these structural requirements. In faba bean (*vicia faba* L.), SA its chemical derivative (acetylsalicylic acid, ASA), at appropriate concentrations could increased rooting efficiency [75]. In Pb^{2+} stress seedlings, SA pretreatment also could significantly increase the length of shoots and roots and partially protect seedlings from Pb^{2+} toxicity [76]. Some researchers have observed that SA promotes later root initiation, emergence and growth, possibly via crosstalk with cytokinin or auxin [77]. However, only a few researchers have noted the role of SA in ARF and the intricate network involving SA and other molecules in this procedure. Our study indicates that SA alone is directly involved in the adventitious rooting process in mung bean hypocotyl cuttings. A dramatic increase of the AR number was observed in SA-treated mung bean hypocotyls cuttings compared with control seedlings (Figure 1a, Figure 2a). The treatments performed with different concentrations of SA confirmed that the effect was dose dependent, with an optimum concentration at 0.4 mM. Those results are consistent with the report of Kling and Riov, who advocated that application of SA increased AR number and yield in mung bean (*Vigna rediata* L.) [5,42]. In addition, SA induced adventitious rooting in a time-dependent manner (Figure 1b). Furthermore, we observed that SA promotes this process through the differentiation of cells to reestablish a new apical meristem (Figure 2b). Accordingly, we confirmed that SA is involved in the adventitious rooting process in mung bean hypocotyl cuttings.

H_2O_2 is a diffusible multifunctional molecule involved in numerous physiological processes in phylogenetically distant species. It is usually regarded as a cytotoxic molecule in cell metabolism. However, there is strong evidence that indicates that H_2O_2 is a useful second messenger in plant growth and development and that it mediates various physiological and biochemical processes, including SAR [78,79], stomata closure [80,81], programmed cell death (PCD)[82,83], wounding signaling[49], and root gravitropism [84]. Other researchers have suggested that H_2O_2 might possibly be an integral component of intracellular signaling transduction. Guan indicated that H_2O_2 plays an important intermediary role in the

ABA signal transduction pathway, which leads to the induction of the *Cat1* gene [85]. Pei noted that Ca^{2+}-channel activation by H_2O_2 is an important mechanism for ABA-induced stomatal closing [71]. H_2O_2 also regulates plant cell expansion through the activation of Ca^{2+} channels via a NADPH pathway [86]. In addition, there is also a considerable cross talk between H_2O_2 and NO transduction [87].

Increasing evidence supports the notion that H_2O_2 plays a causal role in lateral root development and adventitious root formation. Su and others [64] observed that H_2O_2 generated by polyamine oxidative degradation is involved in the development of later roots in soybean plants. Furthermore, Li reported that exogenous H_2O_2 is an essential component in ARF in mung bean and cucumber hypocotyls cuttings and acts as a second massager in IAA-induced ARF [57,58], yet the mechanisms of H_2O_2 in ARF remain elusive.

Early evidence indicates that SA and H_2O_2 are involved in the responses of plants to pathogens [88-90]. Although many works have studied the specific interaction between SA and H_2O_2 in plants, whether SA and H_2O_2 participate in ARF remains unclear. In this study, we observed that SA and H_2O_2 played a synergetic role in regulating ARF in mung bean hypocotyl cuttings (Figure 3). The treatment of hypocotyls with SA (SA 0.4 mM) plus H_2O_2 (10 mM) resulted in an increased response and an increased root number in comparison with hypocotyls treated with SA or H_2O_2 alone. Interestingly, a high level SA and H_2O_2 led to a significant suppress in the number of AR. This might be the exacerbation of oxidative stress, resulting in the inhibitory effect on ARF. Accordingly, we can speculate that SA and H_2O_2 have additive effect in adventitious rooting process. These results indicate that there is a potential interaction between SA and H_2O_2 as signaling molecules.

However, it is still unclear whether H_2O_2 involves signal transduction in relation to the development of SA-induced adventitious rooting. The development of adventitious roots are blocked in hypocotyls treated with DMTU or DPI (Figure 4), implying that endogenous H_2O_2 plays a pivotal role in adventitious rooting. These results suggest that DMTU and DPI achieve inhibition via a decrease in the H_2O_2 level during the adventitious rooting process. However, when DMTU was used as a trap for H_2O_2, the number of SA-induced ARF was significantly reduced, suggesting that SA requires H_2O_2 to initiate adventitious rooting. DPI, a specific inhibitor of membrane-linked NADPH oxidase, which is one of the main sources of H_2O_2 formation in plant cells, inhibited NADPH oxidase activity and also inhibited ARF in mung bean hypocotyls. Furthermore, the application of a specific concentration of SA alleviated the inhibitory effect of DPI (Figure 4b). The results suggest that H_2O_2 may function as a downstream signaling molecule or involved in a parallel pathway in the SA-induced formation and development of AR in mung bean seedlings.

In recent years, considerable evidence has accumulated suggesting that physiologically relevant concentrations of SA treatment can enhance H_2O_2 levels, and H_2O_2 has been proposed as being functionally downstream of SA in plants based on the evidence that SA can participate in regulating the antioxidant enzymes, such as catalase (CAT), superoxide

dismutase (SOD) and ascorbate peroxidase (APX) [61,78,91-94].. In adventitious rooting process, Tewari reported that SA could induce the hydrogen peroxide accumulation in *Panax ginseng* [95]. Furthermore, 40 day-old adventitious roots of *Panax ginseng* treated with 0.2 mM SA could cause an increase in the carbonyl and hydrogen peroxide contents [76]. In contrast, other studies report that SA accumulation could be induced by elevated H_2O_2 levels [97,98]. In addition, H_2O_2 activates SA biosynthesis via stimulation of BA2H (benzoic acid 2-hydroxylase) activity [97], which is also observed in tobacco cells. Furthermore, other studies have indicated that H_2O_2 does not function downstream of SA in regulating PR protein expression [99]. In view of the existing contradictory results derived from in vitro and in vivo studies, the method by which SA enhances H_2O_2 production in vivo and the relative significance of such SA-enhanced H_2O_2 remains unclear.

However, there is no information, as far as we know, regarding the effects of SA on H_2O_2 generated in the adventitious rooting process of mung bean. The interaction between exogenous SA and H_2O_2 on ARF in mung bean hypocotyl cuttings was further confirmed by the vivo H_2O_2 content determination. The present findings indicate that the enhanced endogenous H_2O_2 level was triggered by SA treatment in a time-dependent manner (Figure 5a). A peak H_2O_2 content was detected in mung bean hypocotyls incubated with SA for 12 h after the primary root was removed. These results indicate that the rapid increase in H_2O_2 content during the first 12 h of incubation could be attributed to the SA stimulus. Nag and others [14] demonstrated that in mung bean hypocotyls cuttings, the induction, initiation and expression phases are 0-24, 24-72 and after 72 h, respectively. We believe that the enhancement of H_2O_2 production by SA treatment is useful for cell division and root primordium in the induction phase. Nevertheless, hypocotyl cuttings treated with SA did not present differences in O_2^- content throughout the entire experimental period compared with the water-treated seedlings (Figure 5b). These data suggest that H_2O_2 is involved in SA-triggered adventitious rooting, but O_2^- is not. Histochemical and cytochemical method also support the result that SA could induce the H_2O_2 accumulation in mung bean hypocotyls. Taken together, these date confirm that H_2O_2, and not O_2^-, is the limiting factor in SA induced adventitious rooting in mung bean hypocotyls.

These results confirm a tight physiological link between SA and H_2O_2 in adventitious rooting process, but the sources H_2O_2 of remains to be clarified. In plants, a wide range of abiotic and biotic stresses result in H_2O_2 generation from a variety of sources. H_2O_2 is removed from cells via a number of antioxidant mechanisms, both enzymatic and nonenzymatic. To further determine the source of H_2O_2 enhancement by SA treatment, the activity of NADPH oxidase and the major antioxidant enzymes was also investigated in this paper.

Plasma membrane NADPH oxidase is composed of membrane-bound and cytosolic proteins [100,101]. NADPH oxidase is an enzyme that can use cytoplasmic NADPH to transfer an electron to molecular O_2 to form O_2^-, followed by dismutation of O_2^- to H_2O_2 [102,103]. It has been reported that SA treatment was most effective in increasing NADPH oxidase

activity in wheat seedlings [104]. To address this issue, we focused our attention on the hypothesis that adventitious root formation induction by SA signals may be involved in the NADPH oxidase pathway. In the end, we observed that there was no difference in the NADPH oxidase activity between the water and SA treatments (date not shown). So, we thought that SA-induced H_2O_2 accumulation had no relationship with the NADPH oxidase signal pathway.

To further investigate the status of the antioxidant defense system in SA-treatment seedlings, the activity of the major ROS-scavenging enzymes, SOD, CAT, APX and glutathione reductase (GR) were investigated. SOD converts highly reactive O_2^- to the more stable molecular-H_2O_2. Previous results reported by Rao demonstrated that SA could increase SOD activity in *Arabidopsis thaliana*, which led to increases in the H_2O_2 content [61]. In the current paper, higher SOD activity in SA-treated hypocotyls was observed to be time dependent. In addition, the highest SOD activity was observed at 6 h after incubation with SA, and SOD activity increased by 69% compared with the control seedlings (Figure 7a). The enhanced SOD activity in SA-treated hypocotyls might participate in the formation of H_2O_2. The existing relationship between SA and SOD activity clearly suggests that hypocotyls treated with SA may have enhanced H_2O_2 largely due to the activation of enzymes capable of generating H_2O_2. H_2O_2 can be removed by CAT or by the APX of the ascorbate-glutathione antioxidant cycle [105]. Previous results indicate that SA inhibits CAT activity and thereby causes an increase in H_2O_2 levels [32,78,106]. In the present study, lower CAT activity was detected in SA-treated hypocotyls compared with the water-treated hypocotyls (Figure 7b). These results are in agreement with the hypothesis that SA enhances H_2O_2 levels by inactivating the enzymes that are capable of degrading H_2O_2. It can be concluded that the accumulation of H_2O_2 observed in SA-treated hypocotyls also could be the result of the inhibition of CAT activity. High GR activity maintains the pool of glutathione in the reduced state, allowing GSH to be used by DHAR to reduce DHA to AA [107]. Nevertheless, there was no difference in APX and GR activity between the SA and water-treated cuttings (Figure 7c, 7d). Therefore, APX and GR activity were not the cause of H_2O_2 accumulation in the present study. It can be concluded that the accumulation of H_2O_2 observed with SA treatment mainly resulted from the enhancement of SOD activity and the inhibition of CAT activity. This mechanism may explain the increase of in vivo H_2O_2 content at 12 h after SA incubation. Comparisons in Figure 6 indicate that SA-enhanced H_2O_2 levels were related to the increased activities of SOD and were dependent on CAT changes, but that was not the case with GR activity and APX activity. Thus, the conversion of O_2^- by SOD does appear to be the main reason for altered H_2O_2 levels.

In conclusion, this study contributes to defining a distinctive role for SA during the adventitious rooting process in mung bean hypocotyls. In addition, hypocotyls treated with SA presented a transient elevation in H_2O_2 content. Furthermore, the results of this study indicate that SA enhanced H_2O_2 via changes to the status of the antioxidant system. Based on this evidence, we suggest that H_2O_2 could be involved in the

signaling pathway transduction of SA that triggered ARF in the examined mung bean hypocotyls. However, the underlying mechanism by which SA and H_2O_2 induced adventitious root formation requires further investigation.

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Conceived and designed the experiments: WY KX DN. Performed the experiments: WY LG XM. Analyzed the data: WY CZ GL. Contributed reagents/materials/analysis tools: XM GL DN. Wrote the manuscript: WY.

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