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ORIGINAL PAPER

Heme oxygenase is involved in H_2O_2-induced lateral root formation in apocynin-treated rice

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Abstract
Key message **Apocynin is a natural organic compound structurally related to vanillin. We demonstrated that hydrogen peroxide and heme oxygenase participated in apocynin-induced lateral root formation in rice.**
Abstract Apocynin, also known as acetovanillone, is a natural organic compound structurally related to vanillin. Information concerning the effect of apocynin on plants is limited. In this study, we examined the effect of apocynin on lateral root (LR) formation in rice. Treatment with apocynin induced LR formation and increased H_2O_2 production, but had no effect on nitric oxide production. Diphenyleneiodonium chloride, an inhibitor of H_2O_2 generating NADPH oxidase, was effective in reducing apocynin-induced H_2O_2 production and LR formation. Apocynin treatment also increased superoxide dismutase activity and decreased catalase activity. H_2O_2 application was able to increase the number of LRs. Moreover, H_2O_2 production caused by H_2O_2 and apocynin was localized in the root area corresponding to the LR emergence. Treatment with H_2O_2 and apocynin also increased heme oxygenase (HO) activity and induced *OsHO1* mRNA expression. Lateral root formation and HO activity induced by H_2O_2 and apocynin were reduced by Zn protoporphyrin IX (the specific inhibitor of HO). Our data suggest that both H_2O_2 and HO are required for apocynin-induced LR formation in rice.

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Introduction

Hydrogen peroxide is a constituent of oxidative metabolism and is itself a reactive oxygen species (ROS). The accumulation of H_2O_2 increases the probability of hydroxyl radical formation via a Fenton-type reaction. This leads to the phenomenon known as oxidative stress (Foyer and Noctor 2000). Initially, H_2O_2 was only considered damaging to cells (Gechev et al. 2006). More recently, H_2O_2 emerged as ubiquitous signaling molecule (Gechev et al. 2006). H_2O_2 is involved in many developmental and physiological processes (Gapper and Dolan 2006; Kwak et al. 2006). It acts as a signal molecule in the formation of adventitious roots, lateral roots (LRs), and root hairs (Su et al. 2006; Dunand et al. 2007; Li et al. 2007, 2009a, b; Huang et al. 2011). Nitric oxide (NO) is now emerging as an important signaling molecule in many important physiological processes in plants (Lamattina et al. 2003; Besson-Bard et al. 2008). There is increasing evidence indicating that NO also plays a critical role in root development such as LR formation (Correa-Aragunde et al. 2004; Guo et al. 2008; Chen and Kao 2012).

Heme oxygenase (HO) is a ubiquitous and highly active enzyme, which catalyzes the degradation of heme to produce carbon monoxide (CO), free iron, and biliverdin IXα (BV) (Kicuchi et al. 2005). HO is a small family with several members. It has been found that HO1 is clearly the one most highly expressed, followed by HO2, with both HO3 and HO4 expressed at low levels (Matsumoto et al. 2004). In plants, HO1 has been shown to be associated with LR formation (Cao et al. 2007; Guo et al. 2008; Chen et al.

2012; Han et al. 2012). The expression of HO1 has been shown to be induced by H_2O_2 (Yannarelli et al. 2006; Chen et al. 2009) and NO (Noriega et al. 2007).

Apocynin (4-hydroxy-3-methoxyacetophenone, acetovanillone) is a compound originally isolated from the medicinal plant *Picrorhiza kurroa*, a small perennial herb that grows in the Himalayas. It not only acts as an inhibitor of phagocyte NADPH oxidase but also as a ROS production stimulator in non-phagocytic cells (Vejrazka et al. 2005). Riganti et al. (2008) demonstrated that apocynin induces NO synthesis in N11 mouse cells. In maize leaves, apocynin increased NO production (Tossi et al. 2009). This is the only work showing that apocynin increases NO production in plants. To date, it is not known whether apocynin increases H_2O_2 in plants. Neither do we know whether apocynin promotes LR formation.

In this work, we first examined the effect of apocynin on the formation of LRs and production of NO and H_2O_2 in rice. In our recent study, we demonstrated that HO is involved in LR formation in the rice root system (Chen et al. 2012). Thus, the possible role of H_2O_2, NO, and HO in regulating apocynin-induced LR formation in rice is also examined.

Materials and methods

Plant material and growth conditions

Seeds of rice (*Oryza sativa* L., cv. Taichung Native 1, an Indica type) were sterilized with 3 % sodium hypochlorite for 15 min and washed extensively with distilled water. To obtain more uniformly germinated seeds, rice seeds in a Petri dish (20 cm) containing distilled water were pretreated at 37 °C for 1 day under dark conditions. Uniformly germinated seeds were then selected and transferred to a Petri dish (20 cm) containing two sheets of filter paper moistened with distilled water for 2 days. Two-day-old seedlings were then transferred to Petri dishes (9 cm) containing distilled water, apocynin, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), diphenyleneiodonium chloride (DPI), zinc protoporphyrin IX (ZnPPIX), at the desired concentration. Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts; thus, seedlings grown in distilled water were used as the control. Each Petri dish contained five seedlings and each treatment was replicated four times. The seedlings were allowed to grow at 27 °C in darkness. The seminal roots of rice seedlings at the times specified were used for the analysis of LR formation, HO activity, and *OsHO1* transcripts.

LR formation

To show LR formation in seminal roots for each treatment, the number of LRs longer than 1 mm per seedling was counted. For some experiments, photographs of seedlings were taken. Representative photographs of seedlings were shown in figures.

Detection of endogenous NO and H_2O_2

The NO and H_2O_2 were detected by fluorescence microscopy. Nitric oxide and H_2O_2 imaging were conducted according to Xiong et al. (2009) and Shin and Schachtman (2004), respectively. For NO detection, rice roots were incubated in 20 μM of the cell-permeable fluorescence probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) in 20 mM HEPES (pH 7.5) for 15 min, whereas for H_2O_2 detection, rice roots were incubated in 50 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, dissolved in 0.0025 % dimethyl sulfoxide) in the dark for 30 min. A Nikon SMA 1500 stereoscopic fluorescence microscope was used for fluorescence image. Fluorescence was visualized by excitation and extinction at 495 and 515 nm, respectively.

For the in situ histochemical detection of H_2O_2, root segments stained with CM-H_2DCFDA were cut into approximately 1-cm segments. The segments were embedded in 5 % agar and then longitudinally cut into section of 30–50 μm using vibrating microslicer (DTK-1000; Dosaka EM Co. Ltd., Kyoto, Japan). The longitudinal sections were observed with an Axio Scope A1 fluorescence microscope, following the manufacturer's instructions (Carl Zeiss, Jena, Germany). Axio Scope A1 images were acquired with an AxioCam camera and processed with Axiovision software.

Enzyme extraction and assays

For the extraction of superoxide dismutase (SOD) and catalase (CAT), ten roots were homogenized with 100 mM sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000×*g*. SOD activity was determined according to Paoletti et al. (1986). The reaction mixture (2.73 mL) contained 100 mM triethanolamine–diethanolamine buffer (pH 7.4), 7.5 mM NADH, EDTA/$MnCl_2$ (100/50 mM, pH 7.4), 10 mM of 2-mercaptoethanol, and enzyme extract (0.2 mL). The reaction was started by adding NADH. The reaction was allowed to proceed for 10 min. The absorbance was measured at 340 nm. One unit of SOD was defined as the amount of enzyme that inhibits by 50 % the rate of NADH oxidation observed in blank sample. CAT activity was

assayed according to Kato and Shimizu (1987). The decrease in H_2O_2 was defined as the decline in the absorbance at 240 nm, and the activity was calculated using extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2. One unit of CAT was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per min.

HO activity was analyzed basically as described (Xuan et al. 2008). For extraction of HO, 25 roots were homogenized with 3 mL of 25 mM HEPES–Tris (pH 7.4) containing 250 mM mannitol, 1 mM EDTA, 1 % (w/v) polyvinylpyrrolidone, 10 % (v/v) glycerol, and 1 mM dithiothreitol. The whole isolation procedure was carried out at 4 °C. The homogenate was centrifuged at 15,000×*g* for 30 min, and the resulting supernatant was used for determining the activity of HO as described (Xuan et al. 2008). The reaction mixture (1 mL) contained 2 mM deferrioxamine in 100 mM HEPES–NaOH (pH 7.2), 100 μM NADPH, 10 μM Hm, 0.15 mg mL^{-1} bovine serum albumin, 50 μg mL^{-1} (4.2 μM) spinach ferredoxin, 0.025 units mL^{-1} spinach ferredoxin-$NADP^+$ reductase, 5 mM ascorbate and enzyme extract (250 μL). The reaction was started by adding NADPH and allowed to proceed at 37 °C for 30 min. The absorbance of BV was measured at 650 nm. The increase in BV concentration was determined by the extinction coefficient 6.25 mM^{-1} cm^{-1} at 650 nm. One unit of activity for HO was defined as the amount of enzyme that produced 1 μmol of BV per 30 min. Rice roots contained very low protein. Thus, SOD, CAT, and HO activities were expressed on a dry weight (DW) basis.

Semi-quantitative RT-PCR

Total RNA was isolated from the roots of seedlings by the TRIzol reagent method (Invitrogen, CA, USA). To prevent DNA contamination, RNA was treated with Turbo DNase I (Ambion, TX, USA) for 30 min at 37 °C before performing RT-PCR. Control PCR amplifications involved RNA as a template after DNase I treatment to verify the elimination of contaminated DNA. Reverse-transcription reactions involved 200 ng of total RNA and the SuperScript III first-strand synthesis RT-PCR system (Invitrogen, CA, USA).

We searched the rice genome annotation project (http://rice.plantbiology.msu.edu/) for the sequence of rice heme oxygenase 1 (*OsHO1*, LOC_Os06g40080) with Arabidopsis *HY1* (Davis et al. 1999) (*AtHO1*) used as a reference. Gene-specific primers were designed from the 5′ UTR of the rice *OsHO1* gene. The sequences used and the predicted amplicon are shown in Table 1. The RT-PCR conditions were 94 °C denaturation for 5 min, then 27–30 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s, 72 °C extension for 5 min, and finally 16 °C. PCR was optimized for a number of cycles to insure product intensity within the linear phase of amplification. For all treatments, RT-PCR was performed three times with three batches of total RNA isolated independently. PCR products were resolved by electrophoresis in 3 % agarose gel and stained with ethidium bromide. The gel images were captured with the use of a SynGene gel documentation system and analyzed by Genetools (Syngene, MD, USA). The rice *OsUbiquitin* gene was used for normalization.

Statistical analysis

Data were analyzed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Effect of apocynin on the formation of LRs

To examine the effect of apocynin on LR formation, 2-day-old seedlings were treated with various concentrations of apocynin for 3 days. In comparison with water treatment, apocynin (1–30 μM) was effective in inducing LR formation (Fig. 1). Apocynin at 10 μM was proved to be an optimal concentration for the promotion of LR formation.

Hydrogen peroxide but no NO is involved in apocynin-induced LR formation

To examine whether apocynin-induced LR formation was the result of the production of H_2O_2 or NO, 1 μM diphenyleneiodonium chloride (DPI, a NADPH oxidase inhibitor) or 100 μM cPTIO (a NO-specific scavenger), was applied along with 10 μM apocynin. The effect of

Table 1 Primers used in semi-quantitative RT-PCR assay

Gene	TIGR locus name	Primer	Sequence (5′–3′)	Products (bp)
OsHO1	LOC_Os06g40080	HO1-5′	CATTCCAATCCACTCCCACCA	209
		HO1-3′	AAG GTG CTC GAC GAT GGCGAC	
OsUbiquitin	LOC_Os03g13170.1	Ubi-5′	CGCAAGTACAACCAGGACAA	101
		Ubi-3′	TGGTTGCTGTGACCACACTT	

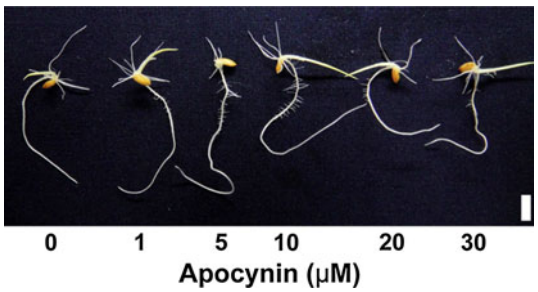

Fig. 1 Effect of apocynin on LR formation in rice. Two-day-old rice seedlings were treated with apocynin (1–30 μM) for 3 days. Experiments were repeated four times with similar results. Representative photograph of rice seedlings were shown. *Bar* 1 cm

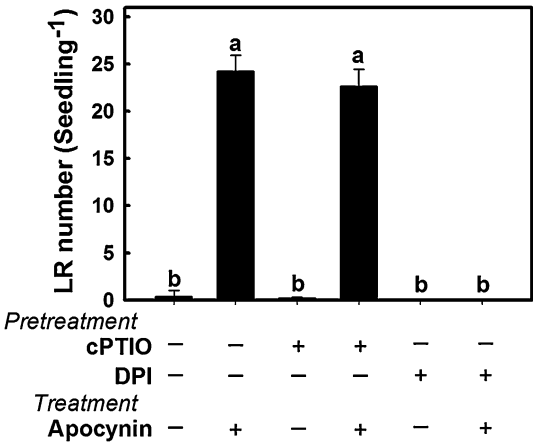

Fig. 2 Effect of cPTIO and DPI on LR number of rice seedlings treated with apocynin. Two-day-old rice seedlings were pretreated with 100 μM cPTIO or 1 μM DPI for 3 h and then treated with 10 μM apocynin, respectively for 3 days. *Bars* indicates standard errors ($n = 20$). *Values* with the *same letter* are not significantly different at $P < 0.05$

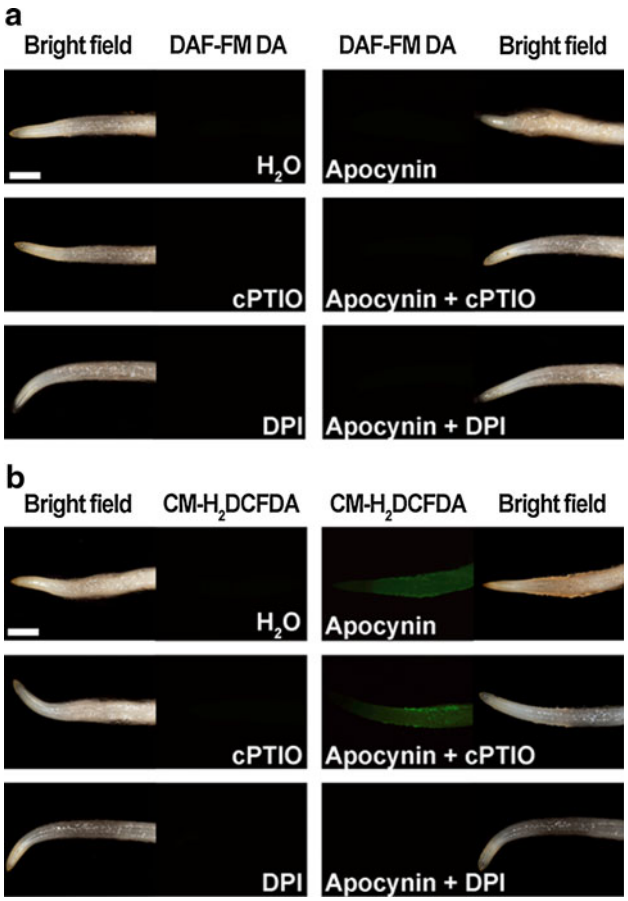

Fig. 3 Effect of cPTIO and DPI on NO production (**a**) and H_2O_2 production (**b**) in rice seedlings treated with apocynin. Two-day-old rice seedlings were treated H_2O, 100 μM cPTIO, 1 μM DPI, 10 μM apocynin, 10 μM apocynin + 100 μM cPTIO, 10 μM apocynin + 1 μM DPI for 24 h. Experiments were repeated four times with similar results. Representative photograph of rice seedlings were shown. *Bars* 1 mm

apocynin on LR formation was significantly inhibited by DPI but not by cPTIO (Fig. 2). Clearly, the effect of apocynin may be attributed to H_2O_2 produced.

In vivo detection of H_2O_2 or NO in rice seminal roots was carried out using fluorescence probes. CM-H_2DCFDA and DAF-FM DA were used to follow H_2O_2 and NO production, respectively. As shown in Fig. 3a, apocynin did not induce green fluorescence of NO. However, apocynin induced green fluorescence of H_2O_2 (Fig. 3b). Interestingly, when DPI at 1 μM was added along with 10 μM apocynin, the apocynin-induced H_2O_2 fluorescence was completely suppressed (Fig. 3b).

Effect of apocynin on SOD and CAT activities

To examine the effect of apocynin on SOD and CAT activities, 2-day-old rice seedlings were treated with 10 μM apocynin for 24 h. As compared with water treatment, apocynin was effective in increasing SOD activity (Fig. 4a) and decreasing CAT activity (Fig. 4b).

Effect of exogenous H_2O_2 on LR formation

To examine the effect of H_2O_2 on LR formation, 2-day-old seedlings were treated with various concentrations of H_2O_2 for 3 days. In comparison with water treatment, H_2O_2 (1–2.5 μM) was effective in inducing LR formation (Fig. 4). Hydrogen peroxide at 1 μM was proved to be an optimal concentration for inducing LR formation.

Localization of H_2O_2

In the longitudinal section examination of seminal roots, H_2O_2 production in H_2O_2- and apocynin-treated roots was observed to be localized in the root area corresponding to the LR emergence (Fig. 6).

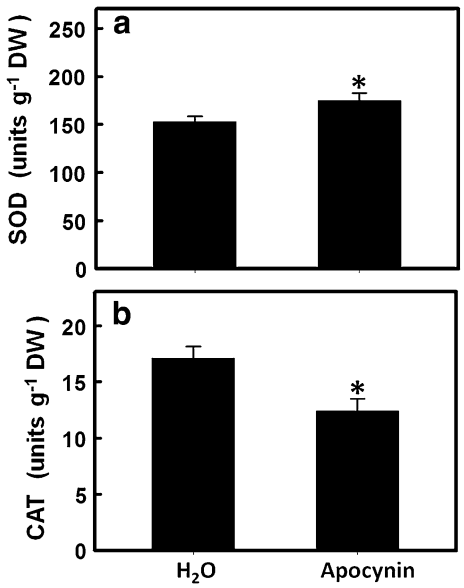

Fig. 4 Effect of apocynin on the activities of SOD (**a**) and CAT (**b**) in rice. Two-day-old rice seedlings were treated with 10 μM apocynin for 24 h. *Bar* indicates standard errors ($n = 4$). *Asterisks* represent values that are significantly different between H_2O and apocynin treatments at $P < 0.05$

Effect of apocynin and H_2O_2 on LR formation and HO activity

To examine whether zinc protoporphyrin IX (ZnPPIX) affects apocynin- and H_2O_2-promoted LR formation and HO activity, 2-day-old rice seedlings were treated with apocynin and H_2O_2 for 1 or 3 days. As compared with water treatment, apocynin and H_2O_2 were effective in inducing LR formation (Fig. 7a) and increasing HO activity (Fig. 7b). It has been shown that ZnPPIX (a potent HO1 inhibitor) inhibits HO activity in plants (Liu et al. 2007; Xuan et al. 2008). Application of ZnPPIX alone had no effect on LR formation and HO activity (Fig. 7a, b). However, pretreatment with 200 μM of ZnPPIX inhibited apocynin- and H_2O_2-increased LR formation and HO activity (Fig. 7a, b).

Apocynin and H_2O_2 increase *OsHO1* mRNA expression

To examine whether apocynin and H_2O_2 affect the expression of *OsHO1*, 2-day-old seedlings were treated with apocynin and H_2O_2 for 24 h, which increased the mRNA level of *OsHO1* (Fig. 8).

Discussion

Lateral roots play important roles in increasing the absorptive capacity of roots to absorb water and mineral nutrients as well as to anchor the plants in the soil (Hao and Ichii 1999; López-Bucio et al. 2003; Wang et al. 2006). Recently, Den Herder et al. (2010) suggest that root architecture, including LR, could be considered as one of the promising features of crops in a new green revolution. Information regarding the effect of apocynin on plants is scarce. It has been shown that apocynin confers antioxidant protection in maize leaves (Tossi et al. 2009). However, the effect of apocynin on LR formation remains largely unknown. In the present study, we observed that apocynin promoted LR formation in rice (Fig. 1). Moreover, we found that HO plays a role in H_2O_2 signaling leading to LR formation in apocynin-treated rice.

In animal system, apocynin induces NO synthesis (Riganti et al. 2008). Tossi et al. (2009) also demonstrated that apocynin increases NO production in maize leaves. In contrast, we have shown that, in rice roots, apocynin is unable to induce NO production (Fig. 3a).

Apocynin is often used as a specific inhibitor of NADPH oxidase (Riganti et al. 2006). However, Vejrazka et al. (2005) found that apocynin not only acts as an inhibitor of phagocyte NADPH oxidase but also as H_2O_2 production stimulator in non-phagocytic cells. In the present study, the in vivo detection of H_2O_2 in rice roots was carried out using a fluorescence probe, CM-H_2DCFDA (Fig. 3b). The probe specificity has been checked using ascorbic acid, an H_2O_2 scavenger (data not shown). Interestingly, we observed that apocynin treatment resulted in an accumulation of H_2O_2-dependent CM-H_2DCFDA fluorescence in rice roots (Fig. 3b).

In several model systems investigated in plants, the accumulation of H_2O_2 appears to be mediated by the activation of a plasma membrane-bound NADPH oxidase complex (Pei et al. 2000; Orozco-Cardenas et al. 2001; Zhang et al. 2001). DPI is known to inhibit plasma membrane NADPH oxidase (Orozco-Cardenas et al. 2001). DPI (1 μM) can prevent the increased production of H_2O_2 (Fig. 2b) in seminal roots of rice induced by apocynin (Fig. 2b). These data strongly suggest apocynin-induced accumulation of H_2O_2 was mediated, at least in part, through the activation of NADPH oxidase. However, apocynin has been shown to be an inhibitor of phagocyte NADPH oxidase (Vejrazka et al. 2005). It is not known whether apocynin induces H_2O_2 production in other plant species. In this regard, further work is thus required.

The increase in SOD activity and the decrease in CAT activity could result in H_2O_2 accumulation. We demonstrated that apocynin enhanced SOD activity and deactivated CAT activity in rice roots (Fig. 4a, b). Thus, apocynin-induced H_2O_2 accumulation is also mediated through the increase in SOD activity and the decrease in CAT activity in rice roots. These results suggest that apocynin may exert other effects besides its ability to

induce NADPH oxidase, for instance, it increases SOD activity and decreases CAT activity.

The involvement of H_2O_2 in the formation of adventitious roots, LRs, and root hairs has been reported (Su et al. 2006; Dunand et al. 2007; Li et al. 2007, 2009a, b; Huang et al. 2011). Our data demonstrate that (a) H_2O_2 per se is able to induce LR formation (Fig. 5) and increase endogenous H_2O_2 level (Lin and Kao 2001) in rice seminal roots, (b) apocynin-promoted LR formation (Fig. 1) and H_2O_2 production (Fig. 3b) could be blocked by DPI (Figs. 2, 3), (c) H_2O_2 production occurred 24 h after apocynin treatment, whereas LR primordia were observed 42 h after apocynin treatment, indicating apocynin-caused H_2O_2 production is prior to apocynin-promoted LR formation, and (d) apocynin- and H_2O_2-induced H_2O_2 production were localized in root area corresponding to the emergence of LRs (Fig. 6). Thus, this evidence supports the suggestion that H_2O_2 mediates apocynin-induced LR formation.

Our recent results revealed that HO is involved in NO and auxin-induced LR formation in rice (Chen et al. 2012). We found that apocynin and H_2O_2, which increased HO activity (Fig. 7b) and *OsHO1* mRNA level (Fig. 8), could induce LR formation in rice roots (Figs. 1, 5, 6). In addition, apocynin and H_2O_2-promoted LR formation and HO activity could be blocked by ZnPPIX, a potent HO inhibitor (Fig. 7a, b). Collectively, these data indicated that HO might be involved in apocynin- and H_2O_2-promoted LR formation in rice roots. More recently, similar function of HO1 from maize in the induction of LR formation was also reported (Han et al. 2012). It is known that CO and BV are generated by HO. It has been shown that CO could mimic or mediate the effect of auxin on the promotion of LR in rapeseed and tomato seedlings (Cao et al. 2007; Guo et al.

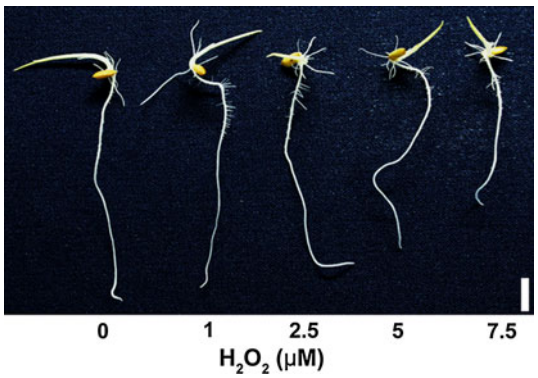

Fig. 5 Effect of H_2O_2 on LR formation in rice. Two-day-old rice seedlings were treated with H_2O_2 (0–7.5 μM) for 3 days. Experiments were repeated four times with similar results. Representative photograph of rice seedlings were shown. *Bar* 1 cm

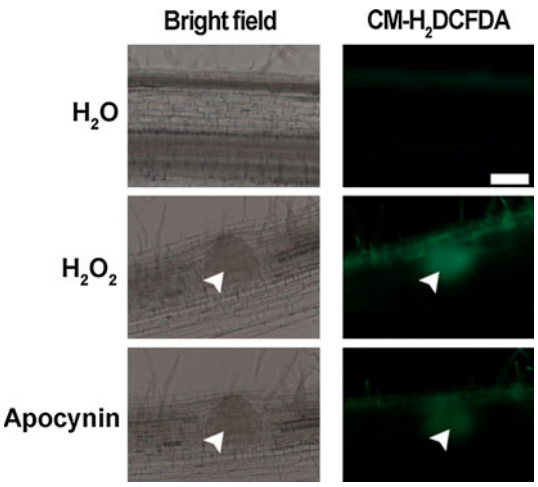

Fig. 6 H_2O_2 localization in H_2O-, H_2O_2-, and apocynin-treated rice roots. Two-day-old seedlings wee treated with H_2O, H_2O_2, or apocynin for 42 h. The concentration of H_2O_2 and apocynin were 1 μM and 10 μM, respectively. *Arrowheads* indicate LR primordia. Experiments were repeated four times with similar results. Representative photographs of rice seminal roots were shown. *Bar* 0.2 mm

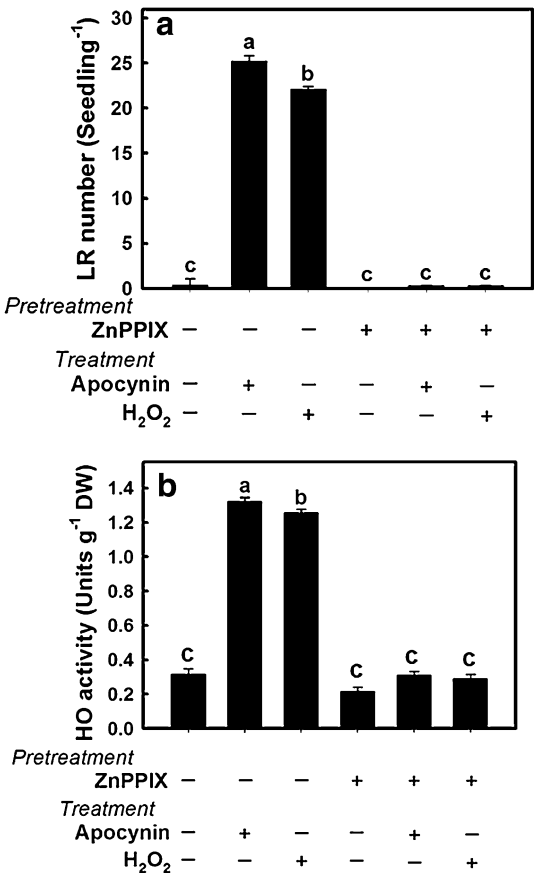

Fig. 7 Effects of ZnPPIX on H_2O_2- and apocynin-increased LR number (**a**) and HO activity (**b**) in rice. Two-day-old seedlings were pretreated with or without ZnPPIX for 3 h and then treated with H_2O, H_2O_2, or apocynin, respectively, for 1 day (**b**) or 3 days (**a**). The concentrations of H_2O_2, apocynin, and ZnPPIX were 1, 10, and 200 μM, respectively. *Bar* indicates standard errors ($n = 4$). *Values* with the *same letter* are not significantly different at $P < 0.05$

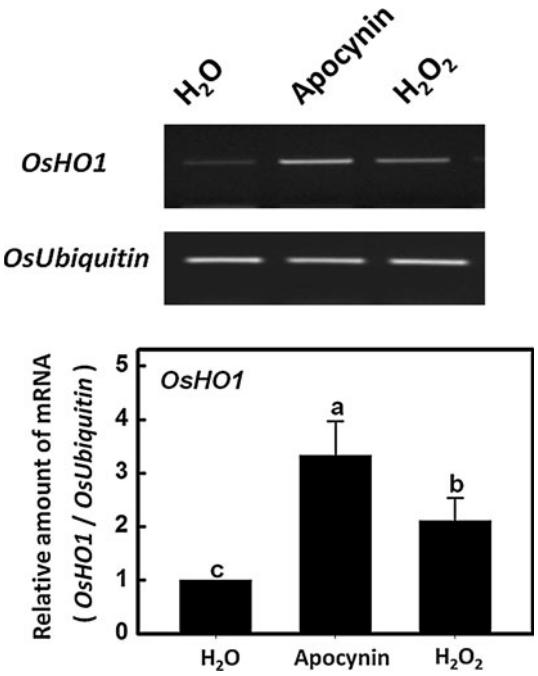

Fig. 8 Effect of H_2O_2 and apocynin on *OsHO1* mRNA level in rice roots. Two-day-old seedlings were treated with H_2O, 1 μM H_2O_2, or 10 μM apocynin for 1 day. Semi-quantitative RT-PCR analysis of mRNA levels relative to that of *OsUbiquitin*. *Bar* indicates standard errors ($n = 3$). *Values* with the *same letter* are not significantly different at $P < 0.05$

2008). Our previous results revealed that HO is involved in NO- and auxin-induced LR formation in rice (Chen et al. 2012).We also provided indirect evidence to show that CO increases HO activity in rice (Chen et al. 2012). More recently, we reported that HO is required for BV-induced LR formation in rice (Hsu et al. 2012a). It is interesting and necessary in the future to investigate the specific role of HO1/CO and HO1/BV in the apocynin-induced LR formation process.

Jasmonic acid and methyl jasmonate are a class of plant hormones, which mediate various aspects of developmental and stress response (Wasternack 2007). Recently, we have shown that HO is required for methyl jasmonate-induced LR formation in rice (Hsu et al. 2012b). Data in Figs. 7 and 8 suggest that H_2O_2 is not as effective as apocynin in promoting the LR formation, enhancing the HO activity and inducing the HO1 transcription. Therefore, the effect of apocynin on the LR formation could also be mediated by some other pathway unrelated to H_2O_2, such as methyl jasmonate.

In summary, this is the first study investigating the effect of apocynin on LR formation in the rice root systems. Our data strongly support that H_2O_2 is responsible for apocynin-promoted LR formation and HO possesses a central role in determining apocynin- and H_2O_2-induced LR formation in rice. Apocynin has also been shown to confer antioxidant protection in maize leaves (Tossi et al. 2009). It is not known whether apocynin could protect against oxidative stress in rice seedlings, so further work in this direction seems warranted.

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