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Heme oxygenase is involved in cobalt chloride-induced lateral root development in tomato

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Abstract In animals, heme oxygenase (HO), a rate-limiting enzyme responsible for carbon monoxide (CO) production, was regarded as a protective system maintaining cellular homeostasis. It was also established that metal ions are powerful HO-inducing agents and cobalt chloride ($CoCl_2$) was the first metal ion identified with an inducing property. Previous study suggests that $CoCl_2$ stimulates adventitious root formation in tomato and cucumber cuttings. In this test, we discover that both $CoCl_2$ and an inducer of HO-1, hemin, could lead to the promotion of lateral root development, as well as the induction of HO-1 protein expression, HO activity, or *LeHO-1/2* transcripts, in lateral root initiation zone of tomato seedlings. The effect is specific for HO since the potent HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX) blocked the above actions of $CoCl_2$, and the inhibitory effect was reversed partially when 50% CO aqueous solution was added. However, the addition of ascorbic acid (AsA), a well-known antioxidant, exhibited no obvious effect on lateral root formation. Molecular evidence further showed that $CoCl_2$-induced the up-regulation of target genes responsible for lateral root formation, including *LeCDKA1*, *LeCYCA2;1*, and *LeCYCA3;1*, was suppressed differentially by ZnPPIX. And these decreases were reversed further by the addition of CO. All together, these results suggest a novel role for HO in the $CoCl_2$-induced tomato lateral root formation.

Keywords Cell cycle regulatory gene · Cobalt chloride · Heme oxygenase · Lateral root development · *Lycopersicon esculentum*

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Introduction

Lateral root formation, which originates exclusively from pericycle founder cells located opposite xylem poles, is a major determinant of root systems architecture. Meanwhile, the density of lateral root (LR) development also performs the essential tasks of providing water, nutrients and physical support to plants. Therefore, understanding the regulation of lateral root development is of vital agronomic importance. It was well known that lateral root formation depends on both genetic determinants and postembryonic developmental processes that are mainly under the influence of plant hormone auxin and environmental factors, including water and nutrient availability (Casimiro et al. 2001; Malamy 2005). Mutations that render plants less sensitive to auxin reduce lateral root numbers. Meanwhile, other

plant hormones brassinosteroids and abscisic acid (ABA), and some signaling molecules such as nitric oxide (NO) have all been confirmed to affect lateral root formation via auxin, either by modulating its transport or signaling (Brady et al. 2003; Bao et al. 2004; Correa-Aragunde et al. 2004).

It is clear that activation of the cell cycle must accompany lateral root initiation. Normally, auxin induces lateral root initiation by modulating the expression of cell cycle regulatory genes such as cyclins and Cyclin Dependent Kinases (CDK) in the pericycle cells (Casimiro et al. 2003). In higher eukaryotes, activation of a CDKA/cyclin D complex at the G1-to-S transition leads to hyperphosphorylation of the transcriptional repressor retinoblastoma (Rb) protein (Boniotti and Gutierrez 2001). Inactivated Rb releases the transcriptional factor E2F/DP, which in turn triggers the expression of S-phase-specific genes (Kosugi and Ohashi 2002). Additionally, the Kip-related proteins (KRPs), one of the classes of CDK-inhibitory proteins, are involved in inactivating CDK/cyclin complexes thus prevent the G_1-to-S phase transition (de Veylder et al. 2001). For example, the expression of *KRP2* was strongly downregulated in xylem pole pericycle cells when roots are subjected to auxin treatment (Himanen et al. 2002; Casimiro et al. 2003). Previous results further showed that activation of auxin-dependent cell cycle regulatory genes encoding *CYCA2;1*, *CYCA3;1*, *CYCD3;1*, *CDKA1*, and the cell cycle inhibitor Kip-Related Protein *KRP2* in tomato seedlings, was dependent on NO, further supporting the idea that the NO modulation of cell cycle regulatory genes involved in G_1-to-S phase transition during lateral root initiation (Correa-Aragunde et al. 2006). However, the molecular, biochemical and physiological events participating between signal transduction and lateral root formation are less understood.

Cobalt, a component of vitamin B_{12}, has long been regarded as an essential element for animals and microorganisms but has not been recognized as such for plants. In contrast, it was shown that cobalt was able to display negative effects on plant growth and metabolism in different degrees, depending on the concentration and status of cobalt in rhizospheree and soil. Meanwhile, stimulation of adventitious root formation induced by low levels of $CoCl_2$ was confirmed firstly in tomato and cucumber cuttings (Gad and Atta-Aly 2006). In fact, although most heavy metals are phytotoxic affecting plant growth and development, chronic exposure could also induce a specific 'stress-induced morphogenic response' (SIMR) phenotype, characterized by enhanced formation of lateral root and adventitious root, as well as the inhibition of root elongation (Potters et al. 2007). For instance, the induction of lateral root development was discovered in *Triticum aestivum* and *Arabidopsis*, when subjected to chromium (Cr) and Cu stress, respectively (Hasnain and Sabri 1997; Pasternak et al. 2005).

Heme oxygenases (HOs, EC 1.14.99.3) catalyse the oxidative cleavage of haem to carbon monoxide (CO), biliverdin (BV), and free iron. In fact, HO has been widely studied in animal tissues, and its major role is associated with haem degradation and its participation in the antioxidant machinery. Three isoforms of HO, HO-1, HO-2 and HO-3 have been identified, which are products of distinct genes (Maines 1997). HO-2 and HO-3 are constitutively expressed whereas HO-1 is highly inducible. The HO-1 gene expression is extremely sensitive to up-regulation by disparate conditions and a number of pathological states including hypoxia, endotoxic shock, atherosclerosis, and inflammation. It was also established that metal ions are powerful HO-inducing agents and cobalt chloride ($CoCl_2$) was the first metal ion identified with an inducing property (Maines and Kappas 1976). In plants, however, the role of HOs was originally focused on its association with the pathway leading to phytochrome chromophores metabolism and functioning in light signaling (Davis et al. 2001; Shekhawat and Verma 2010). During last 5 years, furthermore, HO was demonstrated to be associated with antioxidant machinery in plants when subjected to various abiotic stresses, including salt stress (Xie et al. 2008), UV-B radiation (Yannarelli et al. 2006), and cadmium (Cd) toxicity (Noriega et al. 2004; Han et al. 2008). In addition, the HO-dependent signaling network is related closely to the plant hormones auxin (Xuan et al. 2008), and ABA (Cao et al. 2007a), and other signaling molecules such as H_2O_2 (Yannarelli et al. 2006; Chen et al. 2009) and NO (Noriega et al. 2007; Xuan et al. 2008).

Our previous result showed that CO was able to induce lateral root formation in rapeseed seedlings probably mediated by NO signal (Cao et al. 2007b). Similar response was also confirmed in tomato and Arabidopsis (Guo et al. 2008). Furthermore, we

provided pharmacological, physiological and molecular evidence, showing that HO/CO represents a new signal system with significant impact on auxin-induced adventitious rooting process in cucumber (Xuan et al. 2008). In this context, the analysis of HO/CO-regulated mechanism leading to lateral root promotion is expanded. Pharmacological and molecular evidence is presented to support the ideas that, besides the induction of adventitious root formation (Gad and Atta-Aly 2006), $CoCl_2$ was able to induce lateral root development by the up-regulation of HO.

Materials and methods

Chemicals

All chemicals were obtained from Sigma unless stated otherwise. $CoCl_2$ (analytical reagent, AR), purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, was used at concentrations of 1, 10, 20, 50, and 100 μM. Hemin was used at 10 μM as an HO-1 inducer. Ascorbic acid (AsA), a well-known antioxidant, was applied at concentrations of 10, 100, and 1000 μM. Zinc protoporphyrin IX (ZnPPIX), a potent inhibitor of HO-1, was used at 200 μM. The preparation of CO aqueous solution was carried out according to the method described in our previous reports (Han et al. 2008; Xuan et al. 2008). Also, 50% saturation of CO aqueous solution was used in our experimental condition according to its maximal inducible effect on lateral root formation.

Plant material and growth conditions

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Suhong 2003) were kindly supplied by Jiangsu Agricultural Institutes. Selected identical seeds were surface-sterilized in 5% sodium hypochlorite for 10 min, rinsed extensively and imbibed in water for 3 days. Seedlings with radicles 2–3 mm long were transferred to Petri dishes (90 mm diameter) containing filter paper soaked with distilled water for 1 day, then immersed with 6 ml of the various treatments as indicated. Seedlings were grown in an illuminating incubator and maintained at 25°C ± 1°C for 4 days with a 14-h photoperiod at 200 μmol m^{-2} s^{-1} intensity. Afterwards, tomato seedlings were incubated with $CoCl_2$ or AsA with various concentrations, ZnPPIX (200 μM), hemin (10 μM), 50% saturation of CO aqueous solution alone, or the combination treatments for the indicated times. Then, lateral root (LR) number (the number of LR per seeding), LR density (the number of LR per cm primary root [PR]), PR length and total root length (the combination of LR and PR length) were quantified with IMAGE J 1.34s (http://rsb.info.nih.gov/ij/). LR number only included those roots that were >1 mm in length. Additionally, LR primordia were observed after 3 days of treatments by root squash preparations and quantified by a light microscope (model Stemi 2000-C; Carl Zeiss, Germany). Additionally, the root apical meristems of tomato seedlings at indicated times were cut off and the shoots were removed by cutting below the root-shoot junction in order to obtain samples of only lateral root-inducible segments for the corresponding biochemical and molecular determinations (Correa-Aragunde et al. 2006).

HO activity determination

HO activity from excised tomato samples was analyzed using the method described in our previous reports (Han et al. 2008; Xuan et al. 2008). One unit of activity was calculated as the quantity of enzyme needed to produce 1 nmol BV per 30 min. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Western-blot analysis for HO-1

Rabbit polyclonal antibody was made against the mature LeHO-1 with a molecular mass of 26 kD. Homogenates obtained for HO activity assays were also analyzed by western blotting. Fifty micrograms of protein from homogenates were subjected to SDS–PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad) according to the method described in our previous report (Xuan et al. 2008). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding of antibodies was blocked with 5% non-fat dried milk in phosphate buffered saline (PBS) (pH7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies diluted 1:2000 in phosphate-buffered saline plus 1% non-fat milk. Immune complexes

were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG. The color was developed with a solution containing 3,3′-diaminobenzidine tetrahydrochloride as the horseradish peroxidase substrate.

Semi-quantitative RT–PCR analysis

Total RNA isolation and RT reaction were carried out as previously described (Xuan et al. 2008). Primers used were as follows: for *LeHO-1* (accession no. AF320028), forward (5′-TGGTTTAGGCAGCAGG-3′) and reverse (5′-CGCCGTCCCATTTGTA-3′), amplifying a 211-bp fragment; for *LeHO-2* (accession no. AF320029), forward (5′-TCACTTTCCTTGCTC CAT-3′) and reverse (5′-CATACCCATCATCTTTC ATT-3′), amplifying a 454-bp fragment; for *LeCDKA1* (accession no. Y17225), forward (5′-GCTTATTGT CATTCTCATAGAGTTCTT-3′) and reverse (5′-TC GTTGAAGCACTCATGCTCAAGGGC-3′), amplifying a 520-bp fragment; for *LeCYCD3;1* (accession no. AJ245415), forward (5′-TTATCTTTCATTGAT CATATTATGAGG-3′) and reverse (5′-CTAGGTA ATCTAGAGAACAAGATATCG-3′), amplifying a 525-bp fragment; for *LeCYCA2;1* (accession no. AJ2 43452), forward (5′-TATGAAGAAATTTGTGCACC TCGTG-3′) and reverse (5′-GGATTGGCCACCGA GACTTAAAATCAGC-3′), amplifying a 554-bp fragment; for *LeCYCA3;1* (accession no. AJ243453), forward (5′-GAATTTTGAGATCAGTAGTCCCAC C-3′) and reverse (5′-GCTTGGTGAACATCTTTGG GGCTCG-3′), amplifying a 472-bp fragment; for *LeActin* (accession no. BT012695), forward (5′-AA GAGCTATGAGCTCCCAGATGG-3′) and reverse (5′-TTAATCTTCATGCTGCTAGGAGC-3′), amplifying a 272-bp fragment. To standardize the results, the relative abundance of *LeActin* was determined and used as the internal standard.

The cycle numbers of the PCR were adjusted for each gene to obtain visible bands on agarose gels. Aliquots from the PCR were loaded on 1.5% agarose gels and stained with ethidium bromide. Specific amplification products of the expected size were observed, and their identities were confirmed by sequencing.

Statistical analysis

Where indicated, results are expressed as the mean values ± SE of at least three independent experiments. Statistical analysis was performed using SPSS 16.0 software.

Results

Effects of $CoCl_2$ on tomato lateral root development and HO gene expression

Figure 1 shows several parameters of root growth, LR number/density, primary root (PR), and total root length, in tomato seedlings. In comparison with $CoCl_2$-free control, concentrations between 1 and 100 μM $CoCl_2$ increased LR numbers and/or LR density ($P < 0.05$ or $P < 0.01$), with a maximal response at 20 μM $CoCl_2$ on LR number and 100 μM $CoCl_2$ on LR density, respectively. Meanwhile, PR length and total root length were affected as well. 50 and 100 μM $CoCl_2$ decreased PR length/total root length remarkably ($P < 0.01$) while no apparent difference occurred among the other concentrations of $CoCl_2$. Side effects of higher concentrations ($\geq$50 μM) of $CoCl_2$ (etiolated cotyledons appeared, photograph not shown) was also observed.

To get better understanding of the association of HO with the $CoCl_2$-induced lateral root formation, we undertook a detailed study on the $CoCl_2$-induced *LeHO-1/LeHO-2* transcription. The results in Fig. 2a demonstrate that $CoCl_2$ induced HO expression in a dose-dependent manner. Semi-quantitative RT–PCR revealed that treatment of 50 or 100 μM $CoCl_2$ for 0.75 and 12 h brought about the highest induction of *LeHO-1/LeHO-2* expression, which was consistent with their maximal inducible effects on lateral root density (Fig. 1), and the level of *LeActin* was unaffected throughout all the experiments. Densitometric analysis (Fig. 2b) showed that *LeHO-1/LeHO-2* mRNA level were increased by 40.2 and 200.8% after 100 μM $CoCl_2$ treatment (12 h) respect to $CoCl_2$-free control values, while a 58.5% and 147.3% enhancement was observed at 0.75 h of treatment. In comparison with the responses of 100 μM $CoCl_2$, treatment with 20 μM $CoCl_2$ provoked a relative weaker up-regulation of *LeHO-1/LeHO-2*, because we observed an augmentation of the relative gene expression by 50.2% and 113.3% at 0.75 h, 17.2% and 195.0% at 12 h, respectively. Therefore, this fact indicated that *LeHO-1/LeHO-2* gene expression was modulated approximately by $CoCl_2$ treatment in a dose-dependent fashion.

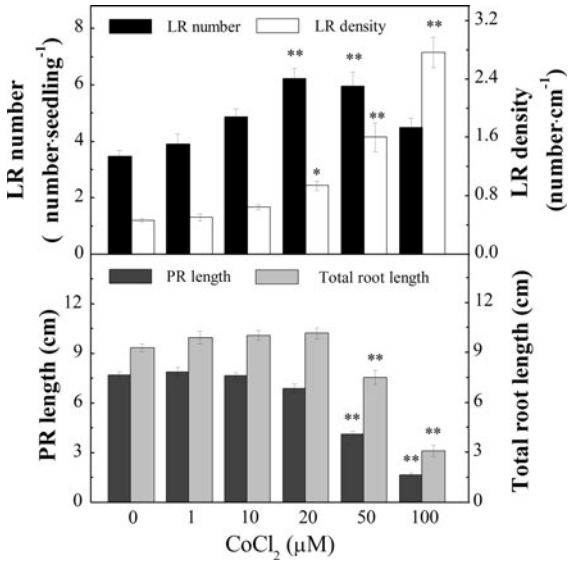

Fig. 1 Effects of $CoCl_2$ on lateral root (LR) number, density, primary root (PR) length, and total root length. Tomato (*Lycopersicon esculentum*) seedlings were treated with various concentrations of $CoCl_2$ for 4 days. The number of LRs, the length of the PRs and all LRs (>1 mm) per seedling were analyzed. LR density and total root length were then calculated. Mean and SE values were calculated from three independent experiments ($n = 30$). * or ** indicates a significant difference in comparison with the control (0 μM $CoCl_2$) at $P < 0.05$ or 0.01 (Duncan's multiple test)

A biphasic burst of HO activity is observed in responses to $CoCl_2$ treatment

To test above hypothesis, tomato seedlings were treated with 20 μM $CoCl_2$, an effective concentration for lateral root formation and no side effect was observed, and HO activity was investigated. The time course experiment (Fig. 3) showed that, in comparison with control samples, a biphasic burst of HO activity appeared as early as 3 h after treatment, followed by a higher peak at 12 h, and a gradual decrease until 48 h. Meanwhile, a weaker increased level of HO activities in the control sample was observed. We also noted that the enhancement of HO activity induced by 20 μM $CoCl_2$ apparently preceded lateral root formation.

HO is required in the formation of lateral root formation induced by $CoCl_2$

To test the hypothesis that HO was involved in $CoCl_2$-induced lateral rooting process, the potent HO-1 inhibitor ZnPPIX in both animals and plants (Lamar et al. 1996; Iyer et al. 2003; Song et al. 2008; Xuan et al. 2008) was applied. In our test, treating tomato seedlings with this compound prevents the induction action of $CoCl_2$ on lateral root formation ($P < 0.05$, Fig. 4). However, when exogenous 50% saturation of CO aqueous solution was added together with ZnPPIX, the lateral root number inhibited by ZnPPIX treatment was relieved and returned to a similar extent to that displayed in tomato seedlings treated with 50% CO aqueous solution alone. Meanwhile, application of $CoCl_2$ plus CO resulted in a similar response to that obtained with $CoCl_2$. In contrast, only a slight but no significant effect could be observed in responses to the addition of ZnPPIX

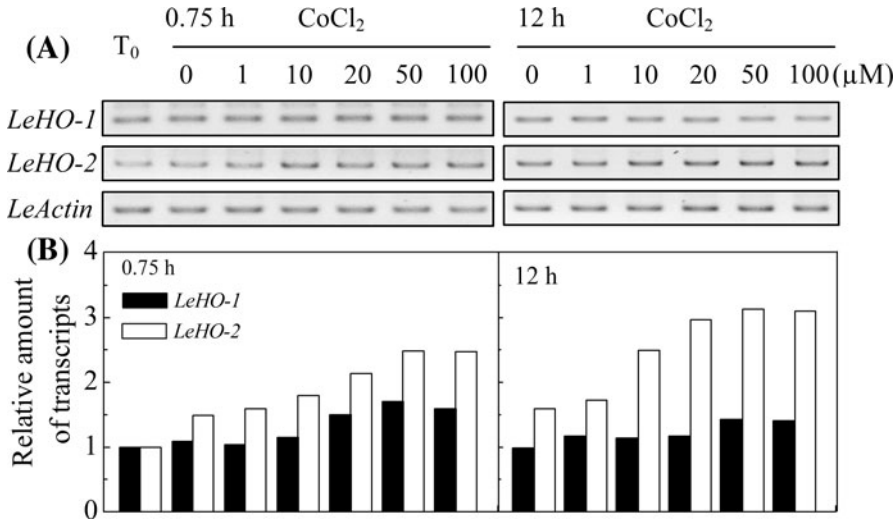

Fig. 2 $CoCl_2$ dose-dependently induces HO transcription in tomato seedling roots. (**a**) *LeHO-1* and *LeHO-2* expression was analyzed by semi-quantitative RT–PCR after 0.75 or 12 h treatments of 0, 1, 10, 20, 50, and 100 μM $CoCl_2$. (**b**) Quantitative analysis of *LeHO-1* and *LeHO-2* transcript levels at 0.75 and 12 h. The data were obtained by densitometric analysis. The values represent relative transcript levels with respect to the data at time zero

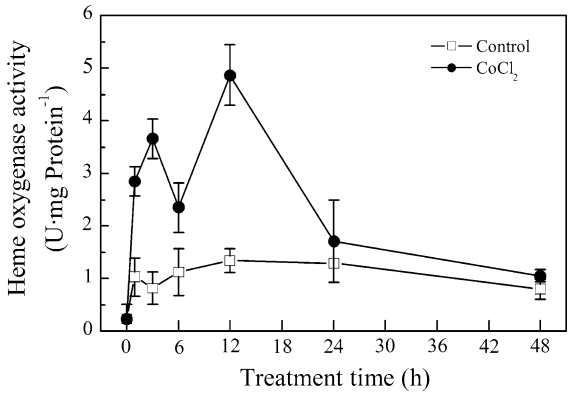

Fig. 3 Time course of HO activity in tomato seedling roots under $CoCl_2$ treatment. Tomato seedlings were treated with 20 μM $CoCl_2$ for 48 h. Then, HO activity was measured at the indicated times. Mean and SE values were calculated from three independent experiments

with or without CO aqueous solution compared with $CoCl_2$-free control sample. As a positive control, the application of a HO-1 inducer hemin produced the similar inducible response in lateral root formation, in comparison with the action of $CoCl_2$. Thus, above results suggested the important role of HO-1/CO in $CoCl_2$-induced lateral root formation. When the whole root system was analyzed, total root length in various treatments were slightly modified (Fig. 4b). Anatomical studies shown in Fig. 4c also illustrated that changes of lateral root primodia (LRP) numbers after 3 days of various treatments matched approximately the responses of LR numbers.

In order to further confirm the HO requirement during $CoCl_2$-induced lateral root formation, immunoblot analysis combined with enzyme activity determination were performed. Rabbit polyclonal antibody raised against to mature LeHO-1 recognized a single band of approximately 26 kD in roots of tomato seedlings, a mass consistent approximately to that previously reported for mature HO1 of pea and *Arabidopsis* plants (Fig. 5a, Muramoto et al. 1999; Linley et al. 2006). In the presence of added ZnPPIX, $CoCl_2$-induced HO-1 protein expression (3 h, Fig. 5b) and HO activity (3 and 12 h, Fig. 5c) in tomato seedling roots were significantly lower than those of $CoCl_2$-treated alone samples, respectively. When the above sample was treated simultaneously with CO aqueous solution, the decreased HO activity only after 12 h of treatment was reversed. Additionally, as expected, application of ZnPPIX alone for 3 h

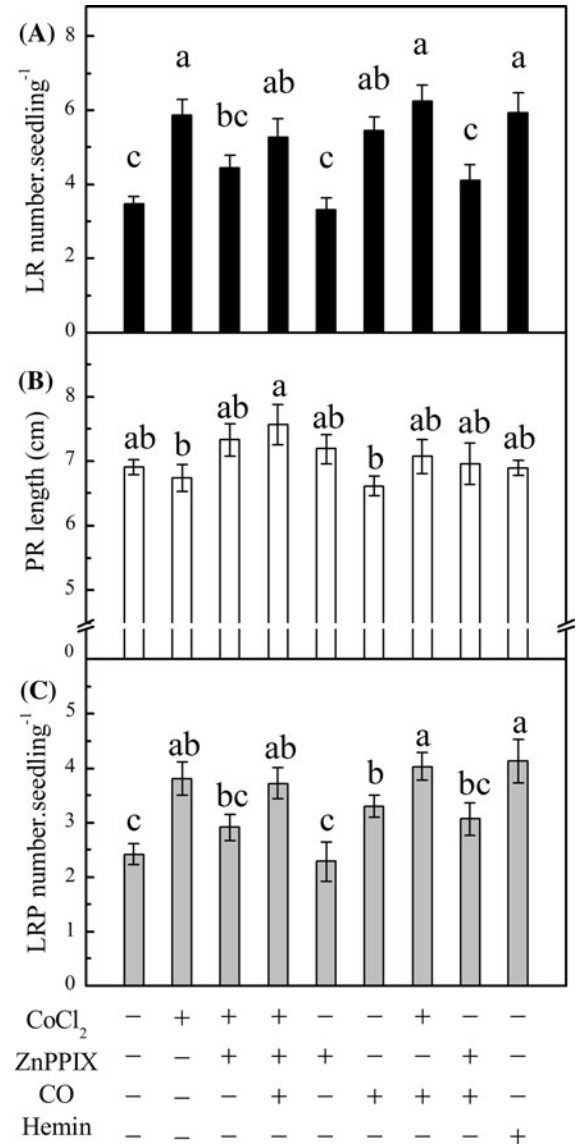

Fig. 4 Application of CO aqueous solution to $CoCl_2$-treated tomato seedlings reverses the ZnPPIX responses. Tomato seedlings were incubated with $CoCl_2$ (20 μM), ZnPPIX (200 μM), hemin (10 μM), 50% saturation of CO aqueous solution alone, or the combination treatments, then LR number (**a**) and PR length (**b**) were recorded after 4 days. In another set of experiments, LR primordia (LRP, **c**) were observed by root squash preparations and quantified by bright-field microscopy after 3 days of various treatments. Mean and SE values were calculated from three independent experiments ($n = 30$). *Bars* denoted by the same letter did not differ significantly at $P < 0.05$ (Tukey test)

produced a decrease in the HO-1 protein expression and HO activity ($P < 0.05$). While, the inducible effect of hemin on HO expression was also observed.

Together, all above results were consistent with lateral root formation in tomato seedlings treated for 4 days as described above (Fig. 4a).

Ascorbic acid fails to influence lateral root formation

Normally, HO expression is up-regulated under conditions that cause oxidative stress. Thus, to assess the possible role of HO in lateral root development, AsA, a non-enzymatic scavenger of free radicals and peroxides, was applied in our experimental conditions. Results of Fig. 6 show that AsA with various concentrations could not change the inducible response of $CoCl_2$ in lateral root formation. Meanwhile, except the significant inhibition of higher dose of AsA (1000 μM) on PR length, no difference was observed in the AsA-treated alone samples respect to the control plant. Above results suggest that the induction of lateral root formation be specific for $CoCl_2$ rather than its-induced oxidative stress.

Effects of ZnPPIX, CO, and $CoCl_2$ on the expression profiles of cell cycle regulatory genes

In the following experiment, the influence of $CoCl_2$, ZnPPIX, and CO aqueous solution applied alone or their combination on the expression of cell cycle regulatory genes was analysed. Semi-quantitative RT–PCR analysis was carried out on RNA extracted from tomato seedling roots treated for 48 h. Figure 7 shows that $CoCl_2$ up-regulated the expression of the *LeCDKA;1* and *LeCYCA2;1* during 24 h of treatment and then declined. Some inducible effects of $CoCl_2$ are sustained in time, inducing the expression of *LeCYCD3;1* and *LeCYCA3;1* up to 48 h. Whereas, in comparison with $CoCl_2$-free control sample, only *LeCDKA;1* and *LeCYCA2;1* were decreased slightly during the beginning period (less than 24 h) of ZnPPIX alone treatment. Meanwhile, the $CoCl_2$-induced expression of *LeCDKA;1*, *LeCYCA2;1* and *LeCYCA3;1* expression was prevented or delayed differentially when ZnPPIX was added simultaneously. These findings provided preliminary evidence and suggested that endogenous HO and its products might modulate the expression of cell cycle regulatory genes, which are also involved in $CoCl_2$-induced lateral root development. Further results

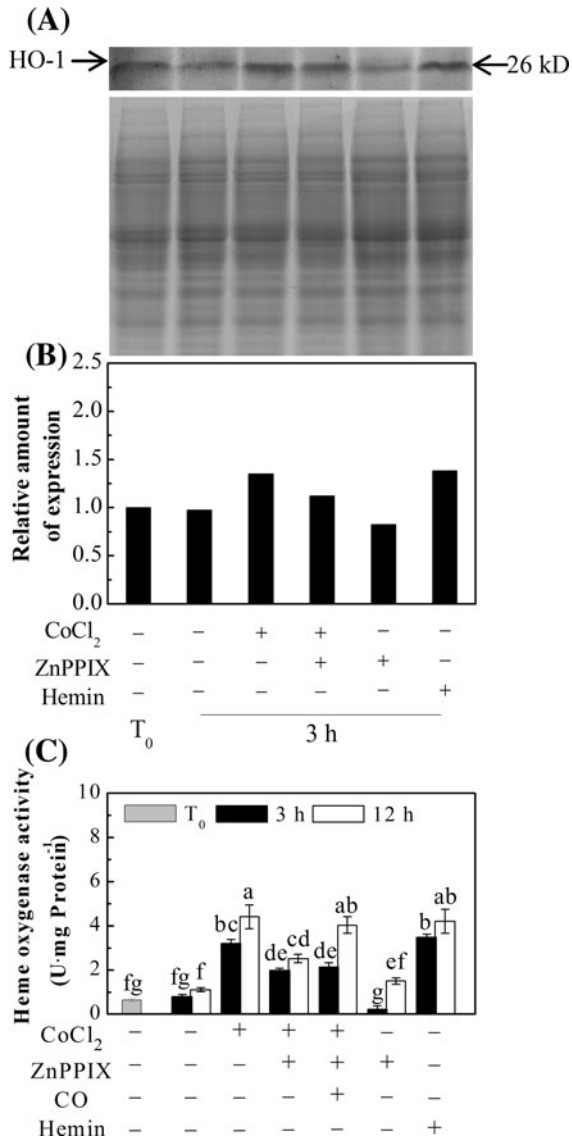

Fig. 5 Effects of $CoCl_2$, ZnPPIX, hemin, and CO on HO-1 protein expression and HO activity. Tomato seedlings were incubated with $CoCl_2$ (20 μM), ZnPPIX (200 μM), hemin (10 μM), 50% saturation of CO aqueous solution alone, or the combination treatments, then HO-1 protein expression was determined by western blotting after 3 h treatment (**a**). Meanwhile, Coomassie Brilliant Blue-stained gels are present to show that equal amounts of proteins were loaded. Relative HO-1 protein expression taking $CoCl_2$-free control sample at time zero as 1 U (**b**). Additionally, HO activity (**c**) was analyzed after 3 or 12 h treatment, respectively. Mean and SE values were calculated from at least three independent experiments. *Bars* denoted by the same letter did not differ significantly at $P < 0.05$ (Tukey test)

illustrated the restoration effects of CO aqueous solution on the ZnPPIX-induced inhibition of cell cycle genes expression, further strengthening the

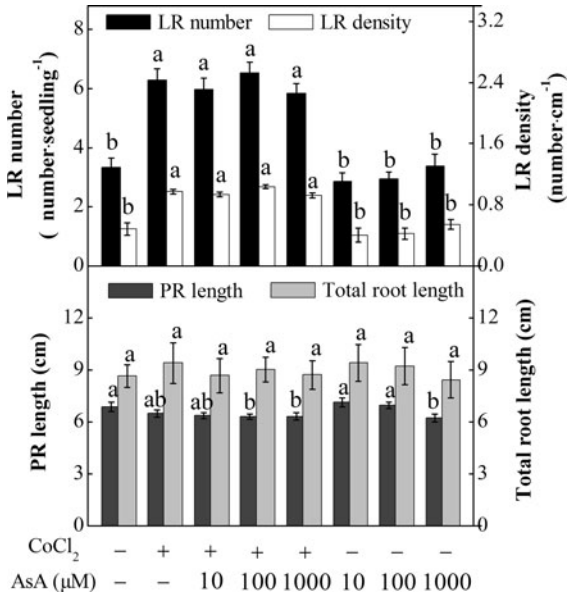

Fig. 6 Effects of ascorbic acid (AsA) and $CoCl_2$ on lateral root (LR) number, density, primary root (PR) length, and total root length. Tomato seedlings were treated with $CoCl_2$ (20 μM), various concentrations of AsA alone, or the combination treatments for 4 days. The number of LRs, the length of the PRs and all LRs (>1 mm) per seedling were analyzed. LR density and total root length were then calculated. Mean and SE values were calculated from three independent experiments ($n = 30$). Within each set of experiment, *bars* denoted by the same letter did not differ significantly at $P < 0.05$ (Duncan's multiple test)

hypothesis that CO produced by HO might be responsible for $CoCl_2$-induced lateral root formation.

Discussion

To the best of our knowledge, the observation that the application of $CoCl_2$ to tomato seedlings led to one of the SIMR phenotypes, the induction of lateral root formation (Fig. 1), is new. In fact, similar inducible response has been observed in adventitious root formation in tomato and cucumber cuttings conferred by low doses of $CoCl_2$, and these effects were associated with the increased ethylene production (Gad and Atta-Aly 2006).

Further data support a lineal signal transduction cascade involving up-regulation HO downstream of $CoCl_2$ response. The following results support these conclusions: (1) exogenous application of $CoCl_2$ and hemin induced lateral root formation (Figs. 1 and 4); (2) $CoCl_2$ induces HO expression in a dose- and time-dependent manner (Figs. 2 and 3); (3) The potent HO-1 inhibitor ZnPPIX, not only decreased HO-1 protein expression (3 h) and HO activity (3 and 12 h), but also blocked inducible responses of $CoCl_2$ on lateral root formation (4 days), both of which could be reversed partially when CO aqueous solution was added (Figs. 4 and 5). Thus, this pharmacological evidence supports the possibility that $CoCl_2$ and up-regulation HO might be on a linear signaling pathway in the process of lateral root formation in tomato plants.

In animals, ample evidence illustrated that HO-1 is highly induced by a variety of agents or stimuli causing oxidative stress, such as H_2O_2, glutathione depletors, ultraviolet irradiation and hyperoxia. In fact, metal irons are other powerful HO-inducing agents and cobalt was the first metal ion identified with an inducing property. For example, it is well established that $CoCl_2$ produces strong increase in rat liver HO activity (Maines and Kappas 1976; Numazawa et al. 1989), and it has been shown that HO-1 knockout mice exhibit reduced stress defences when exposed to oxidative challenge (Poss and Tonegawa 1997). HO induction by cobalt in Chinese hamster ovary cells is also oxidative stress-dependent and mediated by the stress response element/nuclear factor erythroid-2 transcription factor pathway (Gong et al. 2001). All above factors induced HO-1 expression by a general mechanism: enhancement of HO activity by de novo enzyme synthesis associated with an increase in HO-1 mRNA level. In agreement with these results, we discovered that the application of $CoCl_2$ could result in the induction of *LeHO-1/2* transcripts and HO-1 protein expression in tomato seedlings in a dose- or time-dependent manner (Figs. 2 and 5). Interestingly, a biphasic burst of HO activity was also observed (Fig. 3). However, combined with the results that AsA, a well-known antioxidant, failed to influence lateral root formation in our experimental conditions (Fig. 6), we assumed that HO-1 induction would be beneficial for lateral root formation by enhancing the release of CO, a signaling molecule responsible for lateral root proved in rapeseed seedlings reported by our research group (Cao et al. 2007b).

On the other hand, the response of HO induction by $CoCl_2$ treatment could be used to explain how $CoCl_2$ stimulates adventitious root formation in

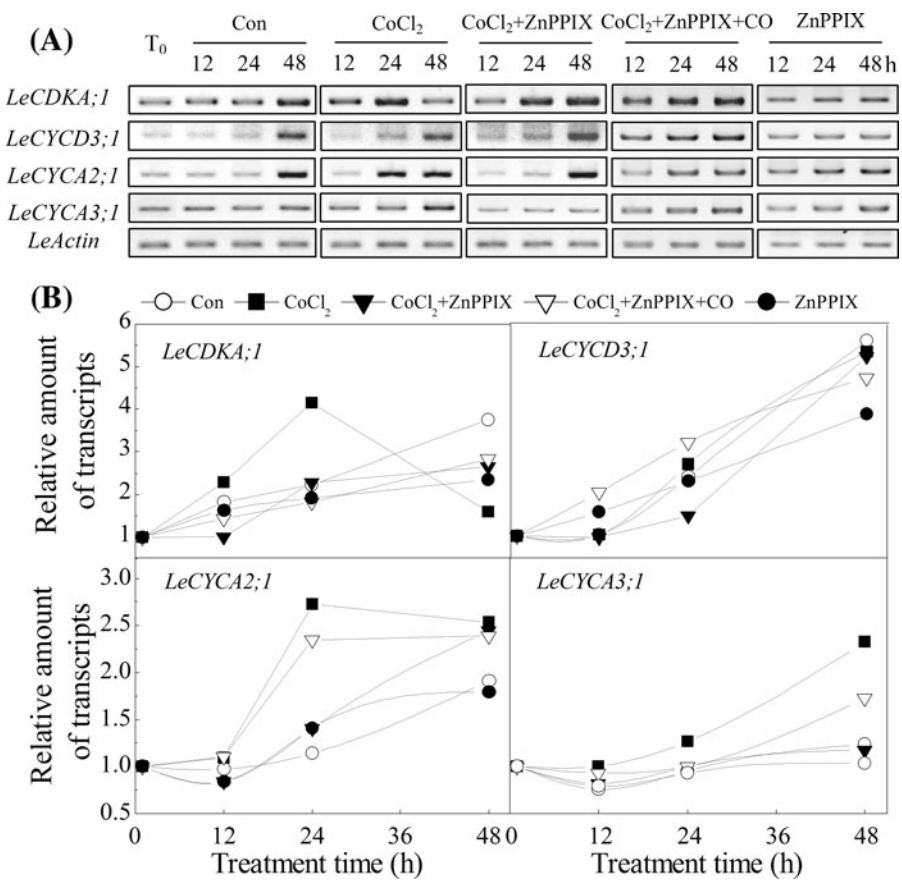

Fig. 7 Effects of $CoCl_2$, ZnPPIX, and CO on the expression profiles of cell cycle regulatory genes. Tomato seedlings were incubated with $CoCl_2$ (20 μM), ZnPPIX (200 μM), 50% saturation of CO aqueous solution alone, or the combination treatments for 2 days. *LeCDKA;1*, *LeCYCD3;1*, *LeCYCA2;1*, and *LeCYCA3;1* expression was analyzed by semi-quantitative RT–PCR at indicated times after various treatments (**a**). Quantitative time course analysis of *LeCDKA;1*, *LeCYCD3;1*, *LeCYCA2;1*, and *LeCYCA3;1* transcript levels under different treatment conditions (**b**). The values were obtained by densitometric analysis. The values represent relative transcript levels with respect to the data at time zero

tomato and cucumber cuttings (Gad and Atta-Aly 2006), because we have confirmed that HO/CO might be involved in cucumber adventitious rooting process by modulating the expression of one DnaJ-like gene (*CSDNAJ-1*) and two calcium-dependent protein kinase genes (*CSCDPK1* and *CSCDPK5*, Xuan et al. 2008). Additionally, our result showing that the up-regulation of *LeHO-2* expression is more sensitive to $CoCl_2$ than that of *LeHO-1* by using semi-quantitative RT–PCR analysis (Fig. 2) indicated that both *LeHO-1/2* might belong to the inducible type of HOs (HO-1) which could be inhibited by the addition of ZnPPIX (Fig. 5c), or *LeHO-2* acted as an inducible HO-2 enzyme in plants. However, HO-2 in animals—only referred to as the "constitutive" isozyme—does not seem to be inducible by either inflammatory or oxidative stress (Bauer et al. 1998), although the promoter of the *HO-2* gene contains a glucocorticoid response element (Maines et al. 1996). Certainly, further biochemical and molecular evidence should be provided to confirm these possibilities.

It was well known that cell cycle regulatory genes are the regulatory genes of lateral root formation. Thus, above link goes further in the analysis of the HO-mediated molecular mechanisms leading to lateral root formation triggered by $CoCl_2$. Results illustrated that $CoCl_2$ treatment induced higher expression of the *LeCDKA;1*, *LeCYCD3;1*, *LeCYCA2;1*, and *LeCYCA3;1* genes during the first 24 h period of treatment (Fig. 7), and these were consistent with the number and density of lateral root observed after another 3-d treatment (Fig. 1). Further evidence showed that besides the inhibition of lateral root formation (Fig. 4) and HO expression (Fig. 5), the addition of the potent HO-1 inhibitor ZnPPIX decreased the $CoCl_2$-induced transcription of *LeCDKA;1*, *LeCYCA2;1* and *LeCYCA3;1* in tomato seedlings, in comparison with $CoCl_2$-free control sample (Fig. 7). Moreover, the application of CO

aqueous solution was able to up-regulate differentially the expression of cell cycle regulatory genes as well as the increased HO activity (12 h, Fig. 5c). Meanwhile, the inhibition of lateral root formation conferred by ZnPPIX plus $CoCl_2$ treatment also recovered ($P < 0.05$, Fig. 4). Thus, we deduced that cell cycle regulatory genes, which have been proposed to be involved in the auxin- and NO-induced lateral root development in tomato seedlings (Correa-Aragunde et al. 2006), might be targets for the HO/CO-involved lateral root formation induced by $CoCl_2$. In animals, it was proven that HO-1 could regulate the cell cycle in a cell-specific manner by using an inhibitor of HO activity tin-mesoporphyrin (SnMP), and its inducers heme or $SnCl_2$. For example, it increases vascular endothelial (EC) but decreases smooth muscle cells (SMC) cycle progression (Li Volti et al. 2002). E2F, one of transcription factors modulated by CO, has been proven that could regulate the expression of many genes involved in cell proliferation, and govern the transition of cells from the G_1 to S phase (Dulak and Józkowicz 2003). Since one of the HO catalytic products, CO can induce lateral root formation in rapeseed (Cao et al. 2007b), it thus suggested that CO is specifically stimulating some pericycle cells triggering cell cycle activation leading to lateral root formation.

The involvement of up-regulation of HO in plant hormone responses has been well elucidated. For example, HO mediates auxin-induced adventitious root formation (Xuan et al. 2008) and ABA-induced stomatal closure in *Vicia faba* (Cao et al. 2007a). Gad and Atta-Aly (2006) provided evidence that exposing tomato and cucumber cuttings to 0.25 ppm of cobalt ion, not only induces adventitious root formation, but also significantly induced ethylene production, which was recently proven to regulate lateral root development by interaction with auxin (Negi et al. 2008). While, increasing cobalt concentration up to 1.0 ppm strongly inhibited the ethylene precursor 1-aminocyclopropan 1-carboxylic acid (ACC) conversion to ethylene. Thus, it would be interesting in the future to investigate the possible link among $CoCl_2$-induced lateral root development, HO up-regulation, and ethylene signalling.

As stated above, it was demonstrated that HO expression can be enhanced by $CoCl_2$ treatment both in animals (Maines and Kappas 1976) and plants. Our results further confirm a novel role for HO in the $CoCl_2$-induced tomato lateral root formation by the modulation of expression of cell cycle regulatory genes, which will surely contributed to our understanding of the molecular mechanisms that regulate root morphogenesis.

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