Plant Mol Biol (2014) 85:49–61
DOI 10.1007/s11103-013-0168-3

Interaction between HY1 and H_2O_2 in auxin-induced lateral root formation in *Arabidopsis*

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Received: 5 May 2013 / Accepted: 16 December 2013 / Published online: 24 December 2013

Abstract Haem oxygenase-1 (HO-1) and hydrogen peroxide (H_2O_2) are two key downstream signals of auxin, a well-known phytohormone regulating plant growth and development. However, the inter-relationship between HO-1 and H_2O_2 in auxin-mediated lateral root (LR) formation is poorly understood. Herein, we revealed that exogenous auxin, 1-naphthylacetic acid (NAA), could simultaneously stimulate *Arabidopsis HO-1* (*HY1*) gene expression and H_2O_2 generation. Subsequently, LR formation was induced. NAA-induced *HY1* expression is dependent on H_2O_2. This conclusion was supported by analyzing the removal of H_2O_2 with ascorbic acid (AsA) and dimethylthiourea (DMTU), both of which could block NAA-induced *HY1* expression and LR formation. H_2O_2-induced LR formation was inhibited by an HO-1 inhibitor zinc protoporphyrin IX (Znpp) in wild-type and severely impaired in *HY1* mutant *hy1-100*. Simultaneously, HY1 is required for NAA-mediated H_2O_2 generation, since Znpp inhibition of *HY1* blocked the NAA-induced H_2O_2 production and LR formation. Genetic data demonstrated that *hy1-100* was significantly impaired in H_2O_2 production and LR formation in response to NAA, compared with wild-type plants. The addition of carbon monoxide-releasing molecule-2 (CORM-2), the carbon monoxide (CO) donor, induced H_2O_2 production and LR formation, both of which were decreased by DMTU. Moreover, H_2O_2 and CORM-2 mimicked the NAA responses in the regulation of cell cycle genes expression, all of which were blocked by Znpp or DMTU, respectively, confirming that both H_2O_2 and CO were important in the early LR initiation. In summary, our pharmacological, genetic and molecular evidence demonstrated a close inter-relationship between HY1 and H_2O_2 existing in auxin-induced LR formation in *Arabidopsis*.

Keywords *Arabidopsis* · Auxin · Hydrogen peroxide · HY1 · Lateral root formation

Fei Ma, Lijuan Wang and Jiale Li contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0168-3) contains supplementary material, which is available to authorized users.

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Introduction

Lateral roots (LR) are major components of root system architecture, responsible for water and nutrients uptake, as well as providing anchorage. However, LR formation is widely affected by environmental stresses and phytohormones (Fukaki and Tasaka 2009). Among these factors, auxin plays a central role in LR development. For example, exogenous auxin can increase the number of LR (Blakely et al. 1988), and a series of auxin-related mutants of *Arabidopsis* impair LR formation (Péret et al. 2009). Further studies revealed that both leaf-derived and root tip-localized auxin are essential for LR formation during early seedling development in *Arabidopsis* (Bhalerao et al. 2002).

Application of the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA), however, efficiently arrests LR development (Casimiro et al. 2001). An increasing body of evidence further confirmed that a series of cell cycle regulatory genes are involved in auxin-triggered LR initiation, including G_1/S cell cycle marker genes *Histone H4*, *E2Fa*, and *KRP2*, and the G_2/M-specific genes *CDKB1;1* and *CYCB2;1*, etc. (Himanen et al. 2002, 2004; Casimiro et al. 2003). Additionally, some transcriptional factors, including LATERAL ORGANBOUNDARIES DOMAIN 16 (LBD16), LBD18, and LBD29 were also involved in LR formation (Fukaki and Tasaka 2009; Feng et al. 2012).

It is confirmed that hydrogen peroxide (H_2O_2) plays various important roles in signal transduction beside its toxic effects. Several enzymatic sources are responsible for H_2O_2 generation, including NADPH oxidase (Rboh, respiratory burst oxidase homologue), xanthine oxidase, amine oxidase and cell wall peroxidase, etc. (for review, see Neill et al. 2002). As a signaling molecule, H_2O_2 could participate in multiple plant responses against stresses and development processes, including systemic acquired resistance (Chen et al. 1993), hypersensitive response (HR) against pathogen attack (Levine et al. 1994; Torres et al. 2002), programmed cell death (PCD; Fath et al. 2002), stomatal closure (Pei et al. 2000), root gravitropism (Joo et al. 2001), cell elongation (Foreman et al. 2003), adventitious rooting (Li et al. 2007; Bai et al. 2012), and LR formation (Su et al. 2006; Wang et al. 2010; Chen et al. 2013), etc. Specially, the involvement of H_2O_2 in auxin signaling was also reported (Joo et al. 2001; Song et al. 2007; Bai et al. 2012).

Haem oxygenases (HOs; EC 1.14.99.3) degrade haem to produce equimolar amounts of biliverdin IXa (BV), free iron (Fe^{2+}) and carbon monoxide (CO); the BV is subsequently reduced to bilirubin (BR) (for review, see Otterbein et al. 2003). Three types of HOs were found in mammals: inducible HO-1, constitutive HO-2 and HO-3. HOs have been identified from various higher plants (Shekhawat and Verma 2010; Cao et al. 2011; Han et al. 2012). In *Arabidopsis*, for example, four types of HOs were classified into two subfamilies: HY1, HO-3 and HO-4 are belonged to HO-1 subfamily; HO-2 is the only member of HO-2 subfamily, which is not the real HO, due to its inability in binding or degrading haem (Gisk et al. 2010). Apart from it's central role in light signaling (Gisk et al. 2010; Shekhawat and Verma 2010), HO-1 and one of its products CO could participate in plant responses against multiple stresses and developmental processes, including ultraviolet (UV; Yannarelli et al. 2006; Xie et al. 2012), salinity and drought (Cao et al. 2011; Xie et al. 2011), heavy metal exposure (Han et al. 2008; Fu et al. 2011; Cui et al. 2011, 2012), oxidative insult (Chen et al. 2009; Xu et al. 2012; Jin et al. 2013), *α-Amy2/54* gene expression (Wu et al. 2013), and root organogenesis (adventitious and LR development; Guo et al. 2008; Xuan et al. 2008, 2012; Chen et al. 2012; Han et al. 2012; Lin et al. 2012; Hsu et al. 2013).

Although previous studies presented evidences about the roles of HO-1 and H_2O_2 in LR development, their interrelationship in auxin-mediated LR formation is largely unknown. Understanding of this physiological process, in which cross-talk between HO-1 and H_2O_2 exists, is of critical importance. The aim of this investigation is to examine the interaction between Arabidopsis HO-1 (HY1) and H_2O_2 in mediating auxin-induced LR formation. This work may increase our understanding of the mechanisms underlying auxin-mediated root organogenesis in plants.

Materials and methods

Chemicals

All chemicals were obtained from Sigma–Aldrich (St Louis, MO, USA) unless stated otherwise. 1-naphthylacetic acid (NAA) was used with the indicated concentrations. *N*-1-naphthylphthalamic acid (NPA), from Chem Service, was used as the auxin transport inhibitor at 10 μM (Casimiro et al. 2001). Hydrogen peroxide (H_2O_2) and catalase (CAT) was purchased from Shanghai Medical Instrument, Co., Ltd., China National Medicine (Group), Shanghai, China. Dimethylthiourea (DMTU) (Levine et al. 1994) and ascorbic acid (AsA) (Bai et al. 2012), two membrane-permeable scavengers of H_2O_2, were used at 5 mM and 200 μM, respectively. Diphenylene iodonium (DPI), an inhibitor of NADPH oxidase (Desikan et al. 2006; Bai et al. 2012), was used at 5 μM. Haemin (10 μM), purchased from Fluka, was used as an HO-1 inducer (Cui et al. 2012). Bilirubin (BR), another by-product of HO-1, was used at 10 μM (Jin et al. 2013). Zinc protoporphyrin IX (Znpp), an inhibitor of HO-1, was used at 50 μM (Xuan et al. 2008; Xie et al. 2011). Carbon monoxide-releasing molecule-2 (CORM-2), a donor of carbon monoxide (Xie et al. 2011), was used at 20 μM. The concentrations of the above-mentioned chemicals were determined in pilot experiments from which the significant induced responses were obtained.

Plant materials and growth conditions

Arabidopsis thaliana (Col-0) seeds, including wild-type (WT), *hy1-100* (CS236), *ho2* (SALK_025840), *ho3* (SALK_034321), and *ho4* (SALK_044934; Xie et al. 2011); and *arf7 arf19* (CS24630; Okushima et al. 2005) were surface-sterilized and rinsed for three times with distilled water, then cultured in petri dishes on solid half-strength Murashige and Skoog (MS) medium (pH 5.8) with 1 % (w/v) sucrose. Plates containing seeds were kept at 4 °C for 2 days, and then transferred into a growth

chamber with a 16/8 h (23/18 °C) day/night regime and 150 μmol m^{-2} s^{-1} irradiation.

Uniform five-day-old seedlings were transferred to homogenous mediums containing indicated chemicals for the indicated times or another 5 days. Afterw ards, the photographs were taken. Lateral root (LR) density (LRs/cm primary root; only emerged and visible LRs were included) and length (cm/seedling; total length of all LRs of a given primary root) covering the whole primary root were determined using Image J software. LR primordia (LRP) were observed by a light microscope (model Stemi 2000-C; Carl Zeiss, Germany). According to the previous study (Himanen et al. 2002), only the lateral root-inducible segments were used for H_2O_2 determination and RNA extraction; therefore, the root apical meristems were cut off, and the shoots were removed by cutting below the adventitious root primordia.

Determination of H_2O_2 content by spectrometer method

The content of H_2O_2 was measured according to Bellincampi et al. (2000). Samples were ground to a fine powder with liquid nitrogen and homogenized with 0.2 M $HClO_4$ at 4 °C. The extract was held for 5 min followed by centrifugation at 10,000*g* for 10 min. The supernatant was added to the assay reagent (500 μM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 μM xylenol orange, and 200 mM sorbitol). After incubation for 45 min, absorbance of the Fe^{3+}–xylenol orange complex (A_{560}) was detected. Standard curves were obtained by adding variable amounts of H_2O_2 to basal medium mixed to assay reagent.

Confocal analysis of H_2O_2 production

Production of H_2O_2 was assayed by confocal microscopy using 20 μM 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Calbiochem, La Jolla, CA, USA) (Desikan et al. 2006; Bai et al. 2012). Samples were collected at the indicated times and loaded with H_2DCFDA for 30 min before washing in 20 mM HEPES buffer (pH 7.8) three times for 15 min each (Xie et al. 2011). All images were captured using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany, excitation at 488 nm, emission at 500–530 nm). All manipulations were performed at 25 ± 1 °C. Data are presented as relative units of pixel intensities calculated by the ZEN software.

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was isolated from 100 mg of fresh-weight samples by using Trizol reagent (Invitrogen, Gaithersburg, MD). cDNA was synthesized from 2 μg of total RNA using 1 μM oligo(dT) primer and AMV reverse transcriptase XL (TaKaRa). PCR was performed using 2 μL of a twofold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 U of *Taq* polymerase (TaKaRa) in a 20-μL reaction volume.

The primer sequence information was listed in Supplementary Table S1. Relative expression levels of *HY1* (accession number BT002327) are presented as values relative to the corresponding control samples at the indicated times, after normalization to *Actin7* (accession number NM_121018) transcript levels. Aliquots from the PCR were separated on 1.5 % agarose gels and visualized using ethidium bromide (EB). The specific amplification products of the expected sizes were observed, and their identities were confirmed by sequencing. EB-stained gels were scanned and analysed using Quantity One V4.4.0 software (Bio-Rad, Hercules, CA, USA).

Real-time RT-PCR analysis

Real-time quantitative RT-PCR (qRT-PCR) reactions were performed using a Mastercycler® ep *realplex* real-time PCR system (Eppendorf, http://www.eppendorf.com/) with SYBR® *Premix Ex Taq*™ (TaKaRa, http://www.takara-bio.com/) according to the manufacturer's instructions. The primer sequence information was listed in Supplementary Table S2. Relative expression levels of *Histone H4* (accession number NM_100639), *E2Fa* (accession number AJ294534), *CYCB2;1* (accession number NM_127316), *CDKB1;1* (accession number NM_115278), *KRP2* (accession number NM_114923), *LBD16* (accession number NM_129804), *LBD18* (accession number NM_180105), and *LBD29* (accession number NM_115681) are presented as values relative to the control samples at the indicated times, after normalization to *Actin7* (accession number NM_121018) transcript levels.

Statistical analysis

Where indicated, the values are shown as the mean values ± SE of at least three independent experiments with similar results. Statistical analysis was performed using SPSS 16.0 software. For statistical analysis, *t* test ($P < 0.05$ and $P < 0.01$) or Tukey's multiple range test ($P < 0.05$) was chosen as appropriate.

Results

NAA induces *HY1* expression, H_2O_2 generation and LR formation

As shown in Fig. 1a and Supplementary Fig. S1, the addition of the synthetic auxin 1-naphthylacetic acid (NAA) ranging from 25 to 100 nM, increased *Arabidopsis* LR density in a dose-dependent manner. Additionally, 100 nM

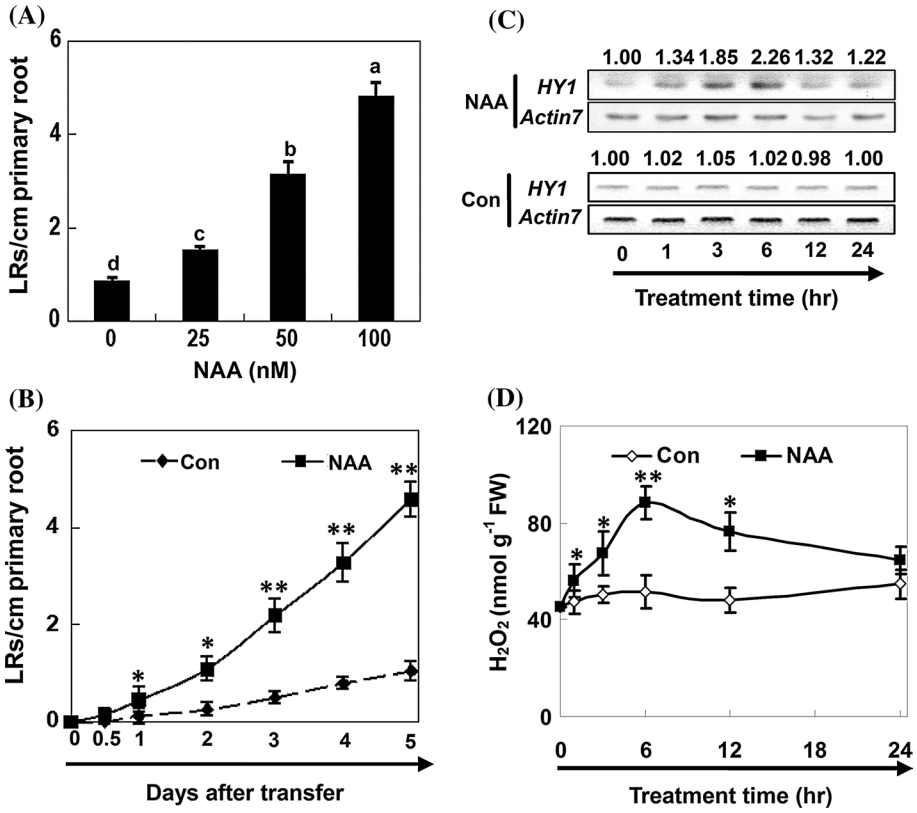


Fig. 1 NAA induces *HY1* expression, H_2O_2 generation and LR formation. Five-day-old wild-type seedlings were treated with H_2O (0 nM NAA or Con), the indicated (**a**) or 100 nM NAA (**b**–**d**) for 5 days (**a**) or the indicated times (**b**–**d**). Afterwards, the emerged LR density (**a**, **b**), *HY1* expression (**c**), and H_2O_2 content (**d**) were determined. The number above the band (**c**) indicates relative expression levels of *HY1* analyzed by semi-quantitative RT-PCR with respect to the data at time zero, after normalization to *Actin7*. Mean and SE values were calculated from at least three independent experiments. *Bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test (**a**). Within the same treated time points, *bars* with *asterisks* were significantly different in comparison with Con at $P < 0.05$ or $P < 0.01$ according to *t* test (**b**, **d**)

NAA time-dependently induced LR formation, respect to the weaker response in the control samples (Fig. 1b). Therefore, 100 nM NAA was used throughout our experiments.

Subsequent results showed that, NAA significantly induced *HY1* expression (Fig. 1c) and H_2O_2 generation (Fig. 1d) also in a time-dependent manner, with a maximal response at 6 h of treatment, respectively, then followed by a gradual decrease until 24 h. Remarkably, the time-courses of NAA-induced *HY1* expression and H_2O_2 generation showed the similar tendencies, and apparently preceded LR formation. Moreover, auxin-response mutant *arf7 arf19*, which could reduce sensitivity to auxin and impair LR formation (Okushima et al. 2005), obviously accumulated less H_2O_2 and decreased LR formation under both normal condition and NAA treatment, compared with wild-type (Supplementary Fig. S2). These results clearly suggested that there was a possible inter-relationship between HY1 and H_2O_2 in NAA-induced LR formation.

Removal of H_2O_2 prevents NAA-induced *HY1* expression and LR formation

In order to investigate the possibility of an interaction between HY1 and H_2O_2 in NAA-induced LR development, two membrane-permeable scavengers of H_2O_2, dimethylthiourea (DMTU) and ascorbic acid (AsA) were applied together with NAA. The results showed that, both DMTU (5 mM) and AsA (200 μM) dramatically blocked the induction of endogenous H_2O_2 contents triggered by NAA (Fig. 2a, b; Supplementary Fig. S3). Decreased levels of *HY1* transcript were also observed (Fig. 2c). Afterwards, NAA-triggered LR formation evaluated by LR density and length, was respectively impaired (Fig. 2d). It is noteworthy that, when applied alone, DMTU and AsA not only markedly inhibited H_2O_2 production, but also down-regulated *HY1* transcript and inhibited LR formation, suggesting that endogenous H_2O_2 might be necessary for LR formation under the normal growth conditions. However, a co-treatment of the seedlings with catalase (CAT), which can not permeate the epidermal layer of roots (Joo et al. 2001), had no effect on the LR formation, respect to the NAA-treated alone samples (Supplementary Fig. S4). The results obtained from the above suggested that removal of H_2O_2 prevents NAA-induced *HY1* expression and LR formation.

Previous results suggested that NADPH oxidase might be one of the potential sources of superoxide anion and even H_2O_2, and required for auxin-induced adventitious root formation (Bai et al. 2012). To assess the possible source of H_2O_2 in our experimental conditions, diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, was used. As expected, DPI at 5 μM, significantly prevented NAA-induced H_2O_2 production (Fig. 3a, b; Supplementary Fig. S3) as well as LR formation (Fig. 3c, d). Meanwhile, DPI-treated alone also decreased H_2O_2 content and LR formation,

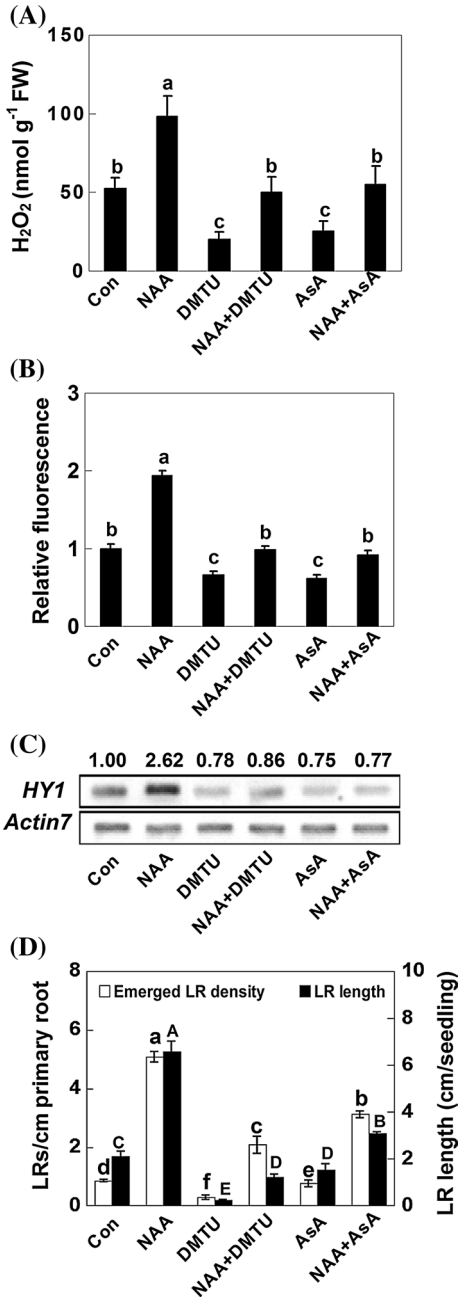


Fig. 2 Removal of H_2O_2 prevents NAA-induced *HY1* expression and LR formation. Five-day-old wild-type seedlings were treated with H_2O (Con), NAA (100 nM), DMTU (5 mM), AsA (200 μM) alone or their combinations. Afterwards, H_2O_2 content (spectrometer method, 6 h; **a**) and its production (LSCM detection, 6 h; **b**), *HY1* expression (semi-quantitative RT-PCR, 6 h; **c**), the emerged LR density and length (5 d; **d**) were determined. The number above the band (**c**) indicates relative expression levels of *HY1* with respect to Con, after normalization to *Actin7*. Mean and SE values were calculated from at least three independent experiments. Within each set of experiments, *bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

both of which were similar with inhibitory responses of AsA or DMTU treatment alone (Fig. 2). Above results clearly confirmed that NADPH oxidase might be, at least partially, responsible for the generation of auxin-induced H_2O_2, and involved in auxin-triggered LR formation thereafter.

Pharmacological and genetic evidence confirms that HY1 is involved in H_2O_2-induced lateral root formation

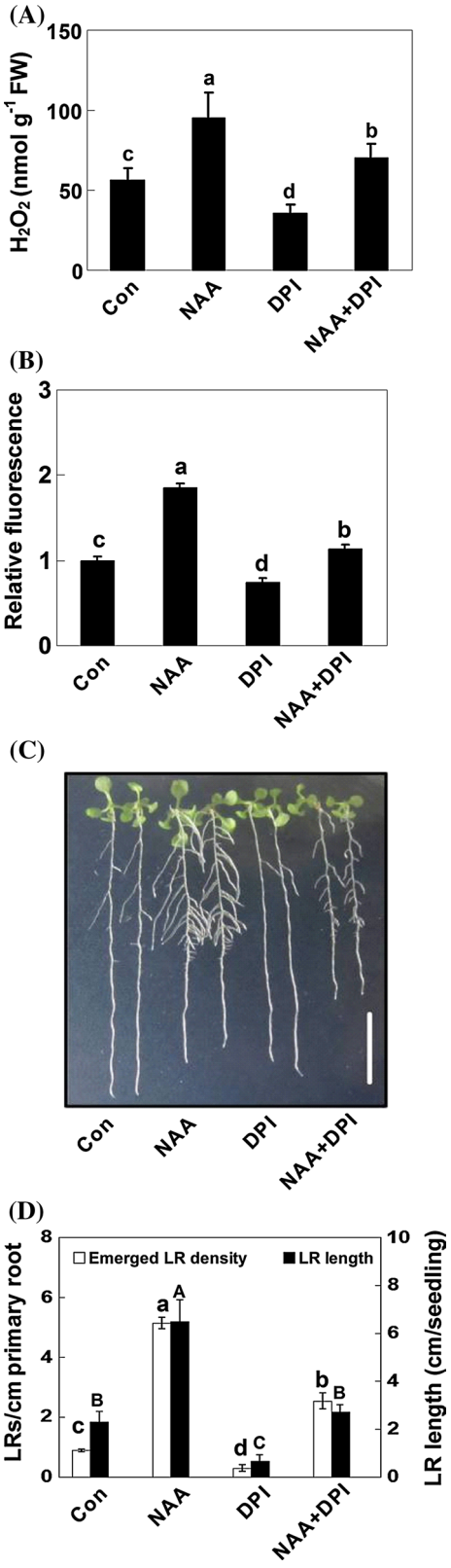
As expected (Wang et al. 2010), exogenous H_2O_2 with concentrations between 0.25 to 1 mM obviously triggered *Arabidopsis* LR formation in a concentration-dependent manner, with a maximal biological response at 0.5 mM ($P < 0.01$; Supplementary Fig. S5). Similarly, a dose-dependent induction of LR triggered by haemin, an inducer of HO-1, or carbon monoxide-releasing molecule-2 (CORM-2), the donor of CO, was also confirmed (Supplementary Fig. S6a, b). A maximal response was observed when 10 μM haemin or 20 μM CORM-2 was applied, respectively ($P < 0.01$). Genetic data demonstrated that in *hy1-100* mutant, supplementation with the HY1 inducer haemin failed to induce LR formation (Supplementary Fig. S6c). However, the phenotypes of *hy1-100* could be restored by the addition of CORM-2, respect to the wild-type plants. Additionally, no significant changes were observed when either Fe^{2+} or bilirubin (BR) was applied alone (Supplementary Fig. S7). Above genetic evidence confirmed that HY1 and its catalytic product CO could be responsible for LR development (Guo et al. 2008).

To further confirm a close link between HY1 and H_2O_2 in LR formation, genetic and pharmacological approaches were combined used. Subsequent work showed that, 0.5 mM H_2O_2 markedly up-regulated *HY1* transcript and LR formation, both of which were blocked by the addition of Znpp, an inhibitor of HO-1 (Fig. 4a, b). Znpp applied alone, also caused considerable decreases in *HY1* transcript and LR formation, in comparison with the control samples. Above results suggested that H_2O_2-induced LR formation might, at least partially, act in an HY1-dependent fashion.

Above deduction was further confirmed by genetic evidence. For example, *HY1* mutant *hy1-100* showed reduced sensitivity to 0.5 mM H_2O_2, since the mutant plants exhibited fewer and shorter lateral roots, compared with the strong inducible responses in the wild-type (Fig. 4c).

HY1 is also required for NAA-induced H_2O_2 production and LR formation

Our previous study showed the involvement of *Arabidopsis* HY1 in salt acclimation, which is caused and/or mediated



by RbohD-derived reactive oxygen species (ROS) synthesis (Xie et al. 2011). To further assess the putative interaction between HY1 and auxin-induced H_2O_2 production

◀ **Fig. 3** Effects of NADPH oxidase inhibitor DPI on NAA-induced H_2O_2 generation and LR formation. Five-day-old wild-type seedlings were treated with H_2O (Con), NAA (100 nM), DPI (5 μM) alone or their combinations. Afterwards, H_2O_2 content (spectrometer method, 6 h; **a**) and its production (LSCM detection, 6 h; **b**), LR phenotypes (*bar* = 1 cm) and corresponding parameters (5 days; **c**, **d**) were determined. Mean and SE values were calculated from at least three independent experiments. Within each set of experiments, *bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

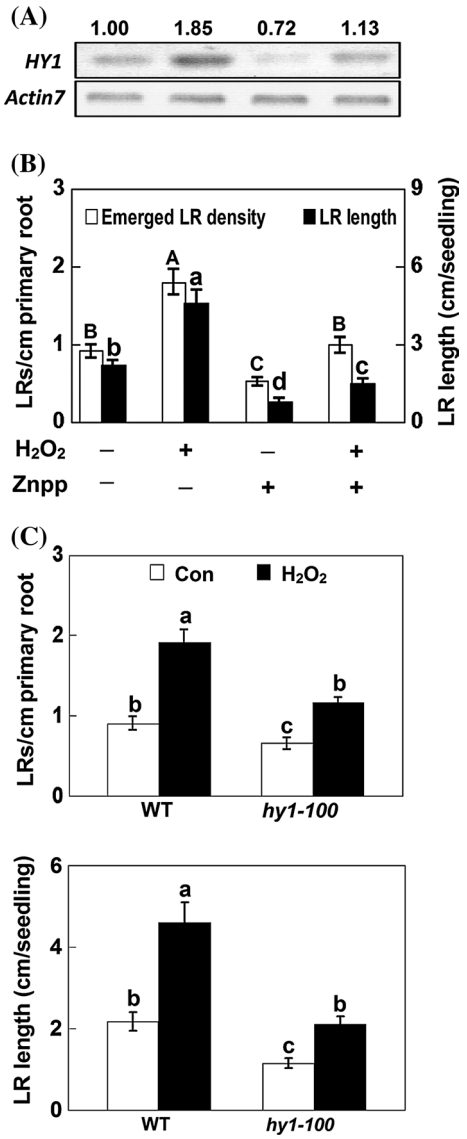


Fig. 4 Pharmacologic and genetic evidences reveal that HY1 is involved in H_2O_2-induced LR formation. Five-day-old wild-type and *hy1-100* mutant seedlings were treated with H_2O (Con), H_2O_2 (0.5 mM), Znpp (50 μM) alone or their combinations. Afterwards, *HY1* expression (semi-quantitative RT-PCR, 6 h; **a**), the emerged LR density and length (5 days; **b**, **c**) were determined. The number above the band (**a**) indicates relative expression levels of *HY1* with respect to the control sample, after normalization to *Actin7*. Mean and SE values were calculated from at least three independent experiments. Within each set of experiments, *bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

and LR formation, wild-type plants were treated with NAA together with Znpp. Haemin (10 μM) responses were recorded as a positive control. Experimental results showed that respect to the responses of NAA, haemin treatment led to a lesser degree in the induction of LR formation, while the effects of both on the induction of *HY1* transcripts and stimulation of H_2O_2 production were similar (Fig. 5). However, treatment with Znpp nearly fully arrested the NAA-induced *HY1* gene expression and H_2O_2 production, and blocked the induction of LR formation. The significant inhibitory responses also occurred in Znpp-treated alone samples. However, no additive effects were observed for haemin plus NAA treatment except an obvious increase in LR density.

Genetic evidence further showed that, *hy1-100* accumulated less H_2O_2 in response to NAA, compared to the wild-type (Fig. 6a, b; Supplementary Fig. S3), which was also consistent with the reduced LR formation in mutant plants (Fig. 6c). Incubation of wild-type seedlings with CORM-2 not only increased H_2O_2 production (Fig. 7a, b; Supplementary Fig. S3), but also stimulated LR formation (Fig. 7c). By contrast, these CORM-2-induced responses were sensitive to DMTU, a scavenger of H_2O_2. Together, these results clearly suggested that HY1 and one of its products, CO, at least partially, are required for H_2O_2 production and the development of LR in auxin signal transduction.

Both CO and H_2O_2 reverse the inhibition of LR formation achieved by NPA

Similar to previous reports (Casimiro et al. 2001), NPA, an auxin transport inhibitor, was able to prevent LR formation (Fig. 8). Both CORM-2 (20 μM) and H_2O_2 (0.5 mM) efficiently reversed the inhibitory effect of NPA pretreatment on LR formation, evaluated by the changes of LR primordium (LRP) density (Fig. 8b), LR density (Fig. 8c), and LR length (Fig. 8d), but to a lesser degree than those of NAA treatment. Additionally, we also noticed that NAA-, H_2O_2-, and CORM-2-induced LR primordia exhibited a similar anatomic structure (Fig. 8a). These results revealed that both CO and H_2O_2 could reverse the inhibition of LR formation achieved by NPA.

Changes of cell cycle regulatory genes

Previous experiment (Himanen et al. 2002) developed a NPA pretreatment-dependent synchronized LR induction system in *Arabidopsis*. Subsequent real-time RT-PCR analysis revealed that NAA (in particular), H_2O_2, and CORM-2 up-regulated the expression of representative cell cycle regulatory genes (Himanen et al. 2002, 2004), including *Histone H4*, *E2Fa*, *CYCB2;1*, and *CDKB1;1* genes, after 12 h of treatments; whereas, *KRP2* (encoding a CDK inhibitor) transcripts were down-regulated (Fig. 9). Above changing tendencies were differentially blocked when HY1 was inhibited by Znpp, or H_2O_2 was scavenged by DMTU, respectively. Significant

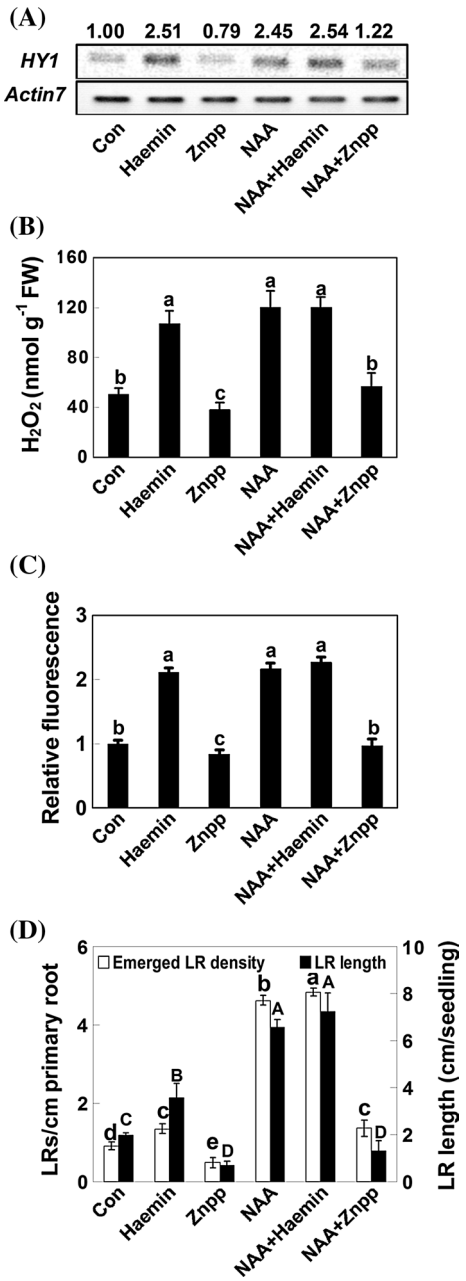


Fig. 5 NAA-induced *HY1* expression, H_2O_2 generation and LR formation are inhibited by Znpp. Five-day-old wild-type seedlings were treated with H_2O (Con), haemin (10 μM), Znpp (50 μM), NAA (100 nM) alone or their combinations. Afterwards, *HY1* expression (semi-quantitative RT-PCR, 6 h; **a**), H_2O_2 content (spectrometer method, 6 h; **b**) and its production (LSCM detection, 6 h; **c**), the emerged LR density and length (5 days; **d**) were determined. The number above the band (**a**) indicates relative expression levels of *HY1* with respect to Con, after normalization to *Actin7*. Mean and SE values were calculated from at least three independent experiments. Within each set of experiments, *bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

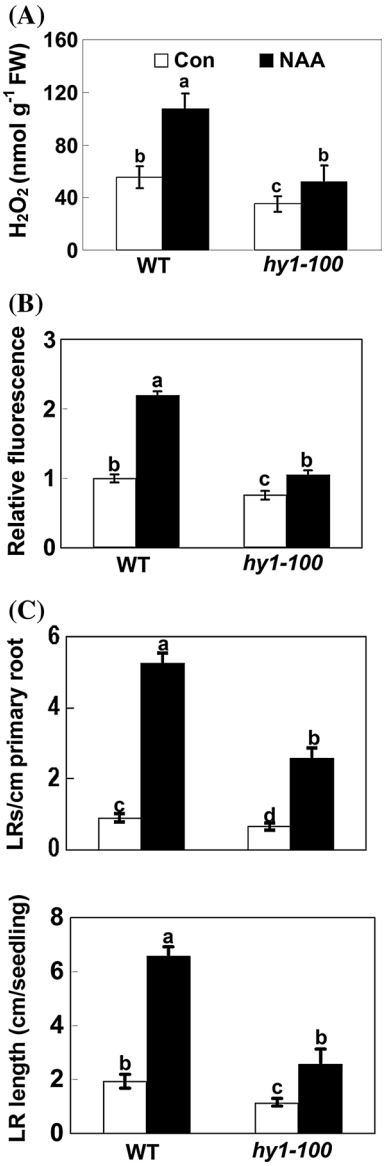


Fig. 6 NAA-induced H_2O_2 generation and LR formation are impaired in *hy1-100* mutant. Five-day-old wild-type and *hy1-100* mutant seedlings were treated with H_2O (Con) or NAA (100 nM). Afterwards, H_2O_2 content (spectrometer method, 6 h; **a**) and its production (LSCM detection, 6 h; **b**), the emerged LR density and length (5 days; **c**) were determined. Mean and SE values were calculated from at least three independent experiments. *Bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

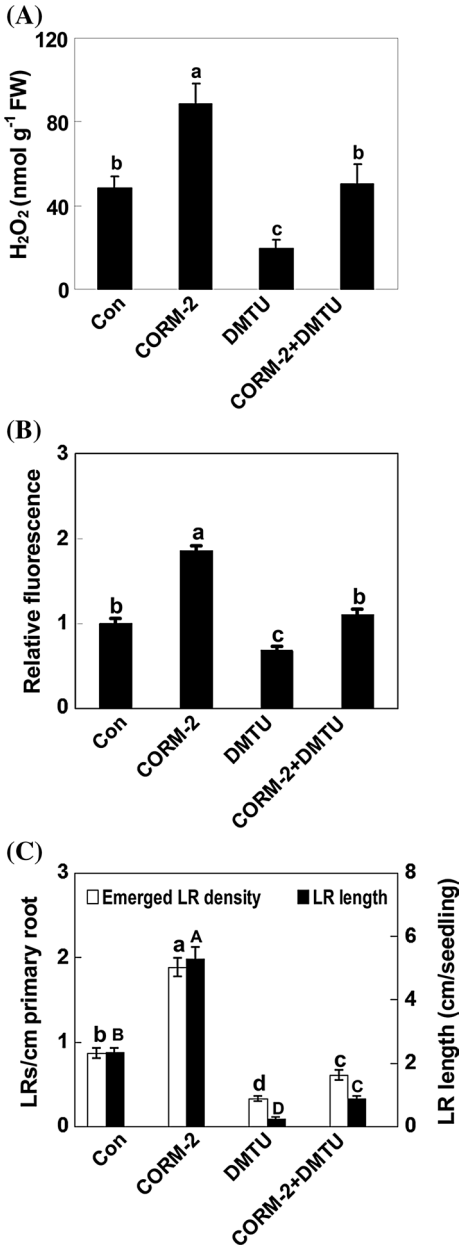


Fig. 7 CORM-2 promotes H_2O_2 generation and LR production. Five-day-old wild-type seedlings were treated with H_2O (Con), CORM-2 (20 μM), DMTU (5 mM) alone or their combinations. Afterwards, H_2O_2 content (spectrometer method, 6 h; **a**) and its production (LSCM detection, 6 h; **b**), the emerged LR density and length (5 days; **c**) were determined. Mean and SE values were calculated from at least three independent experiments. Within each set of experiments, *bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

decreases in *Histone H4*, *E2Fa*, *CYCB2;1*, and *CDKB1;1* transcripts and increases in *KRP2* transcripts were also observed in Znpp- or DMTU-treated alone samples. Genetic evidence further revealed that above increased or decreased transcript levels of cell cycle regulatory genes triggered by NAA or H_2O_2 treatment in wild-type, were attenuated in *hy1-100*. Moreover, the inducible effects of NAA on levels of *LBD16*, *LBD18*, and *LBD29* transcripts, which have biological functions in LR formation (Fukaki and Tasaka 2009; Feng et al. 2012), were also impaired in *hy1-100* respect to wild-type (Supplementary Fig. S8). These findings gave preliminary evidence and suggested that HY1 and endogenous H_2O_2 might occur downstream of auxin signaling leading to LR formation by modulating the expression of cell cycle regulatory genes, and LBDs might participate in this process.

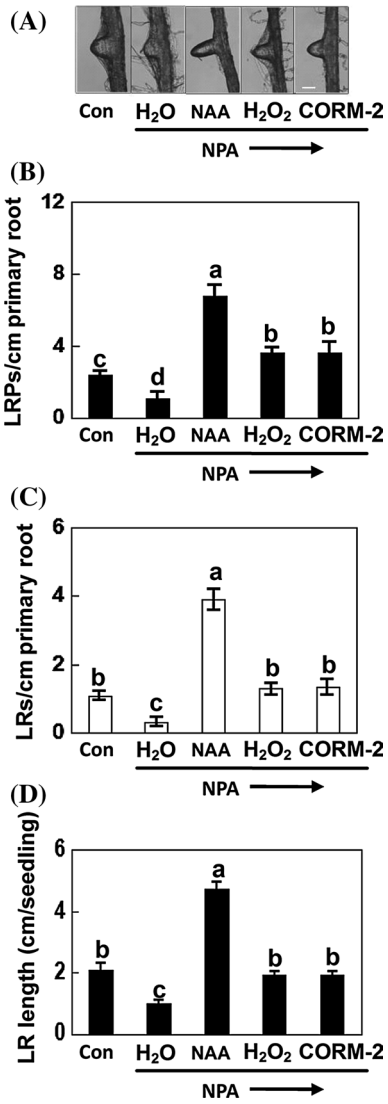


Fig. 8 Both H_2O_2 and CO rescue the auxin depletion-induced inhibition of LR formation. Three-day-old wild-type seedlings were incubated with NPA (10 μM) for 2 days, and further transferred to H_2O, NAA (100 nM), H_2O_2 (0.5 mM) or CORM-2 (20 μM) for 3 days. Afterwards, the corresponding photographs of LR primordia (LRP) were taken (*bar* = 100 μm; **a**), and the emerged LRP density was counted (**b**). After another 2 days, the emerged LR density (**c**) and length (**d**) was determined. Seedlings without chemical treatments were used as the control (Con). Mean and SE values were calculated from at least three independent experiments. *Bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

Discussion

Our pharmacological results previously revealed that HO-1 is involved in auxin-induced LR formation in rapeseed (Cao et al. 2007, 2011) and maize (Han et al. 2012). H_2O_2 is also required for auxin-induced adventitious root formation in mung bean (Li et al. 2009; Bai et al. 2012). However, to our knowledge, there is no direct report about whether H_2O_2 is clearly involved in auxin-induced LR formation. Moreover, the inter-relationship between HO-1 and H_2O_2 in auxin-mediated LR development is largely unclear.

A requirement for H_2O_2 in auxin-induced LR formation

First, we provided evidence for a previously uncharacterized role for H_2O_2 in auxin-induced LR formation. This conclusion is based on several pieces of evidence: (i) a rapid increase of H_2O_2 production triggered by NAA was obviously preceded the beginning of LR formation (Fig. 1b, d); (ii) exogenously applied H_2O_2 induced LR formation in a dose-dependent fashion (Supplementary Fig. S5), whereas removal of endogenous H_2O_2 by using its membrane-permeable scavengers DMTU and AsA, significantly blocked the induction of LR formation triggered by NAA (Fig. 2a, b, d); (iii) the inhibitory effect on LR formation induced by NPA, an auxin transport inhibitor, was partially restored by feeding with exogenous H_2O_2 (Fig. 8). Further evaluating the potential source of H_2O_2 revealed that auxin-induced H_2O_2 and thereafter LR formation could be, at least partially, attributed to NADPH oxidase activity. The effects of the NADPH oxidase inhibitor DPI on auxin-induced H_2O_2 and LR development were significant and implicated NADPH oxidase in these responses (Fig. 3). (iv) upon NAA treatment, auxin-response mutant *arf7 arf19* accumulated less H_2O_2 and decreased LR formation, respect to those in wild-type plants (Supplementary Fig. S2).

Although we can not exclude the possibility that the chemical agents used in the present investigation may not specifically target H_2O_2, our results collectively showed a casual inter-relationship between the endogenous H_2O_2 production and the induction of LR formation triggered by auxin in *Arabidopsis*. This conclusion is in agreement with the observation that H_2O_2 is involved in auxin-induced adventitious root formation (Li et al. 2009; Bai et al. 2012) and root gravitropism (Joo et al. 2001). However, the possibility of H_2O_2 functioning upstream of the auxin signaling pathway reported by Zhao et al. (2012), in which they found that auxin redistribution induced by cadmium is closely associated with H_2O_2, could not be easily ruled out.

Involvement of *HY1* up-regulation in auxin-induced LR formation

Similar to previous reports in rapeseed (Cao et al. 2007, 2011), rice (Chen et al. 2012), and maize (Han et al. 2012), further pharmacological and genetic evidence support a linear signal transduction cascade involving up-regulation of *HY1* and/or increased CO production downstream of auxin response in *Arabidopsis* LR formation.

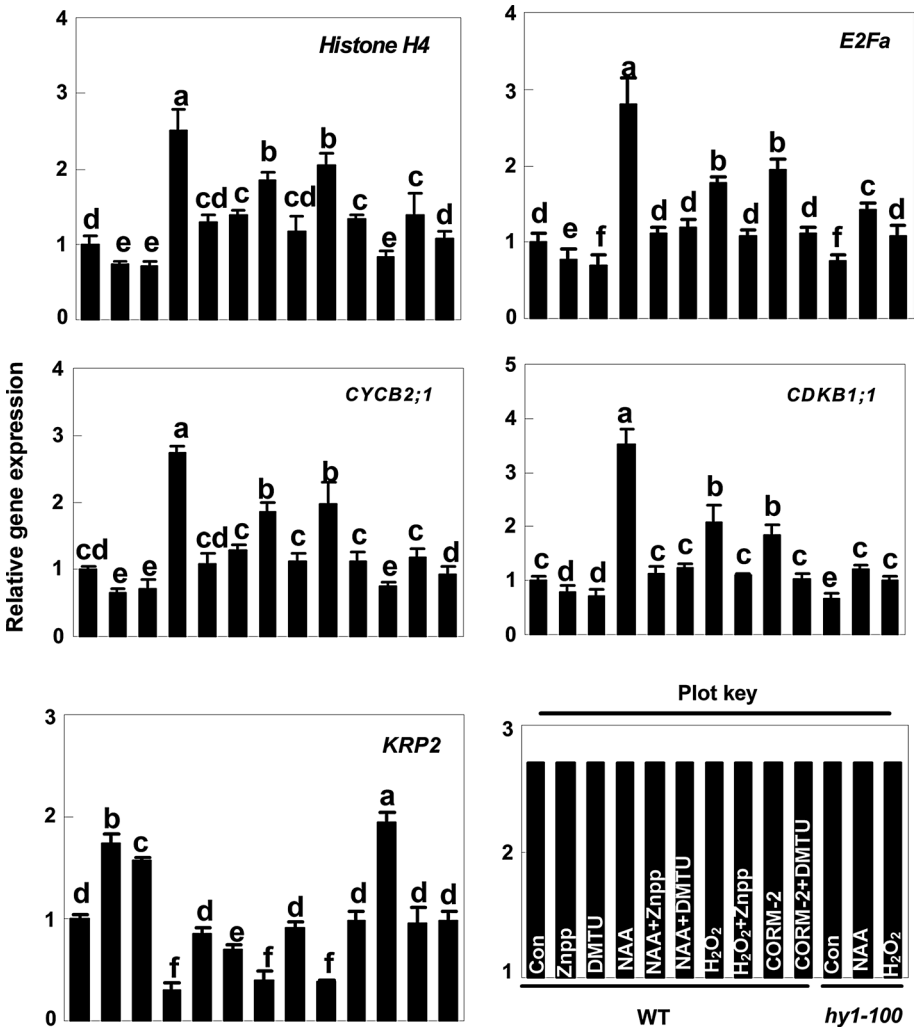


Fig. 9 Changes of cell cycle regulatory genes in a lateral root induction system (LRIS) described by Himanen et al. (2002). After germination, wild-type and *hy1-100* seedlings were incubated with NPA (10 μM) for 3 days, and then transferred to H_2O (Con), Znpp (50 μM), DMTU (5 mM), NAA (100 nM), H_2O_2 (0.5 mM), CORM-2 (20 μM) alone or their combinations. After 12 h of various treatments, the indicated gene expression was determined by real-time RT-PCR, and presented as values relative to Con, after normalization to *Actin7*. Plot key illustrated each *bar* shown in the figure. Mean and SE values were calculated from at least three independent experiments. *Bars* with *different letters* indicate statistical difference at $P < 0.05$ according to Tukey's multiple range test

The following results support this conclusion: (i) NAA-induced *HY1* transcripts is one of the earliest responses during 12 h of treatment. Afterwards, the appearance of LR was observed (Fig. 1b, c); (ii) when *Arabidopsis* seedlings were co-treated with NAA and the HY1 inhibitor Znpp, the induction of *HY1* transcript and LR formation were approximately reversed (Fig. 5a, d); (iii) among *hy1-100*, *ho2*, *ho3*, and *ho4* mutants, the *hy1-100* mutant was hypersensitive to NAA in the induction of LR formation (Supplementary Fig. S9). (iv) CORM-2, an effective donor of CO, partially reversed the inhibitory effect of NPA pretreatment on LR formation (Fig. 8). On the basis of these findings, we conclude that NAA responses, at least partially, dependent on the up-regulation of *HY1*.

Our conclusion is consistent with the results reported by Guo et al. (2008), in which they reported *LeHO-1* loss-of-function tomato mutant *yg-2* exhibiting a phenotype of impaired LR development, which could be restored by exogenously applied CO. However, they also suggested CO-induced auxin signaling in LR formation process. Therefore, it remains to be elucidated how auxin interacts with HY1/CO in the signaling pathway leading to LR development.

An interaction between HY1 and H_2O_2 in auxin signaling

It is well established that HO-1/CO and H_2O_2 in plants usually have similar physiological roles. Therefore, it is most likely that there existed an interaction between HY1 and H_2O_2 in auxin response leading to LR formation.

In the subsequent study, *HY1* up-regulation in response to H_2O_2 in *Arabidopsis* seedlings is demonstrated (Fig. 4a), and vice versa (Figs. 5b, c, 7a, b). Similar inducible responses were observed in soybean (Yannarelli et al. 2006), wheat (Chen et al. 2009), *Arabidopsis* (Xie et al. 2011), and rice (Chen et al. 2013). Importantly, these processes were correlated to the biological response of LR formation (Figs. 4b, 5d, 7c). In fact, the up-regulation of *HY1* gene expression in response to H_2O_2 may explain how H_2O_2 promoted lateral and adventitious root formation in

soybean (Su et al. 2006), cucumber (Li et al. 2007), mung bean (Li et al. 2009), and Arabidopsis (Wang et al. 2010), because HO-1/CO has been confirmed to be a novel inducer of root organogenesis (Xuan et al. 2008).

Using pharmacological and genetic approaches, we revealed that H_2O_2 synthesis is, at least partially, required for auxin-induced HY1-mediated LR formation. Firstly, time-course analysis revealed that NAA could simultaneously stimulate *Arabidopsis HY1* gene expression and H_2O_2 generation. Subsequently, LR formation was induced (Fig. 1b–d). NAA-induced *HY1* gene expression and thereafter LR formation were sensitive to two H_2O_2 scavengers, DMTU and AsA (Fig. 2). H_2O_2-induced *HY1* transcript and LR formation were strongly down-regulated by Znpp (Fig. 4a, b). These results clearly suggested that HY1 is involved in auxin-induced H_2O_2-mediated LR formation. Similar results were also suggested in rice seedlings (Chen et al. 2013). Further genetic evidence revealed that seedlings of *hy1-100* mutant subjected to H_2O_2 treatment, displayed impaired LR formation, respect to the wild-type plants (Fig. 4c).

On the other hand, both NAA-induced H_2O_2 generation and LR formation required the participation of HY1. For example, the HO-1 inhibitor Znpp, which could decrease *HY1* expression (Fig. 5a), obviously blocked NAA-induced H_2O_2 production (Fig. 5b, c) and thereafter LR formation (Fig. 5d). In comparison with the wild-type, *hy1-100* mutant was less sensitive to NAA-triggered H_2O_2 production (Fig. 6a, b) and LR formation (Fig. 6c). CORM-2 resulted in the inducible effects on H_2O_2 production and LR formation, both of which could be abolished by the scavenging of H_2O_2 with DMTU (Fig. 7). This result parallels the situation encountered in animals, in which oxidative stress could induce CO production, in turn, CO-dependent H_2O_2 production through both mitochondrial and non mitochondrial played an important role in signal transduction (Piantadosi 2008). In *Arabidopsis* salt acclimation, HY1-mediated ROS formation is also needed (Xie et al. 2011). Combined with the present data, we suggested that there is a close interaction between HY1 and H_2O_2 involved in auxin-induced LR formation.

Previous study (Himanen et al. 2002) proved that during early LR initiation, auxin mediates cell cycle reactivation through regulating the expression of multiple cell cycle genes. Consistent with this, mimicking the effects of NAA, both H_2O_2 and CORM-2 treatments for 12 h increased the levels of *Histone H4*, *E2Fa*, *CYCB2:1*, *CDKB1:1* transcripts; while the expression of *KRP2*, one of CDK-inhibitory proteins, was down-regulated (Fig. 9). The effects of NAA, H_2O_2 and CORM-2 were impaired by Znpp and DMTU, respectively. Genetic evidence further revealed that above increased or decreased transcript levels of cell cycle regulatory genes triggered by NAA or H_2O_2 in wild-type, were attenuated in *hy1-100*. Therefore, our results clearly demonstrated that both H_2O_2 and CO were important in the early LR initiation, and cell cycle regulatory genes might be the target genes of the actions of H_2O_2 and CO triggered by auxin, thus leading to LR development.

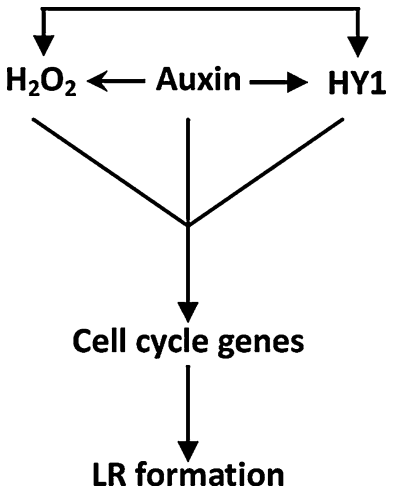


Fig. 10 Schematic representation of a proposed model for regulating LR formation by auxin involving HY1 and H_2O_2. Auxin induces up-regulation of *HY1* and generation of H_2O_2, and then enhances LR formation through regulating related cell cycle regulatory genes expression. Especially, there exists an interaction between H_2O_2 and *HY1*

In summary, the present study suggested that both HY1 and H_2O_2 act as downstream signal of auxin to induce *Arabidopsis* LR formation through the modulation of related cell cycle regulatory genes. This signal transduction pathway is not linear, and the interaction between HY1 and H_2O_2 plays an essential role in this process (Fig. 10). Moreover, taking into account that auxin, HO-1, and H_2O_2 participate in both development process and stress responses, this study may extend our understanding of the complex system integrating developmental and environmental signals.

Acknowledgments This work was supported by the Fundamental Research Funds for the Central Universities (grant no KYJ200912), and the National Natural Science Foundation of China (J1210056 and J1310015).

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