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Full Length Research Paper

Nitric oxide interacts with reactive oxygen species to regulate oligosaccharide-induced artemisinin biosynthesis in *Artemisia annua* hairy roots

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This work was to characterize the generation of nitric oxide (NO) and reactive oxygen species (ROS) in *Artemisia annua* roots induced by an oligosaccharide elicitor (OE) from *Fusarium oxysporum* mycelium and their relationship with artemisinin production. In the hairy root cultures, the OE at 0.3 mg total sugar/ml evoked a rapid production of nitric oxide synthase (NOS)-dependent NO and hydrogen peroxide (H_2O_2). The NO donor, sodium nitroprusside (SNP) potentiated OE-induced H_2O_2 production. The OE-induced NOS activity was suppressed by NADPH oxidase inhibitor diphenylene iodonium (DPI) and H_2O_2 scavenger catalase (CAT), while the induced NADPH oxidase activity was decreased by the NOS inhibitor and NO scavenger. Moreover, the OE-induced NO and ROS were involved in stimulating the artemisinin accumulation in the hairy roots. These results suggest that NO and ROS could play a role in the elicitor-induced responses and secondary metabolism activities in *A. annua*.

Key words: Artemisinin, hairy roots, nitric oxide, oligosaccharide elicitor, reactive oxygen species.

INTRODUCTION

Nitric oxide (NO) is a free gaseous radical with a wide variety of physiological implications in animal and plant cells (Lamattina et al., 2003; Neill et al., 2003). Several recent studies have suggested its involvement in the regulation of plant growth, development and defense responses (Flores et al., 2008; Hong et al., 2008). As NO production in plant tissues and cells usually occurs in response to pathogen invasion (Delledonne et al., 1998) and challenges by fungal elicitors (Foissner et al., 2000) and abiotic stresses (Garcês et al., 2001), its most possible and prominent role is signaling and regulating plant defense or stress responses. It is widely believed that the synthesis of secondary metabolites in plants is part of the defense responses of hosts to elicitor and pathogenic attack. Recently, some studies have shown that NO is involved in fungal elicitor-induced production of secondary metabolites such as ginseng saponin (Hu et al., 2003), hypericin (Xu et al., 2005) and puerarin (Xu et al., 2006). These observations suggest the existence of a NO-mediated signaling pathway in biosynthesis of induced secondary metabolites in plant cells. In our previous reports (Wang et al., 2001; 2002), oligo- or polysaccharides derived from endophytic fungi have been tested for the elicitation of artemisinin production in hairy root cultures of *Artemisia annua*. Furthermore, the oligosaccharide-induced artemisinin production could be potentiated by the NO donor sodium nitroprusside (SNP) (Zheng et al., 2008).

On the other hand, production of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) during so-called “oxidative burst” has been considered as a central event in triggering of plant defense responses (Brausegem et al., 2001). We showed earlier, that *A. annua* hairy roots responded to the oligosaccharide elicitor with an induced peroxidase

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activity reaching maximum activity 1 d after the treatment (Wang et al., 2002). As peroxidase catalyzes the oxidation of a variety of electron donor substrates by H_2O_2 (Passardi et al., 2004), the induced peroxidase in *A. annua* hairy roots indicated the possible involvement of ROS burst. In addition, NO has been shown to induce or potentiate some of the responses which are mediated by ROS signal, such as defense gene activation, the hypersensitive cell death and phytoalexin biosynthesis (Delledonne et al., 1998; Durner et al., 1998). An increasing number of reports suggest that ROS generation is accompanied by NO synthesis and that NO collaborates with ROS in plant disease resistance initiation and execution (Delledonne et al., 2001; Hong et al., 2008). However, there has been so far neither report concerning the simultaneous burst of NO and ROS in elicited *A. annua*, or regarding the intercross role of those free radicals on artemisinin biosynthesis.

Artemisinin, an endoperoxide sesquiterpene lactone isolated from *A. annua* L. (Asteraceae), is a potential drug effective against multidrug resistant strains of malarial parasite, *Plasmodium* (Klayman, 1985). Since its chemical synthesis is difficult and expensive, the plant sources including cultures of shoots and hairy roots have been considered an attractive alternative in the production of this secondary metabolite (Woerdenbag et al., 1993; Weathers et al., 1994). The enhanced production of artemisinin either in hairy roots or in the whole plant of *A. annua* is highly desirable. Therefore, as a follow-up to our previous characterization of exogenous NO action (Zheng et al., 2008) and fungal elicitor (Wang et al., 2001; 2002), the present work was carried out to investigate NO production induced by oligosaccharide olicitor (OE) from *Fusarium oxysporum* mycelium and its relationship with H_2O_2 production. The intercross role of both signal molecules on artemisinin biosynthesis was elucidated with the aid of exogenous donors and specific inhibitors.

MATERIALS AND METHODS

Plant cell and hairy root cultures

Transformed roots of *A. annua* were established by co-culture of leaf discs with *Agrobacterium rhizogenes* strain R1601 (Liu et al. 1997). For experiments in flasks, 0.1 g hairy roots were incubated in 50 ml hormone-free Murashige and Skoog medium (MS) containing 3% (w/v) sucrose in 250 ml Erlenmeyer flasks and grown at 25°C on a rotary shaker (120 - 130 rpm) under 16 h light (100 µmol photons/m^2 s) per day. An *A. annua* cell line was induced from hairy roots (Baldi and Dixit, 2008). To establish the suspension cultures in 125 mL Erlenmeyer flasks, 3.0 g fresh weight of cells from the solid culture were transferred to 25 ml liquid MS medium with 3% (w/v) sucrose and 0.1 mg/l of each of 1-naphthaleneacetic acid and 6-furfurylaminopurine. The flasks were incubated on a rotary shaker (120 - 130 rpm) under 16 h light (100 µmol photons/m^2 s) a day at 25°C. Cells from 21 day old suspension culture were used for further experiments.

The experiments for studying the eliciting responses of *A. annua* hairy roots were all conducted in roots collected from the shake-flask culture. The fresh weight (FW) of roots in the culture was obtained by filtration through a Whatman filter paper under vacuum, and the dry weight (DW) by drying the fresh roots at 50°C in an oven until constant weight.

Elicitation and inhibition experiments

The fungal oligosaccharide elicitor was extracted from the mycelium of a root pathogenic fungus, *F. oxysporum* f. sp. *niveum* as described previously (Zheng et al., 2008). OE at 0.3 mg total sugar/ml was added to the culture on day 20 post inoculation which was amid a rapid growth phase, a favorable time for elicitor treatment (Wang et al., 2001).

Sodium nitroprusside (SNP) was used as an NO donor and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) as an NO scavenger. Nω-nitro-L-arginine methyl ester (L-NAME) was used as the NO synthase (NOS) inhibitor. On the other hand, exogenous H_2O_2 and $O_2^{\bullet-}$ generator (xanthine + xanthine oxidase, X + XO) were used as ROS donors. NADPH oxidase inhibitor diphenylene iodonium (DPI) and a scavenger of H_2O_2, catalase (CAT), were used to pretreat hairy root cultures before the elicitation. They were all prepared at 50 - 100 times of the final concentrations in the culture and sterilized by filtration.

For tests on the effect of NO on OE-induced responses and artemisinin synthesis, SNP (50 µM), L-NAME (500 µM), cPTIO (500 µM) were added to the culture at 30 min prior to the OE treatment. Their dosage used in the experiments was chosen based on our previous study (Wang and Wu, 2004). For tests on the effect of ROS, the scavenger CAT (1 unit/ml) and NADPH oxidase inhibitor DPI (50 µM) were applied to the culture at 30 min prior to the OE treatment. 25 mM H_2O_2 and $O_2^{\bullet-}$ generator (0.5 mM X + 1 unit XO) were added at the same time with the OE application, respectively. Their dosages used in the experiments were chosen based on the previous reports (de Pinto et al., 2006; Zhao et al., 2007). Hairy root cultures were harvested 4 days after the final elicitor treatment (on day 24) for measurement of root weight and artemisinin content. All treatments were performed in triplicate and results are represented by their mean ± standard deviation (SD).

Measurement of OE-induced NO production

NO concentration in cells was quantified by binding 4,5-diaminofluorescein diacetate, (DAF-2 DA, Sigma) in a fluorometric assay as described by our previous report (Wang and Wu, 2004). NO production in the cells (intracellular) was also observed by fluorescence microscopy on an Olympus BX51 microscope mounted with an exciter filter BP 460 - 490 nm and a barrier filter BA 515 nm.

Measurement of OE-induced hydrogen peroxide

Hydrogen peroxide (H_2O_2) in the culture medium was measured by luminol chemiluminescence as described by Wang and Wu (2005) on a luminescence analyzer (BPCL Ultra-weak, Beijing, China). In brief, 50 ml of sample medium was mixed with 750 ml of phosphate buffer (0.05 M, pH 7.9), followed by auto-injection of 200 µl of luminol (0.3 mM in phosphate buffer) and 100 ml of $K_3[Fe(CN)_6]$ (14 mM in water). Fluorescence intensity was recorded after the last injection at an integration time of 5 s, and the intensity value was calibrated to the actual H_2O_2 concentration with pure H_2O_2 liquid.

Enzyme extraction and assays

NOS activity determination was performed according to Murphy and

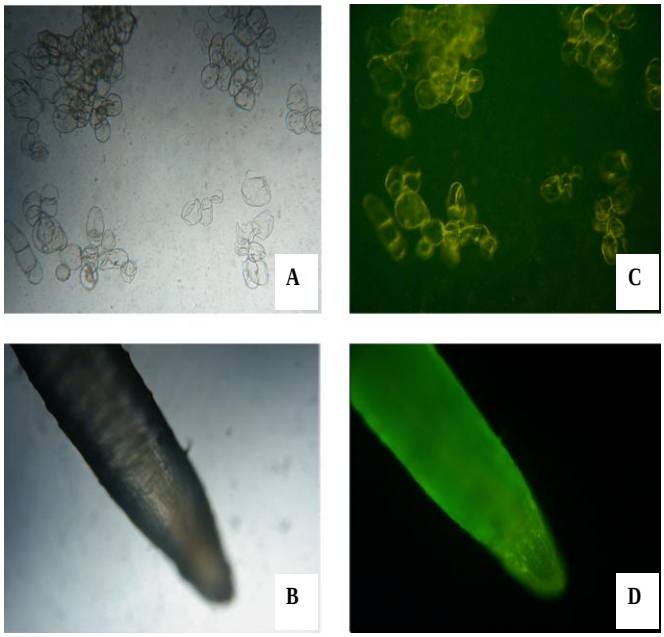

Figure 1. Bright-field image of elicitor-treated *A. annua* cells (A) and hairy roots (B); fluorescence microscopy (460 - 490 nm excitation and 515 nm emission) of DAF-2 DA-stained cells (C) and hairy roots (D) after 30-min elicitor treatment (at 0.3 mg total sugar/ml). All cells and hairy roots were from 20-day-old shake-flask cultures.

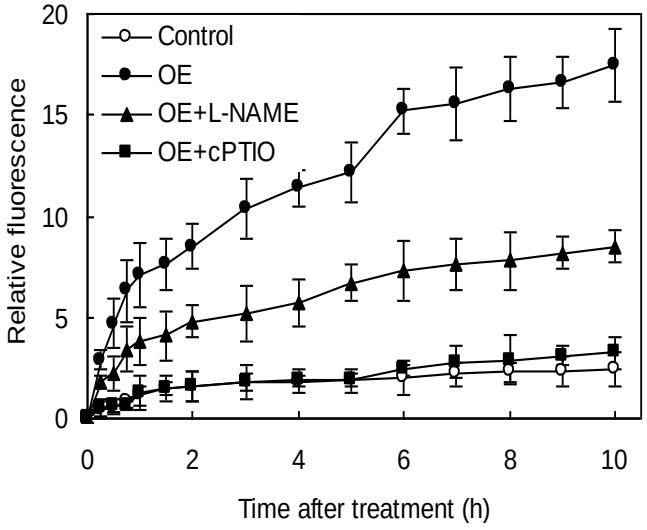

Figure 2. Time course of oligosaccharide elicitor (OE)-induced NO accumulation in hairy root cells of *A. annua.* OE at 0.3 mg total sugar/ml was added to 20 day-old cultures. L-NAME (500 µM) and cPTIO (500 µM) were added 30 min before the addition of OE. Control received the same volume of water only. Values are means of triplicate results and error bars show standard deviations.

Noack (1994) with some modifications. About 1 g of roots was homogenized in 2 mL of homogenization buffer (50 mM triethanolamine hydrochloride (pH 7.5) containing 0.5 mM EDTA, 1 mM leupeptin, 1 mM pepstatin, 7 mM gluathione, and 0.2 mM phenylmethylsulfonyl fluoride). After centrifuging at 10,000 × g for 20 min (4°C), the supernatant was collected and recentrifuged at 100,000 × g for 45 min. The supernatant was used for NOS determination. NOS activity was analyzed by haemoglobin assay as previously described (Murphy and Noack, 1994). Protein concentration was determined as described by Bradford (1976).

The extration and assay on NADPH oxidase followed the method reported by Martinez et al. (1998). Fresh mass of cells (200 mg) from the suspension cultures was homogenized in 50 mM sodium phosphate buffer (pH 6.0) containing 1% polyvinylpolypyrrolidone and 10 mM β-mercaptoethanol. The homogenate was then centrifuged at 12,000 g for 30 min at 4°C, and the supernatant was collected for the enzyme assay. Each 50 µl of the extract was mixed with 2 ml of assay buffer (50 mM sodium phosphate buffer, pH 6.0, and 0.1 mM magnesium chloride) containing 100 µM NADPH in a quartz cuvette and measured at 340 nm for 3 min. The enzyme activity (nmol of NADPH oxidized/g FW/min) was calculated using a millimolar extinction coefficient of 6.22 for NADPH at 340 nm.

Analysis of artemisinin and artemisinic acid content

The extraction and analysis of artemisinin from hairy roots was based on the procedure as described by our previous report (Wang et al., 2001). The isolation and determination of artemisinic acid from hairy roots was performed according to the method of Zhang et al. (2005). Calibration curves were made with a genuine standard (Sigma) and artemisinic acid kindly supplied by Prof. L Yang (Chinese Materia Medica Institute , Chinese Academy of Medical Sciences).

RESULTS

Oligosaccharide elicitor-induced NO burst in *A. annua* hairy root cultures

The fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA) has been widely used to identify and quantitatively measure NO production in plants or cell cultures (Foissner et al., 2000; Zhao et al., 2007). The OE-induced NO production in the *A. annua* cell and hairy roots could be directly observed by fluorescence microscopy, showing the green fluorescence of DAF-2 DA-stained cells (Figure 1). Figure 2 shows the time courses of fluorescence intensity in the DAF-2 DA-stained hairy root cells after various treatments by OE and NO-related reagents. OE-treated cells showed a stronger signal of NO production than nontreated cell cultures. The induced fluorescence increase was effectively blocked by the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) at 500 µM (Figure 2), proving that the fluorescence increase detected in the cells was mainly a result of NO production. When pretreated with N_{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS activity, NO production decreased correspondingly, suggesting the occurrence of a NOS-like enzyme in *A. annua* cells after OE treatment.

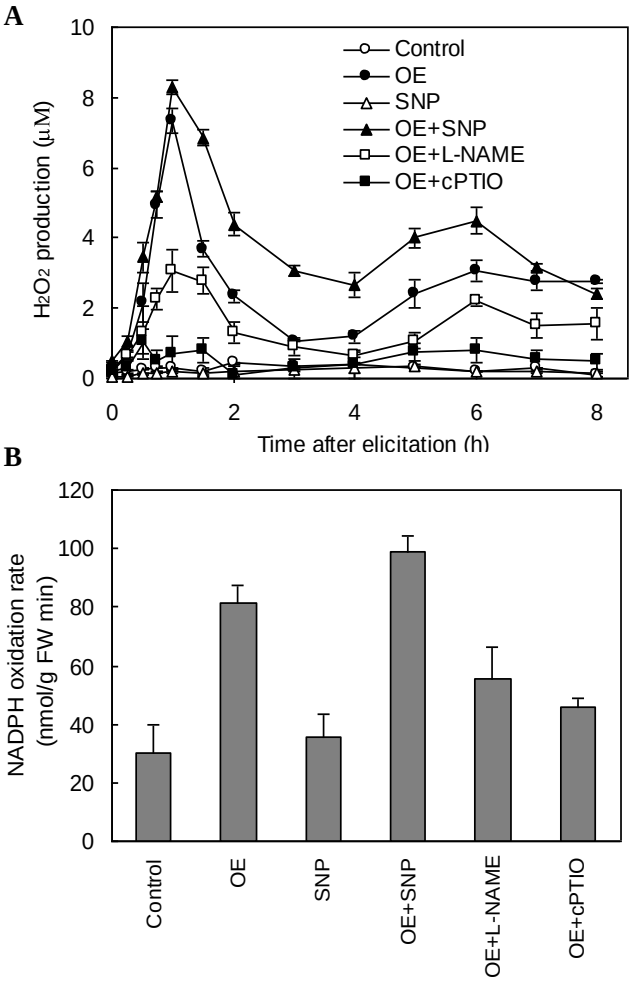

Figure 3. Elicitor-induced H_2O_2 generation (A) and NADPH oxidase activity (B) in *A. annua* hairy root cultures (same procedure and dosage as specified in Figure 2). SNP (50 μM) were added 30 min before the addition of OE. NADPH oxidase activity was determined 4 d after the treatments. Values are means of triplicate results and error bars show standard deviations.

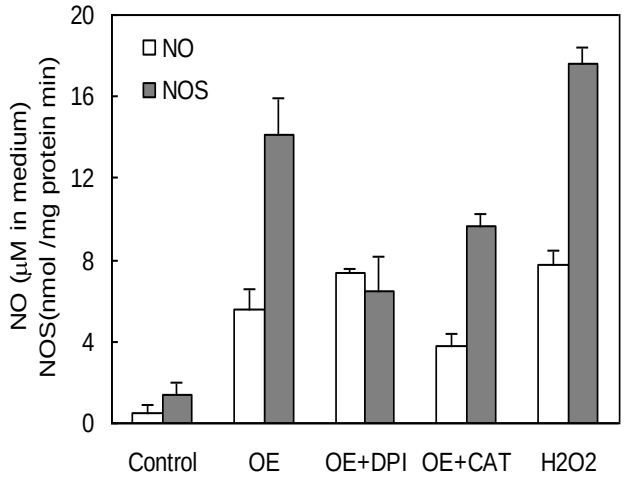

Figure 4. Effect of reactive oxygen species (ROS) on OE-induced NO and NOS activity in *A. annua* hairy roots. OE at 0.3 mg total sugar/ml was added to 20 day-old cultures for treatment of 4 days. CAT (1 unit/ml), DPI (50 μM) were applied to the culture at 30 min prior to the OE treatment. 25 mM H_2O_2 was added at the same time with the OE application. Control received the same volume of water only. Values are means of triplicate results and error bars show standard deviations.

OE-induced oxidative burst in *A. annua* hairy root cultures

The OE treatment induced rapid production of H_2O_2 in *A. annua* hairy root cultures, reaching a sharp peak of 7.3 μ M around 1 h, and another lower and broad peak (3.1 μ M) around 6 h (Figure 3A). The OE-induced H_2O_2 production was decreased by both NO inhibitors, L-NAME and cPTIO. The NO donor SNP at 50 μM in the absence of OE did not induce any H_2O_2, while its combination with OE resulted in a significant increase in the H_2O_2 production (OE+SNP versus OE). As shown in Figure 3B, OE-treated roots had higher NADPH oxidase activity than the controls and SNP stimulated OE-induced increase of NADPH oxidase activity. However, the pretreatment of L-NAME and cPTIO result in the abolishment of the increase of NADPH oxidase activity in the OE-treated roots. The results are indicative of the relationship between NO and the OE-induced oxidative burst.

Effect of ROS scavenger, NADPH oxidase inhibitor and H_2O_2 on NO burst in OE-induced *A. annua* hairy root cultures

To test whether OE-induced ROS production is required for a NO burst in *A. annua* hairy root cultures, we measured NO production and NOS activity in elicitor plus ROS scavenger CAT or NADPH oxidase inhibitor DPI or exogenous H_2O_2 alone-treated root cultures. As shown in Figure 4, both H_2O_2 and OE treatment significantly stimulated NOS activity and NO generation. However, both DPI and CAT treatments reduced OE-induced NOS production, whereas DPI promoted NO production slightly. These results suggest that ROS affect NO production in OE-treated *A. annua* hairy root cultures.

OE-induced artemisinin biosynthesis in *A. annua* hairy root culture

The OE stimulated artemisinin content in hairy roots to 1.0 mg/g DW, a 66.7% over the control after 4 days of treatment. Meanwhile, the content of artemisinic acid, the precursor of artemisinin, was decreased by 70.6 % to 2.5 mg/g DW during the elicitation (Figure 5). Although the

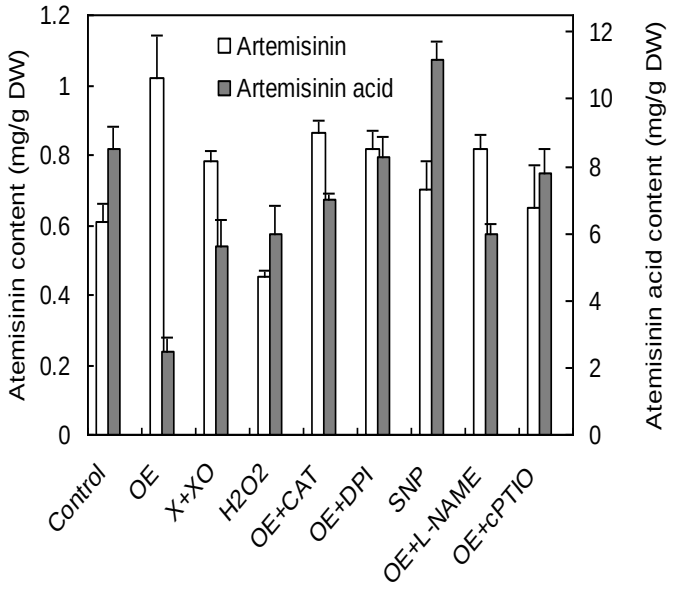

Figure 5. Effect of reactive oxygen species (ROS) and NO on OE-induced artemisinin and artemisinic acid contents. Artemisinin NO and NOS activity in *A. annua* hairy roots. The $O_2^{\bullet-}$ generation system (0.5 mM xanthine + 1 unit xanthine oxidase, X + XO) was added simultaneously with the OE application. The same procedure and dosage as specified in Figure 3, 4. Values are means of triplicate results and error bars show standard deviations.

NO donor SNP slightly but significantly stimulated the content of artemisinic acid, it had no effects on artemisinin contents of the hairy roots. Moreover, the OE-induced artemisinin accumulation was significantly repressed by both the NOS inhibitor L-NAME and the NO scavenger cPTIO, whereas the OE-induced reduction of artemisinic acid production was alleviated by those NO inhibitors. This set of results is indicative of the involvement of endogenous NO in artemisinin biosynthesis during the elicitation of fungal oligosaccharide.

To test whether OE-induced ROS production is required for artemisinin biosynthesis in *A. annua* hairy root cultures, NADPH oxidase inhibitor DPI and the scavenger of H_2O_2 CAT were used to pretreat cell cultures before the elicitation. Exogenous H_2O_2 and $O_2^{\bullet-}$ generator (xanthine + xanthine oxidase, X + XO) were used for investigation on the effect of ROS. As shown in Figure 5, H_2O_2 treatment reduced the contents of both artemisinin and artemisinic acid, whereas low dose of $O_2^{\bullet-}$ (0.5 mM X + 1 unit XO) increased artemisinin content but reduced artemisinic acid content. When OE was applied to root cultures, DPI and CAT treatments all reduced elicitor-induced artemisinin accumulation but increased artemisinic acid content, suggesting that the OE-induced artemisinin biosynthesis requires endogenous $O_2^{\bullet-}$ and H_2O_2 production.

DISCUSSION

Fungal oligosaccharides have been reported previously to induce multiple defense responses in plants, including the ROS production, lipid peroxidation, ion fluxes, ethylene synthesis, hypersensitive cell death, phytoalexin accumulation and expression of pathogenesis-related (PR) genes (Heath, 2000). Although there have been some reports regarding the effects of chitosan oligosaccharides on the synthesis of nitric oxide in macrophages and human umbilical vein endothelial cells (Yu et al., 2004; Liu et al., 2009), few reports dealing with NO production induced by oligosaccharide in plants could be found. Leitner et al. (2008) reported that pathogen-derived β-(1,3)-β-(1,6)-glucan elicitor and symbiotic signalling oligosaccharides (Nod-factors) could induced NO accumulation in stems of *Medicago truncatula*. Our previous study showed that oligosaccharide fractions from *F. oxysporum* mycelium could stimulate NO production in *A. annua* hairy roots (Zheng et al. 2008). More importantly, our present study demonstrated the elicitor-induced NO production and its interacting with oxidative burst as well as their different ways of affecting on artemisinin biosynthesis. To the best of our knowledge, this is the first report to demonstrate the distinct functions and interrelationships between NO and ROS in plant elicited by fungal oligosaccharides.

Our present study indicates that, the OE-induced NO production partly depended on H_2O_2 production, but negatively affected by $O_2^{\bullet-}$ accumulation (OE+DPI in Figure 4). The treatment of exogenous H_2O_2 also demonstrated the stimulation on the NOS activity. Moreover, the OE-induced NOS activity was partially suppressed by both the H_2O_2 scavenger CAT and $O_2^{\bullet-}$ inhibitor DPI. This suggests that ROS is implicated in mediating the elicitor-induced NO production. In plant guard cells, it has been shown that H_2O_2 treatment can induce NO synthesis (He et al., 2005). ROS were identified as the upstream signals that lead to NO production in guard cells during stomata closure induced by chitosan in abaxial epidermis of *Pisum sativumin* (Srivastava et al., 2009). On the other hand, although NO generated by the NO donor SNP could not induce any enhancement of the activity of NADPH oxidase, the stimulation of OE-induced H_2O_2 production by NO inhibitors observed in our study suggests that intracellular NO plays a regulatory role in the oxidative burst (Figure 3). The inhibition of H_2O_2-scavenging enzymes such as CAT, ascorbate peroxidase (APX) activity and the stimulation of H_2O_2-producing enzyme guaiacol peroxidase (POD) by NO could be attributed to the enhancement of elicitor-induced H_2O_2 production (Małolepsza and Różalska, 2005; Zhao et al., 2007). The mutual stimulation between NO and H_2O_2 during elicitation in this study are consistent with other reports (Tada et al., 2004; Zago et al., 2006; Zhao et al., 2007). In animals, NO displays cytotoxic activity with ROS but

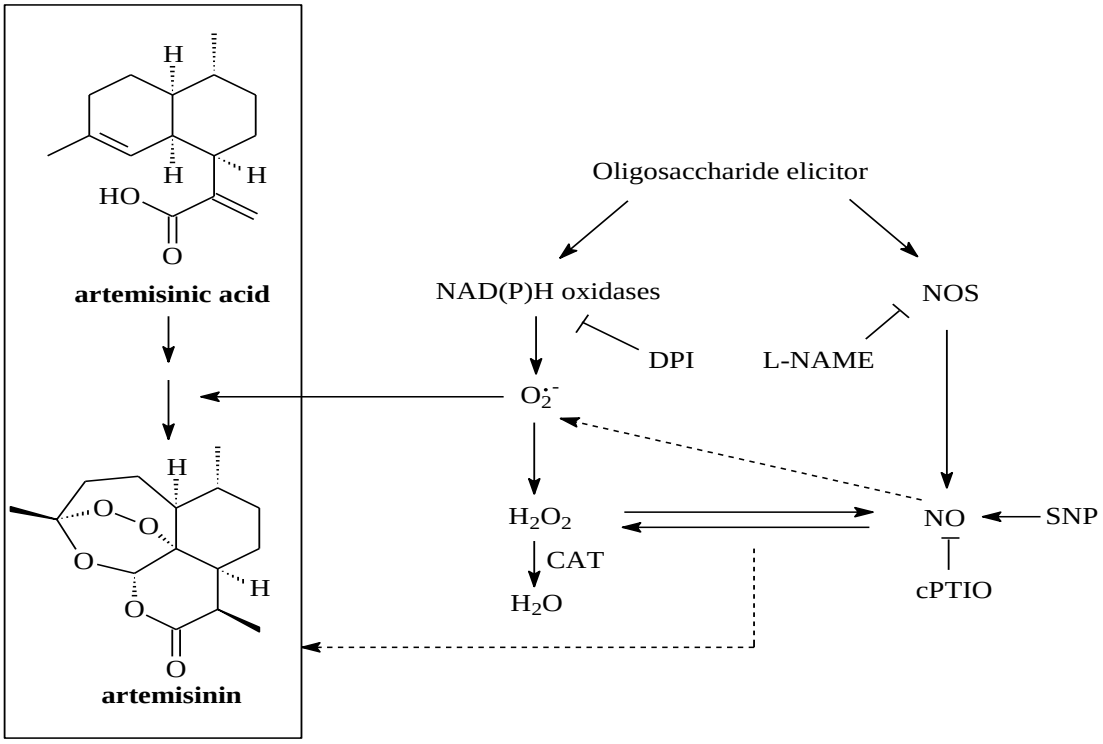

Figure 6. A simplified hypothetical model for oligosaccharide elicitor (OE)-induced NO and ROS production and the involvement of both in the signal transduction of OE-induced the bioconversion from artemisinic acid to artemisinin (dashed lines indicate the more uncertain steps; |—, point of inhibitor application).

may also play a cytoprotective role in oxidative stress (Kroncke et al., 1997). The studies on NO and ROS interaction in plant disease resistance have led to two opposite conclusions on NO role as a prooxidant or an antioxidant (Beligni et al., 2002; Delledonne et al., 1998; 2001). This dual role of NO may depend on the relative timing and intensity of NO and ROS released in different plant–pathogen systems. In our study, with the increase of ROS by the possible changes of cellular redox state in *A. annua* cells after the oligosaccharide elicitation, NO can act synergistically with ROS to potentate thehypersensitive response (HR).

It is widely believed that the synthesis of secondary metabolites in plants is part of the defense responses of plants to elicitor and pathogenic attacks. In view of the allelopathic effect of artemisinin (Duke, 1987), it is interesting to note that the accumulation of such an allelochemical can be stimulated whereas the content of artemisinic acid decreased during the elicitation of the fungal oligosaccharide (Figure 5). Artemisinic acid, a precursor in the biosynthesis of artemisinin, can be 8 - 10 times higher than artemisinin in some chemotypes of *A. annua* (Abdin et al. 2003). Zhang et al. (2005) suggested that the rate-limiting step in artemisinin biosynthesis seems to be between artemisinic acid and artemisinin. They applied gibberellic acid (GA_3) to *A. annua* plants in vegetative stage and induced the conversion of artemisinic acid to artemisinin. Our results indicated that, such conversion can be activated by fungal oligosaccharide elicitor. EI-Feraly et al. (1986) converted artemisinic acid to another artemisinin precursor artemisinin B by single oxygen ($^1O_2$) generated through sensitized photo-oxygenation. It has been demonstrated the oxidation reaction of the $\Delta^{4,5}$ double bond in both artemisinic acid and dihydroartemisinic acid in vivo was involved in the biological transformations to the 1,2,4-trioxane system of artemisinin (Brown and Sy, 2004). This study clarified the points that $O_2^{\cdot-}$ from the elicitor-induced oxidative burst played a role in stimulating the conversion from artemisinic acid to artemisinin whereas H_2O_2 alone suppressed the biosynthesis of both metabolites. Our present study has also shown that elicitation of such biotransformation by the fungal oligosaccharide is strongly dependent on the OE-induced NO production (Figure 5). This finding, together with the interaction between the induced NO and ROS mentioned afore, indicates a role for them as new signal components within the signaling system leading to activate the transformation of artemisinic acid to artemisinin.

Based on our experimental results and the above discussion, we propose a simplified hypothetical model as shown in Figure 6 for the network of OE-induced responses in the *A. annua* hairy root cultures. According to this model, the fungal oligosaccharide is the initial

trigger of the responses in which NO (from NOS) and $O_2^{\bullet-}$ (from NADPH oxidase) are at the upstream of the pathways. NO and ROS ($O_2^{\bullet-}$, H_2O_2) may function independently or synergistically to signal further downstream responses, leading to the activation of the biotransformation for artemisinin accumulation. Although detailed mechanisms are not clear, these ROS and NO, as well as their interactions, could modulate not only the oxidation during the bioconversion of artemisinic acid, but also some enzymes and genes in the artemisinin biosynthetic pathway. Furthermore, this study provides new insights into the regulation of artemisinin production in *A. annua* root cultures, which could reveal more effective strategies such as SNP priming and combining elicitation for the production of this pharmaceutically important compound by *A. annua* hairy root cultures.

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