Nitric oxide: A potential key point of the signaling network leading to plant secondary metabolite biosynthesis*

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Abstract The endogenous signaling network of plants plays important roles in mediating the exogenous factor-induced biosynthesis of secondary metabolites. Nitric oxide (NO) has emerged as a key signaling molecule in plants recently. Numerous studies demonstrated that the main signaling molecules such as salicylic acid (SA), jasmonic acid (JA), reactive oxygen species (ROS), and NO were not only involved in regulating plant secondary metabolite biosynthesis but also interacted to form a complex signaling network by mutual inhibition and/or synergy. The recent progress in the signal network of plant secondary metabolite biosynthesis has been discussed in this paper. Furthermore, we propose a hypothetical model to show that NO might act as a potential molecular switch in the signaling network leading to plant secondary metabolite biosynthesis.

Keywords plant secondary metabolites, biosynthesis, signaling network, nitric oxide, salicylic acid, jasmonic acid, reactive oxygen species.

Production of the secondary metabolites with distinct and complex structures in plants by cell cultures has been one of the most extensively explored areas in recent years owing to the enormous commercial value of those compounds, the scarcity of some plant species in the world, and the high expense of the chemical synthesis of those compounds. However, the numbers of compounds that are producible commercially by cell culture technology are very few. One of the main limitations is the low productivity of the desired compounds[1,2]. Investigation on the mechanism of secondary metabolite biosynthesis in plants is, therefore, necessary and of great importance.

Biosynthesis of secondary metabolites involves a series of biochemical reactions (biosynthetic pathways) that are controlled by related genes and is easily affected by the environmental factors[3]. It is clear that the environmental factors per se do not participate directly in secondary metabolite biosynthetic pathways. Therefore, some special secondary messengers should exist in plant cells to accept and transduce the environmental stimuli. It has been reported that external factors such as the elicitors from pathogenic microorganism triggered not only defense responses and secondary metabolite biosynthesis but also multiple signal molecules and/or signaling pathways in plants. Ion fluxes across the plasma membrane, synthesis of reactive oxygen species (ROS), jasmonic acid (JA) biosynthesis, salicylic acid (SA) accumulation, and phosphorylationand dephosphorylation of proteins, have frequently been discussed as putative components of the signal transduction chain(s) leading to the elicitor-induced defense responses of plants[4—6], among which JA, SA, and ROS have been reported to be involved in secondary metabolite biosynthesis[7—9].

Nitric oxide (NO) is a small, water and lipid soluble gas, first described in mammals as an inter- or intracellular messenger, which has various functions ranging from dilation of blood vessels for neurotransmission to defense immune responses. NO has emerged as a key signaling molecule in plants recently[10, 11]. Studies have shown that NO accumulation is essential for mediating the biosynthesis of secondary metabolites in plants. Furthermore, NO has been demonstrated to act upstream of SA, JA, and ROS and regulate the synthesis of those signals in plant cells. Studies also showed that NO, SA, JA, and ROS not only were involved in regulating plant secondary metabolite biosynthesis but also interacted by

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mutual inhibition and/or synergy in plant cells[12—14], which implies that a complex signaling network leading to secondary metabolite biosynthesis may exist in plants. The recent progress in signaling of plant secondary metabolite biosynthesis has been discussed in this paper. Furthermore, a hypothetical model is proposed to show that NO may act as a potential molecular switch in the signaling network leading to plant secondary metabolite biosynthesis based on the newest development obtained.

1 NO is essential for plant secondary metabolite biosynthesis

1.1 Chemical properties of NO

Nitric oxide ($NO^{\cdot}$) is a gaseous free radical. It contains an unpaired electron in its Π_2 orbital, but remains uncharged. However, because of its free radical nature, it can adopt an energetically more favorable electron structure by gaining or losing an electron, so that NO can exist as three interchangeable species: the radical ($NO^{\cdot}$), the nitrosonium cation (NO^{+}), and the nitroxyl radical (NO^{-})[15, 16]. Once produced, it can move from one cell to another or within a cell. However, being a reactive free radical, it has a relatively short half-life, in the order of a few seconds. Typically, NO rapidly reacts with O_2 to form nitrogen dioxide (NO_2), and rapidly degrades to nitrite and nitrate in aqueous solution. Thus, the range of its effects is limited to the cell in which it is generated, or to the cells in the near neighborhood[15, 16].

1.2 Involvement of NO in plant secondary metabolite biosynthesis

Nitric oxide was first described in mammals as an inter- or intracellular messenger, having various functions ranging from dilation of blood vessels for neurotransmission to defense immune responses[17]. Recent studies have shown the existence of NO in plants. A number of experiments have provided the evidence that NO has multiple functions in plants, such as the stimulation of seed germination and root growth, induction of plant defense response, and defense gene activation[18—20]. The role of nitric oxide in plant defense signaling has been extensively investigated, and studies show that nitric oxide is an important element for plants responding to biotic and abiotic stresses[21, 22]. Durner et al.[21] reported that injection of tobacco seedling with mammalian nitric oxide synthase (NOS) induced the transcription of pathogenesis-related genes. Furthermore, direct addition to tobacco cells of the NO donor GSNO also induced the transcription of these genes. Application of NO via NO donor SNP induced the expression of *pal*, *prl*, *chi* and other defense genes in rice cells[23]. Moreover, NO scavengers prevented the effects of NO donor on gene activation[21, 23]. These studies indicated that NO may act as an essential signal molecule in triggering the defense response of plant cells.

In addition to being involved in plant defense responses, NO has also been demonstrated to play important roles in plant secondary metabolite biosynthesis recently. Modolo et al.[24] reported that external application of NO stimulated the accumulation of isoflavonesdaidzein and genistein in soybean. Treatment with fungal elicitor prepared from the cell walls of *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm) not only enhanced NOS activity in soybean tissue, but also stimulated soybean phytolaxin production[24], implying that NOS might be involved in fungal elicitor-induced isoflavone accumulation of soybean. Our recent studies showed that NO content in *Taxus chinensis* suspension cells treated with the elicitor derived from the cell walls of *Penicillium citrinum* significantly increased 2 h after treatment and reached the highest levels at about 6 h, being 10 fold of the control[10], which indicated that the elicitor may induce NO generation of the cells. NO specific scavenger 2-4-carboxyphenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO) suppressed not only the elicitor-induced NO generation but also the elicitor-triggered Taxol production of *Taxus chinensis* cells[10]. The results showed that NO generation was essential for the elicitor-induced Taxol biosynthesis of the cells. Similar results have been reported in other plants[25, 26].

Although NO generation has been well demonstrated to be a common response of plant cells to biotic and abiotic stresses, the sources of NO in plants have not been known so far. In animals, biosynthesis of NO is primarily catalyzed by the enzyme nitric oxide synthase (NOS) that oxidizes L-arginine to L-citrulline and NO[27]. NOS activity was also reported in high plants[28]. NOS activity was detected in roots and nodules of *Lupinus albus*, and inhibited by the NOS inhibitor L-NMMA (N^{G}-monomethyl-L-argi-

nine)[28]. The elicitor-induced release of NO from tobacco was inhibited by the NOS inhibitor L-NMMA[29]. In our experiments, the elicitor-induced NO generation of *Taxus chinensis* cells was strongly inhibited by NOS inhibitor PBITU[14], which suggested that NOS or NOS-like enzyme might exist in *Taxus chinensis* cells and be responsible for the elicitor-induced generation of NO. However, the fungal elicitor-induced NOS-like activities and NO generation in *Hypericum perforatum* cells did not match kinetically and the elicitor-triggered NOS-like activity (production of citrulline) was much lower than NO production[13]. Thus, the results suggested that the elicitor-induced NO in *Hypericum perforatum* cells did not entirely depend on NOS or NOS-like enzymes, other NO-generating system(s) might exist in the cells and be responsible for elicitor-induced NO generation.

Numerous studies have shown that fungal elicitors may induce defense responses such as expression of defense genes and hypersensitive reactions (HR) of tobacco, soybean, and rice[23, 24]. Furthermore, the fungal elicitor-induced defense reactions could be inhibited by NO specific scavenger[21—23], which suggested that NO was necessary for elicitor-induced defense responses of plants. The biosynthesis of secondary metabolites is widely believed to be one of the results of plant defense responses to external stress[3]. It is, therefore, deduced that NO may stimulate the secondary metabolite biosynthetic pathway by triggering the defense responses of plants[13, 14].

2 Relationship between NO and jasmonic acid (JA) in regulating plant secondary metabolite biosynthesis

2.1 Involvement of JA in secondary metabolism of plants

Jasmonic acid (JA) is one of the important signal molecules in plants[30], which plays key roles in defense response signal transduction of plants to stresses[31—33]. JA and its derivatives such as methyl jasmonate (MeJA) have also been widely tested as chemical inducer for secondary metabolites of plant cells[34]. JA biosynthesis and secondary metabolite accumulation are two early reactions of plants to external stimuli such as fungal elicitors[35]. JA is known to be derived from the octadecanoid pathway, which involves the peroxidation of linolenic acid by lipoxygenase (LOX). It has been reported that JA and MeJA accumulated rapidly in tobacco and other plant cells after exposure to fungal elicitor[31—33]. JA and its octadecanoid precursors have also been implicated as intermediate signals in elicitor-induced secondary metabolite accumulation in plants[31—33]. In parsley cells phenylpropanoid biosynthetic genes were induced by octadecanoids, and the elicitor-induced gene expression was blocked by a LOX inhibitor[36]. Similar results have also been reported in rice and other plants[37, 38]. A correlation between elicitor-induced accumulation of endogenous JA and secondary metabolite production has been shown in cells of California poppy[31—33]. In *Catharanthus roseus* cells both JA biosynthesis and the expression of terpenoid indole alkaloid biosynthetic genes were induced by fungal elicitor[39]. External application of the precursor of jasmonate precursor α-linolenic acid or MeJA alone induced the expression of terpenoid indole alkaloid biosynthetic genes, and the fungal elicitor-induced gene expression could be blocked by JA biosynthesis inhibitors[37]. These results suggested that the jasmonate biosynthetic pathway was an integral part of the elicitor-triggered signal transduction pathway leading to the biosynthesis of secondary metabolites in plant cells. Our recent results showed that the elicitor prepared from *Aspergillum niger* triggered hypericin production of *Hypericum perforatum* cells and that the elicitor-induced hypericin production was blocked by JA inhibitors[14], showing that JA was essential for the elicitor-induced secondary metabolite biosynthesis in the cells.

2.2 Regulation of NO on JA signaling

Fungal elicitor prepared from *Aspergillum niger* cell walls triggered NO generation, JA biosynthesis, and hypericin production of *Hypericum perforatum* cells[14]. The fungal elicitor-induced JA biosynthesis could be inhibited by NO specific scavenger while the NO-induced secondary metabolite production of *Hypericum perforatum* and *Catharanthus roseus* cells might be blocked by JA inhibitors[14]. The results suggested that JA may act downstream of NO to mediate secondary metabolite biosynthesis. Moreover, external application of NO triggered JA biosynthesis in *Hypericum perforatum* cells, which indicated that NO might activate the JA signaling in the cells. Together, the data implied that fungal elicitor may stim-

ulate secondary metabolite biosynthesis of plant cells through JA signaling pathway, while NO might act upstream of JA signaling to control the elicitor-induced secondary metabolite production.

2.3 Mutual amplification between NO and JA

Treatment of NO enhanced JA levels of transgenic NahG *Pueraria thomsonii Benth* cells[12], showing that NO might trigger JA biosynthesis of the cells. Application of JA could also stimulate NO generation of *Pueraria thomsonii Benth* cells[12, 25], which indicated that JA may enhance NO synthesis of the cells. Thus, the data suggested that a mutually amplifying reaction between JA and NO might exist in plant cells. Furthermore, the LOX activity was significantly induced by NO, which implied that NO might stimulate JA biosynthesis by activating the octadecanoid pathway in plants. The nitric oxide synthase (NOS) activities of the transgenic NahG *Pueraria thomsonii Benth* cells treated with JA were not significantly increased as compared with the control cells, showing that NOS was not involved in JA-induced NO generation of the transgenic cells[40].

3 Relationship of NO, SA, and JA in plant secondary metabolite biosynthesis

3.1 Regulation of NO on SA signaling in plant secondary metabolite biosynthesis

External treatment of salicylic acid (SA) induced puerarin biosynthesis in *Pueraria thomsonii Benth* suspension cells[12, 40], showing that SA may stimulate the secondary metabolite biosynthesis through a specific signaling pathways in the cells. The fungal elicitors prepared from the cell walls of *Penicillium citrinum* triggered both NO generation and SA accumulation[12, 40]. Treatment of NO alone enhanced SA contents of the cells. Furthermore, the fungal elicitor-induced SA accumulation could be inhibited by NO specific scavenger[12, 40]. It is, therefore, deduced that NO may activate SA signaling and mediate the fungal elicitor-induced puerarin production in a way at least partially dependent on SA signaling.

3.2 Inhibition of SA on NO-induced JA biosynthesis of *Pueraria thomsonii Benth* cells

Treatment of NO, JA, and SA alone triggered secondary metabolite biosynthesis in plant cells[10, 35, 41, 42], which suggested that all the molecules might be involved in plant secondary metabolite biosynthesis. However, the relationship and/or interactions of NO, JA, and SA in plant secondary metabolite biosynthesis are still not well characterized so far. Our results showed that treatment of NO may stimulate SA contents in wild *Pueraria thomsonii Benth* cells but did not affect JA levels in the cells, which was in agreement with the results obtained in *Arabidopsis* by Durner et al.[21, 43]. Interestingly, external application of NO significantly enhanced LOX activities in wild *Pueraria thomsonii Benth* cells, though JA biosynthesis was not affected by NO[12, 40]. In *Arabidopsis*, NO induced the expression of JA biosynthesis genes *LOX2*, *AOS*, and *OPR3* but did not enhance JA levels[43]. The data implied that NO might be involved in the regulation of JA biosynthesis in *Pueraria thomsonii Benth* cells, but some unknown factors in plants inhibited the effects of NO on JA biosynthesis. This deduction was further confirmed by the results obtained in transgenic NahG *Pueraria thomsonii Benth* cells in which JA levels were significantly enhanced by NO[21, 43]. Since the SA accumulation and/or signaling were impaired in the transgenic NahG plant cells, it was, therefore, proposed that the failure of NO on JA biosynthesis might be related to SA in the cells. In order to test this hypothesis, we determined the effects of SA on JA biosynthesis induced by NO in transgenic NahG *Pueraria thomsonii Benth* cells. The results showed that external application of SA blocked the NO-induced JA biosynthesis in transgenic NahG cells[12, 40], which suggested that the effects of NO on JA biosynthesis might be inhibited by SA in the cells.

JA and SA are two common signaling molecules in plant defense reactions. The relationship between SA and JA in plant defense responses has been well characterized[44]. Although some reports showed that SA and JA might act synergistically in inducing plant defense reactions, numerous studies have demonstrated the antagonistic action between SA and JA in plants[45, 46]. However, the molecular basis of the interaction between SA and JA in plant defense responses is still largely unknown. Different results about the relationship between NO and JA have been reported in distinct plant systems. For example, fungal elicitors triggered NO generation and JA biosynthesis in *Pueraria thomsonii Benth* cells and treatment of NO via its donor stimulated JA levels in the

cells[12, 40]. Conversely, application of NO did not affect JA biosynthesis in *Hypericum perforatum* cells[12, 40]. Considering that NO may stimulate SA accumulation in plant cells and that SA may inhibit JA biosynthesis, the effects of SA on NO-induced JA biosynthesis should, therefore, be determined when investigating the relationship between NO and JA in plant cells.

3.3 Both SA and JA acted downstream of NO to mediate plant secondary metabolite biosynthesis

NO triggered JA biosynthesis in transgenic NahG *Pueraria thomsonii Benth* cells that were impaired in SA accumulation. JA biosynthesis inhibitors suppressed the NO-induced secondary metabolite biosynthesis in the cells[12], suggesting that JA might act downstream of NO to mediate the NO-induced secondary metabolite biosynthesis. External treatment of NO triggered JA biosynthesis, SA accumulation, and secondary metabolite production in wild *Pueraria thomsonii Benth* cells[12], but the NO-induced secondary metabolite production was not entirely dependent on JA signaling. In other words, some other signaling pathways might be involved in NO-induced secondary metabolite biosynthesis besides JA. It has been reported that treatment of SA may stimulate pueririn production of *Pueraria thomsonii Benth* cells[12]. In transgenic NahG *Pueraria thomsonii Benth* cells, expression of NahG gene not only impaired SA accumulation but also inhibited NO-induced secondary metabolite production[12, 40], which suggested that SA may act downstream of NO to mediate the secondary metabolite production of the cells.

The antagonistic action between SA and JA in plant defense responses has been well characterized[47—49]. SA levels in transgenic NahG *Pueraria thomsonii Benth* cells were gradually decreased when the expression of NahG gene was increased[12, 40]. However, the NO-induced puerarin production was not affected by the abolishment of SA accumulation and/or signaling[12, 40]. Meanwhile, the JA levels of the cells increased gradually as the SA contents decreased[12, 40]. The results suggested that JA signaling pathway might be activated to substitute the SA signaling to mediate the NO-induced secondary metabolite biosynthesis in the cells when SA accumulation was impaired.

4 Relationship between NO and ROS in plant secondary metabolite biosynthesis

4.1 Involvement of ROS in plant secondary metabolite biosynthesis

Oxidative burst is a common reaction of tobaccos and soybeans to pathogenic microorganism and elicitors[6, 50]. The reactive oxygen intermediates (ROI), including superoxide anions (O_2^-) and H_2O_2, generated by oxidative burst are considered to be important signaling molecules in plants. ROI has been reported to be implicated in the cross-linking of cell wall proteins[51], in signal transduction as a regulator of pathogenesis-related (PR-1) gene expression[52, 53], in plant cell death process[54], in direct killing of invading pathogens, and in the induction of hypersensitive reaction (HR)[50, 53, 54]. Recent studies show that ROI produced by oxidative burst might be involved in the elicitor-induced secondary metabolite biosynthesis of plant cells[18, 19].

Fungal elicitor induced not only oxidative burst and ROS generation but also the secondary metabolite biosynthesis in *Catharanthus roseus* cells[8, 55]. Inhibitors of oxidative burst suppressed both elicitor-induced ROS generation and secondary metabolite biosynthesis, showing that oxidative burst and ROS were essential for fungal elicitor-induced secondary metabolite biosynthesis in the cells[8, 55].

It has been reported that the production of reactive oxygen species is mostly attributed to a membrane-bound NAD(P)H oxidase and the cell well-bound peroxidases[56, 57]. In *Taxus chinensis* cells, the elicitor-induced O_2^- and H_2O_2 generation was suppressed by membrane NAD(P)H oxidase inhibitor DPI, indicating that the fungal elicitor might trigger ROS generation through the membrane NAD(P)H oxidase[10].

O_2^- and H_2O_2 are two important reactive oxygen intermediates[7, 9], since superoxide anions (O_2^-) have high toxicity for plant cells and their half-life period is shorter than 1 s. Numerous studies have focused on H_2O_2, the most stable form of ROI, as the oxidative burst signal leading to plant defense responses[7, 9]. For example, the fungal elicitor-induced PAL activation in tobacco cells could be blocked by

H_2O_2 inhibitor catalase (CAT), indicating that H_2O_2 might be essential for fungal elicitor-induced phenylalanine ammonia-lyase (PAL) in the cells[12]. However, the fungal elicitor-induced secondary metabolite biosynthesis in parsley cells could be suppressed by O_2^- scavenger superoxide dismutase (SOD)[58]. Moreover, treatment of O_2^- via its donor KO_2 alone induced the secondary metabolite production, while application of H_2O_2 alone had no effects on secondary metabolite biosynthesis of the cells and treatment of CAT did not inhibit the fungal elicitor-induced secondary metabolite biosynthesis[58]. The data suggested that O_2^- might be the signal molecule involved in secondary metabolite biosynthesis of parsley cells. Our results showed that O_2^- from oxidative burst induced by fungal elicitor was necessary for catharanthine production of *Catharanthus roseus* cells and that H_2O_2 was essential for mediating Taxol production of *Taxus chinensis* cells induced by the fungal elicitor prepared from cell walls of *Penicillium citrinum*[13]. The inconsistent results obtained with different systems regarding the nature of individual ROI species that mediated elicitor-induced secondary metabolite production may reflect differences in experimental details or species specificity of the signaling mechanisms.

4.2 Dependence of NO-mediated secondary metabolite biosynthesis on ROS signaling

The fungal elicitors prepared from the cell walls of *Penicillium citrinum* induced both NO generation and ROS generation of *Taxud chinensis* cells[13]. The inhibitors of NO and ROS suppressed not only the elicitor-induced NO generation and ROS accumulation but also the elicitor-triggered Taxol production, showing that both NO and ROS were involved in the fungal elicitor-induced secondary metabolite biosynthesis of the cells[13]. Furthermore, NO inhibitors cPITO and PBITU inhibited the fungal elicitor-induced ROS generation in the cells, suggesting that oxidative burst and ROS accumulation might act downstream of NO to mediate the secondary metabolite biosynthesis[13,59]. External treatment of NO via its donor triggered Taxol production and the NO-induced Taxol biosynthesis could be blocked by oxidative burst inhibitor diphenylene idonium (DPI). The results showed that the NO-induced Taxol production was dependent on oxidative burst and ROS generation. However, NO and the fungal elicitor could still induce Taxol biosynthesis even though the accumulation of reactive oxygen species was completely abolished in *Taxus chinensis* cells. The data showed that NO may mediate the elicitor-induced Taxol biosynthesis of *Taxus chinensis* suspension cells through both ROS-dependent and -independent signal pathways.

5 The signaling network leading to plant secondary metabolite biosynthesis

The signaling molecules such as JA, SA, ROS, and NO have been well demonstrated not only to be involved in plant secondary metabolite biosynthesis but also to interact in mediating plant secondary metabolite production by mutual inhibition and/or synergy[8,12,13,24,26,55]. It is, therefore, obvious that the different signaling molecules act as a network to mediate plant secondary metabolite biosynthesis. Although SA, JA, and ROS may stimulate plant secondary metabolite biosynthesis through distinct signaling pathways, they are all interacted with NO in mediating plant secondary metabolite production[12,14,40,43,60], which implies that NO may act as the key-point in the network. A hypothetical model is proposed to summarize the possible relationship and/or interactions between the signaling molecules in mediating plant secondary metabolite biosynthesis (Fig. 1). It can be seen from the model that NO acts upstream of JA, SA, and ROS and may, therefore, control the synthesis of these signaling molecules, which strongly suggests that NO might act as a molecular switch of the signaling network.

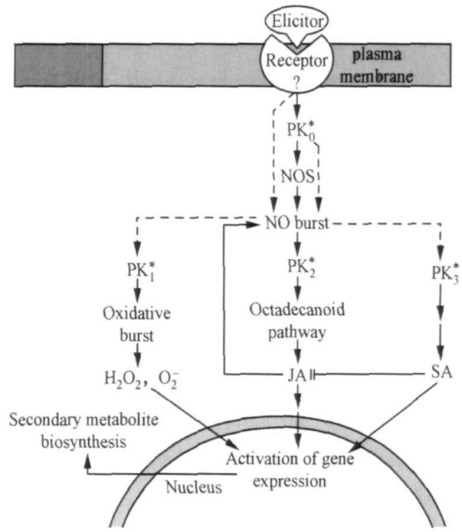

Fig. 1. A hypothetical model proposed to show the relationship and/or interactions between nitric oxide and other signal molecules.

The signaling mechanism of plant secondary metabolite biosynthesis has been extensively explored in recent years owing to its importance in ecological functions of plants and for human health. Although much progress about the signaling mechanism of plant secondary metabolite biosynthesis has been made, we are still at the early stage in understanding the biochemical mechanisms of the signaling network leading to secondary metabolite production in plant cells. More details about the signaling network of plant secondary metabolite biosynthesis are still needed to be further investigated.

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