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Involvement of nitric oxide signaling in mammalian Bax-induced terpenoid indole alkaloid production of *Catharanthus roseus* cells

XU MaoJun† & DONG JuFang

Department of Biotechnology, Zhejiang Gongshang University, Hangzhou 310035, China

Bax, a mammalian pro-apoptotic member of the Bcl-2 family, has been demonstrated to be a potential regulatory factor for plant secondary metabolite biosynthesis recently. To investigate the molecular mechanism of Bax-induced secondary metabolite biosynthesis, we determined the contents of nitric oxide (NO) of the transgenic *Catharanthus roseus* cells overexpressing a mouse Bax protein and checked the effects of NO specific scavenger 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO) on Bax-induced terpenoid indole alkaloid (TIA) production of the cells. The data showed that overexpression of the mouse *Bax* in *C. roseus* cells triggered NO generation of the cells. Treatment of cPITO not only inhibited the Bax-triggered NO burst but also suppressed the Bax-induced TIA production. The results indicated that the mouse Bax might activate the NO signaling in *C. roseus* cells and induce TIA production through the NO-dependent signal pathway in the cells. Furthermore, the activities of nitric oxide synthase (NOS) were significantly increased in the transgenic *Bax* cells as compared to those in the control cells, showing that the mouse Bax may induce NOS of *C. roseus* cells. Treatment of the transgenic *Bax* cells with NOS inhibitor PBITU blocked both Bax-induced NO generation and TIA production, which suggested that the mouse Bax might trigger NO generation and TIA production through NOS. However, the NOS-like activities and NO generation in the transgenic *Bax* cells did not match kinetically and the Bax-induced NOS-like activity was much later and lower than NO production. Moreover, the Bax-induced NO generation and TIA production were only partially inhibited by PBITU. Thus, our results suggested that the Bax-induced NO production and secondary metabolite biosynthesis in *C. roseus* cells was not entirely dependent on NOS or NOS-like enzymes.

Bax, nitric oxide, *Catharanthus roseus* cells, terpenoid indole alkaloid (TIA)

Plants produce a vast and diverse assortment of small organic compounds, the great majority of which do not appear to participate directly in plant growth and development and are traditionally referred to as secondary metabolites. These natural products have been widely used as drugs, dyes, flavoring agents, and polymers nowadays. Although plants are renewable resources, some species are becoming more difficult to obtain in sufficient amounts to meet increasing demands. 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 processes of these compounds[1]. Cell culture is also advantageous in that useful metabolites are obtained under a controlled environment, independence of climatic changes and soil conditions. In addition, the products are

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† Corresponding author (email: maojunxu@163.com)
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free of microbe and insect contamination. However, the numbers of compounds that are producible commercially by cell culture technology are still very few[1,2]. One of the main limitations is the low productivity of the desired compounds.

Genetic manipulation has been considered to be the powerful tools for enhancing the production of the secondary metabolites in plant cells recently[3]. One of the most frequently used strategies for achieving this goal is the engineering of single steps (the "rate-limiting" enzymes) in a particular secondary metabolite biosynthetic pathway to increase metabolic flux to target compounds[1,3]. Although the way the secondary metabolite biosynthesis is being made apparent by concerted molecular, genomic and genetic approaches, engineering the secondary metabolite pathways of plants has often been limited by the need for coordinated regulation of multiple gene activities[4]. The regulatory architectures of the biosynthetic pathways of secondary metabolites, and the ways in which they are integrated into the broader metabolic networks, are less well understood so far, often making it hard to predict the results of overexpressing a single structural gene within a particular pathway[5,6]. Moreover, the value of this approach has often been limited in plant cells, because the effects of modulating single enzymatic steps are often absorbed by the system in an attempt to restore homeostasis[7]. Thus, attention has been recently shifted away from reductionist strategies of single structural gene engineering and towards more complex approaches in the context of the whole cell[3,8]. For example, some transcription factors have been tested to control multiple gene expression of secondary metabolite biosynthetic pathways of plant cells[3,8]. However, the regulatory strategy based on the context of whole cell has not been reported until now.

In previous studies, we reported that expression of mouse Bax, a mammalian pro-apoptotic member of the Bcl-2 family, in transgenic *Catharanthus roseus* cells stimulated defense responses and enhanced the transcription of multiple genes involved in TIA biosynthetic pathway and TIA production of the cells[9], which strongly implied that the mouse Bax might be a potential regulatory factor for secondary metabolite biosynthesis in plant cells. However, the molecular basis of the effects of the mouse Bax on plant secondary metabolites is still largely unknown.

The biosynthesis of secondary metabolites comprises a series of biochemical reactions catalyzed by multiple enzymes. The protein encoded by the mammalian apoptotic gene *Bax* is not an enzyme involved in plant secondary metabolite biosynthesis directly. It is obvious that the mouse Bax *per se* does not participate in catalyzing the biosynthetic reactions of secondary metabolites, although overexpressions of Bax do stimulate the biosynthesis of secondary metabolites in plant cells. Therefore, an unknown mechanism should exist in plants to mediate the mouse Bax-induced secondary metabolite biosynthesis.

A substantial body of evidence has suggested that the biosynthesis of secondary metabolites is regulated by special signal pathway(s) in plants. Nitric oxide (NO), an important signaling molecule, has multiple functions in plants, such as stimulation of seed germination and root growth, induction of plant defense response and activation of defense genes[10−13]. In previous studies, we demonstrated that NO played key roles in the signaling network leading to plant secondary metabolite biosynthesis[14−16]. Recent studies showed that overexpression of the mouse Bax might trigger the endogenous signaling events in tobacco cells[17]. We, therefore, hypothesize that the mouse Bax might stimulate plant secondary metabolite accumulation through special signaling pathway(s) in plants. In order to test this hypothesis, we determined the effects of the mouse Bax on NO generation and the inhibition of NO specific scavenger on Bax-induced TIA production of *Catharanthus roseus* cells. The results show that overexpression of mouse Bax induces nitric oxide synthase (NOS) and stimulates NO generation. NO scavenger inhibits not only the Bax-induced NO accumulation but also the Bax-triggered TIA production of *Catharanthus roseus* cells. Thus, our data suggest that the mouse Bax may induce TIA production through the NO-dependent signaling pathway.

1 Materials and methods

1.1 Plant materials and cell culture

The plant cell line for the study was induced from the young stems of *Catharanthus roseus* growing in Zhejiang Province in China with MS medium[18], supplemented with 2.0 mg/L of α-naphthaleneacetic acid (NAA), 2 mg/L of indole acetic acid (IAA), 0.1 mg/L of kinetin, 20 g/L sucrose and 8 g/L agar. The suspension culture of the cell line was initiated from the callus culture on a liquid medium similar to that for the callus culture but with 30 g/L sucrose and excluding the agar.

The medium was adjusted to pH 5.8 and then sterilized at 121℃ for 20 min. The suspension culture was maintained in 250 mL Erlenmeyer flasks with a liquid volume of 100 mL in each flask capped with Magenta B-Caps (Sigma 38648), and incubated on an orbital shaker incubator in the dark at 120 rpm and 25℃.

1.2 Plant transformation

The ORF of mouse *Bax* gene (a gift of Dr. Haegeong Moon, Gyeongsang National University, Korea) was inserted into the β-estradiol-inducible vector pER8 (a gift of Dr. Nam-Hai Chua, the Rockefeller University, USA). The pER8 vector consists of three transcription units. In the first transcription unit G10-9, a strong constitutive promoter controls the *XVE* fusion gene, which encodes a chimeric transcriptional activator consisting of the DNA-binding domain (DBD) of the bacterial repressor LexA, the acidic transactivating domain of VP16, and the carboxyl region of the human estrogen receptor. The *Xho* I and *Spe* I fragment of pER8, which contains the G10-9 and *XVE*, was ligated to the *Xho* I- and *Spe* I-tagged *Bax* cDNA. The resulting construct, pER-Bax, was used for *Agrobacterium*-mediated transformation of *Catharanthus roseus* callus, as described by Clark[19]. Transformants were selected on MS medium containing hygromycin (20 μg/mL). The callus transformed with empty vector without Bax was also generated for the transgenic control line.

1.3 Measurement of cell growth

Cell growth was determined by measuring the dry cell weight (DCW) of the culture. In order to do this, the biomass in the culture broth was separated from the liquid by filtration and then dried at 40℃ under vacuum for about 2 days to constant weight to obtain the dry cell weight.

1.4 Alkaloid extraction and determination

Alkaloid extraction and determination was carried out as the method reported[20]. Cells in the culture broth were separated from the liquid by filtration and then dried at 40℃ under vacuum for about 2 days. Dry cells powder was extracted twice with methanol, and the extraction solutions were combined and concentrated under vacuum. The residues were dissolved in water, quickly extracted with ethyl acetate phase three times after the pH of aqueous solution was adjusted to 10. The ethyl acetate extracts were combined and concentrated under vacuum. The residues were dissolved in 1 mL of methanol and analyzed by HPLC. HPLC analysis conditions: Shimadzu LC-6A apparatus with Shimadzu C-R3A integrator and SPD-6A UV-detector monitoring at 280 nm; Kromasil C18, 250 mm × 4.6 mm dimension and 5 μm packing (Alltech, Deerfield, IL). Samples were eluted with a mobile phase of methanol/ acetonitrile/ammonium acetate(0.025 mol/L)/triethylamine (15∶40∶45∶0.1, *v/v*) at a flow rate of 1 mL/min. Alkaloids were identified by TLC, co-elution and HPLC-Diode Array Detection (Hewlett-Packard 1100 LC-DAD HP) compared with standards. Alkaloid production was calculated by using an equation: alkaloid production (mg/L) = alkaloid content (mg/g) × biomass (g/L).

1.5 Determination of NO

Nitric oxide is a small, water and lipid soluble gas, which can move freely through plasma membranes[10,21]. Therefore, the concentrations of NO in the cell culture medium have been frequently used to represent those in the plant cells[22,23]. Five-day-old *Catharanthus roseus* cells were resuspended at 0.05 g (fresh weight)/mL in double distilled water. Greiss reagent method and the haemoglobin assay were used to determine NO released by the cells[11,24]. Briefly, 1 mL filtrate of *Pueraria thomsonii Benth* suspension cells from various treatments was obtained via passage through a 0.22 μm micro-sieve, 1 mL Greiss reagent [1%(*w/v*) sulfanilamide/0.1% (*w/v*) N-(1-naphthyl)-ethylenediamine dihydrochloride in 5 % (*v/v*) phosphate acid] was added for 30 min at room temperature, and the absorbance was quantified spectrophotometrically at 550 nm. Different concentrations of $NaNO_2$ were used to prepare a standard curve. NO released to the culture medium was also determined by monitoring the conversion of HbO_2 to metHb as described by Delledonne et al. (1998)[11]. HbO_2 was added to the filtrate to a final concentration of 10 μmol/L. After 2 min, the changes in the absorbance of the medium at 421 and 401 nm were measured and NO levels were calculated by using extinction coefficient of 77 $(mmol/L)^{-1}\cdot cm^{-1}$ [A_{401}(metHb)-A_{421}(HbO_2)].

1.6 Determination of NOS activity

The NOS activity of the cells with various treatments was determined by the citrulline assay method modified from Rees et al. (1995)[25]. In brief, 1.0 g of the cells, together with 50 mg of polyvinylpolypyrrolidone, was homogenized in 1.0 mL of cooled extraction buffer (50 mmol/L Tris, pH 7.4, containing 320 mmol/L Suc, 10 μg

/mL leupeptin, 10 mmol/L reduced glutathione, 10 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10 000 *g* for 10 min at 4℃. NOS activity was determined by measuring the formation of L-[U-^{14}C]-citrulline using L-[U-^{14}C]-Arg as the substrate. 40 μL of the supernatant was added to 100 μL of the assay buffer [40 mmol/L HEPES, pH 7.2, containing 10 μmol/L FDA, 10 μmol/L FMN, 1 mmol/L dithiothreitol, 1.25 mmol/L $CaCl_2$, 50 μmol/L 6(R)-5,6,7,8-tetrahydrobiopterin, 10 mg/L calmodulin, 1 mmol/L NADPH, and 1 μmol/L (50 nCi) L-[U-^{14}C]Arg; Amersham Pharmacia Biotech, Buchinghamshire, UK]. After incubation for 30 min at room temperature, the reaction was stopped by adding 1 mmol/L Dowex-Ag 50 W suspended in 100 nmol/L HEPES containing 10 mmol/L EDTA (1∶1.5, *v/v*). The resin was removed by centrifugation (10 000 *g* for 10 min at 15℃). The supernatant (400 μL) was added to 3 mL of scintillation liquid and was counted in a counter (LS 6000; Beckman, Fullerton, CA). Protein content was determined by the Coomassie Blue-binding method (Bradford, 1976)[26] using protein reagent (Bio-Rad, Hercules, CA), and bovine serum albumin as standard. The activity of NOS was shown as pmol L-[U-^{14}C] citrulline $h^{-1} \cdot (\text{mg protein})^{-1}$.

1.7 Chemical induction of Bax expression

17-β-estradiol was purchased from Research Biochemicals International (Natick, MA, USA). The chemicals were prepared as 1, 4 and 10 mmol/L stock solutions in dimethyl sulfoxide (DMSO), and stored at −20℃ in small aliquots. DMSO alone had no effects on transgene expression. Chemical treatments were carried out by directly growing the cells on a medium supplemented with inducers, or adding the inducers to the culture medium to the desired concentrations at the selected times.

2 Results

2.1 Mouse Bax-induced indole alkaloid production

pER8 is a chemically inducible promoter, the expression of which is highly dependent on the concentrations of β-estradiol (EST) applied[9]. As shown in Figure 1, the contents of TIA (per cell basis) of the transgenic *Catharanthus roseus* cells treated with EST are significantly increased as compared to those of the control. Since EST *per se* has no effects on TIA contents of the cells (Figure 1), the results suggest that the enhancement of TIA production is due to the expression of mouse Bax induced by EST in the transgenic cells.

It has been reported that overexpression of mammalian Bax is lethal in the budding yeast *Saccharomyces cerevisiae*[27,28]. The death-promoting effect of Bax on plant cells has also been found in tobacco and *Arabidopsis* plants[17,29,30]. The results of the present work show that overexpression of the mouse Bax significantly decreases the biomass of *Catharanthus roseus* cells (Figure 1(c)), indicating that the mouse Bax inhibits the growth of the cells. Subsequently, early expression of mouse Bax (day 0) has no significant effects on the production of catharanthine and total TIA (Figure 1(e)), although expression of mouse Bax may increase the contents of catharanthine and total TIA on per cell basis. In order to reduce the adverse effects of Bax on cell growth and TIA production, we check the effects of adding EST at different culture time on the production of catharanthine and total TIA of the transgenic *Catharanthus roseus* cells. As shown in Figure 1(e), the production of catharanthine and total TIA of the cells treated with EST on day 10 to day 25 is significantly higher than that of control cells, indicating that the adverse effects of Bax on TIA production can be suppressed by inducible expression of the mouse Bax in the late culture phase of the cells. The highest production of catharanthine and total TIA is obtained by adding EST on day 20, about 3.5 and 4.2 fold of control respectively (Figure 1(e)).

2.2 Nitric oxide burst of *Catharanthus roseus* cells triggered by the mouse Bax

Nitric oxide (NO) generation is one of the early responses of plant to biotic and abiotic stresses[10,11,22]. Treatment of fungal elicitors induced NO burst in suspension cells of *Hypericum perforatum, Taxus chinensis*, *Pueraria thomsonii* Benth, and *Sophora flavescens* Ait[31−33]. Moreover, NO has been demonstrated to be involved in the fungal elicitor-induced secondary metabolite biosynthesis of plant cells[31,33]. In the present work, the effect of mouse Bax on NO generation of *Catharanthus roseus* cells is investigated. Treatment of the transgenic Bax *Catharanthus roseus* cells with EST stimulates NO generation (Figure 2(a)), while EST *per se* has no effects on NO generation of the wild type cells and the transgenic control cells (Figure 2(b) and (c)). Thus, our results indicate that expression of the mouse Bax induced by EST may trigger NO burst of *Catharanthus roseus* cells.

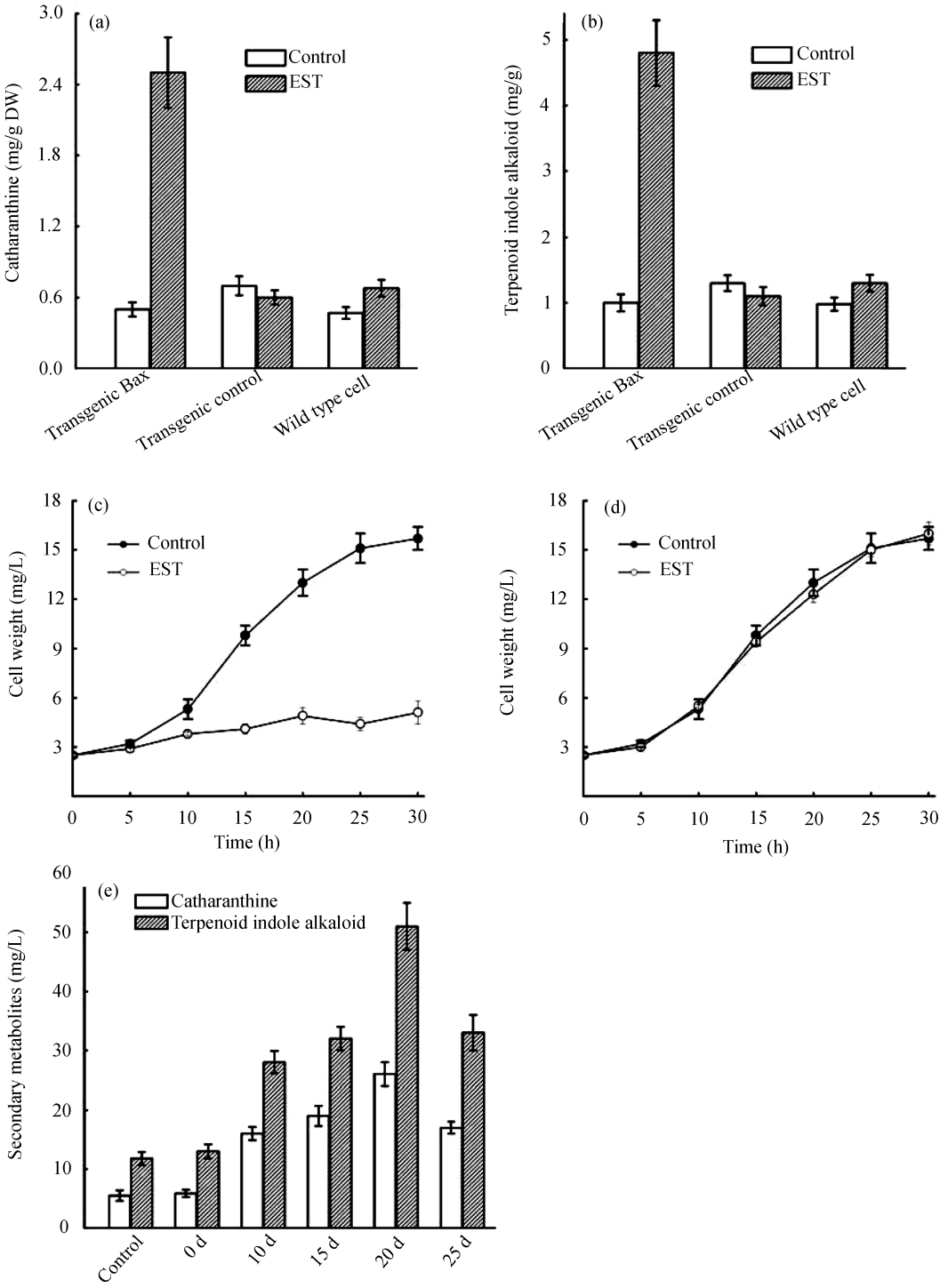

Figure 1 Effects of the mouse Bax on the growth and the production of catharanthine and total TIA of *Catharanthus roseus* cells. (a) Effects of EST on catharanthine contents of the cells; (b) Effects of EST on TIA contents of the cells; (c) Effects of EST on the growth of the transgenic *Bax* cells; (d) Effects of EST on the growth of the transgenic control cells; (e) Effects of EST on catharanthine and total TIA production of the transgenic *Bax* cells. Five-day-old cells treated with 30 μmol/L EST on day 0 ((a), (b), (c) and (d)) or as indicated in the figure (e) were harvested 25 d ((a) and (b)) or 30 d (e) later to determine catharanthine and total TIA production. The control received vehicle solvent only. Values are means of three independent experiments. Bar represents the standard error.

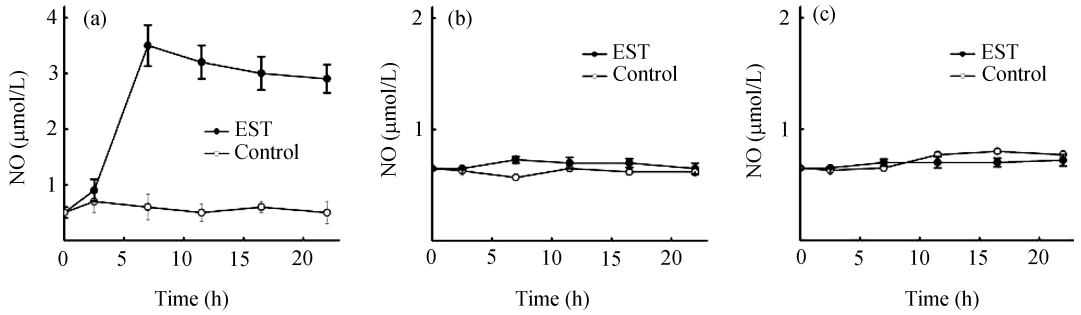

Figure 2 NO burst of *Catharanthus roseus* cells induced by the mouse Bax. (a) Effects of EST on NO contents of the transgenic *Bax* cells; (b) Effects of EST on NO contents of the wild type cells; (c) Effects of EST on NO contents of the transgenic control cells. The culture medium of five-day-old cells treated with 30 μmol/L EST on day 0 was harvested as the time indicated in the figure to determine NO contents. The control received vehicle solvent only. Values are means of three independent experiments. Bar represents the standard error.

2.3 Mouse Bax-induced nitric oxide synthase (NOS) of *Catharanthus roseus* cells

NO generation of plants under stresses has been well documented. However, the source of NO in plants is still largely unknown. A family of enzymes, the nitric oxide synthase (NOS), is instrumental in the generation of NO in animal systems[10,21]. The NOS-like activities have been determined in soybean and tobacco cells[34,35], although the NOS gene has not been cloned in plants so far. In order to investigate the mechanism of the mouse Bax-induced NO generation, we determined the effects of Bax on NOS activities of *Catharanthus roseus* cells. The results show that NOS activities of *Catharanthus roseus* cells treated with EST are significantly enhanced as compared to those of control and that EST *per se* has no effects on NOS activities of the wild type cells and the transgenic control cells (Figure 3(a), (b), and (c)). Therefore, our data suggest that inducible expression of mouse Bax by EST may trigger NOS activation of *Catharanthus roseus* cells. Furthermore, the Bax-induced NO generation of the cells can be suppressed by NOS inhibitor PBITU (Figure 4(a)), which implies that NOS might be involved in the mouse Bax-induced NO generation. However, data of Figure 4(a) also show that treatment of PBITU can not entirely block the Bax-induced NO generation of *Catharanthus roseus* cells, showing that the mouse Bax may simulate NO generation of the cells through both NOS-dependent and -independent pathways.

2.4 Involvement of NO signaling in mouse Bax- induced TIA production

In order to test whether NO is involved in the mouse Bax-induced TIA production, we checked the effects of NO specific scavenger 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO) and NOS inhibitor S,S-1,3-phenylene-bis(1,2-ethanediyl)-bis-

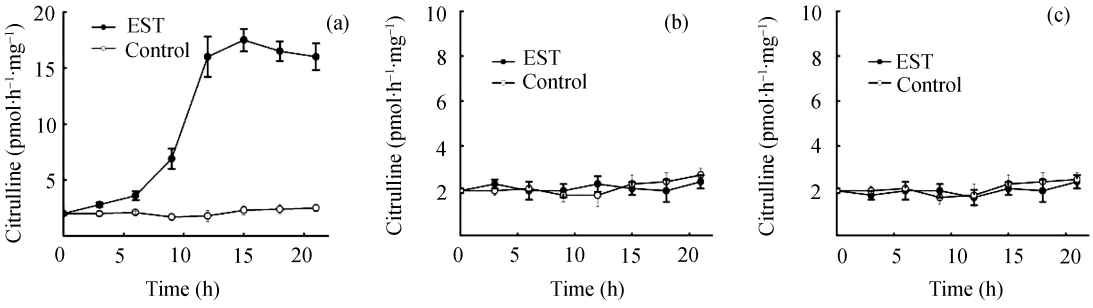

Figure 3 The mouse Bax-induced NOS activities of *Catharanthus roseus* cells. (a) Effects of EST on NOS activities of the transgenic *Bax* cells; (b) Effects of EST on NOS activities of the wild type cells; (c) Effects of EST on NOS activities of the transgenic control cells. Five-day-old cells treated with 30 μmol/L EST on day 0 were harvested as the time indicated in the figure to determine NOS activities. The control received vehicle solvent only. Values are means of three independent experiments. Bar represents the standard error.

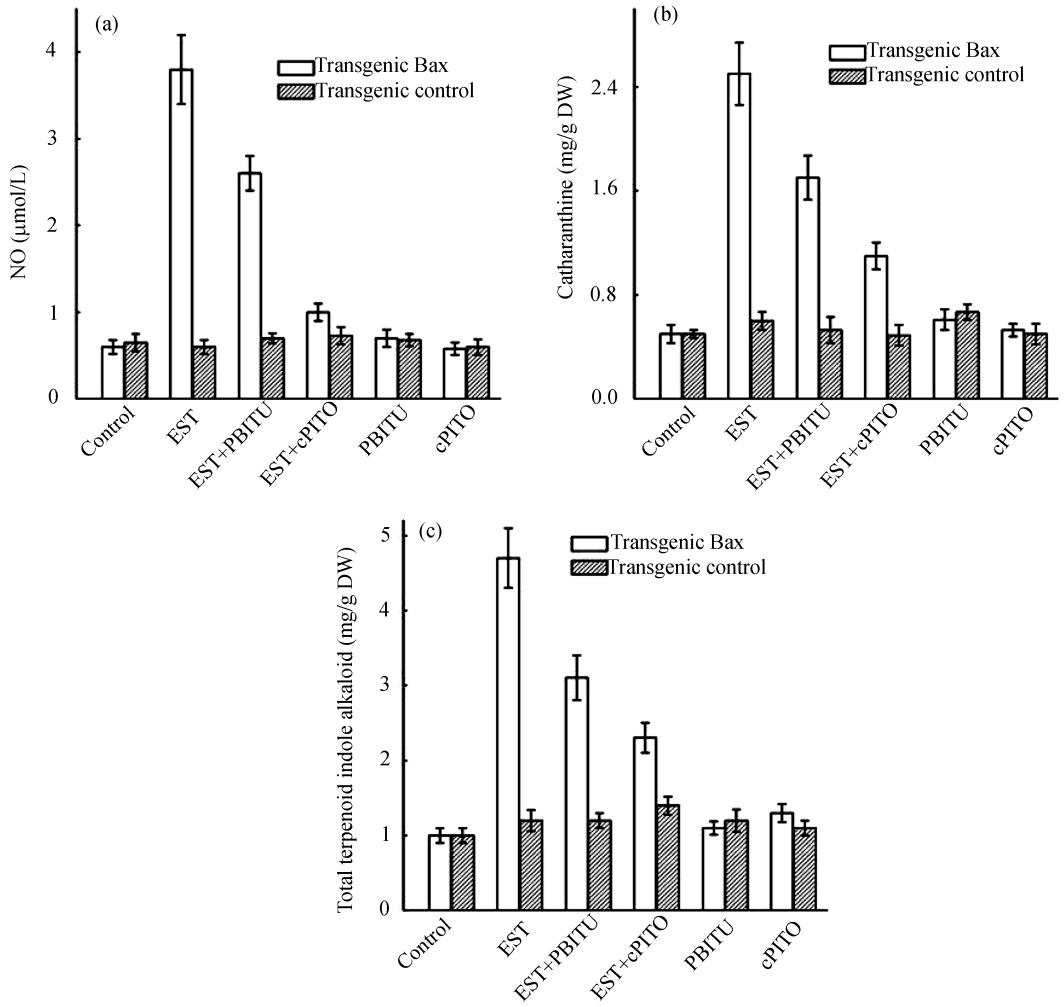

Figure 4 Suppression of Bax-induced NO generation and TIA production by NO inhibitors. (a) Effects of NO inhibitors on Bax-induced NO generation of the cells; (b) Effects of NO inhibitors on Bax-induced catharanthine production of the cells; (c) Effects of NO inhibitors on Bax-induced TIA production of the cells. Five-day-old transgenic *Bax* cells and the transgenic control cells treated with 30 μmol/L EST, 0.5 mmol/L PBITU, and 0.5 mmol/L cPITO on day 0 were harvested 10 h or 20 days later to determine NO contents and TIA production respectively. cPITO and PBITU were added 30 min before EST. The control received vehicle solvent only. Values are means of three independent experiments. Bar represents the standard error.

isothiourea (PBITU) on Bax-induced catharanthine and total TIA production of *Catharanthus roseus* cells. Treatment of cPTIO and PBITU suppresses not only the Bax-triggered NO generation but also the Bax-induced catharanthine and total TIA production (Figure 4(a)), while cPTIO and PBITU *per se* have no effects on catharanthine and total TIA production (Figure 4(b) and (c)). The results demonstrate that NO signaling is essential for the mouse Bax-induced catharanthine and total TIA production of *Catharanthus roseus* cells. Data of Figure 4 also show that the mouse Bax can still stimulate catharanthine and total TIA production even though the NO generation is totally suppressed by cPITO, showing that the Bax-induced TIA production might be not entirely dependent on NO signaling.

3 Discussion

Previous studies showed that expression of mammalian Bax in plant cells enhanced secondary metabolite accumulation[9]. Since the product encoded by mammalian *Bax* is not the enzyme involved in the biosynthesis of secondary metabolites directly, we, therefore, hypothesize that the mammalian Bax may trigger the endogenous signaling pathways of plants and induce the biosynthesis of secondary metabolites through specific signaling pathway(s). To test this hypothesis, we determined the contents of NO of the transgenic Bax *Catharanthus roseus* cells and the effects of NO scavenger on Bax-induced TIA production. Inducible expression of

mouse Bax triggers NO generation of *Catharanthus roseus* cells, showing that the Bax may activate NO signaling in the cells. Furthermore, treatment of NO inhibitors suppresses the Bax-induced TIA production of the cells. Thus, our data strongly suggest that NO is an essential signaling molecule for the mouse Bax-induced TIA production of *Catharanthus roseus* cells.

Biosynthesis of plant secondary metabolites is regulated by multiple endogenous signaling pathways[7,27,29]. The signaling molecules such as jasmonic acid (JA), salicylic acid (SA), reactive oxygen species (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[20,31,36,37]. It is, therefore, obvious that the different signaling molecules function 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 all interact with NO in mediating plant secondary metabolite production[31,38,39], which implies that NO may act as the key-point in the signaling network. The results of the present work indicate that the mouse Bax may trigger NO generation and induce TIA production of *Catharanthus roseus* cells through NO-dependent signaling pathway. Considering the key roles of NO in the signaling network leading to plant secondary metabolite biosynthesis, it is proposed that the mouse Bax may have extensive effects on the endogenous signaling of plants. However, the relationship between mammalian Bax and other signaling events still needs to be further investigated.

NO generation has been documented to be a common reaction of plant to biotic and abiotic stresses[10,21]. However, the source of NO in plants is far from being fully understood 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. During the last few years, several groups have provided evidence for the existence of NOS-like activity in plants, although the NOS gene has not been cloned in plants until now. NOS-like activities have been identified in several plant species such as tobacco, soybean, maize, pea, *Lupinus albus* and *Mucuna hassjoo*[34,35,40,41]. Mammalian NOS inhibitors have been reported to suppress elicitor-induced NO generation in soybean, tobacco, and *Taxus chinensis* cells[20,27,29], showing the existence of NOS-like enzymes in plants. Data of the present work show that expression of mouse Bax triggers NOS activities of *Catharanthus roseus* cells (Figure 3). Furthermore, treatment of NOS inhibitor suppresses the Bax-induced NO generation (Figure 4). The results suggest that the mouse Bax may stimulate NO generation of the cells by activating NOS-like enzymes. However, the results also show that the Bax-induced NOS-like activities and NO generation of the cells do not match kinetically and the Bax-induced NOS-like activity (production of citrulline) is much lower and later than NO production (Figures 2 and 3). Therefore, our results suggest that the Bax-induced NO generation of *Catharanthus roseus* cells is not entirely dependent on NOS or NOS-like enzymes. Other NO-generating system(s) may also exist in the cells and be responsible for Bax-induced NO production. This conclusion gets further support from the finding that treatment of NOS inhibitor PBITU can only partially suppress the Bax-induced NO generation of *Catharanthus roseus* cells (Figure 4(a)). In addition to NOS, other enzymes, such as nitrate reductase (NR), were also found to be implicated in NO synthesis in plants[42−45]. Furthermore, the non-enzymatic production of NO has recently been demonstrated in seeds[46,47]. Whether the NR and non-enzymatic catalysis are involved in the mouse Bax-triggered NO generation of *Catharanthus roseus* cells still needs to be further investigated.

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