Science in China Series C: Life Sciences

© 2007 Science in China Press
Springer-Verlag

Enhancing terpenoid indole alkaloid production by inducible expression of mammalian Bax in *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, triggers hypersensitive reactions when expressed in plants. To investigate the effects of Bax on the biosynthesis of clinically important natural products in plant cells, we generate transgenic *Catharanthus roseus* cells overexpressing a mouse Bax protein under the β-estradiol-inducible promoter. The expression of Bax in transgenic *Catharanthus roseus* cells is highly dependent on β-estradiol concentrations applied. Contents of catharanthine and total terpenoid indole alkaloid of the transgenic cells treated with 30 μmol/L β-estradiol are 5.0- and 5.5-fold of the control cells. Northern and Western blotting results show that expression of mammalian Bax induces transcriptional activation of *Tdc* and *Str*, two key genes in terpenoid indole alkaloid biosynthetic pathway of *Catharanthus roseus* cells, and stimulates the accumulation of defense-related protein PR1 in the cells, showing that the mouse Bax triggers the defense responses of *Catharanthus roseus* cells and activates the terpenoid indole alkaloid biosynthetic pathway. Thus, our data suggest that the mammalian Bax might be a potential regulatory factor for secondary metabolite biosynthesis in plant cells and imply a new secondary metabolic engineering strategy for enhancing the metabolic flux to natural products by activating the whole biosynthetic pathway rather than by engineering the single structural genes within the pathways.

Catharanthus roseus cells, terpenoid indole alkaloid, metabolic engineering, Bax

Higher plants are important sources of many clinically important natural products (secondary metabolites). 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, independent of climatic changes and soil conditions. In addition, the products are free of microbe and insect contamination. However, the numbers of compounds that are producible commercially by cell culture technology are still very few[2]. One of the main limitations is the low productivity of the desired compounds. Further investigation on the methodology and mechanism of the production of secondary metabolites by plant cell cultures is, therefore, necessary and of great importance.

It has been reported that the biotic and abiotic stresses such as pathogenic infection and UV light may activate the secondary metabolite biosynthetic pathways and stimulate secondary metabolite accumulation in plants[3]. Thus, the use of elicitors from pathogenic microorgan-

Received June 28, 2006; accepted July 20, 2006
doi: 10.1007/s11427-007-0030-4
†Corresponding author (email: maojunxu@163.com)
Supported by the National Natural Science Foundation of China (Grant No. 30572331), the Natural Science Foundation of Zhejiang Province (Grant No. 302785), and the Key Scientific Project of Zhejiang High Education Commission

ism and other external factors has been one of the most effective strategies for improving the productivity of useful secondary metabolites in plant cell cultures[4–6]. However, the practical utilization of the external factors for secondary metabolite production is greatly limited by its poorly reproducible performance.

Plants respond to the biotic and abiotic stresses by activating various defense reactions including hypersensitive cell death and the accumulation of the secondary metabolites[3,7]. Therefore, activation of plant defense responses at the molecular level might be an efficient strategy for improving the secondary metabolite production of plant cells. Bax is a death-promoting member of the Bcl-2 family in mammalians. It has been reported that overexpression of mammalian Bax in tobacco and *Arabidopsis* induces reactions similar to the hypersensitive responses of plants to pathogenic attack[8,9], which implies that mammalian Bax may trigger the defense responses of tobacco and *Arabidopsis* cells. We, therefore, hypothesize that expressing mammalian Bax in plant cells might stimulate the biosynthesis of secondary metabolites. In order to test the hypothesis, we developed the transgenic *Catharanthus roseus* cells overexpressing a mouse Bax protein under β-estradiol-inducible promoter and determined the effects of Bax on the biosynthesis of catharanthine and total terpenoid indole alkaloid of *Catharanthus roseus* cells.

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 of China with MS medium[10], supplemented with 2.0 mg/L α-naphthaleneacetic acid (NAA), 2 mg/L indole acetic acid (IAA), 0.1 mg/L 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 r/min and 25℃.

1.2 Plasmid construction and plant transformation

The ORF of the mouse *Bax* (a gift of Dr. Haegeong Moon, Gyeongsang National University, Korea) was inserted into the β-estradiol-inducible vector pER 8 (a gift of Dr. Nam-Hai Chua, The Rockefeller University). The pER8 vector consists of three transcription units. In the first transcription unit G10-9, a strong constitutive promoter controls the *XVE* fusion gene. The latter 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-*Spe* I-tagged *Bax* cDNA. The resulting construct, pER-Bax, was used for *Agrobacterium*-mediated transformation of *Catharanthus roseus* callus, as described by Clark[11]. 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 Protein extraction and Western blotting

Protein extraction and Western blot analyses of cell protein were performed as the method reported[12]. Total cell protein (10 μg) was electrophoresed on a 15% SDS-polyacrylamide gel. After transferring protein to nitrocellulose membranes, immunoblotting was performed by using antisera raised in rabbits against either Bax (1:1000, Alexis Biochemicals, San Diego, CA), or PR1 (1:1000, Alexis Biochemicals, San Diego, CA). Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) was used as the secondary antibody.

1.4 RNA extraction and Northern analysis

We extracted RNA and performed Northern analysis as previously described[13]. Unless indicated otherwise, 10 μg RNA samples were loaded onto the gels. All Northern blots used ^{32}P-labeled cDNA probes. For *Tdc* we used a cDNA clone that was of full length and homologous to the cDNA cloned by De Luca et al.[14]. The full-length cDNA clone for *Str*[15] and a cDNA corresponding to *Rps9*[13] (40S ribosomal protein S9) were isolated from *Catharanthus roseus* by our laboratory.

1.5 Chemicals and induction

The 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°C 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.

1.6 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.

1.7 Alkaloid extraction and determination

Alkaloid extraction and determination were carried out according to the method reported[16]. 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 for 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, 250×4.6 mm dimension and 5 μm packing (Alltech, Deerfield, IL). Samples were eluted with a mobile phase of methanol/acetonitrile/0.025 mol/L ammonium acetate/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 the following equation: alkaloid production (mg/L) = alkaloid content (mg/g) × biomass (g/L).

2 Results

2.1 Expression of the mouse *Bax* in *Catharanthus roseus* cells stimulates indole alkaloid biosynthesis

In order to test the effect of Bax on the biosynthesis of natural products in plant cells, we generated transgenic *Catharanthus roseus* cells overexpressing the mouse Bax protein under a tightly regulated β-estradiol-inducible promoter[17]. As shown in Figure 1(a), the expression of *Bax* was highly dependent on β-estradiol concentrations, which indicates that the expression of Bax in *Catharanthus roseus* cells could be controlled by the application of β-estradiol. Treatment of the transgenic *Catharanthus roseus* cells with 30 μmol/L β-estradiol at the early culture stage (day 0) significantly increased catharanthine and total terpenoid indole alkaloid contents on per cell basis (Figure 1(b)). Since β-estradiol per se had no effects on terpenoid indole alkaloid contents of *Catharanthus roseus* cells (Figure 1(c) and (d)), the data indicated that the expression of Bax was responsible for the enhancement of terpenoid indole alkaloid contents in transgenic *Catharanthus roseus* cells. In other words, expression of the mouse *Bax* triggers indole alkaloid biosynthesis of *Catharanthus roseus* cells.

2.2 Expression of the mouse *Bax* induces transcriptional activation of the genes in terpenoid indole alkaloid biosynthetic pathway of *Catharanthus roseus* cells

Catharanthine is derived from terpenoid indole alkaloid biosynthetic pathway in *Catharanthus roseus*[18]. To evaluate the mechanism of Bax on catharanthine biosynthesis, we examined the expression of terpenoid indole alkaloid biosynthetic genes in transgenic *Catharanthus roseus* cells treated with β-estradiol. In *Catharanthus roseus*, *Tdc* and *Str* genes encode Trp decarboxylase and strictosidine synthase respectively, which play key roles in terpenoid indole alkaloid biosynthetic pathway[19—21]. Both genes are present as single copies in the *Catharanthus roseus* genome[20]. As shown in Figure 2, transcripts of *Tdc* and *Str* are significantly increased in the transgenic cells treated with β-estradiol. Since β-estradiol per se had no effects on *Tdc* and *Str* transcription in wild *Catharanthus roseus* cells (Figure 2) and the transgenic control cells (data not shown), the results indicated that the mouse *Bax* triggers the expression of indole alkaloid biosynthetic genes in transgenic *Catharanthus roseus* cells. Thus, our data strongly suggested that the mouse *Bax* may activate the terpenoid indole alkaloid biosynthetic pathway of *Catharanthus roseus* cells.

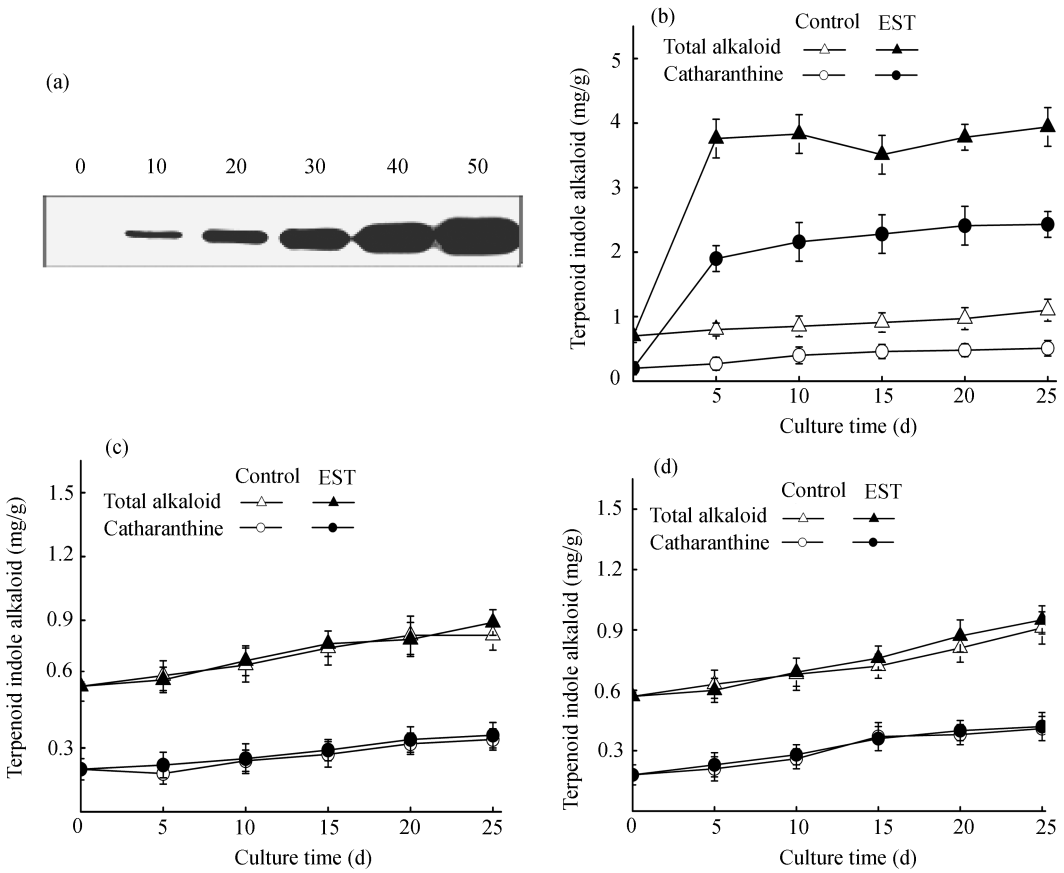

Figure 1 Inducible expression of mouse *Bax* in *Catharanthus roseus* cells and the effects on terpenoid indole alkaloid biosynthesis. (a) Western blot analysis of Bax protein in transgenic *Catharanthus roseus* cells treated with β-estradiol. The micromolar concentrations of β-estradiol are given at the top. Protein was extracted 6 h after β-estradiol treatment. (b) Effects of Bax on catharanthine and total terpenoid indole alkaloid contents of transgenic *Catharanthus roseus* cells. The transgenic cells were treated with 30 μmol/L β-estradiol on day 0. Control received vehicle solvent only. Cells were harvested on the day indicated, catharanthine and total terpenoid indole alkaloid contents were then determined. Values are means of 3 independent experiments. Bar represents the standard errors. (c) Effects of β-estradiol on catharanthine and total terpenoid indole alkaloid contents of wild cells. The wild cells were treated with 30 μmol/L β-estradiol on day 0. Control received vehicle solvent only. Cells were harvested on the day indicated, catharanthine and total terpenoid indole alkaloid contents were then determined. Values are means of 3 independent experiments. Bar represents the standard errors. (d) Effects of β-estradiol on catharanthine and total terpenoid indole alkaloid contents of transgenic control cells. The transgenic control cells (transformed by the empty vector without mouse *Bax*) were treated with 30 μmol/L β-estradiol on day 0. Control received vehicle solvent only. Cells were harvested on the day indicated, catharanthine and total terpenoid indole alkaloid contents were then determined. Values are means of 3 independent experiments. Bar represents the standard errors.

2.3 Expression of *Bax* induces PR1 accumulation in *Catharanthus roseus* cells

It has been reported that fungal elicitor triggers defense responses of plants and activates the biosynthesis of secondary metabolites in the cells[5,22], which prompts us to investigate if mouse Bax could trigger the defense reactions of *Catharanthus roseus* cells. Accumulation of the defense-related protein PR1 is one of the best characterized responses of plants to pathogenic attack[23]. In our work, PR1 protein accumulated in transgenic *Catharanthus roseus* cells 20 h after β-estradiol treatment, while PR1 accumulation was not observed in wild type cells (Figure 3) or in the transgenic control cells (data not shown), which suggested that expression of Bax induces the defense responses of transgenic *Catharanthus roseus* cells.

2.4 Inducible expression of the mouse *Bax* enhances the production of catharanthine and total terpenoid indole alkaloid

Overexpression of *Bax* is lethal in the budding yeast *Saccharomyces cerevisiae*[24,25]. The death-promoting effect of Bax on plant cells has also been found in tobacco and *Arabidopsis* plants[8,9,26]. In this work, we found that overexpression of the mouse *Bax* decreased the biomass of *Catharanthus roseus* cells (Figure 4(a)), indicating that Bax inhibited the growth of the cells.

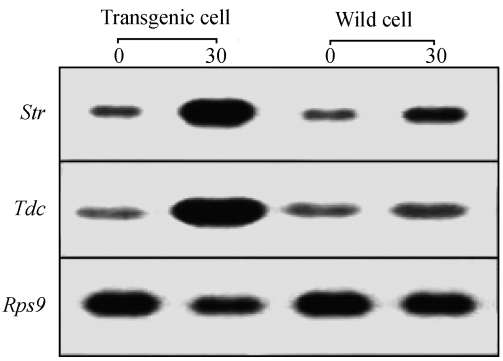

Figure 2 Bax induces the transcriptional activation of terpenoid indole alkaloid biosynthetic genes. Transgenic and wild *Catharanthus roseus* cells were exposed to 30 μmol/L β-estradiol or DMSO (0 μmol/L β-estradiol) for 30 h, then the cells were harvested and total RNA was isolated. Northern blots were hybridized with *Tdc*, *Str*, and *Rps9* cDNAs. The micromolar concentrations of β-estradiol are given at the top.

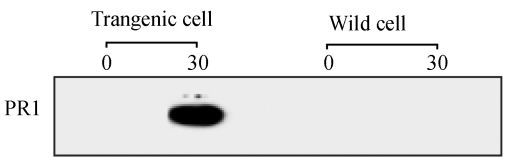

Figure 3 Analysis of PR1 protein accumulation in *Catharanthus roseus* cells. Wild and transgenic *Catharanthus roseus* cells were treated with 30 μmol/L β-estradiol and DMSO (0 μmol/L β-estradiol) respectively. Total cell proteins were extracted 20 h after treatment.

Subsequently, the stimulation of Bax on catharanthine and total terpenoid indole alkaloid production was highly suppressed (data not shown). However, the results also showed that the inhibitory effects of Bax on cell growth were highly dependent on the time and concentrations of β-estradiol application (Figure 4(a) and (b)). Adding 30 μmol/L β-estradiol on day 20 had almost no inhibition on cell growth (Figure 4(b)). Therefore, the highest catharanthine and total terpenoid indole alkaloid production were obtained by applying 30 μmol/L β-estradiol on day 20, which was about 5- and 5.5-fold of the control cells (Figure 4(c) and (d)).

3 Discussion

Catharanthine, a secondary metabolite of *Catharanthus roseus*, can be chemically coupled with vindoline to form the clinically important anti-cancer drug vinblastine, which provides a novel and effective way to commercially produce vinblastine, since vindoline is abundant in *Catharanthus roseus* plants and catharanthine can be produced by *Catharanthus roseus* cell cultures[27,28]. The production of catharanthine by various *Catharanthus roseus* cell cultures has been extensively explored in recent years. Commercial application of plant cell culture for the production of catharanthine, however, is still limited due to the low product yield.

Genetic manipulation has been considered to be the powerful tools for enhancing the production of the secondary metabolites, e.g. indole alkaloids, in plant cells recently[29]. 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[30]. Although the way the secondary metabolites biosynthesize 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 coordinate regulation of multiple gene activities[31,32]. 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 or multiple genes within a particular pathway. 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[33]. Thus, attention has been recently shifted away from reductionist, single structural gene engineering strategies to more complex approaches in the context of the whole cell[29,34]. Recently, some studies use transcriptional factors to control multiple gene expression of secondary metabolite biosynthetic pathways of plant cells[35,36]. However, the regulatory strategy based on the context of whole cell has not been reported until now.

The results of the present work showed that expressing the mouse Bax in *Catharanthus roseus* cells enhanced catharanthine and total terpenoid indole alkaloid contents, showing that Bax might stimulate indole alkaloid biosynthesis. Furthermore, our data showed that inducible expression of Bax significantly induced the transcription of *Tdc* and *Str* in transgenic *Catharanthus roseus* cells, which suggested that mouse Bax might activate the terpenoid indole alkaloid biosynthetic pathway in the cells. Thus, our results suggest that Bax might be a potential regulatory factor for secondary metabolite biosynthesis in plant cells and imply a new secondary metabolic engineering strategy for enhancing the metabolic flux to natural products by activating the whole biosynthetic pathway rather than by engineering the sin-

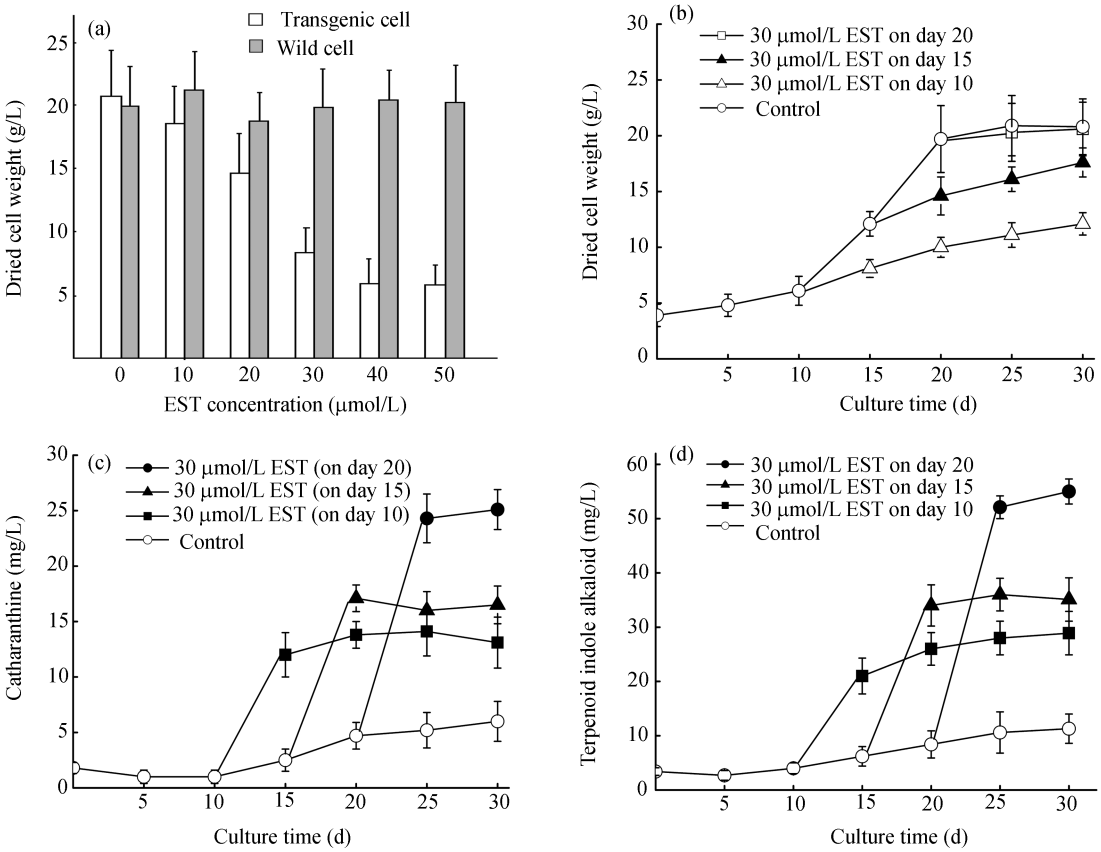

Figure 4 Effects of Bax on cell growth and total indole alkaloid production of *Catharanthus roseus* cells. (a) Effects of β-estradiol concentrations on cell growth. Transgenic *Catharanthus roseus* cells and wild cells were treated with different β-estradiol concentrations on day 0. Cells were harvested on day 25. Values are means of 3 independent experiments. Bars represent standard errors. (b) Effects of the addition time of β-estradiol on cell growth. 30 μmol/L β-estradiol was added into transgenic cells at the time indicated. Values are means of 3 independent experiments. Control receives vehicle solvent only. Bars represent standard errors. (c) Effects of the addition time of β-estradiol on catharanthine production. 30 μmol/L β-estradiol was added into transgenic cells at the time indicated. Values are means of 3 independent experiments. Control receives vehicle solvent only. Bars represent standard errors. (d) Effects of the addition time of β-estradiol on total terpenoid indole alkaloid production. 30 μmol/L β-estradiol was added into transgenic cells at the time indicated. Values are means of 3 independent experiments. Control receives vehicle solvent only. Bars represent standard errors.

gle structural genes within the pathways.

Secondary metabolites of plants not only are important pharmaceutical and source compounds for derivative synthesis, but also play important roles in ecological interactions, especially in protection against biotic and abiotic stresses. However, many secondary metabolites are not necessary for normal plant metabolism[7]. Thus, the biosynthesis of secondary metabolites is tightly controlled in plant cells in ordinary conditions, which might be the main reasons resulting in low production of secondary metabolites of the cultured plant cells. It was reported that both biotic and abiotic stresses might induce secondary metabolite biosynthesis of plant cells. Plants respond to biotic and abotic stresses by activating various reactions. One of the common responses of plants to stresses is the transcriptional reprogramming of plant cells from "normal" primary metabolism to defense-oriented secondary metabolism in the context of whole cell[7,33]. Therefore, activation of plant defense responses at the molecular level may not only trigger the secondary metabolite biosynthetic pathways, but also affect the regulatory systems in the cells to facilitate the accumulation of secondary metabolites. The results of the present work show that expression of the mouse Bax triggers the accumulation of defense-related protein PR1 in *Catharanthus roseus* cells. Since the accumulation of PR1 is one of the best characterized responses of plants to stresses[23], our data indicate that the mouse Bax may activate the defense response of *Catharanthus roseus* cells at the molecular level. Thus, the present work suggests that expression of the mouse Bax in *Catharanthus roseus* cells may stimulate indole alkaloid biosynthesis in the context of whole cells.

Together, the present work shows that the inducible

expression of mouse Bax may enhance the transcriptional level of the key genes in terpenoid indole alkaloid biosynthetic pathway and trigger indole alkaloid biosynthesis. Furthermore, our data indicate that expression of the mouse Bax may activate the defense responses of *Catharanthus roseus* cells at the molecular level. Thus, the results of the present work suggest that the mouse Bax might be a powerful regulatory factor for improving secondary metabolite biosynthesis in plant cells based on the context of whole cell. The molecular basis of the effects of the mouse Bax on secondary metabolite biosynthesis of plant cells, however, needs to be further investigated.

We thank Haegeong Moon and N.H. Chua for giving mouse Bax cDNA and pER 8 promoter respectively.

1 Verpoorte R, Van der Heijden R, Van Gulik W M, et al. In: Brossi A, ed. The Alkaloids, Vol 40. New York: Academic Press, 1991. 2－187

2 Roberts S C, Shuler M L. Large-scale plant cell culture. Curr Op Biotechnol, 1997, 8: 154－159

3 Yu S W，Ou-Yang G C. The material basis of plant pathogenic resistance. In: Yu S W, Tang Z C, eds. Plant Physiology and Molecular Biology (in Chinese). Beijing: Science Press, 1999. 770－783

4 Ebel J, Scheel D. Elicitor recognition and signal transduction. In: Boller T, Meins F, eds. Genes Involved in Plant Defense. New York: Springer-Verlag, 1992. 183－205

5 Ciddi V, Srinivasan V, Shuler M L. Elicitation of *Taxus* sp. cell cultures for production of taxol. Biotechnol Lett, 1995, 17: 1343－1346

6 Li C, Yuan Y J, Ma Z H, et al. Changes of physiological state of suspension cultures of *Taxus chinensis* var. mairei induced by oligosarchride. J Chem Indus Engineer (in Chinese), 2002, 53(11): 1133－1138

7 Buchanan B B, Gurissem W, Jones R L. Biochemistry and Molecular Biology of Plants, 1st ed. American Society of Plant Physiolgists, 2000. 1250－1316

8 Baek D, Nam J, Koo Y D, et al. Bax-induced cell death of *Arabidopsis* is meditated through reactive oxygen-dependent and -independent processes. Plant Mol Biol, 2004, 56: 15－27

9 Lacomme S, Cruz S S. Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc Natl Acad Sci USA, 1999, 96: 7956－7961

10 Murashige T, Skoog F A A. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant, 1962, 15: 473－479

11 Clark M S. Plant Molecular Biology—A Laboratory Manual. 1st ed. Berlin Heidelberg: Springer-Verlag, 1979. 305－340

12 Cruz S S, Chapman S, Roberts S C, et al. Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl Acad Sci USA, 1996, 93: 6286－6290

13 Menke F L, Parchmann S, Mueller MJ, et al. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol, 1999, 19: 1289－1296

14 De Luca V, Marineau C, Brisson N. Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: Comparison with animal dopa decarboxylases. Proc Natl Acad Sci USA, 1989, 86: 2582－2586

15 Pasquali G, Goddijn O J, de Waal A, et al. Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. Plant Mol Biol, 1992, 18:1121－1131

16 Xu M J, Dong J F. Nitric oxide stimulates indole alkaloid production in *Catharanthus roseus* cell suspension cultures through a protein kinase-dependent signal pathway. Enzyme Microb Technol, 2005, 37: 49－53

17 Zuo J R, Niu Q W, Chua N H. Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J, 2000, 24: 265－274

18 Canel C, Lopes-Cardoso M I, Whitmer S, et al. Effects of over-expression of strictosidine synthase and tryptophan decarboxylase on alkaloid production by cell cultures of *Catharanthus roseus*. Planta, 1998, 205: 414－419

19 Kutchan T M. Alkaloid biosynthesis [mdash]: The basis for metabolic engineering of medicinal plants. Plant Cell, 1995, 7: 1059－1070

20 Meijer A H, Verpoorte R, Hoge J H C. Regulation of enzymes and genes involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. J Plant Res, 1993, 3: 145－164

21 Goddijn O J M, Lohman F P, De Kam R J, et al. Nucleotide sequence of the tryptophan decarboxylase gene of *Catharanthus roseus* and expression of *tdc-gusA* gene fusions in *Nicotiana tabacum.* Mol Gen Genet, 1994, 242: 217－225

22 Xu M J, Dong J F. Elicitor-induced nitric oxide burst is essential for triggering catharanthine synthesis in *Catharanthus roseus* suspension cells. Appl Microbiol Biotechnol, 2005, 67: 40－44

23 Linthorst H J M. Pathogenesis-related proteins of plants. Crit Rev Plant Sci, 1991, 10: 123－150

24 Simonen M, Keller H, Heim J. The BH3 domain of Bax is sufficient for interaction of Bax with itself and with other family members and it is required for induction of apoptosis. Eur J Biochem, 1997, 249: 85－91

25 Zha H, Aime-Sempe C, Sato T, et al. Proapoptotic protein Bax heterodimerizes with Bcl-2 and homodimerizes with Bax via a novel domain (BH3) distinct from BH1 and BH2. J Biol Chem, 1997, 271: 7440－7444

26 Kiwai-Yamada M, Jin L, Yoshinage K, et al. Bax-induced plant cell death can be down-regulated by overexpression of *Arabidopsis* Bax Inhibitor-1 (AtBI-1). Proc Natl Acad Sci USA, 2001, 98: 12295－

1300

27 Zhao J, Zhu W, Hu Q. Selection of fungal elicitors to increase indole alkaloid accumulation in *Catharanthus roseus* suspension cell culture. Enzyme Microb Technol, 2001, 28: 666－672

28 Xu M J, Dong J, Zhu M Y. Effect of nitric oxide on catharanthine production in *Catharanthus roseus* suspension cells. Biotechnol Bioeng, 2005, 89: 367－371

29 Capell T, Christou P. Progress in plant metabolic engineering. Curr Opin Biotechnol, 2004, 15:148－154

30 Sato F, Hashimoto T, Haciya A, et al. Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci USA, 2001, 98: 367－372

31 Rothe G, Hachiya A, Yamada Y, et al. Alkaloids in plants and root cultures of Atropa belladonna overexpressing putrescine N-methyltransferase. J Exp Bot, 2003, 54(390): 2065－2070

32 Moyano E, Jouhikainen K, Tammela P, et al. Effect of pmt gene overexpression on tropane alkaloid production in transformed root cultures of Datura metel and Hyoscyamus muticus. J Exp Bot, 2003, 54(381): 203－211

33 Hahlbrock K, Bednarek P, Ciolkowski I, et al. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. Proc Natl Acad Sci USA, 2003, 100s: 14569－14576

34 Broun P, SomervIlle C. Progress in plant metabolic engineering. Proc Natl Acad Sci USA, 2001, 16: 8925－8927

35 Bovy A, de Vos R, Kemper M, et al. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. Plant Cell, 2002, 14(10): 2509－2526

36 Yu O, Shi J, Hession A O. Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry, 2003, 63: 753－763