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Effects of β-cyclodextrin and methyl jasmonate on the production of vindoline, catharanthine, and ajmalicine in *Catharanthus roseus* cambial meristematic cell cultures

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Abstract Long-term stable cell growth and production of vindoline, catharanthine, and ajmalicine of cambial meristematic cells (CMCs) from *Catharanthus roseus* were observed after 2 years of culture. *C. roseus* CMCs were treated with β-cyclodextrin (β-CD) and methyl jasmonate (MeJA) individually or in combination and were cultured both in conventional Erlenmeyer flasks (100, 250, and 500 mL) and in a 5-L stirred hybrid airlift bioreactor. CMCs of *C. roseus* cultured in the bioreactor showed higher yields of vindoline, catharanthine, and ajmalicine than those cultured in flasks. CMCs of *C. roseus* cultured in the bioreactor and treated with 10 mM β-CD and 150 μM MeJA gave the highest yields of vindoline (7.45 mg/L), catharanthine (1.76 mg/L), and ajmalicine (58.98 mg/L), concentrations that were 799, 654, and 426 % higher, respectively, than yields of CMCs cultured in 100-mL flasks without elicitors. Quantitative reverse transcription (RT)-PCR showed that β-CD and MeJA upregulated transcription levels of genes related to the biosynthesis of terpenoid indole alkaloids (TIAs). This is the first study to report that β-CD induced the generation of NO, which plays an important role in mediating the production of TIAs in *C. roseus* CMCs. These results suggest that β-CD and MeJA can enhance the production of TIAs in CMCs of *C. roseus*, and thus, CMCs of *C. roseus* have significant potential to be an industrial platform for production of bioactive alkaloids.

Keywords *Catharanthus roseus* · Cambial meristematic cells · β-Cyclodextrin · Methyl jasmonate · Terpenoid indole alkaloids

Pengfei Zhou and Jiazeng Yang contributed equally to this work.

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Introduction

The medicinal plant *Catharanthus roseus* (L.) G. Don is of enormous pharmaceutical interest because it contains more than 130 terpenoid indole alkaloids (TIAs), some of which exhibit strong pharmacological activities, such as vindoline, catharanthine, and ajmalicine (Guo et al. 2005; van der Heijden et al. 2004). Vinblastine and vincristine, two bisindole alkaloids derived from coupling vindoline and catharanthine, were the first natural anticancer drugs and are still among the most valuable agents used to treat cancer (Guo et al. 2005; van der Heijden et al. 2004). Ajmalicine is a potent antihypertensive reagent (Liu et al. 2012).

Bioactive alkaloid content in *C. roseus*, however, is extremely low (e.g., 1 g of vinblastine was extracted from approximately 500 kg of dry leaves of *C. roseus*), while undesired alkaloids are abundant; thus, the extraction and purification of the valuable TIAs from *C. roseus* leaves have historically been laborious and expensive (Noble 1990). Commercial production has used a semi-synthetic route to couple

vindoline and catharanthine (van der Heijden et al. 2004). However, *C. roseus* leaves are still the primary commercial resource of the dimeric alkaloids vincristine and vinblastine (Salim and De Luca 2013). Great progress in plant biotechnology in recent decades has made the culturing of plant cells and tissues an attractive alternative for the economical production of valuable anticancer alkaloids.

Cultured plant cells can be either undifferentiated or differentiated. High and stable production of vindoline and catharanthine has never been achieved in most differentiated cell cultures of *C. roseus*, such as callus and hairy root cultures (Zhao and Verpoorte 2007). Callus cultures are multicellular mixtures that have undergone dedifferentiation (Grafi et al. 2007; Thorpe 2007). Because deleterious genetic and epigenetic changes often occur during this process, callus exhibits poor growth properties with low and inconsistent yields of secondary metabolites (Baebler et al. 2005; Roberts 2007). The absence of key biosynthetic genes in cultured plant cells and tissues is another cause of low yields of secondary metabolites (Kutchan 1998). For example, although hairy roots have been found to grow faster and larger than other cell cultures in large-scale bioreactors (Nguyen et al. 2013), and many alkaloid biosynthetic genes in hairy roots of *C. roseus* are stable over time, *C. roseus* hairy roots have not produced vinblastine and vincristine because the biosynthetic genes of the precursor, vindoline, are absent (Salim and De Luca 2013; Peebles et al. 2009). Conversely, innately undifferentiated cambial meristematic cells (CMCs) of *Taxus cuspidata*, whose marker genes and transcriptional programs were consistent with stem cell identity, were found to produce large yields of paclitaxel (264 mg/kg) in culture in a 3-L airlift bioreactor (Lee et al. 2010). Despite numerous studies on the use of differentiated cell cultures of *C. roseus* for TIA production, undifferentiated cell cultures of *C. roseus* remain unstudied. In our previous work, CMCs from *C. roseus* were isolated and cultured, and 66.6 % of cells were present as singletons or small aggregates (2–3 cells) (Zhou et al., unpublished) (Fig. S1 in the Supplementary Materials). These small and abundant vacuoles may be responsible for the relative tolerance of *C. roseus* CMCs to shear stress, reduced aggregation, and thin cell walls, characteristics that are highly beneficial for the development of a commercial culturing system in bioreactors (Lee et al. 2010; Joshi et al. 1996).

Elicitors such as β-cyclodextrin (β-CD) and methyl jasmonate (MeJA) have been previously used to enhance alkaloid production in *C. roseus* cell and hairy root cultures. β-CD was found to elicit the biosynthesis of phytoalexin by inducing a defense response (Wasternack 2007). MeJA, a plant stress hormone, accumulates in high levels in plant tissues damaged by abiotic or biotic stress and can activate many biosynthetic pathways (Vázquez-Flota et al. 2009). In *C. roseus*, MeJA induced the accumulation of ajmalicine in cell suspension cultures, both ajmalicine and catharanthine in hairy root cultures, and vindoline in shoot cultures. This response was induced by upregulating transcription of genes coding for enzymes involved in TIA biosynthesis, including geraniol synthase (GES), geraniol 10-hydroxylase (G10H), tryptophan decarboxylase (TDC), strictosidine synthase (STR), strictosidine β-D-glucosidase (SGD), and desacetoxyvindoline-4-hydroxylase (D4H) (Fig. 1) (Goklany et al. 2009; van der Fits and Memelink 2000; Simkin et al. 2013). Expression of the *ORCA3* gene for a transcription factor is induced by jasmonates that also regulate the expression of *G10H*, *TDC*, *STR*, *SGD*, and *D4H* in cell suspension cultures (van der Fits and Memelink 2000; Simkin et al. 2013; Montiel et al. 2011; Vazquez-Flota et al. 1997). In addition, both loganic acid O-methyltransferase (LAMT), which is involved in secologanin biosynthesis, and iridoid synthase (IRS), which is capable of generating the iridoid ring scaffold, have been shown to play important roles in the biosynthesis of bioactive TIAs in *C. roseus* (Murata et al. 2008; Geu-Flores et al. 2012; Miettinen et al. 2014).

In the present study, excellent growth properties and stable production of TIAs were found in CMCs of *C. roseus* after 2 years of culture. We investigated the production of vindoline, catharanthine, ajmalicine, and total alkaloids in *C. roseus* CMC cultures that were treated with β-CD and MeJA individually or in combination in both Erlenmeyer flasks and 5-L bioreactors. Transcription of the key biosynthetic genes and the transcription factor *ORCA3* gene was also analyzed using quantitative reverse transcription (RT)-PCR. Analysis of levels of gene transcription and TIA accumulation together provide a better understanding of the regulation of TIA biosynthesis. The results indicate that *C. roseus* CMCs combined with low-cost bioreactor technology may provide a method for increasing commercial production of vindoline, catharanthine, and ajmalicine.

Materials and methods

Chemicals

Vindoline, catharanthine, ajmalicine, β-CD, and MeJA were purchased from Aladdin (Aladdin Reagents Co., Shanghai, China). Trizol, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) were obtained from Takara (Takara Bio., Kyoto, Japan). All other chemicals were of analytical grade.

Plant materials and culture conditions

CMCs of *C. roseus* were established using the modified methods of Lee et al. (2010). Twigs collected from wild-grown *C. roseus* were rinsed in running tap water for 30 min, disinfected with 75 % ethanol for 30 s, and then rinsed

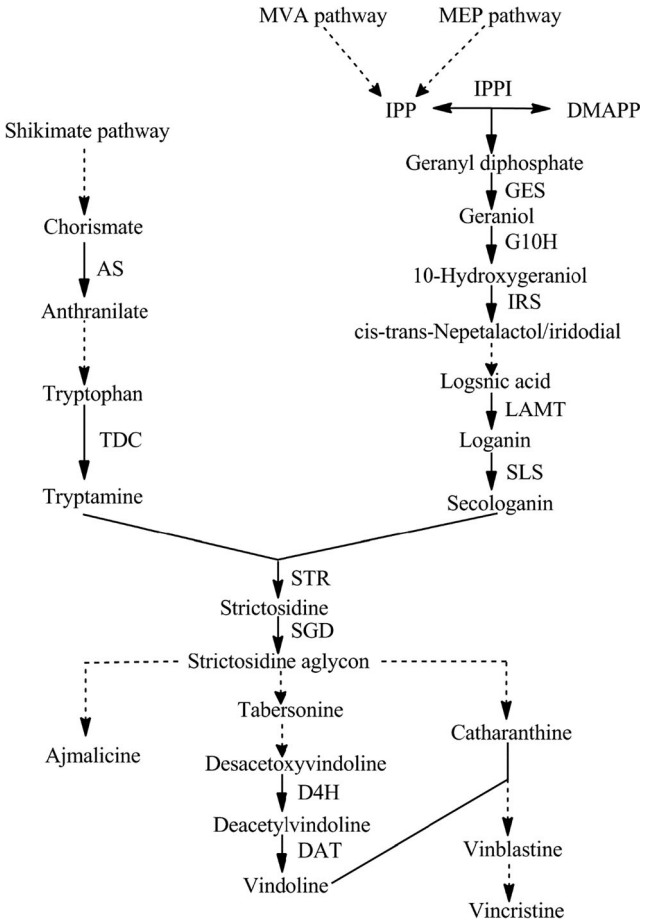

Fig. 1 Terpenoid indole alkaloid biosynthetic pathways of *C. roseus* (Goklany et al. 2009; Murata et al. 2008; Geu-Flores et al. 2012; Miettinen et al. 2014) Enzyme abbreviations: *MVA*, mevalonic acid; *MEP*, methylerythritol 4-phosphate; *IPPI*, isopentenyl diphosphate isomerase; *GES*, geranial synthase; *IRS*, iridoid synthase; *G10H*, geraniol 10-hydroxylase; *LAMT*, loganic acid methyltransferase; *TDC*, tryptophan decarboxylase; *STR*, strictosidinesynthase; *SGD*, strictosidine β-D-glucosidase; *D4H*, desacetoxyvindoline 4-hydroxylase. *Single arrows* denote single steps and *dotted arrows* denote multiple or unidentified steps

five times with sterilized distilled water (dH_2O). Twigs were then put in 0.05 % $HgCl_2$ for 6 min, rinsed five times with dH_2O, and rinsed once with dH_2O containing 150 mg/L citric acid. Cambium, phloem, cortex, and epidermal tissues were peeled from the xylem, and the epidermal tissue was placed on pH-adjusted (5.75) Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1.0 mg/L α-naphthalene acetic acid (NAA), 30 g/L sucrose, and 4 g/L gelrite. After 7 to 12 days, cell division was found only in cambium, and after 18 days, dedifferentiated cells (DDCs) began to generate from the layer consisting of phloem, cortex, and epidermis. Twenty-four days post-culture, a visible split was observed between cambium cells forming a flat plate of cells and the DDCs of the phloem, cortex, and epidermis that proliferated in an irregular manner. After separation of the cambial cells from the other cell types, the cambial cell layer was transferred to 250-mL Erlenmeyer flasks containing 100 mL of MS medium supplemented with 2.0 mg/L NAA, 10 g/L sucrose, and 4 g/L gelrite. CMCs of *C. roseus* were cultured at 25 °C under continuous dark and were maintained by serial subculture every 12 days. Two months prior to the experiments, CMCs were transferred to 100 mL of MS solid medium in a 250-mL Erlenmeyer flask and grown at 25 °C with a 12/12-h light/dark photoperiod. Initial suspension cultures were established by inoculating CMCs into MS liquid medium supplemented with 2.0 mg/L NAA and 20 g/L sucrose at concentrations of 50.0 g fresh weight/L. CMC growth was determined by grams of dry weight (DW)per liter:

$$\text{Growth rate} = \frac{\text{dry cell weight}}{\text{initial dry cell weight}} \times 100\%$$

Establishment of a long-term culture system of *C. roseus* CMCs

Suspension cultures of *C. roseus* CMCs were incubated on a HZT-2 gyrotory shaker (Donglian Electronic & Technol. Dev. Co., Beijing, China) at 25 °C and 120 rpm under continuous light. CMCs were subcultured every 12 days in 250-mL Erlenmeyer flasks by transferring 15 mL of 12-day-old suspension into 85-mL fresh MS medium. Suspension cultures were continuously maintained for 2 years. Roughly every 3 months, the cultures were harvested for determination of growth characteristics and TIA production. Cell viability was assayed using the fluorescein diacetate staining method (Durante et al. 2011).

Treatment with 10 mM β-CD and 150 μM MeJA individually or in combination

First, 12-day-old suspensions of *C. roseus* CMCs were centrifuged at 300×*g* for 10 min, and the medium was discarded. CMCs (10.0 g fresh weight) were transferred to 500-mL Erlenmeyer flasks containing 200-mL MS liquid medium supplemented with different concentrations of β-CD (0, 2, 4, 6, 8, or 10 mM) and maintained at 25 °C with continuous light and orbital shaking at 120 rpm. Cultures were harvested after 12 days. Cell viability was assayed using the fluorescein diacetate staining method (Durante et al. 2011). Twelve-day-old suspensions of CMCs (50.0 g fresh weight/L) were then cultivated with continuous light in 100-, 250-, and 500-mL Erlenmeyer flasks and in 5-L stirred hybrid airlift bioreactors at 25 °C (containing 50-, 100-, and 200-mL and 4-L liquid medium, respectively), each supplemented with 0 or 10 mM β-CD. CMCs in flasks were maintained at 25 °C in constant light and 120 rpm on a HZT-2 gyrotory shaker (Donglian Electronic & Technol. Dev. Co.). The bioreactor used was a 5BGG-7000A hybrid airlift bioreactor (Baoxing Bio-

engineering Equipment Co., Shanghai, China) equipped with two sets of turbine impellers, an Ingold pH probe (Mettler-Toledo Inc., Columbus, OH, USA), an Ingold polarographic dissolved oxygen probe (Mettler-Toledo Inc.), a flowmeter, and inlet/outlet air sterilization filters (PTFE membrane, 0.2-μm pore size, Cole-Parmer). The total and working volumes of the bioreactor were 5 and 4 L, respectively. All the experiments were performed in continuous light at 25 °C and a constant pH of 5.8, which was controlled by automatic addition of either 2 M NaOH or HCl solution. Temperature in the bioreactor was controlled using a circulating bath, and the aeration and agitation rates were 0.3 vvm (volume of air per volume of medium per min) and 110 rpm, respectively. Cultures were harvested after 3, 6, 9, 12, and 15 days. Finally, to assess the effects of MeJA and β-CD both individually and in combination, 12-day-old suspensions of CMCs (200.0 g fresh weight) were transferred to 5-L bioreactors containing 4-L MS liquid medium supplemented with 10 or 0 mM (control) β-CD. Cultures were treated with 90 % ethanol or an equal volume of 150 μM MeJA (in 90 % ethanol) on day 6, 9, or 12 and were harvested 24 h after treatment. All CMC cultures were separated from the liquid media by vacuum filtration, washed with distilled water, and freeze-dried. Experiments were performed in triplicate.

Alkaloid extraction and determination

Alkaloid extraction was performed according to the methods of Liu et al. (2013). Alkaloid extracts were dissolved in 1.0 mL of methanol and analyzed by HPLC and ion-pair extraction-spectrophotometry following Yang et al. (2008). Vindoline, catharanthine, and ajmalicine were determined at 25 °C by HPLC analysis using an Agilent 1260 series system (Agilent Technologies, Santa Clara, CA, USA) and a Phenomenex Gemini C18 column (250 mm×4.6 mm, 5 μm) (Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of methanol/acetonitrile/10 mM ammonium acetate (15:40:45, *v*/*v*/*v*) at a flow rate of 1.0 mL/min. The detection wavelength was 280 nm and the injection volume was 10 μL. Before injection, all samples were filtered with 0.22-μm nylon membrane filters. Alkaloids were identified and quantified by comparing retention time and UV absorbance spectra with the commercial standard. Each sample was analyzed in triplicate.

Monitoring gene expression using qRT-PCR

CMC cultures were frozen in liquid nitrogen and ground into powder with a mortar and pestle, and RNA extraction was performed as described by Liu et al. (2013). A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to quantify the content of RNA. A PrimeScriptTM reagent kit with gDNA Eraser (Takara Bio., Kyoto, Japan) was used to treat 1 μg of total RNA with DNase to remove genomic DNA, after which cDNA was synthesized according to manufacturer instructions.

Transcript levels of the 40S ribosomal protein S9 gene (*RPS9*, the housekeeping gene), *GES*, *G10H*, *IRS*, *LAMT*, *TDC*, *STR*, *SGD*, *D4H*, and the transcription factor *ORCA3* gene were monitored in CMC cultures treated with 10 mM β-CD and 150 μM MeJA individually or in combination. Primer sequences for *RPS9*, *LAMT*, *IRS* (Geu-Flores et al. 2012), *TDC*, *G10H, STR*, *D4H*, *GES*, *ORCA3* (Sander 2009), and *SGD* (Goklany et al. 2009) are shown in Table S1 in the Supplementary Materials. Primers for *GES* were designed using the Primer Express 3.0 software (Applied Biosystems, Framingham, CA, USA) with the default parameters.

The qRT-PCR mixture was prepared using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio., Kyoto, Japan), and reactions were performed using a 48-well MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification included a holding stage of 30 s at 95 °C and 40 cycles, each consisting of 5 s at 95 °C followed by 20 s at 60 °C. Melt curve analysis at 60–95 °C was then used to verify the specificity of the amplicons. Expression stability of the qRT-PCR results were assayed using the Bio-Rad CFX Manager Software (Bio-Rad, Hercules, CA, USA). All samples were measured in triplicate.

Measurement of NO

After treatment with β-CD (different concentrations, 0, 2, 4, 6, 8, or 10 mM) or 150 μM MeJA individually, or with 10 mM β-CD and 150 μM MeJA in combination, 1-mL filtrate of each CMC suspension was obtained using 0.22-μm nylon membrane filters (Jinteng Experiment Equipment Co., Tianjin, China). The NO concentration in the liquid medium represented the content of NO produced by the CMCs and was quantified using previously described methods (Xu and Dong 2005; Hu et al. 2003).

Statistical analysis

All values were reported as means±SD. Statistical analyses were performed using independent two-tailed Student's *t* test. All comparisons were made relative to untreated controls. Differences were considered significant at $p<0.05$ (indicated by asterisk; $p<0.01$ indicated by double asterisk).

Results

Growth characteristics and TIA production of *C. roseus* CMCs over 2 years in culture

The determination of growth characteristics and vindoline, catharanthine, and ajmalicine production of *C. roseus* CMCs

over 2 years in culture showed that the growth plateau and steady production of alkaloid metabolites can take up to 6 months to reach (Fig. S2 in the Supplementary Materials). In addition, the growth plateau lasted 18 months, indicating that *C. roseus* CMCs are a robust producer of alkaloid metabolites and tolerant to the oscillating conditions that occur during the sub-culturing process. Moreover, this result was consistent with previous work (Peebles et al. 2009).

Dose-dependent effect of β-CD on CMC growth, NO generation, and TIA production

Increasing concentrations of β-CD did not inhibit CMC growth; on the contrary, CMCs treated with β-CD grew faster than control cells (Fig. S3a in the Supplementary Materials). Vindoline, catharanthine, and ajmalicine production were also induced by β-CD in a dose-dependent manner. Maximal production of vindoline (1.03 mg/L), catharanthine (0.64 mg/L), and ajmalicine (28.64 mg/L) were obtained with 10 mM β-CD, which corresponded to 219, 122, and 164 % of the respective yields in the control cultures (Fig. S3c, d, e in the Supplementary Materials). Maximum total alkaloid accumulation was found on day 12 after treatment with 10 mM β-CD and, at 727.4 mg/L, was 51 % greater than controls. Moreover, 29 % of total alkaloids (216.1 mg/L) were secreted directly into the medium, a level 190 % higher than in the control medium (Fig. S4 in the Supplementary Materials). We also monitored NO generation induced by β-CD and MeJA because common metabolism stimulators (e.g., MeJA and β-CD) associate with the signaling molecule NO, which mediates plant secondary metabolite biosynthesis to defend the corresponding elicitor against pathogenic attack. Unexpectedly, the maximum concentration (9.4 μmol/L) of NO in the medium of β-CD treated cells was nearly threefold that of the control group (3.1 μmol/L) (Fig. S3b in the Supplementary Materials).

Comparison of *C. roseus* CMC growth and TIA production with 10 mM β-CD in Erlenmeyer flasks versus bioreactors

C. roseus CMC cultures in both flasks and bioreactors received 10 mM β-CD to provide optimal growth conditions and achieve maximal yield and biomass quality. Growth assessment revealed that 10 mM β-CD increased growth in treated CMCs compared with control groups (Fig. 2). Maximal growth rates of CMCs treated with β-CD in 100-, 250-, and 500-mL Erlenmeyer flasks and 5-L bioreactors were 523, 608, 649, and 726 %, respectively, while those of corresponding controls were 461, 560, 560, and 600 %, respectively.

The accumulation time course of each alkaloid (vindoline, catharanthine, and ajmalicine) in CMCs treated with 10 mM β-CD is shown in Figs. 3, 4, and 5, respectively. At all time points for each alkaloid, yields were higher in bioreactors than in Erlenmeyer flasks. In both vessels, the patterns of relative contents of each alkaloid were similar to those of the controls. In all controls, vindoline production peaked on day 9, yielding 0.93, 1.43, and 1.62 mg/L in the 100-, 250-, and 500-mL flasks, respectively, and 1.95 mg/L in the 5-L bioreactor. CMCs treated with 10 mM β-CD showed maximal vindoline production on day 6 in the flasks (1.18, 1.88, and 1.94 mg/L in the 100-, 250-, and 500-mL flasks, respectively) and on day 9 in the bioreactors (2.54 mg/L). Thus, β-CD increased vindoline content by about 30 % across all scales of culture. Compared with vindoline, catharanthine production peaked later, occurring on day 12 or 15. Treatment with β-CD increased catharanthine content 21–31 % over controls, yielding 0.352, 0.578, and 0.61 mg/L in the 100-, 250-, and 500-mL flasks, respectively, and 0.783 mg/L in the 5-L bioreactor. Maximum ajmalicine concentrations occurred on day 12 in both β-CD-treated and control groups. Treatment with β-CD, however, increased yields by 15–23 % to 16.19, 24.08, and 26.28 mg/L in the 100-, 250-, and 500-mL flasks and to 36.34 mg/L in the 5-L bioreactors.

Total alkaloid yield depended on the cultivation time and culture volume (Fig. S5 in the Supplementary Materials). Total alkaloid yield increased with increasing culture size at relatively similar rates in 10 mM β-CD-treated groups and in controls. Total alkaloid yield with 10 mM β-CD reached a maximum on day 12 and was 14–49 % higher than in control groups, producing 595.5, 731.2, and 813.3 mg/L in the 100-, 250-, and 500-mL flasks, respectively, and 937.4 mg/L in the 5-L bioreactors.

Overall, treatment of *C. roseus* CMCs with 10 mM β-CD induced increased cell growth and production of vindoline, catharanthine, ajmalicine, and total alkaloids, particularly in cells cultured in the bioreactors.

Individual and combined effects of β-CD and MeJA on regulation of CMC growth, TIA production, and gene expression in 5-L bioreactor cultures

Growth of CMCs did not differ significantly between the control and MeJA-treated groups or between the β-CD- and MeJA/β-CD-treated groups (Fig. 6a), indicating that MeJA had no effect on cell growth. Elicitor addition (MeJA, β-CD, or MeJA/β-CD) sharply increased NO levels in the media, with the highest concentrations (33.0 μmol/L on day 10) occurring in cultures treated with both elicitors (MeJA/β-CD group) (Fig. S6 in the Supplementary Materials). The accumulation time courses of vindoline, catharanthine, and ajmalicine in each of the CMC culture treatments (MeJA, β-CD, or MeJA/β-CD) and in the control are shown in Fig. 6b, c, d. All three elicitor treatments increased alkaloid yields relative to the controls. Maximal vindoline production was reached on day 10 in all groups, with 2.14 mg/L in the control

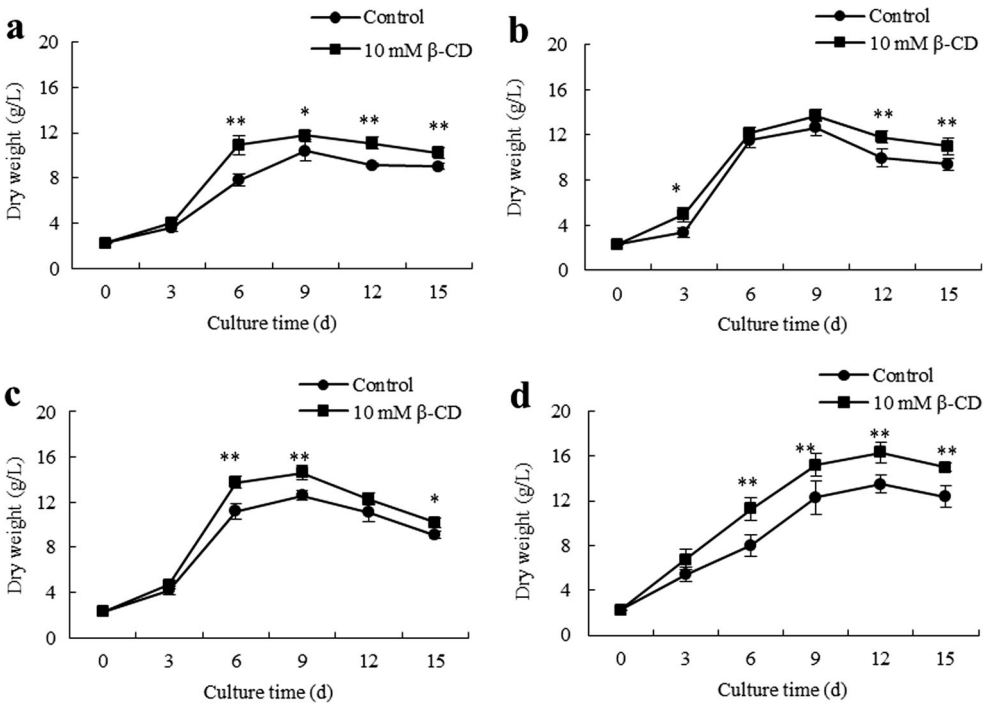

Fig. 2 Effect of 10 mM β-CD on growth of *C. roseus* CMCs in 100-mL (**a**), 250-mL (**b**), and 500-mL (**c**) Erlenmeyer flasks and 5-L bioreactors (**d**). Measurements were taken 3, 6, 9, 12, and 15 days after treatment. Values are means±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

group and 3.42, 2.85, and 7.45 mg/L in the MeJA, β-CD, and combined β-CD/MeJA treatments, respectively. Thus, vindoline yields were 60, 33, and 248 % higher with added MeJA, β-CD, and β-CD/MeJA (Fig. 6b). Maximal catharanthine yields were 0.75, 0.91, and 1.76 mg/L in the MeJA, β-CD, and combined β-CD/MeJA treatments, respectively, representing increases of 34, 61, and 213 % relative to controls (Fig. 6c). Similarly, maximal ajmalicine yields were 32.45, 37.46, and 58.98 mg/L in the MeJA, β-CD, and combined β-CD/MeJA treatments, respectively, which were 15, 32, and 108 % greater than the respective yields in the control group (Fig. 6d).

Transcript levels of the genes involved in the biosynthesis of TIAs (not including *GES*, *IRS*, and *LAMT*) were previously examined in cell suspensions, hairy roots, and shoots of *C. roseus* treated with MeJA (Goklany et al. 2009; van der

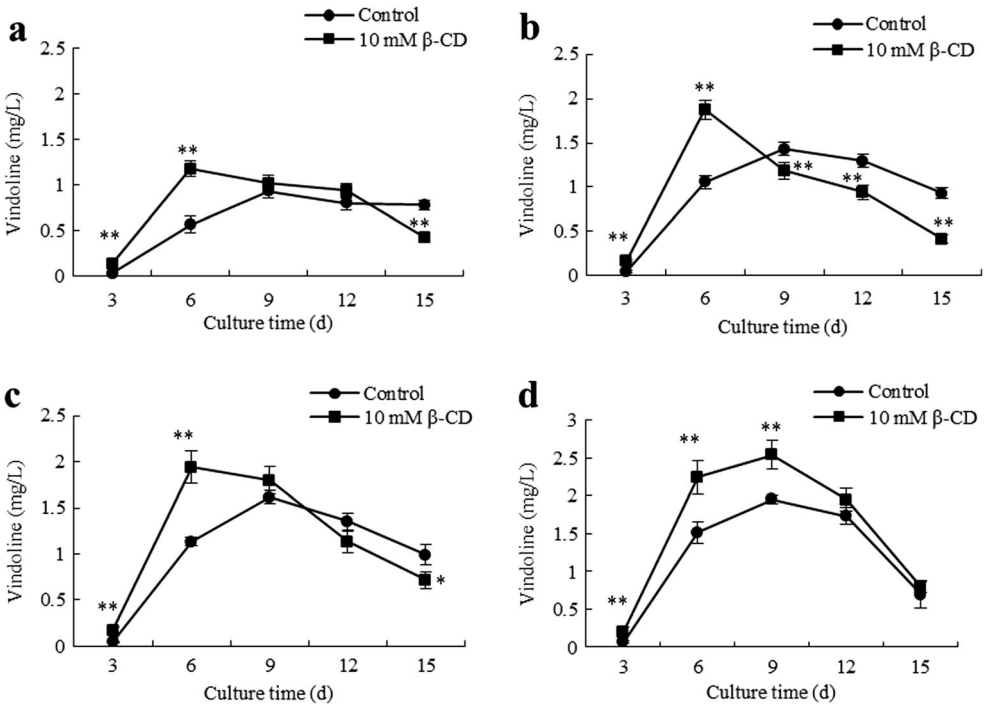

Fig. 3 Effect of 10 mM β-CD on vindoline production of *C. roseus* CMCs in 100-mL (**a**), 250-mL (**b**), and 500-mL (**c**) Erlenmeyer flasks and 5-L bioreactors (**d**). Measurements were taken 3, 6, 9, 12, and 15 days after treatment. Values are means±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

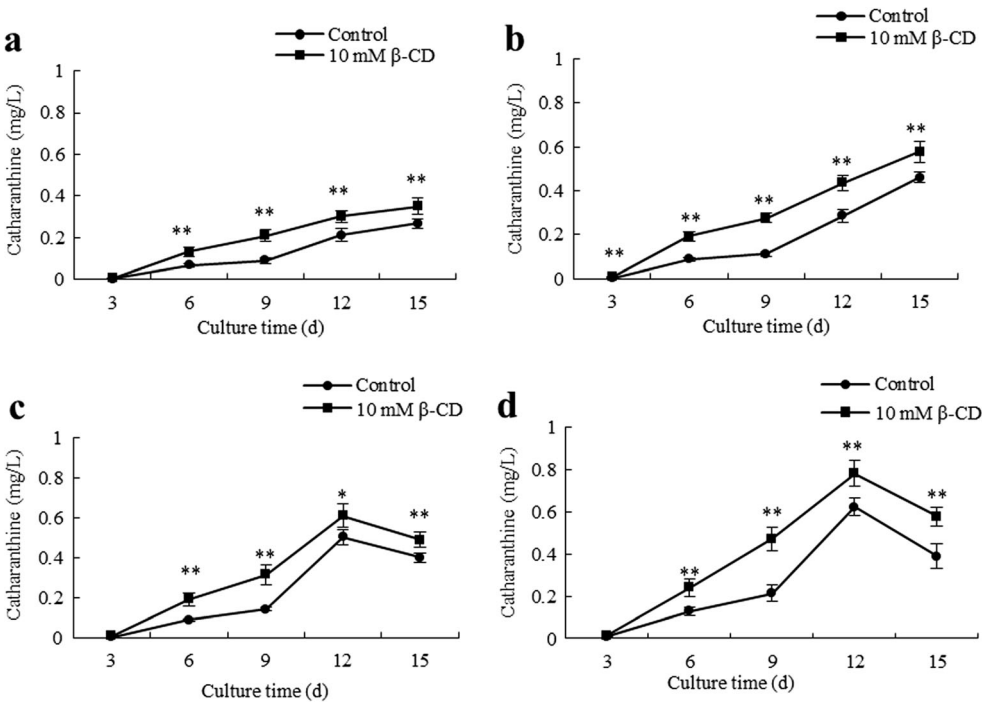

Fig. 4 Effect of 10 mM β-CD on catharanthine production of *C. roseus* CMCs in 100-mL (**a**), 250-mL (**b**), and 500-mL (**c**) Erlenmeyer flasks and 5-L bioreactors (**d**). Measurements were taken 3, 6, 9, 12, and 15 days after treatment. Values are means ±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

Fits and Memelink 2000). The combined effects of β-CD and MeJA on the transcription of TIA biosynthesis genes in *C. roseus* CMC cultures, however, have not been reported. This research examined transcript levels of the genes involved in the methylerythritol 4-phosphate (MEP) pathway (*GES*, *G10H*, *IRS*, and *LAMT*) and the early (*TDC*, *STR*, and *SGD*) and late (*D4H*) MIA-biosynthesis pathways, and of the transcription factor *ORCA3* gene in CMCs of *C. roseus* 24 h after treatment with 10 mM β-CD and 150 μM MeJA individually or in combination (Fig. 7). Both elicitors were found to induce transcription of these genes, and the combination of β-CD and MeJA together was more efficient than either alone. Maximal levels of *LAMT*, *STR*, and *D4H* transcription occurred on day 7 after elicitor addition and were 13.4, 6.2, and 5.2 times higher than those of the control, respectively. Maximal levels of *GES*, *G10H*, *TDC*, *SGD*, and *ORCA3* transcription were

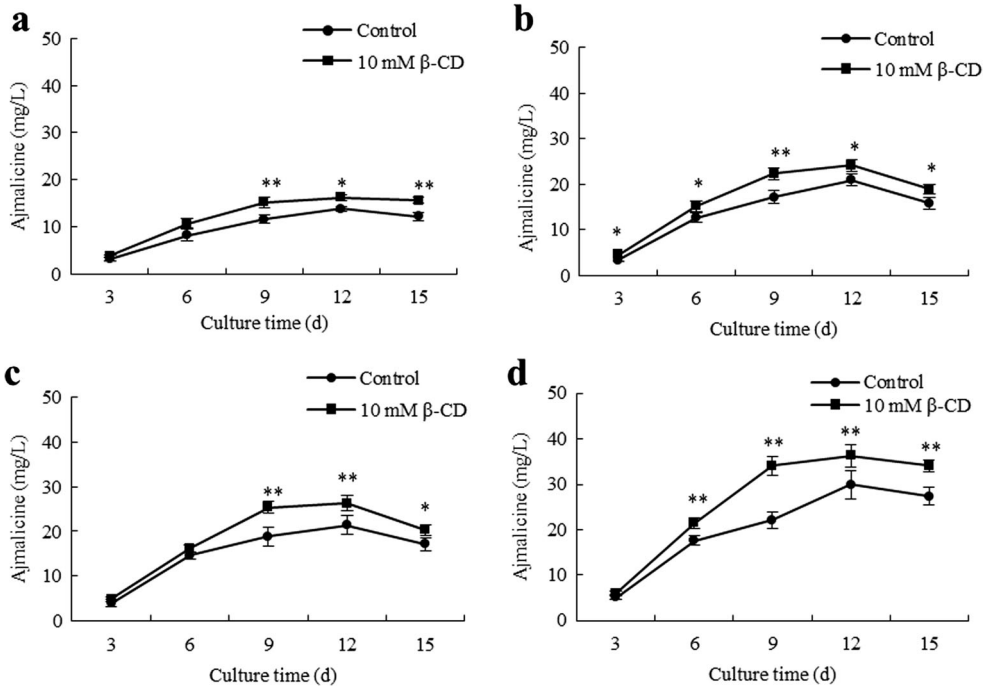

Fig. 5 Effect of 10 mM β-CD on ajmalicine production of *C. roseus* CMCs in 100-mL (**a**), 250-mL (**b**) and 500-mL (**c**) Erlenmeyer flasks and 5-L bioreactors (**d**). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means ±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t*-test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

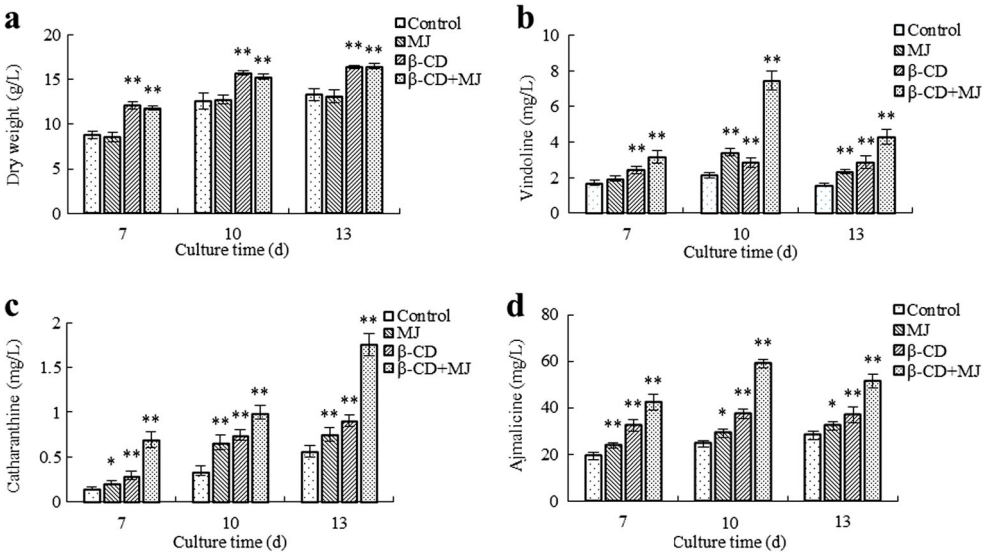

Fig. 6 Effects of 10 mM β-CD and 150 μM MeJA individually or in combination on *C. roseus* CMC growth (**a**), vindoline production (**b**), catharanthine production (**c**), and ajmalicine production (**d**) in 5-L bioreactors. Measurements were taken 24 h after treatment with MeJA on days 6, 9, and 12. Values are means±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

reached on day 10 and were 3, 5.1, 7.2, 6.4, 7.1, and 7.5 times higher than those of the control, respectively. Finally, maximum transcription of *IRS* was sixfold greater than in the control and occurred on day 13.

Discussion

The culturing of plant cells and tissues is known to cause a variety of genetic mutations, primarily via transposable elements. These genetic instabilities often lead to instability in cellular morphology, cell growth, and metabolite production (Hirochika et al. 1996; Phillips et al. 1994). In this study, we found that undifferentiated CMCs from *C. roseus* that we had previously established (Fig. S1 in the Supplementary Materials) were capable of maintaining good cellular morphology (66.6 % of cells were present as singletons or small aggregates) and stable and high production of alkaloid metabolites.

Dramatic decreases in cell viability and enzymatic activities often occur in many plant cell and tissue cultures. Clathering agents such as cyclodextrins have been used to try to preserve cell viability (Rao and Ravishankar 2002). Research has shown that methyl-β-CD can dramatically enhance the production of artemisinin in *Artemisia annua* cell suspension cultures (Van Uden et al. 1993), ajmalicine in *C. roseus* cell cultures (Almagro et al. 2011), and phenolic compounds in hairy root cultures of *Scutellaria lateriflora* (Marsh et al. 2014). The cyclodextrin β-CD, however, is less expensive and more readily available. Previous work showed that β-CD did not affect cell growth or podophyllotoxin content in cell suspension cultures of *Linum flavum* L. or *Podophyllum hexandrum*, indicating that β-CD was neither toxic nor metabolized or used as a carbon source by the cells (Van Uden et al. 1993; Woerdenbag et al. 1990). Cyclodextrins have also been found to increase cell growth and production of taxol and taxanes in cultures of *Taxus chinensis* cells (Alexey 2004). Like previous reports (Durante et al. 2011), our data (Zhou et al., unpublished) suggested that β-CD does not decrease CMC viability. In perfusion culture, CMCs from *T. cuspidata* produced a total of 264 mg of paclitaxel per kilogram of cells, 74 % of which was directly secreted into the medium (Lee et al. 2010). Similarly, *C. roseus* CMCs treated with 10 mM β-CD in our study yielded 727.4 mg/L total alkaloids (51 % more than controls), 29 % (216.1 mg/L) of which were directly secreted into the medium, which represented 190 % more secretion than in controls (Fig. S4 in the Supplementary Materials). These results confirmed that β-CD can not only upregulate transcript levels of alkaloid biosynthesis genes but also promote extracellular transfer of the resulting compounds to reduce feedback inhibition and degradation (Park and Cho 1996; Durante et al. 2011; Almagro et al. 2011).

Among signal mediators, MeJA has a pronounced effect on the production of several secondary metabolites, including paclitaxel (Yukimune et al. 1996), ginsenoside (Hu and Zhong 2008), and ajmalicine (Almagro et al. 2011). In the previous reports (Marsh et al. 2014; Lijavetzky et al. 2008), the combined treatment of cyclodextrin and MeJA led to significantly higher production of aglycones and baicalein in hairy root cultures of *S. lateriflora* and of resveratrol in cell cultures of Monastrell grapevine. Previous work on *C. roseus* indicated that a 100-μM dose of MeJA was optimal to induce ajmalicine production in suspension cultures of leaf-derived cells (Almagro et al. 2011), while our previous work showed that a 150-μM dose of MeJA stimulated maximal production of TIAs in *C. roseus* CMCs (Zhou et al., unpublished). In addition, the combination of methyl-β-CD and MeJA significantly enhanced ajmalicine accumulation in *C. roseus* cultures, as well as aglycone, baicalein, and wogonin yields in hairy root cultures of *S. lateriflora* (Almagro et al. 2011; Sharma et al.

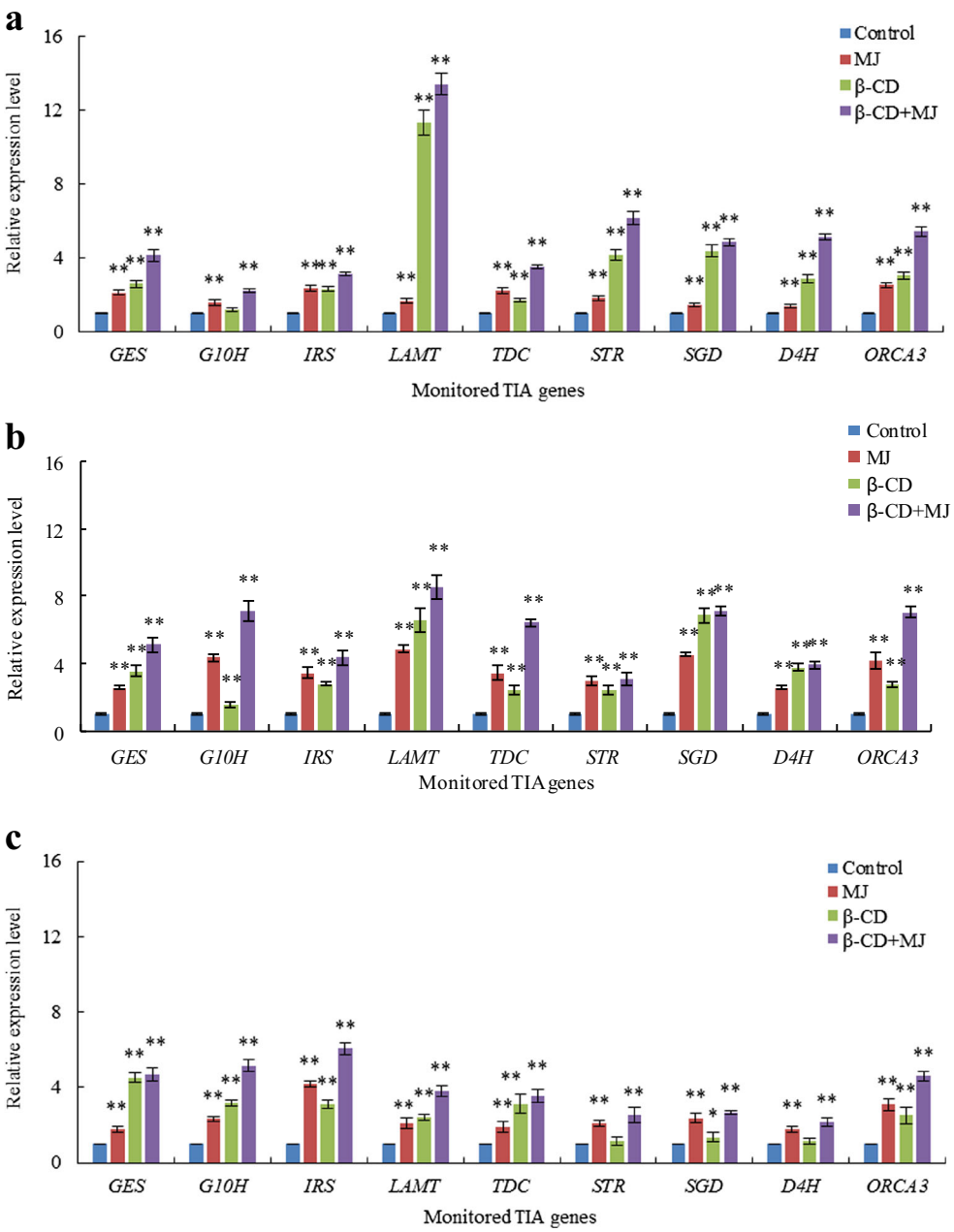

Fig. 7 Effects of 10 mM β-CD and 150 μM MeJA individually or in combination on expression of TIA genes in *C. roseus* CMCs cultured in 5-L bioreactors. Results were evaluated 24 h after treatment with MeJA on days 6 (**a**), 9 (**b**), and 12 (**c**). Values are means±SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's *t* test. Significant differences between treatments and the control are shown as $p<0.05$ (*single asterisk*) and $p<0.01$ (*double asterisk*)

2014). Furthermore, research suggests that these elicitors may regulate many key genes in TIA biosynthesis in *C. roseus* (Goklany et al. 2009; van der Fits and Memelink 2000; Simkin et al. 2013; Montiel et al. 2011); yet the combined effects of β-CD and MeJA on TIA biosynthesis in bioreactor cultures have not been previously examined. Our results demonstrated that 10 mM β-CD was sufficient to induce TIA biosynthesis in CMCs of *C. roseus*. Moreover, CMCs treated with both 10 mM β-CD and 150 μM MeJA in 5-L stirred hybrid airlift bioreactors were found to give the highest yields of vindoline (7.45 mg/L), catharanthine (1.76 mg/L), and ajmalicine (58.98 mg/L), a respective increase of 799, 654, and 426 % relative to yields of CMCs cultured in 100-mL flasks without elicitors. These elicitors also induced transcription of genes involved in alkaloid biosynthesis (e.g., *GES*, *G10H*, *IRS*, *LAMT*, *TDC*, *STR*, *SGD*, and *D4H*) at rates 5.1–13.4 times greater than those of the controls. Transcription of *ORCA3* was also upregulated sixfold, supporting evidence that ORCA3 is a jasmonate-responsive transcriptional regulator of TIA biosynthetic genes (van der Fits and Memelink 2000). MeJA was previously shown to enhance transcription levels of the TIA biosynthetic genes *GES*, *G10H*, *TDC*, *STR*, *SGD*, and *D4H* (Goklany et al. 2009; van der Fits and Memelink 2000; Simkin et al. 2013), but the effects of MeJA on the *IRS* and *LAMT* genes have not been examined. Our results revealed that both MeJA alone and MeJA/β-CD

combined induced *IRS* and *LAMT* transcription, leading to increased accumulation of vindoline, catharanthine, and ajmalicine. Past work on *C. roseus* leaves found that the enzymes involved in vindoline biosynthesis were expressed in more than three cell types (Burlat et al. 2004; St-Pierre et al. 1999). Additionally, some of the enzymes involved in vindoline biosynthesis were found to be absent in cultured cells of *C. roseus*, resulting in low yields of vinblastine and vincristine (Kutchan 1998). In our experiments, however, treatment of *C. roseus* CMCs with β-CD or MeJA individually or in combination dramatically increased transcription of all of these genes. Thus, CMCs of *C. roseus* can be induced to express all of the genes involved in the biosynthesis of these target alkaloids with the addition of the appropriate elicitors.

It is widely believed that NO signaling is involved in mediating the defense responses of plants to elicitor and pathogenic attack and thus is important for plant secondary metabolite biosynthesis (Xu and Dong 2005). Here, we report the first finding that β-CD can induce NO generation in a dose-dependent manner. Although jasmonic acid, MeJA, and salicylic acid may stimulate plant secondary metabolite biosynthesis through distinct signaling pathways, all interact with NO to mediate this production (Zhang et al. 2012). Thus, it is possible that the biosynthetic pathways of vindoline, catharanthine, and ajmalicine in *C. roseus* CMCs can be activated by NO triggering the plant defense response.

Bioreactors play an increasingly important part in many industrial applications, including food, pharmaceutical, and plant cell cultures. Compared with Erlenmeyer flasks, the major advantage of bioreactors is that bioreactors can more accurately control a variety of fermentation factors (e.g., shear stress, oxygen supply, gas composition, and aggregates) (Sharma et al. 2014; Joshi et al. 1996; Xu and Dong 2005; Zhong 2010). In culture, aggregations of cells with different morphologies influence culture growth and cell-cell interactions and are necessary to maintain productivity (Joshi et al. 1996). The higher growth rates and greater TIA production of *C. roseus* CMCs cultured in 5-L stirred hybrid airlift bioreactors compared with those cultured in flasks is likely attributable to greater tolerance to shear stress and good cellar morphology (i.e., singletons or small aggregates) in the bioreactors.

The synthesis of specific metabolites in dedifferentiated cells is often constrained by poor productivity and culture instability (Georgiev et al. 2013). Our experimental results, however, demonstrated that undifferentiated CMCs derived from *C. roseus* were efficient and robust producers of the target alkaloid metabolites. Moreover, treatment with β-CD and MeJA enhanced vindoline, catharanthine, and ajmalicine production. Therefore, CMC cultures combined with low-cost bioreactor technology may increase commercial production of vindoline, catharanthine, and ajmalicine and serve as a tool for further investigation of the biosynthetic pathway of TIAs.

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Conflict of interest The authors declare no conflict of interest.

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