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Full Length Research Paper

Effects of over-expression of allene oxide cyclase on camptothecin production by cell cultures of *Camptotheca acuminata*

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Camptothecin (CPT) is an anticancer and antiviral monoterpene-derived indole alkaloid which can be induced by plant hormone, jasmonates. To improve the production of the pharmaceuticals, the jasmonate biosynthesis related gene allene oxide cyclase from *Camptotheca acuminate* was transferred back into *C. acuminate* using the method of *Agrobacterium*-mediated genetic transformation and over expressed. The results of high performance liquid chromatography (HPLC) analysis indicated that the camptothecin content of transgenic callus was higher than that of non-transgenic callus. The highest camptothecin content in transgenic callus was 3.9310 mg/g DW. However, camptothecin content in both transgenic and non-transgenic calli significantly decreased after further extrinsic methyl-jasmonate's (MeJA's) induction, whereas the content of CPT in transgenic callus was still higher than in non-transgenic one. All the results indicate that endogenic jasmonate's accumulation may be promoted after allene oxide cyclase gene was transformed into *C. acuminate* and over expressed. In this way, jasmonates can affect second metabolism pathway genes' expression and then the camptothecin content improved. Inexpectantly, the mechanism of extrinsic jasmonate to secondary metabolism of *C. acuminate* was different from that of endogenic jasmonates.

Key words: *Agrobacterium tumefaciens*, Allene oxide cyclase, *Camptotheca acuminata*, camptothecin, methyl-jasmonates, transformation.

INTRODUCTION

Camptothecin (CPT), originally isolated from the extract of a traditional Chinese medicinal tree *Camptotheca acuminata* (Nyssacese) (Wall et al., 1966), is a water-insoluble therapeutically valuable monoterpene-derived indole alkaloid. This compound exhibits an ability to specifically inhibit the cleavage and rejoining reaction of DNA topoisomerase I (Topo I) (Hsiang et al., 1985). At present, semi-synthetic water-soluble camptothecin analogues, topotecan and irinotecan, are used as clinical anti-tumor agents throughout the world. They are synthesized from natural camptothecin, which is mainly obtained by extraction and purification from *C. acuminata* plant. Due to the present and potential clinical uses of CPT and its derivatives, it is important to develop sustainable and alternative production sources of these compounds due to low content of these compounds in natural plants.

Terpenoid indole alkaloids (TIAs) biosynthesis in *Catharanthus roseus* is one of the best studied elicitor-induced secondary metabolic pathways (Noé et al., 1984;

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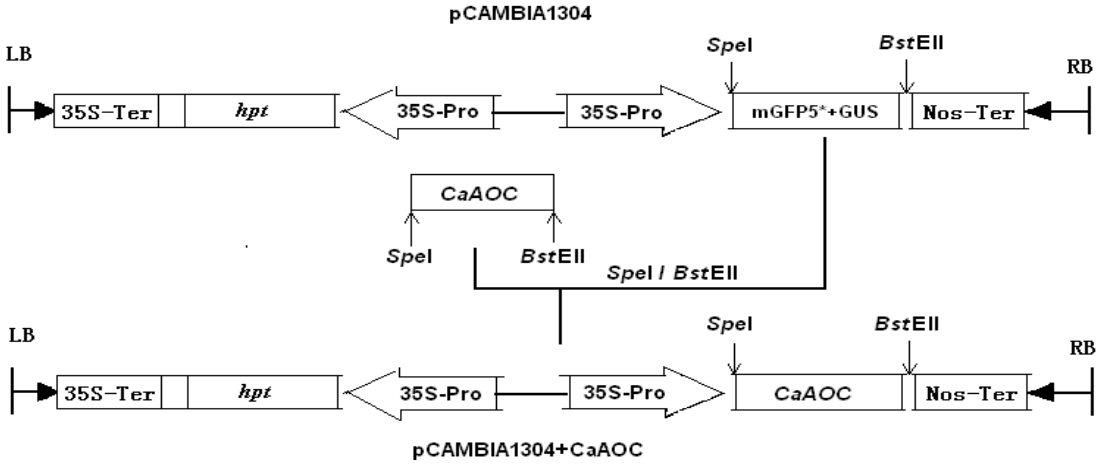

Figure 1. Schematic maps of transformation plasmid (pCAMBIA1304+*CaAOC*). 35S-Pro, cauliflower mosaic virus 35S promoter. *hygromycin*, hygromycin B phosphotransferase gene. Nos-Ter, 3' termination region from *Agrobacterium* nopaline synthase gene. *CaAOC*, *CaAOC* gene. Restriction enzyme (*Spe*I and *Bst*EII), indicated by arrow.

van der fits and Memelink, 2000, 2001; Pauw et al., 2004). Jasmonate (JA) and its derivative, methyl-jasmonate (MeJA) were reported to be general inducers of plant secondary metabolite biosynthesis (Memelink et al., 2001). The strictosidine synthase (STR; EC 4.3.3.2) and the tryptophan decarboxylase (TDC; EC 4.1.1.28) are essential and are key enzymes in TIA biosynthesis, which are coordinately regulated by a JA-responsive transcription factor (ORCA3) in *C. roseus*. It has showed that ectopic expression of the transcription factor (ORCA3) could enhance the production of TIAs in cultured cells of *C. roseus* (van der Fits and Memelink, 2000, 2001). Maybe it is also an alternative approach that the key genes in jasmonate biosynthetic pathway are modified by genetic manipulation for the production of valuable secondary metabolites which is induced by jasmonates.

An essential enzyme involved in JA biosynthesis is allene oxide cyclase (AOC; EC 5.3.99.6). The AOC-catalyzed step is regarded as the crucial point in the biosynthesis of JA due to the establishment of the naturally occurring enantiomeric structure of JA (Cenzano et al., 2007; Maucher et al., 2004). It was reported that the overexpression of an heterologous *AOC* gene from *Hyoscyamus niger* L. in *Nicotiana tabacum* cv. Petit Havana can result in overexpression of nicotine biosynthetic pathway genes and higher yields of nicotine (Jiang et al., 2009). The engineering TIA biosynthesis, as an essential step to ensure the profitable of cell suspensions *in vitro* production systems, will face a major challenge for the next years.

In this study, we attempted to investigate the role of *AOC* gene from JA biosynthesis in promotion of the CPT biosynthetic pathway. An *AOC* gene (defined as *CaAOC*, Database Accession No. AY863428), which has been isolated from *C. acuminata* in previous work was transformed into *C. acuminata*. The result of RT-PCR showed the *CaAOC* expression level in transgenic callus was obviously higher than that of non-transgenic callus. The results of high performance liquid chromatography (HPLC) analysis indicated that the camptothecin content of transgenic callus was higher than that of non-transgenic callus. In addition, the effect of extrinsic MeJA on the camptothecin content was evaluated as well.

EXPERIMENTALS

Construction of *CaAOC* expression vector

The vector pCAMBIA1304 (CAMBIA, Canberra, Australia) used in this study contains a 1.9 kb GUS expression cassette and a hygromycin B phosphotransferase gene (*hpt*) driven by the CaMV35S promoter. To construct pCAMBIA1304+*CaAOC*, the GUS expression cassette of pCAMBIA1304 was replaced with the *Spe*I-*Bst*EII *CaAOC* coding sequence amplified with F*CaAOC*p1304 (5'-GG ACTAGT ATG GCT GCT TCA TCA ACT TC-3', the *SpeI* digestion site is underlined) and R*CaAOC*p1304 (5'-GA GGTTACC TCA GTC AGT GAA GTT AGG AA-3', the *Bst*EII digestion site is underlined), using cDNA of *C. acuminata* (Figure 1). The plasmid was introduced into the disarmed *Agrobacterium tumefaciens* strain EHA105. A positive clone, after confirmation by PCR and enzymatic digestion analysis for the presence of the *CaAOC* gene, was used for plant transformation.

Plant material and *C. acuminata* transformation

A. tumefaciens EHA105 harboring the vector was grown at 28°C in Luria Bertani (LB) medium (Sambrook et al., 2002) supplemented with 100 µg/ml kanamycin, 40 µg/ml streptomycin, 25 µg/ml rifampicin and 100 µM acetosyringone. For infection, the concentration of *Agrobacterium* suspension in inoculation MS (Murashige and Skoog, 1962) liquid medium with 100 µM acetosyringone was adjusted to an OD_{600} of 0.6. Mature seeds of *C. acuminata* were collected from the plants growing in the campus of

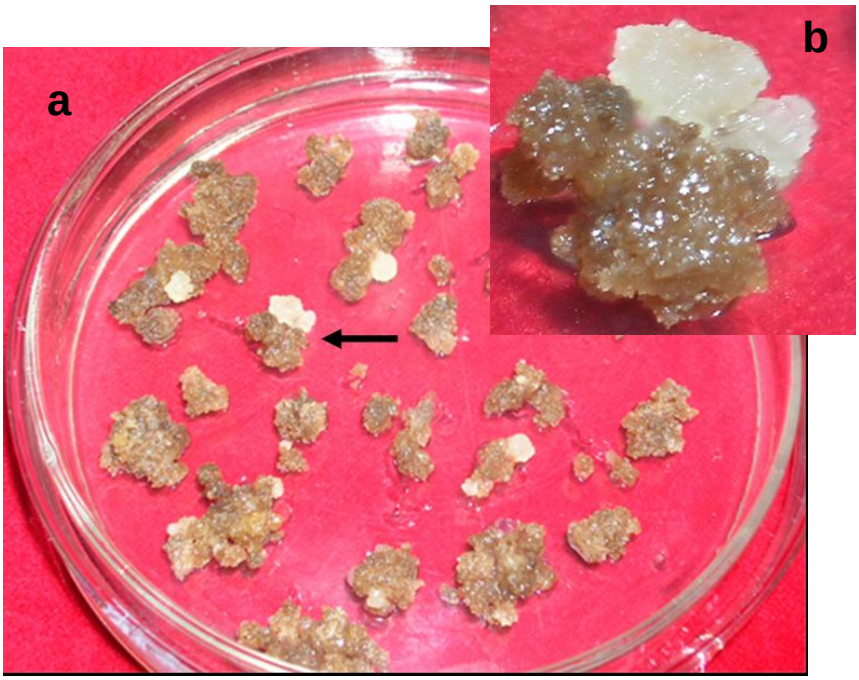

Figure 2. Selection of transgenic cell lines. a, Transformed *Camptotheca acuminata* cell lines in selection medium MS_2. b, Transformants selected in MS_2.

Fudan University, Shanghai, China. The decoated seeds were surface sterilized with 0.1% (w/v) of mercuric chloride for 5.0 min and rinsed thoroughly five times in sterile distilled water. Then, the wounded seed embryos were inoculated with *Agrobactetium* for 5.0 min, blotted twice on sterile filter paper and placed on MS_1 callus induction medium (MS solid medium supplemented with 5 µg/ml naphthalene-acetic acid, 0.5 µg/ml 6-benzyladenine, 0.3 µg/ml 2,4-dichlorophenoxy-acetic acid, and 30 mg/ml of sucrose) for two days as a co-culture stage.

Selection and culture of *C. acuminata* cell lines

After the co-culture stage, the wounded seed embryo pieces was transferred onto selection medium MS_2, the same medium as MS_1 but supplemented with 250 µg/ml carbenicillin (Carb) and 20 µg/ml hygromycin B. After initiation of the independent callus initiated from seed embryo in about three to five weeks (Figure 2), it was maintained as usual in darkness (28°C) and was sub-cultured every four weeks to the same fresh selection medium MS_2. Nine independent transgenic cell lines, maintained in selection medium MS_3 (MS_1 supplemented with 20 µg/ml hygromycin B), were selected for examination.

Molecular analysis of transgenic cell lines

Total genomic DNA was extracted from fresh cell lines by cetyltrimethylammonium bromide (CTAB) method as described (Doyle and Doyle, 1990). PCR primers used for detecting the *hpt* gene were Fhpt (5'-CGA TTT GTG TAC GCC CGA CAG TC-3') and Rhpt (5'-CGA TGT AGG AGG GCG TGG ATA TG-3'). Primers for the detection of transformed *CaAOC* were F35Sp1304 (5'-GCA CAA TCC CAC TAT CCT TCG CAA-3') and R*CaAOC* (5'-CGT ACA CGT CAC CTT TCT TCT CCG G-3'). Plasmid DNA from the *Agrobacterium* carrying pCAMBIA1304+*CaAOC* was used as a positive control. For detection of the *hpt* gene and the *CaAOC* gene, the template was denatured at 94°C for 3 min followed by 34 amplification cycles (50 s at 94°C, 50 s at 60°C and 50 s at 72°C) and finally at 72°C for 8 min. Products (10 µl) were analyzed on 1% agarose gel.

MeJA treatment

To study the changes of the transcription of *CaAOC* in transgenic cell lines and non-transgenic cell lines under MeJA treatments, the 15-day-old *C. acuminata* cells, sub-cultured on fresh medium in a new Petri dish, were soaked in a solution of 100 µM MeJA, respectively. The cells were grown at 25 ± 2°C in the dark for 60 min, then each sample was divided into two parts, one was stored -80°C using for total RNA isolation, the other was oven-dried at 60°C in the dark for three days to a constant weight and CPT content was determined.

RT-PCR analysis of *CaAOC* in transgenic cell lines

After MeJA induction, the nine transgenic cell lines, accompanied with control treated by distilled water, were subjected to analysis for the transcription of *CaAOC* using One Step RT-PCR (Takara, Shiga, Japan) with the primers *CaAOC*-RT-F (5'-AAACCTCAGACTCA-CCACCACA -3') and *CaAOC*-RT-R (5'-TTCCACGGTCT-CGTTCGTT -3'). 18S rRNA was used as internal control with the primers 18S-F (5'-ATG ATA ACT CGA CGG ATC GC-3') and 18S-R (5'-CTT GGA TGT GGT AGC CGT TT-3'). RT-PCR was carried out following the manufacturer's protocol, and the thermal cycle condition used was 30 min at 50°C, 2 min at 94°C, followed by 22 cycles of amplification (30 s at 94°C, 30 s at 60°C and 60 s at 72°C). Products (5 µl) were analyzed on 1% agarose gel.

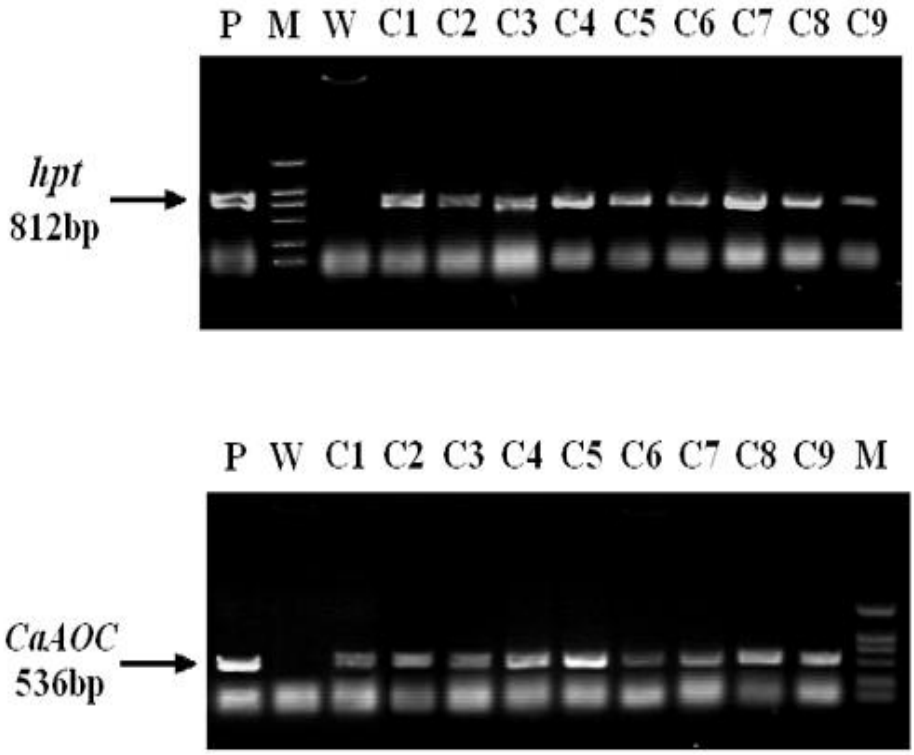

Figure 3. PCR analysis of the transgenic cell lines. M, DNA size marker DL2000 (100 to 2000 bp). P, plasmid (positive control). W, non-transgenic cell lines (negative control). *CaAOC*, *CaAOC* gene (536 bp). Hpt, hygromycin B phosphotransferase gene (812 bp). Arabic numbers indicate the independent transgenic cell lines.

Camptothecin extraction and HPLC analysis

The CPT was extracted from dried samples by supersonic oscillator method as described (Yan et al., 2002). After filtering, the solutions were directly analyzed by HPLC (Model 2690; Waters, Milford, MA, USA) as described (Wiedenfeld et al., 1997).

RESULTS

C. acuminata transformation and molecular analysis

The embryos with papery cotyledons from *C. acuminata* were transformed with *A. tumefaciens* strain EHA105 harboring pCAMBIA1304+*CaAOC*. Transformed cell lines were excised and put onto selection medium MS_2 and maintained in selection medium MS_3. The putative transformants were analyzed by PCR to test the integration of the exogenous gene. In order to avoid the effect of inherent *AOC* gene in *C. acuminata*, a section of the 35S promotor sequences was used to explore *CaAOC* gene as a forward primer. PCR was performed with the DNA from transformed plants using *hpt* and *CaAOC* gene primers. The products were analyzed on 1% agarose gel, the expected sizes of the amplified fragments were 812 and 536 bp (Figure 3). The untransformed plants did not show any signals.

RT-PCR analysis of *CaAOC* in transgenic *C. acuminata* cells

It was reported that the transcription of *AOC* gene nearly reached their higher levels at 60 min after treated with MeJA (Agrawal et al., 2003a, b; Stenzel et al., 2003). To examine the changes of *CaAOC* gene expression under extrinsic MeJA treatments, the 15-day-old *C. acuminata* calli were used for RNA isolation after treated for 60 min. The result shows that the transcript of *CaAOC* was induced by 100 µM MeJA in the nine transgenic calli and non-transgenic control callus (Figure 4). Under extrinsic MeJA induction, the expression of *CaAOC* mRNA in transgenic calli was higher than that in the non-transgenic control.

Camptothecin content analysis in transgenic *C. acuminata* cells

The profiles of camptothecin concentration in transgenic cell lines were determined by HPLC. The capacities of transgenic cell lines to biosynthesize camptothecin are shown in Figure 5. The content of camptothecin in transgenic calli (it ranged from 3.9310 mg/g to 1.0028 mg/g of dry weight) was much higher than that in non-

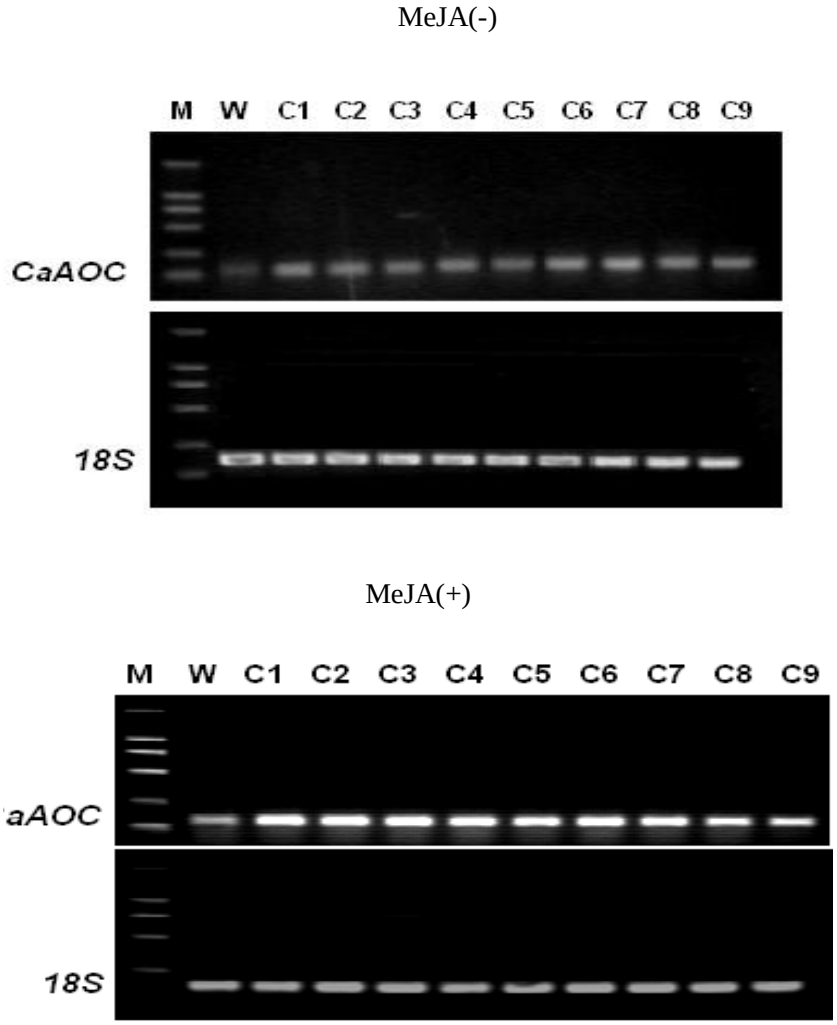

Figure 4. RT-PCR analysis of the transgenic cell lines after 100 μM MeJA treatments. *CaAOC*, *CaAOC* gene. *18S*, *18S rRNA*. W, non-transgenic cell lines. Arabic numbers indicate the independent transgenic cell lines. MeJA(-), Treatments without MeJA; MeJA(+), treatments with MeJA.

transgenic control which was nearly detected. The 3.9310 mg/g was higher than those values described in the literature before and was comparable to the results of young flower buds and seeds from *C. acuminata* plant.

DISCUSSION

Increasing evidences indicate that jasmonates can regulate the synthesis of secondary metabolites in many species by an ubiquitous plant defense response (Keinänen et al., 2001; Goossens et al., 2003; Sasaki-Sekimoto et al., 2005; Wang et al., 2010), and the transgenic manipulation of endogenous JA levels in tomato and tobacco proved to be feasible (Jiang et al., 2009; Keinänen et al., 2001; Chen et al., 2006). Our results indicate that *CaAOC* gene had been integrated in the genome and the transcript level was increased. The CPT accumulation in nine transgenic cell lines was higher than in non-transgenic cell lines. Hairy roots derived from the wild-type strains of *Agrobacterium rhizogenes* have also been developed in *Ophiorriza pumila* and *C. acuminata*, the yields of the camptothecin in the two species were of approximately 1 mg/g DW. The CPT content in *C. acuminata* callus in optimization condition was 2.36 mg/g DW. The highest CPT content of the transgenic callus reached 3.9310 mg/g DW in this present research, which was higher than those reported. And the content in organs of the plant seems to be somewhat smaller than that in transgenic calluses but is also in the same range.

It could be deduced that the CPT accumulation was associated with increased levels of transcripts that encode *CaAOC* enzyme in the jasmonate pathway. The cloning and overexpression of the JA-responsive

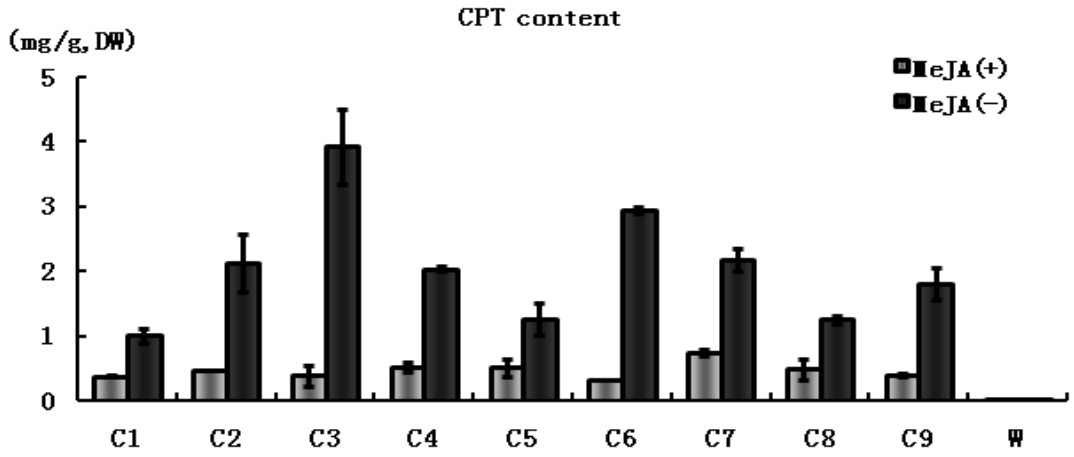

Figure 5. The CPT concentration of transgenic *Camptotheca acuminata* cell lines and non-transgenic cell lines. W, Non-transgenic cell lines. C1 to C9, Nine independent transgenic cell lines. MeJA(-), Treatments without MeJA; MeJA(+), treatments with MeJA.

transcription factors (ORCA) had been shown to regulate the jasmonate-responsive activation of several mono-terpene indole alkaloids-synthesizing genes in cell cultures (Gantet and Memelink, 2002; Vom Endt et al., 2002; Pasquali et al., 2006). The transformation of *CaAOC* in *C. acuminata* might also promote the accumulation of JAs. The transcription factors (ORCA) responded to JAs, and regulated the activation of several CPT biosynthesis pathway genes. Then the CPT production in transgenic callus was enhanced. This might be a way that metabolic engineering and physiological manipulation was used as complementary to improve alkaloid yields.

Extrinsic JAs can elicit accumulation of secondary metabolites in many plants. In this present study, the results show that the transcript of *CaAOC* was induced in the nine transgenic calli and non-transgenic control callus of *C. acuminata* by 100 µM MeJA (Figure 4), but the CPT accumulation was not simultaneously associated with the increased expression of *CaAOC* in transgenic calli. It was reported that the extrinsic MeJA could significantly enhance the production of secondary metabolites such as the anti-cancer drug paclitaxel (Taxol) in cell suspension cultures of *Taxus* and the TIAs in cells of *C. roseus* (Wang et al., 2010; Ketchum et al., 2003; Magdi and Rob, 2005; Sabah et al., 2007). And MeJA and JA could increase CPT production six and 11 times in *C. acuminata* cells, respectively (Song and Byun, 1998). Our results is not consistent with those cases, but the same as the result previously reported that extrinsic MeJA did not elicit CPT yields in *C. acuminata* cells. What's more, the transgenic lines exhibited significantly diverse CPT concentrations without MeJA treatment, while with MeJA treatment, these lines seemed to perform lower but much closer values in CPT concentration (Figure 5). Herein, possible explanations might lay on that: (1) endogenous MeJA had different affection mechanism on the yields of TIAs with extrinsic one; (2) in the different time course, MeJA showed different affection mechanism, inhibition or inducement; (3) MeJA had another affection mechanism on the yields of CPT in *C. acuminata* compared with that on the yield of TIAs in *C. roseus* and (4) over accumulation of exo-genous MeJA might have preferentially triggered other secondary metabolism which competes with CPT biosynthesis. On this occasion, all the transgenic lines in MeJA treatment preferred to produce CPT at a restricted and balanced concentration.

Regulation of JA biosynthesis is quite a complex process. It is less understood that the functional role of the subsequent transcriptional up-regulation of JA-biosynthetic enzymes. Here, we offered an alternative to elicitors as a means to enhance the CPT production. The signaling property of JAs is interesting not only for what it reveals about how primary and secondary metabolism is reconfigured in response to stress, but also for practical applications in the production of economically useful compounds.

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Abbreviations

AOC, Allene oxide cyclase; **CPT,** camptothecin; **HPLC,**

high performance liquid chromatography; **hpt,** hygromycin B phosphotransferase gene; **JA,** jasmonate; **MeJA,** methyl-jasmonate; **TIAs,** terpenoid indole alkaloids.

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