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Effect of Three Plant Hormone Elicitors on the Camptothecin Accumulation and Gene Transcript Profiling in Camptotheca Acuminata Seedlings

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Abstract: Camptothecin (CPT) is an antitumor alkaloid first isolated from Chinese tree *Camptotheca acuminata* Decaisne (Nyssaceae). Several attempts have been made to improve CPT production in plant, among which elicitation was considered as a useful method. In this study, camptothecin (CPT) and its derivative 10-hydroxycamptothecin (HCPT) accumulation, as well as related gene transcript profiling in *C. acuminata* seedlings, in response to abscisic acid (ABA, 100 μM), methyl jasmonate (MJ, 100 μM) and salicylic acid (SA, 1 mM) were examined. Results showed that all three elicitors enhanced both camptothecin and 10-hydroxycamptothecin accumulation, among which abscisic acid (ABA) exhibited the most effective elicitation with the increment of 1.44-fold for CPT (1.81 mg/g DW) and 1.21-fold for HCPT (2.6 mg/g DW) respectively. Our expression results showed a positive correlation between gene expression and alkaloid accumulation, suggesting that alkaloid accumulation may be the result of the synchronous up-regulation of several genes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and tryptophan decarboxylase (TDC) involved in CPT biosynthesis under the treatment of various elicitors. Our work provided useful information of further understanding molecular regulation mechanism of CPT biosynthesis and its metabolic engineering in *C. acuminata* in the future.

1. Introduction

Camptothecin (CPT) is an antitumor alkaloid first isolated from *Camptotheca acuminata* Decaisne (Nyssaceae), a native tree of Southern China, in 1966 [Wall et al., 1966]. CPT and its derivatives have received considerable attention after 1985 when Hsiang discovered that camptothecin, by a unique mechanism, inhibited the enzyme topoisomerase I [Hsiang et al., 1985]. 10-Hydroxycamptothecin (HCPT), with low content in *C. acuminata*, has higher anti-cancer activity and less toxicity compared to CPT, and its two analogs, Topotecan and Irinotecan (CPT-11), have been approved by the FDA for treatment of refractory ovarian and colorectal cancer with rapidly increasing clinical need [Wall et al., 1998]. The combined sales of irinotecan and topotecan only in 2003 had reached nearly $1 billion [Oberlies and Kroll, 2004]. The annual CPT production throughout the world is only 600kg, far from meeting the demand of the market. Since extraction from the limited natural plants is still the main reliable source of CPT production at present [Sirikantaramas et al., 2007], how to enhance the CPT accumulation in *C. acuminata* is of huge economic significance.

CPT is a modified monoterpene indole alkaloid, belonging to the class of terpenoid indole alkaloids (TIAs) (Fig. 1). All TIAs are derived from the common precursor strictosidine, which is condensed by the indole tryptamine and the monoterpenoid secologanin

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[Kutchan et al., 1995]. Tryptamine is synthesized via the shikimate pathway, and secologanin part is synthesized via the MEP pathway [Yamazaki et al., 2004]. Strictosidine is then converted to strictosamide, but the remaining details and precise intermediates between strictosamide and CPT are not completely defined [Lorence et al., 2004].It had been proven that at least some genes involved in CPT biosynthesis of *C. acuminata* were responsive to MeJA and SA, such as *TDC*, *DXR* and *HDR* [Lo´pez-Meyer and Nessler, 1997; Yao et al., 2008; Wang et al., 2008], suggesting that these signal molecules could stimulate the secondary metabolite accumulation by eliciting the expression of biosynthesis pathway genes.

Elicitation, treatment with plant defense response elicitors, is one of the most effective strategies for enhancing secondary metabolite production in plant tissue and cell cultures [Eilert et al., 1987]. Many abiotic elicitors and biotic elicitors have been widely used on alkaloids-or-phytoalexins-producing plants with good effects [Yan et al., 2005; Kai et al., 2011; Christie et al., 2009; Zulak et al., 2007; Liu et al., 2010].The most common and effective elicitors used in plant tissue cultures include yeast extract (Kai et al 2012), and some important signal molecules in plant defense mechanisms, e.g., jasmonic acid (JA) and its relatives such as methyl jasmonate (MeJA), and salicylic acid (SA) and its analogues [Ge et al., 2005; Fujimoto et al., 2011; Li et al., 2004]. As plant hormones and intracellular messengers, MeJA and SA can induced certain plant defense responses and secondary metabolite accumulation to ensure their survival, persistence and competitiveness [Namdeo et al., 2007].

The promotion of some common elicitors such as MeJA, JA, SA, YE and chitosan on CPT biosynthesis have been studied in cell suspension culture [Burnett et al., 1993; Song et al., 1998], but there are few research on comparison of effect on CPT accumulation and expression profiles of CPT biosynthesis genes of C. acuminata in seedlings treated by various elicitors. In this paper, we reported the elicitation effect of ABA, MeJA and SA on camptothecin production and expression profiles of CPT biosynthesis genes in C. acuminata seedlings, as an initial step, to investigate the potential application role in the future.

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2. Materials and Methods

2.1 Plant cultivation

The mature seeds of *C. acuminata* were collected from the Kangjian Park in Xuhui district, Shanghai, China. The seeds were dried by airing and made a small incision to facilitate seed germination. Then they were sowed in the mixed soil (vermiculite: black soil: perlite= 12: 2: 1) under 25ºC with 16-h light period and watered every two days. The 26-day-seedlings were used for elicitation.

2.2 Elicitors preparation and treatment

MeJA (Sigma, St. Louis, Mo.) was diluted with distilled water to the final concentration of 100μM [Ge et al., 2005]. ABA (Sigma, St. Louis, MO, USA) and SA (Zhanyun chemical Co., Ltd., Shanghai, China) were dissolved in distilled water to the concentration of 100 μM and1mM independently. 100ml of each elicitor solution and distilled water (negative control) were sprayed evenly to the seedlings. After 12h, 24h, 48h, 96h and 7d, the seedlings were harvested and separated to leaves, stems and roots for RNA extraction and HPLC analysis.

2.3 RNA extraction and Real-time quantitative PCR

0.05g fresh weight of each sample was used for RNA extraction with the RNA pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. RNA was quantified by spectrophotometer at an absorbance of 260nm. 0.5μg total RNA was used to carry out reverse transcription with the M-MLV RTase cDNA Synthesis Kit (TaKaRa Biotech (Dalian) Co., Ltd., Dalian, China) in an Eppendorf MasterCycler Gradient 96 PCR (Eppendorf, Hamburg, Germany) following the manufacturer's instructions. The first strand cDNA was diluted to a final volume of 100 μL with nuclease-free water.

The qRT-PCR amplification was carried out in ABI Prism 7300 Real-time PCR system (Applied Biosystems, USA). Each reaction contained a mixture of 2μL 10 × PCR buffer (Biocolor BioSci & Tech Co., Ltd., Shanghai, China), 1.6μL 25mM Mg^{2+} (Biocolor), 2μL 2μM dNTPs (Biocolor), 0.4μL of each primer (10μM), 0.2μL SYBR (Generay Biotech Co., Ltd., Shanghai, China), 0.2μL Taq DNA polymerase (Biocolor) and 12.2μL nuclease-free water.

All the primers used were listed in table 1. The reaction mixture was incubated for 5 min at 94ºC and for 40 cycles of 30s at 95ºC, 20s at 55ºC and 20s at 72ºC. The relative gene expression was quantified by using the comparative C_T (threshold cycle) method. Expression of *18S rRNA* was used for normalization as an internal control gene.

2.4 Alkaloid extraction and HPLC detection

The seedlings were washed with distilled water after harvest and blotted to remove excess water. Then they were dividing into cotyledon, hypocotyl and root and dried in the oven for 1d at 50ºC, respectively. The dried tissues were ground into powder and 0.1g powder was extracted in 10mL methanol at 50ºC for 4hrs and then a 30min's ultra-sonication. The extracts were clarified by centrifugation at 12,000rpm for 10 min at 4 ºC and the supernatants was collected. Then 10mL methanol was added to the remaining residues and the extraction procedure was repeated to re-dissolve the alkaloid thoroughly. The supernatants of the two extractions were combined and concentrated to 1mL using a vortex evaporator, and passed through a 0.22mm nylon filter.

HPLC analyses were performed to determine the contents of CPT and HCPT on a Sepax sapphire C18 reversed-phase symmetry column (4.6×250mm, 5μM). The mobile phase consisted of 35% acetonitrile (HPLC grade) and 65% double distilled water. The flow rate was 1.0 mL/min and the injection volume was 20 μL. The chromatogram was monitored at 220 nm on a HITACHI Diode Array Detector L2455. CPT (Sigma St. Louis, MO, USA) was dissolved in methanol with a concentration of 1 mg/mL. The accuracy and reproducibility of HPLC analysis were confirmed by analyzing different quantities of the sample including the control group. Samples were quantified using standard curves fit with linear regression.

2.5 Statistical analysis

All the data were repeated in three independent times and analyzed by SPSS software. Standard deviation of the mean was estimated to measure the precision of the estimate of the mean. The statistical significance of the treatments and control differences were analyzed using variance, and the difference was significant ($P<0.05$).

3. Results

3.1 Alkaloids production and gene expression in different organs

CPT and HCPT contents in cotyledon, hypocotyl and root of *C. acuminata* seedlings were quantified respectively by HPLC (Fig. 2), and the results showed that there was higher CPT content in cotyledon and hypocotyl than root while higher HCPT in cotyledon and root than hypocotyl. Totally, cotyledon possessed the highest alkaloid content of 3.12 ‰.

To investigate the expression pattern of known CPT biosynthesis genes in different organs of *C. acuminata*, total RNA were extracted from cotyledon, hypocotyl and root, and subjected to qRT-PCR analysis for the transcript levels of the following genes: *HMGS*, *HMGR3*, *DXR*, *HDR*, *IPI1* and *IPI2* from the monoterpenoid pathway; *EPSPS*, *Asα1*, *Asα2*, *TSB* and *TDC* from the indole pathway; and 18S rRNA which served as the internal control gene (Fig. 3). The results showed that most of tested genes (except for *DXR*) exhibited similar pattern response to elicitors, with the highest expression level in cotyledon, and relatively lower levels in hypocotyl and root, which is quite consistent with the alkaloids distribution. On account of higher alkaloids production and gene expression levels, cotyledon was chosen for the following induction study.

3.2 Effect of ABA on the metabolites accumulation and transcript profile

As shown in Fig. 4, ABA elicitor treatment caused quick and obvious changes in the abundance of CPT and HCPT. The maximum increment of each metabolite was 1.44-fold (1.81mg/g DW) for CPT and 1.21-fold (2.6mg/g DW) for HCPT over the control at 12h after treatment. The stimulation of both metabolites also last to day 7 whereas the control group had gradually declined with the seedlings aged. No recognizable morphological changes of the seedlings were observed after ABA treatment (data not shown).

qRT-PCR analysis revealed a significant change in transcription levels of most detected genes，with the exception of *HDR* gene, in response to ABA treatment (Fig. 5). *HMGS*, *HMGR3*, *IPI1*, *IPI2*, *EPSPS*, *Asα1*, *TSB* and *TDC* transcription values were gradually up-regulated and reached the highest level on day 2. Genes with the greatest increments are *HMGS*, *HMGR3* and *TDC*, and their maximum mRNA transcript levels reached 8.6-, 7.7- and 20.3-fold higher than the control, respectively. For *DXR* and *Asα2* genes, the transcription levels rose more slowly and reached the peak value on day 7. Interestingly, *HMGR3*, *IPI1* and *IPI2* genes showed a second peak on day 7, in accordance with the second increase of the alkaloids concentration on day 7.

3.3 Effect of MeJA and SA on the metabolites accumulation and transcript profile

As shown in Fig. 6, MeJA and SA treatments could also boost the abundance of the two metabolites, but not as effective as ABA. The maximum increment induced by MeJA was 1.13-fold (1.345mg/g) for CPT and 1.30-fold (2.55mg/g) for HCPT at 24h after treatment and with a second peak at day 7, which is similar to ABA with two peaks upon treatments. SA elicitor caused a 1.19-fold (1.33mg/g)increase of CPT and 1.20-fold (2.25mg/g) increase of HCPT at 48h after treatment. No recognizable morphological changes of the seedlings were observed after MeJA and SA treatment (data not shown).

Different to ABA treatment, MeJA and SA treatment didn't enhance the expression of most of the detected genes involved in the CPT biosynthesis pathway (data not shown). Only *IPI1* gene from monoterpenoid pathway were remarkably induced by MeJA after 4 days of the treatment, with its maximum mRNA transcript levels reaching 8.4-fold higher than the control (Fig. 7). The expression level of *Asα1* gene from the indole pathway reached 3.9-fold higher than the control 1 day after SA treatment (Fig. 7).

4. Disscussion

As a well-known senescence-triggering plant hormone, ABA plays important roles throughout plant life and development. ABA is also reported to regulate biosynthesis of secondary metabolites in some plant cell cultures. For instance, Indole alkaloids in *C. roseus* cell culture can be stimulated by ABA [Zhao et al., 2000]. ABA also stimulates taxol production in *Taxus spp* cell culture [Luo et al., 2001].

In order to visualize detailed regulatory mechanisms underlying the secondary metabolic conversions in response to ABA in *C. acuminata* seedlings, a number of related gene transcripts as well as the metabolites accumulation profiling were examined in present study. The results indicated the increased CPT and HCPT accumulation in the tested samples was apparently because of the activation of both the monoterpene pathway and indole pathway. Meantime, serveral camptothecin biosynthetic gene such as 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), 3-hydroxy-3-methylglutaryl coenzyme A reductase 3 (HMGR3), anthranilate synthase alpha 2 (ASA2), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and tryptophan decarboxylase (TDC) displayed the most substantial increases.

MeJA and SA had been widely used to induce secondary metabolite such as terpenoids and TIAs biosynthesis recently [Ge et al., 2005; Peebles et al., 2009; Pan et al., 2010]. In hairy root cultures of *Salvia*

miltiorrhiza and Catharanthus roseus, JA treatment had obvious elicitation effect on metabolites and related biosynthetic genes (Kai et al 2012, Peebles et al., 2009;?). In present study, the induction of MeJA and SA was not so dramatic, and it may be due to different plant species.

In earlier studies, much more attention has been pay to CPT production rather than HCPT in metabolic regulation [Song et al., 1998; Li and Liu., 2003; Li et al.,2004; Kim et al.,2006], which exhibits stronger pharmacological activities and less cytotoxicity resulting in better medicinal value than CPT. In our study, HCPT was observed to have similar response pattern as CPT but higher background content in cotyledon and root. Since there is only a hydroxyl group difference between CPT and HCPT, indicating that there is possibly a single hydroxylase responsive for this conversion. Isolation and functional identification of this hydroxylase would provide the possibility to obtain the more valuable anti-cancer alkaloid HCPT by metabolic engineering in CPT-producing plants including *C. acuminata*.

Conclusion:

CPT and HCPT accumulation and related biosynthetic gene transcripts in *C. acuminata* seedlings can be stimulated by all three plant hormone elicitors, among which ABA treatment was the most effective. Our expression results showed a positive correlation between gene expression and alkaloid accumulation, suggesting that alkaloid accumulation may be the result of the synchronous up-regulation of several genes involved in CPT biosynthesis under the treatment of various elicitors.

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Figure legends

Fig. 1 Camptothecin biosynthetic pathway in *Camptotheca acuminata* Dotted line arrows indicate multiple steps between intermediates.

HMGS: hydroxy methylglutaryl coenzyme A synthase, HMGR: hydroxy methylglutaryl coenzyme A reductase, DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, HDR: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IPI1: isopentenyl diphosphate isomerase 1, IPI2: isopentenyl diphosphate isomerase 2, EPSPS: 5-enolpyruvyl shikimate 3-phosphate synthase, Asα1: anthranilic acid synthetase α1, Asα2: anthranilic acid synthetase α2, TSB: tryptophan synthase beta, TDC: tryptophan decarboxylase.

Fig. 2 CPT and HCPT contents in different tissues of *C. acuminata*

Fig. 3 Transcription level of CPT biosynthesis genes in different organs of *C. acuminata*

HMGS: hydroxy methylglutaryl coenzyme A synthase, HMGR3: hydroxy methylglutaryl coenzyme A reductase3, DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, HDR: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IPI1: isopentenyl diphosphate isomerase 1, IPI2: isopentenyl diphosphate isomerase 2, EPSPS: 5-enolpyruvyl shikimate 3-phosphate synthase, Asα1: anthranilic acid synthetase α1, Asα2: anthranilic acid synthetase α2, TSB: tryptophan synthase beta, TDC: tryptophan decarboxylase.

Fig. 4 CPT and HCPT contents of *C. acuminata* seedlings after ABA treatment

Fig. 5 Transcription level of CPT biosynthesis genes in *C. acuminata* after ABA treatment

Fig. 6 CPT and HCPT contents of *C. acuminata* seedlings after MeJA and SA treatment

Fig. 7 Transcription level of *IPI* and *Asα* in *C. acuminata* after MeJA and SA treatment

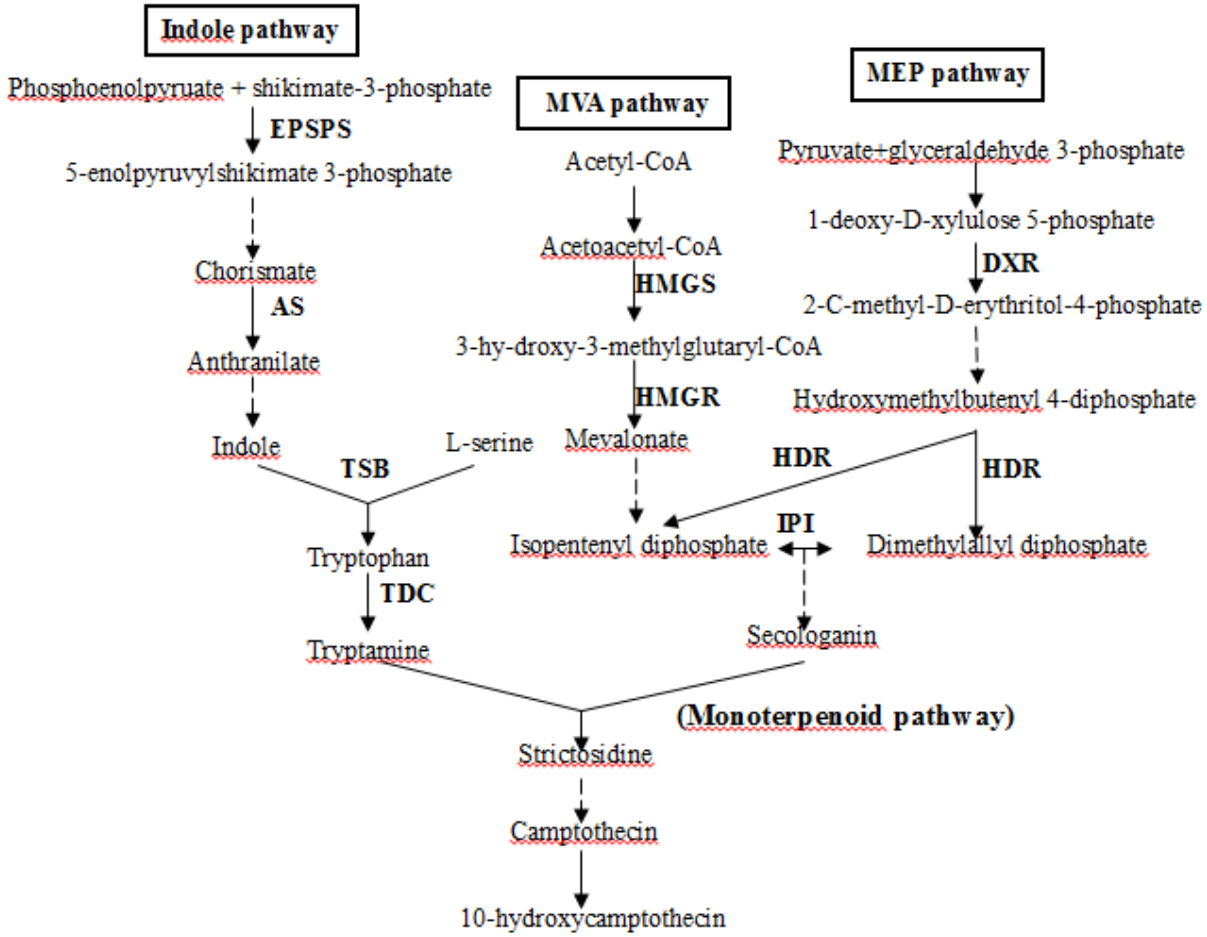

Indole pathway
Phosphoenolpyruate + shikimate-3-phosphate
EPSPS
5-enolpyruvylshikimate 3-phosphate
Chorismate
AS
Anthranilate
Indole
TSB
L-serine
Tryptophan
TDC
Tryptamine
MVA pathway
Acetyl-CoA
Acetoacetyl-CoA
HMGS
3-hy-droxy-3-methylglutaryl-CoA
HMGR
Mevalonate
MEP pathway
Pyruvate+glyceraldehyde 3-phosphate
1-deoxy-D-xylulose 5-phosphate
DXR
2-C-methyl-D-erythritol-4-phosphate
Hydroxymethylbutenyl 4-diphosphate
HDR
HDR
IPI
Isopentenyl diphosphate
Dimethylallyl diphosphate
Secologanin
(Monoterpenoid pathway)
Strictosidine
Camptothecin
10-hydroxycamptothecin

Fig. 1

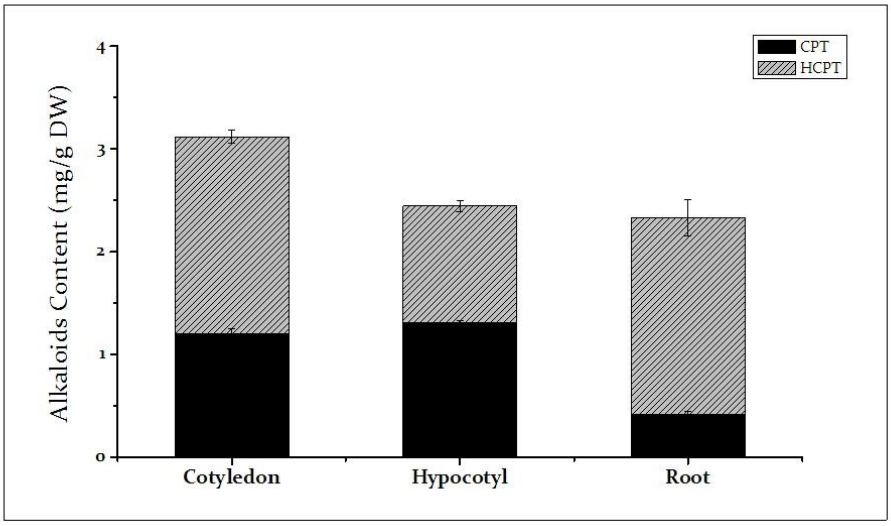

CPT
HCPT
Alkaloids Content (mg/g DW)
0
1
2
3
4
Cotyledon
Hypocotyl
Root

Fig. 2

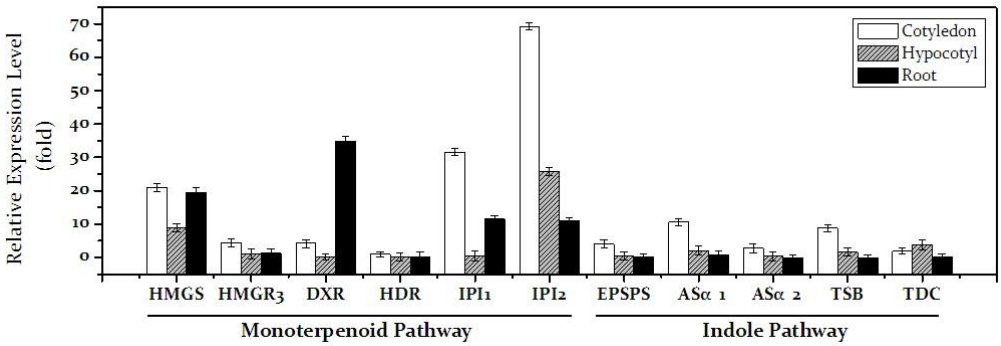
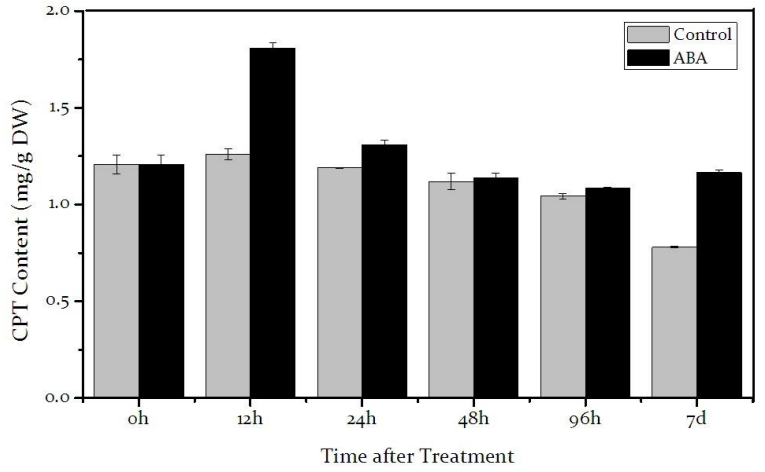

Relative Expression Level (fold)
70
60
50
40
30
20
10
0
Cotyledon
Hypocotyl
Root
HMGS
HMGR3
DXR
HDR
IPI1
IPI2
EPSPS
ASα 1
ASα 2
TSB
TDC
Monoterpenoid Pathway
Indole Pathway

Fig.3


CPT Content (mg/g DW)
2.0
1.5
1.0
0.5
0.0
Control
ABA
0h
12h
24h
48h
96h
7d
Time after Treatment

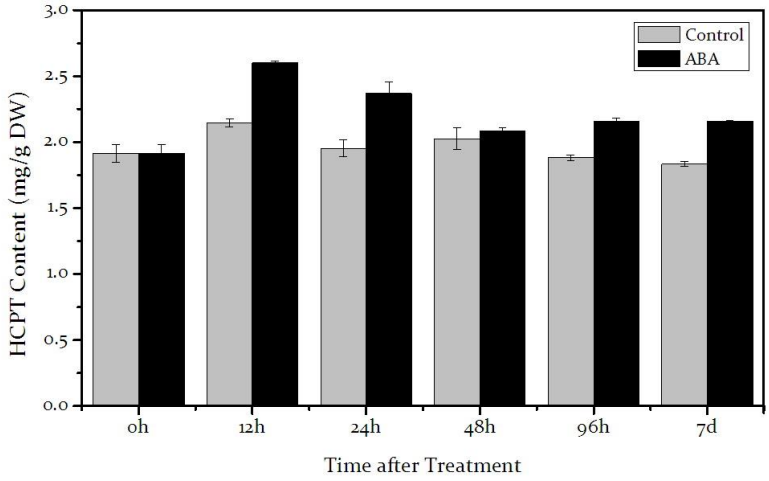

HCPT Content (mg/g DW)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
ABA
0h
12h
24h
48h
96h
7d
Time after Treatment

Fig.4

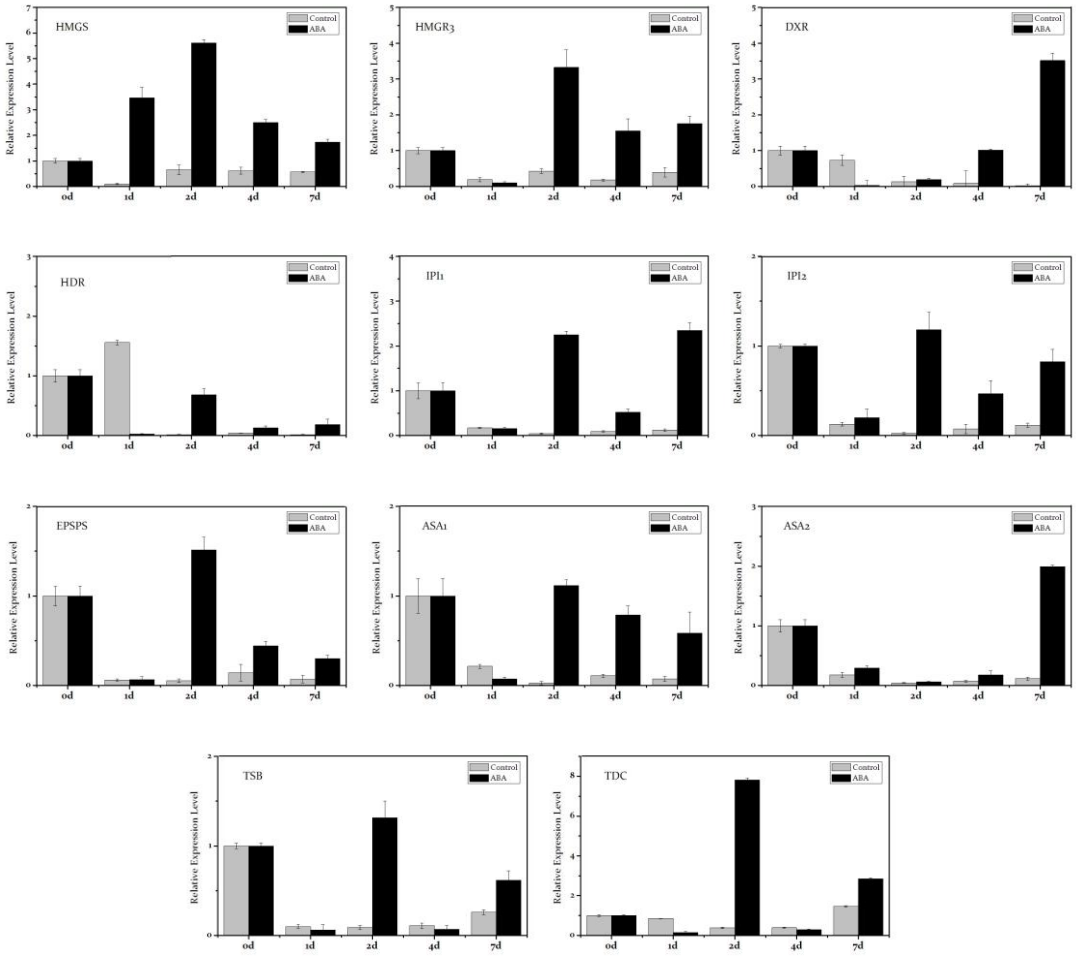


Fig.5

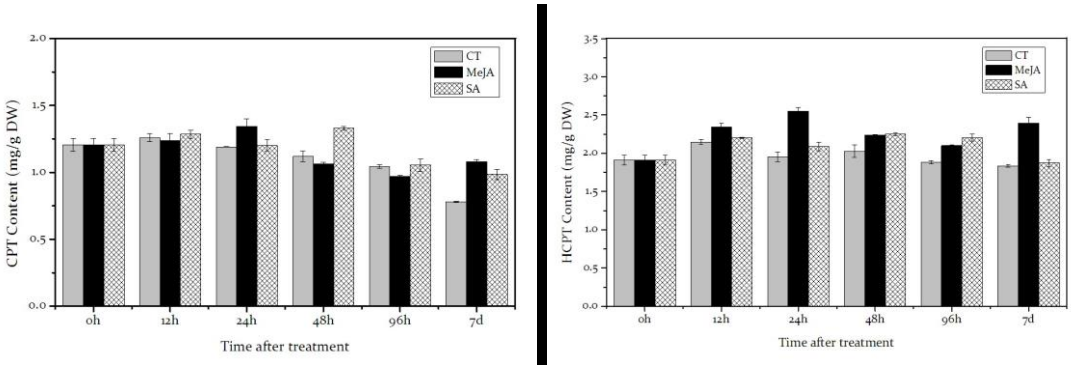


Fig.6

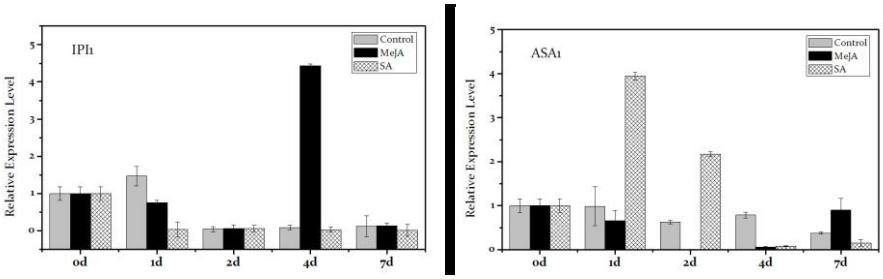


Fig.7