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ORIGINAL PAPER

Isolation and functional analysis of the *Catharanthus roseus* deacetylvindoline-4-*O*-acetyltransferase gene promoter

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Abstract Madagascar periwinkle (*Catharanthus roseus*) produces many therapeutically valuable terpenoid indole alkaloids (TIAs), such as vinblastine and vincristine derived from the monomers vindoline and catharanthine. Deacetylvindoline-4-*O*-acetyltransferase (DAT) is a key enzyme for the terminal step of vindoline biosynthesis. In this research, the *DAT* gene promoter was cloned, sequenced, and analyzed. An approximately 1,773 bp genomic DNA fragment of *DAT* promoter was obtained. Sequence analysis revealed that *DAT* promoter contained several potential regulatory elements which were involved in the regulation of gene expression. To investigate its function, the promoter fragments with 5′ deletions and gain-of-function deletions were fused to *GUS* reporter gene, and their expressions were analyzed by transient expression in *C. roseus* cell suspensions. The regulatory activity of *DAT* promoter was identified with fluorescence quantitative assays. Three TGACG motifs and one inverted motif (CGTCA) between −808 and −1,086 bp in the *DAT* promoter were found to be involved in methyljasmonate signal transduction pathway.

Keywords *Catharanthus roseus* · Deacetylvindoline-4-*O*-acetyltransferase · GUS activity · Metyljasmonate · Promoter

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Abbreviations

DAT	Deacetylvindoline 4-*O*-acetyltransferase
TIAs	Terpenoid indole alkaloids
GUS	*β*-Glucuronidase
MeJA	Methyljasmonate
MUG	4-Methylumbelliferyl-*β*-D-glucuronide
4-MU	4-Methylumbelliferone

Introduction

Due to the pharmacological activities, alkaloids are valuable nitrogen-containing basic compounds in plants and constitute one of the largest groups of natural secondary metabolites. *Catharanthus roseus* contains important anti-tumor terpenoid indole alkaloids (TIAs) such as vinblastine and vincristine. Vinblastine and vincristine are derived from the coupling of vindoline and catharanthine monomers. The terminal step of vindoline biosynthesis is catalyzed by DAT. Transcription of *DAT* gene can be activated by light treatment and is predominantly expressed in young leaves of mature plants (St-Pierre et al. 1998). Previous reports show that *C. roseus* cell suspensions could not produce vinblastine and vincristine due to it inability to make vindoline (Eilert et al. 1987).

However, they can accumulate the catharanthine and tabersonine precursors of vindoline. Normally, tabersonine is converted into vindoline through six enzymatic steps. For part of their biosynthesis, these steps happen in specialized cells, e.g., idioblast and laticiter cells, which are located in stems and leaves (St-Pierre et al. 1999). However, there is no sufficient evidence about the *DAT* gene expression in *C. roseus* suspension cells to date.

Jasmonic acid (JA) and its volatile methyl-ester (MeJA), collectively called jasmonates, are fatty acid derivatives synthesized via the octadecanoid pathway (Turner et al. 2002; Wasternack et al. 2006). Jasmonates is a plant-signaling molecule that plays key roles in defense against certain pathogens and insects by controlling the biosynthesis of protective secondary metabolites (Vom Endt et al. 2007). Several *cis*-acting elements in various gene promoters that mediate the JA responsiveness have been identified in higher plants, including a jasmonate- and elicitor-responsive element (JERE) in the *C. roseus STR* promoter (Menke et al. 1999), a GCC box in the *Arabidopsis PDF1.2* promoter (Brown et al. 2003), G-box sequences or G-box-like in the potato *PIN2* promoter (Kim et al. 1992), the soybean *VSPB* promoter (Mason et al. 1993), the *Arabidopsis VSP1* promoter (Guerineau et al. 2003), and the tomato *LAP* promoter (Boter et al. 2004). TGACG motifs had been confirmed to be essential for responsiveness to MeJA such as the inducibility of the promoter of *Agrobacterium tumefaciens* T-DNA nopaline synthase gene (Kim et al. 1993, 1994), the barley *lipoxygenase 1* gene (Rouster et al. 1997), and the rice *OsOPR1* gene (Sobajima et al. 2007). In *C. roseus*, the expression of *DAT* genes is also induced by MeJA (Van der Fits and Memelink 2000). However, the responsive elements of *DAT* gene to MeJA have not been investigated.

In this research, the *C. roseus DAT* promoter was isolated and characterized. The promoter activity of the *DAT* gene was examined in *C. roseus* cell suspensions by fusing its promoter into the *GUS* reporter gene based on *Agrobacterium*-mediated transient assay. In addition, the TGACG motifs were identified as MeJA-responsive element of the *C. roseus DAT* promoter.

Materials and methods

Promoter isolation and analysis

The Universal Genome Walker Kit (Clontech, USA) was used to obtain promoter regions of *C. roseus DAT* gene. Genomic DNA was isolated from young leaves using cetyltrimethylammonium bromide (CTAB). The genomic DNA was digested with *Dra*I, *Stu*I, *Pvu*II, and *Eco*RV to make a blunt end. Genome walker adaptors were ligated to the digests and the ligated products were then used as template to amplify promoter regions of *DAT* gene. Using the Genome walker library, primary and nested PCRs were performed with the *DAT* gene-specific primers and Genome Walker adaptor primer in kit. The gene-specific primers (outer and inner primers; Table 1) were designed according to *DAT* cDNA sequence. All PCR reactions were amplified using the Advantage 2 polymerase mixture (Clontech, USA) as per manufacturer's instruction. The nested PCR products were purified from 1.2% (w/v) agarose gel and subcloned into the pMD18-T vector (Takara, Japan). Then these cloned vectors were sequenced and the putative *cis*-regulatory elements were analyzed with PLACE (Higo et al. 1999) and PLantCARE databases (Lescot et al. 2002).

Determination of the *DAT* promoter transcription start site

The transcription start site (TSS) was determined using 5′-rapid amplification of cDNA ends (5′-RACE) technique following SMART RACE cDNA Amplification Kit (Clontech, USA) protocol. Total RNA was isolated using Trizol Reagent (Watson Biotechnologies, China). The reverse transcriptase, moloney murine leukemia virus (MMLV), was provided in the kit. In the first strand cDNA synthesis, the PCR of cDNA was performed using SMART III and CDS III/3′-PCR primers. For amplification of ds cDNA, one adaptor primer (5′-PCR Primer IIA) and two nested gene-specific antisense primers (DAT RACE1, 2; Table 1) were used in subsequent PCR reactions. The second PCR products were subcloned into pMD18-T vector. The isolated positive clones were sequenced and aligned with the *DAT* promoter sequence to determine the TSS.

Construction of *DAT* promoter-GUS fusions

For construction of the *DAT* promoter-GUS fusion genes, a series of *DAT* promoter fragments (from −1,665 to +74 bp, including ATG) of various lengths were obtained by PCR-amplification with designed primers (Table 1). A *Bam*HI restriction site was introduced into the forward primers and an *Nco*I restriction site was introduced into the reverse primer. Then the amplified sequences were digested by *Bam*HI and *Nco*I and cloned into the *Bam*HI and *Nco*I digested binary vector pCAMBIA1301 (CAMBIA Company, Australia) to replace the CaMV 35S promoter in front of the *GUS* gene. The five constructions were described as DAT1 (−1,665 to +74), DAT2 (−1,039 to +74), DAT3 (−428 to +74), DAT4 (−252 to +74), and DAT5 (−120 to +74).

To gain the functions of the promoter, two *cis*-regions from −1,039 to +74 were employed, in which one was a deleted fragment from −428 to −252 and another from −252 to −120. Then they were inserted upstream of the GUS gene, respectively.

Cell cultures and MeJA induction treatments

All experiments were conducted based on the cell suspension cultures of *C. roseus*. Cell suspensions were

Table 1 The primers used for PCR

Primer no.	Primer sequence
Outer primer	R: 5′-AATTTCAATGCCGTCGGGATTTTCGT-3′
Inner primer	R: 5′-GGATTGAGGGGTTGGAGAAGAGGGTT-3′
DAT RACE 1	R: 5′-ATAGCATGCAGAAGCAGCCCAATC-3′
DAT RACE 2	R: 5′-GAGAGCGAATTTTGTAGCTGCTCACG-3′
DAT 0 (+74)	R: 5′-CATGCCATGGAGGGTTTGATCAACGTTTTGG-3′
DAT 1 (−1,665)	R: 5′-CGCGGATCC CTTAGGTGACGCTTCCGAAC-3′
DAT 2 (−1,039)	R: 5′-CGCGGATCC CTTAGGTGACGCTTCCGAAC-3′
DAT 3 (−428)	R: 5′-CGCGGATCC TTCTTGCTGTGAATGTTCGTTT-3′
DAT 4 (−252)	R: 5′-CGCGGATCC TTTCCAATACTTTCTCAACT-3′
DAT 5 (−120)	R: 5′-CGCGGATCC TAGGAACTAAAACCAAAACCATT-3′
Md F (−428 to −252)	F: 5′-TTCTTGCTGTGAATGTTCGTTTCCAAT ACTTTCTCAACT-3′
Md R (−428 to −252)	R: 5′-AGTTGAGAAAGTATTGGAAACGAA CATTCACAGCAAGAA-3′
Md F (−252 to −120)	F: 5′-AATGGTTTTGGTTTTAGTTCCTAAGTTG AGAAAGTATTGGAAA-3′
Md R (−252 to −120)	R: 5′-TTTCCAATACTTTCTCAACTTAGGAACT AAAACCAAAACATT-3′
Md F (−1,086 to −808)	F: 5′-CATCCACTATTAGAAAATACCCGTGTTA ATATGCTTAGAATTGC-3′
Md R (−1,086 to −808)	R: 5′-GCAATTCTAAGCATATTAACACGGGTATTTTC TAATAGTGGATG-3′
DAT-F	F: 5′-CTTCTTCTCATCACGTACCAACTC-3′
DAT-R	R: 5′-ATACCAAACTCAACGGCCTTAG-3′
Rps9 F	F: 5′-TCGCAACTATGGTAAGACCT-3′
Rps9 R	R: 5′-CTGTTCATCCTCCTCAAAAG-3′

F forward primer, *R* reverse primer

established as described by Pasquali et al. (1992). The *C. roseus* seeds were purchased from PanAmerican Seed Company (Cherry Red, USA) and the plants were grown in green house at 26°C. Cell suspension cultures grown in 60 mL MS containing 2 mg/L 1-naphthaleneacetic acid (NAA) and 0.2 mg/L kinetin (KT) in 250 mL Erlenmeyer flasks in continuous light at 25°C on a gyratory shaker at 120 rpm. Subculturing was performed once a week by eightfold dilution in 50 mL fresh medium. Cells were collected by vacuum filtration on a 80 μm nylon filter, frozen in liquid nitrogen, and stored at −80°C.

Cell suspension cultures were treated for 8 h with 100 μM MeJA (Sigma, USA) diluted in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO only.

RT–PCR analysis

The effects of MeJA to *DAT* transcripts in *C. roseus* suspension cells were quantified by semi-quantitative RT–PCR and real-time RT–PCR. The specific primers for their corresponding genes were analyzed, which include DAT-F and DAT-R for the *DAT* gene, and Rps9-F and Rps9-R for the *Rps9* gene (Table 1).

The cDNA for real-time PCR was synthesized from RNA samples using Prime Script™ Reverse Transcriptase Reagent according to the manufacturer's instructions (TaKaRa, Japan), using oligo (dT) as primer. The real-time PCR analysis was performed in Peltier Thermal Cycler PTC200 (Bio-Rad, USA), using the cDNAs as templates. SYBR Premix Ex Taq (TaKaRa, Japan) was used in the PCR reactions to quantify the amount of dsDNA. The relative C_t, the threshold of cycle value, was used to estimate the initial amount of template present in the reactions.

Agrobacterium-mediated transient expression

A. tumefaciens strain EHA105 containing the binary pCAMBIA1301 was used for transformation. The *GUS* reporter gene in pCAMBIA1301 contains an intron inside the coding sequence to ensure that expression of glucuroidase activity is derived from eukaryotic cells. The amplified fragments were respectively inserted into plasmid pCAMBIA1301 separately as *Nco*I-*Bam*HI fragments at corresponding restriction sites in place of the CaMV 35S promoter region. The constructs were introduced into *A. tumefaciens* strain EHA105 by the liquid nitrogen freeze-thaw method. Individual colonies of transformed

A. tumefaciens EHA105 were picked and grown to a final OD_{600} of 0.6 in 10 mL of Luria–Bertani media containing kanamycin (100 mg/L) and rifampicin (100 mg/L) at 28°C and suspended at 250 rpm. Bacteria were then centrifuged at 2,000×*g* for 10 min and remaining media were removed. The *A. tumefaciens* pellet was washed once with MS liquid media to remove residual antibiotics before being resuspended in 20 mL of MS liquid media containing 100 μM/L acetosyringone and added to a 125 mL Erlenmeyer flask containing 20 mL of newly subcultured *C. roseus* cells in MS media. The *A. tumefaciens*-inoculated flask grew under 28°C for 48 h. After 48 h of cocultivation, *C. roseus* cells were washed twice with MS liquid media. Cells were collected and stored as mentioned before.

GUS activity assay

GUS activity was quantitatively analyzed according to the method of Jefferson et al. (1987). *C. roseus* transient transgenic cell lines were homogenized in protein extraction buffer (0.05 M $NaPO_4$, pH 7.0, 0.1% SDS, 10 mM EDTA, 20% methanol, 10 mM β-ME, Triton X-100), and centrifuged for 10 min at 13,000 rpm. Aliquots of the supernatant were reacted with MUG, and the enzymatic products were measured by fluorometry. Protein concentrations were measured using Bradford protein assay reagent (Bio-Rad, USA). GUS enzyme activity was expressed as nanomolar 4-MU produced per minute per milligram protein.

Results

DAT promoter sequence analysis

The promoter region of *DAT* gene was cloned from *C. roseus* genomic DNA. Then the cloned *DAT* promoter region was determined as 1,773 bp long, and the sequence is shown in Fig. 1. Furthermore, the cloned *DAT* promoter region was analyzed using the PLACE and the PLantCARE databases. Several core fragments were identified, which are homologous to the *cis*-acting elements of other plants and of great importance for the promoter functions (Fig. 1).

Three kinds of elements, which had been found regulated by hormones in the 5′-upstream region of some plant genes, are present in *C. roseus DAT* promoter: the ABRE (CACGTG and GCAACGTGTC) involved in the abscisic acid responsiveness; the TGA (AACGAC) as auxin-responsive element; and the TGACG-motif (TGACG) as *cis*-acting regulatory element involved in the MeJA responsiveness.

Several elements in the *DAT* gene promoter have previously been identified in other plants and shown to be responsive to light, for example, ATCT-motif (AATC TAATCC), LAMP-element (CCAAAACCA), GT1-motif (GGTTAA), Box 4 (ATTAAT), Box I (TTTCAAA), and as-2-box (GATAatGATG).

Several putative regulatory motifs, which are homologous to the *cis*-acting elements involved in the activation of defense genes in plants, exist in the promoter region of the *DAT* gene. They consist of one HSE (AAAAAATTTC) element, one ARE (TGGTTT) element, two MYB-binding (CAACTG) sites, and four W-box (TGAC) elements.

TSS of the *C. roseus DAT* promoter

The TSS of *DAT* promoter was determined by 5′-RACE using total RNA extracted from *C. roseus* cells. The TSS is an adenine (A) at −13 bp upstream of translation initiation cordon. The distance between putative TATA-box and TSS is 34 bp, which is consistent to the distance 32 ± 7 of previous data (Joshi 1987).

Function analysis of the *DAT* promoter in *C. roseus* suspension cells

To characterize the function of the *DAT* promoter, a series of 5′-promoter deletions fused to *GUS* reporter gene were delivered into *C. roseus* suspension cells for transient expression assay (Fig. 2). The expression level of full length promoter (coordinates −1,665 to +74, construct 1) was 1.7 nmol MU/min/mg protein, and this value was considered to represent full (100%) promoter activity compared to the other deletion constructs. The activity of 5′-end deletion construct 2 (coordinates −1,039 to +74) and 3 (coordinates −428 to +74) was decreased 13.2 and 18.3%, respectively. However, further deletion down to −252 (construct 4) resulted in 45.7% decrease of the protein activity. Deletion to −120 (construct 5) resulted in 80% loss. These data suggested that the sequence between −1,665 and −428 from the TSS, in this context, may not be essential for maximal promoter activity. However, successive deletion of about 150 and 300 bp from 5′-end of construct 3 markedly decreased the promoter activity. It reveals that the sequence between the 428 and 120 bp upstream of the TSS constrains *cis*-acting elements which are necessary for the transcription. The promoter region (−120 to +74, construct 5) with minimal activity containing the TATA-box is considered as the minimal promoter.

Since the region from −1,689 to −1,039 had little influence on the promoter activity, two upstream regions of −1,039 to +74 deleted from −428 to −252 (construct 6) and deleted from −252 to −120 (construct 7) were further analyzed (Fig. 2). Quantitative GUS assay showed the promoter activity decreased significantly without the two deleted segments. The protein activities of constructs 6 and

Fig. 1 DNA sequence of *C. roseus DAT* promoter. A 1,789 bp fragment with respect to TSS is from −1,773 to +16. The TSS is indicated as +1. Important *cis*-elements are indicated under the *line*

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-1773  GTCTCCTTTAAAAAATAAAAAATCTCTTTCAAGAAAGTAGTTAAAGAACCTTTCCAAAAA
-1713  AGAAAAGTACTTAAAGAAAAATGATTAAATTTCAATTTCGTCCTTCAAAATATAGAACTA
-1653  TCTCAATCGAGGACTTTGATTTAAAAAGTTTTTAATTTCATCCTTCAAAATGGAAAAAAA
-1593  TTTGTGTCCATCAAGTCCTTAACCTGGCTTCTATTAAAGTTTTGACAATCAAACTCCATT
                                                    W-box
-1533  CAATCAATTTCATGAATTGAGAAGTCTTGGTCTTTTTGTCCACAAAAGAACCTGTAAAAA
-1473  AAATAAAAATAAAAGGTTAGAAGACTAACACTTTTGGATTAAATAAAAAGTCCTTGGCAA
-1413  TGACATCAAAATTTTACACGTGCTATCATAGGCATGTCAAATTAATTCTAATATTCTTCC
       W-box          G-box                  Box-4
-1353  TAAAAATATACTTAGAGCAAGCAAGTGTCAATCCCACAAAGACGCATAAATTTAATCCTT
                      ABRE                                ATCT-motif
-1293  TAAGATAATGAACTAATCACAAAGTAATGGTTTTAAGGGGTTTGAGGTTCATTAAAATAA
                                ARE
-1233  ATAACTAACTAAAATGAACTAAAGTTAACTAAGAAATCAAATTAAACGATTAAAAGAATC
-1173  AAGGGAAAAAAAAGTTAGCAATGCACATGCAATTGATTATATTTTTATGGAATCGAATCTA
                                                    ATCT-motif
-1113  TCATCCACTATTAGAAAATACCCTTATAATGACGCAGAAACGTGTCACCATACAACTTTA
                                TGACG-motif  ABRE
-1053  TAGTGACGGTTTGCCTTAGGTGACGCTTCCGAACAGCCTAAAGTCAAGTAGTAATAGACC
       TGACG-motif       TGACG-motif
 -993  AATAACGCTTTAAAGCGTTACCATACACGATCTATGCTTTAAAGGTCATTAAAAACATAT
 -933  ACCTACATTTTTTAGAGTACCAACTGACATTCTAATGGTAAAAAGATTTAAACGTTTTCA
                     MYB-core                              BoxI
 -873  AAATTATATTACGGTTAAAAGTATCAGCGTAGATGTATGATGATGCTCTAAAAGCGTCAC
                GT1-motif                          CGTCA-motif
 -813  TATAGTGTTAATATGCTTAGAATTGCTACTATAGATTACCTATTACTACGCTTTTAGACT
 -753  TAGCGCTAACATATATATCATTATGCTATTTTATCTCATAAATGCTCAATGCATTAATAT
 -693  ATTGCTGCACTATTAAAATATCACTATAGGTCACTTCTAAAAAAAATTTGAATCATATTA
                                                    HSE
 -633  TTAATATTTTTTGTTAGACATATTTATAGATTTAAGATACAATAGATTACAAAACTGTAA
 -573  AGGAAATGTAATTTGTCATTAGAACGACAGAGTCCAATTAGAATTGAACATTGAAAGTTC
                           TGA-element
 -513  TAGTTCCCTTTAAATAATATATTTGAACATCTATTATGTGTACATTAATTCAATAACATT
 -453  CCCTTTTCGTATTTATTATGCTAATTTCTTGCTGTGAATGTTCGTTTTTTCTTTATGAGT
 -393  TTAACAAAGATTTAGATAATTATGAGAGATTTTAAAGTATTTTTTCAATTTTTAACATTG
                     As-2-box
 -333  AAATAATTTAGAAATGAAATATAATTATTAGTAATTCTCACACTTTGAAGAGAATATTTT
 -273  TATTGATTTATGTAAAATTTATTTCCAATACTTTCTCAACTAATATTCATTACATGACTA
                                                          W-box
 -213  CACATCTACTTTATCTTTGACTTTTAGTTGTTATATATATATATATATATATTTAATA
                           W-box                TA-rich region
 -153  CTCTTCAAAAGAAAAAACAACTGATTTATATCATAGGAACTAAAACCAAAACCATTCTGG
                           MYB-core                        LAMP
  -93  CCACAAAAAAAATGAAAGTATCATAGGAACGTTGGGTGGGGTGGGGCGCCTGGATAGCT
  -34  TATATATGTGTTGTGTGTTGGGGTCTGAATATATAACAGCAAGCAAAATG
         TATA-box                        +1
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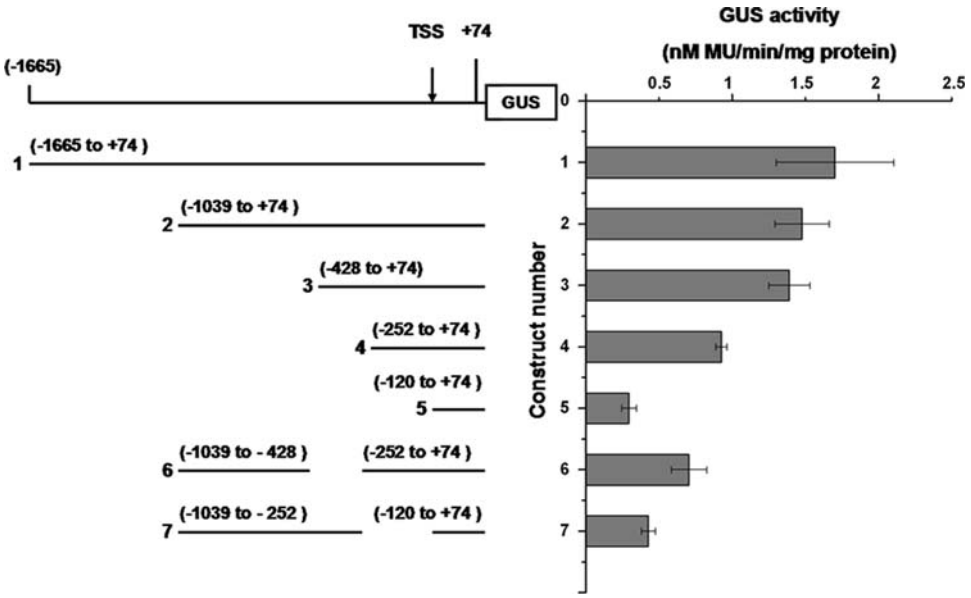

Fig. 2 Deletion and gain-of-function analysis of the *DAT* promoter. The *left panel* represents a series of deletion constructions containing different lengths of *DAT* promoters fused to GUS reporter gene. Cells were harvested for GUS assays at 48 h after transfection. At the top, the relative position of the transcription start site and the *DAT* promoter coordinates are indicated. The *right panel* shows the corresponding GUS activities of each construct. The GUS activities were determined in the *C. roseus* cell transient expression assay. Data are mean ± SD from three independent experiments

7 were 47.7 and 28.9% of the construct 2, respectively. These results suggested that at least two enhancing sequences are present in the regions from −428 to −252 and from −252 to −120. Further, the latter is more important because the protein activity of construct 6 is 1.7-folds of construct 7. This result is consistent with the sequence analysis mentioned before that the region from −252 to −120 includes the transcription enhancer, TA-rich motif.

Effects of MeJA treatments on *DAT* gene promoter

In previous studies, the transcription of *DAT* gene had been found to be strongly induced in response to MeJA (Van der Fits and Memelink 2000). In order to investigate MeJA on the promoter inducibility at different time points, we analyzed *DAT* mRNA level by semi-quantitative RT–PCR and real-time RT–PCR after treating the suspension cells MeJA. As shown in Fig. 3, the mRNA level of *DAT* was low in untreated cells. MeJA induced *DAT* mRNA accumulation with maximum level at 8 h. However, the expression of *Rps9* mRNA, encoding 40S Rps9, was not affected by MeJA. Therefore, the peak levels of *DAT* mRNA arrived at 8 h after treatment, and the highest levels of protein accumulation may be at a time point later than 8 h.

Sequence analysis showed that three TGACG motifs and one inverted motif (CGTCA) were present at the location from −1,086 to −808 in *DAT* promoter. It is well known that TGACG-motif is a *cis*-acting regulatory element involved in the MeJA responsiveness in barley grain

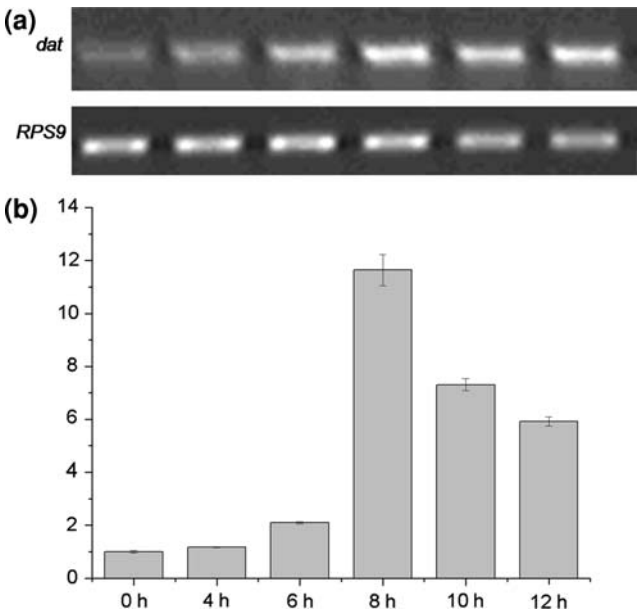

Fig. 3 Response of *DAT* mRNA expression to the treatment of 100 μM MeJA at different time points by semi-quantitative (**a**) and real-time RT–PCR (**b**)

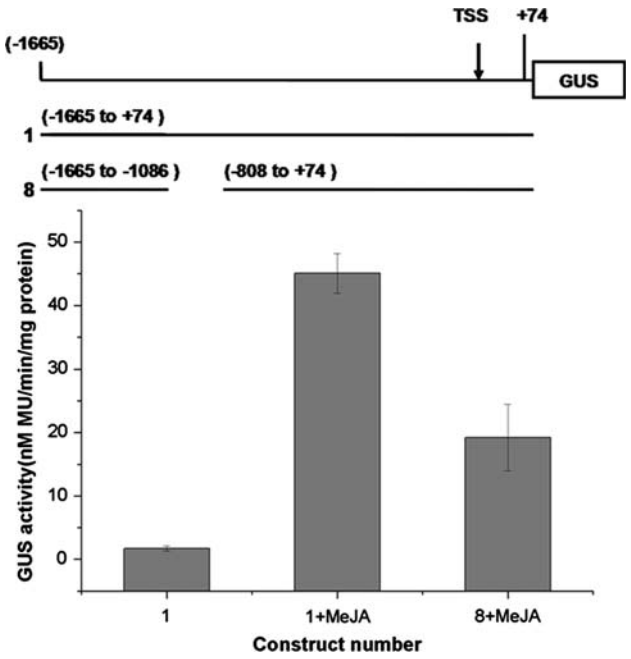

Fig. 4 Effects of MeJA on *DAT* promoter expression in transient transgenic *C. roseus* cell suspensions. Cells were incubated for 8 h with DMSO (*1*), or 100 μM MeJA (*1 + MeJA*, *8 + MeJA*) and detected for GUS activity. Data are mean ± SD from three independent experiments

(Rouster et al. 1997). To determine whether the TGACG-motif sequence from *DAT* promoter is involved in MeJA signaling, we performed loss-of-function analysis with the deletion of major *cis*-elemnts from −1,086 to −808 (construct 8, Fig. 4). After incubation with 100 μM MeJA for 8 h, a significant increase in the expression of GUS activity was observed in both constructs 1 + MeJA and 8 + MeJA (Fig. 4). GUS activity increased approximately 25-folds when 100 μM of MeJA was used to induce the *C. roseus* cells in the full promoter construct. However, when the segment from −1,086 to −808 was deleted, the GUS activity increased only 11-folds, two times lower than that of construct 1 + MeJA. The results indicate that the TGACG motifs may play a key role in MeJA-responsive expression of the TIAs biosynthetic gene *DAT*.

Discussion

Cloning and characterization of *DAT* promoter

To explore the regulation mechanism of *DAT* expression, we isolated and characterized the 1,773 bp promoter of *DAT* gene, which encodes deacetylvindoline-4-*O*-acetyltransferase, a key enzyme in TIAs biosynthesis. The TSS is mapped to an adenine at −13 bp upstream of the start cordon and 34 bp downstream of the TATA-box. The

sequences located between −428 and −120 bp upstream of the *DAT* gene, especially from −252 to −120, were found crucial for the basal expression of the promoter.

Transient expression assays are the rapidly expanding techniques, which have opened up new strategies for transgenic studies without delay and environmental risks associated with the production of stable transgenic plants (Omidvar et al. 2008). The general trend for relative promoter activities is the same for both transiently and stably transformed *C. roseus* cells (Van der Fits and Memelink 1997). In the *Agrobacterium*-mediated transient expression system, each transgenic cell line is a mixed population comprising thousands of independent transformants. Expression level of transgene can reflect an average largely independent of T-DNA chromosomal position and copy number. Therefore, measuring transient activity of promoters introduced by *A. tumefaciens* represents an easy and credible method to assay promoter activity.

It is well known that the late stage of vindoline biosynthesis, which is catalyzed by DAT, is light-regulated in developing seedlings and the transcriptional expression of *DAT* increase progressively after light treatment (St-Pierre et al. 1998). In this experiment, lots of elements responded to light, which had been identified in other plants promoter previously, were also found in *DAT* promoter in *C. roseus*. The results obtained here could explain the molecular mechanisms of light signal to stimulate the accumulation of *DAT* transcripts and protein in *C. roseus*. Thus, we conclude that light is very important for vindoline pathway to produce the alkaloids.

Effect of MeJA on *DAT* promoter activity

Accumulating evidences show that MeJA can induce the expression of most genes in TIAs pathway in *C. roseus*, including *STR*, *G10H*, *TDC*, and *SGD* (Van der Fits and Memelink 2000; Suttipanta et al. 2007). The JERE in *STR* promoter was demonstrated to associate with AP2/ERF-domain transcription factors (ORCA2 and ORCA3) (Menke et al. 1999; Van der Fits and Memelink 2001). Overexpression of ORCA3 increased the expression levels of several genes involved in TIAs pathway in *C. roseus*. While the *DAT* expression is not regulated by ORCA transcription factors, implicating that it is controlled by other JA-responsive transcription factors and elements in *DAT* promoter participating in MeJA responsiveness. The result obtained here is very helpful for deeper understanding of the complex regulatory mechanism in TIA pathway.

The TGACG-motif had been previously identified as a binding site for transcription factors involved in the MeJA signal transduction pathway in the promoter of *lipoxygenase 1* gene in barley (Rouster et al. 1997), and for bZIP-transactivating factors (Benfey et al. 1989; Schindler et al. 1992). In *lipoxygenase 1* gene promoter, two complementary sequences (CGTCA and TGACG) are located between −319 and −297 (Fig. 5). The presence of the two TGACG motifs in the *lipoxygenase 1* promoter, although in opposite orientation, suggests that they could be involved in the binding of transcription factors. Site-directed mutagenesis on these two sequences could abolish the MeJA response, and thus they are identified as MeJA-responsive elements (Rouster et al. 1997). In this research, sequence analysis of *DAT* promoter showed that three serial TGACG motifs are located in the fragment from −1,086 to −1,028, and their complementary sequences, CGTCA, were found in 209 nucleotides after them (Fig. 5). Furthermore, MeJA inducibility was largely deduced by the deletion of the 278 bp fragment containing three TGACG motifs and one CGTCA motif. Compared to previous published data, the distance between TGACG motifs and CGTCA motif is relatively longer. These palindromic sequences might provide a binding domain for transcription factors (Rouster et al. 1997). The TGACG-motif was not found in the other known promoter sequences of *C. roseus* TIAs biosynthetic pathway genes, such as, *Str*, *Tdc*, and *G10H*. The result indicates that this novel *cis*-element responded to MeJA may be different in *DAT* promoter.

Fig. 5 Comparison of the MeJA-inducible fragments between promoters *C. roseus DAT* and barley *lipoxygenase 1* (*Lox1*). The sequence of *lipoxygenase 1* promoter obtained from Rouster et al. (1997)

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References

Benfey PN, Ren L, Chua NH (1989) The CaMV 35S enhancer contains at least two domains which can confer different

developmental and tissue-specific expression patterns. EMBO J 8:2195–2202

Boter M, Ruíz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. Genes Dev 18:1577–1591

Brown RL, Kazan K, McGrath KC, Maclean DJ, Manners JM (2003) A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of *Arabidopsis*. Plant Physiol 132:1020–1032

Eilert U, De Luca V, Kurz WGW, Constabel F (1987) Alkaloid formation by habituated and tumorous cell suspension cultures of *Catharanthus roseus*. Plant Cell Rep 6:271–274

Guerineau F, Benjdia M, Zhou DX (2003) A jasmonate-responsive element within the *A. thaliana vspl* promoter. J Exp Bot 54:1153–1162

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements(PLACE) database. Nucleic Acids Res 27:297–300

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion maker in higher plants. EMBO J 6:3901–3907

Joshi CP (1987) An inspection of the domain between putative TATA-box and translation start site in 79 plant genes. Nucleic Acids Res 15:6643–6653

Kim SR, Choi JL, Costa MA, An G (1992) Identification of G-box sequence as an essential element for methyl jasmonate response of potato proteinase inhibitor II promoter. Plant Physiol 99:627–631

Kim SR, Kim Y, An G (1993) Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (*nos*) promoter. Plant Physiol 103:97–103

Kim Y, Buckley K, Costa MA, An G (1994) A 20 nucleotide upstream element is essential for the nopaline synthase (*nos*) promoter activity. Plant Mol Biol 24:105–117

Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325–327

Mason HS, DeWald DB, Mullet JE (1993) Identification of a methyl jasmonate-responsive domain in the soybean *vspB* promoter. Plant Cell 5:241–251

Menke FL, Champion A, Kijne JW, Memelink J (1999) A novel jasmonate-and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J 18:4455–4463

Omidvar V, Siti Nor Akmar A, Marziah M, Maheran AA (2008) A transient assay to evaluate the expression of polyhydroxybutyrate genes regulated by oil palm mesocarp-specific promoter. Plant Cell Rep 27:1451–1459

Pasquali G, Goddijin OJ, de Waal A, Verpoorte R, Schilperoort RA, Hoge JH, Memelink J (1992) Coordinated regulation of two indole alkaloid biosynthetic genes from *Catharanthus roseus* by auxin and elicitors. Plant Mol Biol 18:1121–1131

Rouster J, Leah R, Mundyt J, Cameron-Mills V (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. Plant J 11:513–523

Schindler U, Beckmann H, Cashmore AR (1992) TGA1 and G-box binding factors: two distinct classes of *Arabidopsis*, leucine zipper proteins compete for the G-box-like element TGACGTGG. Plant Cell 4:1309–1319

Sobajima H, Tani T, Chujo T, Okada K, Suzuki K, Mori S, Minami E, Nishiyama M, Nojiri H, Yamane H (2007) Identification of a jasmonic acid-responsive region in the promoter of the rice 12-oxophytodienoic acid reductase 1 gene *OsOPR1*. Biosci Biotechnol Biochem 71:3110–3115

St-Pierre B, Laflamme P, Alarco AM, De Luca V (1998) The terminal *O*-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. Plant J 14:703–713

St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell 11:887–900

Suttipanta N, Pattanaik S, Gunjan S, Xie CH, Littleton J, Yuan L (2007) Promoter analysis of the *Catharanthus roseus* geraniol 10-hydroxylase gene involved in terpenoid indole alkaloid biosynthesis. Biochim Biophys Acta 1769:139–148

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14:153–164

Van der Fits L, Memelink J (1997) Comparison of the activities of CaMV 35S and FMV 34S promoter derivatives in *Catharanthus roseus* cells transiently and stably transformed by particle bombardment. Plant Mol Biol 33:943–946

Van der Fits L, Memelink J (2000) ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289:295–297

Van der Fits L, Memelink J (2001) The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J 25:43–53

Vom Endt D, Soares e Silva M, Kijne JW, Pasquali G, Memelink J (2007) Identification of a bipartite jasmonate-responsive promoter element in the periwinkle *ORCA3* transcription factor gene which interacts specifically with AT-hook DNA-binding proteins. Plant Physiol 144:1680–1689

Wasternack C, Stenzel I, Hause B, Hause G, Kutter C, Maucher H, Neumerkel J, Feussner I, Miersch O (2006) The wound response in tomato-role of jasmonic acid. J Plant Physiol 163:297–306