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Expression analysis of the recombinant *Catharanthus roseus* deacetylvindoline 4-O-acetyl transferase in tobacco plants

Bui Thi Ha[1,2], Nguyen Thi Ngoc Lan[1], Nguyen Thi Tam[1], Le Van Son[3], Chu Hoang Mau[1*]

[1]Thai Nguyen University of Education, Vietnam
[2]Thai Nguyen University of Medicine and Pharmacy, Vietnam
[3]Institute of Biotechnology, Vietnam Academy of Science and Technology, Vietnam

***Corresponding author: chuhoangmau@tnu.edu.vn**

Abstract

Catharanthus roseus (L.) G. Don contains about 130 types of alkaloids, including vincristine and vinblastine, which are outstanding drugs for cancer. The *C. roseus* deacetylvindoline-4-O-acetyl transferase (CrDAT) is a key enzyme which catalyzes the second to the final reactions in the vindoline way. The low content of indole alkaloid in *C. roseus* plants and the high cost of indole alkaloid production have promted many research to improve indole alkaloid yield in this plant. The aim of this work was to express recombinant CrDAT in tobacco, a model plant, to create the basis for the overexpression of the gene encoding CrDAT (*GenBank* LN809930) in *C. Roseus* plants. In this study, the 35S-DAT-cmyc structure was transferred to tobacco and the transgenic tobacco lines was generated. The T1 generation was then analyzed by Western blot method and ELISA analysis. Southern blot assays confirmed that the *CrDAT* gene was completely introduced into tobacco genome by *Agrobacterium*-mediated transformation. The recombinant CrDAT protein of 51.5 kDa in size was successfully expressed at the seven transgenic tobacco lines. The recombinant CrDAT protein content of transgenic tobacco lines were 2.75 - 5.35 (μg. mg^{-1} of total protein) range and the recombinant CrDAT protein content of the T0-1 line was highest (5.35 μg. mg^{-1} of total protein).

Keywords: *Agrobacterium*-mediated transformation, recombinant CrDAT protein, transgenic tobacco**,** viblastine, vincristine.
Abbreviations: CrDAT_*C. roseus* deacetylvindoline-4-O-acetyl transferase; ELISA_Enzyme-Linked Immunosorbent Assay; MIAs_monoterpenoid indole alkaloids; D4H_deacetoxyvindoline 4-hydroxylase; rCrDAT_recombinant CrDAT; T0, T1_generations of transgenic plants; TDC _tryptophan decarboxylase; WT_ the wild-type tobacco plants (non transgenic plant).

Introduction

Catharanthus roseus (L.) G. Don is a tropical perennial subshrub, which is widely distributed in almost all Southeast Asia countries, including Vietnam, North and South America, India and Australia (Plaizier, 1981; Van Bergen, 1996). This plant comprises many terpenoid indole alkaloids, in which vincristine and vinblastine are the most important alkaloids for the pharmaceutical industry. Vincristine and vinblastine were discovered as a result of a drug-screening program performed in the late 1950s. They have been applied in the treatment of some diseases such as Hodgkin's disease, lymphosarcoma, choriocarcinoma, neuroblastoma, chronic leukemia and cancer (Robert et al., 2004; Maryam et al., 2013).

In *C. roseus* plants, vincristine and vinblastine are produced by the coupling of two monoterpenoid indole alkaloids (MIAs), vindoline and catharanthine (Qu et al., 2015). While cathranthine is produced in every plant parts, vindoline is only produced in green leaves and its formation requires light as an elicitor. There are seven genes identified as the source of the essential enzymes for converting tabersonine into vindoline, including the *DAT* gene. The DAT is a key enzyme that catalyzes the final reaction forming vindoline. The production of vindoline requires the participation of at least two types of cells, which are idioblasts and laticifers and the intercellular transport of intermediate path found in the leaves, stems and buds (St-Pierre et al., 1999; Qu et al., 2015). Although light is not necessary for the formation of two types of cells and laticifers idioblasts but light stimulates the transcription of CrDAT along with other alkaloid biosynthesis genes as deacetoxyvindoline 4-hydroxylase (D4H) and tryptophan decarboxylase (TDC) (Vazquez-Flota et al, 1998; Vazquez-Flota et al., 2000).

The production of indole alkaloid in *C. roseus* cells treated with fungal elicitors tends to accumulate rapidly as a result of increase of enzyme activity (Eilert et al., 1987). Desacetoxyvindoline 4-hydroxylase and deacetylvindoline 4-O-acetyltransferase catalyze the late biosynthesis reactions, which transform tabersonine into vindoline (St-Pierre et al., 1999). The CrDAT has a molecular weight of 50 kDa, including 9 tryptic fragments. The 1320-nucleotide *CrDAT* gene encodes DAT that contains 439 amino acids. In previous studies, the *CrDAT* was successfully isolated from *C. roseus* plants with pink and white flowers in Vietnam. The *CrDAT* sequence data was submitted and registered in

GenBank with accession numbers LN809930 and LN809931 (Bui et al., 2015a, b). Because of the low content of vincristine and vinblastine in *C. roseus* plant, transgenic technology was used to enhance the expression of *CrDAT*. The *CrDAT* sequence isolated from pink flower periwinkle was used as a transgene. Additionally, the 35S-DAT cmyc structure was transferred into tobacco plants to assess activity of the promoter which controlled the expression of the *CrDAT* transgene. In this study, our aim was to express recombinant CrDAT in model tobacco plants to create the basis for the overexpression of gene encoding DAT in transgenic *C. roseus* plants.

Results

CrDAT transformation and generation of transgenic tobacco plants

The pBI121 transgenic vector contained *CrDAT* gene and CaMV 35S promoter was 13 kb in size with two sites cutting by a pair of XbaI / *Sac*I restriction enzymes (Fig 1).
The *Agrobacterium*- mediated transformation experiment was repeated three times. A total of 249 pieces of approximately $1cm^2$ tobacco leaf were used as materials for transformation. After inoculation for 30 minutes, the leaf explants were blotted dry and placed on co-cultivation medium in the dark for three days. The leaf explants were then washed and transferred to selective medium supplemented with antibiotics and BAP. There were 205 explants produced shoot-buds within 2-3 weeks. The shoot-buds were separated from the leaf explants, then elongated shoots were put onto rooting medium for induction of roots. The 113 plantlets were transplanted in pots, among which 65 plants grown up healthy in a greenhouse.

Analysis of CrDAT expression in transgenic tobacco plants

The leaves of the 4-week-old transgenic tobacco plants were used to test the presence of *CrDAT* transgene by PCR using primers *CrDAT-F-XbaI / CrDAT-R-SacI*. The PCR products were determined by 1% agarose gel electrophoresis (Fig 2).
Among the total of the 19 transgenic tobacco lines, seven lines (at 3, 7, 9, 10, 11, 14, 18 electrophoresis lanes) were negative and twelve lines (at 1, 2, 4, 5, 6, 8, 12, 13, 15, 16, 17, 19 electrophoresis lanes) were positive for PCR reactions. The PCR products were about 1.3 kb in size which were similar to the size of *CrDAT*. The PCR results demonstrated the presence of the introduced *CrDAT* transgene in twelve tobacco lines. The nine positive lines were used for Southern blot analysis to confirm the structure of pBI121- CrDAT was transferred into tobacco plants (Fig 3). The results showed that all of the nine transgenic tobacco lines were positive for Southern blot and the transgenic tobacco lines T0 have symbol is T0-1, T0-2, T0-4, T0-5, T0-6, T0-8, T0-12, T0-13 and T0-15. Of which the seven lines contained a single copy and two lines T0-5 and T0-15 had two copies. The seven lines with a single copy were selected for the analysis of recombinant protein expression, as T0-1, T0-2, T0-4, T0-6, T0-8, T0-12 and T0-13.

Analyzing the expression of recombinant CrDAT protein

The results of analysis of recombinant CrDAT protein in the seven Southern positive transgenic tobacco lines are presented in Fig 4. The product of the *CrDAT* gene in the seven transgenic tobacco lines (T0-1, T0-2, T0-4, T0-6, T0-8, T0-12, T0-13) were determined on nitrocellulose membrane. All seven lines had a single band with a molecular weight of approximate 51.5 kDa. This band was completely missing in the non-transgenic tobacco plants. Hence, it is strongly suggested that *CrDAT* was successfully transformed and expressed in the transgenic tobacco lines. To assess the level of recombinant protein expression, ELISA technique was used to determine the content of the recombinant CrDAT protein, the results are shown in Fig. 4B. The recombinant CrDAT protein content of the transgenic tobacco lines were 2.75 - 5.35 ($\mu g.\ mg^{-1}$ total protein) range and the recombinant CrDAT protein content of the T0-1 line was highest (5.35 $\mu g.\ mg^{-1}$ total protein).

Discussion

Transgenic vector 35S- CrDAT-cmyc contained identified c-myc peptide sequence was used as antigen for Western blot reactions to detect recombinant protein. Several pairs of antigen - antibody has been built to be used for the purpose of selecting transgenic plants, but the c-myc tagged has been an efficient and economical choice for many current studies (Ahn et al., 2014). The results of many research have reflected the expression of recombinant proteins, which were detected in tobacco plants by the c-myc structure. Additionally, the effect of using vector PBI121_DAT on the model plant was initially tested and evaluated, which was fundamental for design and transformation the vector containing *CrDAT* gene to periwinkle plants. The *CrDAT* gene was expressed and tested for activities in tobacco plants which do not have CrDAT enzyme activities. Findings of Magnotta et al. shown that while the specific activities of CrDAT in transformed tobacco was at least 10-fold lower than the level of this enzyme activities found in *C. roseus* leaf extract (Magnotta et al., 2007). Makhzoum et al. (2011) used different promoter *CrDAT* (pDAT 812, pDAT 2.3, pDAT 2.3/ CrDAT and pCAMBIA1305.1) to test the effect of *CrDAT* gene activities and select *CrDAT* gene promoter controling levels of specific expression or create recombinant vector pBluescript II SK + (pBSIISK +) / CrDAT. This research also demonstrated that the *CrDAT* promoter were identified as having light sensitivity. The other authors have indicated experimentally that the *DAT* gene expression related to the response of light (Vazquez-Flota et al, 1998; St-Pierre et al., 1995; Hernandez-Dominguez et al., 2004). The characterized three TGACG motifs involved in MJ signaling in the first 1.8 kb of the *DAT* promoter (Wang et al., 2010), while the in silico analysis of the *DAT* promoter region also identified numerous motifs known to be light responsive elements. Many other motifs has been known to be involved in the acclimation and response to biotic and abiotic stresses. These factors participated in the regulation of many processes such as synthesis of storage proteins, regulation of the light response of carbohydrate metabolism genes, several defense mechanisms, and gibberellin and

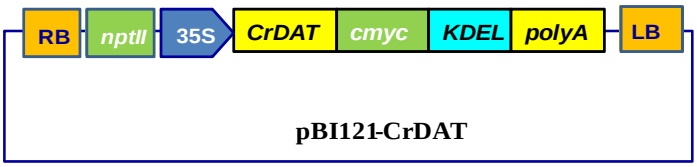


Fig 1. Construction of 35S-DAT-cmyc *vec*tor used for *Agrobacterium*-mediated transformation. RB: right T-DNA border; *nptII*: kanamycin resistance; 35S: 35S promoter (Cauliflower mosaic virus 35S promoter); *CrDAT*: *C. roseus deacetylindoline 4-O-acetyl transferase* gene isolated from the periwinkle plants; cmyc: nucleotide sequence encoding cmyc peptide; KDEL: nucleotide sequence encoding the KDEL peptide; LB: left T-DNA border.

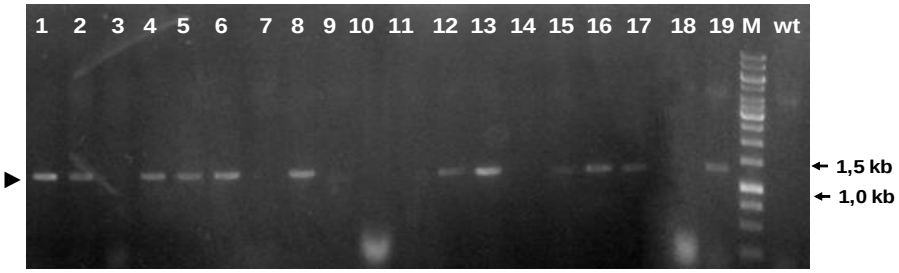


Fig 2. PCR amplification of *CrDAT* in the nineteen transgenic tobacco lines. M: DNA marker 1kb; wt: the wild type tobacco plant; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19: transgenic tobacco lines. There are twelve lines (1, 2, 4, 5, 6, 8, 12, 13, 15, 16, 17, 19) shown the typical pattern of the single-stranded DNA components with approximate size of 1.3 kb. The seven lines (at 3, 7, 9, 10, 11, 14, 18 electrophoresis lanes) were negative for PCR reactions.

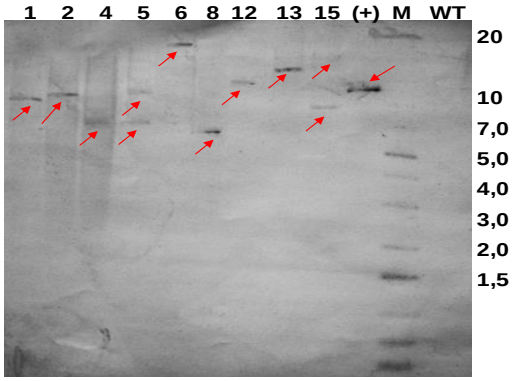
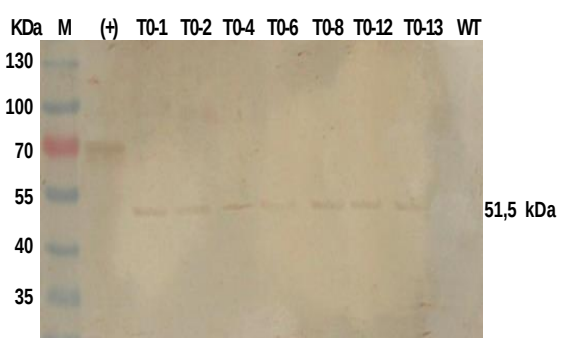
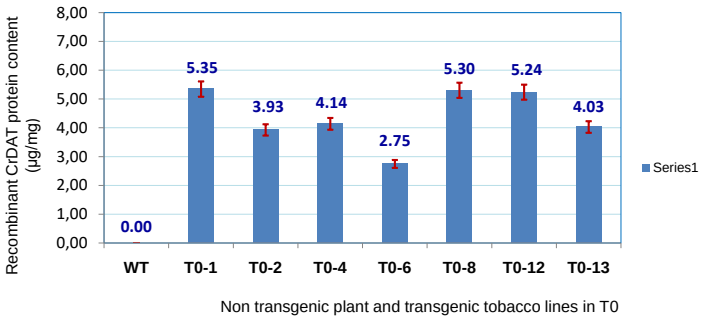


Fig 3. The result of Southern blot hybridization of the nine transgenic tobacco lines. M: DNA marker 1 kb; (+): Plasmid pBI121-CrDAT was cut by *Sac*I; The lanes 1, 2, 4, 5, 6, 8, 12, 13 and 15 are the transgenic tobacco plants T0-1, T0-2, T0-4, T0-5, T0-6, T0-8, T0-12, T0-13 and T0-15 respectively; WT: the wild-type tobacco plants (non transgenic plant).



A



B

Fig 4. Western blot and ELISA analysis of lines expressing CrDAT protein. A- Western blot analysis of seven transgenic tobacco lines. M: Protein marker ; (+): positive control; T0-1, T0-2, T0-4, T0-6, T0-8, T0-12, T0-13: transgenic tobacco lines; WT: non transgenic plant.

B- Comparison of recombinant CrDAT protein production (µg. mg^{-1} total protein) in WT and the transgenic tobacco lines. WT: non-transgenic plants and T0-1, T0-2, T0-4, T0-6, T0-8, T0-12, T0-13: transgenic tobacco lines. Error bars represent the standard

deviations.

auxin responses (Yanagisawa et al, 1999; Plesch et al., 2001). In our study, the 35S promoter was used to control *CrDAT* gene expression in tobacco K326, and the recombinant CrDAT protein with approximate 51.5 kDa in size was completely examined by Western blot. Previous studies of Magnotta et al. (2007) have shown that the DAT coding region was expressed in a hairy root system, albeit under the control of a constitutive (35S) promoter (Magnotta et al., 2007). Therefore, it was clear that the coding region of *CrDAT* gene, in conjunction with elements in the 5′ upstream region, played a role in its cell or tissue specific expression. However, *cis* and *trans* factors had specific roles in the *CrDAT* gene expression under the influence of different factors need to be analyzed to clarify.

Materials and methods

Plant materials

35S-DAT-cmyc transgenic structure involved *CrDAT* gene (cDNA) was used for transformation into tobacco plants (Bui et al., 2015), *Nicotiana tabacum* L. (K326 cutivar) was provided by the Plant Cell Biotechnology Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology as wild type.

Transferring the 35S-DAT-cmyc construct into the tobacco

Agrobacterium-mediated transformation via leaf infection and regeneration of tobacco plants was carried out as previously described by Topping (1998). Total DNA was isolated from young leaves based on the method of Shaghai-Maroof et al. (1984).

Analysis of transgenic tobacco plants by PCR and Southern blot

PCR analysis was used to confirm the presence of the introduced *CrDAT* gene in regenerated tobacco plants. The *CrDAT* gene primer sequences: *CrDAT-F-XbaI:* 5′ GCTCTAGATGGAGTCAGGAAAAATATCGGTTG-3′ (forward primer) and *CrDAT-R-SacI*: 5′ CGAGCTCTTAATTAGAAA-CAAATTGAAGTAGCTG-3′ (reverse primer).

The DNA from PCR positive transgenic plants was subjected to Southern blot analysis (Southern, 1975). DNA genome of the transgenic plants was cut by *BamH*I and SacI enzymes and electrophoresis 1% agarose gel. Mutated the DNA on the gel with an alkaline solution (NaOH 0.5 M; 1.5 M NaCl), and then transferred the DNA from the gel onto a nitrocellulose membrane using absorbent hybrid transmission Southern. The CrDAT gene segments by PCR cloning (to create probes) were marked with Biotin Labeling Kit DNA DecaLabel (Thermo Scientific) using biotin-11-dUTP and conducted in accordance with the manufacturer's instructions. Hybrid membranes were put into plastic trays containing pre-hybrid solution (6xSSC, 5xDenhardt's buffer, 0.5% BSA, 50% Deion formamide) and incubated for 2 to 4 hours at 42 ^{o}C on a shaker, and then replace this solution with the hybridization solution added salmon sperm DNA. The probe was already marked, hybridized overnight at 42 ^{o}C by gentle shaking. Washed 2 times in solution 2x SSC + 0.1% SDS for 10 min at room temperature, and washed again 2 times in solution 0.1x SSC + 0.1% SDS for 30 min at 60 °C. Discard the liquid, dry filter paper. The experiments for determining the presence of *CrDAT* transgene were conducted using chromogenic detection Biotin Kit according to the manufacturer's instructions (Thermo Scientific).

Western blot method and ELISA analysis

To extract total protein, 0.5 g of transgenic tobacco plant leaves were grinded in liquid nitrogen and dissolved in 1.0 mL of PBS with 0.05 % Tween 20 (PBS-T), then centrifuged at 13000 g for 15 min. Proteins were denatured and separated by electrophoresis on 10 % SDS-PAGE (Laemmli, 1970), then transferred to nitrocellulose membranes using Pierce G2 Fast Blotter (25 V, 1.3 mA for 20 min). Membranes were then blocked in the blocking solution (5 % skimmed milk in PBS-T) overnight, then incubated with primary antibody (c-myc) for 3 h by shaking at room temperature, followed by three times washing with PBS, then incubated with secondary antibody for 2 h. Mouse monoclonal antibody to c-myc (Santa Cruz Biotech) was diluted in 5% milk in PBS-T at 1:700. For secondary antibody, anti-mouse IgG antibody attached HRP (Horse Radish Peroxidase) was diluted in 5% milk in PBS-T at 1 : 4000. The results were determended by using TMB (3, 3′, 5, 5′ - tetramethyl benzidine) or DAB (3, 3′ - diamino benzidinetetra hydrochloride).

Enzyme-Linked Immunosorbent Assay method

The content of recombinant CrDAT protein was determined by the Enzyme-Linked Immunosorbent Assay (ELISA) method (Sun et al., 2006). Recombinant CrDAT (rCrDAT) was determined using anti-cmyc antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase and TMB solution. rCrDAT tagged cmyc can be read on a spectrophotometer at 630 nm wavelength. ScFv protein, positive control, was diluted with coating buffer to construct a standard curve. The recombinant CrDAT protein content is calculated by $\mu g.\ mg^{-1}$ total protein.

Conclusion

The 35S-DAT-cmyc structure was successfully transferred to the tobacco plant by *Agrobacterium*- mediated transformation and generated transgenic tobacco lines. Southern blot assays confirmed that CrDAT gene was completely introduced into tobacco genome. The recombinant CrDAT protein of 51.5 kDa in size was successfully expressed at seven transgenic tobacco lines. The recombinant CrDAT protein content of transgenic tobacco lines were 2.75 to 5.35 ($\mu g.\ mg^{-1}$ of total protein) range and the recombinant CrDAT protein content of T0-1 line was highest (5.35 $\mu g.mg^{-1}$ of total protein). The analysis results of expression of the recombinant CrDAT protein in tobacco plants provided the basis for the study on overexpression of *CrDAT* gene in *C. roseus* plants.

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