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Expression of tryptophan decarboxylase in chloroplasts of transgenic tobacco plants

Qiu-Rong Li[2,3], Stefano Di Fiore[3], Rainer Fischer[3], and Miao Wang[1,*]

[1]*Institute of Applied Ecology, The Chinese Academy of Sciences, Shenyang 110016, The People's Republic of China*

[2]*General Hospital in Nanjing Millitary District, Nanjing 210002, The People's Republic of China*

[3]*Botanical Institute of Rheinisch-Westfalisch Technische Hochschule Aachen, D-52074 Aachen, Germany*

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Abstract. Tryptophan decarboxylase (TDC) is a key enzyme in the biosynthesis of terpenoid indole alkaloids (TIAs) from plants such as *Catharanthus roseus*. We transformed tobacco plants with *tdc* gene and expressed chloroplast-targeted versions of TDC protein. TDC was shown to be over-expressed in chloroplasts of tobacco plants by Western blot analysis and TDC enzymatic assay. The effective expression of TDC lead to accumulation of tryptamine in transgenic tobacco plants. T_1 plants showed higher levels of TDC expression than T_0 plants. In order to confirm that TDC was correctly targeted and expressed in chloroplasts, they were isolated from T_1 transgenic tobacco plants, and the different fractions of purified chloroplasts were analyzed. Transgenic plants displayed the phenotype characteristic of necrotic syndromes, which was related to the accumulation of tryptamine.

Keywords: Chloroplast; *Nicotiana tabacum*; Terpenoid indole alkaloids; Tryptophan decarboxylase.

Introduction

Tryptophan decarboxylase (TDC, EC 4.1.1.28) is the key enzyme in the early step of the TIA biosynthetic pathway and links primary metabolism to secondary metabolism by converting tryptophan into tryptamine. Tryptamine is subsequently condensed with secologanin, resulting in strictosidine, the precursor to pharmaceutically important alkaloids such as vinblastine and vincristine (Hashimoto and Yamada, 1994). *Catharanthus roseus* (Madagascar periwinkle, the family of Apocynaceae) produces these important alkaloids (Meijer et al., 1993). Vinblastine and vincristine are mainly extracted from *C. roseus*, but there are only trace amount of the alkaloids. Cell cultures of *C. roseus* had been considered to be sources of medicinally important TIAs, but they suffered from low productivity (Pasquali et al., 1992; Roewer et al., 1992; Moreno et al., 1995). TDC enzymatic activity is one of the bottlenecks in the TIA biosynthetic pathway. A cDNA clone encoding TDC was first isolated from *C. roseus* (De Luca et al., 1989). A high accumulation level of tryptamine was observed by expressing TDC in transgenic tobacco plants (Songstad et al., 1990). However, there was no report found in targeting and expressing recombinant TDC enzymes in subcellular compartments to study TIA biosynthesis. TDC is a cytosolic soluble enzyme in TIA-producing plants such as *C. roseus*. Tobacco is an ideal host plant, and endogenous TDC activity is not detected in it because it does not contain *tdc* gene in its genome or the enzymes of downstream to metabolize tryptamine, so the biosynthesis of tryptamine in the leaves of transgenic tobacco plants expressing targeted TDC was used as a direct evidence of in vivo TDC function in the chloroplasts. The chloroplast represented an important subcellular compartment because the chloroplast is the site of L-Tryptophan biosynthesis. In this paper, we manipulated the reaction catalyzed by TDC to increase the level of tryptamine through engineering the TIA biosynthetic pathway in plants. TDC was expressed in the chloroplasts of transgenic tobacco plants to study the effect of targeting TDC to chloroplast on the in vivo functionality in tobacco. The special phenotypes of transgenic plants expressing recombinant TDC in the chloroplast were observed.

*Corresponding author. Tel: 0086-024-23051460; Fax: 0086-024-23843313; E-mail: wangmiaojue@163.com

Materials and Methods

Plant: *Nicotiana tabacum* L. cv. Petite Havana SR1.

Bacteria: *Agrobacterium tumefaciens* GV3101 (pMP90RK, Gm^R, Km^R, Rif^R) (Koncz and Schell, 1986).

Manipulation of DNA was performed by standard techniques (Sambrook et al., 1986). *Tdc* was amplified by PCR using the pTSK plasmid DNA (Leech et al., 1998) with the sense primer 5′-CGCGAGCTCCATGGGCAGCATTGATTCAAC-3′ and the antisense primer 5′-CCCAAGCTTGTCGACGGCTTCTTTGAGCAAATCATC-3′. These primers were designed to amplify *tdc* downstream to the 5′ untranslated region (UTR) and upstream to the stop codon of the cDNA sequence (Genebank accession number M25151) in the pTSK plasmid DNA. 5′ *Sac*I and *Nco*I

and 3′ *Sal*I as well as *Hind*III restriction sites were introduced respectively by the primers to allow cloning of the amplified *tdc* DNA. The antisense primer was also designed to delete the stop codon at the 3′ end of the *tdc* sequence.

T*dc* was cloned as a SacI/HindIII fragment into pUC18 (Messing, 1983) and subcloned as an NcoI/SalI fragment into a pGEM (Pharmacia, Freiburg, Germany) derivative to target the chloroplast. TDC expression cassette targeting chloroplast was summarized in Figure 1. TDC targeted-chloroplast was generated by cloning *tdc* as a *Nco*I/*Sal*I fragment between the 5′ cDNA sequence of the potato granule-bound starch synthase chloroplast-targeting signal and the 3′-*c-myc*/His6 tags (Spiegel et al., 1999). The *c-myc* was cloned at the C-terminus to facilitate the detection of expression of recombinant TDC. Epitope was recognized by the 9E10 anti *c-myc* monoclonal antibody (Evan et al., 1985). The construct was subcloned as an *Eco*RI/*Xba*I fragment into the pSS plant expression vector (Voss et al., 1995) between 35S double enhanced promoter of cauliflower mosaic virus (CaMV) and terminator sequence of CaMV.

The TDC plant expression cassette was transformed into electrocompetent *Agrobacterium tumefaciens* GV3101 cells according to the standard technique (Horsch et al., 1985). Recombinant TDC agrobacteria were selected on YEB-agar plates with rifampicin, kanamycin, and carbenicillin. The colony was checked by PCR. Tobacco leaf discs were transformed with recombinant agrobacteria. Tobacco plants were regenerated from transgenic calli, transferred to soil pots, and grown in greenhouse for ten weeks. Fully expanded leaves were collected for Western blot analysis and tryptamine accumulation. The plants were self-pollinated and allowed to set seed. Seeds from selected T_0 transgenic plants were used to generate T_1 plants, and the chloroplasts were isolated from T_1 plants to confirm TDC correct targeting.

The crude extracts of leaves were prepared by homogenizing fresh tobacco leaves in liquid nitrogen with extraction buffer consisting of 100 mM NaPi (pH 7.5), 2 mM EDTA, 4 mM DTT and 5% (mg/ml) polivinilpolipyrrolidone in a 1:1.5 w/v ratio. Tissue debris was removed by centrifugation at 15,682 *g* at 4°C for 20 min. The supernatant as the leaf crude extract was used for further analysis.

The polyacrylamide concentration was 10% in SDS gels and transferred into nitrocellulose membranes. TDC protein bands were detected by the 9E10 anti *c-myc* antibody. The GAM^{H+L-AP} conjugated antibody was used as the secondary antibody. The blotted membranes were developed in the one-step solution (Nitro Blue Tetrazolium/BCIP) and scanned into computer.

Tryptamine is the product of the TDC-mediated decarboxylation of tryptophan. The assay of TDC enzymatic activity is based on the direct fluorometry of tryptamine. The analysis of tryptamine accumulation in crude extracts of transgenic tobacco leaves was a quantitative assay to evaluate in vivo TDC enzyme activity. Tryptamine accumulated in vivo in leaves of transgenic plants was detected according to the following method (Sangwan et al., 1998). The leaves were ground in liquid nitrogen and extracted with the buffer as described above. Aliquots (10-100 μL) of leaf crude extracts were mixed with tryptamine assay buffer (100 mM Tris-HCl, 5 mM β-mercaptoethanol, 10% glycerol pH 8.0) up to 1 mL. Two mL of 4 mM NaOH were used to stop the reaction. Five mL of ethyl acetate were added to the solution, vortexed for 30 s, and centrifuged at 262 *g* for 2 min. The organic phase was subjected to fluorometric analysis using an Aminco Bowman AB2 luminescence spectrometer (Spectronic Instruments, Rochester, NY). Tryptamine was detected at 280 nm excitation and 340 nm emission wavelengths and measured in triplicate. For each sample, fluorescence intensity and integrated values of the tryptamine emission scan were recorded in triplicate against a blank of extract buffer in tryptamine assay buffer and against a negative control from the crude extract of wild-type tobacco leaves in tryptamine assay buffer. The concentration of tryptamine in the crude extract of transgenic plants, expressed as $\mu g \cdot mg^{-1}$ of total soluble protein (TSP), was extrapolated each time from a calibration curve from standard solutions of tryptamine in the leaf crude extracts of wild type plants.

The chloroplasts were isolated from transgenic tobacco plants using the Percoll step gradient protocol (Orozco et al., 1986). T_1 transgenic tobacco plants were kept in the dark for 24 h prior to leaf harvesting. Fresh young leaves were collected, and mid-veins were removed. Leaves were weighed, cut into small pieces, and homogenized with the ice-cold buffer (50 mM Hepes-KOH pH 7.5, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM sodium ascorbate, 2 mM EDTA, 0.025% w/v BSA) for 3 s set at high speed. The crude homogenate was filtered through four layers of Miracloth and centrifuged at 7,455 *g* for 3 s. The supernatant was discarded, and the crude organelle pellets were resuspended in 2 mL of homogenization buffer and layered

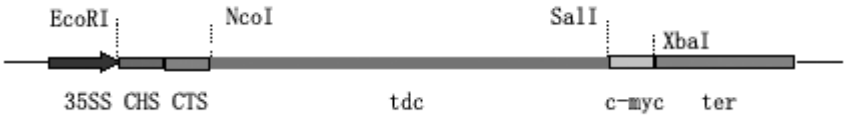


Figure 1. Expression cassette for targeting TDC to chloroplast. 35SS, double enhanced 35S promoter of the cauliflower mosaic virus; ChS, 5′ UTR of chalcone synthase; CTS, chloroplast targeting signal of the potato granule bound starch synthase; c-myc, C-terminal c-myc tag; ter, terminator.

onto the top of 30 and 80% (v/v) Percoll step gradients. The gradients were pulse centrifuged at 7,455 *g* at 4°C. The chloroplast pellet was gently resuspended in 200 µL of buffer (50 mM Hepes-KOH pH 7.5, 0.33 M sorbitol). The integrity of isolated chloroplasts was checked under light microscopy. They were then freeze-thawed and divided in two aliquots, and one of them was centrifuged at 106,000 *g* for 1 h to separate the stroma from the pellets. All the fractions were subjected to western blot analysis and enzymatic assay.

Results

Of 25 independent primary transgenic plants, 15 showed the functional active TDC expression in the leaves of transgenic tobacco plants. Expression of TDC targeted to chloroplast of T_0 and T_1 transgenic tobacco plants was identified by Western blot, and in vivo enzymatic activities were identified by fluorometric assays in leaves. TDC bands were observed by western blot analysis (Figure 2A, B). TDC is a homo-dimeric enzyme, and its monomer has a molecular mass between 49 and 55 kDa. The transgenic T_0 and T_1 tobacco plants showed the bands that migrated with a M_r of 50-52 kDa. The recombinant TDC targeted to chloroplast was effectively expressed by Western blot analysis.

The T_0 and T_1 plants were analyzed for tryptamine accumulation. It was shown in Figure 3 that tryptamine levels in the T_1 plants were higher than in the T_0 transgenic plants. The average level of tryptamine in T_0 transgenic plants was 50.6 ± 8.1 µg mg^{-1} total soluble protein (TSP). They were 99.36 ± 12.2 for plants expressing chloroplast-targeted TDC in T_1 plants. The tryptamine level in T_1 plants was about onefold higher than that in the T_0 plants. Enzyme activities were not detectable in untransformed tobacco plant, demonstrating in vivo functionality of the transgenic encoded TDC enzymes by expressing in chloroplasts.

Chloroplasts were isolated from leaves of two T_1 transgenic plants in which TDC were effectively expressed in order to confirm correct TDC targeting. The different fractions of chloroplasts purified from tobacco plants were subjected to Western blot analysis (Figure 4) to study TDC distribution in chloroplasts. It was shown that TDC mainly accumulated in a fraction of the stroma from chloroplasts in the plants of two lines (A and B). TDC bands were faint in membrane pellets and homogenate in the transgenic plants (line of B). TDC protein was not detected in any fractions of chloroplasts from wild type plants.

All fractions (homogenate, stroma, membrane pellets) of chloroplasts purified from leaves of TDC transgenic plants were subjected to TDC enzymatic assay. The results were shown in Figure 5.

TDC enzymatic activity was high in the stroma and in the homogenate from the leaves of tobacco by expressing TDC targeted to the chloroplast. It was undetectable in the membranes pellets. It was higher in stroma than in the homogenate (line B). TDC enzymatic activity was not

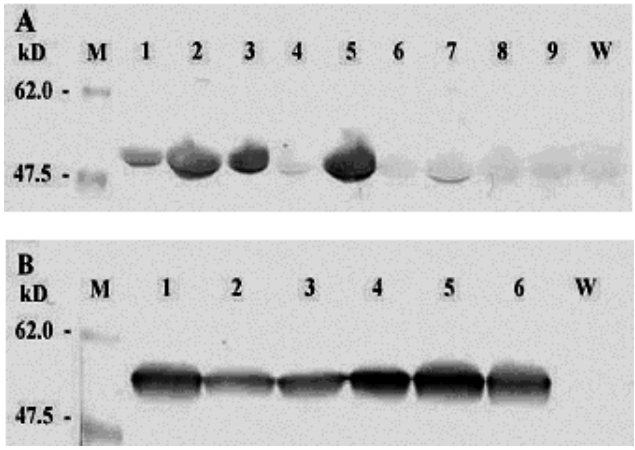
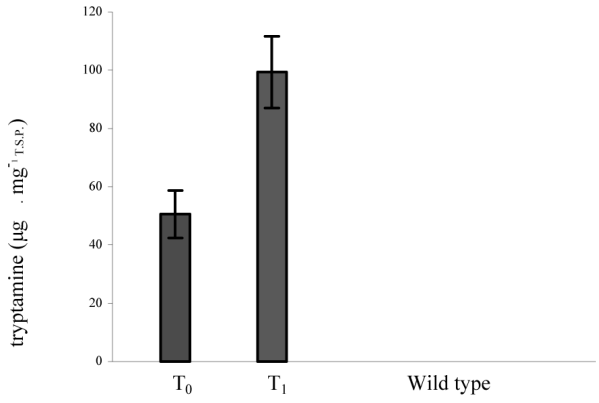


Figure 2. Western blot analysis of the leaf crude extract of transgenic T_0 and T_1 Tobacco plants expressing targeted TDC to the chloroplast (A and B). M: pre-stained protein marker. W: wild type plants. Lane 1-9 (A), leaf crude extract of T_0 plants. Lane 1-6 (B), leaf crude extract of T_1 plants. The 9E10 anti c-*myc* antibody was used to detect the recombinant TDC.



Level of tryptamine in T_0 and T_1 tobacco plants

Figure 3. In vivo function of TDC in the crude extract of T_0 and T_1 tobacco leaves expressing TDC in chloroplast. The amount of tryptamine accumulation level is expressed as µg·mg^{-1} of total soluble protein (T.S.P).

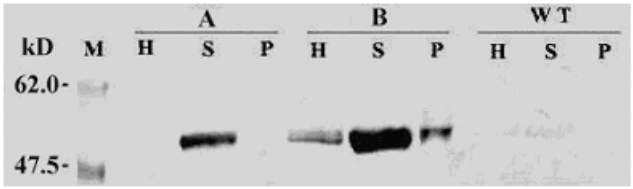


Figure 4. Western blot analysis for different fractions of isolated chloroplasts from T_1 transgenic tobacco plants with TDC targeted to the chloroplasts. The different fractions: homogenate (H), stroma (S) and membranes pellet (P) in line A, line B and wild type. M: pre-stained protein marker. WT: wild type plants. The anti c-*myc* antibody was used for the detection of expression of TDC targeted to chloroplast.

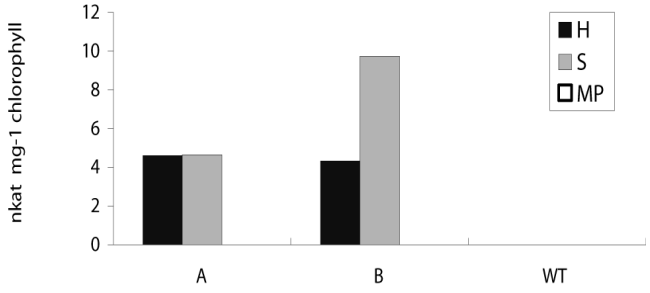


Figure 5. TDC enzymatic assay (nkat mg^{-1} chorophyll) in the different fractions of chloroplast from T_1 transgenic tobacco plant leaves for TDC targeted to chloroplast.

detected in any fractions of the wild type plant. These results demonstrated that recombinant TDC was correctly targeted to the chloroplast and TDC localized in the stroma.

T_0 and T_1 transgenic tobacco plants expressing TDC targeted to the chloroplast exhibited severe abnormalities and showed special symptoms. At first small necrotic areas occurred in the old leaves before the flowers developed. The necrotic areas increased, and younger leaves also had small necrotic areas at the edges as the plant developed. Most of the leaf surface was necrotic, bent, and dry when the plants flowered (Figure 6A-C). They were fully fertile. The transgenic plants, which accumulated high levels of tryptamine, did have such symptoms.

Discussion

A series of genes in the biosynthesis of terpenoid indole alkaloids have been cloned and used in various experiments of the plant. In particular, the genes encoding tryptophan decarboxylase have been expressed in plants and plant cells. In accordance with the results reported by others we found that the expression of *tdc* gene in tobacco plants resulted in the production of tryptamine (Wang et al., 2002). Strategies are employed to ensure gene products get directed to specific cell compartments, allowing a site-specific accumulation (Facchini, 2001). This is very important to understanding TIA biosynthesis at the molecular level and to increasing vinblastine production through engineering TDC in chloroplasts of tobacco plants. In this study transgenic plants were used to further test the hypothesis that TDC can be effectively expressed in chloroplasts of tobacco. The significant accumulation of tryptamine demonstrated that tryptophan was endogenously converted to tryptamine in transgenic plants. Transgenic lines in T_1 plants expressing chloroplast-targeted TDC produced higher levels of tryptamine than T_0 plants, by approximately onefold. Accumulation levels of tryptamine were consistent with the results of Western blot analysis. Accumulation levels of enzymatically active TDC and its product were achieved by targeting TDC to the chloroplast of transgenic plants and genetically altering the accumulation of tryptamine. The chloroplasts were isolated from two T_1 transgenic tobacco plants. All the fractions (homogenate, stroma, membrane pellets) were subjected to Western blot and TDC enzymatic activity, confirming recombinant TDC was correctly targeted in chloroplast and allowing us to evaluate the expression of TDC in chloroplast. The recombinant TDC can function in tobacco plants by being targeted to chloroplasts. Western blot and TDC enzymatic activity analysis of chloroplasts purified from T_1 transgenic plants showed that that the majority of TDC accumulated in the stroma and that TDC was correctly targeted to the chloroplast and was related to the stroma.

TDC expression in chloroplast resulted in severe abnormalities in the plants. Necrotic symptoms were observed in the leaves of TDC transgenic plants. The mechanism creating phenotypic effects on transgenic tobacco plants is unclear. Results from the present study suggest that accumulation tryptamine does influence the phenotypes of transgenic tobacco. The necrotic symptoms were observed only in the transgenic plants, which accumulated high levels of tryptamine. It was shown previously that TDC from *C. roseus* was expressed in tobacco plants, resulting in altered biochemical and physiological phenotypes. Transgenic seedlings also displayed a root-curling phenotype that directly correlated with the depletion of the tryptophan pool (Guillet et al., 2000). TDC catalyzes a rate-limited step in the biosynthesis of indole acetic acid (IAA),

Figure 6. Special symptoms on the leaves of transgenic tobacco plants expressing TDC in the chloroplast. The necrosis spots started on the leaf surface of transgenic plants (A), and the necrotic areas increased as the plant grew (B). The plants with the symptoms still flowered and were fully fertile (C).

one of the major hormones, and increased levels of this hormone could be devastating to plant development (Reinecke and Bandurski, 1990). In contrast, tobacco transformed with 35S:TDC was reported to grow and develop normally in the presence of high levels of tryptamine. Similarly, canola (Chavadej et al., 1994) and potato (Yao et al., 1995) transformed with TDC accumulated high levels of tryptamine without any apparent adverse effects. A connection between TDC activity and an abnormal phenotype in transgenic plants cannot be established from this data. Our explanation for the phenotypes is the movement of subcellular compartments in plants expressing TDC and high levels of accumulated tryptamine. TDC is localized in the cytosol and targeted to express in the chloroplast, so movement of tryptamine into the chloroplast may be one of reasons.

Further biochemical analysis and characterization of the subcellular localization of TDC and subsequent reactions leading to the accumulation of tryptamine will be important goals for understanding TIA biosynthesis pathways.

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色胺酸脫羧酶在經轉殖之煙草植物葉綠體的表達

李秋榮[2] Stefano Di Fiore[3] Rainer Fischer[3] 王 淼[1]

[1]中國科學院瀋陽應用生態研究所

[2]南京軍區總醫院

[3]Botanical Institute of Rheinisch-Westfalisch Technische Hochschule Aachen D-52074 Aachen, Germany

色胺酸脫羧酶（TDC）是萜烯類吲哚生物鹼合成代謝的關鍵酶。我們將 *tdc* 基因轉入煙草植物在葉綠體內標定表達，應用 Western 雜交法和 TDC 酶活測定對其表達進行分析。結果表明，TDC 在轉基因煙草葉綠體內高效功能性表達，產生色胺大量累積，T_1 植物的 TDC 表達水平高於 T_0 植物。分離轉基因植物的葉綠體，通過對其分離不同部分的 TDC 表達分析，進一步確定了 TDC 在煙草植物中葉綠體的正確標定表達。轉基因煙草在生長過程中出現葉片壞死症狀，該特殊表型可能與色胺在植物體內大量累積有關。

關鍵詞：色胺酸脫羧酶；萜烯類吲哚生物鹼；煙草；葉綠體。