Short Communication

Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, is Secreted into the Storage Cavity of the Glandular Trichomes

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Tetrahydrocannabinolic acid (THCA) synthase is the enzyme responsible for the production of tetrahydrocannabinol (THC), the psychoactive component of marijuana (Cannabis sativa L.). We suggest herein that THCA is biosynthesized in the storage cavity of the glandular trichomes based on the following observations. (i) The exclusive expression of THCA synthase was confirmed in the secretory cells of glandular trichomes by reverse transcription–PCR (RT–PCR) analysis. (ii) THCA synthase activity was detected in the storage cavity content. (iii) Transgenic tobacco expressing THCA synthase fused to green fluorescent protein showed fluorescence in the trichome head corresponding to the storage cavity. These results also showed that secretory cells of the glandular trichomes secrete not only metabolites but also biosynthetic enzyme.

Keywords: Biosynthesis — Glandular trichome — Localization — Marijuana — Tetrahydrocannabinol — Tetrahydrocannabinolic acid synthase.

Abbreviations: CBGA, cannabigerolic acid; GFP, green fluorescent protein; THC, tetrahydrocannabinol; THCA, tetrahydrocannabinolic acid; THCAS, tetrahydrocannabinolic acid synthase gene.

Marijuana (Cannabis sativa L.) is an annual herb that has been cultivated and used for thousands of years (Fairbairn 1976). It contains unique secondary metabolites called cannabinoids, a group of terpenophenolics. To date, about 70 cannabinoids have been isolated from marijuana (Mechoulam and Ben-Shabat 1999) including Δ⁹-tetrahydrocannabinol (THC), a well-known psychoactive component. Besides its psychoactivity, THC possesses analgesic, anti-inflammatory, appetite stimulant and anti-emetic properties, making this cannabinoid a very promising drug for therapeutic purposes (Baker et al. 2003, Guzman 2003).

It is known that cannabinoids such as THC are originally generated in acidic forms. In fresh tissues, the concentration of THC is much lower than that of Δ⁹-tetrahydrocannabinolic acid (THCA). THCA is later decarboxylated by a non-enzymatic reaction during storage or smoking (Yamauchi et al. 1967). Despite the long history of marijuana research, the cannabinoid biosynthetic pathway was elucidated only recently. Studies on biosynthetic enzymes over the last decade have revealed the THCA biosynthetic pathway as shown in Fig. 1. Cannabigerolic acid (CBGA), a precursor of THC, is the product of the alkylation of olivetolic acid with geranyl pyrophosphate by an enzyme called geranylpyrophosphate:olivatolate geranyltransferase (Fellermeier and Zenk 1998). Then, CBGA is converted into THCA by a novel enzyme called THCA synthase (Taura et al. 1995a). THCA synthase catalyzes a unique oxidative cyclization of the geranyl group of CBGA. Recently, we have successfully cloned THCA synthase and characterized its structural and functional properties (Sirikantaramas et al. 2004). Biochemical characterization demonstrated that THCA synthase is a flavinylated oxidase that requires molecular oxygen and produces THC and hydrogen peroxide. In addition, we have already constructed transgenic tobacco hairy roots expressing THCA synthase that can produce THC upon feeding of CBGA, suggesting a strategy for the biotechnological production of THC (Sirikantaramas et al. 2004). However, the metabolic engineering of the cannabinoid pathway requires a detailed understanding of the biosynthetic mechanism including regulation and trafficking of the enzymes involved in the pathway. In this study, we describe the cell-specific localization and possible physiological function of THCA synthase, the enzyme responsible for THC production.

With regard to the localization of cannabinoids in C. sativa, it has been reported that THC is accumulated only in the secretory cavity of the glandular trichomes (Fujita et al. 1967, Fairbairn 1972). In addition, we have reported that the leaf bud tissue, which is rich in glandular trichomes, contains a potent THC synthase activity (Taura et al. 1995a). Thus it appears possible that THC is biosynthesized in the glandular tri-
Cannabinoid biosynthesis in glandular trichomes of Cannabis plants. To investigate this possibility, we performed a semi-quantitative reverse transcription–PCR (RT–PCR), using THCA synthase-specific primers, with cDNA samples from the glands and the tissue after gland removal. We used seed coats as a starting material since they provide a large number of glands. As a result, a much larger amount of PCR product, amplified by THCA synthase-specific primers, was detected for the sample from glandular trichomes (Fig. 2). In contrast, only a trace amount of PCR product was detected with the cDNA from gland-removed tissue. The control 18S rRNA gene fragment was amplified equally for both samples. These results suggested that THCA synthase is expressed exclusively in the glandular trichome.

We previously have reported the characteristics of the amino acid sequence of THCA synthase (Sirikantaramas et al. 2004). The sequence contains a 28 amino acid signal peptide and eight putative N-glycosylation sites. In addition, a recombinant THCA synthase from an insect cell culture was secreted into the medium, suggesting the trafficking of THCA synthase from the endoplasmic reticulum to the outside of the cell. However, the destination of the protein in plants might be different from that in insect cells. Therefore, we performed a stable transformation to produce transgenic tobacco BY-2 cells harboring THCAS to confirm whether THCA synthase is secreted from plant cells. It is noted that we used tobacco as a host because it is difficult to transform C. sativa plant stably. Interestingly, enzyme assays with cell extract and culture medium from transformed BY-2 demonstrated that most of the THCA synthase activity (∼95%) was secreted into the culture medium. This result suggested that THCA synthase is a secreted biosynthetic enzyme.

The results of reverse transcription–PCR (RT–PCR) and heterologous expression suggest that THCA synthase is localized in the apoplastic space of the glandular trichome. Thus, we next prepared transgenic tobacco plants expressing a green fluorescent protein (GFP)-tagged THCA synthase (THCA synthase–GFP) to visualize the expression of THCA synthase in the glandular trichome. The control GFP-expressing transgenic tobacco cells showed green fluorescence only in the nucleus and cytoplasm. In contrast, GFP-tagged THCA synthase shows green fluorescence all over the trichome head, demonstrating the localization of the fusion protein in the storage cavity (Fig. 3). We also confirmed THCA synthase activity in the leaf tissues containing glandular trichomes, suggesting that THCA synthase–GFP is functionally expressed. Taken together, a sorting pathway for THCA synthase from...
secretory cells into the storage cavity is suggested. This finding is interesting since the glandular trichome, which is believed to secrete small molecules such as resinous compounds or volatile oils, can also secrete biosynthetic enzyme into the storage cavity.

To obtain further evidence that THCA synthase is sorted into the storage cavity of the glandular trichome, we directly withdrew the contents of the cavity of *C. sativa* using a stretched glass pipette without destroying the secretory cells, and analyzed the enzyme activity. As expected, THCA synthase activity (~0.01 picokatal) was readily detected for the storage cavity content of individual glandular trichomes. In addition, HPLC analysis showed the presence of CBGA, the substrate of the enzyme, and THCA in the same sample. These results indicated that THCA synthase is actually localized to and functions in the storage cavity of glandular trichomes of *Cannabis* plants. Therefore, the storage cavity is not only the site for the accumulation of cannabinoids but also the site for THCA biosynthesis.

The finding that THCA synthase is secreted into the storage cavity raises an interesting question: why must this enzyme be secreted for the biosynthesis of THCA? In general, secondary metabolites are toxic and must be expelled from the cytoplasm after their biosynthesis. For example, anthocyanin is imported into the vacuole by a specific transporter to prevent cellular damage (Goodman et al. 2004). In the case of cannabinoids, it has been reported that THC is toxic and induces the death of mammalian cells in vitro (Guzman et al. 2001). However, there is no report about other cannabinoids. Since THCA is biosynthesized outside the cell, it is possible that acidic cannabinoids, such as THCA, are also cytotoxic. We, therefore, examined the toxicity of CBGA and THCA, in suspension-cultured *C. sativa* cells and tobacco BY-2 cells, and compared it with that of olivetolic acid, a phenolic moiety in cannabinoids. In 10-day-old suspension-cultured cells of *C. sativa*, 24 h treatment with CBGA and THCA at 50 µM caused 100% cell death as demonstrated by trypan blue staining, whereas olivetolic acid did not have any effect on the cells (Fig. 4A). The same result was observed in 7-day-old tobacco BY-2 cells treated with these two cannabinoids and olivetolic acid. Moreover, we confirmed that CBGA and THCA induce cell death...
via apoptosis since DNA laddering was detected by gel analysis of genomic DNA from treated plant cells (Fig. 4B). These results clearly showed that cannabinoids are toxic substances. We also confirmed that both CBGA and THCA induced apoptosis not only in plant cells but also in insect (Spodoptera frugiperda, SP) cells (data not shown), suggesting that cannabinoids act as plant defense compounds, like many secondary metabolites biosynthesized in the glandular trichome. Since cannabinoid-producing glandular trichomes are distributed in physically fragile young tissues of the Cannabis plant, THCA and CBGA would protect these tissues from predators such as insects. This is the first report suggesting the physiological importance of THCA and CBGA as apoptosis-inducing defense compounds.

As previously reported, the THCA synthase reaction produces hydrogen peroxide as well as THCA during the oxidation of CBGA (Sirikantaramas et al. 2004). Since the Cannabis plant produces a very large amount (~2% of dried tissue) of THCA (Shoyama et al. 1975), a toxic amount of hydrogen peroxide might also be accumulated in the storage cavity of the glandular trichome as a result of the THCA synthase reaction. Thus, THCA synthase might contribute to the self-defense of Cannabis plants by producing both THCA and hydrogen peroxide. In addition, since these reaction products are toxic to C. sativa itself, THCA synthase must be secreted from secretory cells into the storage cavity to avoid cellular damage.

Cannabis sativa contains various cannabinoids that share a similar structure to THC. For example, cannabidiolic acid and cannabichromenic acid are isomers of THC having a similar structure to THCA. For example, cannabidiolic acid reduces hydrogen peroxide as well as THCA during the oxidation of CBGA (Sirikantaramas et al. 2004). Since the Cannabis plant produces a very large amount (~2% of dried tissue) of THCA (Shoyama et al. 1975), a toxic amount of hydrogen peroxide might also be accumulated in the storage cavity of the glandular trichome as a result of the THCA synthase reaction. Thus, THCA synthase might contribute to the self-defense of Cannabis plants by producing both THCA and hydrogen peroxide. In addition, since these reaction products are toxic to C. sativa itself, THCA synthase must be secreted from secretory cells into the storage cavity to avoid cellular damage.

THCA synthase activity was measured as follows. The enzyme solution (50 µl) was added to the standard reaction mixture consisting of 200 µM CBGA, 0.1% (w/v) Triton X-100 and 100 mM sodium citrate buffer (pH 5.5) in a total volume of 200 µl. The reaction mixture was incubated at 30°C for 2 h. The reaction product, THCA, was detected by HPLC as described previously (Taura et al. 1995a).

A stretched glass pipette was used to collect the secretory products from individual capitulate-stalked glands from the seed coat of C. sativa. The secretory products were analyzed for cannabinoid content and THCA synthase activity by HPLC (Taura et al. 1995a).

GFP fluorescence in transgenic tobacco was observed under a Nikon TE-2000 microscope (Tokyo, Japan) with a GFP-B filter set (excitation filter, 480 ± 20 nm; barrier filter, 535 ± 50 nm). Images were taken with a Nikon D100 digital camera.

Olivetolic acid was chemically synthesized by carboxylation of olivetol with methyl magnesium carbonate (Mechoulam and Ben-Zvi 1969). CBGA was chemically synthesized by a two-step procedure: condensation of olivetol and geraniol to synthesize cannabigerol (Mechoulam and Yagen 1969), followed by carboxylation of cannabinoid with methyl magnesium carbonate. THCA was purified from dried leaves of C. sativa as described previously (Taura et al. 1995b).

Ten-day-old suspension cultured cells of C. sativa or 7-day-old tobacco BY-2 cells at a 5-fold dilution were incubated with various concentrations of CBGA, THCA or olivetolic acid (5–50 µM) for 24 h. The viability of the cells was tested by addition of trypan blue solution as described previously (de Pinto et al. 1999). DNA was isolated from C. sativa and tobacco BY-2 cells with the CTAB method (Murray and Thompson 1980). Then, electrophoresis was performed in 1.0% (w/v) agarose gels.
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**References**


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