

# Production of Tetrahydrocannabinol by Transgenic Cultured Cells

Futoshi TAURA

*Graduate School of Pharmaceutical Sciences, Kyushu University*

## Research aims

It is known that tetrahydrocannabinol (THC), a major pharmacologically active constituent of marijuana, is generated in its acidic form, tetrahydrocannabinolic acid (THCA), in the fresh tissues of *Cannabis sativa*. I have previously demonstrated that THCA is biosynthesized by a novel enzyme, THCA synthase, which catalyzes stereoselective cyclization of geranyl group of cannabigerolic acid (CBGA) to form THCA (Fig. 1). Since CBGA is easy to be synthesized from commercially available chemicals, and since THCA is easy to be decarboxylated by heating, THCA synthase would open the way to the novel biotechnological production system of THC. In this study, I attempted to establish an efficient expression system of THCA synthase, which is available for the biotechnological production of THC.

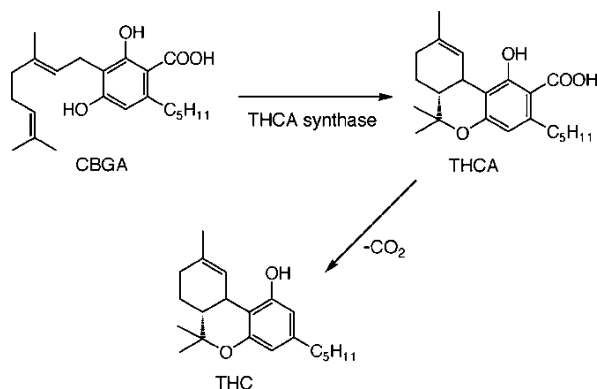
## Methods

**Expression of THCA synthase in a yeast culture**—The entire coding sequence of THCA synthase was subcloned into the expression vector pPICZ-B (Invitrogen) containing alcohol oxidase promoter. The resulting vector was digested with *SacI*, and transformed into a yeast *Pichia pastoris* SMD1168 (Invitrogen). The transformed colonies were selected on minimal plates containing zeocin, and subcultured on the same plates. The transformed *Pichia* harboring THCA synthase gene was inoculated in liquid BMGY complex medium containing glycerol, and cultured for 2 days at 30°C with gentle shaking. Then, the cultured cells were col-

lected by centrifugation, and resuspended in BMMY complex medium containing methanol to induce the expression of the recombinant THCA synthase. The THCA synthase activities in the culture medium and cell extract were measured by HPLC.

**Expression of the recombinant THCA synthase in tobacco hairy roots**—Full-length THCA synthase cDNA was subcloned into the plant expression vector pBI121 (Clontech). The resulting construct was introduced into *Agrobacterium rhizogenes* strain 15834 by tri-parental mating. The tobacco (*Nicotiana tabacum* cv. Xanthi) stems were infected with *A. rhizogenes* using needles. Hairy roots generated from the infected sites were cultured on solidified B5 media containing cefotaxime and kanamycin. For the bioconversion of CBGA into THCA, ~2 cm tips of the root expressing THCA synthase were inoculated in 30 ml of liquid B5 medium in 100-ml flasks and pre-cultured for 2 weeks at 25°C with gentle shaking (90 rpm). Then, 1 mg of CBGA was added to the medium, and the hairy roots were further cultured under the same conditions to produce THCA.

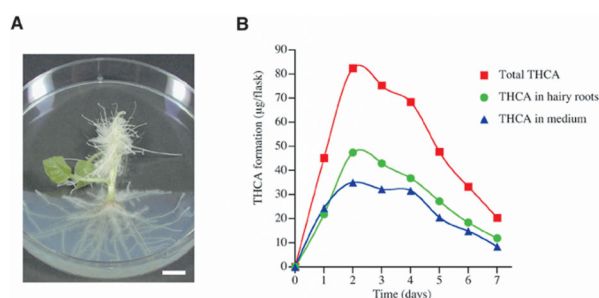
**Expression of the recombinant THCA synthase in tobacco BY-2 culture**—pBI121 expression vector harboring THCA synthase was introduced into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating. The tobacco BY-2 cells were transformed by infection with *Agrobacterium*. Transformed cells were selected on solidified BY-2 media containing cefotaxime and kanamycin. For the bioconversion of CBGA into THCA, transformed BY-2 callus expressing THCA synthase were inoculated in 30 ml of liquid BY-2 medium in 100-ml flasks and pre-cultured for 1 weeks at 25°C with gentle shaking (90 rpm). 1 mg of CBGA was added to the culture flask or the cell-free culture supernatant, and incubated under the same condition to produce THCA.



**Fig. 1.** Scheme of THC formation. THCA synthase catalyzes oxidative cyclization of the monoterpene moiety of CBGA to form THCA. THC is derived from THCA by non-enzymatic decarboxylation.

## Results

**Heterologous expression of THCA synthase in yeast**—We first tried a yeast expression of THCA synthase because the yeast system is easy to construct. Interestingly, enzyme assays demonstrated that the THCA synthase activity in the medium (32 picokatal/l) was much higher than that (1.8 picokatal/l) of the cell extract, indicating that most of the recombinant enzyme was secreted. The secretion level of the recombinant THCA synthase was ~9-fold improved (0.30 nanokatal/l) by adding some supplements such as casamino



**Fig. 2.** Development of THCA-producing tobacco hairy roots. A, induction of hairy roots from tobacco stem infected with *Agrobacterium rhizogenes* harboring THCA synthase cDNA. B, Production of THCA in suspension cultures of transgenic tobacco hairy roots expressing recombinant THCA synthase. The roots were inoculated in 30 ml of liquid Gamborg B5 medium and pre-cultured for two weeks. Then, 1 mg of CBGA was added to the culture, and the accumulation of THCA in the aliquot of hairy roots and medium was measured by HPLC.

acid and proteinase inhibitors, which are known to stabilize the secreted proteins in yeast cultures. The maximum expression level was estimated to be less than 0.2 mg/l culture based on the specific enzyme activity of the purified native THCA synthase (1.9 nanokatal/mg protein). The yeast expression demonstrated that THCA synthase cDNA actually encodes an active enzyme. However, the expression level was unexpectedly low, and should be improved for further investigation.

*Heterologous expression of THCA synthase in tobacco hairy roots*—For the expression in plants, THCA synthase gene was cloned into a pBI121 vector, and introduced into the tobacco genome using *Agrobacterium rhizogenes*. The transformants appeared as rapidly growing hairy roots from tobacco stem infected with *Agrobacterium* because of the co-integration of bacterial root-forming genes (Fig. 2A). I confirmed that the hairy roots possessed THCA synthase activity (1.2 picokatal/mg protein).

I next investigated whether transformed hairy root can produce THCA. When the hairy roots were cultured in liquid B5 medium (30 ml) supplemented with 1 mg of CBGA, the maximum level of THCA (82 µg, 8.2% conversion from CBGA) was produced 2 days after the addition of CBGA (Fig. 2B). Although the hairy roots did not secrete THCA synthase, about half of the THCA produced was found in the culture medium, indicating CBGA uptake and THCA release by tobacco cells. This is the first evidence that THCA synthase can produce THCA not only in *Cannabis sativa* but also in transgenic plant. However, since the conversion rate was not improved by various modifications for culture and reaction conditions, I suspected that the expression system is still insufficient for the cannabinoid production.

*Production of THC by using the recombinant THCA synthase secreted from transgenic BY-2 cells*—To improve the expression level of the recombinant THCA synthase, I used tobacco BY-2 cell as the third expression host because this cell line grows more rapidly than any other plant cell or tissue cultures including hairy roots. Enzyme assays with cell

extract and culture medium from transformed BY-2 liquid cultures demonstrated that most THCA synthase activity (>95%) was secreted into the culture medium as described for the yeast expression. The most active cell line afforded a THCA synthase activity of ~8.7 nanokatal/l culture medium. This enzyme activity suggested the amount of the expressed THCA synthase is more than 4 mg/l as the specific activity of the purified native THCA synthase is ~1.9 nanokatal/mg protein. Thus the expression level was greatly improved by using BY-2 cells as host.

For the production of THCA by the BY-2 culture, I first attempted the bioconversion by feeding of CBGA directly into the cell suspension culture. However, the conversion rate was unexpectedly low (8~10%) in spite of the high expression of THCA synthase. I suspected that, in the culture, metabolic enzymes other than THCA synthase degrade CBGA, because the decrement of CBGA was much faster than THCA production. Therefore, I next removed the cells from the culture by filtration through nylon screen, and added CBGA into the supernatant. As a result, up to 45% conversion into THCA was achieved after 6-hour incubation with the culture supernatant as enzyme. The enzymatically synthesized THCA was quantitatively decarboxylated by heating at 120°C, and resulting THC was readily purified by passing a short silica gel column with benzene as a mobile phase. Thus, in this study, I have established a novel biotechnological production system of THC based on transformed tobacco BY-2 cell cultures.

## Conclusion

In the present study, I have expressed the recombinant THCA synthase in three different heterologous expression systems, a yeast expression system and plant expression systems using different hosts (tobacco hairy roots and BY-2 cells). The yeast and tobacco hairy root systems produced active THCA synthase, however, the expression level of the enzyme was not enough for effective THCA production. On the other hand, the transformed BY-2 culture secreted a large amount (>4 mg/liter) of the enzyme. The supernatant obtained from the culture could efficiently convert CBGA into THCA, which is later decarboxylized into THC. Therefore, I have established here a novel production system of THC based on the tobacco BY-2 cells expressing THCA synthase.

## References

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