Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology

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Abstract: Cannabinoids, consiting of alkylresorcinol and monoterpene groups, are the unique secondary metabolites that are found only in *Cannabis sativa*. Tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabichromene (CBC) are well known cannabinoids and their pharmacological properties have been extensively studied. Recently, biosynthetic pathways of these cannabinoids have been successfully established. Several biosynthetic enzymes including geranylpyrophosphate:olivetolate geranyltransferase, tetrahydrocannabinolic acid (THCA) synthase, cannabidiolic acid (CBDA) synthase and cannabichromenic acid (CBCA) synthase have been purified from young rapidly expanding leaves of *C. sativa*. In addition, molecular cloning, characterization and localization of THCA synthase have been recently reported. THCA and cannabigerolic acid (CBGA), its substrate, were shown to be apoptosis-inducing agents that might play a role in plant defense. Transgenic tobacco hairy roots expressing THCA synthase can produce THCA upon feeding of CBGA. These results open the way for biotechnological production of cannabinoids in the future.

Key Words: *Cannabis sativa*, cannabinoid, tetrahydrocannabinol, tetrahydrocannabinolic acid synthase, biosynthesis, glandular trichome and biotechnology.

1. INTRODUCTION

Cannabis sativa L. (Cannabinaceae), better known as marijuana or hemp, belongs to a group of herbaceous shrubs with 1-2 m in height. Various preparations of *C. sativa* including marihuana, hashish, charas, dagga and bhang, have been estimated to be consumed by 200-300 million people around the world and, therefore, currently represents the most widely used illicit drugs. It is believed that *C. sativa* had been cultivated since 4000 B.C. in Russia. At present, this species has been cultivated widely in the world as a resource of fiber, oil and drug. The seeds are now medicine in Japan as listed in Japanese Pharmacopoeia [1]. The drug was mentioned in the early Hindu and Chinese worked on medicine, and its use had been slowly spread through Persia to the Arabs. A number of historical records on marijuana use suggested that it has several interesting pharmacological activities. History of *C. sativa* as a medicine has been reviewed recently in greater detail elsewhere [2].

Cannabinoids, a group of terpenophenolic compounds, is the unique secondary metabolites that are found only in *C. sativa*. Chemical structures of some cannabinoids are shown in Fig. (**1**). To date, almost 70 natural cannabinoids have been isolated [3]. Among these cannabinoids, Δ^9-tetrahydrocannabinol (THC) is the main pharmacologically active principle in marijuana [4] and produces various psychoactive effects. THC has been used as an anti-vomiting drug in cancer chemotherapy and as an appetite stimulant, especially for AIDS patients [5]. On the other hand, cannabidiol (CBD), the isomer of THC, has no psychotropic effect. However, it possesses a variety of pharmacological activities. CBD reduces aggressive behavior in the L-pyroglutamate-treated rat, spontaneous dyskinesias in the dystonic rat, and turning behavior in the 6-hydroxyldopamine-treated rat caused by apomorphine [6]. Cannabichromene (CBC) and related compounds possess anti-inflammatory, anti-fungal and anti-microbial activities [7]. Therefore, cannabinoids are considered to be promising agents for the treatment of various kinds of diseases.

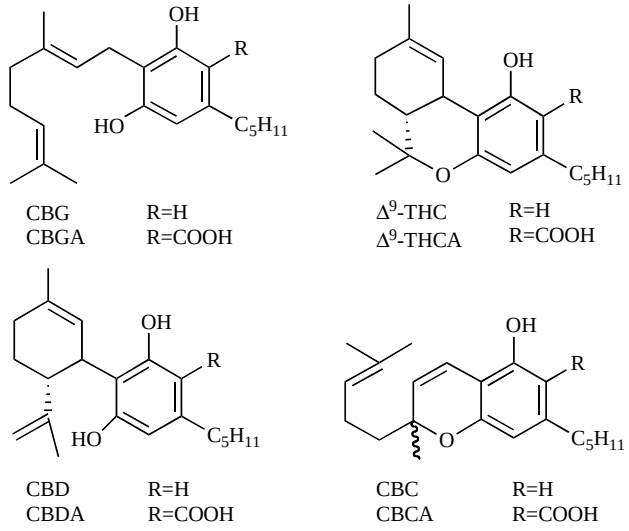

Fig. (1). Major cannabinoids in *C. sativa*.

In the early 1990s, two types of receptors, CB_1 [8] and CB_2 [9] against cannabinoids, and their endogenous ligands

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called endocannabinoids, anandamide [10] and 2-arachidonoyl glycerol [11] have been found in brain and macrophage, respectively. It is becoming evident that the endocannabinoid system is a major actor in the physiology and biochemistry of mammalians. Cannabinoid receptors are coupled to several regulatory pathways, including the signal transduction system of cAMP-protein kinase A [12]. The endocannabinoids play central roles in appetite, feeding and suckling, extinction of memory and protection of numerous physiological pathways. Since THC can bind and activate both receptors similar to those of endocannabinoids, a new field research on *C. sativa* has been developing recently.

In this paper, we review the studies on cannabinoid biosynthetic pathway including molecular cloning, characterization and localization of THCA synthase, which is the first gene in cannabinoid biosynthetic pathway to be cloned. Possible potential for biotechnological production of cannabinoids is also discussed.

2. BIOSYNTHESIS OF CANNABINOIDS

Scientific research on *C. sativa* has been conducted since early 1960s when Gaoni and Mechoulam (1964) first isolated THC [4]. We confirmed that only four cannabinoid acids which are tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabichromenic acid (CBCA) and cannabigerolic acid (CBGA), are major original cannabinoids in the fresh *C. sativa* [13]. Furthermore, we found several artificial pathways of cannabinoids. All cannabinoid acids easily loss carboxylic acid groups by short time heating resulting in their neutral active forms [14]. THCA is further oxidized under the existence of oxygen to give cannabinolic acid [13]. CBCA is cyclized to produce cannabicyclolic acid by irradiation [15]. Despite the long history of *C. sativa* research, the cannabinoid biosynthetic pathway was elucidated only recently (Fig. (**2**)). In this section, we review a number of studies in cannabinoid biosynthetic pathway.

2.1 Biosynthetic Pathway Leading to Cannabigerolic Acid (CBGA)

In plant terpene biosynthesis, two independent pathways, the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways, have been reported. Fellermeier *et al*. (2001) conducted ^{13}C-glucose incorporation experiment to demonstrate that the terpenoid moiety of cannabinoids is biosynthesized predominantly through plastidial methylerythritol phosphate pathway [16]. In addition, polyketide pathway is predicted to be responsible for the biosynthesis of olivetolic acid (OLA) [16]. It is proposed that OLA is formed by aldol condensation of one molecule of hexanoyl-CoA and three molecules of malonyl-CoA [17]. Raharjo *et al*. (2004) performed homology-based PCR cloning strategy to identify a novel polyketide synthase catalyzing the biosynthesis of OLA [18]. However, when expressed heterologously in bacteria, the protein showed chalcone synthase and chalcone synthase-like activities instead of the desired activ-

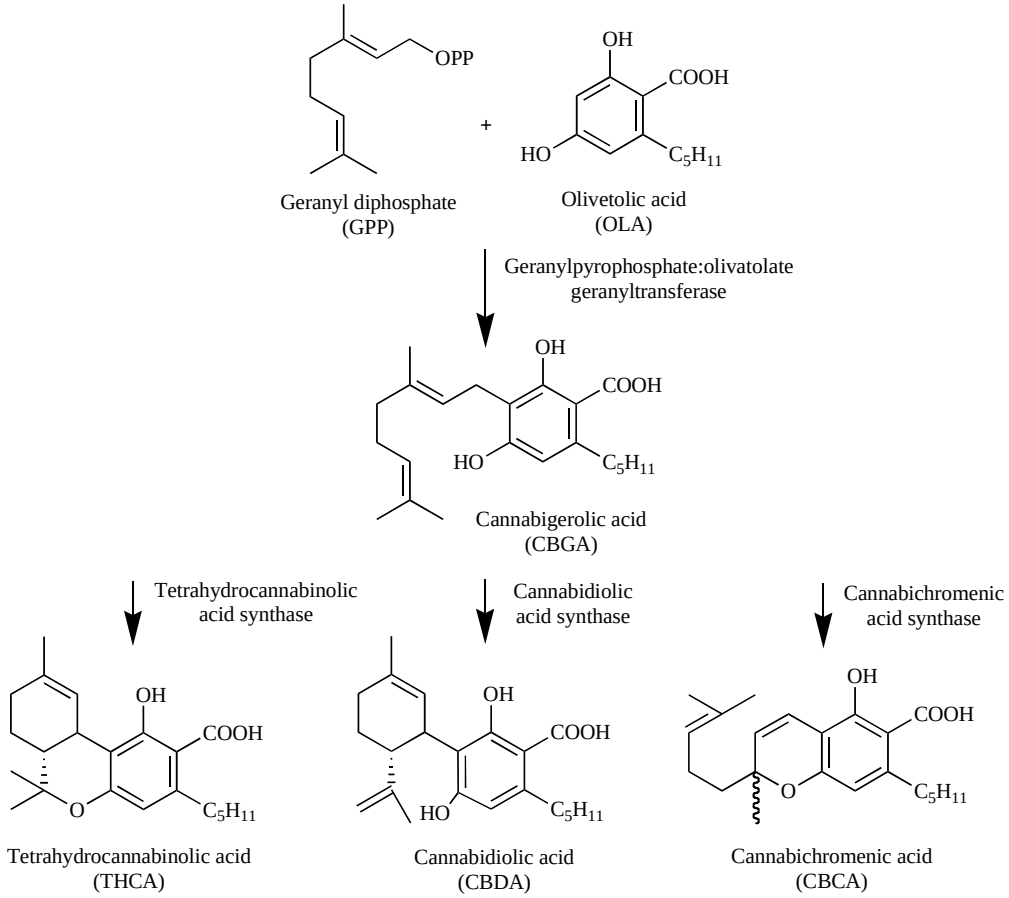

Fig. (2). Biosynthetic pathway of cannabinoids.

ity. Therefore, biosynthetic mechanism leading to OLA formation has remained elusive.

Fellermeier and Zenk (1998) have purified and characterized a novel enzyme called geranylpyrophosphate:olivetolate geranyltransferase from young rapidly expanding leaves of *C. sativa* [19]. This enzyme catalyzes the alkylation of OLA with geranyl pyrosphate (GPP) leading to the formation of cannabigerolic acid (CBGA), the central precursor of various cannabinoid. In addition, this enzyme accepts not only GPP but also nerylpyrophosphate, the isomer of GPP, as a cosubstrate leading to another cannabinoid, cannabinerolic acid.

2.2 Post-CBGA Biosynthetic Pathway

With the availability of drug type (tetrahydrocannabinolic acid-rich) and fiber type (cannabidiolic acid-rich) of *C. sativa*, we selected the suitable type for purification of three cannabinoid synthases including, tetrahydrocannabinolic acid (THCA) synthase [20], cannabidiolic acid (CBDA) synthase [21] and cannabichromic acid (CBCA) synthase [22, 23] which convert CBGA to THCA, CBDA and CBCA, respectively. These cannabinoid acids are accumulated in fresh *C. sativa* and non-enzymatically decarboxylated into their active neutral forms, THC, CBD and CBC, respectively, during storage or smoking [24,25]. Recently, our group have reported the molecular cloning, characterization and localization of THCA synthase [26,27]. This is the first gene involved in cannabinoid biosynthesis to be cloned.

2.2.1 THCA Synthase

cDNA Cloning and Characterization

THCA synthase oxidatively cyclizes the monoterpene moiety of CBGA to form THCA. This reaction is similar to that catalyzed by mototerpene cylcases in monoterpene biosynthesis. Monoterpene cyclases cyclize GPP into various monoterpenes [28], but these reactions are not accompanied by oxidation, contrary to the THCA synthase reaction. Recently, molecular cloning and characterization of THCA synthase have been performed by our group to reveal the mechanism of the THCA synthase [26]. Using degenerate primers based on the partial amino acid sequences of the purified THCA synthase for PCR reaction [17], cDNA encoding THCA synthase (*THCAS*) was successfully cloned.

THCAS consisted of a 1635-nucleotide open reading frame encoding 545 amino acid residues. PSORT analysis of the deduced amino acid sequence suggested a signal peptide sequence in its first 28 amino acids, being consistent with the result obtained by the N-terminal sequencing of the native enzyme. These results indicate that the mature THCA synthase consists of 517 amino acid residues. However, the theoretical molecular weight (58,597 Da) calculated from the amino acid sequencing of mature enzyme is clearly lower than that of purified native enzyme (~75 kDa) on SDS-polyacrylamide gel electrophoresis (PAGE). It is easily suggested that THCA synthase is modified by glycosylation because the mature THCA synthase possesses eight possible Asn-glycosylation sites.

We found a very important finding that THCA synthase is not related to monoterpene cyclases [29-35] although having a geranyl moiety in CBGA molecule, but had high homology with berberine bridge enzyme (BBE) characterized by Dittrich and Kutchan [36]. It is interesting that both enzymes that share high homology are involved in totally different biosynthetic pathways. BBE, a covalently flavinylated enzyme, catalyzes oxidative cyclization of (S)-reticuline to (S)-scoulerine found in benzylisoquinoline alkaloid biosynthetic pathway [37]. This finding suggests THCA synthase might be a flavoenzyme. In addition, motif analysis of THCA synthase revealed a sequence of Arg-Ser-Gly-Gly-His, which is the flavin-binding site in flavoproteins [38,39]. To characterize THCA synthase, we overexpressed the recombinant enzyme using a baculovirus-insect cell system. After insect cells were infected with baculovirus harboring *THCAS*, the higher THCA synthase activity was observed in the culture medium than cell extract indicating that most of the recombinant enzymes were secreted to outside the cells. The N-terminal amino acid sequence of the recombinant enzyme was identical to that of the native enzyme, indicating that the first 28 amino acid signal peptide was correctly cleaved in insect cells. The recombinant THCA synthase exhibited yellow coloring and autofluorescence by irradiation at 366 nm on SDS-PAGE, suggesting THCA synthase is a flavoprotein. In addition, several analyses have been performed to demonstrate that THCA synthase contained covalently attached FAD cofactor with a molar ratio of FAD to protein of 1:1. Site-directed mutagenesis demonstrated the binding site of FAD to be His-114 [26].

The structural similarity between THCA synthase and BBE suggests that the oxidative cyclization of the substrate by THCA synthase proceeds through a mechanism similar to the BBE reaction [39]. We confirmed that THCA synthase requires molecular oxygen and succeeded the determination of hydrogen peroxide release and THCA production at the molar ratio of 1:1. Finally, we concluded that THCA synthase oxidizes CBGA *via* a mechanism similar to that of BBE and that the reaction mechanism was proposed as shown in Fig. (**3**). The reaction is initiated by the hydride transfer from the C-3 position of CBGA to the reactive N-5 position of the isoalloxazine ring of FAD [40]. This step produces a reduced flavin and an ionic intermediate, the configuration of which is competent for cyclization. The next step is the stereospecific and electrophilic ring closure to form THCA, and the hydride ion is transferred from the reduced flavin to molecular oxygen, resulting in hydrogen peroxide formation and re-oxidation of the flavin.

Since THCA synthase showed no homology to other proteins that crystal structures have been determined, we performed the crystallization and preliminary X-ray characterization of THCA synthase [41]. Further studies would provide an insight into structure-function relationship of the enzyme active site.

Localization of THCA Synthase and Possible Physiological Function of THCA

It is well known that cannabinoids are accumulated only in the secretory cavity of the glandular trichomes [42,43]. We have confirmed that the young leaf tissue having rich glandular trichomes possess strong THCA synthase activity [20]. From this finding it is easily suggested that THCA might be biosynthesized in the glandular trichomes of *C. sativa*. Using semi-quantitative RT-PCR analysis, we

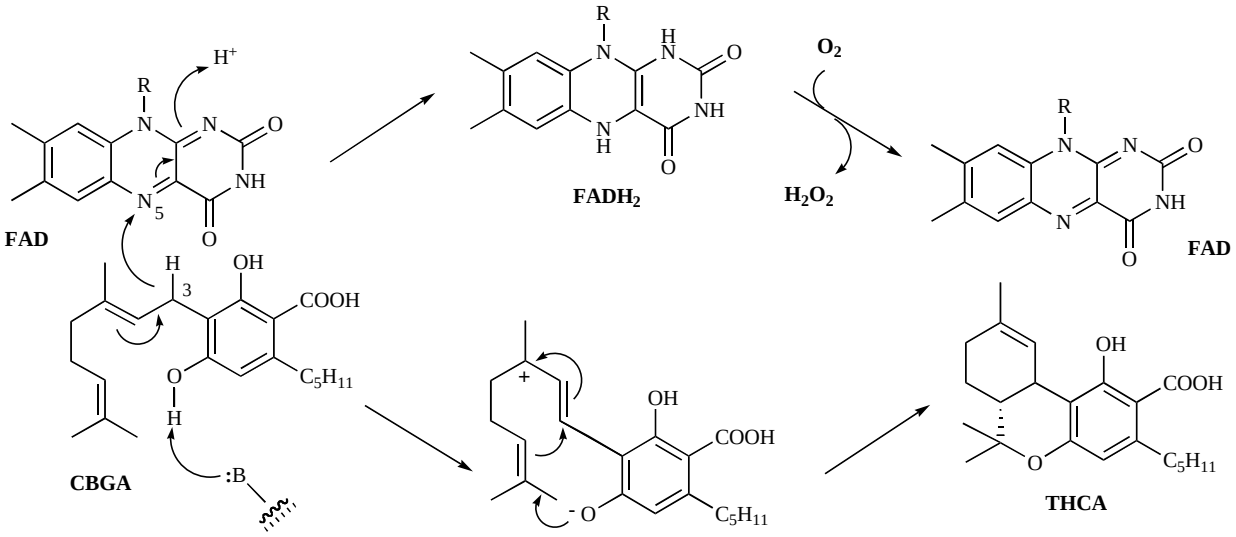

Fig. (3). Proposed reaction mechanism of THCA synthase.

Two electrons from the substrate are accepted by enzyme-bound FAD and then transferred to molecular oxygen to re-oxidized FAD. THCA is synthesized from the ionic intermediate *via* stereoselective cyclization by the enzyme. *R*, the rest of FAD molecule. *B*, the proposed basic residue of the enzyme.

showed that THCA synthase is exclusively expressed in the secretory cells of glandular trichomes [27]. Transgenic tobacco expressing THCA synthase fused to green fluorescent protein showed fluorescence in the trichome head corresponding to the storage cavity (Fig. (**4**)) [27]. These results showed that secretory cells of the glandular trichomes secrete not only metabolites but also biosynthetic enzyme and storage cavity is not only the site for the accumulation of cannabinoids but also the site for THCA biosynthesis. To our knowledge, THCA synthase is the first biosynthetic enzyme that is sorted into the secretory cavity.

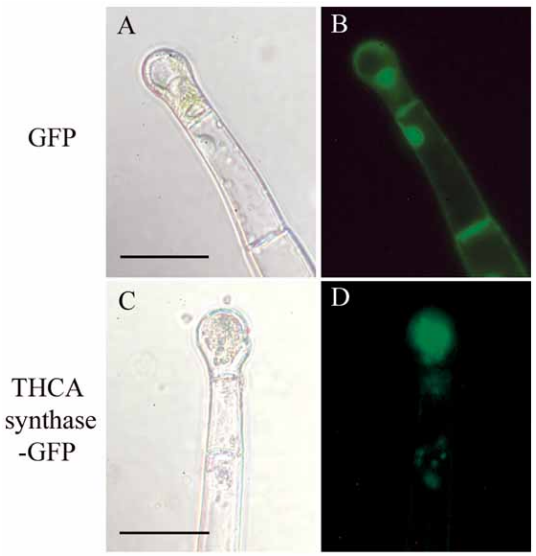

Fig. (4). Localization of THCA synthase-GFP in storage cavity of glandular trichomes of transgenic tobacco plant.

The expression of control GFP visualized under phase contrast microscopy (A) or fluorescence microscopy (B). The expression of THCA synthase-GFP visualized under phase contrast microscopy (C) or fluorescence microscopy (D). Bars = 0.05 mm.

Regarding the physiological function of cannabinoids, it has been reported that THC is toxic and induces the death of mammalian cells *in vitro* [44]. However, there is no report about other cannabinoids. We showed that cannabinoids, CBGA and THCA, are toxic substances and act as apoptosis-inducing defense compounds [27].

C. sativa contains various cannabinoids that share a similar structure to THCA. For example, CBDA and CBCA are isomers of THCA having different ring structures. We have reported that these cannabinoids are biosynthesized from the common substrate CBGA, *via* a mechanism similar to that of the THCA synthase reaction [21,22]. Therefore, these cannabinoids might also be biosynthesized in the storage cavity of the glandular trichome, and involved in self-defense in the *C. sativa*. It is of interest why *C. sativa* produces such a variety of cannabinoids and whether these cannabinoids have different physiological functions. Evolution of cannabinoid synthase genes leading to production of various cannabinoids is an intriguing aspect.

2.2.2 CBDA Synthase and CBCA Synthase

CBDA synthase was purified from the fiber type that contains CBDA as the main constituent [21]. This enzyme catalyzes oxidative cyclization of CBGA to CBDA in a similar manner as the reaction of THCA synthase. It has been suggested that CBDA synthase stereoselectively synthesizes (-)-CBDA based on CD analysis. However, molecular cloning and characterization of this enzyme have not yet been reported. Thus, detailed mechanism has remained unresolved.

CBCA synthase was also purified from the fiber type [22]. CBCA is one of the major constituents in both drug type and fiber type. It is believed that CBCA is an optically inactive compound for long time since they have no optical rotation [45]. However, when CBGA was catalyzed by purified CBCA synthase, a clear CD cotton effect appeared [23]. From this finding, it is easily suggested that CBCA might be

produced by the optically unequal cyclization. When compared three cannabinoid synthases, only CBCA synthase produce an optically active enantiomers [23]. We suggested that the difference in enzymatic behaviors of those three cannabinoid synthases might be due to the products that CBDA and THCA have two and three asymmetric centers, respectively, in their molecules, however, CBCA has only one. Further investigation is required to confirm this suggestion.

3. FUTURE PROSPECTS: BIOTECHNOLOGY AND DRUG DEVELOPMENT

The recent cloning of the gene encoding THCA synthase promises to open opportunity for biotechnological production of cannabinoid [26]. Our group has previously established transgenic tobacco hairy roots producing THCA synthase that can produce THCA upon feeding of CBGA (Fig. (**5**)) [26]. Then, THCA can readily decarboxylized to THC by heating [14]. Development based on this system would lead to the biotechnological production of THC since CBGA is easy to synthesize [46,47]. Further molecular studies on cannabinoid biosynthesis are necessary to develop a production system without feeding of any precursors. So far, THCA synthase is the only one in the pathway to be cloned and characterized. As previously reported, homology-based cloning strategy does not seem to be effective for cloning of polyketide synthase [18]. Recently, metabolomic and proteomic studies in *C. sativa* have already reported by the Verpoorte group [48,49]. The studies on integration of metabolomics and transciptomics have already proven to be powerful tools to discover unknown genes involved in biosynthesis of secondary metabolites [50-52]. These studies would open the way leading to novel gene identification in the cannabinoid biosynthetic pathway.

Genetic engineering in *C. sativa* has been impeded by plant transformation and regeneration. Feeney and Punja (2003) have successfully performed genetic transformation of *C. sativa* suspension culture by *Agrobacterium tumefaciens* [53]. However, it has been known that suspension culture does not produce cannabinoids such as THCA and CBDA. Moreover, these cannabinoids are toxic to *C. sativa* itself making genetic engineering in suspension culture difficult. Transgenic *C. sativa* construction would be one of the main goals for metabolic engineering, However, to our knowledge, regeneration of *C. sativa* is very difficult to perform and has not been reported yet except somatic embryogenesis from callus [54]. Thus, it would be very interesting to perform biomimetic production of cannabinoids in suitable heterologous plants. We have shown that cannabinoids are very toxic and must be produced in a specific organ such as glandular trichomes. Organ-specific gene expression must be considered for engineering the pathway.

In many countries, including Japan, possession and cultivation of *C. sativa* are criminal offences. The accuracy of identification of drug material in seized samples is crucial. Previously, the determination method of a type of *C. sativa* by using anti-THCA monoclonal antibody (MAb) was developed in our laboratory [55,56]. Since the MAb possesses wide cross-reactivity against all cannabinoids, but only limited to cannabinoids, *C. sativa* and its preparations can be distinguished from other plants [57]. Furthermore, all metabolites of THC were detected by this MAb which will be able to use as the first screening for marihuana users [58]. Another typical determination method for *C. sativa* relied on the pollen protein using anti-pollen IgE was reported [59]. This method can specifically detect the pollens of *C. sativa* in a mixture of pollens of other plants. The cloning of *THCAS* opens an additional and reliable methodology for identification the drug type and the fiber type of *C. sativa*. Since there were two variants of the *THCAS* in those types, it is possible to distinguish between the drug type and the fiber type by analyzing the DNA polymorphism of *THCAS*. A specific PCR marker of *THCAS* for identification of drug-type strains has been successfully developed [60, 61].

In order to open a new channel for the field of drug development, we have also been investigating in the pharma-

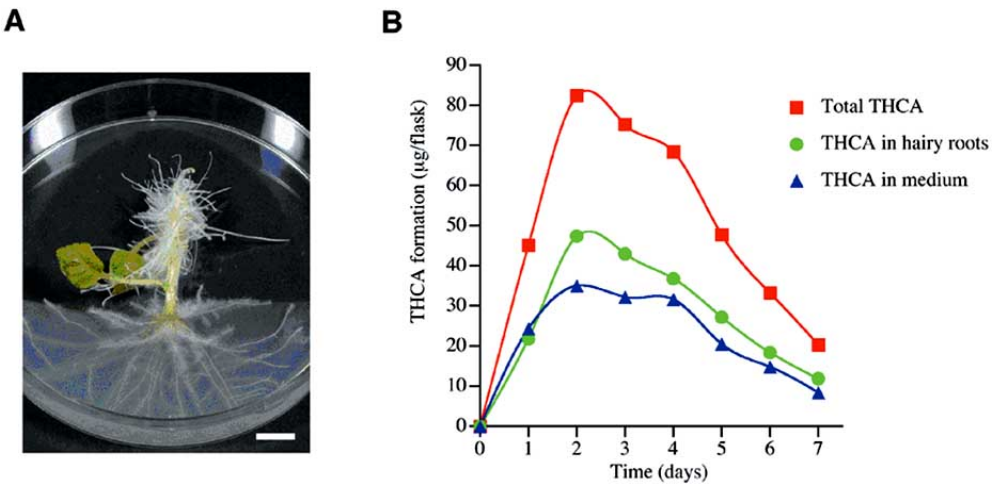

Fig. (5). Development of THCA-producing tobacco hairy roots.

(A) The transgenic tobacco hairy roots expressing *THCAS*. The white bar indicates 1 cm. (B) Production of THCA in the transgenic hairy root. The roots were inoculated in 30 ml of liquid Gamborg B5 medium in 100-ml flasks and pre-cultured for 2 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 [20]. Data are the means of triplicate determinations.

cological field. Egashira *et al*. recently investigated the impairment related to spatial memory [62]. Furthermore, it became evident that the arachidonic acid cascade plays a key role in dependence on and withdrawal from abused drugs such as cannabinoid, opioid and psychostimulants [63]. Therefore, the endocannabinoid system, through possible mediation by the arachidonic acid cascade, serves as a modulator of the reinstating effects of methamphetamine-priming and cues [63]. Extending the current view on the treatment of drug dependence, endocannabinoid-activating substances as well as cyclooxygenase inhibitors may be promising as antirelapse agents [64]. These new pharmacological phenomenon could be combined with the biotechnological applications as discussed earlier. Then, the drug development using cannabinoids might be realized in future.

At present, most research has focused on physiological and pathophysiological roles of the endocannabinoid system. However, exploring the pharmacology of plant cannabinoids is a challenging task. Many problems including comprehensive understanding of biosynthetic enzymes need to be solved in order to make biotechnological application for cannabinoid production possible.

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ABBREVIATIONS

THC	=	Tetrahydrocannabinol
CBD	=	Cannabidiol
CBC	=	Cannabichromene
THCA	=	Tetrahydrocannabinolic acid
CBDA	=	Cannabidiolic acid
CBCA	=	Cannabichromenic acid
CBGA	=	Cannabigerolic acid
OLA	=	Olivetolic acid
GPP	=	Geranyl pyrophosphate
THCAS	=	cDNA encoding THCA synthase
PAGE	=	Polyacrylamide gel electrophoresis
BBE	=	Berberine bridge enzyme
MAb	=	Monoclonal antibody

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