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Original article

Cloning and over-expression of a cDNA encoding a polyketide synthase from *Cannabis sativa*☆

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Abstract

A polyketide synthase has been suggested to play an important role in cannabinoid biosynthesis in *Cannabis sativa* L. This enzyme catalyzes the biosynthesis of olivetolic acid, one of the precursors for cannabinoid biosynthesis. Using a reverse transcriptase-polymerase chain reaction (RT-PCR) based on the DNA homology of chalcone synthase (EC 2.3.1.156) and valerophenone synthase (EC 2.3.1.156) of hop (*Humulus lupulus*), a cDNA encoding a polyketide synthase in *C. sativa* was identified. The coding region of the gene is 1170 bp long encoding a 389 amino acid protein of a predicted 42.7 kDa molecular mass and with a p*I* of 6.04. The gene shares a high homology with a chalcone synthase gene of *H. lupulus*, 85% and 94% homology on the level of DNA and protein, respectively. Over-expression of the construct in *Escherichia coli* M15 resulted in a 45 kDa protein. The protein has chalcone synthase activity as well as valerophenone synthase activity, a chalcone synthase-like activity. Using *n*-hexanoyl-CoA and malonyl-CoA as substrates did not give olivetol or olivetolic acid as a product.

Keywords: Cannabinoids; *Cannabis sativa*; Chalcone synthase; Polyketide synthase; Stilbene synthase

1. Introduction

The biosynthesis of cannabinoids in *Cannabis sativa* is almost fully understood. Cannabinoids such as Δ^9-tetrahydrocannabinolic, cannabidiol, and cannabichromene are well known to be derived from their acid forms [5,9,21]. The acid cannabinoids are formed from cannabigerolic acid. These conversions are catalyzed by tetrahydrocannabinolic acid synthase, cannabidiolic acid synthase, and cannabichromenic acid synthase (Fig. 1). All these enzymes have been characterized [14]. Cannabigerolic acid results from prenylation of olivetolic acid with geranyl diphosphate (GPP). The activity of the prenyltransferase in the *C. sativa* leaves has also been identified [5]. However, the steps leading to olivetolic acid have been poorly studied.

Cannabis sativa is in the same plant as hops (*Humulus lupulus*), the family cannabaceae. The biosynthesis of the main secondary metabolites in hop, the bitter acids such as humulone and cohumulone, and the cannabinoids in cannabis are similar. Both occur in glandular hairs of female flowers. Considering the biosynthetic pathway, an important step of hop bitter acid biosynthesis is a prenylation where phloroisovalerophenone (PIVP) and phloroisobutyrophenone (PIBP) are prenylated by dimethylallyl diphosphate to yield deoxyhumulone and deoxycohumulone respectively [27]. In *C. sativa* prenylation with GPP occurs as an important step. The first step of hop bitter acid biosynthesis is the condensation of one molecule of isovaleryl-CoA or isobutyryl-CoA with three molecules of malonyl-CoA to yield a tetraketide, which then folds to PIVP or PIBP [15,16]. This step is catalyzed by a valerophenone synthase (VPS), a chalcone synthase-like enzyme, belonging to the polyketide synthase (PKS) group. Considering that *C. sativa* and *H. lupulus* are taxonomically very close and the similarity in the

Abbreviations: CHS, chalcone synthase; PIBP, phloroisobutyrophenone; PIVP, phloroisovalerophenone; PKS, polyketide synthase; STCS, stilbene carboxylate synthase; STS, stilbene synthase.

☆ The nucleotide sequence reported in this paper has been submitted to GenBank (www.ncbi.nlm.nih.gov) with accession number AY082343.

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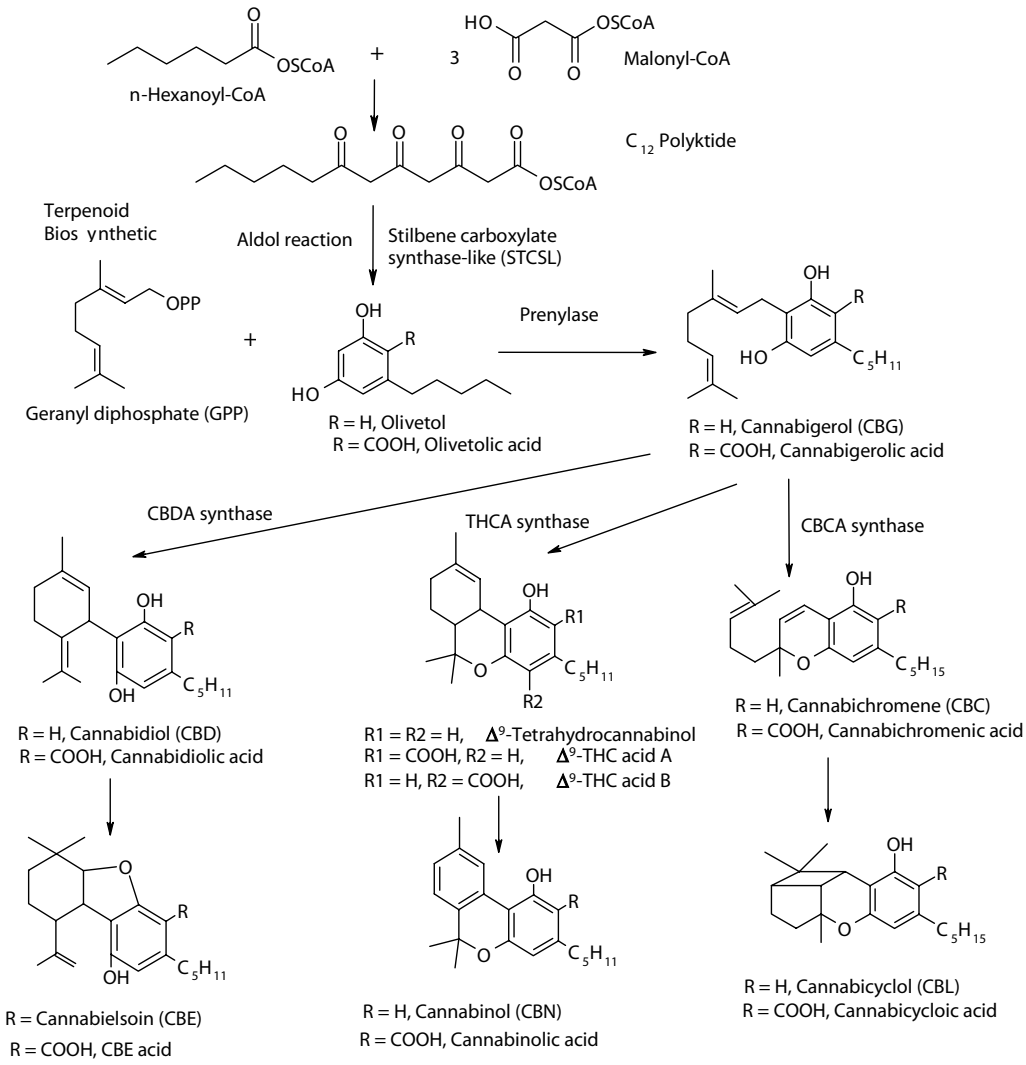

Fig. 1. Biosynthesis of cannabinoids in *Cannabis sativa*. The cannabinoids acids are formed in the biosynthetic process, while corresponding decarboxylation products are formed later by decomposition, e.g. under the influence of heat.

prenylation step, olivetolic acid is also be expected to be synthesized by a PKS.

Polyketide synthases play an important role in the biosynthesis of secondary metabolites such as resveratrol, a candidate for cancer chemoprevention [7] and naringenin, the precursor for flavonoids. This enzyme class includes chalcone synthase (CHS) and stilbene synthase (STS). Both CHS and STS catalyze the reaction between one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA, forming a tetraketide. The CHS cyclization follows a Claisen condensation, while STS cyclization follows an Aldol condensation accompanied by decarboxylation [18]. In some cases STS activity without decarboxylation was found. This enzyme is called stilbenecarboxylate synthase (STCS). Such a STCS has been characterized from *Hydrangea macrophylla* [4]. In *C. sativa*, based on the structure of olivetolic acid, the PKS involved in its biosynthesis should be STCS-like using *n*-hexanoyl-CoA and malonyl-CoA as substrates.

At the DNA level, about a hundred cDNAs and genes encoding CHS (*chs*) have been characterized from 40 plant species, as well as five cDNAs and genes encoding STS (*sts*) from five plant species [18,23]. A phylogenetic tree constructed from 34 CHS and four STS amino acid deduced sequences revealed that the STS formed no separated cluster but grouped with CHS from the same related plants. This fact supports the opinion that *sts* has evolved from *chs* during evolution [23,24]. Therefore, identification of a plant PKS gene (*pks*) can be done by homology-based techniques using the sequences of *pks* genes from closely related species.

In *H. lupulus*, at least four *pks* genes have been identified. Three of them are *chs* genes (*chs1, chs2, chs3*) [12] and one encodes a VPS (*vps*) [15]. In the present study, we cloned and over-expressed a *pks* cDNA of *C. sativa*, using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique based on the sequences of the *chs* and *vps* of *H. lupulus*, the plant most closely related to *C. sativa*.

Over-expression of a protein in *E. coli* as a His-tag protein is an easy way to get pure protein for functional studies. In the case of PKS enzymes, some reports showed that over-expressed PKS in *E. coli* has different specificities from PKS

from the plant, even cross-reactivity, e.g. CHS with both CHS and STS activities, was reported [25]. Therefore, several substrates should be used in activity assays. The highest activity for one substrate may be considered as representing the real activity of the enzyme.

2. Results and discussion

2.1. Sequence of a cDNA encoding a polyketide synthase (PKS)

Based on the fact that the sequences of *sts* genes are closer to *chs* sequences of closely related plants than to other *sts* genes [24], we designed the primers for RT-PCR based on the sequences of *vps* (accession number AB047593) and *chs1* (accession number AJ304877) from hops. We expected several *pks* genes in *C. sativa* that could potentially be amplified. However, only one cDNA fragment of 570 bp was obtained. A homology study using a BLAST program database search showed that the sequence of the cDNA fragment has a high homology of 85% to the *chs* of *H. lupulus*. The cDNA sequence was used as a probe for Southern blot analysis, which showed that at least four *pks* genes are present in *C. sativa* (results not shown).

The RACE-PCR of 5′ and 3′ ends gave ca. 830 bp and ca. 840 bp products, respectively. These fragments were partially sequenced and combined with the sequence of the internal fragment, resulting in a sequence of cDNA containing an open reading frame (ORF) of 1170 bp (Fig. 2). This ORF encodes 389 amino acids polypeptide with a calculated molecular mass of 42.7 kDa and a p*I* of 6.04. The homology of the deduced amino acid sequence of *C. sativa* PKS with other PKSs is shown in Fig. 3. The highest homology is 94% with CHS1 of *H. lupulus*. It is 74% with the VPS from *H. lupulus*. The homology with other enzymes in the STS group is relatively high, e.g. 73% with STS of *Arachis hypogaea* (accession number AB027606) and 74% with STCS of *Hydrangea macrophylla* (accession number AF456445). However, it is possible that the encoded protein is a CHS, because it also shares 88% homology with CHS from *Sinapsis alba* (accession number X14314).

It has been shown by means of site-directed mutagenesis of a CHS of *S. alba* that only one amino acid (Cys164) is essential for the active site [11,23]. Fig. 3 shows that the PKS enzymes of *C. sativa*, CHS of *H. lupulus*, and STS of *A. hypogaea* have a conserved Cys residue in the active site. The VPS of *H. lupulus* has the Cys active site residue at position 166 while CHS of *S. alba* has it at position 169. The sequence of other amino acids in this area is also conserved for both CHS and STS except for the positions –2 and –3 from the Cys. All CHSs contain a Gln-Gln in this position, while the STS of *Pinus sylvestris* and *A. hypogaea* contain Gln-His and His-Gln, respectively [19]. Valerophenone synthase of *H. lupulus* contains Gln-Leu [15]. *Cannabis sativa* PKS contains a Gln-Gln like other CHSs, but surprisingly,

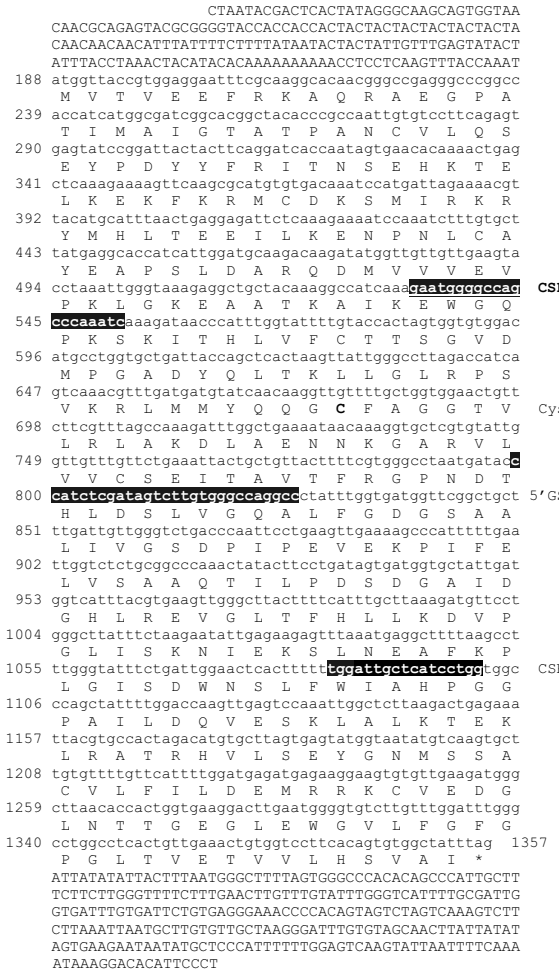

Fig. 2. Nucleotide and deduced amino acid sequence of the full length cDNA of a *Cannabis sativa* polyketide synthase. The marked nucleotides show the position of the primers in the whole sequence. 5′ GSP and 3′ GSP primers are in the same position but with different orientation. The translational stop codon is marked with asterisk (*), Cys 164 might be the active site of the enzyme.

the only published STCS sequence contains a Gln-Gln as well [4]. The other amino acids that are involved in the active site such as His303, Asn336 and Phe215 [8] were found to be conserved at similar positions. Therefore it is difficult to judge whether the *C. sativa* PKS is a CHS, a STS, or a STCS.

2.2. Overexpression of the gene

We over-expressed the obtained cDNA using plasmid pQE30, which contains a histidine tag (His-tag) in front of the multiple cloning site. It allowed the easy purification of the over-expressed protein using Ni-NTA affinity chromatography. This system has been successfully used for over-expression of other PKSs such as VPS of hop [15].

Over-expression of the constructed gene in *E. coli* M15 was determined by a time course study. It was found that 6 h after induction with isopropyl-D-thiogalactopyranoside, the amount of the protein was not increasing significantly further. After purification using a Ni-NTA affinity column, still

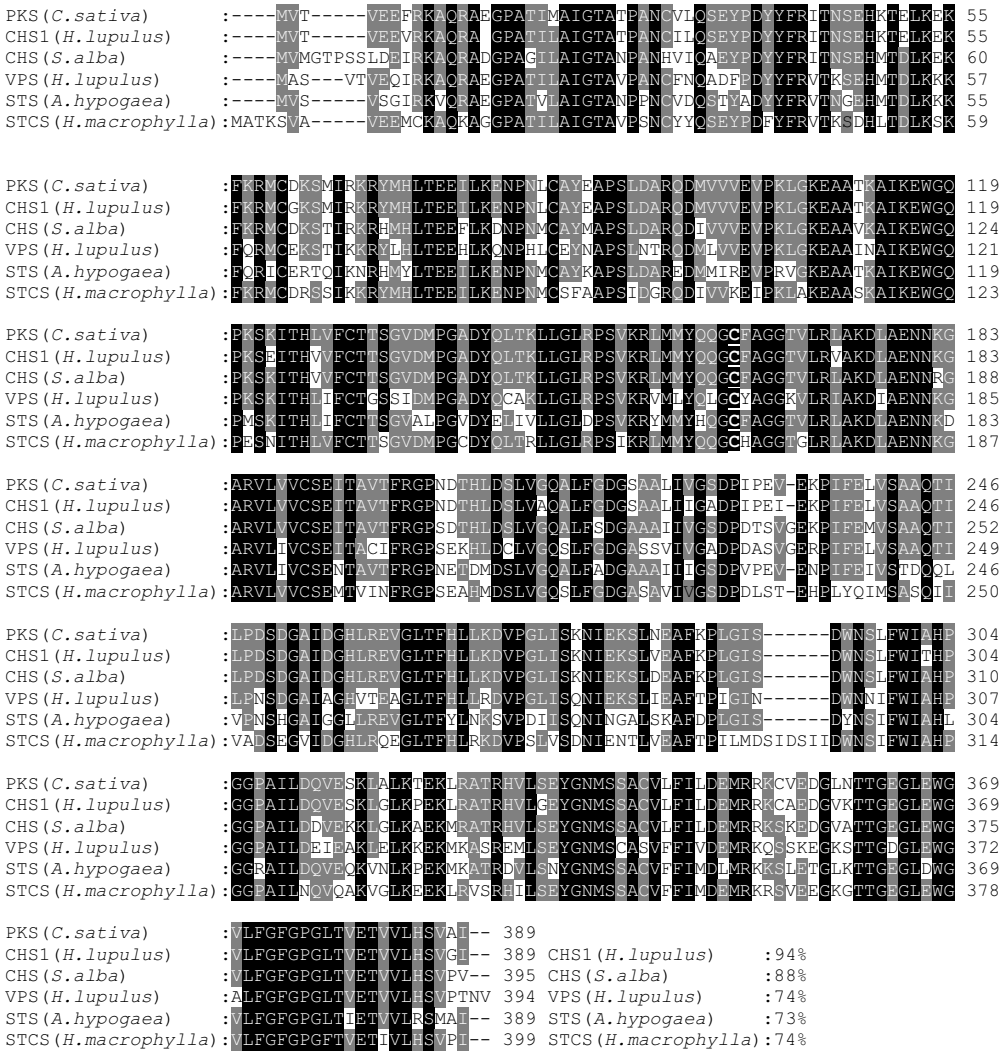

Fig. 3. Comparison of the amino acid sequence of *Cannabis sativa* polyketide synthase and other polyketide synthases: chalcone synthase of *Humulus lupulus*, chalcone synthase of S*inapsis alba*, valerophenone synthase of *Humulus lupulus*, stilbene synthase of *Arachis hypogaea*, and stilbene carboxylate synthase of *Hydrangea macrophylla*. Identical amino acids in the six proteins are marked black. Proteins with four or five identical amino acid are marked grey. The percent numbers show similarity of the polyketide synthases with *Cannabis sativa* polyketide synthase.

more than one band was observed on the SDS-PAGE gel, but one protein band (ca. 45 kDa) was the most prominent (Fig. 4). This size is the same as the size of one subunit of PKS. Using western blot analysis, we confirmed that the band was a PKS. The western blot was performed using antibodies against CHS (*P. silvestris*). This antibody can also detect other PKS proteins due to the high homology of the amino acid sequences (more than 60%) [18].

2.3. Activity assay of the enzyme

Based on the deduced amino acid primary sequence, the cloned gene might encode a CHS, but an activity study was necessary to confirm this. Due to the high homology of PKS genes, still the possibility was present that the gene encodes a STCS-like enzyme. The HPLC assay of CHS is the most suitable way to test the activity [8].

Using *p*-coumaroyl-CoA and malonyl-CoA as substrates, we tested the over-expressed protein for the presence of CHS and STS activity. Naringenin and resveratrol were expected as the products respectively as shown in Fig. 5, naringenin (R_t = 5.9 min) was found as the product, but no resveratrol was detected. This was confirmed by using naringenin and resveratrol (R_t = 4.6 min) reference compounds. The peak of naringenin was not found in the control reactions: enzyme with malonyl-CoA only, enzyme with *p*-coumaroyl-CoA only and a mixture of malonyl-CoA and *p*-coumaroyl-CoA without the enzyme. The large peak (R_t = 8.1 min) present in all reaction mixtures is from the enzyme mixture. Another extra peak (R_t = 6.4 min) is observed when the reaction mixture contains *p*-coumaroyl-CoA. According to the retention time of some reference compounds, this peak is *p*-coumaric acid, which results from the hydrolysis of *p*-coumaroyl-CoA during the incubation.

Cross-reactivity was found when CHS of *Pueraria lobata* and STS of *Arachis hypogaea* were over-expressed in the *E. coli* [13,25]. Over-expressed *C. sativa* PKS in *E. coli* did not have cross-reactivity. Some studies report that PKSs have

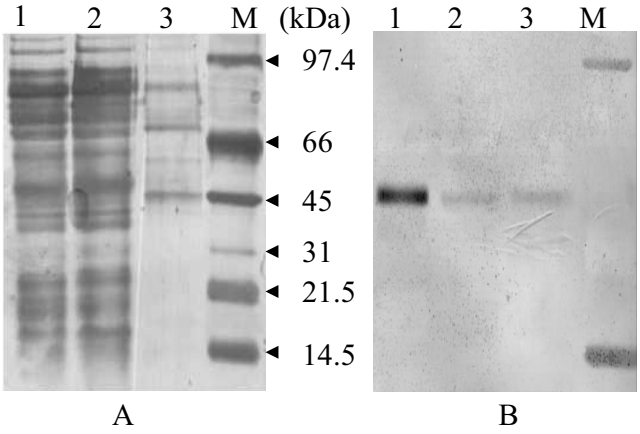

Fig. 4. SDS-PAGE (A) and western blot (B) analysis of over-expressed polyketide synthase. The protein was over-expressed in *Escherichia coli* and analyzed as: crude protein (1) cell lysate supernatant (non-bound protein of Ni-NTA purification step) (2) and pure protein (result of the purification of the crude protein using Ni-NTA column) (3) M is the marker. The two detected markers on panel B were phosphorylase b (rabbit muscle) and lactalbumin (bovine milk).

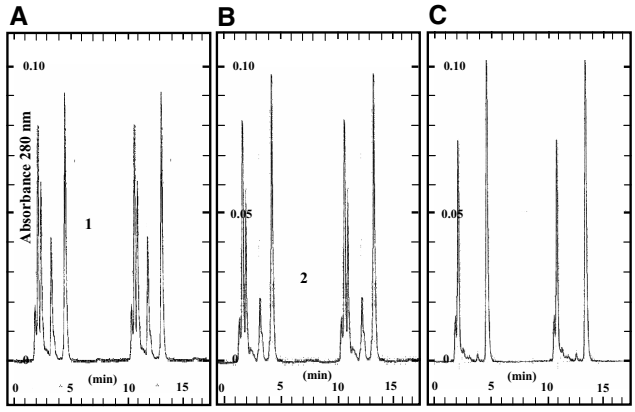

Fig. 5. HPLC profile of a chalcone synthase assay of over-expressed *Cannabis sativa* polyketide synthase. The assay was performed in the presence of both malonyl-CoA and *p*-coumaroyl-CoA (A) in the absence of malonyl-CoA (B) or *p*-coumaroyl-CoA (C). Nar.= Naringenin. Peak 1 (R_t = 5.9 min) is naringenin. Peak 2 (R_t = 6.4 min) is probably *p*-coumaric acid, which is a result from the hydrolysis of *p*-coumaroyl-CoA during the incubation.

a broad substrate specificity [1,20,28]. Isovaleryl-CoA and isobutyryl-CoA, both substrates for VPS in *H. lupulus*, were tested to replace *p*-coumaroyl-CoA. The reaction products of PIVP and PIBP were identified by comparing with reference compounds. This shows that the *C. sativa* PKS also has VPS activity. Surprisingly over-expressed hop CHS in *E. coli* did not give VPS activity [12]. The *C. sativa* PKS has 30×10^{-9} kat g^{-1} specific activity as CHS while VPS specific activity were 15×10^{-9} and 10×10^{-9} kat g^{-1} using isovaleryl-CoA and isobutyryl-CoA, respectively. Testing the *C. sativa* PKS for STCS-like or olivetolic acid synthase activity were done using *n*-hexanoyl-CoA and malonyl-CoA as substrates, but neither olivetolic acid nor olivetol could be detected. The observed product has a UV spectrum similar to the UV spectra of PIVP and PIBP. The presence of this product is probably due to a CHS reaction of *n*-hexanoyl-CoA, and thus might be phlorocaprophenone.

If the enzyme is CHS, its activity might have correlation with the level of flavonoids present in the plant. We determined the level of apigenin and luteolin, two flavonoids present in *C. sativa*, in roots, stems, lower leaves, upper leaves, flowers, seeds, and glands, while at the same times the transcription levels of the gene in those tissues were measured using northern blot analysis. Very low levels of transcription were found in all tissues tested (results not shown), while significant amounts (up to 0.8%) of flavonoids were found in the leaves. It seems that this PKS does not directly connect to flavonoid biosynthesis.

3. Conclusion

We have presented nucleotide sequences of a *pks* cDNA cloned from *C. sativa*. The protein encoded by the open reading frame contains 389 amino acids and belongs to the PKS family. Previously genes encoding PKSs have been cloned and studied from many other plants and it has been reported that they are involved in the biosynthesis of secondary metabolites. However, this is the first time that a *pks* has been cloned and characterized in *C. sativa*. A cDNA encoding a PKS with STCS activity, involved in olivetolic acid biosynthesis, was targeted. However according to sequence analysis, it seems that the cDNA encodes a CHS instead of STCS. When the protein was over-expressed in *E. coli, C. sativa* PKS shows both CHS and CHSL activities. The activity of STS or STCS was not detected. Therefore, this PKS might be a CHS that has a broad specificity of substrate including aliphatic substrates such as isovaleryl-CoA, isobutyryl-CoA, and *n*-hexanoyl-CoA.

4. Methods

4.1. Materials

Seeds of *Cannabis sativa* 'Four-way' (The Sensi Seed Bank, Amsterdam, The Netherlands) were grown in a protected greenhouse under legal permission. Flowers, leaves, stems and roots of female plants were harvested after 14 weeks, while young leaves were harvested 6 weeks after germination. After harvesting the material was frozen in liquid nitrogen and kept at –80 °C until used. The antibody against *P. sylvestris* CHS was generously provided by Prof. J. Schröder (University of Freiburg, Germany). Malonyl-CoA, *n*-hexanoyl-CoA, isovaleryl-CoA, isobutyryl-CoA, naringenin, resveratrol and olivetol were purchased from Sigma (St. Louis, MO, USA). *p*-Coumaroyl-CoA was synthesized according to [22] while PIVP and PIBP were synthesized according to [6].

4.2. Characterisation of the cDNA

Total RNA was isolated from young leaves using a TRIzol kit (Invitrogen Carlsbad, CA, USA) according to the manu-

facturer's instructions. Total RNA was used as a template for RT-PCR using primers: CSF (5′-GAATGGGGYCAG-CCCAAGTC-3′) and CSR (5′-CCACCIGGATGI-GIAATCCA-3′), performed by a SuperScript one-step RT-PCR kit (Invitrogen) to synthesize and amplify a cDNA with a Perkin Elmer DNA Thermal Cycler 48. Reverse transcription was performed at 50 °C for 30 min followed by deactivation of reverse transcriptase at 95 °C for 5 min. The PCR conditions were denaturation for 30 s at 95 °C, 1 min annealing at 50 °C, 1 min extension at 72 °C, 30 times. The final step was extension at 72 °C for 10 min. A 570 bp DNA internal fragment was obtained and directly sequenced on both strands (BaseClear, Leiden, The Netherlands). The 5′ and 3′ end of the cDNA were obtained by RACE-PCR using the primers: 5′ GSP (5′-GGCCTGGCCCACAAGAC-TATCGAGATGG-3′) and 3′ GSP (5′-CCATCTCGATA-GTCTTGTGGGCCAGGCC-3′) and performed by a SMART™ RACE kit (ClonTech, Palo Alto, CA, USA). The first cDNA strand was synthesised at 42 °C for 1.5 h. Amplification for both cDNAs was performed by 30 cycles of PCR in following conditions: 94 °C for 30 s, 68 °C for 1 min and 72 °C for 1 min. Both RACE-PCR products were sequenced (BaseClear). Sequence homology was verified by database searching at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and an open reading frame was predicted with a program from the same site. The deduced amino acid sequence of open reading frame was searched for homology in the SWISS-PROT database and analyzed by the ExpasyBlast program (http://www.expasy.ch). For cloning of the full cDNA, the primers: CHSF (5′- GGGGGGGATCCGTTACCGTGGAGGAATTTCGC-AAG-3′) and CHSR (5′-GGGGGGAAGCTTCTAAAT-AGCC-ACAC-TGTGAAGGACCA-3′) were generated based on the sequence of the ORF. These primers were used for RT-PCR to synthesize and amplify a cDNA in the same conditions as that mentioned before. Reverse transcription was performed at 50 °C for 30 min, followed by deactivation of reverse transcriptase at 95 °C for 5 min. The PCR was performed with 30 cycles of 30 s denaturation at 95 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C, followed by one step of final extension at 72 °C for 10 min. A DNA fragment of ca. 1.2 kb was obtained. The fragment was purified using a MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The DNA fragment and plasmid pQE30 (Qiagen) were cut using *Bam*HI and *Hin*dIII (Isogen Bioscience, Maarssen, The Netherlands) then ligated with each other. The ligation product was used to transform *E. coli* MH1 (own collection).

4.3. Over-expression and purification of the PKS protein

The recombinant plasmid was isolated from transformed *E. coli* and purified using the QIAquick purification kit (Qiagen). The recombinant plasmid containing the *pks* cDNA was used to transform *E. coli* M15 [pREP4] (Qiagen). Transformed *E. coli* M15 were grown on LB agar plates containing ampicillin (100 μg ml^{-1}) and kanamycin (25 μg ml^{-1}). Over-expression, extraction and purification of the protein on Ni-NTA columns were done according to the protocol of the QIAexpressionist (Qiagen) [17]. Extraction was done both in native and denaturing condition. The protein concentration in extracts resulting from these processes was determined according to Bradford [3] using BSA (Sigma) as a standard.

4.4. SDS-PAGE and western blot analysis

SDS-PAGE was performed according to [10]. Approximately 3 μg of protein (crude, supernatant and Ni-NTA-purified) were loaded into the separate wells of a 12% of SDS-PAGE gel and run at 100 V using a Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA). The gels were silver stained according to [2]. Proteins separated by SDS-PAGE were blotted onto an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA). Blotting was performed using a BioRad TransBlot Electrophoresis Cell Apparatus (Bio-Rad) for 1 h at 4 °C at 100 V, according to the manufacturer's instructions. After blotting, the dried membrane was incubated for 1 h in blocking solution consisting of 1% (w/v) BSA in PBST (10 mM NaH_2PO_4, 150 mM NaCl adjusted to pH 7.2, and 0.5% (v/v) Tween-20). The CHS antibody (diluted 1:1000 in PBST, 100 μl cm^{-2}) was incubated with the membrane for 1 h. It was then washed twice for 10 min in PBST then blocking solution. Detection of the protein on the membrane was performed using alkaline phosphatase conjugated anti-rabbit IgG antibody (Promega Corp., Madison, WI, USA) diluted 1:5000 in PBST. After 30 min incubation, the membrane was washed twice for 10 s in PBST solution. It was then exposed to the staining solution until the signal reached the desired contrast. The staining solution consisted of 200 μl NBT/BCIP stock solution, 250 μl $MgCl_2$ 1 M, and 9.3 ml TBS buffer (0.1 M Tris, 0.1 M NaCl, pH 9.5). The NBT/BCIP was 18.75 mg ml^{-1} 5-bromo-4-chloroindoxyl phosphate (BCIP) (Sigma), 9.4 mg ml^{-1} 4-nitro blue tetrazolium (NBT) (Sigma) in DMSO 67% (v/v). All the reactions were performed using 5 ml solution in sealed plastic bags.

4.5. Enzyme assay

Protein activities were tested using both the crude protein and Ni-NTA-purified protein from a native enzyme extraction. Assays were performed using the HPLC assay for CHS [26]. Fifty microgram of the crude protein extract or 10 μg of purified protein extract were mixed with *p*-coumaroyl-CoA (10 nmol) and malonyl-CoA (20 nmol) then added assay buffer (0.5 M potassium phosphate pH 6.8, 2.8 mM 2-mercaptoethanol and 2% (w/v) BSA) until 500 μl. The incubation was carried out at 30 °C for 1 h. At the end of the incubation period, the mixture was extracted using ethyl acetate and analyzed by HPLC. For STCS, a same amount of *n*-hexanoyl-CoA replacing *p*-coumaroyl-CoA, and for the specificity study, isovaleryl-CoA and isobutyryl-CoA were used instead of *p*-coumaroyl CoA.

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