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PKS Activities and Biosynthesis of Cannabinoids and Flavonoids in *Cannabis sativa* L. Plants

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Polyketide synthase (PKS) enzymatic activities were analyzed in crude protein extracts from cannabis plant tissues. Chalcone synthase (CHS, EC 2.3.1.74), stilbene synthase (STS, EC 2.3.1.95), phlorisovalerophenone synthase (VPS, EC 2.3.1.156), isobutyrophenone synthase (BUS) and olivetol synthase activities were detected during the development and growth of glandular trichomes on bracts. Cannabinoid biosynthesis and accumulation take place in these glandular trichomes. In the biosynthesis of the first precursor of cannabinoids, olivetolic acid, a PKS could be involved; however, no activity for an olivetolic acid-forming PKS was detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant, from plant tissues revealed differences in their distribution, suggesting a diverse regulatory control for these biosynthetic fluxes in the plant.

Keywords: Cannabinoids — *Cannabis sativa* — Flavonoids — Glandular trichomes — Olivetol synthase — Polyketide synthase.

Abbreviations: APCI, atmospheric pressure chemical ionization; BAS, benzalacetone synthase; BBS, bibenzyl synthase; BUS, isobutyrophenone synthase; CBCA, cannabichromenic acid; CBD, cannabidiol; CBDA, cannabidiolic acid; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinodiol; CHS, chalcone synthase; Δ^8-THC, Δ^8-tetrahydrocannabinol; Δ^9-THC, Δ^9-tetrahydrocannabinol; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Δ^9-THVA, Δ^9-tetrahydrocannabivarinic acid; DMSO, dimethylsulfoxide; DOXP/MEP, deoxyxylulose phosphate/methyl-erythritol phosphate; DTT, dithiothreitol; GPP, geranyl diphosphate; HEDS/HvCHS, homoeriodictyol/eriodictyol synthase; LC-MS, liquid chromatography–mass spectrometry; PiBP, phlorisobutyrophenone; PiVP, phlorisovalerophenone; PKS, polyketide synthase; PVPP, polyvinylpolypyrrolidone; Rt, retention time; STCS, stilbene carboxylate synthase; STS, stilbene synthase; TFA, trifluoroacetic acid; VPS, phlorisovalerophenone synthase.

Introduction

Cannabis sativa L. is an annual dioecious plant from Central Asia. It has been used since ancient times as a medicinal plant, but also it is the source of hemp fibers and of a vegetable oil used as food. Hemp fibers are produced from *C. sativa* plants that are very low in cannabinoids, the compounds related to the recreational and medicinal uses of this plant. For plant breeding purposes, a zero level of cannabinoids would be an interesting trait. Such plants could be grown for fiber production and for the vegetable oil. Moreover, such plants would be the ideal blank controls in studies of the medicinal value of cannabis. Knocking out the cannabinoid biosynthetic pathway would thus be an interesting option. Cannabinoids are the best known group of natural products in *C. sativa*, and 70 of these have been found so far (ElSohly and Slade 2005). Several therapeutic effects of cannabinoids have been reported (reviewed in Williamson and Evans 2000) and the discovery of an endocannabinoid system in mammalians marks a renewed interest in these compounds (Di Marzo and De Petrocellis 2006, Di Marzo et al. 2007). The cannabinoid biosynthetic pathway has been partially elucidated (Fig. 1). It is known that geranyl diphosphate (GPP) and olivetolic acid are initial precursors, which are derived from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fellermeier et al. 2001) and from the polyketide pathway (Shoyama et al. 1975), respectively. These precursors are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (Fellermeier and Zenk 1998) to yield cannabigerolic acid (CBGA); which is further oxido-cyclized into cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabichromenic acid (CBCA; Morimoto et al. 1999) by the enzymes cannabidiolic acid synthase (Taura et al. 2007), Δ^9-tetrahydrocannabinolic acid synthase (Sirikantaramas et al. 2004) and cannabichromenic acid synthase (Morimoto et al. 1998), respectively. On the other hand, the first step leading to olivetolic acid, an alkylresorcinolic acid, is less known and it has been proposed that a polyketide synthase

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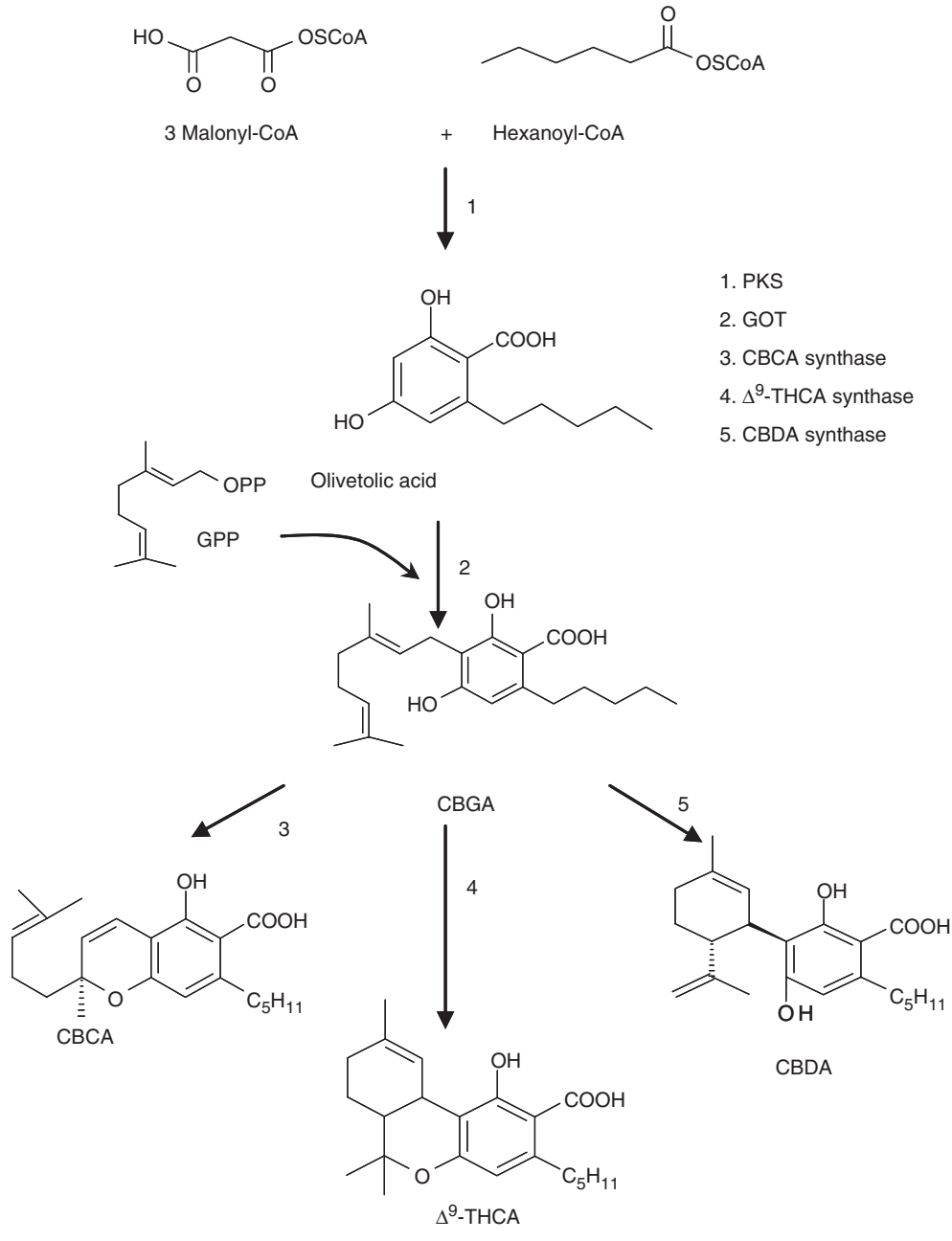

Fig. 1 General pathway for biosynthesis of cannabinoids. PKS, polyketide synthase; GPP, geranyl diphosphate; GOT, geranyl diphosphate:olivetolate geranyltransferase; CBGA, cannabigerolic acid; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; CBDA, cannabidiolic acid; CBCA, cannabicromenic acid.

(PKS) could be involved in its biosynthesis. Raharjo et al. (2004a) found in vitro enzymatic activity for a PKS from leaves and flowers, though yielding olivetol and not olivetolic acid as the reaction product. Olivetolic acid is the active form for the next biosynthetic reaction step of the cannabinoids. Later, a *PKS* mRNA was detected from leaves, which expressed activity for the PKS enzymes chalcone synthase (CHS), phlorisovalerophenone synthase (VPS) and isobutyrophenone synthase (BUS), but not for the formation of olivetolic acid (Raharjo et al. 2004b).

The PKS enzymes are a group of condensing enzymes that catalyze the initial key reactions in the biosynthesis of a myriad of secondary metabolites (Schröder 1997). In plants, several PKS enzymes have been found which participate in the biosynthesis of compounds from secondary metabolism. CHS, stilbene synthase (STS), VPS, BUS,

bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS) and stilbene carboxylate synthase (STCS) are some examples of the type III group of PKS enzymes as they have been classified (Austin and Noel 2003, Eckermann et al. 2003, Klingauf et al. 2005). Type III PKS enzymes use a variety of thioesters of coenzyme A as substrates from aliphatic-CoA to aromatic-CoA, from small (acetyl-CoA) to bulky (*p*-coumaroyl-CoA) or from polar (malonyl-CoA) to non-polar (isovaleryl-CoA). For example, CHS (Kreuzaler and Hahlbrock 1972) and STS (Rupprich and Kindl 1978) condense one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA, forming naringenin-chalcone and resveratrol, respectively. VPS (Paniego et al. 1999) and biphenyl synthase (Liu et al. 2007) use isovaleryl-CoA and benzoyl-CoA, respectively, as starter substrates instead of *p*-coumaroyl-CoA.

The obvious goal for engineering of zero-cannabinoid plants is thus knocking out the first step of the biosynthesis catalyzed by a type III PKS enzyme. Because often several PKS genes are found in plants, we studied the occurrence of different type III PKS enzyme activities in the plant. Here, we report the PKS activities found in different tissues of cannabis plants and show a correlation between the production of polyketide-derived secondary metabolites and the activity of these PKS enzymes in the plant.

Results and Discussion

Activities of PKS enzymes present in plant tissues from Cannabis sativa

For positive control of PKS activity, CHS from *Pinus sylvestris*, STS from *Arachis hypogaea* and VPS from *Humulus lupulus* were used (Supplementary Table S1 online). The activities of these enzymes were similar to those previously reported for STS (58.6 pKat mg^{-1} protein) from peanut cell cultures (Schoppner and Kindl 1984), CHS (30 pKat mg^{-1} protein) from *Phaseolus vulgaris* cell cultures (Whitehead and Dixon 1983) and VPS (35.76 pKat mg^{-1} protein) from hop (Okada et al. 2000), respectively. Negative control assays consisted of a standard reaction mixture adding 50 µl of water as starter and extender substrate. The final pH for CHS and benzalacetone synthase (BAS) assays was 8.0, which is optimum for naringenin (Schröder et al. 1979, Whitehead and Dixon 1983) and benzalacetone (Abe et al. 2001, Abe et al. 2007) formation, while for the rest of the PKS assays it was maintained at 7.0. Due to limited availability of substrates and standards, for detection of STS-type activity in cannabis protein extracts we decided to perform the assay using the starter substrate *p*-coumaroyl-CoA for resveratrol formation as a general indicator from STS activities. For detection of CHS-type activities, the assay was carried out with *p*-coumaroyl-CoA as starter substrate, and naringenin-chalcone formation was an indicator of CHS-type activity. For detection of VPS and BUS activities, the assays were achieved with the starter substrates isovaleryl-CoA and isobutyryl-CoA, respectively.

The protein extracts were from different plant tissues of four varieties of *C. sativa* plants, two drug (Skunk and Fourway) and two fiber (Kompolti and Fasamo) types. For the analysis of the assays of PKS enzyme activities by HPLC, we started with the eluent system reported by Robert et al. (2001), which was slightly modified, as described in Materials and Methods (method 1). Narigenin [retention time (Rt) 33.55 min] and resveratrol (Rt 26.36 min) had a good separation in this solvent system; however, the retention times of olivetol, PiVP and PiBP (Supplementary Table S2) were longer than that of naringenin. Four elution gradients were tested in order to reduce the retention times of these standards, and method 5 was used subsequently for the analysis by HPLC and liquid chromatography–mass spectrometry (LC-MS).

In Fig. 2, the activities found in the different plant parts of four varieties are presented. The products formed from the different incubations were confirmed by means of LC-MS (Supplementary Fig. S1). If we consider the plant parts first, it is clear that each plant part has another spectrum of activity. Olivetol synthase activity is found in leaves and bracts, i.e. correlates with the occurrence of cannabinoids in the plant. In roots it is the CHS activity that is most important. Interestingly, roots also have STS activity, but not olivetol synthase activity, though mechanistically they concern the same reaction. Apparently, there is an enzyme in roots that only accepts coumaroyl-CoA as substrate, whereas in leaves both olivetol synthase and CHS enzyme activities are present. The roots also have STS activity, but not olivetol synthase, again showing a clear preference for the coumaroyl-CoA as substrate for the PKS enzymes present. STS activity is also found in fruits and seedlings, where no olivetol synthase activity is found. BUS and VPS activity show a similar pattern, except for male flowers where high activity is found for VPS, but not for BUS. BUS and VPS are found in the same plant tissues as olivetol synthase, but also in seedlings where no olivetol synthase activity is present. CHS activity is found in all plant tissues.

Comparing leaves for gender, no significant difference was found in PKS activities ($P < 0.05$). In terms of specific activity, olivetol synthase is the highest of all measured activities (in the bracts ~25–40 pKat mg^{-1} protein). BUS activity is generally somewhat higher than the other activities in bracts and leaves. A comparison between fiber-type and drug-type plants does not show any major differences in activities. Apparently, every plant tissue has a specific set of enzymes that is capable of producing different compounds. The bracts and leaves showed all activities,

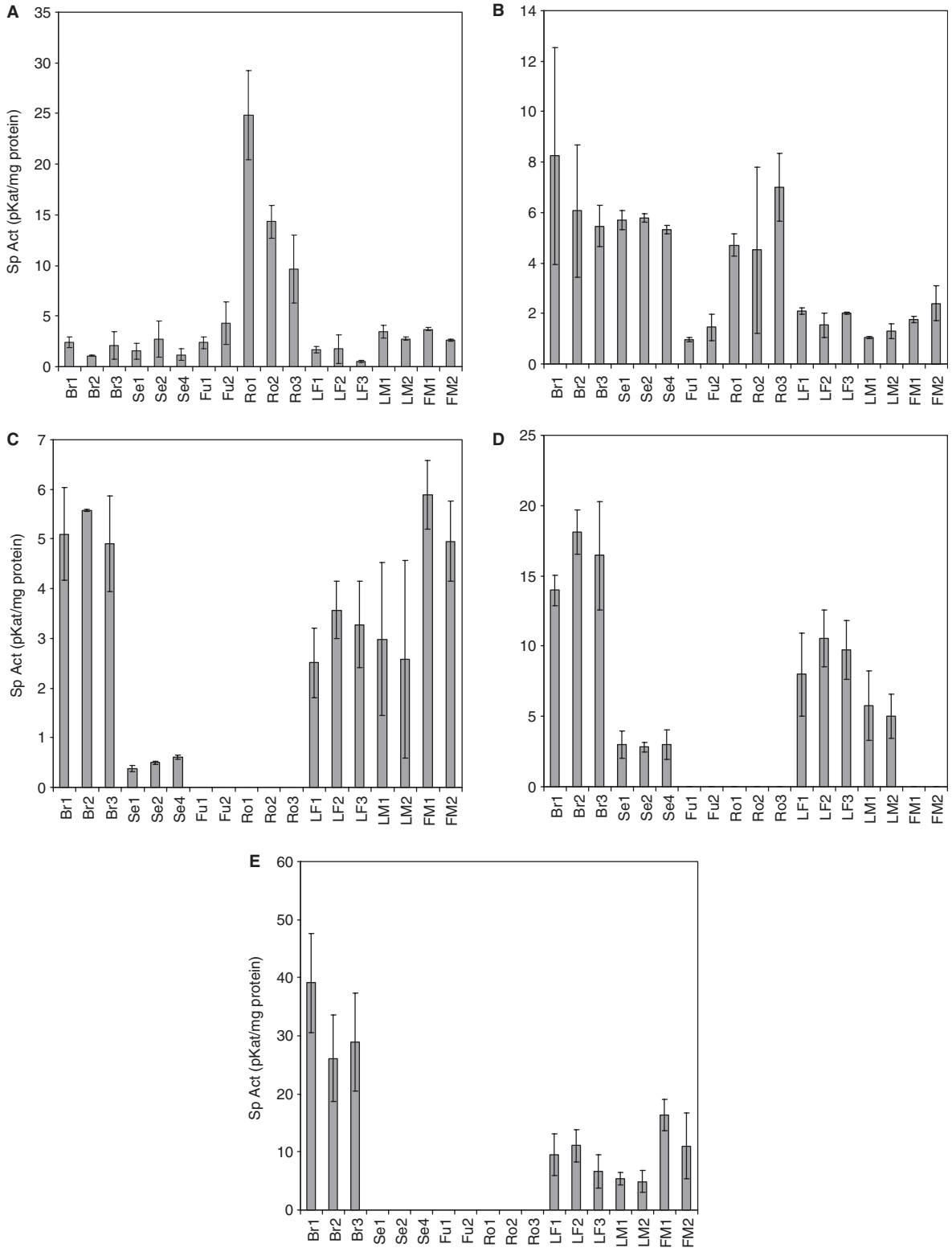

Fig. 2 PKS activities in several crude extracts from different cannabis tissues. (A) CHS; (B) STS; (C) VPS; (D) BUS; (E) olivetol synthase. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety; 4, Fasamo variety. Bracts of 29-day-old flowers. Values are expressed as means of three replicates with standard deviations.

though in different ratios. The compounds found so far in the plant are flavonoids, stilbenoids and cannabinoids (Flores-Sanchez and Verpoorte 2008), but the PKS enzymes present might be capable of producing various other alkyl-substituted phloroglucinol and resorcinol derivatives.

The PKS activities were also followed through the development of the glandular trichomes on the female flowers (Fig. 3). The enzymes showed different patterns: the STS activity was more or less stable, reaching the highest level at days 35 for the 'Skunk and Kompolti' varieties and 37 for the 'Fourway' variety; whereas the other activities showed maximum activity at earlier stages of plant development. In particular, olivetol synthase, BUS and VPS showed the highest activity at 21–29 d. In the case of CHS activity, the peak is not as pronounced (significant, $P < 0.05$). Both varieties (Skunk and Fourway) show similar patterns of the PKS enzyme activities with time, as well as for the variety Kompolti on day 35. No activity for an olivetolic acid-forming PKS was detected during the time course of the growth and development of glandular trichomes on female flowers. However, HPLC and LC-MS analyses confirmed formation of olivetol (Rt 18.21 ± 0.24 min and m/z 181.2 $[M + H]^+$; Supplementary Fig. S1) using hexanoyl-CoA as starter substrate.

Raharjo et al. (2004a) suggested that olivetol was formed by a PKS, and Kozubek and Tyman (1999) proposed that alkylresorcinols, such as olivetol, are formed from biosynthesized alkylresorcinolic acids by enzymatic decarboxylation or via modified fatty acid-synthesizing enzymes, where the olivetolic acid carboxylic group would be expected also to be attached either to ACP (acyl carrier protein) or to CoA. Thus, in the release of the molecule from the protein compartment in which it was attached or elongated, simultaneous decarboxylation of olivetolic acid may occur, otherwise the olivetolic acid would be the final product. PKS isolation and identification of genes forming alkylresorcinolic acids (Gaucher and Shepherd 1968, Gaisser et al. 1997, Funa et al. 2007) and stilbene carboxylic acids [Eckermann et al., 2003; Schröder Group (http://www.biologie.uni-freiburg.de/data/bio2/schroeder/stilbenecarboxylates.html)] have been reported. Conversion of tetraketides (free acids or lactones) synthesized in vivo by stilbene carboxylic acid synthases (Schröder Group) or by chemical synthesis (Money et al. 1967) into the carboxylic acids at a suitable pH (mildly acidic or basic conditions) has also been suggested. Raharjo et al. (2004a) did not observe any effect on the formation of olivetol by either the incubation time of the PKS assays or the mildly acidic conditions used. Enzymatic decarboxylation in vitro and in vivo, and purification of carboxylic acid decarboxylases has been reported from liverworts (Pryce 1972, Pryce and Linton 1974), lichens (Mosbach and Ehrensvard 1966) and microorganisms (Pettersson 1965, Huang et al. 1994, Dhar et al. 2007, Stratford et al. 2007). We did not observe formation of olivetol by an enzymatic or chemical decarboxylation from olivetolic acid (Supplementary Table S3). Although the recovery for the standards orcinolic acid and 2,4-dihydroxy-benzoic acid was >95%, no orcinol or resorcinol (1,3-dihydroxy-benzene) was detected; methyl-olivetolate was used as a negative control of decarboxylation. Purification of this olivetol-forming PKS is required in order to characterize it and analyze the mechanism of reaction. In addition, no activity was detected with benzoyl-CoA at pH 7.0, 7.5 or 8.0, and no BAS activity was found. Small amounts of derailment by-products were detected from the PKS assays.

Cannabinoid profiling by HPLC

Fig. 4 shows the variations in the cannabinoid content with respect to tissues and the varieties (chemotypes) of the plants analyzed. In chemotype I or drug-type cannabis plants, an eight times higher concentration of Δ^9-THCA was detected in female flowers than male flowers and there was no significant difference between the two varieties analyzed ($P < 0.05$). No significant differences were found in the Δ^9-THCA contents in male flowers, fruits and male or female leaves in the chemotype I plants analyzed ($P < 0.05$). Previous studies confirmed that there is no significant difference in the cannabinoid content in leaves of the two genders from the same variety (Holley et al. 1975, Kushima et al. 1980). As we expected, in chemotype III or fiber-type plants the Δ^9-THCA content in flowers (Kompolti) was less (7-fold) than the content in drug-type plants (Skunk and Fourway varieties). The Δ^9-THCA contents in female leaves and male flowers were four times less than the contents in the drug-type cannabis plants analyzed. Δ^9-THVA was only detected in male and female flowers, and fruits. The concentration of this cannabinoid in female flowers was more than seven and three times higher than the contents in fruits and male flowers, respectively. No significant differences were found among the three varieties of the plants analyzed ($P < 0.05$). The CBGA contents in female flowers and male and female leaves were not significantly different. The content of this cannabinoid in fruits was six times less than in female flowers. The CBGA concentration detected in male flowers was not significantly different from that of fruits, and no significant difference was observed in the three varieties analyzed ($P < 0.05$). CBDA was identified in flowers and leaves. In chemotype I plants, the CBDA content from female flowers was 2.6 times higher than in male flowers. The CBDA contents from leaves were not significantly different from those of male flowers, and no significant differences were observed in the two drug-type plants; but in male leaves from the variety Fourway the CBDA content was significantly different ($P < 0.05$). On the other hand,

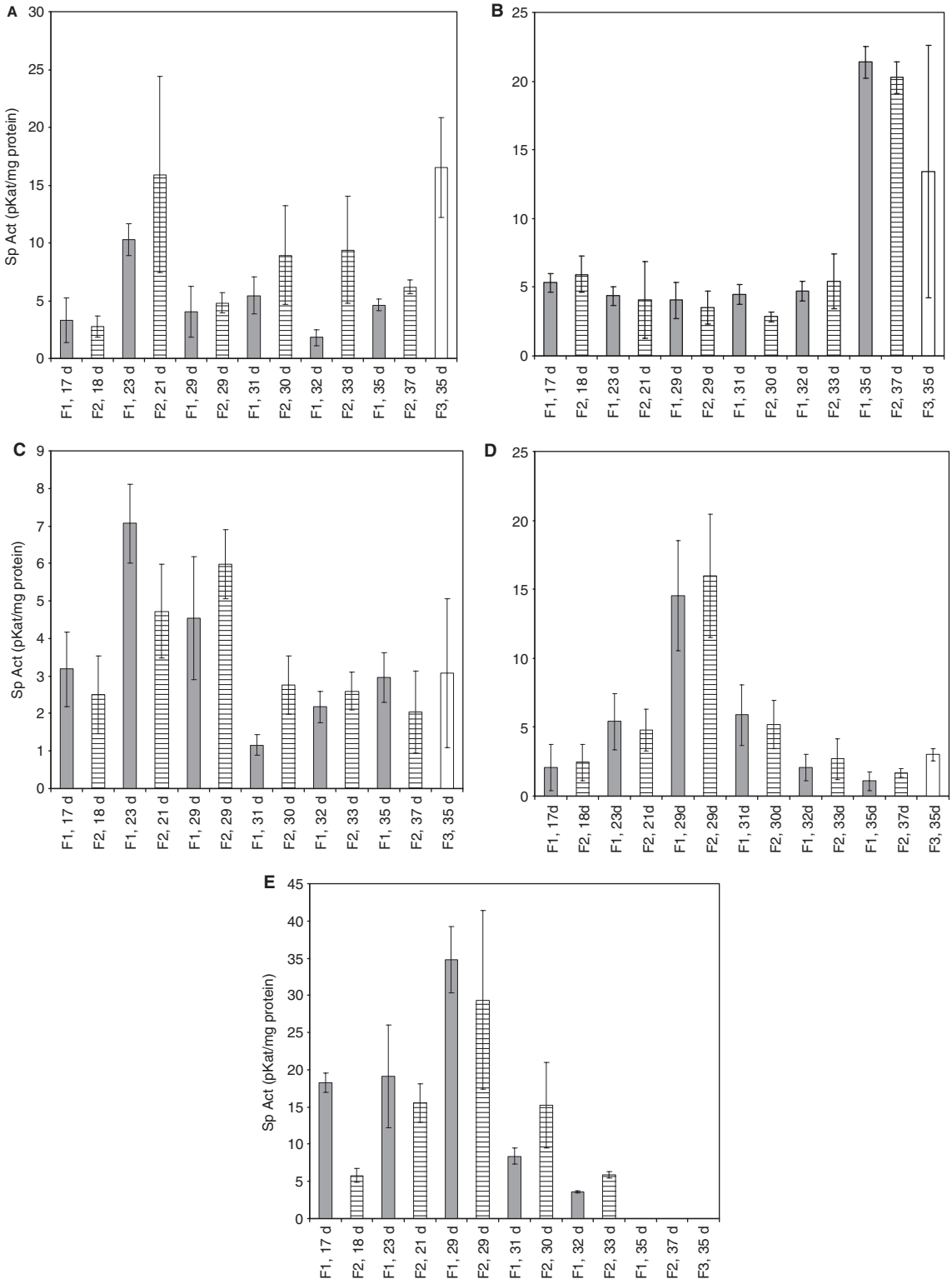

Fig. 3 PKS activities during the development of glandular trichomes on female flowers. (A) CHS; (B) STS; (C) VPS; (D) BUS; (E) olivetol synthase. F1, Skunk variety; F2, Fourway variety; F3, Kompolti variety. Values are expressed as means of three replicates with standard deviations.

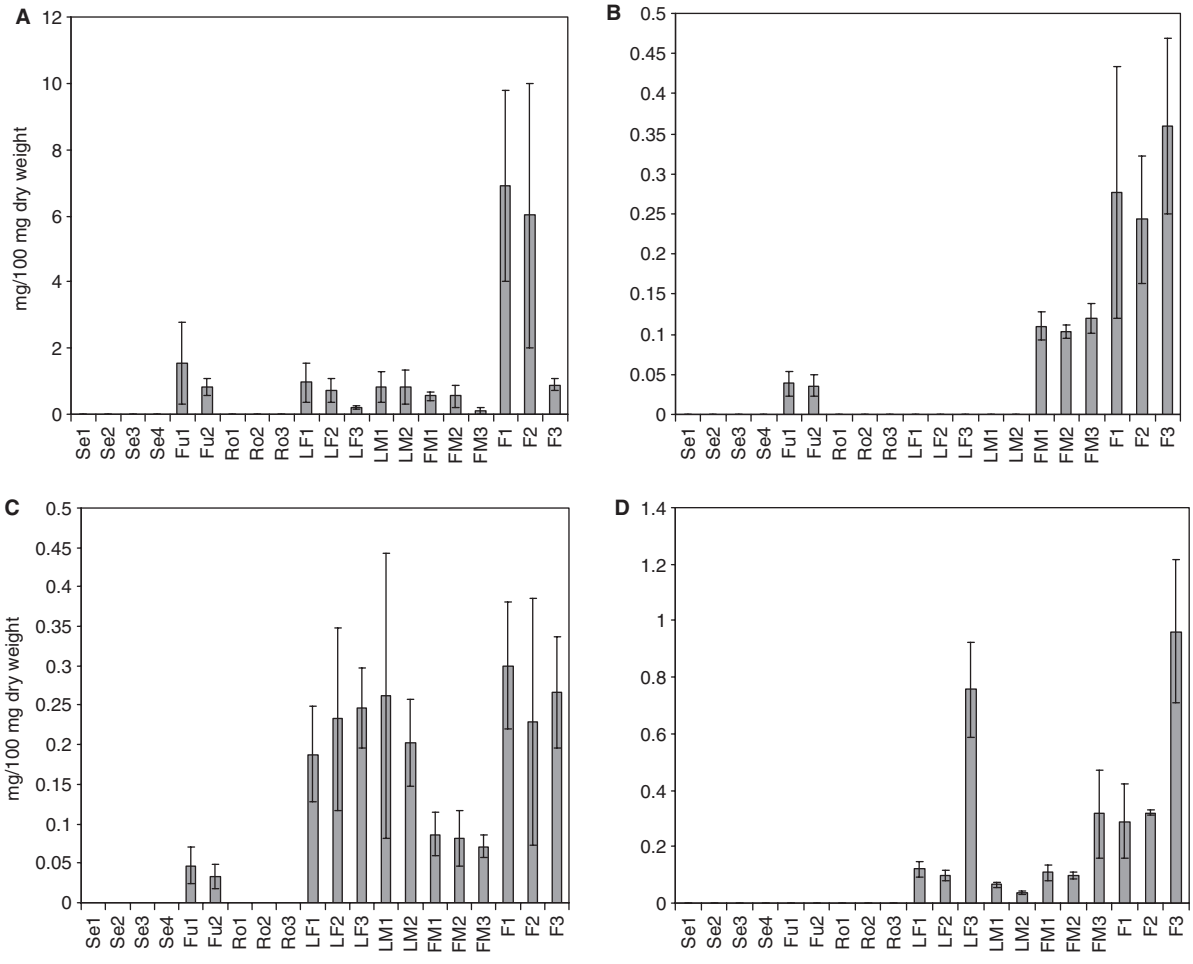

Fig. 4 Cannabinoid content in different cannabis plant tissues. (A) Δ^9-THCA; (B) Δ^9-THVA; (C) CBGA; (D) CBDA. Br, bracts; Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower; F, female flower. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety; 4, Fasamo variety. Female flowers were from 35-day-old female flowers. Values are expressed as means of three replicates with standard deviations.

the CBDA content in the fiber-type plants was significantly different from the CBDA content in drug-type plants ($P<0.05$). The CBDA content in female leaves from the variety Kompolti was six times higher than the contents from the varieties Fourway and Skunk, while the contents in male and female flowers from Kompolti plants were three times higher than the CBDA contents from Fourway and Skunk plants.

The increment on the concentration of cannabinoids corresponds to the development and growth of the glandular trichomes on the bracts (Table 1 and Fig. 5A). No significant differences were found in the CBGA contents from the three varieties of cannabis plants analyzed (Skunk, Fourway and Kompolti; $P<0.05$). Although the cannabinoid content in the individual gland trichomes can vary with age, type and location (Turner et al. 1977, Turner et al. 1978), a correlation exists between glandular density and cannabinoid content at each stage of bract development (Turner et al. 1981). As CBGA is the precursor of Δ^9-THCA and CBDA, its concentration decreased as it was observed during the time course of glandular trichome development. The Δ^9-THCA content increased at day 31 and the contents of this cannabinoid were not significantly different in the two drug-type plants (Fourway and Skunk). On the other hand, Δ^9-THVA accumulation started only after day 24 for Skunk and day 25 for Fourway. In the fiber-type plant Kompolti, the CBDA content at day 31 was seven times higher than the CBDA contents in the drug-type plants. Natural (plant decarboxylation) or artificial degradation (oxidation, isomerization, UV light) of cannabinoids occurred to a lesser extent in our plant material (Table 1). Neither cannabinoid acids nor their decarboxylated forms were found in seedlings and roots in the four varieties of cannabis plant analyzed (Skunk, Fourway, Kompolti and Fasamo). In male flowers, minimal amounts of cannabinoids have been detected (Potter 2004) due to the

Table 1 Cannabinoid content from different *Cannabis* tissues

Tissue	Cannabinoids[a] (acid forms)	Δ^9-THC	CBG	CBD	CBN	Total
Bracts:						
Br1, 24 d	5.19	0.42 ± 0.03	–	–	–	5.61
Br1, 31 d	8.40	0.12 ± 0.03	–	–	0.08 ± 0.002	8.60
Br2, 25 d	1.80	–	–	–	–	1.80
Br2, 31 d	5.97	0.08 ± 0.02	–	–	0.05 ± 0.041	6.10
Br3, 31 d	1.26	0.13 ± 0.01	0.16 ± 0.011	–	–	1.55
Fruits:						
Fu1	1.63	0.04 ± 0.02	–	–	–	1.67
Fu2	0.87	0.10 ± 0.03	–	–	–	0.97
Leaves:						
Female						
LF1	1.26	0.43 ± 0.31	–	–	0.06 ± 0.01	1.75
LF2	1.04	0.36 ± 0.06	–	–	–	1.40
LF3	1.21	0.10 ± 0.09	0.23 ± 0.02	0.12 ± 0.001	–	1.67
Male						
LM1	1.13	–	–	–	–	1.13
LM2	1.07	0.37 ± 0.04	–	–	0.06 ± 0.010	1.50
Flowers:						
Female						
F1	7.78	0.15 ± 0.006	–	–	0.09 ± 0.005	8.02
F2	6.79	0.39 ± 0.004	–	–	–	7.18
F3	2.48	0.10 ± 0.03	–	–	–	2.58
Male						
FM1	0.86	–	–	–	–	0.86
FM2	0.84	–	–	–	–	0.84
FM3	0.63	–	–	–	–	0.63

[a] Concentration expressed in mg 100 mg^{-1} DW (Δ^9-THCA, Δ^9-THVA, CBDA and CBGA)
Br, bracts; Fu, fruits; LF, female leaf; LM, male leaf; F, female flower; FM, male flower. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety.

presence of glandular trichomes on the underside of the anther lobes; these are called antherial capitate-sessile glands (Mahlberg et al. 1984).

Flavonoid profiling by HPLC

As standards for most flavonoid glycosides are not commercially available, we proceeded to hydrolyze the samples in order to analyze the aglycones. Apigenin, luteolin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were used as internal standards. The recovery percentage of aglycones from standards was >90% (Supplementary Table S4). Typical profiles corresponding to a standard mixture of the selected flavones and flavonols with our samples are shown in Supplementary Fig. S2, and analyses by LC-MS confirmed the identity of the aglycones (Supplementary Fig. S3).

Flavonoid content varied from one plant tissue to another (Fig. 6). No flavonoids were detected in roots. Orientin content in flowers and leaves did not differ significantly in different genders and varieties of the cannabis plant. The orientin contents in seedlings and fruits were 14 times less than the contents in leaves, and they were not significantly different for the chemotypes of the cannabis plants analyzed in this study. Vitexin contents in fruits were similar and the lowest of all plant tissues. Higher vitexin contents were detected in seedlings, and no significant differences were observed in the three varieties. Similarly, no significant differences in the contents of vitexin and isovitexin were found in the three varieties of cannabis plants analyzed ($P<0.05$). The lowest isovitexin and quercetin contents were detected in fruits, and the highest amounts of quercetin in male flowers. No significant differences were observed in the content of this aglycone in the other tissues by gender and variety ($P<0.05$). However, the quercetin content in male flowers from fiber-type plants (Kompolti) was significantly different from that of the drug-type plants ($P<0.05$). The luteolin content in male flowers was significantly different from the content in leaves, but no significant difference was found

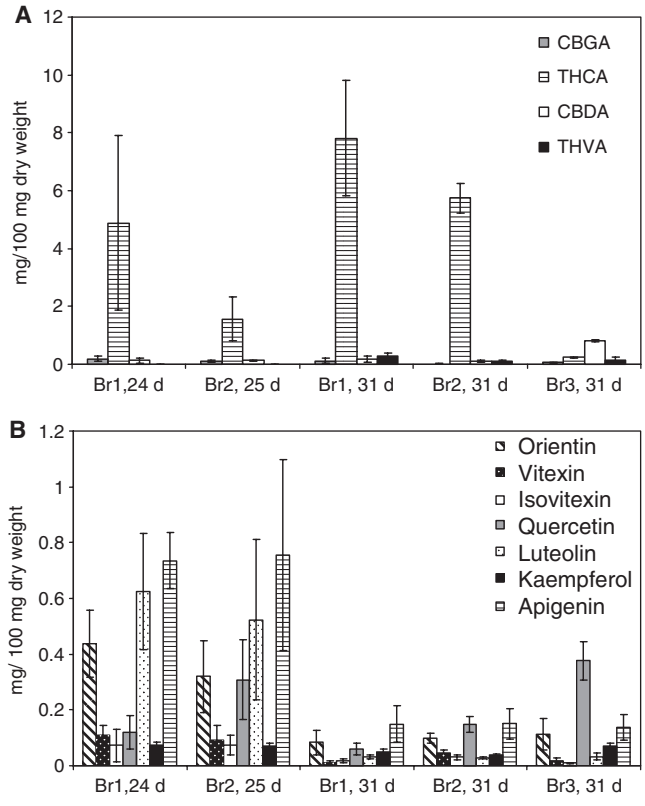

Fig. 5 Cannabinoid content (A) and flavonoid content (B) in bracts during the growth and development of glandular trichomes on female flowers. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety.

among the three varieties of the male flowers or leaves (Skunk, Fourway and Kompolti; $P<0.05$). The kaempferol distribution also did not show any significant difference for the three varieties. The contents of this aglycone in seedlings and female flowers were 17 times higher than the contents in fruits. In leaves, the apigenin contents were not significantly different by gender but the contents in flowers were significantly different by gender ($P<0.05$). The lowest contents of this aglycone were detected in fruits. Luteolin and vitexin contents are similar to results reported by Vanhoenacker et al. (2002), but apigenin and orientin contents are higher in our samples. Though Raharjo (2004) only reported apigenin and luteolin in leaves and flowers of *C. sativa* Fourway plants, the contents were different from our results, probably because of differences in plant tissue age. In contrast to the cannabinoid accumulation during the growth and development of glandular trichomes, the flavonoid content decreased (Fig. 5B and Table 2). The isovitexin content was not significantly different during the growth and development of glandular trichomes on the bracts ($P<0.05$). No significant differences were found in the flavonoid contents between the varieties Skunk and Fourway at days 24 and 25, respectively. However, the contents of the aglycones vitexin and quercetin were significantly different at day 31 ($P<0.05$). The kaempferol content in bracts of the variety Kompolti (fiber-type) was significantly different compared with the varieties Skunk and Fourway (drug-type) at day 31 ($P<0.05$). The isovitexin content in bracts from fiber-type (Kompolti) was 2.5 times less than the content in the bracts from drug-type plants, whereas the quercetin contents in bracts from fiber-type (Kompolti) plants were six times and twice higher than the contents in bracts from the varieties Skunk and Fourway (drug-type), respectively. Some studies suggest that flavonoid distribution could have a chemotaxonomic value in cannabis plants (Clark and Bohm 1979, Vanhoenacker et al. 2002).

PKS activities and secondary metabolites in C. sativa

In plant tissues from *C. sativa*, in vitro PKS activities of CHS, STS, BUS and VPS, as well as activity for an olivetol-forming PKS were detected. Content analyses of cannabinoids and flavonoids, two secondary metabolites present in this plant (Flores-Sanchez and Verpoorte, 2008), revealed differences in their distribution, suggesting a diverse regulatory control in these biosynthetic fluxes in the plant. Apigenin, luteolin and kaempferol are widespread compounds in plants (Valant-Vetschera and Wollenweber 2006). Quercetin and kaempferol have a role in fertility of male flowers (Vogt et al. 1995, Napoli et al. 1999), and higher levels of these two flavonols in cannabis male flowers (Fig. 6) support this role. Moreover, protection from UV-B (280–315 nm) by flavone or flavonol glycosides has been reported (Lois and Buchanan 1994, Rozema et al. 2002) and their occurrence in aerial cannabis tissues should be vital. Furthermore, roles as growth regulators have been suggested (Ylstra et al. 1994, Gould and Lister 2006). Quercetin, apigenin and kaempferol can modulate auxin-mediated processes (Jacobs and Rubery 1988), and this role should not be excluded in cannabis. It has been reported that luteolin and apigenin derivatives acted as feeding deterrents to *Lepidoptera* larvae (Erhard et al. 2007). On the other hand, it is known that cannabinoids are cytotoxic compounds (Rothschild et al. 1977, Roy and Dutta 2003, Sirikantaramas et al. 2005) and they can act as plant defense compounds against predators such as insects. Moreover, a regulatory role in cell death has been suggested as cannabinoids have the ability to induce cell death through mitochondrial permeability transition (Morimoto et al. 2007). The accumulation of cannabinoids in bracts during the growth and development of glandular trichomes from flowers (Fig. 5A) could be related to floral protection and, consequently, during seed maturation, the cannabinoid content may decrease. Lower contents of cannabinoids were detected in fruits (seed and cup-like bracteole) than in female flowers (Table 1). Moreover, no significant

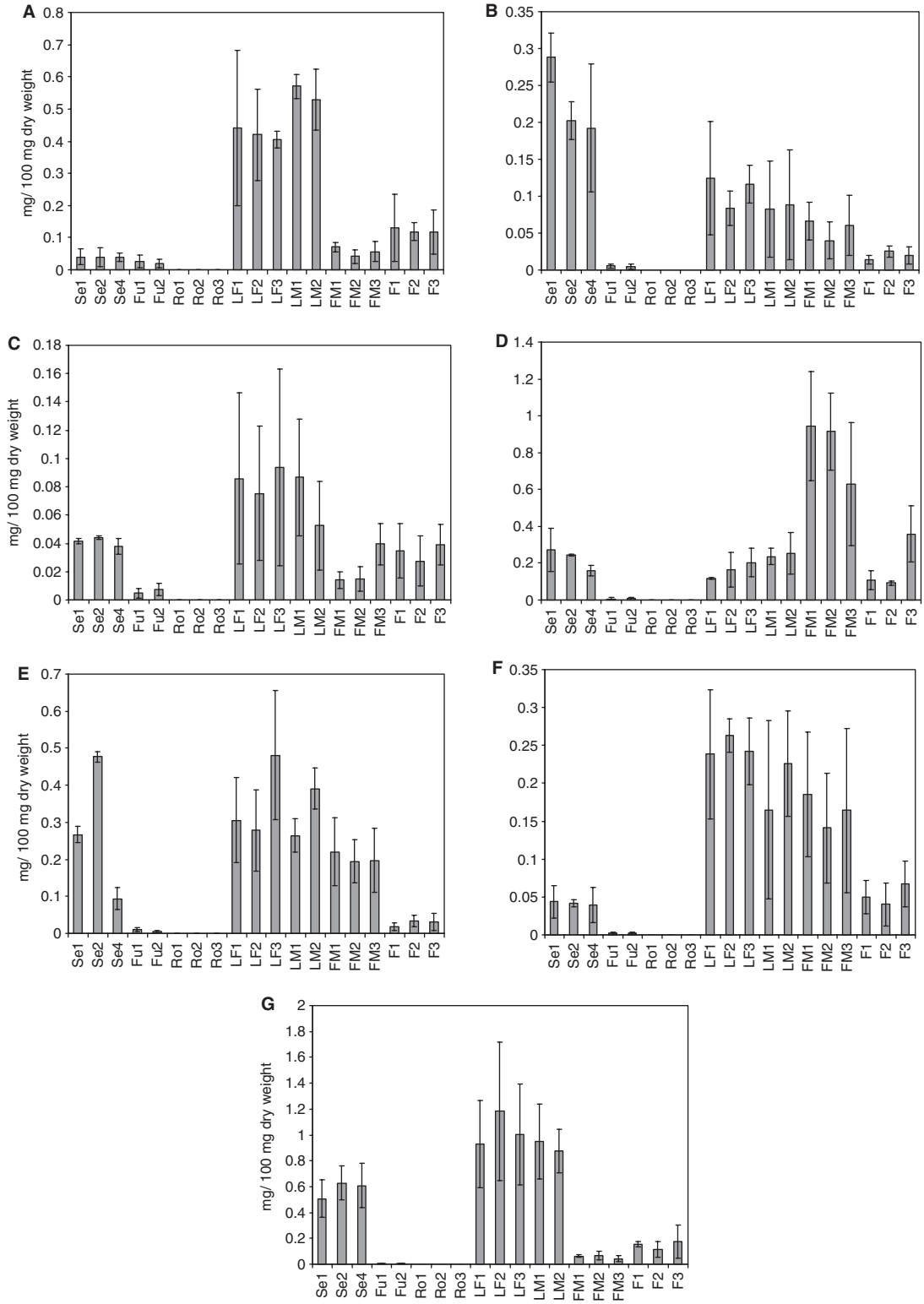

Fig. 6 Flavonoid content in different cannabis plant tissues. (A) Orientin; (B) vitexin; (C) isovitexin; (D) quercetin; (E) luteolin; (F) kaempferol; (G) apigenin. Se, seedlings; Fu, fruits; Ro, roots; LF, female leaf; LM, male leaf; FM, male flower; F, female flower. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety; 4, Fasamo variety. 35-day-old female flowers. Values are expressed as means of three replicates with standard deviations.

Table 2 Flavonoid content in different plant tissues from *C. sativa*

Tissue	Flavonoid total content (mg 100 mg^{-1} DW)
Bracts:	
Br1, 24 d	2.18
Br1, 31 d	0.40
Br2, 25 d	2.14
Br2, 31 d	0.54
Br3, 31 d	0.74
Fruits:	
Fu1	0.06
Fu2	0.05
Seedlings	
Se1	1.46
Se2	1.67
Se4	1.17
Leaves:	
Female	
LF1	2.24
LF2	2.37
LF3	2.54
Male	
LM1	2.36
LM2	2.42
Flowers:	
Female	
F1	1.56
F2	1.41
F3	1.19
Male	
FM1	0.51
FM2	0.46
FM3	0.81

Br, bracts; Fu, fruits; Se, seedlings; LF, female leaf; LM, male leaf; F, female flower; FM, male flower. 1, Skunk variety; 2, Fourway variety; 3, Kompolti variety; 4, Fasamo variety. Bracts from 24-, 25- and 31-day-old flowers.

differences were found between the cannabinoid and flavonoid contents in fruits, and they were not significantly different between the varieties Fourway and Skunk ($P<0.05$). It seems that cannabinoid accumulation is correlated with maximum activities for an olivetol-forming PKS (Figs 3, 5A) and the CHS activity preceded the accumulation of flavonoids at day 24 (Figs 3, 5B). A significant STS-type activity was detected at day 35 (Fig. 3). Although significant enzymatic activities for VPS and BUS were also detected in crude protein extracts, no acylphloroglucinols have been identified in cannabis so far (Flores-Sanchez and Verpoorte 2008). Acylphloroglucinols and activities of VPS and BUS have been detected in *Humulus lupulus* (Paniego et al. 1999) and *Hypericum perforatum* (Hoelzl and Petersen 2003, Klingauf et al. 2005). It is known that PKS enzymes can use a broad range of substrates efficiently (Springob et al. 2000, Samappito et al. 2003, Novak et al. 2006) and probably the cannabis PKS enzymes also have this notorious in vitro substrate promiscuity. Zuurbier et al. (1988) showed that CHS and STS can have VPS- and BUS-type activities, and the VPS and BUS activities identified in this study could be from CHS or olivetol-forming PKS, or even from STS. Although significant CHS and STS activities were detected in crude protein extracts from roots (Fig. 2), no flavonoids were identified in these tissues (Fig. 6). There are no reports about isolation or detection of flavonoids and stilbenoids in roots (Flores-Sanchez and Verpoorte 2008) and this seems to contradict the CHS- and STS-type activities detected in roots. Low expression of the *CHS-type PKS* gene in roots and the absence of flavonoids in this plant tissue were previously reported (Raharjo 2004, Raharjo et al. 2004b). Stilbenoids have been isolated from cannabis leaves and resin (Flores-Sanchez and Verpoorte 2008), but they could not be identified in the methanol : water fractions from leaves and bracts by LC-MS analysis; this could be due to the low STS-type activity (Fig. 3). Gehlert and Kindl (1991) found a relationship between induced formation by wounding of stilbenes and the PKS BBS in orchids. Stilbenoid functions in plants include constitutive and inducible defense mechanisms (Chiron et al. 2001, Jeandet et al. 2002), plant growth inhibitors and dormancy factors (Gorham 1980).

It is known that induction of enzymatic activity in early steps of a biosynthetic pathway precedes the accumulation of final products (Fig. 7). The cannabinoid content in female flowers from drug-type plants (Skunk and Fourway varieties) was five times higher than the flavonoid content, but in female flowers from fiber-type plants (Kompolti variety) it was only twice the amount (Tables 1, 2). During the development of the glandular trichomes on the flowers, the activity of the olivetol-forming PKS at day 29 was seven times higher than the CHS activity (Fig. 3) and it was correlated with the cannabinoid accumulation (Table 1). Although, STS activity detected during the time course was low, it increased at the end, being five and 18 higher than the CHS and olivetol-forming PKS activities, respectively. This STS activity can be associated with precursor formation in stilbenoid biosynthesis. The profiling shown here suggests the presence of three PKS activities, one CHS type, one STS type and another for olivetol biosynthesis. Using the present approach, we believe that we have clear evidence for the presence of different PKS profiles in the different tissues; which are further supported by the phytochemical analyses of these tissues. The role of olivetol synthase in the plant needs further clarification. Its activity coincides with

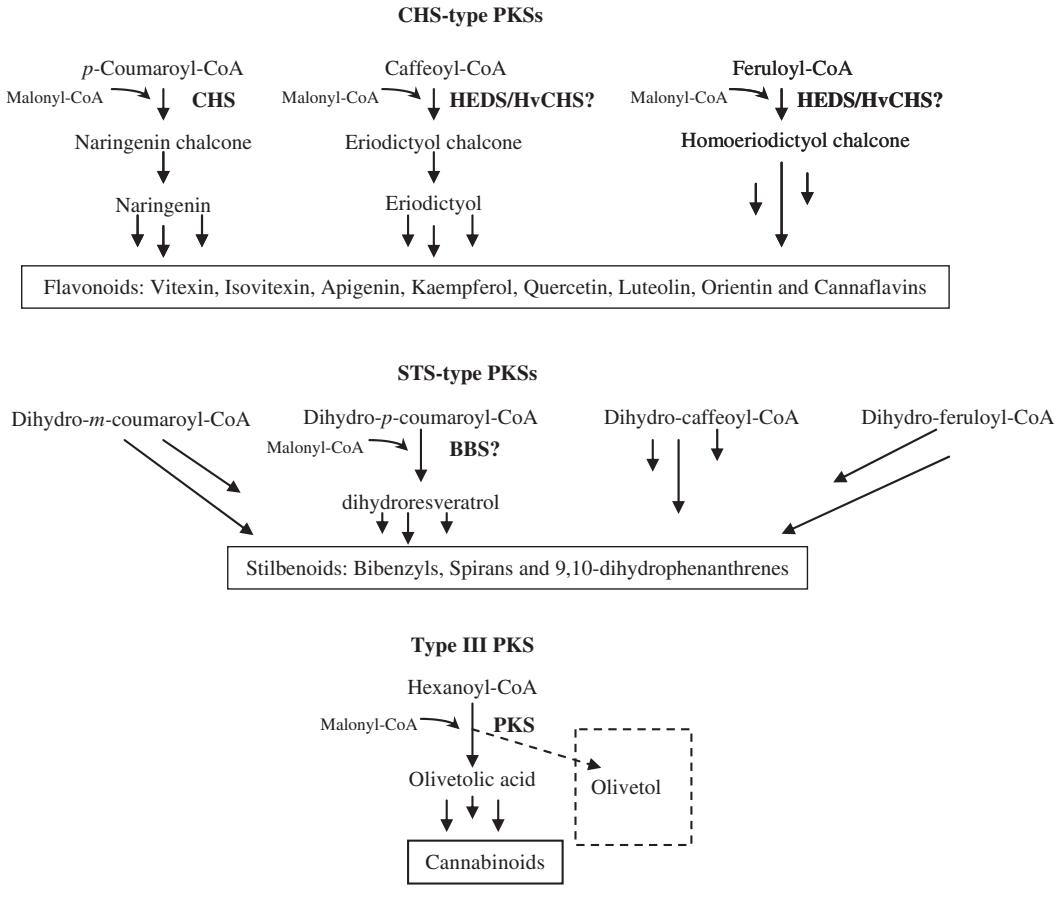

Fig. 7 Proposed reactions for PKS enzymes in the biosynthesis of precursors from flavonoid, stilbenoid and cannabinoid pathways in cannabis plants. The dashed square represents the compound found in crude extracts.

the occurrence of the cannabinoids. On the other hand, no activity of olivetolic acid synthase has been found in cannabis so far. This raises the question about whether the in planta activity of this enzyme could be different from its in vitro activity. Further studies are required to identify the substrate specificities of these individual PKS enzymes in cannabis plants. Purification and characterization of the PKS enzymes will be necessary to determine their catalytic potential and regulation, which may lead to the identification of their role in the plant.

Materials and Methods

Plant material

Seeds of *C. sativa*, drug-type varieties Skunk and Fourway (The Sensi Seed Bank, Amsterdam, The Netherlands), and fiber-type varieties Kompolti and Fasamo (Dr. D. Watson, HortaPharm, Amsterdam, The Netherlands), were germinated and 9-day-old seedlings were planted in 11 LC pots with soil (substrate 45 L, Holland Potgrond, Van der Knaap Group, Kwintsheul, The Netherlands) and maintained under a light intensity of 1,930 lux, at 26°C and 60 ± 7% relative humidity (RH). After 3 weeks, the small plants were transplanted into 10 liter pots for continued growth until flowering. To initiate flowering, 2-month-old plants were transferred to a photoperiod chamber (12 h light, 27°C and 37 ± 11% RH). Five day-old seedlings, young leaves from 13-week-old plants, female flowers and bracts at different stages of development, and male flowers from 4-month-old plants were harvested. Three-month-old male plants were used for pollination of female plants. The fruits were harvested 18 d after pollination. Roots from 4-month-old female plants were harvested and washed with cold water to remove residual soil. All vegetabl material was weighed and stored at –80°C.

Chemicals

Benzoyl-CoA, hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, malonyl-CoA, resveratrol, naringenin and 2,4-dihydroxybenzoic acid were obtained from Sigma (St Louis, MO, USA). Olivetol was acquired from Aldrich Chem (Milwaukee, WI, USA) and 4-hydroxybenzyledeneacetone (*p*HBA) from Alfa Aesar (Karlsruhe, Germany). Orcinolic acid (orsellinic acid) was from AApin Chemicals Ltd (Abingdon, UK) and resorcinol (1,3-dihydroxy-benzene) from Merck Schuchardt (München, Germany). *p*-Coumaroyl-CoA was synthesized according to Stöckigt and Zenk (1975), and phlorisovalerophenone (PiVP) and phlorisobutyrophenone (PiBP) were previously synthesized in our laboratory (Fung et al. 1994). Olivetolic acid was obtained

from hydrolysis of methyl olivetolate (Horper and Marner 1996), and methyl olivetolate was a gift from Professor Dr. J. Tappey (Virginia Military Institute, USA). The cannabinoids Δ^9-THCA, CBGA, Δ^9-THC, Δ^8-THC, CBG, CBD and CBN were isolated from plant materials previously in our laboratory (Hazekamp et al. 2004). Δ^9-THVA was identified based on its relative retention time and UV spectra (Hazekamp et al. 2005), and its quantification was relative to Δ^9-THCA. The flavonoids kaempferol, orientin and luteolin were purchased from Extrasynthese (Genay, France), and vitexin, isovitexin and apigenin from Sigma-Aldrich (Buchs, Switzerland). Quercetin, apigenin-7-*O*-Glc and luteolin-7-*O*-Glc were from our standard collection. All chemical products and mineral salts were of analytical grade.

Protein extracts

Frozen plant material was homogenized in a mortar with nitrogen liquid, the powder was thawed in polyvinylpolypyrrolidone (PVPP) and extraction buffer [0.1 M potassium phosphate buffer, pH 7, 0.5 M sucrose, 3 mM EDTA, 10 mM dithiothreitol (DTT) and 0.1 mM leupeptin], squeezed through Miracloth and centrifuged at 14,000 r.p.m. for 20 min. Per each gram of fresh weight, 0.1 g of PVPP and 2 ml of extraction buffer were used. The crude protein extracts were desalted using Sephadex G-25 M (PD-10) columns and eluted with the same extraction buffer without addition of leupeptin. All steps were performed at 4°C.

PKS assays

PKS activity was measured by the conversion of starter CoA esters and malonyl-CoA into reaction products.

The standard reaction mixture, in a final volume of 500 µl, contained 50 mM K-Pi buffer (pH 7), 20 µM starter-CoA, 40 µM malonyl-CoA, 0.5 M sucrose and 1 mM DTT. The reaction was initiated by addition of 250 µl of crude desalted extracts (100–440 µg of protein) and was incubated for 90 min at 30°C. Reactions were stopped by addition of 20 µl of 4 N HCl, extracted twice with 800 µl of ethyl acetate and centrifuged for 2 min. The combined organic phases were evaporated in a vacuum centrifuge and the residue was kept at 4°C. Samples were resuspended in 100 and 40 µl of methanol for analysis by HPLC and LC-MS, respectively.

VPS was isolated previously in our laboratory (Paniego et al. 1999), and CHS and STS were a gift from Professor Dr. J. Schröder (Freiburg University, Germany).

Protein determination

Protein concentration was measured as described by Peterson (1977) with bovine serum albumin as standard.

HPLC analysis

The system consisted of a Waters 626 pump, a Waters 600S controller, a Waters 2996 photodiode array detector and a Waters 717 plus autosampler (Waters, Milford, MA, USA), equipped with a reversed-phase C18 column (250 mm×4.6 mm, Inertsil ODS-3, GL Sciences, Tokyo, Japan). A 80 µl aliquot of sample was injected; the gradient solvent system consisted of methanol and water, both containing 0.1% trifluoroacetic acid (TFA). Method (1) 0–40 min, 20–80% methanol; 40–43 min, 80% methanol; 43–48 min, 80–20% methanol; 40–50 min, 20% methanol. Method (2) 0–30 min, 40–60% methanol; 30–33 min, 60% methanol; 35–38 min, 60–40% methanol 38–40 min, 40% methanol. Method (3) 0–40 min, 40–60% methanol; 40–43 min, 60% methanol; 43–44 min, 40–60% methanol; 44–45 min, 40% methanol. Method (4) 0–40 min, 50–100% methanol; 40–43 min, 100% methanol; 43–44 min, 100–50% methanol; 44–45 min, 50% methanol. Method (5) 0–20 min, 50–80% methanol; 20–30 min, 80% methanol; 30–35 min, 80–50% methanol; 35–40 min, 50% methanol. The flow rate was 1 ml min^{-1} at 25°C; olivetol, methyl olivetolate, olivetolic acid, PiVP, PiBP, naringenin and resveratrol were detected at 280 nm, orcinolic acid at 260 nm, orcinol at 273 nm and 2,4-dihydroxy-benzoic acid at 256 nm. *p*HBA was detected at 320 nm. Calibration curves with the respective standards were made.

LC-MS analysis

For the confirmation of the identity of enzymatic products, 20 µl of samples were analyzed in an Agilent 1100 Series LC/MS system (Agilent Technologies, Palo Alto, CA, USA) with positive/negative atmospheric pressure chemical ionization (APCI), using elution system method 5 with a flow rate of 0.5 ml min^{-1}. The optimum APCI conditions included a N_2 nebulizer pressure of 45 p.s.i., a vaporizer temperature of 400°C, an N_2 drying gas temperature of 350°C at 10 liters min^{-1}, a capillary voltage of 4,000 V, a corona current of 4 µA and a fragmentor voltage of 100 V. A reversed-phase C18 column (150 mm×4.6 mm, 5 µm, Zorbax Eclipse XDB-C18, Agilent) was used.

Extraction of compounds

Extraction was carried out as described by Choi et al. (2004) with slight modifications. To 0.1 g of lyophilized and ground plant material was added 4 ml of MeOH : H_2O (1 : 1, v/v) and 4 ml of $CHCl_3$, vortexed for 30 s and sonicated for 10 min. The mixtures were centrifuged in the cold at 3,000 r.p.m. for 20 min. The MeOH : H_2O and $CHCl_3$ fractions were separated and evaporated. The extraction was performed twice. The extracts were resuspended on 1 ml of MeOH : H_2O (1 : 1) and $CHCl_3$, respectively, for the subsequent cannabinoid and flavonoid analyses.

Cannabinoid analysis by HPLC

The column used was a Grace Vydac (WR Grace, Columbia, MD, USA) C_{18} (250 mm×4.6 mm, Mass Spec 5 µm) with a Waters Bondapak C_{18} guard column (2 mm×20 mm, 50 µm). The solvent system and the operational conditions were the same as previously reported by Hazekamp et al. (2004). For preparation of samples, 100 µl of the $CHCl_3$ fraction from extraction was evaporated using N_2 gas. The samples were dissolved in 1 ml of EtOH and 20 µl was injected in the HPLC system. Cannabinoids were detected at 228 nm. Calibration curves with their respective standards were made.

Flavonoid analysis by HPLC

A reversed-phase C18 column (250 mm×4.6 mm, Inertsil ODS-3) was used. The solvent system and the operational conditions were as described by Justesen et al. (1998) with slight modifications. The mobile phase consisted of MeOH : H_2O (30 : 70, v/v) with 0.1% TFA (A) and MeOH with 0.1% TFA (B). The gradient was 25–86% B in 40 min followed by 86% B for 5 min and a gradient step from 86 to 25% B for 5 min at a flow-rate of 1 ml min^{-1} and at 25°C. A 20 µl aliquot of resuspended hydrolyzed samples was injected. Retention times for aglycones were as follows: apigenin 23.02 min, kaempferol 21.95 min, luteolin 18.37 min, quercetin 16.37 min, isovitexin 5.32 min, vitexin 4.71 min and orientin 3.64 min; and for apigenin-7-*O*-Glc 10.7 min and luteolin-7-*O*-Glc 7.42 min. Flavones and flavonols were detected at their maximal UV absorbance (quercetin, 255 nm;

kaempferol, 265.8 nm; apigenin, isovitexin, vitexin and apigenin-7-*O*-Glc, 270 nm; and orientin, luteolin and luteolin-7-*O*-Glu, 350 nm). The flow rate was 1 ml min^{-1} at 25°C. Calibration curves with their respective standards were made. The standards apigenin and vitexin were dissolved in MeOH : dimethylsulfoxide (DMSO) (7 : 3), orientin in MeOH : DMSO (8 : 2, v/v), apigenin-7-*O*-Glc and luteolin-7-*O*-Glc in MeOH : DMSO (9 : 1, v/v) and the remainder only in MeOH.

The optimum APCI conditions for LC-MS analyses were as described above.

Acid hydrolysis for flavonoids

A 500 µl aliquot of the MeOH : H_2O fraction from the extraction was hydrolyzed at 90°C for 60 min with 500 µl of 4 N HCl to which 2 mg of the antioxidant *tert*-butylhydroquinone was added. Hydrolysates were extracted with ethyl acetate three times. The organic phase was dried over anhydrous $NaSO_4$ and evaporated with N_2 gas.

Statistics

All data were analyzed by MultiExperiment Viewer MEV 4.0 software (Saeed et al. 2003). For analyses involving two, and three or more groups, paired *t*-tests and analysis of variance (ANOVA) were used, respectively, with $\alpha = 0.05$ for significance.

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