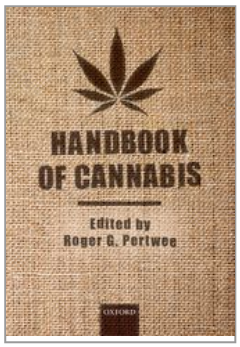

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The Chemical Phenotypes (Chemotypes) of Cannabis

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Abstract and Keywords

Chemical phenotypes (chemotypes) of *Cannabis* are defined by the content and composition of their cannabinoid fraction. This chapter describes the current diversity of chemotypes and discusses their underlying genetic mechanisms and breeding histories. The role of chemotype in *Cannabis* classification is addressed. Specific morphological features of the glandular trichomes associated with certain chemotypes are illustrated. The purity, content, and yield levels of the cannabinoids of clones selected in a plant-breeding program at GW Pharmaceuticals are presented. Several of these clones are currently in use for pharmaceutical raw material production. Their crude floral extracts and purified cannabinoids are being investigated for therapeutic potential. The chapter closes with some thoughts on the prospects of molecular breeding to modify cannabinoid biogenesis and further expand the cannabinoid portfolio

Keywords: biogenesis, cannabinoid, Cannabis, chemotype, phenotype, plant-breeding, trichomes

5.1 Introduction

Cannabinoids belong to a class of terpenophenolic compounds that, with some reported exceptions in the plant kingdom (Bohlmann and Hoffmann 1979; Raederstorff et al. 2012; Toyota et al. 1994, 2002), is largely unique to the genus *Cannabis*. In a review, ElSohly and Slade (2005) estimated the total number of cannabinoids at 70, but this number is dynamic and subject to definitions and limitations. Since then, ElSohly's group has added about 35 new cannabinoid terpene esters, cannabigerol-, and cannabichromanone-related substances. In

the GW Pharmaceuticals (GW) laboratories, a range of fatty acid esters, cannabitriol esters, cannabitriol ethers, terpene esters, dimers and prenylated products of cannabinoids have been identified. These, with the proven and expected existence of several cannabinoid alkyl homologues, would bring the total number of cannabinoid-related compounds significantly in excess of 130 (A. Sutton, personal communication). Only a few of them are considered major, in the sense that they commonly occupy substantial proportions of a plant's total cannabinoid fraction. The large majority of the cannabinoids occur in trace proportions. Many of them appear to, or are expected to, induce specific physiological effects in mammals and are therefore of potential pharmaceutical interest. Pharmaceutical research, and product development especially, requires an ample availability of the compounds of interest. Economic and efficient horticultural production of cannabinoids is realized by the cultivation of uniform female crops with high yields of botanical raw material (BRM, the combined fraction of stem leaves and floral bracts and bracteoles), high cannabinoid content, and well-defined cannabinoid profiles that are strongly dominated by a single compound. These criteria provide the rationale and targets for a medicinal *Cannabis* breeding program. The economic production of the naturally minor cannabinoids particularly would not have been possible without committed breeding work. The focus of this chapter is on the currently available range of chemotypes, as expressed by selected female clones obtained through conventional breeding methods. These are discussed in terms of underlying genotype, breeding history, production level, and, in some instances, highly characteristic trichome morphology. A genetic model for chemotype inheritance is presented. Finally, the increasing molecular biological interest in *Cannabis* is addressed as this development may result in advanced breeding approaches, novel cannabinoid variants, and chemotypes beyond the current range.

5.2 Chemical phenotype and *Cannabis* classification

The genus *Cannabis* L. is unambiguously recognizable by botanical criteria. Within the genus, the variability of chemotypical and other characteristics is impressive and there is a long history of **(p.90)** taxonomic controversy on the number of species to be recognized. Cannabinoids belong to the more conspicuous and spectacular attributes of the genus and cannabinoid chemotypes have been employed to classify groups within the genus, both casually and in formal taxonomy. Informally many authors refer to plants with high tetrahydrocannabinol (THC) content and low cannabidiol (CBD) content as "drug-type" and those with low THC content and high CBD content as "fiber-type" (e.g., Kojoma et al. 2006; Lydon and Teramura 1987). Although this may sound logical, such terminology is problematic. There are no strict natural relationships between fiber characteristics and cannabinoid content or –composition; only artificial associations for which exceptions occur (de Meijer and Keizer 1996).

Small and Cronquist (1976) attributed taxonomic importance to chemotype and used the THC:CBD ratio as a criterion to discriminate within the single species *C. sativa* L., two subspecies *sativa* and *indica* and, per subspecies, two varieties: one domesticated and one wild. Hillig and Mahlberg (2004) discriminated *C. sativa* and *C. indica* as separate species. However, their definitions of the categories *sativa* and *indica* deviate significantly from Small's and other (e.g., Anderson 1980; Schultes et al. 1974) taxonomic systems. They used chemotype-related criteria such as the B_T- and B_D allele frequency (encoding tetrahydrocannabinolic acid and cannabidiolic acid synthase, respectively), THC content, and the level of propyl cannabinoids. The great difficulty with such criteria is that they have, directly or indirectly, been subjected to human selection for ages. Furthermore, cannabinoid ratios are governed by simple genetic mechanisms and in segregating populations, or even in single plant progenies, morphologically similar (sister) plants can be found with strongly contrasting chemotypes. This makes the cannabinoid chemotype unsuitable as a taxonomic criterion.

Agreement on *Cannabis* taxonomy has never been reached and none of the proposed systems appears practically applicable as, under investigation, actual plants usually end up as "intermediate" between categories. A monospecific concept, with no further subspecific division, has implicitly been adopted in virtually all, nontaxonomic, publications on *Cannabis*. Also, in this author's opinion, the genus should be considered as monospecific, i.e., comprising only the single species *C. sativa* L. The reasons for this view are simple. All groups of plants belonging to the genus are perfectly interfertile and the morphological diversity within the genus shows a diffuse and continuous pattern. Hence, neither biological nor morphological criteria are available for the discrimination of more than one species. However, the issue remains of how to adequately indicate the different groups of plants within this single species. The current pattern of *Cannabis* diversity is primarily due to intentional actions of humans and reflects a long, intense, and divergent process of domestication which has blurred any natural evolutionary pattern of diversity. It is even questionable if truly wild *Cannabis* still exists, therefore a characterization of groups within the genus/species in nontaxonomic terms appears most appropriate. For instance, groups could be defined by their type of utilization: ("crop-use groups": fiber hemp, drug strains, seed hemp), their (usually secondary) geographic provenance, their domestication status (landraces (locally adapted, traditional varieties), cultivars of diverse nature, weedy escapes) and key agronomic features (chemotype, fiber content, etc.). Without any formal taxonomic intention, this provides a coherent idea of a group phenotype, a complex of commonly associated features resulting from domestication. To avoid taxonomic impasse and confusion, the use of "cultonomic" rather than natural taxonomic criteria has been recommended for domesticated plants in general (van den Berg 1999, 2004). Cultonomic classification has been formalized in the

International Code of Nomenclature for Cultivated Plants (ICNCP, Brickell et al. 2004) and provides two categories, “Group” and “cultivar.” The Group is a category for assembling cultivars on the basis of some defined similarity and, along with other users’ criteria, chemotype would be a suitable attribute to **(p. 91)** specify Groups. The implementation of a system according to the ICNCP would be useful to all who need to refer to *Cannabis* plant materials.

5.3 Defining chemotype

5.3.1 Components of chemotype

For a systematic approach, it is important to discriminate qualitative and quantitative aspects of chemotype. The cannabinoid composition, i.e., the mutual ratio of the different cannabinoids, represents the qualitative chemotype and is generally controlled by simple genetic mechanisms, shows discrete distribution patterns in progenies and populations, and is hardly affected by the environment (de Meijer et al. 2003). The quantitative aspects of chemotype are controlled by different, polygenic mechanisms, show Gaussian distributions in progenies and populations, and are greatly affected by environmental factors. The yield of a certain cannabinoid in a horticultural production system can be considered as a complex characteristic composed of four components: **(p.92)** the total above ground dry matter yield, the proportion of BRM (leaf and inflorescence), the total cannabinoid content in the homogenized BRM, and the proportion (purity) of the target cannabinoid in the total cannabinoid fraction. The first three components are quantitative in nature. The purity, or the mutual ratio of cannabinoids, has generally a monogenic background. Fig. 5.1 shows the differences in distribution patterns of the polygenic trait total cannabinoid content and the monogenic trait cannabinoid composition. For male and female *Cannabis* plants, the same principles for chemotype inheritance apply and the cannabinoid compositions (ratios) are similarly expressed. However, the dry matter yields, the BRM proportions, and the total cannabinoid contents reach lower values for the males than for the females. This is due to the typical male morphology: fewer floral bracts and bracteoles that carry the trichomes where the cannabinoid production takes place. Data presented in this chapter relate to mature female plants.

5.3.2 Production procedures and conditions

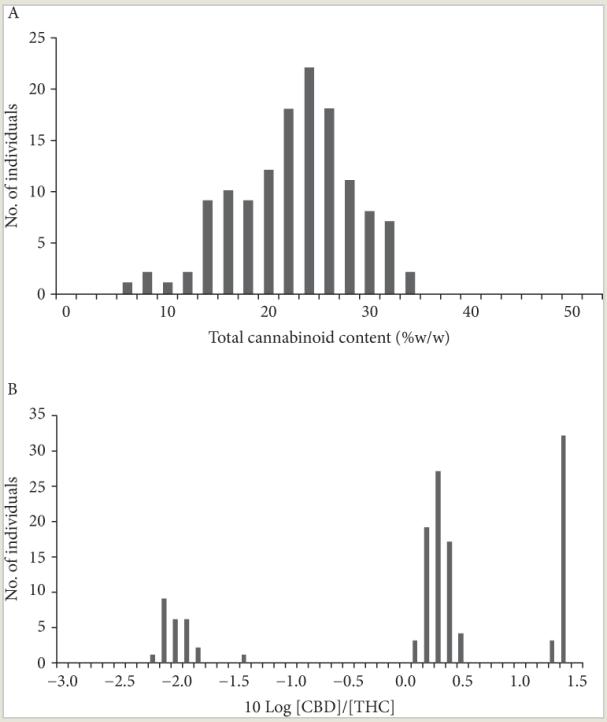


Fig. 5.1 The Gaussian distribution of the polygenic trait total cannabinoid content (A) and the discrete distribution of the monogenic trait cannabinoid profile (log [CBD]/[THC]) (B), in a segregating progeny of 130 sister-individuals.

The plasticity of the quantitative components of chemotype requires some specification of the production environment. Data referred to in this chapter (e.g., Table 5.1) are based on the procedures and conditions in the GW glasshouse. For propagation of the production clones, shoot-cuttings are taken from mother plants. These are treated with a rooting hormone and incubated for 2 weeks under permanent light. Then, cuttings are transplanted to 5 L pots of compost and kept under permanent light (80 W/m^2 photosynthetically active radiation (PAR)) for a 3-week period of vegetative development. Crops are then spaced to 10 plants/m^2 under a 12 h photoperiod for flower induction, flowering, and maturation for a further 8–9 weeks. The average light intensity at crop level in the winter period is around 400 and in the summer period around 600 µmol.m^{-2}.s^{-1} (*c*.80 and 120 W/m^2 PAR, respectively). Temperature is kept at 25°C throughout the growing period. The compost used is an adjusted Begonia growth mix with a neutral pH. The structure is **(p.93)** medium-coarse with added perlite for aeration and free draining. After the generative period the above-ground plant material is collected, air dried, and the BRM separated from the stems and branches. Total cannabinoid contents (% w/w) are determined for the dry, homogenized (milled) BRM fraction. Compositions (ratios) and purities are expressed as the weight proportions (% w/w) of the individual cannabinoids in the total cannabinoid fraction.

Table 5.1 Achieved production levels of current clones representing nine different chemotypes. BRM indicates the total dry yield of leaf and floral tissue at maturity; C_{tot} is the total cannabinoid content in the BRM; purity is the proportion of the target cannabinoid in the total cannabinoid fraction; yield is the resulting quantity of the target cannabinoid produced. Performance of the propyl cannabinoid clones is still suboptimal and breeding aimed at yield improvement ongoing

Chemotype (main cannabinoid)	Clone (code)	BRM (g/m^2)	C_{tot} (%w/w)	Target cannabinoid	
				Purity (%w/w)	Yield (g/m^2)
CBG	M378	792	11.2	99.9	89
CBGV	M350	507	10.4	87.4	46
THC	M87	650	15.3	96.8	96
THCV	M264	609	14.5	81.7	72
CBD	M255	810	14.5	88.7	104
CBDV	M276	475	9.5	71.0	32
CBC	M394	731	2.9	93.4	20
CBCV	M206	283	1.8	52.6	3
Cannabinoid-free	M299	620	0.0		

5.4 Genetic determination of chemotype

5.4.1 Cannabinoid biogenesis

Cannabinoids are terpenophenolic products. The monoterpenoid precursors, predominantly geranylpyrophosphate (GPP) and to a lesser extent nerylpyrophosphate (NPP), originate from the deoxyxylulose (DOX) pathway (Fellermeier et al. 2001). The phenolic precursors (5-*n*-alkyl-resorcinolic acid homologues) are generated by the polyketide pathway (Raharjo et al. 2004). In the cannabinoid polyketide pathway, acyl-activating enzyme-1 (AAE1; Stout et al. 2012) binds coenzyme A (CoA) to different short-chain fatty acids. The most common phenolic precursor, 5-*n*-pentyl-resorcinolic acid (olivetolic acid, OA) results from the condensation of *n*-hexanoyl-CoA with three molecules of malonyl-CoA. In a two-step reaction, first a tetraketide intermediate is formed by olivetol synthase (OLS; sequenced by Taura et al. 2009), recently renamed as tetraketide synthase (TKS; Gagne et al. 2012). Subsequently, the tetraketide intermediate is cyclisized by the recently identified olivetolic acid cyclase (OAC; Gagne et al. 2012). Also the less common 5-*n*-propyl-resorcinolic acid homologue (divarinolic acid, DA) can be formed from *n*-butanoyl-CoA and three molecules of malonyl-CoA, probably by the same promiscuous enzyme system. Other resorcinolic acid alkyl homologues from C_1 through to C_7 are produced in minute quantities.

The phenolic and terpenoid moieties are subsequently condensed into terpenophenolics (cannabinoid acids) by the prenyltransferase enzyme geranylpyrophosphate:olivetolate transferase (GOT; Fellermeier and Zenk 1998). GOT was sequenced by Page and Boubakir (2011). Most commonly, geranylpyrophosphate (GPP) is condensed with OA to produce cannabigerolic acid (CBGA). With lower affinity, GOT condenses also NPP with OA to produce CBGA’s optical isomer cannabinerolic acid (Taura et al. 1995a). Based on Shoyama et al. (1984) it can be deduced that GOT is promiscuous and also accepts resorcinolic acid homologues other than OA, but probably with lower affinity and/or turnover. Incorporation of these OA alkyl homologues results in the corresponding homologues of CBGA (i.e., CBGA-C_1 through to CBGA-C_7) and cannabinerolic acid.

The variability among cannabinoid structures is mainly attributable to the incorporation of different resorcinolic acid variants. Recently however, Pollastro et al. (2011) reported on a cannabinoid prenyl variant, a “sesqui-CBGA,” which is apparently the condensation product of the sesquiterpene farnesylpyrophosphate and OA. According to Samuelsson (1999), unlike monoterpenes, these sesquiterpenes are not derived from the DOX pathway, but from the mevalonate pathway (MVA).

The various homologues of CBGA and cannabinerolic acid are the central intermediates in the cannabinoid pathway. Three different enzymatically catalyzed oxidative cyclizations lead to three categories of cannabinoid end

products: the various alkyl homologues of tetrahydrocannabinolic acid (THCA-C_5), cannabidiolic acid (CBDA-C_5), and cannabichromenic acid (CBCA-C_5). Per enzymatic conversion, CBGA and cannabinerolic acid yield the same cyclization product (Morimoto et al. 1998; Taura et al. 1996). Kinetic parameters of THCA synthase were characterized by Taura et al. (1995b) and the gene was sequenced by Sirikantaramas et al. **(p.94)** (2004); Taura et al. (1996) characterized the kinetic parameters of CBDA synthase and the gene was sequenced by Taura et al. (2007); kinetic parameters of CBCA synthase were characterized by Morimoto et al. (1998) but it remains to be sequenced.

A hydroxy-methoxy substitution reaction of the CBGA type intermediates results in cannabigerolic acid monomethyl ether (CBGAM; Shoyama et al. 1970). Most commonly occurring is the C_5 homologue CBGAM; the C_3 homologue cannabigerovarinic acid monomethyl ether (CBGVAM) is less common and other homologues occur as traces. Although obviously genetically controlled, as yet a gene/enzyme combination for this methoxylation has not been identified.

Post harvest, under the influence of heat, a nonenzymatic decarboxylation reaction takes place which results in neutral cannabinoid molecules (e.g., THCA → THC). Under the influence of UV light and the presence of oxygen these neutral structures can further degrade. Alkyl homologues of cannabinol (CBN), cannabielsoin (CBS), and cannabicyclol (CBL) are the degradants of the corresponding alkyl homologues of THC, CBD, and CBC, respectively.

The large number of possible CBGA alkyl homologues, the various parallel pathways from CBGA type structures, and the various nonenzymatic conversions together lead to a large number of compounds classified as cannabinoids. However, in wild-type *Cannabis* plants and their processed products only a few of these are found to occupy substantial proportions of the total cannabinoid fraction. These are: THCA-C_5 and its degradants THC-C_5 and CBN-C_5; THCA-C_3 (THCVA, tetrahydrocannabivarinic acid) and its degradant THC-C_3 (THCV, tetrahydrocannabivarin); CBDA-C_5 and its degradant CBD-C_5; CBCA-C_5 and its degradant CBC-C_5. All other cannabinoids are generally classified as minor.

5.4.2 A model for chemotype inheritance

The inheritance of chemotype has been investigated in the course of a long-term medicinal *Cannabis* breeding program, commenced at HortaPharm B.V. (The Netherlands) and continued at GW Pharmaceuticals (UK). A key technique in this program has been the self-fertilization of female plants after a chemically induced partial masculinization. In contrast to the natural outbreeding propagation system, this enables the creation of homozygous inbred lines, contrasting crosses between homozygous female plants and the systematic study of chemotypical segregation patterns in the cross progenies. Besides production clones of different chemotype (Table 5.1), the program has also resulted in a genetic model for the regulation and inheritance of chemotype (Fig. 5.2).

Evidence for this model has been published by de Meijer et al. (2003, 2009a, 2009b) and de Meijer and Hammond (2005).

The formation of the phenolic moieties incorporated in cannabinoids (resorcinolic acids) can be obstructed by a monogenic factor. In the homozygous state, this factor induces a cannabinoid-free chemotype (de Meijer et al. 2009b). We postulated a single locus "O" with a mutant null allele *o* that blocks the resorcinol synthesis and a functional wild-type allele *O* that does not interfere. The null allele has a strong but incomplete dominance over the functional one. In segregating progenies, the *O*/*o* genotypes have only one-tenth of the cannabinoid content of *O*/*O* genotypes. The dominance of the knockout factor reflects the nature of a dominant repressor of a pathway gene rather than a fatal mutation in a structural pathway gene itself.

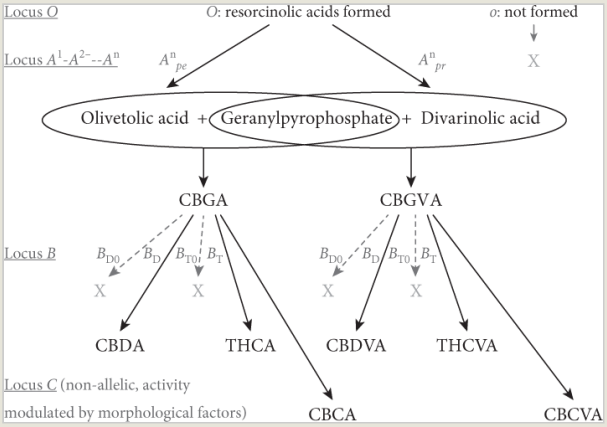


Fig. 5.2 A genetic model for chemotype regulation. Locus *O* determines if cannabinoids are formed. The multiple locus *A* determines the alkyl homologue ratio. Wild-type alleles at locus *B* control the ratios CBDA:THCA and/or CBDVA:THCVA whereas mutant alleles induce CBGA and/or CBGVA accumulation. Locus *C* is fixed but its chemotypical effect can be strongly modulated by morphological factors.

A postulated multiple locus "*A*" determines which of the resorcinolic acids is formed, olivetolic acid and/or divarinolic acid. Ongoing breeding experiments (unpublished) strongly suggest that this genetic factor is oligo- or polygenic with locus *A* carrying alleles $A_{pe}{}^{1\text{ to n}}$ and $A_{pr}{}^{1\text{ to n}}$. The $A_{pe}{}^{1\text{ to n}}$ alleles encode for the more common olivetolic acid synthesis and the subsequent formation of cannabinoids with a pentyl side chain. The $A_{pr}{}^{1\text{ to n}}$ alleles encode for the less common **(p.95)** divarinolic acid synthesis and the subsequent formation of propyl cannabinoids. The codominant *A* alleles contribute additively but not equally to the chemotype (propyl:pentyl cannabinoid ratio); some having major, and others minor effects. Cannabinoid alkyl homologues other than the propyl- and pentyl ones do occur (C_1 through to C_7 homologues have been detected in *Cannabis* extracts). So far these homologues have only been detected in insignificant proportions and therefore the corresponding pathways are not covered by the model.

Olivetolic acid and divarinolic acid condense with geranylpyrophosphate into CBGA and cannabigerovarinic acid (CBGVA) respectively. There are no signs of allelism at this level, the enzyme GOT appears to be promiscuous and prenylates resorcinolic acids regardless of the alkyl side chain length. In spite of GOT's promiscuity for resorcinolic acid substrates, experiments by Shoyama et al. (1984) suggest that the enzyme's substrate affinity might be differential, with a preference for the C_5 homologue. CBGA and CBGVA are classified as true cannabinoids and form the substrates for a number of enzymatic conversions into cannabinoid end products: CBGA is converted into THCA, CBDA, CBCA, and CBGAM, respectively; CBGVA into THCVA, CBDVA (cannabidivarinic acid), CBCVA (cannabichromevarinic acid), and CBGVAM, respectively.

A monogenic locus "*B*" that controls the conversions of CBGA/CBGVA into THCA/THCVA (allele B_T) and CBDA/CBDVA (allele B_D) regardless of the alkyl side chain is postulated (de Meijer et al. 2003). Alleles B_T and B_D are codominant, i.e., heterozygous individuals (genotype B_T/B_D) express a chemotype composed of substantial proportions of both THCA/THCVA and CBDA/CBDVA. The ratios CBDA:THCA and CBDVA:THCVA are highly progeny specific and can deviate strongly from 1/1. This has been attributed to sequence variation in the B_T and B_D alleles, leading to synthases with differential catalytic properties. At the extremes of the locus *B* allelic range we find recessive, minimally functional, and nonfunctional alleles. In the homozygous state these induce a chemotype characterized by a high proportion of the accumulated precursor CBGA and/or CBGVA (de Meijer and Hammond 2005). Two of such alleles have been **(p.96) (p.97)** found in the form of B_D mutants and are indicated as $B_{D0}{}^1$ and $B_{D0}{}^2$. A B_T mutant, indicated as $B_{T0,}$ has subsequently been found. It also induces a substantial CBGA and/or CBGVA accumulation along with a minimal THCA/THCVA production (unpublished data).

Independently of the THCA and CBDA synthase genes, a locus "*C*" regulates the conversion of CBGA/CBGVA into CBCA/CBCVA (de Meijer et al. 2009a). Locus *C* is fixed; it shows no allelism. Nevertheless, *Cannabis* chemotypes can vary greatly in the proportion of CBCA/CBCVA that they contain. The ontogenetic (developmental) variation in CBCA proportion has been commonly observed (e.g., Morimoto et al. 1997, 1998; Rowan and Fairbairn 1977; Shoyama et al. 1975). Apparently, CBCA synthase best competes with THCA synthase and CBDA synthase for the common CBGA/CBGVA substrate in the early juvenile stage. It would be problematic to exploit this feature for commercial CBCA or CBCVA production but, as an alternative strategy, we found different morphological mutations (reflecting underlying mono- and polygenic mechanisms) that enhance the activity of CBCA synthase throughout the life cycle of the plant. These mutations have in common the reduction of the presence of stalked glandular trichomes to the advantage of sessile trichomes (Fig. 5.3E) and are indicated as "PJC" genes (prolonged juvenile chemotype) in our model. The common "wild-

type” status, not inducing this prolonged juvenile chemotype, is referred to as “pjc.”

A fourth conversion, the methoxylation of CBGA and CBGVA results in the monomethyl ethers CBGAM and CBGVAM, respectively (Shoyama 1970). These compounds are not very prominent in the cannabinoid profile. Small and Beckstead (1973) reported the consistent presence of small amounts of CBGAM in plants from north-eastern Asia. We found that the presence of methoxylated cannabinoids is irregular but obviously inheritable. The methoxylation of CBGA and CBGVA does not appear to be controlled by the loci *B* and *C*. We found CBGAM and CBGVAM proportions up to 5% of the total cannabinoid fraction of certain lineages and hypothesized that such plants carry an active allele *M* in the homozygous state at a locus *M*, whereas plants devoid of these compounds carry the wild-type, inactive allele *m*. A breeding experiment aimed at the clarification and possible utilization of this mechanism has recently commenced and the role of CBGAM and CBGVAM in chemotypes will not be addressed further.

Obviously there is a gap between a genetic model that predicts and explains the outcome of breeding experiments and the actual events at the molecular level. Increasingly the different chemotypes are being investigated in transcriptome and gene expression studies which further clarify the mechanisms of chemotype regulation. For example, the powerful effect of the cannabinoid knockout factor at the monogenic locus *O* in heterozygous individuals was initially hard to explain. Recently it was found that the OLS (TKS) gene sequence of cannabinoid-free plants is identical to the wild-type sequence but that the gene is not expressed, probably due to a dominant monogenic repressor (unpublished data). In addition, the hypothesis that the accumulation of CBGA and CBGVA is due to normally expressed but minimally functional and nonfunctional alleles at locus *B*, is now supported by transcriptome analysis. Our CBGA/CBGVA-rich plants were found to express sequence variants of THCA and CBDA synthase, with radical amino acid substitutions in the conserved domains (unpublished data).

5.5 Results of chemotype breeding

5.5.1 Chemotype breeding

Chemotype manipulation is a target in the context of fiber/seed hemp breeding (suppression of THCA content), recreational drug breeding (high THCA content), and pharmaceutical drug breeding (various cannabinoid profiles). The most common chemotypes are CBDA and THCA predominant and can be encountered in all crop groups. Other, more specific chemotypes result from **(p.98)** breeding programs such as the one initiated at HortaPharm and continued at GW, committed to increasing the purity and content of a range of different cannabinoids for commercial development. At an early stage in this program, a key technique allowing mass-scale self-fertilization and mutual crossing of female plants was developed. Source materials of various provenances and their first inbred generations were screened through gas chromatographic (GC)

analyses. Selected progenitor genotypes, often with deviant profiles, were preserved in seed collections and clone libraries and used for line selection to obtain true-breeding (homozygous) inbred lines. Novel, recombinant cannabinoid profiles were established by crossing homozygous materials with different pure profiles, followed by self-fertilization. The newly inbred parental clones were then added to the library and, per chemotype, mutually crossed, in order to produce vigorous heterotic hybrids for production.

5.5.2 Currently available pure chemotypes

5.5.2.1 THCA-predominant chemotype

THCA predominance can be considered as a “wild-type” condition. In terms of the genetic model, it results from wild-type alleles at the loci *O*, *A* and *B* and a wild-type status (pjc) at the loci that induce the morphological features associated with prolonged CBCA catalysis: O/O-$A_{pe}{}^{1\text{ to }n}$ / $A_{pe}{}^{1\text{ to }n}$-B_T/B_T-pjc_{mono}/ pjc_{mono}-pjc_{poly}. THCA predominance is not exclusively associated with drug strains. Individuals of drug type landraces can be CBDA predominant or show a mixed CBDA/THCA profile, whereas certain fiber hemp strains of Far-Eastern provenance often comprise THCA-predominant individuals. Common relationships between cannabinoid chemotype and fiber yield or quality parameters are artificial and by no means natural. The purity of THCA, i.e., its proportion in % w/w in the total cannabinoid fraction reaches levels of 96–98%, with a residual fraction composed of traces of THCVA, CBCA, and CBGA. Modern, specifically

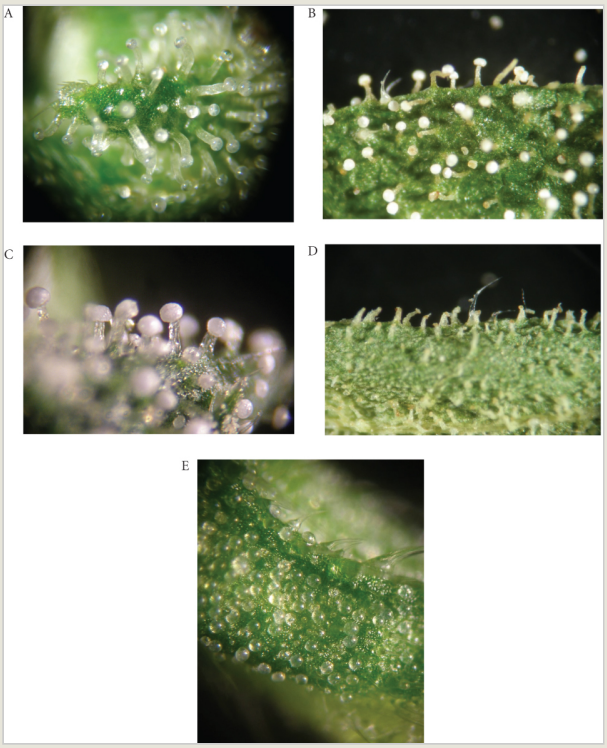


Fig. 5.3 (See also Color Plate 5.) Glandular trichomes associated to different chemotypes. (A) CBDA- and/or THCA-predominant plants carry stalked trichomes with large transparent heads. CBGA-predominant clones with underlying $B_{D0}{}^2/B_{D0}{}^2$ (B) and B_{T0}/B_{T0} (C) genotype both show white opaque trichome heads. (D) Cannabinoid-free chemotypes carry trichomes with shriveled heads. (E) Optimized CBCA-predominant clones lack stalked trichomes and show a high density of sessile trichomes.

bred THCA-predominant drug clones express total cannabinoid contents up to 25–30% w/w of the dry, “manicured” inflorescences. The total cannabinoid content of THCA-predominant drug landrace materials and fiber strains is much lower, 2–5% and <2%, respectively.

In marijuana strains, even landraces (e.g., from Thailand and South Africa), locus *B* has usually reached a fixed homozygous status (B_T/B_T) resulting in populations that are entirely composed of THCA-predominant individuals. In contrast, traditional hashish landraces (e.g., from Morocco, Pakistan, Afghanistan) are usually polymorphic at locus *B* and comprise THCA- and CBDA-predominant and -intermediate individuals. A plausible explanation for this difference is the fact that marijuana (dried inflorescences) is still an intact and recognizable tissue which allows seed retention. This enables generation from individuals selected for an appreciated smoking quality, i.e., high THCA purity. With a monogenic factor inducing a desirable phenotype, it is quite simple to select against the undesired allele (B_D) and create a fixed homozygous population. Hashish is traditionally collected in the field as a bulk crop extract and post harvest, when the overall quality is assessed, it is no longer possible to select the seeds from particular plants.

Numerous THCA-predominant clones and seed progenies circulate on the recreational market. Due to their illicit nature, these materials are not formally registered so their identity and stability are not guaranteed. The many different names (e.g., Haze, Skunk, Northern Lights, White Widow) cannot be considered as unequivocal cultivar names. They refer to more or less coherent groups, all THCA predominant, but with differences in the terpene entourage, morphology, phenological development, photoperiod requirements for flower induction, etc. A small number of THCA clones have been through the Plant Breeders Rights registration procedure and received European Breeders Rights. Examples are the cultivar “Medisins” (HortaPharm) and GW’s clones used for the raw material production of Sativex®. **(p.99)**

5.5.2.2 CBDA-predominant chemotype

As with THCA predominance, CBDA predominance is also a common wild-type condition. It results from wild-type alleles at the loci *O*, *A*, and *B* and a wild-type (pjc) status at the loci that induce the morphological features associated with prolonged CBCA catalysis: $O/O\text{-}A_{pe}{}^{1\text{ to }n}/A_{pe}{}^{1\text{ to }n}\text{-}B_D/B_D\text{-pjc}_{mono}/\text{pjc}_{mono}\text{-pjc}_{poly}$. CBDA predominance is common and usually fixed in modern hemp fiber and seed cultivars. It is common, but not usually fixed, in fiber and hashish landraces. The purity of CBDA, i.e., the proportion in % w/w in the total cannabinoid fraction reaches levels of 85–90%, with a residual fraction composed of CBDVA, CBCA, CBGAM, THCA, and CBGA. In specially bred CBDA drug clones the total cannabinoid content reaches levels as in THCA clones: up to 25–30% w/w of the dry, “manicured” inflorescences. In hashish landraces and fiber strains, individuals reach much lower levels of 1–5%. The consistent

presence of a 4–5% w/w proportion of THCA in the total cannabinoid fraction of CBDA-predominant plants is intriguing but has rarely been addressed in the literature. Lydon and Teramura (1987) ruled out CBDA as photochemically converted into THCA. Possibly, besides the main cyclization of CBGA to CBDA, CBDA synthase might be able to perform a second minor conversion of CBGA to THCA. Evolutionarily this would indicate that the CBDA synthase gene has evolved from an ancestral THCA synthase gene (vice versa, THCA-predominant plants contain practically no CBDA). Alternatively, this THCA could be the product of “inactive” THCA synthase homologues that were reported by Kojoma et al. (2006) for CBDA-predominant and -intermediate CBDA/THCA chemotypes. However, these homologues were *c*.40 SNPs (single nucleotide polymorphisms) different from the active sequence published by Sirikantaramas et al. (2004), so it is questionable if they really have retained any catalytic ability. Furthermore, the small amount of THCA in CBDA plants occurs invariably in the form of both the cis and the trans isomer in a 1:3 ratio, whereas in true THCA-predominant plants solely the trans isomer is found (A. Sutton, personal communication). Although not a perfect 1:1 racemic ratio, this finding suggests as a third possibility that a nonenzymatic reaction occurs in these plants. A possible approach to clarify this issue could be an in vitro assay with each of the heterologously expressed proteins of the THCA and CBDA synthase sequence variants. Practically, the consistent presence of some THCA in CBDA-predominant chemotypes can be problematic in the case of fiber and seed cultivars with a relatively high overall cannabinoid content. For these, a limit of 0.2% THC is legally enforced in the European Union (EU). As THCA occupies 4–5% of the cannabinoid fraction of a CBDA-predominant chemotype this limit will be reached at a total cannabinoid content of 4% w/w. In CBD-rich pharmaceutical extracts too, the associated presence of psychoactive THC can be undesirable.

Around 60 fiber cultivars with a CBDA-predominant chemotype are registered in the EU. Plants Breeders Rights have also been obtained for GW’s CBDA-rich clones used for the raw material production of Sativex®.

5.5.2.3 CBGA-predominant chemotypes

Fournier et al. (1987) were the first to report on a CBGA-rich plant in a normally CBDA-predominant French fiber hemp population. Accumulation of this otherwise minor compound is a mutant condition induced by an absence of sufficiently active THCA and/or CBDA synthase. According to the genetic model, CBGA predominance results from wild-type alleles at the loci *O* and *A*, mutant (“null”) alleles at locus *B* (de Meijer and Hammond 2005) and a wild-type status (pjc) at the loci that induce the morphological features associated with prolonged CBCA catalysis: $O/O\text{-}A_{\mathrm{pe}}{}^{1\ \mathrm{to\ n}}/A_{\mathrm{pe}}{}^{1\ \mathrm{to\ n}}\text{-}B_0/B_0\text{-pjc}_{\mathrm{mono}}/\mathrm{pjc}_{\mathrm{mono}}\text{-pjc}_{\mathrm{poly}}$. We have obtained two different CBGA-predominant chemotypes with a residual presence of CBDA from fiber hemp source populations. The inbred generation of a marijuana clone revealed a second CBGA-rich chemotype with a **(p.100)**

residual presence of THCA. It was postulated that the first category is attributable to minimally functional CBDA synthase (alleles $B_{D0}{}^{1}$ and $B_{D0}{}^{2}$, which was subsequently found), and the second category to minimally functional THCA synthase (allele B_{T0}). The purity of CBGA-predominant plants can vary, depending on the impact of the mutation involved. Genotypes that are homozygous for the alleles $B_{D0}{}^{1}$, $B_{D0}{}^{2}$, and B_{T0}, typically express CBGA proportions of 90%, nearly 100%, and 85% respectively, in the cannabinoid fraction. Regardless of the underlying genotype, all CBGA-rich chemotypes share the morphological feature of white opaque glandular trichome heads (Fig. 5.3B and C). It is remarkable that Gorshkova et al. (1988) characterized this same morphological phenotype as indicative of an absence of cannabinoids. There is no obvious inhibitory feedback in cannabinoid metabolism if the normal end products CBDA and/or THCA are absent or are only poorly formed. After some committed breeding effort the absolute cannabinoid contents of CBGA-predominant plants now reach levels similar to high-content THCA and CBDA plants (Table 5.1). In 2003, Plant Breeders Rights were obtained for the Italian, CBGA-rich fiber hemp cultivar Carma. The average CBGA proportion in the cannabinoid fraction of this cultivar is *c*.55%, with a residual fraction of mainly CBDA (G. Grassi, personal communication).

5.5.2.4 CBCA-rich and -predominant chemotypes

CBCA is often considered a minor cannabinoid and usually occurs only in proportions of 0–5% in the cannabinoid fraction of most mature *Cannabis* plants of all chemotypes. It is more prominent in juvenile profiles (e.g., Morimoto et al. 1997, 1998; Rowan and Fairbairn 1977; Shoyama et al. 1975). Morphological mutants were found in Afghan hashish and Korean fiber landraces that maintain somewhat higher proportions of CBCA (15–30% of the cannabinoid fraction) throughout the course of the life cycle (de Meijer et al. 2009b). These mutations have in common the suppression of the formation of bracts and bracteoles and thereby, that of stalked glandular trichomes. This leads to a relative abundance of sessile trichomes on the floral tissues (Fig. 5.3E). Besides these inheritable morphological factors we have never found any indication that the variation in CBCA content is attributable to allelism at a “biochemical locus” encoding an active and an inactive CBCA synthase (de Meijer et al. 2009b). The breeding strategy to obtain pure CBCA plants was therefore based on “stacking” the different morphological mutations and obstructing the competitive pathways from CBGA to THCA and/or CBDA. This was realized by establishing a $B_{D0}{}^{2}/B_{D0}{}^{2}$ genotype at locus *B*. In selected clones, CBCA purities at maturity of up to 95% of the cannabinoid fraction were achieved, with CBCA-C_1, CBCA-C_3, cannabicyclol (CBL, a CBCA degradant), THCA (cis and trans isomers), and CBGA as additional trace compounds. In terms of the genetic model, these optimized clones have the genotype: O/O-$A_{pe}{}^{1\ to\ n}/A_{pe}{}^{1\ to\ n}$- $B_{D0}{}^{2}/B_{D0}{}^{2}$ -PJC_{mono}/ PJC_{mono}-PJC_{poly}. As an inherent effect of the absence of bracts and bracteoles that carry the highly productive stalked trichomes, such plants can only attain a

relatively low overall cannabinoid content. So far, the maximum content in a dry homogenized BRM appears to be 3–3.5% w/w.

Unlike other chemotypes, the CBCA-rich chemotype shows a certain sensitivity to its environment. At high light intensities, the total cannabinoid content may increase at the expense of the CBCA purity (de Meijer et al. 2009a). This is probably due to the fact that CBCA synthase quickly reaches its catalytic maximum and then leaves a surplus of the CBGA substrate unconverted.

CBCA-rich clones have not been submitted for Plant Breeders Rights but the chemotype is categorically protected by a patent (WO2009/125198).

5.5.2.5 THCVA- and other propyl cannabinoid-rich chemotypes

Generally, propyl cannabinoids occur in low proportions (< 2%) of the total cannabinoid fraction. In situ, THCVA appears the only compound that is occasionally found in more substantial **(p.101)** proportions. It can reach various levels, up to 70% of the cannabinoid fraction, in plants from populations belonging to different crop-use groups. These often originate in China (fiber and seed landraces) and Southern Africa (marijuana landraces). In terms of the genetic model, THCVA-rich plants carry a number of A_{pr} alleles at the multiple locus A and are homozygous B_T/B_T at locus B. Geographical isolation is a possible explanation of why in situ the A_{pr} alleles do **(p.102)** not usually occur in combination with a B_D/B_D and B_0/B_0 genotype or with the morphological PJC factors in order to produce CBDVA-, CBGVA-, or CBCVA-rich chemotypes, respectively. In our breeding program we were successful in producing CBDVA- and CBGVA-predominant clones whereas the breeding of CBCVA-predominant plants is still in an early stage. Further improvement of the propyl cannabinoid purity in these chemotypes is ongoing and promising. For THCVA, a level of over 92% of the cannabinoid fraction has already been achieved by stacking A_{pr} alleles from different progenitors in hybrid offspring (Fig. 5.4C). THCA is the main residual cannabinoid in THCVA-predominant plants and its presence is undesirable as it requires chemical purification to avoid the presence of psychoactive, and possibly THCV-counteracting, THC in THCV-based medicines. The purities currently achieved for CBGVA, CBDVA, and CBCVA are **(p.103)** 87%, 73%, and 76% and in chemotypes with these compounds, the corresponding pentyl homologues also form the main residual cannabinoid (Fig. 5.4B, D, and F). The characteristic trichome morphology of CBGA- and CBCA-predominant chemotypes (Fig. 5.3B, C, and E) is also associated to the CBGVA- and CBCVA-predominant chemotypes.

The molecular basis of the C_3/C_5 regulation at the postulated locus *A* remains to be clarified. We compared flower transcriptomes of C_3-rich- and pure C_5 segregant bulks of segregating, single plant progenies. In the phenolic pathway, meaningful variation in gene sequence or gene expression was not found at the level of the candidate genes AAE1, TKS, and OAC (data unpublished). This suggests that the C_3:C_5 ratio is regulated by still unknown genes involved in the production of the short-chain fatty acid precursors: butanoate and hexanoate. **(p. 104)**

Currently, no Plant Breeders Rights or patents are known for THCVA- or other propyl-rich materials.

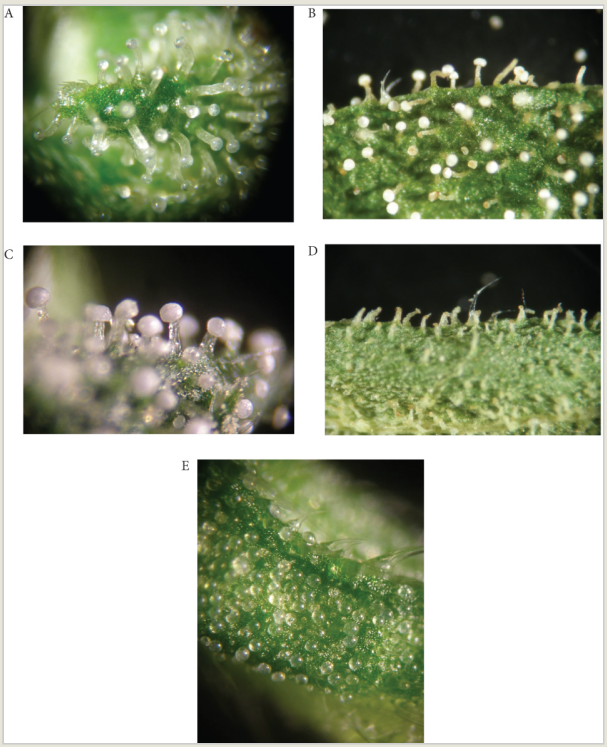


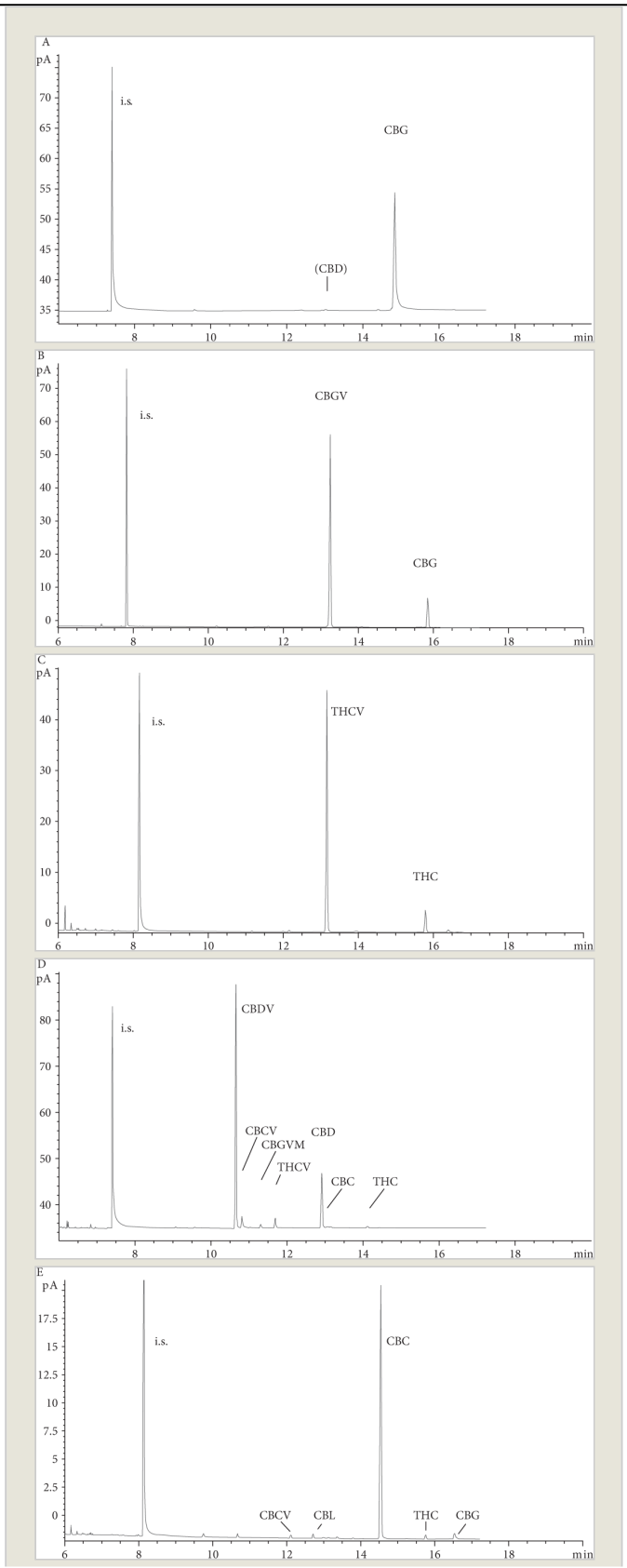
Plate 5 (See also Fig. 5.3.) Glandular trichomes associated to different chemotypes. (A) CBDA- and/or THCA-predominant plants carry stalked trichomes with large transparent heads. CBGA-predominant clones with underlying $B_{D0}{}^2/B_{D0}{}^2$ (B) and B_{T0}/B_{T0} (C) genotype both show white opaque trichome heads. (D) Cannabinoid-free chemotypes carry trichomes with shriveled heads. (E) Optimized CBCA predominant clones lack stalked trichomes and show a high density of sessile trichomes.

5.5.2.6 Cannabinoid-free chemotype

Our cannabinoid-free chemotype appears to be the consequence of an obstacle in the phenolic pathway towards the resorcinolic acids (de Meijer et al. 2009b). In terms of our genetic model, these plants carry a mutant allele in the homozygous state at the single locus *O*. It is conceivable that other mechanisms also induce an absence of cannabinoids. Gorshkova et al. (1988) reported on a cannabinoid-free chemotype attributable to a total absence of glandular trichomes. Nonfunctional trichomes, an obstacle in the terpenoid pathway towards geranylpyrophosphate or a mutation in GOT, thereby disabling the terpeno-phenolic condensation, would also obstruct cannabinoid production, but as yet there are no reports on cannabinoid-free chemotypes induced by such mechanisms.

The cannabinoid knockout gene expressed in our clones was derived from a low-cannabinoid-content fiber cultivar. In certain specimens of this cultivar there were no detectable cannabinoids. When crossed with high-content plants, an F_1 with low cannabinoid levels (ca 1/10 of that of the high-content parent) was produced, whereas F_2 generations obtained from self-fertilized F_1 individuals segregated in three discrete chemotypes: cannabinoid-free, low-content (as F_1), and high-content individuals in a monogenic 1:2:1 ratio. The severe reduction in the cannabinoid content of the heterozygous groups suggests that the knockout factor is not a mutated structural pathway gene. It is more likely to be a regulator, in this case probably a dominant repressor of OLS/TKS (F. Licausi, unpublished data). Repeated backcrossing of the first-generation cannabinoid-free plants with high-content materials has resulted in a range of cannabinoid-free plants with a dense, branched habitus, high trichome density, and a strong fragrance. Knockout homologues with a strong resemblance to the Sativex® THCA and CBDA clones (Fig. 5.5) plus a series of cannabinoid-free clones predominant in each one of the monoterpenes: pinene, myrcene, terpinolene, carene, and limonene, have all been bred through backcrossing. The fact that these clones contain terpenes (mono- and sesqui-) in normal quantities, demonstrates that neither the **(p.105)** **(p.106)** terpenoid pathway nor the functionality of the trichomes is affected by the knockout factor. Such materials could play a role in clarifying cannabinoid-terpenoid interactions (“entourage effects”; Russo 2011). The incorporation of purified cannabinoids (or combinations thereof) into various cannabinoid-free BRMs would enable a systematic study of the possible differential physiological effects of pure cannabinoids versus cannabinoids extracts. Another obvious application of the knockout factor is in breeding cannabinoid-free fiber hemp and seed hemp. The absence of the usual terpenophenolic end products together with the presence of most of the pathway enzymes has made our cannabinoid-free plants useful as chemical-analytical reference material and as crude enzyme sources for in vitro assays.

Patent protection has been obtained for cannabinoid-free clones and their use as a reference plant (WO2008/146006).



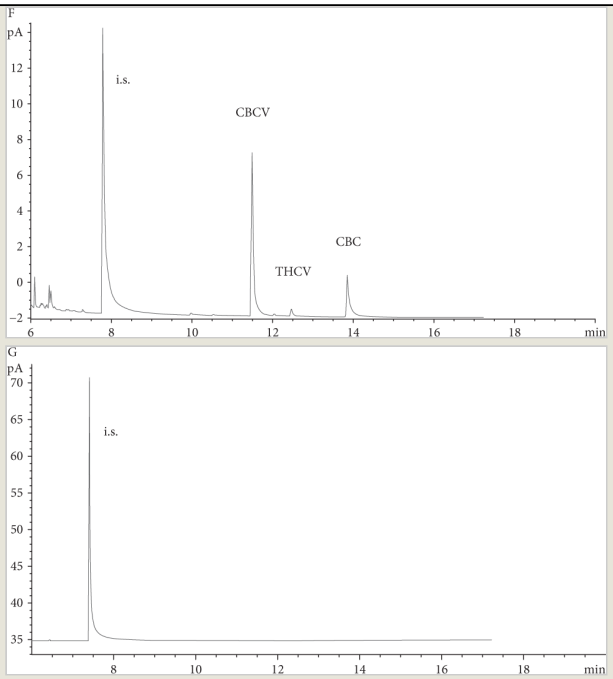


Fig. 5.4 Chromatograms of unusual chemotypes obtained using a gas chromatograph with flame ionization detector (GC-FID). Peaks indicate cannabinoids in decarboxylated form. Data originate from different GC runs and between chromatograms retention times cannot be compared. I.s. = added internal standard. (A) clone M281, CBG purity ≤ 99.9%; (B) clone M350, CBGV purity 87%; (C) clone M408, THCV purity 92%; (D) clone M277, CBDV purity 73%; (E) clone M394, CBC purity 95%; (F) seedling 2012.16.3.26.6, CBCV purity 76%; (G) clone M299, cannabinoid-free.

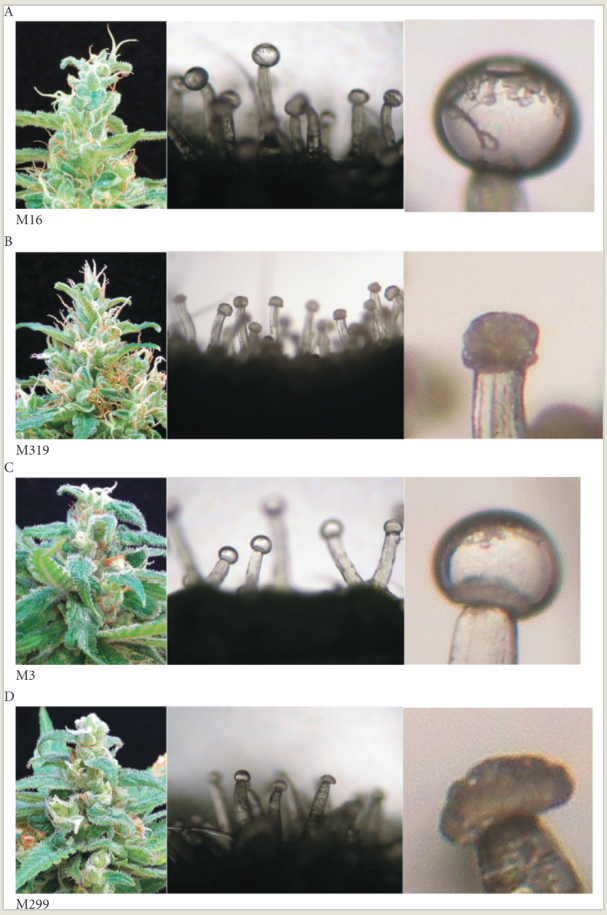


Fig. 5.5 (See also Color Plate 6.) Macro- and microscopic photos of clones used for Sativex® raw material production, M16 (CBD) and M3 (THC), and their respective cannabinoid-free homologues M319 and M299. The homologues were selected from backcross progenies (e.g., M299 = M3 × (M3 × (M3 × knockout progenitor))) and share 87.5% genetic identity with the corresponding "original."

5.5.3 Chemotype and evolutionary fitness

Cannabinoids can reach extremely high concentrations in above-ground plant tissues. Various theories, all relating to the defense against biotic and abiotic stress, attribute ecological benefits to the presence of cannabinoids in general, or to the presence of certain cannabinoids in particular (Appendino et al. 2008; Lydon et al. 1987; Morimoto et al. 2007; Pate 1983). Our breeding experiments and the crop production of different chemotypes take place in a protected indoor environment, but they should still reveal some relationship between chemotype and fitness, if it does exist. The chemotype segregating progenies obtained from a single self-fertilized parent, where all individuals are highly related sister plants, are particularly suitable to compare the strengths or susceptibilities of contrasting chemotypes. However, compelling associations between chemotype (including the cannabinoid-free plants) and features such as seed set, plant size, and infestation with insects or fungi have so far not been observed.

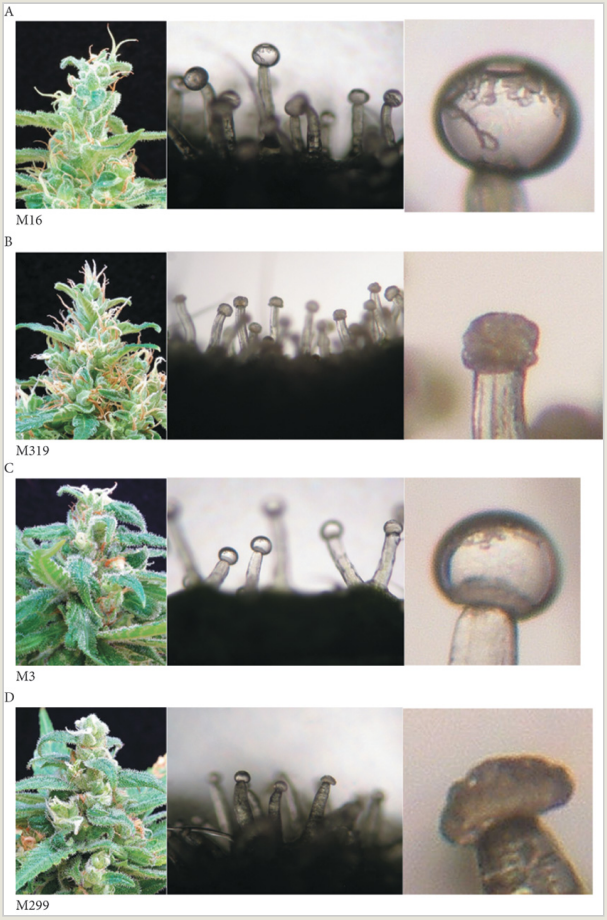


Plate 6 (See also Fig. 5.5.) Macro- and microscopic photos of clones used for Sativex® raw material production, M16 (CBD) and M3 (THC), and their respective cannabinoid-free homologues M319 and M299. The homologues were selected from backcross progenies (e.g., M299 = M3 × (M3 × (M3 × knockout progenitor))) and share 87.5% genetic identity with the corresponding "original."

5.6 Molecular studies of chemotype regulation

5.6.1 Historic overview

A more molecular approach to the underlying genetics of *Cannabis* chemotype perhaps commenced with the in vitro testing of crude *Cannabis* enzyme extracts (e.g., Shoyama et al. 1984). This was followed by the purification and characterization of the important pathway enzymes THCA synthase, CBDA

synthase, and CBCA synthase by Taura et al. (1995b, 1996) and Morimoto et al. (1998), respectively. THCA synthase and CBDA synthase were also the first pathway genes to be sequenced, by Sirikantaramas et al. (2004) and Taura et al. (2007), respectively. THCA synthase is polymorphic and Kojoma et al. (2006) published sequence variants found in plants with a THCA-predominant, CBDA-predominant, and mixed THCA/CBDA chemotype. A number of these sequence variants encodes an active synthase whereas, in the CBDA-predominant plants, only inactive or minimally active THCA sequences occur, with many amino acid substitutions compared to the active ones. Different molecular markers (PCR products) associated with CBDA or THCA synthase sequences have been developed (e.g., de Meijer et al. 2003; Kojoma et al. 2006; Pacifico et al. 2006; Rotherham and Harbison 2010). Very recently, Shoyama et al. (2012) elucidated the structure–function relationship of the active THCA synthase protein.

One step upstream in the pathway, the prenyl transferase GOT, that catalyzes the condensation of the resorcinolic acids with geranylpyrophosphate into CBGA type products, was sequenced by Page and Boubakir (2011). **(p.107)**

Further upstream in the phenolic pathway, three crucial genes for the *n*-alkyl-resorcinolic acid synthesis have now been sequenced. Stout et al. (2012) identified the acyl activating enzyme AAE1 that binds coenzyme-A to the short-chain fatty acids hexanoate, butanoate, and malonate. The process of the condensation of the fatty acid-CoA substrates into resorcinolic acids by a polyketide type mechanism has long been unclear, but was recently clarified by Gagne et al. 2012. For this two-step reaction both a TKS and an OAC are required. TKS had already been sequenced by Taura et al. (2009) under the name olivetol synthase (OLS). OAC was sequenced by Gagne et al. (2012). In 2011 the first entire draft genomes for *Cannabis*, based on two different THCA-predominant drug strains, were published by McKernan et al. (see http://www.medicinalgenomics.com). Van Bakel et al. (2011) published the transcriptomes (expressed genes) of various tissues of a high-content THCA drug strain and a low-content CBDA-predominant oil seed cultivar as well as the draft genomes of these accessions and a low-content fiber cultivar.

Although the cannabinoid pathways have now largely been elucidated, there are still some important issues to be resolved: the CBCA synthase sequence, the regulation of the methoxylation of CBGA type structures into monomethyl ethers, and the mechanism of the cannabinoid alkyl side chain regulation. In order to study these topics, F_2 progenies, obtained from self-fertilized parents which segregate the relevant chemotypes, would be more promising plant materials than unrelated drug and fiber strains.

5.6.2 Prospects for novel chemotype breeding

As has been presented here, conventional plant breeding, with the inclusion of the cannabinoid knockout, has resulted in nine fairly pure chemotypes. Additionally, an increase in the proportions of CBGA type monomethyl ethers (CBGAM and CBGVAM) might be possible. Since the conventional approach appears to have reached its limit, it is opportune to explore the prospects of molecular techniques for a further expansion of the chemotype portfolio. With cannabinoid contents up to 25–30% of the floral dry weight already achieved, there is no urgency to improve the quantitative aspects of chemotype, a situation quite different from that with plants producing opiates, artemisin, taxol, etc. Innovations such as (1) chemotypes containing substantial proportions of truly novel terpenophenolic compounds (e.g., with branched alkyl- or aromatic side chains); (2) chemotypes with dramatically increased proportions of known, but currently very minor substances (e.g., rich in methyl- or butyl cannabinoids, rich in farnesyl cannabinoids); (3) chemotypes lacking naturally associated, undesirable compounds (e.g., CBDA-predominant plants devoid of THCA) would be more valuable. Page and Boubakir (2011) mention the possibility of mutating their sequenced prenyltransferase gene (GOT) in order to enhance cannabinoid production or to obtain cannabinoid-free plants by gene inactivation. Van Bakel et al. (2011) speculate that the identification of candidate pathway genes may eventually result in the development of cannabinoid-free hemp cultivars and CBCA-rich materials. McKernan et al. (2011, http://www.medicinalgenomics.com) hope that their draft genome, once further annotated, will help to enhance the expression of some, currently minor, cannabinoids. These ambitions are remarkably modest and largely aimed at existing chemotypes.

Modification of the phenolic pathway achieved by exploiting recent advances in *Cannabis* molecular biology could be an interesting new direction. Currently available homologues with the same aromatic structure but a different alkyl side chain, such as THC and THCV, can show totally different activity. The *Cannabis* gene(s) regulating the C_3:C_5 cannabinoid ratio, once identified, would be an interesting target for site-specific mutagenesis. This may lead to a substantial production of resorcinolic acids with alkyl chains other than propyl or pentyl. Alternatively, there **(p.108)** might be a transgenic option to modify this pathway. Some other plant species do produce resorcinols, resorcinolic acids, and even terpenophenolic acids, often in a different form from that of *Cannabis*. It is likely that such plants carry genes homologous to certain cannabinoid pathway genes. Transfer to, and heterologous expression of, such genes in *Cannabis* may result in variant precursors and products. One uncertainty with both site-specific mutagenesis and heterologous expression is that the desired incorporation of alternative precursors into cannabinoid end products requires substrate promiscuity of the enzymes downstream. Shoyama et al. (2012) suggest that their structure–function study of THCA synthase allows the

development of mutants with altered substrate preference or catalytic activity. This could lead to novel THCA derivatives. In the plant, suitable substrates other than CBGA and cannabinerolic acid homologues do not occur naturally, but they could result from an artificially modified phenolic pathway. Altered catalytic activity of THCA (and CBDA) synthase would also be of practical interest were it to result in new cyclization products.

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