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The inheritance of chemical phenotype in *Cannabis sativa* L. (II): Cannabigerol predominant plants

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Summary

This paper aims to clarify the genetic mechanism that is responsible for the accumulation of cannabigerol (CBG) in certain phenotypes of *Cannabis sativa* L. CBG is the direct precursor of the cannabinoids CBD, THC and CBC. Plants strongly predominant in CBG have been found in different fibre hemp accessions. Inbred offspring derived from one such individual were crossed with true breeding THC predominant- and CBD predominant plants, respectively. The segregations in the cross progenies indicate that CBG accumulation is due to the homozygous presence of a minimally functional allele, tentatively called B_0, at the single locus *B* that normally controls the conversion of CBG into THC (allele B_T) and/or CBD (allele B_D). The fact that CBG accumulating plants have so far been found in European fibre hemp populations that are generally composed of B_D/B_D plants, and the observation that the here investigated B_0 allele possesses a residual ability to convert small amounts of CBG into CBD, make it plausible that this B_0 is a mutation of normally functional B_D. Therefore, B_0 is considered as a member of the B_D allelic series encoding a CBD synthase isoform with greatly weakened substrate affinity and/or catalytic capacity.

Introduction

Chemotypical diversity in Cannabis with reference to cannabigerol

Quantitative and qualitative aspects of cannabinoid accumulation together determine the chemical phenotype, or chemotype, of *Cannabis* (Hillig, 2002; Mandolino, 2004). To discriminate between these distinct aspects it is adequate to consider the yield of a certain cannabinoid as a complex trait (de Meijer et al., 2003). Quantitative components such as the total amount of dry biomass, the proportion of floral tissue and the total cannabinoid content in the floral tissue are polygenic, not related to specific metabolic pathways and are heavily affected by the environment. In contrast, the cannabinoid composition strictly depends on the metabolic pathways followed by the plant to convert common precursors into specific end-products. This paper focuses on chemotype in the qualitative sense of the proportions of the pertinent cannabinoids within the total cannabinoid fraction.

Cannabigerol (CBG) commonly occurs as a minor compound in proportions of up to 10% of the cannabinoid fraction (unpublished data). In contrast with this frequent presence of small proportions of CBG, Fournier et al. (1987) reported on a new chemotype, initially found as a single individual in a French fibre hemp population (normally predominant in cannabidiol, CBD), having CBG as the major constituent, occupying 94% of the cannabinoid fraction. Grassi (personal communication) found an individual with a CBG proportion of 80–85% in the cannabinoid fraction in a Southern Italian hemp accession. Recently, we observed CBG predominance (85%) in an individual from the Ukrainian fibre cultivar USO-31 (Virovets, 1996). In each of these CBG predominant plants, CBD was the single significant complementary cannabinoid.

Although *Cannabis* chemotypes can be strongly predominant in a single compound, no plant so far analysed has achieved a 100% proportion for its major cannabinoid in the total cannabinoid fraction. Accurate analysis of extracts from plants predominant in a single cannabinoid always shows a minor presence of a choice of residual precursors; cis- and delta 8-isomers; degradants; alkyl homologs and other end-product cannabinoids. Each cannabinoid appears to have a specific maximum proportion that can be reached.

The biosynthetic relationship of CBG with other cannabinoids

The most common cannabinoids are cannabidiol (CBD; Adams et al., 1940; Mechoulam & Shvo, 1963), delta 9-tetrahydrocannabinol (THC; Gaoni & Mechoulam, 1964a), cannabichromene (CBC; Gaoni & Mechoulam, 1966) and cannabigerol (CBG; Gaoni & Mechoulam, 1964b).

In the *Cannabis* plant, cannabinoids are synthesised and accumulated as carboxilic acids (e.g., cannabigerolic acid, CBGA). In this paper, these compounds will be indicated by the abbreviations for their neutral forms.

CBG is the direct precursor for THC (Taura et al., 1995), CBD (Taura et al., 1996) and CBC (Gaoni & Mechoulam, 1966; Morimoto et al., 1997, 1998). The different conversions of CBG are enzymatically catalysed, and for each reaction an enzyme has been identified: THC acid synthase (Taura et al., 1995), CBD acid synthase (Taura et al., 1996) and CBC acid synthase (Morimoto et al., 1997, 1998). CBD- and THC synthase are highly similar in respect of their affinity for CBG (K_m values 134 and 137 μM, respectively) and their catalytic capacity (turnover number k_{cat}, 0.19 and 0.20 s^{-1}, respectively) (Taura et al., 1995, 1996). The affinity of CBC acid synthase for the CBG substrate is higher ($K_m = 23\ \mu M$) but in contrast, its catalytic capacity is lower ($k_{cat} = 0.04\ s^{-1}$) (Morimoto et al., 1998).

The mentioned cannabinoids have a pentyl side chain, but propyl homologues do occur (Vree et al., 1971; de Zeeuw et al., 1972). The propyl homologues of CBD, THC, CBC and CBG are indicated as cannabidivarin (CBDV), delta 9-tetrahydrocannabivarin (THCV), cannabichromevarin (CBCV) and cannabigerovarin (CBGV), respectively. Shoyama et al. (1984) found that CBGV is the key intermediate for propyl cannabinoids, just as CBG is for the pentyl ones. They also demonstrated that an enzyme extract from a 'pentyl *Cannabis* strain' possesses the ability to convert the propyl substrate CBGV as well. Therefore, the later identified THC-, CBD- and CBC acid synthases are apparently not selective for the length of the alkyl side chain of the CBG(V) molecule.

Fournier et al. (1987) supposed that in their CBG predominant plant, the biosynthesis downstream of CBG was blocked. They also presumed that this feature was due to a recessive allele, because of the low frequency of CBG predominant plants in the open-pollinated progeny of their single mutant plant. The 4% of second-generation plants showing CBG predominance were considered to result from self-fertilisation of the initial, monoecious, mutant.

In a previous paper (de Meijer et al., 2003), it was concluded that the inheritance of CBD and THC composed chemotypes is controlled by a monogenic, co dominant mechanism. A single locus, referred to as *B*, with two alleles, B_D and B_T, encoding for CBD and THC synthase respectively, was postulated. According to this model, a true breeding CBD predominant plant has a B_D/B_D genotype at the *B* locus, a true breeding THC predominant plant has a B_T/B_T genotype and plants with substantial proportions of both CBD and THC are heterozygous B_D/B_T. Although the experiments did not cover the subject, plants accumulating the precursor CBG were presumed to have a mutated allele, tentatively called B_0, in the homozygous state, encoding for a defective synthase enzyme.

One implication of the similarity in the kinetic properties of CBD- and THC synthase (Taura et al., 1995, 1996) would be that in heterozygous B_D/B_T genotypes both the conversions CBG → CBD and CBG → THC would occur at similar rates and lead to mixed CBD/THC chemotypes with CBD/THC ratios close to 1.0. However, de Meijer et al. (2003) found that different cross combinations of THC and CBD predominant parents gave progeny specific CBD/THC ratios, ranging from ca. 0.5 to 1.5 in the resulting F_1 hybrids. These specific CBD/THC ratios were fairly stably inherited by the F_2 heterozygotes obtained through self-fertilisation. It was suggested that B_D and B_T are each part of a wider allelic series, encoding several isoenzymatic forms of CBD synthase and THC synthase respectively, with differential kinetic properties and resulting in specific CBD/THC ratios in heterozygotes. The hybrid progenies evaluated by de Meijer et al. (2003) were obtained from only three different CBD- and three different THC predominant parental sources. It is conceivable that the range of the ratios of heterozygous CBD/THC ratios could be extended through interaction between a very weak and a highly active

Table 1. Characteristics of the cross-parents

Code	Generation	Source population[a]	Predominant cannabinoid	Purity[b] (%)	Total cannabinoid content (%)
2001.25	S_1 inbred line	Southern-Italian fibre hemp	CBG	79.6	1.5
55.24.4.34.7.24	S_4 inbred line	South-Indian marijuana landrace	THC	91.8	10.5
M68	S_1 inbred clone	Afghani hashish landrace × Skunk	CBD	93.3	6.4
99.1.9.30.3	S_2 inbred line	German fibre landrace × (Haze × Skunk)	CBD	92.7	6.5
99.2.21.30.21	S_2 inbred line	Afghani hashish landrace × Haze	CBD	93.5	14.3

[a] 'Skunk' and 'Haze' are modern, fairly true-breeding marijuana strains.
[b] The proportion of the major cannabinoid in the total cannabinoid fraction.

isoform of either synthase. In a homozygous genotype, the sole presence of a weak isoform of either CBD synthase or THC synthase could lead to a substantial accumulation of the precursor CBG next to the conversion product CBD or THC.

Aim of this work

In the context of a medicinal *Cannabis* breeding programme (de Meijer, 2004), a full range of homozygous inbred lines predominant in CBD(V), THC(V), CBC(V) or CBG(V) has been bred. This paper reports on the production of improved CBG predominant inbred lines. It aims to verify the genetic mechanism of a recessive B_0 allele responsible for the accumulation of CBG, as proposed by de Meijer et al. (2003) by examining the segregations of chemotypes during the breeding process.

Materials and methods

Plant materials

The parental materials used to produce the cross progenies in this study are described in Table 1. The CBG predominant, 2001.25 inbred line was obtained from Dr G. Grassi (Istituto Sperimentale per le Colture Industriali, Bologna, Italy). The complementary cannabinoid fraction of this monoecious line consisted solely of CBD. As shown in Table 1, the total cannabinoid content of the CBG source material was low and in order to increase the CBG yield potential, basic crosses were performed with CBD or THC predominant materials of good breeding value. All inbred offspring of these latter materials preserved the parental CBD or THC predominant chemotype and can therefore safely be considered as B_D/B_D and B_T/B_T genotypes, respectively (de Meijer et al., 2003). Also the CBG predominant line must have been homozygous, since its inbred offspring expressed invariably the same chemotype. The cross-progenies obtained were subjected to line selection (selective self-fertilisation). The pedigrees of the progenies considered are listed in Table 2. Basic crosses and line selections were performed as described elsewhere (de Meijer, 2004).

Table 2. Pedigrees and codes of the progenies studied for chemotype segregation

Seed parent[a]	Pollen parent[a]	F_1 code	F_2 code[b]
M68 (CBD)	2001.25 (CBG)	2002.2	2002.2.4
2001.25 (CBG)	99.1.9.30.3 (CBD)	2002.13	2002.13.22
2001.25 (CBG)	99.2.21.30.21 (CBD)	2002.14	2002.14.10
55.24.4.34.7.24 (THC)	2001.25 (CBG)	2002.95	2002.95.34

[a] The major cannabinoid of the parental plants is indicated in brackets.
[b] The underlined ciphers in the F_2 codes indicate the single F_1 individual that was self-fertilised to produce the F_2 generation.

The assessment and the expression of cannabinoid composition

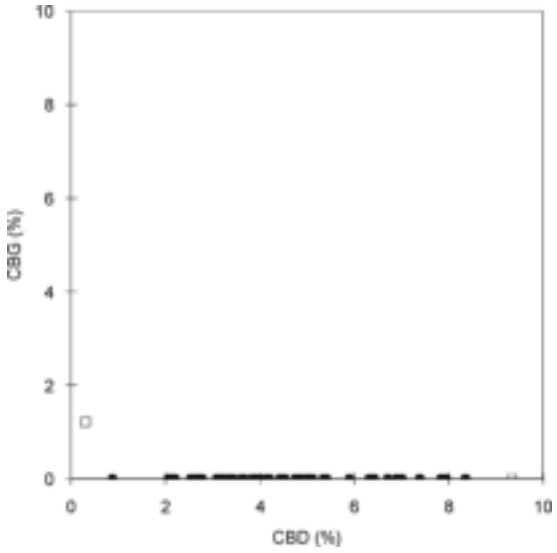
Mature floral clusters were sampled from every individual plant. Sample extraction and GC analysis took place as described by de Meijer et al. (2003). The identities of the compounds detected were confirmed by GC-MS and by comparison of the retention times with those of pure standards. Cannabinoid peak areas were converted into dry weight concentrations using a linear calibration equation obtained with a CBD standard range. As the response of the flame ionisation detector that was used is proportional to the number of C─H bonds in the analytes, a correction factor of 29/30 was applied

for CBG with its 30 C—H bonds, as opposed to the 29 C—H bonds in CBD and THC. The absolute contents of the individual cannabinoids were expressed as weight percentages of the dry floral tissue. The cannabinoid composition was expressed as the weight proportion of the individual cannabinoids in the total cannabinoid fraction.

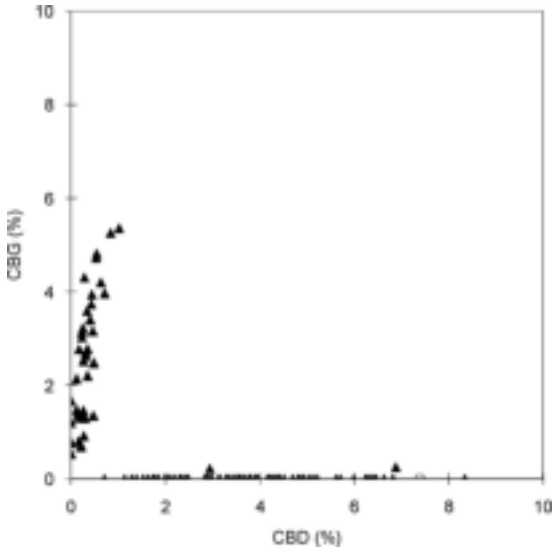
Results

Crosses between the CBG predominant parent and three different CBD predominant parents

The CBG and CBD predominant parents and their entire hybrid offspring had, on average, proportions of (CBG + CBD) of 96%. The remaining cannabinoid fraction consisted of CBC and THC, which will not further be discussed. Figure 1a shows a CBG versus CBD content scatter plot for the 2002.2 F_1. The F_1 is chemotypically uniform, with all the plants, like the parent M68, having a strongly CBD predominant chemotype. The 2002.2.4 F_2, being the inbred offspring of a single F_1 plant, segregates into two distinct chemotypes, one CBG predominant the other CBD predominant (Figure 1b). The other F_2 progenies, from comparable crosses between CBG and CBD parents, showed a similar pattern of segregation. The segregation ratios in the different F_2s are presented in Table 3, with the χ^2 values for the conformity to a 1:3 ratio for CBG predominant chemotypes versus CBD predominant chemotypes. This 1:3 ratio was accepted at $P = 0.05$, for all the F_2s tested. For each of the F_2s, the cluster comprising CBG predominant plants, showed a strong possitive correlation between the absolute CBG content and the absolute CBD content (r values 0.78; 0.87 and 0.90, respectively). The inbred F_3s, based on F_2 plants with the highest CBG proportions and contents, all showed a fixed CBG predominant chemotype. The CBG proportion in the F_3 and further inbred generations from crosses with the CBG line 2001.25, eventually reached a maximum of between 86 and 94%. As far as practical breeding objectives are concerned, Figure 1b shows that the total cannabinoid content of the CBG predominant plants of hybrid origin is strongly improved on the initial CBG source line 2001.25. Figures 1a and 1b illustrate that among plants of one progeny, there is a large variation in absolute cannabinoid content, ranging, from ca 0.8 to 8.5%, irrespective of the cannabinoid composition. Within progenies, these absolute cannabinoid contents show a normal distribution.



(a)



(b)

Figure 1. (a) CBG content versus CBD content scatter plot of the 2002.2 F_1 (*solid circles*). The positions of the parents, one CBG predominant and the other CBD predominant, are indicated by open squares. (b) CBG content versus CBD content scatter plot of the 2002.2.4 F_2 (*solid triangles*). The position of the single F_1 plant, self-fertilised to obtain this F_2 is indicated by an open circle.

Cross between the CBG predominant parent and the THC predominant parent

The chemotypes in the 2002.95 cross progeny, between the CBG- and the THC predominant parent were determined by three cannabinoids: CBG, THC and CBD.

Table 3. Chemotype segregation in the F_2

F_2	No. of plants analysed	CBG predominant	THC predominant[a], or CBD predominant[b]	χ^2 Value[c]	1:3 accepted $P = 0.05$
2002.2.4	115	35	80	1.81	Yes
2002.13.22	47	6	41	3.75	Yes
2002.14.10	44	13	31	0.48	Yes
2002.95.34	48	8	40	1.78	Yes
All crosses	254	62	192	0.05	Yes

[a]Chemotype only present in the CBG × THC cross progeny 2002.95.34.
[b]Chemotype only present in the CBG × CBD cross progenies.
[c]χ^2 values were calculated to test the conformity to the model of one Mendelian locus with a recessive allele, accumulating CBG when in the homozygous state, and a dominant allele, B_D or B_T, encoding CBD- or THC synthase, respectively. The threshold for acceptance at $P = 0.05$ is 3.84.

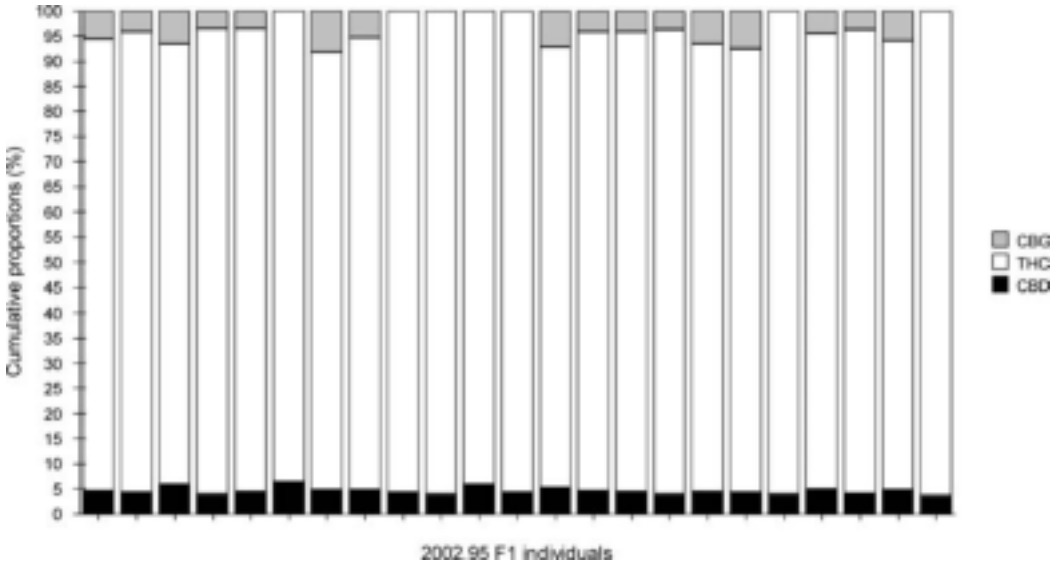
Together they accounted for 98% of the cannabinoid fraction. The main complementary component was CBN, a THC degradation product, and there was also an occasional trace of CBC. Omitting these minor compounds, the cannabinoid composition of the 2002.95 F_1 individuals is presented in the stack bar diagram of Figure 2a. All F_1 plants were strongly predominant in THC (accounting for 85–95% of the cannabinoid fraction) and consistently had a small proportion of CBD (4–6%), whereas CBG was detected in some plants but absent in others. The 2002.95.34 F_2, obtained from a single self-fertilised F_1 plant, fell into two distinct groups: CBG predominant plants and THC predominant plants for which χ^2 tests accepted a 1:3 ratio (Figure 2b; Table 3). The CBG predominant cluster was chemotypically uniform, with all the plants having CBG in a proportion ranging from 80 to 87% and with CBD as the single complementary cannabinoid. The absolute CBG content and the absolute CBD content in this cluster were strongly positively correlated ($r = 0.95$). The THC predominant group was more heterogeneous and showed a variable presence of CBG and CBD. As CBD, unlike CBG, was consistently present in all F_1 plants (Figure 2a), the presence/absence of CBD was employed as a criterion to further subdivide the individuals of the THC predominant group in the stack bar diagram of Figure 2b. A tripartite distribution appears for the 2002.95.34 F_2 with a cluster of 11 THC predominant plants, devoid of CBD and occasionally with some CBG; a cluster of 30 THC predominant plants, consistently with CBD and frequently with some CBG; and the clearly distinct group of nine CBG predominant plants, with CBD as the single complement. With a χ^2 value of 2.16, a 1:2:1 segregation ratio is accepted (threshold for acceptance at $P = 0.05$: $\chi^2 < 5.99$). Apparently, the residual ability to synthesise CBD allows the discrimination of three chemotypes in a CBG × THC F_2 like 2002.95.34, as opposed to only two in a CBG × CBD F_2 like 2002.2.4 (previous section).

Inbred F_3 progenies from F_2 plants with the highest CBG proportion showed a fixed CBG predominant chemotype, as is shown in Figure 2c for the 2002.95.34.6 F_3. The CBG purity in the F_3 and further inbred generations eventually reached a maximum of 86–88% and CBD was the single complementary cannabinoid, just as in the progenies derived from crosses between 2001.25 and the CBD predominant materials.

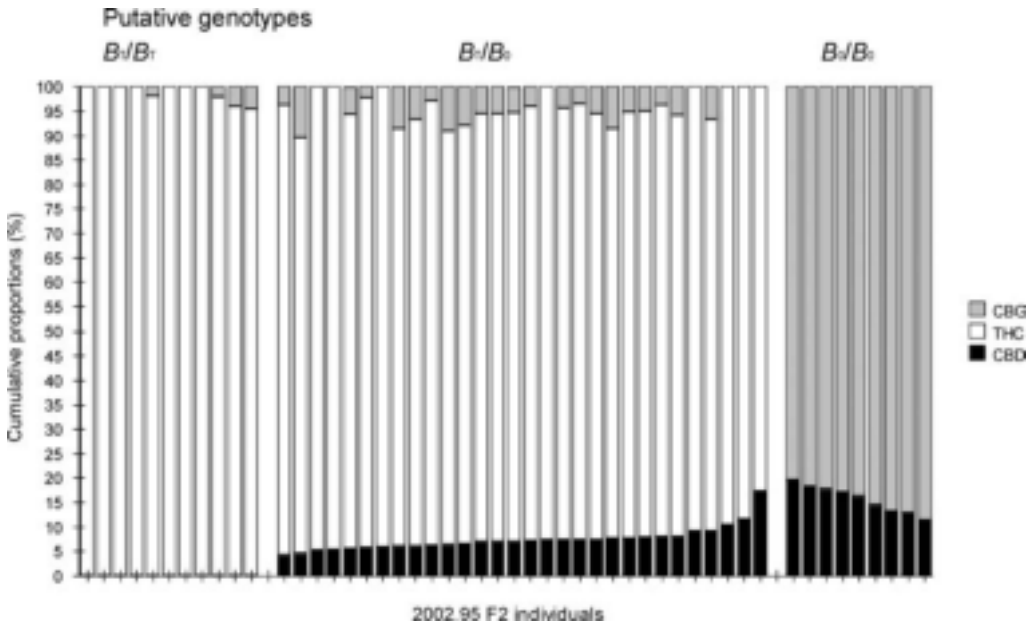
Discussion

The observed chemotype segregations agree with the genetic model proposed by de Meijer et al. (2003) which postulated a single locus *B*, with two common alleles, B_D and B_T, encoding for CBD and THC synthase respectively, and with a rare allele, B_0, encoding for a defective synthase enzyme. According to this model, true-breeding CBD predominant plants have a B_D/B_D genotype, true-breeding THC predominant plants are B_T/B_T and plants accumulating the precursor CBG were presumed to have a B_0/B_0 genotype. Consequently, a cross between a CBG predominant plant and a true breeding CBD predominant plant would result in a uniform F_1 with a B_D/B_0 genotype. Analogously, a cross between a CBG predominant plant and a true breeding THC predominant plant would yield an F_1 with a B_T/B_0 genotype. The exclusive presence of either CBD (Figure 1a) or THC predominant plants (Figure 2a) in the F_1s can be explained by the presumption that the B_0 allele is suppressed in a heterozygous combination with a B_D or B_T allele because the

defective synthase encoded by B_0, is overruled by functional CBD- or THC synthase. The frequency of the CBG predominant chemotype in the F_2s, agrees well with the expected proportion of 25% B_0/B_0 genotypes (Table 3). Besides CBG, these B_0/B_0 genotypes have a proportion of about 15% CBD (Figures 1b and 2b) which indicates that the defective synthase encoded for by B_0 has a residual ability to convert a small amount



(a)



(b)

Figure 2. (a) Stack bar diagram showing the cannabinoid composition of the 2002.95 F_1 plants. The individuals are arranged in random order. (b) Stack bar diagram showing the cannabinoid composition of the 2002.95.34 F_2 plants. The individuals are arranged in three clusters, primarily on the basis of either THC- or CBG predominance; the THC predominant plants are further subdivided on the basis of absence or presence of CBD. Putative genotypes have been assigned to the three groups. (c) CBG content versus CBD content scatter plot of the 2002.95.34.6 F_3 (*solid stars*). The position of the single F_2 plant, self-fertilised to obtain this F_3 is indicated by an open triangle.

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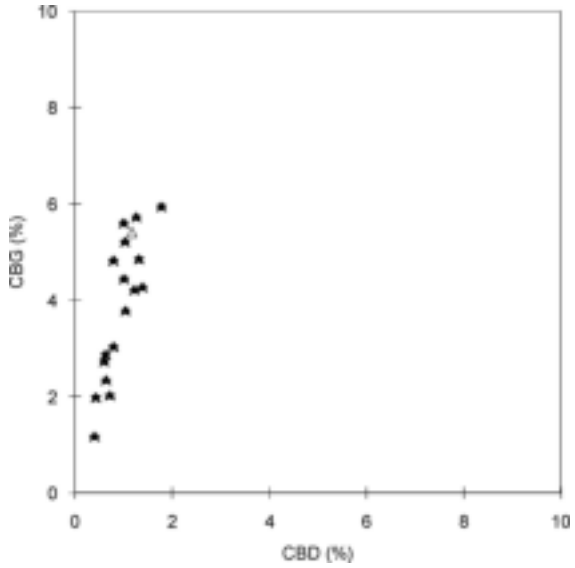

(c)

Figure 2. (Continued)

of CBG into CBD. In the F_2s from crosses between a CBG predominant plant and a CBD predominant plant, all the plants other than B_0/B_0, are strongly CBD predominant and cannot further be discriminated, although they should comprise the two different genotypes B_D/B_0 and B_D/B_D. In the F_2 from the cross between a CBG predominant plant and a THC predominant plant, all plants other than B_0/B_0, are strongly THC predominant. However, within such an F_2 it appears possible to differentiate the expected remaining genotypes, B_T/B_0 and $B_T/B_{T,}$ on the basis of the residual ability of the synthase, encoded for by the B_0 allele, to convert small amounts of CBG into CBD (Figure 2b). As a result, the monogenic segregation ratio of 1:2:1 for B_0/B_0:B_T/B_0:B_T/B_T could be verified and confirmed. CBG accumulation can indeed, as proposed by de Meijer et al. (2003), be considered as caused by the homozygous presence of a defective allele, B_0, at locus *B*. B_0 is fully recessive in interaction with B_D. B_T also suppresses B_0 in a heterozygous combination, but B_0's residual ability to convert a small amount of CBG into CBD, allows the differentiation of B_T/B_0 and B_T/B_T genotypes.

It was acknowledged by de Meijer et al. (2003) that the distribution of chemotypes, as observed in cross progenies obtained from CBD and THC predominant parents, could be alternatively explained by a model of two closely linked chemotype loci, one encoding CBD synthase, and the other THC synthase. With such a model, the CBD predominant parents should carry defective alleles at the THC locus (*thc/thc-CBD/CBD*) and the THC predominant parent should be defective at the CBD locus (*THC/THC-cbd/cbd*). In the rare event of a crossing-over in heterozygous genotypes, the alleles could rearrange, resulting in the doubly homozygous genotypes *THC/THC-CBD/CBD* and *thc/thc-cbd/cbd*. The *THC/THC-CBD/CBD* genotype would express a mixed CBD/THC chemotype but would not segregate on selfing. The *thc/thc-cbd/cbd* genotype with only inactive alleles would accumulate the precursor CBG. For the CBD × CBG crosses this model predicts a uniform *thc/thc-CBD/cbd* F_1 with a CBD predominant chemotype. The THC × CBG cross would result in a uniform *THC/thc-cbd/cbd* F_1 with THC predominant chemotype. The F_2s from self-fertilised F_1s would segregate CBD- or THC plants versus CBG predominant plants in a 3:1 ratio. Since these chemotype distributions are identical to the ones predicted by a monogenic model our results do not rule out the possibility of a model with two closely linked loci. It appears however, that the experimental data and the distribution of chemotypes in *Cannabis* populations agree better with a model of one allelic locus. In our experiments, all CBG predominant plants contain a complementary proportion of 10–15% CBD in the cannabinoid fraction. According to a linked loci model, these plants are *thc/thc-cbd/cbd* and the minor presence of CBD should then be explained as a residual expression

of the *cbd* allele. As a consequence, the common THC predominant plants with a *THC/THC-cbd/cbd* genotype should show a readily detectable proportion of CBD as well, a feature that is notably absent. The single locus model attributes the residual CBD synthase activity to the B_0 allele. It is expressed in B_0/B_0-, B_T/B_0 – and hidden in B_D/B_0 genotypes, but it has no implications for the THC predominant B_T/B_T genotype. Negative evidence against a linked loci model is provided by the fact that there are no reports on plants with a fixed CBD/THC chemotype. A monogenic model excludes such plants but according to a model of two closely linked loci, they should appear after a crossing-over in a heterozygous genotype, with the same likelihood as the CBG predominant plants.

Since B_0 can be regarded as a 'CBG allele', it may appear paradoxical that CBD is a more suitable indicator than CBG to distinguish B_T/B_0 from B_T/B_T in the segregating CBG × THC F_2. The parental THC and CBG predominant lines used for this cross were true-breeding (i.e., homozygous) for chemotype. Therefore the F_1 plants must have been uniform for the genotype underlying their chemotype. Nevertheless, the F_1 showed an incidental presence of minor proportions of CBG, making this feature, unlike the consistent presence of minor proportions of CBD, unsuitable to demarcate segregant groups in the F_2. Apparently, small amounts of CBG can be found in both B_T/B_0 and B_T/B_T genotypes but its presence is obligatory for neither. The presence of detectable CBD is most uncommon in plants of the B_T/B_T genotype and, as a result of B_0's residual metabolic activity, the norm in B_T/B_0. If in a B_T/B_0 plant, B_0 has converted some CBG into CBD, that feature cannot ever be masked by the activity of B_T since the reaction CBG → CBD is irreversible. CBG, being the direct precursor for CBD, THC and CBC, is a common, if occasional, minor constituent in a variety of *Cannabis* genotypes and chemotypes. Possibly, the rate at which CBG is produced, through the enzymatic condensation of olivetolic acid with geranylpyrophosphate (Fellermeier & Zenk, 1998), can occasionally exceed the rate at which it is subsequently converted into CBD, THC and CBC. Such an imbalance may be related to developmental stage; sampling of several *Cannabis* drug strains throughout the generative stage has shown a gradual decrease of this minor CBG proportion with the maturation of the inflorescences (unpublished data).

So far, plants strongly predominant in either CBD or THC have been considered true-breeding for chemotype (de Meijer et al., 2003). The presence of the recessive B_0 allele has changed this situation. A strongly THC predominant chemotype can be due to two different genotypes, B_T/B_T and B_T/B_0, and a strongly CBD predominant chemotype to the genotypes B_D/B_D and B_D/B_0. Therefore, plants strongly predominant in CBD or THC should no longer necessarily be regarded as homozygous and true-breeding for chemotype.

The CBG predominant segregant groups of all four F_2s showed a strong positive correlation between the absolute CBD content and the absolute CBG content, reflecting that in these plants, a fairly fixed proportion of the available CBG substrate is converted into CBD. This can be interpreted as support for our hypothesis that a single enzyme, a weak CBD synthase isoform, encoded by a single allele, B_0, is responsible for both the accumulation of CBG and for its limited conversion into CBD.

The virtually defective synthase encoded for by the B_0 allele possesses a residual ability to convert small amounts of CBG into CBD, which suggests that B_0 is a mutated form of the B_D allele. In addition, the fact that CBG accumulating plants have so far been found in European fibre hemp populations, generally composed of B_D/B_D plants, make it more likely that B_0 has evolved from B_D than from B_T. Evidence for the close relation between B_0 and B_D is provided by the fact that a B_D- and B_T sequence based molecular marker that perfectly identifies B_D and B_T alleles, cannot discriminate B_0 from 'normally functional' B_D (G. Mandolino, personal communication). Therefore, B_0 can also be considered as a member of a wider B_D allelic series (de Meijer et al., 2003), which encodes a CBD synthase isoform with a strongly reduced affinity for the CBG substrate and/or a much lower catalytic capacity. This does not necessarily apply for all possible B_0 alleles; it may be possible that B_T has also mutated into B_0' alleles encoding for non-functional or barely functional THC synthase isoforms. As yet, there are no reports of such mutants.

Irrespective of the cannabinoid composition, a large variation in the absolute cannabinoid content, (which has a polygenic background and is independent from the cannabinoid composition), was found among plants of all the evaluated progenies. Such variations in cannabinoid content, as illustrated in the scatter plots of Figures 1a and 1b, are the result of environmental factors interacting with, probably several, unknown genes that determine the availability of general, basic cannabinoid precursors. Other unknown genes may also determine traits such as the density and activity of the trichomes where the cannabinoid biosynthesis takes

place. As a result of the basic crosses, the cannabinoid content of the CBG predominant F_2 plants of hybrid origin was clearly improved on the initial CBG source line 2001.25. However, it is still modest and these improved plants should be considered as an intermediate result. Applying the backcross principle, we have crossed the B_0/B_0 F_3s with the highest absolute cannabinoid content once again with THC- and CBD plants of good breeding value. Selective self-fertilisation is being performed in order to re-establish the B_0/B_0 genotype in more productive plants. The chemotype distributions so far observed in this procedure are in agreement with the presented model.

The previous version of the genetic model for chemotype inheritance (de Meijer et al., 2003) which was restricted to CBD- and THC composed chemotypes has now been considered in relation to other observations. One implication of the model is that the distribution of CBD predominant, THC predominant and mixed CBD/THC chemotypes directly reflects the B_D and B_T allele frequencies within a population. Hillig and Mahlberg (2004) have performed a chemotaxonomic analysis of 157 *Cannabis* populations on the basis of these frequencies, where in accordance with our chemotype concept, they considered the absolute cannabinoid content as a separate trait. Inspired by legislation, breeders of industrial hemp have a strong focus on the average, absolute THC content in populations, expressed as a w/w percentage of the floral dry matter. This THC content is the resultant product of the total cannabinoid content and the relative proportion of THC in the total cannabinoid fraction. Therefore it behaves as a polygenic trait. If, in addition, bulk-sampling protocols are applied to assess the average THC contents of accessions, the simple genetic background of cannabinoid composition, and its obvious implications for plant breeding, remain completely unnoticed, as is discussed by Hillig and de Meijer (2004) in a comment on Small and Marcus (2003).

In conclusion, the presented results provide the first evidence for a third allele B_0, at the previously described locus *B* and form an extension of the genetic model for the inheritance of *Cannabis* chemotype by de Meijer et al. (2003). The here presented B_0 allele appears to have evolved from the B_D allele, and encodes a CBD synthase isoform with a greatly weakened catalytic capacity. Our data cannot rule out an alternative model with two closely linked loci but indications are presented that this is a less likely explanation. The extended chemotype inheritance model provides readily applicable possibilities for *Cannabis* breeding: the breeding of low cannabinoid content, B_0/B_0 industrial hemp, practically devoid of THC, as well as the breeding of high content B_0/B_0 clones for CBG rich, pharmaceutical raw material production. In a B_0/B_0 genotype, the pathway CBG → THC is completely obstructed, and the pathway CBG → CBD is largely so. Using this genotype, breeding experiments to study the genetic control of the biogenesis of CBC, which is CBG's third major conversion product, should be possible. As previously only Fournier et al. (1987) have reported on a CBG predominant individual, this chemotype and its underlying B_0/B_0 genotype, appear to be very rare in *Cannabis* populations. Nevertheless, the B_0 allele frequency may be higher than expected. B_0's recessive nature, *Cannabis*' out breeding character and the common application of bulk sampling protocols for cannabinoid assessment in fibre hemp will effectively prevent the discovery of B_0's presence.

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