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Sex chromosomes and quantitative sex expression in monoecious hemp (*Cannabis sativa* L.)

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Abstract Hemp (*Cannabis sativa*) has a highly variable sexual phenotype. In dioecious hemp, the sex is controlled by heteromorphic sex chromosomes according to an X-to-autosomes equilibrium. However, in monoecious hemp, the sex determinism remains widely unknown and has never been related to a quantitative approach of sex expression. The present paper aims to contribute to the comprehension of the sex determinism in monoecious hemp by assessing the genotypic variability of its sex expression and establishing its sex chromosomes. Five monoecious and one dioecious cultivars were grown in controlled conditions under several photoperiods. The monoecy degree of 194 monoecious plants was recorded at each node by a figure ranging from 0 (male flowers only) to 6 (female flowers only). The genome size of 55 plants was determined by flow cytometry. The DNA of 115 monoecious plants was screened with the male-associated marker MADC2. The monoecy degree varied significantly among monoecious cultivars from 3.36 ± 2.28 in 'Uso 31' to 5.70 ± 0.81 in the most feminised 'Epsilon 68'. The variation of monoecy degree among cultivars remained consistent across trials despite a significant "cultivar × trial" interaction and partly agreed with their earliness. The genome size of monoecious plants (1.791 ± 0.017 pg) was not different from that of females (1.789 ± 0.019 pg) but significantly lower than that of males (1.835 ± 0.019 pg). MADC2 was absent from all monoecious plants. These results strongly support that cultivars of monoecious hemp have the XX constitution and that their sex expression has a genetic basis.

Keywords *Cannabis sativa* · Genome size · Monoecy · Photoperiod · Sex chromosomes · Sex expression

Introduction

In a context of increased attention for alternative crops, the potential of the non-food species hemp (*Cannabis sativa* L.) has been raised in the production of fibre, bio-composites or paper pulp (van der Werf et al. 1996; Struik et al. 2000; Ranalli and Venturi 2004).

Agriculturally, growing hemp is significantly affected by its photoperiodism and reproductive features. Firstly, hemp is a short-day plant with a critical photoperiod of approximately 14 h (Amaducci

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et al. 2008a; Borthwick and Scully 1954; Lisson et al. 2000). By modulating the flowering time, the photoperiodic conditions can have a key influence on the crop yield (van der Werf et al. 1994). Secondly, the species is naturally dioecious and characterised by sexual dimorphism, in plant size and precocity in particular. Monoecious cultivars of hemp have been developed from plants bearing hermaphrodite flowers or bisexual inflorescences (Moliterni et al. 2004). These cultivars display several agronomical advantages in comparison to the dioecious ones, such as higher seed yields, higher crop homogeneity and easier mechanical harvest due to synchronised maturity (Mandolino and Carboni 2004). However, their sexual phenotype is unstable. The monoecious state presents a continuous distribution between the male and female extreme phenotypes (Bocsa and Karus 1998), and the multiplication of monoecious hemp seeds requires a strict elimination of the sporadically occurring dioecious male plants in order to prevent a gradual return to the dioecy. In addition, the sexual phenotype of hemp is affected by external factors, such as hormonal treatments, the photoperiod or nitrogen status (Freeman et al. 1980). In spite of the diversity and plasticity of the intersexual forms of hemp, significant variations of the sex expression were observed among monoecious hemp cultivars in field trials, and higher seed yields were obtained with the early and mid-early feminised cultivars, suggesting that the sex expression of monoecious plants could affect the seed yields (Faux et al. 2013). Investigating the potential genetic basis of the sex expression in hemp appears therefore valuable for the improvement and cultivation of monoecious hemp.

Hemp is a diploid species ($2n = 20$) that includes sex chromosomes (Hirata 1924). The chromosomes XX are found in female plants, and XY are present in male plants, with the Y chromosome larger than the X one and larger than the autosomes (Yamada 1943 cited by Sakamoto et al. 1995). The genome sizes of diploid female and male plants have been estimated using flow cytometry as $1{,}636 \pm 7.2$ and $1{,}683 \pm 13.9$ Mbp, respectively, and the size difference between them (47 Mbp, i.e., 2.8 % of the genome size of male plants) has been attributed to the large long arm of the Y chromosome (Sakamoto et al. 1998). The sex determinism in dioecious hemp would be based on an X-to-autosomes equilibrium and not on a Y-active mechanism (Westergaard 1958; Ainsworth 2000). On the opposite, the sex determinism of monoecious forms of hemp remains widely unknown. Although Hoffman (1961 cited by Truta et al. 2007) assumed the existence of XX, XY and YY forms, Menzel (1964) observed presumably XX chromosomes in monoecious hemp plants, and recent studies did not reveal the presence of male-associated DNA markers (MADC, for male-associated DNA in *Cannabis*). However, the karyotype was limited to the cultivar 'Kentucky' (Menzel 1964) while very few monoecious plants have been screened with MADC markers to date, i.e., 20 plants of cultivars 'Bialobrzeskie' and 'Beniko' (Mandolino et al. 1999) and 6 plants of cultivars 'Fibrimon' and 'Fleischmann' (Torjek et al. 2002). Mandolino et al. (2002) stated that there is no specific report on the chromosome set in monoecious hemp. Furthermore, all of these studies including monoecious hemp cultivars considered the monoecious state as a qualitative trait. In this context, assessing the genotypic variability of the sexual phenotype expressed as a quantitative variable and establishing the composition in sex chromosomes in a large number of monoecious hemp plants would allow setting preliminary information that can be of interest for further studies on the sex determinism of monoecious hemp.

Flow cytometry constitutes an indirect but advantageous technique to show dissimilarities in chromosomal composition. Indeed, flow cytometry is able to analyse quickly large populations of cells (Dolezel and Bartos 2005) and highlight very small genome-size differences (Costich et al. 1991; Vagera et al. 1994; Dolezel and Göhde 1995). Given the larger size of the Y chromosome of hemp (Sakamoto et al. 1998), the presence, in monoecious hemp, of a Y chromosome similar to that found in male plants could be inferred from the comparison of the genome sizes of monoecious, female and male plants. In addition, several DNA markers closely linked to the male phenotype have been developed in hemp (Sakamoto et al. 1995; Mandolino et al. 1999; Torjek et al. 2002; Peil et al. 2003; Sakamoto et al. 2005; Rode et al. 2005). Among them, the MADC2 marker proved to be completely associated to the male phenotype and should therefore be located on the Y chromosome in a region excluded from recombination during meiosis (Mandolino et al. 2002). The amplification of this marker by monoecious plants would indicate the existence of a male-associated fragment in the sex chromosomes of monoecious hemp.

The present paper aims to contribute to the comprehension of the sex determinism in monoecious hemp. Two specific objectives are pursued: firstly, assessing the genotypic variability of the sexual phenotype expressed as a quantitative variable and, secondly, establishing the constitution in sex chromosomes of monoecious hemp through the evaluation of a large number of plants from distinct cultivars. The characterisation of the sexual phenotype under several photoperiodic conditions allowed to take the sensitivity of the trait to the photoperiod into account. Flow cytometric analyses and the MADC2 DNA marker (Mandolino et al. 1999) were used to address the second specific objective. Finally, we discussed the implications of the results of the present study for future investigations on the sex determinism in monoecious hemp.

Materials and methods

Genetic material

Six hemp cultivars were used, one dioecious, 'Carmagnola', and five monoecious covering a wide range of earliness: 'Uso 31' (very early), 'Fedora 17' (early), 'Santhica 27' and 'Felina 32' (mid-early), and 'Epsilon 68' (late). The dioecious cultivar was obtained from Assocanapa, Carmagnola (Italy), and all of the monoecious ones were obtained from the Fédération nationale des Producteurs de Chanvre (FNPC), Le Mans (France).

Growth conditions

Three trials were conducted successively, the first two in a greenhouse and the third one in a phytotron to apply short photoperiods. Sowing was performed on 13 September 2011, 2 February 2012 and 18 may 2012 in each trial, respectively. The trial 1 included 20 plants from each of the five monoecious hemp cultivars, and both trials 2 and 3 included 10 plants per monoecious cultivar, with the exception of 'Santhica 27' in trial 1 with 15 plants and 'Epsilon 68' in trial 3 in which a male plant was found. In total, 95, 50 and 49 plants of monoecious hemp were grown in trials 1, 2 and 3, respectively. In addition, trial 3 included 10 plants of the dioecious cultivar 'Carmagnola'.

The set-point temperatures were 25/20 °C day/night in all of the trials. The photoperiod was firstly 16/8 h during 22, 60 and 20 days and then shortened to 14/10, the natural daylength and 12/12 h in order to promote flowering in trials 1, 2 and 3, respectively. In trial 2, the natural daylength increased from 13 h at the time of photoperiod shortening (2 April) to 14.5 h at the end of the trial (27 April). In the greenhouse, the plants received the natural daylight, and the daylength was extended by Philips HPLR lamps (400 W). The daylight intensity decreased from on average 580–170 µmol m^{-2} s^{-1} between September and November (trial 1) and, conversely, increased from on average 250 to 520 µmol m^{-2} s^{-1} between February and April (trial 2). The light intensity used to extend the natural daylength was approximately 40 µmol m^{-2} s^{-1} in both trials 1 and 2. In the phytotron, lighting was performed by Philips HPI-T plus lamps (400 W, $\sim$135 µmol m^{-2} s^{-1}). There were 4 or 5 plants per lamp in both greenhouse and phytotron.

For each trial, the sowing was performed in dimpled germination plates. After 10 days, the seedlings were transplanted into 30 cm (height) by 18 cm (diameter) pots. The substrate consisted of a 3:1:1 mixture of soil, sand and loam with 2.8 g per pot of Osmocote® (14:13:13 NPK with slow release of nutrients for up to six months) added just before transplanting. The plants were watered by capillarity twice per week.

Plant phenotyping

The sex of the dioecious hemp plants was noted. In the monoecious hemp, the time of flowering, flowering duration and sex expression were recorded. The flowering time was defined as the time at which closed male flowers or white styles were easily visible at leaf axils. The sex expression was characterised by the degree of monoecy modified from the scale of Sengbusch (1952) by varying from 0 to 6 according to the ratio between male and female flowers (Table 1). The monoecy degree was recorded at each flowering node independently once a week during the flowering duration, i.e., from the first to last flower appearance. However, the plants were checked until the end of flowering, i.e., when male flowers were withering and green seeds were present at most nodes. The experiments stopped when this developmental stage was

Table 1 Sex-expression scale in monoecious hemp modified from Sengbusch (1952)

Monoecy degree	Sex ratio
0	100 % of male flowers
1	80–99 % of male flowers (strongly masculinised node)
2	60–80 % of male flowers (masculinised node)
3	40–60 % of female and male flowers
4	60–80 % of female flowers (feminised node)
5	80–99 % of female flowers (strongly feminised node)
6	100 % of female flowers

reached, i.e., 63, 85 and 55 days after sowing in trials 1, 2 and 3, respectively. All of the monoecious plants were phenotyped, i.e., 194 plants.

Estimation of the genome sizes using flow cytometry

The nuclear genome size of monoecious and dioecious hemp plants was measured using flow cytometry, simultaneously with *Glycine max* (soybean) and *Zea mays* (maize) as internal reference standards to compare the peaks from distinct samples of hemp nuclei. Both reference standards were chosen from those recommended by Praça-Fontes et al. (2011) for their genome size, which is a priori relatively close and distant from that of hemp for soybean and maize, respectively (Sakamoto et al. 1998). Otto's buffers (Otto 1992; Dolezel and Göhde 1995) were used for nuclei isolation as recommended by Loureiro et al. (2006) for plant species with low nuclear DNA content (between 1.30 and 1.96 pg per 2C).

Fresh leaf samples of hemp (20 mg), soybean (20 mg) and maize (50 mg) were collected from mature leaves, mixed and chopped for 120 s with a razor blade in a plastic Petri dish on ice containing 1 ml of the Otto I buffer used for nuclei isolation. A larger proportion of maize leaf tissue was needed to obtain sufficient nuclei. The resulting suspension rested for 15 min with shaking every 5 min to facilitate the nuclei extraction. The mixture was thereafter filtered twice through 30 µm (Sakamoto et al. 1998) and 10 µm nylon meshes successively (Partec GmbH, Münster, Germany). A volume of 200 and 500 µl of Otto I buffer was poured on the first and second filter, respectively, to recover as many nuclei as possible. The solution was then centrifuged for nuclei precipitation (2,500 rpm, 5 min). The resulting pellet was diluted in 100 µl of Otto I buffer before being filtered through a 10 µm mesh. A volume of 800 µl of modified Otto II buffer (20 ml of 0.4 M $Na_2HPO_4.12H_2O$, 1 ml of PI, 1 ml of RNAse and 40 µl of β-mercaptoethanol), used as staining buffer, was added upon the filter. After 10 min, the solution was analysed with the LSRFortessa cell analyser (Becton–Dickinson Benelux N.V., Erembodegem) from the de Duve Institute (Brussels, Belgium). For each sample, a minimum of 10,000 events were analysed at a maximum rate of 300 events s^{-1}. The fluorescence was measured using the 575/26 BP filter, specific to the wavelengths emitted by propidium iodide. The median, standard deviation and coefficient of variation (CV) of the peaks were extracted using the BD FACsDiva Software. For each sample, the genome size of the hemp nuclei was estimated by dividing the median of fluorescence of the hemp peak by that of each standard peak multiplied by the genome size of the standard. Genome sizes of 2.41 ± 0.170 and 5.57 ± 0.146 pg per 2C DNA for soybean and maize, respectively, were used to calibrate the hemp peaks (Praça-Fontes et al. 2011).

The protocol (nuclei isolation and flow cytometry) was developed on plants from trials 1 and 2, while the presented results were obtained from 56 plants from trial 3: 10 'Carmagnola' (6 female and 4 male), 9 'Uso 31', 9 'Fedora 17', 8 'Santhica 27', 10 'Felina 32' and 9 'Epsilon 68'. The cytometric analyses were spread out among six dates as a result of logistical constraints linked to the use of the flow cytometer. For each plant on each date of analysis, two distinct leaf samples were probed independently at the flow cytometer.

PCR with male-associated DNA marker

Leaf DNA was extracted according to Murray and Thompson (1980) with modifications as detailed below. Frozen leaves were ground to a fine powder in liquid nitrogen. The resultant powder was mixed with 900 µl of extraction buffer (10 % Tris–HCl, 10 % EDTA, 2 % CTAB, 50 % NaCl, 2 % PVPP and 1 % β-mercaptoethanol) and heated at 55 °C for 1 h with upside-down shaking every 10 min. A volume of 500 µl of a 24:1 mixture of chloroform-isoamyl alcohol (v/v) was added, shaken for 10 s and

centrifuged (13,000 rpm for 10 min). The upper phase was collected, and the extraction with chloroform-isoamyl alcohol was repeated once. The final upper phase was collected, mixed with 350 µl of isopropanol at 4 °C, shaken for 10 s, rested for 30 min and centrifuged (13,000 rpm for 10 min). The supernatant was drained, and the resultant DNA pellet was mixed with 500 µl of 70 % ethanol at −20 °C and centrifuged (13,000 rpm for 5 min). This last step was repeated once before dissolving the pellet in 50 µl TE buffer (10 mM Tris–HCl pH8, 1 mM EDTA pH8). The extracted DNA was kept at −20 °C.

The presence of the male-associated DNA marker MADC2 (Mandolino et al. 1999) was tested. The reaction volume was 12.5 µl and contained 6.25 ng of genomic DNA, 0.4 µM of the MADC2 primer (Eurogentec SA, Seraing, Belgium), 80 µM of each dNTP, 1.6 mM of $MgCl_2$, 1.25 U of GoTAQ and 1X of GoTAQ buffer (Promega Corp, Madison, USA). The PCR amplifications were performed with a PTC 100 thermal cycler (MJ Research, Waltham, Mass): 10 min at 95 °C, 40 cycles (30 s at 95 °C, 45 s at 58 °C and 2 min at 72 °C) and 4 min at 72 °C. The amplification products and a DNA ladder (Smartladder, Eurogentec SA, Seraing, Belgium) were separated on 1 % agarose gel run at a constant voltage of 90 V. The gels were stained with ethidium bromide and displayed under UV light.

The MADC2 primer was preliminarily tested on ten plants of the dioecious cultivar 'Carmagnola' (five of each sex) and then applied on 115 plants of monoecious hemp (from 21 to 26 plants per cultivar).

Statistical analyses

The statistical analyses were carried out to assess (i) the effects of the cultivar and trial on the flowering time, flowering duration and sex expression in monoecious hemp and (ii) the effects of the sex (monoecious, female or male), cultivar and date of analysis on the genome size of hemp nuclei.

The effect of the experimental factors on the flowering time and flowering duration was assessed by using the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk} \quad (1)$$

where y_{ijk} was the flowering time or flowering duration of the kth hemp plant of cultivar i in trial j; μ was the general mean response; α_i, β_j and $(\alpha\beta)_{ij}$ were the fixed effects of cultivar i, trial j and their interaction, respectively; e_{ijk} was the error associated to the kth plant of cultivar i in trial j. The model (1) was tested with the GLM procedure from the SAS statistical package (SAS Institute Inc. 2012).

The sex expression, quantified by the degree of monoecy (Table 1), is a discrete variable with seven response levels and was therefore statistically analysed using the GLIMMIX procedure with a multinomial response distribution (DIST = MULTI) and cumulative logit link function (LINK = CUMLOGIT) (SAS Institute Inc. 2012). The analysis was performed with the model (2) below, which integrates random effects of the "plant" and "node" nested to the plant in addition to the "cultivar" and "trial" fixed effects:

$$y_{ijknp} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + d_{ijk} + f_{ijkn} + e_{ijknp} \quad (2)$$

where y_{ijknp} was the monoecy degree of the nth node of the kth hemp plant of cultivar i in trial j observed at time p; μ, α_i, β_j and $(\alpha\beta)_{ij}$ had the same meaning as in model (1); d_{ijk} was the random effect associated to the kth plant of cultivar i in trial j; f_{ijkn} was the random effect associated to the nth node of the kth plant of cultivar i in trial j; e_{ijknp} was the error associated to the nth node of the kth plant of cultivar i in trial j observed at time p. The option MAXOPT = 100 was used to obtain the convergence of the model.

The statistical analysis of the genome size was firstly simplified by incorporating the "sex" effect of the dioecious cultivar into the "cultivar" effect, which consequently included seven levels (one for each of the five monoecious cultivars and two for each sex of the dioecious cultivar), and secondly by using the mean genome size computed for each plant on each analysis date. The effect of the experimental factors on the genome size was tested in the GLM procedure (SAS Institute Inc. 2012) by using the structure of model (1), where y_{ijk} was the genome size of the kth hemp plant of cultivar or sex i analysed at the jth date, α_i, β_j and $(\alpha\beta)_{ij}$ were the fixed effects of cultivar or sex i, date j and their interaction, respectively, and e_{ijk} was the error associated to the kth plant of cultivar or sex i analysed at the jth date. An entire mixed model, which included the fixed effects of "sex", "cultivar", "date" and their interactions as well as the random

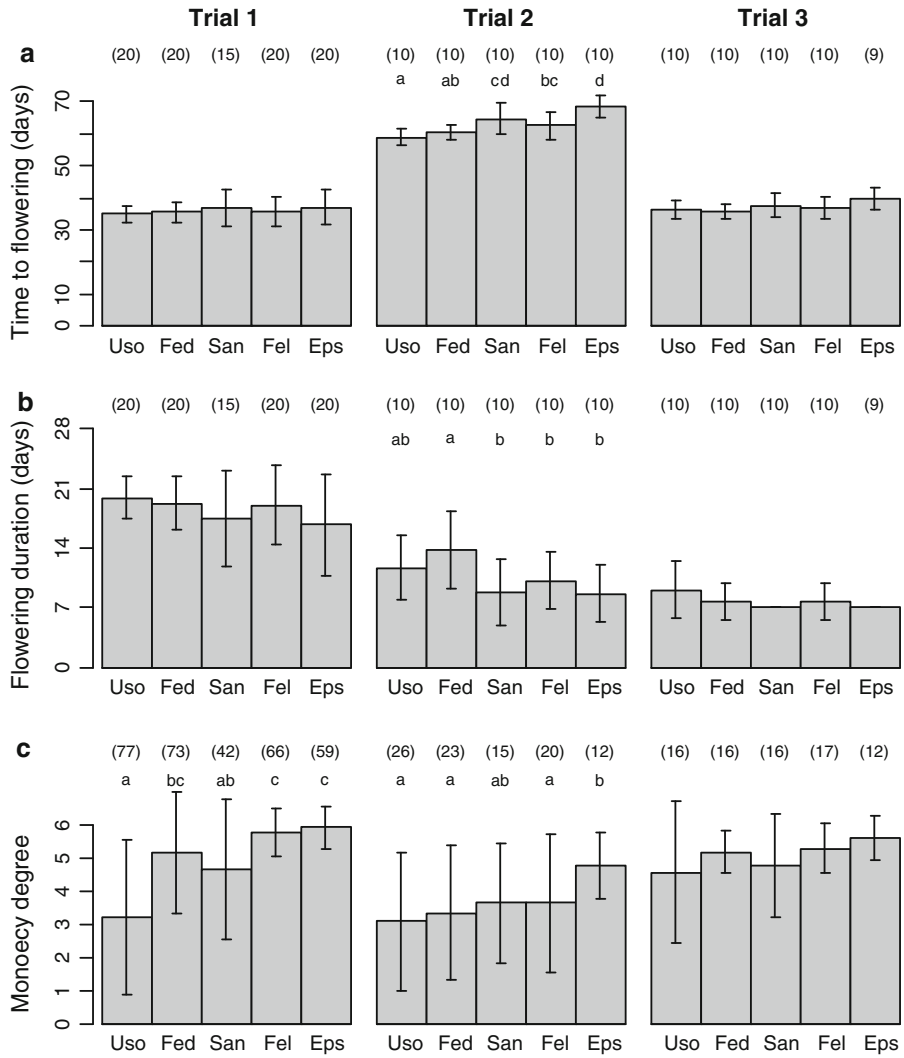

Fig. 1 Flowering phenology and sex expression in five monoecious hemp cultivars. **a** Time to flowering, **b** flowering duration and **c** degree of monoecy (Table 1) (mean ± SD). The photoperiod was 16/8 h during 22, 60 and 20 days and then 14/10, the natural daylength and 12/12 h in trials 1, 2 and 3, respectively. In trial 2, the natural daylength increased from 13 h at the time of photoperiod shortening (2 April) to 14.5 h at the end of the trial (27 April). 'Uso' to 'Eps' refer to the cultivars 'Uso 31, 'Fedora 17', 'Santhica 27', 'Felina 32' and 'Epsilon 68', respectively. Significant differences among cultivars ($P < 0.05$) were indicated by *different letters* above the *bars* when the "cultivar" effect was significant ($P < 0.05$). The number of data points is given between *brackets* for each "cultivar × trial" treatment. For the monoecy degree, each data point corresponds to the mean value computed on all flowering nodes of a given plant at a given observation time

"plant" effect and its interaction with "date", was firstly tested, and we verified that the interpretation of the fixed effects was the same as in model (1).

For each observed variable—flowering time, flowering duration, monoecy degree or genome size—the MEANS procedure and the CONTRAST statement of the GLM or GLIMMIX procedures were used to compute means and pairwise comparisons, respectively.

Results

Flowering phenology and records of the sex expression

The plants started to flower 34, 57 and 35 days after sowing in trials 1, 2 and 3, respectively (as a reminder, the photoperiod was shortened 22, 60 and 20 days after sowing in trials 1, 2 and 3, respectively). The

Table 2 Flowering phenology: ANOVA table for the time of flowering and flowering duration

Dependent	Source	DF	SS	MS	F value	Prob F
Time of flowering	Cultivar	4	544.1	136.0	9.1	<0.0001
	Trial	2	25,517.4	12,758.7	853.6	<0.0001
	Cultivar × trial	8	232.7	29.1	1.9	0.0560
Flowering duration	Cultivar	4	239.8	60.0	4.2	0.0031
	Trial	2	4,184.7	2,092.3	145.2	<0.0001
	Cultivar × trial	8	77.4	9.7	0.7	0.7160

appearance of new flowers was noted until 55, 77 and 49 days after sowing in each trial, respectively. The mean flowering time was 36.1, 63.0 and 37.3 days, and the mean flowering duration was 18.6, 10.6 and 7.7 days in trials 1, 2 and 3, respectively (Fig. 1a, b).

The differences in flowering time among trials were significant only between trial 2 and both trials 1 and 3 ($P < 0.001$; Table 2). The cultivar significantly affected the flowering time in trial 2 only ($P < 0.001$). However, the ranking of cultivars according to their flowering time was similar across trials, resulting in no significant "cultivar x trial" interaction and an overall significant "cultivar" effect ($P < 0.001$). The relative flowering times of the five monoecious cultivars were in agreement with their earliness as stated by the FNPC: 'Uso 31' was the earliest one, followed by 'Fedora 17', 'Felina 32', 'Santhica 27' and 'Epsilon 68', the latest one.

The differences in flowering duration were significant among all three trials ($P < 0.001$; Table 2). Similarly to the flowering time, the flowering duration was affected by the cultivar in trial 2 only ($P < 0.05$), and no significant "cultivar x trial" interaction was found. The flowering duration was on average longer in 'Fedora 17' and 'Uso 31', the earliest cultivars, than in 'Santhica 27' and 'Epsilon 68', the latest ones, and intermediate in 'Felina 32' over the three trials.

As a result of the flowering phenology, the sex expression was recorded four times in trial 1 at 7 days intervals (34, 41, 48 and 55 days after sowing), three times in trial 2 (57, 62 and 70 days after sowing) and only twice in trial 3 (38 and 45 days after sowing). From the first to the last observation time, the number of phenotyped nodes per plant varied from 1 to 14 in trial 1 (mean ± SD = 5.8 ± 2.9), from 1 to 16 in trial 2 (8.4 ± 3.6) and from 1 to 12 in trial 3 (5.7 ± 2.7).

Sex expression

The dioecious cultivar 'Carmagnola', grown in trial 3, included six female plants, characterised by racemes with leafy bracts and only female flowers, and four male plants, characterised by hanging panicles with few or no leaves and only male flowers (Fig. 2a, b). All of the plants from the five monoecious hemp cultivars showed inflorescences similar to those of female plants from dioecious hemp and bore female and male flowers arising in variable amounts (Fig. 2c).

The sex expression of the monoecious hemp plants was affected by the cultivar and trial ($P < 0.001$) as well as by their interaction ($P < 0.01$) (Table 3). The plants were more masculinised in trial 2 (mean monoecy degree ± SD = 3.55 ± 1.95), i.e., in the trial in which the duration of the long-photoperiod treatment was longer, than in both trials 1 (4.88 ± 1.98) and 3 (5.05 ± 1.34) ($P < 0.001$; Fig. 1c). However, the monoecy degree varied significantly among cultivars only in trials 1 ($P < 0.001$) and 2 ($P < 0.05$) and among trials only in three cultivars, 'Fedora 17' ($P < 0.01$) and both 'Felina 32' and 'Epsilon 68' ($P < 0.001$), resulting in a significant "cultivar x trial" interaction. Nevertheless, the ranking of the cultivars according to their monoecy degree remained consistent across the trials (Fig. 1c). The cultivar 'Uso 31' was the most masculinised one (mean monoecy degree ± SD = 3.36 ± 2.28). It was followed by 'Santhica 27' (4.47 ± 1.97), 'Fedora 17' (4.78 ± 1.91), 'Felina 32' (5.28 ± 1.39) and 'Epsilon 68' (5.70 ± 0.81), the most feminised cultivar. Thus, the rankings of cultivars according to earliness and sex expression agreed with each other, with the exception of 'Fedora 17' and 'Santhica 27', the former being earlier and more feminised than the latter. The differences in monoecy degree between the cultivars were highly significant ($P < 0.001$) between 'Uso 31' and both 'Felina 32' and 'Epsilon 68' and between

Fig. 2 Inflorescences found in dioecious and monoecious hemp: **a** female and **b** male plants from the dioecious cultivar 'Carmagnola' and **c** a plant from the monoecious cultivar 'Uso 31'

'Epsilon 68' and both 'Fedora 17' and 'Santhica 27'. The differences were also significant ($P < 0.05$) between 'Uso 31' and both 'Santhica 27' and 'Fedora 17' as well as between 'Santhica 27' and 'Felina 32'.

Genome size

The analysis of each sample by flow cytometry allowed the detection of three peaks that corresponded to hemp, soybean and maize nuclei successively. The CVs of the peaks ranged between 1.5 and 3.4 % for maize, 2.6 and 4.7 % for soybean and 2.6 and 5.0 % for hemp.

Despite absolute values that were very close to each other, the genome size of the hemp nuclei was significantly affected by the sex of the plant, defined as monoecious, female or male, regardless of the reference standard (Fig. 3; Table 4). The genome size of the male 'Carmagnola' plants was significantly larger ($P < 0.001$) than that of all of the other plants, i.e., both female 'Carmagnola' plants and the plants from all of the monoecious cultivars. The female plants were not significantly different from the monoecious plants, nor were the monoecious cultivars significantly different from each other. The mean genome sizes estimated with soybean as the reference standard were 1.791 ± 0.017, 1.789 ± 0.019 and 1.835 ± 0.019 (pg) in monoecious, female and male plants, respectively, and the corresponding sizes estimated with maize as the reference standard were 1.838 ± 0.020 (pg), 1.835 ± 0.017 and 1.883 ± 0.018, respectively. The date of analysis significantly affected the genome size of the nuclei (Table 4). However, no interaction was observed between the fixed effects ("cultivar or sex" and "date").

Table 3 Sex expression in monoecious hemp: test of fixed effects on the monoecy degree (Table 1)

Effect	Num DF	Den DF	F value	Prob F
Cultivar	4	159.99	8.90	<0.0001
Trial	2	170.51	15.05	<0.0001
Cultivar × trial	8	157.79	2.66	0.0092

Male-associated DNA marker

The MADC2 primer amplified one male-associated band of 390 bp—the MADC2 marker—in the male plants of the dioecious cultivar 'Carmagnola', as expected (Mandolino et al. 1999). This male-associated band was absent from all of the female 'Carmagnola' plants (Fig. 4a). Moreover, the MADC2 marker was absent from all of the plants belonging to monoecious hemp cultivars (Fig. 4b for cultivar 'Fedora 17').

Discussion

Flowering phenology

The variation of flowering time among trials was in agreement with the short-day photoperiodic reaction

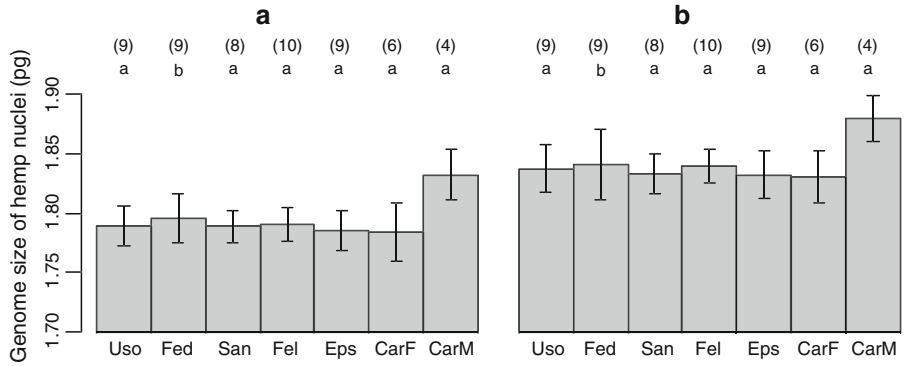

Fig. 3 Genome size of monoecious and dioecious hemp nuclei (mean ± SD) estimated using **a** soybean and **b** maize as the reference standard. 'Uso' to 'Eps' refer to the five monoecious cultivars 'Uso 31, 'Fedora 17', 'Santhica 27', 'Felina 32' and 'Epsilon 68', respectively, and 'CarF' and 'CarM' refer to female and male plants of the dioecious cultivar 'Carmagnola'. The values are the overall mean genome size to which the residual values obtained from an ANOVA including "date" as cofactor were added (Costich et al. 1991). *Different letters* above the bars indicate significant differences ($P < 0.001$). The number of data points is given between *brackets* for each cultivar

Table 4 Flow cytometry: ANOVA table for the genome size (GS) of hemp nuclei estimated using soybean or maize as the reference standard

Dependent variable	Source[a]	DF	SS	MS	F value	Prob F
GS—soybean	Cultivar or sex	6	0.017	0.003	8.78	<0.0001
	Date	5	0.009	0.002	5.89	0.0003
	(Cultivar or sex) × date	16	0.004	0.000	0.86	0.6157
GS—maize	Cultivar or sex	6	0.019	0.003	7.98	<0.0001
	Date	5	0.010	0.002	5.33	0.0006
	(Cultivar or sex) × date	16	0.006	0.000	0.98	0.4949

[a] The analysis was performed considering seven levels for the "cultivar or sex" factor: one for each of the five monoecious cultivars and two for each sex of the dioecious cultivar

of hemp: the plants started to flower earlier in the trials in which the photoperiod was shortened earlier (trials 1 and 3). The late flowering observed in trial 2 should be due to a longer photoperiod-induced phase (PIP) of development, the duration of which is determined by the number of hours exceeding the critical photoperiod [approximately 14 h in hemp (Amaducci et al. 2008a; Borthwick and Scully 1954; Lisson et al. 2000)] and the genotypic sensitivity to the photoperiod (Lisson et al. 2000). Thus, the genes conferring the photoperiod sensitivity were likely further stimulated by the long 16-h day treatment of trial 2, so that the flowering was delayed and the variation of flowering time among cultivars was more significant in this trial.

The differences in flowering duration observed among trials (on average 18.6, 10.6 and 7.7 days, respectively) could be due to the photoperiod experienced by the plants after the flowering time, which was 14 and 12 h in trials 1 and 3, respectively, and increased from 13 to 14.1 h during the flowering period in trial 2 (between 60 and 77 days after sowing). In field trials, Amaducci et al. (2008b) observed that the flowering duration of hemp plants was shorter in late sown treatments, which flowered under decreasing daylengths. However, a relatively high difference in flowering duration was observed between trials 1 and 2, which experienced relatively close photoperiods. It is possible that the flowering duration was affected by the time of photoperiod shortening—which occurred 14 days before and close to the flowering time in trials 1 and 2, respectively—or that the response of the flowering duration to the photoperiod is not linear. In soybean, a short-day species, the flowering duration is affected by photoperiods above a critical threshold, which varies according to the genotype (Summerfield et al. 1998).

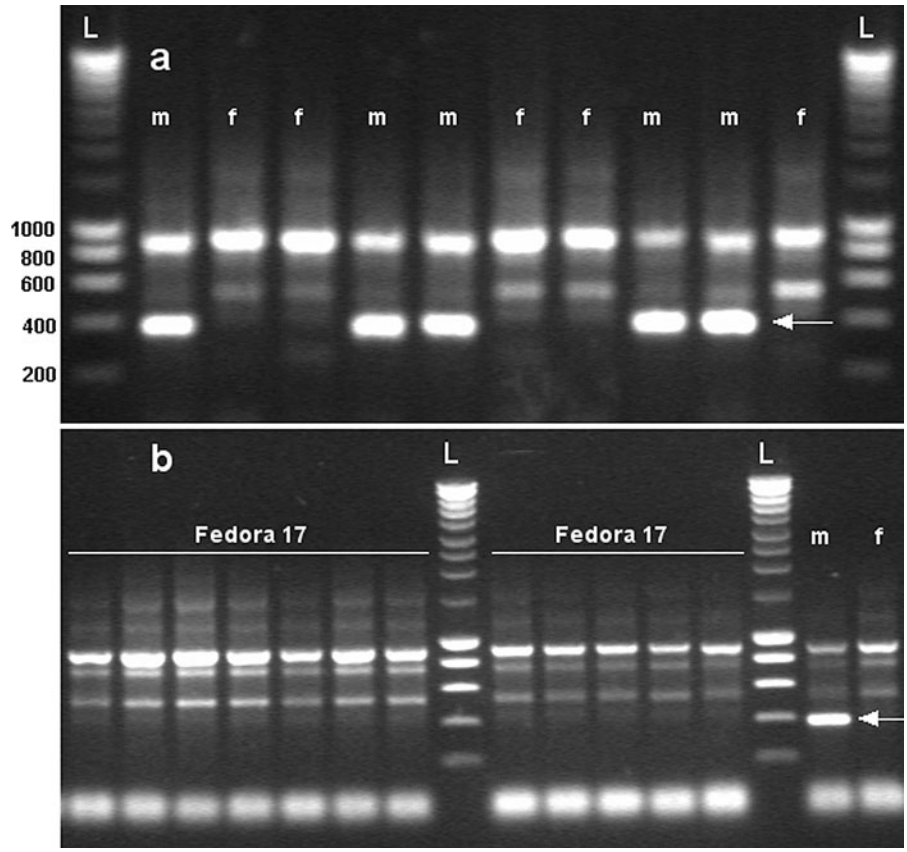

Fig. 4 PCR products obtained with the male-associated DNA marker MADC2 applied on **a** ten plants of the dioecious hemp cultivar 'Carmagnola', male (m) and female (f), and **b** twelve plants of the monoecious hemp cultivar 'Fedora 17'. The arrows indicate the male-specific band of 390 bp long. L: Smartladder (bp)

In addition, the flowering duration was longer in the earliest cultivars ('Uso 31', 'Fedora 17') than in the latest ones ('Santhica 27', 'Epsilon 68'). Amaducci et al. (2008b) observed a large genotypic variation in the flowering duration among hemp cultivars; however, no link between earliness and flowering duration was drawn. Additional trials in controlled conditions are needed to explain the response of the flowering duration to the photoperiod and genotype.

The sex expression in response to the genotype

Both previous field trials (Faux et al. 2013) and the present study revealed (i) a significant effect of the cultivar on the sex expression of monoecious hemp plants and (ii), despite a significant "cultivar x trial" interaction in the present study, the same ranking of cultivars according to their sex expression. The consistency of the genotypic effect on the sex expression across environments supports that the sex expression in monoecious hemp has a genetic basis.

The rankings of cultivars according to their sex expression and earliness partly agreed with each other. In dioecious hemp, the association of earliness and maleness is known: the male plants flower generally earlier than the female ones (Bocsa and Karus 1998; Struik et al. 2000). Borthwick and Scully (1954) attributed the excess of male over female plants observed in long photoperiods to the lower sensitivity to the photoperiod of the male plants. In monoecious hemp, Amaducci et al. (2008b) suggested that early plants have male characteristics. These observations support the hypothesis that both traits share a common basis in their genetic determinism. Effects of exogenous hormones on sex have been reported in hemp: gibberellin induces predominantly male plants, while auxin, cytokinin and abscisic acid induce the feminisation of the plants (Heslop-Harrison 1956; Mohan Ram and Jaiswal 1972; Chailakhyan and Khryanin 1978, 1979; Freeman et al. 1980). In addition, Chailakhyan and Khryanin (1978, 1979) observed that plants treated with gibberellin produced floral buds earlier than the controls, whereas the auxin

treatment delayed the flowering. In the long-day model plant *Arabidopsis thaliana*, gibberellin is required for early flowering and flowering under non-inductive short days (Wilson et al. 1992). Investigating the role of gibberellin in hemp flowering appears therefore to be an interesting pathway to dissect the relation between earliness and maleness. However, this does not rule out the involvement of other hormones. Besides, given the sensitivity to the photoperiod of both hemp flowering and sex expression, it would be worthwhile to integrate the photoperiod in the study of the hormonal regulation of hemp flowering.

The sex expression in response to the environment

The pattern of variation of the sex expression among trials suggested that the "trial" effect on the sex expression was primarily due to the photoperiod (Fig. 1c). Indeed, a masculinising effect of long photoperiods has been observed in hemp (Borthwick and Scully 1954; Arnoux 1966; Freeman et al. 1980). In the present study, the duration of the 16-h day treatment was the longest in trial 2 (60 days vs. 22 and 20 days in trials 1 and 3, respectively), resulting in a higher production of male flowers in this trial.

However, the significant "cultivar x trial" interaction pointed out, firstly, that the "cultivar" effect on the monoecy degree decreased from trial 1 to trial 3 (Fig. 1c; Table 3). This observation could be explained by the flowering duration or by both the photoperiodic and light conditions. Indeed, the "cultivar" effect on the monoecy degree varied among trials similarly to the flowering duration, suggesting that a long flowering duration would allow a higher differentiation of the sex expression among the monoecious hemp cultivars. In addition, according to Borthwick and Scully (1954), a high light intensity induces a greater production of male flowers on female plants. The light conditions of trials 1 and 2—performed in the greenhouse under natural light artificially extended—would therefore be more favourable to the production of male flowers than those of trial 3—performed in phytotron under artificial light only. However, the photoperiodic conditions were less masculinising in trials 1 and 3 than in trial 2, as a result of their shorter duration of 16-h day treatment (Borthwick and Scully 1954; Arnoux 1966; Freeman et al. 1980). Therefore, the light and photoperiodic conditions would have opposite effects on the sex expression in trial 1. It would be possible that the cultivars were affected differentially by these conditions, resulting in a relatively high genotypic variability of the sex expression in this trial. On the opposite, the light and photoperiodic conditions would be both inductive for the production of male flowers in trial 2, and, conversely, both relatively little inductive in trial 3, inducing thereby less variable genotypic responses. Further studies are clearly necessary to elucidate how the genotypic response of the sex expression is affected by the environment.

Secondly, the sex expression varied significantly among trials only in the most feminised cultivars ('Fedora 17', 'Felina 32' and 'Epsilon 68'). On the opposite, in field trials performed with the same five present cultivars (Faux et al. 2013), the variation of sex expression with sowing date was the highest in the most masculinised cultivars ('Uso 31' and 'Santhica 27'). In view of the effect of the light intensity on the sex expression in hemp (Borthwick and Scully 1954), the inconsistency observed between field and controlled conditions could be due to the light quality and intensity. In the field, the plants grew under natural daylight only, while they received both natural and artificial light in the greenhouse and artificial light only in the phytotron. In addition, the daylight intensity is higher during the field growth season of hemp (from April to September) than during the periods of the present greenhouse trials (from September to April). According to Borthwick and Scully (1954), the cultural conditions could also affect the sex expression of the plants, since differences in the production of male flowers by female hemp plants were observed between field and greenhouse experiments conducted simultaneously under natural daylight and photoperiod.

Flow cytometry: practical considerations

The quality of the nuclei suspension prepared for the purpose of flow cytometry is properly judged by analysing the histogram of the relative fluorescence intensity, which accounts for the relative DNA content (Dolezel and Bartos 2005; Loureiro et al. 2006). The CVs of the peaks obtained in the present study were acceptable according to the 5 % threshold given by Dolezel and Bartos (2005).

Flow cytometry has been widely used to determine the absolute nuclear DNA content of various plant species (Dolezel and Bartos 2005; Praça-Fontes et al. 2011). In this context, the choice of an internal reference standard is considered to be one of the main issues (Dolezel and Bartos 2005). Here, the genome size of hemp nuclei was function of the reference standard—soybean or maize. This observation was due to a difference between the peak ratio between both reference standards (average ratio = 0.44) and their genome size ratio given by Praça-Fontes et al. (2011) (2.41/5.57 = 0.43) and used here to calibrate the hemp peaks. Such discrepancies among laboratories are common issues in flow cytometry (Dolezel et al. 1998; Praça-Fontes et al. 2011). According to Dolezel and Bartos (2005), the genome sizes estimated using soybean as a reference standard were more accurate that those obtained using maize since soybean and hemp have closer genome sizes.

The effect of the date of analysis on the genome size observed in this study (Table 4) was similar to the "run" effect reported by Costich et al. (1991) and Taliaferro et al. (1997). According to Taliaferro et al. (1997), the differences in genome size among dates of analysis could result from nuances associated with sample preparation or machine calibration. Moreover, cytosolic compounds might interact with PI fluorescence, as noted with anthocyanin in *Euphorbia pulcherrima* by Bennett et al. (2008). Hemp produces a variety of secondary metabolites, such as cannabinoids, unique to the species (de Meijer et al. 2003), flavonoids, stilbenoids, terpenoids, alkaloids and lignans (Flores-Sanchez and Verpoorte 2008), which could cause significant variations in the genome-size estimation. In the present study, the significant "date" effect was removed according to Costich et al. (1991) for the computation of the mean genome size of hemp nuclei (Fig. 3).

Genome size of the monoecious hemp

The use of flow cytometry revealed significant differences in genome size between male hemp plants vs. both monoecious and female ones. To our knowledge, the present study constitutes the first report on the genome size of monoecious hemp. Considering a conversion factor of 1 bp = 0.978×10^9 DNA content (pg) (Dolezel and Greilhuber 2010), the genome sizes calculated using soybean as the reference standard were 1,751 ± 16, 1,750 ± 18 and 1,795 ± 18 Mbp for monoecious, female and male hemp plants, respectively. Thus, the genome size of monoecious hemp was similar to that of female plants.

Though higher, the values of genome size obtained in the present study are in the range of those found by Sakamoto et al. (1998) for male and female hemp plants (1,683 ± 13.9 and 1,636 ± 7.2 Mbp, respectively). The difference between both studies could firstly be explained by the use of distinct reference standards (Dolezel and Bartos 2005), i.e., *Arabidopsis thaliana* by Sakamoto et al. (1998) *vs.* soybean and maize in the present study. Secondly, Sakamoto et al. (1998) used the DAPI fluorochrome, which preferentially binds to AT bases and therefore leads to DNA-content estimations that should be interpreted with caution (Dolezel et al. 1992). Thirdly, the genome size of 130 Mbp that was considered for *Arabidopsis thaliana* by Sakamoto et al. (1998) has been reassessed at 157 Mbp by the Arabidopsis Genome Initiative (2000). Nevertheless, the difference in percentage of the genome size between male and female plants found in our study (2.7 %) was very close to the 2.8 % reported by Sakamoto et al. (1998) and to the 2.7 % found by J. Suda (Charles University, Czech Republic, 'pers. comm.').

Male-associated DNA marker

The male-associated band amplified by the MADC2 primer corresponded to the marker described by Mandolino et al. (1999). The adequacy of this marker to discriminate male from female plants in the dioecious cultivar 'Carmagnola' as observed here confirmed the results of Mandolino et al. (1999; 2002).

The present study showed the absence of the MADC2 marker in 115 plants obtained from monoecious hemp cultivars. This result amplified the absence of this marker observed in the monoecious cultivars 'Bialobrzeskie' and 'Beniko' by Mandolino et al. (1999).

General discussion

The species *Cannabis sativa* is characterised by heteromorphic sex chromosomes. Female plants of dioecious hemp have XX chromosomes, and male ones have XY chromosomes. The present study showed a similitude of genome size between

monoecious plants and female plants of dioecious hemp and the absence of the male-associated DNA marker MADC2 in monoecious hemp, which is likely located on the Y chromosome in a region excluded from recombination (Mandolino et al. 2002). These findings suggest that monoecious hemp has basically the same sex chromosomes than female plants of dioecious hemp, i.e., XX chromosomes, and confirm the cytological observations made in the 'Kentucky' cultivar by Menzel (1964).

The sex expression in monoecious hemp varies quantitatively among plants as well as, according to our results, among cultivars and should therefore be a heritable quantitative trait. Considering that monoecious hemp has the XX constitution regarding sex chromosomes, the genetic determinism of sex expression in monoecious hemp should necessarily involve genes that promote the production of male flowers by cytologically female hemp plants. Consistent variations of sex expression and earliness among cultivars were found, and both traits are sensitive to the photoperiod. Therefore, it is possible that genes responsible for the flowering response to the photoperiod are involved in the determinism of the sex expression in monoecious hemp. A very high degree of polymorphism has been noted in *Cannabis* (Faeti et al. 1996; Forapani et al. 2001). Regarding the structure of the genetic diversity, Forapani et al. (2001) suggested the existence of a single and widely shared gene pool, with a proportion of among-cultivar variation strongly dependent upon the examined cultivars. Given the polymorphism revealed in the species and that sex expression in monoecious hemp appears to be a heritable quantitative trait, investigating the sex determinism of monoecious hemp through the identification of quantitative trait loci (QTL) linked to the sex expression seems relevant. To this purpose, the characterisation of distinct hemp cultivars made in the present study can be useful to select contrasting parents for the creation of a segregating population.

In conclusion, the present study provided new insights and established fundamental information for further studies on the sex determinism of monoecious hemp. Firstly, the sex expression in monoecious hemp varies quantitatively and significantly among cultivars. The ranking of cultivars according to their sex expression was maintained across environments and partly consistent with their earliness. Secondly, the genome size of monoecious hemp plants is not significantly different from that of female plants but lower than that of male plants. Thirdly, the male-associated DNA marker MADC2 is absent from monoecious hemp. These results support that monoecious hemp has the XX constitution in sex chromosomes and that its sex expression has a genetic basis. In addition, they suggest that the sex determinism in monoecious hemp could be approached through the identification of quantitative trait loci linked to the sex expression.

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