



## The sexual differentiation of *Cannabis sativa* L.: A morphological and molecular study

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### Summary

*Cannabis sativa* L. is a dioecious species with sexual dimorphism occurring in a late stage of plant development. Sex is determined by heteromorphic chromosomes (X and Y): male is the heterogametic sex (XY) and female is the homogametic one (XX). The sexual phenotype of *Cannabis* often shows some flexibility leading to the differentiation of hermaphrodite flowers or bisexual inflorescences (monoecious phenotype). Sex is considered an important trait for hemp genetic improvement; therefore, the study of the mechanism of sexual differentiation is of paramount interest in hemp research. A morphological and molecular study of *Cannabis sativa* sexual differentiation has been carried out in the Italian dioecious cultivar Fibranova.

Microscopic analysis of male and female apices revealed that their reproductive commitment may occur as soon as the leaves of the fourth node emerge; the genetic expression of male and female apices at this stage has been compared by cDNA-AFLP. A rapid method for the early sex discrimination has been developed, based on the PCR amplification of a male-specific SCAR marker directly from a tissue fragment.

Five of the several cDNA-AFLP polymorphic fragments identified have been confirmed to be differentially expressed in male and female apices at the fourth node. Cloning and sequencing revealed that they belong to nine different mRNAs that were all induced in the female apices at this stage. Four out of them showed a high degree of similarity with known sequences: a putative permease, a SMT3-like protein, a putative kinesin and a RAC-GTP binding protein.

**Abbreviations:** AFLP: Amplified Fragment Length Polymorphism; LINE: Long Interspersed Elements; LTR: Long Terminal Repeat; SCAR: Sequence Characterized Amplified Region

### Introduction

*Cannabis sativa* belongs to the family of Cannabaceae, order Rosales (APGII, 2003). It is a naturally dioecious species with male and female individuals showing unisexual flowers and characterized by sexual dimorphism: male plants are generally taller and slender than female plants and have a shorter life cycle. Unisexual flowers are borne in inflorescences that are terminal at an earlier stage, and terminal or lateral in a later stage.

Male inflorescence consists of hanging panicles sometimes branched, generally with few or no leaves and composed by a variable number of flowers. Male flower has a perianth of five sepals that encloses the androecium, composed by five stamens borne by subtle stalks. The anthers at maturity undergo dehiscence longitudinally, releasing the pollen grains that are mostly wind dispersed (Mohan Ram and Nath, 1964). Female inflorescence is a raceme developing at the apex of the plant or at the axils of leaves or lateral branches. The female flower has a very simple structure as it

is composed by a green bract that completely wraps the rudimental perianth and the ovary. This latter is uniloculate and has a short style that distally differentiates a bifid stigma.

The chromosome set of *Cannabis sativa* is composed by nine pairs of autosomes and one pair of sexual chromosomes: X and Y. The male sex is endowed with an XY pair, and the female one with an XX pair, similarly to what found in other dioecious species such as *Humulus lupulus*, *Silene latifolia*, *Coccinia indica*, *Rumex hastatulus*; however, sex determination in *Cannabis* has been supposed to be based on a X:autosome dosage rather than on an active-Y mechanism (Westgaard, 1958; Grant et al., 1994). The Y chromosome in *Cannabis* is subtelocentric and characterized by a satellite at the extremity of the short arm; besides, the long arm is particularly developed and probably responsible for the difference found between the male and the female genome sizes (1683 and 1636 Mbp, respectively; Sakamoto et al., 1998). The X chromosome is submetacentric, and bears a satellite at the end of short arm. There are no specific reports about the chromosome set of monoecious plants.

As already reported for many other plant sexual chromosomes, *Cannabis sativa* Y chromosome is strongly heterochromatic and rich of repetitive sequences that are likely cause of its marked metaphasic condensation. A high percent of the repeated DNA is made of LINE-like sequences (Boecke, 1989), probably representing traces of transposable elements showing a low level of transcription for the presence of still active ORFs, coding for enzymes involved in the transposition mechanism. *Cannabis sativa* LINE elements (LINE-CS) are represented in the X chromosome and in the autosomes too, but their concentration at the end of Y chromosome is particularly high. This observation led to the hypothesis that these sequences might have a role in maintaining the structure of Y chromosome and that they can contribute to the morphological and structural differentiation of the sex chromosomes, by creating heteromorphic regions in which the recombination is prevented (Sakamoto et al., 2000; Peil et al., 2003).

The phenotypic expression of sex in hemp shows some flexibility. Anomalies in flower development are sometimes observed, such as the appearance of hermaphrodite flowers or the development of mixed inflorescences (bearing both male and female flowers), like those occurring in the monoecious phenotypes. Monoecious varieties have been developed from some of these mutations, and need a strict selection to be

maintained in the variety during the seed multiplication, due to the recessive nature of the trait.

In some hemp genotypes it is possible to obtain total or partial reversion of the sex. It is known that the treatment with masculinizing or feminizing chemical agents is effective in determining the formation of the opposite sex reproductive organs even in plants that are already sexually well differentiated. Chemicals that inhibit the biosynthesis or the activity of ethylene, such as aminoethoxyvinylglycine, silver thiosulphate and silver nitrate, have a masculinizing effect, while the precursors or activators of the biosynthesis of ethylene, like etephon, have a feminizing effect (Mohan Ram & Sett, 1982a, 1982b). The ability to undergo sexual reversion is thought to have a genetic base: some ecotypes such as the Italian Carmagnola are very resistant to any sex reversion treatment, while plants belonging to Fibranova cv. are quite prone to sex reversion (G. Grassi, E. de Meijer, personal communications).

In Italian open field conditions, the life cycle of a typical dioecious variety has a 5–6 months duration, and sexual maturity is attained after 3–4 months, when the earliest unisexual flowers appear. Sexual dimorphism of dioecious hemp is generally apparent only in a much later stage of development, just before the onset of flowering, when a marked elongation of the last internodes occurs in male plants causing them to become taller and slender than female plants.

Sex is considered an important trait for hemp genetic improvement. The Bredemann's strategy of selection for fibre quality implies a relatively early qualitative analysis of fibre in male plants before pollen dispersion (Bredemann, 1938). This analysis is followed by the elimination of lower-quality male plants, not intended for pollination. Therefore, the possibility of early sex identification and the study of the mechanism of sexual differentiation in dioecious varieties are of paramount interest in hemp research. Attempts have been made in the pre-genomic era by multivariate analysis of morphological traits followed by correlation to the sex expression (Lacombe, 1980). Since the Nineties, DNA markers were developed, capable of discriminating the male plants from the female and the monoecious ones (Mandolino et al., 1998, 1999). Such markers (see also the paper by G. Mandolino and A. Carboni in this special issue) can be fruitfully used in the selection schemes for hemp breeding, and in the assessment of the number of male plants in monoecious seed lots.

Sex linked markers, provided that they are tightly and reliably associated to the sexual phenotype, can