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Identification of mutations in three genes responsible for phytocannabinoids biosynthesis and trichome differentiation by using a Reverse Genetics approach in *Cannabis sativa* L.

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ABSTRACT

Functional genomics in *Cannabis sativa L.* is still limited, mainly due to the lack of efficient regeneration systems hindering CRISPR-Cas9 applications. To address this constraint, at CRAG-IRTA (Barcelona, Spain) a chemical mutagenesis platform based on EMS treatment within a TILLING approach was established, generating over 1,600 M2 families suitable for reverse genetics. This resource enabled the identification of mutations in key genes controlling phytocannabinoid biosynthesis, plant architecture, and trichome epidermal differentiation. Four mutant families were selected for molecular and phenotypic characterization: *Olivetolic Acid Synthase* (OLS and 250), *Prenyl Transferase 4* (PT4), and a MYB transcription factor (MYB) involved in trichome differentiation and patterning. After PACE genotyping and targeted sequencing, homozygous and heterozygous individuals were cultivated under accelerated breeding conditions and evaluated for chemotype and morphological traits. This platform represents the first large-scale functional genomics tool for *C. sativa*, providing a valuable resource to dissect gene function and to identify novel regulators of trichome development and cannabinoid metabolism. This work is part of the Ph.D. program *"Innovation and Breeding in Medical Cannabis"* at the University of Modena and Reggio Emilia.

MATERIAL AND METHODS

Plant Material: *Two hundred C. sativa* seeds from the TILLCAN platform were germinated. Four mutant families (*Olivetolic Acid Synthase* – OLS/250, *Prenyl Transferase 4* – PT4, and a MYB transcription factor) plus WT controls were analyzed. Parental lines derived from "Elite" (high-CBD) × EU-certified 'Finola'.

Growing conditions: Seeds pretreated in 0.5% oxygenated solution (24 h) and sown in paperpots (Gramoflor:perlite:vermiculite, 9:3:1). Seedlings grown in phytotron (22 °C, 16 h light), then transferred to Torre Marimon greenhouse, 2 L pots, mineral fertilization (Figure 1A).

Genotyping Target genes: DNA extracted with modified CTAB. PCR assays for sex/chemotype. PACE markers applied to MYB and OLS; PT4 and 250 validated by Sanger sequencing (Figure 1B)

Cross and breeding pollination: Regular and feminized seeds produced via conventional crossing and STS reversion. Flowering induced under short-day (12 h) after 1 month vegetative. Flowers harvested at 68 days; seeds after two weeks (Figure 1 C)

Phenotyping Focus on phytocannabinoids composition and glandular trichome development in inflorescences of MYB population. Phytocannabinoids analyzed by HPLC –UV. Trichome density was evaluated on floral bracts collected from the apical dome (Figure 1 D), with three calyces per sample and three regions per calyx (basal, median, apical) on a total of 14 samples (4 WT, 5 XY, 4 YY) .

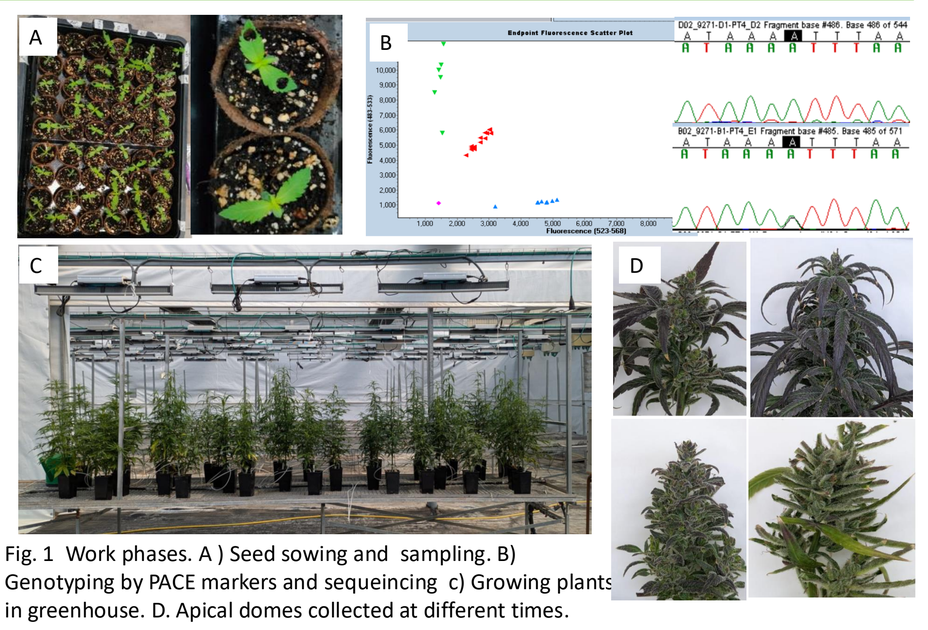

Fig. 1 Work phases. A) Seed sowing and sampling. B) Genotyping by PACE markers and sequeincing c) Growing plants in greenhouse. D. Apical domes collected at different times.

RESULTS

Some of the families developed and bred did not produce genotypes carrying the homozygous dominant allele for the target mutation, while others provided only a limited number of individuals suitable for statistical evaluation. In family 250, only a single XY individual was obtained, limiting the analysis to an exploratory inspection of the distributions (not reported in the following Poster). Mutations within the coding sequences were predicted to be functional in some families (e.g., 250 and PT4) but not in others (e.g., OLS and MYB). Nevertheless, only the MYB family showed a clear and biologically relevant phenotype, while the results of the other families remained inconclusive and difficult to interpret.

OLS

Data from the OLS family showed non-normal distribution for THC and CBG. No significant differences were detected among allelic forms (WT, homozygous, heterozygous) for any cannabinoid (Figure 2). Both ANOVA with Tukey's test and non-parametric analyses (Kruskal–Wallis with Dunn's test) confirmed the absence of significant variation ($p > 0.05$). Regression analysis also indicated no effect of allelic form on cannabinoid levels.

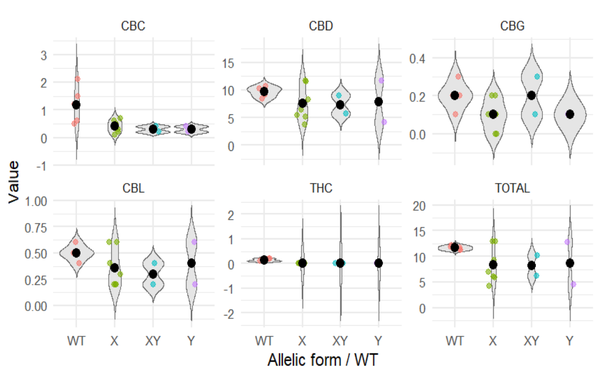

Fig. 2 Cannabinoid distribution, samples and average in different allelic form of OLS family. The Y Axis reported the % on the weight of a dry mass

PT4

The PT4 mutant population showed non-normal data distribution. Although cannabinoid levels differed between WT and PT4, no significant variation was found among allelic forms within the family, except for CBC, which differed between homozygous recessive and heterozygous individuals ($p=0.03$) (Figure 3). Linear regression confirmed no association between allelic form and cannabinoid content ($p > 0.05$).

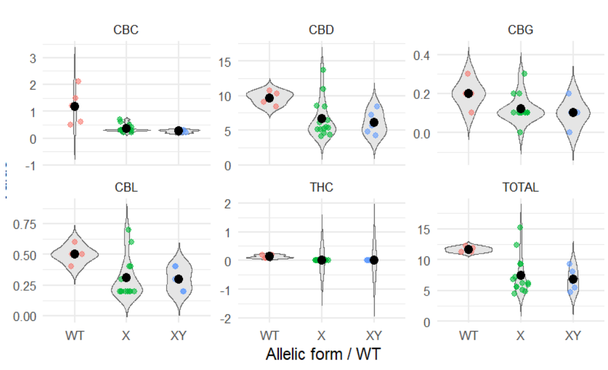

Fig. 3. PT4 Cannabinoid distribution, samples and average in different allelic form vs WT. The Y Axis reported % of the weight on dry mass

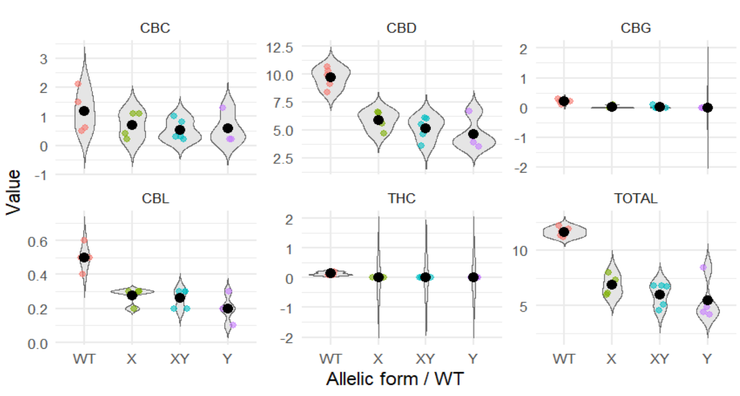

Fig. 3 MYB Cannabinoid content distribution, samples and mean

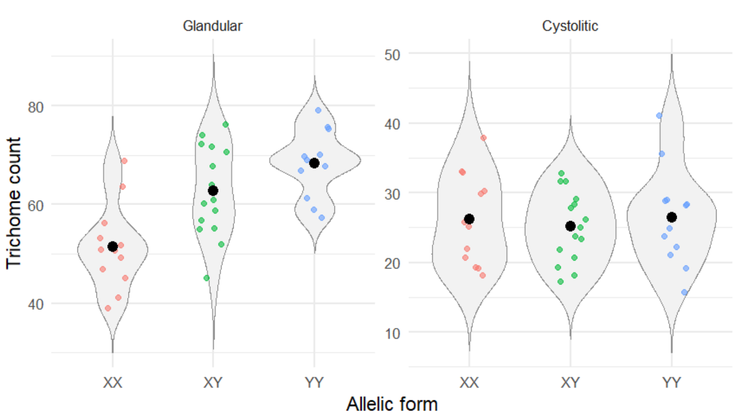

Fig. 4 MYB Violin plots about different type of trichome in different allelic form

MYB

In the MYB family, data were normally distributed except for CBG.

ANOVA (Figure 3) revealed significant differences for Total Cannabinoids ($p = 0.008$), CBD ($p = 0.009$), and CBL ($p = 0.013$), and THC ($p = 0.05$).

Post-hoc analyses indicated reduced Total Cannabinoids and CBD in allelic forms XY and Y, and lower CBL level in Y.Y mutants

For CBG, Kruskal-Wallis confirmed significant differences ($p = 0.042$), while Mann-Whitney highlighted a strong divergence between MYB and WT ($p = 0.0005$). Regression further supported a consistent reduction of Total Cannabinoids, CBD, CBG,THC and CBL in XY and Y.

In the MYB population trichome counts were assessed across three allelic families: WT (XX), heterozygous mutants (XY), and homozygous mutants (YY). The analysis revealed distinct patterns in the distribution of glandular trichomes among the allelic forms, highlighting genotype-dependent variation in trichome type and abundance (Figure 4 and 5)

Interestingly, a novel trichome type was detected in MYB mutants (Figure 5).

Fig. 5 MYB Calix trichome analyses and a novel branched trichome discovered

CONCLUSIONS

This study demonstrates the potential of TILLING as a functional genomics tool in *C. sativa*, providing a non-transgenic strategy for trait discovery in a species recalcitrant to regeneration. MYB mutants were the most informative, showing reduced cannabinoid accumulation despite higher trichome density, suggesting a role in epidermal development with complex effects on metabolism. The detection of a novel trichome type further supports MYB involvement in epidermal patterning and emphasizes the need for backcrossing to stabilize these lines. Overall, TILLING proves effective in linking mutations to phenotypes and offers a foundation for future studies on key regulatory genes for breeding and biotechnology.

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