Full title:

**Exploring Ethylene-Related Genes in Cannabis sativa: Implications for Sexual Plasticity**

Short title:

**Ethylene and *Cannabis* sexual plasticity**

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**Data Availability Statement:**

All scripts and raw data used in this study are available from The Open Science Framework (OSF) under the DOI: DOI 10.17605/OSF.IO/7G9VR at OSF.io. or from the NCBI, under previously published projects.

# Abstract

Sexual plasticity describes an organism's ability to change its phenotypic sex in response to environmental, chemical or physiological cues without a change in sex chromosome karyotype. *Cannabis sativa* L. (cannabis) is a medically important dioecious crop, with a pair of heteromorphic XY sex chromosomes. Cannabis has demonstrated sexual plasticity in response to chemical treatments which target the biosynthesis and signalling of ethylene. To date, ethylene biosynthesis and signalling has not been specifically studied in *C. sativa*, and the role of ethylene-related genes (ERGs) in sexual plasticity in the species has not been investigated. In the present study, we performed gene orthology analysis based on the model species *Arabidopsis thaliana* L. to reconstruct the *C. sativa* Yang Cycle, ethylene biosynthesis and ethylene signalling pathways, whose canonical composition appear conserved in cannabis. Additionally, we employ two transcriptomic datasets containing male, female and chemically induced male flowers to investigate patterns of expression for ethylene-related genes which may be involved in sexual plasticity. Comparison of these transcriptomes highlighted CsERGs whose differential expression was associated with sexual determination and those which we propose are associated with the sexual plasticity. These sexual plasticity ERGs could be classified into two patterns of expression: karyotype concordant (KC) and unique (uERG). We show that CsERGs involved in sexual plasticity are distributed throughout the genome and are not exclusive to the sex chromosomes, suggesting a broad control of sexual plasticity in *C. sativa*.



**Keywords:** ethylene, sexual plasticity, cannabis, STS, biosynthesis, sex expression, sex determination

# Introduction

*Cannabis sativa* L. (cannabis) belongs to the estimated 5-6% of dioecious angiosperm species (Renner, 2014). Despite their rarity, dioecious plants provide an excellent opportunity to study the mechanisms involved in sex expression and the evolution of sex-determining regions and sex chromosomes. Cannabis (2n=20) is predominantly dioecious with a XY sex-determination system; however, monoecious cultivars of *C. sativa* have been bred, especially for the production of seeds and oil used in industrial hemp (Ferfuia *et al.*, 2021). Additionally, dioecious cannabis cultivars have been known to display hermaphroditism (Monthony *et al.*, 2021c), reversion from a sexual to vegetative state (Monthony *et al.*, 2021a), and sometimes complete changes in sex expression in response to certain environmental factors and chemical reagents (Ram & Jaiswal, 1972; Mohan Ram & Sett, 1982; Lubell & Brand, 2018; Moon *et al.*, 2020a). Taken together, these observations suggest that cannabis sex is plastic and not strictly controlled by its XX or XY chromosomal complement.

Sexual plasticity is the ability of an organism to change its phenotypic sex in response to environmental or physiological cues without altering its sex chromosomes (Liu *et al.*, 2017). Vertebrates exhibit sexual plasticity on a continuum from genotypic sex determination (GSD) to environmental sex determination (ESD), with some organisms influenced by both (GSD + ESD; Lambert *et al.*, 2019). Non-mammalian vertebrates, such as fish (Senthilkumaran, 2015; Liu *et al.*, 2017) and frogs (Lambert *et al.*, 2019), as well as insects like aphids and bees (Morgan, 1909; Charlesworth & Mank, 2010), exhibit sexual plasticity triggered by diverse factors. The mechanisms responsible for these changes vary, but can involve gene expression changes related to hormone synthesis and signaling, germ cell development and maintenance as well as

microRNAs (Perry & Grober, 2003; Hayes *et al.*, 2010; Ashby *et al.*, 2016; Cardoso-Júnior *et al.*, 2017). For instance, social factors such as population density, mating competition, and hierarchical structures can affect sexual plasticity in fish (Warner & Swearer, 1991; Liu *et al.*, 2017). Amphibians can exhibit sexual plasticity under normal and extreme conditions (Lambert *et al.*, 2015, 2019), while in some insects, the mode of sexual reproduction can affect offspring sex (Morgan, 1909; Charlesworth & Mank, 2010). Although the term *sexual plasticity* has largely been used to describe fluid sex determination in the animal kingdom (Schlesinger *et al.*, 2010; Senthilkumaran, 2015; Liu *et al.*, 2017), the present study highlights how sexual plasticity can also be used to describe the interaction between GSD and ESD in dioecious plants, such as *C. sativa*.

Similar to amphibians, informal research conducted by enthusiast breeders prior to legalization has demonstrated that chemical exposure can influence sexual plasticity in plants such as *C. sativa*. (Green, 2005; Thomas, 2012). This pre-legalization exploratory work identified silver-containing compounds such as silver thiosulfate (STS), a potent inhibitor of ethylene signalling, as promoting the production of male flowers on female plants (induced male flowers; IMF) and aqueous ethephon ((2-Chloroethyl) phosphonic acid), which rapidly decomposes to produce ethylene gas, as promoting the production of female flowers on male plants (induced female flowers; IFF) as shown in Figure 1. Following the legalization of cannabis in some jurisdictions, the inverse effects of STS and ethephon on cannabis sex have been re-demonstrated, quantified and published by a handful of research groups (Lubell & Brand, 2018; Kurtz *et al.*, 2020; Moon *et al.*, 2020a; Flajšman *et al.*, 2021), pointing to ethylene as being an effector of sexual plasticity. Although the phenotypic outcomes of these treatments in cannabis have been quantified, to date there are no studies which have sought to understand the underlying molecular mechanisms controlling ethylene-related sexual plasticity in cannabis.

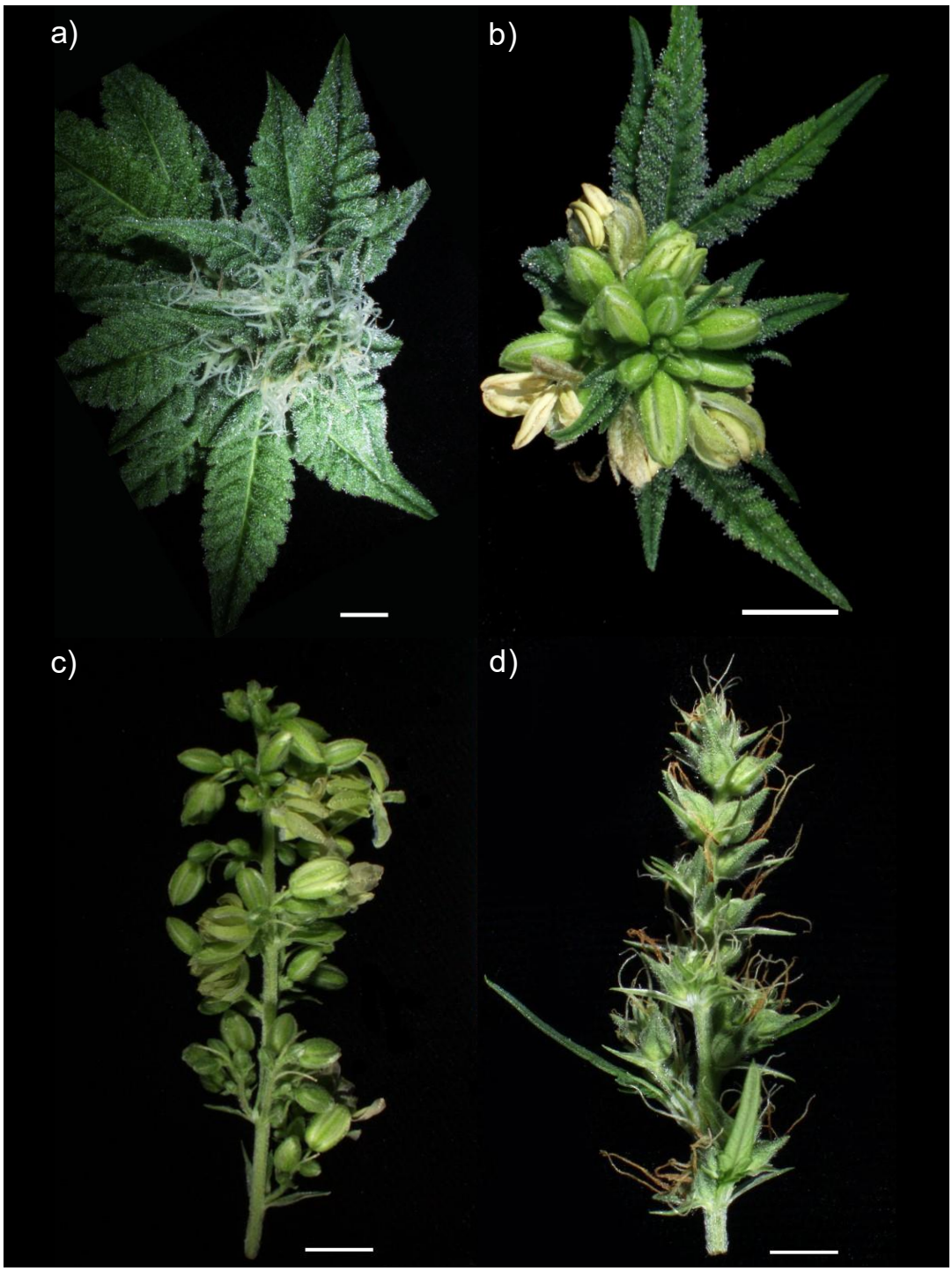


**Figure 1** Representative photos (unpublished data) of four floral phenotypes in *Cannabis sativa* (cv. La Rosca), grown at Université Laval. a) 4-week-old female flowers on a genetically female plant; b) 4-week-old STS-induced male flowers (IMFs) on a genetically female plant; c) 3-week-old male flowers on a genetically male plant and d) ethephon-induced female flowers (IFFs) on a genetically male plant. Scale bars = 5 mm.

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Ethylene was first reported as a plant growth regulator in 1901 and has since been extensively studied (Coulter & Barnes, 1901; Gane, 1934; Kende, 1998; Chang, 2016). It is involved in various biological processes in plants, including seed germination, root development, shoot and root growth, flowering, fruit ripening, leaf and fruit abscission, and responses to abiotic and biotic stresses and senescence (Tieman *et al.*, 2000; Resnick *et al.*, 2006; Argueso *et al.*, 2007; Yang *et al.*, 2015; Dhakal *et al.*, 2019; Binder, 2020). The biosynthesis pathways of ethylene are highly conserved across the plant kingdom, and considerable time and resources have been dedicated to its study (Yang *et al.*, 2015). Ethylene is synthesized via a two-step pathway from S-adenosyl methionine (SAM) derived from the Yang Cycle. The first step is catalyzed by 1-aminocyclopropane-1-carboxilic acid (ACC) synthase (ACS), which converts SAM to ACC and 5'-methylthioadenosine (MTA; Argueso *et al.* 2007; Pattyn *et al.* 2021). ACS is encoded by a multigene family in most plants, with the genes being differentially regulated depending on various environmental and developmental factors (Argueso *et al.*, 2007; Dubois *et al.*, 2018). Arabidopsis has eight identified ACS genes (Argueso *et al.*, 2007). The final step of ethylene biosynthesis is catalyzed by ACC oxidase (ACO), which converts ACC to ethylene, cyanide, and $CO_2$ as by-products (Argueso *et al.*, 2007; Houben & Van de Poel, 2019). Arabidopsis has an estimated five ACO protein family members (Houben & Van de Poel, 2019). In cucumber, ACO plays a key role in sex determination, with four ACO genes identified as rate-limiting in the biosynthetic pathway (Chen *et al.*, 2016a). The genes involved in ethylene signaling are also highly conserved across plant species and can be represented by a largely linear model, with recent studies finding secondary non-canonical pathways that play a minor role (Dubois *et al.*, 2018; Binder, 2020). Ethylene receptors found in the endoplasmic reticulum (ER) are negatively regulated by the Golgi-associated reversion to ethylene sensitivity 1 (RTE1; Zhou *et al.*, 2007) and an ER-localized family

of proteins called auxin-regulated genes involved in organ size (ARGOS; Rai *et al.* 2015; Shi *et al.* 2016). In the presence of ethylene, these receptors, ethylene response sensor 1 (ERS1), ERS2, ethylene resistance 1 (ETR1), ETR2, and ethylene insensitive 4 (EIN4) bind to ethylene to initiate signaling (Dubois *et al.*, 2018). Ethylene functions as an inverse agonist, promoting further ethylene production by altering downstream activity of the biosynthetic pathway and reducing the inhibition of downstream signaling by lowering the activity of constitutive triple response 1 (CTR1) in the presence of a copper ion cofactor (Mayerhofer *et al.*, 2012; Binder, 2020). Inhibition of CTR1 activity by ethylene results in dephosphorylation of EIN2 and cleavage of its c-terminal domain (C-END), allowing for repression of EBF1/2 translation by binding to the 3' untranslated regions of their mRNA (Chang, 2016; Dubois *et al.*, 2018; Binder, 2020). In the absence of ethylene, EBF1/2 target the primary ethylene-responsive transcription factors EIN3 and EIN3-LIKE (EIL1) for protein degradation (Dubois *et al.*, 2018). Repression of EBF1/2 by ethylene allows for expression of EIN3 and EIN3-like transcription factors, which induce expression of ethylene response factors (ERFs), promoting further ethylene biosynthesis. Although the canonical signaling pathways are largely understood, the study of ERFs remains an area that requires further investigation (Olsen *et al.*, 2015; Dubois *et al.*, 2018).

Compared with cannabis, the role of ethylene in sexual plasticity is much more well-understood in the Cucurbitaceae family, where ethylene biosynthesis and signalling have also been shown to be manipulated by STS or ethephon applications (Li *et al.*, 2021). In most cucurbits, ethylene (usually applied as ethephon) has a feminizing effect, increasing the ratio of female flowers relative to males, while the use of STS has the inverse effect, causing a masculinizing effect and results in increased production of male flowers. Numerous studies of sexual plasticity in cucurbits have pointed to the role of ethylene biosynthesis genes such as

*ACS4* and *ACS11*, as well as ethylene signalling genes such as the ethylene receptor *ETR1* as contributing to sexual plasticity (Boualem *et al.*, 2015; Zhang *et al.*, 2017; Li *et al.*, 2020). The manipulation of cucumber sex has allowed growers to control and optimize the ratio of female to male flowers to improve yield and to control production and labour constraints (Oda *et al.*, 2022). In cannabis, control of plant sex is important as medicinally valuable cannabinoids are primarily produced in female flowers. However, without a pathway model for ethylene biosynthesis and signalling in the species, study of cannabis sexual plasticity remains impossible. The highly conserved nature of ethylene biosynthesis and signalling and well-characterized pathways in model species *Arabidopsis thaliana* present a valuable starting point for the reconstruction of the pathway in an under-researched plant such as *C. sativa* where ethylene biosynthesis and signalling has yet to be explored. In the current study, we use homology analysis and RNA-seq data to present the first *C. sativa* putative Yang cycle, ethylene biosynthesis and ethylene signalling pathways, which will serve as an important foundation for expanding our understanding of the role of ethylene in sexual plasticity of this under-researched dioecious species.

# Materials & Methods

## *In silico* identification of ethylene-related gene orthologs in *C. sativa*

*Arabidopsis thaliana* was chosen as the model from which a list of genes related to ethylene biosynthesis and signalling, as well as the production of ethylene precursors derived from the Yang cycle were compiled following a review of the literature (Pommerrenig *et al.*, 2011; van de Poel *et al.*, 2012; Olsen *et al.*, 2015; Chang, 2016; Pattyn *et al.*, 2021). The resulting list of *A. thaliana*

ethylene-related genes (AtERGs; **Error! Reference source not found.**) were used for the identification of orthologous *C. sativa* ERGs (CsERGs). Nucleotide and protein sequences for AtERGs were obtained from the National Center for Biotechnology Information database (NCBI; Bethesda, MD). Ortholog identification was conducted on the *C. sativa* cs10 reference genome (RefSeq assembly accession: GCF_900626175.2; Grassa *et al.*, 2021). A list of putative CsERG orthologs was compiled using two different methods. The first method employed OrthoFinder (Emms & Kelly, 2019) adapted from Carey *et al.* (2021) and the second method consisted of a BLASTP search of each individual AtERG against the cs10 reference genome (Altschul *et al.*, 1990), followed by a manual selection of putative orthologs from the of BLASTP result adapted from Barcaccia *et al.* (2020). This method is described in further detail in the **Error! Reference source not found.**. The chromosome map (Figure 4a) was generated with chromoMap (v4.1.1; Anand & Rodriguez Lopez, 2022) and bar plots of gene distribution (Figure 4b) was generated with ggplot2 (v3.4.1; Wickham, 2016) using the colour palette Spectral from RColorBrewer (Brewer *et al.*, 2002)

## RNA-seq data collection and processing

The RNA-seq reads from Adal *et al.* (2021) and Prentout *et al.* (2020), hereafter referred to as dataset 1 and dataset 2, were downloaded from the Sequence Read Archive (SRA) on NCBI under the BioProject accessions PRJNA669389 and PRJNA549804, respectively. RNA was isolated from male flowers (MFs), female flowers (FFs) and STS-induced IMFs for dataset 1, and from MFs and FFs for dataset 2. To perform a relevant comparison between the 2 datasets, only data from similar tissues were considered, i.e., the young flower buds. The SRA sample accessions used in this study are indicated in **Error! Reference source not found.**. FastQC (Andrews, 2010) was used for validating the quality of the data. Then, reads were aligned to the *C. sativa* cs10

reference genome (version GCF_900626175.2; Grassa *et al.*, 2021) using STAR (v2.7.2b; Dobin *et al.*, 2013) as reported in Cai *et al.* (2020). The STAR index was built with the –sjdbOverhang=100 and the genomeSAindexbases=13 arguments. Splice junctions from the *C. sativa* cs10 RefSeq assembly GTF version GCF_900626175.2 were provided via the --sjdbGTFfile argument. STAR alignments were generated using the default parameters including the --outSAMtype BAM SortedByCoordinate arguments. Quality control of the mapped reads was performed using Qualimap (v2.2.1; Okonechnikov *et al.*, 2015). One IMF sample from dataset 1 was excluded based on abnormal distribution of GC-content among mapped reads which included large shoulders at 30% and 60 % GC content, indicating contamination of the sample (see **Error! Reference source not found.**). Finally, quantification of reads overlapping genetic features was performed with HTSeq (v2.0.1; Putri *et al.*, 2022) based on the cs10 GFF file obtained from NCBI (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/900/626/175/GCF_900626175.2_cs10/GCF_900626175.2_cs10_genomic.gff.gz) in order to generate a gene quantification table required for subsequent differential analysis with DEBrowser (v1.22.5; Kucukural *et al.*, 2019).

## Differential expression analysis of CsERGs

Differentially expressed CsERGs were identified using DESeq2 (Love *et al.*, 2014) with a FDR-adjusted $p$-value < 0.05 on the DEBrowser graphical user interface in RStudio (RStudio Inc., MA, USA) using v4.1.1 of R. The analysis was adapted from DEBrowser's DESeq2 method outlined on the DEBrowser Github (https://github.com/UMMS-Biocore/debrowser). In short, gene quantification tables generated in HTSeq and a manually generated metadata file for the samples were uploaded to DEBrowser. Genes with count per million values less than 1 (CPM < 1) in 7 out of 15 replicates in dataset 1 and 6 out of 12 replicates in dataset 2 were removed. Replicates were then grouped and pairwise comparisons were performed based on the floral type. In DEBrowser

the following DESeq2 parameters were used following the method outline by Kucukural *et al.* (2019) and the DEBrowser Github: *Fit Type- parametric; betaPrior- FALSE; Test Type- Wald; Shrinkage- None*. Differential expression levels were then normalized using a Median Ratio Normalization (MRN) and pairwise comparisons between treatments were considered significantly different in subsequent comparisons if they had an adjusted *p*-value <0.05 according to the Wald test and a |log2foldchange| > 1. Heatmaps comparing differential gene expression were then generated using DEBrowser heatmap function. Tables from pairwise comparisons were generated from DEBrowser and then used for the generation of gene expression bar plots using ggplot2 (Wickham, 2016) in RStudio.

# Results

## ERGs are largely conserved in *C. sativa*

Based on 47 AtERGs previously identified (**Error! Reference source not found.**), ortholog analyses identified 43 putative CsERGs (Table 1). At least one ortholog of each key gene in the canonical ethylene biosynthesis and signalling pathways, as well as the Yang cycle, was identified *in silico* except for auxin-regulated genes involved in organ size (AtARGOS; **Error! Reference source not found.**). To our knowledge this represents this first time that the genes coding for the various enzymes involved in the putative Yang cycle (Figure 2a), ethylene biosynthesis (Figure 2b) and signalling pathway (Figure 3) have been put forward in *C. sativa.*

**Table 1** *Cannabis sativa* ethylene-related genes (CsERGs) identified through ortholog analysis.

| Cs10 ERG Locus[‡] | Name | Chromosome | *A. thaliana* homologs | Pathway | Identification method |
|---|---|---|---|---|---|
| LOC115704991 | CsACO1 | 1 | AT2G19590 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115702109 | CsACO1-LIKE1 | Un[§] | AT2G19590 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115707397 | CsACO2[*†] | 1 | AT1G62380, AT1G12010, AT1G05010 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115699412 | CsACO3[*†] | 8 | AT1G62380, AT1G12010, AT1G05010 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115699408 | CsACO5[*†] | X | AT1G77330 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115704849 | CsACS1 | 1 | AT3G49700, AT4G08040, AT4G11280, AT1G01480 | Biosynthesis | BLASTP |
| LOC115717044 | CsACS2[*†] | 5 | AT4G08040, AT1G01480, AT4G11280, AT3G49700 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115712396 | CsACS3 | 4 | AT5G65800, AT4G37770, AT4G08040, AT3G49700 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115704963 | CsACS7-LIKE | 1 | AT4G26200, AT1G01480, AT4G11280, AT4G08040, AT3G49700 | Biosynthesis | BLASTP, OrthoFinder |
| LOC115696400 | CsACS9[†] | X | AT4G08040, AT5G65800, AT4G37770, AT3G49700 | Biosynthesis | BLASTP |
| LOC115716540 | CsACS9-LIKE | 5 | AT5G65800, AT4G37770, AT4G08040, AT3G49700, AT2G22810 | Biosynthesis | BLASTP |
| LOC115697744 | CsCTR1[*†] | X | AT5G03730 | Signalling | BLASTP |
| LOC115706236 | CsCTR1-LIKE[*†] | 1 | AT5G03730 | Signalling | BLASTP, OrthoFinder |
| LOC115720532 | CsEBF1[*†] | X | AT5G25350, AT2G25490 | Signalling | BLASTP, OrthoFinder |
| LOC115724585 | CsEBF2[*†] | 6 | AT5G25350, AT2G25490 | Signalling | BLASTP |
| LOC115711236 | CsEIL1[*†] | X | AT2G27050 | Signalling | OrthoFinder |
| LOC115719373 | CsEIL3[*†] | 2 | AT1G73730 | Signalling | OrthoFinder, BLASTP |
| LOC115709621 | CsEIN2[*†] | 3 | AT5G03280 | Signalling | BLASTP, OrthoFinder |
| LOC115706079 | CsEIN3[*†] | 1 | AT2G27050, AT3G20770 | Signalling | BLASTP, OrthoFinder |
| LOC115723050 | CsERF1 | 9 | AT3G23240 | Signalling | BLASTP, OrthoFinder |
| LOC115697291 | CsERS1[*†] | 7 | AT2G40940 | Signalling | BLASTP, OrthoFinder |
| LOC115705474 | CsETP1[*†] | 1 | AT3G18980 | Signalling | BLASTP |
| LOC115720323 | CsETP2 | 2 | AT3G18910 | Signalling | BLASTP |
| LOC115702329 | CsETR1[*†] | X | AT1G66340, AT2G40940 | Signalling | BLASTP, OrthoFinder |
| LOC115721785 | CsETR2[*†] | 9 | AT3G04580, AT1G04310, AT3G23150 | Signalling | BLASTP, OrthoFinder |
| LOC115698184 | CsRAN1[*†] | 7 | AT5G44790 | Signalling | BLASTP, OrthoFinder |
| LOC115707134 | CsRTE1[*†] | X | AT2G26070 | Signalling | BLASTP, OrthoFinder |

| Cs10 ERG Locus[‡] | Name | Chromosome | *A. thaliana* homologs | Pathway | Identification method |
|---|---|---|---|---|---|
| LOC115721731 | CsARD1[*†] | 9 | AT5G43850, AT4G14716, AT4G14710, AT2G26400 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115722194 | CsARD2[*†] | 9 | AT4G14716, AT2G26400, AT5G43850, AT4G14710 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115706479 | CsMTI1[*†] | 1 | AT2G05830 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115703014 | CsMTK1[*†] | X | AT1G49820 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115702983 | CsMTK1-LIKE[*†] | X | AT1G49820 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115704327 | CsMTN[*†] | X | AT4G34840, AT4G38800 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115706293 | CsDEP1[*†] | 1 | AT5G53850 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115711364 | CsSAM1[*†] | X | AT1G02500, AT4G01850, AT3G17390, AT2G36880 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115719259 | CsSAM2[*†] | 2 | AT1G02500, AT4G01850, AT3G17390, AT2G36880 | Yang Cycle | BLASTP |
| LOC115711343 | CsSAM3[*†] | 3 | AT2G36880, AT3G17390, AT4G01850, AT1G02500 | Yang Cycle | BLASTP, OrthoFinder |
| LOC115700576 | CsFYF1[*†] | 8 | AT5G62165 | Ethylene Adjacent | BLASTP |
| LOC115706939 | CsFYF2[*†] | 1 | AT5G62165 | Ethylene Adjacent | BLASTP |
| LOC115716986 | CsFYF3[*†] | 5 | AT5G62165 | Ethylene Adjacent | BLASTP |
| LOC115717309 | CsFYF4[*†] | 5 | AT5G62165 | Ethylene Adjacent | BLASTP |
| LOC115701489 | CsORE1[*†] | 8 | AT5G39610 | Ethylene Adjacent | BLASTP, OrthoFinder |
| LOC115709772 | CsORE2[*†] | 3 | AT5G39610 | Ethylene Adjacent | BLASTP |

‡ Hyperlinks to the NCBI cs10 reference genome information page for the specific locus.

* and † ERGs for which transcripts were detected from dataset 1 and/or in dataset 2, respectively.

§ A gene located on an unclassified scaffold.

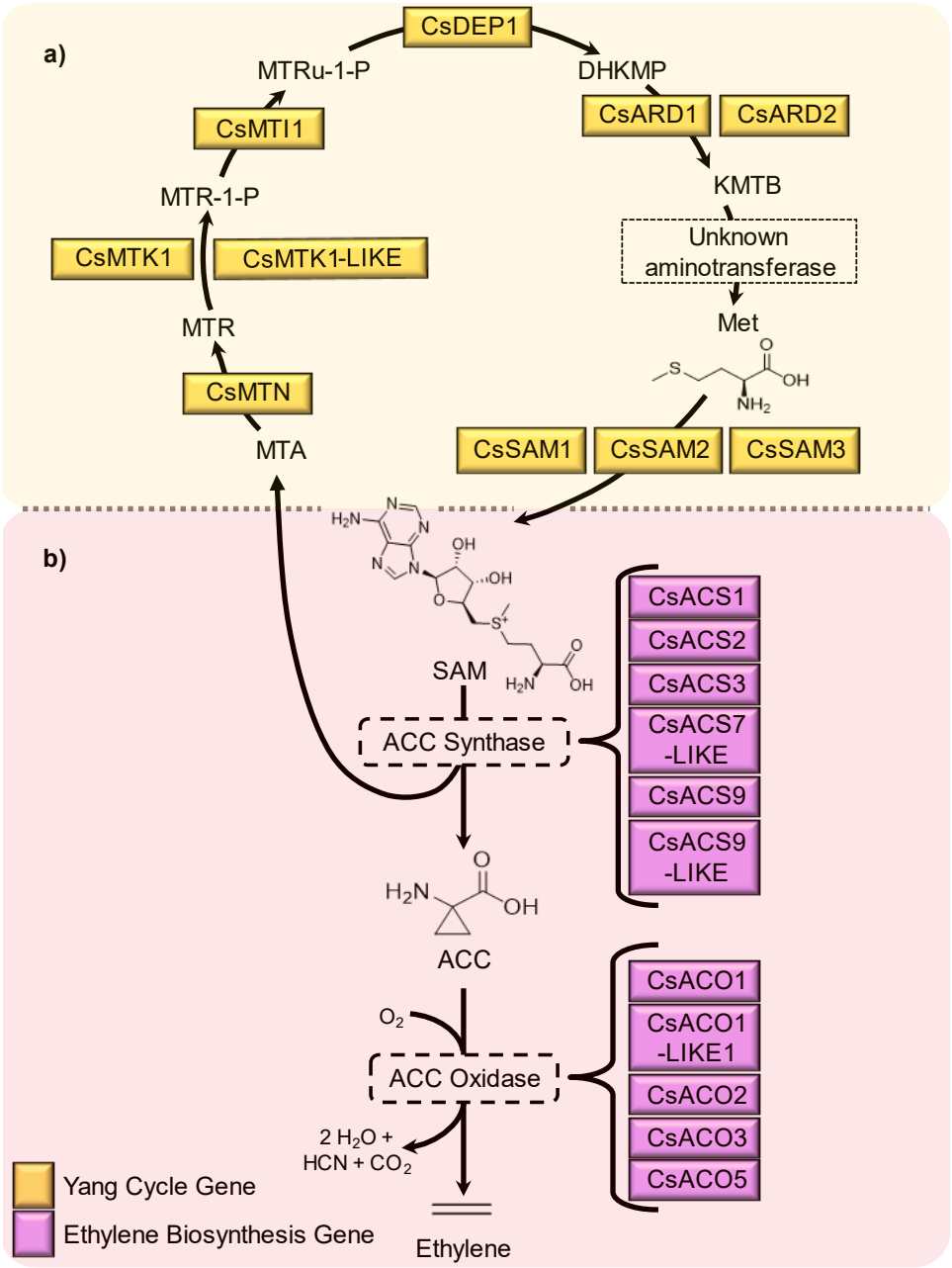


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**Figure 2** The *C. sativa* Yang cycle and ethylene biosynthesis pathway determined by homology analysis with *A. thaliana* genome. (a) Yang cycle; abbreviations: *S*-adenosylmethionine (SAM), 5-methylthioadenosine (MTA), 5-methylthioribose (MTR), 5-methyltioriobose-1-P (MTR-1-P), 5-methylthioribulose-1-P (MTRu-1-P), 1,2-dihydroxy-3-keto-5-methylthiopentene (DHKMP), 2-keto-4-methylthiobutyrate (KMTB), methionine (Met). (b) Ethylene biosynthesis pathway.

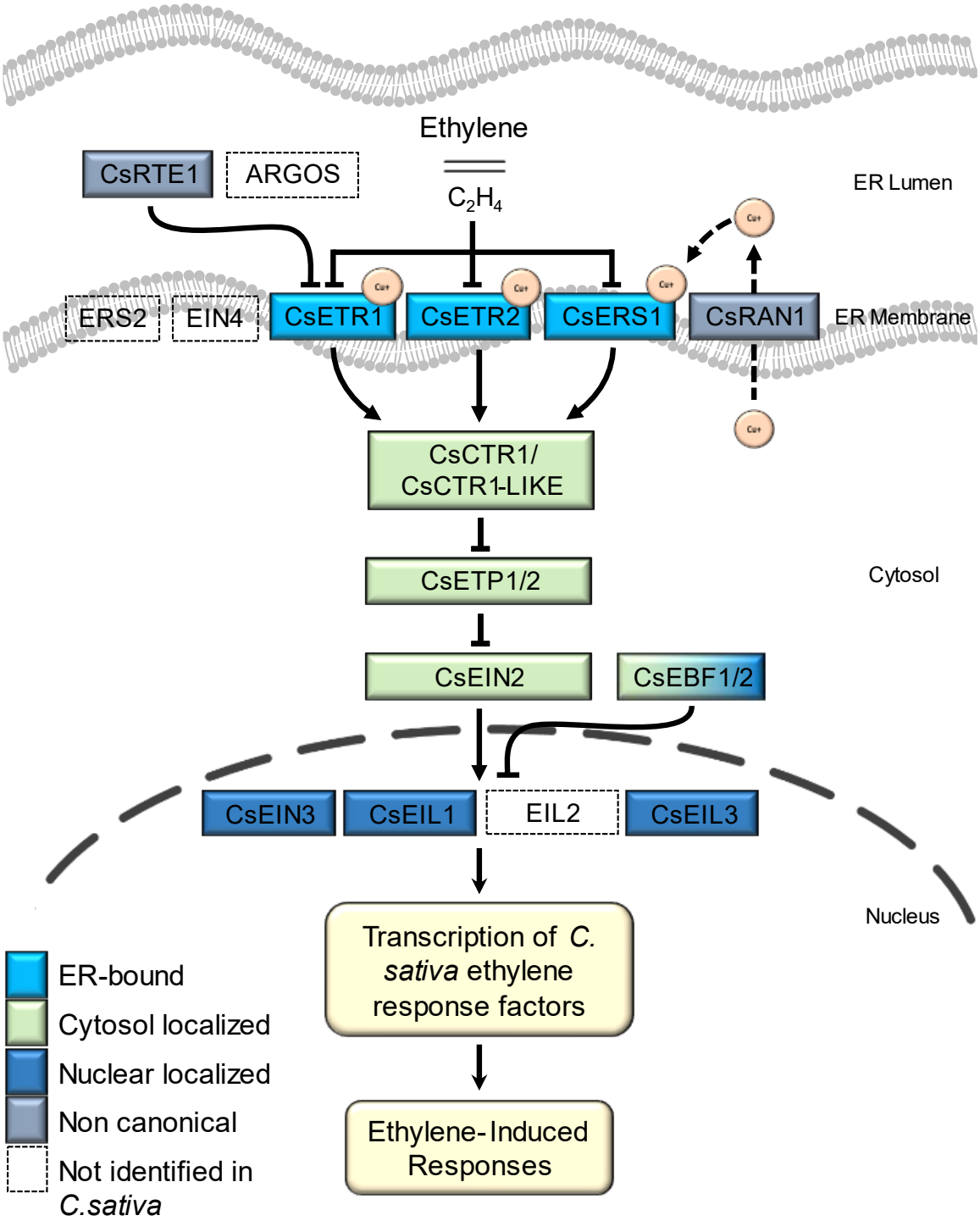


**Figure 3** The *C. sativa* ethylene signalling pathway inferred by homology analysis with *A. thaliana* genome. The pathway was elucidated using the canonical pathway from *A. thaliana*, genes from *A. thaliana* that were not identified in *C. sativa* are outlined by a dashed line. Figure design was based on existing ethylene signalling models (Binder *et al.*, 2010; Dubois *et al.*, 2018; Binder, 2020).

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## ERGs are distributed genome-wide in *C. sativa*

The 43 CsERGs found in the homology analysis were distributed throughout the genome (Figure 4 and **Error! Reference source not found.**). A majority of the CsERGs were located on autosomes (Figure 4b). Yang cycle genes were the most numerous CsERGs present on the sex chromosomes, with 40% of the identified *C. sativa* Yang cycle genes located on the X chromosome. In contrast, none of the genes that were classified as ethylene adjacent (ERGs not belonging to the canonical ethylene biosynthesis or signaling pathways) were located on the sex chromosomes (Figure 4). Of the 43 CsERGs identified all mapped to chromosomes in the reference genome, with the exception of CsACO1-LIKE1, which mapped to an unassigned scaffold.

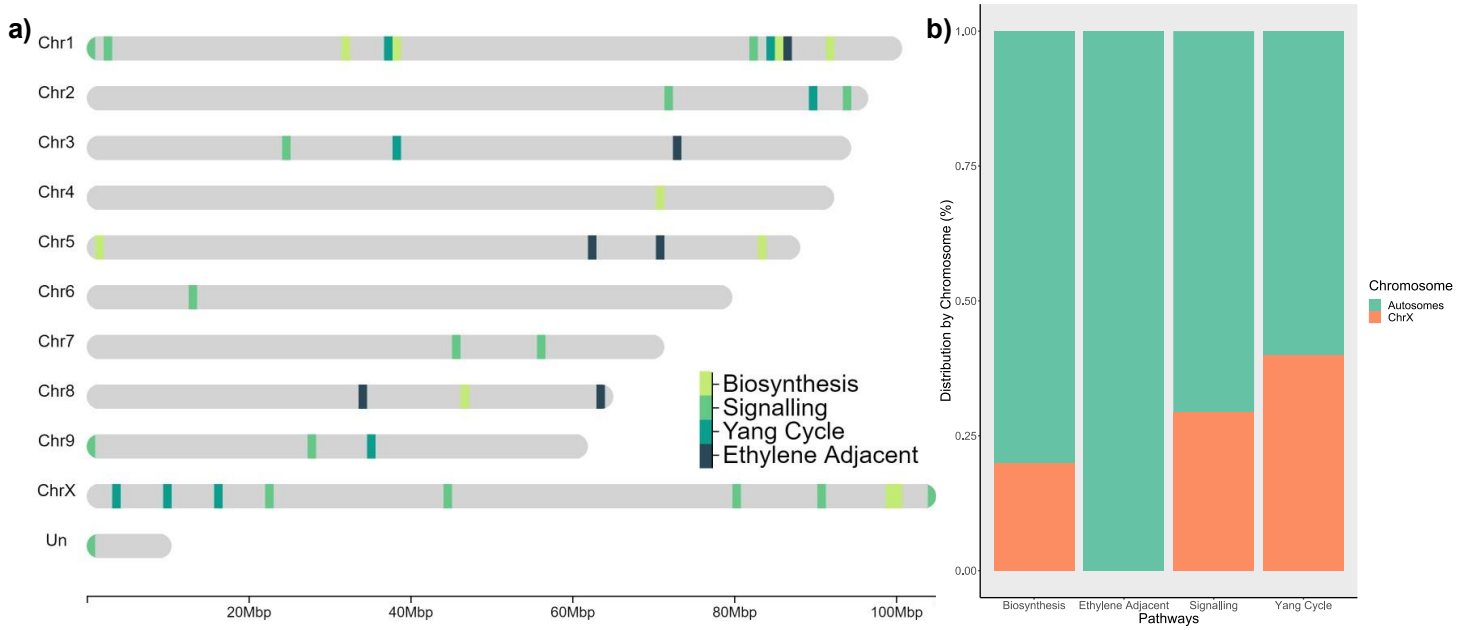


**Figure 4** Position (a) of ERGs in the cannabis cs10 reference genome and the proportional frequency (b) of those genes per chromosome. An interactive version of (a) can be found in the supplemental materials or on the OSF page for this project.

## Differential expression analysis reveals three patterns of expression

Among the 43 putative CsERGs, transcripts of 35 of these genes were expressed in both dataset 1 and dataset 2 (Table 1). Of these, transcripts of 15 CsERGs in dataset 1 and 11 CsERGs in dataset 2 showed differential expression between flower types (**Error! Reference source not found.**). In 7  ERGs common to both datasets, differential expression between MFs and FFs was of similar magnitude (**Error! Reference source not found.**).

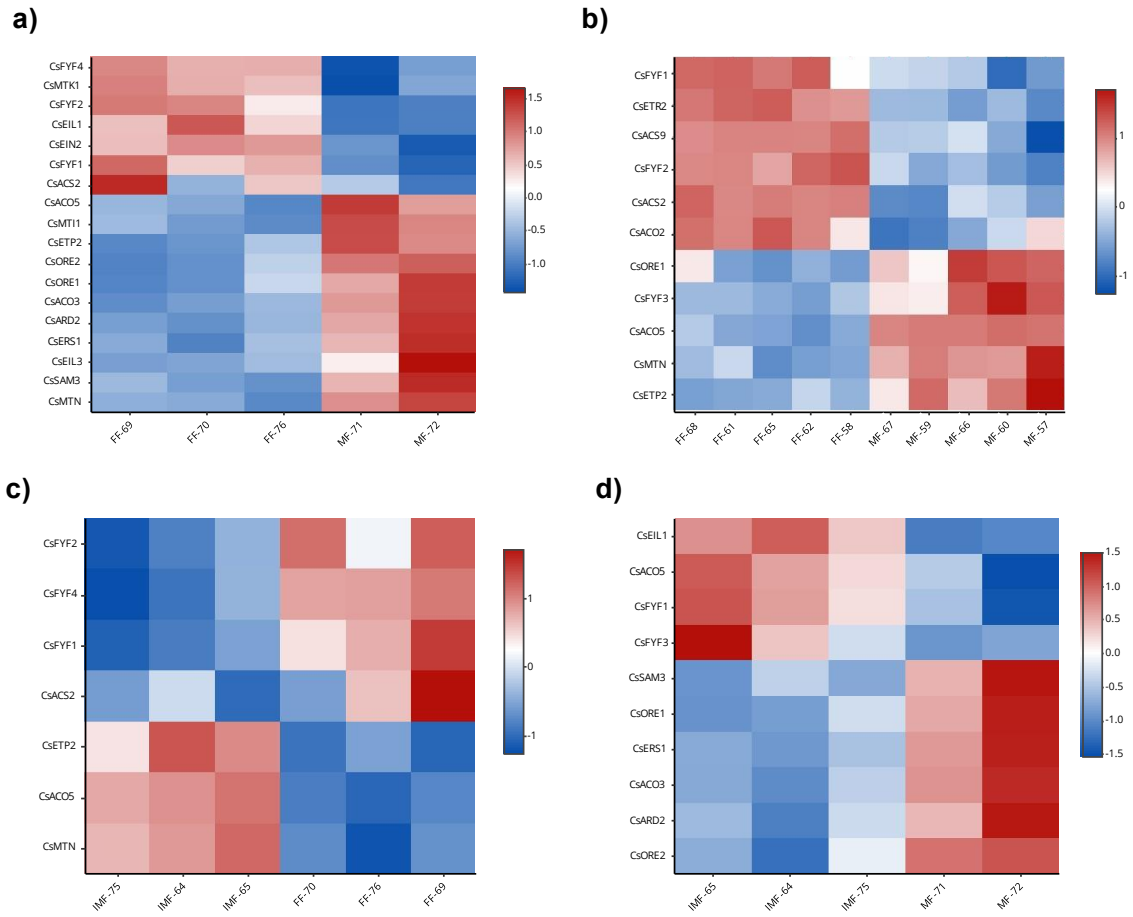


**Figure 5** Heatmaps of differentially expressed CsERGs (identified by locus ID; **Table 1**) for dataset 1 (a, c and d) and dataset 2 (b). X-axis labels are the flower type, followed by the last two digits of the SRA accession numbers, which can be found in **Error! Reference source not found.**. Y-axis contains the CsERG names. Heatmap scale is in log of the fold change in expression for the pairwise comparison of the flower type listed on the x-axis: a) MF vs. FF

comparison in dataset 1; b) MF vs. FF comparison in dataset 2; c) IMF vs. FF in dataset 1; d) IMF vs. MF in dataset 1.

Differential expression analyses of CsERGs in FF, MF and IMF tissues from dataset 1 reveal three distinct patterns of expression which can be used to create a framework for thinking of ethylene-related sexual plasticity in cannabis. We have named these cases of differential expression as follows: karyotype concordant (KC; Figure 7), floral-organ concordant (FOC; Figure 8) and unique ERG (uERG; Figure 9).

**Karyotype concordant (KC) expression**

This first case, karyotype concordant (KC) expression, describes expression patterns where CsERG expression is the same between plants with the sex chromosome karyotype (i.e., FF and IMF). KC genes make up the largest number of genes identified by differential analysis of dataset 1, making up nine genes in total (Figure 7). KC expression represents cases where the pairwise comparison between FF and IMF gene are not significantly different, but where the IMF significantly differs from its biologically male counterpart (MF). In this case of KC expression, both FF and IMF have XX chromosomes and normalized gene counts for a given ERG do not significantly differ between FF and IMF individuals.

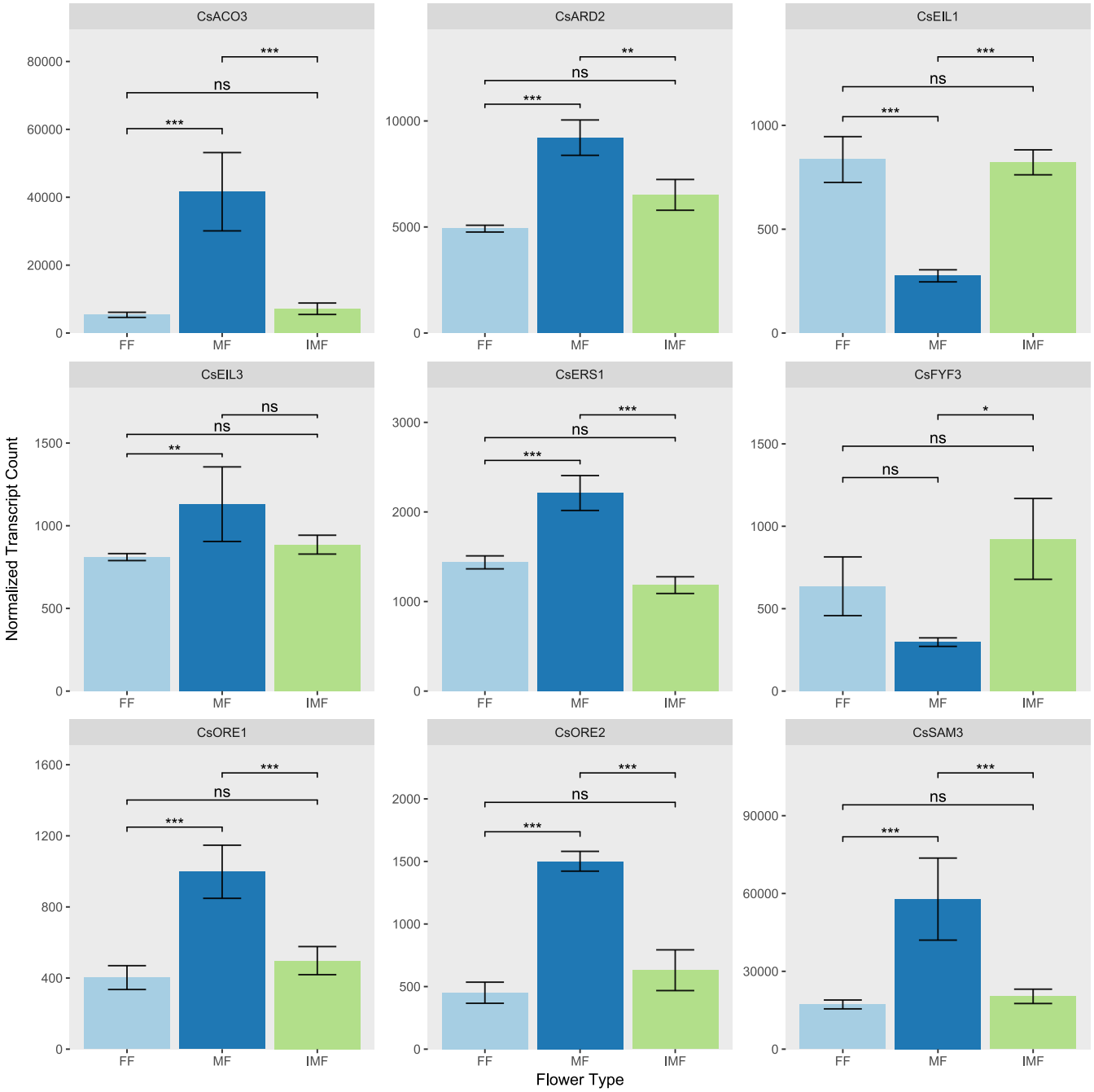


**Figure 6** Differential analysis of *C. sativa* ERGs revealed nine genes with a karyotype concordant (KC) expression pattern. This describes expression patterns shared between organisms with the same sex chromosome karyotype, but not the same sex expression. In these cases, female flowers (FF; XX karyotype) and induced male flowers (IMF; XX karyotype) showed KC expression.

## Floral organ concordant expression (FOC)

In this second case, genes from organisms sharing the same floral organs show similar patterns of expression. From the 15 differentially expressed CsERGs identified in dataset 1, five were identified as belonging to this category (Figure 8). In these five cases, there was no differential expression between male-flowers: i.e., IMFs and MFs. However, in all cases expression significantly differed between MFs and FFs, showing a pattern of expression that is conditioned by the floral organs that are present.

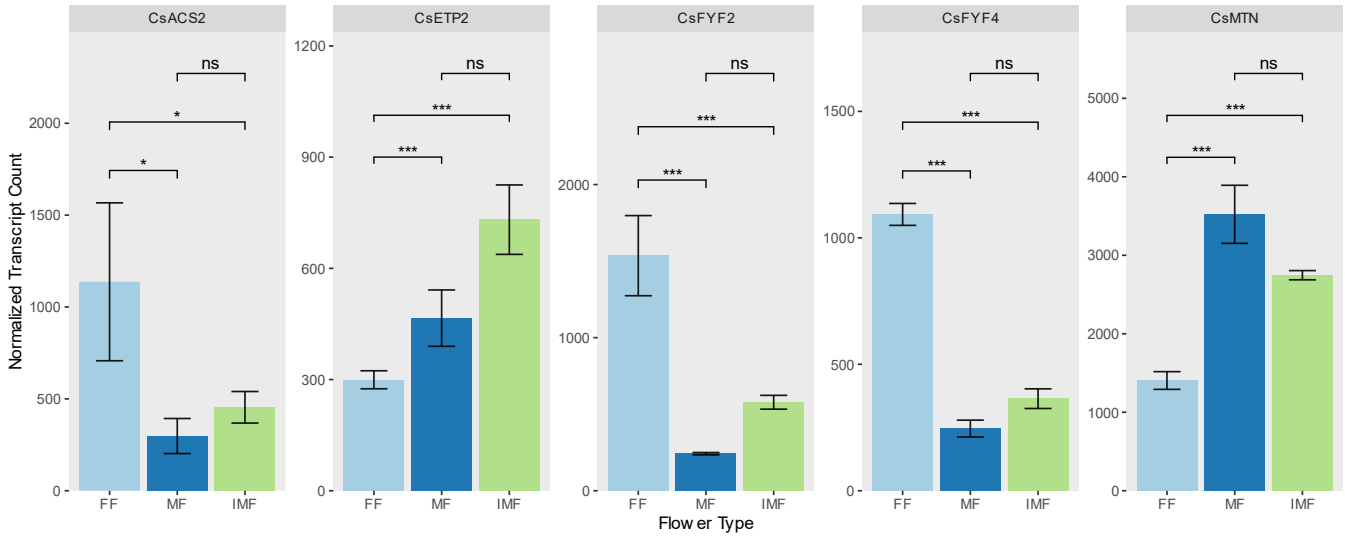


**Figure 7** Differential analysis of *C. sativa* ERGs revealed five cases of genes showing floral-organ concordant (FOC) expression. FOC refer to genes whose expression pattern is shared between organisms with the same floral organ, but not necessarily the same sex-chromosome karyotype designated by ns in transcription levels for male flowers on XY plants and induced male flowers on XX plants.

## Unique ERG (uERG) expression

In this final case, expression of CsERGs in the sex-induced plant (IMF) is not different from plants with shared sex-organs (MF vs IMF) or those with a like sex karyotype (FF vs IMF). Two CsERGs were identified with this pattern of expression.

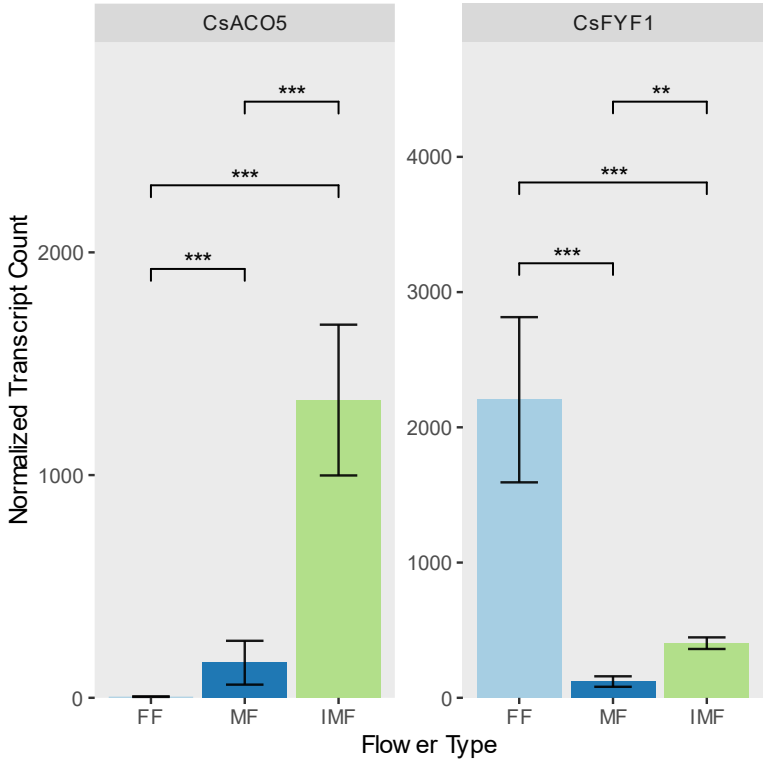


**Figure 8** Differential expression analysis of dataset 1 revealed 2 cases of unique ERG (uERG) expression, which are cases where gene expression in the sex-changed plant does not match either that of the plant with which is shares the same sex chromosome karyotype or the plant with which is shares the same floral organs.

# Discussion

To date, sexual plasticity has been more widely studied in the animal kingdom, whereas in angiosperms most efforts to understand plant sexual plasticity has been focused on the Cucurbitaceae family (Yamasaki *et al.*, 2001; Boualem *et al.*, 2015; Oda *et al.*, 2022). Studies of dioecious sexual plasticity are limited, but *C. sativa* is one dioecious species where sexual plasticity has been frequently observed, both in nature and in research settings, and it is a species for which methods inducing sexual plasticity have been established (Green, 2005; Lubell & Brand, 2018; Kurtz *et al.*, 2020; Moon *et al.*, 2020a; Flajšman *et al.*, 2021; Zheng, 2022). To date, these

reported occurrences of cannabis sexual plasticity have linked the phenomenon to the plant growth regulator ethylene. Existing studies show that inhibition of ethylene in female plants causes the induction of male flowers (Lubell & Brand, 2018) and the treatment of male plants with ethylene causes the induction of female flowers on male plants (Moon *et al.*, 2020a).

Both STS and ethephon affect endogenous ethylene concentrations in plants. STS is hypothesized to inhibit ethylene signaling by interacting with the ethylene receptor on the endoplasmic reticulum, reducing the plant's ability to perceive ethylene (Binder, 2020). The exact mode of inhibition is debated, with two prevailing ideas: in one, silver binds to the receptor's copper ion cofactor site to competitively inhibit signaling (Altman & Solomos, 1995; Rodríguez *et al.*, 1999); in the other, silver ions non-competitively inhibit the receptors from binding ethylene, possibly by binding the active site or reducing the number of binding sites available to ethylene (Beyer, 1976; Blat, 2010; McDaniel & Binder, 2012). Ethephon, on the other hand, increases endogenous ethylene concentrations by breaking down to produce ethylene when applied to plant tissues (Zhang & Wen, 2010). It triggers an autocatalytic increase in ethylene biosynthesis throughout the plant (Hume & Lovell, 1983a).

Studying the role of ethylene in sexual plasticity is an obvious choice for understanding sexual plasticity in dioecious plants, due to its well-known and consistent biosynthesis and signaling pathways, as well as its proven impact on sexual plasticity in the Cucurbitaceae family (Hume & Lovell, 1983b; Manzano *et al.*, 2014; Cebrián *et al.*, 2022). However, given the numerous roles of ethylene in plants, untangling the specific ways with which ethylene affects sexual plasticity also faces some challenges. Here, we presented a framework of three putative CsERG expression patterns, two of which we propose are linked to sexual plasticity in cannabis. We also described the first report of the *C. sativa* Yang cycle and ethylene biosynthesis and

signalling pathways and discuss which key genes in these pathways belong to sex plastic patterns of expression. We highlight how the distribution of these CsERGs whose expression patterns are associated with sexual EGRs are not solely located on sex chromosomes, but rather distributed throughout the genome. Lastly we end by discussing limitations and future opportunities revealed by this study.

## Analyses help distinguish sexual plasticity genes from deterministic genes

*C. sativa* transcriptomes analyses confirmed the expression of 35 of the 43 CsERGs identified in this study (datasets 1 and 2). Additionally, the analysis helped identify candidate sex plastic CsERGs, following the production of IMF using the known ethylene inhibitor STS (Dataset 1). Analyses comparing CsERGs in MF, FF and IMF revealed three sex-linked patterns of expression, which have been named as follows: karyotype concordant (KC; Figure 7), floral organ concordant (FOC; Figure 8) and unique ERG (uERG; Figure 9). Two of these three patterns are of particular interest (FOC and UERG), and below we outline why we believe that CsERGs with these patterns are involved in sexual plasticity of *C. sativa*.

The largest number of CsERGs belong to the KC case, containing a total of 9 genes (Figure 7). In this case, CsERG expression is similar between individuals with the same sex chromosome karyotype. The findings from the current analysis suggest that KC CsERG expression is unchanged in XX plants regardless of the sex organs the plant has. As such, we suggest that genes belonging to the KC case may have sex-specific patterns of expression, but these are unaffected by STS treatments which induces sexual plasticity in *C. sativa*. Taken together, we propose that KC genes are non-sex-plastic.

In FOC expression (Figure 8), the CsERG expression is the same between individuals with shared phenotypic sex but carrying different sex chromosome karyotype (i.e., MF and IMF). Genes with FOC had no significant difference in expression levels between individuals producing male flowers, regardless of their genotypic sex (XX or XY sex chromosome karyotype). However, FOC gene expression in IMFs were significantly different in FF. A total of 5 CsERGs were identified as FOC. Three genes, *CsACS2*, *CsFYF2* and *CsFYF4* showed higher gene expression in FF than the phenotypic male (MF and IMF) plants, while two genes (*CsETP2* and *CsMTN*) had higher expression in the MF and IMF plants than in the FF plants (Figure 8).

In the last case, UERG (Figure 9), we observed a differential expression of CsERGs between all three treatments. Two genes, *CsFYF1* and *CsACO5* showed UERG, with the sex manipulated plant's (IMF) gene expression not matching either of the plant with the same genotypic sex (MF) or the plant with the phenotypic sex (MF). We hypothesize that FOC and UERG are indicative that the genes is a sex-plastic ERG.

## Multiple *CsACS* and *CsACO* genes; a potential regulatory framework for sexual plasticity

An expansive body of literature exists which has established a canonical, highly conserved ethylene biosynthesis, and signalling pathway in a number of widely studied plant species (Binder, 2020; Pattyn *et al.*, 2021). Ethylene biosynthesis and signalling has yet to be studied in cannabis or closely related dioecious species, making *A. thaliana* a logical choice for the first attempts at assembling the canonical *C. sativa* ethylene biosynthesis and signalling pathways. Ethylene biosynthesis is regulated by a set of conserved enzymes that are found in all plant species. The key enzyme in this two-step process is ACC synthase (ACS), which converts SAM, derived from the

Yang cycle, into 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is then converted into ethylene by the enzyme ACC oxidase (ACO). These enzymes are conserved across all plant species, and their regulation is tightly controlled by various factors such as hormonal and environmental cues (Bleecker & Kende, 2000). Ortholog analysis conducted in this study showed that both the Yang cycle, responsible for SAM production and ethylene biosynthesis are conserved in *C. sativa*, with orthologs identified for each key step in these two pathways (**Figure 2**). In the present study, we found that in cannabis, *ACS* and *ACO* belong to multi-gene families like in Arabidopsis. In *C. sativa*, six *ACS* and five *ACO* gene homologs were identified from the eight *ACS* and five *ACO* genes in *A. thaliana* respectively (**Figure 2**). The presence of *ACS* and *ACO* are critical to biosynthesis, but studies have shown that the number of genes in each family can vary between species. For example, in corn (*Zea mays* L.) 5 *ACS* and 15 *ACO* genes have been identified, in rice (*Oryza sativa* L.) those numbers are 6 and 9 respectively and in tomato (*Solanum lycopersicum* L. Karst. ex Farw) there are 9 *ACS* and 7 *ACO* genes (Houben & Van de Poel, 2019; Wu *et al.*, 2022). The importance of having multiple functionally redundant ethylene biosynthesis genes has been demonstrated in multiple plants, with studies showing *ACS* and *ACO* genes are expressed at different levels and in different tissues as a function of the plant organ's needs and developmental stage. In ripening tomatoes van de Poel *et al.* (2012) reported a higher level of *ACS6*, *ACO2* and *ACO4* expression during baseline (system 1) ethylene production during immature fruit development prior to ripening, but that auto-catalytic increases in ethylene during ripening (system 2) were correlated with increases in *ACS2*, *ACS4*, *ACO3* and *ACO5* expression, while abundance of *ACO2* and *ACO4* transcripts decreased at that same stage. The induction/repression of specific *ACS* genes has also been shown to play a role in sexual plasticity of some species in the *Cucurbitaceae* species (Manzano *et al.*, 2014; Boualem *et al.*, 2015; Chen

*et al.*, 2016a; Zhang *et al.*, 2017). One study found that expression of *ACS11* was only observed in the vascular bundles of female flowers of monoecious and in hermaphroditic flowers of andromonoecious plants (plants producing male and hermaphrodite flowers), pointing to the role of *ACS11* in carpel development (Boualem *et al.*, 2015). Sexual plasticity has also been shown in cucumber and melon by way of disruption of a specific *ACO* genes (*ACO2* in cucumber; *ACO3* in melon) resulting in decreased ethylene production in the carpel region of flowers and causing a shift from a monoecy to androecy resulting in unisexual flower production in cucumber and melons (Chen *et al.*, 2016a).

The existence of multiple, functionally redundant *ACS* and *ACO* genes, combined with spatial and temporal patterns of expression allows for the fine tuning of ethylene biosynthesis and giving a plant a high level of control over its development, including effects on a plant's sexual plasticity. In the present study, we demonstrate that *C. sativa* also contains numerous *ACS* and *ACO* genes, spread across its genome (Table 1, Figure 4), which leads us to hypothesize that their expression and regulation is also controlled spatially and temporally in cannabis. In particular, *CsSAM3*, *CsACS2*, *CsACO3* and *CsACO5* showed distinct expression cases (Figures Figure **7**,**Figure 8** and**Figure 9**) suggesting these CsERGs are linked to sexual plasticity. *CsACS2* shows FOC expression, linking it to sexual plasticity and is responsible for the first committed step in ethylene biosynthesis. It has been demonstrated to catalyze the rate-limiting reaction in most cases of ethylene production (van de Poel *et al.*, 2012; Houben & Van de Poel, 2019; Pattyn *et al.*, 2021). This gene is downregulated in IMFs in comparison to female plants, and shows similar patterns of expression to male plants with whom they share the same floral organ despite having an XX karyotype. Since *ACS* is thought to be rate-limiting, and given the association of ethylene with 'feminization' of floral phenotypes we hypothesize that the downregulation of *CsACS2* in IMF

relative to FF, points to reduction in ethylene biosynthesis *in planta* which may play a role in the induction of male flowers in genetically female plants.

*CsACO5* has a unique expression pattern in all three forms of sex expression (UERG; Figure 9), with a significantly larger expression in IMFs compared with MFs or FFs (Figure 9). Here, dosage compensation could play a role with downstream inhibition of ethylene signalling by STS leading to increased transcription of *ACO* as IMF plants which are genetically female, fail to perceive ethylene levels normally required for female floral development. Lastly, *CsSAM3* (produces SAM required for the biosynthesis of ethylene) and *CsACO3* appear to be linked to genotypic sex and uninfluenced by the phenotypic sex changes (KC expression; Figure 7) induced by STS, with activity in XY plants much higher for both than in plants with the XX chromosomal complement. Further study should be performed to investigate if this KC expression pattern is unique to floral tissues or if this karyotype specific pattern of expression is also present in vegetative tissues of flowering plants.

Ethylene is known to affect the sexual plasticity of many plants (Hume & Lovell, 1983b; Hyde *et al.*, 2020), and the present study found patterns of differentially expressed CsERGs between male and female plants, suggesting their implication in cannabis sexual plasticity. However, CsERGs that did not show a difference in expression between male and female plants may have limited or no impact on sexual plasticity at this stage of development, although their regulation may fluctuate before or during floral induction. The study identified 43 CsERGs in the cs10 reference genome but only detected transcripts for 35 genes in two transcriptomic datasets, possibly due to some genes being unexpressed during the specific time points when the floral tissue was collected in their respective studies. The spatial and temporal expression of genes during floral development is well (Trivellini *et al.*, 2016), and future studies should examine gene expression

before and during flowering in response to ethylene and its agonists to better understand the temporal control of sexual plasticity by ethylene. The identification of the *C. sativa* biosynthesis pathway is an important first step toward developing targeted functional studies to understand the role of these biosynthesis genes during sex determination in cannabis.

## Ethylene signalling is conserved in *C. sativa*

Like ethylene biosynthesis, ethylene signalling has been shown to be highly conserved across the plant kingdom (Chang, 2016). Ethylene binds to a family of receptors known as ethylene receptors (ETRs), which are found in the membrane of the endoplasmic reticulum of plant cells (Binder, 2020). These receptors are responsible for transducing the ethylene signal to downstream signalling components. The downstream signalling pathway involves a series of conserved components, including *CTR1*, *EIN2*, and *EIN3*, which are responsible for activating the expression of ethylene-responsive genes (Shakeel *et al.*, 2013). In the present study, we were able to identify the canonical ethylene pathway genes in *C. sativa* responsible for each step of the signalling pathway, making this the first pathway model for the species to date (Figure 3).

The ethylene signalling pathway is of particular interest in the current study as potentially playing a role in cannabis sexual plasticity that is induced by the action of STS, an inhibitor of ethylene signaling. Treatment of genotypically female plants results in male floral phenotypes (i.e., IMFs). Transcriptomes of MF, FF and IMF in this study revealed numerous CsERGs upstream/downstream of or directly involved in the signalling pathway which have FOC expression (*CsETP2*, *CsFYF2* and *CsFYF4*) and UERG (*CsFYF1*). In *Arabidopsis EIN2 TARGETING PROTEIN1 and 2* (*ETP1/2*) acts to suppress ethylene responses in the signalling pathway by carrying out ubiquitinating with Skp1 Cullen F-box E3 ubiquitin ligase (*SCF E3*) of

*EIN2*, marking *EIN2* for proteosome degradation (Qiao *et al.*, 2009; Binder, 2020). Conversely, ethylene acts as a suppressor of *ETP1/2*, thereby allowing ethylene signalling to proceed by preventing the *ETP1/2* mediated degradation of *EIN2* (Qiao *et al.*, 2009; Yang *et al.*, 2015). In the present study, both *CsETP1* and *CsETP2* transcripts were detected, however only *CsETP2* showed sex-plastic differential expression. *CsETP2* expression was lowest in FFs compared to MFs and IMFs (Figure 8). There was no significant difference between *CsETP2* expression in MFs and IMFs, despite IMFs sharing the same sex chromosomes as FF plants. These patterns of gene expression fit with the hypothesized role of ethylene as a feminizing agent. If increased endogenous ethylene is responsible for femaleness, then we should expect lower levels of *CsETP2* in female flower producing plants, as observed.

The differential expression analysis also revealed three *FOREVER YOUNG FLOWER* (*FYF*) genes which belong to the FOC expression (*CsFYF2* and *CsFYF4*) and uERG (*CsFYF1*) expression patterns. FYF genes are expressed in young flowers prior to pollination (Chen *et al.*, 2011) and acts as a suppressor of floral abscission by inhibiting ethylene triggered responses downstream of the signalling pathway (Chen *et al.*, 2022). FYF does this by repressing *Ethylene Response DNA-binding Factors (EDFs)* which are downstream *Ethylene-Responsive Binding Factors* (*ERFs*) that trigger floral senescence and abscission in maturing flowers (Chen *et al.*, 2015). Similar patterns of expression were observed for *CsFYF1*, *CsFYF2* and *CsFYF4* in the present study. In these three genes expression was highest by several orders of magnitude in FF compared to MF and IMF tissues. Typically expression of these genes is highest in early flowering stages and diminishes over time, and it has also shown that different FYF genes are expressed differentially depending on tissues (Chen *et al.*, 2021). However, variations in FYF transcription linked to sex expression have not yet been investigated. These findings suggest that in dioecious

plants FYF expression may vary depending on sex expression, however it remains to be seen if this is a cause or effect of sex plasticity. Alternatively, the reason for high levels of FYF in FF tissues relative to MF and IMF might also be explained by differences in the lifespan of female and male cannabis flowers, the latter which have a much shorter life span and which would encounter senescence sooner. Since FYF is a suppressor of senescence, the presence of lower levels of *CsFYF1/2/4* in MF and IMF flowers may be due to earlier senescence or later developmental stages of the flower compared with FF tissues. An alternative hypothesis for this observed difference could be that higher anticipated levels of ethylene in female plants and flowers may result in more downstream expression of ethylene response factors such as the *EDF*s associated with floral senescence, and in order to prevent premature senescence caused by ethylene, female flowers required higher endogenous concentrations of FYF.

## Distribution of CsERGs is not limited to sex chromosomes

*Cannabis sativa* contains a pair of heteromorphic sex chromosomes, XX for female individuals and XY for males. However, treatment of male plants with ethylene and female plants with the ethylene inhibitor STS has been reported to cause plants to produce flowers opposite than that implied by the genotypic sex of the plant (Green, 2005; Lubell & Brand, 2018; Moon *et al.*, 2020a). This sexual plasticity suggests that the sex-expression of cannabis is not solely influenced by the sex-chromosome complement. However there exist few studies in cannabis which explore the link between sex chromosomes and sex-expression, and to the author's knowledge, no studies which explicitly explore the link between cannabis sexual plasticity and ethylene. In a study comparing the transcriptomes of male and female flowers, Prentout *et al.* (2020) reported that 35% of sex-linked genes mapped to autosomes while 65% mapped to the sex chromosomes of *C. sativa*. The distribution of their reported sex-linked genes in the chromosome is in stark contrast to the

distribution of CsERGs that we identified with only 26.2% of CsERGs mapping to the sex chromosomes. The authors suggest that mapping of their sex-linked genes to autosomes was the result of false positives caused by permissive mapping or incorrect placement of gene in the genome assembly of *C. sativa* (Prentout *et al.*, 2020). Adal *et al.*(2021), however, proposes a different hypothesis suggesting that sex determination may be the result of an X:autosome dosage mechanism, where male flowers may also have a small number of unique Y-chromosome genes affecting their sex. We propose that cannabis sexual plasticity is brought about by ethylene's regulation through differential expression of CsERGs displaying FOC expression and uERG present in autosomal and sex chromosomes of cannabis.

## Limitations and future directions

The present study represents an important first step in to understanding the ethylene-mediated control of sex expression in cannabis. However, in the case of this exploratory study, there are some limitations that we would like to acknowledge. Only two transcriptomic datasets from floral tissues were available which could be used begin identifying changes in gene expression based on floral organ type and plant sex that may be linked to sexual plasticity(Prentout *et al.*, 2020; Adal *et al.*, 2021). In an effort to begin probing the effect of CsERGs during chemically induced sex plasticity, it was important that we also include transcriptomes from IMFs, which were only available in one of the two datasets used (Adal *et al.*, 2021). Additionally, no existing transcriptomic datasets were available for IFFs, which limited our ability to test whether the mechanisms for sex plasticity in genetically male plants are the inverse of those genetically female plants. Future studies on sexual plasticity in cannabis should include IFFs in order to provide a more complete picture of the mechanisms underlying sexual plasticity in the species. Despite these limitations, we were able to use the differential expression findings between MF and FFs in both

studies to find a set of consensuses differentially expressed CsERGs (**Error! Reference source not found.**), which we believe are strong candidate CsERGs with a role in sexual plasticity.

As noted, there were a number of conditions which differed between the datasets employed in this study, which may have caused some differentially expressed CsERGs important to sexual plasticity to not be detected. Transcriptomes from both datasets were generated under different experimental conditions: plants were grown under different conditions, flowers sampled at different stages of growth, and the sampling location on the plants were different. Importantly, plants used to generate the two datasets were not clonal, and each dataset was obtained from a different genotype: one drug-type (cv. MS-17-338; Adal *et al.*, 2021) and one hemp-type cannabis (cv. Zenitsa; Prentout *et al.*, 2020). The limitations of single genotype studies in a highly variable species such as *C. sativa* has been well documented in Monthony et al. (2021b), and thus we propose that future studies that further probe the role of ethylene in sexual plasticity should include a greater number of cannabis genotypes. However, it is important to note that most of the CsERGs identified in the ortholog analysis were present in both datasets, suggesting that the models for ethylene biosynthesis and signalling in cannabis which we have proposed in this study are robust and similar to the canonical pathway in *Arabidopsis thaliana.* The fact that these findings are the same across two datasets with very different experimental conditions (outlined above) can allow us to confidently say that CsERG expression is affected by a plant's karyotype and that the canonical ethylene biosynthesis and signalling pathways is conserved in this species.

There are currently a limited number of biotechnological tools (e.g., *in vitro* culture, CRISPR, VIGS, agrobacterium, etc.) which are optimized in order to functionally validate CsERGs in cannabis (Hesami *et al.*, 2021) and many of these tools have been hampered by the lack of a reliable *in vitro* regeneration protocol for the species (Monthony *et al.*, 2021b). Although such functional

validation is beyond the scope of the current study, it represents an important next step in probing the role of ethylene in cannabis sexual plasticity. Recently progress has been made in establishing methods for gene silencing in cannabis, through the employment of the *Cotton leaf crumple virus* (*CLCrV*) in a virus-induced gene silencing protocol reported by Schachtsiek *et al.* (2019) and through new methods using agrobacterium (Sorokin *et al.*, 2020; Galán-Ávila *et al.*, 2021). However due to their recent nature, these methods have yet to be replicated independently. Given the genotypic variability of cannabis and the role it plays in the replicability of *in vitro* methods (Monthony *et al.*, 2021b), it remains to be seem how effectively these methods are adapted to multiple genotypes across different research settings.

# Conclusion

This study represents the first step in exploring sexual plasticity in the dioecious plant *C. sativa* and the important role that Yang cycle, ethylene biosynthesis and signaling pathways play in sex determination. Here, we present a framework consisting of three distinct gene expression patterns (KC, FOC and uERG) which can be used to categorize the role of CsERGs in sexual plasticity in dioecious plants. These findings are based on our analysis of transcriptomes of male, female and induced male flowers obtained from two different *Cannabis sativa* genotypes produced under two different experimental conditions. From the ortholog analysis undertaken in this study and with support from transcriptomic data, we propose that the canonical Yang Cycle and ethylene biosynthesis and signalling pathways are conserved in *C. sativa* and we identified seven genes belonging to two the FOC and uERG classes which we hypothesize play a role in ethylene-induced sexual plasticity in cannabis. This work lays the groundwork for more detailed study of the role of CsERGs in sexual plasticity in multiple cultivars of cannabis. Given the

commercial importance of cannabis, as well as a number of other dioecious plants such as kiwi, papaya and persimmon, understanding the underlying factors of sexual plasticity and its link with ethylene are important to future efforts in breeding and crop improvement.

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