The Cream of the Crop: Biology, Breeding and Applications of *Cannabis sativa*

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October 1, 2020

Abstract

Cannabis sativa is an extraordinarily versatile species. Hemp and its cousin marijuana, both *C. sativa*, have been used for millennia as a source of fibre, oil and for medicinal, spiritual and recreational purposes. Because the consumption of *Cannabis* can have psychoactive effects, the plant has been widely banned throughout the last century. In the past decade, evidence of its medicinal properties did lead to the relaxation of legislation in many countries around the world. Consequently, the genetics and development of *Cannabis* as well as *Cannabis*-derived products are the subject of renewed attention.Here, we review the biology of *C. sativa,* including recent insights from taxonomy, morphology and genomics, with an emphasis on the genetics of cannabinoid synthesis. Because the female *Cannabis* flower is of special interest as the site of cannabinoid synthesis, we explore flower development, flowering time well as the species' unique sex determination system in detail. Furthermore, we outline the tremendous medicinal, engineering, and environmental opportunities that *Cannabis* bears. Together, the picture emerges that our understanding of *Cannabis* biology currently progresses at an unusual speed. A future challenge will be to preserve the multi-purpose nature of *Cannabis*, and to harness its medicinal properties and sustainability advantages simultaneously.

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Keywords: building materials, *Cannabis sativa*, cannabinoids, crop breeding, flower development, flowering time, hemp, marijuana, sex determination, sustainability

1. Introduction – *Cannabis* in a nutshell

Cannabis sativa is a highly versatile crop with dozens of different uses (Figure 1). There are a multitude of medical applications for *Cannabis* secondary compounds, which have been shown to reduce pain, nausea and neurological conditions like seizures (Whiting et al., 2015), and research on effects on inflammation, depression and cancer is also being conducted (Atalay et al., 2019; Fraguas-Sánchez and Torres-Suárez, 2018; Poleszak et al., 2018; Śledziński et al., 2018, Russo, 2011). Beyond that, fibre varieties of *Cannabis* have high carbon sequestering potential because of their rapid growth. They are therefore utilized for carbon storage in building materials or as biofuel (Finnan and Styles, 2013). For those different reasons, the *Cannabis* industry is gaining more traction and the need for specialized varieties, adapted to local climatic conditions, or suited for specific applications, is steadily increasing.

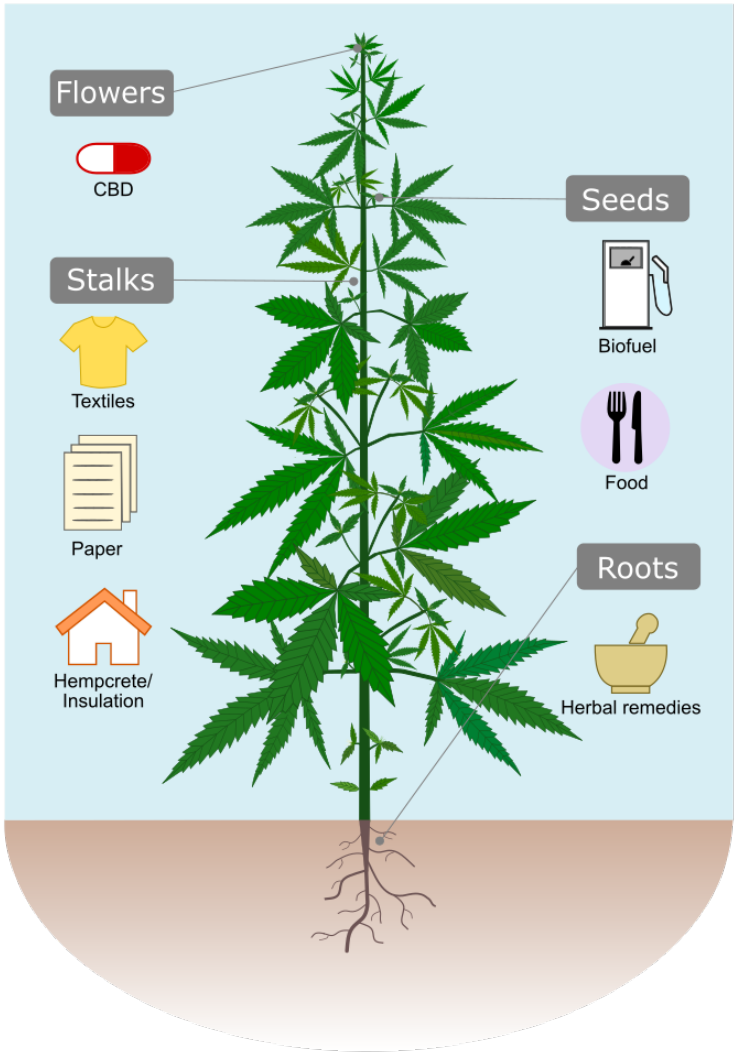

Figure 1: **Cannabis is a multipurpose crop.** The uses for *Cannabis* are manifold. The trichomes present on female flowers produce efficacious phytocannabinoids including cannabidiol (CBD), that has a wide range of medical uses that have been demonstrated in clinical trials. The stalks of the *Cannabis* plant can be processed into textiles, paper and building materials. The roots of the hemp plant have been used in traditional herbal remedies. The seeds can be processed for biofuel or pressed for oil for human consumption. Furthermore, the seed cake remaining after oil is pressed is protein-rich and can be used as animal feed. See also Figure 9 for photos of hemp products.

Cannabis is probably best known for one secondary compound, the psychotropic substance tetrahydrocannabinol (THC). Depending on the THC content of the plant or more specifically the dried inflorescence, *Cannabis* is either classified as marijuana (or drug-type, plants above 0.3% THC) or hemp (fibre-type, below

0.3% THC), which is mainly a legal and not a strict taxonomic classification. A more refined classification of *Cannabis* according to the phytocannabinoid profile into distinct 'chemotypes' can also be useful, with chemotype I and II being marijuana while chemotypes III, IV and V can be seen as hemp (see chapter 3).

Many countries have been easing the ban on medical and even recreational use of THC during the past decade. However, because of the prohibition of *Cannabis* in many countries throughout the last century, it was not bred to the same extent as other high-value crops. Hence, hemp and marijuana lines retain a high level of genetic variability and heterozygosity, that is not found in other crops (Sawler et al., 2015).

Here, we review the biology as well as the applications and future perspectives of *Cannabis* research and breeding. We discuss *Cannabis* taxonomy and cannabinoid synthesis as well as flower development and flowering time control with an emphasis on sex determination in this predominantly dioecious species. We also summarize the currently available genomics resources. Since *Cannabis* is so versatile, we discuss its applications in medicine as well as in the building industry. *Cannabis* ' future role in a sustainable society is summarized as well as the future of cannabinoid production via cell suspension cultures.

2. *Cannabis* systematics

Cannabis is the botanical name of a genus that historically includes three species, *C. sativa*, *C. ruderalis* and*C. indica*. However, since the three species can intercross, they are also often considered one single species, *C. sativa* (Small, 2015). Recent genetic data support the single-species concept and recommend that three subspecies should be recognized: *Cannabis sativa* subsp. *sativa*, subsp. *indica* and subsp. *ruderalis* (Q. Zhang et al., 2018).

Cannabis is a dioecious species, meaning there are male and female individuals (Figure 2a-c). However, through breeding, monoecious lines with male and female flowers on the same plant have also been generated (Figure 2d) (Moliterni et al., 2004).

The genus *Cannabis* is part of the Cannabaceae, a small family of flowering plants with 10 genera and some 120 species (Jin et al., 2020; Yang et al., 2013). The Cannabaceae have been estimated to have originated ca. 70 to 90 million years ago, and are distributed in temperate and tropical regions throughout the world (Figure 3) (Jin et al., 2020; Magallón et al., 2015). Most species of the Cannabaceae are trees or shrubs, *Cannabis* as a herb is, therefore, the exception rather than the rule in the family. However, a trait *Cannabis* shares with many other species in the family is the inconspicuous unisexual flowers (Yang et al., 2013).

The closest relative of *Cannabis* is the genus *Humulus*(Yang et al., 2013), which consists of three species, among which *Humulus lupulus*(hop) is economically important for the beer brewing industry. Both hop and *Cannabis* produce separate male and female flowers, and the trichomes in the female inflorescences are the site of secondary compound production that make both of those plants economically valuable (Page and Nagel, 2006).

Within the angiosperm phylogeny, Cannabaceae are most closely related to the Moraceae (mulberry or fig family) and Urticaceae (nettle family). Together with the Ulmaceae (elms and relatives) they form a group known as the urticalean rosids (Figure 3) (Sytsma et al., 2002). It is interesting to note that unisexual flowers appear to be prevalent in the urticalean rosids, whereas bisexual flowers are by far the dominant system in angiosperms in general (Renner, 2014; Sytsma et al., 2002). The evolution of sex expression and sex determination in this group is an interesting area of future research.

The urticalean rosids belong to the order Rosales, which are eudicots (The Angiosperm Phylogeny Group, 2016). Though the Rosales comprise some 7700 species (Zhang et al., 2011), they contain relatively few well-characterized model plants. The flowering plant super-models *Arabidopsis thaliana* (thale cress, Brassicales) and *Oryza sativa* (rice, monocots) are only distantly related to *Cannabis*, the lineages leading to *Arabidopsis*

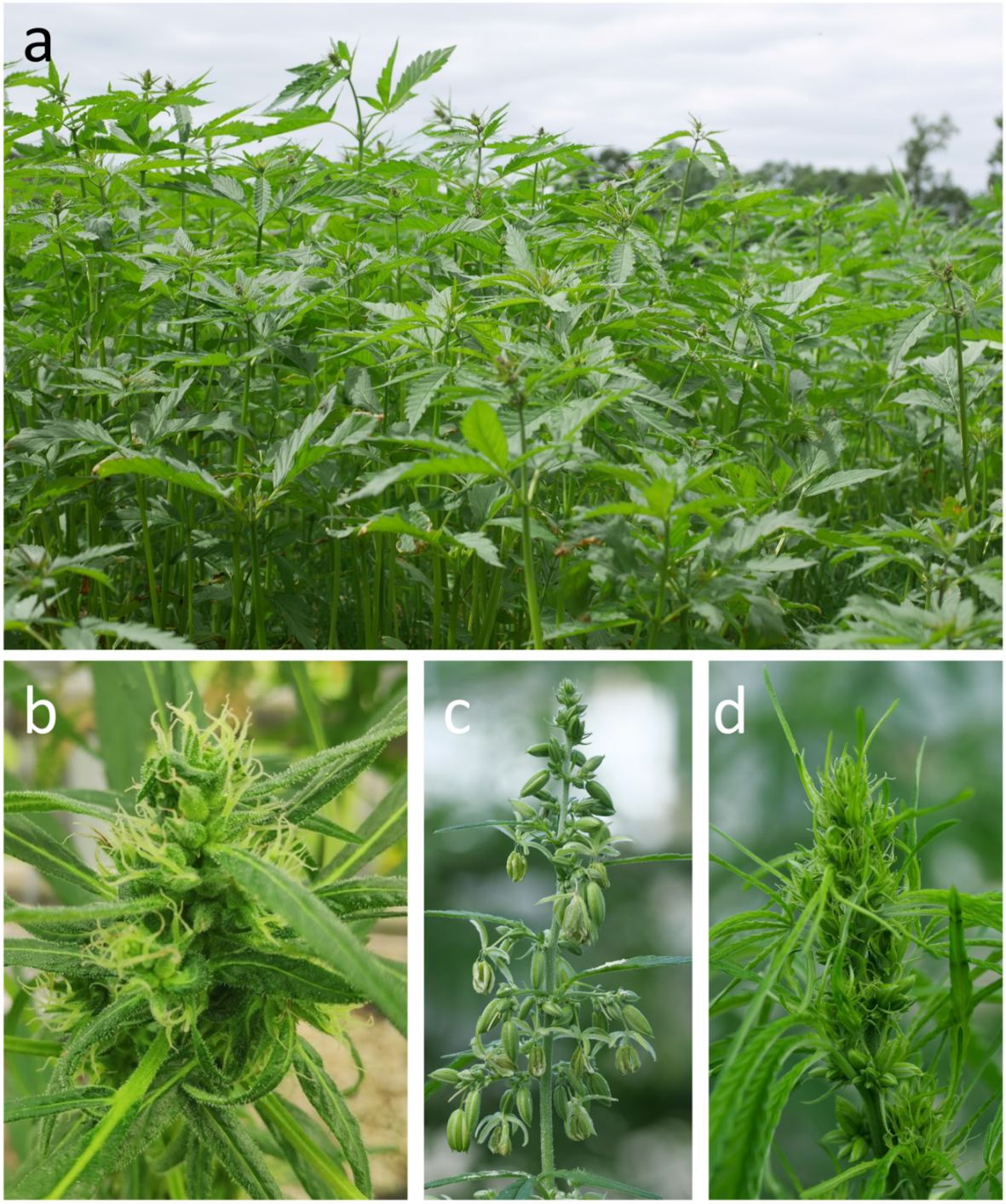

Figure 2: **Hemp varieties of *Cannabis sativa*.** *Cannabis* plants of the hemp cultivar 'Finola' growing in the field (a). The cultivar is dioecious with female (b) and male individuals (c). Monoecious plants of the cultivar 'Felina 32' show male flowers and female flowers in one individual plant (d).

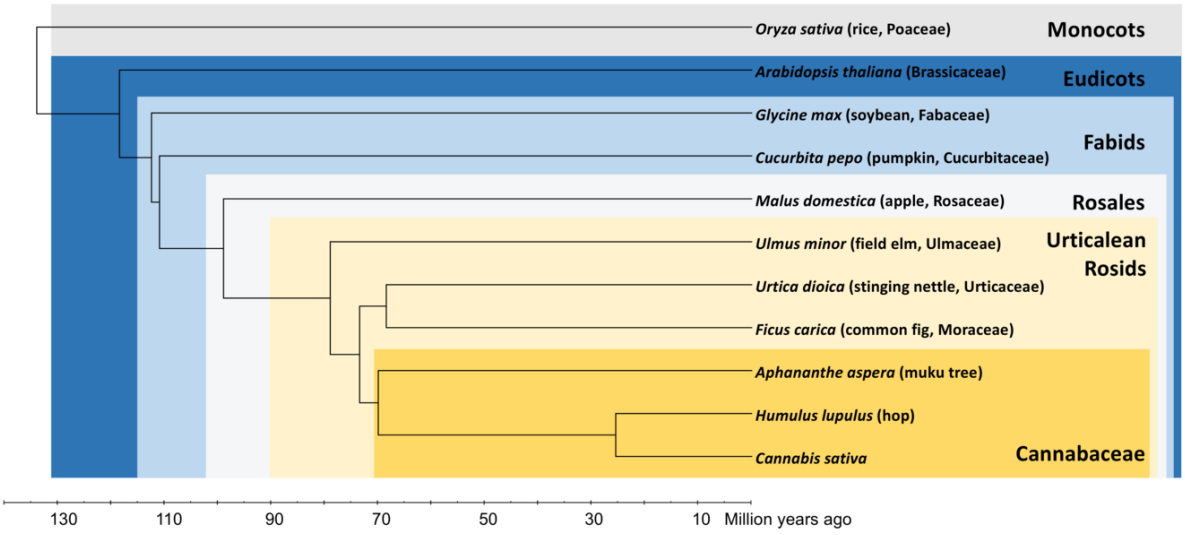

Figure 3: **Phylogenetic position of *Cannabis sativa*.** *Cannabis* belongs to the Cannabaceae, which belong to the order Rosales. Some selected species and their phylogenetic relationship to *Cannabis* are depicted. Taxonomic groups shaded in blue are inclusive of the taxonomic groups shaded in yellow (e.g. Rosales belong to Fabids, which are eudicots). The timescale at the bottom can be used to infer approximate divergence times.

and *Cannabis* separated some 120 million years ago, those leading to rice and *Cannabis* some 130 to 140 million years ago (Figure 3) (Magallón et al., 2015). Among the relatively well-characterized plants that are more closely related to *Cannabis* are many Rosaceae species (rose family, apple, peach and relatives), for which several well assembled and annotated genomes exist (Aranzana et al., 2019; Zhang et al., 2019), the Cucurbitaceae (cucumber, pumpkin and relatives), which serve as an important model for sex determination and sex expression (Li et al., 2019; Schilling et al., 2020a; Zheng et al., 2019) and Fabaceae (bean family) for flowering time regulation (Cao et al., 2017; Schmutz et al., 2010) .

Cannabis sativa itself is phenotypically extremely diverse. *Cannabis* plants vary in numerous traits including height, leaf shape, photoperiod response, tetrahydrocannabinol (THC) and cannabidiol (CBD) content, plant architecture and sex expression (Clarke and Merlin, 2016; Grassi and McPartland, 2017; Raman et al., 2017; Schilling et al., 2020b). The dioecy of many *Cannabis* lines and thus the relatively high levels of heterozygosity further contribute to the fact that even within one cultivar the phenotypic diversity can be substantial (our unpublished observations).

For breeders and farmers, the high level of genetic and phenotypic diversity can be problematic, as a crop is usually best to handle when it possesses a high degree of uniformity in the field. However, at the same time, the existing diversity can be harnessed by breeders to produce new lines for a multitude of different purposes. For plant genetics research, the phenotypic and genetic diversity is a gold mine, as it provides the possibility to study the genetic basis of many traits in *Cannabis* . Some developments in this arena are outlined in the subsequent chapters, but many more are sure to come.

3. Evermore complex: The genetics of phytocannabinoid biosynthesis

One of the commercially most interesting and valuable products that can be generated from *Cannabis* plants are phytocannabinoids. We use the term phytocannabinoids here for plant-derived cannabinoids, and to distinguish them from synthetic cannabinoids or those produced by the human endocannabinoid system. Phytocannabinoids are of great interest for medical applications (see chapter 8 for a detailed discussion) as well as commercial exploitations for recreational use. Hence, one of the major breeding goals involves the accurate prediction and targeted manipulation of phytocannabinoid profiles to ensure the optimal combination of active components in plant extracts (see entourage effect chapter 8) or legal compliance for non-psychoactive products.

While there are over 100 different phytocannabinoids described (Pertwee, 2014), three phytocannabinoids are usually at the centre of attention from a medical and commercial perspective: cannabigerol (CBG), cannabidiol (CBD) and tetrahydrocannabinol acid (THC) (Figure 4). *Cannabis itself* synthesizes phytocannabinoids in the carboxylated form with a carboxylic acid group, i.e. as CBGA, CBDA and THCA. However, to be active in the human endocannabinoid system, phytocannabinoids need to be consumed in their decarboxylated forms, which are usually generated by high-temperature treatment (for example during smoking) (Moreno-Sanz, 2016). Phytocannabinoids are predominantly produced in female inflorescences, more precisely they are secreted from trichomes of perigonal bracts, subtending flowers, and leaves ('sugar leaves') within inflorescences. However, in lower concentrations, phytocannabinoids can also be detected in vegetative leaves at certain times during the growth period (Aizpurua-Olaizola et al., 2016).

Among all phytocannabinoids, THC is the major psychotropic one. However, chemically all molecules mentioned above are very similar in structure and are produced from the same precursor molecules (Figure 4). CBDA and THCA are biochemically synthesized by two closely related enzymes, CBDA and THCA synthase (Shoyama et al., 2012; Taura et al., 1996). CBDA and THCA are both synthesized from CBGA, while CBGA is synthesized from two non-cannabinoids, olivetolic acid and geranyl pyrophosphate by a prenyltransferase (Fellermeier and Zenk, 1998)(Figure 4). Cannabichromenic acid (CBCA) synthase converts CBGA to CBCA (Morimoto et al., 1997) and is closely related to THCA and CBDA synthase (Figure 5), but the CBCA content of most mature *Cannabis* flowers is low (de Meijer et al., 2009a). Interestingly, CBDA synthase-like genes have been found in other plants and fungi (Aryal et al., 2019; Vergara et al., 2019).

Cannabis plants can have very high levels of phytocannabinoids or close to no phytocannabinoids at all, or anything in between (Aizpurua-Olaizola et al., 2016; de Meijer et al., 2009a). This has stipulated the description of different chemotypes that are characterized by their distinct phytocannabinoid profiles. The chemotypes are a very useful concept for chemical classifications and for breeding programmes. It should be kept in mind, however, that they do not necessarily constitute a phylogenetic classification based on evolutionary relationships (de Meijer et al., 2009b; Small and Beckstead, 1973). *Cannabis* plants can roughly be categorized into five different 'chemotypes' (Figure 4). Plants of chemotype I (short 'type I') produce high levels of THCA and only low levels of CBDA and CBGA (Small and Beckstead, 1973). This means the ratio of THCA/CBDA is much larger than 1. In type II *Cannabis* plants THCA and CBDA are both produced in approximately equal amounts (Small and Beckstead, 1973). Both, type I and type II plants, are usually classified as 'marijuana' and can underlie strong regulations, depending on the country or jurisdiction. These plants are bred to produce up to 20 % of their dry mass as phytocannabinoids.

In contrast, type III plants have high CBDA levels and low to very low amounts of THCA.

Chemotype IV and V refer to *Cannabis* plants which have CBGA as their dominant phytocannabinoid or very low levels of phytocannabinoids overall, respectively (de Meijer et al., 2009a; de Meijer and Hammond, 2005)(Figure 4).

In addition to the five different chemotypes, also the hemp-marijuana distinction is used to characterize different *Cannabis* plants (Figure 4). If the THC/THCA content in the dry flower mass is below 0.2-1 %, these

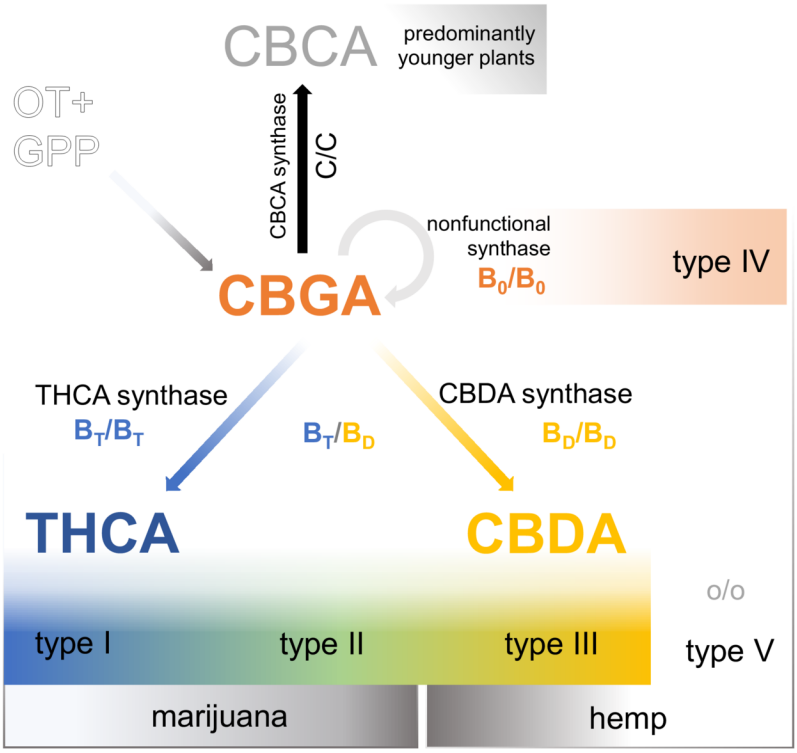

Figure 4: **Phytocannabinoids, synthases, genotypes and chemotypes of *Cannabis*.** Phytocannabinoids are synthesised via a multi-step pathway involving different enzymes. The precursor cannabigerolic acid (CBGA) is first synthesised by a prenyltransferase from the precursor molecules geranyl pyrophosphate (GPP) and olivetolic acid (OA). CBGA is metabolised into tetrahydrocannabinolic acid (THCA) via THCA synthase, into cannabidiolic acid (CBDA) via CBDA synthase or cannabichromenic acid (CBCA) via CBCA synthase. The different synthases are encoded by the B_T (encoding for an active THCA synthase) and BD (encoding for an active CBDA synthase) loci. B_T/B_T plants produce mainly THCA (chemotype I), while B_D/B_D plants produce predominantly CBDA (chemotype III). Presence of B_T and B_D results in chemotype II (THCA and CBDA intermediate). B0indicates that only non-functional THCA and CBDA synthases are present, which results in the accumulation of CBGA (chemotype IV). *Cannabis* varieties with very low overall levels of cannabinoids are categorized chemotype V, which is caused by a homozygous recessive allele of locus O. To complicate matters further, there is also a locus C, which is encoding for CBCA synthase. However, in almost all varieties, CBCA is only produced in young immature flowers. Chemotypes I and II can be considered marijuana, while the other low-THC chemotypes can be considered hemp varieties of *Cannabis*.

plants are usually categorized as hemp, above that as marijuana (depending on the jurisdiction this threshold can vary) (Brunetti et al., 2020; Mead, 2017). The differentiation between hemp and marijuana can typically also be drawn genetically, with hemp and marijuana varieties forming two genetically distinct populations (Sawler et al., 2015). Further, hemp and marijuana can be phenotypically quite distinct with marijuana plants generally being bushier and with a dense set of inflorescences while hemp plants tend to be taller, less branched and with less dense flower structures. However, there are also plants with low THC/THCA content (type III) which strongly resemble marijuana in overall plant and inflorescence architecture (Grassa et al.,

2018). Hence, the terms hemp and marijuana do not necessarily always refer to distinct genetic populations or phylogenetic categories. As the critical distinction between hemp and marijuana is the THC/THCA content, they can also be considered broader categories of chemotypes.

The underlying genetics of the different chemotypes have been studied in quite some detail in the last two decades (de Meijer et al., 2009a, 2009b, 2003; de Meijer and Hammond, 2005; Pacifico et al., 2006; Toth et al., 2020; Weiblen et al., 2015; Welling et al., 2016). However, the complex nature of the *Cannabis* genome with its many transposable elements, low complexity regions and high heterozygosity have made a conclusive analysis of the loci controlling phytocannabinoid production challenging (Grassa et al., 2018; Laverty et al., 2019; McKernan et al., 2018).

Different genetic loci had been postulated which determine a plant's chemotype, they are encoding for the different types of synthases: at locus B two codominant alleles were hypothesized to exist, the allele B_T encodes for the THCA synthase, B_D for the CBDA synthase (Figure 4)(de Meijer et al., 2003). Depending on the presence of either or both loci, the plant will be chemotype I (B_T/B_T), chemotype II (B_T/B_D) or chemotype III (B_D/B_D) (de Meijer et al., 2003; Toth et al., 2020; Welling et al., 2016). Additionally, non-functional alleles of the synthase gene (B_0) are predicted to be associated with chemotype IV, where neither CBDA nor THCA are produced and the precursor, CBGA, accumulates (Figure 4) (de Meijer and Hammond, 2005; Onofri et al., 2015; Welling et al., 2016).

Further, according to this model, CBCA synthase is encoded by an independent locus (C) while another independent locus (O) is relevant for precursor production, with a knockout resulting in overall minimal phytocannabinoid levels (Figure 4) (de Meijer et al., 2009a, 2009b).

The genetic basis of the chemotypes was analysed in detail by producing a cross between high-THC Purple Kush (chemotype I) and low-THC Finola (chemotype III). This resulted in an F1 generation of mainly type II plants, producing both, THCA as well as CBDA (Weiblen et al., 2015). This confirmed earlier findings of crosses between type I and type II plants, resulting in intermediate type II individuals (de Meijer et al., 2003). The segregation pattern of phytocannabinoid profiles in the F2 generation pointed towards a Mendelian inheritance pattern: type I, type II and type III plants were all observed in the F2 generation with the expected distribution of 1:2:1 (de Meijer et al., 2003; Weiblen et al., 2015). A correlation of the expression of either THCA or CDBA synthase with the respective chemotype was also observed and the THCAS/CBDAS locus could be mapped (Weiblen et al., 2015).

However, although these findings were consistent with the idea of codominant alleles at one single locus, it became apparent that the situation is more complex (Grassa et al., 2018; Laverty et al., 2019; Weiblen et al., 2015). New draft genomes generated with third-generation sequencing technology indicated that the THCA and CBDA synthases do not seem to be encoded by alleles of one and the same gene, but rather by distinct loci in marijuana and hemp, respectively, without a clear counterpart in the other genome (Grassa et al., 2018; Laverty et al., 2019). Sequencing of the hemp cultivar 'Finola' and the marijuana cultivar 'Purple Kush' indicates that a functional CBDA synthase gene is present only in in the 'Finola' genome while the 'Purple Kush' genome only encodes for a functional THCA synthase (Laverty et al., 2019). While mapping to approximately the same region in both genomes, the DNA sequences surrounding the respective synthase genes are drastically different from each other. Further, a low albeit still detectable recombination rate between the two loci supports the notion that they are genetically distinct (Laverty et al., 2019). The sequencing of a different *Cannabis* variety ('CBDRx'), which is a chemotype III hemp-marijuana hybrid revealed an even more complex genomic arrangement with a number of pseudo- and functional synthase genes in three different cassettes on the same chromosome (Figure 5) (Grassa et al., 2018).

The CBDA and THCA synthase genes themselves seem to be embedded in cassettes of multiple tandem duplications of putatively non-functional synthase genes, which are regularly interspersed with long terminal repeat (LTR) retrotransposons, making the assembly and analysis of these loci even more challenging (Figure 5) (Grassa et al., 2018; Laverty et al., 2019). This is also the reason why these complex loci could not be resolved in the first published *Cannabis* genome, which relied on short-read sequencing data (van Bakel et

al., 2011). This genomic constitution, where the difference between marijuana and hemp comes down to a large structural variation is, if true, very unusual. Hence, the aforementioned locus "B" with its different alleles might look very different from what was previously assumed to be simple isoforms of a single gene.

The complexity of phytocannabinoid synthases does not end there, though. Copy number variation of CBDA and THCA synthase genes might be involved in phytocannabinoid level and composition (Vergara et al., 2019) and most likely, the number of synthase (pseudo)genes might be different for each cultivar sequenced (Grassa et al., 2018; Laverty et al., 2019; McKernan et al., 2020).

High throughput assays for B_T and $B_D$markers have been developed and show that many plants actually contain both loci (Cascini et al., 2019; McKernan et al., 2020; Toth et al., 2020). Moreover, many B_D/B_D plants, especially those with higher CBDA levels, have THCA levels of above 0.3 % of dry flower mass, despite the absence of a functional B_T allele (Toth et al., 2020). This residual THCA is probably at least to some extent a by-product of the CBDA synthase itself. The THCA and CBDA synthase have a relatively high sequence similarity (83.85 %, Figure 5) and process the same precursor molecule, CBGA (Figure 4). *In vitro* studies have shown that the CBDA synthase produced CBDA and THCA at roughly a ratio of 20:1 (Zirpel et al., 2018). This is similar to ratios observed *in planta* in high-CBD hemp varieties as well (Toth et al., 2020; Weiblen et al., 2015). This potentially results in the problem that, if CBDA production is increased, THCA also increases as a by-product, even if plants do not express a functional THCA synthase. *Cannabis* varieties with very high CBD levels may thus be at risk of exceeding legal THC thresholds.

Understanding the exact genetics underlying the different chemotypes will be important for future targeted breeding approaches. Tight restrictions across the world make it difficult for farmers to grow chemotype III, IV and V varieties, because the presence of residual THC creates regulatory problems and uncertainties. Especially type III plants often have THCA/THC levels slightly above the legal THC limit (Aizpurua-Olaizola et al., 2016; Toth et al., 2020). Hence, one important breeding goal is going to be the generation of zero-THC lines which still produce high levels of CBD in the range of 15 to 20 % of dry flower mass. Whether this is possible to achieve is difficult to say, since even in the absence of a THCA synthase, CBDA synthases produce THCA as a by-product (Toth et al., 2020; Zirpel et al., 2018). This will, therefore, require identification of a CBDA synthase that does produce only very low or no amounts of THCA.*In vitro* experiments show that point mutations can alter the amount of by-products (Zirpel et al., 2018). Natural variation in synthase genes exists and have been linked to altered phytocannabinoid compositions (Onofri et al., 2015). Hence, naturally occurring or artificially generated CBDA synthase varieties could be used for targeted breeding in this direction.

In addition, *Cannabis* varieties used for fibre or seed production could be selectively bred and genotyped to have 0 % overall phytocannabinoids (chemotype V), as currently even the farming of these kinds of varieties is heavily restricted in many countries.

Other phytocannabinoids like CBG(A) and CBC(A) as well as the manifold variants of terpenes produced in *Cannabis* flowers are increasingly coming into focus in the medical research fields (reviewed in Booth and Bohlmann, 2019; Deiana, 2017; Pollastro et al., 2018), hence generating lines with specific phytocannabinoid profiles might be of interest in further research.

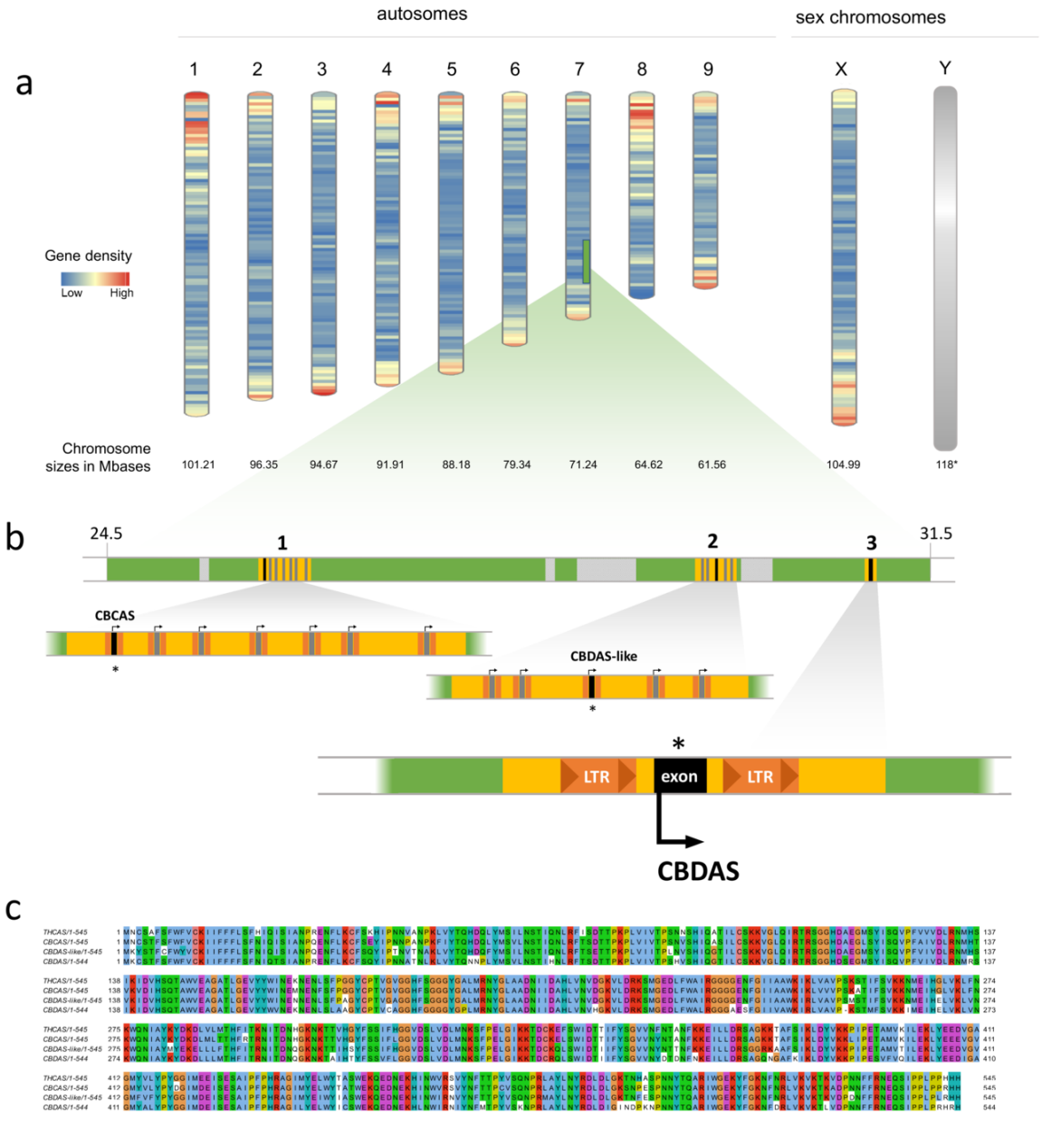

Figure 5: **Genetic location of genes encoding cannabinoid synthases in *Cannabis*.** Preliminary analysis of location and structure of the genes encoding CBCA synthase, CBDA synthase and multiple cannabinoid-synthase-like pseudogene copies in the hemp-marijuana hybrid cultivar 'CBDRx' (Grassa et al., 2018), which is the reference genome for *Cannabis sativa*. The genome has 9 pairs of autosomes and one pair of sex chromosomes, with the main locus of cannabinoid synthases located on chromosome 7 (green box, revised chromosome numbering (NCBI)) (a). Three different cassettes (1, 2 and 3, yellow regions with stripes) have been identified and mapped to a region on chromosome 7 between 24.5 and 31.5 Mb (b). The exact chromosomal arrangement is not clear, since the assembly contains gaps in between the different cassettes (grey boxes). Three cannabinoid synthase genes appear to have a full coding sequence: a CBCA synthase, the CBDA synthase and a CBDA-like synthase (*, black), while the other copies appear non-functional (grey). All sequences, including pseudogenes, have unique expression data associated with them (arrows, NBCI genome browser, unique raw reads). The synthases are encoded by one single exon (black) and surrounded by long terminal repeat retrotransposons (orange). Functional CBDA and THCA synthase share 83.86 % protein sequence similarity, while similarity amongst the other sequences ranges from 82 to 92 % (c). The CBDRx genome does not contain a functional THCAS, the sequence was acquired from uniprot (Q8GTB6). Gene density in (a) shows the ratio of the number of genes per million base pairs, calculated and plotted as an ideogram using RIdeogram (Hao et al., 2020). Gene annotation for the Y chromosome is not readily available, but genes are present (Prentout et al., 2020; McKernan et al., 2020). The CBDRx genome was derived from a female individual (Grassa et al., 2018), the size of the Y (*) chromosome was approximated from McKernan et al., 2020.

4. A hairy topic: Flower development and morphology in *Cannabis*

The flower is the reproductive structure of flowering plants (angiosperms), which represent one of the most successful and diverse groups of organisms on this planet (Krizek and Fletcher, 2005). While the characteristic shape of the *Cannabis* leaf is often used as a symbol for the whole plant, *Cannabis* female flowers are of particular interest because they are the main site of production of pharmacologically active compounds (phytocannabinoids) (Spitzer-Rimon et al., 2019). Understanding the morphology of *Cannabis* flowers and their developmental genetics is therefore especially important.

The typical angiosperm flower consists of four different organ types, which are organized in concentric whorls: sepals, petals, stamens and carpels (Endress, 1992; Krizek and Fletcher, 2005). Sepals are in the outermost whorl and usually green and leaflike in appearance. Petals are in the second whorl and often coloured to attract pollinators. Petals together with sepals are termed the perianth and constitute the non-reproductive part of a flower. Stamens are typically located in the third floral whorl. They are the male reproductive organs and are composed of an anther and a filament. The anthers grow on top of the stalk-like filaments and are the site of pollen production. Finally, carpels develop in the fourth and central whorl of a typical flower. Carpels are the reproductive organs that contain an ovary inside which ovules develop. The tip of the carpel, the stigma, receives the pollen. The style connects the stigma to the ovary (Becker, 2020; Endress, 1992; Krizek and Fletcher, 2005).

Notably, the number, arrangement, and morphology of the floral organs varies substantially between different species of flowering plants (Endress, 2011; Theissen and Melzer, 2007). Most flowers contain, as described above, both carpels and stamens, and are therefore termed bisexual flowers (Renner, 2014). However some 15 % of flowering plant species are monoecious or dioecious and have unisexual flowers that develop only stamens or carpels (Renner, 2014). In dioecious plants, female and male flowers develop on separate individuals. In contrast, in monoecious plants male and female flowers develop on the same individual (Renner, 2014).

Cannabis is primarily dioecious (Moliterni et al., 2004). The male *Cannabis* flower is green-yellow in appearance and has a perianth of five sepals, while petals are completely absent. Further, an individual male flower contains five free stamens, and no female reproductive organs (Figure 6a and b) (Leme et al., 2020; Spitzer-Rimon et al., 2019).

On the other hand, the female flower is enclosed within a green leaflike perigonal bract. The perigonal bract is sometimes also described as a sepal, but morphological studies agree that it is a bract (Leme et al., 2020; Spitzer-Rimon et al., 2019). As such, it is not strictly a part of the flower. Between the perigonal bract and the carpel is a membranous and hyaline perianth which tightly embraces the ovary (Leme et al., 2020; Reed, 1914; Spitzer-Rimon et al., 2019). It is worth noting that this inconspicuous perianth sometimes is not mentioned in the structure of female *Cannabis* flowers or is considered missing as it is not visible from the outside of the flower**.** Most likely, these membranous structures are homologous to sepals (Leme et al., 2020). At the top of the ovary are two filamentous styles. The stigma is brush-like and has epidermal cells elongated into hair-like projections (Reed, 1914; Leme*et al.*, 2020) (Figure 6c and d).

The commercially interesting phytocannabinoids and terpenes are predominantly produced on the perigonal bracts of female flowers, more specifically in glandular trichomes that cover those bracts. Glandular trichomes can be categorized into sessile, stalked and bulbous trichomes (Hammond and Mahlberg, 1973), with bulbous trichomes being metabolically less active (Livingston et al., 2020). *Cannabis* plants also have non-glandular trichomes: hair-like uni- or multicellular trichomes which protect them from biotic and abiotic stresses (Andre et al., 2016; Dayanandan and Kaufman, 1976). However, glandular trichomes are the main site of phytocannabinoid synthesis (Furr and Mahlberg, 1981).

Because phytocannabinoids are cytotoxic in higher concentrations, they have to be secreted and are not stored within cellular compartments. Phytocannabinoids along with other secondary metabolites are secreted from glandular trichomes with a globose head-like structure (Figure 7). This head is formed by an enlarged

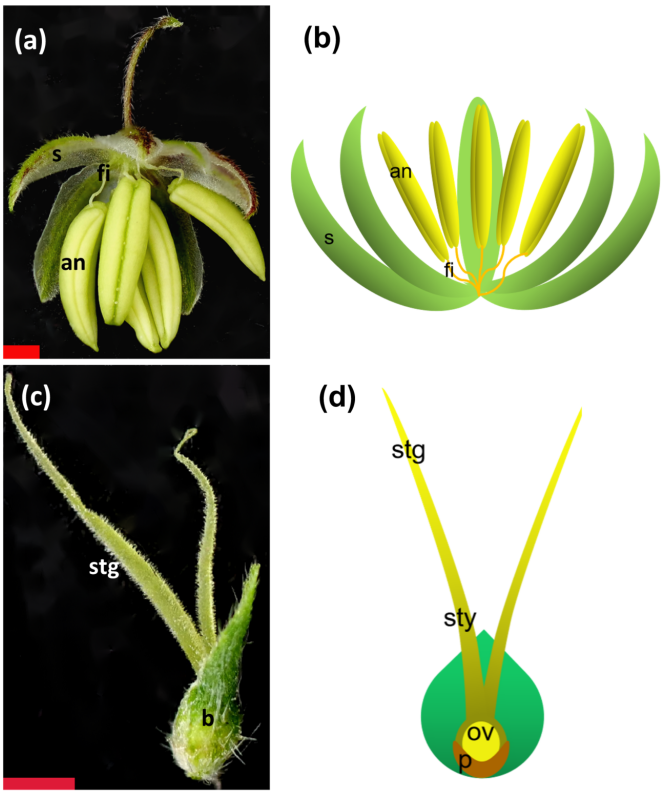

Figure 6: **Mature flowers of *Cannabis sativa*.** Male *Cannabis* flowers have sepals and stamens (a) while female *Cannabis* flowers consist of two carpels enclosed by a perigonal bract (c). Schematic depiction of the structure of *Cannabis* male flower (b) and female flower (d). The bar = 1 mm. an, anther; b, perigonal bract; fi, filament; s, sepal; ov, ovary; p., perianth; stg, stigma; sty, style.

secretory cavity which is surrounded by a culticule that encapsulates the secreted secondary metabolites (Hammond and Mahlberg, 1973). At the base of the head is a layer of secretory cells (Kim and Mahlberg, 1991; Livingston et al., 2020). The head can be sessile, directly on the epidermis and often be found on vegetative leaves (sessile trichomes), or pre-stalked or stalked with the head being elevated above the epidermis (pre-stalked and stalked trichomes), which are mainly found on female inflorescences (Kim and Mahlberg, 1991; Livingston et al., 2020). Additionally, these structures can be distinguished by different levels of autofluorescence, cell numbers as well as phytocannabinoid and terpene profiles (Livingston et al., 2020; Turner et al., 1978). Stalked trichomes seem to be developing from pre-stalked trichomes and contain a terpene profile distinct from true sessile trichomes (Livingston et al., 2020). Transcriptome analysis of floral trichomes of a CBD hemp ('Finola') confirmed high expression levels of genes involved in the synthesis of phytocannabinoids, terpenes and their respective precursor molecules in glandular trichomes, with expression differences between bulbous, sessile, and (pre-)stalked trichomes (Livingston et al., 2020).

It is not clear why predominantly female plants produce glandular trichomes within their inflorescence structures. Illuminating the genetic underpinnings of this sexual dimorphism remains a challenge for further research. Glandular trichomes also develop on male flowers (Leme et al., 2020), albeit at lower density and probably with less phytocannabinoids. Understanding which genetic factors restrict the development

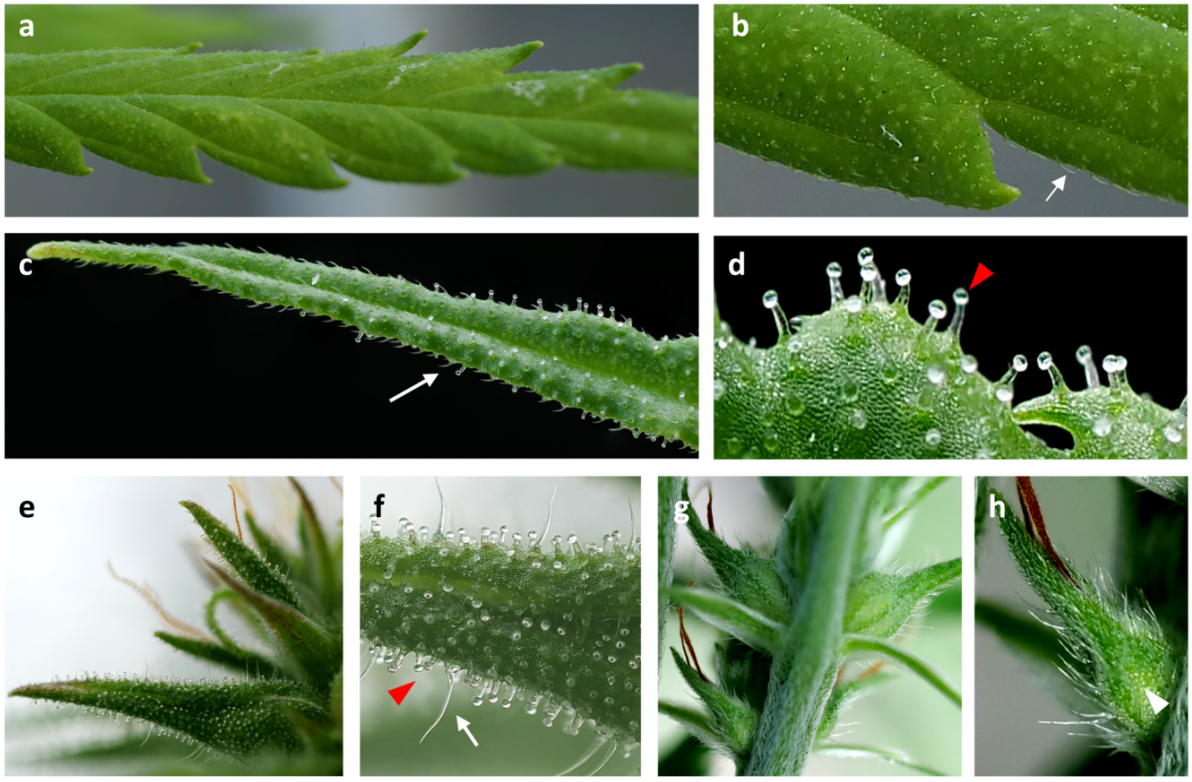

Figure 7: **Different types of trichomes on *Cannabis sativa* hemp varieties.** Trichomes can be found on the majority of female *Cannabis* plant epidermises of the cultivars 'Finola' (a-f) and 'Felina 32' (g, h). Vegetative leaves (a) have mainly non-glandular hair-like trichomes (b). Subtending ("sugar") leaves within an inflorescence (c) do have both, non-glandular trichomes as well as glandular trichomes (d). The perigonal bract of a female 'Finola' flower (e) is covered in glandular stalked trichomes (f), while the majority of trichomes found on the bract of 'Felina 32' flowers (g) are non-glandular and sessile trichomes (h). White arrows: non-glandular trichomes; white arrowheads: sessile trichomes; red arrowheads: stalked trichomes.

of glandular trichomes largely to female inflorescences during flower development would provide a valuable resource for an increase of phytocannabinoid production.

5. The battle of the sexes: Sex determination in *Cannabis*

5.1. The genetics of sex determination

The dioecy of *Cannabis* is genetically controlled (Figure 2). Hemp is diploid (2n = 20), with nine pairs of autosomes and one pair of sex chromosomes. Female plants are homogametic with XX chromosomes and male plants are heterogametic with an XY sex chromosome pair (Moliterni et al., 2004). *Cannabis* thus represents a rare case among the flowering plants in which sex chromosomes have been identified (Charlesworth, 2016).

The diploid genome size of female *Cannabis* plants is estimated to be 1636 Mbp, that of a male plant 1683 Mbp by flow cytometry (Sakamoto et al., 1998). The sex chromosomes of *Cannabis* are the largest in the chromosomal complement, they are estimated to comprise 6.5 % (Y chromosome) and 6.1 % (X chromosome)

of the total length of the genome (Divashuk et al., 2014). Assuming that those estimates roughly correspond to length in base pairs (which is most certainly an oversimplification), the X chromosome would be 102.7 Mbp in size, and the Y chromosome 109.4 Mbp. This is close to the size of the X and Y chromosomes as determined by genome sequencing (Figure 5, Supplementary Table 1). However, the flow cytometry results mentioned above (Sakamoto et al., 1998) indicate that the Y chromosome is 47 Mb larger than the X chromosome (1683 Mb - 1636 Mb = 47 Mb). Flow cytometry analyses of other *Cannabis* varieties yielded very similar results (Faux et al., 2014). It is not clear where the discrepancy between genome sequencing and flow cytometry measurements is coming from. Structural genomic variations between different *Cannabis* plants as well as problems in assembling the sex chromosomes may both play a role.

Detailed analyses of the sex chromosomes revealed that the pseudo-autosomal region on the X chromosome (i.e. the region still recombining with the Y chromosome) is ca. 30 Mb in size, whereas the X-specific region (which is not recombining with the Y chromosome) is ca. 75 Mbp in size (Prentout et al., 2020). Prentout *et al.* also identified ca. 500 sex-linked genes, i.e. alleles that are inherited in a sex-linked fashion (e.g. only from father to daughter, not from father to son for X-hemizygous alleles). It will be especially interesting to analyse the X chromosome alleles that have no Y chromosome counterpart in detail in the future, as they may contribute to sex determination.

Whereas gene density on the X chromosome appears to be similar to autosomes, about 70 % of the genes on the Y chromosomes have been estimated to be lost (Prentout et al., 2020). One explanation for the relatively large size of the Y chromosome despite substantial gene losses seems to be the accumulation of transposons and other repetitive elements (Sakamoto et al., 2000). Prevalence of transposable elements on the Y chromosome might also help to explain difficulties in chromosome assembly and discrepancies in size estimates.

Despite progress in identification and sequencing the sex chromosomes of *Cannabis* , not much is known about the molecular circuits involved in sex determination. Some confusion exists as to what the genetic 'mode' of sex determination is (Kovalchuk et al., 2020; Vergara et al., 2016). Some reports suggest the mode is similar to humans and other mammals, where the presence or absence of the Y chromosome determines the sex: humans carrying a Y chromosome are almost always phenotypically male, those without a Y chromosome are female, with autosomes or extra X chromosomes bearing little consequence on the sex determination (Gamble and Zarkower, 2012; Sakamoto et al., 1998). An alternative view is that the X chromosome to autosome ratio determines the sex (Westergaard, 1958). This would be somehow similar to *Drosophila*, where the number of X chromosomes determines the sex, with the presence or absence of the Y chromosome having limited relevance (Gamble and Zarkower, 2012). In this model, the Y chromosome essentially becomes a 'placeholder', lowering the number of X chromosomes.

Experimental evidence supporting the one or the other mode of sex determination is surprisingly scarce. Warmke and Davidson used colchicine to produce tetraploid *Cannabis* plants (Warmke and Davidson, 1944). Strikingly, a cross between a tetraploid female and a diploid male plant yielded female and female-hermaphrodites, but no male plants, though half of the progeny should have an XXY chromosome constitution (Warmke and Davidson, 1944). This is evidence that the Y chromosome does not play a role as prominent as in humans in sex determination in *Cannabis*, but that an X to autosome ratio model for sex determination might apply.

It is important to note that, even if an X to autosome ratio model applies in *Cannabis*, this does not mean that the Y chromosome is dispensable for the development of male plants. In *Drosophila*, the Y chromosome is not involved in sex determination but encodes genes required for male fertility, such that XO flies are phenotypically male but sterile (Gamble and Zarkower, 2012). Likewise, Y chromosome specific genes in *Cannabis* may well play a critical role in male plant development, even if not involved in *bona fide* sex determination (McKernan et al., 2020).

5.2. Monoecy, dioecy and evolutionary considerations

To add further complications to the study of sex determination in *Cannabis* , monecious varieties exist, in addition to the canonical dioecious varieties. Monoecious cultivars develop male and female flowers on the same plant and are particularly popular for fibre production (Figure 2). This is because in dioecious varieties, male plants flower earlier than female plants, whereas for monoecious cultivars flowering time is more synchronized, thus facilitating the determination of an optimal harvest time (Faux et al., 2016).

Genetically, monoecious cultivars carry two X chromosomes and no Y chromosome, indicating that the XY sex determination system can be 'leaky' in *Cannabis* (Faux et al., 2014; Razumova et al., 2016). In addition, the monoecious cultivars express 'femaleness' and 'maleness' to different degrees, i.e. the ratio of female to male flowers one plant develops differs between cultivars but also between different environmental conditions (Faux et al., 2014). At least some genetic loci relevant for the sex expression in monoecious *Cannabis* plants seem to be located on the X chromosome (Faux et al., 2016). It will be interesting to see whether the distinction between dioecious and monoecious cultivars and between different degrees of sex expression (femaleness or maleness) in monoecious cultivars can be traced back to the same molecular circuits.

From an evolutionary point of view, the sexual system in the entire family of Cannabaceae is complex. In contrast to angiosperms in general, some 85 % of which are bisexual (Renner, 2014), true bisexual flowers are conspicuously rare in Cannabaceae (Yang et al., 2013). Several shifts of the sex determination system occurred in the Cannabaceae, and ancestral character state reconstructions indicate that monoecy, or, with a lesser likelihood, dioecy, is the ancestral state in the family (Yang et al., 2013). Interestingly, one of the closest relatives of *Cannabis sativa* , *Humulus lupulus* (common hop), is dioecious with an XY sex determination system in which the X to autosome ratio determines the sex (Parker and Clark, 1991). This may be taken as additional evidence that also *Cannabis* has an X to autosome ratio sex determination system. However, it should be kept in mind that that *Humulus* and *Cannabis* may have separated some 25 million years ago (Jin et al., 2020) and that sex determination systems can frequently and rapidly change during evolution (Bachtrog et al., 2014). Nevertheless, available data on the sex determination systems in Cannabaceae as a whole may be taken as indication that all family members share an ancestral molecular mechanism for sex determination, but that this mechanism is relatively labile and manifests differently in different species.

5.3. Hormonal and environmental factors affection sex determination

Beyond genetic consideration, the sex expression in *Cannabis* can be shaped by environmental factors. It is well established that silver can be used to induce the formation of male flowers on female plants (Mohan Ram and Sett, 1982). This treatment is used by breeders to self female dioecious plants, which results in offspring with exclusively XX sex chromosomes (Clarke and Merlin, 2016). Because all of the progeny from such a selfed plant will be female, the 'feminized' seeds produced that way are usually much more valuable than conventionally produced seeds (Small, 2015). Silver is a known ethylene inhibitor, and there is also evidence that ethylene induces the development of female flowers on male plants (McDaniel and Binder, 2012; Ram and Jaiswal, 1970). Together, this therefore suggests that ethylene is involved in controlling the sex expression in *Cannabis* .

In addition to ethylene, also other phytohormones have been shown to be capable of altering the sex expression in *Cannabis* (Ainsworth, 2000) . Auxin, for example, can lead to the development of female flowers on male plants. Indeed, male flower development can be entirely repressed by auxin treatment (Heslop-Harrison, 1956). Cytokinin also has a feminizing effect (Chailakhyan and Timiriazev, 1979), whereas gibberellic acid triggers the formation of male flowers on female plants (Ram and Jaiswal, 1972). Together, the hormonal effects are very similar to what is observed for sex expression in the well analysed Cucurbitaceae family (Li et al., 2019; Pawełkowicz et al., 2019; Schilling et al., 2020a). Cucurbitaceae are only distantly related to Cannabaceae, the two lineages separated more than 100 million years ago (Figure 3) (Magallón et al., 2015). Also, many of the studied Cucurbitaceae are monoecious and thus do not possess sex chromosomes (Boualem et al., 2015). Nevertheless, we hypothesize that similar developmental genetic pathways may have

been co-opted to control sex expression in *Cannabis* and the Cucurbits. Melon, cucumber, pumpkin, and their relatives might thus serve as an excellent model to unravel the molecular intricacies of sex expression and sex determination in *Cannabis* .

Cannabis is a short-day plant, and flowering is initiated if day length is below 14 h (see below), but it is well established that sex expression is also affected by day length. It was reported some 100 years ago already that dioecious plants grown under short day conditions only (i.e. without an initial period of long day growth) can develop male as well as female flowers ((Tournois 1911, 1912, as cited by (Heslop-Harrison, 1957), (Schaffner, 1923)). A lot of other environmental factors like nitrogen availability or carbon monoxide also seem to have an influence on sex expression (Freeman et al., 1980; Heslop-Harrison, 1957; Small, 2015). Together, this yields a very complex picture of different environmental factors influencing sex expression in different directions that is far from being completely understood.

In summary, the picture emerges that, although sex determination is genetic, hormonal and environmental influences have a significant effect on sex expression. Because the flowers of female plants are the main source of phytocannabinoids, a more detailed study of the sex determination and sex expression mechanism of *Cannabis* is one of the main areas of future research. For example, creating male sterility would be very beneficial, as phytocannabinoid production is highest in unpollinated female plants. Studying both sex chromosomes, their gene content as well as the molecular intricacies of the sex determination mechanisms will certainly provide valuable insights for breeders as well as researchers.

6. Timing is everything - the complex network of floral initiation and hints for *Cannabis*

6.1. The evolutionary and developmental importance of flowering time

Flowering time control is essential for reproductive success (Shim et al., 2017). Mechanisms to control the time of flowering have evolved given the negative consequences of spontaneous floral initiation: Premature flowering could coincide with the absence of pollinators or dispersers, subsequently causing reduced fertilisation rates and deficient seed dispersal. Conversely, if flowering occurs too late the plant may fail to set seed before harsh conditions hit at the end of the growing season (Gaudinier and Blackman, 2020). Moreover, in dioecious species such as*Cannabis* , the timing of flower emergence is particularly crucial, because if male and female plants do not flower concurrently, pollination cannot occur (Hall et al., 2012). Hence it is evolutionarily beneficial for plants to possess mechanisms to fine-tune their floral initiation (Gaudinier and Blackman, 2020). Analyses in various plant species have demonstrated that flowering time in angiosperms is controlled by internal timekeeping mechanisms as well as environmental signals. Among the major factors controlling flowering time are the plant age, the photoperiod, the circadian clock, ambient temperature, the phytohormone gibberellin and the autonomous pathway (Fornara et al., 2010; Hill and Li, 2016).

The fine-tuning of flowering time is a major goal for plant breeding and crop improvement efforts. Floral transition represents the developmental shift from vegetative to reproductive growth and is a major determinant of yield potential (Jung and Müller, 2009). Alterations in key flowering time genes have been crucial to crop domestication, facilitating the adaptation of crops to local climatic conditions (Gaudinier and Blackman, 2020; Schilling et al., 2018). The success and worldwide expansion of staple crops like wheat and rice can partly be attributed to natural variation in flowering time genes, which enabled local adaptation for cultivation at a wide range of latitudes (Hill and Li, 2016; Langer et al., 2014). As a quantitative short-day plant, *Cannabis* flowering time is particularly determined by the photoperiod. Under long-days *Cannabis* remains vegetative and flowering is only induced when a number of short-day photoperiods have passed. Therefore, in order to cultivate *Cannabis* at new lines of latitude (for example in Ireland where summer daylengths can be over 17h), the adjustment of flowering time genes can be advantageous. Consequently, comprehensive characterisation of the *Cannabis* flowering time pathways is crucial to the integration of this

crop species into modern agriculture.

6.2. Flowering time control in Cannabis - what we know so far

Cannabis has the potential to be a sustainable multipurpose crop. For virtually all applications of *Cannabis* , a better understanding of the genetic factors controlling flowering time would be highly beneficial (Figure 1). The reasons are evident when the flowers or seeds are the main agricultural product, such as hemp oil from seeds or CBD production from flowers. But flowering time also determines the crop purpose in more general terms, with later flowering varieties favouring vegetative stem growth thus suiting fibre production and earlier varieties displaying enhanced flower/seed yield (Salentijn et al., 2019). The interactions between flowering time and fibre quality are complex (reviewed in Salentijn et al., 2019) and the developmental stage at harvest has major implications for fibre quality. Additionally, a better understanding of flowering time is important to generate varieties that are adapted to local climatic and photoperiod conditions.

While various environmental signals including temperature (Amaducci et al., 2012, 2008; Cosentino et al., 2012; Nelson, 1944) prompt floral initiation, *Cannabis* is particularly sensitive to changes in photoperiod (Hall et al., 2012; Salentijn et al., 2019). In as early as 1912 it was observed that flower induction in *Cannabis* is influenced by the photoperiod (Tournois 1912 (cited by Heslop-Harrison, 1957)). *Cannabis* is a facultative short-day plant (Salentijn et al., 2019). This means that while plants will typically flower eventually under long-day conditions, flowering occurs faster in short-day conditions i.e. by experiencing a sequence of days each with a minimum uninterrupted period of darkness. Cultivar-specific variation for the photoperiod at which flowering is induced has been reported, with the optimal photoperiod ranging from 9 to 14 h (Lisson et al., 2000 and references cited therein).

A related question is how many consecutive short days are required to induce flowering. Borthwick and Scully (1954) reported two weeks of a short photoperiod induced flowering in 3-5-week-old plants, and the greater the plant age at the time of switching to short-day the faster the floral transition. Furthermore, Potter (2014) stated flowers can be visible one week after the reduction in day length. Clearly, more research is needed in this area, in particular to explore the variation between cultivars for this trait.

Given that most individuals eventually flower under non-inductive photoperiodic conditions, a more detailed analysis of the age-related and the autonomous pathway and their influence on flowering time is warranted. In addition, it would be especially interesting to observe stressors that can accelerate flowering under non-inductive photoperiodic conditions, and detect whether the same signalling pathways are at play when individuals eventually flower under long days (Takeno, 2016).

6.3. Model plants and candidate genes: *Arabidopsis*, soybean and the hunt for flowering time genes in *Cannabis*

While important efforts to determine the environmental stimuli impacting floral induction in *Cannabis* have been undertaken, the genetic pathways and loci underlying the environmental responsiveness still require elucidation (Salentijn et al., 2019). Huge diversity exists for flowering time in *Cannabis* with phenotypes generally categorised as early-, mid- or late-flowering (Salentijn et al., 2019). Furthermore, photoperiod-insensitive (also known as day-neutral or auto-flowering) cultivars exist (Small, 2018). A recent study suggests that female floral initiation occurs independently of the photoperiod in some *Cannabis* cultivars, while in others shorter photoperiods were required for flower maturation and development (Spitzer-Rimon et al., 2019). Further research is required to substantiate the molecular basis of those observations, and research on model plants may serve as an important primer to understand the gene regulatory network controlling flowering time in *Cannabis*.

Among the model plant species for which comprehensive analyses of flowering time have been conducted are the long-day plant *Arabidopsis thaliana* and the short-day plant *Oryza sativa*(rice). In *Arabidopsis*, the complex flowering time network is well-characterised with several pathways described including the vernalisation,

autonomous, photoperiod, circadian clock, age, ambient temperature and gibberellin pathways (Blümel et al., 2015). One of the key integrators of floral inductive signals in *Arabidopsis* is *FLOWERING LOCUS T* (*FT*), the protein product of which is known as florigen (Turck et al., 2008).

As mentioned previously, *Cannabis* is particularly sensitive to alterations in the photoperiod and as such the photoperiodic pathway of *Arabidopsis* warrants some more detailed discussion. The photoperiodic flowering pathway depends on cross-talk between light perception and the circadian clock, which coordinate to control the expression of the main integrator *FT*(Cao et al., 2017). The first step in the photoperiodic pathway is the perception of light by the photoreceptors (phytochromes and cryptochromes). Phytochromes exist in inactive (Pr) and active (Pfr) forms. Pr is synthesised in the dark, and upon red-light perception is activated to Pfr which translocates to the nucleus. Pfr can interact with transcription factors and induce large-scale transcriptional alterations in response to light (Legris et al., 2019). Pfr then reverts to Pr by far-red light absorption or by light-independent thermal reversion (Klose et al., 2020). Phytochromes have a myriad of roles in regulating plant development and several phytochromes exist in angiosperms. The Brassicaceae possess five phytochromes: phyA to phyE. In *Arabidopsis* phyA and phyB are functionally the most important (Legris et al., 2019).

The photoreceptors subsequently transmit signals to the central node of the photoperiodic pathway: the *GIGANTEA-CONSTANS-FT*(*GI-CO-FT*) signalling cascade. Briefly, the action of the *GI-CO-FT* module in *Arabidopsis* is as follows: the active Pfr form of phyA promotes the stability of the nuclear transcription factor CONSTANS (CO) which activates transcription of *FT*(Putterill et al., 1995; Samach et al., 2000). From the *FT* locus, florigen is produced, a small mobile protein which travels via the phloem from the leaves to the shoot apical meristem to induce the transition from vegetative to reproductive growth (Corbesier et al., 2007). The circadian clock gene *GIGANTEA* (*GI*) allows the degradation of transcriptional repressors that repress the expression of*CO* thus indirectly promoting *FT*(Sawa et al., 2007). The MADS-box transcription factor gene *SOC1* is indirectly upregulated by *CO* via florigen. SOC1, in turn, activates the floral meristem identity gene *LEAFY,* thus promoting flowering (Lee et al., 2008; Yoo et al., 2016).

Importantly, *SOC1* is a major floral integrator of different flowering pathways in *Arabidopsis*. For example, another MADS-box gene, *FLOWERING LOCUS C* (*FLC*), which is involved in the vernalization pathway directly binds to the *SOC1* promoter, and blocks *SOC1* transcriptional activation by CO (Hepworth et al., 2002). *FLC* also represses *FT* transcription in the leaves and blocks florigen transport thus inhibiting flowering (Searle et al., 2006).

GI, *CO* and *FT* seem to be conserved in flowering pathways in many crops, such as wheat, barley, grapevine, pea, tomato, onion and cucurbits (Watanabe et al., 2011 and references therein). Thus, these genes are promising candidates for flowering time control in *Cannabis*. However, the gene functions and mechanisms controlling the flowering pathways may differ between species, and thus must be elucidated in *Cannabis*.

Several of these key regulators of flowering time have been demonstrated to have pleiotropic effects on agronomically valuable characteristics, further emphasising the importance of elucidating the role of these regulators in crop species (Blümel et al., 2015).

Given that *Cannabis* is a eudicot, short-day plant, the commonly used models- the long-day *Arabidopsis* or the monocot rice - may not be the most applicable for comparative analysis. *Glycine max*(soybean) is a short-day crop that belongs to the Fabales and is, therefore, more closely related to *Cannabis* (Rosales) than rice (Poales) or *Arabidopsis* (Brassicales) (Figure 3). Flowering time control in soybean is well studied and may provide important clues about how flowering is regulated in *Cannabis*.

In soybean, the *E* genes and the *JUVENILE* (*J*) gene are involved in flowering time control (Figure 8) (Copley et al., 2018 and references therein). *J* is also named *GmELF3*, it is orthologous to *Arabidopsis EARLY FLOWERING3* (*ELF3*), which is an important part of the circadian clock (Lu et al., 2017). Individuals that carry loss-of-function alleles for *E1* to*E4* exhibit photoperiod insensitive flowering as higher transcript levels of the *FT* genes are present (Figure 8) (Xu et al., 2013).*E1* is a legume specific transcription factor and the remaining genes are orthologous to those involved in flowering time control in *Arabidopsis*:

E2 (also named *GmGIa*) is an ortholog of *GI,* and *E3* (*GmPhyA3*) and *E4* (*GmPhyA2*) are orthologous to *PhyA*.

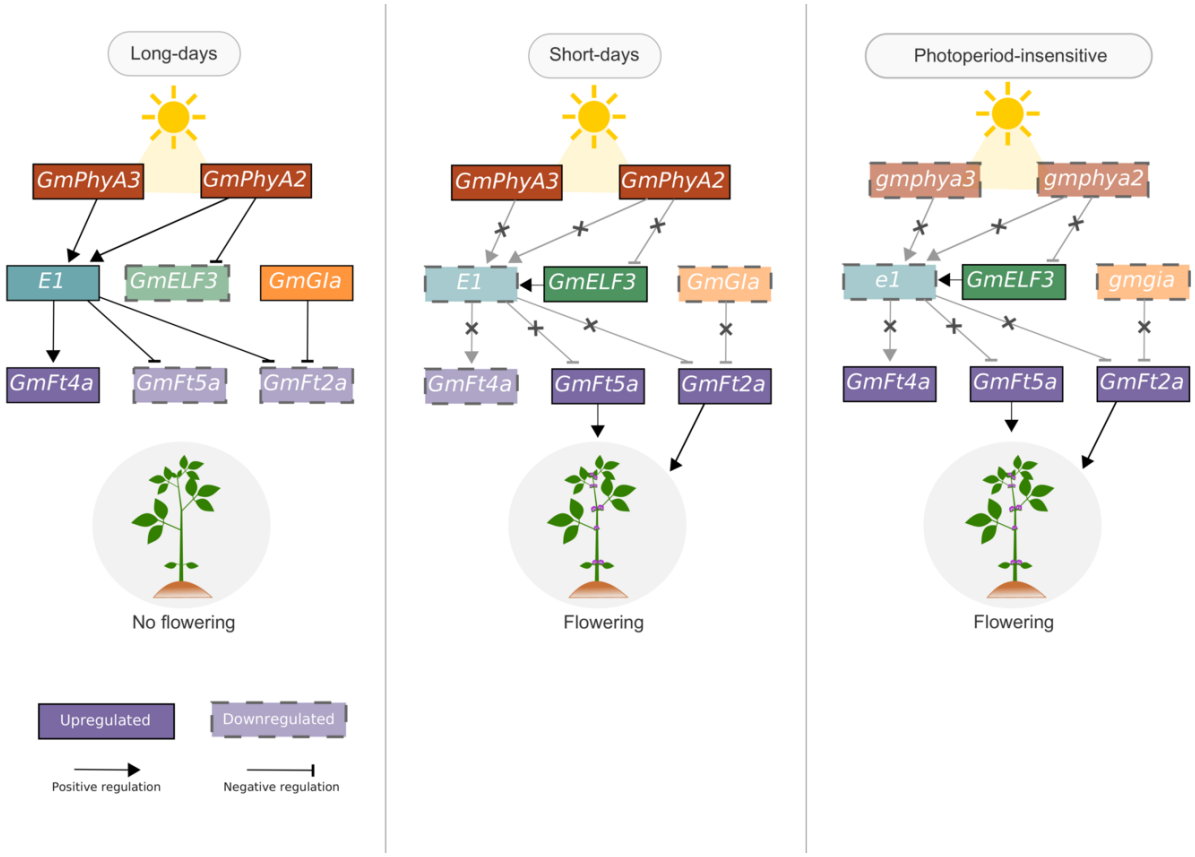

Figure 8: **An overview of the working model for the photoperiodic flowering time pathway in soybean.** Parallel representations of the network under long-day conditions and short-day conditions are shown as well as the genes that when mutated infer photoperiod-insensitive flowering. Under long-day conditions, *Phytochrome A* homologs *GmPHYA3* and *GmPHYA2* promote *E1* expression and inhibit *GmELF3* expression. *E1* up-regulates *GmFT4a* (a change-of-function *FT* that suppresses flowering) and down-regulates *GmFT2a* and *GmFT5a*, all of which are *FT* homologs (Nan et al., 2014; Samanfar et al., 2017; Xia et al., 2012; Zhai et al., 2014). *GmGIa* is a *GI* homolog which inhibits *GmFT2a* (but interestingly not *GmFT5a*) thus delaying flowering under long-day conditions (Watanabe et al., 2011). Under short-day conditions, *GmELF3* is expressed. *GmELF3* represses *E1* by physically associating with the *E1* promoter. This leads to the release of the *E1* suppression of the *GmFT* genes, thus promoting flowering under short days (Lu et al., 2017; Xia et al., 2012). Natural variation in the *GmFT* gene family is at least partially responsible for flowering time variation in soybean, with several polymorphic sites significantly associated with flowering time variation (Jiang et al., 2019). Soybean plants which carry loss-of-function alleles for *E1*, *GmGIa*, *GmPHYA3* and *GmPHYA2* exhibit photoperiod insensitive flowering as higher transcript levels of the *FT* genes are present (Xu et al., 2013). Thus, these genes may represent strong candidates for elucidating photoperiod-insensitivity in *Cannabis*.

Natural variation in the *E* and *J* genes are responsible for the adaptability of soybean to cultivation at various latitudes, including the tropics, with several polymorphic sites significantly associated with flowering

time (Jiang et al., 2019; Lu et al., 2017). Thus, these genes may represent strong candidates for elucidating the natural variation that exists for photoperiod sensitivity in *Cannabis*.

However, though candidate gene searches can be informative, it is worth considering that gene functions may not be similarly conserved across species. As such gene mapping approaches (genome-wide association studies, quantitative trait loci mapping) and functional analyses are still required to elucidate the flowering time network in *Cannabis*. Furthermore, as a wind-pollinated, dioecious species *Cannabis* is primarily outcrossing (Salentijn et al., 2015). In self-pollinating species such as rice, *Sorghum* and *Arabidopsis*, variation in flowering time is controlled by a few large-effect loci (Gage et al., 2020). However, in the model outcrossing species maize, several loci all contribute a small amount to phenotypes of complex traits such as flowering time (Buckler et al., 2009). It remains to be seen how complex traits will be determined in *Cannabis*, but taking inspiration from the successful elucidation of complex traits in maize may motivate the formation of sophisticated multi-parental mapping populations such as Nested Association Mapping (NAM) or Multiparent Advanced Generation InterCross (MAGIC) populations (Gage et al., 2020).

7. The long read: *Cannabis* genetics and genomics

While the medicinal, agricultural, and industrial applications of *Cannabis* are vast, *Cannabis* genomics has lagged in relation to that of other crops. In recent years, however, as legal restrictions have eased and with the advent of third-generation sequencing, the field has picked up significantly. With this, there is now a wealth of novel data readily available for analysis. Here, the currently available genomics and transcriptomics data will be reviewed.

The *Cannabis* genome is diploid (2n=20), consisting of 9 autosomes and a pair of heteromorphic sex chromosomes (X and Y). The haploid genome size is predicted to be 843 Mb and 818 Mb for male (XY) and female (XX) plants respectively, with the larger Y chromosome accounting for the sex-specific difference in genome size (see also discussion above on sex determination) (Sakamoto et al., 1998). The *Cannabis* genome is not large relative to that of other crops like maize and wheat. However, it has been difficult to resolve due to its high heterozygosity and the abundance of repetitive DNA sequences. High heterozygosity levels have been retained in the genome, as *Cannabis* is dioecious and has not been subject to intense breeding (Lynch et al., 2016; Sawler et al., 2015). While this genetic diversity is desirable for selective breeding, it can complicate genome assembly. Highly variable alleles are often misassembled as segmental duplications, whereby both haplotypes are incorporated at distinct loci, inflating the genome assembly size (Claros et al., 2012; Michael and VanBuren, 2020). Additionally, it is estimated that repetitive sequences constitute ~70% of the *Cannabis* genome (Gao et al., 2020; Laverty et al., 2019; Pisupati et al., 2018). Misassembly of multiple repeat elements onto one contig causes assembly collapse, reducing the genome assembly size (Claros et al., 2012; Michael and VanBuren, 2020). These features are particularly challenging when short-read sequencing is applied. While the first draft *Cannabis* genome, from the marijuana Purple Kush (PK) cultivar, was sequenced in 2011, the short-read sequencing of that time could not resolve repeat-rich, low complexity regions (van Bakel et al., 2011). This led to a very valuable yet incomplete genome assembly, consisting of 534 Mb (van Bakel et al., 2011).

Third generation single molecule (or long-read) sequencing, such as Pacific Biosciences (PacBio) and Oxford Nanopore sequencing, can generate long reads which are capable of capturing the regions flanking repeat sequences and segmental duplications. Thus, long-read sequencing greatly facilitates the assembly process and has been revolutionary for plant genomics, enabling chromosome-level assemblies to be achieved (Jiao and Schneeberger, 2017; Michael and VanBuren, 2020). Recently, long-read sequencing, in parallel with genetic and physical mapping, has enabled four chromosome-level assemblies from the CBDRx, PK, Finola (FN), and a wild *Cannabis* (CR) line (Table 1) (Gao et al., 2020; Grassa et al., 2018; Laverty et al., 2019). The CBDRx genome (a female individual) was sequenced using Oxford Nanopore technology and has an assembly size of 876.148 Mb (Figure 5a) (Grassa et al., 2018). In 2019, the first genome-wide annotation was made available for this *Cannabis* genome, making it the reference genome on the NCBI database (Jenkins and

Orsburn, 2019). The PK and FN genomes were sequenced with PacBio single-molecule sequencing (Laverty et al., 2019). The PK (female) and FN (male) assembly sizes are 891.965 Mb and 1009.67 Mb respectively, both of which are significant improvements upon the original draft PK genome from 2011 (Table 1) (van Bakel et al., 2011; Laverty et al., 2019). The CR variety, which is derived from a wild *Cannabis* plant, was also sequenced with PacBio, achieving a genome assembly size of 812.525 Mb (Gao et al., 2020). While linkage maps were generated to resolve the chromosomes for the CBDRx, PK and FN genomes (Grassa et al., 2018; Laverty et al., 2019), Hi-C data was used to create a physical map for CR, enabling the chromosomes to be assembled (Supplementary Table 1) (Gao et al., 2020).

Eight additional genomes have been assembled to varying levels of completeness (Table 1). Amongst these are the genome sequences of a father-mother-daughter trio from the Jamaican Lion (JL) cultivar, which was sequenced using PacBio (McKernan et al., 2020). The parental genome assemblies including gene annotation are available on the NCBI database, while all three genome assemblies are available on the Medicinal Genomics website (*https://www.medicinalgenomics.com/jamaican-lion-data-release/*). In addition to these three genomes, 40 genomes from a diverse range of cultivars were sequenced with Illumina short-read sequencing as part of the Medicinal Genomics 'Cannabis Pan-Genome Project' (McKernan et al., 2020). The whole-genome sequencing (WGS) data generated in this project are available on the NCBI sequence read archive (Supplementary Table 2). These genome sequences will be an invaluable resource for characterising the genetic basis behind the wide phenotypic diversity observed within *Cannabis*. Specifically, they will facilitate the development of a *Cannabis* pan-genome, where gene sets unique to specific cultivars could be defined. Such cultivar-specific genes are often representative of niche phenotypic adaptations that have evolved in response to specific environmental conditions (Montenegro et al., 2017; Tao et al., 2019). Cultivar-specific genes could be key targets for breeding, where new cultivars could be designed with desirable traits for specific production purposes (Tao et al., 2019).

There is also a wealth of additional genomics data available. This includes sequences of organellar genomes, of which there are seven mitochondrial and nine chloroplast genome assemblies available (Supplementary Table 3). The organellar genomes are particularly useful for resolving phylogenetic relationships. The rate of nucleotide substitution of mitochondrial coding sequences is lower than that of the nuclear and plastid genomes, making them useful molecular markers for resolving deep taxonomic relationships (Knoop, 2004; Wolfe et al., 1987). Despite this high intragenic sequence conservation, angiosperm mitochondria can exhibit high variation in genome organisation both within and between species (Cole et al., 2018; Davila et al., 2011; Palmer and Herbon, 1988). Perhaps taking a comparative genomics approach to investigate organisational variation within the mitochondrial genome between different *Cannabis* cultivars would be insightful for resolving relationships within the *Cannabis* genus. In contrast, the chloroplast genome is characterized by both stability in genome organisation and sequence conservation between species (Palmer and Herbon, 1988). Hence the chloroplast genome is often used to resolve phylogenies at the ordinal and familial taxonomic levels (Oh et al., 2016; Vergara et al., 2015; H. Zhang et al., 2018).

Furthermore, genotyping by sequencing (GBS), amplicon sequencing, bisulfite sequencing and Hi-C data are available for a multitude of different hemp as well as marijuana varieties (Supplementary Table 2). GBS is an efficient and cost-effective method to genotype a large number of samples, providing insight into the population structure and genetic diversity within a species (He et al., 2014). There have been at least three population-based studies that have generated GBS data for ~400 samples, representing both hemp and marijuana lines (Lynch et al., 2016; Sawler et al., 2015; Soorni et al., 2017). These studies find that hemp and marijuana often form distinct populations, not segregating based only on the B_T and B_D loci, but on a genome-wide level (Lynch et al., 2016; Sawler et al., 2015; Soorni et al., 2017). Bisulfite sequencing detects DNA methylation and is useful for understanding epigenetic gene regulation (Elhamamsy, 2016; Li et al., 2020). Two bisulfite sequencing datasets are available for analysis (McKernan et al., 2020; Niederhuth et al., 2016). Given that economically important traits like sex expression and flowering time are under strong environmental control, it will be interesting to explore to which extent those traits are epigenetically regulated. This may open the possibility of breeding 'climate smart' *Cannabis* plants, similarly to other crops where epigenetically regulated heat, drought or cold adaption are explored for crop improvement (Varotto

et al., 2020).

Lastly, the 3D organisation of the genome within the nucleus can be mapped with Hi-C data (Rodriguez-Granados et al., 2016). One Hi-C dataset exists for the JL cultivar and is available on NCBI (Gao et al., 2020). Additional Hi-C datasets are available for the Jamaican Lion genomes through the Medicinal Genomics website (https://www.medicinalgenomics.com/jamaican-lion-data-release/). The 3D organization of the genome and its implications for gene regulation are currently being heavily investigated in plants (Santos et al., 2020). The available *Cannabis* Hi-C data are both useful for facilitating genome assembly as well as for understanding epigenetic regulation of gene expression (Burton et al., 2013; Lieberman-Aiden et al., 2009; Xie et al., 2015).

There have also been many studies that have focused on characterising the *Cannabis* transcriptomes (Supplementary Table 2). Perhaps most notably, in 2019, an extensive 'transcriptome atlas' was generated for *Cannabis*(Braich et al., 2019). This study involved RNA-sequencing of 71 samples taken from multiple tissues of the Cannbio-2 cultivar (CN2), at various developmental stages. This transcriptome data will be useful for the annotation of new genome assemblies, as well as for inferring gene functions based on spatiotemporal gene expression patterns. Other studies have characterised the transcriptome of hemp lines grown under salinity and drought stress (Gao et al., 2018; Liu et al., 2016), as well as during bast fibre development (Behr et al., 2016; Guerriero et al., 2017). Three further studies have focused on sequencing the transcriptome of glandular trichomes, with the aim of profiling the expression of genes involved in terpene and phytocannabinoid biosynthesis (Booth et al., 2020; Livingston et al., 2020; Zager et al., 2019). Furthermore, two recent studies have focused on identifying the sex chromosomes based on characterising the expression of sex-linked genes in male and female plants (McKernan et al., 2020; Prentout et al., 2020). The transcriptomes of the PK and FN cultivars sequenced in 2011 are also available (van Bakel et al., 2011).

While wide-spread illegalization of *Cannabis* has stunted genomics research in the past, it is clear that there have been major advances in this field in recent years. With chromosome-level genome assemblies now available, as well as genome-wide annotations and abundant transcriptome data, the resources for future research are plentiful.

8. More than the sum of its parts: Medical applications of phytocannabinoids

Cannabis plants represent a rich source of biologically active compounds, including more than 100 plant-derived cannabinoids (phytocannabinoids) and more than 200 terpenoids (Russo, 2011). Thus far, research into the medicinal effects of *Cannabis* has largely focussed on phytocannabinoids. Among these, the most well-studied are the psychoactive THC, and the non-psychoactive CBD, though other phytocannabinoids such as CBG and CBC also show therapeutic potential (Russo, 2011) (see chapter 3 for details on phytocannabinoid synthesis and genetics).

8.1. *Cannabis* metabolites

Early investigations into the pharmacologic effects of THC led to the discovery of the human endogenous cannabinoid (endocannabinoid) system, which includes endogenous cannabinoid ligands, metabolic enzymes, and the two major cannabinoid receptors, CB1 and CB2 (Izzo et al., 2009). The modes of action of phytocannabinoids are complex, with individual compounds capable of acting at multiple molecular targets. THC modulates the activity of CB1 and CB2 but can also activate the transcription factor PPARγ and the TRP ion channel TRPA1 (Izzo et al., 2009). CBD, meanwhile, has low affinity for CB1 and CB2, but can modulate the activity of various components of the endocannabinoid system. Furthermore, like THC, CBD can target PPARγ and TRPA1, as well as the G-protein coupled receptors GPR55 and GPR18, the TRP ion channels TRPV1, TRPV2 and TRPM8, and the serotonin receptor 5-HT_{1a}(Izzo et al., 2009). By

modulating various signalling pathways involved in multiple diseases, phytocannabinoids have the potential to provide many therapeutic benefits.

The analgesic, antiemetic and anticonvulsant properties of phytocannabinoids are well-established (Whiting et al., 2015). Growing evidence suggests that *Cannabis*metabolites also produce anti-inflammatory, antidepressant, anxiolytic and anti-cancer effects (Atalay et al., 2019; Fraguas-Sánchez and Torres-Suárez, 2018; Poleszak et al., 2018; Śledziński et al., 2018). Synthetically produced cannabinoids can mimic the effects of plant-derived compounds, and many countries have approved synthetic cannabinoids for medicinal use. Dronabinol and nabilone, synthetic forms of THC, are approved for the treatment of chemotherapy-induced nausea and vomiting, and for appetite stimulation in AIDS-associated anorexia (Freeman et al., 2019). However, plant-derived medicines have also been developed, namely Epidiolex, a purified form of CBD for the treatment of severe forms of epilepsy, and Sativex, a *Cannabis* extract containing THC and CBD for the management of pain and spasticity in multiple sclerosis (Freeman et al., 2019).

Additionally, *Cannabis* contains many non-cannabinoid metabolites, including terpenoids, flavonoids, ligand amides and stilbenes (Pollastro et al., 2018b). Of these, the terpenoids have been most extensively studied, and display many therapeutic effects (Russo, 2011). Due to growing evidence that various *Cannabis* bioactive compounds act synergistically to produce therapeutic effects (Russo, 2011), a greater understanding of the pharmacological contributions of different*Cannabis* metabolites will be needed to develop the most effective*Cannabis* -based medicines.

8.2. *Cannabis* synergy

To date, research into *Cannabis*-based medicines has primarily focussed on single isolated cannabinoid compounds. However, some studies show that combinations of various *Cannabis* components display greater biological activity than single compounds, suggesting that whole plant extracts may be more effective than purified phytocannabinoids (Russo, 2011). The increased activity of whole *Cannabis* extracts may be due to the synergism between various cannabinoid and non-cannabinoid components, which has been termed 'the entourage effect' (Russo, 2011). Proposed mechanisms underlying the entourage effect in *Cannabis*include activation of multiple molecular targets, enhanced bio-availability or solubility of compounds, and neutralisation of adverse events (Wagner and Ulrich-Merzenich, 2009). Exploiting *Cannabis* synergy to develop new medicines based on whole-plant extracts may be beneficial for a range of pharmaceutical applications.

In epilepsy, the addition of non-THC, non-CBD *Cannabis*metabolites may enhance the anticonvulsant effects of existing treatments. In a mouse model of epilepsy, the content of minor phytocannabinoid compounds in a high-CBD plant extract treatment affected seizure incidence and survival rates, suggesting that specific combinations of phytocannabinoids may be more effective than single purified compounds (Berman et al., 2018). Furthermore, a meta-analysis of observational clinical studies on epilepsy treatment reported that CBD-rich plant extracts reduced seizure frequency in patients at doses lower than those used in clinical trials of Epidiolex. The CBD-rich plant extract also produced significantly fewer adverse effects, likely due to the lower dose required (Devinsky et al., 2017; Pamplona et al., 2018; Thiele et al., 2018).

The benefits of phytocannabinoids for pain management have been well-established. However, the analgesic effects of *Cannabis* may be enhanced by combining different *Cannabis* bioactive compounds. The analgesic and anti-inflammatory effects of CBD occur only within a very limited dose range, but this bell-shaped dose-response was overcome when CBD was combined with a *Cannabis* extract (Gallily et al., 2015). Another study showed that a *Cannabis* extract high in THC provided no benefit for intractable cancer pain, while nabiximols, a whole extract CBD/THC combination significantly reduced pain in cancer patients (Johnson et al., 2010).

Whole-plant extracts may also be preferable to single phytocannabinoids for the treatment of mood disorders. Several phytocannabinoids, including THC, CBD and CBC, appear to have antidepressant and/or anti-anxiety effects, possibly due to modulation of the endocannabinoid system and/or modulation of serotonin receptors (Crippa et al., 2011; Poleszak et al., 2018; Zanelati et al., 2010). Other non-cannabinoid *Cannabis*

compounds, such as the terpenoid limonene, also exhibit antidepressant effects, and lemon oil, which contains high quantities of limonene displays anti-stress and anxiolytic properties (Komiya et al., 2006; Piccinelli et al., 2015; Russo, 2011). Plant extracts containing both cannabinoids and terpenoids may be the most effective *Cannabis*-based treatment option for psychopharmacological applications.

Synergistic effects are also potentially beneficial for cancer treatment. In breast cancer cell lines and animal models, a *Cannabis* extract produced enhanced anti-tumour effects compared to purified THC, possibly due to the presence of other cannabinoid compounds (Blasco-Benito et al., 2018). Another study reported that whole plant extracts reduced cancer cell survival and proliferation more effectively than pure THC, across a range of cancer cell types (Baram et al., 2019). Notably, one study found that cancer cells were killed most effectively when treated with phytocannabinoids and terpenoids at ratios similar to those found naturally in the plant (Namdar et al., 2019).

The above findings suggest that, for the treatment of various medical conditions, producing a range of *Cannabis* varieties , or chemotypes, with varying phytochemical contents may constitute a more effective approach than developing new synthetic cannabinoid-based medicines. A greater understanding of the synergistic activities of different phytocannabinoids, terpenoids and other *Cannabis*components is needed to identify the most effective combinations for various pharmaceutical applications.

8.3. *Cannabis* breeding for medicine

Cannabis sativa is a versatile multi-purpose crop which requires a simple, low-input cultivation technique, adapts to various ecological conditions, produces sustainable products, and provides raw material for a wide range of applications, including medicine (Amaducci et al., 2015). Research into the synergistic pharmacological effects of*Cannabis* metabolites suggests that ratios of phytocannabinoids, terpenoids and other *Cannabis* metabolites influence a plant's therapeutic potential.

More research is needed to determine the influence of environmental and genetic factors on the phytochemical profile of the *Cannabis*plant. Existing studies show that total phytocannabinoid yields are related to environmental conditions. Phytocannabinoid and terpene levels are affected by factors such as the humidity, rainfall and temperature of the growth environment (Meier and Mediavilla, 1998; Murari et al., 1983; Pavlovic et al., 2019). However, the relative ratios of the different *Cannabis* metabolites are dependent on the genotype (see Chapter 3) (Janatová et al., 2018; de Meijer et al., 2003; Toonen et al., 2006; Vanhove et al., 2011). Identifying the environmental and genetic factors that influence phytochemical production by *Cannabis* could aid in the development of new *Cannabis* cultivars with tailored ratios of various metabolites.

The therapeutic effects of a given plant reflects the proportions of the various pharmacologically active components. The development of *Cannabis* chemotypes containing high levels of specific phytocannabinoids can be achieved through breeding. De Meijer *et al* . produced *Cannabis* chemotypes high in specific single phytocannabinoids, including THC, CBD, CBG and CBC (de Meijer et al., 2009a, 2003; de Meijer and Hammond, 2005). Cannabinoid-free chemotypes were also developed, which could aid investigation into the contributions of non-cannabinoid bio-actives, such as terpenoids, to the pharmacological effects of *Cannabis* (de Meijer et al., 2009b; Russo, 2011). The development of these chemotypes through conventional breeding demonstrates the high diversity of the *Cannabis* genome, which may obviate the need for genetic engineering of *Cannabis*(Russo, 2019).

Understanding the interactions between different *Cannabis*bio-actives remains one of the key challenges to harnessing the full medicinal potential of the *Cannabis* plant. Research into *Cannabis*-based medicines highlights the importance of various *Cannabis* metabolites in producing therapeutic effects. Further research is needed to elucidate the mechanisms underlying the entourage effect observed with whole *Cannabis* extracts, and to assess the contributions of various *Cannabis* metabolites to determine the most effective combinations for various pharmaceutical applications. Applying our knowledge of the entourage effect in *Cannabis* to the development of tailored chemotypes has the potential to provide improved *Cannabis*-based therapies for various medical conditions, which could benefit many patients.

9. Hemp for houses: *Cannabis* as building material

Many hemp varieties of *Cannabis* are fibre crops with multiple inherent qualities as building material. The high tensile strength of hemp fibres, traditionally exploited in rope and fabric applications, also enables mechanical advantages for building construction applications. Additionally, shiv particles of the woody-core are a biobased alternative to mineral aggregates for low-impact concrete. Therefore both the plant fibres and the shiv particles are suitable for developing biobased, environmentally friendly building materials that have been shown to have inherent thermal, hygrothermal and acoustic characteristics (Kinnane et al., 2016; Reilly et al., 2019; Shea et al., 2012).

Building products that integrate hemp are many, but reductively may be grouped into two general categories, hemp concrete and hemp insulation blankets: Hemp concrete are those mixed with a binder to form a porous concrete composite with thermal insulation qualities. Hemp insulation blankets are thermo-formed without an added binder to create a low density, blanket type product. These respectively encompass shiv and fibres, and are distinguished by their dry densities; typically in the range 390-670 kg/m^3 in the case of the hemp-concrete (Collet-Foucault et al., 2004), and about 38 kg/m^3 for the hemp-insulation ('Technichanvre', 2017). The concretes have a wide density range as they are typically bespoke and include varying levels of binders, often lime, but also cement. As noted, both products are recognised for their good thermal properties. The hempcretes have a low thermal conductivity ($\lambda = 0.12$ W/(m*K)) (Walker and Pavía, 2014) relative to other standard concretes (λ = 1-2 W/(m*K)). However, their conductivity is higher than the former insulation wool blanket product (λ =0.04 W/ (m*K)) (Collet-Foucault et al., 2004), which contains up to 90% hemp fibre, are formed in panels or rolls and used for roof, attic and wall insulation.

Hempcrete, in contrast, is a composite material. It is commonly mixed in a ratio by weight of 1:2:3 of hemp: binder: water. In contrast to standard concrete, hempcrete has relatively low mechanical strength. It is therefore typically cast around a load-bearing timber structure. The wet mix is poured between temporary shuttering, and the hempcrete is tamped down to compact it and form the wall. The thickness of these walls typically ranges from 300-600 mm, to ensure structural stability and to meet thermal requirements. These dimensions limit the widespread applicability of hempcrete, particularly in urban infill sites. However, new innovative products are enabling its wider applicability. Increasingly, precast hempcrete blocks are appearing on the market. Exhibiting higher densities, certain of these have load-bearing capability and they generally enable time and labour efficiencies on site. They are also popular for renovation and retrofit projects, primarily because hempcrete is characterised by an open pore structure which allows for the transmission of moisture. Moisture is often present in the walls of old buildings, and breathable insulation allows this water escape, instead of trapping it as do modern-day synthetic insulations which can lead to mould growth, structural and air quality issues.

These are just some example of advantages of biobased materials. However, the construction industry and the agricultural industry diverged during the modern post-war age of development. Synthetic products were developed to meet high demand with a price point that enabled use, and waste, of products during phased redevelopment (Kinnane, 2020). Today synthetic polymer and mineral wool products continue to command almost full market share of the insulation industry, and that demand is increasing as we aim to reduce the operational energy of buildings. Hemp, and biobased materials more generally, remain niche products. Today hemp insulation products are almost twice the price of the mass produced alternatives (Carus et al., 2013), even though plant fibres have a lower cost of processing than synthetic fibres.

However, the building sector, and its considerable environmental impact, is increasingly in focus, and the environmental benefits of biobased materials are giving them greater traction. Hemp, with its fast-growth cycle and multi-purpose advantages is increasingly proposed as a low-impact design solution. Although specific quantification of carbon sequestration remains challenging (Reilly and Kinnane, 2017), authors report levels of between 1.5-2.1 kgCO_2per kg of plant grown and values of energy for production of 0.085-0.19 kgCO_2 per kg of hemp shiv (see Sáez-Pérez et al., 2020 for review). It should be noted however that although hemp exhibits carbon positive credentials, the embodied carbon of any hempcrete is high due to the high quantity

of binders often used, and this is often underestimated by proponents of the material.

10. Also a medicine for the environment? Sustainability aspects of *Cannabis* farming

Considering global warming and consequential efforts to divest from fossil fuels, bioenergy crops and biofuels are gaining increasing interest (Rogelj et al., 2018). *Cannabis* is a high-yielding, annual crop, and has much-untapped potential for contributing to carbon sequestration efforts (Finnan and Styles, 2013). Besides storing carbon in building materials, alternative uses for this crop exist, and as such, there is a high potential for carbon to be stored both short- and long-term in bioenergy, textiles, and paper (Figure 1, Figure 9). Further contributing to the environmental connotations of this species, hemp has been employed in phytoremediation efforts to restore land implicated by heavy metal contaminants (Citterio et al., 2003). Hemp leaves and seeds also provide the basis for human consumption (Figure 9).

Hemp is considerably more efficient (high annual yields with low agrochemical/fertilizer input) than the traditional annual bioenergy crops (sugar beet and oilseed rape) and possesses similar greenhouse gas mitigation potential to the perennial bioenergy crops Miscanthus and willow (Finnan and Styles, 2013). Annual bioenergy crops like hemp can be appealing options for farmers to diversify and explore the bioenergy market without the demands of perennials, namely high establishment costs and long-term commitment (15–20 years) of their land to bioenergy (Finnan and Burke, 2013; Finnan and Styles, 2013).

Hemp biomass has good combustion properties and could be used to generate either heat or electricity (Finnan and Styles, 2013). There are multiple biofuel options: Biogas, solid fuel briquettes, bales, and bioethanol (Kraszkiewicz et al., 2019; Prade et al., 2012, 2011).

As hemp is an annual crop it can be readily integrated into crop rotation cycles, thus not competing with food supplies and can therefore contribute towards sustainable cropping systems (Finnan and Styles, 2013). Moreover, hemp has been reported to improve yields of crops subsequently grown thus complementing food production. Winter wheat planted after hemp had 10–20% yield increases (Bócsa and Karus, 1998), with similar observations recorded for soybean and alfalfa (Adesina et al., 2020). A low input crop, hemp can produce high yields similar to switchgrass and sorghum but with lower nutrient and pesticide requirements (Das et al., 2017). Hemp offers the combined potential of an effective break crop and an efficient energy crop, thus generating income while promoting productivity. Break crops like hemp can be used to disrupt pest cycles and the ability of hemp to tolerate high planting densities suppresses weed growth, thus pesticide and herbicide requirements of subsequently, cultivated crops are reduced (Bhattarai and Midmore, 2014). The hemp root system promotes soil health, as the large taproots penetrate deep into the soil facilitating aeration, but simultaneously forms soil aggregates to prevent soil erosion (Amaducci et al., 2008). Model analysis comparing the relationship between leaf nitrogen status and photosynthesis rate in hemp, cotton and kenaf revealed hemp to have a high photosynthetic capacity, even at low nitrogen levels (Tang et al., 2017). This provides an additional line of evidence that hemp may fulfil a future niche as a sustainable bioenergy crop that can be cultivated over a wide range of climatic and agronomic conditions.

Figure 9: **Products made from hemp.** Non-THC *Cannabis* (hemp) can be processed into a multitude of different products. Hemp husk of fibre varieties (a) can be processed into hempcrete (b), while hemp fibre (c) can be made into rope (d) or insulation material (e). Remains of the plant can be pressed into hemp pellets for heating (f). Composite hemp-plastic material for 3D printing (g) is more sustainable than regular printing plastic. Hemp seeds can be dehulled into hemp hearts for human consumption (h). A variety of hemp products like tea (i), as well as chocolate (k) and candy (l) are also available. (i) courtesy of Christine Schilling.

11. Future prospects: Phytocannabinoids without *Cannabis: In vitro* synthesis using cell cultures

Phytocannabinoids have high potential for medical but also recreational use and therefore their production and extraction are of high commercial interest. However, plant breeding and cultivation come with their

own challenges and phytocannabinoid yield and profiles can highly depend on environmental factors. Cell cultures methods are a powerful tool for the production of high-quality plant material in a manner that is time efficient, seasonally independent, and which can satisfy good manufacturing practice guidelines (Tekoah et al., 2015). This technology has attracted a lot of attention as it can allow the harvesting of high value products produced within cells in suspension or secreted into their surrounding medium (Weathers et al., 2010). Improvement of culture growth kinetics and product yield can be achieved via medium optimisation (Holland *et al.* , 2010; Ullisch*et al.* , 2012; Vasilev *et al.* , 2013) and by selecting for high-producing cell populations using techniques such as fluorescent marker-based cell sorting (Kirchhoff *et al.* , 2012). Cell lines optimised in these ways can subsequently be cryopreserved to ensure consistent production going forward (Ogawa *et al.* , 2012).

Secondary metabolites, including pharmacologically valuable compounds such as paclitaxel and scopolamine or transgenic proteins to be used as vaccines, antibodies, immunomodulators and other therapeutics are already produced in cell suspension cultures on a commercial scale (Mountford, 2010; Paul et al., 2015). Hence, this might be a promising avenue to produce cannabinoids as well (Figure 10).

Of the various types of plant cell culture available, cell suspension cultures are the most commonly used due to their scalability and relatively rapid growth rates (Santos et al., 2016). The use of cell suspension cultures involves growing dedifferentiated plant cells in liquid medium supplemented with hormones to induce culture proliferation (Mustafa et al., 2011). However, the use of suspension cultures for these purposes faces several barriers to successful execution. Firstly, due to their genetic instability, cultures can often lose their ability to produce valuable compounds over time, while low productivity rates sometimes require large volumes of biomass to be grown, therefore increasing costs relative to field-grown plants (Moon et al., 2020; Weathers et al., 2010). Cell suspension cultures tend to form heterogeneous cell clusters rather than proliferating as single cells in culture, leading to increased difficulty of use and inconsistent growth kinetics and product yield. In addition, the scale-up of cell suspension cultures from laboratory to commercial production scale is often associated with a decline in cell productivity (James and Lee, 2006).

In relation to *Cannabis,* the use of *in vitro* bioprocessing techniques has the potential to allow the synthesis of high yields of cannabinoids in a manner that satisfies good manufacturing practice guidelines and guarantees a high-quality product. Metabolic engineering offers the possibility of developing plant or microbial cell lines which exclusively produce a desired cannabinoid, thus circumventing the high costs associated with purifying a desired product during downstream processing. However, achieving these aims poses a number of challenges to which researchers must still find answers.

A study described by Pacifico et al., (2008) assessed the cannabinoid content of callus cultures (which are metabolically identical to and often constitute the starting material for suspension cultures) derived from five different *Cannabis* varieties . The calli did not show any detectable levels of phytocannabinoids at any time during culture, irrespective of the presence or absence of hormones or the phytocannabinoid content of the original plants from which the cultures were derived. As such, cell suspension cultures are unlikely to be an effective biofactory for the production of cannabinoids without some form of intervention.

One method which may overcome the lack of phytocannabinoid production in *Cannabis* suspension cultures is the use of elicitors. These are compounds or a mixture thereof which can be added to the culture medium to stimulate the transient production of a desired secondary metabolite. Elicitors can be either biotic (animal, plant or microbial extracts) or abiotic (metal ions, organic compounds or electric current) and have been used previously with varying degrees of success (Weathers et al., 2010 and references therein). However, since many elicitors are either toxic or stress-inducing, their addition to a suspension culture often leads to a reduction in the vitality of the culture and can even be fatal. Such an approach was used by Flores-Sanchez et al. (2009) in order to try to stimulate phytocannabinoid production in suspension cultures of *Cannabis*. However, no detectable levels of phytocannabinoids were found in response to any of the treatments used, which included a range of biotic and abiotic stimuli. As such, the search for an elicitor which can induce phytocannabinoid production remains ongoing.

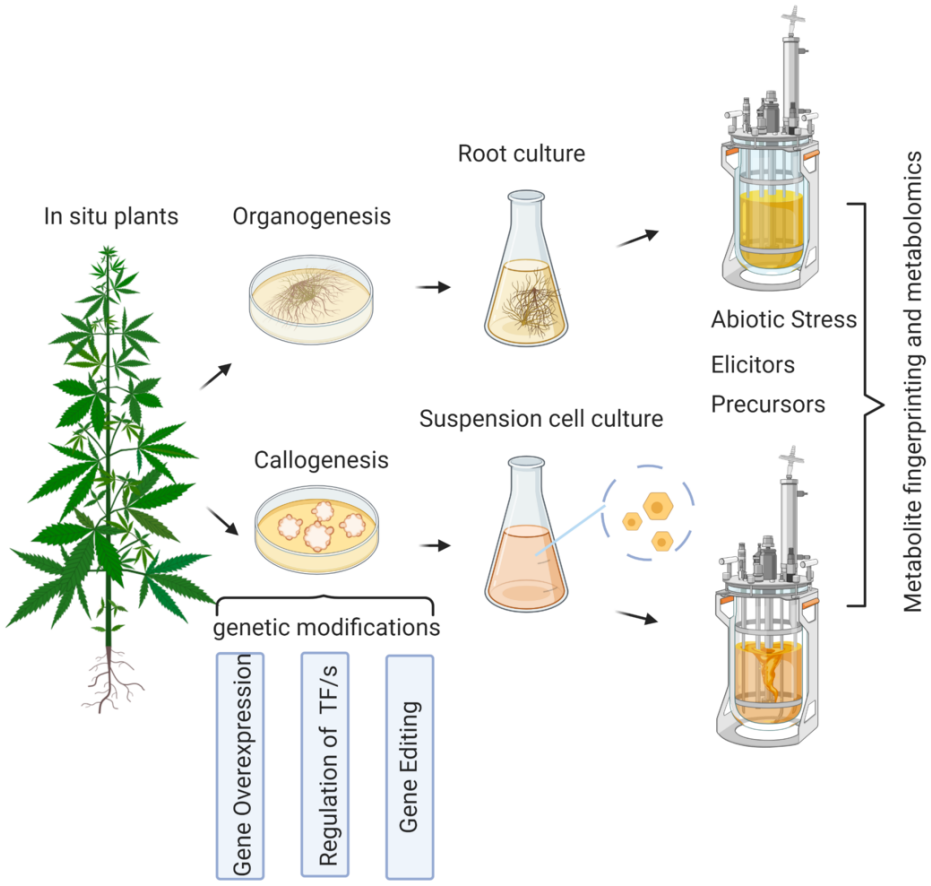

Figure 10: Developments in plant cell and tissue culture methodologies allow the efficient production of metabolites and recombinant proteins at commercial scales. Cell suspension cultures and hairy root cultures are the most commonly used techniques, with protocols described for both in Cannabis . However, efficient, large-scale production of phytocannabinoids in vitro has yet to be developed. Image was made using Biorender (www.biorender.com).

One other point worth noting is that phytocannabinoids are known to be toxic to plant cells when they accumulate at high enough concentrations, which is why *Cannabis* plants use trichomes to compartmentalise these compounds into storage cavities outside the plant. THCA and its precursor molecule CBGA are highly toxic to both *Cannabis* and tobacco cell suspension cultures, inducing 100% apoptotic-like programmed cell death after 24 hours in culture at a concentration of 50 µM (Sirikantaramas et al., 2005). This negative feedback phenomenon is observed in many plant species which produce secondary metabolites and is the reason why many metabolites are sequestered in specialised structures.

However, the development of cell culture methods to avoid cell toxicity is one area where researchers have relative success. Strategies such as two-phase cultures have been shown to enhance the production of secondary metabolites in a range of species (Malik et al., 2013). In these systems, an aqueous phase is used to support cell growth while a non-aqueous phase, typically consisting of a solvent or resin, is employed to act as a sink for the accumulation of the desired product and in some cases facilitates its subsequent

extraction. Two-phase systems have been shown to greatly increase metabolite yield in both plant cell suspension and hairy root culture cultures (Chiang and Abdullah, 2007; Malik et al., 2013; Rudrappa et al., 2004; Sykłowska-Baranek et al., 2019; Wu and Lin, 2003), although to the best of our knowledge such an approach has not yet been attempted in *Cannabis*.

Hairy root cultures are generated by the infection of plant tissues with *Agrobacterium rhizogenes*, a species which can modify the plant's genome by introducing a segment of DNA known as T-DNA which codes for a number of genes affecting the production and regulation of plant hormones (Ono and Tian, 2011). This results in the development of extensive root networks which can be easily cultured *in vitro*, are genetically identical to the mother organ from which they were derived and can also produce the same phytochemicals. Like cell suspension cultures, hairy root cultures have already attracted attention as a means of producing secondary metabolites such as flavonoids (Gai et al., 2015), isoflavonoids (Jiao et al., 2014), artemisinin (Patra and Srivastava, 2014) and lignans (Wawrosch et al., 2014), albeit less commonly than cell suspension cultures due to their increased difficulty of use.

THCA has previously been produced from tobacco hairy root cultures which were transformed to express the enzyme responsible for its production, THCA synthase, under the transcriptional control of the *cauliflower mosaic virus* 35S promoter (Sirikantaramas et al., 2004). When these hairy roots were cultured in liquid medium supplemented with CBGA, the precursor molecule to THCA, 8.2% of this CBGA was converted to THCA after two days of culture, approximately half of which was then secreted into the surrounding medium, thus demonstrating that CBGA uptake and THCA release from these transgenic roots was possible *in vitro,* albeit at low levels.

In *Cannabis*, a protocol for the production of hairy root cultures has already been described which shows that cultures are best established from the hypocotyl of intact seedlings by piercing the epidermis with a syringe and inoculating with *A. rhizogenes*(Wahby et al., 2013). Five varieties of *Cannabis* (three hemp-type and two marijuana drug-type) were used and all were shown to be responsive to infection by *A. rhizogenes*, although with varying morphological responses. Similarly, all eight *A. rhizogenes* strains used could induce a hairy root morphology, albeit with varying degrees of frequency (43-98%, depending on the strain).

An alternative approach described by Farag and Kayser (2015) outlines how hairy root cultures can be developed from *Cannabis* callus cultures without the use of *A. rhizogenes* by growing them in B5 medium supplemented with 4 mg/ml of the auxin NAA. Under these conditions, cannabinoid contents peak at 1.04 µg/g dry weight for THCA, 1.63 µg/g dry weight for CBGA and 1.67 µg/g dry weight for CBDA after 28 days of culture. These low yields highlight the fact that while phytocannabinoids can be produced from hairy root cultures, significant improvements in yield will need to be achieved before this methodology is commercially viable for phytocannabinoid production.

Recent studies have attempted to demonstrate the production of cannabinoids in non-native hosts, with a particular focus on yeast due to the relative ease with which metabolic engineering can be achieved in this model organism. A landmark paper by Luo et al. (2019) described the complete biosynthesis of CBGA, THCA and CBDA and several unnatural analogues in yeast via engineering of the native mevalonate pathway and the introduction of a heterologous hexanoyl-CoA biosynthetic pathway as well as the *Cannabis* genes responsible for the biosynthesis of complete cannabinoids. However, the cannabinoid yields achieved from this system were found to be approximately 100-fold less than those produced by *Cannabis* plants. As such, the efficient production of cannabinoids in either plant or microbial cell culture remains a work in progress.

12. Conclusions: Stay tuned, there is more to come!

The recent progress in *Cannabis* research has been remarkable, and has revealed exciting challenges ahead: The evolution and genetic diversity of phytocannabinoid synthases has proven to be a complex field of research, as is the genetic and environmental control of sex determination and flowering time. The increasing availability of genomic resources will undoubtedly facilitate progress in all those areas, but we predict that

experimental analyses, including detailed morphological, molecular genetics and phenotyping studies will be equally important to understand the developmental and physiological intricacies of *Cannabis*.

Unusual in that it is a multipurpose crop, the full sustainability potential of *Cannabis* can only be fulfilled if it is used as such. Thus, one major challenge will be to design crop ideotypes that harmonise traits of medicinal relevance with those important for carbon sequestration. This will not be an easy task, as the genetic control of different traits is currently unclear. However, the production of e.g. large fibre varieties that do nevertheless develop a dense inflorescence with high CBD content seems not too farfetched. Even if those hypothetical cultivars may not be able to provide the high yields of specialized CBD cultivars they may provide farmers focusing on fibre production with a second source of income.

In summary, the genetic and morphological diversity of *Cannabis* is a treasure trove that we are only beginning to explore. It is important that we capitalise on this treasure to construct a multipurpose swiss knife, and not a series of highly specialised tools.

Acknowledgements

SS is grateful for support by a Postdoctoral Scholarship of the Irish Research Council and Greenlights Medicines under the Enterprise Partnership Scheme (Grant No.: EPSPD/2019/220). CAD is supported by an Irish Research Council-Environmental Protection Agency Government of Ireland Postgraduate Scholarship (Grant No: GOIPG/2019/1987). JS is supported by a Chinese Research Council Postgraduate Scholarship (Grant No: CSC no. 201908300031). EOR and ASP are supported by the Irish Research Council and Greenlight Medicines via an enterprise partnership scheme (Grant no.: EPSPG/2017/376).

RM and SS are grateful to Beate and Peter Melzer, Christine Schilling, and Andreas Schmidt for childcare and general support during the pandemic, and to Jonathan and Theodor Schilling for their patience during those unusual times. This substantially helped with writing this manuscript.

All authors are members of HempHub, a strategic initiative of the UCD Earth Institute, and are grateful for general support through this initiative.

Table 1. Summary of the currently available *Cannabis* genome assemblies.

Cultivar	Cultivar Type	Source	NCBI BioProject	GenBank Assembly ID	WGS Accession	Sequencing Technology	Coverage	Genome Size (Mb)	Assembly Level	No. Scaffolds	Scaffold N50	No. CDS	Publication
CBDRx	Hemp	NCBI	PRJEB29284	GCA_900626175.2	UZAU01	Oxford Nanopore	100x	876.148	Chromosome	221	91,912,889	33,674	Grassa et al., 2018
Finola (FN)	Hemp	NCBI	PRJNA73819	GCA_003417725.2	QKVJ02	PacBio	98x	1009.67	Chromosome	5,303	370,471	n/a	Laverty et al., 2019
Purple Kush (PK)	Drug type	NCBI	PRJNA73819	GCA_000230575.5	AGQN03	PacBio	79x	891.965	Chromosome	12,836	133,904	n/a	Laverty et al., 2019
wild *Cannabis* (CR) [1]	Wild	NCBI	PRJNA562042	GCA_013030365.1	WRXK01	PacBio	153x	812.525	Chromosome	483	82,998,198	n/a	Gao et al., 2020
Jamaican Lion (JL) Mother	Drug type	NCBI, Medicinal Genomics Website [2]	PRJNA575581	GCA_012923435.1	JAATIP01	PacBio	125x	876.736	Contig	1,599	3,283,100	27,358	McKernan et al., 2020
Jamaican Lion (JL) Father	Drug type	NCBI, Medicinal Genomics Website [2]	PRJNA575581	GCA_013030025.1	JAATIQ01	PacBio	125x	1009.16	Contig	1,264	1,668,042	31,591	McKernan et al., 2020
Jamaican Lion (JL) Daughter	Drug type	Medicinal Genomics Website [2]	n/a	n/a	n/a	PacBio	n/a	999.122	Contig	658	3,491,975	n/a	McKernan et al., 2020
Jamaican Lion (JL) DASH	Drug type	NCBI	PRJNA486541	GCA_003660325.2	QVPT02	PacBio	125x	1333.38	Contig	556	3,811,003	n/a	McKernan et al., 2018
Chemdog91	Drug type	NCBI	PRJNA297710	GCA_001509995.1	LKUB01	Illumina	300x	285.933	Scaffold	175,088	2,250	n/a	McKernan et al., 2015
LA Confidential	Drug type	NCBI	PRJNA297710	GCA_001510005.1	LKUA01	Roche 454	50x	595.358	Contig	311,039	2,649	n/a	McKernan et al., 2015
Cannatonic	Drug type	NCBI	PRJNA350523	GCA_001865755.1	MNPR01	PacBio	10x	585.824	Contig	11,110	128,718	n/a	Unpublished
Pineapple Banana Bubba Kush	Drug type	NCBI	PRJNA378470	GCA_002090435.1	MXBD01	PacBio	72x	512.174	Contig	18,355	51,819	n/a	Unpublished

[1] Note that this wild *Cannabis* cultivar is named 'JL' elsewhere, and that it is renamed here to CR to avoid confusion with the Jamaican Lion cultivar.

[2] Assemblies are accessible on the Medicinal Genomics Website available at https://www.medicinalgenomics.com/jamaican-lion-data-release/.

Figure 11: Table 1 - Excel and PDF attached

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Tables

Table 1. Summary of the currently available *Cannabis* genome assemblies.

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Supplementary Material

Supplementary Table 1. Chromosome lengths for each *Cannabis* genome assembly.

Supplementary Table 2. Available *Cannabis* sequencing data from the NCBI sequence read archive.

Supplementary Table 3. Available *Cannabis* organellar genome assemblies.

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