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Using advanced biotechnological techniques to improve cannabis cultivars

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ABSTRACT

This comprehensive review paper explores methods of improvement of *Cannabis sativa* L. propagation and applications. Inherent breeding limitations, genetic instability, and psychoactive compounds have impeded utilization, however, application of biotechnology tools such as molecular breeding, tissue culture, and genetic engineering can advance cannabis research and applications. With recent advancements, cannabis micropropagation can substantially increase multiplication rates while preserving genetic lines. Utilizing gene overexpression, virus-induced gene silencing (VIGS), and RNA interference (RNAi) within cannabis, biochemical pathways, including cannabinoid and terpenoid biosynthesis, and key transcription factors in trichome development and cannabinoid production have been elucidated. Integration of gene editing techniques including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems are promising tools in cannabis for editing biosynthetic pathways to increase enzyme efficiency and the development of novel cannabis traits. In addition, we address gaps, limitations, and rapidly expanding fields in biotechnological methods utilized on cannabis. With concerted efforts, biotechnological tools can aid in understanding the plant and be utilized to increase and improve the desirable properties of cannabis.

1. Introduction

Cannabis sativa L. has multiple uses historically in medicine and industry. Importantly, applications of the plant are influenced by the interplay between the genetic makeup of cannabis and the environmental conditions to which the plant is exposed by influencing the plant's physiology, including the abundance of phytocompounds. By altering both the genetic and environmental factors, new or improved applications of cannabis are possible. This review delves into the inherent challenges limiting cannabis applications, in particular, breeding limitations, such as genetic instability and psychoactive compounds, and currently understudied agricultural propagation methods, as well as the arsenal of biotechnological tools, including genetic engineering and molecular breeding.

Cannabis, scientifically known as *Cannabis sativa* L., belongs to the *cannabaceae* family and is a remarkably diverse and polymorphic plant species originally native to Eurasia (Clarke and Merlin, 2016; Groom et al., 2014). Its global distribution encompasses various habitats, altitudes, soil, and climate conditions (Clarke and Merlin, 2016). Cannabis has a long history of use, valued for its psychoactive and therapeutic properties, as well as its industrial applications (Fig. 1). Uses of cannabis include textile manufacturing, paper production, construction materials, cosmetics, and the food industry (Salami et al., 2020; Schultz et al., 2020). In medicine,

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Fig. 1. Various uses of *Cannabis sativa*.

cannabis has found application in alleviating chronic pain associated with cancer, mitigating the side effects of chemotherapy, addressing issues related to anorexia and AIDS, managing inflammatory conditions, and providing relief from conditions like epilepsy, spasticity in Tourette's syndrome, skin diseases, and multiple sclerosis, among others (Devsi et al., 2020; Gerasymchuk et al., 2022).

The use and exploration of cannabis have often been a topic of debate. Recent legislative changes in many countries have legalized cannabis for medical and recreational purposes (Cox, 2018; Pacula and Smart, 2017), and with the plant's notable therapeutic potential, this has created a pressing need for research efforts in the field of cannabis. Despite being in its early stages, there is a significant and rapidly growing interest in cannabis research evident in the rising number of publications and citations on the subject. Over 100 cannabinoids have been identified, including Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabichromene (CBC), cannabigerol (CBG), cannabinol (CBN), and others. These cannabinoids, along with volatile compounds like terpenes, which contribute to the distinctive aroma of cannabis, are synthesized primarily within the trichomes found on the plant's flowers and leaves (Linder et al., 2022; Pattnaik et al., 2022).

There exists a notable debate within the field of botanical taxonomy regarding the precise number of species that constitute the cannabis genus, however, genomics can settle this debate (McPartland, 2018). Traditionally, informal taxonomic systems have classified cannabis into three distinct groups: *C. sativa* subsp. Sativa (recognized for its high CBD content), *C. sativa* subsp. Indica (known for its high THC content), and *C. sativa* subsp. Ruderalis (a wild-type cultivar with roughly equal levels of THC and CBD) (McPartland, 2018). However, a more refined approach based on chemical characteristics, known as chemotaxonomy, further divides *C. sativa* into three chemotypes primarily differentiated by their THC:CBD ratios (Table 1) (Small and Beckstead, 1973). The THC:CBD ratio serves as a crucial indicator for distinguishing plants with high THC content from those with low THC content. The pioneering work of Fetterman et al., in 1971 was instrumental in developing this concept, enabling the differentiation of fiber- and drug-type plants based on their THC:CBD ratios (Fetterman et al., 1971). Chemotype 1, commonly called the drug type, exhibits a THC:CBD ratio greater

Table 1
Chemotaxonomy classification of cannabis cultivars.

Chemotype	THCA:CBDA	THCA Content	CBDA Content	CBGA Content
I (Drug Type)	>1	>0.3%	N/A	N/A
II (Intermediate Type)	1	N/A	N/A	N/A
III (Fiber Type)	<1	<0.3%	High	N/A
IV	N/A	N/A	N/A	High
V	N/A	Low	Low	Low

Note: CBDA, cannabidiolic acid; CBGA, cannabigerolic acid; N/A, not applicable; THCA; tetrahydrocannabinolic acid.

than 1 and THC content exceeding 0.3% of the total dry weight. Chemotype 2 represents an intermediate type characterized by a THC:CBD ratio close to 1. In contrast, chemotype 3, known as the fiber type, typically possesses low THC content and high CBD content, with a THC:CBD ratio considerably less than 1, often at or below 0.1. Additionally, two distinct chemotypes have been identified: Chemotype 4, characterized by high CBG content; and Chemotype 5, where all cannabinoid levels are low. As a result, a THC:CBD ratio of 0.1 or less renders chemotype 3 non-psychoactive. It's noteworthy that the commercial market offers approximately 600 different cannabis varieties (Rahn et al., 2016), and for many of these varieties, the genetics remain only partially understood (Table 1).

Jin et al. (2020) have established the remarkable diversity of bioactive compounds in cannabis, encompassing 14 cannabinoids, 47 terpenoids (comprising 29 monoterpenoids, 15 sesquiterpenoids, and 3 triterpenoids), 3 sterols, and 7 flavonoids (Jin et al., 2020). These findings were based on an analysis of different plant parts, including inflorescences, leaves, stem barks, and roots, across three distinct chemovars, namely, Grand Doggy Purps, GrandDaddy Purple, and CBD Mango Haze. In the inflorescences of cannabis, the predominant constituents are cannabinoids, accounting for 15.77%–20.37% of the dry weight, followed by terpenoids at 1.28%–2.14%, and flavonoids at 0.07%–0.14%. On the other hand, cannabis leaves are composed of cannabinoids ranging from 1.10% to 2.10%, terpenoids ranging from 0.13% to 0.28%, and flavonoids ranging from 0.34% to 0.44%. Stem barks, in contrast, are distinguished by their sterol content with 0.07%–0.08% and triterpenoid content of about 0.05%–0.15%, while roots contain sterols with 0.06%–0.09% and triterpenoids of 0.13%–0.24% (Fig. 2) (Jin et al., 2020). This comprehensive elucidation of the bioactive compound composition in various parts of the cannabis plant plays a pivotal role in advancing our understanding of the holistic effects of cannabis, including the entourage effect and differences in tissue type.

2. Genetics of cannabinoid synthases in cannabis

Cannabis, which is diploid with nine autosomal pairs and one pair of sex chromosomes (X and Y), (Ming et al., 2011) was recently sequenced with a draft genome showing an estimated size of ~808 Mb–900 Mb (Laverty et al., 2019; Singh et al., 2021; van Bakel et al., 2011). Within the genome, particular focus has been placed on the cannabinoid synthase paralogs that are responsible for the psychoactive and medicinal phytocannabinoids. The cannabinoid synthase paralogs are arranged in tandem arrays and are embedded in long terminal repeat retrotransposons on chromosome 7 (Grassa et al., 2018). The origin of the cannabinoid synthase have been speculated to likely represent isoforms originating from a single genetic locus B (de Meijer et al., 2003), and is supported by patterns of segregation in genome-wide association studies (Welling et al., 2020).

In 2003, de Meijer and colleagues proposed a genetic framework to elucidate the production of THC and CBD within *C. sativa* populations (de Meijer et al., 2003). They conceptualized cannabinoid yield per crop area as a multifaceted trait influenced by factors such as total above-ground biomass, the proportion of biomass consisting of inflorescence, overall cannabinoid content, and the purity of cannabinoids. Given that the production of THC and CBD is closely tied to the presence of enzymes responsible for deacidification, it appeared that the enzymes themselves adhered to straightforward Mendelian additivity at a synthase locus (de Meijer et al., 2003). Individuals harboring two BT alleles tended to produce THCA synthase (THCAS). Conversely, those with two BD alleles tended to produce CBDA synthase (CBDAS). Meanwhile, individuals carrying one BT and one BD allele generated both THC and CBD. Substantiating this hypothesis, the sequencing of THCAS and CBDAS genes revealed an 89% genetic similarity between them (Taura et al., 1995, 1996). Moreover, de Meijer and colleagues figured out that a homozygous genotype, marked by two loss-of-function alleles (B0) at the same synthase locus, regulated the accumulation of the precursor CBG in adult plants (de Meijer et al., 2009). Intriguingly, they observed that the expression of CBC was susceptible to environmental factors like light, and its inheritance did not conform to a simple additive or dominant genetic model (de Meijer et al., 2009). Consequently, de Meijer and colleagues detailed the inheritance patterns not only of THC:CBD ratios but also of CBC production (de Meijer et al., 2003, 2009). They introduced a mathematical model to portray cannabinoid abundance as a potentially intricate quantitative trait. While de Meijer and colleagues probed into the genetic basis of cannabinoids, their examination primarily focused on a straightforward additive model. More complex genetic effects remained largely unexplored until research was conducted by Weiblen and colleagues (Weiblen et al., 2015). This subsequent work identified that the enzymes participating in cannabinoid biosynthesis, which seemingly originated through gene duplication events, represent compelling candidates for targeted breeding initiatives and genetic engineering endeavors. Furthermore, the THC:CBD ratio in most genotypes and populations is fundamentally governed by the cannabinoid synthase alleles present in the plant, while environmental factors and cultivation conditions can influence total cannabinoid levels (Chandra et al., 2017; Toth et al., 2020).

In 2020, Campbell ***et al.*** showed the production of phytocannabinoids are influenced by two additive genetic loci (Campbell et al., 2020). Their analysis utilized data from cultivars with pure THC or CBD chemotypes, as well as hybrid progeny, allowing them to estimate composite genetic effects on cannabinoid concentration variations (Campbell et al., 2020). In contrast to previous research, this

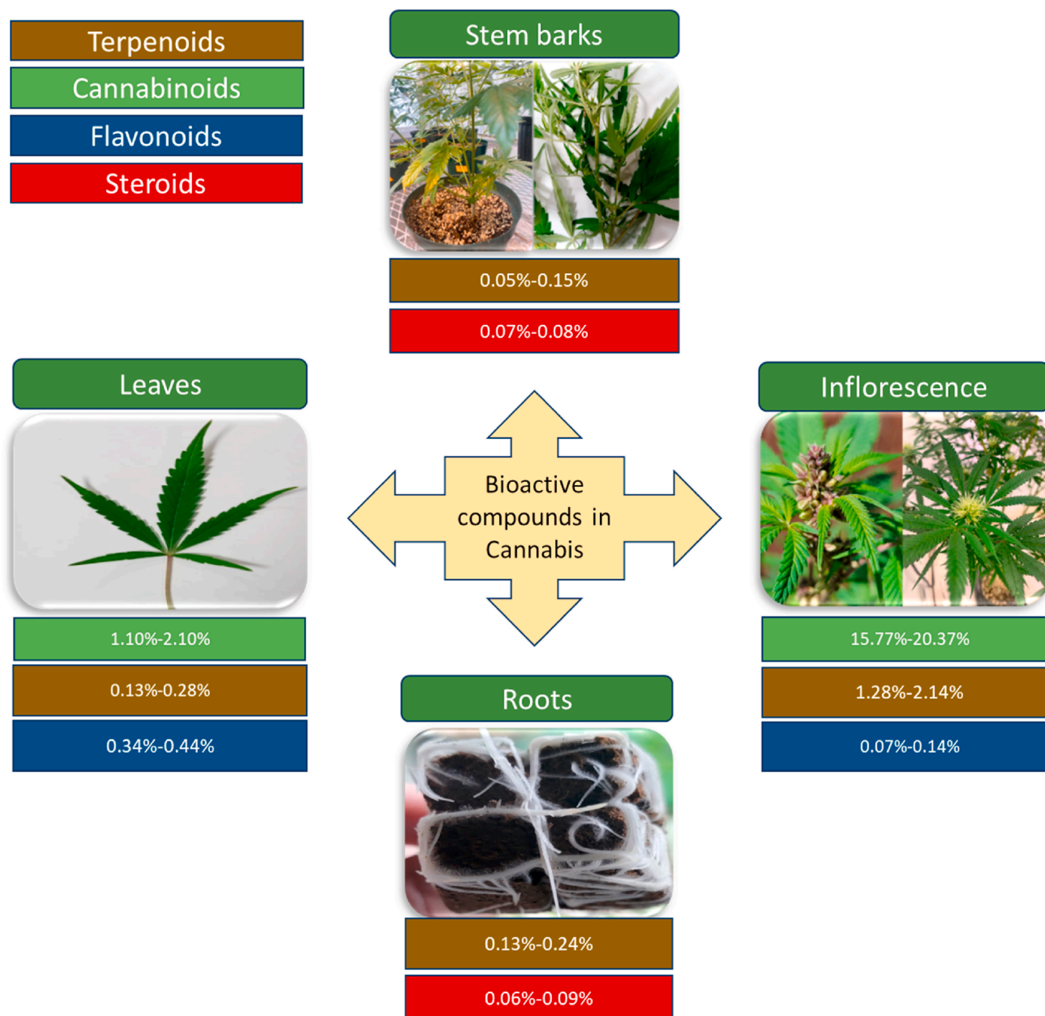

Fig. 2. Schematic diagram demonstrating the content of cannabinoids, flavonoids, terpenoids, and steroids in cannabis inflorescence, leaves, stems barks, and roots (Jin et al., 2020).

study revealed distinct findings concerning the nonadditive aspects of cannabinoid inheritance. THC concentration appeared to be a polygenic trait, with both additive and dominant genetic effects contributing to its expression patterns. On the other hand, CBD concentration may be influenced by cytoplasmic genomes and additive genes. Notably, maternal effects and genetic additive effects seemed to influence CBC expression (Campbell et al., 2020). These findings suggest that the inheritance of cannabinoids is more intricate than previously assumed with cytogenetic and maternal factors potentially influencing cannabinoid ratios and concentrations. Furthermore, emerging evidence confirms and strongly supports a highly complex genetic model (de Meijer et al., 2003, 2009; Staginnus et al., 2014; Weiblen et al., 2015). Variations among genetically uniform F1 offspring from parents with specific alleles displayed considerable variation in cannabinoid levels, highlighting the impact of environmental factors. Furthermore, THC and CBD expression appear to depend on linked loci and gene duplication on chromosome 6 for synthesis. Intriguingly, efforts to reduce THC expression in hemp cultivars have not resulted in its complete elimination. In summary, these findings underscore the complexity of cannabinoid inheritance, surpassing the explanatory power of simple genetic models.

Many studies have suggested that THCAS may have evolved from CBDAS through gene duplication (Onofri et al., 2015; Shoyama et al., 2012; Taura et al., 2007). Both enzymes exhibit similarities in their reaction mechanisms, relying on molecular oxygen to oxidize CBGA and generating hydrogen peroxide as a byproduct. Notably, the domain present in these enzymes bears a striking resemblance to the *C*-terminal berberine-bridge-enzyme (BBE) domain, a critical enzyme in the alkaloid biosynthesis pathway of *Eschscholzia californica* (Onofri et al., 2015). The enzymatic process for both THCAS and CBDAS begins with the transfer of a hydride ion from the CBGA substrate to the isoalloxazine ring of the FAD coenzyme, initiating the reaction. Another piece of evidence supporting their common ancestry is that both enzymes display some degree of promiscuity; each enzyme has the capacity to convert CBGA into CBDA and THCA in varying ratios, with the most common ratios being ~10:1 and ~20:1. Consequently, even in the absence of a *THCAS* gene, a plant can still contain THCA, suggesting CBDAS produces THCA in small amounts. Moreover, it is plausible that only a limited number of amino acid residues determine the product specificity of both enzymes due to their functional similarities in as-

sumed catalytic mechanisms (Taura et al., 2007). Beyond their sequence similarities, THCAS and CBDAS also share comparable biochemical and structural properties. Notably, enzyme promiscuity has led to regulatory considerations, such as those outlined in the Cannabis Act by Health Canada and within the United States for classification of hemp cultivars (Cox, 2018).

Laverty et al. (2019) has presented a comprehensive physical and genetic map obtained from a cross between the drug-type strain Purple Kush and the hemp variety Finola (Laverty et al., 2019). Their research findings indicate that THCAS and CBDAS, which play a crucial role in determining the drug vs. hemp chemotype, are located within large genomic regions (>250 kb) rich in retrotransposons. Importantly, these regions are embedded within approximately 40 Mb of repetitive DNA that undergoes minimal recombination and exhibit significant variation between drug-type and hemp-type alleles (Laverty et al., 2019). Additionally, cytogenetic analysis has hinted at a notable degree of karyotype polymorphisms, encompassing both inter- and intracultivar variations, particularly among hemp varieties (Razumova et al., 2016). These polymorphisms refer to differences in the structure and arrangement of homologous chromosomes, which can be observed through microscopy. This highlights the genetic diversity and complexity present within cannabis, particularly among different hemp cultivars. Phylogenetic analysis of THCAS and CBDAS sequences derived from various cannabis cultivars reveals a lower level of divergence in THCAS compared to CBDAS, lending support to the hypothesis that THCAS may have evolved from CBDAS (Singh et al., 2021).

The publicly accessible database located at http://genome.ccbr.utoronto.ca/has significantly enriches our knowledge of gene sequences for THCA- and CBDA-synthases (*CCBR Genome Browser Home*, n.d.). In addition, it has become clear that the cannabis genome encompasses numerous pseudogenes related to THCA- and CBDA-synthase. These pseudogenes exhibit varying degrees of divergence from the functional genes responsible for determining different chemotypes (Onofri et al., 2015). However, one important study showed that chemotype 1 & 3 cultivar's chemical compositions were not completely characterized by their transcriptome (Yeo et al., 2022). This would suggest that the genome and epigenome do not completely characterize the cannabinoid metabolome, but instead environmental factors play a role.

3. Biosynthesis of terpenes and cannabinoid precursors

The cannabis plant produces a unique category of compounds known as cannabinoids, characterized by their terpenophenolic nature. According to Chandra et al. (2017), a total of 565 constituents have been identified from *Cannabis sativa*, with 120 of them falling under the classification of phytocannabinoids (Chandra et al., 2017). Additionally, these plants undergo biosynthesis, generating a diverse range of lipophilic volatile metabolites through processes like reduction, methylation, and acylation, which involves the removal of hydrophilic components (Pichersky et al., 2006). These plant volatiles (PVs) serve various functions, including regulating interactions with both biotic and abiotic factors. They can attract pollinators, provide protection against pests and pathogens, and fulfill other ecological roles (Dudareva et al., 2013). Terpenoids, among PVs, stand out as the most significant and abundant chemical group. They are further categorized into isoprenes (C5), monoterpenes (C10), and sesquiterpenes (C15). Terpenoids are synthesized through the utilization of dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), which are derived from distinct biosynthetic pathways in different cellular compartments. These pathways share geranyl diphosphate (GPP) as a common precursor with cannabinoids (Nagegowda, 2010; Russo, 2011). Cannabis terpenoids, with their essential role in determining the flavor and fragrance of the plant, have garnered recent attention from researchers (Russo and Marcu, 2017). Comparative studies between terpenoid-rich essential oils and CBD have confirmed the superior bioactivity and medicinal properties of the latter (Gallily et al., 2018). Terpenoids exhibit transient immunosuppressive effects and lower bioactivity levels, such as reactive oxygen species ROS scavenging properties, in comparison to CBD. Moreover, individual terpenoids can potentially be utilized as bioactive molecules themselves, including friedelin, canniprene, cannabisin, and cannflavin A (Russo and Marcu, 2017). For instance, cannabisin B, isolated from hempseed hull, has demonstrated the ability to induce autophagy in human hepatoblastoma HepG2 cells (T. Chen et al., 2013). Terpenoid profiles, like those of cannabinoids, can be used to classify different cannabis chemovars (Fischedick, 2017). Their biosynthesis is regulated by terpene synthases, organized in large gene families, with their activity being spatially and temporally distributed. This makes them ideal targets for genetic engineering (Tholl, 2006). Terpenoids are highly potent metabolites, and even when inhaled at very low doses, they can affect the behavior of animals and humans. Their potential synergy with cannabinoids, known as the "entourage effect", has also been proposed (Russo, 2011). Studies have highlighted the fundamental role that cannabis mono- and sesquiterpenoids play in the potency of flower extracts (Russo and Marcu, 2017). However, a detailed discussion of terpenoids goes beyond the scope of this study.

Female cannabis flowers are known for their biochemical synthesis of cannabinoids within glandular trichomes. In contrast, male flowers typically contain fewer cannabinoids due to a lower density of trichomes (Livingston et al., 2020). Trichomes are primarily found on the bracts and leaves of both male and female plants, as well as on the undersides of the anther lobes in male flowers (Mahlberg et al., 1984). Due to these differences, female cannabis plants are more valuable to cultivators due to more glandular trichomes and as a result more cannabinoids found within the plant. Simultaneously, pollination of female cannabis plants can induce seed development in females resulting in a reduction of energy towards developing female flowers and glandular trichomes. As such, cultivators commonly separate or destroy male cannabis plants, or utilize feminized seeds (Owen et al., 2023).

Despite ongoing research, there is still a lack of clarity regarding the molecular mechanisms underlying cannabinoid biosynthesis (Fellermeier and Zenk, 1998). In summary, cannabinoids share a common initial pathway that involves tetraketide synthase (TKS) (Kearsey et al., 2020). TKS, a type III polyketide synthase (PKS), catalyzes the sequential condensation of hexanoyl-CoA with three molecules of malonyl-CoA, resulting in the formation of 3,5,7-trioxododecaneoyl-CoA (Fig. 3) (Taura et al., 2007). This compound, 3,5,7-trioxododecaneoyl-CoA, then undergoes cyclization and aromatization facilitated by olivetolic acid cyclase (OAC). This transformation leads to the formation of olivetolic acid (OLA) as coenzyme A is detached (Gagne et al., 2012). Subsequently, an aromatic

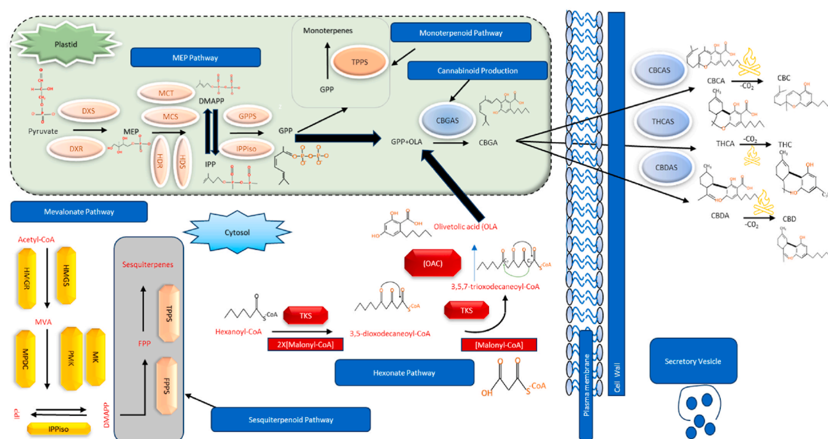

Fig. 3. Biosynthesis of cannabinoids and by-products. Proposed cannabinoid biosynthetic pathway for Δ9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), and cannabichromene (CBC) including by-product formation (pentyl diacetic lactone [PDAL], hexanoyl triacetic acid lactone [HTAL], and olivetol shown in the dotted box) and highlighting the chemical conversion of CBD into THC, long thought to be the source of THC, but this conversion does not occur *in vivo*. Synthesis of geranyl pyrophosphate (GPP) from dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) catalyzed by GPP synthase.

prenyltransferase (PT) inserts a prenyl group into the highly nucleophilic 2-resorcinol position, resulting in the production of cannabigerolic acid (CBGA) (Fellermeier and Zenk, 1998). CBGA serves as a central intermediate that generates cannabinolic acids via cannabinolic acid synthases. These acids are later transformed into their respective neutral forms, THC, CBD, and CBC, through non-enzymatic decarboxylation (Fig. 3) (Fellermeier and Zenk, 1998).

OLA plays a crucial role as the fundamental polyketide nucleus in the synthesis of cannabinoids (Gagne et al., 2012; Z. Tan et al., 2018; Taura, 2009). Initially, it was believed that OLA biosynthesis was solely governed by TKS, where spontaneous cyclization and aromatization occurred after the addition of the third malonyl group (Z. Tan et al., 2018). However, a breakthrough in understanding this process came when Taura and colleagues utilized a cDNA encoding olivetol synthase (OLS) from *C. sativa* (Taura, 2009). Surprisingly, their recombinant OLS did not produce OLA, the expected compound, but instead exclusively generated its decarboxylated form known as olivetol (Fig. 3). This observation was further substantiated by experiments involving crude enzyme extracts obtained from flowers and early-growth leaves, the primary cannabinoid-producing tissues of *C. sativa*, which also exclusively produced olivetol. These findings strongly suggested that OLA biosynthesis does not solely depend on OLS and may involve the participation of other enzymes. However, it's worth noting that olivetol is not detected in *C. sativa*, leading to the hypothesis that olivetol might be an artifact of *in vitro* enzyme assays (Taura, 2009). The puzzling situation of OLA not being producible in a lab setting, while its lab-created by-product olivetol doesn't occur naturally has been clarified by the finding that OAC plays a crucial role. OAC facilitates the intramolecular C2 → C7 aldol condensation of the substrate without decarboxylation (Fig. 3). Kearsey et al. (2020) further confirmed that in the absence of OAC, a nonenzymatic C2 → C7 decarboxylative aldol condensation of the tetraketide intermediate occurs resulting in the formation of olivetol instead of OLA (Kearsey et al., 2020). It's essential to emphasize that OLS and OAC do not directly interact with each other; instead, the metabolite must diffuse from one enzyme to the other through the cytosol. Subsequently, OLA undergoes a conversion into CBGA with the addition of GPP, a process catalyzed by the enzyme APT (Lercker et al., 1992). GPP itself is formed through the condensation of IPP and DMAPP by GPP synthase (Bohlmann and Gershenzon, 2009; Davis and Croteau, 2000). CBGA serves as a central intermediate, giving rise to THCA, CBDA, and CBCA through a series of enzymatic reactions (Fig. 3) (Shoyama et al., 2012; Z. Tan et al., 2018).

It was previously noted that the significant difference between THCAS and CBDAS arises from their primary mode of action during proton transfer (Tahir et al., 2021). CBDAS extracts a proton from the terminal methyl group of CBGA, while THCAS targets the terminal hydroxyl group. This shift in regioselectivity plays a pivotal role in determining the cyclization pattern and ultimately the cannabinoid profile of a given cannabis cultivar (Taura et al., 2007). A more comprehensive understanding of these pathways is essential as it could pave the way for their deliberate manipulation, either within the plant itself or through recombinant vectors, to enable the selective production of specific cannabinoids. Over the past two decades, there has been significant progress in the field of cannabinoid natural product chemistry. However, there remains a substantial amount of research to be undertaken to achieve the production of desired cannabinoids in sufficient quantities and with high purity.

4. *In vitro* tissue culture in cannabis

Micropropagation is an effective alternative to traditional *C. sativa* propagation. It involves growing plants in a controlled, sterile environment using tissue culture techniques. This method allows for the maintenance of cannabis plants in very high density, reducing the need for extensive floor area. It is particularly useful for preserving genetic diversity. Additionally, it enables the preservation of day-neutral genotypes and ensures long-term genetic preservation. A key benefit is the sterile nature of tissue culture, which produces pest-free propagules, reducing biotic pressures, unlike typical propagation methods which require integrated pest and pathogen management systems (Adams et al., 2021; Farahmand et al., 2023).

Micropropagation involves five key stages crucial for establishing a successful protocol ("Plant Propagation Through Tissue Cultures," 1974): Stage 0 (selecting and maintaining parent plant material), Stage 1 (initiating cultures), Stage 2 (multiplying shoots/embryos), Stage 3 (shoot elongation and rooting), and Stage 4 (acclimatization) (Fig. 4). The health of stock plants in Stage 0 significantly influences subsequent outcomes. During Stage 1, environmental factors including media and plant growth regulators (PGRs) are selected. Stage 2 is pivotal to the benefits of micropropagation because it enables for exponential plant multiplication in short periods of time. Horticultural crops often stay in this stage for extended periods to achieve large-scale production. And finally, combining Stages 3 and 4 is preferred in commercial settings for efficiency(Monthony et al., 2021). Research focuses on optimizing conditions for each micropropagation stage due to the complexity of *in vitro* cultivation (Fig. 5).

In cannabis micropropagation, the main focus is on multiplying shoots from early-stage apical and axillary nodes. Similar to traditional greenhouse propagation, nodal cuttings can be cultured *in vitro*. The most effective method involves using PGRs to induce shoot multiplication (SM) from a single nodal explant, resulting in the formation of multiple shoot cultures (MSCs). The most successful SM techniques have reported yields of 9–13 explants per node (Lata et al., 2009, 2016), however, it's worth noting that these methods used freshly initiated tissues from the greenhouse (Stage 1), didn't evaluate Stage 2 performance, and have not been repeatable in

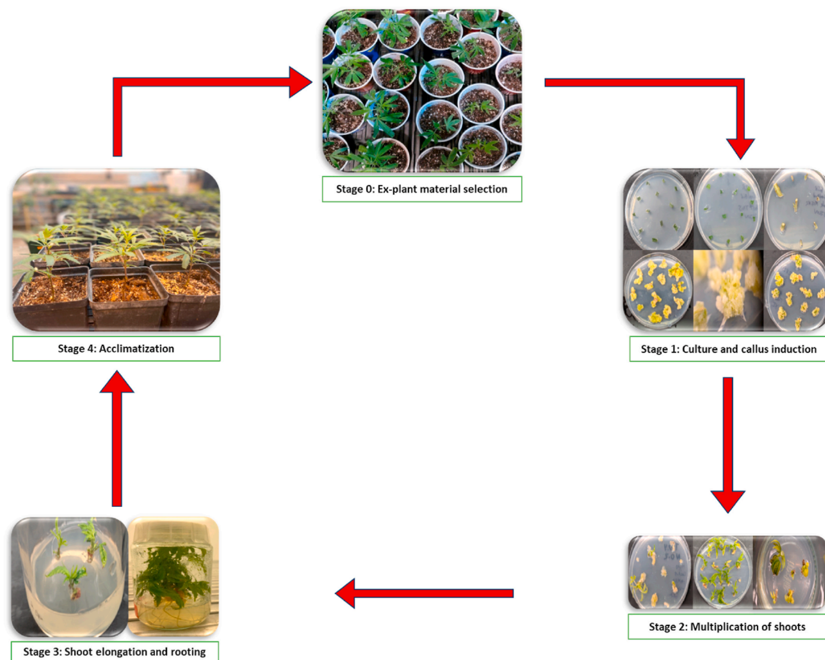

Fig. 4. Schematic diagram showing the five key stages for successful micropropagation protocol in Cannabis plants.

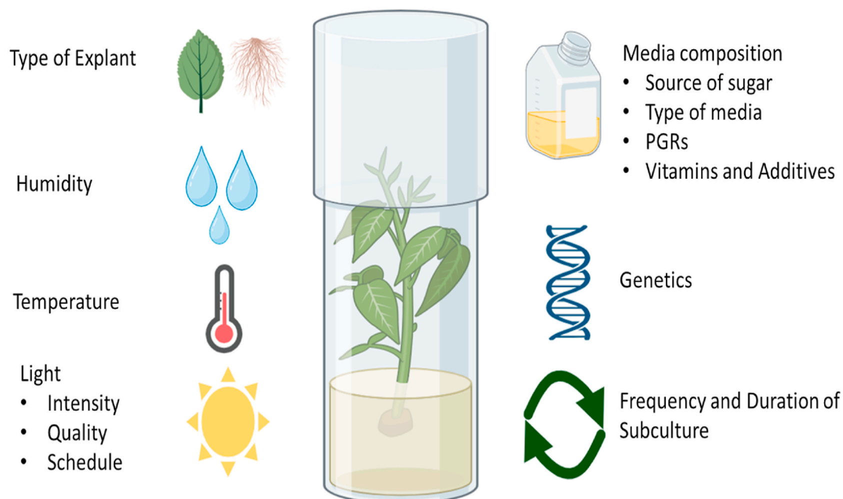

Fig. 5. Factors affecting the efficiency of *in vitro* cultures protocols.

other genotypes (Monthony et al., 2021a). A notable gap in the literature is the scarcity of studies on *C. sativa* micropropagation using long-term *in vitro* grown germplasms, or Stage 2.

While some studies have reported successful and prolific multiple shoot cultures (MSCs) in cannabis, others have observed a different trend. They note that cannabis tends to produce a single shoot with strong apical dominance and limited branching, resulting in a lower multiplication rate (Table 2) (Mestinšek-Mubi et al., 2020; Smýkalová et al., 2019; Wróbel et al., 2022). In a recent study, Mestinšek Mubi et al. focused on two high-CBD cannabis cultivars and found varying shoot multiplication rates on different media recipes (Mestinšek-Mubi et al., 2020). Interestingly, these rates contrasted with previous reports using the same media (Lata et al., 2016). Furthermore, the authors discovered that these media compositions did not significantly enhance shoot proliferation compared to the PGR-free control (MS) in their two tested cultivars (Mestinšek-Mubi et al., 2020). Wróbel et al. [2020] highlighted that conventional nodal propagation protocols led to low multiplication rates, causing a loss of plant material (Wróbel et al., 2022). Another proposed alternative involves using two-node explants (M. S. R. G. Page et al., 2021). However, it's worth noting that while this approach may have its merits, it does reduce the number of explants obtainable from a single plant, ultimately constraining the overall multiplication rate.

To address this challenge, Wrobel et al. in their recent studies proposed a new method for cannabis micropropagation (Wróbel et al., 2022). It involves allowing single shoots to grow for a period of time and then removing the apical meristem in culture (Wróbel et al., 2022). This action disrupts apical dominance, enabling the axillary buds to develop into branches. The shoot tips from these developed axillary branches are subsequently utilized as secondary explants for Stage 2 growth and multiplication. The authors reported a higher survival rate using shoot tips compared to nodal explants (Wróbel et al., 2022). Through this modified shoot tip micropropagation technique on MS + 0.5 mg/L indole-3-acetic acid (IAA) medium, Wróbel et al. (2022) increased the multiplication rate of Stage 2 explants from 0.9 (when micropropagated on MS + 0.25 mg/L TDZ) to 3.0 (Wróbel et al., 2022). Although effective, it's worth noting that this method entails an additional step of removing the apical shoot, which demands more labor and heightens the risk of contamination. Additionally, the explants were initiated into culture from seeds, and it's generally observed that juvenile tissues respond more favorably *in vitro* than mature explants. However, the author states that well-established subcultures were employed in their experiments, with a minimum of ten culture cycles completed before advancing to Stage 3 (rooting). Another group suggested that the inclusion of an auxin antagonist α-(2-oxo-2-phenylethyl)-1H-IAA could also play a role in breaking apical dominance and increasing branching in seedling tissues. This led to a promising multiplication coefficient of up to 1:10 (Smýkalová et al., 2019). This approach shows great potential and warrants further exploration.

Interestingly, studies employing nodal tissues often report modest multiplication rates, ranging from less than one (which leads to a loss of stock plants) to approximately four explants per nodal culture (Mestinšek-Mubi et al., 2020; M. S. R. G. Page et al., 2021; Richez-Dumanois et al., 1986; Smýkalová et al., 2019; Wróbel et al., 2022). From a practical standpoint, these rates may not be suitable for various applications. To augment the multiplication rate through shoot proliferation, alternative strategies to enhance the number of meristems per explant are warranted. One potential avenue is to induce flowering and implement floral reversion. The inflorescence of the cannabis plant constitutes a highly branched compound racemose inflorescence housing numerous meristematic regions (Monthony et al., 2021; Zayed et al., 2016). Initial observations have indicated that some cannabis plants initiate flower development *in vitro*, and more recently, Moher et al. demonstrated that flowering could be consistently induced using a short-day photoperiod, mirroring natural conditions (Moher et al., 2021). Hence, *in vitro* flowering plants present an alternative approach to bolster the number of meristems per plant, potentially amplifying the multiplication rate.

The utilization of inflorescence tissues from *C. sativa* presents a promising alternative to nodal cultures in micropropagation (Monthony et al., 2021; Piunno et al., 2019). Inflorescence tissues capable of transitioning from a flowering phase back to a vegetative stage are commonly described as undergoing floral or inflorescence reversion (Eapen, 1997; Phulwaria and Shekhawat, 2013; Punyarani et al., 2013; Shareefa et al., 2019; Tooke et al., 2005), and has been extensively explored in various species (Eapen, 1997; Phulwaria and Shekhawat, 2013; Punyarani et al., 2013). The exploration of floral reversion in *C. sativa* has been limited. Recently, the first known report of regeneration from floral explants of cannabis demonstrated the potential for *in vitro* floral reversion in two out of three commercially produced cultivars (Piunno et al., 2019). While the study showcased the ability of floral explants to generate phenotypically normal shoots, it did not determine the origin of the shoots, whether from existing meristems or through regeneration from non-meristematic tissues (Piunno et al., 2019). In a subsequent study, Monthony et al. found that the vegetative explants reverting from floral tissues originated from pre-existing meristems beneath the florets, similar to nodal cultures, and pairs of florets showed higher survival rates compared to individual florets, and surpassed multiplication rates of nodal micropropagation (Monthony et al., 2021). Additionally, vegetative explants derived from florets under a long-day photoperiod could be reverted to short-day conditions to induce more *in vitro* flowering. This alternative method may also provide a viable means for the clonal propagation of day-neutral genotypes, which can't be sustained in a continuous vegetative state of growth.

Efficient cannabis regeneration remains a challenge in biotechnology despite recent progress in cell and tissue culture. The species is generally considered recalcitrant (Phillips and Garda, 2019). Various factors, including genotypes, PGRs, explant characteristics, and culture conditions, play crucial roles in *vitro* culture systems (Driver and Kuniyuki, 1984; Phillips and Garda, 2019) (Fig. 5). While studies have focused on some factors, many others remain underexplored. Investigating these variables could establish high-frequency regeneration systems and potentially lead to protocols for somatic embryogenesis and haploid production.

Notably, there are promising PGRs and additives, such as polyamines, brassinosteroids, nanoparticles, and nitric oxide (NO) that have not been incorporated into cannabis micropropagation protocols. NO, recognized as a phytohormone, plays a crucial role in regulating various aspects of plant development (Eapen, 1997; Phulwaria and Shekhawat, 2013).

Recent advancements using sodium nitroprusside (SNP) in culture media have overcome challenges associated with gaseous NO application (Sarropoulou and Maloupa, 2017). Studies have highlighted NO's critical role in organogenesis and somatic embryogene-

Table 2
Media, plant growth regulators, and other methods used to optimize different modes of micropropagation of Cannabis sativa.

Mode of Propagation	Explant	Best Media	Outcomes	Reference
Callogenesis	Roots derived from *in vitro* grown seedling	Gamborg's medium (67-V) + 2,4-D (1.5) + NAA (0.1 mg/L) + IAA (1 mg/L) + Kin (0.25 mg/L) + casein hydrolysate.	Best in root tissues.	Veliky and Genest (1972)
Callogenesis	Leaves, hypocotyl, root, and female/male flowers.	MS + 2,4-D (1) + Kin (0.01–0.1).	High level of callogenesis from leaves.	Itokawa et al. (1975)
Callogenesis	Bracts, calyx	Miller's medium + Murashige's iron source + IAA (0.25, 1 mg/L) + NAA (0.1, 0.25 mg/L) + 2,4-D (0.2 mg/L) + Kin (1, 1.5, 2 mg/L) + casein hydrolysate (1 mg/L).	Callogenesis varied across cultivars and explants.	Hemphill et al. (1978)
Callogenesis	Different parts of seedling	MS salts + B5 vitamins medium + 2,4-D (0.1 mg/L) + Kin (0.5 mg/L).	Callogenesis after 6–8 weeks.	Hartsel et al. (1983)
Callogenesis	Leaf, roots, and stem	MS salts + B5 vitamins medium + 2,4-D (0–5 mg/L) + 2,4,5-T (0–5 mg/L) + NAA (0–5 mg/L) + Kin (0–5 mg/L) + 2iP (0–5 mg/L) + BAP (0–5 mg/L)	Callogenesis in stems and in roots, callogenesis	Loh et al. (1983)
Shoot organogenesis and *in vitro* rooting	Apical and axillary buds	Shoot multiplication: MS + IBA (2 mg/L) + BAP (0.45 mg/L) + 3% glucose + 1% sucrose Rooting: MS + IBA (20 mg/L) + charcoal (2 g/L)	Shoot multiplication + rooting in buds.	Richez-Dumanois et al. (1986)
Callogenesis and shoot regeneration	Leaf & hypocotyl	MS salts + B5 vitamins medium +2,4-D (3–10) + BAP (0.01–1).	Best response for callogenesis: leaves and hypocotyls; and shooting: hypocotyls. Leaves didn't produce shoots.	Ranalli (1999)
Indirect regeneration via callus	leaves, petioles, internodes, and axillary buds	MS medium + 2.0–3.0 mg/L DIC	82.7% callus formation. 6% regenerated plants	S(Lusarkiewicz-Jarzina et al., 2005)
Direct organogenesis and indirect embryogenesis	Stems, roots, and adventitious shoots	Knapp's medium + BAP (NR) + NAA (NR) + IAA (NR) + Activated charcoal (500 mg/L)	Direct organogenesis after 2 weeks, and somatic embryos were obtained.	Plawuszewski et al. (2006)
Shoot multiplication and *in vitro* rooting	Lateral buds	Shoot multiplication: MS + TDZ (0.35 mg/L) + NAA (0.3 mg/L) Rooting: ½ MS or MS + IBA (0.2 mg/L) + NAA (0.15 mg/L)	3 ± 2 lateral shoots appear with 80% rooting rate.	(夏冰 et al., 2007)
Callogenesis, shoot regeneration, and *in vitro* rooting	Cotyledons, stems, and roots	Callogenesis: Daria ind^{+} medium + Kin (1 mg/L), NAA (0.05 mg/L), Shoot regeneration: Daria ind^{+} medium + BAP (0.2 mg/L), NAA (0.03 mg/L). Rooting: Daria ind^{+} medium + IAA (2.0 mg/L)	Highest callogenic rate in cotyledons, stems, then roots. Only stem and cotyledon's regenerated.	Wielgus et al. (2008)
Shoot multiplicaton	Axillary buds	Shoot multiplication: MS medium + TDZ (0.5 μM). Rooting: ½ MS medium +2.5 μM IBA.	100% shooting and 94% rooting.	Lata et al. (2009)
Shoot multiplication	Shoot tips	Shoot multiplication: MS + TDZ (0.2 mg/L) + NAA (0.1 mg/L). Rooting: ½ MS + IBA (0.1 mg/L) + NAA (0.05 mg/L).	3.22 shoots per tip and 85% rooting rate.	Wang et al. (2009)
Indirect regeneration via callus	Young leaves	Callogenesis: MS + TDZ (1.0 μM) + NAA (0.5 μM). Shoot Regeneration: MS + TDZ (0.5 μM). Rooting: MS + IBA (2.5 μM).	93% response rate for callogenesis. 93.3% shooting rate. 96.6% rooting rate.	Lata et al. (2010)
Indirect regeneration via callus induction.	*In vitro* cotyledon and epicotyl	Callogenesis: MS + TDZ (3 mg/L) + IBA (0.5 mg/L) Shoot regeneration: MS + BA (2 mg/L) + IBA (0.5 mg/L).	Highest callogenesis in cotyledons. 2.0 shoots produced from epicotyls.	Movahedi et al. (2015)
Callogenesis and indirect shoot organogenesis	Leaf and hypocotyl	Callogenesis: MS +2,4-D (1 mg/L) + BAP (0.5 mg/L). Shoot regeneration: MS + 2,4-D (0.1 mg/L) + BAP (0.5 mg/L).	Highest callogenesis in leaves. Minimal shooting occurred.	Movahedi et al. (2016b)
Callogenesis	Leaf and hypocotyl	Callogenesis: MS + TDZ (2 mg/L) + IBA (0.5 mg/L).	Highest callogenesis in leaves. No shooting.	Movahedi et al. (2016a)

(*continued on next page*)

Table 2 (*continued*)

Mode of Propagation	Explant	Best Media	Outcomes	Reference
Direct regeneration	*In vitro* cotyledons (2–6 days old)	Shoot regeneration: MS + TDZ (0.4 mg/L) + NAA (0.2 mg/L). Rooting: ½ MS + IBA (0.5, 1, or 2 mg/L).	51.7% shooting in cotyledons. 80% rooting. 75% survived acclimatization.	(Chaohua et al., 2016)
Shoot multiplication	Axillary buds	Shoot multiplication and rooting: MS + mT (2 μM).	100% shooting and 96% rooting.	Lata et al. (2016)
Shoot regeneration	Female inflorescences	Shoot organogenesis: MS + TDZ (10 μM)	~4 shoot clusters formed per explant	Piunno et al. (2019)
Shoot multiplication	Cotyledonary node, epicotyl first node, hypocotyl, epicotyl with first and second node, shoot apical meristem, and shoot apex.	Shoot multiplication: MS + BAP9THP (3.09 mg/L). Rooting: ½ MS + NAA (37.3 μg/L).	Highest shooting in apex, meristem, and cotyledonary node. 59% of shoots rooted.	Smýkalová et al. (2019)
Photoautotrophic micropropagation	shoot tip and nodal cuttings	Rooting: Rockwool blocks with commercial fertilizers.	97.5% rooting rate and 100% survived acclimatization after 3 weeks.	Kodym and Leeb (2019)
Direct shoot regeneration	Leaves, hypocotyl, and cotyledon	Shoot regeneration: ZEA^{RIB} (2.0 mg/L) or ZEA^{RIB} (1.0 mg/L) + NAA (0.02 mg/L).	Shoot regeneration in hypocotyls, cotyledon and leaf had 48.45% 4.70% and 0.42%. Respectively.	Galán-Ávila et al. (2020)
Direct organogenesis	Shoot tips and nodes with one axillary bud	Shoot multiplication: MS + TDZ (0.25 mg/L)	2.5 shoot tips per explant with 69.7% shooting rate.	Wróbel et al. (2022)
Shoot multiplication	Axillary buds	Shoot multiplication: MS + B_5 vitamins + NAA (2.0 μM) + IBA (2.0 μM)	MS media outperformed formula βA and formula βH.	Codesido et al. (2020)
Shoot multiplication and *In vitro* rooting	Axillary buds	Shoot multiplication: MS + BAP (4.0). Rooting: MS + IBA (4.0 μM) or NAA (4.0 μM)	3.63 shoots per explant with 95.83% shooting percentage. 92% of plantlets rooted and acclimatized.	Ioannidis et al. (2020)
Shoot multiplication	Axillary buds	Shoot regeneration: MS + TDZ (0.011, 0.1, 0.11, 0.22, 0.44, 0.88, 1.76 mg/L) + mT (0.012, 0.12, 0.24, 0.48, 0.5, 0.96, 1.93 mg/L) + BAP (1, 2.5, 5 mg/L) + IAA (0.1 mg/L).	No clear best results.	Mestinšek-Mubi et al. (2020)
Shoot multiplication	Stem tips	Shoot multiplication: MS + Mesos with or without vitamins. Rooting: Rockwools in humidity dome.	6.9 cuttings per 12 weeks. Average rooting over 90%. Retipping increased production 9-fold.	Lubell-Brand et al. (2021)
Shoot multiplication	Stem cuttings	Rockwool medium of 5–7 cm cuttings, 150 μmol/m^2/s	Over 90% of plantlet rooting within 2 weeks.	Zarei et al. (2021)
Shoot multiplication and *in vitro* rooting	Apical shoot tip and single-node	Shoot multiplication: 46 μmol/m^2/s in non-vented vessels with hedging. Rooting: 120 μmol/m^2/s in non-vented vessels.	7.2 shoots per explant. 86%, 94%, and 100% rooting in first three cycles.	Murphy and Adelberg (2021)
Callogenesis and shoot regeneration	*In vitro* Leaf explants	Callogenesis and Shoot Regeneration: MS + NAA (0.5 μM) + TDZ (1.0 μM).	100% callus induction rate, but no shooting.	Monthony et al. (2021b)
Shoot multiplication	Seeds and two-node explants	Shoot multiplication: DKW + TDZ (0.5 μM).	1.5-fold higher multiplication rates and 2-fold more canopy area than in DKW than MS media.	(S. R. G. Page et al., 2021)
Shoot multiplication, *in vitro* rooting, and acclimatization	Micropropagated shoot tips and single nodes	Shoot multiplication: MS + + TDZ (5 μM) or 2iP (5 μM). Rooting: ½ MS media + IBA (2.5 μM)	Sucrose and TDZ had greatest fresh weight and quality. Largest shoots in 2iP. Highest *in vitro* rooting with IBA. Highest *ex vitro* rooting with humidity domes.	Stephen et al. (2023)

Note: 2-iP, 2-isopentenyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; DIC, 3,6-dichloroanisic acid; BABI, BDS as modified at Arkansas Bioscience Institute; BAP, 6-benzylaminopurine; Dicamba, 3,6-dichloro-2-methoxybenzoic acid; DKW, Driver and Kuniyuki Walnut; GA_3, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; MS, Murashige and Skoog medium; mT, *meta*-topolin; NAA, 1-naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron.

sis (Hesami et al., 2020; Otvös et al., 2005), suggesting SNP's potential to enhance these processes in cannabis. Additionally, incorporating nanoparticles in culture media has shown promise in improving various regeneration processes by modulating gene expression and antioxidant enzyme activities (Ahmad et al., 2020; Álvarez et al., 2019; Hesami et al., 2019; Hesami et al., 2020a; Nalci et al., 2019; Zaeem et al., 2020). Thus, the potential of nanoparticles as an avenue to augment the *in vitro* regeneration capacity of cannabis warrants further investigation.

Extensive research has examined the effects of sucrose, glucose, and fructose on *in vitro* morphogenic responses of plants (Yaseen et al., 2013). While sucrose has shown to be optimal for certain plants such as *Agave angustifolia* (Reyes-Díaz et al., 2017), *Sapindus trifoliatus* (Asthana et al., 2017), and *Pinus koraiensis* (Peng et al., 2021), other plants like *Vitis Vinifera* (Yancheva and Roichev, 2005), *Brassica napus* (Yadollahi et al., 2011), and *Chrysanthemum* × *grandiflorum* (Hesami et al., 2019) have demonstrated superior *in vitro* morphogenic responses to glucose and fructose. Thus, investigating the impact of different carbohydrate sources is crucial for optimizing cannabis micropropagation.

Developing and optimizing a *de novo* medium for specific purposes is a complex and resource-intensive task. The integration of machine learning algorithms provides a supplementary and efficient perspective for fine-tuning *in vitro* protocols without the need for extensive experimental trials (Hesami et al., 2019; Hesami and Jones, 2020). Recent advancements have successfully applied machine learning algorithms in predicting and optimizing diverse *in vitro* culture processes, including shoot proliferation, callogenesis, somatic embryogenesis, secondary metabolite production, and gene transformation (Akin et al., 2020; Farhadi et al., 2020; García-Pérez et al., 2020; Hesami et al., 2020; Munasinghe et al., 2020; Niazian et al., 2019; Salehi et al., 2020, 2021; Q. Zhang et al., 2020). The integration of experimental methods with machine learning algorithms emerges as a potent approach for crafting tailored protocols for cannabis.

Recent research by Bidabadi and Jain (2020) has shown that mechanical wounding, induced by brushing tissue surfaces, leads to a significant increase in micropropagation (Bidabadi and Jain, 2020). While specific reports on the effect of wounding on cannabis micropropagation are lacking, our own observations suggest that callusing typically initiates at wound sites. This implies that tissue wounding holds promise for enhancing plant regeneration in cannabis, despite low indirect shoot regeneration rates.

Thin cell layer culture represents another promising approach that holds potential for cannabis micropropagation (Tripathi et al., 2018). While this technique has been successfully applied in various recalcitrant plants like *Hedychium coronarium* (Tu et al., 2018), *Withania coagulans* (Tripathi et al., 2018), and *Agave fourcroydes* (Monja-Mio and Robert, 2013), there is currently no documented application of thin cell layer culture in cannabis. This method involves the selection of a thin layer of tissue as the explant, facilitating close contact between wounded cells and the medium composition. Ultimately, this leads to an enhancement in regeneration (Tripathi et al., 2018).

Bioreactors, including both continuous immersion and temporary immersion systems, offer valuable tools for cannabis micropropagation and the study of plant development (Vidal and Sánchez, 2019). These devices prove instrumental in overcoming the challenges posed by certain cannabis genotypes in terms of proliferation, rooting, and acclimation. Additionally, they contribute to cost reduction in large-scale propagation efforts and facilitates photoautotrophic propagation.

Cannabis, a dioecious species with separate male and female plants, places economic emphasis on seedless, unfertilized female flowers (Kovalchuk et al., 2020). However, this characteristic poses challenges, such as the inability to cultivate pollen-producing plants in production facilities and the need for unfertilized plants for accurate phenotyping in breeding strategies. Additionally, self-pollination for inbred line production is inherently difficult (Salentijn et al., 2019). *In vitro* techniques for generating homozygous double haploids offer a robust solution to these challenges (Niazian et al., 2019). These techniques encompass various methods, including wide hybridization-chromosome elimination, parthenogenesis, gynogenesis, and androgenesis (Niazian et al., 2019). While there are no documented reports of haploid production in cannabis, establishing haploid production protocols is crucial for advancing genetic engineering studies in this field.

Conversely, multiple polyploid cannabis strains have been produced (Table 3). While polyploidy cannabis plants may exhibit reduced THC production compared to diploid controls, CBD production is higher compared to diploid controls as well (Parsons et al., 2019). In hemp female flowers, polyploid plants produced 50% less THC and 300% more CBD (Mansouri and Bagheri, 2017). This phenomenon is thought to result from the duplication of certain recessive alleles in polyploid cannabis plants. It's worth noting the implications of autotetraploid induction in subsequent generations are unknown.

Previous studies in cannabis tissue culture focused on refining conditions for better micropropagation rates. However, it's crucial to consider that conditions optimal for *in vitro* propagation may not preserve the genetic integrity of the regenerated genotype (Azizi et al., 2020). Factors like medium composition, PGRs, humidity, subculture frequency, culture duration, temperature, light quality, and intensity collectively contribute to developmental and physiological anomalies in micropropagated plants, known as “somaclonal variation" (Azizi et al., 2020; Monthony et al., 2021). This variation can produce phenotypic plasticity and arises from chromosome mosaicism, spontaneous mutations, or epigenetic modifications (Azizi et al., 2020; Robinson and Robinson, 2020; Rodriguez-Enriquez et al., 2011). Somaclonal variation can enhance diversity and generate new variants for breeding, but may pose an obstacle when the aim is to produce true-to-type clones. Prior research on cannabis tissue culture using ISSR indicates that regenerated plants maintain genetic stability and phenotypic resemblance to parent plants with low mutation occurrences (Chandra et al., 2017; Lata et al., 2010, 2016; Smýkalová et al., 2019). However, recent reports utilizing deep whole-genome sequencing contradict earlier studies with notable intra-plant genetic diversity contributing to phenotypic differences (Adamek et al., 2022).

Somatic embryogenesis and organogenesis, achieved through direct or indirect regeneration, represent the pivotal stages in the development of regeneration protocols. Somatic embryogenesis is particularly advantageous as it necessitates only a single cell for re-

Table 3
Summary of polyploidy induction studies in *Cannabis sativa*.

Chemotype	Polyploidy Induction Efficiency	Survival Rate of Induced Polyploids	Outcomes	References
Unspecified	59.1% after 24 h 42.1% after 48 h	73.3% after 24 h 63.3% after 48 h	0.2% colchicine induced polyploidy with altered morphology. Less CBD in female polyploid leaves.	Mansouri and Bagheri (2017)
I & II	66.7%	37.5%	20–60 μM oryzalin induced polyploidy (chemotype I, II). Tetraploid flowers displayed altered CBD content. Polyploids had altered morphological traits.	Parsons et al. (2019)
III	26–64% after 12 h	Not reported	0.05% colchicine induced polyploidy. With altered morphological traits.	Kurtz et al. (2020)
I & III	Not Reported	Not Reported	Ploidy level is inversely associated with the amount of cannabinoids.	Fernandes et al. (2023).

Note: CBD, cannabidiol; CBDA, cannabidiolic acid.

generation and minimizes chimerism in transformed plants (Niazian et al., 2019). However, somatic embryogenesis has limited success in cannabis (Hesami et al., 2021). The efficiency of cannabis micropropagation is influenced by various factors, including the composition of the growth medium and incubation conditions. Consequently, there is a pressing need to investigate and optimize these factors to establish high-frequency regeneration protocols.

5. Cannabis molecular biology and biotechnology approaches

Current progress in cannabis molecular biology, including systems biology, have significantly enhanced our comprehension of the cannabinoid biosynthesis pathway (Hesami et al., 2020; Hurgobin et al., 2021). These developments have opened the door to employing biotechnological methods for cannabinoid production in various non-native hosts, such as yeast, bacteria, and plant cells, as well as enzymatic systems by constructing a comprehensive biosynthetic pathway by assembling its genes into a single artificial gene cluster (Bharadwaj et al., 2021). Furthermore, transformation and tissue regeneration techniques can be utilized on cannabis to modify the genome. Importantly, recent breakthroughs in computational, molecular, and synthetic biology tools present an opportunity to rapidly understand and exploit the specialized metabolic potential of plants (Arya et al., 2020).

In the context of cannabis cultivation, gene editing techniques represent a highly promising avenue using transformation methods including projectile bombardment or Agrobacterium-mediated transformation (AMT) with *in vitro* tissue culture for regeneration. Once incorporated, these genes can be manipulated using various strategies, including overexpression, virus-induced gene silencing (VIGS), or RNA interference (RNAi), offering researchers a means to precisely control gene activity. However, transgenes often integrate randomly within the cannabis genome, leading to genetic variability. Recent advancements in gene editing tools, such as TALENs, CRISPR-Cas9, and other precision nucleases, have emerged as game-changers in the field. These tools allow for direct and specific alterations of the genome, eliminating the randomness associated with conventional methods.

The THCAS and CBDAS genes were successfully mapped to loci that exhibit close linkage, and multiple copies of CBDAS- and THCAS-related sequences were identified and isolated within the same genomic region. Some are functional while others are pseudogenes or partially functional alleles (Page et al., 2021; Hurgobin et al., 2021; Kovalchuk et al., 2020). Gene editing components are delivered to plant cells by agroinfiltration, through which a gene of interest can be efficiently delivered to plants. Agroinfiltration uses *Agrobacterium tumefaciens*, a bacterial symbiont able to deliver a portion of its DNA, called transfer DNA, or T-DNA into the plant genome. An optimized AMT system has successfully transformed the hairy roots of cannabis (Wahby et al., 2013). Other techniques such as vacuum infiltration and nanoparticle-based approaches can also be employed to deliver molecules of interest into the plant cell (Ahmed et al., 2021; Sorokin et al., 2020), or silencing of undesirable genes in cannabis (Schachtsiek et al., 2019).

Schachtsiek et al. (2019) conducted a study demonstrating the applicability of the VIGS method for reverse genetic research in *Cannabis sativa* aimed at uncovering the functions of unidentified genes. To substantiate their findings, they employed a computational strategy to scrutinize the genomic and transcriptomic resources of *Cannabis sativa* L. (Schachtsiek et al., 2019). Within the genome, they pinpointed specific reporter genes, namely *phytoene desaturase* (PDS) and *magnesium chelatase subunit I* (ChlI), which were anticipated to manifest easily observable phenotypic changes upon gene silencing. Subsequently, they forecasted the targets of small interfering RNAs (siRNAs) and silencing fragments and assessed their effectiveness using a post-transcriptional gene silencing approach. This study demonstrated gene knockdown in *C. sativa* resulting in leaf bleaching, substantiated via quantitative PCR, while chlorophyll A and carotenoid content were decreased concomitantly. In summary, their findings showcase the prospective utility of the CLCrV-based vector system for functional gene investigations in cannabis (Schachtsiek et al., 2019).

An alternative method for reducing targeted gene expression involves the use of RNAi. The effectiveness of this approach hinges on the effective delivery and stability of double-stranded RNA (dsRNA) into the cell. In a recent investigation conducted by Matchett-Oates et al. (2021), the alteration of cannabinoid biosynthesis genes was achieved through the application of RNAi utilizing the agroinfiltration method (Matchett-Oates et al., 2021). Various RNAi constructs targeting CBDAS and THCAS genes were vacuum infiltrated into leaves, downregulating CBDAS and THCAS which was quantified by real-time qPCR. While RNAi technology stands as a valuable tool for probing gene functionality, the persistent challenge remains in attaining stable transformation of foreign genes, particularly in the context of enhancing cannabis traits through genetic engineering.

The attainment of stable transformation is imperative to enable enhancements in cultivars with desirable traits. Nevertheless, the hindrances of a low shoot regeneration rate and the notorious resistance to genetic engineering methods pose significant challenges in optimizing the cannabis transformation protocol. Various investigations into single nucleotide polymorphism analysis within synthase genes have contributed to the understanding of genetic distinctions between chemotype 1 & 3 cannabis, shedding light on their impact on enzyme activity (Borna et al., 2017; Cascini et al., 2019). Promoter cis elements play a central role in governing gene expression during plant development and in response to environmental cues (Hernandez-Garcia and Finer, 2014). Manipulating the expression of THCAS and CBDAS genes holds the potential to increase the yield of target cannabinoids or modify the CBD:THC ratio. Despite the identification of several key genes involved in cannabinoid biosynthesis (Deguchi et al., 2020), their functions have not yet been fully validated, primarily due to limited reports of stable transformation in cannabis (Table 4). Moreover, the regeneration of transgenic plantlets from transformed cells is a time-consuming process that varies among different cannabis varieties (Dreger and Szalata, 2022). Liu et al. (2021) explored the involvement of transcription factors in the biosynthesis of cannabinoids in cannabis plants (Liu et al., 2021). Transcription factors (TF) are known to orchestrate a dynamic regulatory network that governs the timing, magnitude, and spatial distribution of gene expression. In other species, several transcription factors with roles in regulating secondary metabolite biosynthesis in glandular trichomes have been identified including the APETALA2/Ethylene Response Factor (AP2/ERF), WRKY, myeloblastosis transcription factor Aa(MYB)1 or Homeodomain protein 1 Aa(HD1), SlEOT1, SIMYC1, and HI-

Table 4
Summary of *Cannabis sativa* transformation studies.

Chemotype	Tissue	Method	Vector	Outcomes	References
III	Shoot tip	AMT, stable	*PGIP*	50% transformation efficiency.	MacKinnon et al. (2001)
III	Suspension Culture from leaf and stems	AMT, stable	*pNOV3635* containing *PMI*	MB5D1K media induced 100% callus in stem, leaves and petioles. No response for cotyledon.	(Feeney & Punja, 2003, 2003, 2003)
I & III	Hypocotyl & hairy roots	AMT, stable	*GUS, p35SGUS*	88.4% efficiency in hypocotyls and seedlings.	Wahby et al. (2013)
III	Cotyledons & leaves	AMT, transient	*PDS* (VIGS)*Chl1* (VIGS	70% decrease in *ChlI* and 73% decrease in *PDS*	Schachtsiek et al. (2019)
III	Leaves, male & female flowers	AMT, transient	*pEarlyGate101-uidA (GUS) PDS (RNAi)*	Highest rate by 0.015% Silwett L-77, 5 mM ascorbic acid, and 30 s sonication followed by a 10 m of vacuum.	Deguchi et al. (2020)
I	Seedlings	AMT, transient	*pCAMBIA1301* with *uidA (GUS)*	Transient transformation following germination.	Sorokin et al. (2020)
III	Leaves	Nanoparticle vacuum infiltration, transient	*GmMYB29A2, GmNAC42-1, pGWB6*	Expression in 5 days.	Ahmed et al. (2021)
III	Leaves	PMT, transient	*pBeaconGFP_GR-GUS pEVTV_DR5 pBeaconRFP_GUS*	2 million protoplasts per gram with 82% viability and efficiency up to 31%.	Beard et al. (2021)
II	Leaves	AMT, transient	*pRNAi-GG-THCAS. pRNAi-GG-CBDAS. pRNAi-GG-CBCAS. pRNAi-GG-CBDAS-UNIVERSAL.*	Transfection with pRNAi-GG-CBDAS-UNIVERSAL downregulated THCAS (92%), CBDAS (97%), and CBCAS (70%).	Matchett-Oates et al. (2021b)
I	Leaves	PMT, transient	*GFP*	5.7×10^6 protoplasts generated with 23.2% transient expression efficiency of GFP.	Matchett-Oates et al. (2021a)
III	Hypocotyls, cotyledons, meristems	AMT, stable	*pBIN19* with *uidA* (GUS)	63.3% transformation efficiency with 100 mg/L kanamycin, and spontaneous rooting rate of 12.2%.	Galán-Ávila et al. (2021)
III	Immature embryo hypocotyls	AMT, stable	*pG41 s g* with *CsGRF3-CsDIF* chimera and CRISPR/cas9 guide	DMG278 strain had highest rates.	(X. Zhang et al., 2021)
III	Leaves	PMT	*CsCBAS:GFP CsCBDAS:GFP CsTHCAS:GFP*	9.7×10^6 protoplasts per gram leaf produced. 55.3% transformation efficiency of protoplasts.	Kim et al. (2022)
V	Cotyledons	PMT, transient	*CsMYC2*	1.15×10^7 protoplasts per gram produced with 98.5% viability and 75.4% transformation efficiency.	(P. Zhu et al., 2023)
I & III	Hypocotyls	AMT	*RBV; pARSCL504 [pTRANS_230] 35S:Ω:Ruby*	75% transformation efficiency. First *ex vitro* one-step transformation protocol.	Ajdanian et al. (2024)

Note: AMT, agrobacterium-meditaed transformation; CBD, cannabidiol; CBDA, cannabidiolic acid; GFP, green fluorescent protein; PMT, protoplast-mediated transformation.

WRKY1 (H.-Y. Chen et al., 2016; Lu et al., 2013; Matías-Hernández et al., 2017, 2017; Matouk et al., 2017; Spyropoulou et al., 2014; H. Tan et al., 2015; Xu et al., 2018).

It's important to note that in cannabis, the primary site of cannabinoid biosynthesis is within the glandular trichomes that form on female flowers. In one study, a 548 bp segment of the THCAS promoter was characterized and its associated regulatory region, known for driving trichome-specific expression. By employing this promoter fragment in a yeast-one-hybrid screen, three novel TFs - CsAP2L1, CsWRKY1, and CsMYB1 were identified (Liu et al., 2021). Furthermore, compelling evidence demonstrates that these three TFs are involved in the regulation of the THCAS promoter within the plant. It's noteworthy that the O-Box element situated in the proximal region of the THCAS promoter is indispensable for the CsAP2L1-induced transcriptional activation of *THCAS*. Notably, *CsAP2L1* and *THCAS* exhibit remarkably similar temporal, spatial, and strain-specific gene expression profiles, while the expression patterns of *CsWRKY1* and *CsMYB1* are negatively correlated with *THCAS*, suggesting *CsAP2L1* supports *THCAS* expression, whereas *CsWRKY1* and *CsMYB1* acts as negative regulators of *THCAS* expression (Liu et al., 2021).

Gene editing offers breeders the capability to modify the rate-limiting enzymes involved in CBGA biosynthesis, such as GPP synthase, PT, TKS, or OAC (Fig. 6). Overexpression of these enzymes can significantly boost the overall production of cannabinoids and terpenes. Despite GPP's multifaceted impact on plant phenotype, including growth and development (Xi et al., 2016), its regulation and utilization in cannabis remain poorly understood (Gülck and Møller, 2020). In the case of cannabis, specific plastid membrane-localized PT isoforms 1 and 4 are prime candidates for catalyzing the alkylation of GPP and OA to form CBGA (de Bruijn et al., 2020). It's worth noting that PTs are responsible for generating a wide range of specialized metabolites in numerous plant species (Saleh et al., 2009). In this context, increasing the levels of CBGA in cannabis would be an important goal, as it serves as the precursor for various end-point cannabinoids, including CBDA, THCA, and CBCA (Blatt-Janmaat and Qu, 2021). Previous research has primarily focused on THCAS as the rate-limiting enzyme for THCA production, with a similar focus on CBDAS for CBDA (Muntendam et al., 2009;

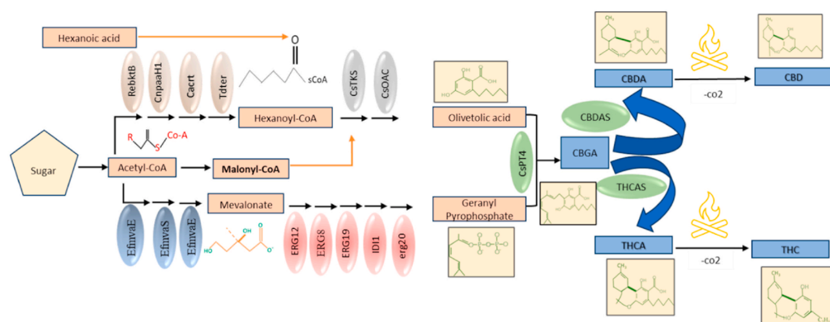

Fig. 6. GPP synthesis involved several genetic modifications. Firstly, *Enterococcus faecalis* genes EfmvaE and EfmvaS were introduced, along with the overexpression of native mevalonate pathway genes (ERG12, ERG8, ERG19, and IDI1) and a mutated ERG20(F96W/N127W gene) (erg20*). To produce hexanoyl-CoA, a heterologous biosynthetic pathway was employed, incorporating genes from *Ralstonia eutropha* (RebktB), *Cupriavidus necator* (CnpaaH1), *Clostridium acetobutylicum* (Cacrt), and *Treponema denticola* (Tdter). Alternatively, hexanoic acid was utilized as a substrate for AAE (encoded by CsAAE1 from cannabis). Expression of genes CsTKS and CsOAC facilitated the production of olivetolic acid, which was subsequently prenylated by CsPT4. The resulting CBGA was then converted into cannabinoid acids THCA and CBDA through the action of cannabinoid synthases THCAS and CBDAS. Upon exposure to heat (ΔT), THCA and CBDA underwent decarboxylation to yield THC and CBD, respectively.

Richins et al., 2018). Recent research findings have revealed the connection between THCAS gene expression and THCA content is not particularly strong (Apicella et al., 2022; Liu et al., 2021). Recent work suggests CBGA production as the bottleneck in terminal cannabinoid synthesis (Apicella et al., 2022). Studies in other plant species, such as orchids, have indicated that geranyl diphosphate synthase (GPPS) plays a pivotal role in limiting the production of monoterpenes (Chuang et al., 2018). Monoterpenes can be synthesized through the largely conserved MEP pathway in plants (Zhou and Pichersky, 2020), suggesting that GPPS likely acts as a rate-limiting factor in monoterpene production in cannabis while also participating in cannabinoid biosynthesis. CsPT1 and CsPT4 enzymes, employ GPP, synthesized by GPPS, to generate CBGA (Luo et al., 2019). This insight has led us to believe that investigating the regulation of genes involved in CBGA production can enhance our understanding of genetic control over cannabinoid production. Both CsPT1 and CsPT4 have been shown to catalyze the alkylation of OA and GPP to form CBGA (Luo et al., 2019; J. E. Page and Boubakir, 2012). They both utilize GPP as the prenyl donor but accept different substrates. CsPT1 demonstrates a degree of promiscuity, accepting multiple substrates, including OA (J. E. Page and Boubakir, 2012).

Both CsPT4 and CsPT1 have been shown to produce CBGA from OA and GPP, particularly in heterologous systems like yeast. Nevertheless, more recent discoveries indicate that CsPT4 is the exclusive enzyme responsible for CBGA synthesis (Gülck et al., 2020; Gülck and Møller, 2020; J. E. Page and Boubakir, 2012). In contrast, *in vitro* assays conducted in yeast and *Nicotiana benthamiana* failed to generate CBG with CsPT1 (Gülck and Møller, 2020; Luo et al., 2019). While previous studies have focused on *in vitro* assays to assess the abilities of CsPT1 and CsPT4 enzymes in CBGA synthesis, there hasn't been an investigation into the hormone-regulated transcriptional control of these genes and how hormonal regulation might be linked to cannabinoid biosynthesis. Both CsPT4 and CsPT1 exhibit abundant expression in the flowers, and the promoters of CsPT1 and CsPT4 display activity in the leaves of young seedlings, with stronger activities detected in the glandular trichomes. The results strongly suggest that both CsPT1 and CsPT4 genes may play pivotal roles as key enzymes in facilitating cannabinoid production. The elevated expression level of CsPT1 in the flowers, along with its upregulation by SA, implies that CsPT1 might be involved in cannabinoid biosynthesis under specific conditions or in the production of other specialized metabolites within the glandular trichomes. It's important to note that all assays assessing CsPT1 enzyme activity were conducted in other organisms, raising the possibility that CsPT1 may function differently in cannabis plants (Gülck et al., 2020; Luo et al., 2019; J. E. Page and Boubakir, 2012). On the other hand, CsPT4 exhibits a Km for OA approximately 10,000 times greater than that of CsPT1 (60 mM for CsPT1 and 6.72 μM for CsPT4) (Gülck et al., 2020; Luo et al., 2019; J. E. Page and Boubakir, 2012). This significant difference in Km values strongly suggests that CsPT4 is more likely to be the enzyme binding to OA for CBGA biosynthesis. Furthermore, when provided with the necessary OA in engineered yeast strains, CsPT4 successfully produced CBGA, whereas CsPT1 failed to do so (Luo et al., 2019). Another intriguing aspect of this study indicates that the promoters of CsPT1 and CsPT4 respond to different hormones, except to ABA and SA. These findings strongly suggest that CsPT1 and CsPT4 could be involved in distinct biosynthesis pathways. Despite these studies, it is still not fully understood how these enzymes function within the glandular trichomes of cannabis female flowers. Further genetic analyses using CsPT mutants, techniques for overexpression, gene silencing, or gene knockout, will provide more explicit evidence regarding the involvement of CsPT1 and CsPT4 enzymes in CBGA biosynthesis *in vivo*.

Luo et al. (2019) successfully engineered *Saccharomyces cerevisiae* to synthesize major cannabinoids, including CBGA, THCA, CBDA, tetrahydrocannabivarinic acid (THCVA), and cannabidivarinic acid (CBDVA), from the simple sugar galactose (Luo et al., 2019). This achievement was made possible by enhancing the native mevalonate pathway to produce GPP and introducing a heterologous hexanoyl-CoA biosynthetic pathway, utilizing the endogenous acyl activating enzyme (AAE) (Dekishima et al., 2011). Additionally, cannabis genes responsible for OLA biosynthesis (Gagne et al., 2012), along with a newly discovered enzyme called olivetolate geranyltransferase (GOT), and corresponding cannabinoid synthase genes were introduced (Taura et al., 2007; Zirpel et al., 2015). To obtain enzymes with GOT activity, the researchers explored various sources, including *Streptomyces* sp. (NphB) and *Humulus lupulus* (HlPT1L and HlPT2). A total of six cannabis enzymes (CsPT2–CsPT7) were selected based on transcript mining. The study established a biosynthetic approach that utilized multiple pathway genes to create cannabinoid analogs. Different

fatty acids were employed to modify these analogs, leading to variations in receptor binding and potency (Reggio, 2009). The researchers went further by integrating their biological system with synthetic chemistry techniques, thereby expanding the range of chemical possibilities. This integrated approach now provides a versatile platform for producing both natural and modified cannabinoids.

Cannabinoids are synthesized and stored within the storage cavity of glandular trichomes, a mechanism that helps prevent the cytotoxicity of these metabolites (Yin et al., 2022). Consequently, increasing trichome density represents a potential strategy to enhance endogenous phytocannabinoid synthesis and storage capacity. One approach involves the overexpression of the MYB1 gene from *Artemisia annua*, which has been shown to increase the average number of trichomes in transgenic cannabis plants compared to those with an empty vector control (Yin et al., 2022). Similarly, in hemp, several MYB genes are predicted to play roles in modulating trichome development and the synthesis of CBDA (Yin et al., 2022). By engineering these genes, it becomes possible to develop cultivars with cannabinoid levels surpassing those found in wild-type plants, thereby generating strains with desired cannabinoid profiles. Moreover, Haiden and his colleagues identified a transcription factor called CsMIXTA in cannabis, specifically belonging to the R2R3-MYB family (Haiden et al., 2022). This factor is likely involved in the formation of trichomes. Similar genes in other plants are known to play a role in initiating glandular trichomes. CsMIXTA is notably more active in flower tissue compared to vegetative tissues, particularly in trichomes from female flowers. During the peak stages of female flower maturation, CsMIXTA is upregulated in coordination with some genes involved in cannabinoid production (Haiden et al., 2022). Through experiments in *Nicotiana benthamiana*, they confirmed that CsMIXTA is located in the nucleus. Additionally, a yeast transcriptional activation assay demonstrated that CsMIXTA possesses transactivation capabilities. When overexpressed in *Nicotiana tabacum*, it led to increased trichome density, larger trichomes, and more branching on glandular trichomes with stalks. These findings indicate that CsMIXTA not only stimulates the initiation of glandular trichomes in epidermal cells but also governs trichome development in tobacco leaves. This study reveals the novel role of the first cannabis transcription factor, potentially crucial for the formation of glandular trichomes. Therefore, utilizing CRISPRa or transformation of a constitutively activated CsMIXTA in cannabis could result in increased trichome density.

In a recent study conducted by Livingston et al. (2020), the use of both light and scanning electron microscopy has proven to be a valuable tool for gaining insights into the development and maturation of trichomes in cannabis (Livingston et al., 2020). This research leads to an intriguing revelation: inflorescences containing a higher ratio of stalked to unstalked capitate trichomes are likely to exhibit a greater overall cannabinoid content (Livingston et al., 2020). Their findings also highlight the importance of visual markers, such as alterations in the color of glandular heads, increased autofluorescence, longer stalks, and larger head diameters, which were discussed in this investigation. These indicators, along with potential future discoveries of biochemical markers, can play a crucial role in ensuring product consistency. However, the irregular timing of development and maturation of stalked-capitate trichomes may present a challenge when attempting to predict maturation. This intricate relationship between the development of glandular heads, the elongation of stalks, and the accumulation of THC in cannabis warrants further in-depth exploration (Punja et al., 2023; Sutton et al., 2023). Importantly, further understanding trichome development can potentially result in manipulation of plants to increase the frequency of stalked-capitate trichomes or facilitating earlier stalk elongation. Ultimately, this could lead to increased THC levels, potentially revolutionizing how we approach the cultivation of cannabis for both medicinal and recreational purposes (Punja et al., 2023).

Another viable approach in cannabinoid production involves the direct modification of enzymes to enhance their activity or to increase their specificity (Zirpel et al., 2018). This can be achieved through structural alterations of the enzyme or by introducing mutations that impact its catalytic activity and specificity. Such modifications hold the potential to yield hemp plants with significantly higher CBD:THC ratios or even plants that produce no THC at all. This approach offers a pathway to tailor cannabinoid profiles to specific desired outcomes, whether for therapeutic, industrial, or regulatory purposes. In a previous study, researchers conducted site-directed mutagenesis to explore various aspects of enzyme functionality, including glycosylation patterns, the BBE domain, the active site, and product specificity, in both Δ9- THCAS and CBDAS enzymes. They achieved recombinant expression of these enzymes in *Komagataella phaffii* and observed the formation of eight different products with a cannabinoid scaffold upon the conversion of the precursor CBGA. Among these products, five remained unidentified, while both enzymes produced three major cannabinoids found in *Cannabis sativa*: THCA, CBDA, and CBCA. To enhance the enzyme properties for biotechnological cannabinoid production, several variants were generated: these includes THCAS variant T_N89Q + N499Q, which lacked two glycosylation sites, and displayed approximately a two-fold increase in activity compared to the wild-type enzyme. Variant T_H494C + R532C, which featured an additional disulfide bridge, showed around a 1.7-fold increase in activity compared to the wild-type enzyme. This variant also exhibited a shifted temperature optimum, from 52 °C to 57 °C. Two CBDAS variants, C_S116A and C_A414V, displayed 2.8- and 3.3-fold increased catalytic activities for CBDA production, respectively. The C_A414V variant additionally demonstrated a broadened pH spectrum and a remarkable 19-fold increase in catalytic activity for THCA production. These studies serve as a foundation for future research and the potential for biotechnological production of cannabinoids (Zirpel et al., 2018).

For crops of substantial economic significance, biotechnological tools like genetic engineering strategies such as transcription activator-like effector nucleases (TALENs) (Barnett, 2018; Sun and Zhao, 2013), zinc-finger nucleases (ZFNs) (Novak, 2019), and clustered regularly interspaced short palindromic repeats (CRISPR) (H. Zhu et al., 2020) have been well-established and integrated into breeding programs for many years. However, the prolonged prohibition of recreational and drug cannabis, coupled with strict regulations, has left these tools largely underdeveloped for this crop. Many of the common techniques that have been successfully employed in other crops need to be adapted and established specifically for cannabis (Barcaccia et al., 2020).

Genetic engineering of cannabis should be targeted towards cannabis strains customized for either medicinal or recreational applications, with enhancements in cannabinoids, terpenes, and resistance to diseases. This approach promotes consistent cannabinoid profiles, reduces the need for pesticides, and contributes to sustainability by minimizing synthetic inputs and resource consumption.

Moreover, biotechnology-driven approaches enable the cultivation of specific cannabis characteristics, including increased yields of cannabinoids, seed oil, and protein (Fig. 7). To attain dependable and unchanging cannabinoid profiles through biotechnology, a combination of genetic modification and advanced breeding methods can be employed (Hesami et al., 2020).

The CRISPR/Cas9 gene editing system is a widely employed tool for making precise genetic modifications in various organisms. This system typically uses the Cas9 enzyme, which is originally derived from *Streptococcus pyogenes*. However, for Cas9 to initiate its editing function, it requires a specific DNA sequence known as a protospacer adjacent motif (PAM), which must match the sequence "NGG" (Barrangou et al., 2007; Jinek et al., 2012). The necessity for the 'NGG' PAM sequence imposes constraints on the available genomic target locations for genome editing. To address this challenge, researchers have engineered various Cas9 variants. Notably, SpCas9-VQR, SpCas9-EQR, Cas9-NG, and xCas9 3.7 are among these variants, each with the ability to recognize distinct PAM sequences such as NGA, NGAG, NG, and NG/GAA/GAT, respectively.

Alternatively, Cas12 nucleases can cleave DNA using shorter crRNAs compared to Cas9 and different PAM sites, including LbCas12a which demonstrates specificity for a T-rich PAM sequence, denoted as 'TTTV'. Researchers have also developed engineered versions of Cas12a with enhanced activities (Kleinstiver et al., 2019; Y. Zhang et al., 2019).

Another breakthrough has been CRISPR-Cas8, a remarkably compact system that operates effectively with a minimal PAM sequence of "TBN-30," where 'B' represents either G, T, or C. In a manner akin to Cas12a, Cas8 induces staggered DNA cuts, resulting in 5′-overhangs. While its efficiency may be somewhat reduced, Cas8 has demonstrated functionality in plant cells. An example of this is its successful editing of the *PDS* gene in Arabidopsis protoplasts when delivered in the form of ribonucleoproteins (Pausch et al., 2020). In general, the diverse range of CRISPR systems and their respective variants offer versatile instruments for precise genome editing in plants, each equipped with distinct characteristics and specific PAM recognition sites. As a result, CAS nucleases have demonstrated the capability to accurately introduce all conceivable mutations at target sites (Nadakuduti and Enciso-Rodríguez, 2021).

A recent study by Zhang et al. (2021) has made notable strides in the creation of edited cannabis plants by refining the AMT method (X. Zhang et al., 2021). Through a strategic combination of the dual reporter (DR) chimera co-expression and CRISPR/Cas9 tools, scientists have achieved a significant enhancement in shoot regeneration efficiency within the immature grain embryo hypocotyls. They introduced modifications to the *PDS* gene, leading to the development of four seedlings exhibiting an albino phenotype (X. Zhang et al., 2021). This outcome provided strong evidence of the stable integration of T-DNA into the cannabis genome. Continued refinement and optimization of this approach hold the key to unlocking the full potential of cannabis.

The DR System is a molecular biology tool used in gene expression studies. It involves the use of two distinct reporter genes, typically a fluorescent protein (e.g. green fluorescent protein, GFP) and a luminescent protein (e.g. firefly luciferase). These reporter genes are fused to the gene of interest's promoter region. The advantage of this system is that it allows researchers to simultaneously measure the expression of a gene of interest using two different readout methods (fluorescence and luminescence) and plays a crucial role in tracking and identifying cells or organisms in which the desired genetic edits have been effectively executed. The blend of dual-reporter chimera co-expression modern technique alongside CRISPR/Cas9 tools signifies a noteworthy leap forward in the realm of genome editing. DR chimera co-expression employs chimeric proteins, combining the catalytically inactive Cas9 (dCas9) with transcriptional activators or repressors. This intricate fusion enables precise control over gene expression levels. By guiding dCas9 to specific DNA regions using guide RNAs without causing DNA cleavage, targeted gene activation or repression becomes possible.

To conduct successful experiments with the CRISPR/Cas system, it's essential to design guide RNAs (gRNAs) that target specific genes and have an efficient regeneration protocol for developing transgenic or edited plantlets (Jacinto et al., 2020). The accuracy of gRNA design relies on having a high-quality reference genome, and cannabis benefits from the availability of 12 different assembled

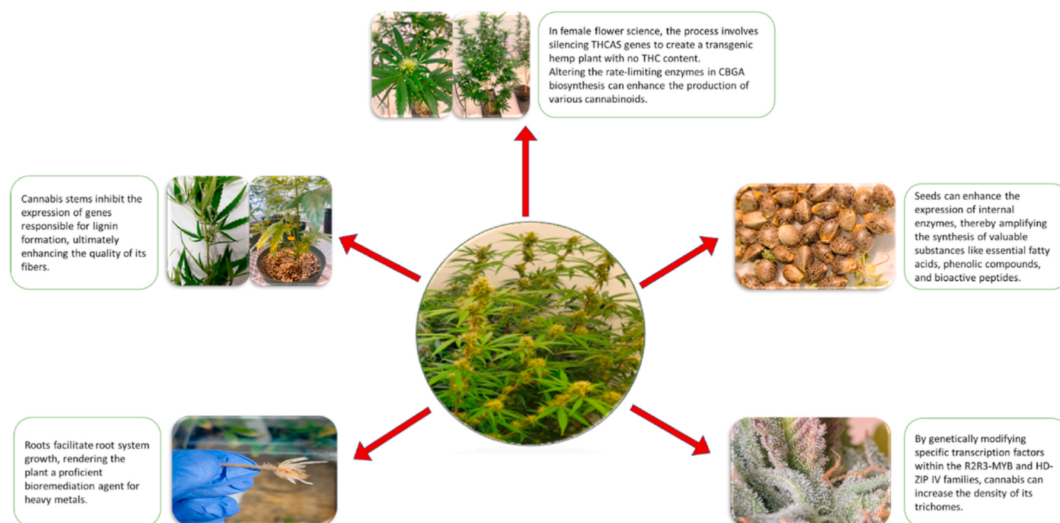

Fig. 7. Gene editing holds the promise to unlock the full potential of cannabis in various fields like bioenergy, textiles, food, and ecological restoration.

and annotated genomes (Hurgobin et al., 2021). However, there are significant disparities in reported genome size, chromosome arrangements, and gene annotations among these different cannabis genome assemblies (Hurgobin et al., 2021; Kovalchuk et al., 2020). Therefore, achieving precise and accurate gRNA design is a critical initial step to ensure high-quality CRISPR/Cas-mediated genome editing in cannabis, minimizing the risk of off-target effects.

One of the significant challenges in the realm of plant gene editing has been the efficient delivery of gene-editing reagents, primarily because established genetic transformation methods (Table 4) are limited to only a handful of plant species (Nadakuduti and Enciso-Rodríguez, 2021). However, there have been notable improvements in Cas effectors and variants; new Cas proteins offer a broader target range, possess smaller sizes, and exhibit heightened specificity. Additionally, new Cas proteins can be tailored for tissue-specific genome editing, allowing for more precise and controlled gene modifications. Furthermore, the application of base editors can complement CRISPR/Cas systems. These innovative tools, such as cytosine, adenine, and glycosylase base editors, have the capability to accurately introduce a variety of mutations at specific target sites.

Lastly, the field has seen notable progress in CRISPR delivery mechanisms for plant gene editing. These innovations bypass the need for laborious tissue culture and regeneration procedures. They include methods like *de novo* meristem induction, delivery through viral vectors, and the promising exploration of nanotechnology-based approaches (Nadakuduti and Enciso-Rodríguez, 2021).

While cannabis exhibits significant variability in crucial traits, the genetic mechanisms governing these traits largely remain a mystery. This lack of understanding poses challenges for the enhancement of desirable characteristics through traditional breeding methods. Given the multifaceted utility of this plant, research efforts are being directed toward identifying ways to stabilize its advantageous traits using CRISPR technology. CRISPR technology offers the potential to not only silence the THCAS gene through a single editing step but also to generate transgenic cannabis plants devoid of THC. This is achieved by identifying and targeting the genes responsible for THC production. Specific guide RNAs are designed to direct the CRISPR-associated protein, usually Cas9, to these genes, inducing a break in the DNA. The cell's natural repair mechanisms may result in gene alterations or deletions. Plants displaying reduced or no THC production are selected through screening. These edited plants can then be propagated and used in breeding programs to develop strains with consistently low or no THC content.

Another challenge in cannabis genome editing is the task of increasing cannabinoid accumulation. Studies have shown that simply overexpressing CBDAS and THCAS doesn't necessarily lead to an augmentation of cannabinoid biosynthesis. Instead, the enzymatic processes involved in CBG production are pivotal in the biosynthesis of CBD and THC (Tanney et al., 2021). An effective strategy for enhancing cannabinoid production, particularly *in vitro*, may involve the upregulation of coding genes responsible for cannabinoid biosynthesis enzymes.

Considering the competition for GPP between the monoterpenoid and cannabinoid pathways (Dusséaux et al., 2020), inhibiting the enzymes involved in the monoterpenoid biosynthesis pathway can divert GPP towards the cannabinoid biosynthetic pathway. Therefore, the contemporary application of CRISPRi, CRISPR Cas13a and dCas13a technologies for inducing silencing or knockdown of terpene synthases emerges as a feasible approach to selectively manipulate the GPP pool.

Cannabinoid production can be scaled up by integrating gene clusters into the cannabinoid biosynthetic pathway within non-native cells sharing a common precursor. Achieving this objective necessitates two critical steps: first, the accumulation of precursors, and second, the incorporation of pivotal genes responsible for the cannabinoid biosynthesis process (Hesami et al., 2023). The coordination of multiplexed genome editing can effectively govern gene expression and regulate the cannabinoid biosynthetic pathway.

6. Conclusions and future directions

In comparison to other profitable crops, cannabis stands out as exceptionally well-suited for bioenergy production, and it is attracting attention for its medicinal and economic prospects. Biotechnology, with a primary focus on continually refining gene editing methods, presents the opportunity to unleash the complete potential of cannabis via genetic enhancements. While endeavors have been undertaken to employ CRISPR technology to induce mutations in hemp or marijuana plants, there is only one documented case of successfully generating albino seedlings, a promising sign for the future of gene editing in cannabis molecular breeding.

A recently emerged CRISPR-based technique known as base editing holds significant promise for customizing alleles and comprehensively characterizing genes, leading to the creation of gain-of-function mutations. A more profound understanding of the cannabinoid biosynthesis pathway can greatly aid in advancing the metabolic engineering of cannabinoids, thus aiding *in vitro* production through methods like cell suspension and hairy root cultures, as well as in foreign systems. Given that callus formation encompasses a mass of undifferentiated cells, it simplifies metabolic processes by repressing multiple genes within the biosynthetic pathway. As a result, CRISPR-based approaches can be effectively deployed in cell suspension cultures to genetically modulate and manipulate the cannabinoid biosynthesis pathway. Importantly, calli have not been able to regenerate shoots through any widely accepted and repeatable protocol (Monthony et al., 2021a). Researchers have made strides to better understand the physiological state of calli through omics technology with the hope of developing shoots and roots from calli in the future (Hesami et al., 2023). Another avenue for enhancing cannabinoid production involves the utilization of hairy root cultures, where CRISPR-mediated genome editing may bolster cannabinoid yields. Moreover, conventional techniques such as the use of elicitors, alteration of medium compositions, and adjustment of environmental conditions can efficiently govern cannabinoid production in both cell suspension and hairy root cultures.

When it comes to cannabis, most genetic engineering studies focus on transient expression (Table 4), indicating the successful introduction of foreign DNA into plant cells. Nevertheless, these cells have shown an incapacity to progress to embryogenic callus, potentially posing a significant obstacle to boosting cannabis regeneration rates. An alternative strategy involves the introduction of developmental regulators into plant cells to stimulate meristem formation. These regulatory genes have demonstrated robust transfor-

Table 5
Summary of OMICS, Bioengineering, and notable differences in Cannabis Enzymes.

Methodologies	Host organism/plant tissue	**Genes/vector/proteins**	Outcomes	References
Transcriptome	Female cannabis flowers	TKS, OAC, Betv1-like proteins, CHI-like proteins.	Type III PKS (TKS) in trichomes necessitates the presence of OAC to produce OA.	Gagne et al. (2012)
Bioengineering	*Saccharomyces cerevisiae*, Komagataella phaffii	nphB, THCAS	Soluble PT *NphB* catalyzes *O*-prenylation reaction producing 2-*O*-geranyl olivetolic acid. Bacterial PT and THCA synthase expressed in yeast can synthesize THCA from OLA and GPP.	Zirpel et al. (2017)
Bioengineering	*E. coli* BL21 (DE3), & JST10 (DE3)	OLA synthase and OAC.	OLA synthase and OAC cyclase expression with *in vivo* synthesis of OLA from a single carbon source.	(Z. Tan et al., 2018)
Bioengineering	*Komagataella phaffii*	CNE1p, FAD1p, PDI1p, Kar2p, Hac1p, Sec53p, CPR5p, Lhs1p, Ero1p or Yap1p.	CNE1p, Kar2p, PDI1p, Hac1p, and FAD1p enhanced THCAS activity levels. 3.05 g/L of THCA after 8 h.	Zirpel et al. (2018)
Bioengineering	*Saccharomyces cerevisiae*	*Cs*TKS, *Cs*OAC, *Cs*AAE1, GOT	Engineered mevalonate pathway resulting in high flux of GPP. Harnessing the promiscuity of enzymes yielded diverse cannabinoid analogs in yeast.	Luo et al. (2019)
meta-transcriptomic analysis	*Nicotiana benthamiana* or *Saccharomyces cerevisiae*	UbiA superfamily chalcone isomerase-like protein	CsaPT4 exhibited CBGAS activity in both *N. benthamiana* and *S. cerevisiae*.	Gülck et al. (2020)
CRISPR/Cas9-mediated targeted mutagenesis.	*Agrobacterium tumefaciens*/DMG278 (*Cannabis sativa* strain)	*CsPDS1, ZmWUS2, NbSTM, NbIPT, OsGRF4, AtGIF1*	Overexpressing developmental regulators enhanced shoot regeneration efficiency. CRISPR/Cas9 editing of *PDS* produced the first gene-edited strain.	(X. Zhang et al., 2021)
In vivo engineering	*Cannabis sativa, Agrobacterium tumefaciens* GV3101	*pRNAi-GG-THCAS pRNAi-GG-CBDAS pRNAi-GG-CBCAS pRNAi-GG-CBDAS- UNIVERSAL*	RNAi resulted in off-target effects. *pRNAi-GG-CBDAS-UNIVERSAL* downregulated THCAS (92%), CBDAS (97%), and CBCAS (70%). Conversely, *pRNAi-GG-CBCAS* upregulated CBCAS (76%) and THCAS (13%).	Matchett-Oates et al. (2021)
Transcriptomics	Female flower development.	GPPS, PT, TKS, OAC	Upregulation of cannabinoid synthase enzymes did not increase cannabinoid production in hemp.	Apicella et al. (2022)
Biotransformation	*Komagataella* phaffii	*THCAS, CBDAS, CBCAS*	Using NADES, CBGA was converted into cannabinoid acids and synthase enzymes had higher activity.	Thomas and Kayser (2022)
Enzyme Evolution	*Nicotiana benthamiana, Saccharomyces cerevisiae*	*HuCoAT6, HuTKS4, HuCBGAS4, CsOAC, CsOLS*	Orthology analyses shows cannabinoid synthesis evolved independently in *H. umbraculigerum* and Cannabis.	Berman et al. (2023)
Bioengineering	*Saccharomyces cerevisiae*	*ScERG12, ScERG8, ScIDI1, ScERG20 EfmvaE, EfmvaS, CsOLS, CsOAC.*	Production of CBGA and CBG from glucose and hexanoic acid. ERG20WW and NphB enhanced olivetolic acid production. Cannabinoid synthase expression in vacuoles is heavily influenced by extracellular pH.	Schmidt et al. (2024)

mation rates in both monocots and dicots, likely by facilitating the transition from stem cells to embryogenic calli (Kong et al., 2020). Through the manipulation of the expression levels or activity of crucial genes and enzymes in the cannabinoid biosynthesis pathway, scientists can significantly elevate the production of specific cannabinoids such as THC or CBD. Approaches such as the overexpression of THC or CBD synthase genes, the suppression of competing pathways, adjustments to precursor availability, and the introduction of new biosynthetic genes are currently under investigation to precisely adjust cannabinoid profiles (Table 5).

As our understanding of cannabis genetics and biotechnological tools advances, we can anticipate more effective and sustainable approaches for producing cannabis with specific characteristics, all while navigating the complexities of the modern cannabis industry.

Statements and declarations

There are no conflicts of interest.

CRediT authorship contribution statement

Salma Shujat: Writing – original draft, Investigation, Conceptualization. **Gregory Ian Robinson:** Writing – review & editing, Project administration. **Farzaneh Norouzkhani:** Visualization. **Igor Kovalchuk:** Writing – review & editing, Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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