



Genome editing-CRISPR/Cas9 for the crop improvement of Industrial *Cannabis sativa* (Hemp)

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Abstract

Industrial *Cannabis sativa* (Hemp) is a multipurpose revolutionary crop widely cultivated for its seeds, which are rich in oil, CBD content, proteins, carbohydrates, fibers, as well as vitamins and minerals. Genome editing, CRISPR/Cas9 acts as highly precise "genetic scissors" that accelerate traditional plant and animal breeding by modifying specific DNA sequences without introducing foreign (transgenic) DNA. The world's first genome-edited rice varieties are DRR Dhan 100 (Kamala) and Pusa DST Rice 1 developed by the Indian Council of Agricultural Research (ICAR), New Delhi, India. Genome editing, CRISPR/Cas9 revolutionizes *Cannabis sativa* (hemp) breeding by enabling precise, targeted modifications in a crop traditionally hampered by dioecy and high heterozygosity. CRISPR/Cas9 technology allows for sequence specific editing of the target genome, thereby allowing for precise control over gene modifications and associated traits, in a low cost and straightforward manner. Gene editing has been successfully used to generate climate-resilient crops for various climatic conditions. However, several limitations are descending its overall potential remains limited. The biological functions of most candidate genes are poorly characterized. For practical crop improvement in *Cannabis sativa*, functional validation serves as a bridge between genomic discovery and breeding applications. The *Cannabis sativa* genome has long been challenging to genetically manipulate due to recalcitrance in tissue culture and transformation, limiting functional validation of genes and targeted trait improvement. The major drawback of gene-editing technologies is off-target, which can cause unwanted editing of other genes, which hinders their wide applicability for crop trait improvement. Despite these advances, transformation and regeneration remain the major bottlenecks limiting widespread genome editing in *Cannabis*. Another disadvantage is the lack of efficient tissue culture methods for regeneration, transformation and regeneration of gene edited hemp crops. There are studies highlighting successful *Cannabis sativa* organogenesis but the commercial scale production is still a problem. However, *in vitro* regeneration of different varieties of hemp is very slow and found recalcitrant. This is another major disadvantage for the application of genome editing, CRISPR/Cas9 in the crop improvement of Industrial *Cannabis sativa*, hemp. Further limitations are caused by the regulatory uncertainty surrounding genetically edited cannabis.

Keywords: *Cannabis sativa*; CRISPR/Cas9 genome editing; Guide RNAs (gRNAs); Hemp; Tissue culture

1. Introduction

Cannabis sativa L. belongs to the family *Cannabaceae* was used as a medicine before the Christian era in Asia, mainly in India, China, Bhutan, Nepal, Afghanistan, Pakistan, Egypt, Africa, Iran, Persians and Latin America [176-231-235]. Many historians believed that Indian Himalayan Region was the centre of origin of *Cannabis sativa* L. and *Cannabis indica* L. [176-189-231]. Cannabis has a long history in India, recorded in legends and religion. It was found in various habitats ranging from sea level to the temperate and alpine foothills of the Indian Himalaya Region from where it was probably spread over the last 10,000 years [176-190-196-231]. Now a days *Cannabis sativa* is a globally domesticated, cultivated

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and introduced species occurring in North and South America, Europe, Africa, Australia, Asia and other parts of world [175-231].

Cannabis sativa L., is classified into two types as Industrial *Cannabis sativa*, hemp or fibre type and Medical *Cannabis sativa* L. (drug or marijuana) based on its THC content [176-189-231]. Medical *Cannabis sativa* (drug or marijuana) contains a very high levels of THC (above 0.3 to 38% of dry weight) [175-235]. On the other hand, Industrial *Cannabis sativa* L. (Hemp) contains very low levels of THC (0 to 0.3% of dry weight[1-55]. Plants are generally classified and regulated as industrial hemp if they contain less than 0.3% THC in the dried flower (this level varies by country) or as a drug type with more than this threshold[176-231]. The U.S. Farm Bills describe hemp as “*Cannabis sativa* L. and any part of that plant, whether growing or not, with a Δ -9 tetrahydrocannabinol (THC) concentration of not more than 0.3 percent on a dry weight basis [176-189-231]. Hemp is a genetically modified plant with less content of Δ -9 tetrahydrocannabinol (THC) concentration [176-189-197-231]. The Industrial *Cannabis sativa* L. (Hemp) contains very low levels of Δ 9-tetrahydrocannabinol (THC) (0 to 0.3% of dry weight) grown outside in a large agriculture farms for the production of fibre, seeds and oil [176-231]. The Industrial *Cannabis sativa* L. (Hemp) is a short-season crop with many applications which is used as a functional food, fibre, 3D printed building material hempcrete, biochar, bioplastic, paper, textile, biofuel, oil, cosmetics, skincare, animal bedding, ropes, medicine and as a replacement for graphene in supercapacitors [176-231].

Industrial *Cannabis sativa* L. (fiber or Hemp) is legalized by **FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA (FSSAI), Government of India, New Delhi** since 2021 grown for seed, fibre, medicine, as the functional food, fiber and hempcrete in Uttarakhand, and Himachal Pradesh States in India [176-189-231]. The hemp plant will continue to find new applications due to its unique properties for sporting goods, musical instruments, portable and wearable energy storage devices, semiconductors, nanomaterials, active packaging, bio-composite orthotic devices, and medical applications [176-189-235]. Because of its variegate phytochemistry, *C. sativa* has been used in many sectors, although the discovery of the presence in the plant of several psychotropic substances (e.g., Δ 9 -tetrahydrocannabinol, THC) caused a drastic reduction of its cultivation and use together with its official ban from pharmacopeias[176-231]. Fortunately, the discovery of new *Cannabis sativa* varieties with low content of THC as well as the biotechnological development of new clones rich in many phytochemical components is another milestone in the *Cannabis* research [176-189-235]. Concerning its content of secondary metabolites, *Cannabis sativa* has been well studied and as of today about 750 compounds have been reported in this plant[1-56]. A range of applications for hemp has emerged, with over 25,000 hemp or hemp-based products and uses reported in the literature [176-189-231-235].

2. Genome Editing by CRISPR/Cas9

Malabadi and Chalannavar (2026) [350], Sharma et al., (2025) [5], Kaur et al., (2020) [37], and Singh et al., (2024) [154] are of the opinion that the recent advent of genome editing tools to produce non-transgenic genome-edited crops with no trace of foreign genetic material has the potential to overcome regulatory hurdles faced by genetically modified crops [1-170]. Kumar et al., (2021) [4], Sharma et al., (2025) [5], Bhattacharya et al., (2021) [6], and Kumar et al., (2019) [39] reviewed that the advanced genome editing techniques and the CRISPR/Cas9 technology can be applied to enhance the tolerance of the main cereal crops (wheat, rice, and maize) against any harsh climates[1-170]. The world's first genome-edited rice varieties are DRR Dhan 100 (Kamala) and Pusa DST Rice 1 developed by the Indian Council of Agricultural Research (ICAR), New Delhi, India (Figure-1) [4-6, 19,20, 23-27, 35, 37, 39, 40, 48, 49, 52, 58, 60, 62, 64, 66, 82, 83, 85, 105, 109, 110, 122, 127, 128]. Kaur et al., (2025) [130], Jyoti et al., (2019) [35], and Mohr et al., (2016) [17] indicated that these climate-smart crops use CRISPR/Cas9 technology to enhance yield, shorten maturity time, and reduce greenhouse gas emissions without utilizing foreign DNA[1-170]. Kaur et al., (2025) [130] reviewed that the current scenario indicates that crop production in tropical areas will be most affected by high temperatures and droughts [1-5-15-68-99-126-163]. Kaur et al., (2025) [130], and Singh et al., (2024) [154] are also of the opinion that climate change is a global concern for agriculture, food security, and human health[1-163]. It affects several crops and causes drastic losses in yield, leading to severe disturbances in the global economy, environment, and community[1-23-46-79-91-110-146-163]. Kaur et al., (2025) [130], and Singh et al., (2024) [154] reported that the most crops with this economic importance, biotechnological tools (i.e., genetic engineering methods including transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) are well developed and have been implemented into breeding programs for decades[1-5-15-68-99-126-163, 350].

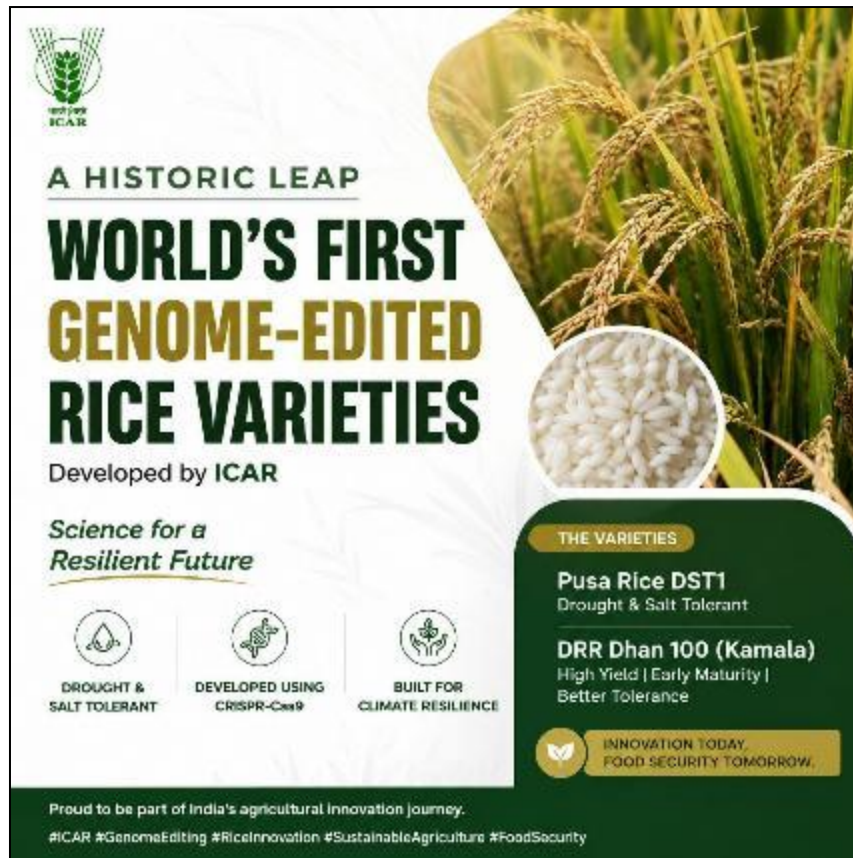


Figure 1 Worlds first genome edited rice varieties developed by Indian Council of Agricultural Research (ICAR), India

3. Hemp: Genome Editing by CRISPR/Cas9

Daundasekara et al., (2026) [235] reviewed that despite its long history of use, genetics and breeding research, industrial hemp has lagged behind that of other major crops [235]. For most of the past century, legal restrictions on cannabis in many regions curtailed scientific research and limited the exchange of genetic resources across borders [166-233-235]. As a result, assessments of genetic variation were historically confined to phenotypic selection, with little to no molecular data being generated [235]. The reclassification and legalization of industrial hemp in several regions, such as under the 2018 U.S. Farm Bill, have transformed the research landscape[235]. Daundasekara et al., (2026) [235] also mentioned that advances in next-generation sequencing, coupled with international efforts to develop reference genomes, have provided unprecedented insights into the biology and evolution of industrial hemp, making it possible to dissect complex trait variations at very small scales, analyze genome-wide diversity, and accelerate the use of marker-assisted and genomic selection methods[235]. Shiels et al., (2022) [166] and Singh et al., (2024) [154] reviewed that developing new cultivars through traditional breeding can often take a lot of time and labour [166]. Singh et al., (2024) [154] and Shiels et al., (2022) [166] reviewed that, molecular breeding strategies such as marker assisted selection have refined the process of breeding, but these strategies are not well developed in this species. Shiels et al., (2022) [166] indicated that the recent development and adoption of genome editing technologies such as CRISPR (clustered regularly interspaced short palindromic repeats) offers a means to improve hemp varieties in a more precise and less time-consuming way[166]. Many hemp varieties do not self-pollinate, and this prevents using this strategy to obtain homozygous plants. Acharya et al., (2019) [19] and Singh et al., (2024) [154] reviewed that gene-editing technology allows breeders to modify genes on both alleles to achieve homozygous lines in one generation[166]. Daundasekara et al., (2026) [235] and Shiels et al., (2022) [166] are of the opinion that potential targets for gene-editing in hemp include genes controlling cannabinoid production and accumulation, fibre deposition, disease susceptibility and seed oil quality[166]. However, genome editing technology, CRISPR/Cas9 to improve the beneficial properties of hemp have not yet been deployed [166-170, 235]. This is mainly due to the long history of the prohibition of recreational/drug type cannabis, along with the strict regulation and lower market value of hemp, these tools are rudimentary, and many common techniques used in other crops have yet to be applied to cannabis [166-170-231, 235]. Recent availability of high-quality *Cannabis* genome sequences from several strains (cannabidiol and tetrahydrocannabinol balanced and CBD/THC rich strains) have paved the way for improving the production of valuable

bioactive molecules for the welfare of humankind and the environment [166-170, 235]. With recent shifts towards the legalization of cannabis for medicinal and recreational purposes in many jurisdictions and the establishment of a legal market for cannabinoids, cannabis production is becoming a large-scale enterprise similar to other major crops [166-170, 235]. Along with the emergence of legal commercial producers, the need for modern technologies for genetic improvement is steadily increasing [166-170, 235]. While biotechnology of cannabis is still relatively new and unrefined, with the advent of affordable large-scale sequencing technologies (i.e., next-generation sequencing (NGS) and the increasing body of candidate genes for traits of interest, we argue that it is time for a paradigm shift toward improving cannabis genetics through genetic engineering [166-170, 235].

CRISPR/Cas9 genome editing revolutionizes *Cannabis sativa* (hemp) breeding by enabling precise, targeted modifications in a crop traditionally hampered by dioecy and high heterozygosity [1-166-170, 235]. It accelerates the development of elite, homozygous lines in a single generation, overcoming reproductive barriers that typically delay conventional breeding [166-170, 235]. Scientists and agricultural biotechnology companies are leveraging CRISPR/Cas9 to address specific bottlenecks in industrial hemp production [166-170, 235]. Researchers successfully target and knock out genes like *CBDAS* (cannabidiolic acid synthase) to create cannabinoid-free hemp lines [166-170]. This trait is critical for farmers to ensure crops consistently test well below strict legal THC thresholds. Conversely, it is used to elevate or isolate novel cannabinoids (e.g., CBG) for pharmaceutical or industrial use [166-170]. *Cannabis* crops are highly susceptible to pathogens like powdery mildew and Hop Latent Viroid (HLVd) [166-170]. Gene-editing is utilized to engineer targeted resistance against these devastating agricultural diseases [166-170, 235]. For industrial applications (e.g., textiles, biocomposites, paper), CRISPR is being researched to optimize cellulose and lignin deposition pathways, creating hemp stalks with superior fiber strength and fineness [166-170]. Scientists are working to introduce traits that allow hemp plants to withstand severe heat, drought, and varying environmental conditions [166-170]. Despite its vast potential, applying CRISPR/Cas9 to *Cannabis sativa* presents notable biological challenges. Hemp is highly recalcitrant to standard plant tissue culture [237-350] and transformation techniques [166-170-231, 235, 237-253]. Protocols rely on *Agrobacterium tumefaciens*-mediated transformation utilizing meristem explants to avoid issues with callus formation. However, transformation frequencies in hemp remain lower than in heavily studied commodity crops like corn or soybeans [166-170, 235]. Furthermore, optimizing *in vitro* regeneration systems and exploring base-editing technologies (such as CBE and ABE) to bypass traditional, lengthy transgenesis pipelines are key areas of current research [166-170]. Leading institutions—such as the University of Wisconsin-Madison, USA are pioneering these precise editing approaches, which promise to transform hemp from an unpredictable cash crop into a highly uniform, resilient, and standardized industrial resource [166-170-231, 235, 236].

Shiels et al., (2022) [166] reviewed that a knockout of the THC acid synthase gene via genome editing is a way to derive THC-free, high-CBD plants which would have huge value in countries with strict laws on THC levels. A patent filed by Canopy Growth Corporation details overexpression of genes regulating trichome development (e.g. GLABROUS INFLORESCENCE STEMS (GIS)) produced trichomes in greater density and abundance, and had a ten-fold increase in THC production over unmodified plants [166]. Using non-transgenic genome editing technology, the target gene(s) can be overexpressed by editing the respective regulatory (enhancer/ promoter) elements upstream of a gene. Fibre quality of cultivated hemp plants can be improved by upregulating the expression of genes involved in the formation of bast fibres (phloem fibres). Several well-known transcription factors including NST1, MYB46 and WILM1 control secondary cell wall deposition and bast fibre development in hemp hypocotyls. The genes SND2, VND1 and NST1 are master regulators of secondary cell wall development. Hemp is susceptible to a range of diseases that can lead to loss in yield and decrease the overall value of the crop [166]. Common pathogens of hemp include fungi, oomycetes, viruses, nematodes, and bacteria. Genome editing technologies offer a way to generate disease resistant varieties with greater precision and in a faster time frame than traditional breeding methods [166]. Targeting resistance (R) and susceptibility (S) genes are one way to increase a plant's resistance. A recent study has identified a powdery mildew resistance (R) gene in a *Cannabis sativa* cultivar, designated PM1, that confers resistance to the pathogen *Golovinomyces ambrosiae* [166]. Improving food quality of seed and seed oil is also possible. Targeting FAD2 genes which are involved in converting oleic acid to linoleic acid and linolenic acid offer a reliable target to upregulate oleic acid production. Mutagenesis studies on the hemp cultivar Finola have shown increased oil quality (high oleic content) and shelf life through targeted mutations of fatty desaturase genes *CsFAD2* and *CsFAD3* [166].

CRISPR/Cas-mediated genome editing has exceptionally improved plant biotechnology [1-175, 235]. This system is robust and offers relatively high target programmability and specificity that can allow accurate genetic modification [1-175]. The CRISPR/Cas system provides a unique opportunity to improve cannabis varieties with desired traits in a sustainable fashion [1-175]. Recently, Zhang et al., 2021 [170] employed the CRISPR/Cas9 system to knock out the phytoene desaturase gene and they reported that four edited cannabis plantlets with albino phenotype have been successfully generated [1-175, 235]. However, the numerous new biotechnological methods such as base editing and prime editing based on CRISPR/Cas platforms would expand cannabis synthetic biology and the toolbox of fundamental

research[1-175, 235]. Zhang et al. (2021) [170] found that cannabis embryo hypocotyls of immature grains collected 15 days after anthesis exhibited the greatest regeneration rate and were also more amenable to agrobacterium transformation[170, 235]. The authors used G41sg vector to deliver sgRNA targeting phytoene desaturase gene (CsPDS1) generating albino plants[170]. This method therefore can play an important role in functional genomics of cannabis genes and release the real potential of gene editing in the production of improved cannabis varieties. This is the first published report of successful gene editing in *Cannabis sativa*, which paves the way for further developments in non-transgenic genome editing technology[170]. Regenerating transfected protoplast cells into complete plants is also challenging. The first report of DNA-free (or non-transgenic) genome editing described the successful regeneration of genome edited lettuce protoplasts into complete plants [170, 215]. The authors incubated preassembled complexes of purified Cas9 protein and guide RNA with plant protoplasts in the presence of polyethylene glycol (PEG), a standard and widely used transfection method [170, 215, 235]. Interestingly, RNA-guided mutations were detected as early as 24 h, suggesting the quick Cas9 activity even before the cell cycle was completed[170, 215]. The transfected protoplasts were mixed with a 1:1 solution of 50% B5 medium and 2.4% agarose to make agarose embeddings plated on 6-well plates [170, 215]. For regenerating plants from genome edited grapevine protoplasts the authors embedded the protoplasts in alginate disks and stimulated the formation of mini-calli in NN-based cultivation medium [170, 215]. Another study demonstrated PEG mediated transient transformation of *Cannabis sativa* protoplasts with a p35S:GFP expression cassette and achieved a transformation efficiency of up to 31%, thus demonstrating the viability of protoplast transformation in this species[170, 215]. Effective protoplast culture protocols provide a platform for whole plant regeneration, and a platform to test sgRNAs in optimizing CRISPR protocols[170, 215]. Shiels et al., (2022) [166] reviewed that cannabis, co-transformation of native homologs of developmental regulators in combinations increased shoot regeneration efficiency up to 1.7-fold with CsGRF3–CsGIF1 chimera and all chimeras containing CsWUS4[166]. Shiels et al., (2022) [166] are of the opinion that even though hemp has large genetic diversity and traditional breeding still offers utility to improve varieties, application of these new plant breeding technologies allows highly specific changes in markedly shorter timeframes[166]. In producing new allelic variation in crop species, CRISPR is the most powerful tool available to breeders, and should be exploited for its full potential[1-166, 235, 350].

A successful CRISPR/Cas system experiment requires designing target-specific guide RNAs (gRNAs) and an efficient regeneration protocol for developing transgenic/edited plantlets [1-175]. The gRNA is categorized as a chimeric RNA including a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). The crRNA consists of a guide (spacer) sequence that accurately navigates the Cas9 protein to the targeted gene. Then, the targeted DNA is cleaved by the RNA-guided DNA endonuclease Cas9[235]. Another key part of CRISPR/Cas-mediated genome editing is the protospacer adjacent motif (PAM), which is a conserved and CRISPR-dependent DNA sequence motif adjacent to the target site (protospacer) and is utilized by the endogenous CRISPR in archaea and bacteria to discriminate invading and self-DNAs. Generally, the application of an effective and precise CRISPR/Cas system is strongly dependent on the selection of the best guide sequence (gRNA target site) [1-175]. The prediction of the presence of off-target sites (i.e., unintended mutations) is one of the most important steps in designing gRNA for CRISPR/Cas-mediated genome editing. The design of the candidate gRNAs starts with the screening of the organism's whole-genome sequence. Therefore, the availability and accessibility of a high-quality reference genome is required, which is the case in cannabis [252]. The genome of cannabis has been de novo assembled nearly a decade ago [253]; however, there are still some major challenges to using the cannabis reference genome. Currently, 12 different genomes (assembled and annotated) are available for cannabis [252]. Having multiple reference genomes can be considered a bonus, but, contradictorily, there are significant differences in reported genome size, chromosome order, and gene annotations among cannabis genome assemblies [166-176]. Therefore, designing a precise and accurate gRNA is an important starting point for highquality CRISPR/Cas-mediated genome editing in cannabis with the minimum off-target activities.

On the basis of literature survey, most of the tissue culture studies on *Cannabis sativa* has reported as recalcitrant [176-231]. Plant tissue culture plays an important role in the modern breeding methods and enhance sustainable agriculture [237-350]. The good news is that the literature on tissue culture studies of *Cannabis sativa* is slowly warming up [118-152]. There are studies highlighting successful *Cannabis sativa* organogenesis but the commercial scale production is still a problem [176-231]. Till today there are no reports on the induction of somatic embryogenesis in *Cannabis sativa*. Direct de-novo organogenesis often appears to yield good results, while indirect regeneration via callus formation generally has regeneration rates that are absent or relatively low [118-152]. The failures of the last 20 years and the challenges faced by the scientists, therefore, prompted the researchers to classify *Cannabis sativa* as a recalcitrant species to plant regeneration and DH induction [175-231].

Daundasekara et al., (2026) [235] reviewed that several candidate genes in *Cannabis sativa* associated with traits such as cannabinoid biosynthesis, sex determination, fiber development, seed quality, disease resistance, and abiotic stress tolerance have been identified through expanding genomic resources[235]. However, the biological functions of most candidate genes are poorly characterized [235]. For practical crop improvement in *Cannabis sativa*, functional

validation serves as a bridge between genomic discovery and breeding applications[235]. Over the past decade, research into the genetic editing and targeted engineering of *Cannabis sativa* has accelerated considerably but remains in its early stages relative to major crops such as rice or maize[235]. The *Cannabis sativa* genome has long been challenging to genetically manipulate due to recalcitrance in tissue culture and transformation, limiting functional validation of genes and targeted trait improvement [235]. However, recent studies have begun to unlock both transient and stable gene manipulation systems that lay the groundwork for future breeding and trait engineering in industrial hemp[235]. The development of CRISPR-based genome editing, together with speed breeding systems that accelerate generation turnover, is opening new possibilities for rapid trait validation and genotype development[235]. Future genome-editing approaches may extend beyond CRISPR/Cas9 systems to include multiplex editing, base editing, prime editing, and transgene-free editing strategies[235]. These emerging technologies offer the potential to precisely modify cannabinoid biosynthetic pathways, improve stress resilience, alter flowering behavior, and enhance seed and fiber quality while minimizing unintended genomic changes [235].

The ultimate goal of functional genomics is the generation of stable and heritable genetic modifications[235]. This was advanced by the first successful stable gene editing in industrial hemp using *Agrobacterium*-mediated transformation combined with CRISPR/Cas9[235]. By optimizing explant choice and culture conditions, including the use of a developmental regulator chimera to enhance regeneration, the study generated transgenic *C. sativa* plants with targeted knockout of the phytoene desaturase gene (CsPDS1) [235]. Edited seedlings exhibited the expected albino phenotype, and T-DNA integration was confirmed in the Cannabis genome, validating both transformation and editing in a stable context[235]. The work represents the first proof of concept for CRISPR/Cas9-based editing in industrial hemp and provides a foundation for future functional genomics and trait improvement [235]. Beyond gene knockouts, CRISPR-based technologies offer opportunities for engineering cannabinoid biosynthesis, modifying flowering behaviors, manipulating sex expression, improving disease resistance, and enhancing stress tolerance[235]. Despite these advances, transformation and regeneration remain the major bottlenecks limiting widespread genome editing in Cannabis [235].

Daundasekara et al., (2026) [235] are of the opinion that transformation efficiencies remain highly genotype-dependent, and many elite cultivars exhibit poor regeneration responses[235]. An improved transformation protocol using a rapid *Agrobacterium*-mediated transformation method has been described: it produced stably transformed *C. sativa* plants from various explants (hypocotyls, cotyledons, and meristems) and achieved higher regeneration and transformation rates than earlier attempts[235]. This methodology not only provides a route for transgene insertion but also enhances the potential for targeted editing using CRISPR/Cas systems by increasing the pool of transformable tissues [235]. In parallel, genotype-independent de novo regeneration systems are emerging, enhancing shoot organogenesis from explants across diverse industrial hemp cultivars without genotype limitations [235]. Scalable regeneration is arguably the rate-limiting step in industrial hemp transformation, and progress here has system-wide implications for how reliably genome editing can be applied across breeding germplasm [235].

However, despite rapid advances in both transient and stable gene manipulation systems, *Cannabis sativa* still lags behind major model and crop species in the implementation of advanced genome engineering technologies[235]. This gap is primarily due to a combination of biological and technical constraints[235]. A key limitation is the strong genotype-dependent recalcitrance to tissue culture and regeneration [237-350], where many elite industrial hemp cultivars show poor callus induction and low shoot regeneration efficiency[235]. These limitations directly reduce the recovery of stable edited lines and hinder scalable genome-editing applications[235]. In addition, low and inconsistent transformation efficiency, particularly in *Agrobacterium*-mediated systems[237-350], further restricts reproducibility across genotypes and laboratories, preventing the establishment of standardized editing pipelines comparable to those in model crops[235].

Another limiting factor is the incomplete functional annotation of *Cannabis* genomes and the lack of well-resolved gene regulatory networks for complex traits such as cannabinoid biosynthesis, sex determination, and stress adaptation[235]. Most candidate genes identified through these approaches remain experimentally unvalidated, creating a significant gap between genomic prediction and functional application [235]. Current gene editing primarily relies on first-generation CRISPR/Cas9 approaches, while advanced technologies such as multiplex genome editing, base editing, prime editing, and transgene-free editing have not yet been widely implemented in Cannabis [162–164, 235]. This limited implementation is largely constrained by regeneration inefficiency and transformation bottlenecks, as well as the absence of genotype-independent delivery systems[235]. Further limitations are caused by the regulatory uncertainty surrounding genetically edited Cannabis. Collectively, the development of VIGS, viral silencing systems, agroinfiltration platforms, transient over-expression tools, and CRISPR/Cas9-based genome editing represents a rapid evolution of functional genomics in *C. sativa* [235]. Through these advances, Cannabis research has been shifting from descriptive genomics toward mechanistic gene function analysis and targeted metabolic engineering [235]. Together,

they mark a transition from simple observing genomic patterns to functional and engineering-based biology[235]. This opens new opportunities for the improvement in cannabinoid content, disease resistance, seed quality, and stress tolerance [235]. Similarly, base editing and prime editing technologies offer the ability to introduce precise nucleotide changes without double-strand breaks[235]. This is highly valuable for fine-tuning enzyme activity within cannabinoid and terpene pathways[235]. These approaches would allow for allele-level engineering rather than complete gene disruption, enabling more predictable metabolic outcomes[235].

In another major development, The USDA's Animal and Plant Health Inspection Service (APHIS) recently issued regulatory status review responses for two new varieties of gene-edited hemp, determining they are not subject to federal regulation under 7 CFR part 340 [236]. Developed by researchers at the University of Wisconsin-Madison, these gene-edited plants were found to pose no increased plant pest risk compared to conventional hemp[236]. This federal clearance marks a significant milestone in streamlining the path from the laboratory to commercial cultivation for precision-bred industrial crops[236]. Badger PMR (powdery mildew resistant) has full resistance to powdery mildew, while Badger Zero (cannabinoid-free) is cannabinoid-free[236]. The primary innovation involves the creation of hemp varieties that are genetically incapable of producing THC (tetrahydrocannabinol), the psychoactive compound found in cannabis[236]. By using CRISPR/Cas9 gene editing technology to switch off the specific genes responsible for THC production, the UW-Madison team has solved a major hurdle for the industry[236]. This genetic certainty prevents crops from "going hot"—a term used when THC levels unexpectedly exceed the legal limit of 0.3%—which previously forced farmers to destroy entire fields to comply with federal law [236]. Beyond the removal of THC, the researchers have also engineered varieties with increased concentrations of CBG (cannabigerol), a non-psychoactive cannabinoid valued for its potential therapeutic benefits [236]. These advancements are expected to stabilize the hemp market by providing growers with reliable, high-value, legally compliant varieties[236]. With the USDA's regulatory green light, these precision-bred hemp lines are now poised to revitalize the industry, offering a more secure and profitable future for farmers across the United States[236].

4. Bioinformatic tools for CRISPR/Cas9 system

Hesami et al., (2021)[168], Malabadi and Chalannavar (2026) [350], Sharma et al., (2025) [5], Kaur et al., (2020) [37], and Singh et al., (2024) [154] reviewed that over the past few years, several bioinformatics tools have been developed to design gRNAs and predict, *in silico*, the off-targets. Generally, these tools exploit a similar algorithm to design gRNA [1-175]. However, some characteristics related to the gRNA spacer region (e.g., number and type of mismatch, GC-content, length, and indels) and the selection of specific Cas-nucleases vary between these tools [1-175]. In general, these tools provide multiple sequences for designing gRNA, which are appropriate for editing similar genome conservative motives of various evolutionarily related genotypes[168]. These tools require the sequence of the targeted gene and the Cas-nucleases type[1-168]. Then they search the genome and provide information related to candidate gRNAs such as the potential protospacers, both non-ranked and ranked with respect to their fitness, and off-target sites [168]. Generally, considering variations in the nucleotide sequences and the increase in individual gene copy number, it is necessary to design multiple gRNAs for each target for an efficient genome editing[168].

Hesami et al., (2021)[168] discussed and reviewed that CRISPR RGEN is a web-based tool that also includes BE-Designer, Cas-OFFinder, Cas-Designer, Microhomology-Predictor, and some other tools that can be employed for post-editing analysis[168]. Implementation of multiple tools increases the flexibility of CRISPR RGEN Tools that can be illustrated by the following examples: The Cas-Designer can detect potential protospacers in the analyzed sequence. It also provides a broad range of nucleases containing mutant forms with various PAM-sequences: SpCas9 (NRG for the off-target sites and NGG for the target sites), SaCas9 (NNGRRT), StCas9 (NNAGAAW), VRER SpCas9 (NGCG), wtSpCas9 (NNGTGA), NmCas9 (NNNGMTT), VQR SpCas9 (NGA), CjCas9 (NNNNRYAC), CjCas9 (NNNVRYAC), BhCas12b (TTN), FnCpf1 (KYTV), FnCpf1 (TTN), AsCpf1 (TTTV), AsCpf1 (TTTN), etc[168]. In the protospacer searches, one may specify if it is essential to find the probability of single nucleotide indels. In this case, the tool notifies about enhancing the time of the search[168]. However, if this option is not applied, the results of the search returns immediately. Other parameters are chosen by default. Cannabis genome should be selected from the list to run the search[168]. Further changes to other options of the Cas-Designer can be applied when it is locally installed on a computer [168]. Hesami et al., (2021)[168] also indicated that the Microhomology-Predictor facilitates the forecast of the type of microhomology-mediated end-joining (MMEJ) and nonhomologous end-joining (NHEJ) by analyzing the microhomology of nucleotide sequences in the sites of double-strand breaks [168]. The Cas-OFFinder tool can be used to screen possible off-target sites in the genome. Furthermore, this tool can be independently applied for previously selected gRNAs to find off-target sites[1-168]. For analysis, it is essential to insert the selected gRNAs sequences, specify the number of indels (both in RNA and DNA) and the adequate number of mismatched nucleotides, similarly Cas-Designer [168]. The BE-Designer tool can be applied for designing gRNAs to edit individual nitrogenous bases. Although Cas-Designer works similarly to BE-Designer, there are some differences. For instance, the selection of the corresponding nucleases and the PAM-

regions is slightly limited in the BE-Designer, and the “window” size should be chosen for searching the targeted substitution of nucleotides (A→G or C→T) and the editing sites[168]. The results would be provided in a table, in which the substituted nucleotides are shown with a specific color [168]. The CRISPOR is another user-friendly tool with a detailed user manual. Selecting optimal protospacers in this tool can be performed in three steps[168]. In the first step, the sequence of the targeted gene should be provided by the user. In the second step, the genome (e.g., cannabis) should be selected. In the third step, the Cas9 nuclease must be selected out of 33 options[168]. The results of the search are provided in both table and graphic formats. The analyzed sequence is represented, and the potential edited sites are highlighted with various colors, relating to low, medium, and high specificity[168]. Moreover, the table of the results includes information regarding the “Cloning/PCR primers”, protospacers sequences, their positions with indicated DNA chains, expression of a specific gRNA, and cloning[168]. Two algorithms are used to assess the efficiency of the proposed gRNAs and, also, specificity is used to rank the proposed gRNAs. In addition to predicting the types of microhomology, it foresees the off-target sites with the number of mismatches [168]. The CRISPR/Cas9 Target online predictor (CCTop) tool is applied to design and screen gRNAs for on-target and off-target sites. It can use a wide range of Cas-9 nucleases from various bacterial strains (e.g., *Campylobacter jejuni*, *Streptococcus pyogenes*, *Treponema denticola*, *Staphylococcus aureus*, *Streptococcus thermophilus*, and *Neisseria meningitidis*), their mutant forms, as well as Cpf1 from *Francisella novicida* and *Lachnospiraceae/Acadaminococcus*)[168]. The most intriguing feature of this tool is its ability to add nucleotides in the form of one or two guanines at the 50 -end of the spacer region to improve in vitro transcription. Additionally, two favorable nucleotides adjacent to the PAM region can be considered in order to prevent them from being with the gRNA)[168]. This tool exploits the CRISPRater prognosis algorithm to rank predicted gRNAs. gRNAs in intergenic, introns, and exons spacers are presented in various colors. This tool shows information in both table and graphic formats [168].

Hesami et al., (2021)[168] reported that the **CHOPCHOP** tool can be used to design and screen gRNAs with the capability to work in complex with the Cpf1 nuclease and Cas9-nuclease and its nickase forms[168]. The search for gRNA can be run either in all 50 - or 30 -non-coding regions, splicing sites, exons, and in the promoter sequence or in a specified coding region, the length of which would be chosen individually[168]. Moreover, the restriction sites in the edited region can be screened by the corresponding enzymes with the manufacturer’s name. After finishing the search, an adjustable color graphic and an interactive table with ranked gRNA target sequences, consisting of DNA chains, the GC-composition, and other information are provided [168].

The Breaking Cas tool can be applied for designing gRNAs and screening the off-target sites) [168]. It is essential to select the form of nuclease from the three types of Cpf1 and the four types of Cas9 with mismatch number and the length of pre-set spacers[168]. It is also possible to select a “Custom” nuclease, which is followed by providing the corresponding lengths of the spacer sequence of gRNA, PAM-sequences, and an acceptable number of mismatches[168].

5. Genome Editing by CRISPR/Cas9

Kaur et al., (2025) [130], Bhoomika et al., (2024) [109], Kumara et al., (2025) [164] Shiels et al., (2022) [166], Santosh Kumar et al., (2020) [110] reviewed that genome editing by CRISPR/Cas9 is revolutionizing plant biology and agriculture in developing improved crops with novel traits[1-130-175]. CRISPR/Cas9 technology allows for sequence specific editing of the target genome, thereby allowing for precise control over gene modifications and associated traits, in a low cost and straightforward manner [1-166-175]. This level of control over DNA sequence change is unprecedented. It is a vast improvement over previous genome modification tools and opens new doors for exciting developments in the fields of medicine and agriculture [1-166-175]. *Agrobacterium*-mediated CRISPR transformation is being widely used for targeted crop improvement to develop gene knockouts, knock-ins, transcriptional regulation, and epigenetic changes in the genome to achieve novel traits[1-166-175]. However, *Agrobacterium*-mediated transformation may pose a bottleneck for regulatory approval because of the introduction of external plasmid DNA into the plant genome[1-166-175].

The newest next-generation genome editing technology encompasses modifying/editing the DNA bases by direct delivery of CRISPR/Cas ribonucleoprotein (RNP) complexes into plant tissue, such as protoplasts, embryos or *in-vitro* grown calli [1-166-175]. The transformed plant tissue is grown in a suitable media to regenerate entire plants followed by screening for the genome edited plant lines[1-166-175]. This approach eliminates the opportunity for plasmid encoded DNA elements to integrate into the plant genome, thereby mimicking natural mutations[1-166-175]. In addition to introducing mutations and deleting entire fragments of DNA elements, CRISPR/Cas technology is also being used to introduce a specific DNA fragment to a precise location in the genome[1-166-175]. A specific donor DNA is included together with Cas9 and sgRNA which spans the flanking regions of the target site with the donor DNA element in between[1-166-175]. The presence of this single-stranded DNA triggers the Homology Directed Repair (HDR) mechanism wherein the donor DNA is used as a template by the DNA repair machinery to repair the cut target

site, and consequently the target DNA sequence gets introduced to the target genome [1-166-175]. This new generation of precision methods has several applications in hemp breeding such as gene knockout/knock-in, base editing, gene- and genome-wide screening, modifying gene regulation, and developing virus resistant plants, as demonstrated in different recalcitrant species such as wheat, maize and grape [1-166-175].

In vitro tissue culture techniques (e.g., callus and cell culture, de novo regeneration, hairy root culture) are the basis of micropropagation [237-350] and breeding in cannabis [166-175-231]. *In vitro culture* methods coupled with genetic engineering techniques (e.g., Agrobacterium-mediated gene transformation and genome editing) as well as polyploidy induction offer opportunities for producing new genotypes and manipulating secondary metabolite production in cannabis [166-175-231]. Although conventional genetic engineering tools (e.g., Agrobacterium-mediated gene transformation and *A. rhizogenes*-mediated hairy root cultures) can alter the production of some secondary metabolites, it seems that the CRISPR/Cas9 system has more potential than these tools to introduce new germplasm and enhance secondary metabolite production in cannabis in a faster manner [1-166-175-231]. Therefore, biotechnological methods can be employed in order to develop improved genetics to help satisfy the demands of producers and consumers [166-175-231].

Efficient and reliable *in vitro* culture procedures [237-350] can be considered as an important prerequisite for successful gene transformation, genome editing, micropropagation, and conservation of cannabis [166-175-231]. Micropropagation is a powerful tool that can develop and propagate new varieties. Polyploidy induction is also a method that can influence secondary metabolites. Although current shoot proliferation-based protocols are relatively developed for mass propagation of cannabis, it is necessary to develop somatic embryogenesis, organogenesis, and haploid production protocols especially for gene transformation and genome editing studies [166-175-231]. *Agrobacterium*-mediated gene transformation has been described in cannabis. Recently, transgenic plant regeneration has been successfully obtained. It seems that using morphogenic genes can help overcome challenges in transgenic plant regeneration. The production of secondary metabolites is another important aspect of *in vitro* culture of cannabis. Although several studies have attempted to manipulate secondary metabolite production in cell suspension culture and hairy root culture of cannabis, small amounts of cannabinoids have been produced [166-175-231]. Therefore, it is necessary to apply new methods such as CRISPR/Cas-equipped *A. rhizogenes*-mediated hairy root culture for increasing or changing secondary metabolite profiles [166-175-231]. Machine learning algorithms can also be considered as robust computational biology for a comprehensive study of secondary metabolites and, also, modeling and optimizing hairy root culture or cell suspension culture for improving its yield [166-175-231]. Generally, cannabis plants still provide the most efficient natural sources of secondary metabolites, and, in particular, cannabinoids and modern biotechnologies will play an important role in further genetic improvement [166-175-231].

6. Conclusion

Cannabis sativa L. is an economically significant multi-use crop valued for fiber, seed, and phytochemical production. Compared with other crops, advancement in *Cannabis sativa* has been slow due to regulatory constraints and genetic resource limitations. Recent advances in technology have transformed the research landscape, supporting a deeper understanding of the genetic architecture underlying key agronomic traits. Tissue culture protocols play an important role in the application of genome editing. Efficient and reliable *in vitro* culture procedures can be considered as an important prerequisite for successful gene transformation, genome editing, micropropagation, and conservation of cannabis. Genome editing tools to produce non-transgenic genome-edited crops with no trace of foreign genetic material has the potential to overcome regulatory hurdles faced by genetically modified crops. A successful CRISPR/Cas system experiment requires designing target-specific guide RNAs (gRNAs) and an efficient regeneration protocol for developing transgenic/edited plantlets. The adoption of genome editing technologies such as CRISPR (clustered regularly interspaced short palindromic repeats) offers a means to improve hemp varieties in a more precise and less time-consuming way. However, genome editing technology, CRISPR/Cas9 to improve the beneficial properties of hemp have not yet been deployed. This is mainly due to the long history of the prohibition of recreational/drug type cannabis, along with the strict regulation and lower market value of hemp, these tools are rudimentary, and many common techniques used in other crops have yet to be applied to cannabis. The major drawback of gene-editing technologies is off-target, which can cause unwanted editing of other genes, which hinders their wide applicability for crop trait improvement. Another disadvantage is the lack of efficient tissue culture methods for regeneration, transformation and generation of gene edited crops due to recalcitrant nature of Industrial *Cannabis sativa*, hemp.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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