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REVIEW

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Genome editing technologies, mechanisms and improved production of therapeutic phytochemicals: Opportunities and prospects

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Abstract

Plants produce a large number of secondary metabolites, known as phytometabolites that may be employed as medicines, dyes, poisons, and insecticides in the field of medicine, agriculture, and industrial use, respectively. The rise of genome management approaches has promised a factual revolution in genetic engineering. Targeted genome editing in living entities permits the understanding of the biological systems very clearly, and also sanctions to address a wide-ranging objective in the direction of improving features of plant and their yields. The last few years have introduced a number of unique genome editing systems, including transcription activator-like effector nucleases, zinc finger nucleases, and miRNA-regulated clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9). Genome editing systems have helped in the transformation of metabolic engineering, allowing researchers to modify biosynthetic pathways of different secondary metabolites. Given the growing relevance of editing genomes in plant research, the exciting novel methods are briefly

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reviewed in this chapter. Also, this chapter highlights recent discoveries on the CRISPR-based modification of natural products in different medicinal plants.

KEYWORDS
biosynthesis pathway, CRISPR/Cas, gene encoding, homozygous mutants, knockout, next-generation sequencing

1 | INTRODUCTION

Plants are known to be producing a diverse spectrum of secondary metabolites, which are either actively ingested by people or utilized by various industries as raw materials. The practical use of such organic elements has increased the usage of medicines derived from plants in the past few years (Soltani Howyzeh et al., 2018). Over a long period of time, traditional biotechnology approaches were used to get a plethora of favorable features, ranging from unraveling the complete metabolic pathway to improved secondary metabolite and production of nutrients, among other things. Furthermore, classical genetic engineering approaches are not suited for introducing large-scale modifications into a plant, as the insertion of DNA construct could be inserted randomly into one or multiple loci within one or more chromosomes, which might result in unfavorable outcomes. As a result, these approaches are ineffective for changing overall metabolic cascades within plants (Naqvi et al., 2010).

The rise of genome management approaches has promised a factual revolution in genetic engineering. Targeted genome editing in living entities permits the understanding of biological systems very clearly, and also sanctions to address a wide-ranging objective in the direction of improving features of plant and their yields. This embraces the establishment of plants with traits conferring resistance to numerous abiotic and biotic stress conditions. Also, novel plants with treasured compositional activities can be established. Following the analysis of next-generation sequencing (NGS), the use of targeted genome-editing technologies, such as CRISPR/Cas9 systems, zinc-finger nucleases (ZFNs), and transcription activator-like endonucleases (TALENs) can integrate synthetic biology into genetic and metabolic engineering in medicinal plants. First, in 1996, ZFNs (protein domains coupled with *Fok*I endonuclease) that slice the DNA specifically at a defined region were discovered (Kamburova et al., 2017; Kim et al., 1996). This chimeric protein possesses a modular assembly, and each of the ZFNs domains identifies a single nucleotides triplet. This approach later led to the introduction of cultured plant cell editing (Gaj et al., 2013; Weeks et al., 2016). Continual studies headed to the expansion of other genome editing tools like TALENs and CRISPR/Cas9. In the design of TALENs, a new protein is re-engineered for specific targets. Nevertheless, the method of designing is streamlined in recent times using the modules of repeat alignments that fundamentally lessen the cloning essential for the design (Kamburova et al., 2017). In contrast, the design and usage of CRISPR/Cas9 are simple. It is an RNA-mediated DNA endonuclease amended from the microbial immune system (W. Jiang & Marraffini, 2015; Mao et al., 2019). Both TALENs and CRISPR/Cas9 systems are effective to function in animals and plants. These editing systems are used effectively for genomes manipulation that could further decipher multifaceted difficulties, comprising the establishment of mutant and transgenic plants. All these have the potential to develop new types of medical plants containing desired-chemical components, as well as novel bio-products (Pouvreau et al., 2018).

In recent years, protein-based editing techniques, such as ZFNs, and TALENs have proven to be effective tools at the transcriptional level for modifying genomes. Both techniques are designed fusion proteins with a DNA-binding domain joined with restriction enzyme FokI via a nonspecific nuclease domain (Palpant & Dudzinski, 2013). These have been utilized effectively in a variety of plant species. The most recent breakthrough in genome editing technology is focused on RNA-guided designed nucleases, which have already shown particular potential because of their ease, effectiveness, and adaptability. The type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated) system derived from Streptococcus pyrogens is by far the most extensively utilized system (Jinek et al., 2012). The technique of the CRISPR/Cas9 system has been identified as a breakthrough in the field of the genome-engineering framework in medical and agricultural studies, with rising evidence of the use of the CRISPR/Cas9 technique in plants with medicinal properties (Ma & Liu, 2016; Weeks et al., 2016).

New CRISPR/Cas9 modifications are being investigated for potential uses, such as suppressing numerous genes, thereby allowing the study of gene families ubiquitous in plants. This will also allow for the creation of new plant varieties with a range of useful features. Furthermore, the CRISPR/Cas9 system, when activated by inducible promoters, can aid in temporal and spatial genomic alterations (Z. P. Wang et al., 2015). As a result, CRISPR/Cas9 has evolved into an efficient, simple, and rapid method for breeding superior plants that further includes both the introduction of desired characteristics and the deletion of unwanted characteristics (Belhaj et al., 2013). This approach has also evolved into a novel synthetic tool in the field of plant biology for constructing minimum plant cells, like engineered cells that lack nonessential constituents. These cells can further be used as a foundation for the development of innovative natural systems (Bortesi & Fischer, 2015).

Many gene editing approaches have been used to regulate secondary metabolite pathways in plants to increase the biosynthesis

of beneficial secondary metabolites for medicinal, nutritional, and commercial processes (Dey et al., 2021). Several studies have been conducted to investigate and validate the efficacy of medical herbalism such as neoechinulin and piperine against various cancers and other diseases such as COPD (Mitra, Anand, Sanyal, et al., 2022; Mitra, Anand, Ghorai, et al. 2022). NGS methods have shown great potential in evaluating the biosynthetic pathways and genetic variation of herbal plants. Novel bioengineered breeding methods, including targeted genome editing techniques, were recognized as an outstanding framework for developing desirable medicinal plants with enhanced secondary metabolites (Niazian, 2019). Accomplishments have been reached in the commercial synthesis of various key metabolites such as reticuline, taxadiene, and naringenin by modifying the biosynthesis pathway of natural products from plants within the microbial systems (Cravens et al., 2019).

This article briefly describes the mechanisms of various genome editing systems and their applications in plant improvements. Also, the beneficial role of the CRISPR/Cas system in various medicinal plants is discussed.

2 | GENOME EDITING TECHNOLOGIES AND THEIR MECHANISMS

A genome editing tool allows the cutting and rejoining of DNA segments in definite sites for effectively modifying the genetic material of cells. Distinct enzymes like restriction endonucleases and ligases are used to cleave and rejoin DNA fragments in small genomes of bacteria and viruses. However, it is very challenging to make use of restriction endonucleases and ligases in manipulating larger genomes, especially plant and animal genomes. The major setback is that restriction enzymes can recognize and target the cleaving of relatively shorter DNA sequences. This specificity may be adequate to work with short DNA segments of microorganisms, however, not effective with large genomes of plants. Initially, "artificial enzymes" were designed for a complex genome as short nucleotide sequences (oligonucleotides). This is so that they may bind selectively to explicit sequences of the target DNA, and possess chemical groups with the capability of cleaving DNA segments.

In recent times, targeted approaches are developed to address these challenges by designing chimeric nucleases, that is, complex proteins comprising 1 or 2 structural entities. Among them, one unit facilitates the catalysis of DNA cleavage. The other selectively binds to precise nucleotide sequences of targeted DNA and provides the nuclease action to the site. These engineered nucleases could be produced in the cells. For this, properly engineered vectors that codes for nucleases are to be transformed into cells. Also, vectors with nuclear localization signals are used to enable the nucleases to move into the cell's nucleus to attain accessibility to the genomic DNA. These genome-editing technologies use programmable nucleases to increase the specificity of the target locus. The establishment of genome editing tools, such as engineered ZFNs, TALENs, and CRISPR/Cas9 has paved the way for crop improvement agendas (Figure 1) (Arora & Narula, 2017; Jankele & Svoboda, 2014; Kamburova et al., 2017; Palpant & Dudzinski, 2013).

2.1 | ZFNs technology

ZFNs are a noticeable tool in the arena of genome editing. ZNFs are the firstly established genome editing tool, which uses engineered nucleases. The functions of Cys2-His2 zinc finger (ZF) domain are the basis for its development (Gaj et al., 2013; Kamburova et al., 2017; Kim et al., 1996; Palpant & Dudzinski, 2013). Thirty amino acids

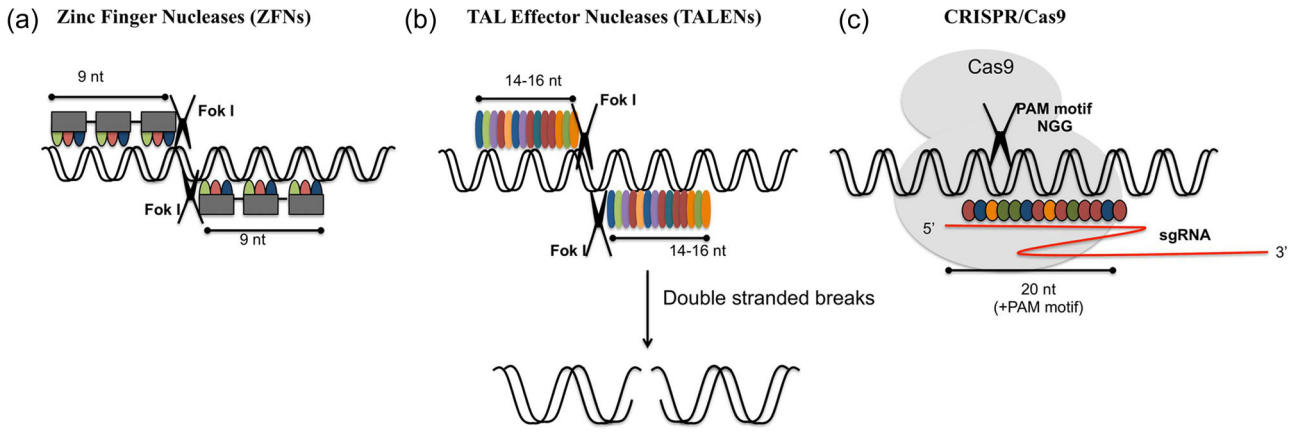

FIGURE 1 Various genome-editing tools. (a) Zinc-finger nucleases (ZFNs) act as dimer. Each monomer consists of a DNA binding domain and a nuclease domain. Each DNA binding domain consists of an array of 3–6 zinc finger repeats which recognizes 9–18 nucleotides. Nuclease domain consists of type II restriction endonuclease Fok1. (b) Transcription activator-like nucleases (TALENs): these are dimeric enzymes similar to ZFNs. Each subunit consists of DNA binding domain (highly conserved 33–34 amino acid sequence specific for each nucleotide) and Fok1 nuclease domain. (c) CRISPR/Cas9: Cas9 endonuclease is guided by sgRNA (single guide RNA: crRNA and tracrRNA) for target specific cleavage. Twenty nucleotide recognition site is present upstream of protospacer adjacent motif (PAM) (Adopted from Arora & Narula, 2017). Copyright © 2017 Arora and Narula. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY).

constitute form the folded configuration of Cys2-His2 ZF proteins. Crystallographic studies have established that the α-helix of the Cys2-His2ZF protein attaches to the double-stranded DNA helix via inserting into its major groove (Kamburova et al., 2017; Pavletich & Pabo, 1991). ZF proteins have the capabilities to identify three tandem sequences in the DNA. Generally, each ZFNs monomer comprises two well-designed active domains, that is, a DNA binding domain, Cys2-His2 ZF at the N-terminal end, and a nonprecise *Fok*I (the type II restriction endonuclease) DNA cleaving site at the C-terminal end. The cleavage domain needs to undergo dimerization for cleaving DNA. The dimerization of *Fok*I site is very important for ZFNs enzymatic activity (Kamburova et al., 2017; Kim et al., 1996). A typical single ZFNs consists of about 3–6 distinct ZF repeats at the DNA-binding domain that can recognize base pairs (bp), between 9 and 18. For example, if a ZF domain can perfectly recognize a 3 bp DNA sequence, then it can produce a 3-finger array, allowing it to recognize a 9 bp target site. Other approaches may make use of 1- or 2-finger modules for generating ZF arrays with more than six individual ZFs. Two individual ZFNs must attach to opposite DNA strands with their C-termini so as to allow two cleavage domains to undergo dimerization and cleave the DNA strand. A single ZFNs dimer with two 3 or 4 ZF domains identifies a target sequence of 18 or 24 bp that practically produces distinctive sites in the genomes of most organisms (Kamburova et al., 2017).

The major disadvantage of this method is that individual ZFs specificities may overlap, and depends on the nearby ZFs and DNA. ZFNs applications involve the modular designing, assembling, and optimization of ZFs against targeted sequences of DNA, followed by the linking of separate ZFs in the direction to target larger DNA sequences. In the recent past, ZF domains have been prepared to identify a large number of triplet nucleotides. This has permitted the choosing and linking of ZFs in sequences that further enables the identification of the specific target sequence of interest (Kamburova et al., 2017). ZFNs are used for manipulating the genomes of several living organisms, including plants and animals. The target-specific endogenous genes inactivation occurs in *Arabidopsis thaliana*, followed by high-frequency alteration of genes in *Nicotiana tabacum*, targeted insertion of herbicide-tolerant genes besides insertional inactivation of a target locus in *Zea mays*, and trait stacking in *Z. mays* (Ainley et al., 2013; Gaj et al., 2013; Kamburova et al., 2017; Osakabe et al., 2010; Townsend et al., 2009; F. Zhang et al., 2010).

2.2 | TALENs technology

TALENs are restriction enzymes that are engineered to cleave specific DNA sequences. They are constructed by joining a distinctive transcription activator-like effector (TALE) proteins domain to a DNA cleavage domain. TALE can be engineered to attach to several desired DNA sequences, thus combining it with nucleases can cleave DNA at a specific location (Boch, 2011; Jankele & Svoboda, 2014). The restriction enzymes hosted in cells can be used for genome editing in situ, which is well-known as genome editing with engineered nucleases (https://en.wikipedia.org/wiki/Transcription_activator-like_effector_nuclease). TALE proteins possess a vital domain and are accountable for DNA binding and nuclear localization signals. Also, TALE proteins have another domain that acts as an activator of transcription of the target gene (Kamburova et al., 2017).

Xanthomonas bacteria, after infecting plant species secretes TALE proteins (Boch & Bonas, 2010). TALE DNA-binding domain encompasses a repetitive highly conserved 33 to 34 amino acid sequence with different 12th and 13th amino acids, and these two loci are denoted as the repeat variable diresidues (RVDs). Due to higher variability, they exhibit a strong correlation with explicit nucleotide recognition (Boch et al., 2009; Moscou & Bogdanove, 2009). The link between DNA recognition and amino acid sequence has permitted the development of precise DNA-binding domains via choosing a grouping of repeat fragments encompassing the suitable RVDs. Particularly, a slight change in the RVDs and incorporating nonconventional RVD sequences may increase genome targeting specificity (Boch, 2011; Juillerat et al., 2015). After identifying TALE protein recognition sequences, the next effort is carried out to create chimeric TALE nucleases. This is done by inserting the sequence that encode the DNA-binding TALE domain in a plasmid vector, earlier employed for creating ZFNs (Christian et al., 2010). An artificial, and chimeric nucleotides-specific nuclease construct, consisting of the DNA-binding domain of TALEs and the catalytic domain of *Fok*I restriction endonuclease is created. This construct helps to create artificial nucleases with DNA-binding domain and different RVDs that can target any nucleotide sequence of interest (Gaj et al., 2013; Kamburova et al., 2017; Nemudryi et al., 2014). The *Fok*I domain acts as a dimer and requires two constructs possessing unique DNA-binding domains for locations in the target genome with appropriate direction and spacing. The amino acid sequence between the TALE DNA-binding domain and the *Fok*I cleavage domain, as well as the number of bases between the two separate TALENs binding sites, seem to be the main factors in the achievement of higher levels of activity (Miller et al., 2011; Mussolino et al., 2011).

The simple link between DNA recognition of the TALE binding domain and amino acid sequence sanctions to engineer proteins effectively. Under this circumstance, the binding domain can create problems due to the inappropriate binding of artificially synthesized genes to the repetitive sequences occurring in the TALEs. To overcome this, one can make use of a publicly accessible software program, DNA Works. This can be specifically used for calculating oligonucleotides appropriate for assembly in a two-stage polymerase chain reaction oligonucleotide assembly, followed by whole gene amplification. Likewise, many modular assembly schemes to generate engineered TALEs constructs are in use (Cermak et al., 2011; T. Li et al., 2011; Weber et al., 2011; F. Zhang et al., 2011).

The TALENs constructs after assembly is inserted into plasmids and transfected into the target cells. At the same time, the expressed gene products pass into the nucleus to bind and cleave the targeted genome sequence. Otherwise, TALENs constructs are delivered to the cells as messenger RNAs that eliminates the probability of genomic integration of the TALENs-expressing protein. TALENs are

generally used for editing genomes via inducing double-strand breaks, which cells respond to with repair mechanisms. Dependent on the application, TALENs can be used for introducing an error, that is, to knock out a target gene or for introducing a novel DNA sequence into the specific gene (https://en.wikipedia.org/wiki/Transcription_activator-like_effector_nuclease). TALENs find it's application in the efficient modification of plant genomes, creating commercially valued food crops with favorable nutritive qualities (Haun et al., 2014; Y. Zhang et al., 2013).

2.3 | CRISPR/Cas9 genome editing technology

In recent times, the CRISPR-associated protein 9 (Cas9) genome editing tool is widely used as a multipurpose tool for performing specific gene targeting and changes, and includes gene insertion or deletion, gene replacement, and single base pair alterations (Dong et al., 2020; Soyars et al., 2018; Y. Zhang & Showalter, 2020; Zong et al., 2017) (Figure 2). Several reviews are providing comprehensive information that on every aspect of CRISPR/Cas tool (Amitai & Sorek, 2016; Arora & Narula, 2017; Gaj et al., 2016; Puchta, 2016; Y. Zhang & Showalter, 2020). The CRISPR/Cas9 arrangement exhibits adaptive immunity in bacteria; it protects genomic DNA from attacking plasmids and viruses via RNA-guided DNA cleavage by Cas proteins (Sorek et al., 2013; Gaj et al., 2016). A cluster of CRISPR-linked genes (Cas) and CRISPR arrays (a genomic locus) are seen in a CRISPR locus, which engraves all immunological memories. A CRISPR array has a series of direct repeat sequences (between 21 and 40 bp) that are interspaced by spacer elements (variable sequences of 25–40 bp), providing immunity against microbial infections. Within the CRISPR locus, short sequences (foreign DNA) are incorporated and expressed to crRNA (CRISPR RNA), which anneals to tracrRNA (*trans*-activating crRNA) and directs the Cas9 protein to degrade pathogenic DNA (Arora & Narula, 2017; Jinek et al., 2012). Later, it was discovered that only a conserved protospacer-adjacent motif (PAM) upstream of the crRNA binding site and a seed sequence within the crRNA is required to recognize the target Cas9 protein. Since then, this tool has been streamlined to manipulate the genome (Cho et al., 2013; Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013). Presently, this system comprises only the single guide RNA (gRNA) having the crucial crRNA and tracrRNA components and Cas9 nuclease (Arora & Narula, 2017; Jinek et al., 2012).

Each gRNA binds to target DNA sequences due to its complementary nature at the PAM region, which ensures proper binding of Cas9 endonucleases. About 80 nucleotides long gRNA scaffold sequence is observed adjacent to the 3′-end gRNA and is also crucial for Cas9 binding. As the gRNA-Cas9 complex is formed, Cas9 creates a double-strand break specifically 3 bp before the PAM sequence. This cut site is mostly repaired by nonhomologous end joining, which is inaccurate and causes insertion/deletion mutations at the cleavage location (F. Jiang & Doudna, 2017; Jiang et al., 2015; Ran et al., 2013; Y. Zhang & Showalter, 2020). These mutations affect protein translation and thus disrupt the functions of a gene. Plant researchers are utilizing CRISPR/Cas9 gene-editing tools for manipulating genetic/metabolic pathways, enhancing numerous agronomic qualities, and obtaining pathogen-resistant plants (Gurumurthy et al., 2016; X. Li et al., 2018; Makarova et al., 2018; C. Wang et al., 2019; Y. Zhang & Showalter, 2020).

3 | CRISPR/CAS-EDITING ORIENTED TO BIOSYNTHETIC PATHWAYS OF PLANT

The biosynthetic pathway of plants starts with the successful implementation of the CRISPR/Cas9-based editing tool in tomatoes which resulted in the production of carotenoid and *γ-aminobutyric*

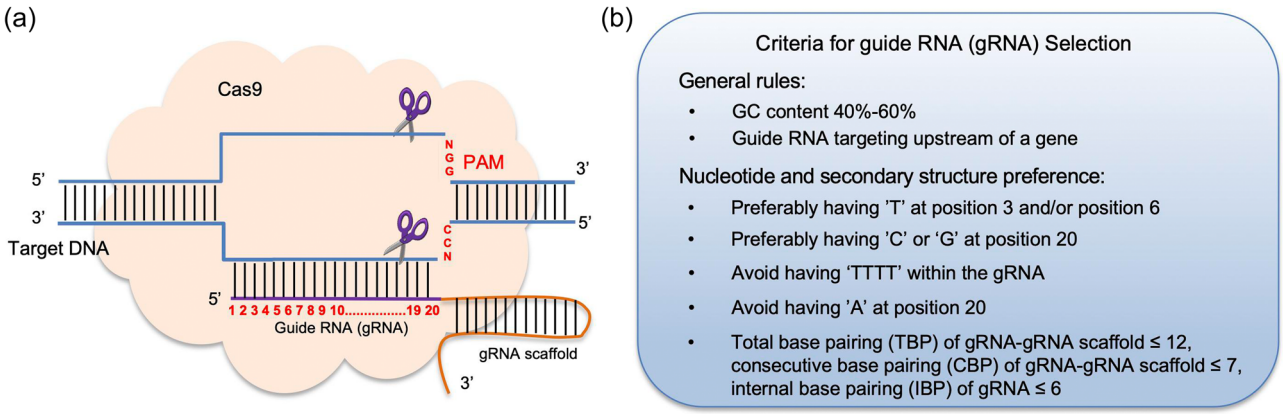

FIGURE 2 The principle of CRISPR/Cas9 mediated genome editing and criteria for guide RNA selection. (a) In the CRISPR/Cas9 system, a 20 nt guide RNA (gRNA) is complementary to the target DNA region in the host genome followed by a gRNA scaffold sequence. Each target DNA sequence ends with a protospacer adjacent motif (PAM), which is often the sequence "NGG." The formation of the gRNA-DNA complex triggers the binding of the Cas9 endonuclease to the complex and generates a double-stranded break (DSB) 3 bp in front of the PAM. (b) General rules for choosing a gRNA sequence to improve its effectiveness (Adopted from Y. Zhang & Showalter, 2020).

acid (GABA) (R. Li et al., 2018). Carotenoid is an essential secondary metabolite that has remarkable dietary significance in higher animals, owing to its physiological abilities. This editing was, however, possible due to the use of two genes namely, Psy1 and CrtR-b2. Mutation of these is facilitated by alterations in the color of the flower or fruit (D'Ambrosio et al., 2018). Isoflavone production has been obtained in soybean, followed by the production of *β-carotene* from bananas. In this case, the fortified banana has lower α-carotene and lutein contents (Kaur et al., 2020). In the latter, the use of the lycopene epsilon-cyclase (LCYε) gene was responsible for altering the metabolic flux for the biosynthesis of β-carotene. CRISPR-based mutagenesis of the *dihydroflavonol-4-reductase-B*, furthermore, tended the Japanese morning glory flower to alter its color into white due to lack of anthocyanin pigment. This mutagenesis, when performed on the same plant using *carotenoid cleavage dioxygenase 4* (CCD4), dehydrated carotenoids, besides downregulating the carotenogenic gene. This, in turn, lowered the levels of carotenoids in petals (Watanabe et al., 2017). From the reports of Shi et al. (2021), an increased concentration of phenolic acid was observed in the bZIP2 knock-out lines of *Salvia miltiorrhiza* (Bunge) which was generated using CRISPR/Cas9 methods. Contrarily, overexpression of bZIP2 in the hairy roots of *S. miltiorrhiza* (Bunge) resulted in a reduced concentration of phenolic. It was also observed that bZIP2 binds to the ABRE2 element in the PAL gene promoter region and reduces its expression (Shi et al., 2021). In *S. miltiorrhiza* (Bunge), overexpression of *SmbHLH60* inhibited biosynthesis of anthocyanin and phenolic acid significantly by transcriptionally suppressing specific genes such as *SmDFR* and *SmTAT*. However, CRISPR/Cas9 mediated knock-out of SmbHLH60 resulted in upregulation of anthocyanin and phenolic acid biosynthesis within the plant (Liu et al., 2022). The CRISPR/Cas9 technique was used to knock out four genes that encode for the enzyme germacrene A synthase (CiGAS). This enzyme further helps in the synthesis of sesquiterpene lactones (STL). Therefore, inhibition of the STL biosynthesis pathway resulted in an upregulation of phenolic concentration as well as increased accumulation of squalene in the taproot of *Cichorium intybus* (L.) (Cankar et al., 2021). The phenylpropanoid pathway is responsible for biosynthesizing lignin monomers, and caffeoyl shikimate esterase (CSE) synthesizes caffeic acid from caffeoyl shikimate. By the use of CRISPR/Cas9, high saccharification efficiency was obtained via creating weak alleles rather than knocking out CSE1 and CSE2 genes. This technique also avoids yield penalty in *Populus tremula* (L.) *x P. alba* (L.) (de Vries et al., 2021).

4 | APPLICATIONS OF THE CRISPR/CAS GENOME EDITING TOOL IN MEDICINAL PLANTS FOR PHYTOCHEMICALS

Being a precise and efficient genome editing technology, CRISPR/Cas9 improves quality, interprets pathways, and upscales valuable secondary metabolites. This is because this technology can modulate the phytochemical profile in medicinal plants, which results in the production of plant-derived products suitable for commercial purposes (B. Li et al., 2017). In the following section, the application of CRISPR/Cas to improve the production of bioactive phytochemicals in different medicinal plants is highlighted (Table 1).

4.1 | *Dendrobium officinale* (Kimura and Migo) (Orchidaceae)

D. officinale (Kimura and Migo) is a famous traditional Chinese herb-tonic that is used as a remedy for yin-deficiency diseases, such as general dryness, hot palms, red face, or even insomnia (Tang et al., 2017; Teixeira da Silva and Ng, 2017). In previous days, the ability of this plant to balance *Yin and Yang* was analyzed to have high usage as tea. Antioxidant, antifatigue, gastroprotective, and neuroprotective are some of the pharmacological attributes of this plant, besides almost 190 phytochemicals (Tang et al., 2017). The aim behind the use of the CRISPR/Cas9 gene knock-out system in this plant was to alter its gene expression. In *D. officinale* (Kimura and Migo), the use of C3H, C4H, 4CL, CCR, and IRX genes associated with the lingo-cellulose biosynthesis pathway resulted in the production of endogenous edited genes (Kui et al., 2017).

4.2 | *Salvia miltiorrhiza* (Bunge) (Lamiaceae)

It is also a Chinese traditional medicinal herb that is used significantly against cerebrovascular, cardiovascular diseases, and diabetes. Its anticancer, antioxidant and anti-inflammatory properties are due to the presence of diterpenoids and phenolic acid (Fan et al., 2019). Zhou et al. (2018) employed CRISPR/Cas9 techniques to alter the rosmarinic acid synthase (SmRAS) gene in the biosynthesis pathway of water-soluble phenolic acid in *S. miltiorrhiza* (Bunge) (Zhou et al., 2018). This study indicates that the content of phenolic acids, such as rosmarinic acid (RA) decreased in altered hairy root lines, particularly in the homozygous mutants. Furthermore, the importance of 3, 4-dihydroxy phenyl lactic acid increased, which is a precursor of RA precursor. The authors proposed that the rosmarinic acid synthase enzyme is important in the production of RA and may be used as a metabolic target to enhance the number of target metabolites. As a result, CRISPR/Cas technique allows researchers to alter the route toward the targeted phyto-metabolites.

B. Li et al. (2017) used CRISPR/Cas9 in *S. miltiorrhiza* (Bunge) to precisely suppress the diterpene synthase (SmCPS1) gene, which is important in the tanshinone biosynthesis pathway. Tanshinones, particularly tanshinone I, tanshinone IIA, and cryptotanshinone were found to be completely absent in homozygous mutants with no influence on the other phenolic acid constituents. Tanshinones, on the other hand, decreased, but remained detectable in chimeric mutants.

TABLE 1 Application of CRISP/Cas in different medicinal plants

Plants	Family	Observation	References
Dendrobium officinale (Kimura & Migo)	Orchidaceae	In the lignocellulose biosynthesis pathway, 5 target genes namely C4H, C3H, CCR, IRX I, and 4CL were altered using the CRISPR/Cas system.	Kui et al. (2017)
Salvia miltiorrhiza (Bunge)	Lamiaceae	• CRISPR/Cas9 techniques were used to alter the rosmarinic acid synthase (SmRAS) gene in the biosynthesis pathway of water-soluble phenolic acid. • CRISPR/Cas9 was used to suppress the diterpene synthase (SmCPS1) gene which is important in the tanshinone biosynthesis pathway and tanshinone I, tanshinone IIA, and cryptotanshinone, were found to be completely absent in homozygous mutants, with no influence on the other phenolic acid constituents.	Zhou et al. (2018); B. Li et al. (2017)
Papaver somniferum (L.)	Papaveraceae	• CRISPR/Cas was used to modify the biosynthesis of benzylisoquinoline alkaloids (BIAs) via targeting the 4′OMT2 gene. • Silencing 4′OMT2 gene by CRISPR/Cas leads to reduced synthesis of S-reticuline.	Alagoz et al. (2016); Hashemi and Naghavi (2016)
Dioscorea zingiberensis (C.H. Wright)	Dioscoreaceae	CRISPR/Cas editing was carried out in the *farnesyl pyrophosphate synthase* (Dzfps) gene, resulting in lower levels of squalene and Dzfps transcript.	Feng et al. (2018)
Nicotiana tabacum (L.)	Solanaceae	• 6 glycosyltransferase genes were suppressed by CRISPR/Cas • Two genes namely *NtPDS* and *NtPDR6* in this plant were knocked out. • CRISPR/Cas inactivated two genes responsible for the synthesis of glycoprotein *α-1,3-fucosyltransferase* and *β(1,2)-xylosyltransferase* genes in BY-2 Cells	Gao et al. (2015); Mercx et al. (2017); Jansing et al. (2019)
Camelina sativa (L.) Crantz	Brassicaceae	• *Fatty acid desaturase 2* (FAD2) genes were knocked out so that the content of oleic acid can be increased in this plant. • FAE1 genes were knocked out, which reduced the C20-C24 very-long-chain fatty acids (VLCFAs) by more than 60%.	Jiang et al. (2017); Ozseyhan et al. (2018)
Artemisia annua (L.)	Compositae	Genes encoding β-farnesene synthase (BFS), caryophyllene synthase (CPS), and squalene synthase (SQS) enzymes were knocked out by CRISPR/Cas to improve artemisinin content in this plant.	Lv et al. (2016)
Rehmannia glutinosa (Gaertn.) DC.	Plantaginaceae	RgPDS gene encoding albino was edited via CRISPR/Cas.	Li et al. (2021)
Trifolium pratense (L.)	Leguminosae	CRISPR/Cas9 was utilized to inhibit the activity of isoflavone synthase, a crucial enzyme in the manufacture of isoflavones (IFS1) which leads to lower levels of the biochanin A, genistein, and isoflavones mononetin.	Dinkins et al. (2021)

4.3 | *Papaver somniferum* (L.) (Papaveraceae)

Biosynthesis of opium poppy with benzylisoquinoline alkaloids (BIAs) results in morphine that has clinical significance (Labanca et al., 2018). Morphine boosts the reward response of the brain, having higher uses in the central nervous system. To target 4′ OMT2, the gene-controlling BIAs biosynthesis that is, type II CRISPR/Cas9-based gene knockout system was used. The use of agrobacterium infection in the *P. somniferum* (L.) produced TRV-based synthetic binary plasmids. This ended up causing manipulation of the biosynthetic flux of BIAs (Alagoz et al., 2016). Hashemi and Naghavi (2016) revealed that silencing 4′OMT2 causes a drop in S-reticuline levels, implying that it plays an important role in morphine production. Furthermore, a unique uncharacterized alkaloid was discovered in the modified plants, demonstrating the CRISPR system's potential for detecting new secondary metabolites (Tong et al., 2019).

4.4 | *Dioscorea zingiberensis* (C.H.Wright) (Dioscoreaceae)

Dioscorea sp. is known to produce diosgenin (steroidal hormones) with anti-inflammatory, antiallergic, cardiovascular, antitumor, and neuroprotection effects. Its rhizomes have significant utilities to isolate diosgenin and also in Dun-Ye-Guan-Xin-Ning tablets (Feng et al., 2018). The first CRISPR/Cas9 editing tool employed in this plant was using *Agrobacterium tumefaciens*-mediated transfection. Promoters, such as 35S and OsU3 drove Cas9 and single-guided RNA (sgRNA) expression cassettes, respectively. In the case of the

secondary metabolite squalene, high mutant frequency has been possible because of editing the *farnesyl pyrophosphate synthase* (Dzfps) gene, resulting in lower levels of squalene and Dzfps transcript (Feng et al., 2018).

4.5 | *Nicotiana tabacum* (L.) (Solanaceae)

This plant produces abundant secondary metabolites with biomedical, industrial, and pharmaceutical significance. When the CRISPR/Cas9 system was initially used to knock out two genes, namely *NtPDS* and *NtPDR6* in tobacco using multiplexing gRNA, it resulted in mutation percentages of 81.8% and 87.5% respectively (Gao et al., 2015). The inactivation of six glycosyltransferase genes in this plant, as mediated by CRISPR/Cas9, resulted in the production of recombinant proteins (Jansing et al., 2019). Gene activation using the CRISPR/Cas9 genome revealed that 7 out of 20 transformations demonstrated a shortened fragment. This signifies deletion between two target sites (Mercx et al., 2016). When the multiplex CRISPR/Cas9 tool was employed to produce glycoproteins without plant-specific glycans, it inactivated *α-1,3-fucosyltransferase* and *β(1,2)-xylosyltransferase* genes in BY-2 Cells of *N. tabacum* (L.). Further, when these cells were transformed with Cas9, sgRNAs, containing three XylT and six FucT sgRNAs, and a selectable marker coding gene, it resulted in knocking out two XylT along with four FucT genes (Mercx et al., 2017).

4.6 | *Camelina sativa* (L.) Crantz (Brassicaceae)

This is a flowering plant of the Brassicaceae family, commonly known as camelina, which has remarkable industrial use due to its distinguishing seed oil fatty acid composition. Its biotechnological improvement has made it suitable for cosmetics, nutrition, and pharmaceuticals (Sainger et al., 2017). Recent scientific investigations have revealed the use of *Camelina* as a medicinal plant, owes to its antioxidant, insulin sensitivity enhancer, anti-inflammatory, and anticancer properties. Oil extracted from *Camelina* is found to be an alternative source of polyunsaturated fatty acid (PUFA) (Dharavath et al., 2016). Despite the limitations of the CRISPR/Cas9-based editing tool in polyploid plant genomes, it was successfully hexaploid *C. sativa* (L.) Crantz to knock out *fatty acid desaturase 2* (FAD2) genes to increase the content of oleic acid (W. Z. Jiang et al., 2017). This was firstly done by reducing undesirable PUFA, namely linoleic and linolenic acid, and secondly, by increasing the content of oleic acid to above 50% from 16% (W. Z. Jiang et al., 2017). Increased contents of oleic acid and decreased PUFA in the seed oil in the hexaploid *C. sativa* (L.) Crantz were obtained via mutagenesis of three FAD two genes by the CRISPR/Cas9 genome-editing tool. Another experiment of CRISPR/Cas9 technology to knock out mutagenesis in *C. sativa* (L.) Crantz resulted in mutations in the FAE1 genes. This reduced the C20–C24 very-long-chain fatty acids (VLCFAs) by more than 60% (Ozseyhan et al., 2018). These mutants, however, demonstrated normal seed physiology along with plant growth, which gained preferences for industrial use.

4.7 | *Artemisia annua* (L.) (Compositae)

The biosynthesis of desired substrates can be directed effectively by removing competing routes. Lv et al. (2016) used this procedure to inhibit genes encoding the following enzymes at branching points, β-farnesene synthase (BFS), caryophyllene synthase (CPS), and squalene synthase (SQS). He further suggested that this technique can be useful to improve the artemisinin content in *A. annua* (L.), which is an important plant having antimalaria properties. Furthermore, artemisinin levels were increased in anti-SQS, anti-BFS, and anti-CPS transgenic plants. As a consequence, CPS, SQS, and BFS appear to be viable targets for the CRISPR system to direct the flow of substrates toward artemisinin (Lv et al., 2016).

4.8 | *Rehmannia glutinosa* (Gaertn.) DC (Plantaginaceae)

R. glutinosa (Gaertn.) DC is a crucial traditional Chinese medicine (TCM) herb with distinct pharmacological and commercial significance. The PDS gene has been employed as a marker gene to test the CRISPR/Cas9 system's application and gene editing effectiveness in *R. glutinosa* (Gaertn.) DC (X. Li et al., 2021). The Agrobacterium-mediated CRISPR/Cas9 system was used to genetically modify *R. glutinosa* and effectively regenerate albino and chimeric albino plants. The NGS verified that the albino phenotype was produced by RgPDS gene target site editing, and it was discovered that base deletion was more prevalent than insertion or replacement. The introduction of the CRISPR/Cas9 system resulted in effective site-directed editing in the genome of the *R. glutinosa* (Gaertn.) DC, which further contributes to the advancement of molecular breeding to develop desirable *R. glutinosa* (Gaertn.) DC phenotypes (X. Li et al., 2021).

4.9 | *Trifolium pratense* (L.) (Leguminosae)

Anthocyanidins, isoflavone, flavan-3-ols, flavanones, flavanols, and flavones are among the phenylpropanoid chemicals produced by *T. pratense* (L). Because of its pharmacological, antibacterial, and environmental effects, the study of isoflavone production and accumulation in legumes has risen to the forefront of biomedical and agricultural research. CRISPR/Cas9 was utilized to inhibit the activity of isoflavone synthase, a crucial enzyme in the manufacture of isoflavones (IFS1) (Subramanian et al., 2005). A hemizygous plant with a 9-bp deletion in the IFS1 gene was retrieved and crossed to produce homozygous mutant plants. The mutant plants have much lower levels of biochanin A, genistein, and isoflavones mononetin. The expression of the upstream genes that produce the precursors for IFS1, specifically chalcone synthase and phenylalanine ammonium

lyase increased, although there were no considerable changes in IFS1 transcriptional activity or in the downstream genes that produce particular isoflavones. The mutant plants have a higher transcription of genes encoding under ethylene response (Dinkins et al., 2021). Therefore, CRISPR/Cas helped in revealing the function of genes involved in the biosynthesis of phenylpropanoid chemicals within red clover.

5 | COMMERCIAL APPLICATION OF CRISPR IN MEDICINAL PLANTS

Over 10,000 medical plant species have been discovered, although the majority of therapeutic plants are found in the wild. Such medicinal plants have various drawbacks, such as significant habitat damage, dispersed distribution, and low yield stability, all of which limit the long-term availability and growth of medicinal herbs. Increasing the domestication of such plants aids in the transformation of wild medicinal herbs into domestic plants. This also helps in the preservation of endangered wild medicinal plant germplasm reserves, and the long-term use of ethnomedicinal ingredients. Furthermore, this strategy can help to domesticate unwanted traits and harmonize the production and administration of therapeutic plants. Economic and biological yields are included in the total yield of medicinal plants. The yield of medicinal components is referred to as the economic yield whereas, the biological yield is primarily the entire dry matter produced by photosynthesis. The application of CRISPR-based gene-editing technology to increase the output of therapeutic plants may be done in two ways. The photosynthesis reactions require the involvement of specific enzymes in the conversion of light energy into stable chemical energy of organic matter. So, at first, the associated enzyme gene is either overexpressed or knocked out for identifying the function of that particular gene (Kim et al., 1996). Therefore, any further beneficial alteration of gene expression could lead to improved photosynthetic products. Secondly, CRISPR may be utilized to modulate the expression of essential functional genes, which regulates the yield and growth of therapeutic components in medicinal plants.

6 | CHALLENGES OF CRISPR/CAS

Methods, such as polyethylene glycol (PEG)-mediated transformation, Agrobacterium-mediated, particle bombardment techniques, and biolistic transformation are currently used for CRISPR/dCas9 delivery. However, because of greater efficiency and reduced cost, agrobacterium-mediated transformation is by far the most frequently used approach. However, the necessity of a binary vector and the integration of a gene into the plant's genome is still a significant disadvantage. On the other hand, PEG-mediated transformation is a time-consuming method because it requires the tedious preparation of protoplasts. Whereas the particle bombardment method does not necessitate the culture of T-DNA-containing binary vectors and isolation of protoplasts, the integration of DNA fragments can occur randomly. The inefficiency of CRISPR/dCas9 delivery and the incapability of plant cells to regenerate into plants continue to be major obstacles to identifying the technology's potential (Agapito-Tenfen et al., 2018; Eckerstorfer et al., 2019). Furthermore, CRISPR/Cas9 requires the PAM sequences for Cas9 to identify the gene of interest. The selectivity of the CRISPR/Cas9 is determined by the PAM sequence. Moreover, if a genome has a limited number of PAM sites, this could restrict the use of CRISPR/Cas9. This restriction applies to genome editing via CRISPR/Cas9 system as well. The new revelation of Cas proteins capable of identifying various PAM sequences may contribute to the functionality of the CRISPR/dCas9 system. CRISPR/Cas9 is likely a potential tool for studying gene functions rather than RNAi technology because it has greater on-target efficiency and fewer off-target effects (Larson et al., 2013).

In the United States, GM plants are usually given a nonregulatory status after the removal of the CRISPR/Cas system (Globus & Qimron, 2018). A browning-resistant mushroom developed via the CRISPR/Cas system was given non-regulatory status because no foreign DNA was detected within its genome (Waltz et al., 2016). Similarly, many such GM plants have been identified under non-regulatory status in the United States. Moreover, only 11.9% of the Chinese population supported GM foods, while 41.4% and 46.7% were neutral or negative, respectively (Cui & Shoemaker, 2018).

7 | CONCLUSIONS AND FUTURE PERSPECTIVE

The targeted genome-editing technologies, such as ZFNs, TALENs, and CRISPR/Cas9 systems have been used for the genetic and metabolic engineering of medicinal plants. Although some successful phytochemicals are produced from these technologies, many challenges persist. First and foremost, these tools are developed for introducing genomic modifications in the absence of DNA breaks. Initially, ZFNs and TALENs were widely chosen. However, the addition of the CRISPR/Cas9 system has transformed the gene-editing approaches because of many appealing characteristics, such as high efficiency, convenience of use, adaptability, and the capacity for multiplexed improvements. Further, in CRISPR/Cas9 technology, DNA cleavage recognition happens via Watson and Crick base pairing, and this significantly simplifies gene targeting. As a result, it is now the most encouraging genome engineering tool, which further showcases a promising future in creating the preferred genetic changes throughout plants. Given the lack of enough sequence data in several medicinal and aromatic plants, the CRISPR/Cas process was only evaluated in very few plants with medicinal properties at this time. However, intensive future studies will certainly unlock the maximum potential of using the CRISPR/Cas framework in many other plants with medicinal properties to classify the genes as well as enzymes involved in the synthesis pathway of various secondary metabolites. New advances show that the CRISPR/Cas9 technology is rapidly becoming the ideal molecular tool in gene editing,

particularly in medicinal plants, which have been modified with the help of CRISPR/Cas9 innovation. Multiplex genetic modification by the targeted elimination of a few genes or the up/downregulation of many genes will concurrently provide valuable agronomic features in target plants within complicated metabolic pathways in which the change of a single gene is not acceptable.

We may anticipate complicated features to be tweaked at will in the coming years as the CRISPR toolkit expands. Therefore, CRISPR/Cas9 has the exciting ability to modify biosynthetic pathways across heterologous medicinal plants using an artificially created and precisely regulated genetic network to enhance the output of biopharmaceutical output. Nevertheless, to get a much higher influence on medical plant biology, additional efforts must be made to enhance the CRISPR/Cas9 procedures so that they are more user-friendly as well as widely available for study and applications.

AUTHOR CONTRIBUTIONS

Sicon Mitra: Literature survey, writing-original draft preparation, prepared the tables and figures, addressed reviewer comments, arranged references. **Uttpal Anand:** Conceptualization, planned and designed the review structure, literature survey, writing – reviewing and editing, revised the manuscript, addressed reviewer comments and final draft. **Mimosa Ghorai, Nishi Kant, Manoj Kumar, Radha, Niraj K. Jha,** and **Mallappa K. Swamy:** Participated in review structure, writing-reviewing and editing, overall manuscript revision, response and suggestions. **Jarosław Proćków:** Revised the review structure, suggestions, completed the critical revision of the entire manuscript, supervised the drafting process of the review and resources. **José M. P. de la Lastra:** Study idea, writing, reviewing and editing, revised the manuscript, supervised the drafting process of the review, resources, project administration, and funding acquisition. **Abhijit Dey:** Came up with the study idea, revised the review structure, suggestions, completed the critical revision of the entire manuscript, supervision, and final draft. The authors **Sicon Mitra** and **Uttpal Anand** contributed equally to this review article and have the right to list their names first in their CVs/any other scientific documents or scientific profile. All authors have read and approved the final version of the manuscript for submission to this journal.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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