Chapter 3
CRISPR/Cas9: A Novel Genetic Tool to Manipulate Plant Secondary Metabolite Pathways

Muthukrishnan Arun, Jayachandran Halka, and Kumaresan Kowsalya

Abstract The CRISPR/Cas9 gene editing method has brought a new era in genetic engineering and genome research. However, CRISPR/Cas9 system incorporation has changed gene editing methods due to its numerous alluring features such as high efficiency, simplicity, adaptability, and the capacity for multiplexing improvements. This system creates minor heritable mutations in the genome, which is significant for elucidating secondary metabolite pathways in medicinal plants. Scientists are increasingly focusing on mining critical genes in metabolic pathways and developing novel synthetic approaches in order to boost the production of potent compounds. However, the use of CRISPR technology in medicinal plants is still in its early stages and also has several bottlenecks such as a lack of genome information and genetic transformation technology. This chapter highlights the applications of CRISPR/Cas9 technology in improving secondary metabolites in various medicinal plants such as *Atropa belladonna*, *Cannabis sativa*, *Cichorium intybus* L., *Dendrobium officinale*, *Dioscorea zingiberensis*, *Salvia miltiorrhiza*, *Rehmannia glutinosa*, *Symphytum officinale*, *Papaver somniferum* L., and *Monochasma savatieri*. It also discuss the future directions of using this approach in other medicinal plants for developing ideal germplasm, creating biotic, and abiotic stress-tolerant plants.

Keywords CRISPR/Cas9 · Secondary metabolite · Medicinal plant · Genetic improvement

3.1 Introduction

Genome editing is a genetic engineering technique in which DNA is inserted, altered, or substituted in the genome of an organism. Genome editing comprises of various techniques such as zinc finger nuclease (ZFNs), transcriptional activator-like

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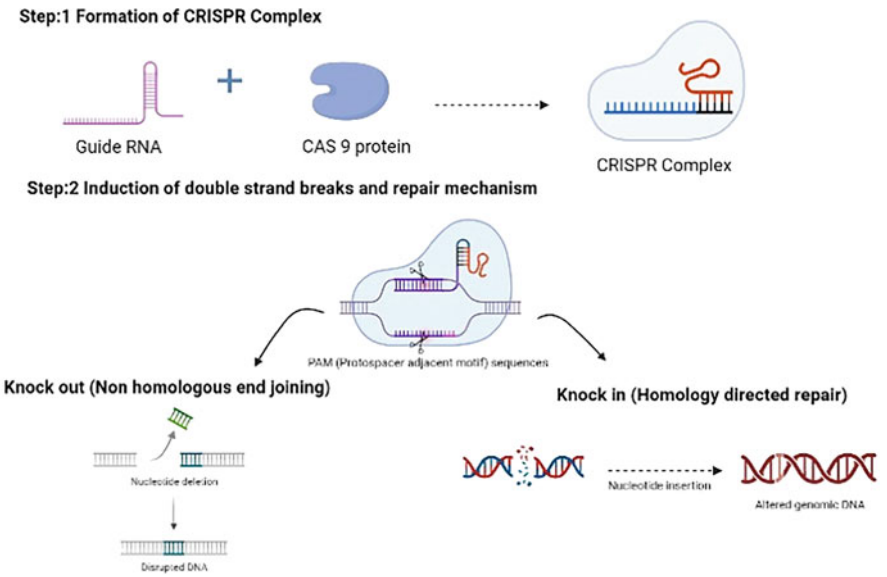

Fig. 3.1 Mechanism involved in CRISPR/Cas9 technique

effector nuclease (TALENs), and the most recently developed clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR associated nuclease 9 system. This technique was developed from the natural bacterial immune system which is distinct and effective in nature. Due to its low cost and easy adaptation, this method is employed for a variety of directed genome editing applications (Chandel 2023). The CRISPR-Cas system is divided into two functional classes based on the structure of the effector nuclease genes. The class I CRISPR system includes the type I, III, and IV systems which are identified by the multisubunit effector nuclease complexes. The class II CRISPR systems are categorized into type II, V, and VI14, according to the factors necessary for pre-crRNA processing and the diversity of the effector protein's domains. The class II CRISPR system has only one effector protein, called Cas9 (Devi 2022; Yildirim and Ekinci 2022). It also enables the scientific community to correlate the parallel relationship between the genetic makeup of plants and their respective biological attributes (Vidya and Arun 2023). CRISPR/Cas9 has two components called Cas9 and sgRNA. The sgRNA along with Cas9 complex cleaves the double-stranded DNA causing double-stranded breaks (DSB). When this break occurs, nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair mechanisms are initiated. Most of the time, the NHEJ repairs the double-stranded breaks. It is a straightforward method to produce mismatches and gene deletions (indel), which results in gene knockout. In the presence of an oligo template, homology-directed repair (HDR) causes selective gene substitution (insertion) called knock-in (Fig. 3.1) (Liu et al. 2017; Halka et al. 2022). Although homologous recombination is the cornerstone of genome

engineering, the effectiveness of editing is limited by its low frequency (Afzal et al. 2020). On top of that, the CRISPR/Cas system recently developed a few advancements such as CRISPR with no PAM (protospacer adjacent motif) limitation, CRISPRi gene knockdown, CRISPR with epigenetic modification, and small-sized new CRISPR systems (Manghwar et al. 2019). Recently, CRISPR/Cas9-based imaging methods were equipped with several new features to improve the fluorescence for effective visualization of chromatin and gene loci. It includes supernova tagging systems, RNA aptamers, halo tags, molecular beacons, bimolecular fluorescence complementation, and RNA-guided endonuclease in situ labeling (Singh and Jain 2022). Moreover, this effective gene editing also established the groundwork in medicinal plants for investigating the molecular activities of genes, producing top-notch germplasm, hastening domestication, and boosting the secondary metabolite's productivity and quality. In addition to that, it advances plant molecular breeding by producing the most desirable features in medicinal plants (Li et al. 2021a). Only a few studies are available investigating the gene function in metabolic pathways of medicinal plants due to inadequate genome data, higher heterozygosity, and lack of genetic transformation technology. Besides, the lack of functional genomics research has impeded the development of restrictions in functional gene mining and genetic enhancement of medicinal plants. Yet, through the CRISPR/Cas9 technology, researchers have recently solved the hidden riddles with self-incompatible genes of *Rehmannia glutinosa* (Scrophulariaceae family) which prevent the production of homozygous lines through self-pollination by knocking out the *RgPDS1* gene (Li et al. 2021b). Hence, this chapter intends to summarize the potential applications of CRISPR/Cas9 in improving clinically significant secondary metabolites in different medicinal plants.

3.2 Applications of CRISPR in Medicinal Plants

CRISPR/Cas is a precise and efficient genome editing technique that improves the quality, infers pathways, and even upgrades valuable secondary metabolites. This technique is helpful in modulating the phytochemical profile of medicinal plants and even increases the production of plant-derived metabolites suitable for commercial purposes (Li et al. 2017). Genetic or metabolic engineering methods are used to manipulate the production of secondary metabolites obtained from plants because these methods are capable of changing a number of the genes involved in the biosynthetic pathway. Any uncertainty in the biosynthetic pathway is likely due to the alterations observed in the transcription level of the whole system. These alterations may change the plant's regulatory system which in turn is found to control secondary metabolite productions in medicinal plants. Further, when the rate-limiting enzyme is overexpressed, there is a drastic change in the transcriptional level for the closely related secondary metabolite genes (Rehman Summia et al. 2021). Further, the applications of CRISPR/Cas for the production of secondary metabolites in medicinal plants are summarized (Fig. 3.2 and Table 3.1).

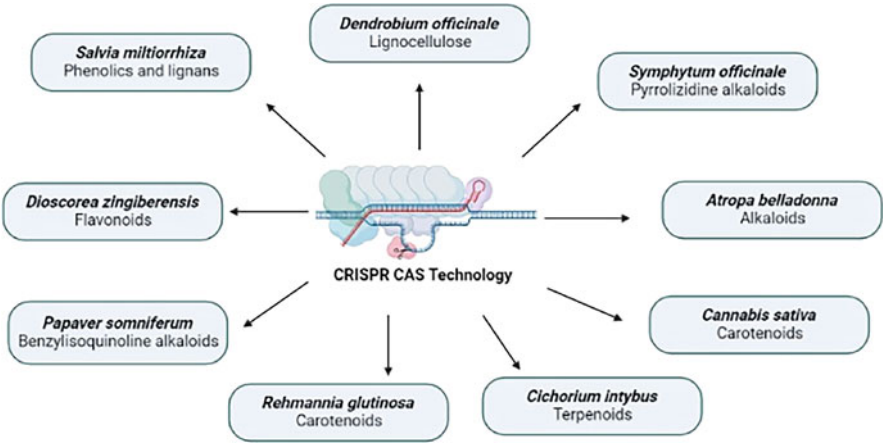

Fig. 3.2 Summary of CRISPR/Cas9 technology in medicinal plants for developing various secondary metabolites

3.2.1 Atropa belladonna

Atropa belladonna L. is one of the most important herbal plants with anisodamine, scopolamine, hyoscyamine, and atropine metabolites. Zeng et al. (2021) developed *A. belladonna* plants for the first time devoid of anisodamine and scopolamine utilizing the CRISPR/Cas9 technology to disrupt *hyoscyamine 6-hydroxylase* (*AbH6H*). It was observed that out of 11 transgenic plants, only 4 plants exhibited mutation and the mutation rate was approximately 63.6%. Thus, hyoscyamine synthesis was considerably increased in the *A. belladonna* plants, but neither anisodamine nor scopolamine was produced. Hasebe et al. (2021) claimed that the disruption of *pyrrolidine ketide synthase* (*PYKs*) reduces the accumulation of tropane alkaloids in *A. belladonna* using CRISPR/Cas 9 technology.

3.2.2 Cannabis sativa

Cannabis sativa is an annual and dioecious crop. Since it is enriched with phytocannabinoids, the demand for this plant is substantially increasing. Zhang et al. (2021) edited the *phytoene desaturase* (*CsPDS1*), a well-linked marker gene, in *C. sativus* using the CRISPR/Cas9 technology. Further, they developed four transgenic plants with albino phenotype by using *Agrobacterium*-mediated transformation system.

Table 3.1 List of medicinal plants improved by CRISPR/Cas9 technology

Species	Target gene	Secondary metabolite	Vector	Promoter	Mutation frequency	References
Salvia miltiorrhiza	*Committed diterpene synthase* (*SmCPS1*)	Tanshinone biosynthesis	pCAMBIA 1300	CaMV35S/ AtU6–26	11.5%	Li et al. (2017)
Salvia miltiorrhiza	*Rosmarinic acid synthase* (*SmRAS*)	Phenolic biosynthetic pathway	pCAMBIA 1300	CaMV35S/ AtU6–26,OsU3	50%	Zhou et al. (2018)
Salvia miltiorrhiza	*Laccase genes* (*SmLACs*)	Phenolic acid and lignin biosynthetic pathway	pCAMBIA 1300	AtUBQ/AtU6	90.6%	Zhou et al. (2021)
Salvia miltiorrhiza	*Basic leucine transcription factor* (*SmbZIP2*)	Phenolic acid biosynthetic pathway	pCAMBIA 2300	CaMV35S/ AtU6–26	12%	Shi et al. (2021)
Dendrobium officinale	*Coumarate 3-hydroxylase (DoC3H), Cinnamate 4-hydroxylase (DoC4H), 4-Coumarate coenzyme A ligase (Do4CL), and Irregular xylem5 (DoIRX)*	Lignocellulose biosynthetic pathway	pCAMBIA 1301-35SN	MtHP, CVMV, MMV, PCISV, CaMV 35S/OsU3	16.7%, 20%, 33.3%, 33.3%, and 6.7% for *C3H*, *C4H*, *4CL*, *CCR* and *IRX*	Kui et al. (2016)
Cannabis sativa	*Phytoene desaturase* (*CsPDS1*)	Carotenoid biosynthesis	pKSE401	AtU6	2.5% and 51.6% for the homozygous and chimeric mutants	Zhang et al. (2021)
Papaver somniferum	*3s-Hydroxyl-N-methylcoclaurine 4′-o-methyltranferase* (*Ps4'OMT2*)	Biosynthesis of benzylisoquinoline alkaloids	pK7WGF2	CaMV35S/AtU6	85%	Alagoz et al. (2016)
Dioscorea zingiberensis	*Farnesyl pyrophosphate synthase* (*Dzfps*)	Biosynthesis of squalene	pCAMBIA 1300	CaMV35S/OsU3	60%	Feng et al. (2018)
Rehmannia glutinosa	*Phytoene desaturase* (*RgPDS*)	Carotenoid biosynthesis	PKSE401	AtU6–26	Produced 45.5% albino phenotype with reduced	Li et al. (2021a, b)
Cichorium intybus	*Germacrene A synthase* (*CiGAS*)	Biosynthesis of sesquiterpene lactones	PEG mediated transfection	–	–	Cankar et al. (2021)

(continued)

Table 3.1 (continued)

Species	Target gene	Secondary metabolite	Vector	Promoter	Mutation frequency	References
Symphytum officinale	*Homospermidine synthase* (*SoHSS*)	PA biosynthetic pathway	pDE	AtU6–26	–	Zakaria et al. (2021)
Atropa belladonna	Disruption of *hyoscyamine 6 β-hydroxylase* (*AbH6H*)	Alkaloid biosynthesis	pCAMBIA 1300	CaMV35S/AtU6	63.6%	Zeng et al. (2021)
Atropa belladonna	*Pyrrolidine ketide synthase (AbPYKS)*	Tropane alkaloids	pMGP237-T1-T4	–	–	Hasebe et al. (2021)
Monochasa savatieri	*Cellulose synthase like D* (*MsCSLD3*)	Phenolic biosynthetic pathway	pRGEB31	CaMV35S/AtU6	6.49%	Bai et al. (2023)

3.2.3 Cichorium intybus *L.*

Cichorium intybus var. sativum is a type of industrial crop grown for extracting prebiotic and low-calorie sweeteners called inulin. The chicory taproot is accumulated with squalene and more phenolic chemicals. These compounds have a punitive taste and could not be eliminated during inulin extraction. Thus, CRISPR/Cas9 technique was able to inactivate the genes responsible for encoding the *germacrene synthase* (*CiGAS*). As a result of blocking the STL biosynthesis pathway, there is a reduced STL level which helps in facilitating inulin extraction without bitter-tasting compounds, and there is a substantial increase in the availability of farnesyl pyrophosphate (FPP) which increases the phenolic content in the chicory roots (Cankar et al. 2021).

3.2.4 Dendrobium officinale

Dendrobium officinale is a valuable medicinal herb, used in medical treatment for more than 2000 years. It possesses a broad spectrum of medicinal qualities, including hepatoprotective (Liang et al. 2018), antitumor (Liang et al. 2019), hypoglycemic (Chen et al. 2020), gastro-protective (Zhang et al. 2019), and anti-inflammatory (Yang et al. 2020) properties. Four genes such as *coumarate 3-hydroxylase* (*C3H*), *cinnamate 4-hydroxylase* (*C4H*), *4-coumarate coenzyme A ligase* (*4CL*), and *irregular xylem5* (*IRX*) in the lignocellulose biosynthesis pathway were successfully altered. Additionally, they measured the mutation rates of several target sites between 10% and 100% using PCR amplification and sequencing techniques (Kui et al. 2016).

3.2.5 Dioscorea zingiberensis

Dioscorea sp. is well recognized for producing diosgenin, which is steroidal hormone with anti-inflammatory, anti-allergic, cardiovascular, antitumor, and neuroprotective actions. Its rhizomes are extremely useful for isolating diosgenin and for making Dun-Ye-Guan-Xin-Ning tablets. Feng et al. (2018) reported that CRISPR/Cas9 editing tool used in this plant was carried out using the *Agrobacterium*-mediated transformation method. High mutant frequency for the secondary metabolite squalene was made possible by excluding the *farnesyl pyrophosphate synthase* (*Dzfps*), which led to decreased levels of squalene.

3.2.6 Monochasma savatieri

Monochasma savatieri is a perennial medicinal plant that is extensively used for treating various diseases. In this study, Bai et al. (2023) successfully transformed *M. savatieri* hairy root mutants through the targeted knockout of the *CSLD2/3* in a phenolic biosynthetic pathway using CRISPR/Cas 9 technique.

3.2.7 Salvia miltiorrhiza

Salvia miltiorrhiza is a Chinese medicinal herb that belongs to the family Labiatae and is widely used for treating cardiovascular and cerebrovascular diseases and diabetes (Ren et al. 2019). Due to the presence of lipid-soluble compounds such as tanshinones and water-soluble phenolic acids such as rosmarinic acid, salvianolic acid, and lithospermic acid, this plant is in great demand (Luo et al. 2014). Another study reported that CRISPR/Cas technique is helpful in knocking out the *SmCPS1*, which is an effective *diterpene synthase* involved in tanshinone biosynthesis. Further, it is reported that three homozygous and eight chimeric transgenic hairy root mutants were produced through *Agrobacterium*-mediated transformation in *Salvia.* Three major predominant tanshinones such as tanshinone I, tanshinone IIA, and cryptotanshinone are completely absent in homozygous mutants. These results demonstrated that this gene is crucial for the formation of tanshinones and laid the groundwork for further research into the production of secondary metabolites in *Salvia miltiorrhiza* (Li et al. 2017). Zhou et al. (2018) successfully generated *S. miltiorrhiza* hairy root mutants by employing CRISPR/Cas9 technology to knock down the *rosmarinic acid synthase SmRAS* in the phenolic acid synthesis pathway. From 16 distinct transgenic hairy root lines, a total of 5 biallelic, 1 homozygous, and 2 heterozygous mutants were produced. The mutants had higher concentrations of the RA precursor 3,4-dihydroxyphe nyllactic acid, whereas phenolic acids like RA, salvianolic acid B (SAB), and salvianolic acid had much lower concentrations.

Recent studies reported that CRISPR/Cas9 dual-locus editing technique was able to eliminate more than 20 genes from the laccase family in *S. miltiorrhiza*. The expression levels of the target laccase genes and critical genes for phenolic acid production were dramatically increased in the editing lines. The formation of hairy roots was also greatly slowed down in the CRISPR lines. These results revealed the function of *SmLACs*, which are crucial for phenolic acid synthesis as well as root growth and lignin formation in *S. miltiorrhiza* (Zhou et al. 2021). Shi et al. (2021) targeted *SmbZIP2*, a new basic leucine zipper transcription factor identified from *S. miltiorrhiza*, using overexpression (OE) and the CRISPR/Cas9 technique. Further, analyzing the transgenic lines showed that the phenolic acid content was increased in the CRISPR/Cas9 lines but decreased in the OE lines. The study showed

that *SmbZIP2* acts as a negative regulator in phenolic acid biosynthesis offering a unique strategy for the production of phenolic acid.

3.2.8 Rehmannia glutinosa

Rehmannia glutinosa is a vital component of traditional Chinese medicine that has special pharmacological and economic importance. In this study, it is successfully modified with the CRISPR/Cas9 cassette by precise editing of the *RgPDS1*. More intriguingly, a few partially albino shoots were able to develop in MS medium in a similar way to wild-type plants, indicating that the chlorophyll and carotenoid content in the leaves of these *PDS* mutants were still present with special emphasis in molecular breeding to generate desired traits into the plant (Li et al. 2021a, b).

3.2.9 Symphytum officinale

Comfrey, also known as *Symphytum officinale* L. Boraginaceae, is a plant with anti-inflammatory, analgesic, and proliferative properties. Its potential role in pharmaceuticals is constrained by the large concentrations of toxic pyrrolizidine alkaloid (PA) throughout the entire plant. Zakaria et al. (2021) claimed that CRISPR/Cas9 technique helps in eliminating *homospermidine synthase* (*HSS*), the first specialized enzyme in the PA biosynthesis pathway. The homospermidine and PA concentrations in the hairy roots (HRs) appeared to have decreased, as evidenced by the successfully acquired HSS-deficient HRs. This work showed the effectiveness of using gene editing as well as the ability to create nontoxic transgenic comfrey varieties.

3.2.10 Papaver somniferum *L.*

Benzylisoquinoline alkaloids (BIAs) are used in the biosynthesis of the opium poppy to create the therapeutically relevant narcotic morphine. Morphine heightens the brain's reward response because it has stronger effects on the central nervous system. CRISPR/Cas9-based gene knockout systems were able to specifically target the gene *4′ OMT2*, which is involved in the manufacture of BIAs (morphine, codeine, s-reticuline, noscapine, thebaine, laudanosine, and papaverine) (Alagoz et al. 2016).

3.3 Limitations of CRISPR/Cas9 Technique

This genome editing technique also possesses a few disadvantages such as off-targets of the desired gene, the identification of the genetic makeup related to particular plant traits, the competent delivery of the CRISPR/Cas9 construct to plant cells, and a reliable plant transformation system. In recent years, these shortcomings have been effectively overcome by the CRISPR/Cas variants and Cas9 orthologs with high precision and specificity (Fig. 3.3) (Vidya and Arun 2023).

3.4 Future Perspectives

This CRISPR/Cas9 technique has emerged as a promising tool in altering the medicinal plant genome, which subsequently escalates their metabolic profile. This can result in user-designed medicinal plants that aid in the large-scale production of commercially important secondary metabolites (Niazian 2019). It revolutionizes the agricultural field by enhancing the nutritional content as well as yield while making them more resilient to biotic and abiotic stresses. These upgraded plant attributes are much more significant and most required to satisfy the demands of a growing global population worldwide (El-Mounadi et al. 2020). On the other hand, a quick method for creating germplasm with disease and herbicide-resistance traits in medicinal plants is made possible by CRISPR/Cas9 technology. Also, researchers can create these systems that can remove harmful genetic elements or induce gain-of-function mutations by carefully altering the genome of therapeutic medicinal plants. CRISPR/Cas9 technique is the most efficient, safest, and cost-effective strategy to control diseases which encourages the sustainable cultivation of medicinal plants (Guo et al. 2022). Moreover, this technique also helps us to unravel the most complex

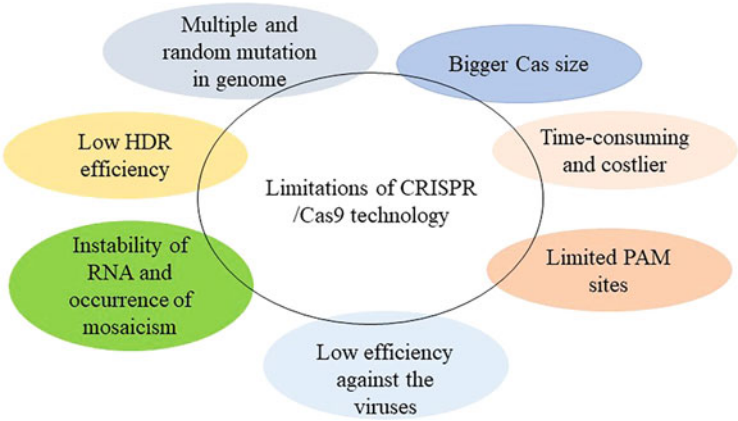

Fig. 3.3 Limitations of CRISPR/Cas9 system

biosynthetic network and multiple regulatory functions of secondary metabolites in medicinal plants (Li et al. 2021a, b). In order to use this technique efficiently, the scientific community must solve the different biosafety and societal problems around this technology in the long term. However, there is a strong need for the re-evaluation of the laws governing genome-edited medicinal plants and the creation of biosafety awareness among the people (El-Mounadi et al. 2020).

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