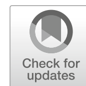
Chapter 8
CRISPR/Cas Genome Editing in Engineering Plant Secondary Metabolites of Therapeutic Benefits



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Abstract Plants hold the ability to produce wide types of bioactive secondary metabolites. Having emerged in the pregenomic era, increasingly more biosynthetic genes are being discovered in plants, leading to the discovery of new types of

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bioactive secondary metabolites. Utilisation of classical techniques is limited that hampers the discovery of pharmacologically important secondary metabolites. However, the development of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated protein)-based tools may alleviate this impasse. This chapter briefly presents existing information about the CRISPR/Cas9 system, and by what implies it was engineered to enhance important secondary metabolite production in plants. CRISPR/Cas systems have been among the most versatile genome editing tools available, revolutionising molecular biology. This chapter intends to highlight and discuss the lasting challenges of CRISPR/Cas-based genome editing and the improvement of secondary metabolite amount in plant natural product engineering. The plants canvassed in this chapter include *Atropa belladonna*, *Brassica napus*, *Camelina sativa*, *Dendrobium officinale*, *Dioscorea zingiberensis*, *Glycine max*, *Humulus lupulus*, *Papaver somniferum* and *Salvia miltiorrhiza*. Additionally, we highlight the prospects of using CRISPR/Cas in plant secondary metabolite engineering.

Keywords CRISPR/Cas9 · Metabolites · Plant natural products · Medicinal plants genome editing

Introduction

For centuries, medicinal plants have been used by us for the production of a plethora of unique metabolites (Calabrò, 2015). Metabolites are biomolecules which formed during the metabolism of an organism. Metabolites have crucial roles in intracellular and intercellular signalling pathways, growth, development and defence against pathogens. Moreover, some metabolites are economically important for the production of drugs, flavourings, pesticides, aromatic compounds etc. (Hussain et al., 2012). There has been evidence that secondary metabolites of plants could serve as potential pharmaceutical leads for reducing human deaths (Dey et al., 2017b). Various natural compounds have been implicated in the reversibility of drug resistance (Das et al., 2021; Guo et al., 2017; Wang et al., 2015). The efficacy of secondary metabolites in medicinal herbalism has also been demonstrated by extensive clinical and preclinical studies (Anand et al., 2020; Kaur et al., 2020a, 2020b).

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For enhancing the production of valuable plant secondary metabolites, several biotechnological interventions have been applied (Dey et al., 2017a).

Plants produce different secondary metabolites that are restricted to specific groups of plants within the plant kingdom. In recent times, the world has shifted towards natural products due to their significant role in medicine without severe side effects. For that, evolving demand for secondary metabolites resulted in boundless interest mainly in the possibility of changing the production of bioactive plant metabolites via targeted metabolic engineering, elicitation, endophytes, bioreactors, precursor feeding, nanotechnology and other specialised biotechnological approaches (Calabrò, 2015; Hussain et al., 2012; Narayani & Srivastava, 2017; Verpoorte & Memelink, 2002). Metabolic engineering can be applied in several ways to increase metabolite yields, including by blocking catabolism, reducing competitive pathways and disabling rate-limiting steps (Verpoorte et al., 1999). Modulations in the production of plant metabolites can be accomplished by targeting regulatory genes for their biosynthetic pathways. Enhancement of secondary metabolite production can sometimes be achieved by targeting a single pathway gene that can act as a transcription factor involved in regulating multiple pathway genes or through knocking out the gene which is responsible for the suppression of natural product production (Staniek et al., 2013; Verpoorte & Memelink, 2002).

There is an increasing demand for genetic engineering strategies; however, conventional genetic engineering approaches have numerous shortcomings, such as being unable to manipulate the genomes of large plants. A variety of tools for genome editing at the transcriptional level such as zinc-finger nucleases (ZFNs), meganucleases (MNs), transcription activator-like endonucleases (TALENs) and CRISPR are helping to resolve the difficulties of specific genome editing of plants (Rehman et al., 2021; Chen & Gao, 2015). ZFNs and TALENs initiate genome editing by employing Fok-I, a protein-based endonuclease for gene knockout through producing double-stranded breaks (DSBs) in the DNA target which further arouse error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) at precise genomic positions (Gaj et al., 2013; Khan, 2019). However, in order to utilise the TALENs and ZFNs techniques, two distinct protein hybrid designs are required which can identify sparsely existing DNA flanks (Li et al., 2011). Furthermore, these techniques are lengthy and less precise, which prompted scientists to develop an efficient genome editing method. Successful gene manipulation of plants has been achieved with CRISPR and associated proteins, for example, CRISPR-associated protein Cas9 (Upadhyay & Sharma, 2014). Figure 8.1 presents the key steps of genome editing through the CRISPR tool in plants.

Background Study of CRISPR-Cas9

The CRISPR/Cas is part of the adaptive immune system of bacteria and a few archaea. Foreign particles like viruses and plasmids are repelled by the CRISPR/Cas mechanism. The foreign genetic material (DNA) will be sliced and reorganised

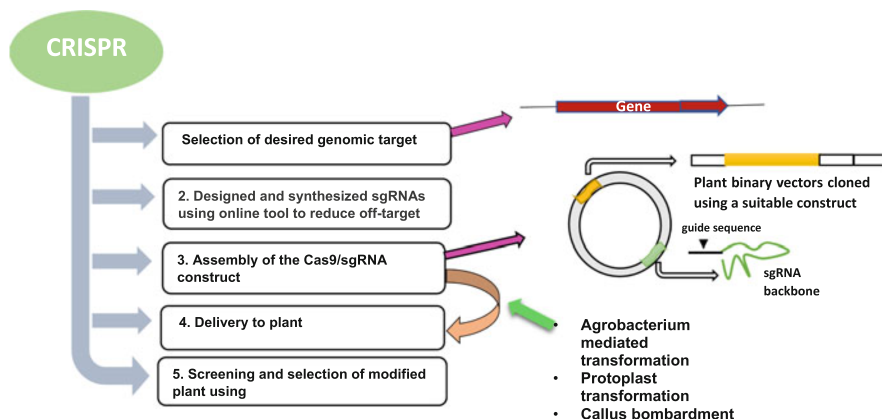

Fig. 8.1 The key steps of genome editing through the CRISPR technique in plants

and its genetic sequence preserved for when the same virus or plasmid invades again, the genetic material will be recognised (Koonin & Makarova, 2013; Koonin & Makarova, 2009). CRISPR/Cas system contains sgRNA (single guide RNA) and Cas9 endonuclease which form a complex that can cleave target DNA sites. CRISPR-Cas9 genome editing system is functioning by introducing DSBs (Jinek et al., 2012). Previous gene manipulating nuclease platforms such as ZFNs and TALENS, could initiate DNA DSBs; however CRISPR-Cas9 differs from ZFNs and TALENS as Cas9 nucleases form a complex with the sgRNAs that guide specific DNA sequences within host nuclei and initiate DSB induction (Jinek et al., 2012). The most likely outcome of a DSB is the initiation of NHEJ mechanisms producing various mutations such as indel and substitutions. By introducing frameshift mutations, NHEJ mechanisms can be used in breeding, mutant library construction and high-throughput mutational screening (Barakate & Stephens, 2016). DSB formation can also initiate HDR, although that is less likely. The CRISPR/Cas technology is a new, popular and powerful gene editing tool and has wide application in gene manipulation in the field of genomic research due to its cost-effectiveness and efficiency (Cui et al., 2018). There are numerous uses of CRISPR/Cas9 in genetically modifying both medicinal and food plants and model organisms (Alagoz et al., 2016; Malnoy et al., 2016; Ricroch et al., 2017; Shan et al., 2014; Singh et al., 2020). CRISPR/Cas9 has successfully edited approximately 32 different plants, but the application of this technology to medicinal plants is still limited. Therefore, this chapter intends to summarise data available in the past pertaining to ten medicinal plant genomes edited with CRISPR. CRISPR-Cas9 toolkit limitations and prospective are also discussed in this chapter.

Secondary Metabolite Engineering of Plants with CRISPR/Cas9

It is believed that there are over lakhs of secondary metabolites, which are classified by structure, function and biosynthesis. Secondary metabolites are divided into alkaloids, terpenoids and steroids, fatty acid-derived compounds, polyketides and nonribosomal polypeptides and enzyme cofactors (McMurry, 2015). CRISPR/Cas offers an alternative production strategy of commercially important plant-derived natural products by modulating the phytochemical profile of medicinal plants with CRISPR/Cas9 technology. Table 8.1 presents application of gene editing with CRISPR/Cas in medicinal plants for the production of valuable secondary metabolites. In Fig. 8.2 we have highlighted different plant metabolites and their genome editing by CRISPR/Cas9 action.

Atropa belladonna L.

Atropa belladonna L., alternatively referred to as deadly nightshade, is used extensively against anti-inflammatory and neuropharmacological disorders. Hyoscyamine is the major tropane alkaloid compound produced by *A. belladonna* which has activity against arrhythmias and organophosphate poisoning. This plant also produces the derivatives of hyoscyamine such as anisodamine and scopolamine (Hashimoto, 1992). Therefore, it is necessary to separate one compound from the others. However, due to their similar structures, this can be difficult to isolate compounds autonomously. So, it is crucial to develop *A. belladonna* without anisodamine and scopolamine, in order to achieve low costs for hyoscyamine separation. Additionally, hyoscyamine content in *A. belladonna* is very low and commercial demand for this compound is high; therefore, Zeng et al. (2021) develop high yields of hyoscyamine from *A. belladonna* plants for the first time, using CRISPR/Cas9 tool. The editing method, CRISPR/Cas9, disrupted the hyoscyamine 6β-hydroxylase (*AbH6H*) gene, initiated homozygous mutations in *AbH6H* and led to elevated hyoscyamine production, as well as decreased hyoscyamine 6β-hydroxylase function without anisodamine or scopolamine (Zeng et al., 2021). The editing method, CRISPR/Cas9, disrupted the hyoscyamine gene, initiated homozygous mutations in *AbH6H* and led to elevated hyoscyamine production, as well as reduced gene function. Hyoscyamine 6β-hydroxylase catalyses the 6β-hydroxylation of hyoscyamine which further produces anisodamine and subsequently converts it to scopolamine (Hashimoto & Yamada, 1986).

Table 8.1 Gene editing with CRISPR/Cas in medicinal plants for the production of valuable secondary metabolites

Plant species	Family	Targeted gene	Promoter for sgRNA	Secondary metabolite(s)	Editing type	Method of Cas9 system delivery	Reference
Atropa bella-donna L.	Solanaceae	*AbH6H*	U6-26	Hyoscyamine	CRISPR/Cas9, targeted mutagenesis	*A. tumefaciens* EHA105-mediated freeze-thaw method	Zeng et al. (2021)
Brassica napus L.	Brassicaceae	*BnaFAD2*	U3	Oleic acid linoleic acid, linolenic acid	CRISPR/Cas9, knockout	*A. tumefaciens*-mediated hypocotyl method	Huang et al. (2020)
		BnaA.FAD2	AtU6	Oleic acid	CRISPR/Cas9, knockout	*A. tumefaciens* GV3101 mediated	Okuzaki et al. (2018)
Camelina sativa (L.) Crntz.	Brassicaceae	*FAD2*	U3, U6	Oleic acid, PUFA	CRISPR/Cas9, targeted mutagenesis	*A. tumefaciens*-mediated floral-dip method	Morineau et al. (2017)
		FAD2	U6	Oleic acid, PUFA	CRISPR/Cas9, knockout	*A. tumefaciens*-mediated floral-dip method	Jiang et al. (2017)
		FAE1	U6- 26	Oleic acid or α-linolenic acid	CRISPR/Cas9, knockout	Floral vacuum infiltration method	Ozseyhan et al. (2018)
Dendrobium officinale Kimura & Migo	Orchidaceae	*C3H, C4H, 4CL, CCR, IRX*	OsU3	Alkaloids, phenanthrenes, polysaccharides, bibenzyls, essential oils, glycosides	CRISPR/Cas9, knockout	*Agrobacterium* mediated	Kui et al. (2017)
Dioscorea zingiberensis C. H. Wright	Dioscoreaceae	*Dzfps*	OsU3	Diosgenin	CRISPR/Cas9, targeted mutagenesis	*A. tumefaciens* GV3101 mediated	Feng et al. (2018)
Dioscorea alata L.	Dioscoreaceae	*DrPDS*	DaU6.3	–	CRISPR/Cas9, knockout	*Agrobacterium* mediated	Syombua et al. (2021)

Glycine max (L.) Merr.	Fabaceae	*IFS*	GmU3 or GmU6 promoter	Isoflavonoids	CRISPR/Cas9, knock-out	*A. rhizogenes*-mediated hairy root culture	Zhang et al. (2020)
Humulus lupulus L.	Cannabaceae	*PDS*	U6-626p and U6-29p	Carotenoid	CRISPR/Cas9, targeted mutagenesis	*A. tumefaciens* mediated	Awasthi et al. (2021)
Nicotiana tabacum L.	Solanaceae	*FucT, XylT*	U6	Alkaloids, flavonoids, terpenoids, phenylpropanoids	CRISPR/Cas9, knockout	*A. tumefaciens* (EHA105, LBA4404) mediated	Mercx et al. (2017)
Papaver somniferum L.	Papaveraceae	*4′OMT2*	AtU6p	Alkaloid	CRISPR/Cas9, knockout	*A. tumefaciens*-mediated leaf infiltration	Alagoz et al. (2016)
Salvia miltiorrhiza Bunge	Lamiaceae	*SmRAS*	U3	Rosmarinic acid phenolic acids, diterpenoids	CRISPR/Cas9, knockout	*A. rhizogenes*-mediated hairy root culture	Zhou et al. (2018)

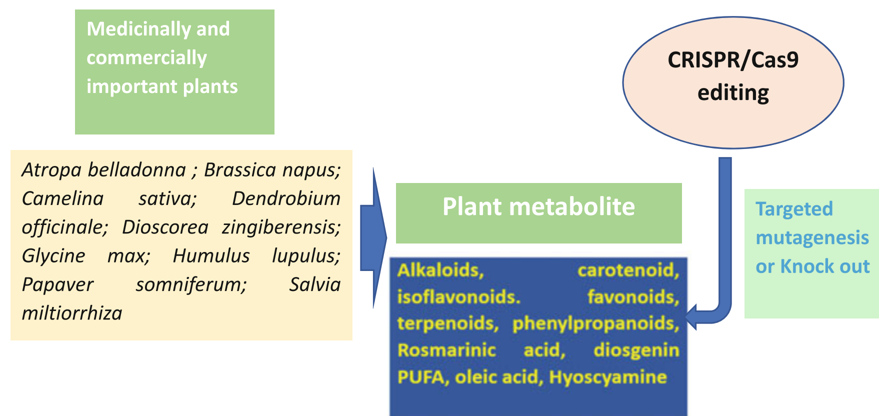

Fig. 8.2 Plant metabolites and their genome editing by CRISPR/Cas9

Brassica napus L.

Rapeseed oil ((*B. napus)* contains three major unsaturated fatty acids, including linoleic acid (18:2), linolenic acid (18:3) and oleic acid (18:1), as well as palmitic acid (16:0) and stearic acid (18:0), which are of high nutritional value (Nesi et al., 2008; Peng et al., 2010). Higher oleic acid content enhances thermal stability of the oil. Oleic acid content of napus is regulated by *FAD2* (fatty acid desaturase 2 gene) loci. Standardised CRISPR/Cas9 technology has generated mutated *BnaA.FAD2* through *Agrobacterium*-mediated transformation in *B. napus*. In an attempt to boost the content of oleic acid in *B. napus* genes using the CRISPR/Cas9 genome editing tool, mutated *BnaFAD2* copies were successfully introduced. Their result suggested that modification by CRISPR/Cas9 system showed significant increase in the content of oleic acid by 80% and a notable decrease in linoleic acid and linolenic acid (Huang et al., 2020). The report also suggested that genome editing of polyploidy species is quite feasible and does not lead to chimeric modifications. One study using CRISPR/Cas9 technology to drive knockout mutations in *FAD2* genes in *B. napus* resulted in an increase in the oleic acid (73%–80%) and reduction in linoleic acid (16%–9%). Though, 4-bp deletion of mutant fad2_Aa allele exhibited normal plant growth. Thus, knocking out via CRISPR/Cas9 technology can also be used to produce mutant plants that are agronomically more advantageous, such as mutant plants that contain higher oleic acid content important (Okuzaki et al., 2018).

Camelina sativa (L.) Crantz

CRISPR/Cas9-based gene editing was effective in enhancing the levels of oleic acid in the hexaploid *C. sativa* plant. Knockout of the *FAD2* genes successfully increased seed oil. A significant increase in oleic acid content (16 % to over 50%) was

observed, accompanied by a drop in linoleic acid (~16% to <4%) and linolenic acid (~35% to <10%). gRNAs targeted all three homologous *FAD 2* genes simultaneously in *Camelina* seeds of T3 and T4 generations (Jiang et al., 2017). An alternative study used CRISPR/Cas9 genome editing technology to improve oleic acid content (from 10 to 62%) and reduce PUFA in the *C. sativa* seed oil using three closely related *FAD 2* genes through targeted mutagenesis. The results of evaluating the mutations in three isologous CsFAD 2 genes over four generations have confirmed a large heritability of mutations (Morineau et al., 2017). In another experiment in allohexaploid *C. sativa*, CRISPR/Cas9 employed knockout mutagenesis by ethyl methanesulfonate which formed fatty acid elongase 1 (*FAE1*) gene mutants that resulted in the reduction in the C20–C24 very long-chain fatty acids (over 60%). Therefore, the inactivation of *FAE1* genes enabled the improvement of oleic acid content or α-linolenic acid in seed oil. Interestingly, knocking out mutant fae1 showed normal growth and seed development (Ozseyhan et al., 2018). Others altered three conserved homologous genes (*CsDGAT1* or *CsPDAT1*) via sgRNA to manipulate TAG (triacylglycerol) composition in the seeds of *C. sativa*. In addition, the seed of both *CsDGAT1* and *CsPDAT1* targeted lines shows wrinkled and shrinking surface (Aznar-Moreno & Durrett, 2017).

Dendrobium officinale Kimura and Migo

One of the largest groups of Angiosperm monocotyledon family, Orchidaceae, finds its uses not only in cosmetics, perfumes and for decoration purposes but also as natural remedies for the treatment of numerous diseases (Sut et al., 2017). The Chinese medicinal herb *Dendrobium officinale*, a species belonging to this family, produces many useful primary and secondary metabolites. There is also evidence to suggest that it heals yin deficiency disorders (Guo et al., 2020; Tang et al., 2017). Major bioactive compounds of *D. officinale* are polysaccharides, besides other compounds such as alkaloids, bibenzyls, essential oils, phenanthrenes and glycosides which have anticancer, antibacterial, antioxidant, anti-inflammatory, antidiabetic, antiviral, anti-ageing and hair growth-promoting properties (Tang et al., 2017; Teixeira et al., 2015). Metabolic profiling of *D. officinale* has led to the identification of several pharmaceutically active ingredients (Jin et al., 2016). The protocol for genome editing through CRISPR/Cas9 is well established for this orchid (Kui et al., 2017). This has not only eased the process of precise genetic manipulation of this plant but also helped in eliminating the controversies associated with transgenic acceptability. This knockout system reached 10–100% editing efficiency by using highly effective promoter (*Cauliflower mosaic virus* 35S), reporter genes β-glucuronidase (GUS), superfolder green fluorescence protein (SG) and vector (pCambia-1301-35SN) by using *Agrobacterium*-mediated transformation system. Five lignocellulose biosynthesis pathway genes, *C3H* (coumarate 3-hydroxylase), *C4H* (cinnamate 4-hydroxylase), *4CL* (4-coumarate), *CCR* (cinnamoyl coenzyme A reductase) and *IRX* (irregular xylem5), were used to

determine the efficiency of genome editing with CRISPR/Cas9 in *D. officinale*. The high efficiency of genome editing marks the entry of *D. officinale* in the new age of reverse genetics to decipher orchid gene functions which will further lead to the sustainable exploitation of this plant for our benefit (Kui et al., 2017).

Dioscorea zingiberensis C. H. Wright

Dioscorea spp. are medicinally important species of plants that are used for the extraction of diosgenin. The rhizomes of this species are used for the isolation of steroidal saponin, i.e. diosgenin, which has therapeutic significance (Pandey et al., 2017). Syombua et al. (2021), for the first time, evaluated the efficiency of CRISPR/Cas9 genome editing tool by targeting the phytoene desaturase gene (DrPDS) of *Dioscorea alata*. PDS (phytoene desaturase) catalyses the conversion of phytoene into both phytofluene and f-carotene (carotenoid precursors) (Mann et al., 1994). Results of this study proved that CRISPR/Cas9 efficiently induced site-specific disruption of the PDS gene with 83.3% editing efficiency and also induced phenotypical changes in yam. It is expected that the established CRISPR/Cas9 system in this species will serve as basal information for establishing editing protocols in other *Dioscorea* species. This will assist in functional genomics studies' trait improvement in other related species like *D. alata*. *D. zingiberensis* (a perennial vine, commonly known as 'yellow ginger') is extensively used in Chinese medicines (Zhang et al., 2018). A range of biological effects like anti-inflammatory, anthelmintic, anti-thrombosis, cardiovascular, hyperlipidaemia and neuroprotection activity have been confirmed. In order to target the farnesyl pyrophosphate synthase gene (Dzfps) (the gene controlling diosgenin biosynthesis), the CRISPR/Cas9 system was used via an Agrobacterium tumefaciens-facilitated transfection method in D. zingiberensis. Farnesyl pyrophosphate (FPP) synthase is important for the production of isoprenoids such as carotenoids, ubiquinones, sterols etc. (Dhar et al., 2013). Cas9 and sgRNA expression cassettes are controlled by the OsU3 and 35S promoters, respectively, which are designed to target the Dzfps gene. Dzfps gene is involved in sequentially catalysing dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP) to produce FPP and finally squalene which is the precursor of diosgenin. Both Dzfps transcript level and squalene content were relatively decreased in mutants with genome editing compared to wild type (Feng et al., 2018).

Glycine max (L.) Merr.

Glycine max (soybean) contains important vegetable oils, proteins and bioactive secondary metabolites. Remarkably, isoflavonoids content in *G. max* is almost 100 times higher as compared to other leguminous plants, which have significant functions in plant disease resistance and human health (Budryn et al., 2018; Kant

et al., 2019; Yu et al., 2003). As a signal molecule, isoflavonoids are involved in activating nod genes (Subramanian et al., 2006). Soybean phenylpropanoid pathway produces isoflavonoids by a sequential but complex process (Zhang et al., 2016). Briefly, the metabolic pathway for isoflavonoid biosynthesis starts by hydroxylation of flavanone which is catalysed by isoflavone synthase (*IFS*) which shares common substrate with flavanone-3-hydroxylase (*F3H*) and flavone synthase II (*FNS II*). *GmIFS1* and *GmIFS2* have been isolated from *G. max* (Jung et al., 2000). Zhang et al. (2020) studied multiplex CRISPR/Cas9 genome editing that was used to target *GmF3H1*, *GmF3H2* and *GmFNSII-1* in the hairy roots of *G. max*. Metabolomic analysis of *GmF3H1*, *GmF3H2* and *GmFNSII-1* triple mutants showed significant enhancement in isoflavone content. Additionally, the viral titre of *Soybean mosaic virus* (SMV) was significantly decreased. According to these findings, improved isoflavone content significantly enhanced soybean's resistance to SMV (Zhang et al., 2020).

Humulus lupulus L.

Humulus lupulus, also called hops, contains phenols, bitter acids, prenylated flavonoids etc., which are considered to be medically significant for those suffering from diseases such as prostate and breast cancer, osteoporosis, menopause and anxiety (Mishra et al., 2020; Srečec et al., 2012). CRISPR/Cas9 was used for the first time to manipulate the gene expression of *H. lupulus*. *Agrobacterium*-mediated transformation [using the binary pKSE401] successfully edited 33.3% of the transformed plants. Targeted editing of a *PDS* gene in carotenoid biosynthesis pathway resulted in endogenous edited genes in *H. lupulus* and decreased concentrations of chlorophyll a/b and carotenoid pigments. The CRISPR/Cas9 system was cited as a precise way to target the genome sequence of hops (Awasthi et al., 2021)**.**

Nicotiana tabacum L.

Nicotiana tabacum, a perennial herbaceous plant that produces tobacco, is reported to be a storehouse of important secondary metabolites such as alkaloids, terpenoids, flavonoids, phenylpropanoids etc. A number of recombinant products have also been produced using *N. tabacum*, including glycoenzymes α-galactosidase, glycohormone erythropoietin, proteases and xylanase (Jutras et al., 2020; Pantaleoni et al., 2014). Plants have been used for the production of pharmacological glycoproteins. These glycoproteins carry N-glycans with β(1,2)-xylose and a core α(1,3)-fucose, which have a significant influence on immunogenicity and allergenicity. CRISPR/Cas9 has been successfully used to knockout six glycosyltransferase genes in *Nicotiana* sp. It was used to produce recombinant proteins without core sugar α-1,3-fucose and β-1,2-xylose (Jansing et al., 2019). Gao et al. (2015) have projected

that CRISPR/Cas9 system is a useful tool for targeted mutagenesis of *N. tabacum* genome mainly due to the high efficiency of this editing system in this species (Gao et al., 2015). Mercx et al. (2017) conducted the study on the knockout lines of *XylT* (β(1,2)-xylosyltransferase) and *FucT* (α(1,3)-fucosyltransferase) in suspended *N. tabacum* BY-2 cells. Expression cassettes for Cas9 and gRNA were driven by the 35S-PPDK and U6 transcriptional promoters, respectively, which knockout four *FucT* and two *XylT* genes. The IgG glycosylation profile was screened by mass spectrometry that displayed the presence of GnGn (69%), Man7 (9.3%) and GnM (4.8%) structure and lack of β(1,2)-xylose or α(1,3)-fucose on the glycosylation moiety (Mercx et al., 2017).

Papaver somniferum L

The benzylisoquinoline alkaloids produced by *Papaver somniferum* have clinical significance in biomedicine (Labanca et al., 2018). A genome editing strategy based on CRISPR/Cas9 genome editing has been applied to this plant successfully. CRISPR-SpCas9 type II was used to knockout *4′OMT2* (gene controlling benzylisoquinoline alkaloids biosynthesis). A resulting DSB was repaired by NHEJ, causing short indels to be introduced, resulting in gene dysfunction. *Agrobacterium*-mediated transformation was carried out utilising TRV (*Tobacco rattle virus*)-based synthetic binary plasmids expressing sgRNA and hCas9 (human-codon optimised Cas9)-encoding synthetic vector to inactivate *4′OMT2*, thus regulating the biosynthesis of the BIAs. An HPLC-ToF/MS (high-performance liquid chromatography-time-of-flight mass spectrometry) study showed that *P. somniferum* plants with gene knockouts had significantly lower production of benzylisoquinoline alkaloids (Alagoz et al., 2016).

Salvia miltiorrhiza Bunge

Salvia miltiorrhiza, a traditional medicinal herb from China, contains diterpene compounds such as tanshinones and rosmarinic acid (RA) (Li et al., 2017a, 2017b; Shi et al., 2019). *S. miltiorrhiza* is extensively used against cardiovascular and cerebrovascular diseases and diabetes. Moreover, this plant has antioxidant and anti-inflammatory properties as well as cardioprotective and anticancer properties. According to earlier reports, an inactivation with the CRISPR/Cas9 system was shown to effectively target the *SmCPS1* (diterpene synthase) gene from the tanshinone biosynthesis pathway. Rosmarinic acid synthase (RAS), an enzyme which catalyses RA biosynthesis, also accumulates lithospermic acid B (LAB) (Sander & Petersen, 2011). *Agrobacterium rhizogenes*-mediated transformation of hairy roots using CRISPR-Cas9 inactivated the *SmCPS1* gene implicated in the production of tanshinone. An analysis of metabolite profiles in homozygous mutants

revealed that tanshinone synthesis is completely absent, but these mutants produce the other phenolics. However, the tanshinone content of the chimeric mutants was reduced (Li et al., 2017a, 2017b). *S. miltiorrhiza* suspension cells were successfully transfected with *Agrobacterium rhizogenes* in order to knockout the *SmRAS* gene using CRISPR/Cas9 genome editing. One homozygous mutant, two heterozygous mutants and five biallelic mutants were generated using the *Arabidopsis* U6 promoter. Mutation in the *SmRAS* genes resulted in a decline in RA and LAB and an increase in 3,4-dihydroxyphenyllactic acid, a precursor of RA (Zhou et al., 2018). *SmPAL1* was targeted and edited in the phenylpropane metabolic pathway using a software designed for CRISPR/Cas9 in the *S. miltiorrhiza*. SmPAL1-g1, SmPAL1-g2 and SmPAL1-g3 are three sequences which are likely CRISPR targets. These sequences achieved 53.3%, 76.6% and 10.0% enzyme digestion efficiencies, respectively (Qiu et al., 2018).

Challenges Associated with the Use of CRISPR/Cas Tools for Plant Natural Product Research

Off-Target Effects

Despite CRISPR/Cas9 being a popular tool among other genome editing tools due to its affordability, precision and simplicity, some negative consequences are also associated with it. The off-target effect is one such type. There are unintentional translocations, insertions, deletions, inversions and point mutations associated with off-target effects. Utilising the CRISPR/Cas9 tool, off-target mutations limit therapeutic implications. In lower group of organisms such as bacteria, a smaller number of off-target effects are reported because of a lower frequency of a spacer-PAM combination in their genome. There are a number of effective solutions to fighting off-target effects, such as mutations in the nuclease domain of Cas9 (Cho et al., 2014), dimerisation of nucleases (Fok1-dCas9) (Tsai et al., 2014) and CRISPR interference (Gilbert et al., 2014). There is considerable effort underway in higher eukaryotes to minimise off-target effects. Improved sgRNAs need to reduce mismatches (Doench et al., 2016) and restrict Cas9 levels in the cells to lessen off-target effects of these tools (Shen et al., 2019).

Editing Accuracy

The dominant error-prone NHEJ pathway for DSB repair is randomly generated small indels at the DSB locus. Unpredictable precision size of the indel leads to frameshift mutations. HDR creates more precise gene editing products. The efficiency of HDR is also reduced multiple folds due to the dominance of NHEJ

pathway, regardless of whether an editing template is accommodated for HDR (Yang et al., 2020). In mammalian cells the efficiency of HDR is much lower (25%) than NHEJ (75%). To conquer this limitation, a few strategies have been effectively utilised to suppress the NHEJ pathway, for example, utilising the small molecule Scr7 on promoting HDR efficiency, as Scr7 binds to the DNA binding domain of ligase IV, KU70 or KU80 and thus inhibits with the of NHEJ pathway events (Li et al., 2017a, 2017b).

Efficient Delivery

The large size and positive charge of Cas9 protein and negative charge of sgRNAs make difficult the delivery of this protein into cell. The efficacious CRISPR/Cas9 application needs strong Cas9 and sgRNA delivery either by plasmid or by mRNA or by ribonucleoprotein (RNP) complex (Rahimi et al., 2020). Inappropriately, few medicinal plants discussed in this chapter lack efficient transformation methods. Nanoparticle-based delivery (polymeric nanocarriers, gold-based nanomaterials, metal-organic frameworks) is fetching a more striking approach due to its specific targeting and minimal exposure to nucleases (Carboni et al., 2019; Chen et al., 2020).

Genome Instability

DSBs may cause chromosomal translocations, instability of the genome which is a dangerous cellular event. CRISPR/Cas9 tool initiates DSB that may put the cells under severe stress. Cas9 expression in *Streptomycetes* leads to deletions in linear chromosomes (Hoff et al., 2018). CRISPR-based editing system (CRISPR-BEST), *CRISPR* interference (CRISPRi) and deaminase-based DNA base editors, that are not based on DSBs, have been conveyed as an effective genome editing tool (Eid et al., 2018; Tong et al., 2020).

Toxicity of Cas9

CRISPR-Cas9 tool is for high-throughput approach, but can be **astonished** by nuclease-induced toxicity because of DNA damage. Toxicity due to Cas9 expression has been reported in bacteria and green algae but has not yet been reported in plants. The presence of high intercellular Cas9 leads to toxicity. Reducing Cas9 levels using weak promoters or by controlling the size and frequency of target gene edits lead to a decreased toxicity (Dow et al., 2015; Morgens et al., 2017).

Indigenous Vs Introduced CRISPR's Role

Bacteria and *Streptomyces* have evolved CRISPR/Cas systems which provide protection against foreign nucleic acids. A future research area of interest could be the interaction between native and introduced CRISPR/Cas.

Perspectives of CRISPR/Cas Genetic Engineering on Plant Natural Products

CRISPR-based genome editing has become a major breakthrough in the twenty-first century due to its effectiveness in knocking out. The success of this application prompted biochemical research companies to completely shift their focus to using CRISPR tools in plants for the production of sustainable agricultural crops and valuable plant natural products (Brinegar et al., 2017). It is crucial to efficaciously edit the genomes of medicinal plants. It enables accurate control of secondary metabolite pathways through regulating metabolic routes and removing rate-limiting constraints and feedback inhibitions. In order to meet the ever-expanding demand of biomedical industries, CRISPR/Cas genome editing may prove to be an exciting tool in metabolite engineering. For plants with a long life cycle, direct CRISPR/Cas-mediated editing can be ineffective. It may be extremely useful to employ microbial cell factories to substitute heterologous expression of secondary metabolic pathways. Earlier, CRISPR/Cas tool was mostly used to modify just one trait in somatic cells of higher plants through NHEJ. However, in recent years, CRISPR/Cas research mainly focused on the utilisation of homologous recombination for chromosomal rearrangement driven genetic traits in plants (Schindele et al., 2020). Incorporating artificial DNA sequences into plant genomes altering plant behaviour due to novel functions by CRISPR/Cas system is a promising approach to improve plant design and synthetic biology (Chen et al., 2019). Genome editing using CRISPR/Cas has immense potential to create biochemical factories, clone long DNA segments and manipulate biosynthetic pathways (Bennett-Baker & Mueller, 2017; Liu et al., 2015). Targeting long genomes with Cas9-aided targeting of CHROMOSOME (CATCH) and isolating those megabase-sized genomic fragments with CRISPR-mediated isolation (CISMR) are powerful tools for synthetic and chemical biology (Bennett-Baker & Mueller, 2017; Liu et al., 2015). Recent studies have shown that inducing artificial polyploidy via the CRISPR/Cas9 mechanism on *A. rhizogenes* hairy root cultures is a novel and promising way to increase secondary metabolite production in a wide range of medicinal plants (Niazian, 2019). As *Agrobacterium*-mediated transformation is expensive and time-consuming, tissue culture-free genome editing offers an alternative, which will likely be more efficient. The specificity of the Cas9-linked base editors has also been improvised through the expansion of the guide sequences sgRNAs and RNP-mediated delivery of the base editors. A genomic editing process has also been improved by enhancing the

frequency of HDR to improve the efficiency and precision of the process. Despite receiving a lot of attention during the past several years, CRISPR/Cas has only just begun to make its way into natural product discovery. Few applications have been reported, and most of the work has focused on proving the feasibility of CRISPR/Cas systems in plants. The current CRISPR/Cas technology should be optimised, developed and innovated further, as no technology is perfect.

Conclusion

A number of plant secondary metabolites could potentially be used to treat several life-threatening human diseases. They provide protection against a wide range of diseases with a wide array of defence properties without causing harmful side effects. They are also relatively affordable and less costly than other methods of treatment. CRISPR/Cas9 has appeared as a breakthrough tool for metabolic engineering in plants. CRISPR/Cas9 modifies desired genomic parts by knocking out, knocking in, mutations etc. It is still in the early stages of CRISPR/Cas9-based genome editing in most medicinal plants as information about whole genomes and mRNAs sequences is lacking. Due to insufficient sequence information, this tool cannot edit key genes involved in plant secondary metabolite pathways. The limitations of this tool and strategic approaches to overcome them have already been discussed. However, a large number of secondary metabolites producing genes have not been modified yet with CRISPR/Cas9 for enhancing metabolite production. The use of higher plants as renewable sources of bioactive metabolites will be extended and enhanced through CRISPR/Cas9. In the future, we expect to achieve controllable and successful biotechnological production of valuable and as yet unknown plant phytochemicals through the continuation of efforts in this area.

References

Alagoz, Y., Gurkok, T., Zhang, B., & Unver, T. (2016). Manipulating the biosynthesis of bioactive compound alkaloids for next-generation metabolic engineering in opium poppy Using CRISPR-Cas 9 genome editing technology. *Scientific Reports, 6*(1), 1–9. https://doi.org/10.1038/srep30910

Anand, U., Nandy, S., Mundhra, A., Das, N., Pandey, D. K., & Dey, A. (2020). A review on antimicrobial botanicals, phytochemicals and natural resistance modifying agents from Apocynaceae family: Possible therapeutic approaches against multidrug resistance in pathogenic microorganisms. *Drug Resistance Updates, 51*, 100695. https://doi.org/10.1016/j.drup.2020.100695

Awasthi, P., Kocábek, T., Mishra, A. K., Nath, V. S., Shrestha, A., & Matoušek, J. (2021). Establishment of CRISPR/Cas9 mediated targeted mutagenesis in hop (*Humulus lupulus*). *Plant Physiology and Biochemistry, 160*, 1–7. https://doi.org/10.1016/j.plaphy.2021.01.006

Aznar-Moreno, J. A., & Durrett, T. P. (2017). Simultaneous targeting of multiple gene homeologs to alter seed oil production in *Camelina sativa*. *Plant and Cell Physiology, 58*(7), 1260–1267.

Barakate, A., & Stephens, J. (2016). An overview of crispr-based tools and their improvements: New opportunities in understanding plant-pathogen interactions for better crop protection. *Frontiers in Plant Science, 7*, 1–7. https://doi.org/10.3389/fpls.2016.00765

Bennett-Baker, P. E., & Mueller, J. L. (2017). CRISPR-mediated isolation of specific megabase segments of genomic DNA. *Nucleic Acids Research, 45*(19), 1–8. https://doi.org/10.1093/nar/gkx749

Brinegar, K., Yetisen, K., Choi, S., Vallillo, E., Ruiz-Esparza, G. U., Prabhakar, A. M., Khademhosseini, A., & Yun, S. H. (2017). The commercialization of genome-editing technologies. *Critical reviews in biotechnology, 37*(7), 924–932.

Budryn, G., Grzelczyk, J., & Pérez-Sánchez, H. (2018). Binding of red clover isoflavones to actin as a potential mechanism of anti-metastatic activity restricting the migration of cancer cells. *Molecules, 23*(10), 1–11. https://doi.org/10.3390/molecules23102471

Calabrò, S. (2015). Plant secondary metabolites. *Rumen microbiology: from evolution to revolution*, *October* (pp. 153–159). doi: https://doi.org/10.1007/978-81-322-2401-3_11

Carboni, V., Maaliki, C., Alyami, M., Alsaiari, S., & Khashab, N. (2019). Synthetic vehicles for encapsulation and delivery of CRISPR/Cas9 gene editing machinery. *Advanced Therapeutics, 2*(4), 1800085. https://doi.org/10.1002/adtp.201800085

Chen, F., Alphonse, M., & Liu, Q. (2020). Strategies for nonviral nanoparticle-based delivery of CRISPR/Cas9 therapeutics. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 12*(3), 1–14. https://doi.org/10.1002/wnan.1609

Chen, K., & Gao, C. (2015). Targeted gene mutation in plants. In *Somatic genome manipulation* (pp. 253–272). Springer.

Chen, K., Wang, Y., Zhang, R., Zhang, H., & Gao, C. (2019). CRISPR/Cas genome editing and precision plant breeding in agriculture. *Annual Review of Plant Biology, 70*, 667–697. https://doi.org/10.1146/annurev-arplant-050718-100049

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., & Kim, J. S. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research, 24*(1), 132–141. https://doi.org/10.1101/gr.162339.113

Cui, Y., Xu, J., Cheng, M., Liao, X., & Peng, S. (2018). Review of CRISPR/Cas9 sgRNA design tools. *Interdisciplinary Sciences: Computational Life Sciences, 10*(2), 455–465. https://doi.org/10.1007/s12539-018-0298-z

Das, T., Anand, U., Pandey, S. K., Ashby, C. R., Assaraf, Y. G., Chen, Z. S., & Dey, A. (2021). Therapeutic strategies to overcome taxane resistance in cancer. *Drug Resistance Updates, 55*, 100754. https://doi.org/10.1016/j.drup.2021.100754

Dey, A., Bhattacharya, R., Mukherjee, A., & Pandey, D. K. (2017a). Natural products against Alzheimer's disease: Pharmaco-therapeutics and biotechnological interventions. *Biotechnology Advances, 35*(2), 178–216. https://doi.org/10.1016/j.biotechadv.2016.12.005

Dey, A., Gorai, P., Mukherjee, A., Dhan, R., & Modak, B. K. (2017b). Ethnobiological treatments of neurological conditions in the Chota Nagpur Plateau, India. *Journal of Ethnopharmacology, 198*, 33–44. https://doi.org/10.1016/j.jep.2016.12.040

Dhar, M. K., Koul, A., & Kaul, S. (2013). Farnesyl pyrophosphate synthase: A key enzyme in isoprenoid biosynthetic pathway and potential molecular target for drug development. *New biotechnology, 30*(2), 114–123. https://doi.org/10.1016/j.nbt.2012.07.001

Doench, J. G., Fusi, N., Sullender, M., Hegde, M., Vaimberg, E. W., Donovan, K. F., Smith, I., Tothova, Z., Wilen, C., Orchard, R., Virgin, H. W., Listgarten, J., & Root, D. E. (2016). Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature Biotechnology, 34*(2), 184–191. https://doi.org/10.1038/nbt.3437

Dow, L. E., Fisher, J., Rourke, K. P. O., Muley, A., Edward, R., Livshits, G., Tschaharganeh, D. F., Socci, N. D., & Lowe, W. (2015). Inducible in vivo genome editing with CRISPR/Cas9. *Nature Biotechnology, 33*(4), 390–394. https://doi.org/10.1038/nbt.3155.Inducible

Eid, A., Alshareef, S., & Mahfouz, M. M. (2018). CRISPR base editors: Genome editing without double-stranded breaks. *Biochemical Journal, 475*(11), 1955–1964. https://doi.org/10.1042/BCJ20170793

Feng, S., Song, W., Fu, R., Zhang, H., Xu, A., & Li, J. (2018). Application of the CRISPR/Cas9 system in *Dioscorea zingiberensis*. *Plant Cell, Tissue and Organ Culture, 135*(1), 133–141. https://doi.org/10.1007/s11240-018-1450-5

Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology, 31*(7), 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004

Gao, J., Wang, G., Ma, S., Xie, X., Wu, X., Zhang, X., Wu, Y., Zhao, P., & Xia, Q. (2015). CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum* CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum. *Plant Molecular Biology, 87*(1–2), 99–110. https://doi.org/10.1007/s11103-014-0263-0

Gilbert, L. A., Horlbeck, M. A., Adamson, B., Villalta, J. E., Chen, Y., Whitehead, E. H., Guimaraes, C., Panning, B., Ploegh, H. L., Bassik, M. C., Qi, L. S., Kampmann, M., & Weissman, J. S. (2014). Genome-scale CRISPR-mediated control of gene repression and activation. *Cell, 159*(3), 647–661. https://doi.org/10.1016/j.cell.2014.09.029

Guo, L., Qi, J., Du, D., Liu, Y., & Jiang, X. (2020). Current advances of *Dendrobium officinale* polysaccharides in dermatology: A literature review. *Pharmaceutical Biology, 58*(1), 664–673. https://doi.org/10.1080/13880209.2020.1787470

Guo, Q., Cao, H., Qi, X., Li, H., Ye, P., Wang, Z., Wang, D., & Sun, M. (2017). Research progress in reversal of tumor multi-drug resistance via natural products. *Anti-Cancer Agents in Medicinal Chemistry, 17*(11), 1466–1476. https://doi.org/10.2174/1871520617666171016105704

Hashimoto, T. (1992). Metabolic engineering of medicinal plants: Transgenic Atropa belladonna with an improved alkaloid composition. *Proceedings of the National Academy of Sciences of the United States of America, 89*, 11,799–11,803.

Hashimoto, T., & Yamada, Y. (1986). Hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. *Plant Physiology, 81*(2), 619–625. https://doi.org/10.1104/pp.81.2.619

Hoff, G., Bertrand, C., Piotrowski, E., Thibessard, A., & Leblond, P. (2018). Genome plasticity is governed by double strand break DNA repair in *Streptomyces*. *Scientific Reports, 8*(1), 1–11. https://doi.org/10.1038/s41598-018-23622-w

Huang, H., Cui, T., Zhang, L., Yang, Q., Yang, Y., Xie, K., Fan, C., & Zhou, Y. (2020). Modifications of fatty acid profile through targeted mutation at BnaFAD2 gene with CRISPR/Cas9-mediated gene editing in *Brassica napus*. *Theoretical and Applied Genetics, 133*(8), 2401–2411. https://doi.org/10.1007/s00122-020-03607-y

Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z., & Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. *Journal of Pharmacy and Bioallied Sciences, 4*(1), 10–20. https://doi.org/10.4103/0975-7406.92725

Jansing, J., Sack, M., Augustine, S. M., Fischer, R., & Bortesi, L. (2019). CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in *Nicotiana benthamiana* for the production of recombinant proteins lacking β-1,2-xylose and core α-1,3-fucose. *Plant Biotechnology Journal, 17*(2), 350–361. https://doi.org/10.1111/pbi.12981

Jiang, W. Z., Henry, I. M., Lynagh, P. G., Comai, L., Cahoon, E. B., & Weeks, D. P. (2017). Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. *Plant Biotechnology Journal, 15*(5), 648–657. https://doi.org/10.1111/pbi.12663

Jin, Q., Jiao, C., Sun, S., Song, C., Cai, Y., Lin, Y., & Fan, H. (2016). Metabolic analysis of medicinal *Dendrobium officinale* and *Dendrobium huoshanense* during different growth years. *PLoS ONE, 11*(1), e0146607. https://doi.org/10.1371/journal.pone.0146607

Jinek, M. K., Chylinski, I., Fonfara, M., Hauer, J., Doudna, & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, (337), 816–821.

Jung, W., Yu, O., Lau, S. M. C., O'Keefe, D. P., Odell, J., Fader, G., & McGonigle, B. (2000). Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. *Nature Biotechnology, 18*(5), 559. https://doi.org/10.1038/75448

Jutras, P. V., Dodds, I., & van der Hoorn, R. A. L. (2020). Proteases of *Nicotiana benthamiana*: an emerging battle for molecular farming. *Current Opinion in Biotechnology, 61*, 60–65. https://doi.org/10.1016/j.copbio.2019.10.006

Kant, R., Tyagi, K., Ghosh, S., & Jha, G. (2019). Host alternative NADH:Ubiquinone oxidoreductase serves as a susceptibility factor to promote pathogenesis of *Rhizoctonia solani* in plants. *Phytopathology, 109*(10), 1741–1750. https://doi.org/10.1094/PHYTO-02-19-0055-R

Kaur, N., Alok, A., Shivani, K. P., Kaur, N., Awasthi, P., Chaturvedi, S., Pandey, P., Pandey, A., Pandey, A. K., & Tiwari, S. (2020a). CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit. *Metabolic Engineering, 59*, 76–86. https://doi.org/10.1016/j.ymben.2020.01.008

Kaur, P., Gupta, R. C., Dey, A., Malik, T., & Pandey, D. K. (2020b). Validation and quantification of major biomarkers in 'Mahasudarshan Churna'—an ayurvedic polyherbal formulation through high-performance thin-layer chromatography. *BMC Complementary Medicine and Therapies, 20*(1), 184. https://doi.org/10.1186/s12906-020-02970-z

Khan, S. H. (2019). Genome-editing technologies: Concept, pros, and cons of various genome-editing techniques and bioethical concerns for clinical application. *Molecular Therapy - Nucleic Acids, 16*, 326–334. https://doi.org/10.1016/j.omtn.2019.02.027

Koonin, E. V., & Makarova, K. S. (2009). CRISPR-Cas: an adaptive immunity system in prokaryotes. *F1000 Biology Reports, 1*, 1–6. https://doi.org/10.3410/b1-95

Koonin, E. V., & Makarova, K. S. (2013). CRISPR-Cas: Evolution of an RNA-based adaptive immunity system in prokaryotes. *RNA Biology, 10*(5), 679–686. https://doi.org/10.4161/rna.24022

Kui, L., Chen, H., Zhang, W., He, S., Xiong, Z., Zhang, Y., Yan, L., Zhong, C., He, F., Chen, J., Zeng, P., Zhang, G., Yang, S., Dong, Y., Wang, W., & Cai, J. (2017). Building a genetic manipulation tool box for orchid biology: Identification of constitutive promoters and application of CRISPR/Cas9 in the orchid, dendrobium officinale. *Frontiers in Plant Science, 7*, 1–13. https://doi.org/10.3389/fpls.2016.02036

Labanca, F., Ovesnà, J., & Milella, L. (2018). Papaver somniferum L. taxonomy, uses and new insight in poppy alkaloid pathways. *Phytochemistry Reviews, 17*(4), 853–871. https://doi.org/10.1007/s11101-018-9563-3

Li, B., Cui, G., Shen, G., Zhan, Z., Huang, L., Chen, J., & Qi, X. (2017a). Targeted mutagenesis in the medicinal plant *Salvia miltiorrhiza*. *Scientific Reports, 7*(January), 1–9. https://doi.org/10.1038/srep43320

Li, G., Zhang, X., Zhong, C., Mo, J., Quan, R., Yang, J., Liu, D., Li, Z., Yang, H., & Wu, Z. (2017b). Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells. *Scientific Reports, 7*(1), 1–11. https://doi.org/10.1038/s41598-017-09306-x

Li, T., Huang, S., Jiang, W. Z., Wright, D., Spalding, M. H., Weeks, D. P., & Yang, B. (2011). TAL nucleases (TALNs): Hybrid proteins composed of TAL effectors and FokI DNA-cleavage domain. *Nucleic Acids Research, 39*(1), 359–372. https://doi.org/10.1093/nar/gkq704

Liu, Y., Tao, W., Wen, S., Li, Z., Yang, A., Deng, Z., & Sun, Y. (2015). In vitro CRISPR/cas9 system for efficient targeted DNA editing. *MBio, 6*(6). https://doi.org/10.1128/mBio.01714-15

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., Velasco, R., & Kanchiswamy, C. N. (2016). DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. *Frontiers in Plant Science, 7*, 1–9. https://doi.org/10.3389/fpls.2016.01904

Mann, V., Pecker, I., & Hirschberg, J. (1994). Cloning and characterization of the gene for phytoene desaturase (Pds) from tomato (*Lycopersicon esculentum*). *Plant Molecular Biology, 24*(3), 429–434.

McMurry, J. E. (2015). Organic chemistry with biological applications. In *Secondary metabolites: An introduction to natural products chemistry* (pp. 1016–1046). Cengage Learning Ltd.

Mercx, S., Smargiasso, N., Chaumont, F., De Pauw, E., Boutry, M., & Navarre, C. (2017). Inactivation of the β(1,2)-xylosyltransferase and the α(1,3)-fucosyltransferase genes in Nicotiana tabacum BY-2 cells by a multiplex CRISPR/Cas9 strategy results in glycoproteins without plant-specific glycans. *Frontiers in Plant Science, 8*, 1–11. https://doi.org/10.3389/fpls.2017.00403

Mishra, A. K., Kocábek, T., Nath, V. S., Awasthi, P., Shrestha, A., Killi, U. K., Jakse, J., Patzak, J., Krofta, K., & Matoušek, J. (2020). Dissection of dynamic transcriptome landscape of leaf, bract, and lupulin gland in hop (*Humulus lupulus* L.). *International Journal of Molecular Sciences, 21*(1). https://doi.org/10.3390/ijms21010233

Morgens, D. W., Wainberg, M., Boyle, E. A., Ursu, O., Araya, C. L., Kimberly Tsui, C., Haney, M. S., Hess, G. T., Han, K., Jeng, E. E., Li, A., Snyder, M. P., Greenleaf, W. J., Kundaje, A., & Bassik, M. C. (2017). Genome-scale measurement of off-target activity using Cas9 toxicity in high-throughput screens. *Nature Communications, 8*, 1–8. https://doi.org/10.1038/ncomms15178

Morineau, C., Bellec, Y., Tellier, F., Gissot, L., Kelemen, Z., Nogué, F., & Faure, J. D. (2017). Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. *Plant Biotechnology Journal, 15*(6), 729–739. https://doi.org/10.1111/pbi.12671

Narayani, M., & Srivastava, S. (2017). Elicitation: A stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. *Phytochemistry Reviews, 16*(6), 1227–1252. https://doi.org/10.1007/s11101-017-9534-0

Nesi, N., Delourme, R., Brégeon, M., Falentin, C., & Renard, M. (2008). Genetic and molecular approaches to improve nutritional value of *Brassica napus* L. seed. *Comptes Rendus—Biologies, 331*(10), 763–771. https://doi.org/10.1016/j.crvi.2008.07.018

Niazian, M. (2019). Application of genetics and biotechnology for improving medicinal plants. *Planta, 249*(4), 953–973. https://doi.org/10.1007/s00425-019-03099-1

Okuzaki, A., Ogawa, T., Koizuka, C., Kaneko, K., Inaba, M., Imamura, J., & Koizuka, N. (2018). CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in *Brassica napus*. *Plant Physiology and Biochemistry, 131*, 63–69. https://doi.org/10.1016/j.plaphy.2018.04.025

Ozseyhan, M. E., Kang, J., Mu, X., & Lu, C. (2018). Mutagenesis of the FAE1 genes significantly changes fatty acid composition in seeds of *Camelina sativa*. *Plant Physiology and Biochemistry, 123*, 1–7. https://doi.org/10.1016/j.plaphy.2017.11.021

Pandey, D. K., Nazir, A., & Dey, A. (2017). Isolation and characterization of phosphate solubilizing bacteria from rhizosphere of *Dioscorea alata* stimulating growth and diosgenin production. *Proceedings of the National Academy of Sciences India Section B—Biological Sciences, 87*(4), 1143–1152. https://doi.org/10.1007/s40011-015-0670-2

Pantaleoni, L., Longoni, P., Ferroni, L., Baldisserotto, C., Leelavathi, S., Siva Reddy, V., Pancaldi, S., & Cella, R. (2014). Chloroplast molecular farming: Efficient production of a thermostable xylanase by *Nicotiana tabacum* plants and long-term conservation of the recombinant enzyme. *Protoplasma, 251*(3), 639–648. https://doi.org/10.1007/s00709-013-0564-1

Peng, Q., Hu, Y., Wei, R., Zhang, Y., Guan, C., Ruan, Y., & Liu, C. (2010). Simultaneous silencing of FAD2 and FAE1 genes affects both oleic acid and erucic acid contents in *Brassica napus* seeds. *Plant Cell Reports, 29*(4), 317–325. https://doi.org/10.1007/s00299-010-0823-y

Qiu, J. R., Su, Y. K., Song, Z. Q., Fang, X. S., Li, J. Y., Zhang, J., & Wang, J. H. (2018). Directing construction of CRISPR/Cas9 vector of SmPAL1 in *Salvia miltiorrhiza* by target efficiency detection in vitro. *Zhongguo Zhong yao za zhi=Zhongguo zhongyao zazhi=China journal of Chinese materia medica, 43*(21), 4226–4230.

Rahimi, H., Salehiabar, M., Charmi, J., Barsbay, M., Ghaffarlou, M., Roohi Razlighi, M., Davaran, S., Khalilov, R., Sugiyama, M., Nosrati, H., Kaboli, S., Danafar, H., & Webster, T. J. (2020). Harnessing nanoparticles for the efficient delivery of the CRISPR/Cas9 system. *Nano Today, 34*, 100895. https://doi.org/10.1016/j.nantod.2020.100895

Rehman, S., Rehman, I. U., Jan, B., Rashid, I., Reshi, Z. A., & Ganie, A. H. (2021). Genome editing: applications for medicinal and aromatic plants. In T. Aftab & K. R. Hakeem (Eds.), *Medicinal and aromatic plants* (pp. 119–144). Academic Press.

Ricroch, A., Clairand, P., & Harwood, W. (2017). Use of CRISPR systems in plant genome editing: Toward new opportunities in agriculture. *Emerging Topics in Life Sciences, 1*(2), 169–182. https://doi.org/10.1042/ETLS20170085

Sander, M., & Petersen, M. (2011). Distinct substrate specificities and unusual substrate flexibilities of two hydroxycinnamoyltransferases, rosmarinic acid synthase and hydroxycinnamoyl-CoA: Shikimate hydroxycinnamoyl-transferase, from Coleus blumei Benth. *Planta, 233*, 1157–1171. https://doi.org/10.1007/s00425-011-1367-2

Schindele, A., Dorn, A., & Puchta, H. (2020). CRISPR/Cas brings plant biology and breeding into the fast lane. *Current Opinion in Biotechnology, 61*, 7–14. https://doi.org/10.1016/j.copbio.2019.08.006

Shan, Q., Wang, Y., Li, J., & Gao, C. (2014). Genome editing in rice and wheat using the CRISPR/Cas system. *Nature Protocols, 9*(10), 2395–2410. https://doi.org/10.1038/nprot.2014.157

Shen, C. C., Hsu, M. N., Chang, C. W., Lin, M. W., Hwu, J. R., Tu, Y., & Hu, Y. C. (2019). Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation. *Nucleic Acids Research, 47*(3), 1–11. https://doi.org/10.1093/nar/gky1165

Shi, M., Huang, F., Deng, C., Wang, Y., & Kai, G. (2019). Bioactivities, biosynthesis and biotechnological production of phenolic acids in *Salvia miltiorrhiza*. *Critical Reviews in Food Science and Nutrition, 59*(6), 953–964. https://doi.org/10.1080/10408398.2018.1474170

Singh, R. K., Prasad, A., Muthamilarasan, M., Parida, S. K., & Prasad, M. (2020). Breeding and biotechnological interventions for trait improvement: Status and prospects. *Planta, 252*(4), 1–18. https://doi.org/10.1007/s00425-020-03465-4

Srečec, S., Zechner-Krpan, V., Petravić-Tominac, V., Košir, I. J., & Čerenak, A. (2012). Importance of medical eff ects of Xanthohumol, Hop (*Humulus lupulus* L.) biofl avonoid in restructuring of world hop industry. *Agriculturae Conspectus Scientificus, 77*(2), 61–67.

Staniek, A., Bouwmeester, H., Fraser, P. D., Kayser, O., Martens, S., Tissier, A., van der Krol, S., Wessjohann, L., & Warzecha, H. (2013). Natural products—Modifying metabolite pathways in plants. *Biotechnology Journal, 8*(10), 1159–1171. https://doi.org/10.1002/biot.201300224

Subramanian, S., Stacey, G., & Yu, O. (2006). Endogenous isoflavones are essential for the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. *Plant Journal, 48*(2), 261–273. https://doi.org/10.1111/j.1365-313X.2006.02874.x

Sut, S., Maggi, F., & Dall, S. (2017). Bioactive secondary metabolites from orchids (Orchidaceae). *Chemistry & Biodiversity, 14*(11), e1700172. https://doi.org/10.1002/cbdv.201700172

Tang, H., Zhao, T., Sheng, Y., Zheng, T., Fu, L., & Zhang, Y. (2017). *Dendrobium officinale* Kimura et Migo: A review on its ethnopharmacology, phytochemistry, pharmacology, and industrialization. *Evidence-Based Complementary and Alternative Medicine, 2017*, 7436259.

Teixeira, J. A., Tsavkelova, E. A., Zeng, S., Cardoso, C., & Cardoso, J. C. (2015). Symbiotic in vitro seed propagation of *Dendrobium*: Fungal and bacterial partners and their influence on plant growth and development. *Planta, 242*(1), 1–22. https://doi.org/10.1007/s00425-015-2301-9

Tong, Y., Whitford, C. M., Blin, K., Jørgensen, T. S., Weber, T., & Lee, S. Y. (2020). CRISPR–Cas9, CRISPRi and CRISPR-BEST-mediated genetic manipulation in *Streptomycetes*. *Nature Protocols, 15*(8), 2470–2502. https://doi.org/10.1038/s41596-020-0339-z

Tsai, S. Q., Wyvekens, N., Khayter, C., Foden, J. A., Thapar, V., Reyon, D., Goodwin, M. J., Aryee, M. J., & Joung, J. K. (2014). Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nature Biotechnology, 32*(6), 569–576. https://doi.org/10.1038/nbt.2908

Upadhyay, S. K., & Sharma, S. (2014). SSFinder: High throughput CRISPR-Cas target sites prediction tool. *BioMed Research International, 2014*. https://doi.org/10.1155/2014/742482

Verpoorte, R., & Memelink, J. (2002). Engineering secondary metabolite production in plants. *Current Opinion in Biotechnology, 13*(2), 181–187. https://doi.org/10.1016/S0958-1669(02)00308-7

Verpoorte, R., Van Der Hejden, R., Ten Hoopen, H. J. G., & Memelink, J. (1999). Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. *Biotechnology Letters, 21*(6), 467–479. https://doi.org/10.1023/A:1005502632053

Wang, P., Yang, H. L., Yang, Y. J., Wang, L., & Lee, S. C. (2015). Overcome cancer cell drug resistance using natural products. *Evidence-Based Complementary and Alternative Medicine, 2015*. https://doi.org/10.1155/2015/767136

Yang, H., Ren, S., Yu, S., Pan, H., Li, T., Ge, S., Zhang, J., & Xia, N. (2020). Methods favoring homology-directed repair choice in response to crispr/cas9 induced-double strand breaks. *International Journal of Molecular Sciences, 21*(18), 1–20. https://doi.org/10.3390/ijms21186461

Yu, O., Shi, J., Hession, A. O., Maxwell, C. A., McGonigle, B., & Odell, J. T. (2003). Metabolic engineering to increase isoflavone biosynthesis in soybean seed. *Phytochemistry, 63*(7), 753–763. https://doi.org/10.1016/S0031-9422(03)00345-5

Zeng, L., Zhang, Q., Jiang, C., Zheng, Y., Zuo, Y., Qin, J., Liao, Z., & Deng, H. (2021). Development of atropa belladonna L. Plants with high-yield hyoscyamine and without its derivatives using the CRISPR/Cas9 system. *International Journal of Molecular Sciences, 22*(4), 1–10. https://doi.org/10.3390/ijms22041731

Zhang, P., Du, H., Wang, J., Pu, Y., Yang, C., Yan, R., Yang, H., Cheng, H., & Yu, D. (2020). Multiplex CRISPR/Cas9-mediated metabolic engineering increases soya bean isoflavone content and resistance to soya bean mosaic virus. *Plant Biotechnology Journal, 18*(6), 1384–1395. https://doi.org/10.1111/pbi.13302

Zhang, X., Jin, M., Tadesse, N., Dang, J., Zhou, T., Zhang, H., Wang, S., Guo, Z., & Ito, Y. (2018). *Dioscorea zingiberensis* C. H. Wright: An overview on its traditional use, phytochemistry, pharmacology, clinical applications, quality control, and toxicity. *Journal of Ethnopharmacology, 220*, 283–293. https://doi.org/10.1016/j.jep.2018.03.017

Zhang, Z., Mao, Y., Ha, S., Liu, W., Botella, J. R., & Zhu, J. K. (2016). A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*. *Plant Cell Reports, 35*(7), 1519–1533. https://doi.org/10.1007/s00299-015-1900-z

Zhou, Z., Tan, H., Li, Q., Chen, J., Gao, S., Wang, Y., Chen, W., & Zhang, L. (2018). CRISPR/Cas9-mediated efficient targeted mutagenesis of RAS in *Salvia miltiorrhiza*. *Phytochemistry, 148*, 63–70. https://doi.org/10.1016/j.phytochem.2018.01.015