REVIEW ARTICLE

Rational Design of Plant Chemical Factories: CRISPR-Based Metabolic Engineering in Medicinal and Aromatic Plants

Saeid Kadkhodaei[1,*], **Hassan Zadabbas Shahabadi**[1] **and Hossein Hosseini-Monfared**[2]

[1] Agricultural Biotechnology Research Institute of Iran, AREEO, Isfahan 84156-83111, Iran
[2] Zanjan Agricultural and Natural Resources Research and Education Center, AREEO, Zanjan, Iran

Abstract

Medicinal and aromatic plants (MAPs) serve as biochemical factories producing valuable secondary metabolites, yet their potential is limited by low yields, tissue-specific accumulation, and co-production of toxic compounds. Traditional improvement methods have achieved only incremental gains, highlighting the need for precision metabolic engineering. CRISPR/Cas genome editing has revolutionized this field by enabling targeted modifications from gene knockouts to single-nucleotide changes. This review examines core strategies in applying genome editing to engineer MAP metabolic pathways, including gene disruption, transcriptional modulation, and multiplex editing to redirect flux, eliminate competing pathways, and remove toxic branches. Case studies demonstrate successes in alkaloid engineering—such as clean chemotypes with pure hyoscyamine in Atropa belladonna (>50% yield increase) and detoxified Symphytum officinale—and terpenoid enhancement with three-fold glycyrrhizin increase in licorice through combined blocking and overexpression. These examples showcase CRISPR's surgical precision for improving pharmaceutical purity, safety, and production efficiency. We discuss integration with multi-omics for systems-level optimization and explore emerging frontiers including base editing, prime editing, and CRISPR-directed evolution for enzyme optimization. As these technologies mature, genome editing will transform MAPs into customizable cellular factories delivering sustainable, high-value bioproducts.

Keywords: CRISPR/Cas9, metabolic engineering, medicinal plants, alkaloid biosynthesis, terpenoid biosynthesis, gene knockout, pathway optimization, secondary metabolism.

1 Introduction: From Natural Abundance to Precision Design

Medicinal and Aromatic Plants (MAPs) are nature's most virtuosic chemists. They function as sophisticated living factories, synthesizing a vast and intricate arsenal of secondary metabolites that

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***Corresponding author:**
✉Saeid Kadkhodaei
s.kadkhodaei@abrii.ac.ir

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form the bedrock of modern pharmacology and numerous industries [1, 2]. For millennia, humanity has harvested these plants for life-saving drugs, essential flavors, and critical industrial materials. Yet, this reliance is inherently fragile. The production of these valuable bioactive compounds is often frustratingly low, confined to specific plant tissues or fleeting developmental windows, and highly vulnerable to environmental changes [3, 4]. A further complication arises from the metabolic networks themselves, which frequently co-produce a cocktail of structurally related compounds. Some of these analogues may be inert, while others can be dangerously toxic, complicating downstream processing, inflating purification costs, and posing a direct risk to consumer safety [5, 6].

Traditional strategies to boost phytochemical yields—including conventional breeding, chemical elicitation, and optimizing cultivation—have provided incremental gains but are ultimately blunt instruments. Breeding is a generational endeavor, limited by available genetic diversity and the complex, polygenic nature of metabolic traits. Elicitation, while sometimes effective, can be inconsistent and may trigger broad, non-specific stress responses that inadvertently increase the accumulation of undesirable byproducts [7]. These fundamental limitations highlight an urgent need for more precise, predictable, and potent tools to unlock the full metabolic potential of MAPs.

Metabolic pathway engineering signifies a paradigm shift in this pursuit. It moves beyond passive optimization to the active, rational redesign of plant biochemistry. The ultimate vision is to transform MAPs into highly optimized "cellular factories," engineered to produce high volumes of specific, high-value compounds in a controlled, sustainable, and economically viable manner [8, 9]. The arrival of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas system has revolutionized plant genome editing, with demonstrated applications in virus resistance [10], biotic and abiotic stress tolerance [11], and beyond. In stark contrast to earlier genetic modification techniques that relied on the random insertion of foreign DNA, CRISPR/Cas offers an unprecedented capacity for precise, targeted modifications—from complete gene knockouts to subtle, single-base changes—directly within an organism's native genetic landscape [12, 13]. This surgical precision allows scientists to dissect and rewire complex metabolic networks with breathtaking accuracy. By targeting pivotal genes, it is now possible to amplify the production of valuable molecules, shut down competing metabolic branches, eliminate the synthesis of toxic contaminants, and even create novel chemical structures, heralding a new era of next-generation metabolic engineering [1, 8, 9].

This review delves into the core strategies and landmark achievements of genome editing for pathway engineering in MAPs. We will explore how targeted gene knockouts, transcriptional modulation, and multiplex editing are being used to redirect metabolic flux and enhance product yields. We will examine in-depth case studies from the biosynthesis of alkaloids and terpenoids, demonstrating how these tools are solving long-standing challenges in phytochemical production. Finally, we will assess the current hurdles and future prospects, outlining a roadmap for developing the next generation of MAPs with custom-designed metabolic outputs for medicine and industry.

2 Core Strategies for Engineering Metabolic Flux

The output of a biosynthetic pathway is dictated by a delicate balance of enzymatic activity, precursor supply, and layers of regulatory control. Successful metabolic engineering hinges on identifying and manipulating the critical nodes within this network to channel resources preferentially towards a desired product [9]. CRISPR-based technologies provide a versatile toolkit to achieve this through several powerful strategies.

2.1 Upregulating Biosynthetic Pathways

A primary objective is to increase the metabolic flow through a target pathway. This is typically achieved by addressing bottlenecks, either by boosting the activity of rate-limiting enzymes or by activating the master regulators that control the entire pathway.

Targeting Rate-Limiting Enzymes: Every biosynthetic pathway has enzymatic steps that act as chokepoints, limiting the overall rate of production. While traditional transgenesis has long been used for overexpression, CRISPR activation (CRISPRa) offers a more refined approach. CRISPRa utilizes a catalytically "dead" Cas9 (dCas9) protein fused to a transcriptional activator, which can be guided to a specific gene's promoter to enhance its natural expression without altering the DNA sequence, thereby preserving its native regulatory context [13]. For terpenoid biosynthesis, for example,

prime targets include upstream enzymes in the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways, such as HMGR and DXS, as well as downstream "tailoring" enzymes like cytochrome P450s (P450s) and glycosyltransferases that finalize the molecule [1]. A powerful demonstration in licorice (Glycyrrhiza uralensis) showed that combining the overexpression of a key P450 enzyme, CYP88D6, with the knockout of competing pathways resulted in a threefold increase in the accumulation of the valuable triterpenoid glycyrrhizin [14].

Modulating Transcription Factors (TFs): Entire biosynthetic pathways are often co-regulated by master TFs from families like MYB, basic helix-loop-helix (bHLH), and WRKY, which act as powerful levers for pathway-wide modulation [15, 16]. Editing these TFs or their binding sites can orchestrate the coordinated upregulation of multiple genes at once. In Salvia miltiorrhiza (Danshen), the TF SmMYC2 positively regulates the biosynthesis of both phenolic acids and diterpenoid tanshinones, making it a key activation target [17]. Conversely, an equally potent strategy is to knock out negative regulators. For instance, the Gibberellin-Induced TF SmMYB71 was found to be a repressor of salvianolic acid biosynthesis in Danshen; its knockout is a clear strategy to increase production [18]. Similarly, in Artemisia annua, the bHLH TFs AabHLH2 and AabHLH3 negatively regulate the synthesis of the antimalarial drug artemisinin, marking them as prime targets for knockout to boost yield [19].

2.2 Downregulating or Eliminating Competing Pathways

Metabolic pathways are not simple linear tracks but complex, branching networks where a single precursor can be diverted into multiple routes. A highly effective engineering strategy is to block these competing branches, forcing the shared precursor down the desired pathway. CRISPR/Cas9-mediated gene knockout is the perfect tool for this, enabling the precise and permanent disruption of enzymes at these critical metabolic junctions [9, 11].

This approach was demonstrated spectacularly in the engineering of triterpenoid saponins in licorice hairy roots [14]. The precursor β-amyrin is a branch point for the synthesis of both desirable glycyrrhizin and less valuable soyasaponins. By using CRISPR/Cas9 to simultaneously knock out two key P450 genes in the soyasaponin branch (CYP93E3 and CYP72A566), researchers effectively dammed this competing pathway. This metabolic redirection not only diminished soyasaponin levels but also funneled the β-amyrin pool toward glycyrrhizin production. When this knockout strategy was combined with the overexpression of the rate-limiting glycyrrhizin enzyme CYP88D6, glycyrrhizin content soared to approximately three times that of the knockout lines alone [14]. This study is a blueprint for how combining pathway upregulation with the elimination of metabolic sinks can yield synergistic gains. A similar principle could be applied in Salvia miltiorrhiza, where TFs like SmbHLH60 and SmMYC2 antagonistically regulate phenolic acid and anthocyanin biosynthesis, presenting an opportunity to knock out regulators of the less desired pathway to enhance the other [17].

2.2.1 Eliminating Undesirable or Toxic Metabolites

Many MAPs produce a cocktail of related compounds, some of which are toxic or have adverse side effects, posing a major barrier to their clinical use. Genome editing provides a revolutionary solution by enabling the surgical removal of genes responsible for synthesizing these toxic molecules, creating "clean" plant lines that are safer, more effective, and cheaper to purify [3].

The most compelling demonstration of this strategy comes from work on comfrey (Symphytum officinale). While comfrey produces valuable wound-healing compounds, it is contaminated with hepatotoxic pyrrolizidine alkaloids (PAs). The biosynthesis of all PAs depends on the enzyme homospermidine synthase (HSS), which catalyzes the first committed step. Using CRISPR/Cas9 in hairy root cultures, researchers successfully knocked out the HSS gene. The resulting mutant lines showed a significant reduction in PA levels, while the production of beneficial metabolites, such as rosmarinic acid, remained unaffected [6]. This landmark study showcases the surgical precision of CRISPR/Cas9 in removing a single toxic branch from a complex metabolic network without collateral damage. A similar principle was applied to eliminate toxic steroidal glycoalkaloids (SGAs) in potato by disrupting the St16DOX gene, a finding highlighted in broader reviews of the field [20]. These examples underscore a powerful application of genome editing: enhancing the safety and quality of medicinal plants and food crops to a degree that is virtually impossible with conventional breeding.

3 Engineering Novelty: Beyond Boosting Yields to Refining Products

Genome editing's potential extends beyond simply tweaking the quantity of existing compounds. It opens the door to creating fundamentally new traits and products by purifying metabolic outputs or generating new chemical diversity.

3.1 Engineering "Clean" and Optimized Product Profiles

The pharmaceutical industry demands highly pure active ingredients, but MAPs often produce a mix of closely related analogues that are difficult and expensive to separate. Genome editing can be used to re-engineer plants to produce only a single desired compound, streamlining the entire production pipeline.

This concept was brilliantly executed in deadly nightshade (Atropa belladonna), a primary source of the anticholinergic drug hyoscyamine [5]. In the plant, a portion of the hyoscyamine is converted into its derivatives, anisodamine and scopolamine, by the enzyme hyoscyamine 6β-hydroxylase (H6H). These derivatives have different clinical uses and must be separated from the primary drug. Using CRISPR/Cas9, researchers targeted and knocked out the AbH6H gene. The resulting homozygous mutant plants exhibited a remarkable new trait: they produced significantly higher levels of hyoscyamine while being completely devoid of both anisodamine and scopolamine [5, 21]. This metabolic blockade not only increased the yield of the target compound but also created a "clean chemotype"—a plant variety perfectly optimized for the industrial production of a single, pure alkaloid. This represents a fundamental redesign of a plant's metabolic output to meet industrial specifications.

3.2 Neofunctionalization and Synthetic Biology

The next frontier lies in creating entirely novel enzymatic functions and products. Advanced tools like base editing and prime editing, which allow for precise nucleotide substitutions without causing disruptive double-strand breaks, are pivotal for this goal [22].

By introducing specific changes to an enzyme's active site, it is theoretically possible to alter its substrate specificity or catalytic activity, a process known as neofunctionalization. One could, for example, modify a P450 enzyme to accept a new substrate or perform a different chemical reaction, leading to the synthesis of a novel compound with new therapeutic properties. This strategy could be applied to key enzymes in terpenoid biosynthesis, such as squalene epoxidases or oxidosqualene cyclases, which forge the foundational carbon skeletons of thousands of different triterpenoids [23, 24]. Subtle edits to these enzymes could unlock entirely new triterpenoid scaffolds for drug discovery.

Looking further ahead, multiplex genome editing—the simultaneous modification of many genes [12]—can be used to reconstruct or domesticate entire metabolic pathways in a heterologous host like Nicotiana benthamiana. The convergence of multiplex editing, multi-omics data integration, and synthetic biology principles will drive the discovery and sustainable production of the next generation of plant-derived medicines [1].

4 In-Depth Case Studies in Pathway Engineering

The power of these strategies is best understood through their practical application. The following case studies detail how CRISPR/Cas9 has been successfully deployed to rewire the biosynthesis of two vital classes of plant secondary metabolites: alkaloids and terpenoids (Table 1).

4.1 Case Study A: Redesigning Alkaloid Biosynthesis for Purity and Safety

Alkaloids are a diverse group of nitrogen-containing compounds with potent physiological effects, forming the basis of many essential medicines. The engineering of alkaloid pathways in Atropa belladonna and Symphytum officinale exemplifies two distinct but equally powerful goals: optimizing the purity of a therapeutic agent and eliminating toxicity.

Optimizing Tropane Alkaloid Production in Atropa belladonna: As detailed previously, the industrial challenge with tropane alkaloids (TAs) is producing hyoscyamine, scopolamine, or anisodamine in pure form. To create a superior strain for hyoscyamine production, Zeng et al. [5] used Agrobacterium tumefaciens-mediated transformation to deliver a CRISPR/Cas9 construct targeting the AbH6H gene, which converts hyoscyamine into its derivatives. They generated stable T0 transgenic plants and screened for mutations. The resulting homozygous abh6h mutant lines showed a complete metabolic overhaul. High-performance liquid chromatography (HPLC) analysis confirmed a total absence of both anisodamine

Table 1. CRISPR/Cas9-mediated metabolic engineering in medicinal plants.

Medicinal Plant	Target Metabolite(s)	Engineering Goal	Gene(s) Targeted	CRISPR/Cas9 Strategy	Key Outcome	Significance	Ref.
Atropa belladonna (Deadly Nightshade)	Hyoscyamine (Target), Scopolamine (Byproduct)	Create a "clean chemotype" for pure hyoscyamine production.	*AbH6H* (Hyoscyamine 6β-hydroxylase)	Knockout of the converting enzyme.	Complete elimination of scopolamine; 50% increase in hyoscyamine accumulation.	First example of redesigning a MAP for a single, pure industrial pharmaceutical.	[5]
Symphytum officinale (Comfrey)	Pyrrolizidine Alkaloids (PAs) (Toxic), Rosmarinic Acid (Beneficial)	Detoxification: Eliminate toxic PAs to improve plant safety.	*SoHSS* (Homospermidine synthase)	Knockout of the first committed enzyme in the PA pathway.	Complete eradication of all PAs in hairy roots; beneficial metabolites unaffected.	Proof-of-concept for using CRISPR to create safe, non-toxic medicinal plant varieties.	[6]
Glycyrrhiza uralensis (Licorice)	Glycyrrhizin (Target), Soyasaponins (Competing)	Redirect flux boost yield: Funnel precursors to glycyrrhizin.	*CYP93E3*, *CYP72A566* (Soyasaponin branch) & Overexpression of *CYP88D6*	Multiplex Knockout of competing pathway + Overexpression of rate-limiting enzyme.	3-fold increase in glycyrrhizin accumulation in hairy roots.	Demonstrates a powerful synergistic effect by combining pathway blocking with upregulation.	[14]
Atropa belladonna (Deadly Nightshade)	Tropane Alkaloids (TAs)	Reduce overall TA accumulation to study pathway regulation.	*PKS* (Pyrrolidine ketide synthase)	Knockout of a key upstream regulatory gene.	Significant reduction in total TA content in hairy roots.	Validated a key gene's role in the TA pathway, providing a target for modulation.	[21]
Model MAPs	Metabolic enzymes	*In situ* directed evolution		EvolvR + biosensors	Localized hypermutation generates enzyme variants; fluorescence-based screening identifies optimized catalysts in live tissues	Enables unbiased enzyme evolution under physiological conditions, circumventing limitations of rational design	[25]

and scopolamine in the leaves, while hyoscyamine content increased significantly—by over 50% in some lines—as the metabolic block prevented its conversion [5]. This work is a quintessential example of pathway engineering to create a "clean chemotype," redesigning a plant to produce a single, high-value product.

Detoxification in Symphytum officinale (*Comfrey*): In contrast, the goal in comfrey was to remove a class of toxic byproducts. Comfrey roots are rich in wound-healing compounds but are contaminated with hepatotoxic pyrrolizidine alkaloids (PAs). The first committed step in PA biosynthesis is catalyzed by homospermidine synthase (HSS). Zakaria et al. [6] used an Agrobacterium rhizogenes-mediated system to generate fast-growing hairy root cultures of comfrey and introduced a CRISPR/Cas9 construct to mutate the SoHSS gene. The strategy was exceptionally effective. Multiple independent mutant lines with frame-shifting mutations in HSS were identified. Subsequent metabolomic analysis by liquid chromatography-mass spectrometry (LC-MS) delivered the remarkable result: PA accumulation was completely eradicated in the mutant lines, while the production of desired beneficial compounds remained unharmed. This study provides definitive proof-of-concept for using genome editing as a "molecular scalpel" to enhance the safety and therapeutic potential of a medicinal plant.

4.2 Case Study B: Modulating Complex Terpenoid Biosynthesis

Terpenoids are the largest class of plant secondary metabolites, with applications ranging from pharmaceuticals to fragrances. Engineering these complex, multi-step pathways requires sophisticated interventions targeting precursors, key enzymes, and regulatory networks.

Multi-Pronged Engineering in Glycyrrhiza uralensis (*Licorice*): The work in licorice demonstrates a masterful, integrated approach to boosting glycyrrhizin production. As previously discussed, researchers combined two powerful strategies: blocking a competing pathway and upregulating a rate-limiting step. Using multiplex CRISPR/Cas9, they knocked out genes in the soyasaponin branch (CYP93E3, CYP72A566) and other competing branches. This knockout background was then combined with the overexpression of CYP88D6, the rate-limiting P450 enzyme in the glycyrrhizin pathway. The result was a synergistic tripling of glycyrrhizin accumulation, showcasing how a holistic engineering approach can achieve dramatic gains that are unattainable with a single modification [14].

Dissecting the Regulatory Code in Salvia miltiorrhiza (*Danshen*): The engineering efforts in Danshen highlight the importance of understanding transcriptional regulation. Researchers have identified a complex web of TFs that control the biosynthesis of valuable phenolic acids and tanshinones. This includes positive regulators like SmMYC2 [17] and negative regulators like SmMYB71 [18] and the antagonistic relationship between TFs like SmbHLH60 and SmMYC2 [17]. While direct knockout experiments in some of these TFs are still developing, this foundational work, integrating transcriptomics with functional studies, has created a detailed roadmap for future genome editing. By precisely knocking out repressors or activating enhancers, scientists can now rationally design interventions to rewire the entire regulatory network for enhanced production of specific target compounds.

5 Future Perspectives: Evolving Enzymes In *Situ*

The next frontier may lie not solely in rational design, but in CRISPR-coupled in planta directed evolution. While current genome editing approaches modify genes based on known sequences and predicted protein structures, enzyme kinetics measured in vitro often differ substantially from those in the crowded, regulated cellular environment. An emerging strategy involves utilizing orthologous DNA polymerases fused to Cas9—so-called EvolvR systems—targeted to specific biosynthetic gene clusters [25]. This approach could potentially induce localized hypermutation specifically within the coding regions of metabolic enzymes during plant growth, generating sequence diversity without genome-wide instability.

Fluorescence-based screening offers a critical avenue for enhancing the recovery of edited events, primarily implemented through either Fluorescence-Activated Cell Sorting (FACS) or manual microscopic selection. FACS facilitates the high-throughput, automated enrichment of protoplasts co-expressing fluorescent reporters and CRISPR components, significantly increasing editing efficiency; however, it necessitates specialized instrumentation and robust protoplast regeneration protocols. Conversely, microscopic selection provides an accessible alternative for species recalcitrant to protoplast culture by allowing visual identification of markers in callus or regenerating shoots, albeit with higher labor requirements.

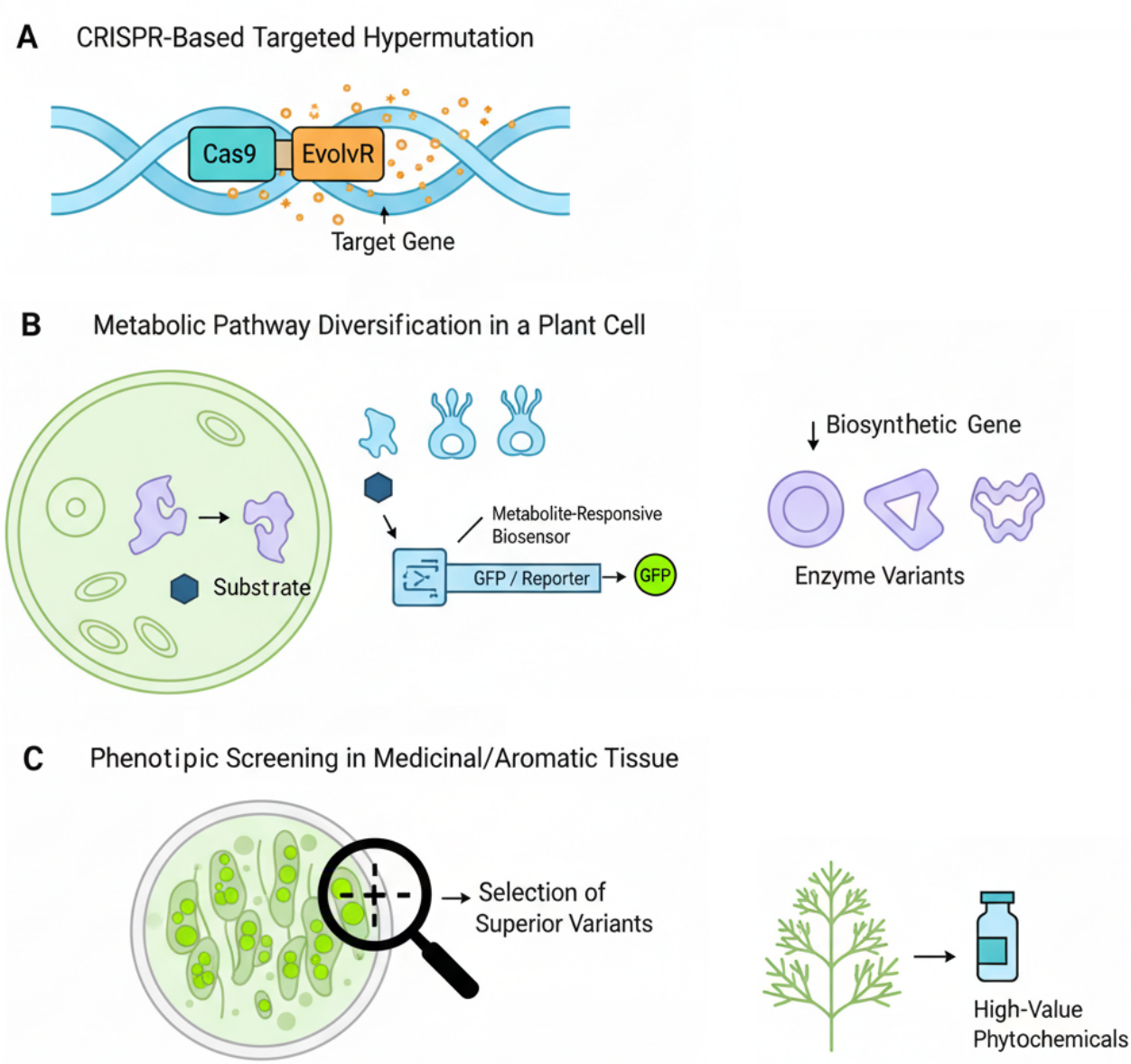


Figure 1. Schematic representation of the cellular evolution platform for CRISPR-based metabolic engineering in medicinal and aromatic plants.

Ultimately, the choice of strategy depends on the specific plant regeneration constraints and requires careful optimization of reporter proteins and screening timing to ensure the effective isolation of edited lines and the subsequent identification of transgene-free progeny via fluorescence segregation.

By coupling targeted mutagenesis with metabolite-responsive biosensors—for instance, engineered transcription factors that trigger fluorescent reporter expression in response to elevated alkaloid concentrations [26]—researchers might screen thousands of enzyme variants within a single leaf or hairy root culture. Cells exhibiting the brightest fluorescence would harbor enzymes evolved for higher catalytic efficiency or altered substrate specificity. This cellular evolution-based approach could bypass the need for complete rational understanding of complex protein folding dynamics, instead allowing the plant cellular environment to serve as the selection pressure for superior metabolic machinery (Figure 1).

1. Targeted Hypermutation Module: The Cas9-EvolvR fusion complex (cyan and gold) targets a specific biosynthetic gene of interest. The Cas9 nickase directs the fused error-prone DNA polymerase to the target locus, inducing localized hypermutation (orange stars) within a defined window of the coding sequence to generate a diverse library of enzyme variants in situ.

2. Pathway Diversification and Biosensing: Inside the engineered plant cell, the library of enzyme variants is expressed, acting on substrates to produce specialized metabolites (e.g., alkaloids or terpenes). A specific metabolite-responsive transcription factor (Biosensor) detects the accumulation of the target end-product. Upon binding the metabolite, the biosensor activates the promoter of a reporter gene (GFP), linking metabolic flux directly to a fluorescent output.
3. High-Throughput Phenotypic Screening: Transformed plant tissues (e.g., hairy roots or leaf discs) are screened for fluorescence intensity. Cells harboring superior enzyme variants with enhanced catalytic activity exhibit strong GFP signals (bright green), enabling the visual identification and isolation of high-performing lines from a heterogeneous population.

6 Conclusion: The Dawn of the Designer Medicinal Plant

The application of CRISPR/Cas systems has irrevocably transformed metabolic pathway engineering in medicinal and aromatic plants. We have moved from the slow, unpredictable outcomes of conventional breeding to an era of precise, rational design. As the case studies in this review illustrate, genome editing provides an exquisite toolkit for solving long-standing challenges in phytochemical production. By knocking out genes with surgical precision, we can create safer medicines by eliminating toxic compounds like pyrrolizidine alkaloids in comfrey [6]. By blocking metabolic branches, we can develop hyper-productive, "clean" plant lines that synthesize a single, pure pharmaceutical, as demonstrated with hyoscyamine in Atropa belladonna [5, 21]. And by combining pathway knockouts with targeted overexpression, we can achieve synergistic increases in yield, as shown with glycyrrhizin in licorice [14].

The journey, however, is not over. Significant challenges remain, including the efficient delivery of editing reagents and regeneration of whole plants from edited cells, especially in non-model MAPs. Furthermore, the metabolic networks we seek to control are profoundly complex, and disrupting one node can have unforeseen consequences elsewhere. The path forward will therefore depend on the tight integration of genome editing with multi-omics technologies [1]. Genomics, transcriptomics, and metabolomics will provide the blueprints needed to identify the most effective targets and to understand the systemic impact of our edits.

The continued development of advanced editing tools like base and prime editors [22] will open up even more sophisticated possibilities, such as neofunctionalizing enzymes to produce entirely new molecules [23, 24]. The vision of the optimized "cellular factory" [8, 9] is no longer a distant dream but an emerging reality. Genome editing is the enabling technology that will allow us to harness the full chemical power of the plant kingdom, delivering the next generation of sustainable, high-value medicines and materials.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Ethical Approval and Consent to Participate

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