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Sustainable production of plant-derived bioactives via genetic engineering and nanotechnology-driven approaches

Sanamdeep Singh [a], Pooja Sharma [a,*], Mohammad Faizan [b], Pravej Alam [c], Thamer Albalwai [c]

[a] Department of Biotechnology, Baba Farid College of Engineering and Technology, Bathinda, Punjab 151002, India
[b] Botany Sections, School of Sciences, Maulana Azad National Urdu University, Hyderabad 500032, India
[c] Department of Biology, College of Science and Humanities, Prince Sattam bin Abdulaziz University, Alkharj 11942, Saudi Arabia

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ABSTRACT

As the global demand for plant-based bioactives rises, fuelled by concerns about synthetic drugs and sustainability, there is urgent need for advanced metabolic engineering solutions. Conventional extraction methods, however, are limited by low yields, seasonal variabilities and environmental impact. This review critically examines sustainable production strategies that combine the potential of CRISPR-based gene editing, nanoparticle mediated delivery systems, elicitation techniques and metabolic flux regulation to enhance the secondary metabolite production in plants. Recent advances have enabled targeted pathway redirection, achieving multifold increases in compounds such as artemisinin, paclitaxel and reservatol in both model and medicinal species. Nanoparticle-mediated elicitation and nano-delivery of CRISPR components further enhance metabolic flux and precise genetic alterations to boost metabolite biosynthesis. Although challenges related to biological and experimental limitations including species-specific responses, incomplete pathway elucidation and off target editing remain. Technological barriers encompass scalability constraints in bioreactors, nanoparticle aggregation, phytotoxicity and regulatory hurdles involving absence of harmonised frameworks for genome-edited and nanomaterial exposed crops for pharma use. This review offers the realistic roadmap for fusion of nanotechnology with metabolite engineering for paving the way for innovations in agriculture, pharmaceuticals and industrial biotechnology, promoting more efficient and environmentally friendly production of bioactive compounds.

1. Introduction

Plants are perpetually exposed to a variety of stresses that interferes with their physiological functions, impede growth and diminish productivity by more than 60% on average [1,2], requiring complex adaptive mechanisms to maintain balance and ensure survival [3]. Biotic stress can be described as detrimental impact that living organism (like pathogens, pests and weeds) have on the plant maturation, development and plant produce [4].Other than that, worsening soil and climate conditions, along with rising human-induced pressures, greatly increase abiotic stress in plants, impacting their growth, development and ability to withstand challenges [5]. To subsist these stressful conditions, plants undergo changes at the transcriptomic and metabolomic levels through signalling pathways that detect environmental and biotic cues. Equipped with receptors and sensors, they can identify threat signals, enabling them to mount defensive reactions to different stresses [6,7].

Plants produce primary metabolites, chemical components, essential for proliferation and development of a plant and bioactive compounds (secondary metabolites) necessary for survival of plant under unfavourable conditions [6,8]. These bioactive compounds include alkaloids, flavonoids, essential oils, glycosides, tannins and resins that have potent bactericide, antimycotic and antivirotic properties therefore helping plants to fight against various disease-causing microbes and inhibit proliferation of phytotoxins in plant's body. Due to their biological function, such as improving human health by minimizing the risk of chronic illnesses, protecting the genome, strengthening the immune system, eliminating toxins, preventing heart disease and helping body to fight cancer, these plant bioactive compounds have been used in manufacturing of traditional medicine form ages. In present time these bioactive compounds are being widely used in cosmetology, agriculture and food sector [9,10].

* Corresponding author.
E-mail address: gunjanpu333@gmail.com (P. Sharma).

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The global pharmaceutical market value for therapeutics based on bioactive compounds has been increasing due to rise in awareness among consumers regarding the side effects caused by medicines made from synthetic components to health and environment such as deforestation [11], soil pollution [12] and water contamination [13,14]. In recent report conducted by MDFR (2024) the global pharmaceutical market value for plant-based therapeutics was valued at $215.4 billion in 2024 and is estimated to reach $375.6 billion by 2032 showing a compound annual growth of (CAGR) of 7.22% [11]. These plant-based therapeutics can be extracted using several methods including steam distillation, enzyme-assisted extraction, solvent extraction, gas chromatography (GC), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), high-performance liquid chromatography (HPLC) and supercritical fluid extraction (SFE) with UAE extracting the highest yield among all methods [15]. The quantity of extraction of these bioactive compounds highly depends on environmental condition like temperature, moisture, soil fertility, light and soil salinity [16]. While advanced methods of extraction results in higher yield but the traditional methods of extraction are cost effective, quick and simple to conduct [17].

One other way to increase the yield of these bioactive compounds is through metabolic engineering which is done by amplification or inhibition of enzymatic activity [18], enhancing carbon flow towards the pathway which produces the targeted metabolite [19] or by interrupting the intermediate steps that results in formation of other desired products [20,21]. This is facilitated through use of nanomaterials e.g., nanomaterials act as catalysts and amplify the enzymatic activity and regulates metabolic flux by managing gene expression. Furthermore, these nanomaterials lead to generation of free reactive oxygen species (ROS) and help in precise delivery of precursor genes resulting in stabilization of free enzymes and optimizing metabolic pathways for increased yield of plant bioactives [22,23]. This method of increasing yield of plant bioactives by triggering physiological changes and defence response by nanomaterials is known as elicitation and nanomaterials are known as elicitors [24].

The present study outlines how these nanomaterials can be used in CRISPR technology facilitating genetic modification to improve plant defence system by improving the yield of bioactive compounds and also focuses on integration of nanotechnology in metabolic engineering, highlighting its potential use to increase the yield of plant bioactives promoting sustainable agriculture offering a fresh perspective on the role of nanomaterials as biochemical catalysts, providing innovative solutions for agriculture, medicine and biotechnology.

2. Role of secondary metabolites in production of plant bioactives

Plant bioactives are compounds are small group of biochemical compounds found in plants and a number of foods that promote good health [25]. The plant-based bio-active's market is projected to be worth 1.78 billion in 2024 and is growing 10.2% annually and expected to reach 4.65 billion by 2031 [26]. These plant bioactives can be classified into several categories, such as alkaloids, capsaicinoids, carotenoids, polyphenols, glucosinolates, phytosterols, tocopherols, and other related compounds [27,28]. These compounds possess potent bactericide, antimycotic and antiviral properties making them valuable ingredient in health & therapeutics, nutraceuticals and sustainable agriculture [29]. However, the commercial exploitation of these molecules is frequently hampered by their low natural abundance, the slow growth rates of host plants and logistical challenges of large scale extraction.

To illustrate the enormous structural and functional diversity of plant-derived bioactives and their wide-ranging applications, major classes of secondary metabolites are overviewed in this section. The representative compounds were selected according to the criteria of core biosynthetic pathways, high commercial relevance and inclusion of compounds actively studied in current biotechnological pipelines. The production maturity of any compound of product is assessed using an adapted Technology Readiness Level (TRL) scale commonly applied to bioprocesses (TRL 1–3: basic research/proof-of-concept; TRL 4–6: laboratory validation and pilot-scale optimization via elicitation, cell suspensions or hairy roots; TRL 7–9: industrial/commercial deployment), highlighting translational readiness and remaining scale-up challenges [30].

At the foundational level of specialized metabolism, phenolic compounds, flavonoids (e.g., kaempferol) and tannins (e.g., tannic acid) utilize the phenylpropanoid and shikimate pathways to exert potent antioxidant and antimicrobial effects by scavenging free radicals and disrupting microbial DNA synthesis. These currently occupy a moderate production maturity, i.e., TRL 5–6 [31] where cell suspension and nano-elicitation strategies have demonstrated successful lab-scale accumulation but face ongoing challenges in achieving the high-density fermentation required for industrial scale-up.

In contrast, terpenoids are derived from the cytosolic mevalonate (MVA) and plastidial methylerythritol phosphate (MEP) pathways. Compounds like limonene, nootkatone (monoterpenes), artemisinin (sesquiterpene) and paclitaxel (Taxol®, diterpene) represent this category of plant bioactives. They exhibit a moderate to high TRL (6−8) depending on the compound [32]. While paclitaxel is a flagship success for industrial-scale plant cell culture (TRL 8), any other antimalarial and anticancer terpenoids are currently being transitioned from microbial chassis to plant-compatible synthetic biology platforms to better accommodate their requirement for specific organelles and P450 matched redox partners [27]. Nitrogenous alkaloids (e.g., vincristine) and steroid-derived glycosides (e.g., ginsenosides) represent the most mature production tier (TRL 8–9). Their high functional specialization, acting through precise receptor binding and ion transport regulation, has already secured their place in commercial in plant and large-scale industrial biofactories. The inclusion of lectins (e.g., MAP30) and bioactive polypeptides (e.g., thionins) addresses the latest frontier in biotechnology [28]. These molecules represent an experimental maturity level (TRL 3–4), where current research focuses on transient expression systems and recombinant DNA technology to harness their unique ability to inhibit viral replication and disrupt microbial ion channels [27].

A study conducted by Elsherif et al. [33] investigated the connection between the synthesis of secondary metabolites and plant bioactives in *Lotus arabicus L.* The research emphasized that when the plant is treated with dried date palm seed powder as an elicitor, there is a notable increase in secondary metabolites like phenolics and flavonoids. This treatment boosted the expression of crucial genes linked with secondary metabolism, resulting in heightened production of bioactive compounds. Essentially, secondary metabolites act as precursors or intermediates in the creation of bioactive compounds, which enhance the plant's medicinal and physiological attributes (Fig. 1).

Despite their beneficial properties these compounds are hard to extract even with sophisticated technology due to insufficient production in plants. Due to this reason, modern approaches such as metabolic pathway engineering using nanotechnology supports biosynthesis through methods like elicitation, metabolic engineering, and gene delivery [17]. The synergy of nanoparticles with hairy root cultures and the fine-tuning of light exposure further boosts production [33]. Furthermore, Synthetic genomics have facilitated the creation of non-viral nano-delivery systems for CRISPR/Cas9 among which non-viral nanocarriers, exosomes and lipid nanoparticles are particularly noteworthy in both *in-vitro* and *in-vivo* settings, offering sustainable solutions for large-scale production [34].

3. Nanoparticle-mediated elicitation in plants

Nanomaterials transform gene expression related to cell division, cell transports, cell organisation and abiotic and biotic stress pathways [35].

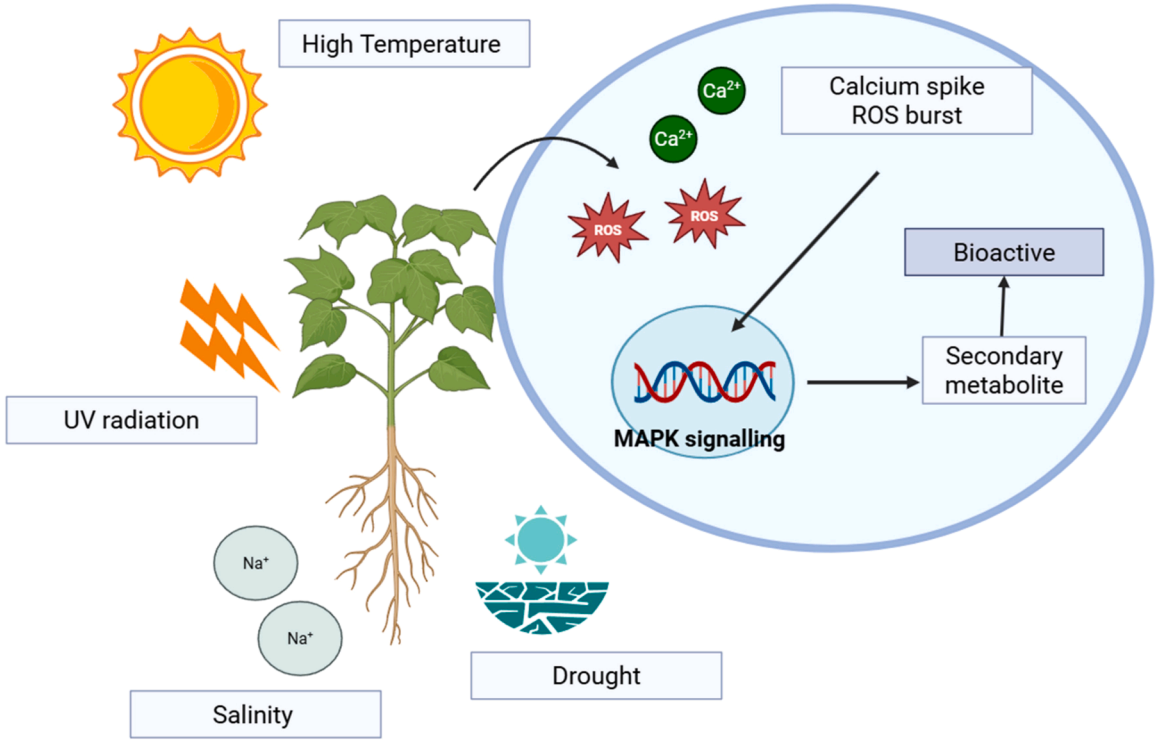

Fig. 1. Due to abiotic stress plants cells produce calcium ions and ROS (reactive oxygen species) which signals nucleus to transcribe MAPK (Mitogen-Activated Protein Kinase) pathway resulting in formation of plant secondary metabolites with acts as precursor to form plant bioactive compounds.

These NPs (nanoparticles) when taken up by plants undergo an oxidative surge, resulting in an overabundance of calcium ions (Ca^{2+}) and free ROS. This activates antioxidant defence system and MAPK cascades, etc., which leads to transcriptional reprogramming of secondary metabolism by production of enzymatic and non-enzymatic antioxidants including plant bioactive compounds to counter the stress condition [36]. This process of inducing physiological changes and activating plant defence systems to enhance the yield of plant bioactive compounds is called elicitation.

Nanomaterials that can initiate various plant responses, such as internal defence mechanisms and synthesis of different plant bioactives are known as elicitors [24]. Nanoparticle-mediated elicitation has emerged as a versatile abiotic strategy for modulating secondary metabolite accumulation, yet outcomes are highly context-dependent and cannot be extrapolated across experimental systems without qualification. Responses vary markedly with plant species, culture type (undifferentiated cell suspensions versus differentiated hairy roots), NP physicochemical properties, dose, exposure duration and culture conditions (pH, agitation and photoperiod). For example, in cell-suspension cultures of *Carthamus tinctorius* maintained under dark conditions (28 °C, 115 rpm), jasmonic acid-loaded Fe_3O_4 NPs at an optimised concentration of 20 mg/L elicited a 2.26-fold increase in chlorogenic acid (peaking at 43.76 mg/g DW after 72 h) together with elevated antioxidant enzyme activities [37]. However, doses $\geq$ 40 mg/L triggered iron-ion dissolution, excessive ROS accumulation and subsequent declines in both biomass and metabolite yields. In another study, hairy root cultures of *Hyoscyamus reticulatus* responded positively to low-to-moderate ZnO NPs concentrations through upregulation of tropane alkaloid biosynthetic genes whereas supra-optimal levels induced cytotoxicity and biomass reduction [38]. Such studies illustrate that enhancement in crop yield or biomass is dose and species dependent and is not universal.

However, several physico-chemical properties of NPs influence bioavailability, uptake and distribution in plant cells, like chemical composition, size, surface-charge, shape and selective uptake by different plant species [39,40]. Past research has shown that NPs with particle size less than 10 nm can be absorbed well by plant roots, e.g., gold NPs (3.5 nm) and CeO_2 (8 $\pm$ 1 nm), in roots of *Vicia faba* and maize, respectively [41,42]. According to Wang et al. [39], silicon-based or natural polymer based NPs (particle size > 100 nm) can be absorbed. For example, SiNPs (200 nm) were absorbed by *Arabidopsis thaliana* root after 6 weeks treatment. These NPs also influence the phytohormone regulation at various levels. Phytohormones are small intrinsic signalling molecules that controls different stress pathways, plant proliferation and maturation. Plants produce abscisic acid (ABA) [43], ethylene [44], salicylic acid (SA) and jasmonic acid (JA) in response to abiotic stress [45]. Treatment of metallic NPs results in overexpression of NCED3, RD22, ACS, ETR, ACO, PR and Cs-PVA genes which sends cascade of signals that results in formation of these hormones ([44]). For example ZnONPs reduced the amount of heavy metals Pb^{2+} and Cd^{2+} in *Latuca sativa* L.var. *Longifolia* up to 44% [46]. Due to increased amount of these phytohormones the secondary metabolite amount of plants also increases [47,48]. This is due to cell division and multiplication caused by phytohormones that causes the increase in content in plants [49]. A group of researchers performed a study on rice with ZnONPs (100 mg/mL) against Arsenic (As) accumulation. They concluded that the concentration of As in bud and root decreased by 40.7% and 31.6% respectively, while improving crop yield. In the same manner, Selenium and Silica NPs decreased lethal effects of Pb and Cd in rice along with helping increase chlorophyll content in leaves [50]. Similarly, the use nanomaterials to modulate intracellular metabolic fluxes (e.g., via targeted delivery of transcription factors or pathway intermediates) demonstrates clear experimental boundaries. Studies employing carbon-based or metal-oxide NPs in *Taxus* or *Artemisia* systems report improved flux towards taxanes or artmisinin only within narrow concentration windows and under tightly controlled bioreactor conditions [51,52]. Hence, this process can be used to increase plant bioactive content making it a useful biotechnological tool for agriculture, medicine and other economic sectors (Fig. 2).

SOD: superoxide dismutase; APX: ascorbate peroxidase; GST:

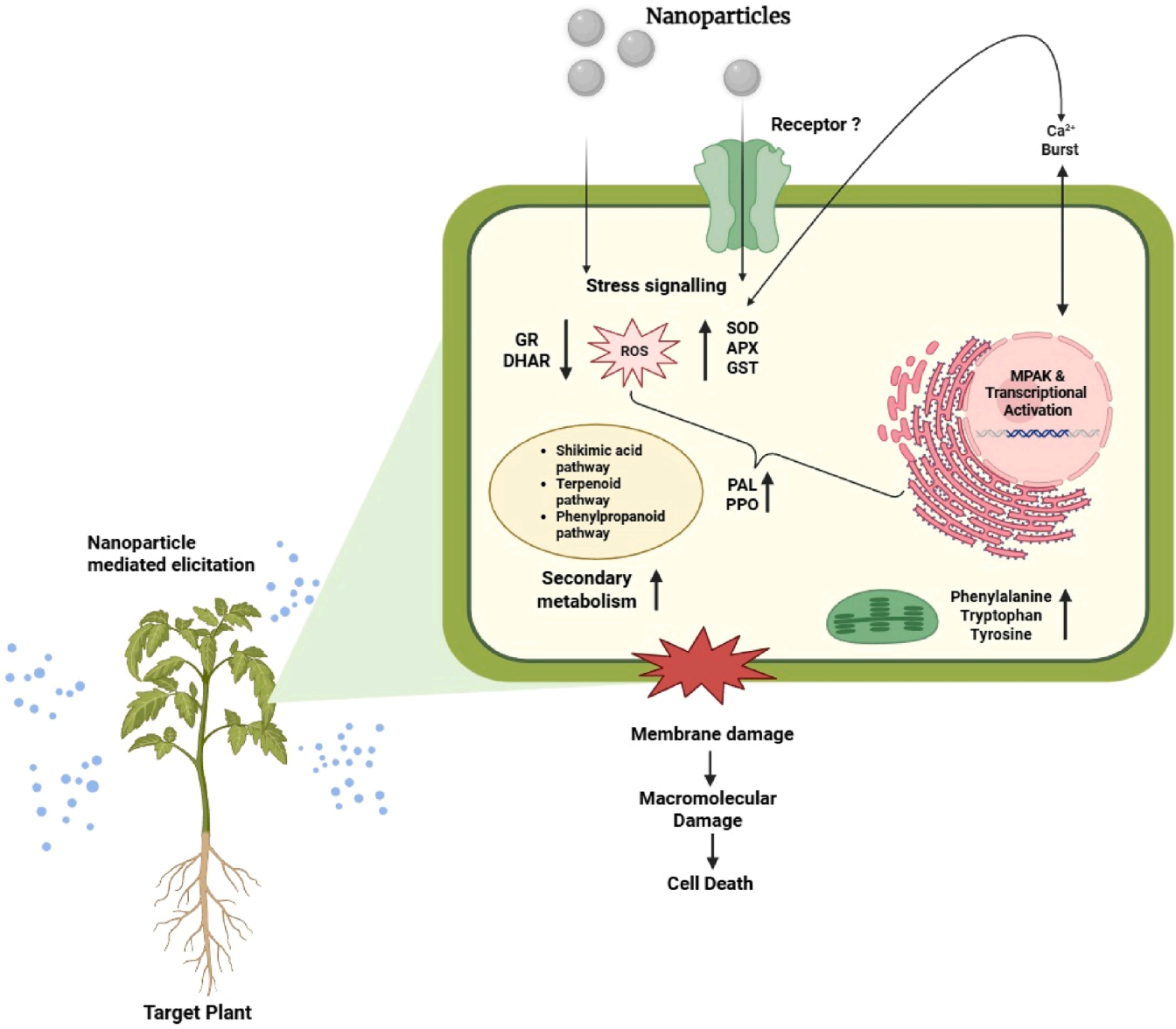

Fig. 2. NMs induce oxidative stress by generating excessive ROS, triggering the antioxidant defence mechanisms, causing lipid peroxidation, damaging membranes, leading to calcium surges [53], activating MAPK signalling pathways, and modifying secondary metabolism in plants. Arrows pointing upwards signify an increase in abundance, while arrows pointing downwards denote a decrease in abundance within the plant cell. **Abbreviations:**.

glutathione transferase; GR: glutathione reductase; DHAR: dehydroascorbate reductase; PAL: phenylalanine ammonia lyase; PPO: polyphenol oxidase [35].

Recent studies emphasize that NP effects are significantly influenced by a plant's genetic background and domestication history. For example, silver nanoparticles (AgNPs) at concentrations of 100–250 ppm significantly enhance germination in wild *Capsicum annum* (up to 90%) but show no comparable effect in domesticated Serrano varieties [54]. This suggest that domesticated plants, possessing larger seed reserves and standardized performance may have a reduced plastic response to nano-priming compared to wild genotypes.

Although NP-mediated elicitations and metabolic modulations offer substantial promise, a critical assessment reveals important biological, experimental and translational constraints that temper expectations for immediate industrial deployment. A consistent pattern documented across recent syntheses is hermetic-dose-response behaviour. Low-to-moderate concentrations (typically 1–50 mg/L, depending upon NP type and plant species) generate controlled ROS bursts that activate defence signalling and secondary metabolite pathways. Whereas, higher concentrations provoke oxidative damage, lipid peroxidation, membrane leakage, DNA degradation and inhibition of key antioxidant enzymes, ultimately reducing cell viability and metabolite productivity [38,52]. These effects are further compounded by pronounced species-specific metabolite capacities and culture-type differences. Undifferentiated cell suspensions generally exhibit greater sensitivity than organised hairy roots, which benefit from partial compartmentalization and differentiated physiology [51]. Experimental reproducibility is further challenged by NP aggregation in culture media, batch-to-batch variability in green-synthesised materials and the influence of uncontrolled variables (such as medium pH and exposure duration). At the translational level, scalability remains limited by high production costs, potential persistence of residual NPs in biomass or effluents and the current absence of harmonised regulatory frameworks governing nanomaterial use in plant bioprocessing [55]. Future integration with CRISPR-based pathway engineering and real-time monitoring systems will be essential to overcome these bottlenecks and realise sustainable, reproducible outcomes.

4. Metabolic pathway modifications using nanotechnology

Catalyst are compounds that increase rate of a reaction without changing its standard Gibbs energy change in a reaction and the processes is known as catalysis [56]. Most of the biological reactions rely on enzymes (i.e., protein catalysts) that dictate reaction specificity and process of using enzymes as catalysts is known as enzyme catalysis [57]. In study conducted by Yang et al., [58] it was found out that enzymatic activity of *Hypsizygus marmoreus* was highly dependent on environmental factors like humidity, CO_2 concentration and light intensity, because change in these environmental factors during growth and developmental phase during cellular respiration affect the expression of enzyme related genes.

Ensuring the regulation of enzyme activity is crucial for maintaining the efficiency of metabolic pathways, particularly in the production of secondary metabolites. Precursors and intermediates are often compartmentalized within organelles or membrane-bound structures to prevent undesirable reactions and increase specificity. This spatial arrangement guides molecules to the appropriate metabolic locations, enhancing enzyme interactions and product formation. In microbial engineering, managing these pathways enables the precise biosynthesis of plant bioactives [59]. Since enzymes have elevated sensitivity to environmental factors' therefore, regulation of these factors is necessary. Nanotechnology can be leveraged as an innovative solution to effectively address these challenges.

Nanoparticles can enhance enzymatic activity of different enzymes by improving stability, catalytic efficiency and kinetic performance, surpassing traditional immobilization methods and can optimize enzyme interactions, leading to higher reaction rates and structured interfacial environments [60]. Nanoenzymes are synthetic enzymes derived from nano-biomaterials, with size ranging from 1 to 100 nm, and they share similar structures and functions with entities like metal complexes, nucleic acids, glucose, and other biomolecules. They are categorized into two groups: (i) enzymes that have been transformed into nanomaterials, known as hybrid nanomaterial enzymes [61] and (ii) nanomaterials that mimic enzymatic properties while reducing biocatalytic activity [62]. These artificial enzymes exhibit multi-enzyme activities, including, superoxide dismutase-like functions, catalase, peroxidase, oxidase and enhancing secondary metabolite production by mimicking natural enzymatic functions while offering superior stability, catalytic efficiency, and adaptability [63].

Peroxidase-like nanozymes breakdown hydrogen peroxide (H_2O_2), producing ROS that causes the biosynthesis of secondary metabolites. In contrast, catalase-like nanozymes mitigate ROS to maintain stable

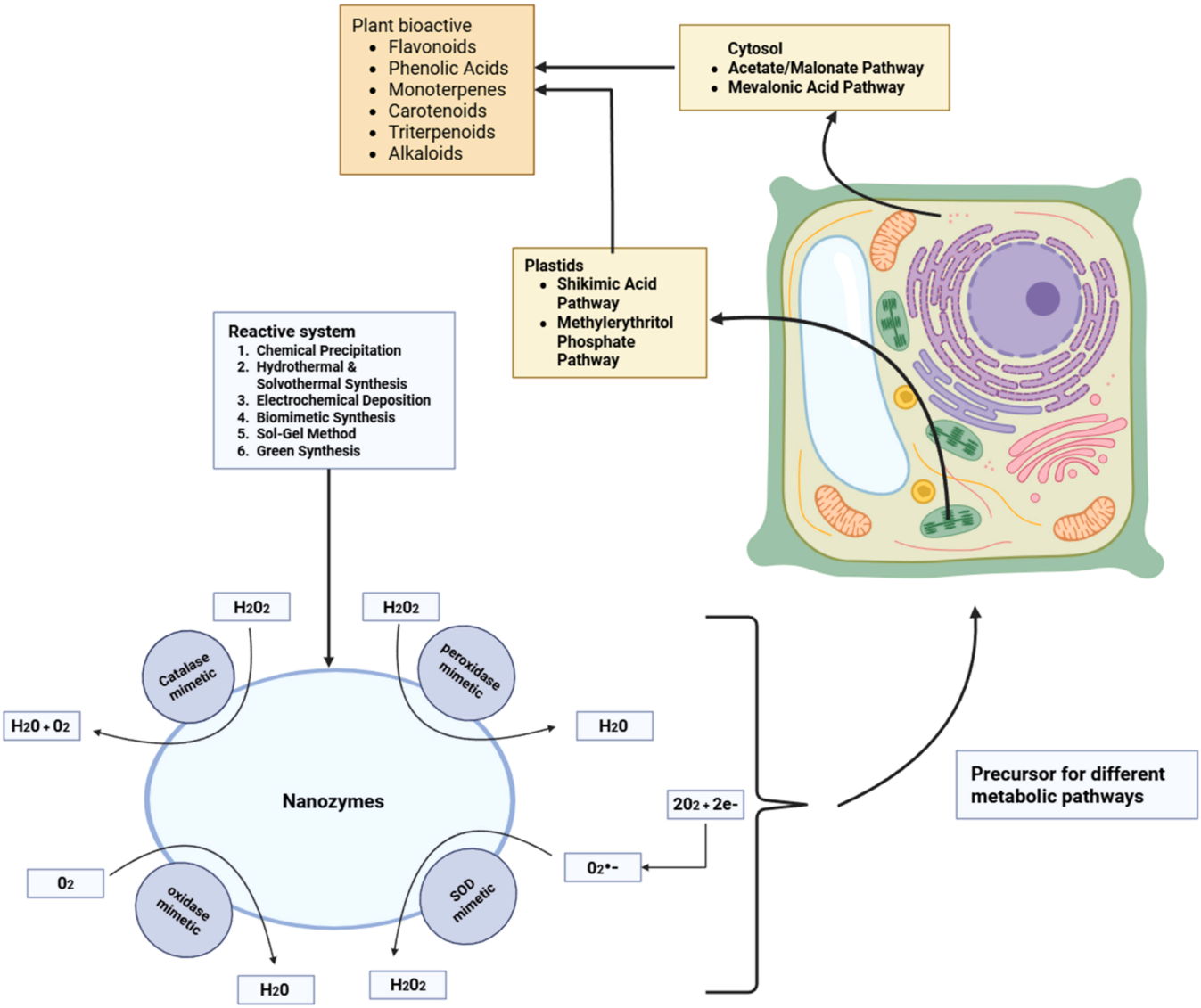

Fig. 3. Nanozymes modulating plant secondary metabolism by influencing key biochemical pathways and acting as precursors for these pathways.

metabolic processes (Fig. 3). For example, oxidase-like nanozymes improve biosensing capabilities by activating molecular oxygen, while superoxide dismutase-like nanozymes manage oxidative stress, thereby optimizing the biosynthetic routes for producing flavonoids, terpenoids and alkaloids. Although certain NPs exhibit nanozyme behaviour that can catalyse ROS generation or scavenging and thereby module metabolic fluxes, a clear distinction must be drawn between *in-vitro* (e.g., hairy-root cultures) and *in plant* (in intact plants) evidence. In cell suspension and hairy-root cultures, nanozyme activity is well documented and frequently translates into sustained metabolites yield increases. For instance, as per a study by Ashraf et al. [37], jasmonic acid-loaded Fe_3O_4 NPs were applied at 20 mg/L to *C. tinctorius* cell suspensions increased chlorogenic acid together with 110.64% higher total phenolics through controlled Fenton-like ROS signalling and sustained intracellular delivery. Such systems benefit from uniform exposure and absence of systematic barriers, allowing quantification of catalytic persistence.

In intact plants, evidence of sustained nanozyme catalytic activity, tissue localization and direct secondary-metabolite yield enhancement remains more limited and context-dependent (primarily stress-alleviation studies). Seed priming with Mn-doped CeO_2 nanozymes (MCNPs $\approx$ 40–70 nm) in wheat demonstrated uptake into seeds as confirmed by single-particle ICP-MS, retained catalase like activity (112.87 U/mg/min) and synergistically reduced ROS accumulation by 4% under cold stress while elevating growth-promoting hormones and β-amylase activity, resulting in 7.1% higher germination index and 6.2–17.2% greater seedling biomass. The particles persisted largely intact with minimal ion dissolution, supporting prolonged redox homeostasis that can indirectly favour secondary-metabolite accumulation under abiotic stress [64]. Similarly, soil-applied MoS_2 NPs (10 mg/kg) in soybean were taken up by roots and translocated to nodules and shoots (9–18% of Mo remaining intact as 32–54 nm particles), where residual nanozymes activity scavenged ROS, elevated thiol antioxidants (glutathione-related compounds up to 522%) and increased grain yield by 35% versus controls through sustained nitrogen-fixation support [65]. Leaf infiltration of Ceo_2 nanozymes (SOD and catalase-mimetic) in *Arabidopsis thaliana* similarly quenched hydroxyl radicals *in-situ*, improving photosynthetic efficiency and salinity tolerance via mesophyll potassium retention [66]. These in planta examples confirm localization and catalytic persistence in living tissues, yet direct, large-scale increases in high-value secondary metabolites under field conditions have not yet been demonstrated at the levels routinely achieved in vitro, underscoring the need for further whole-plant validation. These characteristics allow for precise metabolic engineering in plants and microorganisms, enhancing the quantity of secondary for sustainable applications in bio-manufacturing [63], biotechnology [67,68] and agriculture [69,70].

4.1. Metabolic reprogramming at the single-cell level using nanomaterials

In study conducted by Ashokan et al. [71], it investigates the cell-focused method for modulating metabolism in precision medicine focusing on cancer therapeutics. Study focused on engineering dual-functionalized nanoparticles to selectively transport therapeutic agents to the mitochondria of prostate cancer cells, altering their metabolic conditions. With this type of targeted delivery, the DNA of cancer cell abducted that caused cancer cell death. This approach has the potential to enhance therapeutic precision and overcome drug resistance. In other similar study conducted by Wei et al. [72] it was emphasized in study by the use of similar nanomaterials at single cell level we can accurately detect the metabolic changes in cancer cells, giving adequate information about tumour development and resistance to the therapeutics. Another study conducted by Zhou et al. [73] gave brief insight on application of nanopipette-assisted glycan labelling facilitating precision metabolic interventions at nanoscale. Similar methods can be applied to increase the yield of plant bioactives in plants by modulating metabolic activity at single cell level.

4.2. Mitigating pleiotropy and fitness costs associated with metabolic rewiring

To mitigate pleiotropy and associated fitness costs like flux imbalances, growth penalties and resource trade-offs, arising from CRISPR/Cas or nanomaterial-mediated metabolic rewiring, this section discusses editing strategies that replace blunt knockouts or constitutive overexpression with tunable, spatiotemporal control. Base and prime editing, combined with cis-regulatory promoter modifications and tissue or stress-inducible promoters, enable hypomorphic alleles and localised pathway modulation (e.g., restricting cytokinin or secondary-metabolite gene activity to defence tissues). Thereby minimising developmental disruptions while preserving yield and bioactive accumulations [74]. Synthetic feedback circuits and biosensors can further impose dynamic flux control to prevent toxic intermediate build-up.

For predictive modelling and post-editing monitoring, integrating multi-omics layers like transcriptomics, proteomics, metabolomics into genome-scale metabolic models (GEMs) through constraint-based flux balance analysis (FBA) and proteome-constrained models (PCMs) are recommended [75]. Established pipelines such as the COBRA Toolbox with GIMME or iMAT algorithms construct context-specific models from omics data, stimulate trade-offs, identify genetic intervention points and forecast growth-metabolite balances prior to wet-lab validation. Hybrid mechanistic-AI frameworks (e.g., graph neural networks with explainable AI) further enhance prediction of genotype-environment interactions and pleiotropic outcomes across non-model medicinal species [76].

5. Nano gene delivery for metabolic modulation

The clustered regularly interspaced short palindromic repeats (CRISPR/Cas) system is a scientific tool which is used to edit genes programmable by RNA [77]. CRISPR/Cas works on the principle of its interaction with DNA and cleaving DNA at specific sites [78]. The sgRNA-Cas (single-guide RNA) complex attaches to the target DNA sequence, cuts the DNA and identifies a proto-spacer adjacent motif (PAM) site next to the sgRNA binding location [79]. Most of the times viral vectors are used to transfer these genes to host cell but viral vectors can cause inflammatory reactions and other adverse effects due to recombination with the host genome or pathological insertional mutagenesis [80,81] as shown in Fig. 4. Due to this, non-viral delivery platforms, particularly lipid nanoparticles (LNPs), are gaining significant attention for their ability to facilitate efficient gene delivery this is due to extensive stability [81], increased circulation time [82], reduced toxicity [83] and lowered immunogenicity of LNPs [84,85].

5.1. Lipid nanoparticle delivery of Cas9/gRNA-encoded plasmid DNA

The CRISPR/Cas9 gene-editing system can be introduced using plasmid DNA (pDNA) forms of both Cas9 and gRNA. When delivering pDNA via LNPs for gene editing, several important factors must be considered. Since plasmids need to be transcribed inside the nucleus, LNP formulations of pDNA should enable nuclear entry. This is possible in actively dividing cells when the nuclear membrane is disrupted during mitosis. However, the large size of CRISPR plasmids, exceeding 10,000 base pairs, can reduce encapsulation efficiency. Consequently, the negative charge of inadequately encapsulated pDNA may interfere with the negatively charged cell membranes which need to be. These challenges are addressed by encapsulating pDNA in LNP nanomaterials considered [81,84]. Kulkarni et al. [86] enhanced LNP formulations by utilizing DLin-MC3-DMA, an ionizable cationic lipid to replace saturated helper lipid distearoylphosphatidylcholine (DSPC) with unsaturated phosphatidylcholine (PC) helper lipids, achieving approximately 90% transfection efficiency in embryonic mesenchymal cells. Lipofectamine 2000, RNAiMAX, and other commercial agents have effectively gene edited in U2OS-EGFP and HEK293T cells, with efficiencies reported

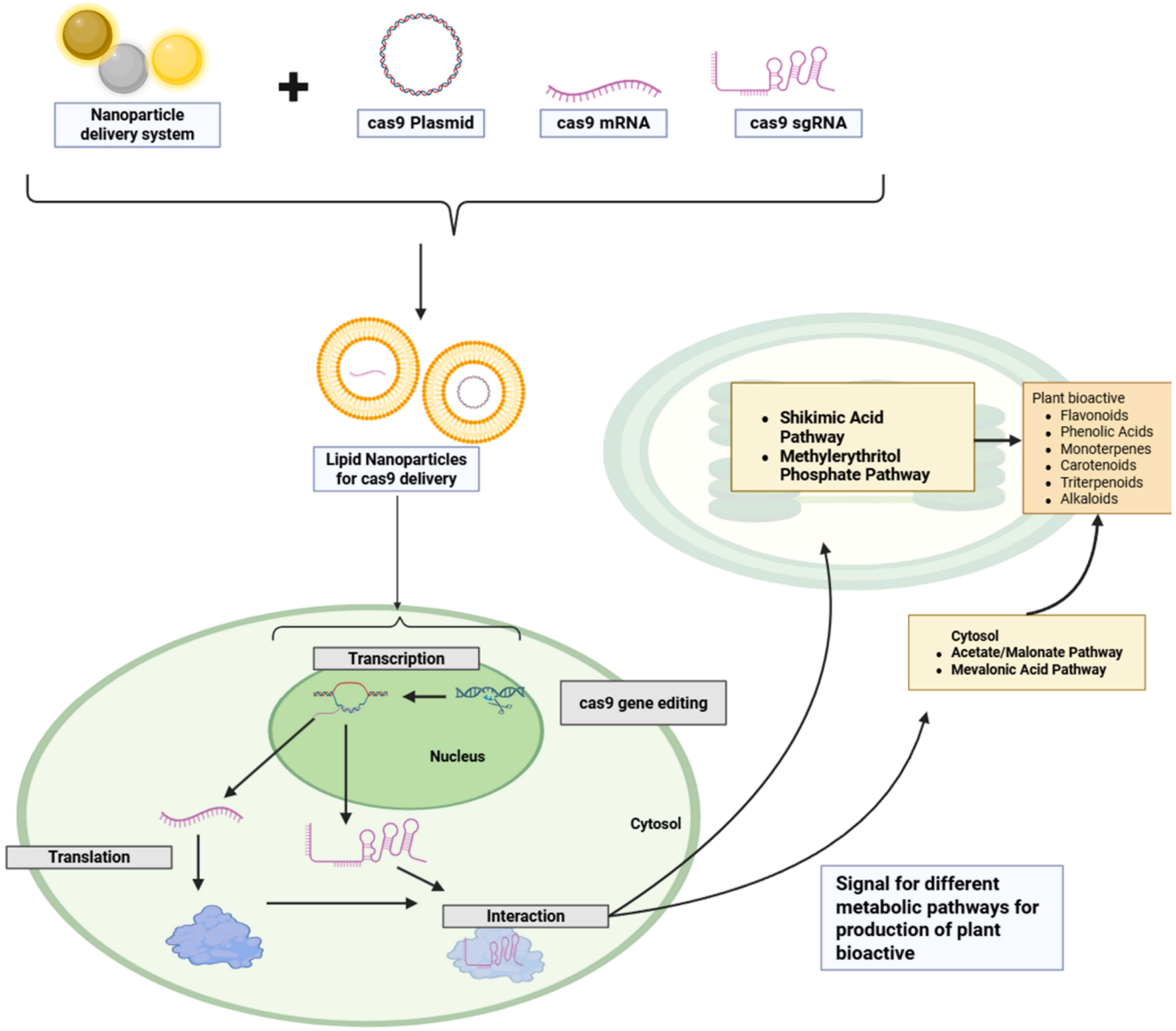

Fig. 4. The cas9 gene containing liposomes enter cell and deliver the cas9 gene in nucleus where it cut DNA at desired site and change small portion of nucleotide sequence to alter the synthesis of specific proteins which signals different cell organelles to initiate different metabolic pathways for production of plant bioactives.

from 18 to 46% [81,87]. Zhang et al. [84] built upon these results by allowing encapsulation of plasmid at lower volumes, utilizing chondroitin sulfate and protamine and creating LNPs with cholesterol, DOTAP, and DOPE, significantly improving transfection and inhibiting PLK-1 expression in A375 tumour models *in-vitro*. Li et al. [88] prepared an ionizable LNP formulation that inhibited the CRISPR/Cas9 pDNA and achieved a 32% mRNA knockdown while also reducing tumour growth in HepG2-Luc xenografts. The continued development of LNPs has collectively improved gene-editing efficiency and has shown their potential as a non-viral delivery system. In a similar study conducted by Im et al. [89], it was suggested that finely tuned LNPs have higher gene editing efficiency due to the most accurate transportation of CRISPR components into targeted cells and enhance bioavailability and stability of these components.

This principle method of CRISPR gene editing can be used in range of processes in metabolic engineering in plants. LNP and cationic liposome/lipoplex systems, widely validated for CRSIPR cargo delivery in mammalian cells, have also been adapted for plant applications, albeit with plant-specific constraints imposed by the cell wall. Efficient delivery across intact cell walls remains challenging and is less documented than for carbon or silica- based carriers. However, cationic lipid formulations have demonstrated utility in regenerable tissues and to a limited extent, walled explants. Typical formulations employ ionisable or permanently cationic lipids (e.g., Lipofectamine LTX/3000 with PLUS reagent or custom lipolpexes), yielding complexes 100–400 nm in hydrodynamic diameter with a net positive surface charge (zeta potential +20 to +45 mV) that facilitates electrostatic condensation of negatively charged pDNA, mRNA or RNP cargoes. In protoplast systems (enzymatically wall-free but regenerable), there carriers enable high-efficiency transgene-free editing. For example, Lipofectamine-mediated delivery of Cas9/crRNA RNP complexes achieved up to a 66.6% editing efficiency at the CsPDS locus in sweet orange (*Citrus sinensis*) protoplasts, with regenerated stable mutant plants recovered after embryo maturation and micrografting [90]. Similarly, lopofectamine-grafted Cas9 RNP produced targeted edits in tobacco (*Nicotiana tabacum*) BY-2 protoplasts and citrus regenerable callus, bypassing plasmid integration and reducing off-target risks [91,92].

For intact tissues, evidence is more limited and often relies on

infiltration or mild physical assistance rather than passive diffusion alone. Cationic lipoplexes have supported transient RNP-delivery via leaf infiltration or spraying in tobacco and citrus explants, yielding detectable indel formation in regenerable leaf discs or callus-derived lines. However, efficiencies drop markedly compared with protoplasts owing cell-wall sieving (pore size ≈ 5–20 nm) and require optimisation of PEG co-treatment or smaller particle subpopulations (<150 nm) for improved penetration. Surface PEGylation or bio-cytotoxicity, yet scalability to whole plants without tissue culture remains at early proof-of-concept stages [92,93]. These examples illustrate that while LNPs/lipoplexes offer a low-immunogenicity, DNA-free route suitable for pharmaceutical-grade bioactives, their translation to agronomic-scale editing of recalcitrant medicinal species will necessitate further engineering of size, charge and targeting ligands.

Among these processes, pathway enzymes and transcription factors are the most crucial. For enzymes involved in metabolic pathways, various regulatory levels can be employed in metabolic editing, including the activation or inhibition of pathways and the management of flux into different branches of the pathway (Table 2). Regarding transcription factors, metabolic editing can focus on different regulatory levels, such as their activity status (active or inactive) and their function as either repressors or activators [94]. Table 3 shows the comparative evaluation of selected nanocarriers and NP elicitors for CRISPR delivery and secondary-metabolite modulation along with the limitations regarding safety data and metabolite targets discussed in the above sections.

Nano-delivery platforms (carbon nanotubes, mesoporous silica, NPs, layered double hydroxides and chitosan-based carriers) have advance beyond conventional *Agrobacterium* or biolistic-mediated methods by facilitating cell-wall penetration and transgene-free RNP delivery, thereby, reducing integration-related regulatory concerns. Nevertheless, these approaches currently support only transient or localised editing in

Table 2
Case studies of modulation of secondary metabolites in medicinal plants using CRISPR/Cas.

Medicinal Plant	Target Gene(s)	Function of Gene(s)	CRISPR/Cas9 Strategy	Observed Effects	References
Atropa belladonna	AbH6H	Conversion of hyoscyamine to anisodamine and scopolamine	sgRNA targeting second exon of AbH6H, regulated by AtU6–26 promoter; SpCas9 controlled by CaMV35S promoter	Edited plants lacked anisodamine and scopolamine; increased hyoscyamine content (up to 4.28-fold)	[78,95,96]
Dendrobium officinale	C3H, C4H, 4CL, CCR, IRX	Biosynthesis of lignocellulose	Three sgRNAs for each target gene, regulated by OsU3 promoter; plant-optimized Cas9 controlled by CaMV35S promoter	Various alterations in product formation, editing efficiency up to 100%	[97,98]
Dioscorea zingiberensis	Dzfps	Biosynthesis of diosgenin	sgRNA targeting first exon of Dzfps, regulated by OsU3 promoter; SpCas9 controlled by CaMV35S promoter	Frameshift mutations, premature termination codons; reduction in squalene levels, compromising Dzfps functionality	[78,99]
Salvia miltiorrhiza	bZIP2	Regulation of phenolic acid biosynthesis	AtU6-sgRNA cassette targeting bZIP2 gene; Cas9 controlled by CaMV35S promoter	Increased phenolic acid content (23–53% higher than wild type); suppression of PAL gene expression	[78,100, 101]
Salvia miltiorrhiza	SmCPS1	Committed diterpene synthase (tanshinones)	Knockout	Reduced tanshinone accumulation	[83]
Salvia miltiorrhiza	SmCPS1uORF	Upstream ORF regulating SmCPS1 translation	uORF disruption	Up to 1.8-fold increase total tanshinones (protein-level)	[102]
Nicotiana tabacum	BBL family	Biosynthesis of nicotine	sgRNA targeting conserved sequences in BBL genes; AtU6–26 promoter; pCas9-TPC vector	Significant reduction in nicotine content (>99.6% decrease in T2 and T3 generations)	[103]
Nicotiana tabacum	NtPDS (model)	Phytoene desaturase (carotenoid pathway)	Knockouts	Albino phenotype; validated editing platform for alkaloids	[104]
Papaver somniferum	4'OMT2	Biosynthesis of benzylisoquinoline alkaloids (BIAs)	sgRNA targeting 5' region of 4'OMT2; Viral vector (pTRV); AtU6 promoter; hCas9 controlled by CaMV35S promoter and nos terminator, knockout	Significant reduction in BIA compound levels (morphine, S-reticuline, thebaine, papaverine, noscapine)	[105,106]
Taraxacum kok-saghyz	1-FFT	Biosynthesis of inulin (impairs rubber production)	sgRNA targeting second exon of 1-FFT; AtU6–26 promoter	Mutation efficiency of 88.9%; genetically modified plants regenerated in six weeks	[107]
Taraxacum kok-saghyz	Tk1-SST + Tk1-FFT	Fructan synthases (inulin biosynthesis)	Double knockouts	Enhanced natural rubber (polyisoprene) accumulation	[108]
Salvia miltiorrhiza	SmRAS	Phenolic acid biosynthesis (rosmarinic acid and lithospermic acid B)	sgRNA targeting SmRAS ORF; AtU6–26 and OsU3 promoters; hSpCas9 controlled by CaMV35S promoter	90% decrease in SmRAS expression in homozygous mutants; phenolic acid reduction	[109]
Cannabis sativa	THCAS, CsTHCAS	Biosynthesis of cannabinoids (THC/CBD)	sgRNA targeting THC acid synthase gene; CRISPR/Cas9; Knockout	Potential for creating THC-free, CBD-rich plants; increased commercial value in strict regulatory environments	[110]
Cannabis sativa	CsPDS1	Phytoene desaturase gene; used as a marker	sgRNA targeting Exon 6 of CsPDS1	Higher editing efficiency in sgRNA3, sgRNA4, and sgRNA5	[111]
Salvia miltiorrhiza	SmLAC7, SmLAC20 (Laccase gene family)	Biosynthesis of salvianolic acid B (SAB)	Dual-locus editing targeting Cu-oxidase domains; sgRNA1 targeting group VII, sgRNA2 targeting groups I-V	Reduction in lignin production and phenolic acid metabolism	[112]
Nicotiana tabacum	FucT, XylT	N-glycan biosynthesis; immunogenic response	Multiplex knockout system targeting four FucT and two XylT genes; sgRNAs aimed at conserved domains	Successful depletion of glycoproteins; no detectable FucT/XylT signals in mutant lines	[113]
Salvia miltiorrhiza	SmCPS1	Biosynthesis of tanshinones	sgRNAs targeting three exons (first, fourth, eleventh); AtU6–26 promoter for sgRNAs; SpCas9 under CaMV35S	42.3% mutation rate; absence of tanshinones (cryptotanshinone, tanshinone I, tanshinone IIA)	[114]
Symphytum officinale	HSS	Pyrrolizidine alkaloid biosynthesis	Three sgRNAs targeting exons three, seven, and eight; AtU6-driven sgRNAs; SpCas9 under CaMV35S	Complete absence of PAs in homozygous mutant lines; reduction of homospermidine by 80%	[115]
Solanum lycopersicum	6 target genes	Various biosynthetic pathways	12 sgRNAs in a single CRISPR/Cas system expression cassette	Successful deletion of six genes, demonstrating efficient multiplex editing	[116]

Table 3
Comparative summary of selected nanocarriers and nanoparticle elicitors for CRISPR delivery and secondary-metabolite modulation.

Material Types	Delivery Route	Plant System	Metabolite Targets	Safety Data and Limitations
Cationic LNPs / Lipoplexes	Protoplast transfection; leaf infiltration	*Citrus sinensis protoplasts; Nicotiana* sp.	Carotenoids (PDS); transient editing	Low cytotoxicity in regenerable tissues; cell-wall barrier limits intact-plant use
Carbon nanotubes	Leaf infiltration / spray	*N. benthamiana*; explants	Transient genome editing	Minimal phytotoxicity at < 10 mg/L; aggregation at high dose
Mesoporous silica NPs	Vacuum infiltration	Various medicinal species	Pathway genes (transient)	High biocompatibility; biodegradable; low residual risk
Chitosan-based NPs	Syringe infiltration	Hairy roots / callus	Secondary metabolite genes	Biodegradable; pH-dependent stability
Fe_3O_4 (JA-loaded)	Cell suspension (elicitation)	*Carthamus tinctorius*	Chlorogenic acid / phenolics	ROS hormesis; toxicity > 40 mg/L; biomass reduction
ZnO NPs	Hairy-root culture	*Hyoscyamus reticulatus*	Tropane alkaloids	Dose-dependent cytotoxicity; species-specific
CeO_2 nanozymes	Seed priming / soil	Wheat / *Arabidopsis* (in planta)	Indirect (ROS homeostasis)	Sustained catalase-like activity; low ion release
MoS_2 NPs	Soil application	Soybean	Thiol antioxidants / yield	9–18% intact particles translocated; low toxicity

explants or leaves via syringe infiltration, vacuum infiltration or spray application with stable germline transmission and whole-plant regeneration achieved in only a limited number of amenable species [78]. Experimental applications in non-model medicinal plants remain sparse and largely restricted to pilot-scale hairy-root or cell-suspension systems.

5.2. Maturity assessment and translational challenges

A clear delineation of maturity levels is essential to guide future research priorities. Using and adapted TRL scale for plant biotechnological processes, most CRISPR/Cas applications for secondary metabolite enhancements currently operate at TRL 4–6. Proof-of-concept studies (TRL 3–4) dominate the literature and are largely confined to hairy-root cultures of species such as *Salvia miltiorrhiza, Dendrobium officnale* and *Taraxacum kok-saghyz*, where gene knockouts successfully alter phenolic, terpenoid or alkaloid profiles under sterile conditions [94,117,118]. Experimental optimisation (TRL 5–6) has been reported in few non-model systems (e.g., *Atropa belladonna* hairy roots (AbH6H)), yet these remain limited to small-volume bioreactors and lack validation under field- relevant conditions [78].

Translational barriers to higher TRLs are multifaceted. Regeneration and stable transformation efficiencies are low in recalcitrant medicinal plants owing to incomplete genome sequences, complex polyploidy and inefficient tissue-culture protocols [93]. Off-target editing, although mitigated by high-fidelity Cas variants and RNP delivery, persist as a concern for regulatory approval of edited lines destined for pharmaceutical or nutraceutical use. Nano-delivery, while promising for bypassing species-specific *Agrobacterium* host-range limitations and enabling direct delivery to intact tissues, faces additional hurdles [92]. These include batch-to-batch variability of nanomaterials, potential phytotoxicity at scale, incomplete understanding of long-term environmental fate and the absence of standardised, cost-effective manufacturing processes suitable for industrial volumes. Furthermore, scaling hairy-root or edited cell-suspension cultures to commercial bioreactors introduces shear stress, oxygen-transfer limitations and metabolite secretion inefficiencies that have yet to be fully resolved.

Realistic prospects of technology transfer (TRL 7 +) within the next 5–10 years will require integrated solutions: high throughput CRISPR library screening for pathway optimisation, NP engineering for improved biocompatibility and organelle targeting, genotype-independent regeneration protocols and harmonised regulatory frameworks that recognise non-transgenic RNP-edited plants, Until these bottlenecks are systematically addressed the integration of CRISPR/Cas with nanotechnology will continue to excel in discovery and proof-of-concept phases but will require substantial further investment to achieve sustainable, industrial-scale production of plant-derived bioactives.

6. Integration of nanoparticles with hairy root culture

When *Agrobacterium rhizogenes* infects a particular site in plant root the infected root area develops small hairy roots (HRs) that follow same photochemical pattern of parent root with higher growth rate and photochemical activity. The HRs in plants is triggered by the root locus gene (rol genes) located within the root-inducing (Ri) transfer DNA (T-DNA) plasmid of *R. rhizogenes*, which integrates into the plant's genome [51]. HRs are noted for their genetic stability, rapid growth, high metabolite production, and their ability to proliferate indefinitely in a medium without hormones. Due to high stability of HRs and lack of contaminants and pathogens, HRs are used to synthesise various plant metabolites in laboratory setting [119]. Plant cell and organ cultures can be initiated using explants such as leaves, stems, roots, and meristems to facilitate the mass production of entire plants or specific tissues, this process of developing plant organs from different cells or tissues is known as plant tissue culture (PTC) [120]. To extract secondary metabolites, either organ cultures or unorganized cultures like callus or suspension are developed, depending on where the compound is synthesized [119,121].

For example, withanolide, a naturally accruing steroid is present in the roots of various *Solanaceous* plants, prompting the establishment of root cultures for a consistent supply of this compound [119,117,122,123]. PTC techniques enable the rapid generation of numerous regenerated plants or tissues in a compact space while maintaining high clonal stability [119], making it a valuable biotechnological method for producing secondary metabolites. But compared to traditional HRs, the production of secondary metabolites can be increased using elicitation, *A. rhizogenes* mediated transformation and metabolic engineering as shown in Fig. 5 [51]. Signals emitted by elicitors are detected by receptors located in the plant cell membrane, which then initiate defence mechanisms that lead to an enhanced production and accumulation of secondary metabolites which can also be done by overexpression of certain genes enhancing productivity and lowering cost of manufacturing of plant bioactive based medicines [119,124,125].

6.1. Methods to enhance secondary metabolites using hairy root culture (HRCs)

A lot of research has been done to study the methods increasing the secondary metabolites production from HRCs to make the metabolites and bioactives more available for pharma as well as cosmetic markets. With the rising demands of green-based ingredients in various sectors, scientists are focusing more on enhancing the yield of the bioactives from plants. Some if these methods are described in Table 4, which are use with HRCs to improve the biosynthesis and yield.

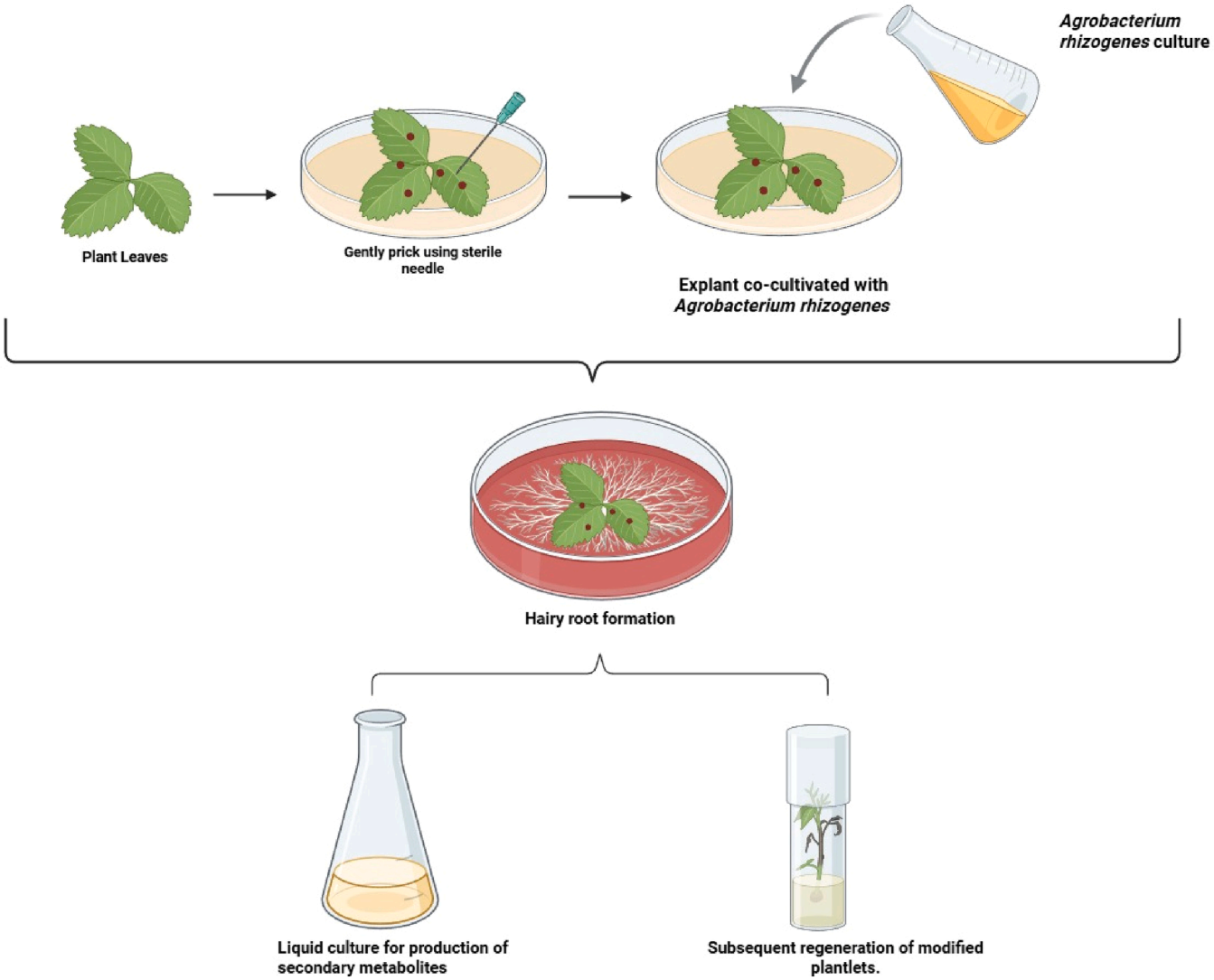

Fig. 5. A visual depiction of the process for inducing hairy roots using Agrobacterium rhizogenes and the subsequent regeneration of modified plantlets.

6.2. *Metabolic engineering in HRCs*

Metabolic engineering employs recombinant DNA technology to alter and improve cellular functions related to transport, enzymes, and regulatory mechanisms. In plants, enzymes encoded by genes within a particular biosynthetic pathway are inserted between the T-borders of the RI plasmid, and the resulting construct is introduced into the host plant's genome (Table 5 and Table 6). Overexpressing in encoding enzymes and other key transcription factors in transgenic HRCs, the production and synthesis of desired plant bioactive are increased and enhanced [150-152].

6.3. *Optimization of culture conditions and light exposure in HR cultures*

The introduction of light-emitting diode (LED) lights in plant cultivation has transformed how the production of phytochemicals. LEDs are both more efficient and easier to operate, enabling fine-tuned adjustments to light, intensity, duration, and spectra [172,173]. Customization of these elements to specific wavelengths, plants can be encouraged to generate greater amounts of beneficial compounds. For instance, certain light wavelengths, such as ultraviolet (UV) and blue light, have been shown to boost the biosynthesis of antioxidants, anthocyanins, and flavonoids whereas red light supports the production carotenoids and chlorophyll [172,174].

Practical integration of LED lighting with nano-elicitation is readily implemented in controlled bioreactors or growth chambers by combining wavelength-specific illumination (blue/red/green) with optimised NP dosing during the exponential growth phase. Additionally, to enhance the production of these plant metabolites the nanoparticles can be synergistically used as done by Mazhar et al. [172]. In this study investigated the combined effects of selenium nanoparticles (SeNPs) and LED light on the production of secondary metabolites in *Santalum album* (sandalwood) through *in-vitro* callus cultures. The callus cultures were exposed to different concentrations of SeNPs (30, 60, and 90 μg/L) along with various LED wavelengths (green ~550 nm, red ~660 nm, and blue ~460 nm), resulting in 16 distinct treatment groups. Analysis using two-way ANOVA and Tukey's HSD test indicated that the combination of blue LED light and 90 μg/L SeNPs notably increased callus growth, fresh and dry weight, and shoot branching. Furthermore, this combination enhanced the activity of antioxidant enzymes (SOD, POD, CAT, PAL) and elevated the levels of secondary metabolites, such as phenolics, saponins, tannins, flavan-3-ols, and tocopherols. The optimized SeNP and LED treatments present a promising strategy for boosting sandalwood-derived bioactive compounds, which could benefit pharmaceutical, cosmetic, and industrial sectors. These protocols are scalable in LED-equipped bioreactors, though species-specific remains essential.

In addition to LED culture conditions like nutrient media, pH and

Table 4
Elicitation and its effect on Metabolite Production in HR Cultures.

Elicitor Type	Elicitor	Target Plant/HR Culture	Key Metabolites Affected	Effects on Metabolite Biosynthesis	Reference (common reference [51])
Biotic (Fungal)	*Phytophthora cinnamon* cell wall	*Rosmarinus officinalis*	Rosmarinic acid	Enhanced production (2.67-fold increase)	[126]
Biotic (Bacterial)	*Staphylococcus aureus*	*Atropa belladonna* HR	Scopolamine	2.8-fold increase after 12 h	[127]
Biotic (Bacterial)	*Pseudomonas fluorescens* P64, P66	*Datura stramonium* HR	Hyoscyamine, Scopolamine	Increased yield after 5–10 days	[128]
Biotic (Hormonal)	Salicylic Acid (SA), Methyl Jasmonate (MeJA),	*Sarcophyton ehrenbergi* HR	Acetylated diterpene, sterol	Sarcophtonolide I increased up to 132-fold	[129]
Abiotic (Permeabilizing)	Dimethyl Sulfoxide (DMSO), XAD-7	*Cichorium intybus* HR	Coumarins	Enhanced growth and metabolite release	[130]
Abiotic (Electric Stimulation)	Electric Current (30–100 mA)	*Pisum sativum* HR	(+)-Pisatin	13-fold increase in elicited HR cultures	[131]
Metallic Nanoparticles	Silver (Ag) NPs	*Hyoscyamus muticus* HR	Total tropane alkaloids	3.42% increase, antimicrobial activity	[132]
Metal Oxide Nanoparticles	Zinc Oxide (ZnO) NPs	*Musa spp.* (Banana) HR	Stress tolerance compounds	Enhanced tolerance to biotic stress	[133]
Magnetic Nanoparticles	FeNPs, Fe_4	*Cichorium intybus* HR	Flavonoids, phenolics	More efficacious elicitation compared to ZnO NPs	[134]
Magnetic Nanoparticles	FeNPs, Fe_4	*Dracocephalum kotschyi* HR	Cirsimaritin, rosmarinic acid, xanthomicrol	Enhanced metabolite accumulation, gene activation (PAL, RAS)	[135]
Magnetic Nanoparticles	FeNPs	*Hyoscyamus reticulatus* HR	Scopolamine, hyoscyamine	Five times increase in alkaloids	[136]
Magnetic Nanoparticles	FeNPs	*Dracocephalum polychaetum* HR	Rosmarinic acid, apigenin, naringin, carvacrol, rutin, thymol, quercetin	1–8-fold increase in metabolite accumulation	[43]
Silicon-Based Nanoparticles	Silica (SiO_2) NPs	*Capsicum frutescens*, *Solanum lycopersicum*	Biomass, root volume	Increased root growth and biomass accumulation	[137,67,117]
Nano-Transporters	Gold (Au) NPs	Various species	Specialized metabolites	Enhanced bioavailability of plant metabolites	[138]
Signal-Inducing Nanoparticles	MAPK-activating NPs	Various species	Alkaloids, defense proteins	Stimulated alkaloid biosynthesis via MAPK signaling	[139–141]
Titanium-Based Nanoparticles	TiO_2 NPs	*Hyoscyamus niger* HR	Scopolamine, Hyoscyamine	Enhanced alkaloid production	[142]
Iron-Based Nanoparticles	FeO NPs	*Hyoscyamus reticulatus* HR	Tropane alkaloids	Five times increase in hyoscyamine and scopolamine	[136]
Copper-Based Nanoparticles	CuO NPs	*Brassica rapa* (Chinese cabbage) HR	Glucosinolates, phenolic compounds	Upregulated biosynthetic genes, enhanced antimicrobial activity	[143]
Silver-Based Nano-Ions	Silver ions (Ag^+)	*Silybum marianum* HR	Silymarin, lipoxygenase, linoleic acid	Increased silymarin content, oxidative stress induction	[144]
Core-Shell Nanoparticles	Ag-SiO_2 NPs	*Artemisia annua* HR	Artemisinin	Increased content, lipid peroxidation, catalase activity	[145]
Gold-Copper Hybrid Nanoparticles	AuCu NPs	*Stevia rebaudiana* HR	Flavonoids, phenolic compounds	Enhanced biomass, total phenolic and flavonoid content	[146]
Iron Oxide Nanoparticles	FeO NPs	*Cichorium intybus* HR	Phenolic compounds	Increased biomass, total phenolics and antioxidant activity	[134]
Titanium-Based Nanoparticles	TiO_2 NPs	*Saponaria officinalis* HR	Flavonoids, polyphenols, SO6 protein	Increased polyphenols, antioxidant enzyme activities	[147]
Nanoparticle-Based Elicitors	SiO_2 NPs, ZnO NPs, FeO NPs	*Polygonum multiflorum* HR	Anthraquinones, phenolics	Enhanced biomass, antimicrobial activity, antioxidant activity	[148]
Biosynthesized Nanoparticles	AgNPs from *Panax ginseng* HR	Various wheat pathogens	Silver ions	Antifungal activity, pathogen sterilization	[149]

Table 5
Single gene overexpression for synthesis of metabolites in HRs.

Species	Major Secondary Metabolites	Hairy Root Characteristics	ORFs of the T-DNA responsible	Reference
Atropa belladonna	Tropane alkaloids (Atropine, Scopolamine)	Stable production via hairy root culture	rolC gene enhances alkaloid biosynthesis	[119,153]
Datura spp.	Tropane alkaloids (Scopolamine, Hyoscyamine)	Hairy roots accumulate higher metabolites than parent plants	rolB gene significantly increases tropane alkaloid yield	[128,154]
Hyoscyamus niger	Tropane alkaloids (Hyoscyamine, Scopolamine)	Rapid growth, profuse root hairs	rolC gene alone efficiently enhances alkaloid production	[139]
Nicotiana tabacum	Pyridine alkaloids (Nicotine)	High growth rate, lateral branching	rolA gene stimulates nicotine biosynthesis	[155]

Ions like NO_3^-, NH_4^+, $KHPO_4^-$, Ca^{2+}, and Mg^{2+} can also influence the production of secondary metabolites as shown in Table 7.

Table 6
Multiple gene overexpression for production of metabolites in HR cultures.

Gene(s) Overexpressed	Function	Target Plant/ HR Culture	Key Metabolites Affected	Effect on Metabolite Biosynthesis	Reference (common: [150])
ZmLc, AtPAP1	Transcription factors regulating flavonoid biosynthesis	*Scutellaria baicalensis* HR	Flavonoids	Increased flavonoid biosynthesis (322% higher than control)	[156]
β-Amyrin Synthase, UDP-Galactose-4-Epimerase	Enzymes regulating terpenoid biosynthetic pathways	*Glycyrrhiza uralensis* HR	Glycyrrhizin	Enhanced glycyrrhizin production via pathway modulation	[50,157]
Squalene Synthase	Key enzyme in triterpenoid biosynthesis	*Centella asiatica* HR	Centelloside	Increased centelloside accumulation, reduced phytosterol content	[158]
Δ24-Reductase, Sterol-Methyltransferase, Squalene Synthase, Cycloartenol Synthase, C26-Hydroxylase	Regulatory genes in sterol biosynthesis	*Trigonella foenum* HR	Diosgenin	Increased diosgenin production; sterol-methyltransferase downregulated	[159]
ORCA3, SGD	Transcription factor regulating alkaloid biosynthesis	*Catharanthus roseus* HR	Catharanthine, Hörhammericine, Lochnericine, Ajmalicine, Serpentine, Tabersonine	Enhanced alkaloid production under jasmonic acid treatment	[160]
Secologanin Synthetase, Geraniol-10-Hydroxylase	Enzymes involved in camptothecin biosynthesis	*Ophiorrhiza pumila* HR	Camptothecin	Increased camptothecin accumulation via co-expression	[161]
Squalene Synthase, Dammarenediol Synthase	Genes regulating ginsenoside biosynthesis	*Panax quinquefolium* HR	Ginsenosides	Improved ginsenoside accumulation in transformed hairy roots	[162]; [163]
SmGGPPS, SmDXSII	Enzymes regulating tanshinone biosynthetic pathways	*Salvia miltiorrhiza* HR	Tanshinones	Greater tanshinone accumulation via multiple gene expression	[164]
DXS, GGPPS, HMG-CoA Reductase	Key genes regulating diterpene biosynthesis	*Salvia miltiorrhiza* HR	Tanshinones	Increased tanshinone production compared to control	[165]
ORCA3, G10H	Alkaloid biosynthetic regulators	*Catharanthus roseus* HR	Catharanthine	Enhanced alkaloid biosynthesis	[166]
FPS, MVD	Regulatory genes for triterpenoid biosynthesis	*Panax ginseng* HR	Triterpenoids, Stigmasterol, Ginsenosides	2.4-fold increase in ginsenosides	[167]
AtDXS, AtDXR	Diterpene biosynthesis regulators	*Salvia miltiorrhiza* HR	Abietanic diterpenes	Increased aethiopinone production	[168]
TAT, HPPR	Regulatory genes for phenolic biosynthesis	*Salvia miltiorrhiza* HR	Rosmarinic Acid	4.3-fold increase compared to conventionally harvested plants	[126]
STR, G10H	Alkaloid biosynthetic enzymes	*Ophiorrhiza pumila* HR	Camptothecin	56% higher accumulation via dual-gene expression	[169]
H6H, PMT	Regulatory genes for tropane alkaloid biosynthesis	*Atropa belladonna* HR	Scopolamine	7.3-fold increase in scopolamine production	[127]
ORCA3, G10H	Alkaloid biosynthetic regulators	*Catharanthus roseus* HR	Catharanthine, Vindoline, Strictosidine, Ajmalicine	Increased production; reduced vinblastine and anhydrovinblastine levels	[170]
T16H, 16OMT	Enzymes involved in vindoline biosynthesis	*Catharanthus roseus* HR	Vindoline	Effective utilization of tabersonine in vindoline synthesis	[171]

7. Challenges and future prospect

CRISPR technology encounters obstacles due to the possibility of unintended genome edits, although wild-type Cas9 and Cas12a have demonstrated high specificity in plants [185]. Its widespread use is restricted by its high cost and the absence of comprehensive whole-genome sequences for medicinal plants [186]. Moreover, a deeper understanding of DNA repair mechanisms is essential for achieving more accurate editing results [185]. Similarly, hairy root systems face challenges with tightly controlled biosynthetic pathways, leading to unpredictable gene expression changes. The presence of multiple rate-limiting steps and the challenge of inserting several genes simultaneously further hinder scalability and reliability [150]. Although nanozymes show promise, they have lower substrate specificity compared to natural enzymes and can only mimic a limited range of catalytic reactions. However, SANs with precise electronic and geometric characteristics provide rapid kinetics and high selectivity, making them viable alternatives [187,188]. Compartmentalization and carbon sourcing can restrict metabolite production, while managing cellular cross-talk is complex and can sometimes lead to the accumulation of unintended metabolites [189,190]. Understanding these regulatory factors is crucial for overcoming biosynthetic bottlenecks [191]. A successful approach must balance cost, scalability, and regulatory compliance. Interdisciplinary strategies that integrate life sciences, chemistry and engineering are vital, while advancements in omics and synthetic biology offer potential for optimizing biosynthetic pathways [190]. Table 8 demonstrates various applications and their details in different approaches taken for their production.

8. Conclusion

The convergence of CRISPR/Cas-mediated genome editing, nanotechnology and optimised cultivation system has demonstrably expanded the toolkit for sustainable production of plant-derived bioactives. Notable studies include multiplex gene knockouts that redirect flux towards high-value terpenoids and alkaloids in hairy root cultures, NP-elicited multi-fold yield increase in cell suspensions and nanocarrier-enabled transgene-free RNP delivery that bypasses traditional transformation limitations. These strategies collectively address long-standing constraints of low natural abundance and environmental

Table 7
Media Optimization for Tropane Alkaloid Biosynthesis in Hairy Root Cultures.

Condition	Effect	Target Species	Reference (common ref: [119])
90 mM nitrogen ($NH_4^+/NO_3^- = 4:1$)	Highest cell yield (4.5 g/L) and maximum alkaloid production (9.9 mg/L)	*Anisodus acutangulus* HR	[175]
Increased NO_3^- concentration	Higher alkaloid percentage and increased hyoscyamine/scopolamine ratio	*Anisodus acutangulus* HR	[176]
NO_3^- inclusion in B5 medium	Enhanced metabolite production	*Various species* HR	[177]
NH_4^+ as sole nitrogen source	Stimulated alkaloid excretion	*Datura stramonium* HR	[178]
Higher NO_3^- than NH_4^+	Increased withanolide A production	*Withania somnifera* HR	[179]
Lower Ca^{2+} concentration	Reduced hyoscyamine synthesis	*Datura stramonium* HR	[180]
Higher Mg^{2+} concentration	Increased hyoscyamine and scopolamine production	*Datura stramonium* HR	[181]
Diluted media (¼MS, ¾MS, ½B5, ¾B5)	Optimized hyoscyamine production	*Datura stramonium* HR	[182]
Sucrose as carbon source	Increased alkaloid and withanolide A production	*Anisodus acutangulus*, *Withania somnifera* HR	[175,183]
pH 6.0	Optimal for withanolide A production	*Withania somnifera* HR	[175,183]
pH 4.5	Optimal for alkaloid biosynthesis	*Anisodus acutangulus* HR	[175,183]
Modified MS medium (1.10 g/L KNO_3, 0.17 g/L KH_2PO_4, 40 g/L sucrose for diploid, 50 g/L sucrose for tetraploid)	Enhanced hyoscyamine production	*Datura stramonium* HR	[184]

dependency, positioning integrated platforms as viable alternatives to wild harvesting or chemical synthesis. However, a balanced assessment reveals persistent limitations that temper immediate translational prospects. Biologically, outcomes remain highly species-specific and culture-system dependent with ROS-mediated toxicity at supra-optimal NP doses and incomplete understanding of complex metabolic networks limiting predictability. Technologically, most CRISPR/nano applications operate at TRL 4–6, confined to controlled *in-vitro* or pilot-scale systems. The scale-up introduces shear stress, oxygen transfer issues, NP aggregation and regeneration inefficiencies in non-model medicinal plants. Regulatory uncertainties surrounding genome-edited crops and environmental fate of nanomaterials further delay commercialisation, particularly for pharmaceutical-grade products. Looking forward, a realistic decade long roadmap must prioritise high-throughput multi-omics integration for predictive modelling; development of biocompatible nanomaterials with standardised green-synthesis protocols; large-scale bioreactor optimisation and international harmonisation of regulatory guidelines for nano-exposed lines. Only through such concerted efforts can the full potential of these technologies be realised, delivering economically viable, environmentally sustainable and socially equitable production of plant-derived bioactives.

Table 8
Applications of different methods to enhance the secondary metabolite biosynthesis in plants and their details.

Application	Description	References
CRISPR/Cas Systems in Plant Research	CRISPR/Cas enables precise gene modifications to enhance secondary metabolite biosynthesis in plants. Programmable CRISPR/dCas systems allow transcriptional regulation without DNA breaks.	[192]
Hybrid Biosynthetic Clusters	Combining genes from related organisms creates chimeric enzymes that enhance substrate specificity and diversify plant-derived bioactives.	[174]
Hairy Root Cultures	*Agrobacterium rhizogenes*-induced hairy roots provide genetic stability and rapid growth for enhanced secondary metabolite production.	[119]
Plant Tissue Culture	Large-scale propagation of bioactive-rich plants ensures a consistent supply of valuable phytochemicals.	[121]
Metabolic Flux Optimization	Genetic modifications improve biosynthetic pathways, increasing flavonoid, alkaloid, and terpenoid production.	[123]
LED-Assisted Cultivation	Specific light spectra regulate phytochemical biosynthesis; blue and UV light stimulate flavonoid production, while red light boosts carotenoid synthesis.	[173]
Integration of Advanced Technologies	Combining CRISPR gene editing, nanotechnology, metabolic engineering, and LED cultivation significantly enhances secondary metabolite yield.	[94]

CRediT authorship contribution statement

Sanamdeep Singh: Conceptualization, Resources, Software, Visualization, Writing- original draft. Pooja Sharma: Conceptualization, Resources, Supervision, Writing- review and editing. Mohammad Faizan: Conceptualization, Writing- review and editing. Pravej Alam: Writing-review and editing.Thamer Albalwai: Writing- review and editing.

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Data availability

No data was used for the research described in the article.

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