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Application of metabolic engineering to enhance the content of alkaloids in medicinal plants

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ABSTRACT

Plants are a rich source of bioactive compounds, many of which have been exploited for cosmetic, nutritional, and medicinal purposes. Through the characterization of metabolic pathways, as well as the mechanisms responsible for the accumulation of secondary metabolites, researchers have been able to increase the production of bioactive compounds in different plant species for research and commercial applications. The intent of the current review is to describe the metabolic engineering methods that have been used to transform in vitro or field-grown medicinal plants over the last decade and to identify the most effective approaches to increase the production of alkaloids. The articles summarized were categorized into six groups: endogenous enzyme overexpression, foreign enzyme overexpression, transcription factor overexpression, gene silencing, genome editing, and co-overexpression. We conclude that, because of the complex and multi-step nature of biosynthetic pathways, the approach that has been most commonly used to increase the biosynthesis of alkaloids, and the most effective in terms of fold increase, is the co-overexpression of two or more rate-limiting enzymes followed by the manipulation of regulatory genes.

1. Introduction

From ancient times to present, humans have turned to nature, especially plants, to find relief and to treat and prevent diseases. Plants produce a huge variety of compounds known as secondary metabolites, many of which exert pharmacological or toxicological effects in humans and animals (Guerriero et al., 2018). Alkaloids are one example of a class of secondary metabolites. They are naturally occurring organic compounds that contain nitrogen atoms and may be divided into the following classes: pyridine, indole, tropane, quinoline, isoquinoline, phenanthrene, phenylethylamine, purine, imidazole, terpenoid, aporphine, pyrrolizidine, indolizidine, piperidine, and pyrrolidine alkaloids (Debnath et al., 2018).

Common examples of alkaloids isolated from plants and used in modern medicine throughout the world are shown in Fig. 1. Reserpine, from *R. serpentina* is a relevant indole alkaloid, known mainly for its antihypertensive and sedative effects. Other beneficial indole alkaloids are the potent antitumor drugs, vinblastine and vincristine, obtained from *C. roseus*. Their structure contains a benzene ring fused to a pyrrole ring (Hamid et al., 2017). Tropane alkaloids include, among others, hyoscyamine and scopolamine, from Solanaceae plants, which are widely used as anticholinergic drugs, and coca alkaloids with anesthetic and psychoactive properties such as cocaine. They are characterized by their bicyclic tropane ring (Kohnen-Johannsen and Kayser 2019). Benzylisoquinoline alkaloids, derived from isoquinoline (a benzene ring fused to a pyridine ring), include the narcotic analgesics morphine and codeine, and antimicrobials sanguinarine, and berberine (Hagel and Facchini 2013). Nicotine, is a pyridine alkaloid which has medicinal uses to treat smoking dependence; other pyridine alkaloids are promising leads for the prevention and the treatment of neurodegenerative disorders and mood disorders (Lin et al., 2020). In controlled doses, plant alkaloids and their synthetic derivatives, exhibit therapeutic effects and are widely used as medicinal agents based on their analgesic, antispasmodic, anticancer, and bactericidal activities.

Since alkaloids are particularly useful as medicines, numerous biotechnological developments have been made to increase the sources

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as well as the content of these compounds to improve commercial production. (Guaadaoui et al., 2014; Espinosa-Leal et al., 2018). Metabolic engineering is one such development, which enhances the cellular processes that are endemic to a specific organism to increase the yield of a particular product (García-Granados et al., 2019). This practice is of significant commercial relevance, for example, in the production of the drugs, artemesinin and peritaxel, the overproduction of L-valine, and the production of other amino acids (Kulkarni 2016).

The objectives of the present review are to describe the metabolic engineering methods that have been used to transform either in vitro or field-grown medicinal plants during the last decade and to summarize the most effective strategies to increase alkaloid production. With this aim, scientific articles from electronic databases (PubMed, Web of Science, Scielo, and Science direct) were selected and an extensive literature search was carried out using different combinations of search terms, such as alkaloid overexpression, transgenic plants, genetic engineering, and metabolic engineering. To assess recent developments in this field, we focused on updated research articles and other reviews on these topics. The following inclusion criteria were applied: alkaloids from genetically transformed plants, studies that reported a fold increase, and detailed methods using metabolic engineering approaches. These works included scientific articles written in English from 2010 to 2021.

2. Plant transformation methods to enhance alkaloids content

Two of the main approaches to introduce DNA into plant cells (nuclear transformation) are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*-mediated transformation, which result in the generation of transgenic plants and transgenic hairy roots, respectively (Ricigliano et al., 2016; Ma et al., 2017). These two methods have been the most used to overexpress alkaloids in medicinal plants. As shown in Table 1, hairy roots transformation is used in most of these studies (52%), followed by whole plant transformation (31%), transient (13%) and cell culture transformation (4%). Transformation efficiency varies significantly depending on factors like transformation method, plant species, specific genotype, explant tissue used, growth conditions, regeneration frequency, among others. In this review, it was found that hairy roots had a higher transformation frequency that ranged from 23% in *C. acuminata* to 95% in *R. serpentina* (Ni et al., 2011; Mehrotra et al., 2013). Whole plant transformation was less efficient owing to the recalcitrant nature of some medicinal plants, for example, *C. roseus* transformation frequency ranged from 3 to 12% (Sharma et al., 2018; Kumar et al., 2018). For *A. belladonna* plants, however, it has been reported a protocol with a high regeneration and transformation frequency (87%) (Song and Walworth, 2013).

Fig. 2 depicts the main methods that have been used in the last years for genetic transformation of medicinal plants. Transient transformation is simpler and efficient. It uses *A. tumefaciens* infiltrations to transiently transform leaves and petals, then followed by a verification of gene function (PCR, qPCR) and the analysis of small amounts of alkaloids before proceeding to the development of transgenic plants. Transient expression was used to overexpress the *CrMYC1* gene in *C. roseus* leaves and it was reported a 3 fold increase in catharanthine and vinblastine content, 2 days after agroinfiltration. Although *CrMYC1* gene needs to be further characterized, it was concluded that it was a suitable candidate for alkaloid enrichment in *C. roseus* via metabolic engineering (Sazegari et al., 2018). It is advisable to check the levels of transcripts and metabolites at different time points to find an optimal time, as well as replicate experiments on a large number of plants with different growth stages, since transient expression could be affected by the developmental and physiological state of the plants (Sazegari et al., 2018).

Hairy roots culture offers an alternative to growing the whole plant for the production and extraction of secondary metabolites. As shown in Fig. 2, they arise as a result of infection of an explant by *Agrobacterium rhizogenes*, resulting in the unlimited growth of hairy roots. In contrast to transgenic plants which are generated after 2–4 months, transgenic hairy roots can be obtained in less than one month. This system offers advantages, for example it does not require plant growth regulators, hairy roots are fast growing, genetically stable and can be successfully scale-up (Kowalczyk et al., 2020). Hairy root cultures enable the modulation of metabolism in several pathways, as in the case of tropane and indole alkaloid biosynthesis. Despite reports that hairy roots fail to synthesize the dimeric vinblastine and vincristine molecules because their biosynthesis remains highly restricted to the leaves (Sharma et al., 2018), some authors have demonstrated the accumulation of these alkaloids in hairy roots (Hanafy et al., 2016; Vu et al., 2022).

Still, the production of vinblastine and vincristine has not been commonly reported in hairy roots. In this review, articles in which metabolic engineering was used to increase indole alkaloids content in transgenic hairy roots, reported an increment on monomeric alkaloids but not dimeric ones (Table 1). The establishment of whole plant

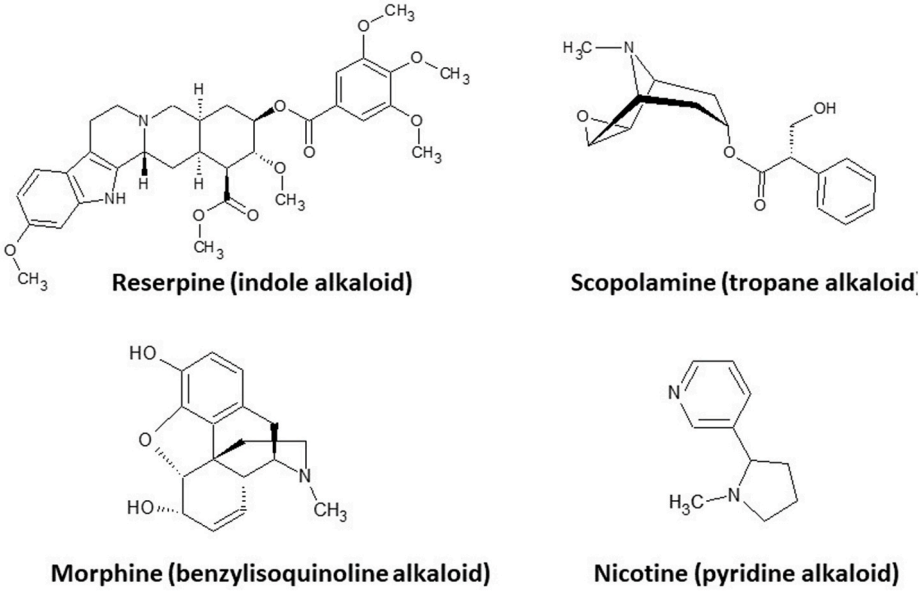

Fig. 1. Examples of relevant alkaloids in medicine and their structures.

transformation protocols for *C. roseus* have been advantageous in this regard. Using this method, it is feasible to increase the content of dimeric indole alkaloids up to 5 times (Sharma et al., 2018). Whole plant transformation is also relevant to assess the impact of a genetic alteration at the whole plant level, it has been shown that complete pathways are closely linked with tissue and organ differentiation of a whole plant (Sharma et al., 2018).

Cell suspension transformation protocols have been well stablished for *C. Roseus*, however recent studies mentioned that cell suspension cultures did not produce alkaloids in a stable manner and their ability to accumulate indole alkaloids declined by prolonged subculture (Wang et al., 2012; Kumar et al., 2018).

For the expression of the gen of interest, a broad range of useful plant vectors, such as binary T-DNA vectors, have been used (Tanaka et al., 2012; Bahramnejad et al., 2019). The gene of interest can be regulated either by constitutive or inducible promoters. Although, in this review, we found that cauliflower mosaic virus (CaMV) 35S represents the most commonly used promoter to drive transgene expression in medicinal plants, some authors have suggested the use of inducible promoters to reduce the uncertainties that result from line variations (Sun and Peebles 2016).

Regardless of the transformation method, it is necessary to confirm the genetic transfer from Agrobacterium to the plant, this is done primarily by PCR. Then, at the mRNA level, a quantitative real-time PCR is performed to confirm the overexpression of the transgene as well as to analyze the relative transcript expression of other genes in the corresponding pathway. Finally, alkaloid extracts are analyzed through high-performance liquid chromatography (HPLC) for a quantification of specific secondary metabolites (Fig. 2). It is expected that a high fold increase of mRNA expression correlates with the increase of alkaloid content, however, when more rate-limiting enzymes and regulatory factors are involved, the correlation might be weak.

3. Metabolic engineering approaches

The basic requirements needed to use metabolic engineering strategies are knowledge regarding the biosynthetic pathway of the compound of interest and the genes that encode the relevant enzymes, particularly, the manner in which they are regulated. Furthermore, some of the fundamental techniques used include: (1) overexpression of the gene that encodes the rate-limiting enzyme of the target biosynthetic pathway, (2) blocking undesired metabolic pathways and expressing heterologous genes, and (3) enzyme engineering (Kulkarni, 2016). Fig. 3 illustrates some of these techniques for which we will provide further discussion.

3.1. Endogenous enzyme overexpression

Once the biosynthesis of secondary metabolites has been defined at the enzyme level, the overexpression of key rate-limiting enzymes involved in metabolism results in the accumulation of specific secondary metabolites. A complex series of reactions including methylation, condensation, isomerization, glycosylation, acylation, as well as others, are catalyzed by such enzymes (Staniek et al., 2013).

Catharanthus roseus produces a wide range of terpenoid indole alkaloids which can be divided into monomeric indole alkaloids (serpentine, strictosidine, ajmalicine, serpentine, catharanthine, vindoline, tabersonine, etc) and dimeric indole alkaloids such as vinblastine and vincristine which are derived from the coupling of catharanthine and vindoline (Dugé de Bernonville et al., 2020). These alkaloids are biosynthesized by the condensation of tryptamine (indole moiety) and secologanin (monoterpenoid) by strictosidine synthase to form strictosidine, a common precursor to all terpenoid indole alkaloids (Peebles et al., 2011). Tryptamine is synthesized by tryptophan via tryptophan decarboxylase. As for secologanin, its biosynthesis starts with geraniol, which is converted to secologanin via multiple enzymatic steps (Kumar et al., 2015). The overexpression of upstream rate-limiting enzymes such as geraniol synthase, secologanin synthase or geranyl(geranyl) diphosphate synthase increases the precursor pool and results in a significant increase of monomeric indole alkaloids (Table 1). Adding to the biosynthetic pathway complexity which involves more than 20 enzymes and even a larger number of gene regulators, the indole alkaloid pathway, is also reported to be induced by stress and methyl jasmonate treatment, thus, the overexpression of enzymes, such as apoplastic peroxidases and MAP kinases, which are known to be activated by a variety of biotic and abiotic stresses, have also resulted in increased biosynthesis of these alkaloids (Jaggi et al., 2011; Raina et al., 2012).

Tropane alkaloids (TA) are produced, from both ornithine and arginine, via putrescine, which is a key precursor. Putrescine can be synthetized by the decarboxylation of ornithine. Overexpression of ornithine decarboxylase (ODC) resulted in a significant increase of putrescine, N-methylputrescine, hyoscyamine, and anisodamine in *Atropa belladonna* hairy roots (Zhao et al., 2020). Downstream, hyoscyamine 6β-hydroxylase is the key enzyme to catalyze the conversion of hyoscyamine to scopolamine. The overexpression of hyoscyamine 6β-hydroxylase (H6H) led to a significant increase of scopolamine in *Datura innoxia* hairy roots (Li et al., 2020).

In the biosynthesis of benzylisoquinoline alkaloids, codeinone reductase (CodR) is a key enzyme involved in the conversion of thebaine to morphine. *Papaver bracteatum* is a medicinal plant with a high thebaine content but low codeine and morphine contents, it was reported that the overexpression of *CodR* in this plant significantly increased the amounts of codeine (11 fold) and morphine (a novel peak 0.28% DW) in transgenic hairy root lines (Sharafi et al., 2013).

The overexpression of a single gene to increase a precursor, may have unintended consequences within the system, a potential problem is feedback inhibition. Anthranilate synthase (AS) is an enzyme within the indole alkaloid pathway, it catalyzes the first committed step in the synthesis of tryptophan, however, it is subjected to feedback inhibition by tryptophan (Peebles et al., 2011). There are, however, various higher plants which can accumulate tryptophan because they have feedback-insensitive AS, therefore, this inhibition problem can be overcome by overexpressing a feedback-insensitive homolog from other plant species or by engineering an endogenous enzyme (Tozawa et al., 2001).

To enhance the production of a specific secondary metabolite by metabolic engineering, it is important to consider the corresponding metabolic pathways, the associated enzymes, optimal subcellular localization, gene regulatory networks, and epigenetic regulation. Valuable tools, such as functional genomics and proteomics, have provided biosynthetic genes that code for regulatory enzymes as well as regulatory genes. In this regard, some of the most important alkaloid pathways have been extensively reviewed recently (Labanca et al., 2018; Huang et al., 2019; Desgagné-Penix 2021; Lichman 2021). A summary of key enzymes to increase alkaloid pathway fluxes are shown in Table 1.

3.2. Foreign enzyme overexpression

Another useful strategy to increase the accumulation of alkaloids in plants is the expression of a foreign enzyme. Foreign enzymes (key rate-limiting enzymes) from other plant species may exhibit higher catalytic efficiency in the conversion of a specific metabolite. For example, in a comparative study, when *hyoscyamine 6β-hydroxylase* genes of *Hyoscyamus niger* (HnH6H) and *Scopolia lurida* (SlH6H) were overexpressed in hairy root cultures of *Scopolia lurida*, the accumulation of scopolamine was much higher in HnH6H-overexpressing hairy roots (10 fold increase) than in SlH6H overexpressing lines (4 fold increase). This suggests that, despite being similar at the amino acid sequence level, the foreign enzyme, HnH6H, was more efficient at converting hyoscyamine to scopolamine compared with the endogenous enzyme, SlH6H (Lan et al., 2018). An additional advantage of using this approach is that,

Table 1
Metabolic engineering approaches to overexpress alkaloids in medicinal plants.

Molecular approach	Alkaloid type	Main secondary metabolite (others)	Times-fold Increase (up to)	Before/ after yield (mg/g DW)	Gene(s)	Plant species	Culture type	Ref
Endogenous enzyme overexpression	indole	Serpentine (ajmalicine)	5	0.76/3.7	*CrPrx*	*Catharanthus roseus*	hairy roots	Jaggi et al. (2011)
	indole	Serpentine (vindoline, catharanthine, vincristine)	3.5	0.017/ 0.060	*CrMPK3*	*Catharanthus roseus*	leaves (transient)	Raina et al. (2012)
	indole	Catharanthine (vindoline)	3	–	*CrGES*	*Catharanthus roseus*	leaves (transient)	Kumar et al. (2015)
	indole	Camptothecin (secologanin)	3	0.80/2.8	*NnCYP72A1*	*Nothapodytes nimmoniana*	leaves (transient)	Rather et al. (2020)
	indole	Catharanthine (vindoline, vinblastine)	3	–	*CrGGPPS*	*Catharanthus roseus*	whole plant	Kumar et al. (2020)
	indole	Vindoline	2.4	1.15/2.72	*CrDAT*	*Catharanthus roseus*	whole plant	Wang et al. (2012)
	tropane	Scopolamine	9	0.060/0.54	*DiH6H*	*Datura innoxia*	hairy roots	Li et al. (2020)
	tropane	Hyoscyamine (scopolamine)	3	0.20/0.63	*SlTRI*	*Scopolia lurida*	hairy roots	Zhao et al. (2017)
	tropane	Hyoscyamine (putrescine, N-methylputrescine, anisodamine)	2	3.0/6.5	*AbODC*	*Atropa belladonna*	hairy roots	Zhao et al. (2020)
	benzyliso-quinoline	Berberine	3	0.68/1.8	*Cj4′OMT*	*Coptis japonica*	whole plant	Inui et al. (2012)
	benzyliso-quinoline	(S)-tetrahydrocolumbamine ((S)-norcoclaurine, (S)-coclaurine, (S)-N-cis-methylcoclaurine, (S)-reticuline)	74	0.0030/ 0.23	*McBBE*	*Macleaya cordata*	whole plant	Huang et al. (2018)
	benzyliso-quinoline	Codeine (thebaine, morphine)	13	0.03/0.4	*PsCodR*	*Papaver bracteatum*	hairy roots	Sharafi et al. (2013)
Foreign enzyme overexpression	indole	Reserpine (ajmalicine)	2	0.60/1.2	*CrTDC*	*Rauwolfia serpentina*	hairy roots	Mehrotra et al. (2013)
	indole	Camptothecin	2	1.1/2.1	*NfSTR*	*Ophiorrhiza rugosa*	whole plant	Singh et al. (2020b)
	tropane	Scopolamine (anisodamine)	10	0.10/1.2	*HnH6H*	*Scopolia lurida*	hairy roots	Lan et al. (2018)
	tropane	Scopolamine	6	0.042/0.26	*mouse ODC*	*Datura innoxia*	whole plant	Singh et al. (2011)
	tropane	Scopolamine (hyoscyamine)	5	0.060/0.30	*VHb*	*Hyoscyamus niger*	hairy roots	Guo et al. (2018)
	pyridine	Anabasine (cadaverine)	3	5.0/15 (ng/mg FW)	*LcL/ODC*	*Nicotiana tabacum*	hairy roots	Bunsupa et al. (2016)
Transcription factor overexpression	indole	Tabersonine (ajmalicine, catharanthine)	40	0.050/2.1	*ORCA4*	*Catharanthus roseus*	hairy roots	Paul et al. (2017)
	indole	Vindoline (catharanthine)	4	0.030/0.14	*ORCA2*	*Catharanthus roseus*	hairy roots	Liu et al. (2011)
	indole	Vindoline (catharanthine)	4	0.040/0.16	*ORCA3*	*Catharanthus roseus*	hairy roots	Tang et al. (2011)
	indole	Serpentine	3	0.13/0.39	*CrWRKY1*	*Catharanthus roseus*	hairy roots	Suttipanta et al. (2011)
	indole	Vinblastine (catharanthine)	3	0.80/2.2	*CrMYC1*	*Catharanthus roseus*	leaves (transient)	Sazegari et al. (2018)
	indole	Camptothecin	3	1.1/3.8	*OpWRKY2*	*Ophiorrhiza pumila*	hairy roots	Hao et al. (2021)
	indole	Anhydrovinblastine (vinblastine, ajmalicine, vindoline, catharanthine)	1.7	0.10/0.17 (FW)	*CrERF5*	*Catharanthus roseus*	petals (transient)	Pan et al. (2019)
	indole	Camptothecin	1.5	1.1/1.7	*ORCA3*	*Camptotheca acuminata*	hairy roots	Ni et al. (2011)
	indole	Camptothecin (tryptamine, loganin)	1.4	0.50/0.70	*OpWRKY3*	*Ophiorrhiza pumila*	hairy roots	Wang et al. (2019)
	tropane	Scopolamine (hyoscyamine, anisodamine)	3	6.2/20 (mg/plant)	*AbSAUR1*	*Atropa belladonna*	whole plant	Bai et al. (2019)
	benzyliso-quinoline	Chelirubine (sanguinarine, chelerythrine)	4	0.10/0.35 (mg/g FW)	*CjWRKY1*	*Eschscholzia californica*	Cell suspensions	Yamada et al. (2017)
	pyridine	Anatabine (nicotine, nornicotine, anabasine)	4	0.48/2.0	*NtERF91*	*Nicotiana tabacum*	whole plant	Sui et al. (2019)
Gene silencing	indole	Vindoline (serpentine)	3	0.046/0.15 (nmol/L)	*CR1*	*Catharanthus roseus*	whole plant	Liu et al. (2017)
	tropane	Scopolamine	6	1.4/8.8	*QPT*	*Duboisia leichhardtii*	hairy roots	Singh et al. (2018)
Co-overexpression	indole	Akuammicine (23 other MIAs)	14	–	*BIS1, ORCA3, CrMYC2a*	*Catharanthus roseus*	Petals (transient)	Schweizer et al. (2018)
	indole	Vinblastine (vindoline, catharanthine)	5	0.030/0.14	*CrTDC, CrSTR*	*Catharanthus roseus*	whole plant	Sharma et al. (2018)
	indole	Catharanthine (vindoline, ajmalicine)	6	0.19/1.2	*G10H, ORCA3*		hairy roots	

Table 1 (*continued*)

Molecular approach	Alkaloid type	Main secondary metabolite (others)	Times-fold Increase (up to)	Before/ after yield (mg/g DW)	Gene(s)	Plant species	Culture type	Ref
						Catharanthus roseus		Wang et al. (2010)
	indole	Vindoline (catharanthine, ajmalicine)	4	0.70/3.0	*ORCA3, G10H*	*Catharanthus roseus*	whole plant	Pan et al. (2012)
	indole	Catharanthine (secologanin, vindoline, ajmalicine, vinblastine)	2	–	*GGPPS, GES*	*Catharanthus roseus*	whole plant	Kumar et al. (2018)
	indole	Total alkaloids (vincamine)	2	1.1/2.1	*CrTDC, CrSTR*	*Vinca minor*	Cell suspensions	Verma et al. (2015)
	indole	Camptothecin	2.4	0.68/1.6	*CrG10H, CrSTR*	*Ophiorrhiza pumila*	hairy roots	Cui et al., 2015b
	indole	Serpentine (catharanthine, ajmalicine, tabersonine)	1.5	1.0/1.5	*ORCA3, CrSDG*	*Catharanthus roseus*	hairy roots	Sun and Peebles (2016)
	indole	Hörhammericine (lochnericine, tabersonine)	1.3	0.80/1.0	*ASA/DXS*	*Catharanthus roseus*	hairy roots	Peebles et al. (2011)
	tropane	Scopolamine (anisodine, anisodamine hyoscyamine)	11	0.011/0.12	*AaPMT, AaTRI*	*Anisodus acutangulus*	hairy roots	Kai et al. (2011)
	tropane	Anisodine (scopolamine, anisodamine hyoscyamine)	19	0.053/0.98	*AaTri, AaH6H*	*Anisodus acutangulus*	hairy roots	Kai et al. (2012)
	tropane	Scopolamine (anisodamine hyoscyamine)	12	0.40/5.2	*NtPMT, HnH6H*	*Atropa belladonna*	whole plant	Xia et al. (2016)
	tropane	Hyoscyamine (scopolamine)	11	0.20/2.2	*NtPMT, HnH6H*	*Atropa belladonna*	hairy roots	Yang et al. (2011)
	tropane	Scopolamine	7	0.16/1.2	*NtPMT, HnH6H*	*Atropa belladonna*	whole plant	Wang et al. (2011)
	tropane	Scopolamine	3	1.1/3.4	*SpPMT, SpH6H*	*Scopolia parviflora*	hairy roots	Kang et al. (2011)
	tropane	Scopolamine	2.5	0.24/0.60	*NtPMT, HnH6H*	*Atropa belladonna*	whole plant	Liu et al. (2010)

when the foreign enzyme does not share high homology, it enables the expression of genes that tend to be silenced if overexpressed as endogenous genes (Rahnama et al., 2013).

The overexpression of heterologous genes has also enabled the manipulation of the metabolic flux toward a specific metabolite. Singh et al. reported a significant increase in scopolamine content by overexpressing the mouse *ornithine decarboxylase (ODC)* gene, a rate-limiting gene of the tropane biosynthesis pathway in *Datura innoxia* plants (Singh et al., 2011). The importance of ODC is in facilitating the availability of putrescine which is both, an alkaloid precursor and an essential

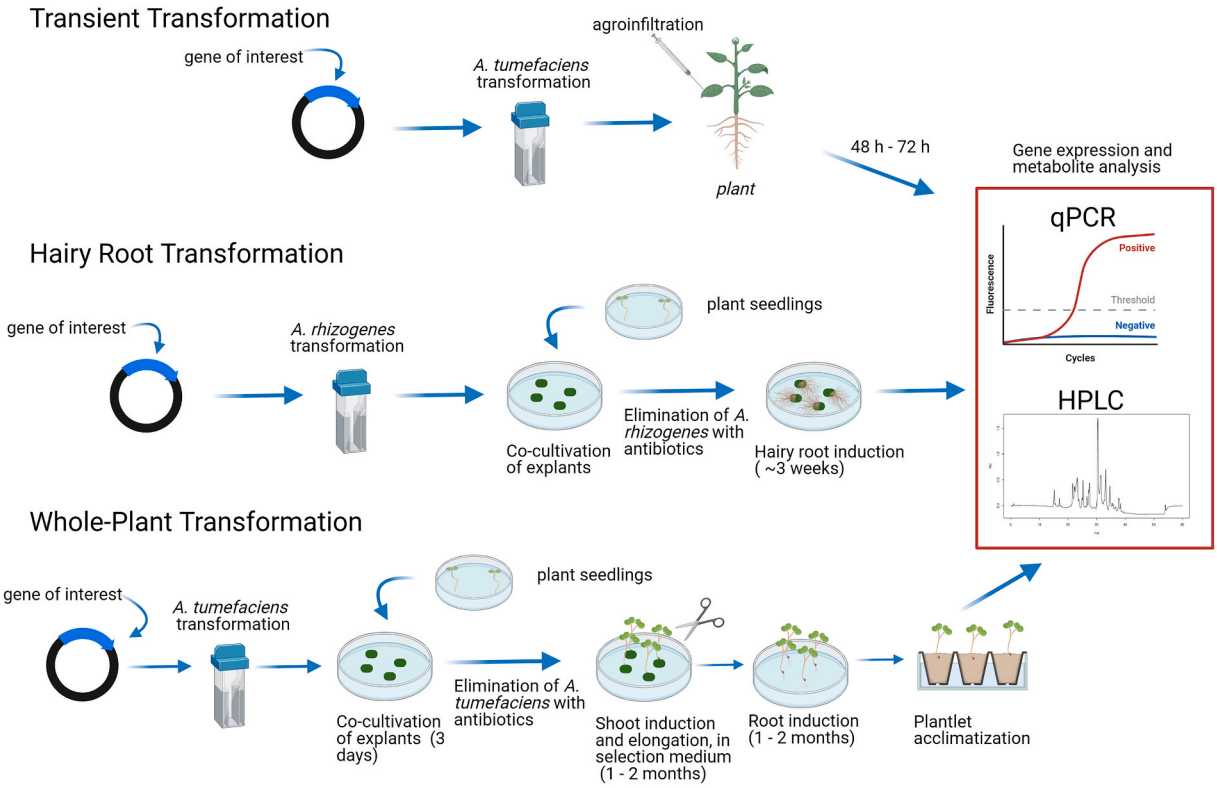

Fig. 2. Genetic transformation methods to increase alkaloid content in medicinal plants (created with BioRender.com).

requirement for cell growth and sustenance, it maintains a balance between primary and secondary metabolites in plant species (Mohapatra et al., 2009). The mouse *ODC* gene has also been overexpressed in *Populus nigra x maximowiczii* (poplar) cells (Bhatnagar et al., 2001; Mohapatra et al., 2009) and transgenic tobacco plants (Kumria and Rajam 2002). In poplar cell culture it was reported that accumulation of putrescine in high amounts didn't affect the native decarboxylase activity, however, enhanced turnover of putrescine could make the plant cells vulnerable to increased oxidative damage. Transgenic tobacco plants were found to be tolerant to salt, however high concentrations of putrescine also affected the normal growth and development of the plants. In contrast, it was reported that transgenic *Datura innoxia* plants overexpressing the mouse ODC gene, not only had increased alkaloid contents but also had a better growth response (Singh et al., 2011).

The expression of heterologous *Vitreoscilla* hemoglobin *(Vhb)* expressed in the hairy roots of *Hyoscyamus niger* enhanced scopolamine content. It was hypothesized that Vhb enhanced the utilization of oxygen, thus accelerating oxygen-requiring metabolic pathways, such as the biosynthesis of tropane alkaloids (Guo et al., 2018). Vhb is a bacterial hemoglobin that interacts with terminal oxidase to provide enough oxygen for cell growth. It has been used to metabolic engineer plants, microorganisms and animals to increase cell density, enhance products biosynthesis and stress tolerance under oxygen-limited conditions (Yu et al., 2021). Table 1 provides more examples of foreign enzymes reported to be useful in alkaloid production.

3.3. Transcription factor overexpression

Transcription factors (TFs) are modular proteins that are essential for regulating gene transcription. They interact with cis-elements to activate or inhibit target genes through a DNA-binding domain (Samad et al., 2017). The overexpression of a single transcription factor (endogenous or foreign) (Decendit et al., 2009) may alter the expression of a number of related genes involved in the production of secondary metabolites. The biosynthesis of terpenoid indole alkaloids is tissue specific and induced by biotic and abiotic stress, indicating a complex transcriptional regulation. In *Catharanthus roseus,* five transcription factors (AP2/ERF TFs): ORCA2, ORCA3, ORCA4, ORCA5, and ORCA6, were shown to form a gene cluster that regulates the biosynthesis of terpenoid indole alkaloids (TIAs) when induced by methyl-jasmonate and ethylene (Singh et al., 2020a). Overexpression of the ORCA4 transcription factor in *C. roseus* hairy roots significantly increased the transcripts levels of important genes in the indole and seco-iridoid pathways (ASA, TDC, G10H and others), as well as the expression of *strictosidine synthase.* This resulted in a significant accumulation of tabersonine, an indole alkaloids that mediate vinblastine biosynthesis (Paul et al., 2017). Despite an increment in alkaloid contents, the overexpression of ORCA4 also upregulated several *ZCT* genes (*Zinc finger C. roseus transcription factor*) which are transcriptional repressors of TIA biosynthesis, suggesting the existence of a negative regulatory loop probably required to modulate the accumulation of alkaloids (Paul et al., 2017).

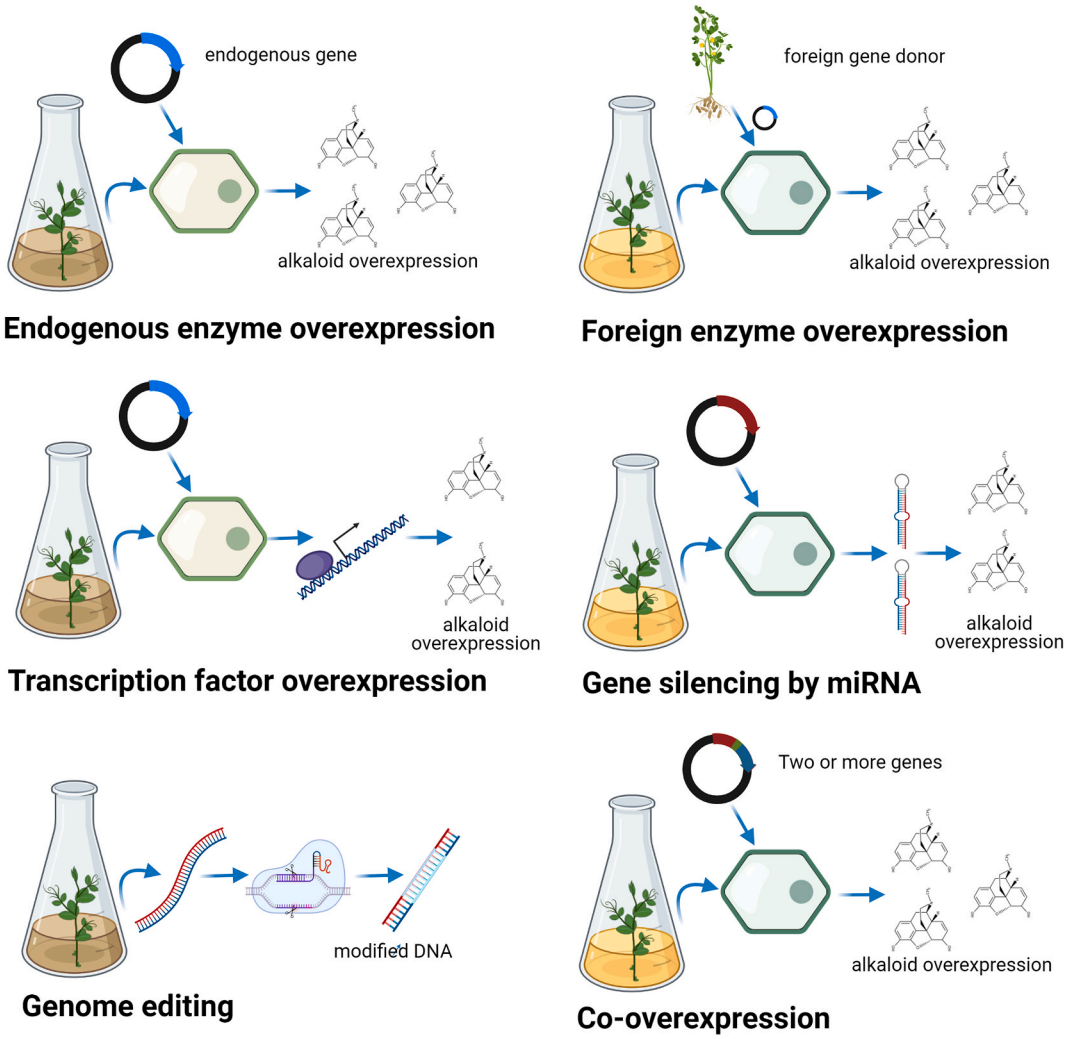

Fig. 3. Metabolic engineering approaches to overexpress alkaloids in plants (created with BioRender.com).

Other important TF that has been discovered in *C. roseus* is CrWRKY1, it is preferentially expressed in roots and induced by the phytohormones jasmonate, gibberellic acid, and ethylene. The overexpression of CrWRKY1 in *C. roseus* hairy roots significantly increased the transcripts levels of several key TIA pathway genes, as well as some *ZCT* transcriptional repressors. The overexpression of CrWRKY1 also repressed the transcriptional activators ORCA2, ORCA3, and CrMYC2. This resulted in a significant accumulation of serpentine in comparison with control roots (Suttipanta et al., 2011). The interaction between TFs can be explored to selectively engineer specific metabolisms, for example the overexpression of CrWRKY1 activates the serpentine branch to completion, while its silencing could guide the pathway towards catharanthine and vindoline (Suttipanta et al., 2011).

As regards tropane alkaloids, novel insights through transcriptome analysis with RNA deep sequencing, have suggested more than 2000 unigenes encoding transcription factors of WRKY, AP2/ERF, JAZ, MYB, bHLH families. This families have been found to play important roles in secondary metabolism biosynthesis (Cui et al., 2015a). As additional pathways, enzymes, regulators such as TFs, and its cross talks are being discovered and characterized, it becomes clear how complex alkaloid metabolism is. Table 1 shows more transcription factors that have been overexpressed to enhance alkaloid content in various plant species.

3.4. Gene silencing

Gene silencing is an approach that can be used to reduce the production of undesirable metabolites (McCue et al., 2018) or to increase the production of certain metabolites by downregulating competing pathways. Gene silencing to increase alkaloid contents may be achieved by RNA silencing methods, such as RNAi (Singh et al. 2018), miRNA overexpression (Li et al., 2015), and Virus Induced Gene Silencing (Liu et al., 2017).

In recent years this approach has been used to increase the contents of indole and tropane alkaloids. Liu et al. (2017) silenced a novel AP2/ERF transcription factor, CR1, in transgenic *C. roseus* plants and showed that 7 genes (*G10H*, *SLS*, *TDC*, *STR*, *SGD*, *DAT* and *PRX*) which code for key enzymes in the indole alkaloid pathway, were upregulated in CR1-silenced plants. This resulted in a higher accumulation of vindoline and serpentine, but not catharanthine in *C. roseus*. As mentioned previously, transcription factors, which can be activators, repressors, or both, are able to regulate the expression of multiple genes, and thus, increase the production of secondary metabolites. CR1 may function, in combination with other TFs, as a negative feedback model to keep balance of indole alkaloid biosynthesis (Liu et al., 2017).

Singh et al. (2018) silenced the quinolinic acid phosphoribosyl transferase (*QPT*) gene in *Duboisia leichhardtii* hairy roots in order to divert the nicotine and tropane alkaloid pathway toward scopolamine biosynthesis, this resulted in enhanced scopolamine contents in transgenic hairy roots. The QPT enzyme catalyzes the nicotinate mononucleotide biosynthesis, which subsequently combines with methylpyrrolinium cation to form nicotine. Both scopolamine and nicotine are synthesized from methylpyrollinum cation, so the strategy was to silence the *QPT* gene to divert the methylpyrollinium cation into scopolamine. Although the QPT enzyme is also relevant to primary metabolism of plants, its silencing did not adversely affect hairy roots development (Singh et al. 2018).

At the post-transcriptional level, miRNAs (microRNAs, which are small non-coding RNAs) regulate gene expression by downregulating their targets, most of which are transcription factors that activate or repress the transcription of their own targets (Samad et al., 2017). Although there is limited research on the manner in which miRNA overexpression regulates alkaloid biosynthesis, overexpression or silencing of a single or multiple miRNAs is a promising approach to regulate key genes and engineer plants with a higher content of secondary metabolites (Gupta et al., 2017). Boke et al. (2015) identified, by deep sequencing, three potential novel miRNAs, pso-miR2161, pso-miR13, and pso-miR408, which may be involved in the biosynthesis of benzylisoquinoline alkaloids (BIA) in *Papaver somniferum*. The target identification analyses showed that pso-miR2161 and pso-miR13 cleaved transcripts of S-adenosyl-L-methionine: 30-hydroxy-N-methylcoclaurine 40-O- methyltransferase 2 (4-OMT) and 7-O-methyltransferase (7-OMT) respectively, these are important enzymes in the BIA pathway. The pso-miR408 possibly targets mRNA from a gene encoding FAD-binding and BBE domain-containing protein, also an important enzyme in the BIA pathway (Boke et al., 2015).

Gene silencing approaches reduce gene expression at the post-transcriptional level. As consequence, a significant decrease in gene expression (knockdown) is observed, however it does not abolish the gene function (Alagoz et al., 2016). It has been suggested that a gene knock-out approach, with gene editing tools, would be preferable for a better understanding of gene function and for increasing the production of specific metabolites.

3.5. Gene editing

CRISPR/Cas9 system, which was honored by the Nobel Prize 2020 in Chemistry, has been considered the best choice for gene editing and targeted mutagenesis in numerous plants (Zakaria et al., 2021). In this system, Cas9 enzyme, a RNA-guided endonuclease, is guided by a single-guide RNA to recognize a targeted site and introduce a double-strand break. Changes to the single-guide RNA sequence can be used to target CRISPR-Cas9 to virtually any DNA region (Westermann et al., 2021). After the generation of double strand breaks, the DNA is modified in two ways: non-homologous end joining which results in nucleotide insertions, deletions and substitutions. And the other is homologous recombination, which can be performed if homologous donor templates are present, facilitating the insertion of DNA fragments (Leitão et al., 2017).

This has enabled the disruption of multiple genes, the characterization of gene families and the identification of genes or regulatory mechanisms, such as transcription factors, that control secondary metabolite pathways (Tang et al., 2017). In addition, it provides a method to generate a new variety of plants (Arshid Shabir 2021). Other advantages of CRISPR-Cas are the complete knock out of a target gene and stable genomic alterations that are transferred to the offspring (Dey 2021).

Comfrey (*Symphytum officinale*) is a medicinal plant with anti-inflammatory and analgesic properties; however, it also contains pyrrolizidine alkaloids, which are regarded as being toxic to humans. Using a CRISPR/Cas9-based approach, Zakaria et al. (2021) introduced mutations into the *homospermidine synthase* (HSS) gene in *S. officinale* hairy roots. HSS catalyzes the biosynthesis of homospermidine, a specific precursor of pyrrolizidine alkaloids. The result was a successful knock-out of the HSS-encoding gene and the complete elimination of pyrrolizidine alkaloids (Zakaria et al., 2021). In another example, the CRISPR-Cas9 system was used to knock out the 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′OMT2) regulatory gene for the biosynthesis of benzylisoquinoline alkaloids in *Papaver somniferum* plants. This resulted in a significantly reduced production of these alkaloids and the biosynthesis of a novel uncharacterized alkaloid (Alagoz et al., 2016).

Few medicinal plants have been edited using the CRISPR-Cas system, thus, its use in the production of alkaloids has been limited. Nevertheless, the use of gene editing systems for metabolic engineering of medicinal and aromatic plants represents a useful methodology that enables increased production of specific metabolites in a controlled manner (Alagoz et al., 2016). Other reviews have summarized the strategies, advantages, and shortcomings of using CRISPR-based screening in eukaryotic cells (Shalem et al., 2015; Housden and Perrimon 2016).

3.6. Gene co-overexpression

Single genes in the alkaloid biosynthetic pathways have been identified and cloned to significantly increase the expression of specific enzymes. However, the nature of biosynthetic pathways is that they are complex with multiple rate-limiting steps. In highly branched pathways, there could be complicated feedback and feed-forward regulation mechanisms, in addition, a precursor can be channeled into a variety of different metabolites away from the desired product (Peebles et al., 2011). Therefore, a successful strategy may involve the overexpression of several rate-limiting enzymes or the manipulation of regulatory genes, which control the expression of multiple genes.

In this review, the most common, and one of the most efficient approaches to increase the accumulation of alkaloids, was the co-overexpression of two or more genes. Transgenic *C. roseus* plants transformed with the *tryptophan decarboxylase (CrTDC)* and *strictosidine synthase (CrSTR)* genes produced a 9-fold increase in vindoline and catharanthine, and a 5-fold increase in vinblastine (Sharma et al., 2018). Hairy root cultures of *C. roseus,* transformed with the *geraniol 10-hydroxylase (G10H)* and *ORCA3* genes, yielded catharanthine at a 6.5-fold higher amount compared with the control (Wang et al., 2010).

As for tropane alkaloids, co-overexpression of putrescine N-methyltransferase (AaPMT) and tropinone reductase I (AaTRI) in *Anisodus acutangulus* hairy roots resulted in a marked production of scopolamine and other tropane alkaloids (Kai et al., 2011). In another study, the co-overexpression of putrescine N-methyltransferase (NtPMT) and hyoscyamine 6β-hydroxylase (HnH6H) caused a significant increase of scopolamine in *Atropa belladonna* plants (Xia et al., 2016). It has been suggested that overexpressing an upstream key enzyme, for example PMT, along with a downstream branch-controlling enzyme (for example TRI or H6H) may act as a push-pull effect in which flux is pushed towards the branch point (Kai et al., 2011).

Gene co-overexpression has also been combined with simultaneous elicitor application. Elicitors, such as growth regulators, biotic, and abiotic stresses, can trigger the expression of key genes and transcription factors (Pan et al., 2016). The overexpression of multiple genes in plants may be done by crossing individual transformants, re-transforming with a new gene, co-transforming with different plasmids (either stable and transient transformation), or by transformation with a single binary plasmid containing multiple expression cassettes (Vidal et al., 2003; Ha et al., 2010). In addition, polycistronic systems using either an internal ribosome entry site or 2A peptide sequences have been used to express multiple genes in a single expression cassette (Ali et al., 2010).

4. Perspectives

Despite the success of the plant metabolic engineering approaches presented here, alkaloids that are used in oncology and anesthesia are in high demand, whereas drug shortages have become a growing public health issue. This is caused, not only by global pandemics, natural disasters, and the destruction of natural habits, but also by the fact that these molecules are structurally complex, difficult to synthesize, and their content in plants is very low (Courdavault et al., 2021).

Consequently, there is a need for new biotechnology tools to address these problems. The CRISPR system is one of such tools, this technology is in rapid development and has enabled considerable advances in plant research. Beyond gene knockouts, CRISPR/Cas systems have been considered as programmable platforms for transcriptional and post-transcriptional regulation. Most Cas proteins are nucleases that cleave the targeted nucleic acid sequence, however there are also nuclease-deactivated Cas (dCas) proteins that remain competent for RNA-guided DNA binding but inadequate to induce DNA double-strand breaks. Inactive Cas proteins (dCas) can be fused with effector proteins including transcriptional activators, repressors, and epigenetic modulators. This platforms, based on CRISPR/dCas, have been demonstrated in mammalian cells and its application could be broaden in plants in the near future (Pan et al., 2021).

An interesting idea for the application of gene editing is the construction of hybrid biosynthetic clusters using genes from close-related organisms in order to produce "hybrid" metabolites, an idea first expressed by Hopwood et al. in 1985 (Leitão et al., 2017). To accomplish this, scientists could engineer genes encoding multidomain condensing enzymes commonly found in secondary metabolic gene clusters (Seyedsayamdost and Clardy 2014; Sundaram et al., 2015; Leitão et al., 2017). By using genome editing tools, researchers could facilitate the generation of chimeric enzymes that present diverse substrate specificity (Leitão et al., 2017).

One of the major concerns of CRISPR technology is the potential "off-target" cleavage that may occur throughout the whole genome, however several studies have revealed a high specificity using wild-type Cas9 and Cas12a in plants (Zhang et al., 2019). It has been suggested that a lack of whole genome sequences has restricted its application for medicinal plants (Dey 2021). In addition, more studies are needed to understand the DNA repair pathways in plants to have more certainty with respect to editing outcomes (Zhang et al., 2019).

Other viable alternatives to meet the increasing demands of medicinal alkaloids include inserting plant genetic blueprints for the biosynthesis of alkaloids into fermentable organisms, such as yeast and bacteria (Guerriero et al., 2018; Srinivasan and Smolke 2020; Yamada et al., 2021). This approach can be used when most of the enzymes involved in a biosynthetic pathway are known. Medicinal alkaloids hyoscyamine and scopolamine have been produced in yeast starting from simple sugars and amino acids. This system required 34 chromosomal modifications, resulting in an integrated whole-cell system that expressed enzymes and transporters in diverse sub-cellular locations. Yeast genomic modifications were performed using a CRISPR technology (Srinivasan and Smolke 2020). The recent discovery of benzylisoquinoline alkaloid transporters from opium poppy (denominated BUPs) and its expression in a yeast strain, hosting segments of the opiate pathway, dramatically increased the yield of dopamine and codeine. The conversion from exogenous Levodopa to codeine requires over a dozen heterologous enzymes in the engineered yeast (Dastmalchi et al., 2019). The employment of a transporter for alkaloid production has also been used in bacteria, *Escherichia coli*, for the production and secretion of high levels of reticuline (Yamada et al., 2021).

In recent years, major advances in tools and insight into plant biosynthetic pathways have emerged. Still, this is an area of ongoing research, therefore, new enzyme function, localization, and regulation of metabolic pathways have yet to be discovered (Shih and Morgan 2020).

5. Conclusions

In this review, effective strategies to increase the alkaloid content in medicinal plants were summarized. One of the most studied plants has been *Catharanthus roseus*, because of the presence of the commercially important indole alkaloids, ajmalicine and serpentine (antihypertensives), and vincristine and vinblastine (antitumoral alkaloids). To increase the accumulation of indole alkaloids, a common and efficient approach is the overexpression of transcription factors such as *ORCA3* and *ORCA4,* and the co-overexpression of two or more key enzymes, such as tryptophan decarboxylase and strictosidine synthase. The indole alkaloid biosynthetic pathway is a complex network that is not fully understood, the discovery of additional enzymes and regulators will open the door for further improvement in alkaloid content.

To increase the yield of tropane alkaloids, such as scopolamine, obtained from Solanaceae plants, the most effective approach is the co-overexpression of two enzymes, an upstream key enzyme, and a downstream branch-controlling enzyme. Most common strategies include the co-overexpression of putrescine N- methyltransferase with hyoscyamine 6β-hydroxylase (in plants and hairy roots), and the co-overexpression of putrescine N-methyltransferase and tropinone

reductase I (hairy roots). It should be noted that, although there are currently some gaps, the tropane alkaloid biosynthesis is more documented and well understood in comparison with the indole pathway. This has allowed the engineering of microorganisms to produce hyoscyamine and scopolamine.

Recent advances in CRISPR technology indicate that, in the near future, it will be possible to accelerate or suppress single or multiple genes in medicinal plants by the use of effector proteins (transcriptional activators, repressors, and epigenetic modulators) fused with dCas (an inactive variant of the protein). This is a promising tool to increase the content of alkaloids, and other secondary metabolites, in plants.

Alkaloids used in oncology and anesthesia are in high demand and their content in plants is very low. The metabolic engineering approaches, presented here, can increase the production of alkaloids, reducing the need to overexploit limited natural resources.

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Author contribution

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ASA:: Anthranilate synthase α subunit
BBE: Berberine bridge enzyme
BIS: Basic Helix-Loop-Helix Iridoid Synthesis
CodR: Codeinone reductase
CR1: (*C. Roseus* AP2/ERF transcription factor)
CYP72A1: Secologanin synthase
DAT: Deacetylvindoline-4-O-acetyltransferase
DXS: 1-deoxy- D-xylulose synthase
ERF: Ethylene responsive factor
G10H: Geraniol 10-hydroxylase
GES: Geraniol synthase
GGPPS: Geranyl(geranyl) diphosphate synthase
H6H: Hyoscyamine-6β-hydroxylase
L/ODC: Lysine/ornithine decarboxylase
MPK3: Mitogen-activated protein kinase 3
MYC: Myelocytomatosis related proteins
ODC: Ornithine decarboxylase
4′OMT: 3′-Hydroxy-N-methylcoclaurine 4′-O-methyltransferase
ORCA: Octadecanoid Responsive Catharanthus AP2-Domain Protein
PMT: Putrescine N-methyltransferase
Prx: Apoplastic peroxidase
QPT: Quinolinic acid phosphoribosyl transferase
SAUR: Small Auxin Up-regulated RNA
SDG: Strictosidine glucosidase
STR: Strictosidine synthase
TDC: Tryptophan decarboxylase
TRI: Tropinone reductase I
VHb: Vitreoscilla hemoglobin
WRKY: Amino-acid sequence WRKYGQK