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REVIEW

Application of genetics and biotechnology for improving medicinal plants

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Abstract

Main conclusion **Plant tissue culture has been used for conservation, micropropagation, and *in planta* overproduction of some pharma molecules of medicinal plants. New biotechnology-based breeding methods such as targeted genome editing methods are able to create custom-designed medicinal plants with different secondary metabolite profiles.**

For a long time, humans have used medicinal plants for therapeutic purposes and in food and other industries. Classical biotechnology techniques have been exploited in breeding medicinal plants. Now, it is time to apply faster biotechnology-based breeding methods (BBBMs) to these valuable plants. Assessment of the genetic diversity, conservation, proliferation, and overproduction are the main ways by which genetics and biotechnology can help to improve medicinal plants faster. Plant tissue culture (PTC) plays an important role as a platform to apply other BBBMs in medicinal plants. *Agrobacterium*-mediated gene transformation and artificial polyploidy induction are the main BBBMs that are directly dependent on PTC. Manageable regulation of endogens and/or transferred genes via engineered zinc-finger proteins or transcription activator-like effectors can help targeted manipulation of secondary metabolite pathways in medicinal plants. The next-generation sequencing techniques have great potential to study the genetic diversity of medicinal plants through restriction-site-associated DNA sequencing (RAD-seq) technique and also to identify the genes and enzymes that are involved in the biosynthetic pathway of secondary metabolites through precise transcriptome profiling (RNA-seq). The sequence-specific nucleases of transcription activator-like effector nucleases (TALENs), zinc-finger nucleases, and clustered regularly interspaced short palindromic repeats-associated (Cas) are the genome editing methods that can produce user-designed medicinal plants. These current targeted genome editing methods are able to manage plant synthetic biology and open new gates to medicinal plants to be introduced into appropriate industries.

Keywords Bio-product · Biotechnology · Genome editing · Metabolic engineering · Plant synthetic biology · Tissue culture

Abbreviations

CENH3	Centromere-specific histone H3
COSTREL	Combinatorial supertransformation of transplastomic recipient lines
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated
DSBs	Double-strand breaks
GM	Genetically modified
GWAS	Genome-wide association
HPLC	High-performance liquid chromatography
NGS	Next-generation sequencing
PTC	Plant tissue culture
PGRs	Plant growth regulators
QTL	Quantitative trait loci
sgRNA	Single-guide RNA
TALENs	Transcription activator-like effector nucleases
TILLING	Targeting-induced local lesions in genomes
ZFNs	Zinc-finger nucleases

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Introduction

Plants are the "green chemical factories" with a wide range of chemical diversity that can support food, feed, medicines, and biomaterial industries (Nogueira et al. 2018). The safe application of organic materials leads to more popularity of plant-derivate medicines in the recent years (Niazian et al. 2017c; Soltani Howyzeh et al. 2018). Despite their mentioned advantages and various applications, medicinal plants are located in the last ring of domestication. Actually, when humans abandoned the nomadic hunting and food gathering life and began to domesticate plants, their focus was on major food supplier crop species, such as wheat, soybean, maize, rice, potato, and sunflower, and valuable medicinal plants have been forgotten for many years (Siahsar et al. 2011). Plant domestication is a dynamic evolutionary process (Kovach et al. 2007) that creates new and valuable forms of plants from wild species through genetic modification. Larger fruits or grains, stronger plant, increased growth of the central stem in comparison to the side stems (determinate growth), loss of seed dispersal and seed dormancy, lower bitter substances in structures, altered photoperiod sensitivity, and harmony in flowering are the major changes that occur during the domestication process of plants (domestication syndrome) (Doebley et al. 2006). Medicinal plants have been deprived of this domestication syndrome and now plant breeders must compensate these shortages by using faster methods of biotechnology. It is time for researchers to discard discrimination and utilize modern biotechnology-based methods to improve medicinal plants. Using next-generation sequencing (NGS) techniques, researchers can obtain the whole genomic and transcriptomic information of medicinal plants. These genomic and transcriptomics data can be combined with proteomics and metabolomics data to produce unidentified natural products of medicinal plants (Zhao et al. 2018). The obtained information of NGS can be used in powerful molecular methods, such as DNA barcoding, for identification of the genetic diversity of medicinal plants and their reliable classification at the genus and species level (Techen et al. 2014). The faster and precise mapping of wild and unknown populations of medicinal plants through RAD-seq, the accurate and fast transcriptome analysis of medicinal plants to identify key genes and enzymes in valuable biosynthetic pathways, and the quick and exact interpretation of TILLING populations, all applicable in medicinal plants through depth and bulk size sequencing of whole genome or NGS-based mapping by sequencing. The application of targeted genome editing methods of TALENs, ZFNs, and CRISPR-Cas9 systems, subsequent to NGS analysis, can introduce synthetic biology to genetic and metabolic engineering in medicinal plants. It can create new varieties of medicinal plants that contain the desired chemical compounds and/or new bio-products (Pouvreau et al. 2018). Although using a creative genetic engineering method such as combinatorial supertransformation of transplastomic recipient lines (COSTREL) can significantly enhance the production of valuable secondary metabolites of medicinal plants (Fuentes et al. 2016), it seems that CRISPR/Cas9 system has greater potential than COSTREL method to create an efficient synthetic biology for medicinal plants in a faster manner.

In the present review, all applied biotechnology-based breeding methods in different medicinal plants along with other potentially applicable methods that can help their faster improvement are highlighted. The principals, advantages, disadvantages, and concerns of applied and other potentially applicable BBBMs are presented. All of these applied/applicable genetics and biotechnological tools in medicinal plants are presented in Fig. 1. As shown, the PTC and NGS are the main cores of genetics and biotechnology for improvement of medicinal plants.

New genetics and biotechnology tools to assess the genetic diversity and genomic and transcriptomic information

The assessment of the genetic diversity (variability) in an unexplored genetic background is very important to schedule an efficient breeding program (Niazian et al. 2017c). The morphological and classic molecular markers such as RAPD, RFLP and ISSR are very efficient for this purpose. However, these are slow, expensive and time-consuming methods with their specified limitations and concerns. Therefore, establishment of faster and efficient methods for genetic diversity elucidation of medicinal plants is important. The DNA microarray is a promising way using hybridization property of target DNA with picomolar size probes, assembled in a solid surface. The microarray technique is very useful in medicinal plants, because its comparative evaluation property allows the recognition of the differences in the expression pattern under different growing conditions. Therefore, it can track and find the genome fragments expressed during different in vitro or field growing conditions. It is also applicable to measure the expression level of specified genes under different growing environments (Ishkanian et al. 2004). The diversity array technology (DArTTM) and subtracted diversity array (SDA) are two altered versions of microarray that are more applicable to organisms with lack of prior sequence information.

Quick and cost-effective methods of next-generation sequencing (NGS) have emerged as powerful tools to discovery, sequencing and genotyping of thousands of markers

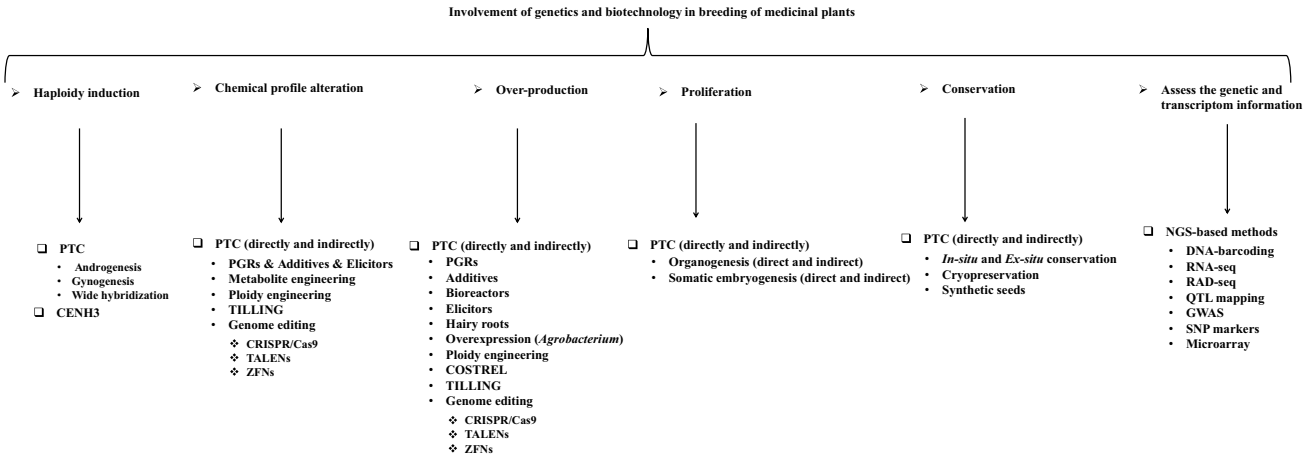

Fig. 1 Different applications of next-generation sequencing (NGS) and plant tissue culture (PTC) techniques in improvement of medicinal plants. *CENH3* centromere histone H3, *COSTREL* combinatorial supertransformation of transplastomic recipient lines, *CRISPR* clustered regularly interspaced short palindromic repeats, *Cas* CRISPR-associated, *GWAS* genome-wide association, *PGRs* plant growth regulators, *TALENs* transcription activator-like effector nucleases, *TILLING* targeting-induced local lesions in genomes, *ZFNs* zinc-finger nucleases)

across any genome of interest in a single step (Davey et al. 2011). The NGS approaches can also evaluate the metabolisms in non-model and unexplored plants (Liu et al. 2017a, b) such as medicinal plants. The NGS technology is applicable for transcriptome profiling or cDNA sequencing (RNA-seq). In RNA-seq, the complete transcriptomes will be sequenced in the target populations. The NGS-based transcriptome analysis is more powerful than microarray techniques to distinguish unknown genes and also to identify the expression differences of homoeologous and paralogous gene copies. This method has been successfully applied in ajowan (*Trachyspermum ammi* L.) medicinal plant to identify the unigenes involved in the biosynthesis of monoterpenoids (Howyzeh et al. 2018). The results of such researches can be used for functional breeding of the desired medicinal plants.

Besides all aforementioned direct applications of NGS in medicinal plants, there are other branches of genetic and biotechnology that can benefit from NGS for faster improvement of medicinal plants (Fig. 2). The NGS technology can contribute to molecular taxon identifier methods such as DNA barcoding. DNA barcoding is an efficient method

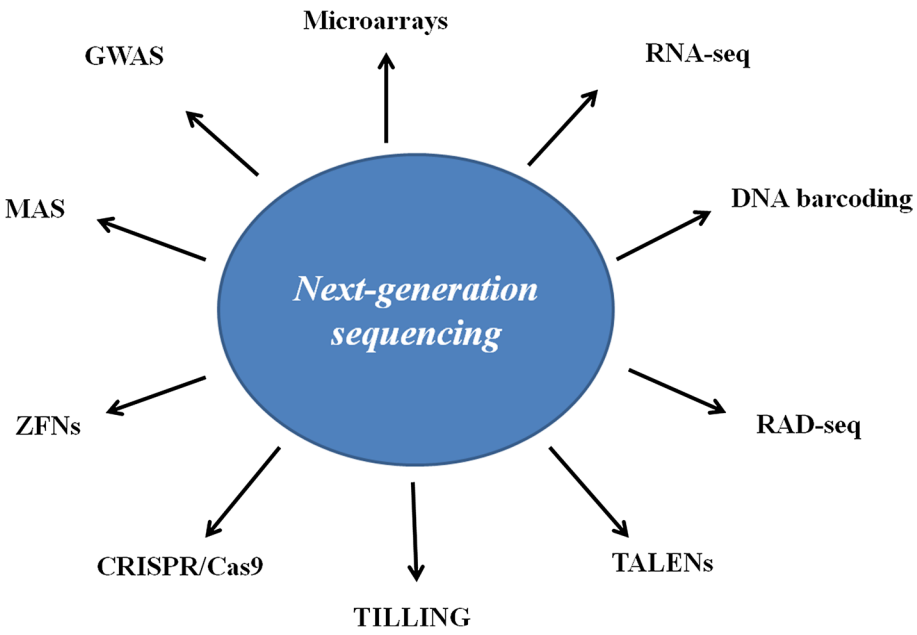

Fig. 2 Different applications of next-generation sequencing technique in genetics and biotechnology for improvement of medicinal plants. *CRISPR* clustered regularly interspaced short palindromic repeats, *Cas* CRISPR-associated, *GWAS* genome-wide association, *MAS*: marker-assisted selection, *TALENs* transcription activator-like effector nucleases, *TILLING* targeting-induced local lesions in genomes, *ZFNs* zinc-finger nucleases

for fast assessment of the genetic diversity of medicinal plants at the genus and species levels (Techen et al. 2014). In wild populations of medicinal plants, without any reference genome, the reduced representation sequencing data of NGS can be implemented in restriction-site-associated DNA sequencing (RAD-seq) method for efficient genotyping of the studied population (Davey et al. 2011; Zargar et al. 2015). The SNP markers are the connection point of NGS and genome-wide association studies (GWAS). The GWAS is applicable to detecting QTLs in plants so that their entire genome is covered through very high density of SNP markers. On the other hand, the discovery of SNP markers via NGS platforms is a routine task (Edwards and Batley 2010). Therefore, the NGS is a useful platform for GWAS studies.

Targeting-induced local lesions in genomes (TILLING) is a reverse genetic screen method that is able to identify the function of mutation-synthesized isogens. In this method, one of the isogens is created through targeted mutagenesis, but the other one is a natural isoform. Therefore, TILLING is able to compare the induced and natural polymorphisms without involvement of transgenic modifications (Varshney et al. 2009).

In addition to all aforementioned applications, the information of NGS can open the way to apply other newly emerged methods of biotechnology, such as genome editing methods of TALENs, ZFNs, and CRISPR/Cas9, which can subsequently connect to another branch of science, i.e., synthetic biology. The combination of high-throughput genome sequencing technology with omics technologies, such as metabolomics, creates functional genomics (phytochemical genomics) to identify the function of the specialized chemical components produced by the identified genes involved in their biosynthetic pathways (Yamazaki et al. 2018). Figure 1 shows the position of NGS technology in improving medicinal plants through different branches of science.

Plant tissue culture (PTC) of medicinal plants

Plant tissue culture is the main core of biotechnology in medicinal plants. Actually, PTC is the most promising savior of medicinal plants that have low yield and are susceptible to biotic stress. PTC leads to success in in situ and ex situ conservation, micropropagation, polyploidy induction, metabolite engineering (gene transformation), and bioreactor applications in the unexploited genome of medicinal plants (Grzegorczyk-Karolak et al. 2018a). PTC can create a stable situation to assess the effect of different experimental conditions and materials on the production of secondary metabolites of medicinal plants (Lalaleo et al. 2018) and endogenous hormone metabolism signaling and transport (Kumari et al. 2018). The in vitro regeneration can provide situations to assess the effect of different PGRs on various physiological and functional properties of medicinal plants, such as alterations of photosynthetic apparatus performance and the stomatal functionality (Rosa et al. 2018). PTC not only saves medicinal plants and enhances the production of their active secondary metabolites, but also opens the way for production of engineered molecules (Espinosa-Leal et al. 2018) and launching synthetic biology strategy. Therefore, PTC can help produce new forms of plant secondary metabolites and/or custom-designed medicinal plants, valuable for food, pharmaceutical, and other industries.

Strategies, such as media optimization, biotransformation, elicitation, *Agrobacterium* transformation and scale-up (Matkowski 2008), directly depend on PTC for enhancing the in vitro production of valuable plant compounds. PTC is a platform for in vitro chromosome doubling and artificial polyploidy induction in medicinal plants, which subsequently has many significant and useful impacts on their quantitative and qualitative performance. The overexpression of the involved key genes in the biosynthetic pathways of secondary metabolites, change in the regulation of responsible genes to produce valuable biologically active substances with higher quality, and transfer of the agronomically valuable genes to reach plants with more vigor depend on PTC. In *Catharanthus roseus*, the overexpression of geranyl (geranyl) diphosphate synthase [*G(G)PPS*] and geraniol synthase (*GES*) genes led to a significant enhancement in monoterpene indole alkaloids of vinblastine and vincristine in transgenic plants (Kumar et al. 2018). The suppression of rosmarinic acid synthase (*SmRAS*) gene in *Salvia miltiorrhiza* led to increase in the level of 3,4-dihydroxyphenyllactic acid and therefore improved the quality of the produced rosmarinic acid in this medicinal plant (Zhou et al. 2018). In *Salvia miltiorrhiza* medicinal plant, the expression of transferred *AtEDT1* transcription factor led to transgenic plants that were tolerant to drought stress (Liu et al. 2017b). PTC is also a platform for the amazing strategy of hairy root cultures, which have higher biosynthetic capacity to produce valuable secondary metabolites of medicinal plants, with higher genetic and biochemical stability in contrast to conventional in vitro cultures (Roychowdhury et al. 2016). In radish (*Raphanus sativus* L.) medicinal plant, the *Agrobacterium rhizogenes*-induced hairy roots produced higher content of phenolic flavonoid and quercetin content compared to auxin-induced roots of non-transformed radish (Balasubramanian et al. 2018). The superiority of hairy root cultures also has been reported for higher production of phenolic acid, flavonoid, and wedelolactone contents in *Sphagneticola calendulacea* (Kundu et al. 2018), promoting the production of tropane alkaloids of hyoscyamine, anisodamine, and scopolamine in *Scopolia lurida* (Lan et al. 2018), and enhancement of flavonoid production in *Isatis tinctoria* (Jiao et al. 2018). The *Agrobacterium rhizogenes*-derived hairy roots are the platforms for large-scale production of

secondary metabolites using bioreactors (Patra and Srivastava 2017), elicitors (Kastell et al. 2018; Xing et al. 2018a), artificial polyploidy (Dehghan et al. 2012), and genome editing method of CRISPR/Cas9 (Li et al. 2017).

All possible direct and indirect applications of PTC in medicinal plants are presented in Fig. 3.

Direct applications of PTC in medicinal plants

Biotechnological-based ex situ conservation

The indiscriminate use and excessive harvesting of medicinal plants have endangered them in their native lands (Niazian et al. 2018a). Environmental damage, deforestation, industrialization, fires, development of land for agriculture, and climate change are other anthropogenic factors that put natural populations of many medicinal plants at risk in the future (Atanasov et al. 2015). Safeguarding via in situ conservation is not sufficient to protect valuable medicinal plants. Therefore, ex situ conservation is very important in medicinal plants. Actually, ex situ conservation is a back-up of specific parts of plant germplasm that might be lost in their native lands (Li and Pritchard 2009). In vitro slow growth cultures provide short-term (1–15 year) storage of clonal plant material with required periodic sub-cultures (Rao 2004) that are the source of contamination. Here, the emphasis is on long-term conservation through cryopreservation. Some important details of the cryopreservation pathway for ex situ conservation of medicinal plants are discussed below.

Cryopreservation

Cryopreservation refers to the long-term storage of biological materials at very low temperature (− 196 °C) (Di et al. 2018). Seeds, bulbs, buds, corms, cuttings, rhizomes, roots, tubers, etc. are the protective organs of plant germplasm. It is obvious that cryopreservation is a valuable tool for ex situ conservation and long-term storage of medicinal plants with recalcitrant seeds. However, retention of biosynthetic potential, genetic stability of somaclones, and germplasm conservation are the three main purposes of cryopreservation in medicinal plants (Bajaj 1988). *Hypericum perforatum* (Urbanová et al. 2006), *Eruca sativa* Mill (Xue et al. 2008), and *Dendrobium candidum* (Yin and Hong 2009) are valuable medicinal plants for which cryopreservation procedure has been developed for long-term storage.

The vitrification, desiccation, and encapsulation–dehydration are the main applied techniques of cryopreservation in medicinal plants (Krishnan et al. 2011). Survival rate, regeneration percent, and genetic stability are the important parameters that should be considered for an efficient cryopreservation technique. Biochemical stability is another important factor for cryopreservation studies in medicinal plants. Ahuja et al. (2002) used vitrification technique for cryopreservation of shoot tips of *Dioscorea floribunda* medicinal plant and reported the genetic stability of cryopreserved shoot tips using molecular, morphological and

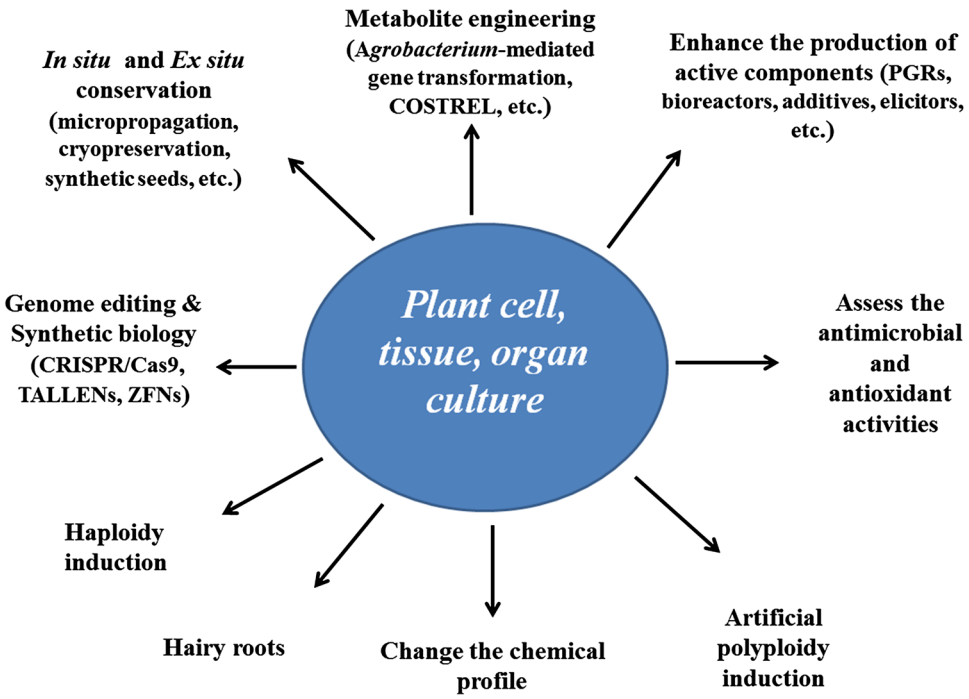

Fig. 3 All direct and indirect applications of plant tissue culture (PTC) in breeding of medicinal plants

biochemical methods. The cryopreserved-derived plants had 87% survival and 30% plant regeneration. The vitrification and encapsulation–dehydration techniques were also applied for cryopreservation the shoot tips of *Dioscorea deltoidea* medicinal plant and HPLC (high-performance liquid chromatography) analysis proved that the diosgenin content of cryopreserved shoot tips was similar to control plants, therefore the biochemical stability of plants derived from cryopreserved shoot tips was not changed (Dixit-Sharma et al. 2005). Ghaffarzadeh-Namazi et al. (2017) investigated different methods of cryopreservation, including desiccation, vitrification with PVS2, vitrification with PVS3 and dimethyl sulfoxide (DMSO) freezing of young leaves-derived callus of *Satureja spicigera* medicinal plant and reported significant differences among investigated methods. They observed the highest regrowth in the callus treated with PVS3 (98.7%).

Synthetic seeds

Field gene banks, seed gene banks, in vitro collections, and cryopreserved tissues are the common forms of plant ex situ conservation (Srivastava et al. 2009). Use of synthetic (artificial) seeds, which refer to the alginate encapsulation of plant explants, are one of the ideal strategies for conservation and micropropagation of medicinal plants, because of their advantages, including genetic stability, ease in handling and transportation, effectiveness in terms of space, labor, time, and cost (Nyende et al. 2003). Naz et al. (2018) compared the conversion response of encapsulated and non-encapsulated nodal segments of *Althaea officinalis* medicinal plant after 6 weeks storage at 6 °C and reported significant higher response of encapsulated than non-encapsulated nodal segments. In addition to conservation, the synthetic seeds are ideal to maintain a screened and selected genotype of medicinal plants that contain a high level of a desired secondary metabolite (Lata et al. 2009). Explant selection, encapsulating agent and matrix are the factors that should be optimized for successful application of synthetic seeds in medicinal plants (Gantait et al. 2015). Unipolar vegetative propagules, microcuttings, differentiating aggregates or bipolar vegetative propagules (somatic embryos and protocorm-like body), in approximate size of 3–5 mm, are the main explants that have been used to produce artificial seeds in different medicinal plants (Gantait et al. 2015). Manjkhola et al. (2005) encapsulated early cotyledonary-stage embryos of *Arnebia euchroma* medicinal plant in 3% sodium alginate and calcium nitrate and reported 60.6% germination and 72% ex vitro survival. Plant growth regulators are an important group of additives used in the encapsulation medium to enhance the efficiency of synthetic seeds in medicinal plants. Saeed et al. (2018) reported that addition of 0.56 mg/L BA + 0.86 mg/L GA_3 + 18.41 mg/L adenine sulfate in MS medium containing 3% Na_2-alginate to encapsulate the nodal segments of *Gymnema sylvestre*, an antidiabetic medicinal plant, led to 88.2 ± 0.48% complete recovery of synseeds to plantlets. Baskaran et al. (2018b) reported 91% regeneration from encapsulated shoot tips of *Urginea altissima* medicinal plants in semi-solid MS medium containing 3% sodium alginate and 100 mM calcium chloride + 2.42 mg/L meta-topolin and 0.37 mg/L NAA. Some recent reports of artificial seed production technology in different medicinal plants taking into account the composition of the capsules and their supplementation in relation to regenerated plant species and secondary metabolite compositions produced in them are presented in Table S2.

Micropropagation

Proliferation is the second direct application of PTC in medicinal plants (Fig. 1). Micropropagation can be done in forms of organogenesis and somatic embryogenesis procedures, in direct and indirect pathways (Fig. 4a). Somatic

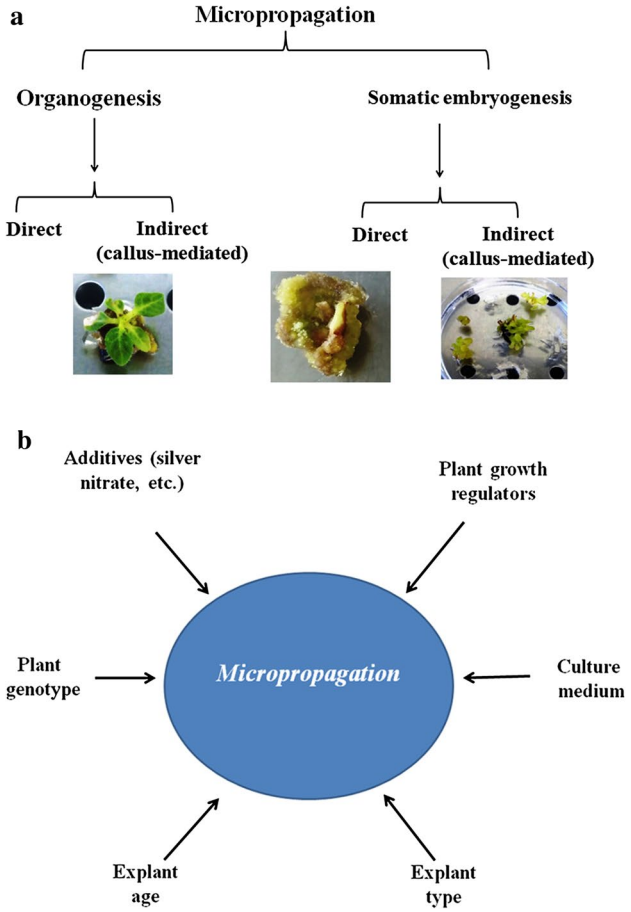

Fig. 4 The micropropagation procedure in medicinal plants. **a** The main pathways of in vitro regeneration in different plants. **b** Factors affecting in vitro regeneration system in plants

embryogenesis is more favorable because of the induced bipolar structures (Shen et al. 2018); however, organogenesis is a two-step procedure that needs different PGRs for shoot and root induction (Hesami and Daneshvar 2018a, b; Hesami et al. 2018a, b). In vitro micropropagation is a multi-variable process and many factors can affect its efficiency (Fig. 4b). Plant genotype, type and size of explant, type and concentration of PGRs, explant age, environmental factors of culture medium (temperature, light intensity and photoperiod), medium's pH, source of carbohydrate, and gelling agent are the main factors that can affect the results of in vitro propagation in different medicinal plants (Nalawade and Tsay 2004). Niazian et al. (2017a) studied the response of 5-, 10- and 15-day-old hypocotyl segments of different ecotypes of ajowan (*Carum copticum* L.) medicinal plant in MS medium supplemented with different concentrations of 2,4-D and Kin PGRs and reported that 15-day-old hypocotyls were the best explant age for somatic embryogenesis. The combination of 2,4-D and Kin has also been reported for induction of embryogenic calli in *Sapindus trifoliatus* (Asthana et al. 2017). PGR-free MS medium has been applied in some medicinal plants for development of induced somatic embryos to mature plantlets (Asthana et al. 2017); however, it seems that the combination of auxin and cytokinin PGRs can help this process in other medicinal plants such as *Swertia corymbosa* (Mahendran and Narmatha Bai 2015) and *Abutilon indicum* (Seth et al. 2016).

In addition to the mentioned factors, there are other additives, such as nanoparticles, casein hydrolysate, picloram, and glutamine, that can be used in basal culture medium to enhance the micropropagation efficiency of medicinal plants. Mahendran et al. (2018) used silver nanoparticles of *Ulva lactuca* extracts (ULAgNPs) in cultured rhizome explants of *Gloriosa superba* L. and reported that application of 0.5 mg/L ULAgNPs along with 2 mg/L BAP + 0.5 mg/L ABA + 0.5 mg/L silver nitrate ($AgNO_3$) + 20% *Ulva lactuca* extracts in MS medium led to the highest percentage of embryo maturation.

As mentioned previously, micropropagation is a multi-variable and complex process; however, there are some computational methods, such as artificial neural network and image processing that can help researchers to overcome its complex nature (Abdipour et al. 2018; Niazian et al. 2018a, b, c).

The successful acclimatization is the final and critical step for successful in vitro regeneration in different medicinal plants, because these are wild plants that have undergone a different growth pathway, and therefore their low acclimatization rate is predictable. Despite these testimonials, most of the in vitro proliferation studies in different medicinal plants do not report the acclimatization percentage of their developed protocol (Table S1).

The overproduction and change in the chemical profile of medicinal plants

In addition to micropropagation, PTC can change the chemical composition of different medicinal plants via the overproduction of concerned phytochemicals, reduce the content of toxic compounds, and produce novel chemical compounds (Gandhi et al. 2015). Media optimization is one of biotechnology's strategies to enhance in vitro production of antioxidants and other valuable defensive secondary compounds of plants (Matkowski 2008). Different culture media, plant growth regulators, and additives are the key actors for this purpose. Monfort et al. (2018) investigated the effect of different culture media (MS, B5 and WPM), different strengths of MS medium, including 2MS, MS, ½MS, and ¼MS, and different combinations of IAA, NAA, IBA, BAP, and thidiazuron (TDZ) PGRs on the nodal segments of basil (*Ocimum basilicum*) and reported that 2MS and ¼MS resulted in increase in the content of methyl eugenol of in vitro regenerated fraction, whereas MS and WPM media led to increase in the volume of linalool and 1,8-cineole fractions. In cell suspension culture of *Dracocephalum moldavica*, the establishment of root-derived callus in MS medium supplemented with 0.5 mg/L 2,4-D and 0.2 mg/L BAP led to higher total phenolic content, rosmarinic acid, and increased radical scavenging activity in comparison to field-grown plants (Weremczuk-Jezyna et al. 2017). Oliveira et al. (2018) applied different concentrations of BA, thidiazuron (TDZ), GA_3, and indole-3-acetic acid on nodal segments of *Cunila menthoides* medicinal plant to develop a direct organogenesis protocol and used UPLC–QTOF–MS^E method to study the metabolome of in vitro regenerated plants. They reported the presence of different phenols, alkaloids, and terpene compounds in regenerated plants which has not been reported before.

In addition to culture media parameters, the environmental parameters can also affect the media optimization strategy. Light is one of these important environmental factors that can affect the production of secondary metabolites. Irshad et al. (2018) applied different light intensities, including 40, 60, 80, and 100 µmol m^{-2}/s on cultures of *Abelmoschus esculentus* and assessed the production of biomass, anthocyanin pigments, and bioactive antioxidants under the applied light intensities. The qualitative and quantitative analysis of callus cultures through HPLC revealed that the highest content of total anthocyanin accumulation, total phenolic content, total flavonoid content, and total antioxidant activity were achieved under 80 µmol m^{-2}/s light intensity.

Plant biostimulants are non-toxic materials and/or microorganisms that can enhance the nutrition-use

efficiency, stimulate plant life processes, and abiotic stress tolerance when added to plant systems (Mustafavi et al. 2018). Elicitors are a group of these materials, in two biotic and/or abiotic forms, which when added to culture medium at different stages of culture can increase the volume of the medicinal plant's defensive secondary compounds (Tonk et al. 2016). In suspension culture of pennyroyal (*Mentha pulegium*) medicinal plant, with yeast extract and salicylic acid used in the medium, the amounts of limonene, menthone, menthol, and α-pinene increased significantly (Darvishi et al. 2016). Liu et al. (2018) compared the effect of salicylic acid and methyl jasmonate elicitors in cell suspension culture of *Gardenia jasminoides* and reported malonyl-4,5-o-dicaffeoylquinic acid for the first time as one of the chlorogenic acid derivatives, according to the results of HPLC–TOF-MS/MS. In a developed in vitro regeneration protocol in *Anemia tomentosa*, application of jasmonic acid led to emergence of 50 different substances in in vitro regenerated plant's extracts, whereas in wild-type plants only 20 substances were identified, according to high-resolution gas chromatography analysis (Castilho et al. 2018).

Bioreactors

The overproduction of plant-derived natural products is not possible because the plant tissues that make these products are usually slow-growing parts. Farm cultivation is not a perfect alternative to supply the demand of high amount biomass of medicinal plants. These are not domesticated plants and have low germination rates and unidentified ecological requirements (Canter et al. 2005). The managing and manipulation of environmental conditions, faster production, and easier and more economical recovery of product from culture medium are the main advantages of in vitro systems in producing secondary metabolites of medicinal plants (Máthé et al. 2015). Suspension cells, hairy roots, and micropropagated plantlets are the main group of in vitro-derived plant materials applicable for production of useful products (secondary metabolites and transgenic proteins) (Weathers et al. 2010). However, the production of secondary metabolites at the commercial level is very difficult. Bioreactor is an eco-sustainable alternative to produce valuable secondary metabolites of medicinal plants in large scale (Werner et al. 2018). Bioreactors are continuous self-contained liquid culture systems that act like biological factories and provide an automated isolation of secondary metabolites in high quality and level (Máthé et al. 2015). Different kinds of bioreactors have been used for PTC, including stirred tanks, airlift, bubble column, orbital shaker, gas-phase bioreactors, and mist or spray reactors. The selection of these bioreactors depends on the starting materials, including suspension cells, hairy roots, or micropropagated plantlets. In addition, there are different kinds of disposable reactors that are cheaper than conventional stainless steel tanks (Weathers et al. 2010). Sitarek et al. (2018a) compared 300 mL, 1 L, 3 L, and 5 L flasks with a 5 L bioreactor in transgenic root cultures of *Leonurus sibiricus* and reported the superiority of the 5 L bioreactor in the production of phenolic acids (chlorogenic acid, caffeic acid) to flask media. The superiority of bioreactor in vitro system in comparison to flask culture has also been reported for production of phenolic acids (cinnamic, salicylic, coumaric, and caffeic acid) and stilbenoid (resveratrol) components in *Scrophularia striata* (Ahmadi-Sakha et al. 2018).

Elicitation and co-culture are the two strategies for the overproduction of secondary metabolites in different bioreactors. Co-culture refers to simultaneous cultivation of two different species, or two different organs of two different or similar species, for example, shoots + roots of a single or different species, and simultaneous cultivation of plant organs with microbes and/or insects. Usually simultaneous cultivation of two hairy roots from two different species and cultivation of hairy root with cell suspension of other specie can lead to higher amount of secondary metabolite production (Mohagheghzadeh et al. 2008). Jesionek et al. (2018) assessed the effects of different biotic and abiotic elicitation strategies including copper and nickel salts, methyl jasmonate, chitosan, ergosterol, and the aphid ethanol extract in bioreactor-grown microshoots of *Rhododendron tomentosum* and reported that the biosynthesis of monoterpenes was affected by the elicitation. The positive effect of co-cultivation of adventitious roots of *Panax ginseng* and *Echinacea purpurea* in a bioreactor has been reported for higher content production of ginsenosides and caffeic acid derivatives (Wu et al. 2008). The comprehensive list of co-culture studies for overproduction of different secondary metabolites in a bioreactor system is presented by Weathers et al. (2010).

Assessment of the antimicrobial and antioxidant activities of medicinal plants

A well-developed in vitro regeneration protocol in medicinal plants is applicable for analysis of their bioactive compounds (Kumar et al. 2017). PTC is a platform to investigate the antimicrobial activities of medicinal plants in a controlled environment. It has many advantages including screening and selection of elite ecotype/genotype of medicinal plants in a shorter time. In addition, in vitro assays can assess the different properties of in vitro regenerated medicinal plants in different developmental stages. Rawat et al. (2018) used 1,1-diphenyl-2 picrylhydrazyl (DPPH) radical-scavenging and ferric-reducing antioxidant power (FRAP) assays

to assess the antioxidant activity of *Angelica glauca* and reported that antioxidant activity of in vitro regenerated plants was higher than control plants. Baskaran et al. (2018a) investigated the effect of BA and NAA PGRs along with organic elicitor of hemoglobin (HB) on antibacterial activity of *Eucomis autumnalis* and reported that the best antibacterial activity against pathogenic bacteria of *Bacillus subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, was achieved when BA and/or HB was used alone in in vitro regeneration medium.

Indirect application of tissue culture in medicinal plants

Metabolic pathway engineering and hairy roots

The *in planta* overproduction of some pharma molecules using only PTC is limited, because these molecules have complicated developmental regulation and also there are different cell, tissues, and organelles that are involved in their synthesis (Sharma et al. 2018a). Metabolic engineering of valuable plant natural products is a promising method in this situation.

Metabolite engineering includes the overexpression and/or competition with the target pathway, overbearing rate-limiting steps, stopping the catabolism pathway of the desired product, and obstruction of other pathways (Matveeva and Sokornova 2018). The overexpression of the involved genes in biosynthesis pathways of secondary metabolites, using *Agrobacterium*-mediated (both *A. tumefaciens* and *A. rhizogenes*) and/or biolistic transformation methods, is the first strategy for plant-based metabolite engineering of medicinal plants.

Agrobacterium tumefaciens is a powerful tool for metabolic engineering of medicinal plants through overexpress and/or downregulation of specific genes and utilization of transcriptional regulators (Khan and ur Rahman 2017). The transformation of *Bacopa monnieri* with tryptophan decarboxylase and strictosidine synthase genes, which originated from *Catharanthus roseus* and involved in terpenoid indole alkaloid pathway, using LBA1119 strain of *Agrobacterium tumefaciens* led to a 25-fold increase in the content of tryptophan in extract of transgenic tissues in comparison with non-transformants (Sharma et al. 2017). Sharma et al. (2018a) used LBA1119 strain of *Agrobacterium tumefaciens* to transfer *tryptophan decarboxylase* and *strictosidine synthase* genes to *Catharanthus roseus*, and reported that the HPLC system confirmed the increased content of terpenoid indole alkaloid metabolite because of transient overexpression of *CrTDC* and *CrST R* genes. *Agrobacterium* is a perfect tool for gene transformation; however, there are some technical problems with this method such as genotype dependency, recalcitrant response, and problems associated with tissue culture. In the case of tissue culture parameters, there are some *in planta Agrobacterium*-mediated transformation techniques that are independent of tissue culture procedure (Niazian et al. 2017b). *Agrobacterium*-mediated transformation is also a multi-variable procedure in which many factors can affect its efficiency (Fig. 5). Factors such as *Agrobacterium*'s optical density (Pandey et al. 2013), *Agrobacterium* killing of antibiotic (Sivanandhan et al. 2016), concentration of acetosyringone (Fernando et al. 2016), and inoculation

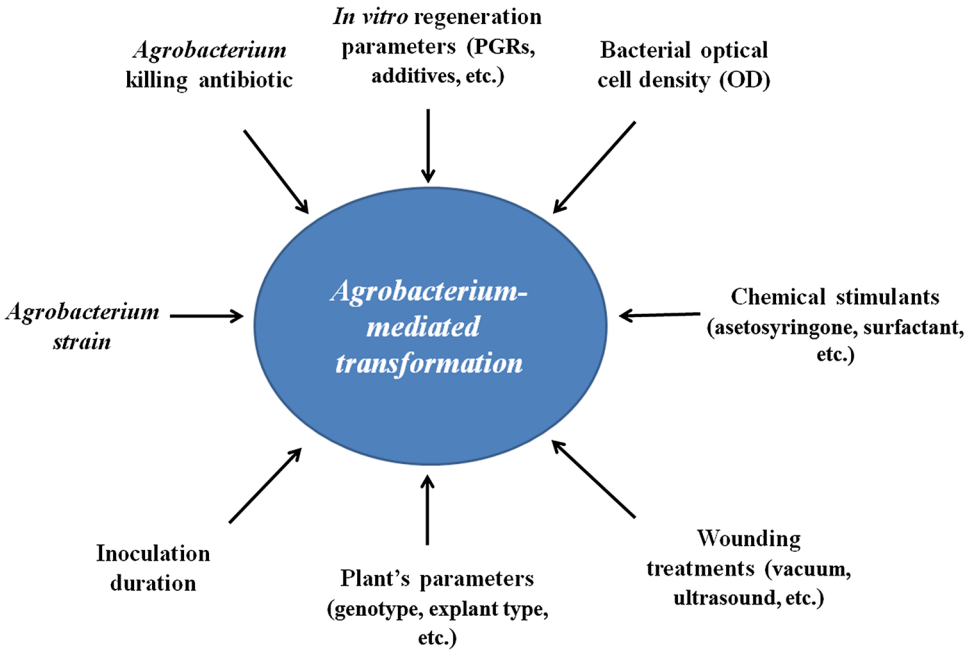

Fig. 5 Factors affecting *Agrobacterium*-mediated gene transformation procedure for metabolite engineering and induction of hairy roots in medicinal plants

duration (immersion time) (Rajesh et al. 2013) can affect the efficiency of *Agrobacterium*-mediated transformation. Some recent *Agrobacterium*-mediated gene transformation studies in different medicinal plants, with optimized gene transformation protocol using reporter and/or selectable marker genes are presented in Table 1.

Plastid (chloroplast) genomes provide some advantages over nucleus transformation for metabolic engineering, including: (i) the capability of multigene transformation in the form of operons; (ii) the higher expression level of transgenes, (iii) maternal inheritance that can enhance transgene containment, and (iv) supertransformation via combination with nucleus transformation in the form COSTREL (Fuentes et al. 2016, 2018).

Transfer of the whole biosynthetic pathway of useful secondary metabolites from original medicinal plants to a second crop with high biomass product, such as tobacco, is a perfect genetic engineering strategy to produce large-scale secondary metabolites. In this case, nucleus transformation of a previously plastid-transformed line can significantly increase the production of the target metabolite (COSTREL method). In case of artemisinin, in the first step, multiple genes involved in the core biosynthetic pathway of artemisinin (responsible genes for PS, ADS, CYP and CPR enzymes) were transferred to the chloroplast of the tobacco plant with gene gun transformation. In the second step, a set of "accessory" genes that can enhance the production of the artemisinin were transformed to transplastomic lines that had higher content of artemisinin. The applied combinatorial supertransformation method led to a significant increase in the production of artemisinin (> 120 mg/kg fresh weight) in tobacco (Fuentes et al. 2016).

Agrobacterium rhizogenes plays an important role in enhancing the secondary metabolite production of medicinal plants through induction of hairy roots, which are subjected to metabolite engineering. The hairy roots are reliable sources for large-scale production of secondary metabolites in various medicinal plants. The hairy root cultures are well known for their genetic and metabolic/biosynthetic stability in long-term in vitro cultures in contrast to cell suspension and callus cultures (Grzegorczyk-Karolak et al. 2018b). The hairy root culture of tobacco (*Nicotiana tabacum*) is one the important platforms for not only direct production of alkaloid nicotine (Zhao et al. 2013), but also for changing the secondary metabolite profiles through genetic manipulation. The comprehensive list of secondary metaboloites engineered through hairy root culture of *N.tabacum* is provided by Kumar and Mitra (2017). Like *A. tumefaciens*-mediated transformation, different parameters should be optimized for successful hairy root induction through *A. rhizogenes*. Ruiz-Ramírez et al. (2018) used cotyledon, hypocotyl, and root segments as explants for *A. rhizogenes*-mediated hairy root induction in *Bonellia macrocarpa* and reported that the highest transformation percent (24.99%) was obtained from hypocotyls and the hypersensitive response of different explants can lead to their different transformation efficiencies. The overexpression of AtPAP1 transcription factor in *A. rhizogenes*-induced hairy roots of *Leonurus sibiricus*

Table 1 Examples of *Agrobacterium*-mediated gene transformation studies in different medicinal plants

Plant species	GOI/SMG/RG	Additive/treatment	Efficiency (%)[a]	References
African basil (*Ocimum gratissimum)*	*–/nptII/gusA*	Acetosyringone	–	Khan et al. (2015)
Blue mallee (*Eucalyptus polybractea*)	*–/hptII/gus*	–	–	Fernando et al. (2016)
Cumin (*Cuminum cyminum* L.)	*–/hptII/gus*	–	1.5	Pandey et al. (2013)
Dendrobium catenatum	*–/nptII/gus&gfp*	Acetosyringone/SAAT/Tween 20, Silwet-77, and TritonX-100	56.5	Chen et al. (2018)
Foxglove (*Digitalis purpurea*)	*–/nptII/gusA*	Acetosyringone	–	Li et al. (2014)
Gentiana tibetica	*–/nptII/uidA*	L-glutamine	22.5	Wójcik and Rybczyński (2017)
Greenviolet (*Hybanthus enneaspermus* L.)	*–/nptII/gusA*	Acetosyringone	28	Sivanandhan et al. (2016)
Indian ginseng (*Withania somnifera* L.)	–/nptII/gusA	Acetosyringone/ vacuum infiltration/SAAT	–	Sivanandhan et al. (2016)
Indian ginseng (*Withania somnifera* L.)	*–/hptII/gus*	Acetosyringone	10.6	Mishra et al. (2016)
Macleaya cordata	*–/nptII/uidA*	Acetosyringone	15.7	Huang et al. (2017)
Orchid (*Dendrobium lasianthera*)	*–/nptII/–*	Acetosyringone	70	Utami et al. (2018)
Pelargonium graveolens	*–/nptII/gusA*	Acetosyringone	69.5	Singh et al. (2017)
Scutellaria ocmulgee	*–/nptII/gusA*	–	–	Vaidya et al. (2016)

GOI genes of interest, *SMG* selection marker genes, *SAAT* sonication-assisted *Agrobacterium*-mediated transformation, *RG* reporter genes

[a]Reported transformation efficiency by author(s) based on the percentage of PCR-positive plants

–: Data not reported by author(s)

led to higher levels of five phenolic acids including chlorogenic acid, neochlorogenic acid, ferulic acid, cafeic acid, and *p*-coumaric acid in the transgenic roots (Sitarek et al. 2018b). A list of medicinal plants in which the secondary metabolites and bioactive compounds have been increased using both *A. tumefaciens* and *A. rhizogenes* is presented in Table S3.

Ploidy engineering

The duplication of the genetic content of different organisms is a process that has amazing impact on their features. Superior vigor and performance are the general and specific characteristics of polyploid plants in contrast to their diploid relatives (Noori et al. 2017). It is well documented that the content of secondary metabolites in polyploid genotypes of a medicinal plant is more than that in diploids (Pradhan et al. 2018); therefore, artificial polyploidy induction can play an important role in breeding and improvement of different medicinal plants, because it not only can increase their general performance (quantitatively), but also it can change their chemical composition (qualitatively) (Salma et al. 2017). The polyploidization of hairy root cultures can create an amazing strategy for scaled-up production of secondary metabolites in different medicinal plants (Banerjee 2018). In Brazilian ginseng (*Pfaffia glomerata*), induced polyploidy led to 31% increase in the content of 20-hydroxyecdysone in comparison to diploid plants (Corrêa et al. 2016). Parameters such as type of applied antimitotic agent, the minimum effective concentration of applied antimitotics, and the optimized exposure duration are the factors that are related to antimitotic agent and are effective in the induction phase. Colchicine, trifluralin, and oryzalin are the most frequently used antimitotic agents in artificial polyploidy studies (Salma et al. 2017); however, colchicine is the most applied one in medicinal plants (Table 2). In *Bletilla striata*, different concentrations of colchicine solution including 0.05, 0.1, and 0.2% (w/v) were applied in different exposure durations of 12, 24, 36, 48, and 60 h for tetraploid induction, and application of 0.2% colchicine for 36 h was identified as the best treatment with the highest percentage of autotetraploid induction (26.7%) (Pan-pan et al. 2018).

Another aspect of biotechnology to engineering the ploidy level of medicinal plants is haploid induction. Haploidy refers to a sporophytic plant with gametophytic chromosome number (Germanà 2011). Biotechnology can implement the conventional methods of tissue culture-based including androgenesis (Kasha 2005), gynogenesis (Piosik et al. 2016), and wide hybridization-chromosome elimination (Forster et al. 2007), to induce haploidy in different plants. Androgenesis pathway, including anther and isolated microspore culture, is the most used method of haploid induction in different medicinal plants (Sharma et al. 2018b). Isolated microspore culture of medicinal plants of *Asteraceae* was first started in 2006, but was associated with various challenges because of the small capitula of the targeted plants (Bal and Touraev 2009). In anther culture of borage (*Borago officinalis* L.), different parameters including culture media, chemical (colchicine and *n*-butanol), and physical stresses (centrifugation and electroporation) were tested and the highest number of doubled haploid plants (65%) were obtained by pretreatment of anthers with 0.2% *n*-butanol for 5 h and then establishing anthers in B5 salt medium containing NLN vitamins supplemented with 200 mg/l colchicine for 4 days (Hoveida et al. 2017). In gynogenesis of black cumin (*Nigella sativa*), different concentrations of BA, Kin, NAA, and 2,4-D PGRs were tested for ovule culture in MS medium and the highest number of haploid plants (41%) were obtained in MS medium supplemented with 2 mg/L 2,4-D (El-Mahrouk et al. 2018). Another method of haploid induction, called CENH3, is independent of tissue culture and can be considered as a universal method in any desired plant species. In this method, the knockout and/or knockdown of native *CENH3* gene, which is responsible for the connection of centromere regions of chromosomes to spindle microtubules, can produce haploid inducer line. The comprehensive explanation of haploidy induction through conventional tissue culture-based and new method of CENH3 is outside the scope of the present review; nonetheless, there are useful reviews that have discussed these issues in great detail (Ravi and Chan 2010; Britt and Kuppu 2016).

Toward genome editing methods and plant synthetic biology

Conventional genetic engineering methods are not suitable for adding large concerted changes into a plant, because the random insertion of DNA construct into one or more loci in one or more chromosomes can lead to undesirable effects. Therefore, these methods are not efficient to change the entire metabolic pathway in plants (Naqvi et al. 2010). Copy number variability is another problem of conventional gene transformation methods that can limit their efficiency in medicinal plants, because metabolic loads are incidental when several genes enter the plant's genome simultaneously. There are advanced genetic technologies, including synthetic promoters, 'tunable' transcription factors, genome editing tools and site-specific recombinases that can help plant biotechnology with more accurate and faster improvement of plants (Liu et al. 2013). Unmanageable transcriptional regulation of endogenous genes is one of the critical problems that has to be solved to mange the secondary metabolite's pathways in medicinal plants; therefore, establishing a targeted regulation of gene expression can create a desirable plant biological system (Xu et al. 2014).

Table 2 The examples of currently published artificial polyploidy induction in different medicinal plants

Plant species	Applied antimitotic agent(s)	Survival rate of induced polyploids	Polyploidy induction efficiency (%)[a]	Method of confirmation	Targeted secondary metabolite/enhanced percentage	References
Ajowan (*Trachyspermum ammi* L.)	Colchicine	59.9	11.53	Chromosome counting–flow cytometry	Thymol/19.53	Noori et al. (2017)
Anise hyssop (*Agastache foeniculum* L.)	Colchicine, oryzalin, trifluralin	56	20	Chromosome counting–flow cytometry	–	Talebi et al. (2017)
Artemisia annua	Colchicine	–	20	Chromosome counting–flow cytometry	Artemisinin/42	Xia et al. (2018)
Bhringraja (*Eclipta alba* L.)	Colchicine	96.67	30.56	Chromosome counting–flow cytometry	Wedelolactone/56.27	Salma et al. (2018)
Bletilla striata	Colchicine	–	26.7	Chromosome counting–flow cytometry	–	Pan-pan et al. (2018)
Brazilian ginseng (*Pfaffia glomerata*)	–	–	–	–	20-hydroxyecdysone/31	Corrêa et al. (2016)
Buckwheat (*Fagopyrum tataricum* L.)	Colchicine	94	54.55	Chromosome counting	–	Wang et al. (2017)
Cannabis sativa	Colchicine	87.78	16.45	Flow cytometry	Tetrahydrocannabionol, -	Mansouri and Bagheri (2017)
Ginger bush (*Tetradenia riparia*)	Colchicine	–	25.6	–	Fenchone/22.98	Hannweg et al. (2016)
Incayuyo (*Lippia integrifolia*)	Colchicine	–	–	Chromosome counting–flow cytometry	Monoterpenes/22.2	Iannicelli et al. 2016
Linum album	Colchicine	80	22	Flow cytometry	Podophyllotoxin/1.66	Javadian et al. (2017)
Patchouli (*Pogostemon cablin-Benth.*)	Colchicine	7.5	28.5	Chromosome counting	–	Widoretno (2016)

[a]Polyploid induction percentage according to ratio of polyploid obtained to the total hardened obtained

–: Data not reported by author(s)

Zinc-finger nucleases (ZFNs), TALENs, and CRIPR/Cas are the three types of engineered sequence-specific nucleases that are able to create site-specific double-strand breaks (DSBs) in target points of DNA sequence of interest. These are fusion proteins that consist of two parts, including a programmable and sequence-specific DNA-binding domain and a nonspecific DNA-cleavage domain (Gaj et al. 2013). In both ZFNs and TALENs, the DNA-cleavage domain is derived from the *FokI* restriction enzyme. In ZFNs, a set of Cys2His2 zinc fingers proteins is responsible for binding to DNA, but in TALENs the TALE proteins of *Xanthamonas* sp. cause binding to DNA. The CRISPR/Cas system consists of a single guide RNA (sgRNA) and a Cas9 endonuclease. The sgRNA, which consists of 20 nucleotides, is responsible for guiding Cas9 cleavage and acting as DNA-binding domain. The DNA-binding domain of all three ZFNs, TALENs, and CRISPR/Cas is customizable and can be programmed to recognize and connect to any sequence of interest (Gaj et al. 2013). The co-transformation of plant tissues with DNA constructs encoding sequence-specific nucleases and desired foreigner DNA leads to precise site-specific integration of foreigner DNA.

The sgRNA of CRISPR/Cas is customizable because of its 20-nucleotide structure; therefore, its complementary sequence can target any gene of interest in a selected genome and, subsequently, different functions including gene mutation, deletion and insertion, and transcriptional activation/repression are applicable through this system (Xu et al. 2014). The type II CRISPR system (CRISPR/Cas9), as one of the newest techniques of transformation, is a promising

BBBMs that can help metabolic engineering of medicinal plants by introducing multiple genes within the same region of the chromosome (Wilson and Roberts 2014).

There are increasing reports of application of CRISPR/Cas9 system in medicinal plants in recent years. In one of them, the CRISPR/Cas9 has been applied to suppression of rosmarinic acid synthase gene (*SmRAS*) in *Salvia miltiorrhiza* (Zhou et al. 2018). The researchers designed the sgRNA that target *RAS* gene, based on the bioinformatics analysis of 11 family members. The authors reported that *Arabidopsis* U6 promoter was more efficient than the rice U3 promoter for driven sgRNA. The HPLC–MS/MS analysis showed that in the hairy root extract of CRISPR/Cas9-obtained mutants, the contents of RA and LAB compounds were decreased, whereas the volume of salvianic acid A sodium (SAAS) and sodium salt form of DHPL were increased. In another report, the CRISPR/Cas9 system has been applied in *Dioscorea zingiberensis* medicinal plant (Feng et al. 2018). The authors reported that targeted mutagenesis in *Dzfps* gene led to reduction in the activity of farnesyl pyrophosphate synthase (FPS) enzyme and, subsequently, the content of squalene was 1.6 times less than wild-type plants. The CRISPR/Cas9 system has been applied on hairy roots of *Salvia miltiorrhiza* using *Agrobacterium rhizogenes* to knock out the *SmCPS1* gene, which is a key gene in the biosynthesis of tanshinone (Li et al. 2017). LC–qTOF-MS and Q-TRAP-LC–MS/MS analyses showed that cryptotanshinone, tanshinone IIA and tanshinone I diterpenoids have been missed in homozygous mutants, whereas phenolic acid metabolites were not influenced. The results of these recent studies show the significance of CRISPR/Cas9 system in medicinal plants, which can simply and purposefully change the chemical profile of useful medicinal plants. The dream of minimal plant cell, a plant cell without any non-essential components, is achievable through CRISPR/Cas9 (Noman et al. 2016).

Although biolistic transformation can provide stable delivery of Cas9 and sgRNA expression constructs into plant genome, *Agrobacterium*-mediated gene transformation is the most efficient transformation method in laboratory condition (Ma et al. 2016). *Agrobacterium*-mediated transformation can be done in various forms (including sonication-assisted *Agrobacterium*-mediated transformation, vacuum infiltration, pollen tube pathway, floral dip, etc.), which scientists should be aware of before conducting an experiment (Niazian et al. 2017b).

Perspectives

The world's population is increasing, especially in developing countries; therefore effort to feed and maintain good health of this growing population is very important. On the other hand, long-term consumption of synthetic medicines has caused many health problems in both advanced and developing countries. In this situation, the valuable secondary metabolites of medicinal plants are promising materials to provide continuous and high quality health, in addition to their commercial value. However, these are endangered plants without any stable production in their growing areas (Niazian et al. 2017c) with extensive wildcrafting and deprived by domestication syndrome (Cordell 2011). Cultivation and the conventional methods of plant breeding are good, but not enough to improve these neglected wildcrafted plants. Biotechnology-based breeding methods can help high-throughput improvement of medicinal plants in faster ways. The powerful method of NGS-based DNA barcoding that only target short regions of genomic DNA and do not need full genome-scale data can help breeders in faster identification and classification of wild populations of medicinal plants (Techen et al. 2014). Plant tissue culture is the core of BBBMs and helps medicinal plants in conservation and micropropagation pathways. It also contributes to the improvement of the chemical profile of medicinal plants in direct and indirect ways. In vitro polyploidy induction and bioreactor are the two other tissue culture-derived techniques that have greatly contributed in the improvement of medicinal plants. *Agrobacterium*-mediated gene transformation (*A. tumefaciens* and *A. rhizogenes*) is another tissue culture-based technique that helps in the comprehensive improvement of medicinal plants through overexpression of key involved genes in the biosynthetic pathway of secondary metabolites and downregulation of genes that produce adverse compounds. Hairy root indiction and transfer of genome editing constructs (CRISPR/Cas9, TALLENs, ZFNs) are other ways that *Agrobacterium* can improve medicinal plants. The modified gene transformation method of COSTREL has great potential for significant enhancement in the production of useful secondary metabolites of medicinal plants (Fuentes et al. 2016). GM-free targeted mutation induction through TILLING is a promising way to enhance the production of valuable secondary metabolites (Nogueira et al. 2018) or produce new useful products from different medicinal plants. Genome editing method of CRISPR/Cas9 emerged as a powerful and promising method for targeted mutation induction in the genome of medicinal plants and subsequent purposeful alteration of their biochemical profile (Feng et al. 2018; Li et al. 2017; Zhou et al. 2018).

An artificial polyploidy induction, subsequent to CRISPR/Cas9-armed *Agrobacterium rhizogenes*-mediated hairy root culture, is a good strategy to create a synthetic biology system and enhance the secondary metabolite production of various medicinal plants (Fig. 6).

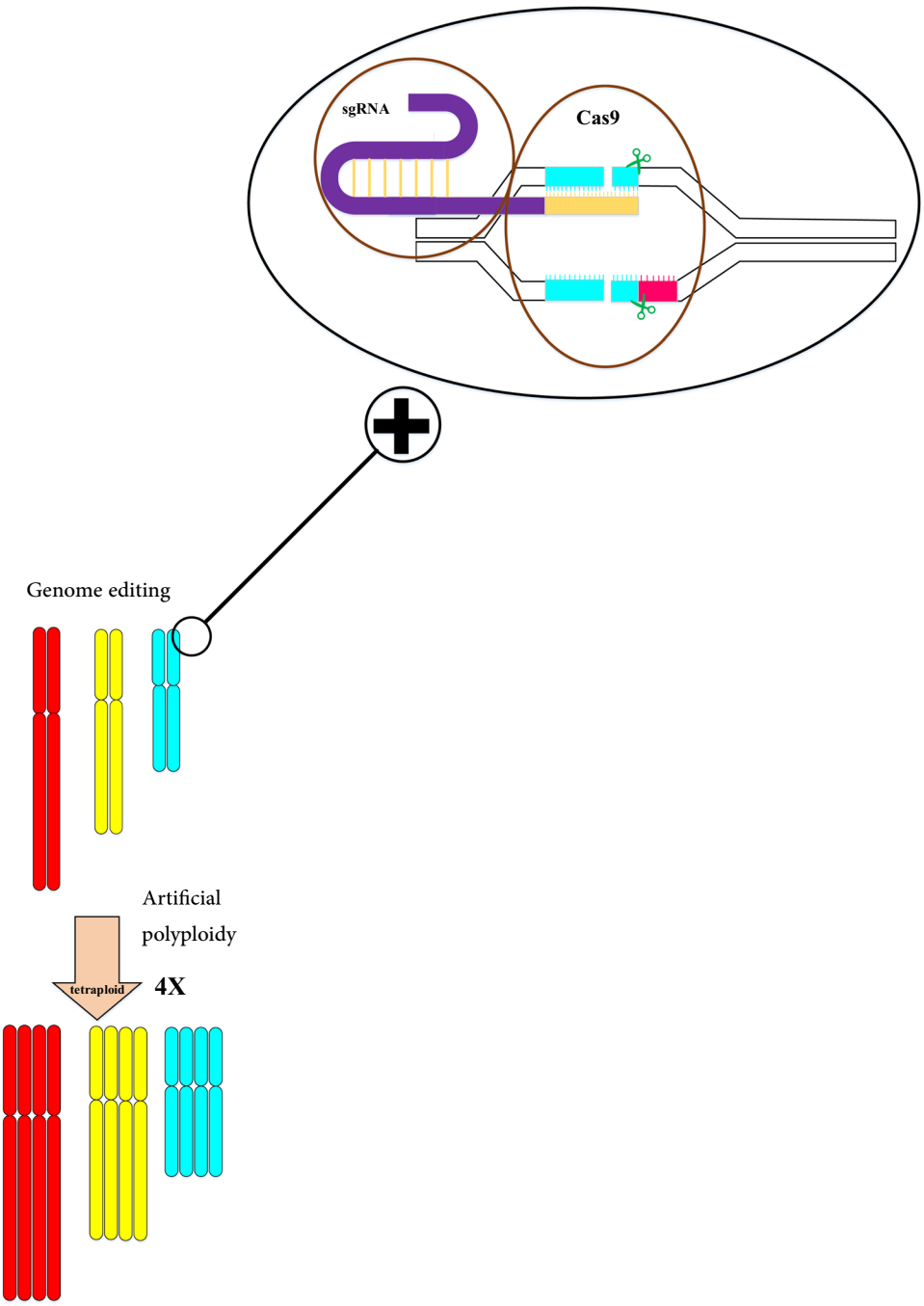

Fig. 6 A proposed strategy to targeted enhancement in production of secondary metabolites in different medicinal plants by combination of artificial polyploidy and *Agrobacterium rhizogenes*-mediated CRISPR/Cas9

Author contribution statement MN conceived the idea and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The author declares that he has no conflicts of interest to disclose.

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