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REVIEW PAPER

Improving transformation and regeneration efficiency in medicinal plants: insights from other recalcitrant species

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Abstract

Medicinal plants are integral to traditional medicine systems worldwide, being pivotal for human health. Harvesting plant material from natural environments, however, has led to species scarcity, prompting action to develop cultivation solutions that also aid conservation efforts. Biotechnological tools, specifically plant tissue culture and genetic transformation, offer solutions for sustainable, large-scale production and enhanced yield of valuable biomolecules. While these techniques are instrumental to the development of the medicinal plant industry, the challenge of inherent regeneration recalcitrance in some species to *in vitro* cultivation hampers these efforts. This review examines the strategies for overcoming recalcitrance in medicinal plants using a holistic approach, emphasizing the meticulous choice of explants (e.g. embryonic/meristematic tissues), plant growth regulators (e.g. synthetic cytokinins), and use of novel regeneration-enabling methods to deliver morphogenic genes (e.g. *GRF*/*GIF* chimeras and nanoparticles), which have been shown to contribute to overcoming recalcitrance barriers in agriculture crops. Furthermore, it highlights the benefit of cost-effective genomic technologies that enable precise genome editing and the value of integrating data-driven models to address genotype-specific challenges in medicinal plant research. These advances mark a progressive step towards a future where medicinal plant cultivation is not only more efficient and predictable but also inherently sustainable, ensuring the continued availability and exploitation of these important plants for current and future generations.

Keywords: Explants, medicinal plants, morphogenic genes, nanoparticles, plant growth regulators, recalcitrance, regeneration, transformation.

Introduction

Among the breadth and diversity of plant species, medicinal plants have held a significant place in human health and culture since ancient times. The World Health Organization estimates that two-thirds of the global population relies on plant medicines for primary healthcare. A quarter of newly developed drugs sold worldwide are based on molecules derived from

Abbreviations: BBM, BABY BOOM; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; GIF, GRF-INTERACTING FACTOR 1; GRF, GROWTH-REGULATING FACTOR; gRNA, guide RNA; MG, morphogenic gene; NP, nanoparticle; PGR, plant growth regulator; SAM, shoot apical meristem; SE, somatic embryogenesis; TC, tissue culture; TDZ, thidiazuron; WUS, WUSCHEL.

plants (Calixto, 2019). With >35 000 identified medicinal plant species, the repertoire of biomolecules of benefit to humankind is yet to be mined to its full potential. The global herbal medicine market compound annual growth rate (CAGR) is estimated to be ~11% over the 2022–2030 period and is expected to be worth ~US$348 billion by 2030 (www.databridgemarketresearch.com). By 2050, the market value is projected to reach US$5 trillion, with China and India to dominate the herb trading market (Booker *et al.*, 2012). Such demand in countries where self-medication through sourcing from the natural environment is current practice has put preservation of native medicinal plant species under significant pressure. As the universal interest in plant-based medicines continues to expand, there is a growing need to generate sufficient supply of medicinal plants and preserve their native populations.

Increasingly, biotechnological approaches are being utilized to satisfy this growing demand. Since the 1990s, plant tissue culture and genetic transformation have been the enabling technologies for crop improvement, and promise to fulfil the same role in medicinal species (Canter *et al.*, 2005). *In vitro* cultivation enables large-scale multiplication of plant tissue and thus yield of desirable biomolecules. Growth in a controlled environment, encompassing both the medium and external conditions, delivers a more consistent product per cropping cycle, improving market value, and provides a platform for germplasm preservation. Tissue culture (TC) medium normally contains plant growth regulators (PGRs) that, when dosed, can induce plants to generate high numbers of multiplication units. Under controlled conditions, micropropagation enables growth at scale. Unfortunately, not all species can acclimate to *in vitro* culture conditions, and this inability to grow and be propagated in tissue culture is called TC recalcitrance (Benson, 2000).

In most species, TC methods are also essential to the success of genetic transformation, the process whereby a beneficial segment of DNA is transferred into the plant's genome (Yildiz, 2012). Genetic transformation offers an ability to amplify biomolecule yields to much higher levels that are well beyond what can be achieved through traditional breeding methods (Pandey *et al.*, 2010). After DNA integration, transformed cells require cues to initiate morphological reprogramming to produce an entire plantlet. Typically, PGRs in the medium stimulate this process. Cellular receptivity to transformation and regeneration is often species or even genotype specific. The inability of a plant to incorporate foreign DNA into its genome is termed transformation recalcitrance, and the failure to form tissue, typically shoots or embryos, post-transformation is defined as regeneration recalcitrance (Fig. 1). Discovery and characterization of the genes and pathways involved in the process of plant morphogenesis, their interplay with phytohormones, and an understanding of the events following a wound response have paved the way towards providing a set of molecular tools to circumvent plant recalcitrance. Multiple recalcitrant crop species have benefited from the controlled expression of morphogenic genes (MGs) to stimulate the regeneration of transgenic plants. Although still in its infancy, the use of MGs to combat regeneration recalcitrance in medicinal species has shown the potential to be broadly applicable (Zhang *et al.*, 2021). In this review, we describe the benefits to TC and transformation and the three main approaches that have been used to overcome regeneration recalcitrance in medicinal plants to date: (i) selection of explants with innate ability to regenerate; (ii) addition of PGRs in TC media; and (iii) the use of enabling technologies involving MGs.

Plant tissue culture: an important biotechnological tool

Plant tissues can be preserved aseptically *in vitro* through TC practices. Although TC requires specialized facilities, equipment, and trained labour, its major advantages over traditional propagation methods include unparalleled scalability free of seasonal constraints within a smaller physical and environmental (e.g. water, fertilizer) footprint, pest-free plants upon release to the glasshouse or field, and is economical in terms of daily maintenance costs. Another benefit is that elite germplasm from heterogenous and outcrossing species can be maintained without the need to pass through fertilization and a seed stage (Kenta *et al.*, 2016). The same advantage is applicable to annuals and tree species alike. For the latter, TC provides maintenance conditions without the development of secondary growth, often considered detrimental to *in vitro* life. In some species and genotypes, the constant exposure to TC conditions can lead to somaclonal variations, genetic and/or epigenetic, that are perpetuated in the culture and can lead to loss of valuable traits in TC-maintained lines (Kenta *et al.*, 2016). However, in some species such as strawberry, somaclonal variation is a deliberate strategy to gain novel traits that can provide tools for crop improvement (Krishna *et al.*, 2016).

In contrast to well-established TC protocols for agricultural crops, medicinal plants often lack standardized procedures, as is the case for *Frangula purshiana*, *Arctostaphylos uva-ursi*, *Physostigma venenosum*, *Strychnos nux-vomica*, and *Ochrosia elliptica*, all dicotyledonous species (Chaturvedi *et al.*, 2007). The wide range of species diversity, genetic variability, complex secondary metabolite mixtures which influence growth and development, the limited research and resources, and in some cases regulatory challenges often hinder the TC progress in medicinal plants. The stages of *in vitro* plant culture are summarized in Fig. 1. Establishing an *in vitro* culture requires that plant tissue(s) are sterilized and placed in media within enclosed vessels. Ideally, TC explants free of embryonic/meristematic cells can produce entire plants under the right media and growth conditions. This remarkable developmental plasticity, which naturally facilitates a species' survival and reproduction success under various natural biotic and abiotic pressures, has enabled the fundamental elements of TC to be developed. This potential of a cell to

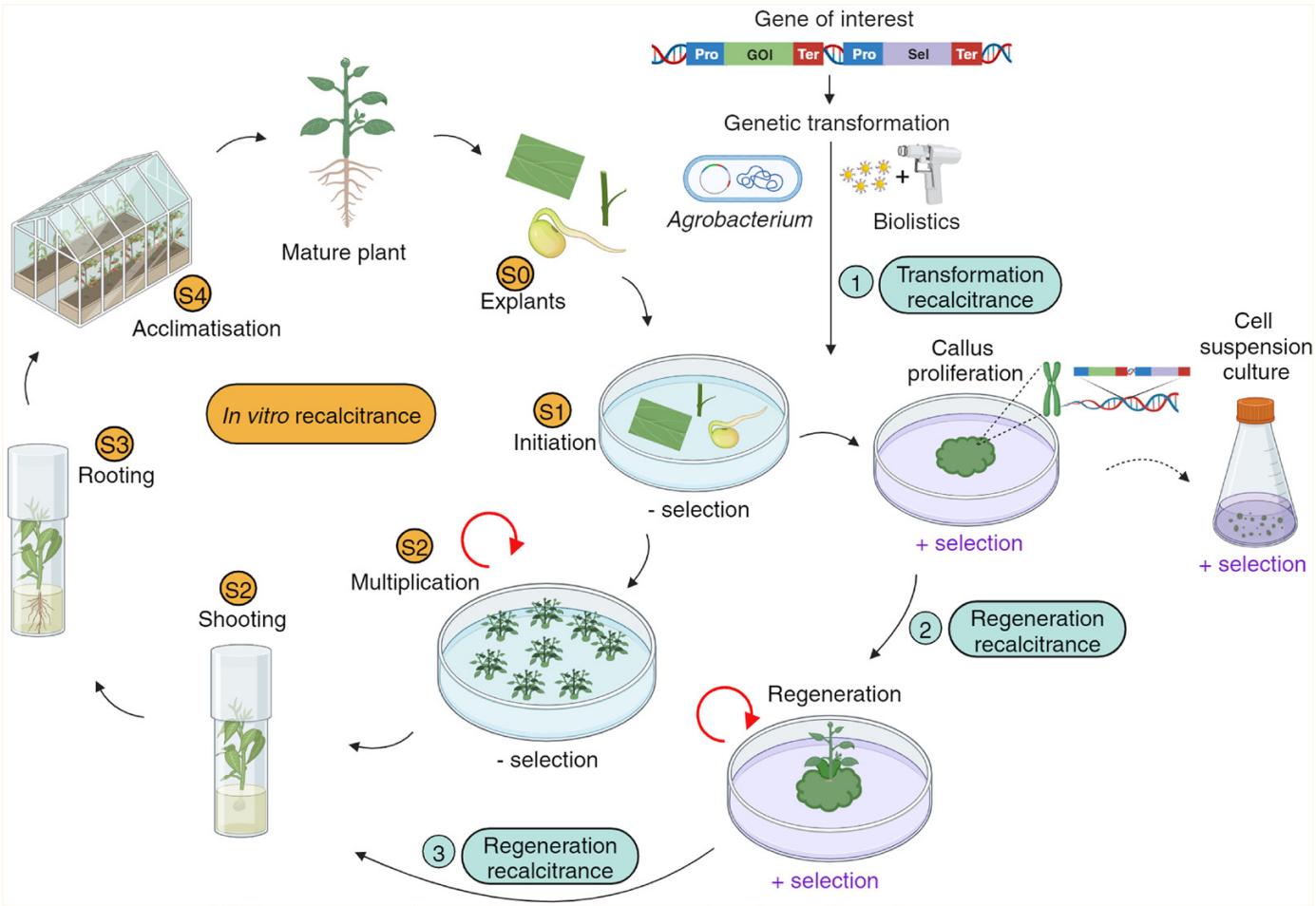

Fig. 1. A schematic of the plant tissue culture and transformation process. Left: a typical plant tissue culture/micropropagation cycle is represented with its different stages: collection of explants from a mature donor plant (Stage 0, S0), sterilization and initiation of explants on shoot proliferation medium (Stage 1, S1), repeated shoot multiplication and elongation (Stage 2-S2), rooting (Stage 3, S3), and acclimatization to *ex vitro* growing conditions (Stage 4, S4). The circular arrow at the multiplication stage represents the iteration of *in vitro* multiplication cycles for large-scale production of TC plantlets. *In vitro* recalcitrance can occur at any stage (S1–S4). Right: genetic transformation of a plant species involves transferring a piece of DNA, such as a gene(s) of interest (GOI), with a selectable marker (Sel), into cells within the explants either through co-cultivation with *Agrobacterium* cells carrying the transformation vector or directly through biolistics. The transformed explants are proliferated on a callus initiation medium with selection pressure (+selection) to select only those cells which have the GOI integrated into their genome. The proliferated calli are then transferred to regeneration medium for embryo development and shoot regeneration with continued selection pressure. The regenerated plantlets enter the usual micropropagation stages (S2–S4). The circular arrow at the regeneration stage represents the continued multiplication of transformed plants. Alternatively, transformed calli can be used to initiate sterile cell suspension cultures, bypassing the need to generate a transgenic plant. *In vitro* recalcitrance can be encountered during genetic transformation (1) or through the process of transgenic plant regeneration at callus proliferation, embryo development, and/or plantlet growth stages (2, 3).

change its cellular identity into any other cell type has been termed cellular totipotency (Condic, 2014). Under the right PGR cues, differentiated somatic cells can re-enact embryonic developmental pathways, a process termed somatic embryogenesis (SE) (Fig. 2). This regeneration capacity of plant species has long been exploited for vegetative plant propagation and biotechnology endeavours (Fehér, 2019). However, the ease of establishment of a plant species in TC is inexplicably variable, with the majority of medicinal plant species lying towards the recalcitrant end of the spectrum, as opposed to being highly regenerative.

Genetic transformation: enabling plant improvement

While TC practices and techniques facilitate the generation of high volumes of genotype-specific clones, they also provide tissue for genetic modification purposes. To deliver the desired piece of DNA into plant cells, traditional transformation methodologies use either physical means (particle bombardment or biolistics) or *Agrobacterium* sp., a bacterium which naturally transfers a DNA segment (transfer or T-DNA) across the plant cell membrane (Fig. 1). Under a selective agent(s)

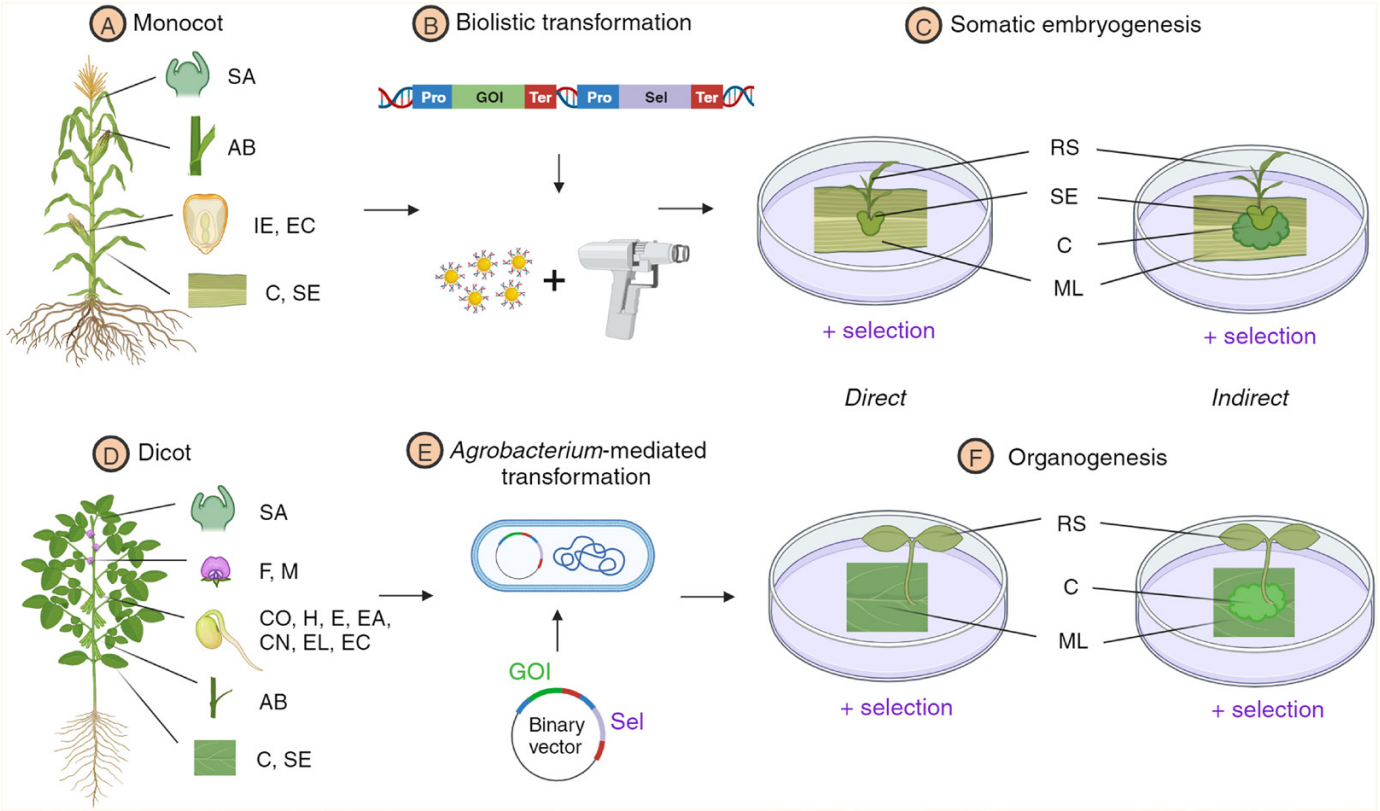

Fig. 2. Tissue culture and genetic transformation differences between monocots and dicots. (A) Preferred choice of explants in monocots are: shoot apex (SA), axillary bud (AB), immature embryo (IE), embryogenic callus (EC), callus (C), and somatic embryo (SE). (B) Biolistic transformation is more frequently used to transform monocot species where a piece of linearized DNA containing a gene(s) of interest (GOI) and one of a limited set of selectable markers (Sel) are coated onto microparticles that are delivered into the explant through high velocity bombardment. (C) Direct or indirect somatic embryogenesis is the predominant regeneration pathway in monocot post-transformation. (D) Preferred choice of explants in dicots are: SA, flowers (F), microspores (M), cotyledons (CO), hypocotyls (H), epicotyls (E), embryonic axis (EA), cotyledonary nodes (CN), embryonic leaflets (EL), EC, AB, C, and SE. (E) *Agrobacterium*-mediated transformation of GOI with one of a broader range of selectable markers in a transformation vector. (F) Dicots exhibit regenerable callus formation from many types of explants due to direct or indirect organogenesis, the products of which can be readily regenerated into entire plants. Regenerated shoot (RS), somatic embryo (SE), callus (C), and mature leaf (ML).

(antibiotic or herbicide), non-transgenic cells are eliminated and, with the appropriate external stimuli in the media, the genetically modified cells can regenerate (Fig. 1). The ability to genetically modify medicinal plant species is not only important to introduce novel traits or modify existing ones, but is also a scientific tool for the purposes of dissecting the molecular basis for the production and regulation of specific biomolecules, for example knowledge which can refine subsequent efforts to enhance their yield. Whilst tissue culture and transformation protocols have been successfully developed in several agriculturally important crops, efforts in medicinal species have been limited (Gómez-Galera *et al.*, 2007). For example, *Aloe vera*, *Ginkgo biloba*, and *Garcinia indica* with well-defined regeneration systems do not have a transformation protocol or may become less regenerative after genetic transformation, as is the case with *Plumbago zeylanica* and *Euphorbia nivulia* (Pandey *et al.*, 2010). Regeneration-recalcitrant species have limitations with respect to the bioengineering applications that can be implemented for the improvement of agromorphological traits and the alteration of their beneficial biomolecule profiles.

In contrast to crop species that have benefited from the concerted knowledge of many years of breeding, medicinal plants generally have a highly heterozygous genome often exacerbated by being obligate outcrossing species. Until the recent application of MG expression, Coker was the sole *Gossypium hirsutum* (cotton) cultivar amenable to transformation (Juturu *et al.*, 2015). Similarly, in *Cannabis sativa* (cannabis), regeneration from calli has been shown to be highly cultivar dependent (Zhang *et al.*, 2021). In this study, transgenic plants were produced in only one cultivar of 100 tested, a result achieved with the combined use of MGs and explants with high potential for totipotency stimulated with exogenous application of potent synthetic phytohormones (Zhang *et al.*, 2021). These examples demonstrate the complexities encountered in recalcitrant species and the diverse approaches required to enable genetic modification and regenerability.

Modern molecular technologies that have been widely applied to agriculture crops are yet to be routinely used in most medicinal plants. These species would benefit from leveraging large-scale sequencing methodologies that have seen dramatic cost reductions in the last decade to provide (pan)genome information, spatiotemporal tissue transcriptome datasets, ideally at cellular resolution, together with an understanding of the epigenome. This information will significantly facilitate bioengineering of medicinal plants, offering, for example, markers for trait selection via traditional breeding approaches and the ability to use gene editing tools such as CRISPR (clustered regularly interspaced short palindromic repeats) effectively by enabling accurate design of guide RNAs (gRNAs) that are target specific and avoid off-target edits (Yang *et al.*, 2021).

Choice of explants

Successful standardization of *in vitro* regeneration protocols depends on the health and accessibility of tissues from a plant donor (S0, Fig. 1). Exploring TC capabilities in a medicinal plant demands consideration of the specific requirements of the taxonomic group to which the species belongs during explant selection and regeneration. For example, gymnosperm medicinal tree species often exhibit varied responses to TC due to a 'phase change' (shift from juvenile to adult state) that results in a significant loss of vegetative propagation capacity, reducing the ability of tissues to regenerate *in vitro* (Pereira *et al.*, 2021). In such cases, production of SEs using juvenile embryo organs has emerged as a preferred regeneration method, maintaining explant juvenility and ensuring a high regeneration rate. Similarly, distinct morphological and developmental differences between monocots and dicots significantly influences their TC responses, the favoured transformation method, and ultimately regeneration efficiency. Monocots provide a limited range of explant types that commonly regenerate through either direct or indirect SE (Fig. 2). In contrast, regeneration in dicots can occur through direct or indirect organogenesis in a wider range of explant types which are readily regenerated into plants. Many medicinal dicots are amenable to TC, and a diverse array of regeneration approaches have been successfully applied compared with monocots, as exemplified by the examples listed in Table 1.

The divergent TC responses between dicots and monocots has led to different transformation approaches being used (Kausch *et al.*, 2019). Initially limited only to dicots, *Agrobacterium*-mediated gene transfer poses a challenge in monocot species due to their non-natural host status (Potrykus, 1990). Monocot transformation recalcitrance was overcome by the introduction of biolistic transformation and protoplast-based systems in which the plant cell wall is enzymically removed prior to DNA introduction (Kausch *et al.*, 2019) (Fig. 2). Additionally, there are far fewer effective selectable markers available in monocots compared with dicots. While aminoglycoside resistance markers such as kanamycin, neomycin, and G418 (geneticin) have proven ineffective in most monocots, they have been used extensively in dicot transformation systems (Jones, 2009). Transformation selection of many monocots has been achieved using herbicide-resistant markers (e.g. phosphinothricin) and through the development of newer antibiotic selection marker systems (e.g. hygromycin).

While plants consist of various tissues and organs, not all are commonly used as explants due to difficulties in viable excision. Despite many tissues displaying totipotency or pluripotency, they are often inhibited from expressing this capability by neighbouring tissues. Isolation and *in vitro* culture of these tissues could free them from being recalcitrant (Bonga, 2017). For example, in *Beta vulgaris*, the guard cells exhibit high totipotency and have the remarkable ability to undergo SE when isolated from leaves (Hall *et al.*, 1996). The choice of explants in medicinal plants becomes limited when the donor population is small, as in the case of endangered species, necessitating the use of mature tissues. Furthermore, factors such as a lack of dedifferentiation capacity (the process of specialized cells reverting to a more primitive state), limited cell division potential, or the presence of specialized metabolites can have an antagonistic effect on regeneration (Benson, 2000). In many cases, regeneration can be enabled through selection of organs that contain undifferentiated cells, such as young tissues of embryonic and meristematic origins.

Mature and immature zygotic embryo explants offer a higher proportion of undifferentiated cells and fewer specialized structures, and accumulate fewer inhibitory compounds. These traits are advantageous for initiating embryonic callus cultures or producing viable shoots through SE in many dicots and monocots (Benson, 2000). Zygotic embryos contain pre-embryogenic determined cells with embryogenic competence (Bhojwani and Dantu, 2013). In many monocots, immature embryos have proven to be efficiently transformable, with their size and growth conditions influencing transformation efficiency, but challenges persist in the consistent production of high-quality immature embryos year-round (Lee and Wang, 2023). Alternatively, mature seeds offer a cost-effective, easy to store, and reliable source of explants such as cotyledons, hypocotyls, epicotyls, and cotyledonary nodes, allowing for continuous supply under controlled conditions, and are used for callus induction and shoot proliferation. Cotyledonary node regions have axillary meristems at the junction between cotyledon and hypocotyl, which can proliferate and regenerate by the formation of multiple adventitious shoots on a culture medium containing cytokinin. A cotyledonary node as an explant offers several advantages such as simple accessibility, speedy response, and immense potential to favour shoot organogenesis and SE. Several examples showing high regeneration with the use of immature and seed-derived explants in various medicinal plants are listed in Table 1. Recently, half-seeds have become the trend for explants as they possess advantages

Table 1. Selected examples showing the different factors affecting the regeneration recalcitrance of medicinal and other plant species

Plant species	Explant type(s), (age), and regeneration pathway	Media+PGRs[a]	Additives[b]		Outcome	Reference
			Silver compound	Others		
Eurycoma longifolia (Tongkat ali)	Cotyledonary node (14 d) Direct organogenesis	MS+1 mg l^{-1} BAP	–	3% sucrose +0.25% gelrite	Highest frequency of multiple shoot induction (76.7%), max. shoot no. (4.87 ± 0.70) per explant observed in BAP cf. KIN or TDZ	Alttaher *et al.* (2020)
Gossypium hirsutum (cotton)	Cotyledonary node (14 d) Direct organogenesis	MS+B5 vitamins+2.5 mg l^{-1} BAP	2 mg l^{-1} $AgNO_3$	3% sucrose +0.8% agar	Max. no. of shoots (22.2) per explant achieved with $AgNO_3$ alone without any PGRs	Prem Kumar *et al.* (2016)
Lallemantia iberica (Dragon's head plant)	Cotyledonary node, cotyledons, and hypocotyl (21 d) Direct organogenesis	MS+1 mg l^{-1} BAP+0.05 mg l^{-1} NAA	–	–	Plant regeneration from all explants with a maximum no. (23 ± 3.60) observed from the cotyledonary nodes cf. other explants	Ebrahimzadegan and Maroufi (2022)
Mucuna pruriens (velvet bean)	Cotyledonary node (7 d) Direct organogenesis	MS+2.5 µM BAP	–	3% sucrose+ 0.8% agar+10.0 µM putrescine +3% PVP	Max. no. of shoots (22.8) and shoot length (6.0 cm) per explant observed in BAP+putrescine cf. other polyamines, e.g. spermine, spermidine	Alam *et al.* (2023)
Ocimum basilicum (sweet basil)	Cotyledonary nodes and leaves (28 d) Cotyledons and hypocotyls (14 d) Direct organogenesis	Cotyledonary nodes, cotyledons, and hypocotyls: MS+4 mg l^{-1} TDZ Leaves: MS+1 mg l^{-1} BAP	–	0.8% agar+ 10 mg l^{-1} citric acid+100 mg l^{-1} ascorbic acid (leaves and hypocotyls)	Regeneration from all explants with highest frequency from cotyledonary nodes (2.6 shoots per explant) cf. other explants	Barberini *et al.* (2023)
Prosopis cineraria (spunge tree)	Cotyledonary node (5 d) Direct organogenesis	Shoot bud initiation: MS+2.22 µM BAP Shoot bud multiplication: MS+2.22 µM BAP+0.46 µM KIN	0.59 µM $AgNO_3$	3% sucrose +0.8% agar	Max. multiple shoot bud regeneration (95.6%) observed in BAP+KIN+$AgNO_3$	Venkatachalam *et al.* (2017)
Salvia plebeian (common sage)	Cotyledonary nodes and shoot tips (16 d) Direct organogenesis Hypocotyls (16 d) Indirect organogenesis	Cotyledonary nodes: MS+1 mg l^{-1} TDZ+0.1 mg l^{-1} IAA Hypocotyls: MS+1 mg l^{-1} TDZ+0.1 mg l^{-1} IAA Shoot tips: MS+1 mg l^{-1} BAP+0.1 mg l^{-1} IAA	–	3% sucrose +0.6% agar	Max. no. of shoots (37.5 ± 1.3) per explant observed in cotyledonary nodes in TDZ+IAA cf. BAP+IAA. Highest no. of globular bodies (17.4) per hypocotyl in TDZ+IAA cf. BAP+IAA	Wu *et al.* (2022)
Vigna mungo (black gram)	Cotyledonary nodes and shoot tips (3–5 d) Direct organogenesis	MS+B5 vitamins+1 mg l^{-1} BAP+0.1 mg l^{-1} TDZ	1 mg l^{-1} $AgNO_3$	3% sucrose +0.8% agar+15 mg l^{-1} ADS	Higher no. of shoots/explants in cotyledonary nodes obtained when BAP+TDZ used in combination with ADS+$AgNO_3$	Mookkan and Andy (2014)
Withania somnifera (Ashwagandha)	Cotyledonary node (7 d) Direct organogenesis	MS+2.5 µM BAP+0.5 µM NAA	–	3% sucrose +0.8% agar	90% regeneration frequency with highest number of shoots (29.3 ± 0.23) per explant and shoot length (5.62 ± 0.17 cm) in BAP+NAA cf. KIN+NAA and 2iP+NAA	Fatima and Anis (2021)

Table 1. Continued

Plant species	Explant type(s), (age), and regeneration pathway	Media+PGRs[a]	Additives[b]		Outcome	Reference
			Silver compound	Others		
Brassica rapa L. ssp. *Pekinensis* (Chinese cabbage)	Cotyledon-petioles, hypocotyls, and roots Direct organogenesis	MS+0.5 mg l^{-1} NAA+4 mg l^{-1} BAP	4 mg l^{-1} $AgNO_3$	3% sucrose +0.7% agar	Higher differentiation rate (81.15%) in cotyledon-petioles cf. other explants, highest adventitious bud differentiation rate (79.2%) in BAP	Li *et al.* (2021)
Cosmos bipinnatus (garden cosmos)	Cotyledons (7 d) Direct organogenesis	MS+5 mg l^{-1} BAP	5 mg l^{-1} $AgNO_3$	3% sucrose +0.8% agar+40 mg l^{-1} ADS	Highest shoot number (5.7) per explant induced on BAP+$AgNO_3$+ADS	Jaberi *et al.* (2018)
Eruca sativa (rocket)	Cotyledons, hypocotyls, and roots (5 d) Direct organogenesis	MS+1 mg l^{-1} TDZ+0.1 mg l^{-1} NAA	5 mg^{-1} $AgNO_3$	2% sucrose+ 0.64% agar	Enhanced shoot regeneration (25.38%) from hypocotyls in TDZ+NAA+$AgNO_3$ cf. 2,4-D and BAP+NAA combinations	Banjac *et al.* (2023)
Ficus religiosa (sacred fig)	Hypocotyls (8–10 d) Direct and indirect organogenesis	Callusing: MS+0.5 mg l^{-1} 2,4-D+0.05 mg l^{-1} BAP (dark) Direct and indirect regeneration: MS+1.5 mg l^{-1} BAP+0.15 mg l^{-1} IBA (light)	–	3% sucrose+ 0.6% agar	Highest callus FW (2.43 g) observed in 2,4-D+BAP cf. IBA+BAP Indirect regeneration: highest regeneration frequency (86.66%) and max. shoot no. (4.13) in BAP+IBA cf. TDZ+IBA and KIN+IBA Direct regeneration: highest regeneration frequency (96.66%) and max. shoot number (6.26) in BAP+IBA cf. TDZ+IBA and KIN+IBA	Hesami and Daneshvar (2018)
Brassica napus (rapeseed)	Hypocotyls (5 d) Indirect organogenesis	Pre-treatment: liquid B5 (for 1 h) Callusing: B5+1 mg l^{-1} 2,4-D+0.1 mg l^{-1} IAA Regeneration: B5+2 mg l^{-1} BAP+1 mg l^{-1} Z	Shooting medim: 5 mg l^{-1} $AgNO_3$	Pre-treatment: 2% sucrose+250 mg l^{-1} NH_4NO_3+750 mg l^{-1} $CaCl_2{\cdot}2\ H_2O$+250 mg l^{-1} xylose Callusing: pre-treatment media+0.6% agar Regeneration:pre-treatment media+0.6% agar	Increased photosynthetic pigments in callus and improved regeneration efficiency from 0–0.8% to 8.3–10% in three of five varieties tested	Al Ramadan *et al.* (2021)
Ficus carica (common fig)	Leaves from *in vitro* shoots Indirect organogenesis	MS+2 mg l^{-1} IBA+0.5 or 1 mg l^{-1} TDZ+0.5 mM phloroglucinol (7 d dark followed by light)	–	3% sucrose+0.8% agar+3 mM MES, sealing with porous tape	Delayed tissue browning and enhanced shoot regeneration (8.1–10.8 multiple shoots per explant) in IBA+TDZ cf. IBA+BAP, 2,4-D+BAP, 2,4-D+TDZ, NAA+BAP and NAA+TDZ	Kim *et al.* (2007)
Prunus persica (peach)	Leaves with petiole (21 d) Indirect organogenesis	WPM+15.5 µM BAP	10 µM STS	3% sucrose+0.5% agar+210 µM cefotaxime+238 µM carbenicillin	Highest regeneration frequency (53%) and no. of regenerating shoots (0.77 ± 0.08) per leaf were observed in BAP+STS cf. KIN+NAA and TDZ+NAA	Ricci *et al.* (2020)

Table 1. Continued

Plant species	Explant type(s), (age), and regeneration pathway	Media+PGRs[a]	Additives[b]		Outcome	Reference
			Silver compound	Others		
Brassica napus (rapeseed)	Microspores Direct organogenesis	Embryo formation: NN-13 (dark) Embryo maturation: B5+0.1 mg l^{-1} GA_3 *Regeneration:* B5	–	Embryo formation: 13% sucrose ABA treatment: 0.5 mg l^{-1} for 12 h Embryo maturation: 2% sucrose+0.7% agar Regeneration: 1% sucrose+0.9% agar	Enhanced embryogenesis (391.4 ± 18.1) in cultures exposed to ABA cf. jasmonic and salicylic acid, increased plant regeneration by 68%	Ahmadi et al. (2014)
	Microspores Direct organogenesis	Embryo formation: NN+0.1, 0.25, 0.5, and 1% PF-68 (dark) Embryo maturation: B5+1 mg l^{-1} Z Regeneration: B5+1 mg l^{-1} IBA	–	Embryo formation: 8% sucrose+0.1% AC Cold treatment (4 °C) for 4 weeks Embryo maturation: 2% sucrose+0.8% agar Regeneration: 2% sucrose+0.8% agar	Four of five recalcitrant populations showed increased shoot regeneration in presence of PF-68, dose–response effect did not show a consistent trend as optimum concentration was influenced by genotype	Barbulescu *et al.* (2011)
Brachypodium distachyon (purple false brome)	Seeds Indirect organogenesis	Callusing: MS+2.5 mg l^{-1} 2,4-D (dark) Regeneration: MS+75 µM FPX	–	Callusing: 3% sucrose+0.3% phytagel+0.6 mg l^{-1} $CuSO_4$ Regeneration: 3% sucrose+0.3% phytagel	Highest regeneration rate (40.7%) in FPX cf. KIN and TDZ	Yu *et al.* (2019)
Gloriosa superba (flame lily)	Leaf Indirect organogenesis	Callusing: MS+3 mg l^{-1} 2,4-D Regeneration: MS+2 mg l^{-1} BAP		3% sucrose+ 0.8% agar	Max. callus regeneration (84.5 ± 3.31%) in 2,4-D cf. BAP, picloram, BAP+NAA and max. no. of shoots per explants (5.25 ± 0.5) in BAP cf. BAP+NAA	Balamurugan *et al.* (2019)
Eremurus spectabilis (foxtail lily)	Roots Indirect organogenesis	Callusing: MS+10 mg l^{-1} BAP+0.1 mg l^{-1} NAA Regeneration: MS+2 mg l^{-1} BAP+0.1 mg l^{-1} IBA	–	Callusing: 3% sucrose+0.8% agar Regeneration: 3% sucrose+0.8% agar+200 mg l^{-1} AC	Highest callus induction frequency (76.67%) and max. shoot proliferation (6.33) in MS medium cf. Schenk and Hildebrandt media. Max. shoot proliferation in intact callus cf. divided callus	Basiri *et al.* (2022)

Table 1. Continued

Plant species	Explant type(s), (age), and regeneration pathway	Media+PGRs[a]	Additives[b]		Outcome	Reference
			Silver compound	Others		
Veratrum dahuricum (mountain corn)	Immature embryos Indirect somatic embryogenesis	Callusing 1: MS+8 mg l^{-1} picloram (dark) Callusing 2: AA+4 mg l^{-1} 2,4-D Regeneration: R2M+3 mg l^{-1} BAP	–	Callusing 1: 146 mg l^{-1} glutamine+ 200 mg l^{-1} casein hydrolysate+3% sucrose+3 g l^{-1} phytagel Callusing 2 and regeneration: 3% sucrose+3 g l^{-1} phytagel	Highest frequency of embryogenic calli (56%) in picloram and green plant regeneration (95%) in BAP	Ma *et al.* (2020)

Monocots are shaded in grey.
[a] MS, Murashige and Skoog; BAP, 6-benzylaminopurine; B5, Gamborg B5; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron; KIN, kinetin; IAA, indole-3-acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; WPM, woody plant medium; NN-13, Nitsch and Nitsch-13; GA_3, gibberellic acid; NN, Nitsch and Nitsch; PF-68, Pluronic F-68; Z, zeatin; FPX, fipexide; AA, amino acid medium; R2M, 190-2 medium.
[b] $AgNO_3$, silver nitrate; STS, sodium thiosulfate; PVP, polyvinyl pyrrolidine; ADS, adenine sulfate; ABA, abscisic acid; NH_4NO_3, ammonium nitrate; $CaCl_2{\cdot}2H_2O$, calcium chloride; AC, activated charcoal; $CuSO_4$, copper sulfate.

of having a greater nutrition supply for shoot regeneration compared with a cotyledonary node alone. They can also be prepared within a shorter time frame, which reduces the total regeneration period and labour costs (Xu *et al.*, 2022).

Another TC approach which takes advantage of the embryogenic process is co-culturing in which two different plant species are grown together in close proximity for promoting SE. In this system, one plant species which exhibits a higher frequency of SE releases specific molecules into the culture medium that stimulate and induce the formation of SEs in the co-cultivated species with a naturally lower rate of SE. Active components identified in embryogenic culture medium include arabinogalactan proteins (AGPs), endochitinases, and lipochitooligosaccharides (von Arnold *et al.*, 2002). The beneficial effect of this strategy has been studied in the regeneration of wheat (*Triticum aestivum*) (Bakos *et al.*, 2003) and to overcome recalcitrance in grapevine (*Vitus* sp.) (Ben Amar *et al.*, 2007) and *Cichorium* species (Couillerot *et al.*, 2012).

The shoot apical meristem (SAM), located at the cotyledon–embryo axis junction, possesses axillary meristems capable of developing into shoots without the need for dedifferentiation or redifferentiation (Sticklen and Oraby, 2005). It offers several advantages, including ease of *in vitro* culture, rapid regeneration, clonal multiplication, competence for genetic transformation, and the ability to be sustained *in vitro* for extended periods without cryopreservation. The strategy underlying the SAM-based transformation system involves multiplying transgenic SAM or germline cells *in vitro* and reprogramming them to differentiate (Baskaran and Dasgupta, 2012). The SAM-based biolistics or *Agrobacterium*-mediated transformation systems have achieved genotype-independent transformation in medicinal plants such as *Catharanthus roseus* (Madagascar periwinkle) (Bahari *et al.*, 2019) and *Tanacetum cinerariifolium* (pyrethrum) (Li *et al.*, 2022).

The use of male and female gametophytes has also been explored due to their ability to produce haploid and doubled haploid plants through gametic embryogenesis, allowing development of homozygous lines from heterozygous parents in a single step. However, not all species are amenable to this type of *in vitro* morphogenesis, and many medicinal species remain recalcitrant. Moreover, determining the optimum developmental stages of microspore explants is essential for maximum *in vitro* response (Benson, 2000). Also, the basis of microspore embryogenesis is the switching of the developmental process from normal gametophytic to sporophytic embryogenesis which requires pre-treatments such as cold or heat shock, carbohydrate, and nitrogen starvation, making the regeneration process more tedious (Sharma *et al.*, 2018). Notwithstanding these challenges, isolated microspore cultures emerge as a promising technique to produce double haploids, surpassing anther and ovule cultures in terms of efficiency. Routinely used in vegetable crops, this method has recently been adapted to medicinal plants with encouraging outcomes, notably the recent

successful induction of microspore-derived embryonic structures in *Artemisia annua* (Purnamaningsih *et al.*, 2024).

Plant growth regulators and other chemical factors

Tissue culture medium provides the essential growth components to the explant, but it can also be considered as an interface for communication with the plant. The medium components can dictate certain growth behaviour, and the molecules intimately involved in reshaping plant development are PGRs. Phytohormones are ubiquitously used as PGRs in TC practices, cytokinins and auxins in particular having an impact on *de novo* shoot organogenesis (Raspor *et al.*, 2021). Cytokinins, auxins, and other phytohormones have a diversity of molecular structures that either exist in nature or are of synthetic origin. For example, >20 different cytokinins and auxins are currently commercially available. Endogenous phytohormones control most aspects of plant growth and development, and modulate responses to abiotic/biotic stresses and other environmental cues. During the establishment and maintenance of meristematic cells of the embryo and SAM, phytohormones fall under the regulation of MG products, which sometime behave in intricate positive feedback loops, as exemplified by WUSCHEL (WUS) and cytokinin (Leibfried *et al.*, 2005).

PGRs are important medium components that have a large impact on developmental and metabolic processes even at low concentrations. Optimizing PGR regimes, including application of novel, potent PGRs and removing the inhibitory interactive effects of endogenous and exogenous hormones, is often a first approach to overcome *in vitro* regeneration recalcitrance in many plant species. In this section, we will cover the roles of media components permitting regeneration capability to recalcitrant explants, but the reader needs to keep in mind that the enabling functions of cytokinin and other molecules are achieved through the involvement of a wide range of molecular players with morphogenic activities. Application of cytokinin is often viewed as the enabling factor in TC regeneration, but this must be viewed in a context of a cascade of events that occur in meristem cells or tissue of embryonic origins that requires the recruitment of morphogenic players that were silenced prior to the application of PGRs.

Exogenous phytohormones

Under *in vitro* cultivation, the fate of an explant hinges on the fundamental golden hormonal regeneration rule: a high ratio of cytokinin to auxin in the medium stimulates the formation of shoots, while a reversed ratio encourages the development of roots. Usually, under the influence of cytokinin, explants can produce elevated numbers of shooting units. Cytokinin can also trigger direct and indirect SE (Fig. 2) and enable regenerability of cells. Use of cytokinins for micropropagation and regeneration is so prevalent that it is often considered the first approach when studying micropropagation or regeneration in a new species (Šmeringai *et al.*, 2023). Use of cytokinin in both micropropagation and calli regeneration protocols has provided excellent results in amenable species. With a wide range of natural and synthetic cytokinins, finding a desirable regeneration response in recalcitrant species is often a trial-and-error approach where a cytokinin's molecular conformation, concentration, type of delivery to the explant, and interplay with other phytohormones form a complex matrix of conditions to test. In the last decade, thidiazuron (TDZ), a synthetic phytohormone (see below in the section on synthetic PGRs), has proven to be tremendously effective in a wide range of medicinal, woody, and other species. It is successfully used to promote *de novo* regeneration and SE initiation, and to stimulate shoot organogenesis and callus induction and proliferation. In the medicinal species *Salvia bulleyana*, direct organogenesis was observed from leaf explants while using TDZ (Grzegorczyk-Karolak *et al.*, 2021) and in embryo explants of cannabis (Galán-Ávila *et al.*, 2020; Zhang *et al.*, 2021). Moreover, recent advancements in our understanding of auxin and cytokinin crosstalk have shed light on the complex world of regeneration phenomena, including SE, for the future of TC and transformation (Asghar *et al.*, 2023). As for auxin, a wide variety of species require exogenous application of auxin in the medium as a pre-requisite to trigger a totipotency reversal in somatic cells. Calli produced under auxin acquire a competency for organogenesis that increases cell susceptibility to SE and shooting upon subsequent cytokinin exposure.

Endogenous phytohormones

Endogenous phytohormones are the native molecules already present in the explant when moved to *in vitro* growing conditions. Endogenous levels of phytohormones can be sufficient on their own to trigger a regeneration response from the right explant type, as seen in the previous section. In other instances, endogenous phytohormones are a hindrance to TC. As such, establishing cultures from vegetative explants such as leaves, petioles, and nodal segments acquired from mature medicinal trees can be difficult as they contain elevated levels of endogenous phytohormones, carbon sources, and other substances that can interfere with the effects of additives present in the growth medium, interfering with their regeneration potential and leading to potential developmental issues. Application of external phytohormones is often ineffective in mitigating the impact of endogenous levels of auxins. For instance, in plants with high endogenous auxin levels, including some medicinal species, the addition of auxin transport inhibitors, auxin antagonists, or auxin biosynthesis inhibitors positively affects shooting induction, as is the case in *Carapichea ipecacuanha* (Koike *et al.*, 2020), and has been used to achieve successful regeneration in otherwise recalcitrant plants such as cannabis (Smýkalová *et al.*, 2019). Applications of auxin transport inhibitors such as

1-naphthylphthalamic acid (NPA), 2,3,5-triiodobenzoic acid (TIBA), 2-(1-pyrenoyl) benzoic acid (PBA), and the flavonoid quercetin have seen increased regeneration rates in model organisms, fruit-bearing trees, and cereals (Yu *et al.*, 2012; Hu *et al.*, 2017; Ohbayashi *et al.*, 2022). In medicinal species, the presence of TIBA in the medium has improved organogenesis from calli in mulberry (Bhau and Wakhlu, 2001), while NPA and TIBA have also shown a positive effect in cannabis (Dreger and Szalata, 2022), and quercetin too has shown increased regenerability in *Oldenlandia umbellate* (Saranya Krishnan and Siril, 2017). To flush out endogenous phytohormones or to load explants with PGRs, pre-treatment of explants in a liquid medium enriched with molecules such as cytokinins can stimulate or promote the regeneration process. For instance, shoot regeneration was successfully achieved in the woody medicinal plants *G. biloba* (Isah, 2020) and *Pterocarpus marsupium* (Ahmad *et al.*, 2018) through pre-treatment with TDZ.

Similarly, endogenous levels of cytokinins play an important role in regeneration efficiency, and the technological approaches to measure endogenous concentration can subsequently be used to optimize the concentration of exogenous cytokinin to be applied to a culture, narrowing the window of the matrix of media conditions to be tested (Smýkalová *et al.*, 2019). Novel rapid methods for quantifying endogenous phytohormones offer a tool for more effective TC protocols to be developed for cultivating recalcitrant species (Erland *et al.*, 2017). A recent study in the woody medicinal plant *Cyclocarya paliurus* has highlighted the importance of seasonal variability of endogenous cytokinins when explants are isolated from perennial plant species (Cheng *et al.*, 2023). The study demonstrated that similar adventitious shooting rates can be obtained across explants from different seasonal origins if the concentration of exogenously supplied 6-benzylademine, a cytokinin, is adjusted to match the endogenous level of phytohormone according to season (Cheng *et al.*, 2023).

Novel synthetic plant growth regulators

Recalcitrance can also be overcome by substituting natural or commonly used PGRs with powerful synthetic counterparts that share similar physiological properties (Benson, 2000). Synthetic PGRs offer several advantages, including light insensitivity and resistance to degradation during autoclaving, exhibit potency levels 10–1000 times higher than natural hormones, and therefore are often required in lower concentrations for activity (Phillips and Garda, 2019). Some auxin-based herbicides such as dicamba, 2,4-dichlorophenoxyacetic acid, and picloram are used to induce SE in various species (Miroshnichenko *et al.*, 2017). TDZ has found extensive application in TC as it demonstrates remarkable potency in propagating recalcitrant woody, legume, and medicinal species *in vitro,* including cannabis (Ali *et al.*, 2022). TDZ's efficacy is well established in TC, facilitating highly efficient regeneration across genotypes and explant types; hence it broadens the scope of transformation protocols to elite genotypes. However, it is worth noting that excessive TDZ concentration and prolonged exposure can lead to issues such as the formation of fasciated and compact shoots, hyperhydricity (shoot vitrification or glassiness), and downstream rooting challenges (Dewir *et al.*, 2018).

Many plant species exhibit varied responses to the different cytokinins, and it becomes necessary to optimize TC protocols for individual species. Topolins in general, and meta-topolin in particular, were identified as a result of the continuous search for superior cytokinins. Meta-topolin and its derivatives are naturally occurring aromatic cytokinins that have shown promising effects in micropropagation of several medicinal plant species and promote induction of multiple shoots, improving physiological and biochemical traits and successful rooting (Ahmad and Anis, 2019). Additionally, several compounds such as brassinosteroids, jasmonates, salicylic acid, phloroglucinol, pluronic F-58, phytosulfokine-alpha, lignosulfonates, fipexide, abscisic acid, and trichostatin exhibit growth-modulating effects and have been used as PGRs in several species (Table 1), offering novel avenues for addressing recalcitrance issues in TC.

Ethylene inhibition and the role of silver compounds

Ethylene, a key regulator of physiological and developmental processes, exhibits contradictory impacts on regeneration, varying with species, genotypes, and explant type. While the concentration of auxins and cytokinins in culture media is precisely controlled, ethylene, being a gas, is typically released during *in vitro* culture, accumulating in closed vessels. Thus, understanding its role is critical for enhancing regeneration and addressing recalcitrance in certain species or tissues (Neves *et al.*, 2021). Ethylene can adversely affect morphogenic responses, contributing to hyperhydricity. Strategies to regulate ethylene, using inhibitors such as salicylic acid, $CoCl_2$, and $AgNO_3$, show promise for improving TC protocols (Bashir *et al.*, 2022). Interestingly, in some cases, ethylene has a positive influence, potentially reversing recalcitrance in genotypes with limited regeneration capacity (Neves *et al.*, 2023).

Silver ions, especially in the form of $AgNO_3$ and silver thiosulfate, are favoured due to their physical, chemical, and biological availability, water solubility, stability, non-toxicity, and specificity to inhibit ethylene action, disrupting its signalling pathway and impacting growth by enhancing polyamine biosynthesis (Pal Bais and Ravishankar, 2002; Kumar *et al.*, 2009; Prem Kumar *et al.*, 2016). Additionally, $AgNO_3$ reduces aminocyclopropane-1-carboxylic acid, a precursor to ethylene, decreasing ethylene production and browning of explants (Gong *et al.*, 2005). As a result of these properties, $AgNO_3$, silver thiosulfate, and other Ag compounds are gaining prominence in refining TC protocols for addressing recalcitrance issues in various plant species, including medicinal plants (Table 1). In a later section, we describe the use of silver nanoparticles ($AgNO_3$) to reduce the impact of ethylene in TC.

Other media components

Apart from phytohormones, other factors within the TC medium such as macro- and micronutrients, vitamins, carbon source, solidifying agents, and other additives all play a role in the *in vitro* growth of explants. Medium permutations affecting the type and concentration of these constituents have been shown to relieve TC recalcitrance (Fig. 1, Stage S1–S3) in multiple species (Long *et al.*, 2022) (Table 1). For example, a doubling of the regeneration rate of indirect somatic embryogenesis was achieved in the recalcitrant rice elite cultivar IR64 with optimization of an established TC protocol by manipulating the type and concentration of carbon source and gelling agent, and by supplementation of the medium with additives such as free amino acids (Sundararajan *et al.*, 2020). Similar increases in regeneration frequency have been observed in medicinal species such as the endangered TC-recalcitrant plant *Oplopanax elatus*, where regeneration frequencies could be increased by similarly modulating both carbon source and concentration and gelling agent in the cultivation medium (Moon *et al.*, 2013; Sahoo *et al.*, 2023). Use of maltose or a combination of maltose and sucrose has proven more effective in increasing regeneration in the medicinal plants *Cymbopogon schoenanthus* (Abdelsalam *et al.*, 2018) and *Kelussia odoratissima* (Ebrahimi *et al.*, 2018), respectively. Through the sugar sensing pathway, use of sucrose in the medium can have an antagonistic effect on cytokinin homeostasis (Ćosić *et al.*, 2021) and other phytohormones (Raspor *et al.*, 2021). Other media additives such as activated charcoal have provided some beneficial effects to ameliorate TC recalcitrance by sequestering and thereby rendering inert chemical inhibitors present in the media or secreted by the explant during their early stages of culture. However, the prolonged presence of activated charcoal in the medium can pose a challenge, as it has the potential to also absorb growth-promoting substances, ultimately diminishing the growth response or regeneration processes (Pinar *et al.*, 2020).

Morphogenic genes

MGs are transcription factors that control cell fate and, consequently, govern plant development. Harnessing MGs can significantly improve and accelerate explant regeneration through their involvement in hormone biosynthesis, perception, and developmental signal transduction pathways, and hence transformation efficiency. Over the past two decades, MGs have been increasingly employed and have unlocked transformability in many recalcitrant crops, as outlined in two recent reviews (Maren *et al.*, 2022; Lee and Wang, 2023).

Overexpression of MGs to stimulate an embryogenic or meristematic response to induce regeneration is classified into two categories: (i) genes that enhance a pre-existing embryogenic response under *in vitro* conditions; and (ii) genes involved in the direct formation of embryo or meristem-like structures without the need for induction conditions (Gordon-Kamm *et al.*, 2019). An example of the first type of inducer is *SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1* (*SERK1*) which has been shown to be an enabler for cells to develop into somatic embryos, a change achieved through the modulation of auxin biosynthesis, transport, and perception (Yan *et al.*, 2023). Previous studies have demonstrated its role in SE in both monocots and dicots (Sivanesan *et al.*, 2022). Constitutive expression of SERK1 has enhanced SE initiation in *Coffea canephora* (Pérez-Pascual *et al.*, 2018), *Oryza sativa* (Hu *et al.*, 2005), and *Arabidopsis thaliana* (Hecht *et al.*, 2001).

Major regulators of SAM formation and maintenance such as the homeobox genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) have also been used as MGs to improve embryonic responses (Lenhard *et al.*, 2002). Expression of At*WUS* in *Medicago truncatula* leaf explants induced callogenesis and the production of highly embryogenic calli, generating plantlets even in the absence of growth regulators in the medium (Kadri *et al.*, 2021). Also, overexpression of *WUS* promoted SE and lateral branching in birch (*Betula platyphylla*) through an increased expression of SE-related genes such as Bp*STM* (Lou *et al.*, 2022), and thus has proven to be a promising tool in developing plant growth regulator-free regeneration systems.

MGs in the second category have been extensively studied in various crops, as reviewed in detail elsewhere (Gordon-Kamm *et al.*, 2019). One such gene is *BABYBOOM* (*BBM*) whose product belongs to the AP2/ERF superfamily of transcription factors (Boutilier *et al.*, 2002). BBM plays a multifaceted role in processes such as cell proliferation, plant growth, and development, and notably it induces embryogenesis in differentiated cells. Its initial success in stimulating SE via ectopic expression without addition of external PGRs was observed in *Brassica napus* (Boutilier *et al.*, 2002). Subsequently, *BBM* and *BBM*-like genes have been utilized in numerous plant species to improve transformation efficiency and regeneration (Jha and Kumar, 2018). Recently, there has been a shift in the use of *BBM* for enhancing transgenic plant regeneration beyond herbaceous plants and crops to include recalcitrant fruit trees. For example, the overexpression of Md*BBM1* in apple has resulted in a remarkable enhancement of apple transformation efficiency (Chen *et al.*, 2022; Xiao *et al.*, 2023).

Beyond the promoter controlling gene expression, several factors influence the outcome of MG expression, including the target cell or tissue type(s), the source of the MG (i.e. whether it is derived from the native or another species), hormone dependency, and co-expression with other MGs. For instance, the gene *LEAFY COTYLEDON1* (*LEC1*) plays a role in SE, and its overexpression can trigger embryo-like structures in vegetative tissues (Zhu *et al.*, 2014). However, in conifers such as *Picea abies* (Norway spruce), overexpressing the LEC1-type gene Pa*HAP3A* which is active during embryo development did not induce embryonic features in vegetative tissues. Instead, when activated during zygotic maturation, ectopic somatic

embryos formed on the surface of zygotic embryos. This highlights that specific cells or tissue types are more receptive to MGs and that the spatiotemporal control of MG expression is an important consideration using this approach (Uddenberg *et al.*, 2016). Additionally, the expression of endogenous genes may produce different developmental responses compared with homologues of other species. For example, ectopic At*BBM* or Bn*BBM* expression in *Nicotiana tabacum* produced developmental responses that differed from those observed using the endogenous tobacco *BBM* gene (Srinivasan *et al.*, 2007).

Several MGs that play an important role in plant regeneration are hormone dependent and are also involved in phytohormone signal transduction. For example, *CUP-SHAPED COTYLEDON* genes (*CUC1* and *CUC2*) contribute to SAM formation during embryogenesis and shoot regeneration (Aida *et al.*, 1997). Overexpressing these genes in transgenic calli from *A. thaliana* hypocotyls promoted adventitious shoots (Daimon *et al.*, 2003). However, when cultured on hormone-free medium, the same transgenic calli did not produce shoots, highlighting the need for an appropriate hormone context for *CUC1* and *CUC2* functionality. On the other hand, *ENHANCER OF SHOOT REGENERATION* genes (*ERS1* and *ERS2*), involved in the cytokinin response pathway, and *MONOPTEROS*, an auxin-response gene, promoted a hormone-independent response in shoot meristem formation when overexpressed (Banno *et al.*, 2001; Ikeda *et al.*, 2006; Ckurshumova *et al.*, 2014). Additionally, the levels of PGRs can influence the phenotypic response of genes involved in morphogenesis. For instance, transgenic *A. thaliana* explants overexpressing *LEC2* produced somatic embryos and calli under low and high auxin concentrations, respectively (Wójcikowska *et al.*, 2013). Similarly, when *WUS* was expressed in the root in the absence of phytohormones, shoots and leaves were observed; somatic embryos arose in the presence of auxin (Gallois *et al.*, 2004). In the same study, floral structures were observed when WUS was induced along with *LEAFY*, a master regulator of floral development, providing evidence that unique phenotypes can be observed when MGs are co-expressed.

In many crops, achieving effective transformation often requires the use of selectable marker genes (Fig. 1) (Zuo *et al.*, 2002). To address this issue, researchers have explored genes, including MGs, that enable the identification of transgenic events without the need for a selectable marker. The maize homeobox gene *KNOTTED1* (*KN1*) is essential for meristem initiation and maintenance, and is normally expressed in shoot meristems. When *KN1* was overexpressed in *N. tabacum* under non-selective conditions (without antibiotics) on a hormone-free medium, a 3-fold increase in transformation efficiency was observed relative to the kanamycin selection treatment, demonstrating its usefulness as a positive selection system for plant transformation (Luo *et al.*, 2006). Similarly, co-expression of maize transcription factor genes *BBM* and *WUS2* enabled regeneration of stable transgenics in the recalcitrant maize inbred line B73 and sorghum (*Sorghum bicolor*) P898012 (Mookkan *et al.*, 2017). *GROWTH-REGULATING FACTOR 4* (*GRF4*) and its cofactor *GRF-INTERACTING FACTOR 1* (*GIF1*) form a transcription factor complex required for pluripotent cell formation in male and female reproductive structures (Lee *et al.*, 2018). Expression of *GRF4–GIF1* substantially increased the efficiency and speed of regeneration in wheat, triticale, and rice, and induced efficient wheat regeneration in the absence of exogenous cytokinins, facilitating selection of transgenic plants, thereby eliminating the need for antibiotic-based selectable markers (Debernardi *et al.*, 2020).

While MGs have proven valuable in transforming and regenerating recalcitrant plant species, they come with a potential drawback—the risk of deleterious pleiotropic effects. When these MGs are expressed strongly and constitutively, they can lead to unwanted changes in plant morphology, reduced fitness, altered metabolism, and even infertility in regenerated plants (Gordon-Kamm *et al.*, 2019). To maximize their benefits while minimizing these drawbacks, an additional step is needed to control MG expression after the transformation or regeneration process has occurred and their usefulness has expired. Several strategies to control the timing and level of expression of MGs have been developed, including their inducible expression, excision from the nuclear genome post-transformation, use of tissue-specific plant promoters, using GRF–GIF chimeras, innovative *Agrobacterium*-mediated delivery methods, and T-DNA border read-through. Some successful examples of the application of these approaches are listed in Table 2, with cannabis among the first medicinal plant species in which use of GRF–GIF chimeras was attempted (Zhang *et al.*, 2021).

Over the past two decades, significant advancements in genetic transformation have been witnessed in major crop species such as rice, maize, wheat, sorghum, soybean, and cotton (Nalapalli *et al.*, 2021). Comprehensive improvements in various aspects of the TC process have led to a high success rate in obtaining transgenic plants, with MGs playing a pivotal role. This transformation success has been particularly evident in monocot crops, where both *Agrobacterium*-mediated and particle bombardment gene delivery methods have been refined to achieve remarkable efficiency (Shrawat and Lörz, 2006). While recent advances in MG research, including genes such as *BBM*, *WUS*, *GRF*, and GRF–GIF chimeras, have effectively addressed transformation and regeneration challenges in many recalcitrant crop species, their application in the realm of medicinal plants has remained limited. This discrepancy presents a dual challenge and opportunity within the fields of plant biotechnology and medicinal plant research. The limited use of MGs in improving medicinal plants can be attributed to several factors such as complex biology and the diverse nature of medicinal plant species, lack of research funding and commercial investment, as well as regulatory and ethical considerations.

Table 2. Different approaches to control MG expression levels to overcome negative pleiotropic effects

Approach	Method	Plant species	MG used	Outcome	Reference
Inducible expression	Dexamethasone-inducible system (GR fusion)	*Capscium annum*	*BBM*	Efficient regeneration of large numbers of fertile transgenic plants	Heidmann *et al.* (2011)
		Arabidopis thaliana	*BBM*	Regeneration of fertile plants during extended cultivation in tissue culture	Lutz *et al.* (2015)
		Theobroma cacao	*LEC2*	Regeneration of secondary transgenics from the leaf-derived secondary embryos	Shires *et al.* (2017)
	Estradiol-inducible system (OLexA promoter)	*Arabidopis thaliana*	*MYB115*	Vegetative to embryonic transition	Wang et al. (2009)
		Brassica rapa var. *rapa*	*WUS*	Fertile transgenic plants without developmental defects	Liu *et al.* (2022)
Excision-based	Desiccation-inducible CRE/LOXP	*Zea mays*	*WUS2+BBM*	Excision of the morphogenic genes to produce healthy, fertile transgenic plants	Lowe et al. (2016)
	Heat shock-inducible FLP/FRT	*Populus tomentosa*	*BBM*	Transgenic plants with phenotypic alterations but fertile	Deng *et al.* (2009)
Tissue-specific promoters	AXIG1 promoter PLTP promoter	*Zea mays*	*BBM+WUS2*	Robust and fertile transgenic plants even without excision of MG	Lowe et al. (2018)
GRF–GIF chimeras	–	*Triticum aestivum* *Oryza sativa* *Citrus* *Cannabis sativa*	*GRF4–GIF1* *GRF3–GIF1*	Fertile transgenic plants without developmental defects	Debernardi *et al.* (2020); Zhang *et al.* (2021)
Agrobacterium-mediated delivery methods	Two *Agrobacterium* strains with each harbouring a distinct T-DNA	*Sorghum bicolor*	*WUS+CRC*	Somatic embryo formation and regeneration of stable transgenic plants with only the selectable marker	Hoerster *et al.* (2020)
T-DNA border read-through	Positioning MG cassettes outside T-DNA left border	*Zea mays*	*WUS2* *WUS2+BBM*	Non-excision method for creating a high-quality transgenic event	Gordon-Kamm *et al.* (2019)

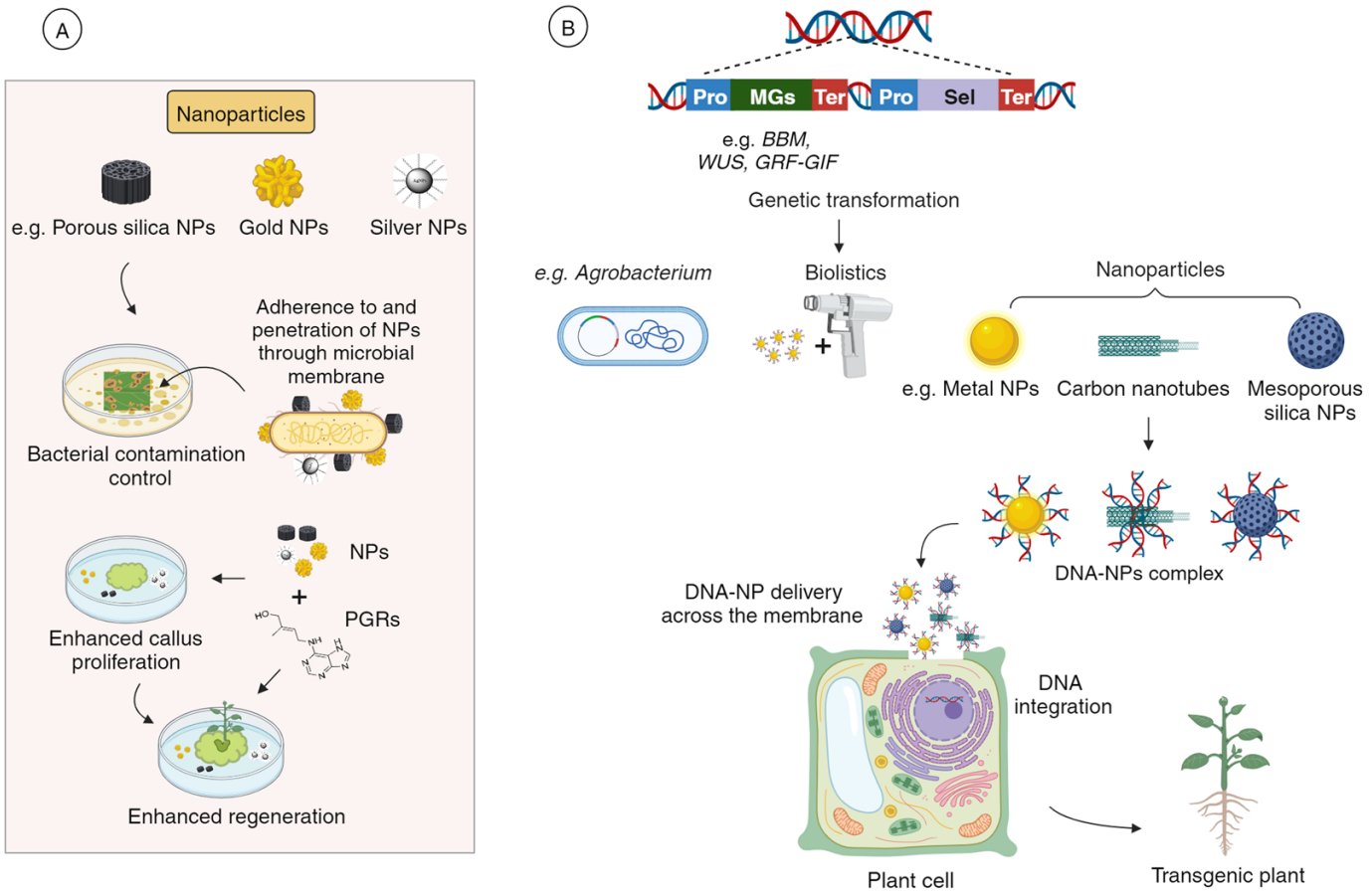

Fig. 3. A schematic of different types of NPs and their application in plant tissue culture and transformation. (A) Role of NPs in eliminating microbial contamination and enhancing regeneration. (B) NPs as vehicles for delivering GOI(s), such as MG cargo, as an alternative to *Agrobacterium*-mediated transformation or biolistic transformation.

Novel techniques to improve transformation efficiency and overcome recalcitrance

Nanoparticles

In recent years, the field of TC has undergone a remarkable transformation with the use of nanoparticles (NPs). Nanoparticles are ultra-small structures measuring <100 nm in size that can be used to deliver molecular cargo through biolistic or transfection methods. They can also act as active agents themselves in TC medium. TC is often challenged by microbial contamination, and NPs provide a promising alternative to antibiotics for addressing this concern (Alfarraj *et al.*, 2023). Endophytes found in many medicinal plants can also become problematic in *in vitro* cultures even though they may not be pathogenic as they can negatively impact plant health and vigour (Wu *et al.*, 2021). While research on the use of NPs against endophytic bacteria is in its early stages, efforts are being made to tackle this issue (Rakhimol *et al.*, 2023). NPs adhere to and penetrate the bacterial cell membrane, bind to the sulfhydryl group of enzymes involved in metabolic activities, and inactivate transport chain mechanisms, thus inhibiting their proliferation in the medium (Ahlawat *et al.*, 2022) (Fig. 3). In addition, NPs generate reactive oxygen species which interact with the bases of microbial DNA and arrest their replication (Kim *et al.*, 2011). These activities not only serve as a microbial deterrent but are also hypothesized to stimulate secondary metabolite production (Sena *et al.*, 2023). NPs have been used as effective elicitors for the biosynthesis of medicinal compounds by causing changes in expression of key genes in diverse metabolite pathways (Ayoobi *et al.*, 2024).

The favourable impact of NPs on overcoming barriers related to callus induction, SE, and organogenesis can be attributed to their ability to regulate key PGRs such as auxin, cytokinin, and gibberellins (Fig. 3). This regulation involves enhancing protein and enzyme activity, as well as improving photosynthesis by enhancing light absorption (Mandeh *et al.*, 2012; Salih *et al.*, 2021). Also, they reduce the rate of transpiration, maintaining cellular osmotic pressure, and facilitate water and nutrient uptake (Arruda *et al.*, 2023). Recent studies have also underscored the effectiveness of NP combinations,

Table 3. Use of NPs in medicinal plants for improved TC response and/or regeneration

Plant species	Explants and TC/regeneration pathway	Nanoparticle type[a]	Size and concentration	Outcome	Reference
-*Bacopa monnieri* (water hyssop)	Full leaf (FL), distal portion of half leaf (DPHL), and proximal portion of half leaf (PPHL) Indirect organogenesis	TiO_2	20–50 nm 2, 4, 6, 8, and 10 mg l^{-1}	Max. callus FW (3.02 g) in PPHL at 8 mg l^{-1} and max. shoots/explant (30.43) in FL at 2 mg l^{-1} TiO_2NP	Aasim *et al.* (2023)
Gloriosa superba (glory lily)	Rhizome Indirect somatic embryogenesis	ULAg	5 nm and 50 nm 0.1, 0.2, 0.3, 0.4, and 0.5 mg l^{-1}	Enhanced embryogenic callus (96%) in 0.1 mg l^{-1}, highest somatic embryo production (95.7%) in 0.4 mg l^{-1}, highest no. of mature embryos (24.3/culture) in 0.5 mg l^{-1}, high-frequency embryo germination (98.1%) in 0.3 mg l^{-1} ULAgNP	Mahendran *et al.* (2018)
Panax vietnamensis (Vietnamese ginseng)	Leaf-derived calli Indirect somatic embryogenesis	Ag	<20 nm 0.4, 0.8, 1.2, 1.6, and 2.0 mg l^{-1}	Max. no. of somatic embryos (140) and embryo-derived plantlets (14.66) in 1.6 mg l^{-1}	Manh Cuong *et al.* (2021)
Prunella vulgaris (common self-heal)	Leaf Callogenesis	Ag and Au	AgNP: 30 μg l^{-1} AgAu (1:3), AgAu (2:1), and AgAu (3:1)	100% callus proliferation in 30 μg l^{-1} AgNP and AgAu (1:2) and AgAu (2:1)	Fazal *et al.* (2016)
Satureja khuzestanica (garden savoury)	Leaf, stem, and root Callogenesis	MWCNT	Outer diameter: 5–15 nm and length: 50 μm 0, 25, 50, 100, 250, and 500 mg l^{-1}	Efficient callus induction in leaf explant cf. stem and root and highest callus proliferation rate (81%) in 100 μg ml^{-}	Ghorbanpour and Hadian (2015)
Scoparia dulcis (goatweed)	Young stem cuttings Indirect organogenesis	Ag, Au, and CuO	AgNP: 13.5 nm, AuNP: 3.5 nm, and CuONP: 25 nm 2, 4, and 6 mg l^{-1}	Optimum callus initiation in 4 mg l^{-1} AgNP, AuNP, and CuONP, and accelerated shoot regeneration in 4 mg l^{-1} CuONP cf. AgNP and AuNP	Rajan *et al.* (2021)
Swertia chirata (chirata)	Shoot apices Direct organogenesis	Biogenic Ag	20 nm 1, 2, and 4 mg l^{-1}	Max. no. of shoots per explant (10.24 ± 1.26), shoot length (2.4 ± 0.43 cm), and shoot regeneration percentage (98.26 ± 3.76) in 4 mg l^{-1}	Saha and Dutta Gupta (2018)
Cannabis sativa[b] (cannabis)	Leaf	Polyethylenimine cationic polymer-modified silicon dioxide-coated gold nanoparticles (PEI-Au@SiO_2)	Gm*MYB29A2*-pGWB6 and Gm*NAC42-1*-pGWB6	Infiltration of DNA-PEI-Au@SiO_2 into leaf tissues resulted in transcription of both genes and localization of fluorescent-tagged transcription factor proteins in the nuclei of leaf cells including trichomes	Ahmed *et al.* (2021)

[a] ULAg, *Ulva lactuca* silver; MWCNT, multi-walled carbon nanotubes; CuONP, copper oxide nanoparticles.
[b] NP used as a cargo delivery is shaded in grey.

demonstrating that blends of different NPs are more potent than single types in promoting callus biomass production and enhancing regeneration, especially when using mature embryo explants (Arruda *et al.*, 2023). Silver NPs bind to ethylene receptors involved in signalling, thus hindering ethylene action, and reduce hyperhydricity. Likewise, the promotion by silver NPs of regeneration from callus cultures derived from diverse plants is linked to their capacity to increase antioxidant reserves (Phong *et al.*, 2023). This dual action potentially mitigates oxidative stress and supports the regeneration process. Several successful although limited medicinal plant examples to date are shown in Table 3.

Genetic engineering in plants is frequently limited by several factors such as the presence of a multilayered and rigid cell wall, cell damage, random DNA integration within the genome (excluding targeted gene edits), and negative effects of high antibiotic concentrations when traditional gene delivery methods are used, reducing transformation efficiency, regeneration, and compromising the genetic stability of resulting plants (Sarmast and Salehi, 2016; Dong and Ronald, 2021). To address these issues, researchers have turned to NP-mediated gene delivery methods (Fig. 3). These methods, free from the external forces utilized in biolistics or electroporation, deliver biomolecules to intact plant cells and offer advantages such as the ability to traverse biological membranes and target specific tissues or cells, protect cargoes (DNA, RNA, proteins, and ribonucleoproteins) from degradation and release them in controlled quantities and intervals (Cunningham *et al.*, 2018; Squire *et al.*, 2023). Delivery of cargo inside liposome NPs, for example, is an efficient method in species with protoplast-amenable regeneration protocols. In cannabis, passive diffusion of silicon polymer-coated gold NPs to which two *Agrobacterium* vectors were fused was successfully used to transiently transform intact leaves with two transcription factors (Ahmed *et al.*, 2021). NPs have therefore emerged as a promising and biocompatible tool for manipulating a plant's genome or for the transient expression of genes of interest.

NP–MG combinations have the potential to make significant advances in the field of plant genetic engineering (Squire *et al.*, 2023). Addressing the pleiotropic effects of MGs, DNA-free direct delivery of transcription factors has emerged as a promising solution. For instance, At*WUS* was successfully delivered into tobacco using cell-penetrating peptides through a method known as delivered complementation *in planta* (Wang *et al.*, 2023). These short peptides, forming cell-penetrating peptide–cargo complexes, enable cytosolic delivery of cargo molecules through the plasma membrane by covalent conjugation, overcoming the need for introducing foreign DNA (Guo *et al.*, 2019). Additionally, nanomaterial-based small-molecule approaches are being explored to mimic endogenous transcription factor proteins, replicating their multidomain structure and gene-regulating functions (Patel *et al.*, 2014). Furthermore, NPs can deliver CRISPR-associated protein 9 (Cas9)/gRNA ribonucleoproteins into regenerative tissues with the aim of generating targeted DNA modifications in transgene-free plants (Demirer *et al.*, 2021). These approaches hold enormous promise for application in plants. Such innovative techniques provide greater precision and control over gene expression, ultimately advancing our ability to manipulate medicinal plants for various purposes.

Overexpression of histone genes

Many economically important crops remain highly recalcitrant to *Agrobacterium* infection. The success of plant transformation depends on complex interactions between the plant and *Agrobacterium*, involving numerous genes from both organisms (Rahman *et al.*, 2023). Several strategies have been attempted to enhance transformation efficiency, such as using highly virulent *Agrobacterium* strains or super binary vectors with extra *Vir* genes, and optimizing plant culture conditions (De Saeger *et al.*, 2021). Despite these efforts, there are limits to improving transformation in recalcitrant crops using these methods. An alternative approach to boosting plant transformation involves modifying the plant itself. This can be achieved by identifying plant genes that play roles in the transformation process. Some candidate plant genes have been identified through genetic screening (Mysore *et al.*, 2000).

One of the identified genes, the *A. thaliana* histone H2A gene *HTA1* (*RAT5*), is involved in the integration of T-DNA into the plant genome. Overexpression of At*HTA1* has been shown to increase *Agrobacterium* transformation efficiency of *A. thaliana* plants (Mysore *et al.*, 2000). Similarly, expression of other histone genes such as *HTR* and *HFO*, whether in their native host or in alternative plant species, has also led to increased transformation susceptibility, suggesting that exploring the manipulation of plant genes involved in the process offers a promising avenue for expanding the range of recalcitrant crops that can be effectively transformed using *Agrobacterium* (Tenea *et al.*, 2009).

Other tissue culture-independent transformation methods

The reproducibility of transformation protocols involving TC is a complex puzzle, particularly in recalcitrant plant species (Gharghi *et al.*, 2023). *In planta* transformation offers a simpler, faster, and TC-independent alternative which involves direct uptake of foreign DNA into plant tissues through techniques such as microinjection, electroporation, or by protoplasts without the use of any vector (Su *et al.*, 2023). Various improvements in *Agrobacterium*-mediated transformation efficiency have been achieved by modifying factors such as pre-culture conditions, chemoattractant concentration (acetosyringone and chloroxynil), and *Agrobacterium* strains (Karthik *et al.*, 2018). Apart from biolistics, other common *in planta* methods include injecting *Agrobacterium* into the SAM, floral dip or spray, pollen uptake, and embryo/seed imbibition (Kaur and Devi, 2019).

Another promising solution comes in the form of a rapid, reliable imbibed seed-piercing method, which has the potential to be applied to fibre-producing crops (Majumder *et al.*, 2020). Pollen magnetofection is being explored to overcome the plant cell wall barrier in some crops which makes them resistant to DNA delivery and recalcitrant to transformation. It involves coupling DNA with magnetic NPs in the presence of a magnetic field (Dobson, 2006). This method takes advantage of the unique characteristics of pollen, which has surface apertures (5–10 μm diameter) with either reduced wall thickness or devoid of walls, facilitating DNA uptake (Ressayre *et al.*, 1998). This technique has been successfully demonstrated to produce transgenic seeds in cotton and other crops such as pepper and pumpkin (Zhao *et al.*, 2017), recalcitrant maize inbred lines (Wang *et al.*, 2022), and okra (Farooq *et al.*, 2022). Despite its advantages, pollen magnetofection has some limitations, not being suitable for certain plant species with incompatible pollen apertures, and it is not effective for introducing genetic material into maternally inherited organelles such as chloroplasts and mitochondria (Lv *et al.*, 2020). Another recent TC-free transformation method has shown great potential for transforming herbaceous, tuberous, and woody species by taking advantage of the shooting regenerability of their roots, tubers, or stem sections, respectively (Cao *et al.*, 2023). In the cut–dip–budding gene delivery system, the method utilizes a scion donor (cut) that is challenged with *Agrobacterium rhizogenes* (dip) to enable the generation of transgenic shoots (budding). It has been successfully applied to various medicinal plants with root-suckering capabilities, in species such as *Clerodendrum* spp (Lu *et al.*, 2024), *Taraxacum mongolicum* (Pugongying), and *Rehmannia glutinosa* (Dihuang) (Cao *et al.*, 2024).

Non-transformation methods

Due to ethical, regulatory, and other concerns regarding the production of transgenic plants, significant effort has been invested in developing methods that do not rely on DNA integration to overcome transformation or regeneration recalcitrance. For example, new *Agrobacterium* strains are being developed that can transiently express but do not integrate T-DNA into the host genome. Additionally, advancements in CRISPR/Cas technology have improved the robustness of this process by allowing genetic changes to be accomplished without any integration of foreign DNA through transient expression of a site-specific nuclease using viral vectors in the form of either mRNA, which is unstable and quickly degrades, or protein, which is not transmitted from parent to offspring (Sedeek *et al.*, 2019). Gene edits can also be implemented through the transfection of gRNA-loaded Cas9 ribonucleoproteins by polyethylene glycol (PEG) in species where regeneration from protoplasts is possible, or by particle bombardment in regenerative explants. Multiple examples in non-medicinal plant species are covered in a recent review (Gu *et al.*, 2021). MGs can essentially be co-delivered in the same way to produce gene edits in regeneration-recalcitrant medicinal plants.

Future advances using artificial intelligence

The numerous environmental and genetic factors on which a successful TC process depends are complex, non-linear, and non-deterministic due to the highly interactive nature of these variables. Their unravelling can be a time-consuming and costly endeavour. To assist with this challenge, artificial intelligence models and optimization algorithms are now being applied to enhance different stages of TC (Hesami and Jones, 2020). For instance, a combination of a generalized regression neural network (GRNN) and a genetic algorithm (GA) was used to model and predict *in vitro* shoot regeneration outcomes of wheat. Metadata collected from previous *in vitro* shoot regeneration studies on the basis of 10 factors, including genotypes, explants, PGR type, and concentration, were considered to develop and optimize genotype-independent regeneration protocols (Hesami *et al.*, 2020). Similarly, other input variables such as digitized images have been used to capture visual data, for example to classify non-embryonic callus and somatic embryos during SE and to recognize different phases of embryo development (Hesami and Jones, 2020). These advances in data-driven modelling demonstrate the potential of artificial intelligence for overcoming genotype-related challenges in medicinal plants and promoting more efficient and widespread crop trait improvement through genetic engineering and TC techniques.

Conclusion

Although MGs have an undisputed impact on explant regeneration, in some instances they require a specific cellular context to enable their morphogenic functions. We have highlighted studies where the right balance of exogenous phytohormones (Daimon *et al.*, 2003) or explant type (Uddenberg *et al.*, 2016) was needed to trigger a regeneration response. Overcoming regeneration in recalcitrant species foremostly requires an understanding of how explant, phytohormones, and MGs, both endogenous and exogenously supplied, interact and enable each other. The use of MGs and NPs to enhance transformation and regeneration in medicinal plants represents a promising field of research, with the potential to radically transform cultivation practices and up-scale the production of valuable therapeutic compounds. While various MGs associated with embryogenesis and meristem development have been identified, their individual and combined effects on medicinal plant transformation need thorough evaluation (Duan *et al.*, 2022). Given the diverse nature of medicinal plants, a universal solution is unlikely, necessitating the exploration of new MG combinations for different species and even within the same species.

Over the past two decades, extensive basic research has elucidated many MGs, with ongoing discoveries providing continued insights for testing and refining their use. These advancements are complemented by the emergence of faster, more affordable, and efficient genome sequencing tools, paving the way for a deeper genetic understanding of medicinal plants. Furthermore, innovative strategies for controlling or limiting MG expression hold promise for enhancing transformation efficiency, making it more routine and accessible for diverse medicinal plant species. Additionally, the integration of artificial intelligence stands to further revolutionize this field by streamlining the research process, offering predictive insights into gene functions and interactions. This, in turn, facilitates CRISPR/Cas-mediated genome modifications in many important species and accelerates cultivar development. Ultimately, progress in *in vitro* cultivation, genetic transformation, and regeneration techniques is essential for ensuring the conservation and sustainable use of medicinal plants for present and future generations. The convergence of cutting-edge biotechnology and computational tools points towards a future where medicinal plant production is more predictable, efficient, and sustainable.

Author contributions

PLB, MOB, and MSD: conceptualization; PLB: reviewing the referenced articles, and writing the original draft, and figure preparation; PLB and SCF: table preparation; MOB and MSD: supervision; PLB, MOB, and MSD: review and editing. All authors approved the final version of the text.

Conflict of interest

The authors declare no conflicts of interest.

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