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Review article

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The emerging role of nanotechnology in plant genetic engineering

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Abstract

Genetic engineering to improve the capabilities of plants is essential given climate change and population growth pressures. Current manipulation methods are laborious and species dependent, which limits advances in agriculture and molecular farming. Therefore, new approaches and tools are needed to broaden the range of transformable species and increase the throughput at which transformation is achieved. Nanotechnology has revolutionized delivery, sensing and imaging in microbial and animal systems, but its application in plants remains scant. However, reports of nano-mediated delivery for the genetic manipulation of plants have emerged, including direct germline editing as well as plastid and mitochondrial genome modification. Here, we review the application of nanotechnology to plant genetic manipulation, including the development of nanocarriers for the delivery of genetic cargos and advances in nano-mediated plant regeneration. Particular focus is given to understanding structure–function relationships for the rational design of nanocarriers, and how these developments can catalyse progress in nucleic acid and protein delivery for plant biotechnology applications.

Sections

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Key points

- Population growth and climate change pose serious challenges to plant-based systems, requiring improvement through genetic manipulation to ensure their maintenance.
- Current manipulation methods are amenable to a limited range of species and with low throughput. Nanotechnology-based strategies could overcome these limitations.
- Advances in understanding nanomaterial structure–function relationships enable the development of first-principle models of the cellular fate of carriers and design heuristics related to size, charge and shape.
- Nanotechnology-mediated delivery of site-specific nucleases and large cargos, such as transcription factors, is a promising strategy to improve the efficiency of genetic manipulation in plants.

Introduction

Plants are used as crops, sources of medicines, fragrances, flavours, engineering substrates for recombinant products and carbon sinks[1–4]. However, population growth, climate change and diseases pose serious challenges to systems reliant on plants, including exacerbation of food insecurity owing to increased demand and falling yields[5,6]. Strategies such as molecular farming and plant genetic engineering could address these issues by expanding the natural capabilities of plants and crops and providing cheap, abundant and rapidly deployable supplies of therapeutics, vaccines and crops[3,7–9].

During the Green Revolution, genetic manipulation through plant breeding improved the yields and nutritional content of crops, temporarily staving off a food security crisis[10]. However, plant breeding is limited by low throughput, lack of genetic specificity and inability to introduce traits not occurring naturally in a gene pool[11]. Nevertheless, key advances in molecular cell biology have enabled targeted genetic engineering of plants with tools such as *Agrobacterium tumefaciens*-mediated transformation (AMT)[12] and biolistic gene delivery[13]. In addition to expediting crop genetic manipulation, these tools enabled plant molecular farming of biologics[3,7] and small-molecule drugs[2], and produced a variety of improved crop species[14,15]. Despite their success, these methods are effective only for a narrow set of species and involve protocols for the transformation, regeneration and modification of non-nuclear genomes that are time and labour intensive.

Plants can be genetically engineered with or without transgene integration, respectively termed stable or transient integration. Transient expression is useful for applications such as molecular farming, where rapid, high-yield production of a product is desired and recovery of progeny is not necessary[8]. In contrast, stable genetic manipulation is preferred for agriculture, where genetically modified progeny are required. Stable transformation involves two primary steps: delivery of genetically active reagents to plant tissue and regeneration of transformed tissue to whole plants. Current delivery methods and regeneration protocols are highly dependent on species and suffer from low throughput, thereby limiting progress in plant bioengineering.

Nanotechnology applied to bioengineering applications has improved the access, imaging, sensing and delivery of molecules across a wide range of biological systems[16–19]. The highly tuneable properties of nanomaterials, such as size, shape, charge and surface chemistry, enable the rational design of nanoscale systems to bypass biological barriers and carry different cargos. In plants, nanotechnology can be applied for biomolecule sensing[20], stress priming[21,22], genetic engineering[23–25] and post-transcriptional manipulation[26,27]. Specifically, nanotechnology for genetic manipulation of plants can enhance delivery and regeneration by improving efficiency and throughput, eliminate the need for regeneration by direct modification of germlines and enable precise manipulation of non-nuclear genomes in a species-independent manner[1]. Here, progress in nanotechnology-mediated genetic engineering of plants is reviewed, focusing on its application in the delivery of genetic material and regeneration of transformed tissue. In particular, emphasis is given to how rational design is enabling breakthroughs in site-specific delivery in plants.

Transformation methodologies

Delivery

The most successful and widely used methods for the delivery of biomolecular reagents to modify plant genomes include AMT[12], biolistic delivery[13] and protoplast transfection[28]. AMT leverages an innate host–pathogen interaction to bypass cellular barriers for DNA vector introduction. AMT can produce transient (non-integrating) or stable (integrating) genetic modifications and is particularly effective for transient expression of recombinant proteins in plants, a cornerstone of fundamental plant biology research and molecular farming[7,29] (Table 1). AMT applied to molecular farming exploits vectors coding for plant virus machinery to drive overproduction of mRNA into proteins, with transformation efficiencies approaching 95% in *Nicotiana benthamiana* and maximum yields of 4 g kg^{-1} of biomass for green fluorescent protein (GFP)[7,30]. Stable expression (genome integration) is also achievable with AMT; however, despite some successes in developing transgenic crops[31–33], challenges remain in generating plants with stable edits. First, genome insertion through AMT is random, potentially interfering with non-target endogenous genes[34]. Furthermore, many plant species, particularly monocots[35], are recalcitrant to AMT — even within the same plant species, different cultivars display varying amenability to AMT[36,37]. For example, indica cultivars of rice are AMT amenable whereas japonica cultivars are recalcitrant[38]. Furthermore, differences in efficiency across *Agrobacterium* strains necessitate engineering of hyper-virulent *Agrobacterium* strains de novo, construction of novel binary and ternary transformation vectors, or modification of plant immune or hormone pathways for amenability to AMT[39]. Finally, to obtain transgenic progeny, plant tissue modified by AMT must be regenerated. Current plant regeneration protocols are time intensive and limited to a narrow set of species and genotypes; cereals, rice and maize, for example, remain challenging to regenerate[40,41]. Moreover, although disarmed strains of *Agrobacterium* are commonly used, AMT is still known to induce necrosis in plant tissues, particularly during co-cultivation steps, thereby compromising downstream regeneration fidelities[42].

For species recalcitrant to AMT, biolistic delivery is an alternative transformation method. Biolistic delivery involves bombardment of plant tissues with micron-sized particles loaded with genetic cargos that rupture cellular barriers to deliver cargo[13]. Similar to AMT, transient and stable expression is possible via biolistic delivery. However, in contrast to AMT, biolistic transformation can modify non-nuclear genomes, including plastid genomes, although with very low efficiencies (less than 1%)[43] (Box 1). Because biolistic delivery relies on a mechanical process, the method is essentially independent of species, enabling the transformation of a wide range of plants. However, nuclear

Table 1 | Plant transformation technologies

Transformation method	Advantages	Limitations
Agrobacterium tumefaciens	Preferred method for transient transformation (expression of recombinant proteins); rapid transformation for model species; direct germline editing for *Arabidopsis thaliana* and green foxtail	Highly species-dependent, not amenable to many species; inability to directly target non-nuclear genomes; regeneration required for stable transformations; limited to DNA delivery
Biolistic gene gun	Species-independent delivery; capable of delivering different cargos (DNA, RNA and proteins); capable of targeting non-nuclear genomes	Causes tissue damage; off-target deletion, duplication or rearrangement of genes; regeneration required for stable transformations
Protoplast transfection	Capable of delivering different cargos (DNA, RNA and proteins); capable of targeting non-nuclear genomes	Limited to species amenable to protoplast generation; regeneration required for stable transformations; large quantities of genetic cargo required
Nanocarriers	Species-independent delivery; potential to circumvent regeneration; capable of targeting non-nuclear genomes	Careful design of carrier required; delivery of large cargos (>10 kb) remains a challenge

transformation through biolistic delivery has low transformation efficiencies (typically on the order of 1%), random insertion of genetic material into the plant genome (often with multiple copies) and tissue damage resulting in off-target gene rearrangement, deletion and duplication[44,45]. Furthermore, as with AMT, regeneration is still required to obtain stable transgenic plants, hampering its application to important crops (cereals, rice and maize).

Protoplast transfection is a common alternative method of delivering cargo to plant cells because it eliminates a primary delivery barrier: the plant cell wall[28]. This strategy involves enzymatically removing the cell wall from plant cells (generating protoplasts) and incubating protoplasts in a solution of polymer and cargo. Transfection agents include polyethylene glycol (PEG) or other commercial transfection products that drive cellular uptake of cargo. Technologies enabling protoplast transformation can deliver a wide variety of cargos, including DNA[46], RNA[47] and proteins[48], and can target non-nuclear genomes[49]. Protoplast transformation is effective in transient expression applications or for species with existing protoplast regeneration protocols, for example, model plants such as *Arabidopsis thaliana* and *N. benthamiana*. Compared to biolistic delivery, protoplast transfection is more efficient in generating nuclear edits; however, it suffers from species dependency and requires regeneration[50]. Protocols for the regeneration of plants from protoplasts only exist for a select number of species such as *Arabidopsis*[50].

Regeneration

With the exception of the floral dip method for the transformation of *Arabidopsis* and green foxtail, genetically transformed tissues must be regenerated into full plants after delivery of genetic cargo to obtain stable transgenic lines[51]. Plant regeneration involves the induction of totipotent tissue, selection of transformed tissue and differentiation into mature plants capable of setting seeds. For the generation of transgenic plants, DNA is introduced to specific tissues or cells, termed explants, which are then cultured in vitro under aseptic conditions. Transformed explants are then selected with a marker, such as antibiotic resistance, which allows cultivation of only the successfully transformed explants into mature plants[52].

Several strategies are available to obtain and propagate genetically identical plants such as organogenesis and somatic embryogenesis. In organogenesis, formation of plant organs can be induced directly from meristematic tissues (shoot or primordial buds) or indirectly from a mass of undifferentiated cells, termed callus. Somatic embryogenesis, in contrast, involves the formation of differentiated embryos from somatic explants or calli. These plant regeneration protocols rely on the totipotency of plant cells and the application of plant growth regulators, namely hormones capable of directing callus production and differentiation. Following differentiation, plants are acclimatized and transferred to the growth substrate for maturation[53,54].

The establishment of plant regeneration protocols through tissue culture was a revolution in the process of plant transformation that currently remains the only method, aside from the floral dip method, to recover transgenic intact plants from transformed tissues[55–58]. However, major regeneration bottlenecks remain, including the long tissue culture periods (on the order of months for major crop species) required to recover transgenic plants from engineered tissues and the necessity for extensive optimization of medium compositions, including the concentration and timing of macronutrients and micronutrients as well as hormone additions[59]. Chimerism, occurring when only portions of a regenerated plant are descended from successfully edited cells, also poses a barrier to regeneration, particularly when tissue culture is coupled with transient, non-selectable transformation approaches (*Agrobacterium* or particle bombardment)[50]. Furthermore, despite ongoing efforts to improve regeneration[59,60], several species remain recalcitrant to tissue culture, and development of new regeneration protocols requires large and multifactorial studies for parameter optimization[31,51,61]. Although certain dicotyledon families, such as *Solanaceae*, *Cruciferae*, *Gesneriaceae*, *Asteraceae*, *Begoniaceae*, *Liliaceae* and *Crassulacea*e, have a greater capacity for regeneration, *Malvaceae* and *Chenopodiaceae*, for example, are less amenable[53]. Tissue culture practices can be further complicated by genotype and cultivar-specific differences in recalcitrance and regeneration potential within the same species[62–64].

For example, *Sorghum bicolor*, the fifth most important cereal crop worldwide, is particularly recalcitrant to transformation owing to the natural production of phenolic compounds that limit differentiation and regeneration, silencing of introduced transgenes by endogenous mechanisms, genotypic-specific differences in compatibility with *Agrobacterium*, and regenerability[65–67]. The first sorghum transformants generated through particle bombardment and *Agrobacterium*-mediated transformation achieved transformation efficiencies of only 0.08–1%[68] and 0.95–2.34%[69], respectively. Transformation efficiencies have now improved to 20.7% for particle bombardment[70] and 33% for *Agrobacterium*[71]; however, each improvement took over a decade to achieve – an effort punctuated by small (often only 1–2%), incremental gains through slight modifications of regeneration conditions. These improvements were not driven by a single factor but by minute modifications to media compositions, explant sources, delivery methods, transformation vectors (particularly promotors that limit native

Box 1

Chloroplast and mitochondrial genome editing

Chloroplast and mitochondria organelles in plants enable photosynthesis and respiration. Because they likely originated from endosymbiotic events that integrated bacteria into eukaryotes, these organelles contain their own genomes. Chloroplast and mitochondrial genomes are three orders of magnitude smaller than nuclear genomes, yet they respectively encode approximately 130 genes involved in photosynthesis and 60 genes involved in respiration[190]. Therefore, these genomes are interesting targets to improve photosynthetic and respiration efficiency. Moreover, these organelles have a role in secondary metabolism, producing proteins and small molecules with potential therapeutic applications. The chloroplast, in particular, is effective at producing recombinant proteins, achieving product yields 10–100 times greater than the nucleus[191].

Chloroplast and mitochondria genomes are inherited maternally in flowering plants; therefore, plant breeding approaches cannot introduce new genes to these genomes. Both organelles present additional delivery barriers in the form of two bilayer membranes that allow internal access to only specific molecules. Chloroplast transformation was first reported in green algae using biolistics[43] but this technique has expanded to only a handful of species. Many species remain recalcitrant to chloroplast modification, perhaps most notably cereal crops, owing to the lack of a reliable selection system to identify transformed organelles and inefficient regeneration[190]. Similarly, mitochondria transformation remains challenging because of the lack of selectable markers with biolistic modification demonstrated only in green algae[192]. Transcription activator-like effector nuclease (TALEN)-mediated base editing of chloroplast and mitochondria genomes has been reported using protoplast transfection but suffers from low efficiencies (1% or less) and is unable to introduce new genes[49]. Nano-mediated techniques are promising for the expansion of species amenable to chloroplast transformation[24,102] and enable some form of mitochondria transformation[95]. Successful nanotechnologies thus far include carbon nanotubes (CNTs) and carbon dots, with their design based on advances in structure–function understanding, exploiting peptides used in native cargo trafficking to organelles[95,102,103] and first-principles modelling of nanoparticle membrane interactions[24,96].

transgene silencing mechanisms), selection protocols, marker genes and contamination management methodologies[66]. Furthermore, except for the model TX430 inbred line, other sorghum cultivar varieties have considerably lower transformation efficiencies (0.7–9.4%)[65]. Even with current protocols, the process of generating a transgenic sorghum line can take anywhere from 9 to 12 months — a multistep process that involves growing plants until immature embryos are formed, co-cultivating immature embryos with *Agrobacterium*, culturing immature embryos under selection with hormones and regenerating whole plants until seed set[51]. In total, embryos are transferred between nine unique media compositions[71]. Given the breadth of the design space that must be optimized to regenerate a single genotype of a single species, regeneration will continue to substantially hamper the genetic manipulation of plants. However, current global challenges require rapid solutions, and the obstacles posed by plant transformation and regeneration underscore the need for new technologies that expedite genetic transformation with broad applicability across various plant species.

Delivery challenges in plants

Delivery barriers

A variety of methods are available to genetically transform microbial and animal cells. Chemical treatment (calcium chloride), electroporation, microinjection, polymer transfection, sonoporation, biolistics and nanotechnology have all enabled genetic delivery to these targets[72]. However, attempts to translate these methodologies to plants failed or were limited in their broad applicability. Transformation through chemical treatment, such as polymer transfection[28], or electroporation[73] has only been successful in protoplasts and not in intact plants. Other methods, such as microinjection[74] and sonoporation[75], were applicable to intact plants but suffered from low efficiencies and were not widely adopted[76]. Engineered nanoparticles (NPs) are an interesting alternative because they provide a (potentially) biocompatible and species-independent tool for plant genetic manipulation. Here, the challenges of nano-mediated delivery in plant systems are briefly discussed with reference to how their use was inspired by similar approaches in animal systems.

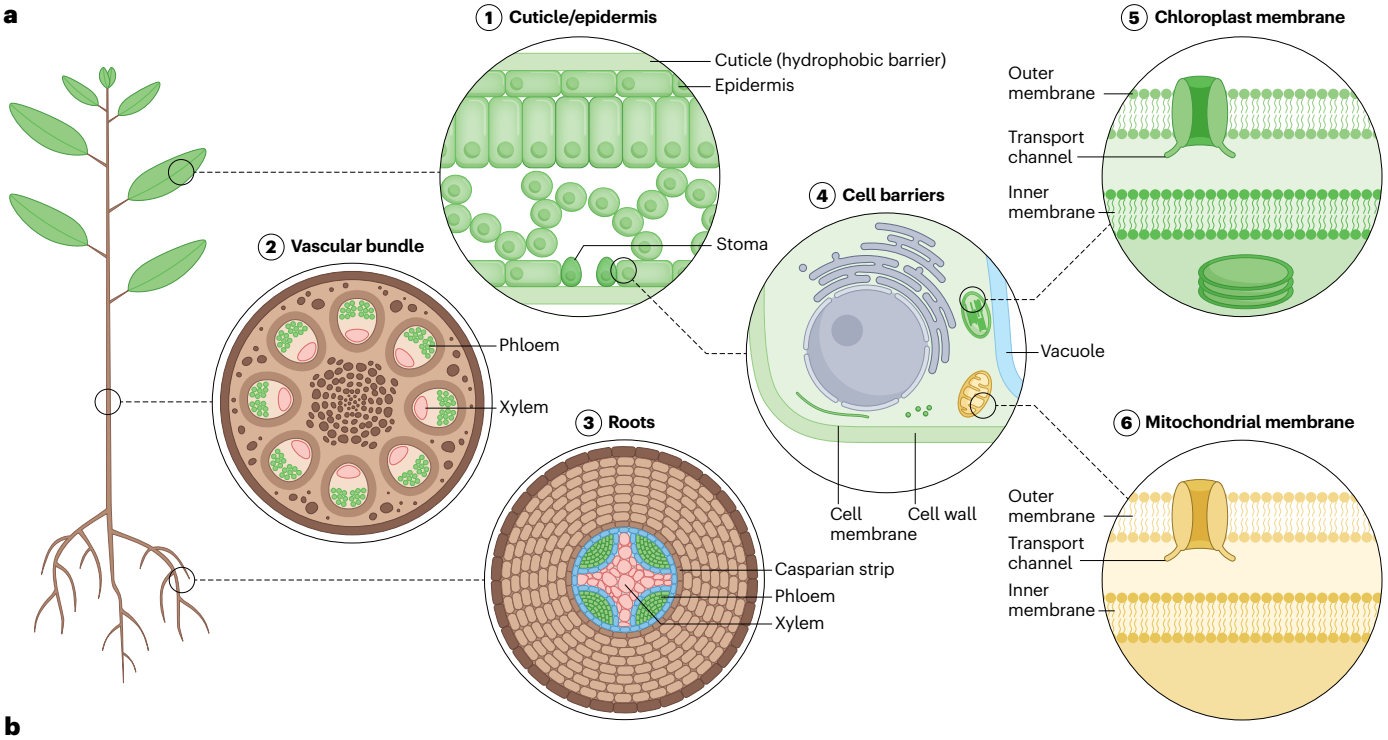
The challenges of nucleic acid and protein delivery in any biological system are similar: a delivery technology must protect cargo from degradation, bypass biological barriers, target specific tissues, cells or subcellular domains, and release cargo at desired quantities and intervals[1,77–79]. The inability to directly translate nanoscale tools that were successful in microbial and mammalian systems to plants is in part caused by the fundamental differences in the biological barriers of each system[1,51,80,81]. Biological barriers include physical barriers, which block access to target sites typically by size exclusion, and localization barriers, which destroy, sequester or remove delivery systems. Physical and localization barriers act over a variety of organizational scales ranging from the subcellular and cellular levels through the tissue, organ or organ system levels (Fig. 1). Therefore, the rational design of a delivery system must account for all types and scales of biological barriers to ensure efficient delivery.

Foliar delivery to intact plants must first overcome the waxy cuticle, a physical tissue-scale barrier similar in function to the epidermis in animal systems. Bypassing the epidermis or cuticle is typically achieved through injection or oral delivery in animals and through vacuum or syringe infiltration in plants[82,83]. Next, physical cellular barriers of the plant must be bypassed, namely the cell wall, cell membrane and, if nuclear or organelle delivery is desired, intracellular membranes. The cell wall is a complex carbohydrate biopolymer providing structure and protection to the cell[84], with a size exclusion limit (SEL) estimated at ~5–20 nm — two orders of magnitude smaller than plant cell or nuclear membrane SELs (~500 nm)[85]. Mammalian delivery systems are designed to bypass membranes but not cell walls; this anatomical difference is likely a primary cause of the failure to directly apply traditional delivery systems to plants. The challenge of delivery past the cell wall is comparable to delivering past the blood–brain barrier (BBB), a physical tissue-scale barrier in mammalian systems. Despite their structural difference (the cell wall is a cellular barrier whereas the BBB is a tissue barrier), the BBB is similar to the cell wall, in that it is highly exclusive, with a normal SEL of 400 Da (ref. [86]).

Therefore, strategies to bypass the cell wall and BBB are similar, including ultrasonic disruption, viral vector-mediated delivery and NP-mediated delivery[87]. Research in bypassing these seemingly different barriers might prove mutually insightful for future technological developments.

Beyond physical barriers, animal and plant systems have localization barriers that sequester foreign delivery systems in cell organelles or non-target tissue. Mammalian delivery must typically overcome lysosomal and organ tissue (liver, spleen, kidney) sequestration[88]. Plant delivery systems must avoid sequestration to the vacuole, the destination of endocytic pathways in plant cells and root tissue[89]. Cell-penetrating peptides and cationic polymers[90] prevent organelle sequestration by disrupting endosomes post-endocytosis in animal systems. Stealth coating with PEG in animal delivery applications also prevents sequestration by blocking adsorption of proteins that signal for clearance of the carrier[91]. Although plant physiological responses to carriers are different from animal immune responses[92], strategies for endosomal escape mediated by cationic polymers could prove useful in plant systems given that some carriers, such as gold nanorods, appear to internalize to plant cells through endocytosis[27].

a



b

#	Barrier	Delivery challenges	Design principles to bypass
1	Cuticle/ epidermis	Hydrophobic barrier that blocks access to internal tissue with pores <5 nm; guard cells prevent access through stoma[82]	Mechanical aid such as vacuum infiltration or syringe infiltration[82]; chemical aid such as foliar surfactant aerosol spray[183]
2	Vascular bundle	Phloem and xylem pathways enable access to distal tissue; pore sizes vary between 200–1,500 nm and 43–340 nm in the phloem and xylem, respectively[184,185]	Negatively charged carriers travel through phloem and xylem pathways, whereas neutral or positive charged carriers are stagnant[98]
3	Roots	The epidermis and Casparian strip guard access to the phloem and xylem, which allow access to distal tissue; carriers are commonly sequestered in root tissue[98]	Positively charged carriers are likely to be sequestrated in root tissue; negative charge enables translocation of carriers to distal tissues[186]
4	Cell barriers	The cell wall is highly exclusive (SEL of 5–20 nm)[84] and prevents access to the cell membrane (SEL of 500 nm)[85], which guards access to the cytosol; carriers can be sequestered to the vacuole[187]	Cylindrical shape and high tensile strength enables carriers to bypass the cell wall[27,106]; optimization of size and zeta potential of carriers enables cell membrane uptake[96]
5	Chloroplast membrane	Chloroplast access requires bypassing outer and inner membranes; TIC–TOC membrane-bound transport channels gate access for proteins[188]	Biomimicry of chloroplast transit peptides enables carrier targeting[181]; optimization of size and zeta potential enables plastid uptake and kinetic trapping of carriers[24]
6	Mitochondrial membrane	Mitochondria access requires bypassing outer and inner membranes; TIM–TOM membrane-bound transport channels gate access for proteins[189]	Biomimicry of mitochondrial transit peptides enables carrier targeting[95]

Fig. 1 | Biological delivery barriers. a, Biological barriers in plant systems (physical and localization) vary across scale and organization. **b**, Advances in understanding the physiological response of plants to carriers have led to the development of design principles to bypass various barriers. Effective delivery tools must overcome all physical and localization barriers[82,183–189]. SEL, size exclusion limit. TIC, translocon on the inner chloroplast membrane complex; TOC, translocon on the outer chloroplast membrane complex; TIM, translocase of the inner membrane complex; TOM, translocase of the outer membrane complex.

Nano-mediated delivery

Nano-mediated genetic manipulation

Regeneration remains a key challenge in stable plant transformation regardless of how cargos are delivered. However, nano-mediated delivery could circumvent regeneration-specific challenges altogether by enabling direct germline editing of plant tissues[93,94]. Additionally, beyond nuclear genome modification, nano-mediated delivery enables the transformation of organelle genomes[24,95].

Design rules

A key limitation of early work in nano-mediated delivery to plants was the lack of an underlying theory to inform carrier design rules or heuristics. The nanocarrier design space is broad, including tuneable parameters such as size, shape, charge, stiffness and surface chemistry; therefore, identifying which levers to turn for specific functional outputs can prove challenging (Fig. 2a). An early model attempting to describe nanocarrier transport in plants involved a mathematical

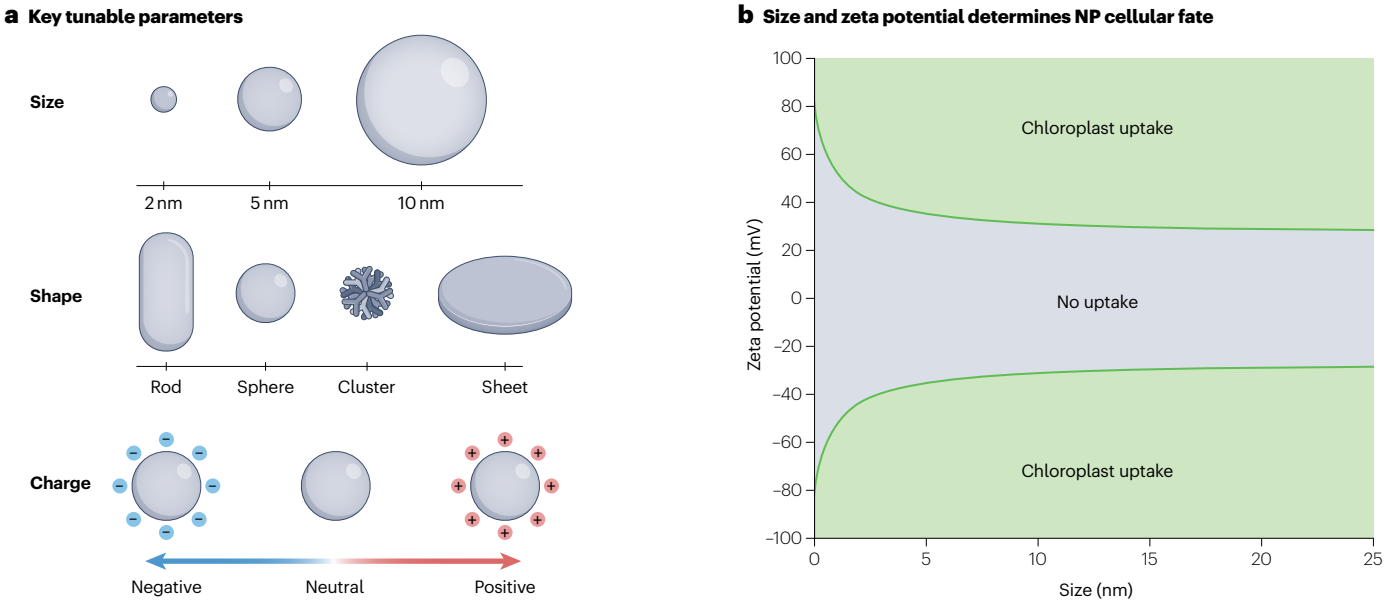


Fig. 2 | Tuneable parameters for NP design. a, Key parameters for the modification of nanoparticle (NP) function include size, shape and charge. **b**, The lipid exchange envelope penetration model predicts that the internal cellular fate of NPs is determined by zeta potential and size. NPs below a particular size and zeta potential are not internalized by chloroplasts. Model from Wong et al.[96] **c**, The energy barrier between phloem surfaces and NPs, as predicted by Derjaguin–Landau–Verwey–Overbeek (DLVO) approximations, is a function of NP surface potential. Negatively charged NPs are repelled from phloem surfaces owing to a DLVO energy barrier, whereas this barrier is absent for neutral or positive NPs. Inset: Interaction energy between a charged NP and phloem surface as a function of separation distance. Model from Su et al.[98] **d**, NP shape influences transport in tissues and cellular uptake. Cylindrical gold NPs tend to assemble in planta either parallel (~0°) or perpendicular (~90°) to the cell wall. Approximately 36% of cylindrical gold NPs assemble at an angle between 0–10° and 80–90° with respect to the cell wall. Data from Zhang et al.[27]. kT, Boltzmann energy.

description of NP cellular uptake and internal cellular fate referred to as the lipid exchange envelope penetration (LEEP) model[96]. LEEP posits that a particle with a sufficient magnitude of charge and size in close proximity to a cellular membrane induces a potential gradient across the membrane, in turn softening the lipid bilayer and opening pores for internalization. Particles below a certain size or charge threshold do not induce sufficient membrane softening and are not uptaken. Once internalized, NPs are predicted to translocate to specific subcellular locations and experience kinetic trapping depending on their size and zeta potential[97] (Fig. 2b). LEEP provides a preliminary framework for predicting NP–plant cell interactions (uptake and fate) based on NP properties (size and zeta potential), and was validated with protoplasts and chloroplasts but not walled cells. Therefore, the broad applicability to walled plants could be limited, although LEEP design rules were successfully applied to deliver cargo to mature, intact plants such as *Nicotiana tabacum* in 2019 (ref. [24]).

In parallel to the development of mathematical descriptions of NP–plant cell interactions, systematic experimental studies have investigated the impact of size, shape and chemistry on NP fate in plants[27,98–101]. Transport of NPs in the extracellular space of plant tissues depends heavily on charge, whereby neutral and positive NPs remain stagnant in planta whereas negatively charged NPs disperse through the xylem or phloem. Delivery efficiency partially depends on the ability of a carrier to traverse plant tissue; therefore, understanding nanocarrier transport rules in planta is essential to design effective delivery tools[98] (Fig. 2c).

Separately, plant exposure to nanomaterials, such as silica NPs, can activate systemic cellular immune responses, which cause physiological changes such as callose deposition to the cell wall[21,22]. Although NP-mediated stress priming can be useful for pathogen defence applications, these responses could limit the ability of carriers to deliver cargos because cells might become recalcitrant to carrier uptake when stressed. Finally, advances in understanding chloroplast and mitochondrial transport proteins in plants are enabling biomimicry approaches to achieve subcellular organelle targeting[49,102]. For example, sequence alignment of proteins that are trafficked to organelles reveals conserved peptide sequences across a variety of species that mark cellular proteins for organelle delivery. Subunits of Rubisco protein and outer envelope chloroplast peptides enable targeting of plastids, whereas mitochondrial cytochrome peptides target mitochondria[102,103]. Decorating NPs with these peptides is a promising approach for organelle targeting. Taken together, these advances provide a foundation for semi-informed carrier design, which might account for the numerous reports of nano-mediated genetic delivery to plants in recent years.

Types of nanocarriers

A wide variety of NPs has been investigated for the delivery of genetic cargos to plants (Table 2), including bio-inspired NPs such as liposomes[104], carbon-based NPs such as carbon nanotubes[23] and carbon dots[26], conjugated polymeric NPs[105] and metallic NPs such as gold[27] and iron oxide[93].

Carbon NPs. Internalization of carbon nanomaterials was demonstrated in 2009 (ref. [106]) but not for delivery applications until 2019. CNTs and carbon dots are the primary carriers for genetic cargos

Table 2 | Nanocarriers for plant delivery

Carrier	Genetic cargo	Advantages	Limitations	Refs.
Carbon carriers				
Carbon dots	siRNA (CYT); DNA (CHL)	Small radius enables carrier internalization; non-nuclear genome targeting	Carriers >8 nm do not internalize and do not deliver cargo	26,181
Single-walled carbon nanotubes	siRNA (CYT); DNA (NUC); DNA (CHL);DNA (MIT)	High tensile strength and small minimum dimension enable carrier internalization; non-nuclear genome targeting; species independent	Limited to small cargos (that is, plasmids <10 kb)	23,24,95,107,174,181
Multi-walled carbon nanotubes	DNA (NUC)	High tensile strength enables carrier internalization; species-independent	Larger carrier than single-walled carbon nanotubes	23
Metallic carriers				
Gold nanospheres	siRNA (CYT)	Efficient delivery (up to 80% target gene knockdown); internalization not required for delivery of siRNA	Carrier internalization remains debated; tested in only one species	27,112
Gold nanorods	siRNA (CYT)	Carrier internalization	Tested in only one species	27
Gold nanoclusters	siRNA (CYT)	Carrier internalization	Tested in only one species	113
Iron oxide clusters	DNA (NUC)	Suitable for pollen transformation	Low efficiency (<1%); low reproducibility; species-dependent	93,94,116,126
Silicon carriers				
Mesoporous silicon	DNA (NUC); protein (NUC)	Simultaneous delivery of multiple cargos; suitable for delivering proteins to intact plants	Reliance on biolistic gun; protein delivery not validated in mature, intact species	121,122,182
Bio-inspired carriers				
Liposomes	DNA (NUC)	Efficient delivery to protoplasts (32% transformation efficiency)	Tested only in protoplasts and not in intact plants	123
Vesicles	Protein (NUC)	Direct delivery of editing proteins	Tested only in callus; low editing efficiency (6%)	104

Genetic cargos are labelled by site of delivery. CHL, chloroplast; CYT, cytoplasm; MIT, mitochondria; NUC, nucleus; siRNA, small interfering RNA.

reported in plants owing to their high tensile strength and small size, suitable for bypassing the cell wall. For example, CNTs covalently grafted with polyethylenimine (PEI) internalize into intact plants, delivering a 4.2-kb DNA plasmid into *N. benthamiana*, arugula, wheat and cotton for transient expression of GFP[23]. Delivering larger plasmids (≥10 kb) with PEI CNTs remains a challenge, requiring careful tuning of surface chemistry and plasmid-to-carrier ratios[107]. Furthermore, the toxicity and biocompatibility of CNT carriers depend largely on the grafted polymer of choice[108], highlighting the importance of developing NP structure–function design rules. Similarly, PEI-functionalized carbon dots can deliver cargos, such as small interfering (siRNA), in a species-independent manner[26]. Interestingly, and in contrast to CNTs, delivery through carbon dots is strongly influenced by the carrier size; carbon dots larger than 8.7 nm display limited siRNA delivery, whereas multi-walled CNTs with a smallest dimension of 12.1 nm can deliver plasmid DNA cargo[23,26]. This discrepancy can be attributed to the differences in shape between carriers but also to differences in delivered cargos.

In addition to nuclear manipulation, carbon NPs have also found use in non-nuclear genome modifications. Using LEEP model design heuristics, CNTs were first tailored for chloroplast targeting by functionalizing the carrier surface with chitosan and plasmid DNA[24]. This surface functionalization optimized the zeta potential (35 mV) and size of the CNT carrier (10 nm smallest dimension) for chloroplast internalization according to LEEP model predictions. This approach enabled manipulation of chloroplast genomes of multiple species as confirmed by fluorescence microscopy of a GFP vector[24], emphasizing the utility of rational design based on fundamental structure–function theories. Notably, this study did not compare the efficiency of CNTs against biolistic delivery; therefore, it remains to be determined whether CNTs could expedite plastid transformation in plants. Moreover, mitochondria-targeting peptide sequences adsorbed to CNT carriers can be used to manipulate mitochondria[95]. Compared to delivery through targeting peptide sequences alone, CNT conjugation is 30-fold more efficient in *Arabidopsis*, likely owing to cargo protection. The species independence of this mitochondrial targeting method remains to be demonstrated.

Metallic NPs. Macro-sized and nano-sized metallic delivery systems have long been used for the delivery of genetic cargo in animal systems, with gold NPs being the most widely investigated for biomolecule delivery. Micro-sized gold has been used for decades in plants for biolistic delivery of molecules[109]. The ubiquity of gold for nucleic acid delivery is largely due to the simplicity of attaching thiolated nucleic acids to gold carriers, protecting cargos from nuclease degradation and enabling finetuning of the quantity of carried cargo[110]. Delivery of plasmid DNA to plants through gold NPs was first reported with the aid of biolistics[111]; compared to micro-gold particles, gold NP biolistics induced less damage to plant tissues and delivered DNA with ~40% better efficiency. However, reports of delivering DNA using gold NPs without the aid of biolistics are scant, and mainly involve delivery of siRNA. For example, poly-disperse spherical gold NPs internalize into *Arabidopsis* protoplasts and deliver siRNA to intact plants knocking down gene expression by 80%[112]. Similarly, gold nanoclusters mediate siRNA delivery to mature *N. benthamiana* leaves with internalization of the carrier[113]. Interestingly, carrier internalization is not a prerequisite for delivering siRNA with gold NPs[27]; a systematic study of gold NP size and shape suggests that spherical gold NPs ranging from 5 to 15 nm do not internalize into cells but embed into the plant cell wall[27]. Despite not internalizing, these carriers still deliver siRNA for gene silencing. Furthermore, although spherical gold NPs >5 nm fail to internalize, gold nanorods with a 13-nm diameter internalize into plant cells, possibly owing to their high aspect ratio. These studies demonstrate that shape is an important parameter to understanding NP transport through plant cells and tissues (Fig. 2d). In animal systems, cylindrical carriers tend to assemble with the smallest dimension parallel to the membrane surfaces of cells, thereby enabling superior transport through tissues and more frequent cellular internalization compared to spherical NPs[114,115].

Delivery through magnetic iron oxide NPs (MNPs) has also been reported. For example, direct transformation of pollen through magnetic field-driven MNPs, also known as magnetofection, was shown to enable modification of a wide range of dicot and monocot species[93]. Pollen transformation is an attractive approach to circumvent regeneration by enabling pollen grains to acquire genomic edits transmitted directly to their progeny. In the original report of magnetofection, plasmid DNA coding for β-glucuronidase (GUS) protein was delivered to the pollen of different species using MNPs and validated by staining of the treated pollen. However, the reproducibility of magnetofection of pollen remains questionable, with different studies failing to reproduce the original result so far, partially owing to the unreliability of the GUS protein reporter system[116]. Subsequently, MNP pollen transformation was reported for maize using a more reliable GFP reporter system[94]. Here, the authors attributed the previously reported reproducibility issue to differences in the pollen structure used across studies, particularly the pollen aperture (flexible, soft regions of the pollen grain through which NPs could pass). Furthermore, the inherent charge of pollen grains[117] can interfere with the transformation process by electrostatically absorbing or repelling NPs, which are usually themselves charged, thereby preventing NP transit to and through pollen apertures. Nonetheless, the range of species amenable to pollen magnetofection is still limited and the technique remains controversial. Systematic studies of NP–pollen interactions across different species will generate more comprehensive knowledge of the parameters that influence NP-mediated pollen transformation.

Silicon NPs. Silicon-based delivery systems were widely reported in animal systems[118–120] prior to their first application in plants, in which mesoporous silica NPs (MSNs) 100–200 nm in diameter were used to deliver DNA plasmids to intact *N. tabacum* with the aid of biolistics[121]. Notably, MSNs must be capped with gold NPs, as uncapped MSNs were not able to deliver plasmids. Using this strategy, genetic manipulation through direct delivery of the site-specific recombinase Cre was achieved, a remarkable result considering the lack of reports on DNA-free genome modifying protein delivery to plants[122]. This method boosted the delivery efficiency by up to 20% but has only been demonstrated in immature maize tissues and onion epidermal tissues, not intact tissues. In general, reports of silicon-mediated genetic manipulation since 2015 are limited likely because it still requires biolistics for delivery and due to the success of carbon-based and metallic-based delivery systems, several of which are biolistics independent.

Bio-inspired NPs. Delivery of genetic cargos to plant cells using bio-inspired NPs, such as liposomes or vesicles, has been limited to protoplast or immature plant tissue. For example, liposome-based protoplast transfection can deliver DNA plasmid coding for site-specific nuclease Cas9 (ref. [123]) and assist the direct delivery of Cas9 (ref. [104]). Using a

commercial liposome transfection agent, Lipofectamine 3000, DNA-free direct delivery of Cas9 ribonucleoprotein (RNP) achieved editing efficiencies of 6% compared to 3% through macro-gold biolistic delivery[104]. Similarly, a cell-penetrating peptide-decorated vesicle system delivered Cas9 RNP to *Arabidopsis* callus, although with an editing efficiency below 1%[25]. Liposomes can also be used to deliver nutrients, such as iron, to cells of intact plants[124]. After foliar application, liposomes based on lecithin with 16–18-carbon chain backbone traversed the waxy cuticle and translocated to roots and distal leaves. These results pose liposomes as promising candidates for genetic delivery not only to protoplasts but also to intact plants; however, further investigation in mature plants is required. Composition will likely dictate liposomal delivery efficiencies, thereby providing an important research focus to enable informed design. Endogenous vesicle trafficking systems involved in plant–fungal interactions could provide a starting point for future work in liposomal-based delivery[125].

Delivery of site-specific nucleases

CRISPR–Cas. CRISPR–Cas radically simplified the process of genetically manipulating an organism. By generating precise double-strand DNA breaks, CRISPR tools enable either knockout of endogenous genes or insertion of new genes[126]. However, gene insertion in plants using CRISPR remains challenging compared to gene knockout. CRISPR–Cas-mediated modification is obtained either by DNA-encoded expression of endonuclease and guide RNA (sgRNA) reagents in cells, or by direct delivery of the CRISPR reagents, either separately or as RNP complexes, to cells. Expression of reagents in transgenic CRISPR–Cas plants has enabled editing of over 40 plant genera[127], including model and crop plants such as *N. benthamiana*[128], peanut[129], wheat[130], banana[131] and citrus[132]. However, stable transgene integration of reagents is undesired owing to possible off-target edits when CRISPR is constitutively expressed, particularly for crop plants where modification must abide by strict regulations. Transient expression of CRISPR reagents[133] is an alternative method; however, plants modified in this manner require regeneration if stably edited lines are desired. Another option to avoid transgene integration is to directly deliver RNPs. Thus far, in plants, direct delivery of RNPs has only been demonstrated using protoplast transfection[48] and particle bombardment[134], typically with sub-10% editing efficiencies depending on the method, genomic target and species. Nano-mediated and non-biolistic delivery of RNPs to whole plants is a promising approach to avoid the challenges of transgene integration and regeneration. Additionally, nano-delivery of CRISPR reagents could enable rapid screening and validation of sgRNA–endonuclease candidates to expedite the process of testing different sgRNA in planta.

Non-biolistic delivery of CRISPR–Cas reagents, particularly endonucleases, is challenging owing to their size, charge and stability. The most widely studied CRISPR endonucleases measure within 100–160 kDa (ref. [135]) and are therefore difficult to deliver past the cell wall. Furthermore, as opposed to DNA and siRNA, RNP function is strongly tied to its structure. As such, RNP attachment chemistries to NP carriers must be carefully designed to account not only for cargo intracellular accessibility but also for cargo function. RNP charge also complicates attachment; Cas9, for example, is highly positive, which could result in electrostatic interactions with NPs that prevent unloading at the site of interest. Finally, RNPs are susceptible to physical and chemical degradation, which reduces efficiency in planta[136,137]. Delivery of small endonucleases, such as Cas14, which are ~40–70 kDa (ref. [135]), is a promising starting point for the non-biolistic delivery of RNPs in plants. However, chemistries that optimize RNP attachment to carriers and function are required. Furthermore, the complexity of the CRISPR–Cas system hinders nanocarrier design for RNP delivery. Lessons can be learned from the nano-delivery of siRNA, which has been reported for a wide variety of NP-based carriers with high efficiencies[26,27,113]. Delivery of sgRNA could use similar strategies, the most promising of which might be chemical (as opposed to physisorption) attachment of sgRNA to nanocarriers to help retain the unique sgRNA structure needed for genome editing function, and these must be validated in either transgenic or transient Cas9-expressing plants. Moreover, cell-penetrating peptide-based protein delivery has achieved non-biolistic delivery of proteins as large as ~40 kDa (ref. [138]), an approach that could be extended for the delivery of small endonucleases.

TALEN. Similar to CRISPR–Cas, transcription activator-like effector nuclease (TALEN) enzymes enable genome editing through the generation of precise double-stranded breaks in DNA[139]. In the TALEN system, targeted gene editing is achieved by fusing TAL effectors to FokI nucleases; TAL effectors anneal to only specific DNA sequences determined by an ~34 amino acid-long binding domain enabling breaks at precise genomic locations by dimerized FokI nucleases. As with CRISPR systems, TALEN editing is primarily achieved through the delivery and expression of coding plasmid DNA. TALEN-based genome editing has been reported in a variety of species, including *Arabidopsis*[140], potato[141], tomato[142], rice[143] and wheat[144]. Similar to RNP delivery in plants, direct delivery of TALEN reagents without biolistics is desirable to address regulatory concerns. However, because TALEN reagents work in pairs, direct delivery is particularly challenging, requiring two simultaneous delivery events. Direct delivery in protoplasts reaches only 1.4% editing efficiency owing, in part, to protease degradation and the necessity of simultaneous delivery of reagents[145]. TALEN delivery in plants could be improved by nanocarriers to protect cargo from degradation and increase the likelihood of simultaneous delivery by maintaining high reagent concentrations near the target site.

Nano-mediated regeneration

Although germline transformation is perhaps the most promising application of nano-delivery to circumvent regeneration and reduce chimerism, nano-mediated approaches could also have a role in simplifying the process of regeneration (Fig. 3). Pathways controlling cell identity are highly sensitive to external conditions and are regulated by manipulating the ratio of auxins to cytokinins, two different classes of plant hormones. High auxin to cytokinin ratios promote totipotency, whereas low ratios foster differentiation and shoot growth[146]. During the regeneration process, auxin and cytokinin hormones, often termed plant growth regulators, are added to plant calli, embryonic tissues or suspension cells at predetermined concentrations and timings. The ability of nanocarriers to protect and release cargo in a controlled manner enables precise temporal and compositional control over plant regeneration conditions. For example, nanomaterial-based regeneration approaches have been used to deliver and detect plant growth regulator hormones necessary for tissue regeneration[147]. Delivery systems include chitosan nanocarriers[148] and bidirectional pH-responsive supramolecular nanovalves[149], both of which delivered the plant growth regulator gibberellic acid. For tissue culture, gold-capped MSNs delivered the auxin plant growth regulator 2,4-dichlorophenoxyacetic acid to *Linum usitatissimum* callus[150]. Compared to growing on auxin-rich medium alone, callus grown in the presence of MSNs loaded with auxin promoted cell embryogenesis by approximately

threefold. NPs have also found application in tissue culture to reduce contamination, stimulate growth (calli induction, organogenesis, shoot growth and rooting) and encourage secondary metabolite accumulation[151].

Nano-mediated approaches could also be used to deliver plant developmental regulators such as *WUSCHEL*, *PLETHORA* and *BABY BOOM*. These transcription factors promote embryogenesis and simplify plant regeneration in various recalcitrant species such as sorghum and indica rice[35,152–154]. Similarly, growth-regulating factors and associated interacting agents have also been shown to improve regeneration efficiency in various monocot and dicot plants[36,155]. In mammalian systems, NPs have been used to deliver[156] and mimic[157] transcription factors, strategies that could inform the use of developmental regulators in plants. Currently, using transcription factors in plant regeneration involves expression through transgene integration in the plant of interest. Subsequent deletion of the transgene is laborious, requiring cross-out through breeding or additional genetic engineering. DNA-free direct delivery of transcription factors could be implemented into the regeneration process; however, like DNA-free delivery of site-specific nucleases, this is challenging owing to the size, charge and stability of these factors. Thus far, there is only one report suggesting that the transcription factor WUSCHEL can be delivered to plants with cell-penetrating peptides[138]. Conveniently, many plant transcription factors responsible for developmental regulation are <70 kDa and might therefore be more amenable to overcoming size exclusion barriers for in-plant delivery than RNPs.

Nano-mediated transformation

Nano-mediated transformations could enable the manipulation of recalcitrant crop species to impart desirable traits such as pest and drought resistance, or improved nutritional content through biofortification. Furthermore, precise and species-independent manipulation with nanotools could advance modern molecular farming technologies such as edible plant biologics and plant suspension cell production methods.

Plants naturally produce essential nutrients and secondary metabolites beneficial for human health[158]. When these micronutrients are produced at low concentrations or are completely absent from crops in a particular region, nutritional deficiencies can become endemic[159]. This problem can be solved by crop biofortification, that is, enhancing crop nutritional value, which can be achieved by classic plant breeding or by metabolic engineering. For example, increased anthocyanin production in tomatoes was obtained through traditional breeding over the course of 20 years ('Sun Black' tomatoes with elevated anthocyanin in their skin)[160], and separately by introducing exogenous genes into tomatoes, including two transcription factors from *Snapdragon*, that improved anthocyanin production[161]. The first commercialized CRISPR–Cas9-generated crops were biofortified tomatoes with increased levels of γ-aminobutyric acid through mutation of an auto-inhibitory domain in a key enzymatic step to increase its activity[162].

Heterologous expression of enzymes or transcription factors that control the expression of several other enzymes in a pathway

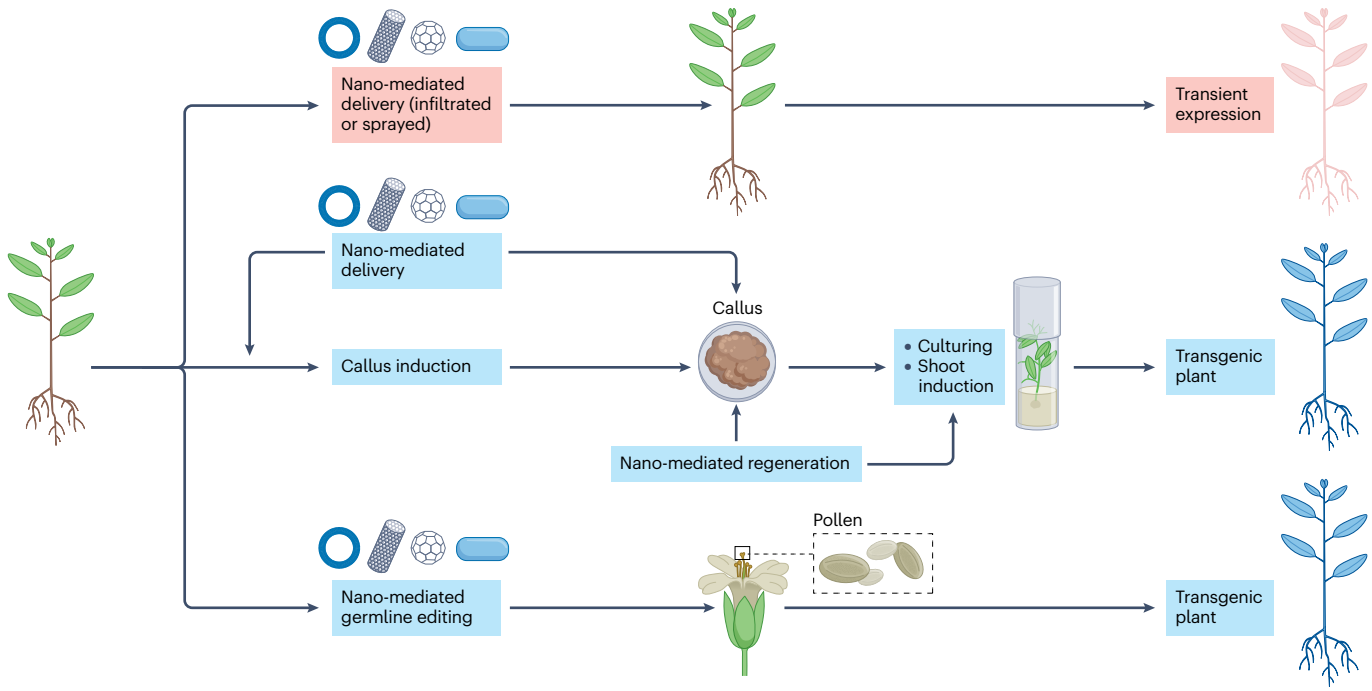


Fig. 3 | Nano-mediated transformation of plants. Nano-mediated delivery enables transient modification of plants in a species-independent manner. Nano-mediated delivery and regeneration enable stable transgenic modification of recalcitrant species. Callus induction could be simplified by nano-mediated delivery of growth-regulating factors, whereas subsequent regeneration could be mediated with nano-delivered hormones. Nano-mediated editing of germlines could circumvent the need for regeneration, improving the throughput of genetically transforming plants. Direct targeting of plant reproductive tissues with nanocarriers could enable stable transformation of progeny capable of passing on transformed traits. Pollen is just an example of a germline tissue that could be substituted in this workflow to accomplish the generation of a transgenic plant without tissue culture.

can also introduce new beneficial metabolites not naturally present in a particular crop, like provitamin A in Golden Rice[161,163]. This is a paramount example illustrating the challenges of biofortification of crops, requiring a multidecade-long effort for re-engineering of expression cassettes alongside compartmentalization and discovery of more active enzymes to increase production of provitamin A[164,165]. Crops such as pepper, cereal grains and specific cultivars of rice remain recalcitrant to modification owing to challenges in delivery and regeneration. These crops, owing to their prevalence in global diets, could benefit from biofortification, including iron and vitamin A fortification. Nano-mediated transformation could be particularly impactful in expanding the array of crops amenable to biofortification and in improving the throughput of the process.

Nano-mediated transformation could also enable the broader use of plants in the production of therapeutics and vaccines, whereby plants could serve as edible vectors for these products. The use of plants as bio-factories to produce therapeutics requires expression of the reconstituted natural product pathways or of a non-native target protein at sufficient levels. Achieving therapeutic efficacy through in planta expression is challenging owing to difficulties in genetic transformation and lack of metabolic pathway mapping[166,167]. Traditionally, elucidation of natural and/or installation of exogenous pathways required long-term multigroup efforts, as exemplified by Golden Rice[163]. Currently, thanks to high-throughput sequencing[168] and multi-omics characterization of pathways for the production of whole, natural products, genetically diverse biosynthetic pathways can be rapidly explored and reconstructed[166,167]. Separately, the adoption of plant suspension cell cultures is desired for the continuous production of valuable therapeutics from totipotent and continuously regenerating cells[169,170], which could circumvent the slow and variable agricultural cycle needed to work with whole plants. NPs could contribute to plant suspension cell culture workflows by providing an abiotic alternative to the delivery of DNA, RNA or proteins without requiring simultaneous optimization of co-cultures for plant cells and *Agrobacterium*.

Another substantial advance in improving the throughput of plant genetic manipulation is the ability to analyse large data sets such as those generated by multi-omics. Machine learning-assisted de novo generation of orthogonal synthetic regulatory elements has helped regulate and express biosynthetic pathways in plants or to analyse the complex metabolomics fingerprints that contain plant natural products[171]. However, this approach sacrifices mechanistic understanding in favour of predictive power. Practically, machine learning has been used to discriminate between genes involved in primary and secondary metabolism in *Arabidopsis*[172] and to breed tomato and blueberry plants for consumer-oriented flavour traits based on the analysis of their metabolic profiles; however, without mechanistic understanding, it can be difficult to translate predictions to other crops or gene targets[173]. In the future, computational advances could be merged with nano-based plant biotechnologies; for example, the slew of new siRNA-based NPs[26,112,174] could help confirm machine learning predictions of gene function in natural product biosynthetic pathways by using NPs to knock down predicted genes to assay their functional metabolic outputs.

Outlook

Here, we reviewed challenges in plant bioengineering and discussed opportunities in the emerging field of nano-based plant biotechnology to advance plant genetic engineering, as well as post-transcriptional manipulation and engineering of non-model plant species. Plant biotechnology research has highlighted a diverse array of nanocarriers capable of delivering different cargos. Defining structure-development design rules through first-principles models, such as LEEP[96,97] and fundamental studies of NP–plant interactions[21,27,98], has proven successful in enabling the rational design of carriers. Nanocarriers, particularly carbon-based and gold-based NPs, offer species-independent delivery of DNA and RNA. Furthermore, NPs can achieve subcellular site-specific delivery to chloroplasts and mitochondria through the targeting of peptide sequences or rational design via LEEP heuristics. Finally, despite reproducibility concerns, preliminary studies of pollen transformation using NPs warrant additional investigation with the long-term potential to eliminate the hurdle of regeneration.

Challenges remain to fully achieve the potential of nano-mediated delivery in plants, including expansion of structure–function design rules to enable direct germline editing and delivery of large cargos such as DNA plasmids larger than 10 kb and functional proteins. Preliminary work in pollen transformation of maize lays the groundwork for future studies to expand the reliability and species amenability of direct germline transformation. A fundamental understanding of pollen–NP interactions is currently lacking; a systematic study of the effect of size, shape and charge on NP localization in pollen grains of different species is necessary to enable pollen transformation. Beyond pollen studies, other germline tissues, such as ovules, or pluripotent tissues, such as meristems, could serve as targets for NP-mediated delivery, also with the end goal of eliminating the need to regenerate tissues. Progress towards the delivery of large cargos remains slow. Biolistic delivery and PEG transfection of proteins, despite their low efficiency, could inform nano-mediated strategies for DNA-free, non-biolistic plant genome editing with site-specific nucleases. Cell-penetrating peptides successfully delivered proteins up to 40 kDa in size and should be investigated further to establish the upper limits of cargo size and develop design rules for cell-penetrating peptides. Taking a cue from animal delivery, homeodomain proteins, which are naturally cell-penetrating to animals[175], could serve as templates for new peptide-based delivery technologies in plants. Coupling the internalization capability of cell-penetrating peptides with the ability of NPs to protect cargos from degradation is a promising approach to improve delivery efficiencies.

Similarly, nano-mediated regeneration could improve the efficiency and throughput of plant transformations given that direct germline editing is currently not technically feasible. For example, nano-mediated delivery of hormones, thus far demonstrated with chitosan NPs[148], could enable precise control over regeneration media or even in vivo hormone composition to influence cell identity. Plant cell identity is tightly regulated by local maxima in auxin concentrations, as demonstrated by hormone transport phenomena in planta[176,177]. Continuous in planta release of auxin using NPs could serve to perturb natural boundary conditions, which regulate the formation of auxin maxima. First-principles investigation of plant hormone transport with the added complexity of NP boundary conditions could provide insight into methods for manipulating auxin maxima to enable finer control of cell identity during regeneration. Separately, direct delivery of developmental regulatory transcription factors can promote regeneration even in recalcitrant species and expand the set of plant species and genotypes amenable to regeneration. Currently, cell-penetrating peptides represent a promising avenue for the direct delivery of transcription factors in plants, yet design rules for delivering large transcription factor cargos are needed.

Box 2

Societal and regulatory considerations

The relationship between the public, governments and genetically modified plants (GMPs) has long been complex. Across the global landscape, public opinion and regulatory approaches differ substantially[193]. Nonetheless, GMP cultivation has expanded 100-fold in the last 25 years, covering over 190 million hectares across 29 different countries[194]. The majority of GMPs are used in industrial applications or animal feed and not for direct human consumption, reflecting public scepticism of GMPs. GMP cultivation is primarily concentrated in the United States, Brazil, Argentina, Canada and India, accounting for 91% of GMP production[193]. Adoption of GMPs in Europe, Africa and Asia, especially South-East Asia, is limited, where regulations are tight or there is an outright ban on the cultivation and/or import of GMPs. New site-specific editing techniques have not been fully addressed by regulatory bodies, but preliminary legislations suggest governments will take a variety of approaches in regulating crops produced using, for example, CRISPR.

Biofortification of crops requires genetic manipulation which, under most regulatory frameworks, would result in crop classification as a GMP. Furthermore, introducing traits, such as pest, disease and drought resistance, important for maintaining yields in a changing climate, would face similar classification. Finally, edible biologics, which carry substantial technical challenges, such as reconstitution of exogenous pathways into non-model plants for the production of therapeutics, would also likely be met with public scepticism and resistance. For example, Golden Rice, a product that involved a multidecade effort to demonstrate lab-scale technical feasibility in 2000 and successful field trials in 2005 (ref. [195]), was developed to address vitamin A deficiencies in at-risk populations in Africa and South-East Asia. Nonetheless, Golden Rice has only been approved in one of the original target region countries so far, the Philippines. Unfortunately, to this day, vitamin A deficiencies remain most prevalent in Africa and South-East Asia and available GMP technologies, which could provide relief, are yet to be adopted[196]. Among the causes for this lack of adoption, the scientific community has pointed to anti-GMP campaigns (which primarily originate from non-governmental organizations in developed countries that disproportionately impact developing ones), the desire to protect local flora, fauna and culture, concerns about privatization of GMP technology, and distrust of foreign governments or corporations[197–199]. For these reasons, GMP advances are slow but nonetheless necessary to ensure food security in the face of climate change. Unification and streamlining of regulatory schemes, in parallel with technical advances, are required to avoid stalling progress in GMP development.

Besides these technical challenges, public resistance to genetic modification of plants presents additional hurdles. The negative perception of genetically modified plants remains difficult to overcome particularly as public perspectives and governmental regulations towards genetically modified plants vary considerably (Box 2). For example, in the European Union, the regulatory process is product based, whereby the genetic state of the end product determines the governing regulations. By contrast, in the United States, regulation is semi-product based, with certain processes prompting automatic triggers for regulation regardless of the genetic state of the end product. Given this heterogeneous landscape, site-specific nuclease edits, particularly through DNA-free delivery, are attractive approaches to avoid the regulatory burdens of traditional genome editing. Nano-based delivery strategies could contribute by providing either non-integrating alternatives for DNA delivery[23] or by enabling direct delivery of RNA and proteins[112,113,138].

NPs applied to plant bioengineering have been used to induce stress priming in crops, sensors, nutrient and nucleic acids delivery vehicles and hormone-dosing tools in regeneration. However, their rapid emergence has made it difficult for regulatory bodies to keep pace with their applications. Although several experts have long pushed for regulatory bodies to impose pre-market safety assessment of nanomaterials[178], to date, comprehensive regulations for nanomaterial use in plant and environmental science are non-existent, especially outside the European Union[179]. Furthermore, the role of NPs in plant genetic manipulations, particularly if high-efficiency protein delivery is achieved, generates an interesting debate about what constitutes a genetically engineered organism in the absence of DNA integration or pathogen application, potentially changing the genetically modified organism landscape[180]. Therefore, future progress in nano-based agriculture includes not only technological advances but also policy development, balancing the full technical potential of NP-based technologies with the development of frameworks designed to evaluate their safety and, when necessary, regulate their usage.

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