Nanoparticle-Mediated Delivery towards Advancing Plant Genetic Engineering

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Genetic engineering of plants has enhanced crop productivity in the face of climate change and a growing global population by conferring desirable genetic traits to agricultural crops. Efficient genetic transformation in plants remains a challenge due to the cell wall, a barrier to exogenous biomolecule delivery. Conventional delivery methods are inefficient, damaging to tissue, or are only effective in a limited number of plant species. Nanoparticles are promising materials for biomolecule delivery, owing to their ability to traverse plant cell walls without external force and highly tunable physicochemical properties for diverse cargo conjugation and broad host range applicability. With the advent of engineered nuclease biotechnologies, we discuss the potential of nanoparticles as an optimal platform to deliver biomolecules to plants for genetic engineering.

Current Biomolecule Delivery Methods for Genetic Engineering in Plants

Food security has been threatened with decreasing crop yields and increasing food consumption in the wake of population growth, climate change, increasing shortage of arable land, and crop usage as raw materials [1,2]. Classical plant breeding to obtain plants with preferred genotypes requires crossing and selection of multiple plant generations, which disallows introduction of traits that do not currently exist in the species. A technique that enables specific horizontal gene transfer stands to greatly benefit the agricultural industry by conferring desirable traits to plants, such as increased yield, abiotic stress tolerance, and disease and pest resistance [3].

Genetic engineering has recently seen major advances in animal systems, though progress has lagged in plants. When compared to the numerous and diverse gene and protein delivery methods developed for animal systems, significantly fewer methods exist for plants (Figure 1, Key Figure). Broadly, modern genetic transformation of plants entails two major steps: genetic cargo delivery and regeneration of the transformed plant, the necessity and difficulty of the latter being highly dependent on what delivery method is used and whether stable transformation is desired. Regeneration procedures involve three parts: the induction of competent totipotent tissue, tissue culture to form calli (see Glossary), and selection and progeny segregation. Regeneration protocols are dominated by complex hormone mixtures, which are heavily species and tissue dependent, making protocol optimization the key to increasing procedure efficacy. The challenge of genetic cargo delivery to plants is attributed to the presence of the multilayered and rigid plant cell wall, otherwise absent in animal cells, which poses an additional physical barrier for intracellular delivery of biomolecules and is one of the key reasons for the slower implementation and employment of genetic engineering tools in plants [4].

Amongst conventional plant biomolecule delivery approaches, Agrobacterium-mediated and biolistic particle delivery are the two most established and preferred tools for plant genetic transformations (Box 1). Current biomolecule delivery methods to plants experience challenges amongst conventional plant biomolecule delivery approaches, Agrobacterium-mediated and biolistic particle delivery are the two most established and preferred tools for plant genetic transformations (Box 1). Current biomolecule delivery methods to plants experience challenges
that hinder their scope of use (Table 1). Methods such as electroporation, biolistics, Agrobacterium-mediated delivery, or cationic delivery typically target immature plant tissue (calli, meristems, or embryos). These methods require the regeneration of genetically modified progeny plants, which can be time-consuming and challenging, whereby efficient protocols have only been developed for a narrow range of plant species. Biolistic particle delivery circumvents the cell wall via mechanical force, but often damages portions of target tissue in the process and yields low levels of gene expression that is often sparse and sporadic. Agrobacterium-mediated delivery is subject to orthogonal challenges, the largest being that Agrobacterium displays narrow host and tissue specificity, even between specific cultivars of the same species [5]. Agrobacterium generally experiences lower transformation efficiency for both delivery and regeneration in monocotyledonous plants (monocots) over dicotyledonous plants (dicots). Additionally, Agrobacterium yields random DNA integration, which can cause disruption of important genes, or insertion into sections of the genome with poor or unstable expression [6]. Random DNA integration, however, can be prevented by utilizing magnification with nonintegrating viruses [7], or by using a plasmid deficient in transfer DNA (T-DNA) insertion [8].

In sum, plant genetic engineering has lagged behind progress in animal systems; conventional methods of biomolecule delivery to plants remain challenged by intracellular transport through cell walls, and in turn limit plant genetic transformation efficacy. To date, plant biotechnology lacks a method that allows passive delivery of diverse biomolecules into a broad range of plant phenotypes and species without the aid of external force and without causing tissue damage. We posit nanotechnology as a key driver in the creation of a transformational tool to address delivery challenges and enhance the utility of plant genetic engineering.

Nanoparticle-Mediated Biomolecule Delivery in Animal Systems

Nanoparticles as Molecular Transporters in Living Systems

Nanotechnology has advanced a variety of fields, including manufacturing, energy, and medicine. Of particular interest is the use of nanoparticles (NPs) (Box 2) as molecular transporters in cells, an area that has largely focused on molecular delivery in animal systems. NPs allow manipulation on a subcellular level, giving rise to a previously unattainable degree of control over exogenous interactions with biological systems. Therefore, the impact of NPs as drug and gene delivery vehicles in animals has been nothing short of revolutionary.

The small size of NPs and their highly tunable chemical and physical properties have enabled NP engineering for NPs to bypass biological barriers and even localize NPs in subcellular domains of CHO and HeLa cells, among others [10–13]. NPs serve as nonviral, biocompatible, and nontoxic vectors that can transport a range of biomolecules [small molecules, DNA, siRNA, miRNA, proteins, and ribonucleoproteins (RNPs)] [14–19] to biological cells. To this end, various features of NPs, including size, shape, functionalization, tensile strength, aspect ratio, and charge, have been tuned for efficient intracellular biomolecule delivery to animal systems. Furthermore, ‘smart’ NPs have been developed to achieve responsive release of cargo for increased control of site-specificity [20]. Various NPs have been manufactured and are responsive to a range of stimuli, including temperature [21], pH [22], redox [23], and the presence of enzymes [24].

Outlook and Implications for Nanocarriers in Plant Science

In contrast to the proliferate studies demonstrating NP-mediated delivery in animals, analogous research in plants is relatively sparse (Figure 1), owing to the transport challenge imposed by the plant cell wall, which renders biomolecule delivery more challenging than for most mammalian systems.
Nevertheless, knowledge gained from biomolecule delivery to animals provides a blueprint for translation to plant systems, and could accelerate advancements in NP-mediated plant biomolecule delivery. NP-mediated delivery may overcome the three foremost limitations of current delivery techniques in plant systems by controlling NP size to traverse the cell wall, tuning charge and surface properties to carry diverse cargo, and greater breadth in utility across plant species.

NP-mediated delivery in animals has successfully carried many types of cargo indiscriminately, whereby certain methods for plants, such as Agrobacterium, can only deliver DNA. For instance, Wang and colleagues report NP-mediated RNP delivery to mammalian cells via lipid encapsulation [25]. Additionally, plastid engineering is not achievable with Agrobacterium, which only targets the plant nuclear genome and cannot target the chloroplast or mitochondrial genomes. Conversely, targeting moieties can be attached to NPs to obtain subcellular localization and modification of the desired genome. Hoshino and coworkers demonstrate the delivery of quantum dots to the nucleus and mitochondria of Vero kidney cells using respective localizing signal peptides [26]. Active targeting and controlled release is not achievable with conventional plant biomolecule delivery methods, but has been demonstrated in animal systems with NP-based delivery. Davis and colleagues designed a polymeric NP with a human transferrin protein-targeting ligand and polyethylene-glycol (PEG) on the NP exterior to deliver siRNA to human melanoma tumor cells, specifically [15]. Additionally, Lai and coworkers accomplished stimuli-responsive controlled release of drug molecules and neurotransmitters encapsulated within mesoporous silica NPs (MSNs) to neuroglial cells [27]. Drawing inspiration from progress in NP-mediated delivery for animal systems, NP-mediated controlled delivery and release of biomolecules without species limitations in plants is a forthcoming goal.

NP-Mediated Biomolecule Delivery to Plants

NP–Plant Interactions

To date, most literature on NP–plant systems focuses on plant-based metallic nanomaterial synthesis [28], agrochemical delivery [29], and NP uptake, showing both valuable and deleterious effects on plant growth [30,31]. Dicot and monocot plants exhibit variable degrees of direct uptake of many NP types, including MSNs [32], carbon nanotubes (CNTs) [33], quantum dots [34], and metal/metal oxide NPs [35–37]. Once uptake, certain types of NPs exhibit phytotoxicity via vascular blockage, oxidative stress, or DNA structural damage [30]. Conversely, NPs have been shown to improve root and leaf growth, and chloroplast production [31]. Tradeoffs between phytotoxicity and growth enhancement as a function of species, growth conditions, NP properties, and dosage are not well understood and call for more studies with a focus on NP physical and chemical properties. Closing the knowledge gap in plant physiological response to NP uptake is important and should be pursued in parallel with the enhancement of plant science using engineered nanomaterials, as the ‘nanorevolution’ in targeted delivery to animals suggests tremendous potential for analogous progress in plants.

Heuristics for Nanocarrier Design

While a complete structure–function landscape of physical and chemical NP properties that drive cargo loading and cellular internalization remains elusive, a heuristic approach to nanocarrier design is a useful starting point. NP uptake and transport throughout plant tissue is limited by pore diameters, setting size exclusion limits (SELs) for various tissues and organs that are discussed extensively in the literature [30,38–43]. The cell wall is commonly thought to exclude particles >5–20 nm, although recently NPs up to 50 nm in diameter have been reported as cell wall-permeable through unclear mechanisms [38,41]. For genetic engineering applications, where cytosolic or nuclear localization is necessary to affect gene function, the plasma and nuclear membranes pose additional barriers to delivery. In practice, the cell wall (SEL <50 nm) plays a dominant role in NP size
internalization limitations, as the cell membrane SEL is much larger (>500 nm) [38]. NP charge and shape greatly influence cell membrane translocation and thus these properties are central to nanocarrier optimization [44]. Plant cellular uptake can occur through energy-dependent (endocytosis) and energy-independent (direct penetration) pathways that are not well understood. It is commonly reported that internalization is faster and more efficient for cationic NPs versus anionic NPs, due to cationic NP binding with the negatively charged cell membrane [44]. This charge preference has been demonstrated in protoplasts and walled plant cells [45,46].

Endosomal escape is critical for subcellular delivery, as vesicle-entrapped NPs can be trafficked for degradation or exocytosis, and remain inaccessible for downstream processing if trapped in the endosome. Subcellular localization of NPs in plants is not well understood but will depend on the uptake pathway, as endocytic proteins and vesicle cargo play a role in endosome fate [47], whereby direct cell penetration bypasses endosomal vesicle formation entirely. Serag and colleagues report CNT internalization in protoplasts through both direct penetration and endocytosis, supporting prior demonstrations in mammalian cells that high aspect ratio NPs undergo vesicle-free internalization [48,49]. However, for Serag and colleagues, direct penetration was only observed for cell wall-impermeable multiwalled CNTs in protoplasts [48,49], motivating further studies for plant cell wall internalization by high aspect ratio NPs. Wong and colleagues have demonstrated passive internalization of single-walled CNTs in extracted chloroplasts [129] through a mechanism dependent on NP size and zeta potential [130]. Cationic, pH-buffering polymers are well-known endosome disruption agents [50] that can function as ligands to improve endosomal escape. Chang and colleagues report energy-independent internalization to walled root cells by organically functionalized spherical MSNs [51]. Notably, endocytosed single-walled CNTs in plants are trafficked to vacuoles but localize in the cytosol when loaded with DNA [33,48].

Most NPs are amenable to surface adsorption (physisorption) of biomolecules as a simple conjugation strategy. However, physisorption may be unstable depending on the specific NP and cargo, and thus electrostatic interactions are preferable for noncovalent cargo loading [52]. Cationic surface chemistry not only enhances endocytic uptake and escape, but is also amenable to electrostatic loading of genetic cargo via attraction with negatively charged DNA and RNA. Covalent NP surface functionalization is typically achieved by one of many ‘click’ chemistries [53]. Notably, covalent attachment of thiolated DNA and proteins to gold NPs has shown recent success [54] but the field remains open to new strategies for covalent bioconjugation, especially for applications in plants. As an alternative to surface functionalization, porous NPs such as MSNs can be internally loaded with macromolecules or small chemicals alike, for controlled intracellular release [55].

NPs with some or all of the properties mentioned above have demonstrated successful biomolecule delivery in plants and are good starting points for choosing the appropriate NP, ligand, and cargo for a given application. However, it should be noted that nanocarrier design is a complex, multivariable optimization process, such that success will likely require tweaking of these heuristics for different systems until a complete NP structure–function relationship is established for plant systems.

Nanomaterials for Plant Genetic Engineering

NPs are valuable materials for intracellular biomolecule delivery, owing to their ability to cross biological membranes, protect and release diverse cargoes, and achieve multifaceted targeting via chemical and physical tunability. Such properties have enabled NPs to revolutionize targeted delivery and controlled release in mammalian systems. However, nanocarrier delivery in plants remains largely underexplored due to the cell wall, which is typically overcome by chemical or mechanical aid (Figure 1). Passive biomolecule delivery to plants is promising for minimally invasive, species-independent, in vivo genetic engineering of plants, especially for transient
Key Figure
Nanoparticle (NP)-Mediated Genetic Cargo Delivery to Animals and Plants

(A) NPs classes commonly employed in genetic cargo delivery

Bio-inspired NPs
- Calcium phosphate
- Chitosan
- Liposomes

Genetic cargo delivered
- DNA
- RNA
- Protein
- RNP

Metallic / Magnetic NPs
- Gold
- Silver
- Iron oxide

Genetic cargo delivered
- DNA
- RNA
- Protein
- RNP

Carbon-based NPs
- Single-walled carbon nanotubes
- Multiwalled carbon nanotubes
- Fullerenes

Genetic cargo delivered
- DNA
- RNA
- Protein
- RNP

Silicon-based NPs
- Silicon spheres
- Mesoporous silica NPs (MSNs)
- Silicon carbide

Genetic cargo delivered
- DNA
- RNA
- Protein
- RNP

Polymeric NPs
- Polyethylene-glycol (PEG)
- Poly(lactic-co-glycolic acid) (PLGA)
- Polyethylenimine (PEI)

Genetic cargo delivered
- DNA
- RNA
- Protein
- RNP

(B) Modes of NP-mediated cargo delivery

Biolistic

Electroporation / sonoporation / optoporation

Magnetofection

Microinjection

Cationic transfection

Incubation

Infiltration

Figure 1. (A) NPs commonly used for biomolecule delivery in both animal and plant systems cover five major categories: bio-inspired, carbon-based, silicon-based, polymeric, and metallic/magnetic. We provide a visual comparison of delivery of various genetic cargo (DNA, RNA, proteins (site-specific recombinases or nucleases), and ribonucleoprotein (RNP)) with each of the five NP types across animal and plant systems. It is evident that NP-mediated delivery has been utilized with a greater variety of genetic cargo in animals than in plants. (B) NP-mediated cargo delivery is conducted via several means. Physical methods include creating transient pores in the cell membrane with electric fields, soundwaves, or light, magnetofection, microinjection, and biolistic particle delivery. Nonphysical methods include the use of cationic carriers, incubation, and infiltration. [64], [86], [87], [88], [89], [90], [91], [92], [58], [93], [94], [95], [96], [97], [98], [99], [101], [100], [63], [102], [54].
expression in somatic tissue (Table 2). The potential of NP-based plant delivery methods is underscored by the limitations of in vitro plant studies in general, wherein regeneration capacity varies widely across species, genotype, and even within a single plant depending on developmental age of source tissue [56]. Currently, stable transformation requires progeny regeneration from embryogenic calli regardless of the delivery method (Table 2). Thus, parallel optimization of delivery and regeneration is necessary to improve efficiency and expand stable transformation capabilities to all plant species.

In 2007, Torney and colleagues were the first to demonstrate NP co-delivery of DNA and chemicals to Nicotiana tabacum plants via biolistic delivery of 100–200-nm gold-capped MSNs [45]. In this study, a chemical expression inducer was loaded into MSN pores (~3 nm) that were subsequently covalently capped with gold NPs. The capped MSNs were then coated with GFP plasmids and delivered by gene gun to N. tabacum cotyledons, wherein GFP expression was triggered upon uncapping and release of the expression inducer [45]. This seminal paper demonstrated proof of concept that strategies common for NP delivery of DNA to mammalian systems can be adapted to plants. Notably, gold MSNs were also used for biolistic co-delivery of DNA and proteins, namely GFP and Cre-recombinase, demonstrating the ability of MSNs to deliver proteins for gene editing [58]. Many delivery strategies still require a gene gun, electromagnetic field, or protoplast PEG-transfection [58–63] as NP structure–function parameters have not yet been fully optimized to passively bypass the cell wall (Table 3). However, for systems where mechanical or chemical aid is necessary for NP internalization, the small size and high surface area of nanocarriers still offers

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**Box 1. Common Gene Delivery Methods in Plants**

**Agrobacterium-Mediated Transformation**

*Agrobacterium tumefaciens* is a soil bacterium that infects a wide range of dicots, causing crown gall disease. The formation of a gall on the host plant is achieved via the stable transfer, integration, and expression of bacterial DNA in infected plants. Engineering of the *Agrobacterium* plasmid by substitution of the gall-inducing virulence genes with genes of interest confers the ability of *Agrobacterium* to transform the host plant. For this reason, *Agrobacterium* has been harnessed as a tool for plant genetic transformation since the early 1980s [107].

Genetic transformation occurs through a process involving T-DNA export, targeting, and insertion into the plant nuclear genome. The export of T-DNA from the bacterium to the plant cell is facilitated by the activity of virulence genes present in the tumor inducing-plasmid of *Agrobacterium*, but are not themselves transferred. These virulence genes are expressed in the presence of phenolic inducers, such as acetosyringone, produced by wounded plant cells. *Agrobacterium* attaches to plant cells, where border sequences on either side of the T-strand (a single-stranded copy of the T-DNA sequence) are cleaved. The T-strand is then carried by a transporter with a nuclear localization sequence and integrated into the plant nuclear genome. Integration occurs at random positions in the genome via nonhomologous recombination, a repair pathway for double-stranded breaks in DNA.

**Gene Gun-Mediated Transformation**

A form of biolistic particle delivery (also called particle bombardment), the gene gun, is a physical method that is commonly utilized for plant genetic transformations. Developed in 1982 by Sanford and colleagues [108], the process involves gold or tungsten microparticles (or microcarriers) coated with genetic cargo that are accelerated by pressurized helium (He) gas into plant cells, rupturing cell walls and membranes. The gene gun consists of three main parts: a rupture disk, macrocarrier (holding microcarrier particles), and stopping screen. The rupture disk is a membrane designed to burst at a critical pressure of He gas. When He gas is accelerated to the desired pressure, the rupture disk bursts, creating a shock wave that propels the macrocarrier towards the plant cells. The macrocarrier’s momentum is stopped by the stopping screen, which allows genetic cargo-loaded microcarriers to pass and enter the plant cells.

Unlike *Agrobacterium*-mediated transformation, biolistic delivery can result in transformation of the nuclear, plastidal, or mitochondrial genomes due to the nonspecific localization of genetic cargo. Consequently, more DNA needs to be delivered with biolistic delivery than *Agrobacterium*-mediated delivery when targeting the nuclear genome.
Table 1. Scope of Use Summary for Plant Biomolecule Delivery Methods

<table>
<thead>
<tr>
<th>Delivery method</th>
<th>Adverse effects of delivery</th>
<th>Target species/tissue</th>
<th>Cargo type and size</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biolistic particle-mediated delivery</td>
<td>Damage to target tissue &amp; cargo, low penetration depth, random integration</td>
<td>Depends on tissue type(^1)/call, embryos, leaves</td>
<td>DNA, siRNA, miRNA, ribonucleoproteins (RNPs), large cargo size</td>
<td>Targeting leaves requires detachment from plant, which limits time to observe delivery effects; targeting embryos requires laborious regeneration protocols, the effectiveness of which is highly species/cultivar-dependent</td>
</tr>
<tr>
<td>Electroporation</td>
<td>Damage to target tissue, nonspecific transport of material through pores may lead to improper cell function</td>
<td>Unlimited/protoplasts(^c), meristems, pollen grains</td>
<td>Nucleic acids (DNA, siRNA, miRNA)</td>
<td>Limited cargo-carrying capacity</td>
</tr>
<tr>
<td>Chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymer-mediated delivery</td>
<td>High charge densities induce cytotoxicity</td>
<td>Species amenable to protoplast regeneration/protoplasts(^c)</td>
<td>Nucleic acids (DNA, siRNA, miRNA)</td>
<td>Regeneration is highly inefficient for most species in transient studies and requires tissue culture</td>
</tr>
<tr>
<td>Biological</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agrobacterium-mediated delivery</td>
<td>Can lead to apoptosis and necrosis, random integration</td>
<td>Narrow range of plant species, especially restricted from monocots(^d)/mature plants, immature tissue, protoplasts</td>
<td>Limited to DNA, large cargo size</td>
<td>Leaf-targeted delivery is transient and gene edits are not transmitted to progeny, but allow diverse biological studies; requires tissue culture (except Arabidopsis) to generate progeny; exhibits high host-specificity</td>
</tr>
<tr>
<td>Viral delivery</td>
<td>Virus integration (can be mitigated by using nonintegrating viruses)</td>
<td>Host plant species restrictions/mature plants, meristems</td>
<td>Nucleic acids (DNA, siRNA, miRNA), very limited cargo size</td>
<td>Highly limited cargo-carrying capacity</td>
</tr>
</tbody>
</table>

\(^a\)While most biomolecule delivery methods to plants can deliver a variety of gene editing reagents, DNA plasmids are arguably the most common cargo of interest; DNA loading capacities are a useful metric for the upper limit for cargo sizes each method can sustain.

\(^b\)While biolistic particle-mediated delivery can theoretically be utilized in unlimited target species, the ability to target species depends on the target tissue (by extension, cell wall structural strength) and capability of available equipment.

\(^c\)The use of protoplasts as target tissue necessitates regeneration protocols and progeny segregation that are time-consuming and are challenged by the limited plant species amenable to protoplast regeneration.

\(^d\)Progress has been made on increasing transformation efficiency in recalcitrant monocots [9].
Cas mutations as small as 1 bp have been conserved through three plant generations [73, 74]. *Nicotiana benthamiana* has shown success for genome editing in both model and crop species, including and multiplexing capabilities over TALENs and ZFNs in plants [69, 70]. Since 2012, CRISPR-Cas has demonstrated increased simplicity, affordability, and multiplexing capabilities over TALENs and ZFNs in plants [69, 70]. Since 2012, CRISPR-Cas has shown success for genome editing in both model and crop species, including *A. thaliana*, *N. benthamiana*, *N. tabacum* (tobacco), *Oryza sativa* (rice), *T. aestivum* (wheat), *Zea mays* (corn), *Solanum lycopersicum* (tomato), and *Sorghum bicolor*, among others [71, 72]. Notably, CRISPR-Cas mutations as small as 1 bp have been conserved through three plant generations [73, 74],
Table 2. Challenges in Plant Genetic Engineering and Proposed Advantages of NP Delivery

<table>
<thead>
<tr>
<th>Desired outcome</th>
<th>Nonheritable* (somatic/transient expression)</th>
<th>Heritable (germline/stable transformation)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Targeted tissue</strong></td>
<td><strong>Leaves</strong></td>
<td><strong>Roots</strong></td>
</tr>
<tr>
<td>Tissue-specific biological and experimental challenges</td>
<td>• Cell wall</td>
<td>• Cell wall</td>
</tr>
<tr>
<td></td>
<td>• Inefficient cellular uptake</td>
<td>• Inefficient cellular uptake</td>
</tr>
<tr>
<td></td>
<td>• Epidermal barrier</td>
<td></td>
</tr>
<tr>
<td>Proposed advantages of NP delivery</td>
<td>• NP-cell wall permeability</td>
<td>• NP-cell wall permeability</td>
</tr>
<tr>
<td></td>
<td>• NP-stomata permeability [57]</td>
<td>• Cationic NP root accumulation [46]</td>
</tr>
<tr>
<td></td>
<td>• Anionic NPs root-to-shoot vascular translocation [46]</td>
<td>• Passive uptake without gene gun or protoplasts</td>
</tr>
<tr>
<td></td>
<td>• Passive uptake or direct mesophyll injection without gene gun or protoplasts</td>
<td>• Tunable NP properties and ligands for subcellular targeting</td>
</tr>
<tr>
<td></td>
<td>• Tunable NP properties and ligands for subcellular targeting</td>
<td></td>
</tr>
</tbody>
</table>

*While these somatic tissues (leaves, roots, protoplasts) are most commonly targeted for transient expression experiments, heritable outcomes may be derived through somatic embryogenesis (dedifferentiation of somatic tissue).
### Table 3. Select Summary of NP-Mediated Genetic Engineering in Plants

<table>
<thead>
<tr>
<th>NP type</th>
<th>Cargo</th>
<th>Plant species; cell/tissue type</th>
<th>Delivery method</th>
<th>Comments</th>
<th>Year</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>With external aid</td>
<td>Gold capped MSNs</td>
<td>GFP plasmid; chemical expression inducer</td>
<td>Biolistic</td>
<td>Co-delivery and controlled release of DNA and chemicals</td>
<td>2007</td>
<td>[45]</td>
</tr>
<tr>
<td>Poly-L-lysine coated starch NPs</td>
<td>GFP plasmid</td>
<td>Dioscorea zingiberensis C.H. Wright calli suspension</td>
<td>Sonoporation</td>
<td>5% transient expression efficiency; some integration occurs</td>
<td>2008</td>
<td>[60]</td>
</tr>
<tr>
<td>Gold-plated MSNs</td>
<td>GFP and mCherry plasmids; GFP protein</td>
<td>Allium cepa epidermis tissue</td>
<td>Biological</td>
<td>DNA and protein co-delivery</td>
<td>2012</td>
<td>[59]</td>
</tr>
<tr>
<td>Magnetic gold NPs</td>
<td>β-glucuronidase (GUS) plasmid</td>
<td>Brassica napus protoplasts and walled cell suspension</td>
<td>Magnetic field</td>
<td>Transient GUS expression</td>
<td>2013</td>
<td>[61]</td>
</tr>
<tr>
<td>Gold-plated MSNs</td>
<td>AmCyan1 and DsRed2 plasmids; Cre protein</td>
<td>Z. mays embryos</td>
<td>Biological</td>
<td>DNA and protein co-delivery; both transient and stable expression</td>
<td>2014</td>
<td>[58]</td>
</tr>
<tr>
<td>Dimethylaminoethyl methacrylate (DMAEM) polymer NPs</td>
<td>Yellow fluorescent protein (YFP) and GFP plasmids</td>
<td>N. tabacum and Ceratodon purpureus protoplasts</td>
<td>PEG transfection</td>
<td>Both transient and stable expression</td>
<td>2017</td>
<td>[62]</td>
</tr>
<tr>
<td>Magnetic Fe₃O₄ NPs</td>
<td>Selectable marker gene plasmids</td>
<td>Gossypium hirsutum pollen</td>
<td>Magnetic field</td>
<td>~1% efficiency for generating stable transgenic seeds</td>
<td>2017</td>
<td>[63]</td>
</tr>
<tr>
<td>In vitro without external aid</td>
<td>Polyamidoamine (PAMAM) dendrimer NPs</td>
<td>Agrostis stolonifera L. calli</td>
<td>Passive</td>
<td>48.5% cells showed transient expression</td>
<td>2006</td>
<td>[66]</td>
</tr>
<tr>
<td>Calcium phosphate NPs (CaP+NPs)</td>
<td>GUS plasmid</td>
<td>Brassica juncea hypocotyl explants</td>
<td>Passive</td>
<td>80.7% stable transformation efficiency</td>
<td>2012</td>
<td>[64]</td>
</tr>
<tr>
<td>Organically functionalized CNTs</td>
<td>YFP plasmid</td>
<td>N. tabacum protoplasts and leaf explants</td>
<td>Passive</td>
<td>Both transient and stable expression</td>
<td>2015</td>
<td>[66]</td>
</tr>
<tr>
<td>In vivo without external aid</td>
<td>Organically functionalized MSNs</td>
<td>mCherry plasmid</td>
<td>A. thaliana roots</td>
<td>46.5% transient expression efficiency</td>
<td>2013</td>
<td>[51]</td>
</tr>
<tr>
<td>PAMAM dendrimer NPs</td>
<td>Double-stranded DNA for RNA interference</td>
<td>A. thaliana roots</td>
<td>Passive</td>
<td>Developmental gene silencing led to systemic phenotypes</td>
<td>2014</td>
<td>[67]</td>
</tr>
<tr>
<td>Polymer functionalized CNTs</td>
<td>GFP plasmid; siRNA for transgenic GFP silencing</td>
<td>E. sativa, N. benthamiana, and T. aestivum leaves</td>
<td>Passive</td>
<td>95% transient silencing efficiency; transient expression in mature leaves</td>
<td>2014</td>
<td>[68]</td>
</tr>
</tbody>
</table>
Box 3. Traditional Genetic Engineering versus Nuclease-Enabled Genome Editing

Genetic engineering refers broadly to manipulating a cell’s genome and gene expression profile. Techniques for genetic engineering may cause recombinant protein expression, up/downregulation of a gene, permanent gene knockout, targeted mutations in the host gene, or insertion of large foreign DNA segments into the host genome. Genome modifications may be transient, permanent, or heritable and involve many types of biomolecules (most commonly RNA, DNA, and proteins) which are sometimes taken up passively by cells but often require enhanced delivery techniques, such as gene guns, microinjection, electroporation, sonoporation, nanoparticle-assisted delivery, and engineered bacteria or viruses. In plants, genetic engineering is hindered by the cell wall, requiring delivery methods that are highly host-specific or limited by challenges in plant regeneration.

Nuclease-enabled genome editing refers to techniques where genes are removed or changed with engineered nucleases, a class of enzymes that perform targeted double-stranded breaks (DSBs) at specific locations in the host genome. When nucleases perform DSBs, the cell undergoes homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) to repair the cut. NHEJ is a random, error-prone repair process that involves realignment of a few bases, such that the high error frequency provides a simplistic pathway for gene knockout. HDR is a nonrandom repair process requiring large stretches of sequence homology, allowing for precise edits by introducing customized homologous recombination sequences for gene knockout, knock-in, and targeted mutations. Prominent tools in genome editing are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR (clustered regularly interspaced short palindromic repeat)-Cas (CRISPR-Cas) systems. In the 1990s, ZFNs became the first nuclease system engineered for selectable genome editing in bacteria [124]. TALEN and CRISPR-Cas genome editing systems were developed for bacteria and eukaryotes more recently, around 2009 and 2012, respectively [125–128]. Composed of protein complexes containing a DNA-binding domain and a DNA-cleaving domain, ZFNs and TALENs rely on protein/DNA recognition to induce endogenous DNA repair. CRISPR-Cas systems are composed of a nuclease protein (Cas) and a guide RNA (gRNA) with sequence homology to the genomic target, and therefore rely on the formation of a ribonucleoprotein (RNP) complex to induce HDR or NHEJ. While all three systems have their drawbacks, CRISPR-Cas has revolutionized the field of genome editing owing to its relatively superior simplicity, efficiency, and multiplexing ability (i.e., simultaneous editing of different genes) over ZFNs and TALENs.

which is promising for stable transgene-free modified crops. As with traditional genetic engineering of plants, many of the limitations for implementing gene editing tools in plants (low editing efficiency, tissue damage, species limitations, cargo-type limitations) originate in biomolecular transport into plant cells. As such, NP-based biomolecule delivery to plants stands to enable higher-throughput plant genome editing via DNA, single guide RNA (sgRNA), and RNP delivery, and thus warrants a discussion on the state of the plant genome editing field.

Global Landscape of Regulatory Uncertainty towards Genetically Engineered Crops

Genetic engineering of crops has evolved to overcome limitations in traditional breeding, as breeding is slow, laborious, and lacks precise control over plant genotype and phenotype generation. Modern biotechnology enables rapid development of crop variants with disease and pest resistance, stress tolerance, higher yield, and enhanced nutritional value. Since 1996, global genetically modified organism (GMO) cultivation has increased 110-fold to 185 mega-hectares in 2016 [75] (Figure 2). The US is a leader in GMO production but highly regulates production of modified crops, which poses, among other challenges, significant financial barriers to commercialization of new crop variants [76]. The US GMO pipeline is product-based but sensitive to plant pests, such that Agrobacterium automatically triggers regulation, while other methods of gene delivery are often deregulated if the product is nontransgenic [76,77]. European Union GMO regulation is process-based and affects any organism whose genome has been modified other than by mating or natural recombination [78], but includes exceptions for certain types of mutagenesis that will likely exempt modern gene editing [79]. The advent of nuclease-based gene editing (Box 3) has set forth a global reevaluation of the legislation surrounding genetically engineered crops, wherein several leading GMO cultivators have exempted nontransgenic genome-edited plants from regulation (Figure 2). Recently, the USDA officially stated that there are no future plans to include genome-edited plants under the current US regulatory umbrella for GMOs [131]. However, due to differences in regulatory philosophy and public opinion, several countries oppose deregulation of nontransgenic genome-edited plants and it remains...
unclear how enforcement of GMO status will proceed worldwide in the future [80]. Despite the heterogenous and dynamic global regulatory landscape, nuclease-based genome editing currently plays a critical role in overcoming regulatory restrictions and ensuring scientific progress, as well as commercial implementation of engineered crop variants.

**Nanocarriers Hold Promise for Nuclease-Based Plant Genome Editing**

Genome editing tools may increase the throughput of plant molecular biology and genetic studies, and as such could shift the paradigm in regulatory oversight of transgenic plants. Species, amenable tissue, expression strategy (DNA, RNA, or protein), and delivery method contribute...
to the efficacy of transgene expression or modification and to the propensity of transgene integration into the host genome. ‘DNA-free’ genome editing techniques are increasingly attractive, especially from a regulatory perspective, to eliminate all risk of transgene integration. Recently, RNP delivery has been demonstrated in A. thaliana and O. sativa protoplasts via PEG-transfection [81] and Z. mays embryos via gene gun delivery [82]; the methods used in both of these studies are primarily throughput-limited by challenges in progeny regeneration. The challenge to realizing efficient, stable gene editing in plants is twofold. First, plant germline cells cannot be transformed by any current method (with the exception of Arabidopsis floral dip [83]) and therefore progeny must be regenerated from embryogenic calli. Second, the cell wall imposes a rigid transport barrier to biomolecule delivery, such that conventional delivery in plants is either destructive and inefficient, or host-specific. Thus, the foremost limitation for broad-scale implementation of plant genome editing originates from an inability to target germline cells, and the absence of an efficient and species-independent bio-cargo delivery strategy. While engineered nucleic systems have begun to reveal remarkable potential for the future of plant genome engineering, novel carriers are required to overcome the restrictions of conventional delivery methods, but could also begin to pave the way for efficient progeny regeneration or direct germline editing in plants.

NPs have begun to facilitate and enhance genome editing through efficient and targeted delivery of plasmids, RNA, and RNPs [84]. In mammalian cells, NPs are routinely used for efficient, direct cytosolic/nuclear delivery of Cas-RNPs in many cell types [85], and RNP delivery has been shown to greatly reduce off-target effects in comparison with plasmid-based CRISPR systems [84]. However, in plants, the cell wall has hindered the development of an analogous system that can passively deliver genome editing cargo to mature plants and across species. Thus, there remains much potential for designing NP carriers with diverse cargo loading capabilities (DNA, RNA, proteins) and optimal geometry/chemistry to efficiently bypass the cell wall and membranes in dense plant tissues without external aid. Previous work [51, 67, 68] shows that some NP formulations are capable of passive internalization in planta with DNA, RNA, or protein cargo. These NP scaffolds, namely CNTs, MSNs, and polymeric NPs, should be further explored for delivering engineered nuclease systems to plants.

**Concluding Remarks and Future Perspectives**

Genetic engineering of plants has greatly accelerated scientific progress and paved the way for crop variants with improved growth characteristics, disease and pest resistance, environmental stress tolerance, and enhanced nutritional value. In parallel, advances in site-specific genome editing technologies have optimized the precision with which genetic engineering of organisms can be accomplished. However, conventional methods of plant genetic engineering and genome editing are limited in scope. This is primarily due to the cell wall that imposes a barrier to efficient delivery of biomolecules, which could potentially be overcome by NPs. Agrobacterium is a preferred method for plant genetic transformation, but is only effective in a limited range of host species and is an automatic trigger for regulatory oversight in the United States. Biolistic particle delivery and PEG-transfection are effective, host-independent transformation methods, but difficulties in regenerating healthy plant tissue and low-efficiency editing are severe drawbacks to their broad-scale and high-throughput implementation. NPs have recently emerged as a novel method of targeted biomolecule delivery in mammalian cells, especially for clinical applications. However, exploration of nanocarriers for biomolecule delivery in plants remains a nascent field, with much potential for the future of plant biotechnology and genome editing (see Outstanding Questions). Preliminary studies show that NPs with proper surface chemistry and physical properties analogous to those developed for animal systems are capable of delivering biomolecules to plants in vivo and in vitro with improvements over conventional methods. However, as of yet, most nanocarriers in plants still require assistance from conventional methods (i.e., gene gun),

**Outstanding Questions**

Are there nanoparticle varieties yet to be discovered for efficient biomolecule delivery in plants, or do we lack knowledge of, or control over, optimal nanoparticle modifications for applications in plant systems?

Can we narrow the current design space to a single nanoparticle type with tunable functionalization for passive delivery in plants, regardless of cargo type, plant species, and tissue variety?

How might we gain a better mechanistic understanding of nanoparticle internalization into plant cells, and how can we harness this knowledge towards rational design of nanoparticles for a range of biological delivery applications?

Will challenges in biomolecule delivery and progeny regeneration always remain decoupled, or will nanoparticle delivery enable significant increase in throughput and efficiency of genetic studies on plant regenerative biology and stable transformation?

How can scientists, the public, and regulatory bodies create a space for open communication to address the risks of introducing crop variants to the environment, while continuing to enable scientific progress and commercialization of sustainable and resilient crop variants?
or are limited to in vitro studies. To our knowledge, the field of plant bioengineering has yet to fully demonstrate a reliable strategy for NP-mediated passive biomolecule delivery to plants. To realize the full scientific and humanitarian potential in genetic engineering of both model and crop species, especially with the advent of nucleic acid-based genome editing, a promising focus will be to optimize NPs as efficient and ubiquitous delivery vessels of diverse biomolecules, tunable across cargo types, species, and tissues, for both transient and stable genetic engineering. However, because germline transformation is currently limited to only one model plant species (Arabidopsis), even a ubiquitous delivery strategy for precise genome editing would be limited by the success of regenerating progeny from somatic tissue. A remarkable, yet conceivable, future accomplishment of NP delivery in plants could be enablement of unprecedented, highly parallel genetic studies that elucidate the precedents for success in tissue regeneration, and the direct manipulation of germline cells.

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