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Nanoparticle-Mediated Genetic Engineering of Plants

Genetic engineering of plants is at the core of sustainability efforts, natural product synthesis, and agricultural crop improvement. The past several decades have brought remarkable progress in biotechnology with the improvement of genome editing and sequencing tools, which stand to advance plant synthetic biology and bioengineering. In agriculture, genetic engineering can be employed to create crops that have increased yields and nutritional value, are resistant to herbicides, insects, diseases, and abiotic stresses, including drought and heat. In pharmaceuticals and therapeutics, genetically engineered plants can be used to synthesize valuable small-molecule drugs and recombinant proteins. Plastids, such as chloroplasts, lack gene silencing pathways and have been demonstrated to have high and stable expression of transgenes. Because chloroplasts are maternally inherited in most plant species, they provide genetic containment in transformed crops. Despite several decades of advancements in biotechnology, many plant species and their plastids remain difficult to genetically transform. Currently, few delivery tools exist that can transfer biomolecules into plant cells and their subcellular compartments, each with limitations. *Agrobacterium*-mediated delivery is the most commonly used tool for gene delivery into plants. However, *Agrobacterium* can only perform gene delivery for a narrow range of plant species, cannot be used for DNA-free editing or for transformation of the chloroplast or mitochondrial genomes, and yields random DNA integration into the plant genome. The other commonly used tool for plant transformation is biolistic particle delivery (also known as gene gun) in which gold microparticles are delivered to plant tissues with a high-pressure gene gun. Biolistics can deliver biomolecules into a wider range of plant species and into plastid genomes but faces limitations of low-level and sporadic expression, random DNA integration, plant tissue damage under high bombardment pressures and exposure to vacuum, and requires use of a substantial amount of DNA. Furthermore, gene gun bombardment is a technique that requires specialized facilities and costly materials that limit its widespread use. The lack of versatile, high-throughput tools for biomolecule delivery into plant cells through the rigid and multi-layered cell wall and double lipid bilayer envelopes of organelles represents a significant bottleneck to plant genetic engineering that may be facilitated by nanoparticle technology.

The "biomolecule delivery problem" in plants is largely due to the presence of the cell wall, which, with a measured size exclusion limit of ~5–20 nm, poses the dominant barrier to the delivery of exogenous biomolecules (Zhang et al., 2019). The plant cell, nuclear, and/or organelle membranes present a much larger size exclusion limit of approximately 300–500 nm and are additional barriers that must be traversed for genetic transformation of the nuclear or plastid genomes (Cunningham et al., 2018) (Figure 1). It is likely that the size similarity of micrometer-sized plant plastids and biolistic gold microparticles make it difficult to deliver DNA biolistically without destruction of the organelle. Here, we opine that nanomaterials delivered to plants in a force-independent manner hold great promise to serve as a delivery toolset of key molecular biology cargoes—DNA, RNA, and proteins—to advance genetic engineering of plants and their plastids. Nanomaterials are defined as materials with at least one dimension below ~100 nm and have unique and tunable physical and chemical properties, which leverage their ability to interact with biological matter with exquisite control and precision.

In the past decade, nanomaterials have gained popularity for biomolecule delivery to plants. Initial studies of nanoparticle-mediated delivery in plants involved biolistic delivery of DNA (Torney et al., 2007) and proteins (Martin-Ortigosa et al., 2012) by gold functionalized mesoporous silica nanoparticles. Several studies have demonstrated the potential of force-independent internalization through cell walls without assistance from mechanical aid, such as biolistics, ultrasound, vortexing, or electroporation. These include: (1) mesoporous silica nanoparticles (MSNs) mediating plasmid DNA delivery when being co-cultured with *Arabidopsis* roots (Chang et al., 2013), (2) layered double hydroxide clay nanosheets mediating the delivery of RNAi molecules through topical application to *Nicotiana tabacum* (Mitter et al., 2017), and (3) DNA origami nanostructures mediating the delivery of small interfering RNA (siRNA) to *Nicotiana benthamiana* (Zhang et al., 2019). These studies validated biomolecule delivery with fluorescence microscopy, phenotypic pest resistance, and with mRNA and protein-level quantification, respectively. Moreover, recent studies have reported the application of carbon nanotubes (CNTs) for the unassisted delivery of plasmid DNA (Demirer et al., 2019b; Kwak et al., 2019) and siRNA (Demirer et al., 2019a) into a variety of model and non-model plant species. Demirer and colleagues (Demirer et al., 2019b) demonstrated that CNTs enable plasmid DNA delivery and GFP transgene expression targeting the nuclear genome with mRNA and protein-level quantification. Kwak and colleagues (Kwak et al., 2019) showed evidence of CNT-based plasmid DNA delivery and YFP transgene transient expression in the chloroplast based on fluorescence microscopy imaging. Interestingly, the surface chemistry, size, and charge of the CNT particles may be guiding the particles to their respective subcellular destinations in extracted chloroplasts, protoplasts (Giraldo et al., 2014; Wong et al., 2016), and intact leaves (Demirer et al., 2019b; Kwak et al., 2019). Positively charged CNTs used for nuclear gene delivery in leaves were functionalized with polyethyleneimine (PEI) polymers and localized in both the nucleus and chloroplasts. In contrast, the surface of the CNTs used for chloroplast gene delivery were functionalized with chitosan (Chi)

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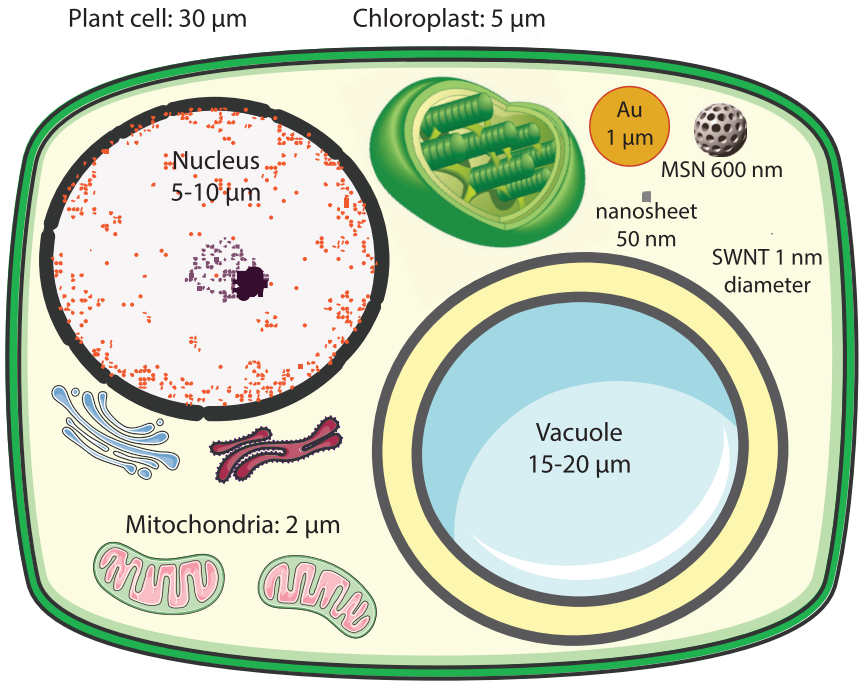

Figure 1. Sizes of Micro- and Nanoparticles Relative to Plant Cells and Organelles.
The plant cell nucleus, chloroplast, and mitochondrion are the transformable elements of the cell. Schematics of gold microparticles used for gene gun transformations, and mesoporous silica nanoparticles (MSN), clay nanosheets, and carbon nanotubes (CNT) drawn approximately to scale. The size exclusion limit is ~500 nm for the plant cell membrane and ~5–20 nm for the plant cell wall.

polymer derivatives. Further studies are needed to assess which nanoparticle physical and surface chemical properties govern nuclear versus plastid subcellular destinations, and how to optimize nanoparticles for different tissue types such as callus or meristem. Excitingly, both studies showed that CNTs, regardless of their surface chemistries, protect the plasmid DNA cargoes from endonuclease degradation and result in transient gene expression, a feature that can be useful for diverse biomolecule delivery efforts.

Future efforts in plant nanobiotechnology depend on developing detailed design heuristics for nanoparticle delivery vehicles. What chemical and physical properties of nanomaterials other than small size allow for efficient transport into the cell remain unclear. One hypothesis is the lipid envelope exchange penetration (LEEP) model, which proposes that successful membrane penetration hinges on the overall surface charge of the particle in addition to its size (Wong et al., 2016). As previously discussed, numerous studies have demonstrated uptake of nanoparticles. But to formulate design guidelines, the transport of nanoparticles throughout the plant as a system must be understood. Studies demonstrating apoplastic and symplastic transport through plasmodesmata are scant, especially when compared with the variety of nanoparticles and model species of plants. For example, MSNs have been shown to enter the cell wall, radially diffuse through plasmodesmata, and migrate via xylem (Sun et al., 2014). Recently, it has also been shown that nanoparticle size and surface modification, e.g., hydrophobicity, alters the transport mechanism of gold nanoparticles in plants (Avellan et al., 2019). The interaction of nanoparticle surface chemistry with heterogeneous plant biological surfaces further complicates unraveling nanomaterial designs for controlling nanoparticle fate. Nanoparticles may need to traverse barriers such as the waxy and hydrophobic cuticle, the dense cell wall composed of glycan networks decorated with proteins and aromatics, and one if not more lipid membranes all while carrying their cargo and remaining aqueously soluble. The breadth of tissues, e.g., leaf, root, seed, and callus, that nanoparticles are able to penetrate is also not well documented. Thus, systematized and cross-disciplinary efforts are required for tuning nanoparticle properties to accommodate plant physiology and intended genetic engineering purposes.

To fully leverage nanoparticles for plant bioengineering, it will be necessary to achieve stable gene expression and transformation to enable generation of fertile transgenic plants. CNT-based DNA delivery yields transient expression and was shown to prevent transgene integration into the plant host nuclear genome, a feature that can be leveraged for transgene-free plant engineering by delivering a CRISPR plasmid with CNTs. If delivered with CNTs, CRISPR plasmids will be expressed transiently to create permanent edits in the plant genome: an advancement that can avoid the drawbacks of multiple copy insertions or undesired constitutive transgene expression, avoid random integration that can lead to disruption of endogenous plant genes and thus functions, and enable transgene-free engineering of vegetatively propagated crops. Separately, because PEI-CNTs and Chi-CNTs both localize in the chloroplasts, CNTs present an enticing opportunity for plastid engineering. Plant cells have three genomes, two of which—chloroplast and mitochondrial—are transformable but largely inaccessible. The "workhorse" genetic transformation tool for plants, *Agrobacterium,* cannot be used for plastid genetic transformation, thus the preferred gene delivery tool for plastids is biolistic delivery, which suffers from cellular damage and low transformation efficiency. While transient heterologous expression of proteins in chloroplast has been demonstrated using Chi-CNTs, it remains to be demonstrated whether stable transplastomic plants can be obtained through homologous recombination of DNA delivered with CNTs. Notably, mesoporous silica nanoparticle-mediated plasmid DNA delivery into the nucleus has been demonstrated in *Arabidopsis* roots (Chang et al., 2013), which could be extended to plastid transformation. Lastly, because biomolecule grafting to most nanomaterials is tunable by the nanomaterial surface chemistry, possibilities emerge for delivery of other important cargoes to plants such as messenger RNA (mRNA), single guide RNA (sgRNA), and protein-polynucleotide complexes such as ribonucleoproteins. Importantly, nanoparticles for gene delivery are generally relatively simple to assemble, requiring little if any non-standard lab equipment. For example, polymer CNT constructs are made

using standard bioconjugation techniques from commercially available PEI and CNT stocks (Demirer et al., 2019c). MSN synthesis requires more advanced chemistry techniques and equipment but can be accomplished using standard inorganic chemistry methods (Chang et al., 2013). The development of efficient methods for genetic element delivery to broad ranges of plant species and subcellular targets could realize the promise that plastids hold as an emerging framework for synthetic biology and synthetic genomics.

Given the emerging promise of nanoparticle-based plant delivery strategies, it is important to dedicate parallel efforts to understanding the figures of merit enabling force-independent molecular transport across the cell wall and organelle membranes. Studies referenced here, and others, have alluded to several factors that affect transport into plant cells: size, shape, aspect ratio, tensile strength, compactness, colloidal stability, and charge, where these factors may not be mutually exclusive contributors to internalization. Although many nano-delivery strategies to-date have been heuristic in nature, the synthetic nature of nanoparticle-based delivery lends itself well to mathematical modeling of the delivery process toward rational design of nanocarriers for plants. Exploration of different materials and form factors will enable a better understanding of the unassisted plant cell internalization landscape. The field of nanobiotechnology as applied to plant genetic engineering is exciting but nascent, requiring interdisciplinary collaborations that include the fields of chemistry, nanotechnology, molecular plant biology, and plant physiology. For plant nanobiotechnology to realize its full potential, a thorough understanding of the control that nanomaterials provide over biomolecule transport into plant cells must be developed while the impact of nanomaterials *in planta* is assessed. Recent publications foreshadow a future suite of nanoparticles with properties finely tuned for their intended application, a deviation from "hammer and nail" delivery approaches where particle and cargo destinations are haphazard. Future advances in nanomaterial chemistry in organic, inorganic, and polymeric nanomaterials could further expedite and advance plant genetic engineering biotechnologies.

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