

Delivering Genes to Plants

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Traditional methods of gene delivery to plants are labor- and time-intensive, are suitable for only a small number of hosts, and have high toxicity and limited practical applicability. This article discusses how nanoparticle-based approaches could enable efficient gene transfer into plants.

Plant synthetic biology has numerous applications in agriculture, as well as in the pharmaceutical and energy industries (Figure 1). In agriculture, genetic engineering of plants can be employed to create crops that are resistant to herbicides, insects, diseases, and drought. The ability to introduce transgenes into plant cells also provides the opportunity to improve the nutrient profile of a crop. In the pharmaceutical industry, genetically engineered plants could be used to synthesize valuable small-molecule drugs. Genetically modified plants could also make biofuel production more efficient, which would provide a major benefit for the energy industry.

A crucial first step of plant genetic engineering, regardless of the application, is to deliver genes into the plant cells. Ever since the first transgenic plants were created in the 1980s, researchers have endeavored to develop and advance new gene delivery systems for plants. This article briefly explains conventional methods of delivering genes to plants and their strengths and limitations. It also describes newer methods of gene delivery that are based on nanoparticle transport, and demonstrates how they may impact the field of plant gene transfer and engineering.

Conventional gene delivery techniques

Conventional methods to deliver genes to plant cells can be grouped into three categories: physical, chemical, or biological approaches (Table 1). The most common and preferred physical gene delivery methods are biolistic particle delivery (also called particle bombardment or gene

gun delivery) and electroporation (the use of electric field pulses to create pores in cell membranes). Chemical delivery methods use polymers and cationic lipids as transfer agents. For example, polyethylene glycol (PEG)-mediated delivery is one of the frequently used chemical delivery methods.

Among the three approaches, however, biological methods are favored over physical and chemical methods, because they have higher transformation efficiencies in plant systems. Gene delivery via *Agrobacterium* is frequently used in plant genome engineering.

Biolistic particle delivery

Biolistic particle delivery was developed in 1982 by Sanford, *et al.* (1). In biolistic delivery, genes are coated and dehydrated onto heavy-metal particles, such as gold or tungsten. High-pressure helium pulses accelerate the particles, propelling them into plant cells at high velocities (Figure 2). Typically, the epidermal tissue of plant cells is targeted. Depending on the experimental parameters, DNA can pass through both the plant cell wall and plasma membrane, and can also penetrate into the nucleus (2). Helium gas pressure, net particle size, and dosing frequency are critical experimental parameters that determine the penetration efficiency, toxicity, and overall gene transfer levels in plants.

Biolistic particle delivery facilitates gene delivery and genome editing by transferring thousands of DNA molecules of sizes up to 150 kilobase (kb) (3). Although the method is inexpensive and easy to perform, researchers have concerns about the integrity of the DNA after transfer, the short-term



and low-level expression of the delivered genes in the plants, and cell damage from the high pressures that plant tissues experience.

Electroporation

In electroporation, strong electric field pulses alter the cell's permeability and generate transient pores in the cell membrane that allow the transport of genes into the plant cell cytoplasm.

Electroporation was developed *in vitro* for protoplast transformation in 1982 (4). However, electroporation has since been shown to successfully initiate transfection (*i.e.*, inserting genetic material into cells) within intact plant cells, *in vivo*, as well (5). In the past decade, electroporation has been standardized for several plant species, including tobacco, rice, wheat, and maize, with the following parameters: a voltage of 25 mV and a current of 0.5 mA applied by a commercially available electroporator over 15 min for a typical cell density of $3\text{--}5 \times 10^6/\text{mL}$ (6).

Although electroporation is fast and inexpensive, it has several limitations when it is used on plant cells. Electroporation is limited to a few plant species — it fails for plant cells that have a thick cell wall. This barrier prevents ubiquitous transfection across all plant species. In addition, strong electric field pulses damage the delivered gene, which creates inaccurate translational end products (7) and can be toxic to cells.

PEG-mediated delivery

Polyethylene glycol, an inert hydrophilic polymer of ethylene oxide, has many applications within the pharmaceutical industry, energy industry, and industrial manufacturing. When used for gene delivery, PEG transfers DNA into plant protoplasts. In this gene-delivery process, DNA is directly incubated with protoplasts, and the delivery of DNA is initiated by adding PEG polymer together with divalent cations (such as Ca^{+2}) to the mixture of protoplasts (6). The addition of the PEG solution destabilizes and softens the plant cell membrane and allows free DNA to enter the plant cytoplasm.

Although PEG-mediated delivery allows for highly efficient simultaneous protoplast transfection, in most plant species, protoplasts cannot be successfully regenerated into whole and fertile plants, rendering the use of PEG-mediated delivery impractical for mature plant transformations.

Agrobacterium-mediated delivery

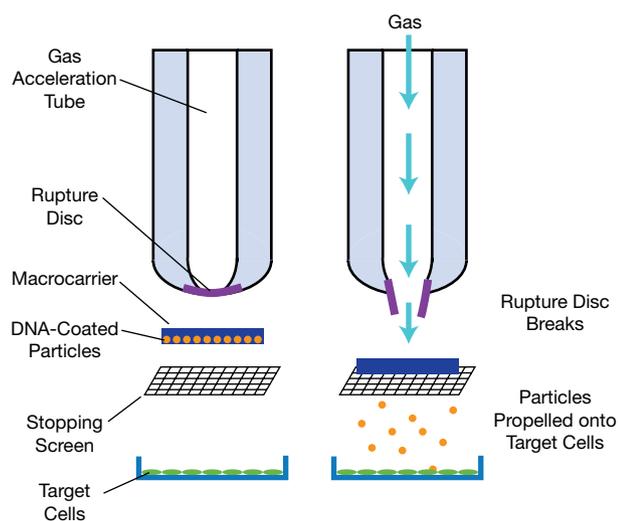
Agrobacterium tumefaciens is a Gram-negative bacterium and a plant pathogen that causes crown-gall disease in some plant species. *Agrobacterium* transfers part of its tumor-inducing plasmid DNA, called transfer DNA (T-DNA), into plant cells through the assistance of virulence



▲ **Figure 1.** Plant synthetic biology has many uses and applications across several industries.

Table 1. Conventional gene delivery methods can be grouped into physical, chemical, and biological categories. Each has its own strengths and limitations.

Conventional Gene Delivery Methods		Strengths	Limitations
Physical	Biolistic Particle Delivery	<ul style="list-style-type: none"> • Easy • Transfers large sizes and amounts of DNA 	<ul style="list-style-type: none"> • Low integrity of delivered DNA • Short-term and low-level expression • Cell damage
	Electroporation	<ul style="list-style-type: none"> • Fast • Inexpensive 	<ul style="list-style-type: none"> • Limited range of plant species • Low integrity of delivered DNA • Toxicity
Chemical	PEG-Mediated Delivery	<ul style="list-style-type: none"> • High-efficiency protoplast transfection 	<ul style="list-style-type: none"> • No regeneration of protoplasts into whole and fertile plants
Biological	<i>Agrobacterium</i> -Mediated Delivery	<ul style="list-style-type: none"> • Low cost • High efficiency • Stable transformation 	<ul style="list-style-type: none"> • Limited host range



▲ Figure 2. A gene gun, or biolistic particle delivery device, can deliver transgenes to cells. First, a macrocarrier is loaded with DNA-coated heavy-metal particles. Within the gene gun, gas pressure builds up against a rupture disk. The pressure eventually reaches a point that causes the rupture disc to break, and the pressure burst propels the macrocarrier into a stopping screen. The DNA-coated particles are propelled through the screen and hit the target cells.

genes. Virulence proteins can act as channels for the transport of T-DNA through the plant cell wall and membrane, and can help integrate T-DNA into the plant nuclear genome.

In the early 1980s, researchers exploited the natural ability of *Agrobacterium* to send genes into plant cells by engineering T-DNA to contain a selectable marker and genes of interest. Research has demonstrated efficient DNA delivery into dicotyledonous plants (*i.e.*, dicots) and a limited number of monocotyledonous plants (*i.e.*, monocots) using *Agrobacterium* (8).

Agrobacterium-mediated gene delivery is attractive for several reasons: the ease of the protocol, its low cost, and its high DNA delivery efficiency. It is possible to deliver large DNA fragments up to 150 kb and obtain stable transfor-

mation through random insertion of the transferred DNA into the plant nuclear genome (9), making *Agrobacterium*-mediated gene delivery a primary choice for mature plant transformations.

However, few monocots appear to be natural hosts for *Agrobacterium*. Thus, *Agrobacterium*-mediated transformation is ineffective in numerous plant species, limiting the host range of this method of gene delivery.

Nanoparticle-based gene delivery methods

Given the aforementioned limitations of conventional gene delivery methods to plants, the plant genome engineering community can benefit from a delivery method that is inexpensive, facile, and robust, and that can transfer genes into all phenotypes of any plant species with high efficiency and low toxicity.

High-aspect-ratio nanoparticles, especially carbon nanotubes (CNTs), exhibit several characteristics of merit (*e.g.*, they can pass through cell membranes and escape endosomal degradation) that may make them useful as gene delivery agents. In the past decade, nanoparticles such as gold and starch, as well as mesoporous silica nanoparticles (MSNs), have been used to deliver genetic material into plants (10). These nanoparticles have been shown to traverse the plant cell wall, cell membrane, and nuclear membrane, but are able to transform plants only with mechanical assistance.

Gold nanoparticles

Carbon matrices embedded with gold nanoparticles can be synthesized by heat-treating the biogenic intracellular gold nanoparticles produced by the fungus *Aspergillus ochraceus*. These nanoparticles have a diameter of 5–25 nm and were used to successfully deliver plasmid DNA into *Nicotiana tabacum* via gene gun by Vijayakumar, *et al.* (11). The same work also demonstrated efficient DNA delivery into the monocot *Oryza sativa* and a hard dicot tree species, *Leucaena leucocephala*, with minimal plant cell damage.



A nano-sized composite carrier has several advantages over commercial micrometer-sized gold particles used in gene gun delivery. First, due to their small size, matrices embedded with gold nanoparticles have higher transformation efficiency. Additionally, they require less gold and plasmid to achieve the same transformation level efficiency. And, this method has relatively low toxicity to plant cells (11). However, gold-nanoparticle-embedded carbon matrices still require the assistance of a gene gun to efficiently deliver genetic material into plant cells.

Starch nanoparticles

Liu, *et al.* (12), synthesized starch nanoparticles in a water-in-oil microemulsion, coated the nanoparticles with poly-L-lysine, and tagged the DNA-loaded starch nanoparticles (50–100 nm in diameter) with a $(\text{Ru}(\text{bpy})_3)^{2+}$ fluorescent label. The DNA-loaded starch nanoparticles were transported into *Dioscorea sp* plant cells with the aid of ultrasound vibrations (120 W at 40 kHz for 5 min). Delivery of the plasmid DNA was verified with optical tracking of the tagged nanoparticles. It is hypothesized that the ultrasonic waves induced instantaneous pore formation in the cell wall, cell membrane, and nuclear membrane, which allowed the transport of DNA-loaded starch nanoparticles into the plant nucleus (13) and yielded a transient transformation.

Gene delivery via starch nanoparticles was initially preferred due to the biocompatible, biodegradable, and nontoxic nature of starch. However, the transfection efficiency is very low (*i.e.*, 5%), with this method, so it is not widely used.

Mesoporous silica nanoparticles (MSNs)

Honeycomb-shaped, surface-functionalized mesoporous silica nanoparticles with 3-nm pores were developed by Torney, *et al.*, to transport DNA into isolated plant cells and intact leaves with a gene gun (14). MSNs were loaded with the gene of interest and the ends were capped with gold nanoparticles through disulfide bonds to prevent the leakage of DNA. In cellular environments, disulfide bonds are reduced, and uncapping releases DNA and triggers gene expression in plants.

This research demonstrated successful transformation of *Nicotiana tabacum* protoplasts and leaves, as well as maize immature embryos, by delivering DNA-loaded MSNs into plant cells with a gene gun.

Carbon nanotubes (CNTs)

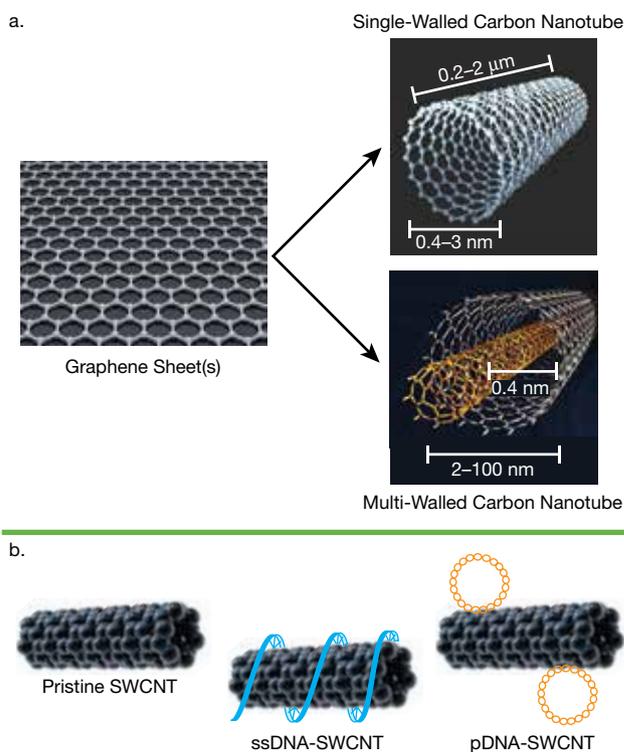
Iijima reported the synthesis and identification of carbon nanotubes in the early 1990s (15). CNTs are allotropes of carbon, and are made by rolling graphene sheets at specific and discrete angles, creating cylindrical and hollow nanostructures that are nanometers in diameter and micrometers in length. Depending on how many graphene sheets are used

during production, CNTs can be single-walled (SWCNT) or multi-walled (MWCNT) (Figure 3a).

Nanotube properties depend on the rolling angle and the radius of the tube, along with the number of graphene sheets used. CNTs have exceptional material properties attributed to their small size, small mass, and symmetric structure (16). These unique mechanical, optical, electronic, chemical, and thermal properties make CNTs of great interest not only in the research community, but also in industry.

CNTs have astoundingly high elastic moduli and reported strengths 100 times higher than the strongest steel available. Because of their high electrical and thermal conductivity, they have been used as additives in electronics, optics, and plastics (17).

In the past decade, several studies reported using CNTs as drug delivery vehicles due to their high surface area (18–20). However, pristine CNTs are not soluble in aqueous solutions owing to their hydrophobicity. Therefore, researchers functionalized CNT surfaces either covalently or non-covalently to solubilize CNTs in aqueous solutions for biological applications. The modified CNTs are soluble in aqueous solutions, and they are biocompatible with body fluids, so they elicit few toxic side effects (21).



▲ **Figure 3.** a. Carbon nanotubes (CNTs) are made from graphene sheets. Single-walled carbon nanotubes (SWCNTs) consist of one layer of graphene, while multi-walled carbon nanotubes (MWCNTs) have several layers of graphene. b. CNTs can be loaded with single-stranded DNA (ssDNA) or plasmid DNA (pDNA).

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The relatively low toxicity of modified CNTs, combined with their ability to penetrate biological membranes, have encouraged scientists to explore CNTs to deliver genetic material into animal cells for cancer therapy and treatment of infectious diseases and central nervous system disorders, as well as in tissue engineering applications.

Kateb, *et al.*, studied the delivery of small interfering RNA (siRNA) and plasmid-DNA-loaded MWCNTs into the murine microglia cell line (*in vitro*) and into the mouse brain (*in vivo*) to manipulate microglia for brain cancer immunotherapy (22). This work demonstrated the efficient loading of genetic material onto MWCNTs and confirmed that these complexes can be internalized by microglia cells without causing significant immune response both *in vitro* and *in vivo*.

Pantarotto, *et al.*, investigated plasmid DNA loading on ammonium-modified SWCNTs, and demonstrated successful transfection and DNA delivery into mammalian HeLa and Chinese Hamster Ovary (CHO) cell lines with modified SWCNTs (23). They reported low nanotube cytotoxicity and efficient gene delivery, with gene expression levels up to 10 times higher than those achieved with DNA alone.

Although there is considerable research dedicated to

interactions between carbon nanotubes and animal cells, and many studies have shown the promise of CNTs to deliver genetic material into animal cells, little attention has been paid to the utilization of CNTs as gene delivery vehicles for plant systems.

Some researchers have investigated the effects of CNTs on plants and achieved significantly higher germination rates for seeds grown in media that contains CNTs (24). It is suggested that CNTs help increase the plant's ability to uptake water, which increases growth in every part of the plant, including the roots and shoots, and increases branching (25). Plants have produced two times more flowers and fruit when grown in soil supplemented with CNTs (26). These studies highlight the potential to use CNTs, and nanomaterials in general, to enhance plant functions.

To the best of our knowledge, Liu, *et al.*, in 2009, were the first to use CNTs as plant gene delivery vehicles (27). They demonstrated the cellular uptake of both SWCNT/fluorescein isothiocyanate (FITC) and SWCNT/single-stranded DNA (ssDNA)-FITC conjugates into *Nicotiana tabacum* cells. This validated the ability of CNTs to penetrate intact plant cell walls and cell membranes without external aid (*e.g.*, a gene gun).

Later, Giraldo, *et al.*, investigated the penetration ability of ssDNA-loaded SWCNTs into *Arabidopsis thaliana* leaves (28). They injected the complex into the underside of the leaves with a syringe, and found that CNTs of a certain size and charge (which was manipulated by surface modification) were able to penetrate membranes without any assistance from a gene gun.

The researchers took advantage of the near-infrared fluorescence emission of individually suspended SWCNTs to study the transport mechanism and localization of the complex in plant cells (28). By imaging leaf tissue cross-sections, they determined that ssDNA-SWCNTs localized in the leaf lamina and veins in both intracellular and extracellular parenchyma tissues, and in chloroplasts inside parenchyma cells. This indicated successful transport through the plant cell wall, cell membrane, and chloroplast membrane. Interestingly, leaf lifespan and chlorophyll content, both indicators of cytotoxicity, are not affected by infiltration with ssDNA-SWCNT solution.

Based on this previous research, our group — the Landry Lab at UC Berkeley — currently works on advancing plant genetic transformation technology by conjugating plasmid DNA and other biomolecular cargoes, such as ribonucleic acids (RNAs), ribonucleoproteins (RNPs), and proteins, onto CNTs (Figure 3b). Our goal is to extend this technology for use in non-model plant species without an external mechanical aid, such as a gene gun or ultrasound. We are loading plasmid DNA (not single-stranded DNA) onto CNTs, and it is harder for plants to internalize these

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larger complexes. However, we have managed some delivery success without using any mechanical aid.

Our preliminary results suggest successful, efficient, and nontoxic delivery of cargoes into plant cells, and transfection of plant protoplasts and leaves through plasmid DNA-loaded-CNTs in a manner that is independent of the plant species, without needing external mechanical aid.

Closing thoughts

Nanomaterials such as carbon nanotubes show great potential to serve as delivery vehicles of genetic material into plant cells and plastids due to their ability to traverse the plant cell wall, cell membrane, and organelle mem-

branes. The development and widespread adoption of CNTs in plant cell biology requires further investigation of internalization mechanisms, limits of what CNTs can carry and deliver efficiently, detailed analysis of cytotoxicity, and fate of CNTs in cells after delivering their cargo.

Our future efforts will explore these aspects of CNT-mediated delivery to develop an inexpensive, facile, and robust delivery method that can transfer genetic material into all phenotypes of any plant species with high efficiency and low toxicity. This, in turn, will give rise to more efficient and powerful plant genome engineering platforms with numerous applications in the agriculture, therapeutics, pharmaceutical, and energy industries.

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