Nanoparticles for protein delivery \textit{in planta}
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Abstract
Delivery of proteins into walled plant cells remains a challenge with few tractable solutions. Recent advances in biomacromolecule delivery using nanotechnology may evince methods to be exploited for protein delivery. While protein delivery remains no small feat, even in mammalian systems, the ability for nanoparticles to penetrate the cell wall and be decorated with a plethora of functional moieties makes them ideal protein vehicles in plants. As advances in protein biotechnology accelerate, so does the need for commensurate delivery systems. However, the road to nanoparticle-mediated protein delivery is fraught with challenges in regard to cell wall penetration, intracellular delivery, endosomal escape, and nanoparticle chemistry and design. The dearth of literature surrounding protein delivery in walled plant cells hints at the challenge of this problem but also indicates vast opportunity for innovations in plant-tailored nanotechnology.

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Introduction
For several decades, breakthroughs in nanomaterial synthesis, production, and characterization have advanced electronics, medicine, and basic research. Nanomaterials are now broadly commercially available, with functionalization approaches that are readily accessible in most laboratories, enabling ease of access and use in a diverse range of applications [1,2]. Although recent nanotechnology-based accomplishments have been made in sensing, delivery, and targeting of nanomaterials \textit{in planta}, both fundamental and applied plant nanoscience lag behind other fields of nanobiotechnology [3–6]. In particular, the delivery of molecular biology cargoes such as DNA, RNA, and proteins to plant cells have become increasingly important goals. Of these goals, protein delivery remains the most difficult to accomplish, and as such, protein delivery strategies using nanomaterial carriers are only nascent in plants [7–10]. The development of gene editing tools motivates \textit{in planta} delivery of proteins that could enable DNA-free gene edited plants and could accelerate the development of both engineered crops and basic plant science. Recent \textit{in planta} protein delivery successes have leveraged protein biolistics for DNA-free gene editing. Although these new protocols have enabled DNA-free genome editing applications in plants, they involve specialized instrumentation and intensive low-throughput screening of hits due to low protein delivery efficiencies [11,12]. Given these limitations, a nanoparticle-mediated protein delivery technology could simplify workflows and streamline plant genome engineering.

To emphasize how challenging protein delivery to walled plant cells can be, we consider that evolution has not generated a “passive” way to bypass the barrier of the cell wall. To our knowledge, no intact plant virus has been found to diffuse across the plant cell wall despite possessing nanoscale (~15–200 nm) dimensions. Viral infection is instead mediated by injury to the plant cell wall on mechanical damage by weather, animals, or fungal attack [13]. Other pathogens have evolved elaborate secretory systems as seen in \textit{Agrobacterium} or anatomical structures such as fungal haustoria to deliver proteins past the cell wall [14,15]. By contrast, certain engineered nanomaterials (ENMs) have been shown to internalize into walled cells, lending credence to their potential application as protein carriers in plants [8,16–18]. Why and how ENMs, defined as constructs synthesized with at least 1 dimension below 100 nm, are seemingly able to pass the cell wall remains an
Barriers to plant protein delivery

The plant cell wall complicates design and analysis of protein delivery

To effectively deliver biomacromolecules into a walled plant cell, the cargo and carrier must bypass two main key barriers: the plant cell wall, and the plasma membrane. Plant cells are surrounded by an extensive network of biopolymers knitted together to form a multilamellar matrix hydrogen cell wall that restricts access to the plasma membrane [22]. The size exclusion limit (SEL) of the cell wall has been probed using a number of methods including gas adsorption [23], topographical EM studies [24,25], and uptake of dye-labeled nanoscale materials of defined sizes [26,27]. Although uptake of some large 100-nm ENMs in walled plant cells has been reported [28,29], evidence suggests a sub-10 nm or 100-kDa protein SEL. Diffusion remains the widely accepted mechanism through which ENMs are purported to bypass the cell wall to access the plasma membrane, although it remains unclear how ENMs near or above the SEL of the cell wall access the symplast. However, without greater evidence, we cannot discount biotransformation and subsequent in situ particle genesis or injurious application methods such as tissue infiltration [30,31] as being the source of detected ENMs above the SEL.

A major impediment to overcoming the cell wall challenge is the over-reliance on diffraction-limited fluorescence microscopy in assessing exogenous particle internalization. The presence of the thin symplast pressed against the perimeter of the plant cell, coupled with the diffraction limit of visible light (~200-nm), makes it difficult to distinguish whether fluorescent signals originate from the symplastic or apoplastic region of the plant cell. In addition, fluorescent labels often overlap with emission wavelengths of endogenous plant autofluorescence. It is possible to address this via plasmolysis induction [26,32,33] to increase cytosolic visualization or by using super-resolution microscopy, although both approaches have their drawbacks—plasmolysis induces drastic morphological changes in cells, and super-resolution microscopy requires specialized equipment and expertise. Going forward, we encourage readers to exercise discretion with conclusions on uptake drawn from diffraction-limited imaging in walled cells without secondary validation.

Membrane penetration and endosomal escape

On passing the cell wall, nanomaterials have been suggested to enter the symplast via a variety of mechanisms including endocytosis [34], plasmodesmata [35], or physical disruption [36] (Figure 1). However, most of these studies have been performed in suspension cells, which do not recapitulate tissue structure and have been reported to possess half-plasmodesmata that expose the cell membrane to the extracellular environment [37]. The most investigated mechanism for cellular uptake of ENMs is endocytosis. Clathrin-mediated endocytosis has been identified as the dominant endocytic process in plant cells and appears to operate analogously to animal cells [38]. Most examples of ENM uptake in walled plant cells do not leverage specific pro-endocytic motifs. Instead, studies take advantage of the natural tendency of ENMs to trigger endocytosis; thus, it remains unclear whether ENM functionalization with a cell-penetrating domain would enhance their cellular entry in plants.

On endocytosis, delivered materials must escape the endosome. Without a method to escape the endosome, endocytosed materials are sequestered into lytic organelles such as the central vacuole or lysosomes and are consequently destroyed [39]. In mammalian systems, polycationic polymers, cell-penetrating peptides (CPPs), or other chemical agents delivered in concert with the cargo have proven successful for cytosolic delivery [40–42]. Despite practical successes, the mechanism through which polycation-mediated endosomal disruption occurs is still under debate [43,44], and it remains unclear if endosome-disrupting tools can be translated for use in plant systems. However, some reports of cytosolic delivery of nucleic acids and proteins using polycation-rich polymers exist in plants [9,19]. For example, recent work by Liu et al. used a commercially available cationic lipid formulation to deliver Cas9 RNP to Nicotiana protoplasts [45].

In considering the barriers for protein delivery in plant cells, we hypothesize the requirements for designing efficient protein delivery systems. First, the protein and...
carriers in toto should be near or smaller than the SEL of the cell wall, which strictly limits the choice of nano-carrier. Second, the protein must be imparted by its carrier with pro-internalization motifs or another mechanism of cell membrane bypass. Thankfully, many ENMs such as single-walled carbon nanotubes (SWNTs) and quantum dots appear to elicit endocytosis [46,47], or carriers can be functionalized with pro-endocytic peptide motifs, such as HIV-1—derived Tat peptide [48]. Finally, pro-endosomolytic moieties that allow endocytosed material to escape degradation and enter the cytosol should be present. A third barrier that we do not fully elaborate on exists for intact plant tissues and organs: the cuticle. In a laboratory setting, the hydrophobic cuticular barrier is often overcome via the application of the carrier/bio-molecule solution. Recent examples have used vacuum infiltration [49], syringe infiltration [20], and foliar spray [50], with or without nonionic surfactants such as Silwet L-77, to deliver the active solution to plant cells. Given that these solutions largely involve formulation rather than ENM design, we will not further digress. Clearly, many variables in not just chemical strategies but also protocols must be considered in developing ENM strategies for protein delivery. In the next section, we will elaborate on biochemical and nanocarrier approaches that may aid in developing in planta protein delivery systems.

Chemistries for plant cellular delivery
The design of an efficacious protein delivery system for plants remains challenging due to variability in protein sizes, protein structural sensitivity to chemical and mechanical perturbations, and the lack of amplified expression that could result from the delivery of DNA or RNA vectors, with the relative impact of each of these considerations being highly dependent on the protein of interest, the carrier, and the desired functional outcome. Despite these complexities, we can consider carrier systems for protein delivery and then separately delve into how proteins might be conjugated to said carriers. Examples of nonbiolistic, mechano-/electro-poration, or protoplast-based methods of protein delivery in plants are scant in literature (Table 1). The Numata group has been prolific in using synthetic CPPs for protein delivery in plants [9,10,19,36,51]. In this study, peptides bearing a protein-binding domain and a CPP domain are complexed with the cargo protein. However, given that carrier—protein complexes measure ~200-nm in radius, their ability to actually bypass the cell wall intact is difficult to explain [9]. One plausible explanation could be activity of noncomplexed pro-endocytic peptides causing uptake of nonbound proteins. Such an effect has been observed in mammalian cells where coinubcation with free CPPs enhances uptake and endosomal escape [44]. Another recent development was by Santana et al.
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**Electroporation in walled plant cells**

- **[77]** Tobacco BY2 culture | ERD14 and ERD10 intrinsically disordered proteins (IDPs) | Electroporation | Confocal FL microscopy |
where inorganic quantum dots were targeted to the chloroplast using an engineered chloroplast transduction peptide [8]. In this case, the size of the carrier in toto was over 24-nm in diameter, suggesting some possible polydispersity in the cell wall SEL while also demonstrating the feasibility of transiting proteinaceous materials using a hard ENM. As their final design lacks an explicit mechanism for endocytosis or endosomal rupture, we cannot say what roles the nanocarrier or peptide play in their successful delivery.

Given our described requirements for ENM-mediated protein delivery, the list of eligible candidates appears short. Where delivery of efficacious quantities of proteins and delivery moieties requires a particle of considerable size to maximize loading, the SEL of the cell wall pushes design toward the smaller end of nanoscale. In this regard, we hypothesize that high aspect ratio nanomaterials, where one dimension is much longer than other dimensions, may provide the necessary surface area for protein conjugation and chemical modification and be plausibly wall-penetrant if one dimension remains considerably smaller than the cell wall SEL. The premier 1-D nanomaterial, SWNTs, possesses a length on the order of 100–1000 nm and a nonfunctionalized diameter of only 1-nm. SWNTs have previously been used for nucleic acid delivery in walled plant cells [3,4] and protein delivery in mammalian systems. Results from Zubkovs et al. demonstrate several protein-conjugation techniques yielding protein-SWNT conjugates that limit perturbations to the cargo’s structure. In this case, ssDNA is used to both solubilize the SWNT and anchor proteins to the surface using alkyne click chemistry [52]. However, SWNTs are not the only viable 1-D nanomaterial; protein delivery to mammalian cells has been demonstrated using inorganic nanowires of similar dimensions to SWNT [53]. Given the diversity of materials that can be fashioned with high aspect ratios, the number of viable protein nanocarriers becomes much broader [54]. With a diversity of untested carriers, further complicating an ENM approach is the development of chemistries to conjugate proteins and other functional motifs to the ENM vehicle. In the next section, we elaborate on considerations for conjugation strategies.

Sticking it to the particle
Size variability is significant across proteins of interest for delivery, with the most common fluorescent reporter, GFP, being much smaller than the most common gene editing nuclease, spCas9 RNP (Figure 2). As proteins alone already approach the cell wall SEL, the addition of a carrier usually increases the complex size beyond the SEL. Furthermore, the role of size on intracellular delivery efficiency remains unclear. For example, Martin-Ortigosa et al. show that BSA undergoes release from mesoporous silica nanoparticle
(MSN) carriers 3.5x more effectively than GFP despite being twice as large [55]. This points to the importance of understanding and modulating both protein-nanocarrier interactions and protein–nanocarrier–host interactions.

Association of a protein with its carrier can be accomplished through covalent bioconjugation or nonspecific adsorption. While nonspecific loading has been the dominant method used in the literature for carrier-mediated protein delivery to plants, the advantages that site-specific bioconjugation have brought to mammalian biology for decades [56] allude to their potential implementation in plants. Bioconjugation chemistry describes a class of fast, high—specificity reactions that site-specifically link biomolecules and has been widely used for covalent and noncovalent delivery strategies. Bioconjugates can be engineered with responsive chemical mechanisms for inducible release on reaching the intracellular target. Protein bioconjugation chemistries have found promise for enhancing drug efficacy, delivery, and specificity in mammals [42,56–58], although adaptations of these chemistries for use in plants have not been widely explored.

In contrast to site-specific conjugation, nonspecific loading strategies rely singularly on nonspecific association such as electrostatics (e.g., PEI-DNA) or local concentration gradients (e.g., diffusion into an MSN pore). A notable caveat is that electrostatic grafting strategies onto nanoparticles used for nucleic acid delivery are not generalizable to proteins. Because strongly cationic environments can cause protein inactivation, targeted conjugation chemistries, encapsulation, or gentler adsorptive methods, as in the case of MSNs, are preferred for protein delivery. That said, efforts have been made to temper the high charge of cationic polymers by modification with hydrophobic moieties or through fluorination [59]. Finally, the reliance on weaker interactions for nonspecific loading may not by default a disadvantage, however, as a weaker interaction with the carrier could translate to effective release of protein cargo into the cytosol.

Alternatives to ENM carriers
Designing nanocarrier-based protein delivery systems for plants presents a major engineering challenge that may require alternatives to those presented previously. Direct covalent or noncovalent modification of proteins with cell-penetrating materials could be a viable alternative. In 2020, Tai et al. used a cholesterol—Coomassie dye conjugate to enable endocytosis-independent cytosolic delivery of proteins in mammalian tissue culture [60]. The result is a generalizable strategy that noncovalently links a small molecular carrier to the protein, generating small, penetrating particles. Other efforts have included covalent modification of the protein itself with larger molecules such as CPPs [9,10,51] or endosomolytic polymers [61]. Others have proposed comprehensive protein engineering strategies such as supercharging or protein resurfacing [62]. However, these approaches are neither trivial nor generalizable. As an additional question for alternative delivery systems, it has not been shown in literature whether or not small molecule endo-osmolytic agents such as chloroquine [63] are effective in plant cells.

Finally, several strategies exist based on enzymatic or mechanical disruption to forcibly overcome the cell wall—such is the logos for protoplast transfection and biolistic bombardment. Protoplast transfection of functional proteins has been widely demonstrated (Table 1), but the limitations of callus regeneration often overshadow the benefits of protein delivery. To date, the biolistic method has been adapted for delivery of protein across the plant cell wall and cell membrane (Table 1) via dehydration of the protein onto 0.6-µm gold particles via lyophilization or air-drying [11]. While similar in practice to biolistic DNA loading, which is widely used, the disadvantage of loading proteins by dehydration is the potential for protein functional deactivation via irreversible disruption of secondary structure—thus the amount of active loaded protein may be low. In addition, the bombardment method itself contains inherent drawbacks, namely tissue damage. In comparison, studies of NPs in plants highlight growth benefits, enhanced immunity but also phytotoxicity and reduced biomass [64,65]. The benefit or harm of NPs in plants remains inconclusive but promising, particularly for short-term use or in applications requiring selection across generations which would reduce or eliminate NP prevalence in the final product.

Concluding remarks
The ability to deliver proteins into walled plant cells could enable diverse plant biotechnology applications, particularly for CRISPR Cas-9-based genome editing applications. In plants, most Cas-9 strategies rely on delivery of plasmids coding for Cas-9 and gRNA using Agrobacterium tumefaciens or gene gun bombardment [66]. Plasmid delivery techniques are hampered by both species specificity and by their potential to incorporate the delivered gene into the host plant genome which may generate off-target effects or trigger regulatory oversight [67]. Conversely, delivery of CRISPR Cas-9 proteins is DNA-free and thus could enable generation of edited plants without risking transgene integration. Recent strides in DNA-free editing have been made by adapting already existing techniques for protein delivery; however, these approaches often require manual selection of hundreds of in vitro transformants and may not be applicable to species with less robust tissue culture protocols or where regeneration remains elusive. A promising direction is in the characterization of
morphogenic transcription factors toward generating edited explants *in situ*, simplifying regeneration [68]. Such technological advancements, when co-delivered with editing nucleases, exemplifies a system with untapped potential for nanoparticle-mediated protein delivery applications.

To advance nanoparticle-mediated protein delivery to plants in such a way that moves toward generalizable platforms for the many applications in agriculture, biotechnology, and academic research, we must engineer complex systems that address a multitude of factors including protein conjugation and release, *in planta* translocation, endosomal escape, subcellular localization, and transformant selection. We emphasize the need for more proof-of-concept studies in reduced biological representations of a whole plant—such as walled suspension cells, leaves, or regenerable tissue in model species such as *Arabidopsis* or *Nicotiana benthamicana*—that provide a simple system in which to test novel delivery strategies or reproduce results across numerous laboratories. Furthermore, the development of more robust microscopic methods (apart from diffraction-limited fluorescence imaging) for reproducibly assaying nanoparticle internalization and localization within plant cells is an area with great unmet need. We also point out that testing pre-existing or novel conjugation chemistries for a wide array of proteins should be pursued in parallel to testing in plant cells, especially as this work would have applications in other fields besides plant science. We hypothesize that a focus on nanomaterial conjugation of smaller proteins with less functional reliance on secondary structure (such as intrinsically disordered peptides) could improve chances of success on translation to *in planta* studies, due to the smaller net carrier size and reduced need to maintain a precise protein fold during conjugation and delivery.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest


5. Demirer et al. demonstrate the single walled carbon nanotube mediated delivery of plasmid DNA for expression in the chloroplast. They demonstrate nanomaterial constructs capable of targeting the chloroplast for GFP expression.


10. Santana et al. engineered CdTe quantum dots with chloroplast transduction peptides for subcellular targeting of nanomaterials in plant cells. These experiments demonstrate the feasibility and bioavailability of protein-conjugated nanoparticles in plant cells after passage through the cell wall.


12. Guo et al. use a synthetic peptide carrier developed over the course of several years in the Numata group to deliver a fluorescent protein, citrine, into walled callus cells. Confirmation of delivery were performed by fluorescence microscopy. Interestingly, citrine alone was visibly endocytosed in some samples.


20. Hu and coworkers screen a diversity of small nanoparticles for their ability to be internalized and translocate through the leaf apoplast.


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60. Tai W, Zhao P, Gao X: Cytosolic delivery of proteins by cholesterol tagging. Sci Adv 2020, 6. In this publication, a novel mechanism for cytosolic delivery of proteins which appears to bypass endocytosis is described. Coomassie dye is used to non-covalently link cholesterol to proteins so that they can passively diffuse through the cell membrane.


70. Maher et al. are able to generate edited explants in situ using an Agrobacterium vector expressing Cas9 and BABYBOOM transcription factors. This method may help expedite the development of edited plants where regeneration of whole plants remains challenging.


