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# Rational nanoparticle design for efficient biomolecule delivery in plant genetic engineering

 Yue Zhao,<sup>†</sup> Calvin Thenarianto,<sup>†</sup> Cansu Sevencan,<sup>id</sup> Sivamathini Rajappa, Di Shen, Suppanat Puangpathumanond, Xiaomin Yao and Tedrick Thomas Salim Lew<sup>id</sup>\*

The pressing issue of food security amid climate change necessitates innovative agricultural practices, including advanced plant genetic engineering techniques. Efficient delivery of biomolecules such as DNA, RNA, and proteins into plant cells is essential for targeted crop improvements, yet traditional methods face significant barriers. This review discusses the multifaceted challenges of biomolecule delivery into plant cells, emphasizing the limitations of conventional methods. We explore the promise of nanoparticle-mediated delivery systems as a versatile alternative. By highlighting the diverse design parameters used to tune the physical and chemical properties of nanoparticles, we analyze how these factors influence delivery efficacy. Furthermore, we summarize recent advancements in nanoparticle-mediated delivery, showcasing successful examples of DNA, RNA, and protein transport into plant cells. By understanding and optimizing these design parameters, we can enhance the potential of nanoparticle technologies in plant genetic engineering, paving the way for more resilient and productive agriculture.

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Department of Chemical and Biomolecular Engineering, National University of Singapore, Singapore 117585, Singapore. E-mail: [tedrick@nus.edu.sg](mailto:tedrick@nus.edu.sg)

<sup>†</sup>These authors contributed equally to this work.



**Tedrick Thomas Salim Lew**

*Tedrick T. S. Lew is an assistant professor in the Department of Chemical and Biomolecular Engineering at the National University of Singapore (NUS). He received his PhD in Chemical Engineering from Massachusetts Institute of Technology in 2020. His lab focuses on developing nanoparticle systems for precision plant engineering and sustainable agriculture. Specifically, his group's expertise includes engineering nanocarriers and nanosensors for plant biotechnology applications. Tedrick has received several awards for his research, including the MRS Bulletin Postdoctoral Publication Prize (2021), NUS Presidential Young Professorship (2022), and Singapore National Research Foundation Fellowship (2023).*

*Tedrick T. S. Lew is an assistant professor in the Department of Chemical and Biomolecular Engineering at the National University of Singapore (NUS). He received his PhD in Chemical Engineering from Massachusetts Institute of Technology in 2020. His lab focuses on developing nanoparticle systems for precision plant engineering and sustainable agriculture. Specifically, his group's expertise includes engineering nanocarriers and*

## 1. Introduction

With growing climate concerns and an increasing world population, enhancing food security and advancing agricultural techniques are at the forefront of problems that many countries and research organizations aim to tackle. Sustainable agricultural practices that aim to increase food production, crop productivity and quality, as well as enhance resistance to stressors such as extreme climate conditions and pathogens are enabled through a multidisciplinary approach that combines plant sciences, genetic engineering techniques and emerging material technologies.

Prior to the development of genetic engineering approaches, conventional breeding techniques such as cross-breeding have been used to confer desirable traits to plants. While more modern breeding techniques such as mutation and transgenic breeding have expanded genetic variation, these methods still involve laborious processes and require a long time to achieve the desired outcomes.<sup>1</sup> Advances in genome editing tools, such as transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) systems and base editing have facilitated more precise modification of plant genome<sup>2,3</sup> Therefore, less time is needed to achieve the desired phenotypes in crops.<sup>4</sup>

These genetic engineering methods require efficient delivery of various biomolecules such as DNA, RNA and proteins into plant tissues. To date, the most established and commonly used methods to achieve this are *Agrobacterium-*

mediated transformation, biolistic (gene gun) delivery and protoplast transformation.<sup>2,5</sup> However, these delivery methods are limited by the lack of precision and applicability across multiple plant species, the potential to cause tissue damage, and a narrow scope of deliverable biomolecular cargoes. Nanoparticles have recently emerged as promising alternatives to address the challenges associated with these conventional delivery approaches. Nanoparticle-mediated delivery is versatile across a broad range of plant species, with the potential to target specific cells or organelles within the plant tissues through surface modifications of the nanoparticles. Furthermore, the physical and chemical properties of nanoparticles can be readily tuned to enhance biocompatibility and enable controlled cargo release within the plant cells.

This review summarizes the current challenges of genetic engineering related biomolecule delivery into plant cells and the recent advances in nanoparticle technologies that address them. Examples of nanoparticle-mediated delivery into plant cells with different types of biological cargoes are highlighted, as well as the specific design parameters that may affect the success of nanoparticle delivery systems. Finally, we review the prospects of nanocarriers' utility in plant genetic engineering applications.

## 2. Opportunities for nanoparticles in the delivery of biomolecules into plant cells

Genetic engineering in animal and plant research is crucial for uncovering biological phenomena and adding new functionalities to living systems. Efficient delivery methods are key for enabling exogenous molecules to function intracellularly. There has been significant progress in the nanomedicine field to develop carriers that can load sufficient amount of drug, deliver to the target location and release effectively. Similar to the aim of nanomedicine, strategies to improve the efficacy of loading and releasing meaningful cargoes to the targeted destination in plants are actively pursued.<sup>6,7</sup> However, compared to delivery in animal systems, the delivery methods for plants remain underdeveloped. This is because the fundamental structure of plant cells is more complex and fine-tuned. Robust physical barriers of plant cells might sequester the delivery system and further affect the delivery efficiency.<sup>8</sup>

### 2.1 Cellular barriers in plants

As illustrated in Fig. 1a, four primary challenges are present in the delivery of biomolecules into plant cells: (1) waxy cuticle: the outermost layer of plants serves as a hydrophobic barrier which prevents the entry of water and foreign substances. Its composition of cutin and wax compounds makes it impermeable to most biomolecules, presenting a challenge for their successful penetration; (2) plant cell wall: a rigid structure primarily composed of cellulose, hemicellulose, and lignin provides structural support and protection to plant cells. This formidable barrier hinders the passage of biomolecules due to its

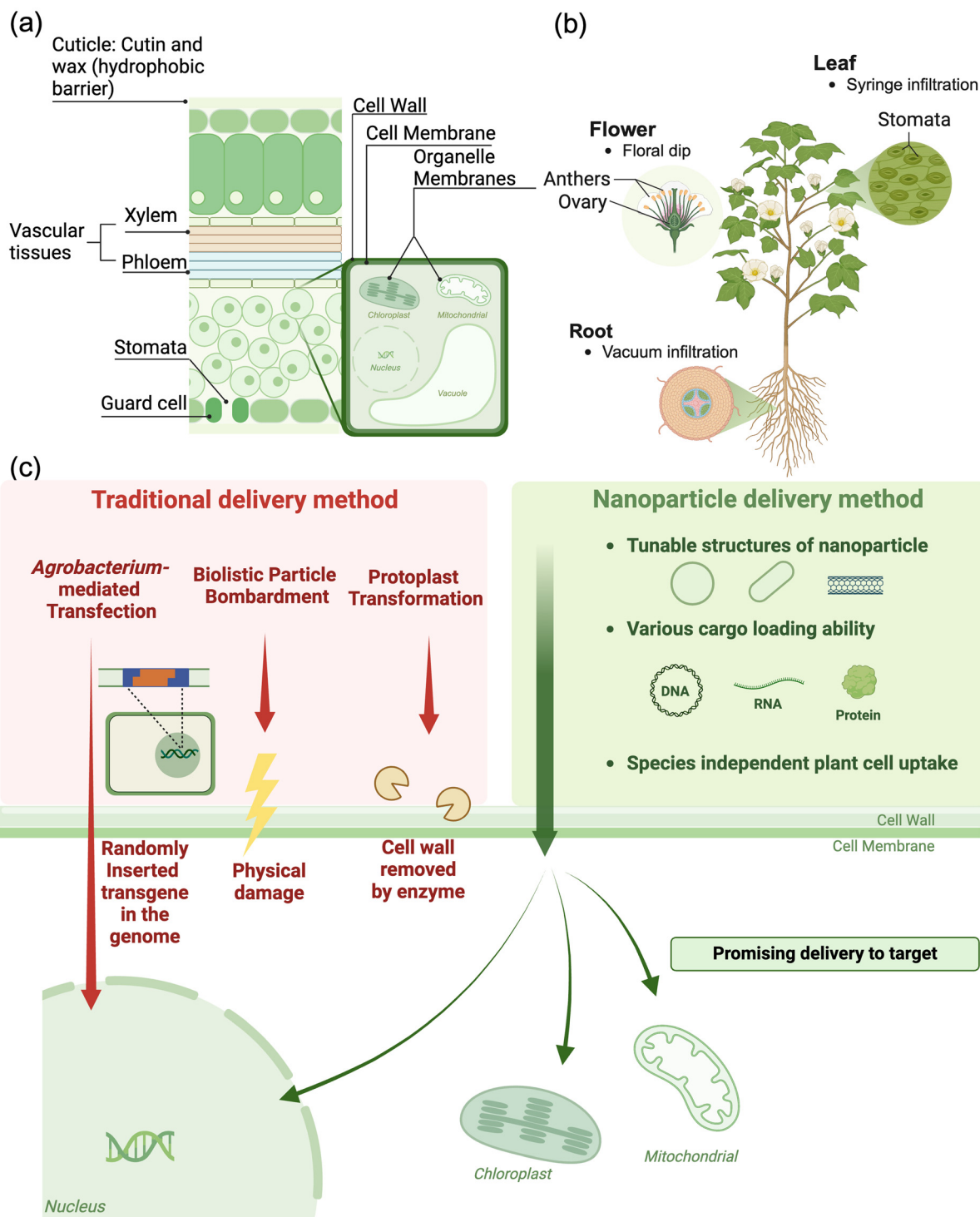
dense and complex network, making it difficult for molecules to traverse; (3) plasma membrane: a membrane that envelops the plant cell and regulates the passage of ions, nutrients, and signaling molecules. Its selective permeability acts as a gatekeeper, controlling the entry and exit of substances. Biomolecules face the challenge of crossing this barrier to access the cellular interior. (4) Organelle membranes: within plant cells, various organelles such as chloroplasts, mitochondria, and endoplasmic reticulum are enclosed by membranes. Targeting biomolecules to specific organelles for precise genetic modifications or therapeutic interventions requires overcoming these additional membranous barriers.

### 2.2 Existing delivery methods

To date, existing delivery methods such as biolistic particle bombardment, protoplast transformation, and *Agrobacterium*-mediated transformation have been developed and widely used. Although they are known as the most successful methods to date, they still face limitations such as potential tissue damage, limited cargo capacity, and low efficiency (Fig. 1c).<sup>9–12</sup> Biolistic particle bombardment propels microprojectiles coated with biomolecules into plant tissues. While useful for transforming challenging plant cells, it can cause cellular damage and result in low transformation efficiency. Protoplast transformation involves plant cells with their cell walls removed by enzymatic digestion, allowing easier transfection with foreign DNA. Despite its simplicity, regenerating plants from transfected protoplasts can be complex and yield limited results. *Agrobacterium*-mediated transformation, a widely used biological delivery method, involves the use of *Agrobacterium tumefaciens*, a natural plant pathogen, to efficiently integrate foreign DNA into plant genomes. Despite its success, this method is constrained by the size of the cargo fragment and the plant species. Additionally, random insertion of the target fragment into the plant genome could potentially induce mutations or affect original gene expression.<sup>13</sup> Overcoming these drawbacks is vital for advancing research in agriculture, biotechnology, and environmental sustainability, enabling the development of genetically modified crops for food security, enhanced crop yield, and adaptation to changing environmental conditions. Guided by the progress in animal systems, nanotechnology could be a possible solution to revolutionize biomolecule delivery into plant cells and unlock the full potential of plant genetic engineering.

### 2.3 Advantages of nanoparticle-mediated delivery

Nanoparticles offer a promising alternative with several advantages over conventional methods.<sup>14–16</sup> Surface modification with aptamers or targeting moieties allow precise delivery to specific organelles, minimizing off-target effects and enhancing the specificity of genetic modifications. Nanoparticle carrier systems are also species-independent in their delivery of nucleic acids and hence are more versatile for use.<sup>17,18</sup> Their small size also allows entry of the biomolecular cargo without disruption to plant cells, keeping the plants viable. Lastly, nanoparticles protect biomolecular cargoes from enzymatic



**Fig. 1** Challenges and possible delivery region for nanoparticle transport into plant cells. (a) Existing physical barriers which might affect nanoparticle transport from the leaf epidermis into the inner mesophyll. (b) Possible tissue regions and related possible delivery methods for biomolecule delivery via nanoparticles. (Floral dip can be used for flower delivery. Syringe infiltration can be applied to leaves. Vacuum infiltration can be used for root delivery.) (c) The comparison between features of traditional delivery systems and nanoparticle delivery methods. (Created in BioRender).

degradation, ensuring safe delivery to their target destinations. As a result, nanoparticles offer a promising solution to overcome the limitations associated with traditional biolistic and Agrobacterium-mediated delivery methods.

Based on these unique properties of nanoparticles, possible delivery regions of plants are briefly illustrated in Fig. 1b and the basic experimental methods for nanoparticle delivery into plants are elaborated below.

## 2.4 Delivery strategies for nanoparticle intracellular delivery

### 2.4.1 Delivery of biomolecules through plant leaves.

Pressurized needleless syringe infiltration into the abaxial side of the leaf is a standard method to deliver nanoparticles into the leaf. Gentle pressure of the syringe allows entry of the nanoparticle solution into the leaf through the stomata without damage to the leaf. The abaxial side of the leaf is chosen as it has more stomata compared to the adaxial side. The size of nanoparticles infiltrated is limited by the size of the stomata opening, 10–20  $\mu\text{m}$  in size. Alternatively, surfactants such as Silwet-77 can be added to the nanoparticle solution and sprayed onto the leaves. The increased wetting of the solution allows entry of the nanoparticles into the leaves without additional pressure, which may allow higher throughput delivery similar to current spray application of pesticides.

A limitation associated with delivery through the leaf, however, is the limited transport of nanoparticles to other organs of the plant. For example, nanoparticles such as SWNTs are unable to pass across the midrib vein using this method.<sup>19</sup> Thus far, only soft-polymeric nanoparticles have been found to be successfully transported away from the original infiltrated leaf to other organs of the plant.<sup>20,21</sup>

### 2.4.2 Delivery of biomolecules through plant roots.

Nanoparticles can be delivered through roots using vacuum infiltration or passively if nanoparticles are present in the liquid growth solution. For vacuum infiltration, the plant is placed in a vacuum chamber with its roots submerged in the nanoparticle suspension. Once vacuum is applied, the negative atmospheric pressure created results in the air inside the apoplast bubbling out while being replaced by the surrounding liquid, along with the nanoparticles in it. A benefit of entry *via* the root system is the ability of the nanoparticles to be transported *via* the xylem and phloem, therefore allowing distribution to the various organs of the plant. Iron oxide nanoparticles<sup>22</sup> and Metal–Organic Frameworks (MOFs)<sup>23</sup> are a few examples of nanoparticles that have been effectively translocated following infiltration *via* the root system. Infiltration *via* the root may also be more efficient to deliver nanoparticles to more plants, as many plants can be grown in the nanoparticle solution.

There are limitations to the root infiltration, as the mechanisms nanoparticle entry *via* the root has not been explored thoroughly yet. The presence of soil or other substrates often interferes with the uptake of nanoparticles, as the nanoparticles are hypothesized to stick to the substrate, lowering the efficiency of infiltration.<sup>22</sup> Furthermore, there may be concerns of environmental contamination or leaching from the nanoparticle growth solution or substrates used.

**2.4.3 Floral dip.** A common method to genetically transform the gametes of *Arabidopsis* involves dipping the flower into *Agrobacterium* sucrose solution containing Silwet-77 surfactant.<sup>24,25</sup> The presence of the Silwet-77 surfactant enhances entry of the *Agrobacterium* into the gametes, replacing the need for vacuum-enhanced delivery. Thus far there has been no reported attempts to deliver biomolecules by floral dip into nanoparticle–biomolecule solutions.

Despite this, floral dip may still be a possible alternative delivery pathway, as previous studies have shown successful delivery of nucleic acids into pollen grains using nanoparticles.<sup>26,27</sup> However, the pollen grains thus far have been collected from the plants before transformation using nanoparticles. Floral dip might be a viable method to achieve pollen transformation without having to remove the pollen grains beforehand. Breakthroughs in pollen transformation using the floral dip method would allow higher throughput transformation of plants, removing the need for pollen collection. Furthermore, as nanoparticles-mediated delivery of biomolecular cargo is often species-independent, this may be a more versatile method for genetic transformation of plants, as the species-dependent *Agrobacterium* is not needed.

## 3. Cargo delivery with nanoparticles into plant cells

The use of nanomaterials as delivery vehicles for biomolecules into plant cells has garnered significant interest in recent years.<sup>28</sup> Nanoparticles are promising cargo carriers into the plant cells due to their small size commensurating with plant cell wall pore sizes, which range between 15–30 nm.<sup>29–31</sup> Consequently, they can overcome the unique barriers imposed by the plant cell wall, facilitating the delivery of exogenous biomolecules such as small interfering RNA (siRNA) for transient gene silencing and functional DNA for target gene expression within plant cells. In this section, we highlight the different biomolecular cargoes which have been successfully delivered by nanoparticles into plant cells.

### 3.1. DNA delivery

DNA can be considered a key that holds necessary information for the development and function of an organism. Genetic engineering aims to achieve precise modification of this 'key' in order to unlock new features to the cell. For instance, by specifically modifying the genome of crops, we can augment their yield potential to surpass the capability of conventional varieties.<sup>32</sup> This precision in genetic manipulation empowers us to meet the increasing demand for agricultural products more efficiently. Such modifications encompass techniques such as gene knockout, which involves disabling undesired genes,<sup>33</sup> or the replacement of specific fragments of the host genome with desired DNA fragments.<sup>34</sup> In the latter case, the desired DNA may need to be supplied from an external source, necessitating efficient and precise delivery methods.

Earlier examples of nanoparticle-mediated DNA delivery systems required external aid, such as a gene gun or a magnetic field. Mesoporous silica nanoparticles (MSN) were the first to be used for co-delivery of DNA and other cargoes using the gene gun, while magnetic nanoparticles demonstrated DNA delivery efficacy with the assistance of an externally-applied magnetic field.<sup>26,35–37</sup> The first example of passive nanoparticle-mediated DNA delivery to *Arabidopsis* roots was achieved using organically functionalized MSNs.<sup>38</sup> The MSNs

had hydrodynamic diameters of around 100–150 nm, and could internalize into the vascular bundles for the delivery of mCherry-encoding plasmid DNA (pDNA) into *Arabidopsis thaliana* roots. These MSNs were found to internalize into the root cells mainly through an endocytosis-independent pathway.

High aspect-ratio nanoparticles have shown promise for DNA delivery to plant cells due to their ability to deliver cargo without a need for mechanical aid.<sup>17,39</sup> Due to their small width, these nanoparticles are able to passively pass through cell wall pores, which have an average size cut off limit of around 20 nm.<sup>40,41</sup> Specifically, single-walled carbon nanotubes (SWNTs) were employed in early studies showing successful DNA delivery to the leaf mesophyll (Fig. 2a and b). One such nanoparticle system was developed for pDNA delivery to chloroplasts.<sup>17</sup> In this study, SWNTs were first functionalized with chitosan (CS-SWNTs) to enable electrostatic binding of cargo pDNA-encoding yellow fluorescence protein (YFP). Cargo release of CS-SWNTs was demonstrated only at alkaline pH, ensuring the selective release of the pDNA cargo in the more basic environment within the chloroplasts. YFP expression overlapping with chloroplast autofluorescence was observed in treated wild-type spinach, tobacco, arugula and watercress leaves, demonstrating successful delivery of pDNA mediated by the SWNT nanocarrier system. Demirel *et al.* also employed SWNTs to deliver plasmid DNA to various plants using SWNTs that could bind electrostatically with GFP-encoding pDNA, resulting in safe delivery of pDNA to the mesophyll cells. In both studies, cationic SWNTs were found to be non-toxic to the plants.

These initial demonstrations inspired follow-up studies on engineering SWNTs for precise delivery of DNA to certain organelles within plant cells. For example, PEI-SWNTs were co-functionalized with a chloroplast-targeting peptide to deliver plasmid DNA to leaf chloroplasts.<sup>44</sup> Increased GFP signal and higher colocalization of GFP within the chloroplasts was observed on leaves treated with targeted nanoparticles, compared to leaves treated with SWNTs lacking modification of the targeting peptide. Although this targeting effect might be useful for highly specific cargo delivery, the treated leaves showed transient increases in reactive oxygen species which may lead to adverse effects on plant health. In a separate study, a mitochondria targeting SWNT system for pDNA delivery was developed by Law *et al.*<sup>45</sup> This was achieved by coating SWNTs in a thiol-reactive layer that allowed the conjugation of mitochondria-targeting and pDNA-binding cationic peptides. *Arabidopsis* plants treated with this nanocarrier showed almost 30-times increase in transient DNA expression, compared to previously reported delivery systems using the same pDNA and peptide complex. Similarly, peptide-enabled delivery of SWNTs were also demonstrated to be successful in delivering DNA into pollen grains protected by sporopollenin, a chemically inert and mechanically robust biopolymer protecting the sperm cells in spores and pollen grains.<sup>27</sup>

Besides SWNTs, Miyamoto *et al.* made use of micellar nanoparticles for nuclear delivery of pDNA into plant cells.<sup>46</sup> Micelles loaded with pDNA were functionalized with cell-pene-

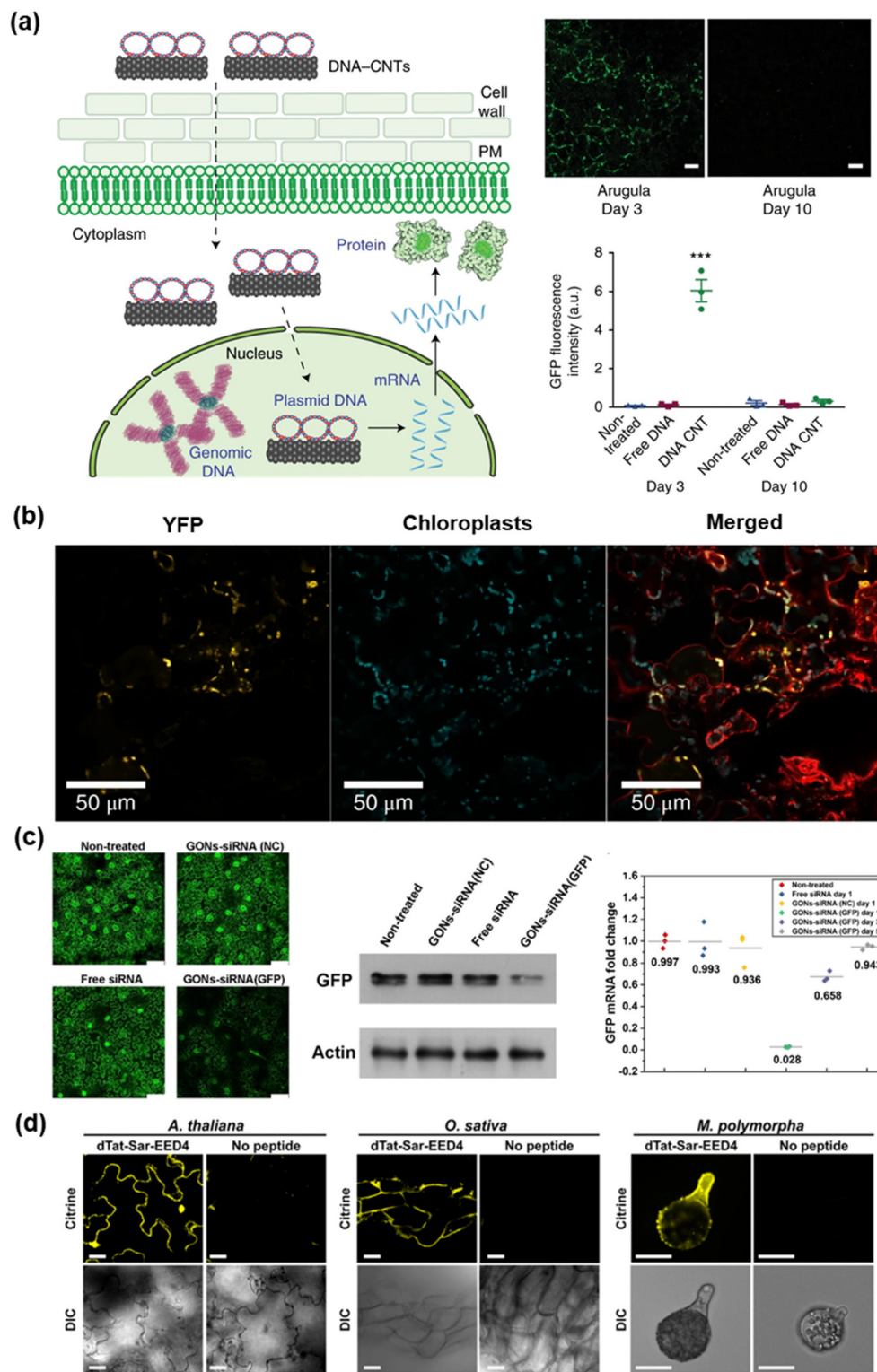
trating (CPP) and endosome-disrupting (EDP) peptides *via* a thiol–maleimide reaction. CPPs are short peptides that can cross cell walls and membranes.<sup>47,48</sup> CPP facilitated higher nanoparticle uptake into the cells while EDP enabled escape of the internalized micelles from endosomes, increasing delivery efficiency of the DNA-encapsulating nanostructures to the nucleus.

### 3.2. RNA delivery

Delivering double-stranded RNA (dsRNA) and siRNA to plant cells can lead to the suppression of specific gene expressions in a process termed as RNA interference (RNAi).<sup>49</sup> Specifically, the type III ribonuclease (RNase) can induce the random cleavage of dsRNA into siRNA.<sup>49–51</sup> The siRNA then associates with the Argonaute protein (AGO) to form an RNA-induced silencing complex (RISC) to recognize and degrade mRNA molecules, suppressing target gene activity.<sup>52,53</sup> RNAi technologies have demonstrated excellent capabilities in elucidating gene function,<sup>54</sup> augmenting the production of valuable biomolecules,<sup>55</sup> and enhancing plant resistance to pests and pathogens.<sup>56</sup>

Nanomaterials can enhance RNA stability and uptake, thereby improving the efficiency of RNAi.<sup>57,58</sup> Consequently, a range of nanomaterials have been developed for RNA delivery. Similar to DNA delivery, SWNTs were engineered to deliver siRNAs for silencing plant genes. Sense and antisense strands of siRNAs were bound to different SWNT nanocomplexes and upon internalization in leaf cells, the two complementary single-stranded siRNAs were released from SWNTs, generating siRNA duplexes that resulted in reduced GFP expression. SWNTs can protect siRNA from enzymatic degradation by plant ribonuclease, improving its longevity.<sup>59</sup> Likewise, surface-modified graphene oxide nanoparticles (GONs) functionalized with polyethyleneimine (PEI) and polyethylene glycol (PEG) can facilitate the delivery of siRNA into plant cells and improve RNAi efficiency through the formation of nearly spherical, small-volume GONs-siRNA complexes (Fig. 2c).<sup>42</sup> DNA nanostructures are recently emerging RNA delivery tools that are easily metabolizable, efficient and non-toxic.<sup>60</sup> A study showed that DNA nanostructures can effectively deliver siRNA, leading to the downregulation of the GFP gene at both the RNA and protein levels. The internalization into plant cells and the efficiency of RNAi are notably influenced by the size, shape, rigidity, and compactness of DNA nanostructures.<sup>61</sup> Moreover, it has been observed that CPPs exhibit the capability to not only transport nucleic acids into plant cells but also to target specific intracellular compartments within them, such as mitochondria and chloroplasts.<sup>62–65</sup> Thagun *et al.* sprayed siRNA/CPP micellar nanocomplexes that can target chloroplasts on the surface of leaves and successfully transported siRNAs into plant cells to induce GFP silencing in chloroplasts.<sup>66</sup>

Although research progress on the RNAi efficacy of the above-mentioned nanocarriers for delivering RNA cargo have been largely encouraging, their practical application in agriculture remains limited. In separate studies, carbon dots (CDs)<sup>67</sup>



**Fig. 2** Nanoparticle-mediated delivery of nucleic acids and proteins into plant cells. (a) Delivery of GFP-encoding plasmid DNA with PEI-SWNT into plant cells, leading to transient GFP expressions over 1-week post-infiltration (scale bar: 50  $\mu\text{m}$ ). Reproduced with permission.<sup>39</sup> Copyright 2019, Springer Nature. (b) Chitosan-functionalized SWNT facilitated chloroplast-selective DNA delivery to the chloroplasts of mature arugula leaf. Reproduced with permission.<sup>17</sup> Copyright 2019, Springer Nature. (c) Functionalized graphene oxide nanoparticles delivered GFP-silencing siRNA into plant cells (scale bar: 80  $\mu\text{m}$ ). Reproduced with permission.<sup>42</sup> Copyright 2022, Wiley-VCH. (d) Peptide nanocomplexes-mediated protein delivery into cells from different plant species. Reproduced with permission (scale bar: 20  $\mu\text{m}$ ).<sup>43</sup> Copyright 2022, American Chemical Society.

and layered double hydroxide (LDH)<sup>68</sup> have been successfully demonstrated in applications aimed at reducing plant pests and diseases. CDs, a type of carbon-based nanomaterial, have gained widespread recognition in the biological field due to their ease of synthesis and functionalization, as well as their minimal toxicity and high biocompatibility.<sup>69</sup> In the realm of plant biology, CDs have been effectively employed to transport siRNA into tomato and *Nicotiana benthamiana* cells, resulting in the downregulation of GFP expression.<sup>70,71</sup> A recent study has demonstrated notable advancements in the utilization of CDs to enhance plant disease resistance.<sup>67</sup> CDs functionalized with poly(ethylene glycol) diamine (PEGDA), can efficiently load Csa3-/OSBP1-dsRNAs through electrostatic attractions to target the key regions of Csa3 and OSBP1 found in *Phytophthora* pathogens. The dsRNA-CDs complex can promote the internalization and uptake of dsRNAs within both plants and pathogens through clathrin-dependent endocytosis for effective control of *Phytophthora* infections in plants.<sup>67</sup> LDH clay nanosheets are a type of inorganic layered material that can enhance the stability of dsRNAs, thereby effectively protecting plants from viruses through the gradual release of dsRNAs.<sup>72</sup> Notably, Mg–Al LDH nanosheets can be successfully internalized into plant cells and chloroplasts, translocating through the plant apoplast and vascular system. Therefore the siRNAs were delivered into plant cells, effectively silencing the target genes.<sup>8</sup> MgAl-based LDH also showed longer persistence on leaf surface, offering sustained protection against plant viruses.<sup>72</sup> However, repeated use of MgAl may cause aluminum toxicity. To address this drawback, Jain *et al.* replaced Al with Fe to produce MgFe-LDH, with less toxicity to plants.<sup>68</sup> The delivery of dsRNA by MgFe-LDH was able to effectively increase the mortality rate of the insect pest *Bemisia tabaci* in cotton.

### 3.3. Protein delivery

Efficient protein delivery into cells underpins most of today's gene-editing and biotechnological applications.<sup>73,74</sup> In plant genetic engineering, it is important to find a delivery method which prevents unwanted or accidental insertion of foreign DNA in the plant genome for biosafety. RNA and proteins are suitable biomolecular cargo candidates for this purpose due to their ability to edit the cell function transiently without affecting the cell's genome integrity. Compared to the more fragile RNA, proteins are relatively more stable and can function without delay,<sup>75</sup> and are able to reach the intended target after internalization due to specifically-designed sequence motifs.<sup>76</sup> They can also be produced on a large scale through biomanufacturing, thereby lowering the cost of production.<sup>77</sup> Based on those advantages, proteins seem more suitable for transient genetic engineering applications. However, proteins usually are unable to pass through the cell membrane, which acts as a highly selective semipermeable barrier.<sup>78–80</sup> The bulky structure and polarity of protein imposes challenges to the design of universal carriers and optimization of experimental conditions for the delivery process. While research efforts have shown moderate success in delivering proteins to mammalian cells,<sup>81</sup> such techniques are often not applicable

for plant cells due to the presence of a thick, multilayered plant cell wall.

Gold nanoparticles have been used to deliver proteins into plant cells *via* particle bombardment. In 2016, CRISPR-Cas9 Ribonucleoprotein complex (RNP) was successfully delivered into immature embryos of corn by biolistic bombardment and achieved targeted gene editing with 0.69% mutation efficiency.<sup>82</sup> Similarly, Cas9 RNP complexes delivered by particle bombardment caused 0.56% mutation efficiency in bread wheat.<sup>83</sup> MSN encapsulating Cre recombinase was also delivered *via* bombardment into the maize cells.<sup>84</sup> After delivery, the Cre enzyme which recombines the loxP sites by removing the DNA fragment flanked can be released from the MSNs. Considering the inherent turgor pressure within the plant cell, applying additional osmolytic reagents is necessary to prevent the inadvertent leakage of cellular contents. Otherwise, potential breaches in the cellular membrane may affect delivery efficiency and cause cell death.<sup>85</sup>

Besides particle bombardment, pressurized leaf infiltration could also facilitate nanoparticle-mediated delivery of proteins past the plant cell walls. Ng *et al.* had engineered fusion-form micellar nanocomplexes that incorporate a cell penetrating peptide (CPP) with a polycationic peptide.<sup>86</sup> After electrostatic mixing to form micellar nanocomplexes, this nanocarrier system successfully delivered various protein molecules from 27–150 kDa by infiltration. Moreover, this delivery process is fast, and the delivered protein could be detected inside the cell as early as 6 hours after infiltration. In a similar study, a peptide-mediated delivery of proteins into the cytosol of plant cells was observed through a macropinocytosis-like mechanism (Fig. 2d). Citrine was successfully delivered into plant cell protoplasts and photosynthetic autotrophs using this method.<sup>43</sup> However, the delivery of functional proteins for gene modification and the generation of the resulting gene-modified plants have not been explored using nanocarriers. Furthermore, the internalization mechanism of protein-encapsulating nanocarrier systems into plant cells is still not sufficiently studied.

Current nanoparticle-mediated protein delivery methods in plants typically require either particle bombardment or complexation with cationic polymers to form nanomicelles. While each method offers its own advantages, they also present a unique set of limitations, side effects, and efficacy concerns. Due to these shortcomings, nanoparticle-enabled protein delivery remains an emerging field in plant biotechnology that requires further exploration.

## 4. Nanocarrier design parameters for efficient delivery into plant cells

Nanoparticles offer multiple design handles which can be facilely tuned to adjust their physical and surface properties. Their design versatility offers unique opportunities to study how their properties influence the interaction between nanoparticles and plant biological barriers. Insights from these studies could offer new insights into designing effective nano-

carrier systems for biomolecule delivery into plant cells. In this section, we discuss the importance of various nanoparticle design parameters in governing nanoparticle internalization efficiency into plant cells and organelles.

#### 4.1. Size

The size of a nanocarrier plays a crucial role in its delivery efficiency, particularly due to the small pores and cellular barriers present in plant tissue.<sup>87</sup> For instance, to penetrate the leaf surface, nanocarriers must traverse waxy cuticle pores (approximately ~2 nm in diameter) or stomata (microscale) to reach the deeper mesophyll.<sup>88–93</sup> Additionally, for cellular uptake, nanoparticles must navigate through cell wall pores (<20 nm) and traverse the cell membrane. Hence, optimizing nanocarrier size is crucial for effective transport and delivery within plant systems.

Extensive studies have investigated the relationship between nanoparticle size and delivery efficiency across various types of nanocarriers (Table 1).<sup>28,39,94,95</sup> For example, Hu *et al.* prepared surface-modified nanocarriers based on carbon dots (CDs, 1.7–6.4 nm), CeO<sub>2</sub> (1.8–15.6 nm) and SiO<sub>2</sub> (18.0 nm).<sup>94</sup> By labeling the nanoparticles with fluorescent dyes, the authors were able to comprehensively observe the localization of infiltrated nanocarriers in *Gossypium hirsutum* (dicotyledon) or *Zea mays* (monocotyledon) leaves. The results showed that the nanocarrier sizes should be lower than 20 nm (*Gossypium hirsutum*) and 11 nm (*Zea mays*) to penetrate

through leaf stomata or cuticle. In a separate study, the localization of spherical and non-spherical nanoparticles (such as SWNTs) within isolated plant cells and chloroplasts was investigated.<sup>28</sup> It was discovered that for non-spherical nanoparticles, the smallest dimension (*e.g.* diameter) significantly influenced their trafficking efficiency more than their length. These findings suggest that nanoparticles primarily pass through the physical pores of plant barriers, with smaller nanoparticles facilitating higher uptake into the mesophyll and cells. This underscores the importance of optimizing nanoparticle size to improve their transport into plant tissues.

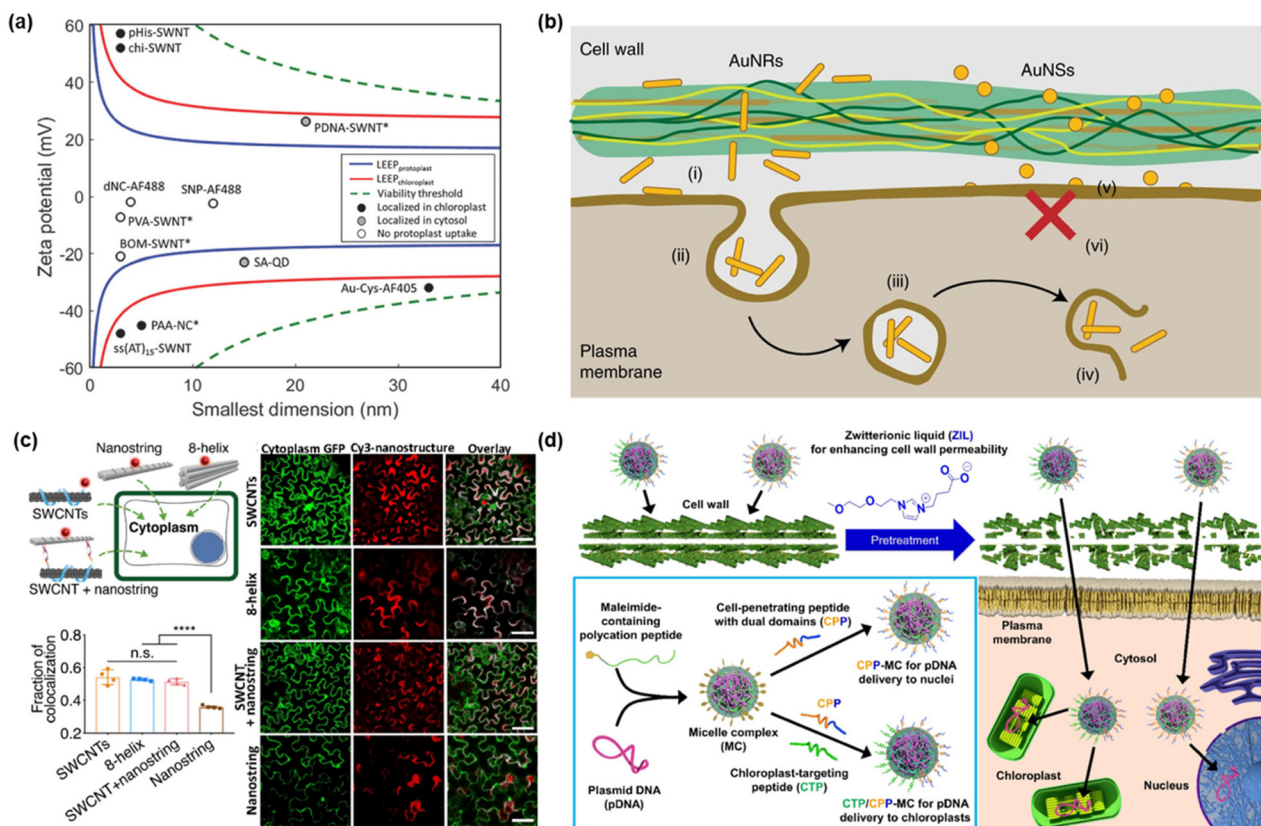
#### 4.2. Surface charge

Besides nanoparticle size, surface charge is another important parameter which influences nanocarrier delivery towards plant cells (Table 1).<sup>28,39,87,94,95</sup> To investigate the effect of surface charge, SWNTs were modified to display surface groups bearing different charges.<sup>28</sup> These functionalized SWNTs were subsequently incubated with isolated protoplasts and chloroplasts. Following overnight incubation, highly charged SWNTs were observed to be more efficiently localized within the interior of protoplasts and chloroplasts. In contrast, nanoparticles with neutral surface charge were absent in plant cells after overnight passive incubation. These findings offer initial insights into the critical role of surface charge in interacting with plant cell and organelle membranes, facilitating nanoparticle penetration into cells.

**Table 1** Sizes and zeta potentials of materials and their penetration abilities

Material <sup>a</sup>	Size (nm)	Zeta potential (mV)	Penetrated location	Ref.
PEI-CD	1.7	23.3	Chloroplast ( <i>Gossypium hirsutum</i> ); chloroplast ( <i>Zea mays</i> )	94
PEI-CD	5.5	36.6	Chloroplast ( <i>Gossypium hirsutum</i> ); chloroplast ( <i>Zea mays</i> )	94
SA-CD	1.9	-13.8	Guard cell ( <i>Gossypium hirsutum</i> ); extracellular space ( <i>Zea mays</i> )	94
SA-CD	6.4	-36.9	Guard cell ( <i>Gossypium hirsutum</i> ); extracellular space ( <i>Zea mays</i> )	94
DiI-ADNC	7.5	14.9	Chloroplast ( <i>Gossypium hirsutum</i> ); extracellular space ( <i>Zea mays</i> )	94
DiI-ADNC	11.7	34.6	Chloroplast ( <i>Gossypium hirsutum</i> ); not penetrated ( <i>Zea mays</i> )	94
DiI-PNC	1.8	-43.5	Chloroplast ( <i>Gossypium hirsutum</i> ); guard cell ( <i>Zea mays</i> )	94
DiI-PNC	10.8	-40.3	Guard cell ( <i>Gossypium hirsutum</i> ); not penetrated ( <i>Zea mays</i> )	94
DiI-PNC	15.6	-52.3	Guard cell ( <i>Gossypium hirsutum</i> ); not penetrated ( <i>Zea mays</i> )	94
FITC-SN	18	-45.8	Not penetrated ( <i>Gossypium hirsutum</i> ); not penetrated ( <i>Zea mays</i> )	94
SA-QD	15	-23	Cell ( <i>Arabidopsis thaliana</i> )	28
Au-Cys-AF405	33	-33	Chloroplast ( <i>Arabidopsis thaliana</i> )	28
SNP-AF488	12	-2.4	No protoplast uptake ( <i>Arabidopsis thaliana</i> )	28
dNC-AF488	4	-1.8	No protoplast uptake ( <i>Arabidopsis thaliana</i> )	28
ss(AT) <sub>15</sub> -SWNT	3	-48	Chloroplast ( <i>Arabidopsis thaliana</i> )	28
chi-SWNT	3	52	Chloroplast ( <i>Arabidopsis thaliana</i> )	28
pHis-SWNT	3	57	Chloroplast ( <i>Arabidopsis thaliana</i> )	28
PVA-SWNT	3	-7	No protoplast uptake ( <i>Spinacia oleracea</i> )	28 and 95
BOM-SWNT	3	-21	No protoplast uptake ( <i>Spinacia oleracea</i> )	28 and 95
pDNA-SWNT	16	32	Cell ( <i>Nicotiana benthamiana</i> , <i>Eruca sativa</i> , <i>Triticum aestivum</i> and <i>Gossypium hirsutum</i> )	39

<sup>a</sup> Abbreviation list: PEI-CD: polyethylenimine-modified carbon dots; SA-CD: succinic anhydride-modified carbon dots; DiI-ADNC: (2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl) prop-2-enylidene]-3,3-dimethyl-1-octadecylindole perchlorate-labeled positively charged aminated dextran-modified CeO<sub>2</sub>; DiI-PNC: (2Z)-2-[(E)-3-(3,3-dimethyl-1-octadecylindol-1-ium-2-yl)prop-2-enylidene]-3,3-dimethyl-1-octadecylindole perchlorate-labeled dextran-modified CeO<sub>2</sub>; FITC-SN: fluorescein isothiocyanate-labeled SiO<sub>2</sub>; SA-QD: streptavidin-modified quantum dot; Au-Cys-AF405: Alexa Fluor 405-conjugated gold-cysteine; SNP-AF488: Alexa Fluor 488-conjugated SiO<sub>2</sub>; dNC-AF488: dextran-modified nanoceria conjugated with Alexa Fluor 488; ss(AT)<sub>15</sub>-SWNT: ssDNA (AT)<sub>15</sub>-modified single-walled carbon nanotube; chi-SWNT: chitosan-modified single-walled carbon nanotube; pHis-SWNT: polyhistidine-modified single-walled carbon nanotube; PVA-SWNT: polyvinyl alcohol-modified single-walled carbon nanotube; BOM-SWNT: bombolitin II peptide-modified single-walled carbon nanotube; PDNA-SWNT polyethylenimine & DNA-modified single-walled carbon nanotube.



**Fig. 3** Influence of nanoparticle design parameters on nanoparticle transport in plant cells. (a) LEEP model relates the size and surface charge of nanoparticles with their localization inside plant protoplasts. Reproduced with permission.<sup>28</sup> Copyright 2018, Wiley-VCH. (b) Morphology-dependent transport of gold nanorods and nanospheres in plant cells.<sup>99</sup> Copyright 2022, Springer Nature. (c) DNA origami-based nanostructures showed stiffness-dependent entry into plant cell cytosol (scale bar: 40  $\mu\text{m}$ ).<sup>61</sup> Copyright 2019, National Academy of Sciences. (d) Peptide nanocomplex-mediated protein delivery into cells from different plant species. Reproduced with permission.<sup>100</sup> Copyright 2022, Wiley-VCH.

Based on experimental observations of nanoparticle localization in protoplasts, an empirical model was formulated to describe the relationship between nanoparticle size and surface charge in governing nanoparticle trafficking efficiency. This Lipid Exchange Envelope Penetration (LEEP) model suggests that highly charged nanoparticles, regardless of the charge polarity, possess the sufficient energy required to bypass threshold bilayer membranes through membrane polarization (Fig. 3a).<sup>28,96</sup> This hypothesis is further supported by the nanoparticle–leaf interaction (NLI) model proposed in another study, which reinforces the role of surface charge in enhancing nanoparticle penetration into plant cells.<sup>94</sup>

### 4.3. Morphology

Nanoparticle shape is another important design parameter for efficient delivery. For mammalian cells, different shapes could influence different uptake pathways, such as by caveolae-, clathrin- or other mediated pathways.<sup>97,98</sup> It is therefore possible that such morphology-dependent mechanisms may also impact nanoparticle uptake into plant cells.

In terms of morphology, commonly used nanoparticles for plant engineering can be broadly classified under two categories: low and high-aspect ratio. Low aspect ratio nano-

carriers include spherical gold nanoparticles, carbon dots and metal-based quantum dots. High aspect ratio nanocarriers include gold nanorods and carbon nanotubes. Nanocarriers of other shapes (nanotriangles, nanobipyramids, nanostars, *etc.*) are also alternatives to explore, where edges of different sharpness and angles could enhance or hinder the entry of the nanoparticle into cells. For example, gold nanotriangles were observed to have the highest cell uptake among differently-shaped gold nanoparticles such as nanostars and nanorods, due to entry *via* multiple endocytosis pathways.<sup>101</sup> As plant systems have different cellular structures, the effects of shape for nanoparticle and hence cargo delivery may vary. Therefore, elucidation into the effects of shape is crucial to gain insights into optimizing parameters for biomolecule delivery.

Gold nanospheres (AuNS) are frequently utilized for biomolecule delivery due to high precision of size control during synthesis and the ease of attaching DNA, RNA, and proteins to the gold surface *via* Au–thiol bonds. This allows for efficient and versatile loading of various biomolecules onto AuNS, which enhances their potential for targeted delivery applications. AuNS have been utilized in studying the delivery of molecules to different plants, such as *Arabidopsis thaliana*,<sup>102</sup> *Nicotiana benthamiana*,<sup>99</sup> and periwinkle.<sup>103</sup> The small size

that they can be synthesized in (5–20 nm) also makes entry *via* the cell wall pores possible. Mini gold nanorods (AuNR) can be synthesized in similar sizes. Hence, both small nanospheres and nanorods could theoretically be utilized and compared as cargo delivery mechanisms into plant cells.

Currently, only one study has directly compared the delivery efficacy of the two gold nanoparticle morphologies.<sup>99</sup> AuNR were found to rotate and orient perpendicularly to the cell membrane in mammalian cell systems.<sup>104</sup> Zhang *et al.* hypothesized that this phenomenon facilitated entry of nanorods into plant cells, as more perpendicularly-oriented AuNR were found compared to randomly oriented ones. TEM imaging revealed the internalization of AuNR within the plant cells, whereas the AuNS failed to enter the cell and remained clustered around the outside of the cell wall. It is hypothesized that the AuNR enters *via* endocytosis, due to the decrease in colocalization fraction of AuNR as compared to AuNS when endocytosis inhibitors are given.

In this same study, although AuNRs were observed to penetrate plant cells *via* TEM, their uptake into plant cells does not necessarily lead to higher cargo delivery efficiency. Notably, siRNA delivery proved more effective using 10 nm AuNS over AuNRs, despite the former accumulating outside the cell wall. It is hypothesized that the accumulated AuNS could act as a cargo reservoir on the cell wall, releasing the siRNA into the cells continuously as other biomolecules replace the surface-bound cargo (Fig. 3b). The ability of AuNS to deliver siRNA is therefore independent of its ability to enter the plant cells. As the result remains counter-intuitive, more studies on the link between nanocarrier entry and cargo delivery will benefit the field of plant nanotechnology.

Another comparative system involves carbon dots and nanotubes, which are allotropes of carbon. Both types of nanoparticles have been utilized to deliver small biomolecules such as siRNA<sup>59,70</sup> or DNA<sup>17,105</sup> to plants. As there has not been a direct comparative study between the two, both nanoparticles can be considered useful for the delivery of small biomolecules into plants. The difficulty of imaging both carbon-based nanoparticles under TEM complicates the comparison, as it is difficult to determine if either nanoparticle has preferable entry into the plant cell.

To address this, two possible methods can be utilized to determine carbon nanoparticle entry into plants. The first method involves plasmolysis in combination with confocal microscopy of fluorescent-tagged nanoparticles. This approach allows for the evaluation of nanoparticle entry into transgene fluorescent plant cells. In the absence of plasmolysis, it becomes challenging to determine whether the nanoparticles reside just outside the cell wall or internally due to their size falling below the resolution limit. Plasmolysis, which involves withdrawing the cytoplasm away from the cell wall, is therefore essential. The colocalisation of the fluorescent-nanoparticles and fluorescent plasmolysed-cytoplasm provides a clearer indication of the entry of carbon nanoparticles. The other method employs self-complementing split-fluorescent protein systems to determine nanoparticle entry.<sup>106</sup> A partial fluorescent

protein, such as GFP 1–10, can be expressed in plant cells using *Agrobacterium*-mediated transformation. Separately, GFP 11, attached as cargo with the carbon nanoparticle, can be delivered into the plant cell. Using a method similar to the CPP-mediated Delivered Complementation in Planta (DCIP) described by Wang *et al.*,<sup>107</sup> the successful expression and recombination of two split GFP protein in the plant cytosol would indicate entry of the nanoparticle into the plant cell. Therefore, these two methods are viable options to compare the efficacy of entry between the different carbon nanoparticles. Given that both carbon nanoparticles have been shown to successfully deliver small biomolecular cargo, shape does not appear to have a significant effect on delivery of small biomolecules by carbon-based nanoparticles.

#### 4.4. Stiffness

Stiffness is another important nanocarrier design parameter that could influence cargo delivery efficiency. The effects of nanocarrier stiffness on cellular uptake in mammalian cells have been extensively studied. For instance, Moses *et al.* assessed the dependence of cellular uptake of liposome-based nanoparticles by human breast cancer (MDA-MB-231 and MCF7) and mammary epithelial cells (MCF10A) on nanoparticle elasticity. They found that cellular uptake increased with decreasing liposome stiffness. This was attributed to softer liposomes being able to traverse the cell membrane through both membrane fusion (major) and endocytosis (minor) pathways, while stiffer liposomal nanoparticles could only do so *via* endocytosis. The hypothesis was later verified using endocytosis inhibitors, which greatly hindered the uptake of stiff nanoparticles while not influencing their soft counterparts.<sup>108</sup>

On the contrary, Jiang *et al.* characterized the uptake of PEGylated polymer-lipid nanoparticles of varying Young's moduli by HeLa cells and discovered that compared to softer nanoparticles, stiffer counterparts were able to pass the cell membranes more easily and as a result exhibited higher cell internalization. Based on molecular dynamics (MD) simulations, this was due to a significant increase in energy required for the cell membrane to wrap the softer nanoparticles when they deform from a spherical to an ellipsoidal morphology during the internalization process.<sup>109</sup> As plant cells have a rigid cell wall that is absent in mammalian cells, nanocarrier stiffness may play an important role in determining the nanoparticle penetration efficiency into plant cells.

Several nanocarriers have been successfully used to bypass the plant cell wall for biomolecule delivery.<sup>39</sup> These include SWNT,<sup>39,110</sup> MSN,<sup>35</sup> gold nanoparticles<sup>39,102</sup> and clay nanosheets.<sup>72</sup> A common feature among these nanocarriers is their high tensile strength, suggesting that delivery efficiency in plant cells is dependent on the stiffness of the nanocarriers. In 2019, Zhang *et al.*, inspired by a previous work done on the stiffness-dependent uptake of colloidal particles in mammalian cells,<sup>111</sup> conducted a study to investigate the effect of DNA nanocarrier stiffness on its uptake in plant cells.<sup>61</sup> Briefly, Cy3-labeled DNA nanostructures of similar shape but varying stiffness were infiltrated into *Nicotiana benthamiana* leaves.

The nanostructures tested in order of increasing mechanical stiffness were 1D DNA nanostrings, eight-helix DNA bundles,<sup>112</sup> DNA nanostrings tethered to SWNT and Cy3-labeled (GT)<sub>15</sub>-SWNT. Internalization of the different nanostructures in the plant cells was assessed using confocal microscopy and colocalization analysis (Fig. 3c). Results revealed that the SWNTs, SWNT-DNA hybrid and DNA bundles were internalized to a greater extent compared to the nanostrings, thus suggesting that nanocarriers of higher mechanical stiffness provide higher delivery efficiencies into plant cells. A possible hypothesis underlying this observation is that nanocarriers with higher Young's moduli are more capable of piercing through the cell wall barrier and entering the cell.<sup>39</sup>

#### 4.5. Surface functionalization with peptides

Besides tuning the physical properties of nanoparticles, functionalizing the surface of nanoparticles with targeting moieties can endow them with enhanced penetration efficiencies and subcellular targeting selectivity. In recent years, CPPs and signal peptides have been incorporated into nanoparticle delivery systems to facilitate transportation of macromolecular cargo in plant cells. They can facilitate the translocation of cargos across cell membranes through various mechanisms including endocytosis and direct penetration.<sup>62–65</sup> For a comprehensive understanding of CPP characteristics, readers can refer to detailed review by S. Deshayes *et al.*<sup>113</sup> Additionally, a signal peptide, also known as a signal sequence, is a short amino acid sequence found at the N-terminus of many newly synthesized proteins in cells.<sup>114</sup> It plays a crucial role in guiding these proteins to their correct location within or outside the cells. The recognition and binding of signal peptides by cellular machinery are essential for the accurate and efficient sorting of proteins to their destinations.<sup>115</sup> The function of signal peptides can be predicted using bioinformatic approaches that screen through the amino acid sequences of organelle-specific proteins. Nanoparticles or micelles functionalized with signal peptides have been shown to successfully target specific plant intracellular compartments, such as mitochondria and chloroplasts.<sup>66,116</sup>

Based on the fundamental properties of CPPs and signal peptides, conjugating a CPP with a signal peptide can serve specific purposes in cellular and molecular biology research, particularly in efficiently and precisely delivering cargo molecules into cells. Chloroplasts and mitochondria work as cellular factories that supply oxygen and energy to plant cells as well as produce a range of small molecules.<sup>66,117</sup> By fusing CPP with a chloroplast-targeting peptide (CTP), positively-charged nanocomplexes can be formed, facilitating the selective delivery of biomolecular cargoes to chloroplasts.<sup>118</sup> The availability of other signaling peptide sequences would enable targeted nanoparticle delivery to different subcellular compartments within the plant cells.<sup>119</sup> By achieving more efficient delivery and targeted localization, the use of CPPs and signal peptides can potentially reduce cytotoxicity associated with some other delivery methods which may involve higher concentrations of cargo or less targeted approaches.<sup>120</sup>

#### 4.6. Co-delivery agents

Co-delivery agents are used for enabling or enhancing the delivery of nanoparticles to the plant cells. In the context of plant cells, these delivery agents are mainly focused on loosening the cell wall as it is arguably the main limitation for nanoparticle-based delivery systems. Ionic liquids (ILs) were early examples of cell wall loosening treatments.<sup>121</sup> However, ILs were known to induce cytotoxicity *via* damaging the plasma membrane.<sup>122</sup> Further tests on plants proved that the ILs negatively affected plant health.<sup>123–125</sup> Zwitterionic liquids (ZILs) were suggested as an alternative treatment option for partially disrupting the cell wall structure without inducing noticeable cytotoxicity.<sup>126</sup> A study led by Miyamoto *et al.* utilized a ZIL (OE<sub>2</sub>imC<sub>3</sub>C) as a co-delivery agent for the delivery of micelle complex with peptide and plasmid DNA cargo (Fig. 3d).<sup>100</sup> First, they compared the effects of ZIL on cell wall structure and cell health with those of commercially available ILs. Optimum concentrations of ZIL showed efficient disruption of cell wall without harming the cells while ILs showed significant cytotoxicity even at lower concentrations. Following this, they administered peptide/DNA containing micelle nanoparticles to ZIL pretreated *Arabidopsis Thaliana* plants. Transfection efficiency significantly improved in plants pretreated with optimal ZIL concentrations compared to non-treated and high-concentration ZIL-treated plants. Additionally, it was shown that micellar nanoparticles with sizes up to 200 nm were able to induce efficient transfection. Similar to ZILs, zwitterionic polymers (ZIPs) were shown to loosen the cell wall without the negative side effects.<sup>127</sup> In addition to this, expansions, a group of endogenous proteins that are involved in the cell wall enlargement process were suggested as a potential cell wall loosening molecule.<sup>128</sup> However, the potential of ZIPs and expansions as co-delivery agents for nanoparticle-based delivery applications is yet to be investigated.

A challenge for the field is to compare and evaluate the efficiency of different types of nanoparticles as delivery systems. As has been discussed in the review, there are many prospective biomolecular cargoes and nanoparticle systems for delivery, each with unique behaviors and effects. Therefore, this review suggests that authors report the following parameters such that meta-analyses of delivery efficiency across nanoparticle types are possible.

- Encapsulation efficiency: mass/amount of biomolecule carried by the nanoparticle against the initial mass of biomolecule introduced in the method.
- Loading efficiency: mass of biomolecule for every nanoparticle present
- Amount of biomolecules delivered: amount of biomolecules delivered into the system or present in the growth/dip/spray solution.
- Delivery method: *via* foliar, root, stem, floral dip or other pathways
- Co-delivery agents (if any): Zwitterionic liquids, Silwet-77 surfactants, or other agents used to enhance delivery.

## 5. Conclusion and outlook

As the field of plant nanotechnology progresses, there are challenges and several possible directions that warrant further study. A more comprehensive understanding of nanoparticle–plant interactions is essential. Although recent developments show that nanoparticles can efficiently deliver biomolecules to intact plant cells, the mechanisms by which they penetrate the cell wall and membrane remain understudied, hindering rational nanoparticle design for efficient internalization. Promising developments in the plant nanotechnology area thus far seem to indicate that nanocarriers have to meet the following criteria for efficient internalization: (1) size is the most important parameter to allow efficient biomolecule delivery. Unless soft-polymeric nanoparticles or cell-wall disrupting molecules are used, at least one nanoparticle dimension should have a size smaller than the cell wall pore size for effective entry. (2) Surface charge: after the nanoparticle is able to traverse past the cell wall, penetration of the cell membranes is required. According to the LEEP model, nanoparticles have to be sufficiently charged to cause a potential drop across the cell membrane, forming pores for entry. Neutral nanoparticle–cargo systems are unable to pass through the cell membrane, hence the surface charge of the nanoparticles must be accounted for. (3) Morphology is another factor to enhance delivery efficiency. As discussed previously, the shape of nanoparticles influences their entry pathways. Optimization of the nanoparticle morphology according to the target cell could lead to enhanced delivery of cargo. (4) Co-delivery agents are also valuable alternatives to consider, especially when cargoes larger than the cell wall pore size are in consideration. Current protein-delivery into plants are still reliant on biolistic methods to force entry. Therefore, co-delivery agents that temporarily disrupt the cell wall may allow larger proteins to enter without the need of harsher biolistic methods.

Another important aspect to consider is the subcellular distribution of nanoparticles within plant cells. Elucidating the mechanisms by which nanoparticles traffic through plant barriers and localize in specific subcellular compartments requires in-depth investigation of how nanoparticle physical and chemical properties affect their transport in plant biological environments. These include controlled and delayed release of biomolecules. Nanoparticles can be designed to release biomolecules over a long period of time for sustained gene expression/knockdown. Currently, there is a lack of studies regarding the protein–corona formed around the nanoparticle after delivery into plants. Exploration on their kinetics and effects will be informative in tuning the controlled release of biomolecular cargo and targeting. Smart nanoparticles can also be designed to release their biomolecular cargo only in the presence of certain stimuli. These stimuli can include temperature<sup>21</sup> or pH,<sup>17</sup> to allow release only in the presence of environmental stressors or specific organelles.

Furthermore, alternative pathways of delivery can also be considered. Microneedle patches (MNPs), widely used for

nanomedicine applications, can also be used to deliver biomolecules.<sup>129,130</sup> MNPs can be applied to various plant regions, including the stem, providing flexibility and ease of use. As the stem contains the xylem and phloem vasculatures, cargo delivery to the stem may facilitate the efficient transport of biomolecules throughout the plant system. MNPs can be engineered for controlled degradation, either releasing their cargo gradually or in bursts.<sup>129</sup> This tunable release mechanism enhances the versatility of nanoparticle delivery systems, allowing for tailored applications based on specific plant needs.

In conclusion, the field of designing nanoparticles for biomolecule delivery into plant cells is still in its early stages but holds great potential for various applications. Based on the papers discussed in this review, further research is needed to develop nanoparticles that can efficiently deliver biomolecules into intact plants and optimize their subcellular distribution. Additionally, the environmental and biological impact of nanoparticles on plants should be carefully studied. With continued advancements in nanotechnology and a deeper understanding of plant cell biology, the development of effective nanoparticle-based delivery systems for plant cells is within reach. By addressing these challenges and advancing our knowledge, we can harness the potential of nanoparticles to revolutionize plant biotechnology, offering new solutions for crop improvement and protection.

## Author contributions

Y.Z., C.T., and T.T.S.L. conceptualized and designed the study. Y.Z. and C.T. prepared the original draft of the manuscript. C. S., S.R., D.S., S.P., and X.Y. assisted with figure preparation, data curation and collection. All authors edited and reviewed the manuscript.

## Data availability

No new primary research results were generated as part of this review.

## Conflicts of interest

The authors declare no competing interest.

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