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Nanomaterials as tools in plant transformation: A protoplast-centric perspective

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ABSTRACT

Genetic engineering of plants can boost disease resistance, enhance crop traits, and ultimately improve agricultural productivity. Several approaches to plant bioengineering have been successful in recent decades. Nanomaterials (NMs) can be customized and fabricated with targeting capabilities, making them well-suited for bioengineering applications. These NMs include organic, inorganic, and composite materials with many different structures, including nanofibers, nanoparticles (NPs), and nanomembranes. Protoplasts are often used as target cells because they lack a cell wall and are more likely to endocytose NM. In this review, the efficacy of NMs in delivering genetic material to protoplasts is examined. The challenges associated with protoplast generation and optimization of protocols for transformation are explored and the possible advantages of NMs in this process are identified. The chemical properties of these NMs in relation to their potency is briefly discussed. Ultimately, this technology is evolving and our understanding of NMs and the requirement for migration through the cellular membrane is still missing several key pieces of information. The next decades will likely produce important new insights that will have important impacts in this field.

1. Introduction

Agriculture develops and cultivates crops to provide sufficient sustenance for a rapidly increasing population (Steinwand and Ronald, 2020) but climate change and extreme weather create biotic and abiotic stresses and reduce crop yields (Challinor et al., 2014). It is therefore necessary to modify existing plant species with traits that can withstand these stresses. Historically, this has been achieved using multigenerational breeding, but this process is slow and can be extremely costly. Genetic bioengineering can generate modified plants quickly and with specific combinations of traits that may not be possible with traditional breeding methods (Yan et al., 2022; Su et al., 2023). Genetic bioengineering relies on the delivery of nucleic acids into a target cell, thereby changing its genome. One of the most significant challenges in gene delivery to plant cells is the multilayered, rigid cell wall, primarily composed of cellulose microfibrils (Cunningham et al., 2018; Liu et al., 2009). Although some approaches have been successful in crossing the cell wall barrier and introducing foreign DNA to plants, it remains a significant challenge for plant biotechnologists. For this reason, some approaches have used protoplasts, plant cells lacking a cell wall, as the target cells for gene manipulations. By eliminating the cell wall barrier, protoplasts theoretically become more amenable to genetic manipulation, although there are still many barriers to successful gene transfer. This review will focus on some advantages and challenges of genetic engineering using protoplasts.

One of the more state-of-the-art approaches to plant engineering has been the application of nanomaterials (NM). This approach has many benefits, including increasing precision and decreasing development timetables. However, NM can be a problematic tool, accompanied by technological and environmental challenges. Throughout this review, we will examine how NM has been used to genetically engineer protoplast target cells – and we will discuss how some of these technological and environmental challenges have been overcome or can be addressed.

Abbreviations: NMs, nanomaterials; NPs, nanoparticles; MSNs, Mesoporous Silica nanoparticle; MNPs, Magnetic nanoparticles; RNTs, rosette nanotubes; CNT, carbon nanotubes; SWCNTs, Single-wall carbon nanotubes; MWCNTs, multi-walled carbon nanotubes; PEG, polyethylene glycol.

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2. Current bottlenecks using conventional plant transformation

Plant bioengineering is sometimes referred to as "transformation" and involves delivering biomolecules into plant cells or tissues and regenerating the transformed cells or tissues into whole plants. It is important to understand the processes that contribute to transformation so that it can be optimized and targeted to specific traits and plant species (Yan et al., 2023). Since this process is complex, it can encounter several roadblocks (Fig. 1). First, tissue culture methods often suffer from low regeneration rates, prolonged timelines, and genotype dependency, which can hinder the successful development of transgenic plants (Mangena et al., 2017; Singh and Prasad, 2016). Additionally, these methods may require extensive optimization and can be labor-intensive, posing significant challenges in scalability and efficiency (Aesaert et al., 2022; Aregawi et al., 2022). Unfortunately, the results may not be as planned at the end of the conventional plant transformation process. The regenerated plants obtained from tissue culture can exhibit insertional effects and frequent unintended variation (termed somaclonal variation) because of mutations or genome instability (Fossi et al., 2019), which may reduce the value of the regenerated plants. Careful selection, monitoring, and genetic characterization are essential to manage and mitigate the impact of somaclonal variation in plant tissue culture and genetic modification programs (Duta-Cornescu et al., 2023). This approach often requires significant time and specialized skills. Consequently, strategies are urgently needed to overcome these difficulties and improve the effectiveness of gene delivery methods.

Many direct (Agrobacterium-mediated gene delivery methods) and indirect (particle bombardment, electroporation, silicon carbide whiskers, polyethylene glycol (PEG)-mediated transformation, pollen tubes, and NPs) approaches have been used to deliver various biomolecules into plant cells and tissues (Saifi et al., 2020; Su et al., 2023). To date, particle bombardment and Agrobacterium-mediated gene delivery are the most widely used methods for genetically modifying plants (Anjanappa and Gruissem, 2021). However, challenges remain in plant transformation using these methods (Ramkumar et al., 2020).

Agrobacterium-mediated gene delivery can be time-consuming and labor-intensive, requiring specialized experimental skills, tissue culture, and random genome insertion, and ultimately the process can cause necrosis in some plant tissues (Mangena et al., 2017; Singh and Prasad,

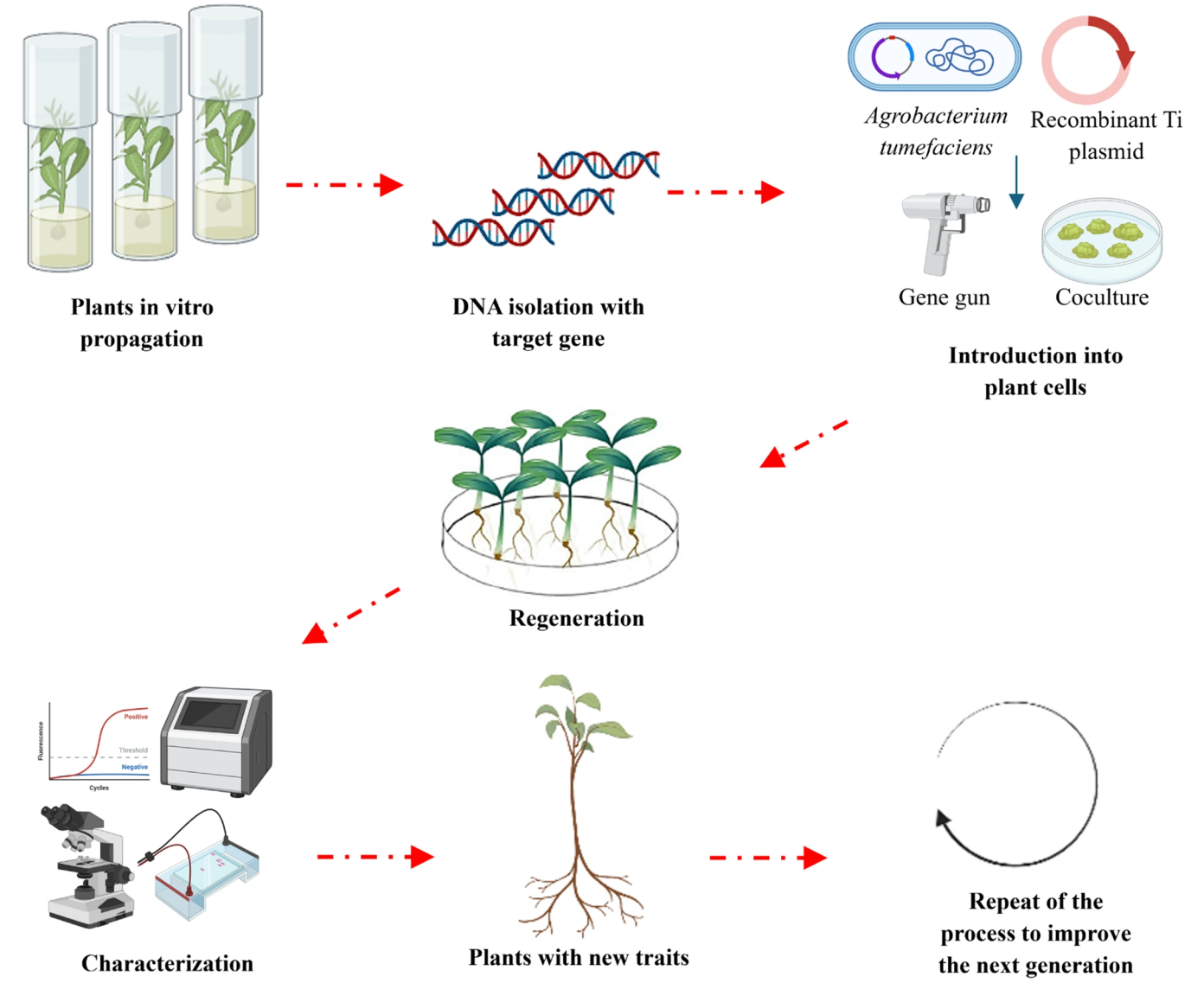

Fig. 1. Generating plants through traditional gene transformation involves several steps: isolating suitable cells, introducing the target gene, growing the transformed cells or tissues under controlled conditions, and regenerating whole plants from these tissues or single cells. This process is known for its extensive duration and labor-intensive nature. (Created with BioRender.com).

2016). Moreover, Agrobacterium-mediated transformation effectiveness can vary depending on the plant genotype, as it involves several interactions between Agrobacterium and the plant's inherent protective pathways (Lacroix and Citovsky, 2013). Therefore, this method poses significant challenges for applying them to transformation-recalcitrant plant species and crop genotypes (Altpeter et al., 2005; Delporte et al., 2014; Anjanappa and Gruissem, 2021). The first genetically engineered plant was an antibiotic-resistant tobacco using Agrobacterium-mediated transformation (Bevan et al., 1983), creating a foundational breakthrough in plant biotechnology. However, the tobacco plant produced by this early approach was genotypically dependent, which limited its immediate impact on agricultural practices and highlighted the need for more universally applicable transformation methods. In recent years, the combined utilization of some developmental regulators and growth regulating factors with advancements in tissue culture protocols, such as optimized medium formulations and hormone treatments, have shown significant promise in overcoming genotype dependence and improving the efficiency of plant regeneration (Aesaert et al., 2022; Aregawi et al., 2022).

Particle bombardment, also known as biolistic delivery (or gene gun) provides several advantages for delivering genetic materials into plant cells, such as versatility and the ability to bypass tissue culture. However, biolistic delivery methods also come with limitations, including random and multiple-copy insertions of target DNA, low transformation efficiency, and issues with tissue specificity that may prevent effective targeting of specific tissues or cell types. Moreover, using these highly pressurized particles causes physical damage to the plant cells and their chromosomes (Altpeter et al., 2005; Liu et al., 2019). Top of Form

2.1. NPs as gene-delivery vehicles in plant genetic transformation

An NM refers to any structure with at least one dimension within the nanoscale range (Fig. 2). Therefore, nanoparticles (NPs) are usually spheroid or cylindrical structures with a diameter of about 10–200 nm. NMs can include NPs, nanofibers, nanomembranes, nanoemulsions, nanocomposites, and different biological materials (Fig. 2). Nanofibers are long thread-like structures with a thickness in the nanometer range and can take many forms, including nonporous nanofibers, mesoporous nanofibers, hollow nanofibers, and core-shell nanofibers. Each nanofiber has a unique structure based on the material used for its synthesis. Nanomembranes are thin films composed of organic or inorganic materials with a thickness below 100 nm and a large aspect ratio. Nanofilms are versatile materials that can adhere to surfaces or free-floating in solutions. Nanoemulsions are mixtures containing either dispersion or suspensions of materials in different phases of water or oil. Nanocomposites are solid materials composed of multiple phases, where at least one dimension of each phase is less than 100 nm. Many biological molecules are therefore considered to be bio-nanomaterials, since many proteins, viruses, and nucleic acid constructs (such as aptamers) fall within the nanoscale range. While all these materials can be classified as NMs, not all are suitable for gene delivery.

NMs that are used for gene delivery are categorized based on their composition, structure, and gene delivery mechanisms. The most common types of NPs include carbon-based NMs (e.g., carbon nanotubes (CNT), graphene, and carbon nanofibers), inorganic-based NMs including non-carbon NPs, nanostructured materials (e.g., metals (Cu, Ag, and Au NPs), and metal oxides), organic-based NMs (e.g., polymer NPs, nanocellulose, and nanostarch), composite-based NMs (e.g., composite nanofibers and mixed metal oxides), and bio-based NMs (e.g., nanobacteria and enzymes) (Jeevanandam et al., 2018; Tuominen and Schultz, 2010).

Employing NMs involves several key considerations. Altering a material's size to the nanoscale can significantly change its chemical properties, and the environmental impact of releasing such materials is not yet fully understood (El-Kalliny et al., 2023). The health impacts of some of these materials are an area of active research worldwide. The

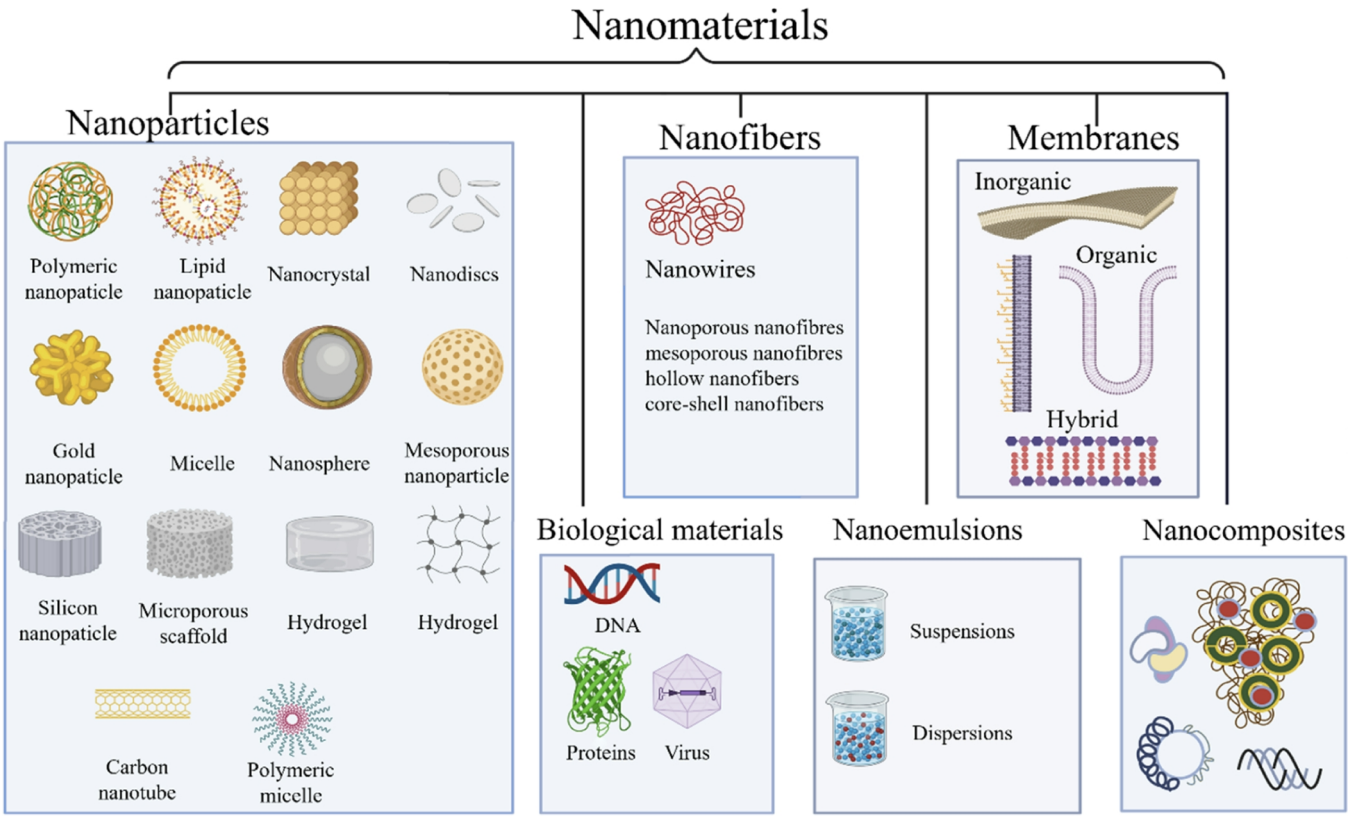

Fig. 2. Overview of the different types of NMs (NMs) and their structures. NMs can include NPs, nanofibers, nano-membranes, nanoemulsions, nanocomposites, and various biological materials. (Created with BioRender.com).

direct environmental effects of NPs in the agriculture industry may seem limited and short-term (Lead et al., 2018), but the long-term impact on ecosystems and biodiversity remains unknown. Despite these caveats, the potential benefits of NPs in improving food quality and safety make them a promising alternative or complementary approach to conventional methods.

NM-based methods have proven effective in delivering biomolecules and chemicals into mammalian cells (Fortuni et al., 2019; Wang et al., 2014). Many gene delivery approaches that have used NMs have focused on NPs because they are relatively easy to manipulate and fabricate. Thus, this review will focus on NPs. However, it's important to note that any NM could theoretically deliver cargo to plant cells as long as it can cross the plasma membrane. In the case of plant cells, NMs can overcome some of the drawbacks of conventional biomolecule delivery approaches because they improve targeting and delivery precision, enhance stability and protect the DNA, increase uptake efficiency (1000 times less DNA is needed compared to conventional DNA modification techniques), reduce immune responses (by coating NPs with materials like PEG), reduce labor and cost, increase scalability, enhance entry into plant cells without external assistance, and have minimal environmental impact (Milewska-Hendel et al., 2017; Wang et al., 2019). For example, since NPs can directly modify germlines (e.g., plant embryos or gametes), they can eliminate the need for regeneration in plant tissue culture(Zhao et al., 2017). Moreover, NPs can target specific cell compartments, thus enabling precise manipulation of not only nuclear genomes but also non-nuclear genomes, such as the genetic information contained in chloroplasts or mitochondria (Fig. 3) (Cunningham et al., 2018). NMs can serve as protective carriers, shielding nucleic acids from enzymatic degradation (especially in the case of RNA) and other environmental stresses and enhancing their ability to penetrate cells (Mitter et al., 2017). As discussed above, successful and efficient regeneration of transformed plants depends on various parameters, including genotype and explant type. Therefore, tissue culture-independent transformation strategies using NP-based delivery systems could offer a viable solution

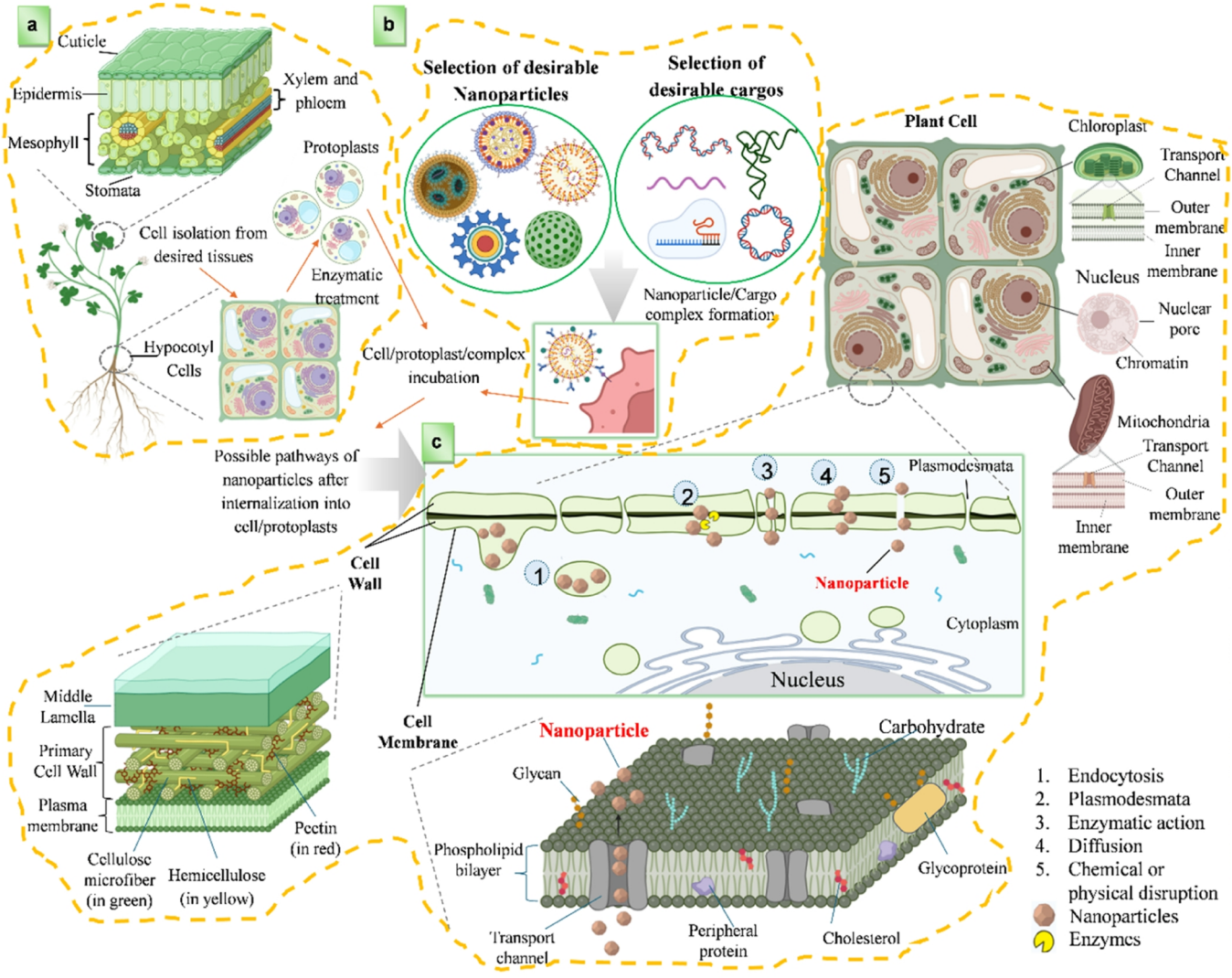

Fig. 3. Schematic illustration of NP-mediated gene transfer in plant cell/protoplasts: a) Examples of different tissues commonly employed for cell and protoplast isolation. The figure also depicts the enzymatic digestion of the cell wall. b) Types of NPs and cargos for forming NP/cargo complexes. c) The cargo/NP complex may be internalized through different barriers and via different pathways, including 1) endocytosis, 2) plasmodesmata transport channels, 3) enzymatic action, 4) diffusion, and 5) chemical or physical disruption. Once the cargo/NP complexes pass through the cell wall and plasma membrane, they can interact with various organelles within the cell, such as chloroplasts, mitochondria, and the nucleus. This interaction may involve the complexes passing through the pores of organelles or the release of biomolecules from the complexes, allowing them to enter the organelles independently. The chloroplast and mitochondria possess distinct outer and inner membranes, each with varying permeabilities to external substances. (Created with BioRender.com).

to overcome this bottleneck (Lv et al., 2020).

There are many types of NPs and each type possesses unique characteristics and functions, offering distinct advantages and limitations in plant genetic engineering (Table 1). For example, mesoporous silica nanoparticles (MSNs) have garnered attention due to their solid framework, large surface area, high thermal stability, and biocompatibility. Their porous structure and functionalizable surface enhance these properties, making MSNs particularly effective (Hajiahmadi et al., 2020; Torney et al., 2007). While MSNs facilitate the controlled delivery of DNA and other materials, their relatively smaller pore size can restrict the accommodation of larger DNA segments. To mitigate this limitation, polymer coatings like polyethyleneimine (PEI) are often employed (Jat et al., 2020; Napierska et al., 2009).

Carbon nanotubes (CNTs), particularly single-walled carbon nanotubes (SWCNTs), offer significant advantages as gene-delivery vehicles due to their biocompatibility, high aspect ratio, large surface area relative to volume, and remarkable tensile strength (Hendler-Neumark and Bisker, 2019; Mohanta et al., 2019). Their high surface area allows for substantial DNA loading, making them effective in gene transfer into plant cells (Burlaka et al., 2015; Demirer et al., 2019b; Kwak et al., 2019). SWCNTs also protect DNA from degradation and can deliver small interfering RNA (siRNA) and plasmid DNA for gene silencing (Demirer et al., 2018, 2019a, 2019b). However, the application of CNTs in plants is constrained by cell wall barriers. Multi-walled carbon nanotubes (MWCNTs) and methods like combining CNTs with cellulase have overcome these barriers, allowing CNTs to penetrate cell walls and protoplast membranes (Serag et al., 2011).

Magnetic nanoparticle-based transformation, or magnetofection, utilizes materials such as Fe_3O_4 and Fe_2O_3, which have extensive surface area, tiny dimensions, minimal sedimentation tendency, excellent thermal resistance, and low toxicity (Cardoso et al., 2018). This approach uses a magnetic field to introduce genetic material into plant cells (Scherer et al., 2002). Although successful in a study involving cotton, magnetofection faces limitations such as the incompatibility with certain pollen apertures and the inability to target maternally inherited organelles like chloroplasts and mitochondria (Ruf and Bock, 2017).

Cell-penetrating peptides (CPPs) are effective biotransmitters for delivering various molecules, including DNA and proteins (Chen et al., 2007; Chuah and Numata, 2018; Chugh and Eudes, 2008; Golestanipour et al., 2018; Lakshmanan et al., 2013). They offer notable advantages, such as the ability to penetrate cell walls and membranes, extracellular stability, and the release of DNA inside cells (Chuah and Numata, 2018). However, several challenges remain unresolved. It is still largely unknown how the peptide interacts with DNA, including the mechanisms of peptide–DNA association and dissociation. Complexes formed with these cell-penetrating peptides are often inefficient in releasing DNA, making it very difficult to control intracellular release. Finally, since these complexes are composed of unique peptide configurations, it is difficult to track them after internalization (Chuah and Numata, 2018; Lv et al., 2020).

Demirer and colleagues in 2018 and 2019 showed that carbon nanotubes effectively transport small RNA molecules to intact plant cells through electrostatic adsorption. This method protects RNA from degradation and enhances gene silencing (Demirer et al., 2018, 2019b). Correspondingly, Zhao et al. (2017) showed that magnetic nanoparticles loaded with DNA could be introduced into pollen, allowing for the immediate production of stable transgenic seeds without requiring plant regeneration (Zhao et al., 2017). Despite the promising advancements and unique benefits of carbon-based nanoparticles, ongoing research is necessary to address their limitations and optimize their application in plant genetic engineering.

Zhang et al. (2020) explored the problem of delivering siRNA through lignin-rich and complex cell walls of plants. Their study showed that DNA nanostructures could be effective carriers for siRNA, facilitating gene silencing in mature plants (Zhang et al., 2020). Furthermore, Dutta et al. (2021) achieved targeted delivery to specific cells and organelles by modifying the surface properties of nanocarriers, such as charge (Dutta et al., 2021). Notably, chitosan-functionalized single--walled carbon nanotubes (SWCNTs) were used to transfect transgenes into chloroplasts and induce GFP expression without integration into the nuclear genome (Kwak et al., 2019) which suggests organelle specificity. This may be an important property that can be exploited in situations where only chloroplast genes need to be modified.

The use of CRISPR/Cas9 and related systems in genetic engineering has increased exponentially because specific genes can be targeted with precision. This system is also very cheap and can be manipulated with very few specialized tools. Encapsulating the CRISPR system into an NP is a logical next step. However, there are considerable challenges with delivering large and unstable molecules like Cas9 within the small confines of an NP. Mahmoud et al. (2022) and Nagy et al. (2023) have successfully demonstrated the efficient and safe delivery of the CRISPR/Cas system into maize and citrus protoplasts using cationic polymers, suggesting that it is possible to carry large payloads using this approach (Mahmoud et al., 2022; Nagy et al., 2023). It remains to be seen whether combining other cargo, in conjunction with the CRISPR/Cas system, in these "high loading capacity" NPs is possible.

Nevertheless, the efficient loading of biomolecules (e.g., DNA, RNA, proteins, or chemicals) onto or into NPs is crucial. The inefficient loading of cargo into NPs remains an underappreciated problem in this field and often debilitates promising NP strategies. Several techniques are considered efficient at loading cargo, resulting in NPs with a high payload-to-NP ratio and increased stability. For example, Wang *et al.* examined different types of NPs as RNA carriers to enhance RNA silencing in rice plants. They showed that the dsRNA complexes formed with (Chitosan (CS), polyethyleneimine (PEI), protamine, carbon quantum dot (CQD), polyamidoamine (PAMAM), and chitosan/SPc complex (CSC)) exhibited formation efficiencies of 46, 43, 60, 31, 58 %, respectively (Wang et al., 2023).

Although NPs offer several potential benefits over conventional methods, achieving a high transformation efficiency remains challenging. Transformation efficiency is affected by NP size, shape, surface properties, and delivery methods. Smaller NPs with a negative charge typically have greater uptake by mammalian cells. Rod-shaped NPs, rather than spherical NPs, tend to enter some cells more efficiently (Chithrani et al., 2006; Chithrani and Chan, 2007). However, this issue also depends on the species and tissue source of the target cell, requiring significant optimization in any biological system, such as plants. Furthermore, as mentioned earlier, when a material is processed at the nanoscale, its chemical properties can sometimes change – with profound effects on target cells. NPs may lead to cellular stress or toxicity, potentially impacting cell viability and transformational efficiency.

NPs can enter plant cells through various mechanisms, including diffusion, endocytosis, or direct uptake via ion channels and plasmodesmata transport channels (Fig. 3c) (Hong et al., 2021; Liu et al., 2020a). Once inside the plant cells, the NPs release the encapsulated biomolecules–into the cytoplasm or endolysosomes. The NPs are designed to protect the biomolecules from degradation by cellular nucleases and facilitate their delivery into plant cells (Jat et al., 2020). Biomolecule release from NPs can be achieved through controlled release mechanisms or by breaking down the NP complexes under specific conditions– such as pH changes that occur within the endolysosomes (Sembada and Lenggoro, 2024). Controlling the release of the cargo is essential if specific organelles or pathways are targeted within the cell.

2.2. The advantage of using a protoplast gene delivery system with NPs

Without the cell wall and cuticle, which act as physical barriers (Fig. 3c), it becomes much easier to introduce the cargo-NP complex (Fig. 3b) into the protoplasts. The composition and structure of the plant cell wall exhibit significant variation not only between different plant

Table 1
A comparative illustration of NP based-transformation methods and their advantages and limitations.

NP type	advantages	limitations	Successful examples of delivered DNA in plant cells
Carnon Nanotubes (Single-wall carbon nanotubes (SWCNTs); MWCNTs: multi-walled carbon nanotubes (MWCNTs))	• Efficient uptake by plant cells • Strong mechanical stability • High surface area for cargo attachment • Potential for multi-gene delivery • Surface functionalization to protect the biomolecules from degradation or denaturation • Time-saving, and cost-effective delivery of biomolecules • biocompatibility, high length-to-diameter ratio, and outstanding tensile strength • Capacity to be loaded with large quantities of DNA for transfer into plant cells • Ability to move through protoplast membranes, including short ones ($\leq$100 nm) • High efficiency, absence of detectable toxicity, and no integration of the transgene into the nuclear genome, suitable for a wide range of plant species, including model and non-model plant species **References:** Burlaka et al., (2015); Demirer et al., (2021); Demirer et al., (2019b); Dunbar et al., (2022); Liu et al., (2009); Wu et al., (2008)	• Multiple rounds of selection and breeding because of concerns about the potential adverse impact on health • Complex surface functionalization • Difficulties in achieving precise targeting • Long-term stability issues • limited water dispersibility, tendency to form entangled bundles, and inert properties, may restrict their applications in plant systems **References:** Hansen and Lennquist, (2020); Nygaard et al., (2009); Yang et al., (2012)	• Penetration of cargo into the cell wall and cell membrane of intact tobacco BY2 cells using carbon nanotubes (Liu et al., 2009) • Genetic material delivery into mesophyll protoplasts, callus cells, and leaf explants of tobacco plants using carbon nanotubes (Burlaka et al., 2015)
Rosette nanotubes (RTs)	• Biocompatibility • Versatile in structure or easy modification with different functional groups or molecules • High Stability under a wide range of conditions, including changes in pH and temperature • Encapsulation capability to release drugs or other therapeutic agents. • Adjustable properties, including dimensions, morphology, and surface chemistry **References:** Cho et al., (2020); Ede et al., (2016); Journeay et al., (2008)	• Complex Synthesis which may limit their large-scale production and commercial viability. • Biodegradation Elevated production costs resulting from the complexity of synthesis methods and the expense of raw materials**References:** Cho et al., (2020); Ede et al., (2016); Journeay et al., (2008)	• Successful delivery of rosette nanotubes into the viable wheat microspores under mild conditions and in the absence of an external force (Cho et al., 2020)
Mesoporous Silica NP (MSNs)	• High cargo loading capacity • biocompatible and biodegradable nature • Protection of genetic material • Surfaces with functionalization capability and adjustable textural properties (Tunable pore size 2–20 nm) and large surface area (>1000 m^2/g)) • Controlled release mechanisms • Easy functionalization **References:** Cai et al., (2024); Chang et al., (2013); Hajiahmadi et al., (2020); Li et al., (2012); Torney et al., (2007)	• Size limitations for efficient penetration • Potential toxicity and biocompatibility concerns • Complex synthesis **References:** Jat et al., (2020); Napierska et al., (2009)	• Delivery of foreign DNA into intact *Arabidopsis* roots by MSNs without the aid of mechanical force (Chang et al., 2013) • Successful transport biomolecules into isolated tobacco cells and intact leaves by MSNs (Torney et al., 2007)
Metal-based NPs	• Efficient delivery into plant cells • Stable and long-lasting in the environment • Potential of surface modification for targeting • Versatile in cargo delivery • Simplicity of synthesis, biocompatibility, and well-defined surface chemistry • high surface area-to-volume ratio, accessible DNA-compatible geometry within monolayers, and tunable hydrophilic characteristics **References:** Rosi et al., (2006); Vijayakumar et al., (2010)	• Some metallic NPs may pose environmental concerns • Potential toxicity in high concentrations • Complex surface functionalization **References:** Balážová et al., (2020); Du et al., (2017); Rastogi et al., (2017)	• Delivery of DNA into plants using Au NPs (Vijayakumar et al., 2010) • Gold NPs for gene transfer in rice cells (Rai et al., 2012)
Magnetic NPs (MNPs)	• Large surface area, small size, low sedimentation rates, high thermal stability, and low toxicity • Efficient and precise targeting using external magnetic fields • Potential of controlled release • Biocompatible in many cases • Versatile in cargo delivery	• May require specialized equipment for magnetic guidance • Limited to specific applications • Potential aggregation issues **References:** Ruf and Bock, (2017); Zhao et al., (2017)	• introducing biomolecules into canola protoplasts facilitated by an external magnetic field (Hao et al., 2013) • Delivery of exogenous DNA into pollen grains of cotton plants through pollen magnetofection and obtaining high pest resistance in the cotton offspring (Zhao et al., 2017)

(*continued on next page*)

Table 1 (*continued*)

NP type	advantages	limitations	Successful examples of delivered DNA in plant cells
	References: Dobson, (2006); Scherer et al., (2002); Wang et al., (2022); Zhao et al., (2017)		
quantum dots (QDs)	• Bright and stable fluorescent markers • Suitable for tracking and imaging applications • High cargo loading capacity **References:** Jamieson et al., (2007); Schwartz et al., (2020)	• Potential cytotoxicity • Concerns about long-term environmental impact Limited cargo versatility**References:** El-Kalliny et al., (2023); Gad et al., (2020); Schwartz et al., (2020)	• Apply siRNA molecules directly to tobacco and tomato leaves to achieve highly efficient gene silencing (Schwartz et al., 2020)
DNA nanostructures (DNs)	• Easily taken up by cells through caveolin- or clathrin-mediated endocytosis • Biocompatible, non-toxic, and easy to metabolize • Internalization into plant cells without force is possible • Fast, cost-effective, nondestructive and scalable • Outstanding biological stability and preventing degradation by nucleases • High biocompatibility with the target tissues and does not trigger a protective immune response • Versatile, adaptable, and straightforward to implement in plants **References:** Kim et al., (2020); Li et al., (2019); Zhang et al., (2019), (2020)	• May require adapting, testing, and optimizing nanostructures for use in different plant species and tissues • The choice of cargo may also impact the final properties and behavior of NMs, so systematic testing of several candidate nanostructures may be needed **References:** Jat et al., (2020); Li et al., (2019); Zhang et al., (2019), (2020)	• Utilizing DNA nanostructures for siRNA delivery into tobacco plants via infiltration (Zhang et al., 2019) • Engineering DNA nanostructures for siRNA delivery in plants (Zhang et al., 2020)
Layered double hydroxides (LDHs), Clay nanosheets	• Ease of synthesis • Contains highly positive charges • Non-toxic, biodegradable, and resistant to removal through washing. • efficiently host dsRNA and enable its absorption from the formed complexes, facilitating continuous release and resulting in the targeted DNA's silencing • safer and can decompose in ambient air • Efficient protection of such vulnerable cargo as DNA and RNA, keeping them safe on the leaf surface • Controlled release of cargo, ensuring sustained effects **References:** Bao et al., (2016); Liu et al., (2020b); Mitter et al., (2017); Yong et al., (2021)	• Current understanding of cargo and NP internalization and their distribution within plants remains incomplete and requires further investigation **Reference:** Mitter et al., (2017)	• Using LDHs to introduce a FITC–DNA short fragment into BY2 and Arabidopsis root cells (Bao et al., 2016) • LDH nano-sheets serve as carriers for the delivery of RNA molecules and obtaining protection against viruses (Mitter et al., 2017) • Efficient dsRNA delivery into tomato pollen using sheet-like clay NPs to silence a target gene (Yong et al., 2021)
Peptide NPs(CPP)	• Cell wall/membrane penetrating capability, extracellular stability, and intracellular DNA release • Rapid and effective cell-penetrating agents for delivering cargo into the cytosol of plant cells with cell walls • Carry cargo in either a covalent or noncovalent manner • cell internalization of CPP/cargo complexes by either direct membrane translocation or endocytosis **References:** Chen et al., (2007); Chuah and Numata, (2018); Chugh and Eudes, (2008); Golestanipour et al., (2018); Lakshmanan et al., (2013)	• The detailed mechanism of peptide–DNA association and dissociation remains unclear • Inefficient release of DNA from peptide–DNA complex within cells, resulting in reduced expression of foreign genes. • Peptide–DNA internalization and tracking mechanisms remain unknown and limit the design of efficient peptide gene delivery vectors. **Reference:** Chuah and Numata, (2018)	• high potential and efficient delivery of the plasmid DNA in walled plant root cells (Chen et al., 2007), embryos (Chugh and Eudes, 2008), and leaf cells (Lakshmanan et al., 2013)

species but also within different tissues of the same plant (Houston et al., 2016; O'Neill and York, 2018). Some plant species have more porous cell walls that allow for easier NP penetration, while others may have more rigid and resistant walls (Sembada and Lenggoro, 2024). The variability in cell wall permeability complicates the delivery of genetic material to an entire plant or multiple tissues simultaneously. This challenge would be particularly relevant when creating a stably transformed plant that expresses an altered gene in every tissue. For this reason, removing the cell wall entirely—such as in the case of protoplasts—can be advantageous. As a result, protoplasts become invaluable genetic engineering tools, allowing for more efficient manipulation of plant genetic material. If protoplasts are cultured with suitable media, they can regenerate cell walls, undergo cell division, and even regenerate into whole plants (Fig. 4) (Du and Bao, 2005; Gandhi and Khurana, 2001). This protoplast regeneration capability is a significant advantage for plant transformation and breeding. However, protoplast regeneration into whole plants is complex, time-consuming, requires specialized expertise, and is not always successful. Despite the complexities, protoplast regeneration is a powerful tool for plant biotechnology, allowing for the creation of genetically modified plants and propagating plants with desirable traits (Fig. 4). All attempts to penetrate the plant cell wall with MWCNTs, MSN, TiO2, and CeO2 NPs have been unsuccessful (Larue et al., 2012; Rodea-Palomares et al., 2011; Tan et al., 2009; Torney et al., 2007). Burlaka and colleagues confirmed that while

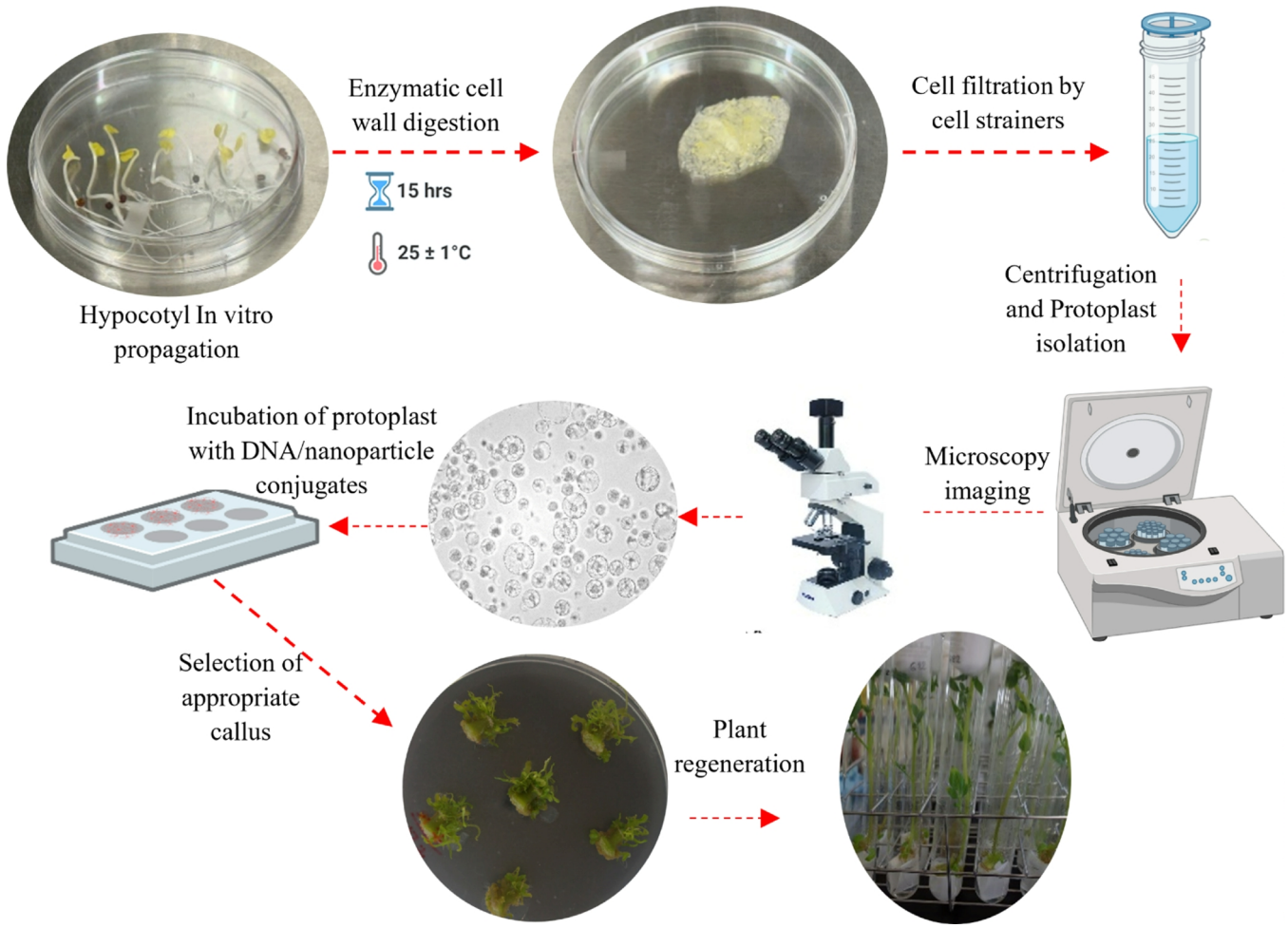

Fig. 4. Schematic illustrating the NP-mediated transformation of protoplasts and subsequent plant regeneration process. Protoplasts are achieved by removing the cell walls from plant cells and isolating the resulting from the plant tissues. NPs loaded with genetic material or biomolecules are introduced to the protoplasts. These NPs facilitate the delivery of the desired genes into the protoplasts. The transformed protoplasts are cultivated under controlled conditions to promote cell division and growth. The transformed protoplasts undergo organogenesis or embryogenesis, resulting in the formation of plant embryos or shoots. The plant embryos or shoots are cultured under specific conditions to promote their growth into mature plants. Transgenic plants with the desired traits are harvested for further study or agricultural applications.

MWCNTs can effectively serve as nanocarriers only for transforming plant protoplasts, SWCNTs face limitations due to the cellulose wall, which impedes their penetration into the cells (Burlaka et al., 2015).

For the regeneration process to be successful, it is necessary to start with healthy protoplasts. Therefore, the protoplast isolation method (Fig. 3a and Fig. 4) must be optimized for each plant cell type: the density of protoplasts in the culture, enzymatic conditions for removing cell wall (such as enzyme concentrations and purity, and digestion time), pH, temperature, buffers, and optimal D-mannitol concentration. Buffers for the plant protoplast isolation are critical for maximizing the viable protoplasts. Buffers typically include various components such as KCl, $CaCl_2$, osmolytes (mannitol, sorbitol, or salts), pH buffer (2-(N-morpholino) ethanesulfonic acid (MES)), Bovine serum albumin (BSA), and β-mercaptoethanol. These components help maintain osmotic pressure, stabilize pH, prevent enzyme degradation, and maintain a reducing environment (Reed and Bargmann, 2021).

The CPW salt formulation, originally developed by Frearson *et al.* (1973), is a widely used basal salt solution. This formulation was later enhanced with osmolytes and enzymes to improve protoplast isolation (Hao et al., 2013; Jie et al., 2011; Tomiczak et al., 2015). The modified buffers create an environment conducive to NP stability and effective delivery of genetic materials into plant cells. For instance, a W5 solution (2 mM MES or 5 mM glucose, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl; pH 5.7) was used for MSNs and CNTs in tobacco protoplasts, while an MMG solution (0.4 M mannitol, 4 mM MES (pH 5.7), and 15 mM $MgCl_2$) was used for quantum dots (QDs), gold and silica NPs, nanoceria, and single-walled CNTs in *Arabidopsis* protoplast (Burlaka et al., 2015; Chang et al., 2013; Lew et al., 2018). Careful consideration and optimization of the buffer conditions (e.g. through mechanical stresses, pH, ingredient interactions, thermal stability, and ionic strength) may be necessary to suit the requirements of both NPs and plant cells and enhance compatibility and achieve successful NP dispersion and delivery in plant cell cultures (Lv et al., 2020; McClements, 2020).

Several successful instances exist where genetic material has been effectively delivered into protoplasts using NPs. For example, a process that used CNTs showed an 85 % transformation efficiency indicating that a significant proportion of protoplasts were successfully transformed (Demirer et al., 2019b). Similarly, fluorescein isothiocyanate (FITC) signals can be delivered by PEG-modified magnetic gold particles into over 95 % of canola protoplasts (Hao et al., 2013). Lew et al. (2018) found that the localization of DNA-coated SWCNTs within the protoplasts is mainly influenced by the size and the charge of NPs. Studies have demonstrated that the physical properties of both NPs and target cells are important in mediating their interaction. Thus, the morphology of protoplasts influences NP internalization efficiency (Lew et al., 2018), while surface properties and dimensions of MSNs influence their

internalization by tobacco mesophyll protoplasts (Jat et al., 2020).

Foreign DNA, RNA, or protein (Fig. 3b) can be introduced into protoplasts using either chemical (e.g., PEG) or physical (electroporation) disruption treatment (Fig. 3c) but each of these approaches has challenges. Electroporation increases the plasma membrane permeability using an externally applied pulsed electric field that allows nucleic acid to enter the cellular cytoplasm (Fisk and Dandekar, 2004). However, the use of a high electric field voltage over 1 kV/cm can significantly reduce cell viability and transformation/transfection rate (Reed and Bargmann, 2021). Depending on the source of the protoplasts (i.e. plant species and cultivar, as well as the source tissues such as the leaf, young shoots, petals, or seedling organs such as hypocotyls or cotyledons, embryos, pollen grains, and suspension cells), physiological condition (young and actively growing tissues are suitable), the voltage applied, the composition of the enzymatic mixture and the duration of enzymatic reaction, losses of 50 % viable protoplasts can occur (Chadegani et al., 1989; Silva et al., 2010; Stajič, 2023).

One of the earliest techniques for gene transfer and plant transformation was PEG-mediated protoplast transformation. PEG is a membrane permeabilization agent through interacts with lipids in the cell membrane to facilitate entry into target cells. Due to its effectiveness in many plant species, ease of preparation, and relatively stable efficiency, this method has been broadly applied in molecular and cellular studies in plants (Wang et al., 2015). However, PEG-mediated transformation can also activate undesirable cellular responses, cause DNA damage, induce ROS overproduction, cause severe oxidative stress, and result in cell plasma membrane permeabilization. The effect of PEG can vary depending on the concentration used, the cell type, and the specific application (Pathirana et al., 2017). For this reason, hybrid NPs have been designed to combine the effectiveness of PEG with reduced cytotoxicity by incorporating more biocompatible constituents, such as lipids. For example, a combination of PEG and cationic lipid NPs has been used to successfully deliver DNA into citrus protoplasts (Mahmoud et al., 2022).

Mahmoud *et al.* reported an improved protocol for plasmid encapsulation using Lipofectamine™ LTX with PLUS Reagent to introduce donor DNA into sweet orange protoplasts with minimal cytotoxic effects on cells (Mahmoud et al., 2022). In another report, several parameters of plasmid delivery into maize protoplasts were optimized to achieve highly efficient CRISPR/Cas9 delivery using Cationic Polymer Poly (2-hydroxypropylene imine) (Nagy et al., 2023). Their findings indicated that an incubation period of 30 minutes for polyplexes with protoplasts and an N/P ratio of 9.2, was optimal. Moreover, the study demonstrated that using 2 μg of the polymer poly(2-hydroxypropylene imine) (PHPI) resulted in the highest delivery efficiency for 1 μg of plasmid. This finding differs from the *Arabidopsis* protoplast infection results using Poly (2-(N,N-dimethylamino) ethyl methacrylate), in which a longer incubation time of 12 hours and N/P ratio of 15 was applied (An et al., 2022).

Once a protoplast has been transformed, it is necessary to regenerate the cells into tissues and whole plants. Since protoplasts have had their cell walls enzymatically disrupted (Fig. 3a), they can survive for a limited period, typically up to 72 hours. However, this time frame can vary depending on the specific plant species and culture conditions (Silva et al., 2010). For most protoplasts, the regeneration process takes about 72 hours (Nagata and Takebe, 1970). This process requires specific growth conditions, including appropriate macro-, and micro-nutrients like MS or Gamborg B5 medium, as well as plant growth regulators, osmotic agents, gelling agents, and other supplements (Du and Bao, 2005; Gandhi and Khurana, 2001; Reed and Bargmann, 2021). Moreover, temperature and light conditions play roles in the regeneration success (Brown et al., 1987; Kaur et al., 2006). The efficiency of regeneration might be influenced by how the NPs are transformed and internalized during the process. For instance, treating tobacco protoplasts with conjugated polymer NPs for siRNA delivery did not immediately impact cell wall regeneration during the first 24 hours; however, notable effects were observed 48 hours post-treatment (Silva et al., 2010). As the protoplasts are induced to regenerate, the developmental stage of the protoplast calli capable of shoot induction becomes important (Li et al., 2021).

Measuring the transformation or internalization efficiency of NPs into target cells is often done using a fluorescent reporter. However, this measurement can be challenging because many plant cells are autofluorescent due to cellular components like chlorophyll, lignin, and other plant pigments. To mitigate the influence of autofluorescence, it is necessary to use careful wavelength selection, time-gated imaging, quenchers, filters, and background subtraction. For example, near-infrared images of arugula and *Nicotiana benthamiana* protoplasts, using a 720 nm excitation laser with a 200 ms exposure and with a 1070 nm long-pass filter, can minimize interference from chlorophyll autofluorescence (Demirer et al., 2019b). It is also possible to circumvent some of these autofluorescence issues by selecting plant tissues with weak autofluorescence, such as young and undifferentiated plant tissues or tissues with lower chlorophyll content (e.g., hypocotyls or roots). It is also possible to optimize growth conditions to minimize the accumulation of autofluorescent compounds or use fluorescent labels that do not interfere with the natural autofluorescence of the plant tissue. For instance, since tobacco protoplasts are autofluorescent in the green spectrum (Chang et al., 2013), it is possible to use MSNs labeled with rhodamine B isothiocyanate (R-MSNs, red fluorescence) to show the internalization of NPs.

2.3. Future perspective

Researchers and scientists continue to optimize NP-mediated gene delivery into plant cells, including protoplasts. As these methods are perfected, NP-mediated gene transformation in plants can play a significant role in improving crop yields, enhancing disease resistance, and contributing to sustainable agriculture. However, careful consideration of safety, ethical, and regulatory aspects is equally important for developing this technology. NP-mediated biomolecule delivery holds significant promise for improving transformation efficiencies in plant biotechnology. It can contribute to more efficient and precise genetic modification of plants for various purposes, including crop yield, pest resistance, and nutritional content.

The integration of CRISPR/Cas9 with nanomaterials (NMs) marks a significant breakthrough, facilitating precise gene editing within protoplasts with minimal off-target effects. The use of CRISPR/Cas9-nanoparticle complexes in protoplasts facilitates and accelerates the transient transformation, silencing, and modification of genes to study and address current and anticipated biotic and abiotic challenges, thereby promoting the development of nutrient-rich crops. These advancements present profound opportunities in plant genetics, with emerging technologies poised to revolutionize agricultural science.

Researchers have engineered numerous crops using the CRISPR/Cas9-nanoparticle complex system (Demirer et al., 2021; Mahmoud et al., 2022; Nagy et al., 2023). They enable precise delivery of therapeutic molecules, enhance genetic modifications, and increase crop resilience. While integrating nanomaterials with CRISPR DNA plasmids and delivering them into plant cells has shown promise, the CRISPR-Cas9 technology for genome editing in plants remains challenging due to the large size and high charge density of CRISPR DNA plasmids and Cas9 proteins. Efficient delivery of CRISPR components requires identifying optimal chemistries for loading large CRISPR plasmids onto nanoparticles and addressing the stability and charge issues of Cas9 proteins. Potential solutions include covalent attachment of Cas9 RNPs to nanoparticles using cleavable linkers or near-infrared light--triggered release mechanisms (Demirer et al., 2021). Protoplasts represent a unique solution to the challenges posed by the large size of CRISPR components, as they lack the size exclusion limits found in intact plant cells. The plant cell wall serves as a significant barrier to the entry of nanoparticles (NPs) and macromolecules, typically allowing passage

only for substances around 20–50 nm, depending on the plant species and cell type (Jonsson *et al.*, 2022; Avellan *et al.*, 2019; Miyamoto *et al.*, 2022). For instance, efficient NP delivery into leaf cells is limited to hydrodynamic sizes of approximately 20 nm for cotton and 11 nm for maize (Hu *et al.*, 2020). In contrast, protoplasts, devoid of a rigid cell wall, can facilitate the delivery of larger CRISPR constructs, overcoming the barriers that intact cells impose. While the lipid bilayer of the cell membrane has a larger size exclusion limit of around 300–500 nm, allowing larger cargoes to enter, protoplasts can efficiently accommodate and internalize CRISPR components without the restrictions faced by intact cells. This unique characteristic enhances the potential for effective gene editing applications in protoplast systems, paving the way for innovative agricultural solutions.

In the use of nanoparticles for the transformation of plant cells, a critical issue is the investigation of nanoparticle toxicity on cells. The uptake and release of resazurin in plant cells may be affected by the presence of the cell wall; for example, there is only one report of using resazurin for assessing plant cell viability (Byth et al., 2001). Unlike intact plant cells, where the cell wall can hinder the diffusion of such molecules, protoplasts provide a more accurate environment for evaluating cellular health and metabolic activity. This distinction is essential and must be considered in future studies to ensure reliable assessments of cell viability and the impact of nanoparticles on plant cells. Further research is essential to build upon existing developments and ensure their widespread adoption in agricultural settings.

2.4. Concluding remarks

Researchers have conducted several groundbreaking experiments in NM-based plant genetic engineering using protoplasts, highlighting the unique advantages of NPs in revolutionizing plant biotechnology. NPs can penetrate the plant cell wall without external forces, making them particularly effective for protoplast applications. Their highly tunable physicochemical properties enhance their capability with precision and efficiency to deliver biomolecules, such as nucleic acids, proteins, and small molecules into protoplasts.

Moreover, certain NM formulations exhibit long-term stability, protecting genetic cargo from degradation and prolonging its activity within protoplasts. This characteristic is especially beneficial for experimental settings and future applications in field testing. The versatility of NPs allows for targeted delivery of payloads to specific cellular compartments within protoplasts, facilitating sophisticated manipulation of plant cellular processes.

In conclusion, the advancements in NM-based genetic engineering using protoplasts provide a solid foundation for the widespread implementation of these methods. As nanotechnology continues to evolve, it holds promise for developing innovative strategies to enhance crop productivity, improve stress tolerance, and engineer desirable traits in plants for agriculture and beyond.

Author's contribution

All authors drafted and edited the manuscript.

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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 paper.

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No data was used for the research described in the article.

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