Trends in Plant Science
Opinion Article

Developing Frameworks for Nanotechnology driven DNA-Free Plant Gene-Editing

Neelam Gogoi,[1*] Hendry Susila,[1] Joan Leach,[1,2] Markus Müllner,[3-5] Brian Jones,[4,6] Barry J Pogson[1*]

[1]ARC Training Centre for Future Crops Development, Research School of Biology, College of Science, The Australian National University, Canberra, ACT 2601, Australia.
[2]Australian National Centre for the Public Awareness of Science, The Australian National University, Canberra, ACT 2601, Australia.
[3]Key Centre for Polymers & Colloids, School of Chemistry, Faculty of Science, The University of Sydney, NSW 2006, Australia.
[4]Sydney Institute of Agriculture, The University of Sydney, NSW 2006, Australia.
[5]The University of Sydney Nano Institute (Sydney Nano), The University of Sydney, NSW 2006, Australia.
[6]School of Life and Environmental Sciences, Faculty of Science, The University of Sydney, Camperdown, NSW 2006, Australia.

Correspondence: neelam.gogoi@anu.edu.au (N. Gogoi), barry.pogson@anu.edu.au (B.J.Pogson)

Keywords: Nanotechnology, DNA-free, CRISPR/Cas, plant gene-editing, RRI

GLOSSARY

Agrobacterium tumefaciens-mediated delivery: It involves a multi-step process to introduce foreign genes into plants by utilizing naturally occurring soil bacterium, *Agrobacterium*. The bacterium transfers a DNA segment known as transfer DNA (T-DNA) from its Ti plasmid to integrate into the host plant nuclear genome to get the desired traits.

Carbon nanotubes (CNTs): These are one-dimensional nanomaterials made entirely of thin tubes of carbon. Their diameters are in the range of nanometre and length could vary from 100-1000 micrometres.

CRISPR/Cas: It is an acronym for clustered regularly interspaced short palindromic repeats (CRISPR) associated protein (Cas). Famously known as “genetic scissors”, it is a Nobel prize winning technology based on RNA-guided endonuclease which can be used to selectively edit genes of interest. Currently, all RNA-guided gene editing uses the CRISPR/Cas system and its possible predecessor, TnpB.Engineered nanomaterials (ENMs): These are specifically designed nanomaterials having at least one dimension in 1-100 nm scale and unique properties that are incorporated for their specific applications.

Environmental Protection Agency (EPA): The EPA in the United States has developed regulations and guidance for the safe use of nanomaterials, including requirements for labelling and reporting.

European Commission: The EU has implemented regulations and initiatives to promote the safe and sustainable development of nanotechnology, such as the REACH regulation (Registration, Evaluation, Authorization, and Restriction of Chemicals).

Gene-Editing: It is a technique to precisely target and modify an organism's endogenous genome to achieve desired traits.

Gene-Modification: It is technique to achieve targeted manipulation of a genome by insertion of an exogenous/foreign DNA.

International Organization for Standardization (ISO): ISO develops international standards related to nanotechnology, including guidelines for risk assessment and safe handling.

Lipid exchange envelope penetration (LEEP) model: It is a mathematical model which explains the entry of a nanoparticle inside a plant cell.

Nanotechnology: It is defined by the National Nanotechnology Initiative as ‘the manipulation of matter with at least one dimension sized from 1 to 100 nanometers. At this scale, known as the nanoscale, properties of materials change compared to their bulk counterparts giving them unique attributes.

Organization for Economic Cooperation and Development (OECD): The OECD has published guidelines for the testing and assessment of nanomaterials, providing a framework for evaluating their safety and environmental impact.

Particle bombardment: Also known as biolistics, it is commonly used to shoot the exogenous DNA coated on metal submicron particles (usually gold) inside the target cell using a gene gun or biolistic device.

Protoplasts: They are isolated plant cells without their cell walls, typically isolated through enzymatic digestion.

Protoplast transformation: It is a technique used to directly introduce DNA into protoplasts.

Responsible research and innovation (RRI): It is a rapidly emerging framework for developing research projects with a focus on potential effects of innovations on society and environment. RRI fundamentally emphasizes collaboration among diverse stakeholders, including researchers, citizens, policymakers, industry, and third-sector organizations to align R&I with societal values, needs, and expectations.

Ribonucleoprotein (RNP): It is a complex of ribonucleic acid (RNA) and RNA-binding protein. Preassembled CRISPR/Cas RNP is a popular DNA-free gene editing tool.

Somaclonal variation induced mutations: These mutations originate in plants generated from tissue culture methods. The variation among the regenerated plants can occur from the culture of protoplasts, callus, meristems, leaf and stem tissues.

Sustainable agriculture: It is a way of farming with a goal to meet the needs of the present generation without compromising the needs of future generations by finding a balance between environmental, social, and economic factors.

TALENs: It is an acronym for transcription activator-like (TAL) effector nucleases. It is a widely used gene editing tool which can precisely target any desired sequence without recognition of a protospacer adjacent motif (PAM) site within a genome.

Zeta (ζ) potential: It is useful in the assessment of surface charge of particles in an aqueous suspension. It is defined as the electrokinetic potential of a colloidal dispersion.

Abstract

The bottlenecks of conventional plant gene-editing methods gave an innovative rise to nanotechnology as a delivery tool to manipulate gene(s) of interest. Studies suggest a strong correlation between the physicochemical properties of nanomaterials and their efficiency in gene delivery to different plant species/tissues. In this opinion we highlight the need for a deeper understanding of plant-nanomaterial interactions to align their full capabilities with the strategic goals of plant gene-editing. Additionally, we emphasise DNA-free plant gene-editing approaches to potentially mitigate concerns surrounding genetically modified organisms (GMOs). Lastly, we propose a strategic integration of the principles of responsible research and innovation (RRI) in R&D. We aim to initiate a dialogue on developing collaborative and socio-technical frameworks for nanotechnology and DNA-free plant gene-editing.

Need for a transformative shift in agriculture practices

With an estimation of one in eleven individuals globally going hungry every day as revealed by five United Nations agencies, [1] , global food security and agricultural production are at a critical juncture in this changing climate.[2] This raises a few fundamental questions. Is it time to rethink agriculture? How could we achieve substantive change? What needs to be done differently? It is estimated that current agricultural practices account for 29% of global carbon emissions, 50% of habitable land use, and 70% of freshwater depletion.[3] It can be argued that to limit the environmental impact of agriculture development of new approaches to old problems would be required. One possible avenue could be to populate R&D's focus with new multidisciplinary technologies that can bring structural changes in agriculture addressing both productivity and climate change. The recent advancement in RNA-guided gene-editing facilitated by nanotechnology offers a compelling avenue to consider for a paradigm shift.[4] The convergence of the two cutting-edge technologies could, in theory, overcome a number of longstanding bottlenecks. Bringing nanotechnology and precision gene-editing together could, for example, enable precision edits to be made directly in elite cultivars and breeding lines or to broaden the scope of genome editing to encompass species that are currently difficult to transform. Deployment of such technology-smart approaches for increasing plant resilience and yield are clear priorities for improved environmental outcomes.

What role can nanotechnology play in advancing plant gene editing?

The conventional plant gene-editing practices;[4] *Agrobacterium*-mediated delivery, protoplast transfection and particle bombardment, while foundational in our current genetically modified (GM) crops and gene-editing research, are hampered by several key bottlenecks. *(i)* Integration of any exogenous/foreign DNA into the target genome challenges regulatory and public concerns. *(ii)* Recalcitrant with most target species and varieties thereof.[5] For example, commonly available wheat transformation protocols, mostly *Agrobacterium* are largely limited to non-commercially viable genotypes (Bob White, Fielder, Cb037), while most elite wheat varieties remain difficult to transform or have limited success. *(iii)* A long route from transformation to regeneration with labour-intensive and technical protocols.[5] For example, the transformation process of wheat embryos to the regeneration of plantlets ready for transplantation into soil requires 11-12 weeks, and transplanted plants need another 12-14 weeks to mature. Additionally, somaclonal variation induced mutations during regeneration adds complexity. *(iv)* Very low transformation efficiencies. For example, most legume crops, such as chickpea,[6] are difficult to transform, with the transformation efficiency ranging from 0.6% to 2.1%. *(v)* The presence of a rigid plant cell wall adding another layer of complexity.[7] Particle bombardment [7] is efficient in at crossing the plant cell wall, however, the use of mechanical force disrupts membrane and chromosomal integrity, diminishing efficiency and reproducibility. Protoplast transformation [8] through polyethylene glycol (PEG)-mediated transfection negates issues with cell wall penetration, as the cell wall has been enzymatically removed. However, the process of regenerating the cell wall and regenerating whole plants from single isolated somatic cells remains a major challenge for many species. *(vi)* Intellectual property (IP) restrictions limit easy and accessible use. For example, the wheat transformation method by Japan Tobacco Inc. (http://www.jti.co.jp) is licensed and restricted for widespread use. Therefore, it is imperative to explore more efficient and streamlined processes for the integration of targeted genome modifications in plants.[9]

Here, nanotechnology, dealing with materials of dimensions sub-100 nanometre has the ability to offer new avenues for innovation by manipulating the composition, architecture and charge of nanomaterials.[3] Such engineered nanomaterials (ENM) (Key Figure 1) can address multiple issues associated with plant cell modifications, for example, to facilitate plant cell wall and plasma membrane penetration, to carry functional loads of different types of cargos, including large biological macromolecules, provide protection from enzymatic degradation outside and inside the cell, reduced genotype and tissue dependency, and in some cases sidestepping the need for tissue culture.[4,10] ENMs when manipulated into a diverse range of shapes, sizes, and surface properties can also effectively engage with the plant cell wall and different tissues.[11] Table 1 outlines various types of ENMs successful so far in delivering a diverse array of genes into plant protoplasts, leaves and roots. There are some successful demonstrations of ENM-mediated delivery in plants, potentially because of significant technical knowledge gaps on the impact of design factors (size, shape, surface property) on translocation, cellular uptake, and the functional utilization of cargo within plant cells. Since successful ENMs-mediated plant transformation is dependent on multiple parameters, a systematic step-by-step analysis and optimisation of the associated technical components is crucial.

Size of ENMs: The particle size is one of the key factors influencing the mode and efficiency of uptake into target cells. In general, large ENMs usually takes longer to associate and diffuse through the plant tissue. On reaching the outer surface of the plasma membrane, ENMs can initiate membrane wrapping through the process of endocytosis.[12] However, the interaction between ENMs and any cell membrane is also size-dependent. Small ENMs exhibit low binding affinity, while excessively large ENMs fail to undergo the necessary membrane wrapping for internalization. Studies have established varied size exclusion limits (SELs) for ENMs uptake by plants with cell walls and cuticles showing SEL ≤50 nm, [13,14] while cell membranes and plasmodesmata have SEL <500 nm and <10 nm, respectively. This indicates that ENP's rigidity is also a critical factor influencing delivery across the plant cell wall.[15] Polymeric NPs, with lower stiffness, can circumvent SEL constraints and can be readily absorbed by leaves compared to hard metal and metal oxide particles of similar size. This underscores the pivotal role of the size of ENMs on the internalization process within plant cells and necessitates a comprehensive study

Shape of ENMs: The uptake mechanism of ENMs varies depending on their geometry and plant cell type which can direct its contact curvature and angle and/or type of entry. Spherical ENMs predominantly undergo clathrin-mediated endocytosis, while cylindrical shapes engage in phagocytosis or micropinocytosis. Simulation studies have revealed that small-aspect ratio ENMs with flat tips employ a "rocket mode" of entry through a tip-first technique, while high-aspect NMs with round tips utilize a "submarine mode" through a side-first approach.[12] High-aspect ENMs, such as carbon nanotubes (CNTs) generally do not initiate membrane wrapping [16] but instead elicit uptake through a lipid exchange envelope penetration (LEEP) model. In LEEP uptake, charged nanoparticles can interact with the surface charges on the membrane, causing it to become more fluid and flexible leading to temporary pores. This process is believed to be thermodynamically favourable, allowing the nanoparticles to passively penetrate the membrane. [17] A recent study on sub-20 nm spherical gold nanoparticles showed they can deliver siRNA into plant leaf cells without being internalised.[18] They act as a reservoir associated to the cell wall and delivering siRNA upon exchange of surrounding biofluids whilst providing protection from nuclease degradation. Given the vast array of nanomaterials available, more in-depth study is required to understand the influence of ENM shape on cell wall and plasma membrane penetration efficiencies.

Surface property of ENMs: There is a critical role of surface chemistry (for example, charge and type of functional groups) of ENMs in mediating interactions with phospholipid layers of plant cell wall and plasma membrane. Charged ENMs with the capacity to engage in favourable electrostatic interactions with lipid bilayers exhibit higher transfection efficiency compared to their neutral counterparts.[12] Charged ENMs with a high magnitude of zeta (ζ) potential (~30 mV) have an even greater chance of associating with plasma membrane and translocating into plant cells. Positively charged ENMs can induce localized disruptions through strong electrostatic interactions due to its negative transmembrane electric potential at the cell adhesion point. A higher positive charge density amplifies this disruption, explaining the toxicity of highly cationic ENMs. A positive ζ potential also plays a significant role in higher loading of DNA/RNA due to their negatively charged phosphate groups and protecting against enzymatic degradation through the proton-sponge effect within the cell. In comparison, the influence of negative ζ potential of ENMs in association and translocation in plant remains under-explored. On the other hand, surface modifications, for example with polyethylene glycol (PEG) [16] functionalization increase the stability and hydrophilicity of ENMs. Polymers like polyethyleneimine (PEI) [13,19-21] attached to the surface of an ENM have shown to increase the cargo load and transfection efficiency but, can lead to cytotoxicity with lower molecular weight polymers demonstrating lesser toxic effect. Another critical factor here is the influence of DNA/RNA to ENMs ratio. Higher loading of DNA/RNA as compared to ENMs may neutralize the surface charge of the ENMs, leading to both colloidal instability and reduced transfection efficiency.[13,16] Additionally, ENM's hydrophilic and hydrophobic properties also play a pivotal role in determining its interactions with the different components of plant membranes. For instance, hydrophilic star polymers interacted with leaf cuticles, whereas hydrophobic star polymers interacted with trichomes and adhere more strongly to lipophilic leaf cuticle.[22] Therefore, it is reasonable to conclude here that much remains to be explored in terms of the role of the surface of ENMs in promoting efficient delivery to different parts of a plant.

ENMs interaction with different plant cell type and their fate post-delivery: The role of different plant cell types (leaf, pollen, callus) and membranes (chloroplast, plasma membrane of isolated protoplasts) has significant driving behaviour in the fate of ENMs delivery. For foliar delivery, ENMs need to either cross a waxy cuticle layer prior to entering into the apoplastic network or enter via stomata into the network of intercellular spaces, spongy mesophyll and other internal leaf cells. Nanoformulation of ENMs with lower surface tension (~22 mN/m) are reported to be crucial in foliar delivery.[23] The species-determined anatomical structure also plays a role in the efficiency of foliar delivery. A higher stomatal density, for example, can provide more micron-sized pathways for the entry of ENMs. In pollens, scanning electron microscopy revealed that the germ pore in the outer wall (exine) of a pollen grain gave access to the inner wall (intine) and a potential pathway for pre-germination ENM uptake [14,20,24]. Access to the germ cells within the pre-germinated pollen grains or in the pollen tube post gemination offers a feasible means of directly modifying the male gamete prior to fertilisation.[25] However, designing ENMs that are capable of delivery cargos into the sperm cells without comprising the pollen viability is a critical challenge here. Isolated protoplasts are another cell type that have been successfully used with ENMs-mediated delivery. Liposomes are often preferred for protoplast delivery due to their biocompatibility, targeting capabilities, and encapsulation capacity[4] but this method retains the disadvantages inherent in the transformation of single somatic cells. Targeted delivery to organelles such as chloroplast [19] and mitochondria [26] has been shown to achieve by decorating the surface of ENMs with targeting peptides.

While it is understood that the mode of translocation affects the behaviour of ENMs inside the cells, the mechanisms determining the final subcellular localisation and levels of accumulation remain poorly understood. Systematic studies to determine how ENMs interact with the target cell type and the mechanisms of transfer and intracellular trafficking will aid in future ENM design. A “plant digital twin” a virtual representation of a plant's surfaces, organs, and vascular system, could revolutionize targeted nanoparticle delivery. This virtual in silico approach would allow to rapidly design and test nanoparticle strategies as described above for multiple functions.[15] The successes and design flexibility of ENMs, however, posit that nanotechnology has considerable potential for use in targeted genome modification in plants.

How DNA-free approach could contribute to accelerating gene-editing processes?

The decades-old debate around the suitability and safety of plant biotechnology continues to influence regulatory frameworks and public opinion globally. Currently, the journey of a new genetically modified (GM) crop containing a exogeneous DNA takes an average of 13 years and US $136 million from the lab-to-field-to-market.[27]. Although current breeding practices use backcrossing to segregate stably integrated DNA from a genome, the elimination of integrated exogenous DNA adds an extra level of complexity and regulatory risk. In many jurisdictions, when DNA-free approaches are used in conjunction with the manipulation of endogenous genome sequences the resulting plants might be subject to different regulatory regimes. This allows for a different regulatory conversation about these technologies than GM technologies. For instance, CRISPR-edited mushrooms developed at Pennsylvania State University in 2015 were approved by the United States Department of Agriculture (USDA) without the usual GMO oversight.[28] It is, therefore, crucial to set socio-technological boundaries that reliably differentiate targeted DNA-free genome editing from the traditional GM approach to trait improvement.

Combining preassembled CRISPR/Cas ribonucleoprotein (RNP) or CRISPR/Cas mRNA-gRNA genome editing with ENM-delivery avoids the need to stably integrate the genome editing machinery into the target genome. The plants are deemed to have substantial equivalence to those bred by traditional practices. The use of preassembled CRISPR/Cas RNPs also has the advantage that it eliminates the initial work involved in designing appropriate species-specific transcription units to drive the expression of the engineered nuclease and guide RNAs. While the use of ENMs has the potential to increase the efficiency of plant gene-editing; transient, protein or RNA-based genome editing is also preferred for plants with long reproductive cycles or clonally propagated species.

Successful DNA-free gene-editing has been achieved using CRISPR-Cas9/Cas12a RNPs, TALEN proteins, and TALEN mRNA across diverse plant species (Table 2). Reported studies mostly involved transfecting protoplasts of different species with preassembled CRISPR-Cas9 RNPs. Whereas somatic cell-based approaches have been successful, they still face the major challenges of low efficiency and/or *de novo* whole plant regeneration from transformed cells. For instance, indel mutagenesis efficiency through direct delivery of RNPs into the protoplast of grapevine was 0.1% and apple was 0.5-6.9%.[29] Another study reports that regeneration of plantlets from transformed protoplast of different species required at least 3 months following an extensive protoplast transfection protocol.[30] Here, ENMs-facilitated transfection of CRISPR/Cas RNPs into plant reproductive tissues (such as pollen) [25] shows promise. This approach bypasses the challenging and time-consuming plant regeneration process, eliminates the risk of exogenous DNA integration into the plant genome and therefore does not require backcrossing for the removal of unwanted DNA sequences. While current RNP transformation efficiency is relatively low, studies have demonstrated that ENMs can enhance the delivery of plasmid DNA (pDNA) or RNA into plant cell.

Although published work indicates that combining ENMs-facilitated transfection and genome editing has considerable potential, effort is required to develop the technology for routine and efficient use. For example, CRISPR/Cas RNPs bring the complexity of a large-sized cargo (Cas9/Cas12: 1000-1500 amino acids) for ENMs delivery. This can lead to aggregation of nanoparticles and impede the efficient application of ENMs. Recently, designs for smaller Cas effectors such as Cas12f or 14: 400-700 amino acids and Cas12j or Φ: 700-800 amino acids are reported,[31] although their efficacy in plants is yet to be fully investigated. Establishing firm design principles for DNA-free plant genome editing and understanding the compatibility of these methods with ENM will require considerable ongoing effort. As the convergence of nanotechnology and DNA-free plant gene-editing offers emerging avenues for crop improvement and possibly favourable public perception, the effort is arguably warranted.

What are the socio-technical aspects of nanotechnology and DNA-free plant gene-editing?

Technologies are very powerful tools; their design is the result of a wide number of decisions and assumptions about how and in what context the technology will be used. Nanotechnology, on the one hand, promises potential path making solutions for DNA-free plant gene editing. It also will come up against limits to the current design of nano solutions as many were conceived for alternative use contexts. [3] New decisions and contexts for the design of nano for gene editing will need to be made; this is an excellent opportunity for design to take on board issues of responsible research and innovation. Nanotechnology sometimes raises serious concerns due to its 'newness' or unfamiliarity as well as potential issues that emerge downstream of the initial use of the technology. Therefore, assessing the socio-technical implications of new nanotechnology applications by evaluating impact on the environment, health, and safety is prudent and articulating these issues clearly important to working with stakeholders on the future of the technology. Adopting an approach governed by responsible research and innovation (RRI) [32] could be a strategic key. RRI provides a comprehensive framework underpinned by the principles of anticipation, inclusion, reflection, and response for conducting research that aligns with societal values and needs. Its pillars; Public Engagement, Gender Equality, Ethics, Open Access, Science Education, and Governance; (Figure 2 (A)) offer a roadmap that could ensure that nanotechnology driven plant gene-editing research is conducted with full consideration of its societal and environmental impacts. While RRI offers a systematic approach to anticipating and engaging public concerns, private companies and academia often lack incentives for implementation and may struggle with practical application. Therefore, it is critical to introduce and engage the pillars of RRI, not only to guide public engagement, but also to guide the innovation phases which could be categorised as; 1) scientific and technical R&D, 2) product oversight, and 3) post-commercialisation marketability and use (Figure 2 (A)). In this conceptualisation, RI is not a ‘nice to have’ academic concept, but a necessary part of the innovation process.*Scientific and Technical R&D:* It is crucial to brainstorm initial ideas for designing safer ENMs considering the environment, human health and economic well-being entailed by their development. A rigorous risk assessment for characterising toxicity of ENMs and their potential exposure to any biological system should be at the forefront of R&D. This could be achieved by a proactively adhering to a pyramid of guiding principles for safer technology [33] (Figure 2 (B)). Principle 1► Size, shape and surface: This guides to carefully consider the interplay between size, surface, and structure, researchers and engineers can develop safer nanomaterials that minimize potential risks while maintaining desired performance characteristics. Principle 2► *Alternative material*: This guides to replace hazardous ENMs with less toxic materials, such as bio-based alternatives or synergistic combinations for redesign with a thorough analysis of the potential impacts on its performance, safety, and cost-effectiveness. Principle 3► Functionalization: This guides to employ chemical modification techniques to alter the properties of ENMs and mitigate potential risks without compromising their intended function. Principle 4► Encapsulation: This guides to confine potentially hazardous ENMs within a protective barrier to prevent direct contact with environment or human health and/or provide long-term stability. Principle 5► Reduced quantity: This guides to explore ways to reduce the quantity of the hazardous ENMs while maintaining product performance. Additionally, the design and risk control information should be communicated and documented among all those involved in the process.

Product Oversight: Nanotechnology and DNA-free plant gene-editing are relatively new technologies in the agriculture sector and the engagement with publics and policymakers is important to capture concerns as well as opportunities for new contexts of use. Researchers should actively engage in clear communication with policymakers to understand the process and identify shared objectives. This could be achieved by 1) *Open dialogue:* Researchers need to be transparent about their research ideas and innovations to identify any possible questions that it could raise. Engaging in frequent conversations with policymakers and public would help. Researchers need to understand that audiences don't need to be science experts, but they should be informed about the key issues and decision points involved. 2) *Understanding policy making process:* Research need to equip themselves with knowledge of the policy making process. They also need to learn how to write clear and concise briefs for policymakers or consider collaborating with science communicators who can translate research for them. They can enrol for online courses on policy communication like the American Association for the Advancement of Science (AAAS) (https://www.aaas.org/programs/office-science-policy-and-society). 3) *Respecting the process:* Above all, researchers need to recognize that policymaking is a complex process with diverse perspectives. They need to be prepared to address legitimate concerns with respect and time. Additionally, there are several international organizations and regulatory bodies, for example, ISO, OECD, EU and EPA, that have established guidelines and regulations for safe nanotechnology development. A stringent assessment following these guidelines would help researchers to build a comprehensive risk-benefit analysis and earning confidence among policymakers and publics (Figure 2 (A)).

Post-Commercialisation Marketability and Use: Societal concerns remain a significant issue in bringing any products into the market. Only a handful of gene edited plants have successfully been marketed, such as the gene edited soybean variety that produces healthier cooking oil.[34] Public perception towards a technology significantly impacts its adoption to the market. Arie Rip argues that scientists' fear of this public apprehension towards nanotechnology, which he termed as 'nanophobia-phobia', [35] meaning fearing the fear of nanotechnology could hinder innovation. That is, researchers may 'second guess' public appetite for new technologies and get it wrong. Hence, it is important to prioritise open communication with the stakeholders and public. Researchers can benefit from early inclusion of key stakeholders (academic, industry, and government researchers) in R&D phases to get valuable insights, address technical challenges, anticipate potential downstream barriers for overall development of desirable, safe, and effective nanotechnology. Furthermore, post-commercialisation evaluation for long-term impact of the technology to environment, health and society should also be implemented. (Figure 2 (A)) Therefore, by prioritizing compliance with regulations and transparency with stakeholders, researchers can help to ensure that nanotechnology is developed and commercialized in a responsible manner .

Concluding Remarks and Future Perspective

The recent application of nanotechnology in gene-editing of plants has opened pathways for more comprehensive R&D addressing the current challenges of gene delivery in plants. There is a plethora of ENMs that can be designed to smartly interact with different cargoes and tissues of plant to achieve a successful delivery. A definitive conclusion regarding the optimal frameworks for efficiently delivering ENMs to plant cells remains elusive. A deeper understanding of the design principles of ENMs to direct their maximum interaction with the cargo and plant tissue of interest is of paramount importance. Additionally, incorporation of DNA-free gene-editing tools like CRISPR RNPs and TALENs proteins could provide faster and streamlined approaches. While nanotechnology and DNA-free plant gene-editing offers these exciting possibilities, the assessment of associated risk is also necessary. Here, the challenges at the scale of nanotechnology are substantial and multifaceted (see Outstanding Questions).

Going beyond the realms of nano and genes and integrating the principles of RRI throughout the R&D process will help researchers negotiate the winding road from the lab-to-field-to-market. RRI encourages open communication fostering transparency and inclusivity to help researchers anticipate challenges to building public trust and help researchers be trustworthy. This demands a collaborative team to navigate the socio-technical aspects (see Outstanding Questions) of such multidisciplinary research. Scientific experts proficient in cutting-edge technologies like nanotechnology and plant gene-editing and social scientists with expertise in regulatory processes and the engagement of multiple publics and interested parties should work together. Such a holistic effort would maximize the transformative capabilities of nanotechnology and DNA-free plant gene-editing developing practical frameworks to produce plants with enhanced yield, climate resilience, and nutritional value at low-cost and lesser time.

Acknowledgement

N.G. and H.S. thank the *Australian Research Council Training Centre for Accelerated Future Crops Development IC210100047* for the fellowship. The views expressed herein are those of the authors and are not necessarily those of the Australian Government or Australian Research Council. N.G. acknowledges feedback from Muhammad Arslan Mahmood for designing the key figure. M.M. acknowledges the Australian Research Council for a Future Fellowship (FT200100185) and Discovery Project (DP220100452), respectively. M.M. is a grateful recipient of the University of Sydney Research Accelerator (SOAR) Prize. B.J. acknowledges funding received from the University of Sydney, Faculty of Science, EiPGE Compact.

Conflict of Interest

The authors declare no competing interests.

References

1. FAO; *et al.* (2024) *The State of Food Security and Nutrition in the World 2024 – Financing to end hunger, food insecurity and malnutrition in all its forms. Vol. 2024*) FAO ; IFAD ; UNICEF ; WFP ; WHO ;
2. Von Braun, J. *et al.* (2021) Food systems: seven priorities to end hunger and protect the planet. *Nature* 597, 28-30. 10.1038/d41586-021-02331-x
3. Hofmann, T. *et al.* (2020) Technology readiness and overcoming barriers to sustainably implement nanotechnology-enabled plant agriculture. *Nat. Food* 1, 416-425
4. Yan, Y. *et al.* (2022) Nanotechnology Strategies for Plant Genetic Engineering. *Adv. Mater.* 34, 2106945. 10.1002/adma.202106945
5. Biswal, A.K. *et al.* (2023) An efficient transformation method for genome editing of elite bread wheat cultivars. *Front. Plant Sci.* 14. 10.3389/fpls.2023.1135047
6. Das, B. *et al.* (2019) Robust Genetic Transformation System to Obtain Non-chimeric Transgenic Chickpea. *Front. Plant Sci.* 10. 10.3389/fpls.2019.00524

7. Poddar, S. *et al.* (2023) Optimization of highly efficient exogenous-DNA-free Cas9-ribonucleoprotein mediated gene editing in disease susceptibility loci in wheat (Triticum aestivum L.). *Front. Plant Sci.* 13. 10.3389/fpls.2022.1084700

8. Najafi, S. *et al.* (2023) DNA-free genome editing in grapevine using CRISPR/Cas9 ribonucleoprotein complexes followed by protoplast regeneration. *Hort. Res.* 10. 10.1093/hr/uhac240

9. Ahmar, S. *et al.* (2021) Advantage of Nanotechnology-Based Genome Editing System and Its Application in Crop Improvement. *Front. Plant Sci.* 12. 10.3389/fpls.2021.663849

10. Demirer, G.S. *et al.* (2021) Nanotechnology to advance CRISPR–Cas genetic engineering of plants. *Nat. Nanotechnol.* 16, 243-250. 10.1038/s41565-021-00854-y

11. Sharma, P. and Lew, T.T.S. (2022) Principles of nanoparticle design for genome editing in plants. *Front. Genome Ed.* 4, 846624

12. Behzadi, S. *et al.* (2017) Cellular uptake of nanoparticles: journey inside the cell. *Chem. Soc. Rev.* 46, 4218-4244. 10.1039/c6cs00636a

13. Demirer, G.S. *et al.* (2019) High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. *Nat Nanotechnol* 14, 456-464. 10.1038/s41565-019-0382-5

14. Yong, J. *et al.* (2021) Sheet-like clay nanoparticles deliver RNA into developing pollen to efficiently silence a target gene. *Plant Physiol.* 187, 886-899. 10.1093/plphys/kiab303

15. Lowry, G.V. *et al.* (2024) Towards realizing nano-enabled precision delivery in plants. *Nat. Nanotechnol.* 10.1038/s41565-024-01667-5

16. Kwak, S.-Y. *et al.* (2019) Chloroplast-selective gene delivery and expression in planta using chitosan-complexed single-walled carbon nanotube carriers. *Nat. Nanotechnol.* 14, 447-455. 10.1038/s41565-019-0375-4

17. Wong, M.H. *et al.* (2016) Lipid Exchange Envelope Penetration (LEEP) of Nanoparticles for Plant Engineering: A Universal Localization Mechanism. *Nano Lett.* 16, 1161-1172. 10.1021/acs.nanolett.5b04467

18. Zhang, H. *et al.* (2022) Nanoparticle cellular internalization is not required for RNA delivery to mature plant leaves. *Nat. Nanotechnol.* 17, 197-205. 10.1038/s41565-021-01018-8

19. Santana, I. *et al.* (2022) Targeted Carbon Nanostructures for Chemical and Gene Delivery to Plant Chloroplasts. *ACS Nano* 16, 12156-12173. 10.1021/acsnano.2c02714

20. Zhao, X. *et al.* (2017) Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers. *Nat Plant* 3, 956-964. 10.1038/s41477-017-0063-z

21. Ali, Z. *et al.* (2022) DNA–Carbon Nanotube Binding Mode Determines the Efficiency of Carbon Nanotube-Mediated DNA Delivery to Intact Plants. *ACS Appl. Nano Mater.* 5, 4663-4676. 10.1021/acsanm.1c03482

22. Zhang, Y. *et al.* (2021) Star Polymer Size, Charge Content, and Hydrophobicity Affect their Leaf Uptake and Translocation in Plants. *Environ. Sci. Technol.* 55, 10758-10768. 10.1021/acs.est.1c01065

23. Hu, P. *et al.* (2020) Nanoparticle Charge and Size Control Foliar Delivery Efficiency to Plant Cells and Organelles. *ACS Nano* 14, 7970-7986. 10.1021/acsnano.9b09178

24. Kanwal, M. *et al.* (2022) Pollen: A Potential Explant for Genetic Transformation in Wheat (Triticum aestivum L.). *Agronomy* 12, 2009. 10.3390/agronomy12092009

25. Gogoi, N. *et al.* (2022). Wheat pollen uptake of CRISPR/Cas9 RNP-PDMAEMA nanoassemblies results in targeted loss of gene function in progeny. Cold Spring Harbor Laboratory

26. Law, S.S.Y. *et al.* (2022) Polymer-coated carbon nanotube hybrids with functional peptides for gene delivery into plant mitochondria. *Nat. Commun.* 13. 10.1038/s41467-022-30185-y

27. Landry, M.P. and Mitter, N. (2019) How nanocarriers delivering cargos in plants can change the GMO landscape. *Nat. Nanotechnol.* 14, 512-514. 10.1038/s41565-019-0463-5

28. Ahmad, A. *et al.* (2023) GMOs or non-GMOs? The CRISPR Conundrum. *Front. Plant Sci.* 14, 1232938. 10.3389/fpls.2023.1232938

29. Malnoy, M. *et al.* (2016) DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins. *Front. Plant Sci.* 7. 10.3389/fpls.2016.01904

30. Woo, J.W. *et al.* (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33, 1162-1164. 10.1038/nbt.3389

31. Nguyen, G.T. *et al.* (2022) Miniature CRISPR-Cas12 endonucleases - Programmed DNA targeting in a smaller package. *Curr. Opin. Struct. Biol.* 77, 102466. 10.1016/j.sbi.2022.102466

32. Cummings, C.L. *et al.* (2021) Barriers to responsible innovation of nanotechnology applications in food and agriculture: A study of US experts and developers. *NanoImpact* 23, 100326. 10.1016/j.impact.2021.100326

33. Morose, G. (2010) The 5 principles of “Design for Safer Nanotechnology”. *J. Clean. Prod.* 18, 285-289. 10.1016/j.jclepro.2009.10.001

34. Song, H. *et al.* (2023) Bioengineering of Soybean Oil and Its Impact on Agronomic Traits. *Inter. J. Mole. Sci.* 24, 2256. 10.3390/ijms24032256

35. Rip, A. (2019) Folk theories of nanotechnologists. 10.4324/9780429465734-4

36. Dunbar, T. *et al.* (2022) Carbon Nanotube-Mediated Plasmid DNA Delivery in Rice Leaves and Seeds. *Inter. J. Mole. Sci.* 23, 4081. 10.3390/ijms23084081

37. Ghaghelestany, A.B. *et al.* (2020) Gene transfer to German chamomile (L chamomilla M) using cationic carbon nanotubes. *Sci. Hortic.* 263, 109106. 10.1016/j.scienta.2019.109106

38. Golestanipour, A. *et al.* (2018) Gene Delivery to Tobacco Root Cells with Single-Walled Carbon Nanotubes and Cell-Penetrating Fusogenic Peptides. *Mol. Biotechnol.* 60, 863-878. 10.1007/s12033-018-0120-5

39. Burlaka, O.M. *et al.* (2015) Plant genetic transformation using carbon nanotubes for DNA delivery. *Cytol. Genet.* 49, 349-357. 10.3103/S009545271506002X

40. Hao, Y. *et al.* (2013) Magnetic gold nanoparticles as a vehicle for fluorescein isothiocyanate and DNA delivery into plant cells. *Botany* 91, 457–466

41. Fu, Y.-q. *et al.* (2012) Delivering DNA into plant cell by gene carriers of ZnS nanoparticles. *Chem. Res. Chinese U* 28, 672-676

42. Zolghadrnasab, M. *et al.* (2021) Ultrasound-mediated gene delivery into suspended plant cells using polyethyleneimine-coated mesoporous silica nanoparticles. *Ultrason. Sonochem.* 73, 105507. 10.1016/j.ultsonch.2021.105507

43. Hajiahmadi, Z. *et al.* (2019) Enhancement of tomato resistance to Tuta absoluta using a new efficient mesoporous silica nanoparticle-mediated plant transient gene expression approach. *Sci. Horticult.* 243, 367-375. 10.1016/j.scienta.2018.08.040

44. Chang, F.-P. *et al.* (2013) A simple plant gene delivery system using mesoporous silica nanoparticles as carriers. *J. Mater. Chem. B* 1, 5279. 10.1039/c3tb20529k

45. Torney, F. *et al.* (2007) Mesoporous silica nanoparticles deliver DNA and chemicals into plants. *Nat. Nanotechnol.* 2, 295-300. 10.1038/nnano.2007.108

46. Liu, Q. *et al.* (2020) Clay nanosheet-mediated delivery of recombinant plasmids expressing artificial miRNAs via leaf spray to prevent infection by plant DNA viruses. *Hortic. Res.* 7, 179. 10.1038/s41438-020-00400-2

47. Mitter, N. *et al.* (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3, 16207. 10.1038/nplants.2016.207

48. Bao, W. *et al.* (2016) Layered Double Hydroxide Nanotransporter for Molecule Delivery to Intact Plant Cells. *Sci. Rep.* 6, 26738. 10.1038/srep26738

49. Amani, A. *et al.* (2018) Ultrasound-enhanced gene delivery to alfalfa cells by hPAMAM dendrimer nanoparticles. *Turk. J. Biol.* 42, 63-75. 10.3906/biy-1706-6

50. Finiuk, N. *et al.* (2017) Investigation of novel oligoelectrolyte polymer carriers for their capacity of DNA delivery into plant cells. *J. Plant Biotechnol.* 131, 27-39. 10.1007/s11240-017-1259-7

51. Jiang, L. *et al.* (2014) Systemic gene silencing in plants triggered by fluorescent nanoparticle-delivered double-stranded RNA. *Nanoscale* 6, 9965-9969. 10.1039/c4nr03481c

52. Silva, A.T. *et al.* (2010) Conjugated polymer nanoparticles for effective siRNA delivery to tobacco BY-2 protoplasts. *BMC Plant Biol.* 10, 291. 10.1186/1471-2229-10-291

53. Pasupathy, K. *et al.* (2008) Direct plant gene delivery with a poly(amidoamine) dendrimer. *Biotechnol. J.* 3, 1078-1082. 10.1002/biot.200800021

54. Delgado-Martín, J. *et al.* (2022) Carbon Dots Boost dsRNA Delivery in Plants and Increase Local and Systemic siRNA Production. *Inter. J. Mol Sci.* 23, 5338. 10.3390/ijms23105338

55. Wang, B. *et al.* (2020) Carbon Dots Enable Efficient Delivery of Functional DNA in Plants. *ACS Appl. Bio Mater.* 3, 8857-8864. 10.1021/acsabm.0c01170

56. Schwartz, S.H. *et al.* (2020) Carbon Dots for Efficient Small Interfering RNA Delivery and Gene Silencing in Plants. *Plant Physiol.* 184, 647-657. 10.1104/pp.20.00733

57. Islam, M.R. *et al.* (2024) DNA Delivery by Virus-Like Nanocarriers in Plant Cells. *Nano Lett.* 24, 7833-7842. 10.1021/acs.nanolett.3c04735

58. Naqvi, S. *et al.* (2012) Calcium phosphate nanoparticle mediated genetic transformation in plants. *J. Mater. Chem.* 22, 3500. 10.1039/c2jm11739h

59. Norfaezah, J. *et al.* (2024) DNA-free CRISPR/Cas9 genome editing system for oil palm protoplasts using multiple ribonucleoproteins (RNPs) complexes. *Ind. Crops Prod.* 208, 117795. 10.1016/j.indcrop.2023.117795

60. Azariadis, A. *et al.* (2024) A Walk on the Wild Side: Genome Editing of Tuber-Bearing Solanum bulbocastanum. *Plants* 13, 1044. 10.3390/plants13071044

61. Banakar, R. *et al.* (2022) Efficiency, Specificity and Temperature Sensitivity of Cas9 and Cas12a RNPs for DNA-free Genome Editing in Plants. *Front. Genome Edit.* 3. 10.3389/fgeed.2021.760820

62. Lin, C.-S. *et al.* (2022) DNA-free CRISPR-Cas9 gene editing of wild tetraploid tomato Solanum peruvianum using protoplast regeneration. *Plant Physiol.* 188, 1917-1930. 10.1093/plphys/kiac022

63. Liu, Y. *et al.* (2022) Establishment of a DNA-free genome editing and protoplast regeneration method in cultivated tomato (Solanum lycopersicum). *Plant Cell Rep.* 41, 1843-1852. 10.1007/s00299-022-02893-8

64. Subburaj, S. *et al.* (2022) A DNA-Free Editing Platform for Genetic Screens in Soybean via CRISPR/Cas9 Ribonucleoprotein Delivery. *Front. Plant Sci.* 13. 10.3389/fpls.2022.939997

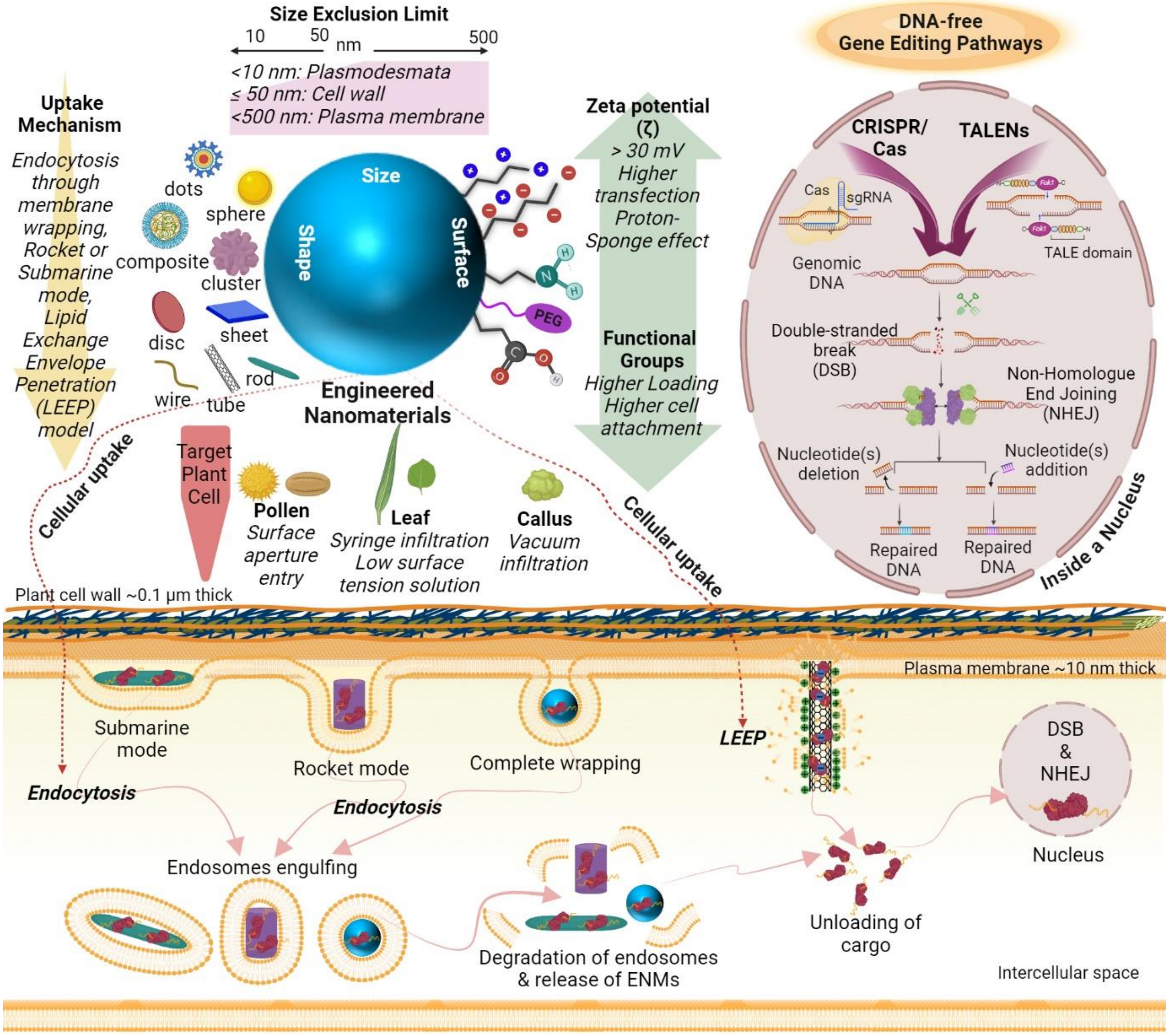
65. Sidorov, V. *et al.* (2022) Heritable DNA-free genome editing of canola (Brassica napus L.) using PEG-mediated transfection of isolated protoplasts. *In Vitro Cell Dev. Biol. Plant* 58, 447-456. 10.1007/s11627-021-10236-7

66. Zhao, X. *et al.* (2021) Amylose starch with no detectable branching developed through DNA-free CRISPR-Cas9 mediated mutagenesis of two starch branching enzymes in potato. *Sci. Rep.* 11. 10.1038/s41598-021-83462-z

67. Badhan, S. *et al.* (2021) First Report of CRISPR/Cas9 Mediated DNA-Free Editing of 4CL and RVE7 Genes in Chickpea Protoplasts. *Inter. J. Mol. Sci.* 22, 396. 10.3390/ijms22010396

68. Wu, S. *et al.* (2020) Establishment of a PEG-mediated protoplast transformation system based on DNA and CRISPR/Cas9 ribonucleoprotein complexes for banana. *BMC Plant Biol.* 20. 10.1186/s12870-020-02609-8

69. Brandt, K.M. *et al.* (2020) A Streamlined Protocol for Wheat (Triticum aestivum) Protoplast Isolation and Transformation With CRISPR-Cas Ribonucleoprotein Complexes. *Front. Plant Sci.* 11. 10.3389/fpls.2020.00769
70. Liu, W. *et al.* (2020) Lipofection-mediated genome editing using DNA-free delivery of the Cas9/gRNA ribonucleoprotein into plant cells. *Plant Cell Rep.* 39, 245-257. 10.1007/s00299-019-02488-w
71. Xu, J. *et al.* (2020) Cas9-mediated editing of 1-aminocyclopropane-1-carboxylate oxidase1 enhances <i>Petunia</i> flower longevity. *Plant Biotechnol. J.* 18, 287-297. 10.1111/pbi.13197
72. Murovec, J. *et al.* (2018) DNA-Free Genome Editing of Brassica oleracea and B. rapa Protoplasts Using CRISPR-Cas9 Ribonucleoprotein Complexes. *Front. Plant Sci.* 9. 10.3389/fpls.2018.01594
73. Andersson, M. *et al.* (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiol. Plant.* 164, 378-384. 10.1111/ppl.12731
74. Liang, Z. *et al.* (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8. 10.1038/ncomms14261
75. Kim, H. *et al.* (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nat. Commun.* 8, 14406. 10.1038/ncomms14406
76. Svitashev, S. *et al.* (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nat. Commun.* 7, 13274. 10.1038/ncomms13274
77. Subburaj, S. *et al.* (2016) Site-directed mutagenesis in Petunia × hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. *Plant Cell Rep.* 35, 1535-1544. 10.1007/s00299-016-1937-7
78. Stoddard, T.J. *et al.* (2016) Targeted Mutagenesis in Plant Cells through Transformation of Sequence-Specific Nuclease mRNA. *Plos One* 11. 10.1371/journal.pone.0154634
79. Zhang, Y. *et al.* (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* 7, 12617. 10.1038/ncomms12617



Key Figure 1. Design Principles for Engineered Nanomaterials (ENM) and DNA-free Gene-Editing pathways. There are three major aspects of nanomaterials that regulate their efficiency as a delivery vehicle of gene-editing: Size, Shape and Surface. Size of ENMs: Size exclusion limit (SEL) is the maximum size of nanoparticle that can enter a particular membrane. Its <10 nm for plasmodesmata, ≤50 nm for cell wall, and <500 nm for plasma membrane. Nanoparticles which are bigger than SEL are not capable of entering a cell. Shape of ENMs: Different shapes of nanoparticle tend to follow different uptake mechanism into a cell. Spherical ENMs (sphere, dos, composite, clusters, disc) tend to enter through membrane wrapping initiated endocytosis. Tube like ENMs can follow different mode depending on their tips; flat tips follow rocket mode (tip-first), round tips follow submarine mode (side-first), carbon nanotubes are shown to follow lipid exchange envelope penetration model. Surface of ENMs: Surface charge and properties of ENMs can tune their efficiency to enter a plant cell. Nanoparticles with surface zeta potential (charge) ≥ 30 nm are found to be most effective in transfecting a plant cell, with positive charge more effective than a negative charge. The higher surface charge also stabilises the nanoparticle and protects cargo from enzymatic degradation through proton-sponge effect. Surface properties can be further tuned by functionalisation using different functional groups to increase cell attachment, hydrophilicity, cargo loading. Additionally, the role of target plant cell is important while designing ENMs. Pollen grains have surface apertures (thinner cell membrane) that render easier transfection of nanoparticles. Leaf tissues on the other hand require some mechanical force, such as syringe infiltration of nanoparticles to transfect inside the cell. Lowing the surface tension of the nanoparticle solution using surfactants helps in bypassing the leaf waxy cuticles. Other tissues, such as callus are shown to transfect nanoparticles using vacuum infiltration. The cellular uptake of the nanoparticles may occur through different mechanisms depending on its properties. Mostly, endocytosis mode of entry, wherein, spherical nanoparticles usually undergo complete membrane wrapping, whereas different aspect ratio nanoparticles prefer either submarine-mode (for high aspect ratio) or rocket-mode (for low aspect ratio) of entry. Once inside a cell, a nanoparticle carrying the cargo (e.g, CRISPR/TALENs) would undergo endosomes engulfing and then degradation process as the pH changes in the cytoplasm to release the cargo which enters the nucleus. Inside the nucleus, DNA-free gene-editing process can be achieved using tools like CRISPR/Cas, clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) or TALENs, transcription activator-like effector nucleases. CRISPR/Cas in the form of preassembled ribonucleoproteins (RNP) or TALENs protein can direct the double stranded break (DSB) in the targeted DNA which can be repaired through non-homologues end joining (NHEJ) mechanism without integrating any exogenous DNA into the genome. *Created with BioRender.com*

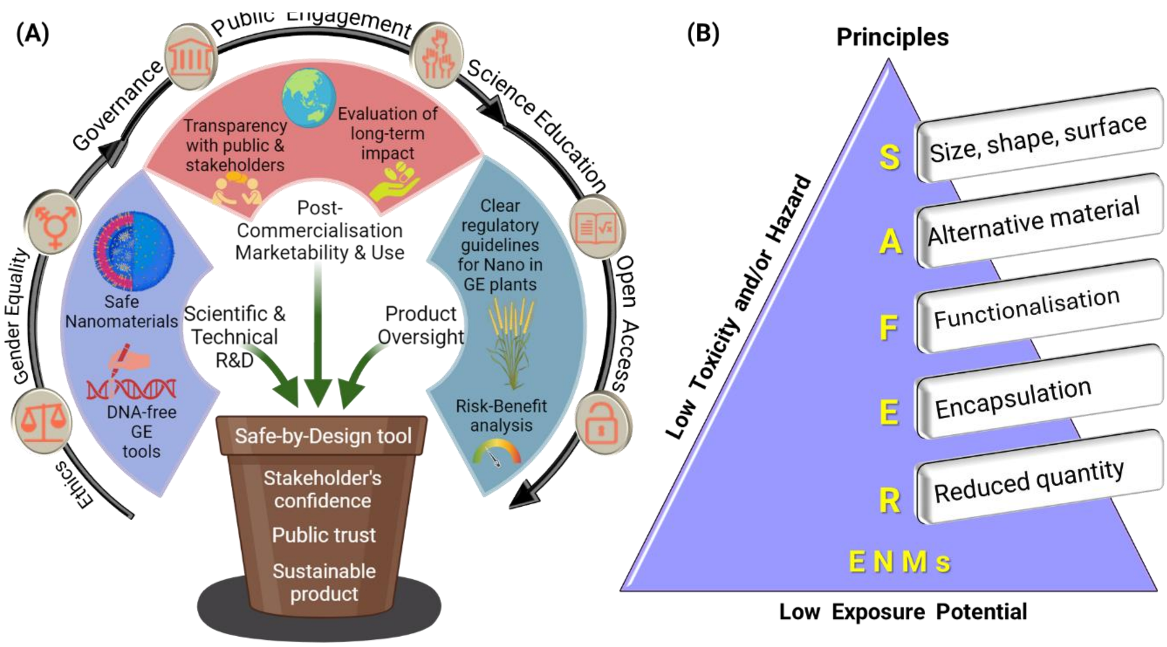


Figure 2. The role of principles of Responsible Research and Innovation (RRI) in nanotechnology mediated plant gene-editing (A) The six pillars of RRI navigating the three innovation phases to grow plants for the future.; 1) Scientific and technical research and development (R&D) for safe and sustainable designs and 2) Product oversight to perform risk-benefit analysis and put in place clear regulatory guidelines, 3) Post-Commercialisation Marketability and use, constant and transparent evaluation post commercialisation addressing public and stakeholders' concerns. (B) The principles to design safer engineered nanomaterials (ENMs) with low toxicity and/or hazard and exposure potential.[33] *Created with BioRender.com*

Table 1. Different types of Engineered Nanomaterials (ENMs) mediated delivery into plant cells.

Category of ENMs	**Attributes and Advantages of ENMs**	**ENM type, shape & size**	**Genetic material & Delivery technique**	**Plant species & Type of Tissue**	**Major Findings**	**Refs.**
Carbon nanotube (CNT)-based NMs	*High aspect ratio*: Helps in high cargo loading & high transfection efficiency *Reactive surface*: Could be functionalised & shields from enzymatic degradation	Single-walled CNT (SWCNT) Tube-like, L: ~ 500-1000nm D: ~ 1-5 nm	Plasmid DNA encoding GFP, YFP, GUS CRISPR/CasP DS Syringe Infiltration	*Oryza sativa* (Rice), Leaf and seed (SAM) Mature embryo	• PEI-functionalised CNT for transient expression of GFP, YFP, GUS in leaf and embryo tissues • CRISP/Cas9 targeting PDS were infiltrated in seed and embryo • Very low frequency of mutation was observed	Dunbar et al., 2022 [36]
			Plasmid DNA encoding GFP and CY3 Foliar application	*Arabidopsis thaliana*, Leaves	• PEI-functionalised SWCNT delivers electrostatically bound plasmid DNA driven by a plastid specific promoter to chloroplast by plant biorecognition approach • Increased leaf H_2O_2 levels to 0.2970 ± 0.0341 μmol gFW−1 after 1 day of exposure • SWCNTs induced transient oxidative damage	Santana et al., 2022 [19]
			Plasmid DNA encoding GFP	*Arabidopsis thaliana*, Roots	• CNT- polymethacrylate maleimide network (SWNT-PM) modified with functional peptide, Cytcox and KH9 • Vacuum infiltration of the whole seedling • GFP localisation in nucleus and mitochondria with 30 times higher efficiency	Law, S.S.Y. *et al.* (2022) [26]

			siRNA Syringe Infiltration	*Nicotiana benthamiana*, Leaf	• 95% efficiency of gene silencing at mRNA level in intact plant • Delay in siRNA nuclease degradation	Demirer et al, 2020 [10]
			ssDNA-Fluorescein isothiocyanate (FITC) Ultrasound	*L chamomilla M* (German chamomile), Suspension cells	• Synthesis of polyethyleneimine functionalised SWCNT • 47% transfection efficiency into Chamomile cells • High potential of SWCNTs protects from ultrasound damages • Ultrasound waves create pores in the cell wall to facilitate transfer of gene transfer.	Ghaghelestany et al, 2020 [37]
			Plasmid DNA encoding YFP Syringe Infiltration	*Eruca sativa, Nasturtium officinale*, *Nicotiana tabacum* & *Spinacia oleracea,* Leaf *Arabidopsis thaliana,* Mesophyll Protoplasts	• Synthesis of chitosan functionalised SWCNT • Selective gene delivery to Chloroplast • Transgene expression • Easy and cost effective • Application in mature plants • Effective in different plant species	Kwak et al, 2019 [16]
			Plasmid DNA encoding GFP Passive delivery	*Nicotiana tabacum*, Root cells	• Synthesis of arginine functionalised SWCNT • Fast, easy and safe plasmid DNA carrier • Successful GFP expression confirmed by fluorescence microscopy and western blotting analysis.	Golestanipour et al, 2018 [38]
			Plasmid DNA encoding GFP Syringe Infiltration	*Nicotiana benthamiana*, *Eruca sativa* (arugula), *Triticum aestivum* (wheat) and *Gossypium hirsutum* (cotton), Leaf *Eruca vesicaria* (Arugula), Protoplast	• Synthesis of polyethyleneimine functionalised SWCNT • Efficient delivery of plasmid DNA with strong transient expression of GFP • 85% transformation efficiency in arugula protoplasts. • Lipid exchange envelope penetration (LEEP) model of internalisation SWCNT-DNA • Independent technique applicable in monocot and dicot plant species.	Demirer et al, 2019 [13]
			Plasmid DNA encoding YFP Passive delivery	*Nicotiana tabacum,* Protoplast, callus, and leaf discs	• Synthesis of noncovalently functionalized SWCNTs • 16% efficiency in transient transformation of protoplasts • 8% stable transformation frequency for the Callus • 6% stable transformation frequency for the Leaf explants	Burlaka et al, 2015 [39]
		Multi-walled CNT (MWCNT) Tube-like, L: ~ 2-13 nm D: ~ 1000 nm	Plasmid DNA encoding GFP, Passive delivery	*Nicotiana benthamiana*, *Eruca sativa* (arugula), *Triticum aestivum* (wheat) and *Gossypium hirsutum* (cotton), Leaf	• Synthesis of polyethyleneimine functionalised MWCNT • Thicker diameter compared to SWCNT • Loading and delivery of plasmid DNA is very less with MWCNT in comparison to SWCNT	Demirer et al, 2019 [13]
			Plasmid DNA encoding YFP, Passive delivery	*Nicotiana tabacum* (Tobacco), Protoplast, callus, and leaf discs	• Synthesis of noncovalently functionalized MWCNTs • 13% efficiency in transient transformation of protoplasts • 3% stable transformation frequency for Callus • 2% stable transformation frequency for the Leaf explants	Burlaka et al, 2015 [39]

Metallic and/or Magnetic NMs	*High surface to volume ratio*: Helps in high cargo loading & functionalisation *Tunable optical properties*: Helps in bioimaging *Bio-compatible*: Reduced toxicity and nuclease degradation *Tunable size/shape*: trigger cargo release & easy transfection *Magnetic Field*: Acts as external aid for easy and faster transfection	PEI-Magnetic Iron oxide NPs (MNPs) Spherical, D: ~ 100-120 nm	Plasmid DNA encoding GUS gene External magnetic field	*Gossypium herbaceum L.* (Cotton), *Capsicum annuum L.* (pepper), *Cucurbita moschata* (pumpkin) *Cucurbita pepo* L. (cocozelle), and *Lilium brownie* (lily), Pollen grains	• Successful delivery of exogenous GUS gene through surface aperture of pollen facilitated by magnetofection phenomenon • Generation of transformed plants from transgenic seeds through pollination with the magnetofected pollen • Stable inheritance of exogenous gene in the offspring • The system is simple, fast, culture-free and versatile with genotype independence	Zhao et al, 2017 [20]
		PEG-Magnetic Gold NPS (mGNPs) Spherical, D: ~ 20-30 nm	Fluorescein isothiocyanate (FITC) and Plasmid DNA encoding GUS gene External magnetic field	*Brassica napus* (Canola), Protoplasts and Walled cell suspension	• 95% delivery efficiency with the delivery mGNP-FITC nanoparticles into protoplasts • Successful expression of GUS gene with the delivery mGNP-GUS nanoparticles into protoplasts and walled cell suspension • Non-cytotoxic and biocompatible • No physical damage to cells due to small size	Hao et al, 2013 [40]
		Zinc NPs Spherical, D: ~ 3-5 nm	Plasmid DNA encoding GUS gene Ultrasound	*Nicotiana tabacum* (Tobacco), Leaf	• Synthesis of positively charged poly-L-lysine (PLL) functionalised ZnS nanoparticles • Stable expression of GUS gene observed in transgenic plant. • 88.5% regeneration efficiency obtained in 131 regenerated plants • ZnS NPs provide cost-effective and non-destructive delivery to intact plant cell walls	Fu et al, 2012 [41]
Mesoporous silica NMs (MSN)	*Mesoporous structure*: High cargo loading & controlled release at target site *High bio-compatibility*: Very low toxicity and nuclease degradation *Large surface area*: High cargo loading *Reactive surface*: Ease to functionalise & shields from enzymatic degradation	PEI-MSN Spherical, D: ~ 100 ± 8 nm	Plasmid DNA encoding GUS gene Ultrasound	*Nicotiana tabacum* (Tobacco), BY-2 suspension cell culture	• Tobacco cells can sustain upto 320 W of ultrasonic intensity • DNA: PEI-MSN mass ratio of 1:1 resulted in 43.43 μg/mg adsorption capacity • Highest transfection efficiency obtained for ultrasonic intensity of 60 W for 20 min	Zolghadrnasab et al., 2021 [42]
		MSNs Spherical, D: ~ 40 nm	Plasmid DNA encoding GUS gene and Plasmid DNA encoding crylAb gene Passive delivery	*Solanum lycopersicum* (Tomato), Spray and injection into leaf and injection into shoot.	• Synthesis of aminopropyl triethoxysilane (APTES) functionalized MSNs with high porosity • Delivery and transient expression of GUS gene • Delivery of crylAb gene confirmed by an enhanced resistance in the tomatoes against *T. absoluta*. • Biocompatible, fast and energy efficient • Efficient in multiple plant tissues	Hajiahmadi et al, 2019 [43]
		Fluorescein- or rhodamine-doped MSNs (F/R-MSNs) Spherical, D: ~ 50 nm	Plasmid DNA Passive delivery	*Nicotiana tabacum* (Tobacco), Protoplast *Arabidopsis thaliana*, Root	• Synthesis of three organically functionalised F/R-MSNs, viz., N-trimethoxysilylpropyl N,N,Ntrimethylammoniumchloride (TMAPS), 3-aminopropyl-trimethoxysilane (APTMS,) and (3-trihydroxysilyl)propylmethyl phosphonate (THPMP) • Successful DNA delivery into protoplast and intact plant tissue • Gene expression detected in epidermal layer and deep tissues of cortical and endodermal root by fluorescence microscopy and antibody labelling • Energy-independent internalisation process	Chang et al, 2013 [44]
		Type-I MSNs,	Plasmid DNA, Lucifer Yellow dye	*Nicotiana tabacum* (Tobacco),	• Synthesis of fluorescein-doped MSNs (Type-I MSNs) and organic ligand	Torney et al, 2007 [45]

		Type-II MSNs Spherical, D: ~ 100-200 nm	Passive delivery	Mesophyll Protoplast	functionalised Type-I MSNs by triethyleneglycol (TEG) (Type-II MSNs) • 26±7% endocytosis efficiency of Lucifer Yellow dye into protoplast • No uptake of DNA-Type-I MSNs and 7±3% internalisation of DNA-Type-II MSNs into protoplast • Role of Surface properties crucial in plant cell endocytosis	
Clay NMs	*Large surface area*: High cargo loading *Bio-compatible & bio-degradable*: Ideal for biomolecules *Multi-layered:* Protection from degradation & controlled release *Reactive surface*: Ease to functionalise *Cost-effective:* Easy to upscale	Layered double hydroxide (LDH) nanoparticles Nanosheet, D: ~ 30-120 nm	Fluorescein isothiocyanate isomer I (FITC), dsRNA encoding GUS gene Passive delivery	*Solanum lycopersicum* (Tomato), Pollen grains	• Synthesis of LDH nanoparticles of different sizes; 30, 50, 80, 120 nm • Different internalisation efficiency at different development stages of pollen with maximum at early bicellular stage • LDH-50 NPs facilitate delivery of functional GUS-dsRNA with 89% decrease in mRNA levels • % gene silencing varies with size, dose of dsRNA ratio of LDH–dsRNA complexing and duration of treatment	Yong et al, 2021 [14]
		Layered double hydroxide Nanosheet	Artificial miRNAs, plasmid DNA (13 kb) Passive delivery on Spraying	*Solanum lycopersicum* (Tomato), Leaf	• Topical spraying on leaves accumuated miRNA for 7 days and pDNAs for 35 days • Decrease in TYLCV infection and increase in H_2O_2 levels.	Liu et al., 2020 [46]
		BioClay nanoparticles Nanosheet D: ~ 50 nm	dsRNA encoding GUS gene Passive delivery on Spraying	*Arabidopsis thaliana*, *Nicotiana tabacum* (Tobacco) and *Vigna unguiculata* (Cowpea), Leaf	• Synthesis of positively charged LDH nanoparticles of average size 50 nm • Topical spraying of dsRNA loaded LDH NPs as Bioclay • Sustained release of dsRNA from Bioclay upto 30 days after spraying • The uptake of dsRNA triggers RNAi in plant cells against homologous RNA	Mitter et al, 2017 [47]
		Layered double hydroxide lactate nanosheets (LDH-lactate-NS) Nanosheet D: ~ 30-60 nm	Tetramethyl rhodamine isothiocyanate (TRITC), Fluorescein isothiocyanate isomer I (FITC), ssDNA-FITC Passive delivery	*Arabidopsis thaliana*, Leaf *Nicotiana tabacum* (Tobacco), BY-2 cell suspension	• Synthesis of positively surface charged delaminated LDH lactate nanosheets (LDH-lactate-NS) • Green fluorescence in FITC and red fluorescence in TRITC loaded LDH-lactate-NS after their internalisation in intact plant cells via non-endocytic pathway • LDH-lactate-NS delivers ssDNA-FITC in the nucleus of BY-2 cells	Bao et al, 2016 [48]
Polymeric NMs	*Chemical versatility:* Can be designed in varied shapes with functionalities to increase bio-compatibility and bio-degradability *pH controlled delivery*: -Ease to render cargo delivery at different pH conditions *Positive surface charge*: Easy binding of nucleic acids & enhanced	Poly [2-(dimethylamino)ethyl methacrylate], PDMAEMA Spherical D:~ 10-15 nm	CRISPR/Cas9 RNP Endocytosis	*Triticum aestivum* L (Wheat) Pollen grains	• Linear polymer, PDMAEMA complexed RNP targeting SR50 gene conferring resistance to rust pathogen • The first demonstration of the uptake of protein-polymer nanocomplexes into wheat pollen grains. • The transfection efficiency of PDMAEMA-RNP was found to be 20% higher than the control (only RNP) • In planta experiment generated loss-of-mutation progency.	Gogoi et al. 2022 [25]
		Polyamidoamine (PAMAM) dendriplexes Spherical or elliptical D: ~ 123-240 nm	ssDNA-FITC (Fluorescein isothiocyanate), ssDNA encoding GUS gene Ultrasound	*Medicago sativa* L. (Alfalfa), Leaf and petiole explants	• Hyperbranched PAMAM (hPAMAM) and Generation 2 hPAMAM (hPAMAM-G2) with a core of diethylenetriamine and 45 surface amino groups • Synthesis of DNA and PAMAM dendriplexes at N/P ratios 0.25, 0.5,1, 2, 3, 4, 5, 10, and 20 • Complete neutralization achieved at N/P=4 and smallest size achieved at N/P=10 • Transfection and expression of *gus*A gene with highest efficiency of 1.4% at an N/P=10	Amani et al, 2018 [49]

	transfection efficiency				- Increased transfection efficiency of 3.86% due to a combined effect of ultrasound and hPAMAM–DNA	
	Low cost: Easy to upscale	Poly (dimethylaminoethylmethacrylate (PDMAEM)-based polyplexes	Plasmid DNA encoding YFP gene Passive delivery	*Allium cepa* (Onion), *Nicotiana tabacum* L. (tobacco) and *Ceratodon Purpureus* (Moss), Protoplast	- Synthesis of various block copolymers; TN83/6, TN 84/5, LM-8-DM and DLM-9-DM and formation of polyplexes with plasmid DNA encoding YFP - TN 83/6 and TN 84/5 possessed highest DNA binding capacity - Successful transient and stable protoplast transformation observed by using TN 83/6, TN 84/5 and DLM-9-DM	Finiuk et al, 2017 [50]
		Polymeric fluorescent dendriplexes G1 = 129.9 ± 1.1 G2 = 136.5 ± 3.4 G3 = 136.6 ± 2.1	CXR dye-labeled DNA (blue), dsRNA:SHOOT MERISTEMLESS (STM) and WEREWOLF (WER) Passive delivery	*Arabidopsis thaliana*, Root	- Assembly of G1, G2, and G3 dendrimers with a perylene-3,4,9,10-tetracarboxydiimide (PDI) chromophore core with plasmid DNA - G1 has the lowest and G3 has the highest delivery efficiency - In vivo delivery of G2-dsRNA (STM) and G2-dsRNA (WER) results in reduced shoot apical meristem (SAM) size and increased lateral roots, respectively - Cationic surface renders DNA and dsRNA entry into plant cells	Jiang et al, 2014 [51]
		Conjugated polymer nanoparticles (CPNs)	siGLO Red (a commercial red fluorescent dye)-labelled siRNA, siRNA targeting NtCesA-1 genes Passive delivery	*Nicotiana tabacum* L. (tobacco) BY-2, Protoplast and BY-2 suspension cell	- Successful delivery of CPNs into protoplast within 2 hours, but not in intact plant cells - CPNs deliver siRNAs knocking out NtCesA-1 genes in protoplast - Good viability of protoplast with 90-96% at 8h, 85% at 24h and 60% at 48h - Easy cell wall regeneration of protoplast after transformation	Silva et al, 2010 [52]
		Generation 4 dendrimers (G4-PAMAM) D: ~ 4.5 nm	Plasmid DNA encoding GFP gene Direct and non-invasive delivery	*Agrostis stolonifera* L., cv., Penn-A-4 (Turfgrass), Callus cells	- Synthesis of tetramethyl rhodamine isothiocyanate (TRITC) functionalised G4-PAMAM - Complexation of DNA-PAMAM forming a "supramolecular mass" - Physiochemical properties and nanosize of G4-PAMAM facilitate direct and non-invasive - Delivery of DNA with 48.5 % efficiency in transient expression.	Pasupathy et al, 2008 [53]
Carbon Dots (CD)	Fluorescent nanoparticles Good biocompatibility Low toxicity Easy surfaces functionalisation Tunable emission range	PEI -carbon dots	dsRNA Spraying	*Cucumis sativus cv.* (Cucumber), Leaf cells	- Hydrodynamic size is ~5 nm - 50-fold increase in dsRNA delivery by CD as compared to naked dsRNA Increase in production od systematic siRNA with CD delivery	Delgado-Martin et al., 2022 [54]
		Peptide funtinalised CD	Plasmid DNA encoding GFP gene	*Arabidopsis thaliana*, Leaf chloroplast	- CDs engineered with targeting peptides increase pDNA delivery efficiency - CDs with molecular baskets act as reservoirs - Increased H_2O_2 levels observed with decrease in lea chlorophyl content	Santana et al., 2022 [19]
		PEI-CDs	Plasmid DNA encoding GFP gene Leaf infiltration, root soaking	*Oryza Sativa japonica* (rice), *Triticum aestivum* (wheat), *Phaseolus radiatus* (mung bean) Leaves and roots	- It can deliver in multiple cells of monocots and dicots plants - 96% positive GFP transformation reported in wheat leaf cells - CDP deliver hygromycin resistance gene and induce resistance in rice	Wang et al., 2020 [55]

		PEI-CDs	siRNA Spraying	*Nicotiana benthamiana*, *Solanum lycopersicum* (Tomato), Leaf	• Highest level of gene silencing observed with CDs of size 3.9 nm • GFP transcript and protein levels reduced to 80% • A visible bleaching phenotype was observed.	Schwartz et al., 2020 [56]
Miscellaneous Kinds	*High aspect ratio*: Helps in high cargo loading *Reactive surface*: Could be functionalised & shields from enzymatic degradation	Tobacco mild green mosaic virus (TMGMV)-like nanocarriers	Single-stranded DNA bonded to Cy3 and Plasmid DNA encoding GFP	*Arabidopsis thaliana*, Protoplasts Intact leaf	• Native and inactivated TMGMVs coated with polycationic biopolymer poly(allylamine) hydrochloride (PAH) generate highly charged nanomaterials 56.20 ± 4.7 mV • TMGMV-PAH delivered ssDNA into protoplast cells without using mechanical aid • Inactivated TMGMV-PAH demonstrate pDNA delivery and expression in *Arabidopsis* leaf epidermal cells *in vivo*	Islam et al., 2024 [57]
	Large surface area: High cargo loading *Bio-compatible & bio-degradable*: Ideal for biomolecules *Low cost*: Easy to upscale	Calcium phosphate (CaP) NPs Spherical, D: ~ 20-50 nm	Plasmid DNA encoding GUS gene Passive delivery	*Brassica juncea L. cv. Pusa Jaikisan,* Hypocotyl explants from seeds acid mediated dissolution followed by endosomal	• Synthesis of CaP nanoparticles in aqueous core of reverse micellar droplets by the reaction between calcium and phosphate ion • 80.7% transformation efficiency of CaP-DNA as compared to 8% of naked DNA • GUS integrates into genomic DNA by non-homologous recombination process • Different advantages of CaP NPs • escape of DNA facilitated by calcium ions osmotic imbalance • Compaction of DNA by calcium ions rending its cytosolic stability • Nuclear uptake of DNA facilitated by nuclear pore complex of calcium ions	Naqvi et al, 2012 [58]

Table 2. DNA-free gene-editing delivery methods in a variety of plant cells

Plant species	Mode of delivery & Type of Tissue	Type of SDN & Targeted gene	Major Findings	Refs.
Elaeis guineensis (Oil palm)	PEG transformation of protoplast	CRISPR/Cas9 RNP Eg PDS	• Multiplex genome editing using multiple RNPs • Assessment of different amounts of Cas9 and heat stress using *E. guineensis phytoene desaturase* (*EgPDS*) as the target gene. • Cas9 amount at 40 µg, and treatment at 39ºC increase the genome editing efficiency to 100%.	Norfaezah et al., 2024 [59]
Solanum bulbocastanum	PEG transformation of protoplast	CRISPR/Cas9 RNP A gene from nitrate and peptide transporter family	• The first transgene-free protocol for genome editing of *S. bulbocastanum* • 2-8 bp deletions showing loss-of-function mutation • Regeneration to full plants	Azariadis et al. 2024 [60]
Vitis vinifera (Grapevine)	PEG transformation of protoplast	CRISPR/Cas9 RNP GFP	• Successful knockout of GFP reporter gene with 17% protoplast transformation efficiency • Regeneration of GFP- plant with 23-34% efficiency	Najafi et al., 2023 [8]
Triticum aestivum L. (Bread Wheat)	Biolistic bombardment of immature embryo cells PEG transformation of protoplast	CRISPR/Cas9 RNP Pi21 Tsn1 Snn5	• Heat treatment of transfected tissue. • Protoplast transfection at 30ºC yielded higher editing rates • Biolistic transformation at 30 & 37ºC regenerated 11.8-50% edited plants	Poddar et al., 2023 [7]
Triticum aestivum L. (Bread Wheat)	Nanoparticle mediated delivery into pollen grains	CRISPR/Cas9 RNP SR50	• Successful knockout of SR50 gene with loss-of-function mutation in M2 generation • Linear polymer, PDMAEMA complexed RNP targeted SR50 gene • The first demonstration of the uptake of protein-polymer nanocomplexes into wheat pollen grains.	Gogoi et al. 2022 [25]
Nicotiana benthamiana, *Thlaspi arvense* (pennycress), *Glycine max* (soyabean), *Setaria viridis*	PEG transformation of protoplast	CRISPR/Cas9 RNP & Cas12a RNP PDS	• Temperature sensitivity of different Cas was tested. Highest activity observed at 22 and 26 ºC • Resulted in 34-85.2% mutagenesis efficiencies • Bi-allelic and mono-allelic mutation observed in regenerated plants	Banakar et al., 2022 [61]
Solanum peruvianum (Tomato)	PEG-calcium transformation of protoplast	CRISPR/Cas9 RNP and plasmid SpRDR6, SpSGS3 SpPR-1, SpProSys, SpMlo1	• A diploid/allotetraploid protoplast regeneration method • 60% efficiency without any marker gene selection • Diploid and tetraploid heritable mutants were obtained in all targeted genes and were heritable in next generation	Lin et al., 2022 [62]
Solanum lycopersicum (Tomato)	PEG transformation of protoplast	CRISPR/Cas9 RNP SP SP5G	• Optimisation of protoplast isolation and shoot regeneration • Mutation efficiency was 31.8% targeting both gene simultaneously • Mutation efficiency was 60% in one allele in either of the gene	Liu et al., 2022 [63]
Glycine max (Soyabean)	PEG transformation of protoplast	CRISPR/Cas9 RNP CPR5	• Protoplast transformation, concentration and ratio of Cas9 &gRNA were optimised for soyabean • +5 nt & -30 bp InDels with mutation frequency of 4.2-18.1% in GmCPR5 locus	Subburaj et al., 2022 [64]
Brassica napus L. (Canola)	PEG transformation of protoplast	CRISPR/LbCas12a RNP HSFA1	• Frequency of edits was 40% with targeted deletion of 2-9 nucleotide • Out of 30 regenerated plants, 12 were edited. • 6 of the edited plants were biallelic mutated and another were monoallelic.	Sidorov et al., 2021 [65]
Solanum tuberosum L. (Potato)	PEG transformation of protoplast	CRISPR/Cas9 RNP Sbe1 (BE1T3, BE1T4) Sbe2 (BE2T3, BE2T4)	• Mutation frequency was 72% for potato starch with no detectable branching • All alleles of Sbe1 were mutated in Group 1 & 2 • 2-3 alleles of Sbe2 in group 2 and all alleles of Sbe1&2 mutated in Group 3	Zhao et al., 2021 [66]
Cicer arietinum (Chickpea)	PEG transformation of protoplast	CRISPR/Cas9 RNP 4CL RVE7	• RVE7 sgRNA show ~77.3% indel percentage indicating editing efficiency • 4CL sgRNA very low (2%) indel percentage	Badhan et al., 2021 [67]

Musa spp. Cavendish (Banana)	PEG transformation of protoplast	CRISPR/Cas9 RNP PDS	• Optimised and established protoplast transformation in banana • Mutation efficiency of 0.92% observed	Wu et al., 2020 [68]
Triticum aestivum L. (Bread Wheat)	PEG transformation of protoplast	CRISPR/Cas9 RNP GW2-B PinB-D ASN2-A	• Demonstrates a simplified protoplast isolation method • GW2-B:19.2% in Bob-white and 36% in Chinese spring • ASN2-A: 16.4% in Bob-white and 12.9% in Chinese spring • PinB-D: 0% in both	Brandt et al., 2020 [69]
Nicotiana tabacum L. (Tobacco)	Lipofection using Lipofectamine 3000 and RNAiMAX in BY2 cell protoplast	CRISPR/Cas9 RNP pporRFP	• First lipofection-mediated RNP transfection approach in plant cells • Lipofectamine 3000 showed 66% efficiency and RNAiMAX- 48% efficiency in protein delivery • It confers higher % of targeted mutagenesis frequency than PEG- and biolistic-mediated protein delivery	Liu et al., 2020 [70]
Petunia cv. (Mirage Rose)	PEG transformation of protoplast	CRISPR/Cas9 RNP PhACO	• Targeted gene encoding the enzyme for ethylene biosynthesis • High transcript levels linked to high ethylene levels detected in later stage of flowering • Less ethylene production and increased flower longevity in T0 & T1 mutant lines.	Xu et. al, 2020 [71]
Brassica (B. oleracea, B. napus, and B. rapa) (Cabbage, rapeseed and Chinese Cabbage)	PEG transformation of protoplast	CRISPR/Cas9 RNP FRI PDS	• 4 sgRNAs targeting FRI and PDS genes in all 3 species • No mutations in rapeseed protoplasts • Cabbage: 0.09 to 2.25% mutation frequencies • Chinese cabbage: 1.15 to 24.51% mutation frequencies • Local mutations (indels) with only 7.5 mg each of Cas9 and sgRNA	Murovec et al., 2018 [72]
Solanum tuberosum (Potato)	PEG transformation of protoplast	CRISPR/Cas9 RNP GBSS	• GBSS encodes granule bound starch synthase enzyme • RNP (crRNP, synthetically produced) showed 9% mutation frequency • IVT-RNP (*in vitro* transcriptionally produced) showed 25% mutation frequency • 80% of mutated shoots had unintended deletions • Mutations in all four alleles of regenerated shoots confirmed knockout of GBSS function	Anderson et al, 2018 [73]
Triticum aestivum L. (Bread Wheat)	Biolistic bombardment of immature embryo cells with gold particles= 0.6 µm PEG transformation of protoplast	CRISPR/Cas9 RNP TaGW2 and TaGASR7	• No off targeted mutations • Protocol takes 7-9 weeks producing independent mutants from 100 RNP transfected immature embryos with very high 4.4% mutant production efficiency	Liang et al., 2017 [74]
Nicotiana attenuate (tobacco), *Glycine max var. (soyabean)*	PEG transformation of protoplast	CRISPR/Cpf1 (Lb &As) RNP AOC FAD2-1A, FAD2-1B	• Cpf1 induces multiple nucleotide deletions at two targeted loci simultaneously in soyabean • Indels observed at FAD2-1A with 11.7 % frequency and at FAD2-1B with 9.1% frequency with LbCpf1 which more than AsCpf1 • Cpf1-RNP induced indels in tobacco target site[75]	Kim et al., 2017 [75]
Chardonnay (Grapevine), *Golden delicious* (Apple)	PEG transformation of protoplast	CRISPR/Cas9 RNP MLO-7 (Grapvine), DIPM-1, DIPM- 2, DIPM-4 (Apple)	• MLO-7 is a susceptible gene targeted to increase powdery mildew resistance • DIPM-1, DIPM- 2, and DIPM-4 genes targeted to increase fire blight disease resistance in apple • Highest mutation frequency: 3:1 ratio, MLO-7; the 3:1 ratio, DIPM 1; 1:1 and 3:1 ratio, DIPM 2; 1:1 ratio, DIPM 4 for Cas9: sgRNA ratio	Malnoy et al. 2016 [29]
Zea mays (Maize)	Biolistic bombardment of immature embryo cells	CRISPR/Cas9 RNP LIG ALS2 MS26,MS45	• DNA-free and selectable marker free mutagenesis at high frequencies (2.4 – 9.7 %) • No off-target mutation • Obtained 90% as one mutated allele with 10%as biallelic mutations	Svitashev et al. 2016 [76]
Petunia x hybrida cv. (Madness)	PEG transformation of protoplast	CRISPR/Cas9 RNP Petunia nitrate reductase (NR)	• Targeted mutation can occur in 4 out of 6 specific NR sites • T7E1 assay showed site-specific mutations at 2.4 to 21% frequencies at four NR1, 2, 4 and 6 sites • Deep DNA sequencing showed 63:37 average ratio of deletion to insertion collectively by the 4 NR sites	Subbhuraj et al, 2016 [77]
Nicotiana benthamiana	PEG transformation of protoplast	TALENs mRNA ALS2	• 4 *A.thaliana* UTRs fused to coding sequence of TALEN pair • Targeted mutagenesis 12 -fold lower than DNA controls. • UTRs increased frequency of mutagenesis	Stoddard et al, 2016 [78]

Triticum aestivum L. (Bread Wheat) *Triticum turgidum L. var. durum* (Durum Wheat)	Biolistic bombardment of immature embryos PEG transformation of protoplast	CRISPR/Cas9 DNA and RNA TaGASR7-A1 TaGASR7-B1 TaGASR7-D1	• Transient expression of CRISPR/Cas9 DNA or guide RNA • Mutagenesis through CRISPR DNA is higher compared to RNA • T0 generation with no detectable transgene was obtained through CRISPR RNA with no off-target effects	Zhang et al 2016 [79]
Arabidopsis thaliana, *Nicotiana attenuate* (tobacco), *Oryza sativa* (rice), *Lactuca sativa* (lettuce)	PEG transformation of protoplast	CRISPR/Cas9 RNP BRI-1, PHYB (Arabidopsis) AOC (tobacco) P450, DWD1 (rice) BIN2 (lettuce)	• RNPs induced targeted gene modifications in 6 genes in 4 plants • Small insertions/deletions at targeted sites with germline-transmissibility • 46% frequency in achieving targeted mutagenesis in the whole regenerated plants	Woo et al., 2015 [30]