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Nanotechnology to advance CRISPR–Cas genetic engineering of plants

Gozde S. Demirer[1,9,✉], **Tallyta N. Silva**[2,3], **Christopher T. Jackson**[1], **Jason B. Thomas**[4], **David W. Ehrhardt**[4], **Seung Y. Rhee**[4,✉], **Jenny C. Mortimer**[2,3,5,✉], **Markita P. Landry**[1,6,7,8,✉]

[1]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, CA, USA.

[2]Joint BioEnergy Institute, Feedstocks Division, Emeryville, CA, USA.

[3]Environmental Genomics and Systems Biology Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA.

[4]Department of Plant Biology, Carnegie Institution for Science, Stanford, CA, USA.

[5]School of Agriculture, Food and Wine, Waite Research Institute, University of Adelaide, Adelaide, South Australia, Australia.

[6]Innovative Genomics Institute (IGI), Berkeley, CA, USA.

[7]California Institute for Quantitative Biosciences, QB3, University of California, Berkeley, CA, USA.

[8]Chan Zuckerberg Biohub, San Francisco, CA, USA.

[9]Present address: Department of Plant Biology and Genome Center, University of California, Davis, CA, USA.

Abstract

CRISPR–Cas genetic engineering of plants holds tremendous potential for providing food security, battling biotic and abiotic crop stresses caused by climate change, and for environmental remediation and sustainability. Since the discovery of CRISPR–Cas technology, its usefulness has been demonstrated widely, including for genome editing in plants. Despite the revolutionary nature of genome-editing tools and the notable progress that these tools have enabled in plant genetic engineering, there remain many challenges for CRISPR applications in plant biotechnology. Nanomaterials could address some of the most critical challenges of CRISPR genome editing in plants through improvements in cargo delivery, species independence, germline transformation and gene editing efficiency. This Perspective identifies major barriers preventing CRISPR-mediated plant genetic engineering from reaching its full potential, and discusses ways that nanoparticle technologies can lower or eliminate these barriers. We also describe advances

✉**Correspondence** should be addressed to G.S.D., S.Y.R., J.C.M. or M.P.L., gsultandemirer@gmail.com; srhee@carnegiescience.edu; jcmortimer@lbl.gov; landry@berkeley.edu.

Competing interests

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that are needed in nanotechnology to facilitate and accelerate plant genome editing. Timely advancement of the application of CRISPR technologies in plant engineering is crucial for our ability to feed and sustain the growing human population under a changing global climate.

Plants are essential for food security and the production of therapeutics, bioenergy and biomaterials. To meet global demand sustainably, improving plants beyond their natural reserves and abilities is a promising strategy. Plant improvements began millennia ago with crop breeding to domesticate wild plants and increase their yield and nutrient density. The Green Revolution of the 1950s generated high-yielding varieties of semi-dwarf wheat and rice, and exemplified how plant breeding can boost yields to support a rapidly growing population[1]. Nevertheless, breeding requires extensive time and labour, is genetically non-targeted, can reduce plant fitness due to the potential co-introduction of undesirable traits and cannot introduce traits that do not exist in the crossed species.

Compared with conventional plant-breeding approaches, genetic-engineering methods, which rely on tools such as particle bombardment and *Agrobacterium tumefaciens* transformation, have broadened the range of traits that can be introduced and improved in plants[2] (see Box 1 for definitions of the main terms used in this Perspective). However, these genetic-engineering tools insert genes into a random location in the plant genome and may cause undesirable outcomes. Recently developed nuclease-based genome-editing methods, such as TALEN (transcription activator-like effector nucleases) and CRISPR (clustered regulatory interspaced short palindromic repeats)–Cas, are precise, rapid, genetically targeted and can introduce novel traits into specific locations in the genome[3].

CRISPR–Cas genome-editing technology was awarded the Nobel Prize in Chemistry in 2020. CRISPR–Cas genome editing has and continues to be extensively studied in animal systems, including the first clinical trials with patient T cells[4]. CRISPR–Cas cargoes are commonly delivered to animal cells using ex vivo methods such as electroporation or in vivo vehicles such as viruses. In recent years, nanoparticles have emerged as an alternative vehicle suitable for delivering CRISPR editing components to mammalian cells. For example, cationic lipid-based nanoparticles can be used to encapsulate CRISPR components for cell delivery, albeit with some concerns regarding toxicity and non-specific cell uptake[5]. Additional advances have also demonstrated the use of gold nanoparticles that can be assembled with CRISPR–Cas complexes for delivery in mice[6].

In the field of plant biology and agriculture, CRISPR–Cas technology holds much potential for transforming plant functional genomics research, improving crop resilience to abiotic and biotic stresses, and rapidly introducing new desirable traits into crops. However, the widespread application of CRISPR technologies in plants faces several barriers. Obstacles include CRISPR cargo delivery challenges, limitations of plant tissue and cell culture, and lack of methods that work across plant species. In addition, our limited understanding of plant genetic and metabolic networks hinders the development of plant varieties with desired traits. Introducing CRISPR-engineered plants to the market may face further obstacles in many countries because of regulations and societal acceptance.

Over the past decade, the intersection of plant biotechnology and nanomaterials has been fruitful. Early use of nanomaterials in plants has focused on the synthesis of plant-based metallic nanoparticles, the delivery of agrochemicals, and nanoparticle uptake and toxicity studies. More recently, nanomaterials have been used to deliver genes and proteins into plant cells for genetic-engineering applications[7–10]. Nanomaterials can reach previously inaccessible plant tissues, cellular and subcellular locations. In addition, nanomaterials can enable the targeted delivery and release of cargoes, and can protect cargoes from degradation. Finally, nanoparticles can often perform these tasks in a species-independent manner. While there is much potential for nanomaterials to address many central bottlenecks of CRISPR-based genome editing, several nanotechnology-specific advances are needed to realize the potential of these tools in plant biology. In this Perspective, we discuss how current nanomaterial systems have addressed some challenges of CRISPR in plants, and what nanotechnology-specific advances are needed to circumvent the remaining barriers to plant CRISPR genome editing.

CRISPR genome editing in plants

In this section, we will first discuss some of the major accomplishments of CRISPR genome editing in plants, followed by the current limitations of plant CRISPR genetic engineering.

Accomplishments in plant genome editing with CRISPR plasmid and protein delivery.

Genome editing in plants using the CRISPR–Cas system (Fig. 1) has widely been reported[11]. Plasmids encoding the CRISPR–Cas reagents have been delivered into plant cells by various methods for either stable or transient transformation. CRISPR–Cas expression using transient methods is preferable, as transient methods do not rely on chromosomal integration of the CRISPR–Cas reagents but rather in their temporary expression using plant machinery. Transient expression of the CRISPR–Cas complex has been shown to result in fewer off-target mutations in bread wheat[12], no heritable DNA integration and hence a reduction of the regulatory burden. However, transient expression methods through plasmid delivery have had limited applicability so far, as most plant species are not susceptible to these DNA-delivery methods.

DNA-free editing, in which the CRISPR–Cas complex is introduced directly into plant cells, is an alternative genome-editing strategy (Fig. 1b). Preassembled ribonucleoproteins (RNPs) have been successfully delivered via protoplast transfection[13] or particle bombardment[12]. However, these editing efficiencies are still low (≤10%) outside of a few well-studied species[14]. Recently, an RNA virus was used to deliver CRISPR–Cas reagents, leading to heritable mutations of multiple genes simultaneously and editing efficiencies of 90–100% in infected somatic tissue[15]. A drawback is the limited host range associated with any virus-based tool. Comprehensive reviews of CRISPR–Cas applications in agriculture and plant biotechnology have been summarized elsewhere[16].

Current limitations of CRISPR genetic engineering in plants.

For full realization of the promise of CRISPR genome editing, we need simple, accurate and efficient methods to deliver CRISPR reagents to diverse plant species. A major barrier

to all forms of plant genetic engineering is the need to induce somatic embryogenesis in species where germline transformation is not an option. Somatic embryogenesis is a time-consuming and technically challenging process, particularly in monocot species. Furthermore, even within a species, different genotypes respond differently to tissue culture. To expedite transformation, morphogenic regulators have been introduced into plants[17] to induce embryo formation from somatic cells[18] and, recently, to induce de novo meristem formation to circumvent tissue culture[19]. The latter has only been demonstrated in eudicots, but holds great promise if applied to monocots, particularly to species and genotypes recalcitrant to transformation.

A second critical barrier to plant CRISPR genetic engineering is a physical barrier to the delivery of CRISPR reagents—the plant cell wall, a rigid and thick extracellular matrix composed primarily of polysaccharides and absent in most other biological systems in which CRISPR has seen many successes. The use of protoplasts, plant cells in which the cell wall is removed by enzymatic digestion, can overcome this barrier, facilitating the delivery of CRISPR reagents. However, regeneration of full plants from protoplasts is technically challenging and has not been demonstrated in most species[20]. For intact plant cells, particle bombardment allows reagents to breach the cell wall, although it increases the chance of irreversible cell damage and suffers from a low editing efficiency.

Another challenge that prevents successful gene insertion through homology-directed repair (HDR) is the need for simultaneous delivery of donor templates as well as the CRISPR–Cas complex. Studies showing successful HDR in plants are very limited. A low HDR editing efficiency in plants is due in part to difficulty in delivering sufficient concentrations of the donor template into the nucleus at the cut site and the short stability of the donor template inside the plant cell[21]. Further advancements require the ability to deliver preassembled RNPs and donor templates in a manner that has not yet been reported in plants. CRISPR base editors are an alternative approach, which avoids the need for donor templates. Base editors consist of a catalytically disabled Cas endonuclease with a cytosine or adenine deaminase domain, allowing more precise edits[22]. However, these base editors only allow the conversion of cytosine-to-thymine or adenine-to-guanine bases, limiting the method to specific target mutations. Recently, a promising prime editing technology that introduces all 12 base-to-base conversions has been applied to rice and wheat[23], overcoming the limitations of CRISPR base editors. Prime editing is composed of an engineered prime editing guide RNA (pegRNA) and a prime editor. The latter has a Cas9 nickase fused to a reverse transcriptase enzyme that performs the editing following pairing with the pegRNA. Further studies involving a broader range of species and target genes are still required to unravel the technique’s full potential.

The requirement of Cas9 for a G-nucleotide-rich protospacer adjacent motif (PAM) site close to the cut site is another factor limiting high-efficiency genome editing in plants. For example, there may be a lack of G-rich regions close to the target site of the gene of interest, such as untranslated and promoter regions of plant genomic DNA that are generally AT-rich. An alternative is the use of different nucleases, such as Cas12a (formerly Cpf1), which recognizes T-rich PAMs and requires a shorter single guide RNA (sgRNA)[24]. Cas12a is a promising strategy for nanotechnology-mediated genome editing as it is approximately 40

kDa smaller than Cas9, resulting in a smaller cargo that could be beneficial for nanoparticle-based delivery.

Successful CRISPR–Cas genome editing further relies on selection of the correct sgRNA to optimize Cas specificity to the plant genomic target and cleavage efficiency, and to avoid off-target editing[25,26]. Existing bioinformatics tools have poor correlation with in planta sgRNA editing efficiency[27]. Methods for the transient screening of sgRNA efficiency have been demonstrated in tobacco and wheat[28,29] but need development for other species.

Even with the capability to deliver CRISPR reagents to crops and make specific heritable mutations with no off-target effects, a remaining problem is that the functions of most plant genes are unknown[30]. A first step for both understanding gene function and performing targeted mutagenesis is having a well-annotated genome. Most major crop species have had their genomes sequenced, but these genomes vary in quality and many orphan crop genomes have yet to be sequenced[31]. In addition to genomic knowledge, information about gene function at the transcriptomic, proteomic and metabolomic levels is just as important for crop engineering[32]. As a further complication, there are complex biological interactions between these -omics levels. This may result in another type of off-target effect, wherein mutating the target gene indirectly affects the regulatory and metabolic connections to other genes[33]. With increases in data availability, quality assessment and access, plus high-throughput experimental validation in plants, we will more easily make predictions on gene function that can be used for crop CRISPR engineering[30].

Nanotechnology to address CRISPR challenges in plants

Initial studies of plant biomolecule delivery and genome editing with nanomaterials used larger (over 100 nm) particles necessitating their biolistic delivery to plants[7,34] (Fig. 2). Subsequent developments have demonstrated that certain smaller nanoparticles can be delivered into plant cells without biolistic delivery, and that nanoparticles can deliver DNA and RNA cargoes to many plant species and target tissues[8–10,35–38]. See Table 1 for a roadmap summary for how nanotechnology could address key outstanding CRISPR challenges in plants.

Delivery.

Delivery is a critical challenge in plants, as common abiotic transfection techniques (heat shock, electroporation, lipid- and polymer-mediated delivery) that are used for microbes and animals are typically ineffective in intact plants. Nanotechnology’s biggest contribution to plant genetic engineering is in enabling efficient delivery into diverse plant species and tissues (Fig. 1a). In addition to targeted delivery, controlled cargo release and cargo protection from degradation, certain nanoparticles allow imaging of cargo delivery and release processes in planta given their intrinsic or engineered fluorescent properties[39].

While delivering DNA and proteins into plant cells using nanomaterials has been successful, nanomaterial-mediated CRISPR–Cas genome editing in plants has not yet been reported due to the unique physicochemical properties of CRISPR reagents and the high delivery efficiencies needed to enable CRISPR genome editing in plants. DNA plasmids encoding for

the CRISPR–Cas complex are substantially larger than reporter plasmids that are commonly used in nanoparticle proof-of-principle studies. Owing to the difference in plasmid size and net charge, nanomaterial researchers need to identify optimal chemistries for loading CRISPR DNA plasmids onto nanoparticles. In the case of Cas9 protein and RNP delivery, challenges stem from the large size of Cas9, its high local charge density and its low stability in ionic solutions. One possible solution is to covalently attach Cas9 RNPs onto the nanoparticle surface via a conjugation chemistry that can be cleaved in the vicinity of the target. Several promising approaches involve enzymatically cleavable linkers and the use of tissue-penetrable near-infrared light to trigger release of the RNPs from the nanoparticle surface. Additionally, nanomaterial-mediated delivery is still less efficient than biotic delivery approaches; thus, increasing the delivery efficiency of CRISPR reagents is needed to enable genome editing for practical applications.

Tissue culture.

Nanomaterial-based technologies that are developed for plant germline transformation could circumvent the need to regenerate plants from tissue culture, a major plant biotechnology limitation, and produce edited offspring directly. Towards circumventing regeneration, transformation of the pollen (the male gametophyte) is a promising method as edited plants can be generated directly through pollination with edited pollen. So far, there has been limited success in transforming pollen through either physical or biological methods due to the tough outer layer of pollen combined with its fragile viability following transformation. The unique mechanical properties of nanoparticles, such as the high tensile strength of carbon nanotubes, could be leveraged for CRISPR editing of pollen, though optimization is necessary to maximize the intake of nanoparticles by pollen without sacrificing its viability. Researchers can attempt the delivery of CRISPR DNA or RNPs through ~5-μm-diameter pollen surface apertures, where the outer pollen wall (exine) is thinner. A detailed investigation of pollen surface properties is needed to engineer nanoparticles for penetration of the pollen coat.

An alternative approach to pollen transformation is to edit the germline cells in intact flowers, where both the male and female gametophytes (ovules) reside. Administration of CRISPR reagents to flowers via nanoparticles has the potential to edit cells in all of the following locations: pollen, ovules and the embryo. Another promising tissue target for nanomaterials is the shoot apical meristem, from which whole edited plants can be generated with a reduced tissue culture and regeneration burden. However, most of these plant organs are buried deep inside plants; therefore, how to penetrate through multiple plant tissue layers remains to be solved.

Species dependence.

One of the biggest bottlenecks for the widespread application of plant CRISPR genetic engineering is the inability of transformation tools to be effective for a wide range of plant species. There are several reasons for this plant species dependence: (1) the inability to deliver cargoes to all species, (2) challenges of in vivo sgRNA validation and (3) the PAM site requirement of nucleases with unsuitable genomic composition in certain species. Nanoparticles have facilitated many aspects of delivery into a diverse range of plant species,

including *Arabidopsis*, tobacco, maize, wheat, arugula, spinach, cotton and watercress[7–9,38]. These promising studies suggest that the entry of nanoparticles into plant cells is likely a mechanical phenomenon, and thus is not heavily affected by plant genetics or signalling pathways.

In addition to delivery benefits, nanotechnology might offer a way to identify high-efficiency sgRNAs by rapidly screening sgRNAs for efficacy in planta. Since nanomaterial-mediated RNA delivery platforms are based on chemisorption of the polynucleotide cargo to the nanoparticle, and are indifferent to the polynucleotide cargo type[10,35,36], these nanoparticles could deliver sgRNA into Cas transgenic plants for high-throughput and rapid testing of sgRNA efficacy in vivo. Nanomaterial surface and conjugation chemistries will need to be optimized to enable tight binding of sgRNA for delivery into plant cells without irreversibly disturbing the three-dimensional structure of the sgRNA molecules, which is necessary for their function. Similar to in vivo sgRNA efficacy testing with nanomaterials, it is also possible to devise high-throughput tools to survey alternative nucleases with differing PAM sites to address specific PAM site limitations of current nucleases, or possibly to deliver mRNA molecules for DNA-free expression of CRISPR tools.

Low HDR efficiency.

Nanotechnology could increase the HDR efficiency in plants through multiple approaches. Nanomaterials that enable efficient delivery of double- or single-stranded donor (template) DNA to the plant cell nucleus is one of these promising ways. Also, approaches that bring the donor DNA and Cas RNP into the proximity of the double-stranded break site in the plant genome can increase the HDR efficiency. Borrowing from a recent animal study[40], negatively charged nanoparticles can be used to increase the HDR efficiency in plants. Here, nanoparticles stabilize the Cas–sgRNA complex and carry a modified donor DNA interacting with Cas RNPs to shuttle the template to the nucleus. This approach has been shown to enhance the HDR efficiency approximately twofold to fourfold in human T cells, and could provide substantial enhancement in plants. The nanoparticle size should be designed appropriately to allow the stable carrying of RNP and donor DNA, while still being suitable for plant cell entry, which is a challenging balance.

To improve HDR editing efficiencies in plants, nanomaterials can also be exploited to achieve time-staggered delivery (and expression) of Cas, sgRNA and donor DNA. Many nanomaterials have already been used in animal systems for the sequential delivery of genetic material and drugs[41]. Translating these technologies into plants can circumvent some of the limitations of HDR. As RNA is not stable long-term inside plant cells, sgRNA could be delivered when Cas reaches its maximum cellular levels of expression. To achieve this, nanoparticles can aid either through the sequential delivery or controlled release of cargoes and/or by delaying the degradation of donor DNA and sgRNAs in plant cells. Promisingly, nanoparticle-mediated delivery platforms demonstrated for siRNA delivery may be indifferent to the polynucleotide type[10,35,36] and could thus be re-purposed for the direct delivery of donor DNA.

Generalizability of nanotechnology platforms for use in diverse plant systems.

Differences in nanoparticle uptake and transport across plant tissues might affect the generalizability of nanotechnologies for plant genetic engineering with CRISPR. Leaves and roots are the most common nanomaterial uptake pathways in plants. In leaves, a waxy, hydrophobic cuticle with small pores (<5 nm) reduces the time nanomaterials spend on the leaf surface and inhibits their entry[39]. Instead, nanoparticles are more likely to enter leaves through the stomata, larger pores measuring tens of microns that regulate water and gas exchange. While stomata can comprise up to 5% of a leaf's surface, the location and number of stomata vary between plant species and their size can fluctuate depending on surrounding environmental factors[42]. The variability in leaf anatomy and morphology is an important consideration for nanotechnology generalizability between different plant tissues and species.

Regarding nanoparticle uptake by roots, there are conflicting reports of nanoparticle entry and translocation[43–45]. Most recently, Milewska-Hendel and colleagues demonstrated that gold nanoparticles did not translocate into or within roots by either apoplastic or symplastic pathways in barley[46]. These shortcomings are potentially due to additional barriers within root tissue, such as the root cortex and the Casparian strips of root endodermal cells. While research into the mechanisms for nanoparticle movement within tissues suggests that properties such as nanoparticle size, charge, stiffness and aspect ratio may play an important role[36,46], these properties may also influence the ability of nanomaterials to deliver large proteins, such as Cas9, through the cell wall's measured size-exclusion limit of ~5–20 nm (ref.[47]). Bypassing this barrier and double-membranous organelles such as mitochondria, nuclei and chloroplasts is critical for intracellular localization within organelles. Further work is also needed to explore other variable factors that could affect nanoparticle transport between plant cells, such as pH and osmotic conditions, which will likely influence both the transport and the stability of nanomaterial–cargo complexes within a plant cell.

Regulatory considerations

In this section, we will discuss the regulation of genetically engineered crops and societal challenges that they face. We will also analyze the safety and regulatory aspects of nanotechnologies.

Regulation of genetically engineered crops and societal challenges.

The regulation of genome-edited crops is a complicated issue. While regulations are important for public safety, genetically engineered crops may be over-regulated given that they pose no higher risks to human health and the environment than conventionally bred crops[48]. At present, safety testing for a single genetically engineered crop in the United States lasts over a decade and costs several million US dollars before being introduced to the market, stifling innovation[49]. Furthermore, regulatory practices vary widely worldwide. The new 'SECURE' rule (that is, Sustainable, Ecological, Consistent, Uniform, Responsible, Efficient) introduced in 2020 by the US Department of Agriculture updates and streamlines regulatory processes to sustainably speed innovation in genetically engineered crop development[50]. This guidance places genetically engineered crops under

regulatory oversight only if they contain foreign DNA from agricultural pathogens. In the case of CRISPR–Cas mutagenized plants, while vector DNA from pathogenic *Agrobacterium* is typically used to introduce the Cas protein, after the target gene has been mutated, the bacterial vector can be removed from the plant host genome with crossing. Thus, these CRISPR–Cas mutagenized crops are regulated on a 'product-basis' and do not fall under oversight by US regulatory agencies[51] but remain dependent on the lengthy process of plant breeding. A few CRISPR-edited crops have recently been introduced into this regulatory pipeline including corn, soybeans, mushrooms and camelina[51,52]. Canada, Argentina, Brazil, Japan and Australia have similar regulatory frameworks[53]. In the European Union, however, CRISPR-edited crops are regulated on a 'process-basis' and thus as conventional genetically engineered crops, based on the notion that the procedure to generate CRISPR crops does not occur in nature[54]. Notably, this rationale is inconsistent with other rules. For example, gamma irradiation is commonly used to generate new germplasms that are not regulated, and gamma irradiation does not occur naturally[55].

Safety and regulatory aspects of nanotechnologies.

As nanotechnologies for plant systems evolve, there must be parallel efforts to better understand their safety implications and create effective regulatory policies. Early studies of nanotechnologies such as carbon nanotubes emphasized their non-biodegradable nature and likened their appearance to needle-like, carcinogenic asbestos fibres[56]. Other studies have instead correlated the toxic effects of nanoparticles with the presence of heavy-metal impurities introduced during their synthesis[57]. While many of these initial concerns have been ameliorated by subsequent research, toxicity and other safety concerns are still being addressed within the nanomaterial community.

While regulation broadly defines risk based solely on nanomaterial size, systematic studies of nanomaterial toxicology have shown that not all nanomaterials are equally toxic, with disparities being based largely on nanomaterial physical and chemical properties instead of size[58]. For example, the European Commission defines nanomaterials by a size range of 1–100 nm, without explicit consideration of size distribution, nanoparticle surface chemistry, synthetic route and purity or other material properties. Nanomaterials represent a broad class of substances of different sizes, shapes and compositions, determined not by a single value but by a distribution of values. This diversity of physicochemical properties makes classification of nanomaterials difficult. The lack of consensus about how to define and categorize nanomaterials has created a fragmented regulatory market that places a burden on researchers attempting to move research from the laboratory to the field. Many of the nanotechnologies commonly used for genetic engineering are regulated in the United States under the Toxic Substances Control Act, which places the burden of proof on producers and importers of chemicals to demonstrate safety[59]. However, to our knowledge, there has been no government oversight of nanomaterial use in laboratories, greenhouses or in the field to date.

As such, for nanomaterial applications in CRISPR genetic engineering, we need to better understand the lifecycle of nanomaterials after cargo delivery to ensure that edited plants, their litter, progeny and consumers are free of nanomaterials. This path will allow for the

development of new nanotechnologies with minimal safety concerns and offers exciting opportunities for the remarkable reduction of regulatory barriers.

Conclusion

CRISPR–Cas plant genome editing has shown success in several plant species following traditional transformation and regeneration procedures. However, there are still many challenges regarding the range of plant species that can be genetically engineered through this approach, the time and labour input required for plant regeneration, and the types of CRISPR edits that can be routinely and efficiently achieved in plants. We have discussed how nanomaterials could make an impact on addressing each of these challenges. Nonetheless, many outstanding questions surrounding the use of nanomaterials for plant genome editing persist (Table 2). First, an upper limit for the nanoparticle-loaded cargo size and amount has not yet been established for CRISPR DNA and protein cargoes, and this is likely to be dependent on the nanoparticle type and surface chemistry. Second, despite the successful delivery of certain nanoparticles into the plant chloroplasts, it remains unknown whether or not these nanoparticles can carry CRISPR reagents to plant plastids and mitochondria for modification of their genomes. Third, more studies are needed to establish the compatibility of nanoparticles with plant tissue culture and regeneration protocols, in the cases where germline transformation is not plausible. Last, would the regulation of plants engineered via nanoparticles be different from traditionally engineered plants? To answer this question, the persistence of nanoparticles in the offspring of edited plants should be determined.

As seen in Table 2, there are various outstanding questions, which call for attention from a diverse set of researchers, industry and policymakers for progress in nanomaterial-mediated plant genetic engineering. Further development of nanoparticle chemistries and a better understanding of plant–nanomaterial interactions, delivery routes, and health and environmental risks are all key steps in the path towards widespread applicability. To facilitate this transition, the plant nanotechnology community could draw on lessons from the field of nanomedicine, which has overcome much to translate benchtop research into clinical applications. In both medicine and plant agriculture, researchers and regulators must consider complex biological environments, human exposure, limited reproducibility and the challenges of scale-up and cost. Building on these lessons, we encourage the agriculture sector to develop a unified pathway to foster academia–industry collaborations that include stakeholders such as fertilizer and pesticide producers, speciality chemical companies and large- and small-scale farmers.

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Box 1 |

Useful terminology

Agrobacterium tumefaciens.

This is a Gram-negative soil bacterium that can insert a small segment of its DNA, known as transfer DNA (T-DNA), into the plant genome at an undefined location.

Apoplastic pathway.

This pathway is one of the two main pathways for transport in plants. Molecules move through the apoplast, which is the area between the plasma membrane and cell wall/ intercellular spaces.

Casparian strip.

This is a band of cell-wall material deposited in the walls of endodermis cells of root tissue and provides an extracellular diffusion barrier within the plant roots.

DNA-free editing.

This type of editing relies on the introduction of a preassembled RNP complex into the plant cell, which avoids the introduction of any exogenous DNA into the plant.

Donor template.

This is the DNA fragment that is targeted to be inserted by HDR into the plant genome through CRISPR machinery. It can be single- or double-stranded.

Eudicots.

These are species of flowering plants with a pair of cotyledons in the seed embryo. Leaf venation is branched, pollen has three grooves (tricolpate), vascular bundles are arranged in rings and typically there is one main root.

Germline transformation.

This is modification of the genome of the female or male gametes that will pass the modification to the offspring.

Homology-directed repair (HDR).

This is a DNA repair mechanism used by the cell when a DNA sequence with homologous regions is present in the nucleus. It allows introduction of specific point mutations or entire genes.

Meristem.

This is a type of plant tissue that consists of undifferentiated cells capable of cell division. In plants, meristematic cells can develop into all the other tissues and organs.

Monocots.

These are species of flowering plants that have a single cotyledon in the embryo. Leaf venation is usually parallel, pollen has a single groove (monosulcate), vascular bundles are complexly arranged and the root system is typically fibrous.

Morphogenic regulators.

These are genes involved in controlling developmental processes that pattern and shape cells, tissues and organs.

Non-homologous end joining (NHEJ).

This is a DNA repair mechanism in which the break ends are directly ligated without the need for DNA sequence homology. It can generate random insertions or deletions in the target site.

Protospacer adjacent motif (PAM).

This is a DNA motif, a few nucleotides long, upstream of the CRISPR RNA binding region in the genome. It is required for Cas9 recognition of the target sequence.

Particle bombardment.

This is a DNA-delivery method, also called gene gun or biolistic transformation, which can deliver DNA, RNA or proteins into cells using high-velocity gold particles to penetrate the cell wall.

Protoplast.

This is a plant cell that has had its cell wall removed, generally by the application of a cocktail of cell-wall-degrading enzymes, such as pectinases and cellulases.

Ribonucleoprotein (RNP).

This is a complex formed by RNA-binding proteins conjugated with RNA.

Single guide RNA (sgRNA).

This is an engineered single RNA combining CRISPR RNA and trans-activating CRISPR RNA.

Somatic embryogenesis.

This is a tissue culture process in which embryos are formed from dedifferentiated plant somatic cells.

Stable transformation.

This is the genetic modification of plant somatic or germline cells, where a modified progeny is generated.

Symplastic pathway.

This pathway is one of the two main pathways for transport in plants. Molecules move through the cytoplasm of cells via cellular connections called plasmodesmata.

Transgene.

This is a non-native DNA segment containing a gene sequence that has been isolated from one organism and introduced into a different organism.

Transient transformation.

This is the genetic modification of plant somatic cells, either without DNA integration into the genome or without generating modified progeny.

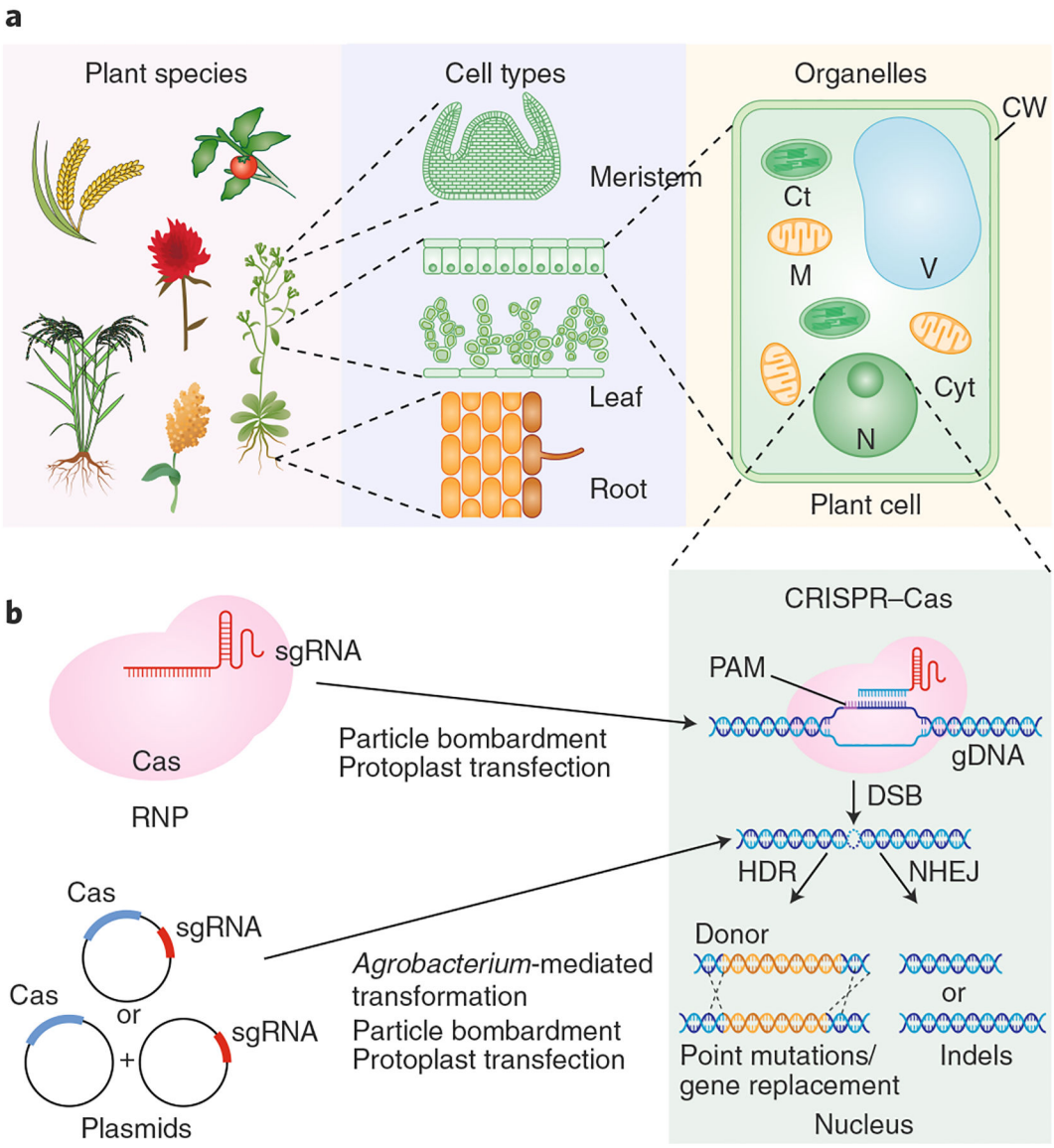

Fig. 1 |. CRISPR–Cas reagent delivery to diverse plant species, cells and organelles.

a, Examples of plant species, cell types and organelles that can be targeted by the CRISPR–Cas system. **b**, The CRISPR–Cas genome-editing system is derived from bacterial adaptive immunity. It consists of the Cas endonuclease, which site-specifically cleaves double-stranded DNA, and an sgRNA that hybridizes to approximately 20 nucleotides of the target sequence via Watson–Crick base pairing. A PAM upstream of the sgRNA binding site in the genome is also required. In the nucleus, the Cas protein and the sgRNA form an RNP complex and Cas undergoes conformational changes that enable DNA binding and cleavage. Once a double-stranded break (DSB) is generated, one of two plant DNA repair mechanisms is triggered. HDR, in which a DNA donor template with homology to the target sequence is provided, leads to point mutations or gene replacement. NHEJ, which is error-prone and generates small insertions or deletions (indels), is much more commonly demonstrated than HDR. RNPs can be delivered into the plant cell by particle bombardment

or protoplast transfection. Alternatively, plasmids containing genes that encode Cas and the sgRNA are delivered into the cell through *Agrobacterium*-mediated transformation, particle bombardment or protoplast transfection. Ct, chloroplast; Cyt, cytoplasm; CW, cell wall; gDNA, genomic DNA; M, mitochondria; N, nucleus; V, vacuole.

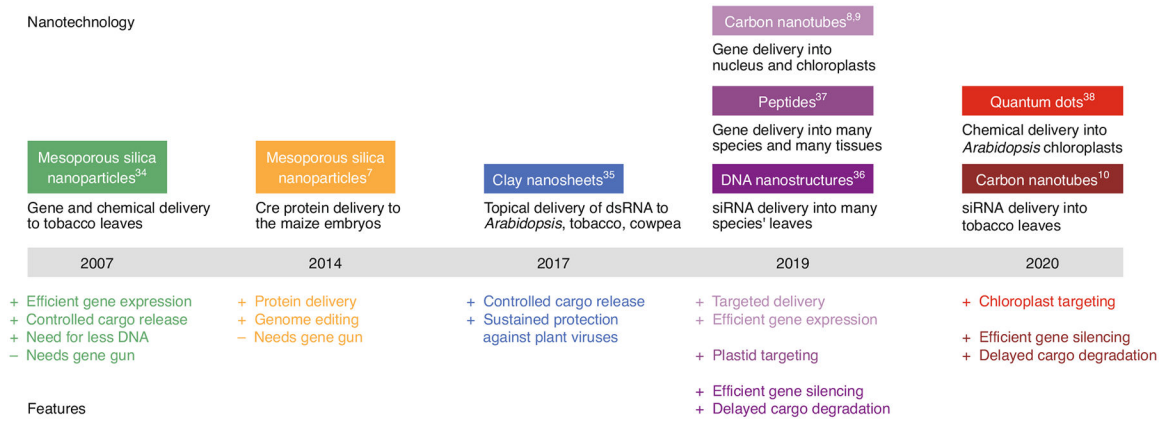

Fig. 2 |. Schematic showing nanomaterials developed for plant biotechnology, delivery and genetic engineering.

Nanotechnology (upper) used in plants and its features (lower). The first use of nanomaterials for plant delivery was by Torney and colleagues, where mesoporous silica nanoparticles biolistically delivered a gene to tobacco leaves. The same group used mesoporous silica nanoparticles for plant genome editing in 2014, by delivering Cre protein into maize embryos. In 2017, studies with RNA demonstrated the ability of nanomaterials to protect RNA cargoes from degradation. Parallel to RNA delivery, DNA delivery without biolistics was shown using carbon nanotubes and peptides. These studies showed that nanoparticles can be used to express genes in different subcellular locations, such as the nucleus and chloroplast, and that the nanotechnologies are compatible with a diverse set of plant species.

Table 1 |

Roadmap for nanotechnology to address CRISPR challenges in plants

Challenges	Insights on nanoparticle solutions
Delivery	
Large size, high local charge density and low stability of Cas9 protein.	Covalent attachment Cas9 RNPs onto nanoparticle surfaces via cleavable chemical linkers to release the RNP in the target's vicinity: enzymatically cleavable linkers and the use of tissue-penetrable near-infrared light to trigger release of RNPs from the nanoparticle surface.
Donor DNA delivery.	Nanoparticle delivery is indifferent to the polynucleotide type and could be used for direct delivery of donor DNA into plant cells.
Tissue culture and regeneration	
Inability to transform plant germline cells.	Use of high tensile strength nanomaterials to transform pollen through large pollen surface apertures.
	Combined use of nanomaterials and other physical approaches such as microinjection for the transformation of flowers and shoot apical meristem.
Species dependence	
Inability to deliver cargoes to all species.	Entry of nanoparticles into plant cells is likely a mechanical phenomenon and may not be affected by plant genetics or signalling pathways.
Challenges of in planta sgRNA validation.	Nanoparticles could deliver sgRNA into Cas transgenic plants for high-throughput and rapid testing of sgRNA efficacy in planta.
Unsuitable genomic composition in certain species for the PAM site.	Devise high-throughput nanoparticle tools to survey alternative nucleases with differing PAM sites to address PAM site limitations of current nucleases.
Low HDR efficiency	
Limited simultaneous reach of Cas and sgRNA to the plant nucleus.	Use of negatively charged nanoparticles to stabilize the Cas-sgRNA complex and carry a modified donor DNA interacting with Cas RNPs to shuttle the template to the nucleus.
Different timescales of Cas protein and sgRNA synthesis and stability in cells.	Time-staggered delivery of Cas protein, sgRNA and donor DNA with nanoparticles: sgRNA to be delivered when Cas reaches its maximum cellular expression levels either through sequential delivery, controlled cargo release or delayed sgRNA degradation in cells.

Table 2 |

outstanding questions of nanomaterial-mediated CRISPR editing in plants

Broad Categories	Specific outstanding Questions
Technological unknowns	What is the upper limit of DNA and protein size and amount that can be efficiently delivered by nanomaterials?
	Can nanoparticles target CRISPR reagents to chloroplasts and mitochondria, especially in grass species?
	Can nanomaterials enable pollen transformation through an optimum balance between the pollen's tough exine and its susceptibility to damage following transformation?
	How does the frequency of off-target editing compare between canonical and nanomaterial-mediated CRISPR delivery?
	Are nanomaterials compatible with current plant tissue culture and regeneration protocols?
Safety and regulation	Would the regulation of edited plants using nanoparticles be different from traditionally edited plants?
	Do nanoparticles persist in downstream generations of edited plant offspring?
	What are the environmental lifecycles and safety implications of nanomaterials on microbes and animals?
Broad use	When will these nanotechnologies be widely available and routinely used in plant biotechnology?
	Does the future of nanotechnology in plants include commercially available nanoparticles or will people need to make their own?
	What is the overall cost of obtaining nanoparticle-mediated CRISPR-edited plants? How does this compare with conventional methods?
Novel applications in the future	Will nanomaterial-mediated CRISPR be effective in creating customized genetic mosaics for experimentation?
	Can somatic CRISPR manipulations be done in the field?
	Can gene replacement and allele swapping be done in plants?
	Can we induce transformation on an as-needed basis?