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Review

Nanocarrier-Mediated Delivery of miRNA, RNAi, and CRISPR-Cas for Plant Protection: Current Trends and Future Directions

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ABSTRACT: Current trends in plant genetic transformation technologies, i.e., designing and applying molecules like miRNA, RNAi, and CRISPR-Cas, largely enable researchers to target specific sites in the plant genome to avert the growing biotic and abiotic threats to plants. However, the delivery of these molecules through conventional techniques brings an array of drawbacks such as low efficiency due to the cell wall barrier, tissue damage that leads to browning or necrosis, degradation of these biomolecules by physiological conditions (high temperature, harsh pH, and light), and plant-specific protocols. The advancements in nanotechnology offer an excellent alternative for the safe and highly efficient delivery of biomolecules such as miRNA, CRISPR-Cas, and RNAi without damaging the plant tissues. Nanoparticle (polymeric, metallic, magnetic, silica, carbon, etc.)-based delivery of biomolecules can be efficiently utilized especially for plant protection applications. Herein, we present a comprehensive overview of current trends (with a focus on the previous five years) in nanoparticle-based delivery of miRNA, RNAi, CRISPR-Cas and simillar biomolecules for plant protection applications. In addition, a future perspective focuses on the research gaps and unexplored potentials of nanoparticles for the delivery of biomolecules.

KEYWORDS: *nanoparticles, plant protection, controlled delivery, chitosan, silica, DNA origami*

INTRODUCTION

With the increase in the global population, which will exceed 10 billion people by 2050, guaranteeing food security and energy production, in addition to mitigating the impacts already caused, is a major challenge for world leaders.[1] Innovations in recent decades have made the use of labor, land, capital, and inputs more efficient, leading to a considerable increase in global agricultural production.[2] However, many losses of agricultural productivity still occur due to factors such as climate change, plant diseases, pests, and rapid urbanization, which has been putting unprecedented pressure on soils and leading to losses of organic matter, excessive application of fertilizers, erosion, contamination, salinization, acidification, and loss of genetic diversity.[3]

The advancements in genetic engineering technologies enabled researchers to precisely intervene at the point of interest in plants for the desired molecular manipulation.[4] For this purpose, several molecular tools are being used, including miRNA, RNAi, zinc fingers, TALENs, and CRISPR.[5] Among these, CRISPR is a newly introduced approach with enhanced precision compared to the others.[6] These molecular tools are actively in service for plant protection. Significant progress has been made in the utilization of the mentioned molecular tools for the development of plant variants that are resistant to pests and diseases.[7−9]

The genetic transformation of plants for disease or pest resistance through gene or protein delivery involves two key steps: delivery of cargo molecules and regeneration of the genetically transformed plants. The regeneration of transformed plants is a difficult step (tissue browning, necrosis, and *in vitro* recalcitrance), and protocols are highly dependent on plant species.[10,11] For the delivery of biomolecules, two commonly used conventional approaches are in practice, including *Agrobacterium*-mediated and biolistic particle delivery.[12,13] These biomolecule delivery approaches come with several challenges that hinder the applicability of a certain method. In addition, these approaches are carried out in calli, meristems, or embryos and require a prolonged and challenging regeneration procedure.[4] These approaches are time-consuming, and the efficiency of the product decreased due to the substeps involved (subculturing for regeneration, *in vitro* recalcitrance, tissue browning, etc.). Similarly, in the biolistic particle approach, the mechanical barrier of the cell wall has been easily circumvented due to the involved mechanical force.[13] However, in the process, the efficiency of the system decreases due to the damage to the target tissue. In addition, these systems require species-specific and sometimes tissue-specific protocols. All of these disadvantages make these

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approaches less effective and time-consuming for the production of disease- or pathogen-resistant genotypes.

The evolution of nanotechnology in the past decade has revolutionized genetic engineering technologies for both animals and plants.[14] In the field of plant genetic engineering, many processes have been developed to protect crops against pests and pathogens. A variety of nanoplatforms, commonly studied for applications in the fields of materials, electronic devices, and medicine, have been attracting interest for applications in agriculture.[15] In addition to their application as nanocarriers for pesticide substances and plant nutrition, nanoparticles can act as vectors of genetic material to cells, overcoming the limitations of conventional transformation methods.[16] The nanomaterials can perform the delivery of different biomolecules such as DNA, miRNA, siRNA, ribonucleoproteins, etc. These systems are considered non-cytotoxic vectors and present biocompatibility.[17,18]

Herein, we discuss the major advancements made in the delivery of biomolecules such as miRNA, RNAi, and CRISPR for plant protection applications. In addition, we present a detailed future perspective with a focus on highlighting the major gaps in this area.

CURRENT GENETIC TECHNOLOGIES FOR PLANT PROTECTION

miRNA. Plants are primary producers and are facing challenges posed by different abiotic and biotic stress factors. It is estimated that the approximately 20−40% loss of major crops is mainly due to biotic stress caused by bacteria, fungi, viruses, nematodes, etc. These plant diseases are transmitted from one plant to another either by physical contact or by insects and decrease the crop yield and quality. To overcome these challenging stress conditions, plants have self-defense mechanisms against different kinds of stress factors. For many years, in plant pathology, the general elicitors (oligosaccharide or glycoproteins) could activate the defense response. For instance, pathogen-associated molecular patterns (PAMPs), for instance, lipopolysaccharides, trigger the basal resistance response in plants as an elicitor.[19] Additionally, plants have been employed with various other defense mechanisms such as immune receptor signaling, RNA silencing, ubiquitin, metabolic regulation, and hormone-mediated defense. Gene technology is a primary tool for developing genetically modified plants that are resistant to some insects, pesticides, drought, cold, etc. For example, in Australia, pesticide use has been decreased by 80% upon introduction of genetically modified cotton plants that are resistant to caterpillars of *Helicoverpa armigera*.

In RNA silencing, a non-protein-coding RNA (ncRNA) regulates messenger (mRNA) translation through base-pairing complementary. RNA silencing plays a vital function in plant immunity against causal infectious agents, including viral or bacterial.[20] In eukaryotic organisms, RNA silencing induced by small RNAs (sRNAs) is a fundamental regulator of evolutionarily conserved mechanism and gene expression.[21] In plants, sRNAs have different lengths, i.e., 21−24 nucleotides, and these sRNAs are grouped into two major classes, small interfering RNAs (siRNAs) and microRNAs (miRNAs), which are developed from double-stranded RNA (dsRNA) or single-stranded hairpin RNA precursors.[22,23] miRNAs play a crucial role in plant environmental interactions and in the development of plasticity against biotic or abiotic stresses as well as in parasitic and symbiotic interactions.

miRNA Biogenesis and Mode of Action. miRNAs are classes of small noncoding RNAs that obstruct the expression gene (Figure 1). miRNAs are endogenous ∼20-nucleotide

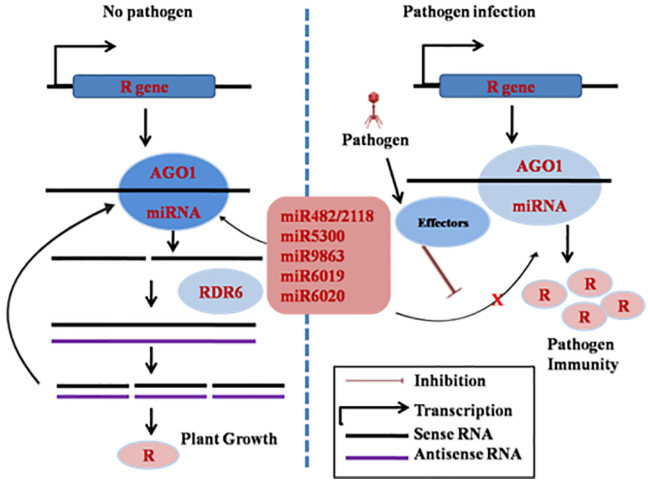

Figure 1. Schematic representation of microRNA-mediated regulation of the R gene in plants and the basic principle of miRNA gene regulation along with AGO1 and RDR6 proteins.[24]

noncoding RNAs synthesized from the long transcripts that can form a hairpin structure and are widely involved in the development of plant growth by modulating the expression of target genes at the transcriptional and post-transcriptional levels. They are formed during various processes, including transcription, methylation, precursor processing, and arrangement of the miRNA-induced silencing complex (miRISC).[24] miRNA expression is stimulated by different transcription factors. For instance, negative-no-TATA-less 2 triggers the transcription of protein miRNA genes and helps in the effective establishment of miRNA biogenesis.[25] Cell division cycle 5 (CDC5) is cell cycle serine/threonine-protein kinase that acts as a reliable transcription factor partner with miRNA genes.[26]

The biogenesis of miRNAs in insects, animals, and plants functions similarly, excluding the cellular compartmentalization and protein levels.[27] In organisms such as plants and animals, miRNAs are transcribed to primary miRNA (pri-miRNA) transcripts, thereby removing the hairpin stem from the miRNA precursor, and then cleave the hairpin loop, to form the duplex passenger stand called miRNA.[28] The mature miRNA loaded on Argonaute (Ago) protein from the RNA-induced silencing complex (RISC) binds to the target mRNA and represses gene expression through inhibition of the translation and decay of mRNA molecules. In the case of plants, the cleavage of primary miRNA(pri-miRNA) transcripts is employed for the nuclear RNAase dicer-like (DCL 1) and its accessory proteins HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE) and,[29] and also in plants, the Drosha gene is absent.[30] Previously, two review, Eamens et al.[31] and O'Brien et al.,[25] explained the biogenesis of miRNAs in *Arabidopsis thaliana*. According to the studies, *DCL1* cut the Pri-miRNA in the nucleus, with the help of HYL1 and dsRBP, resulting in a short pre-miRNA dsRNA molecule. The two 3′ nucleotide overhangs of the released miRNA duplex are methylated by the activity of the sRNA-explicit methyltransferase HUA enhancer1 (HEN1). Then duplex miRNA is transported to the cytoplasm. The cytosolic mature single-stranded miRNA binds to the Argonaute family of proteins (AGO1) to from RISC,

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Table 1. Advanced Gene Technologies Used in Agriculture for the Development of Resistance against Pathogenic Infections

gene technology	plant/crop	counter defense mechanism	refs
microRNA (miRNA)/ artificial miRNA	tomato (*Solamnum lycopersicum*)	regulation of the expression of genes involved in abiotic and biotic stress	47, 48
miRNA	*Achyranthes bidentata*	promotes root growth and development and enhances transport activity in various stresses by integrated miRNA-mRNA	49
miRNA	maize (*Zea mays*)	enhances resistance against *Fusarium* ear rot (FER)	50
miRNA160	potato (*Solanum tuberosum*)	resistance against *Phytophthora infestans* infection	51
miRNA162/ miR162, miR168, miR403	*Nicotiana tabacum* BY2	regulation of viral suppressors (VSRs) in early infection of *Tombusvirus* p19 and *Cucumovirus* 2b	52
MiR166k−166h	rice (*Oryza sative*)	resistance against rice blast diseases caused by the pathogenic fungus *Magnaporthe oryza*	53
miR482/2118 family	tomato (*S. lycopersicum*)	enhancement of plant innate immunity in legumes and Solanaceae by regulation of nucleotide binding site-leucine rice repeat (NBS-LRR) receptors	54, 55
RNA interference	barley, wheat (*Triticum astivum* L.), and *Arabidopsis*	enhancement of the resistance to *Fusarium* head blight (FHB) and *Fusarium* seedling blight (FSB) by host-induced gene silencing of the fungal chitin gene	56
	barley and wheat	reduces powdery mildew fungal disease by downregulation of the TaMlo gene via virus-induced gene silencing (VIGS)	56, 57
	squash, papayas, and potatoes	resistance to tobacco mosaic virus (TMV) diseases by coat protein gene silencing	58
	peppers and tomatoes	resistance to cucumber mosaic virus (CMV)	59
	plums and beans	resistance to plum poxy virus (PPV) and bean golden mosaic virus (BGMV)	60
	tomato	resistance to tomato yellow leafcurl virus (TYLCV)	59, 61
	cassava	cassava brown streak (CBSD) disease by silencing the CP genes of Ipomovirus	62
CRISPR-Cas9	*Arabidopsis* and *Nicotiana benthamiana*	resistance to RNA virus [e.g., cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV)] infections caused by the expression of *Francisella novicida* Cas9 (FnCas9) and single-guide RNA (sgRNA)	63
	N. benthamiana	resistance to begomoviruses and tobacco rattle virus (TRV)	64
	tomato	tomato yellow leafcurl virus (TYLCV)	65
	Arabidopsis and *N. benthamiana*	resistance to geminiviruses, beet severe curly top virus (BSCTV), bean yellow dwarf virus (BeYDV)	66−68
	Theobroma cocao	resistance to *Phytophthora tropicalis*	67
	rice	resistance to rice blast disease caused by disturbing OsERF922 and OsSEC3A genes	69
	citrus	resistance to citrus bacterial canker (CBC) caused by *Xanthomonas citri*	70, 71
	Brassicaceae family, including *A. thaliana* and several *Nicotiana* species	resistance to cauliflower mosaic virus (CaMV)	72

and the resulting RISC complex blocks the expression of the targeted mRNA by cleavage.[32]

Biological Function and Applications of miRNA in Plants. MicroRNAs (miRNAs) play a vital role in plants under abiotic stress (such as salinity, temperature, drought, and reactive oxidative species); for example, upregulation of miR393 has been recorded when *Arabidopsis* seedlings are exposed to different abiotic stress conditions[33] (Table 1). Furthermore, in *Arabidopsis* miR397b, miR389a, miR319c, and miR402 are regulated to varying degrees upon exposure to abiotic stresses. It is reported that miR395 and miR399 are triggered during sulfur and phosphate starvation in *Arabidopsis*.[28]

RNAi. RNA silencing or RNA interference (RNAi) of the most natural significant resistance mechanisms is employed for plant protection against diseases activated through regulation of sequence-specific gene expression.[34] Plants need to persevere through a scope of unfriendly conditions caused by different abiotic and biotic stresses that differently influence the overall advancement of plant growth and yield.[35] Specifically, biotic burdens caused by life forms largely rely upon the accessibility of reasonable conditions that allow explicit life forms to taint their hosts. Among various types of biotic stress that have existed since antiquated time, fungi, bacteria, viruses, and nematodes can cause infection. In response to different pathogen aggressors, plants initiate a particular arrangement of signaling pathways or guard components, for enhanced resistance or upgraded elasticity based on the host−pathogen interactions.

Mechanism and RNAi Pathway Components. RNA silencing is initiated by the production of small RNA (sRNA) through a sequence of key components, namely, AGO proteins, DCL proteins, and RNA-dependent RAN polymerases (RDRs) (Figure 2). DCL proteins form sRNAs through a dsRNA precursor and afterward join into RISCs. On the basis of their source and arrangement, these sRNAs have

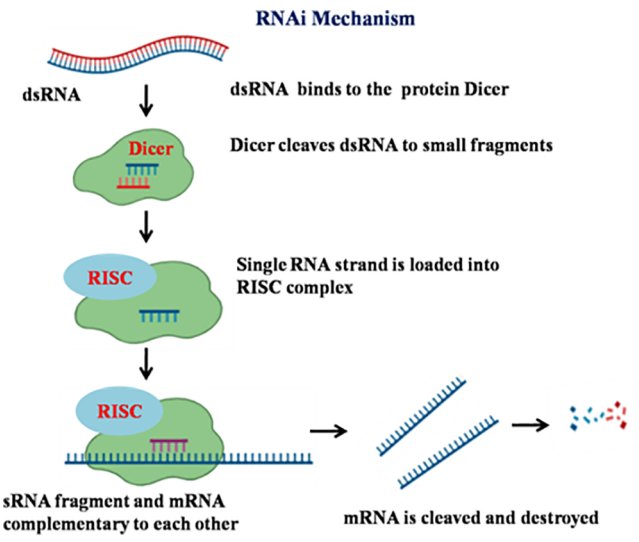

Figure 2. Schematic representation of the RNAi mechanism and mode of action in the gene silencing pathway.

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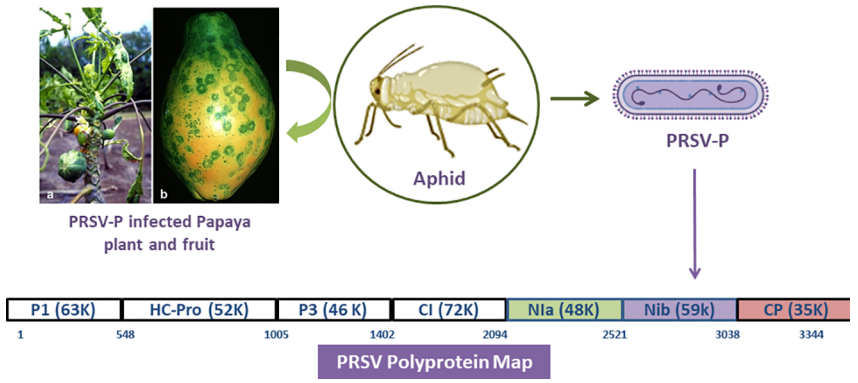

Figure 3. Schematic representation of CRISPR-Cas9 system-mediated immunization to PRSV NIa/Nib gene silencing in papaya.

been classified as miRNAs or siRNAs. In plants, various DCL proteins have been identified and are classified into four groups: DCL1−DCL4. Of these, DCL3 is an important component that participates in the RNA-directed DNA methylation pathway (RdDM) and its processes for RDR2 construct dsRNA into siRNA.[36] The plant's RNAi pathway shows two major steps in gene silencing: transcriptional (TGS) and post-transcriptional (PTGS). In plant cells, AGO4, AGO6, and AGO9 play an active role in TGS, while AGO1−AGO5, AGO7, and AGO10 are contributing in PTGS.[37] Moreover, the AGO4 group is involved in the RdDM pathway and dsRNA that is produced by RDR2 and DCL3 protein.[38]

RDRs are the first recognized components of a plant's sRNA biogenesis pathways and are illustrated by a special RNA-dependent RNA polymerase catalytic domain (RdRp). In the plant kingdom, three different RDRs are exclusively studied as RDR1, RDR2, and RDR6, which take part in an important task in the collaboration with other components of RNAi machinery (i.e., DCLs, AGOs, and RDRs proteins) and defend against invading foreign nucleic acids.[35] RDRs are functionally different from each other and are further categorized into individual groups. RDR1 participates in the intensification of exogenous nucleic acids and priming for defense against plant diseases.[39] RDR2 plays a vital role in the RdDM pathway that is essential for the biogenesis of nuclear RNAi and siRNA.[40] RDR6 activates DNA methylation of the transcribed region without RDR2. Currently, the function of RDR6-RdDM has been studied in the transposable element methylation and epigenetic silencing mechanisms.[41]

Biological Function and Applications of RNAi in Plants. In higher eukaryotic organisms (plants and animals), the pattern of expression of any gene can be downregulated in a significant manner without altering the expression of other genes using RNAi technology.[34] For example, the effect of the collision of global warming and insufficient nutrients and water on crop yield can be mitigated by influencing the architecture of the root system in a consistent circulation of roots in the soil, which rapidly mobilizes the uptake of nutrients and water.

Host-induced gene silencing (HIGS) has demonstrated that plant trans-gene-derived artificial sRNAs are capable of inducing gene silencing in fungi, nematodes, insects, and oomycetes, indicating that the artificial sRNAs from the plant host are successfully transferred to pests and pathogens.[42] On this basis, applying sRNAs or dsRNAs from RNAi targeting fungi DCL genes on the surface of plant organs can attenuate fungal pathogenicity and growth and significantly inhibit gray mold disease and start an era of environmentally friendly fungicides.[43] In transgenic plants, the expression of dsRNA against certain insect target genes has been developed as effective strategy to offer protection against pests such as aphids in agricultural practice. For example, feeding or injecting dsRNA into aphids that targets key genes involved in metamorphosis and insect herbivory is the best approach for aphid control, which usually results in a series of physiological, developmental, reproductive, behavioral, or lethal effects.[44]

The application of dsRNA for the control of plant pests and viruses has become an advanced alternative approach to genetically modified crops, though a main limitation is the convenient use of dsRNA as an early spray for virus protection. In addition, concerns include but are not limited to the transfer in plants of dsRNA to provide stable protection against the target virus and resistance to extreme environmental conditions.[45]

Recently, spray-induced gene silencing (SIGS) has proven to be the latest advancement in plant protection nanotechnology. A study suggested that protection against viruses is spatiotemporally dependent and requires more than 20 days to develop maximum resistance when dsRNAs are integrated into layered double hydroxide (LDH) bioclay.[46] The dsRNA embeded on the degradable nontoxic LDH bioclay can be easily detected upon spraying of the leaves even after application for 30 days. This can be a safer and classical example of the use of sustainable nanotechnology for the incorporation of RNAi in crop protection.[46]

CRISPR-CAS9

Cluster regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (CRISPR-Cas9) has evolved as a vigorous and flexible genetic editing apparatus for generating targeted DNA double-strand breaks (DSBs).[73] This method overcomes the impediment of conventional breeding and accelerates the advancement of improved plant assortments.[74] Recently, CRISPR-Cas9 technology has been recommended for breeding virus-resistant plants by transferring a viral gene sequence instead of a resistant gene sequence through gene silencing methods like RNA interference (RNAi) and microRNA (miRNA).[75,76]

Mechanism and Key Steps. Initially, the CRISPR-Cas9 protein was reported in a bacterial adaptive immune system (*Streptococcus pyogenes*). According to Khatodia et al.,[77] CRISPR-Cas9 is a dual-factor system comprising the Cas9 nuclease protein and a specific single-guide RNA (sgRNA). Moreover, it also requires a protospacer adjacent motif (PAM) sequences (5′-NGG-3′) to stimulate DSBs at the object site.

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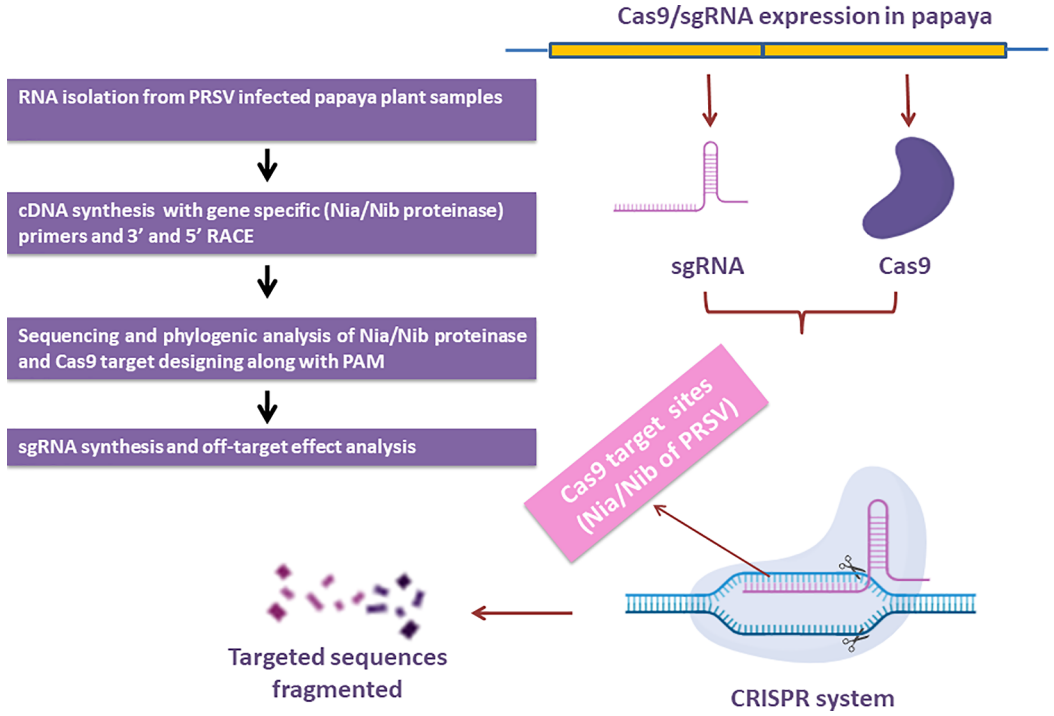

Figure 4. Papaya ringspot virus infection symptoms and transformation and the PRSV polyprotein gene map.

DSBs can be repaired by nonhomologous end joining (NHEJ) or homology-directed repair (HDR). For example, the development of ringspot viral resistance in papaya using CRISPR-Cas9 consisted of three key steps: (a) target design for Cas9 nucleases, (b) sgRNA design and off-target effect determination, and (c) Cas9/sgRNA transformation in papaya (Figure 3).

SELECTION OF CAS PROTEINS

In the CRISPR-Cas protein system, the selection of the type of nuclease proteins is an essential primary key step. Currently, several Cas proteins such as SpCas9 (NGG), FcCpf1 (TTN), AsCpf1 (TTTN), etc., are commonly used enzymes that selectively recognize the protospacer adjacent motif (PAM). Among them, SpCas9 is a widely used CRISPR-associated RNA endonuclease enzyme isolated from *S. pyogenes*. SpCas9 has broad PAM compatibility and high DNA target specificity, although SpCas9 has two major limitations, i.e., PAM compatibility and off-target effects, that hamper therapeutic application.

sgRNA Design and Off-Target Effect Determination. PAM adjacent Nia/Nib gene complementary sgRNA was designed by using different tools such as E-CRISP (http://www.e-crisp.org/E-CRISP/), CRISPR design (https://zlab.bio/guide-design-resources/), and Cas-OFFinder (http://www.rgenome.net/cas-offinder/).[78] Off-target activity Cas9 induces mutations at sites that are present other than target sites. It is one of the main concerns for clinical and therapeutic applications. To overcome these problems, the sgRNA/dCas9 complex off-target sites were determined by using the web-based tool ChiP-seq. To edit targeted Nia/Nib gene sequences in papaya, the initial expression of the Cas9 and gRNA is an essential step. A multiple-gene expression vector system like pENTR vectors with *AtU6* or *TaU6* promoter constructs is used for the expression of sgRNA, and the cas9 gene in the host plant. The formation of CRISPR-Cas9 complex and target reorganization steps are presented in Figure 4. When the PRSV genome enters hot (papaya), the active CRISPR-Cas9 system cleaves the target site (NIa/NiB) into small pieces. This process arrests further PRSV multiplication and actively inhibits further infections.[79]

Applications in Plants. The development of rice ethylene response factor 922 and mildew resistance locus O (MLO)-resistant gene was achieved with CRISPR-Cas9 by targeting S geneand.[80] Tomelo, a nontransgenic tomato variety that is resistant to powdery mildew disease caused by *Oidium neolycopersici*, has been developed using CRISPR-Cas9.[81] Resistance against blast pathogen *Magnaporthe* has been developed in rice by targeting the OsERF922 gene using CRISPR-Cas9 technology. On the contrary, the development of virus-resistant plants was achieved by targeting the viral genome of the susceptible host.[80] Many plants have been developed for geminivirus resistance by targeting ssDNA modifications using CRISPR-Cas9 tools[66] (Table 1). CRISPR-Cas together with RNAi was considered as the method with the most potential for managing citrus pathogens.[82] Using the CRISPR-Cas gene editing system, a tomato variety resistant to bacterial speck disease, Pto DC3000, was created.[83]

CRISPR-Cas protein can be grouped into various delivery systems for incorporation into plant cells, including plant virus vector-mediated delivery, protoplast transfection, *Agrobacterium*-mediated gene transfer, biolistic delivery, and the recently emerging nanoparticle platforms, such as the chitosan platform through vacuum infiltration.[84] Cas9 can be delivered in the form of a plasmid, protein, or mRNA by vectors of liposomes, PEI, Cas9-peptide complexes, gold nanoparticles, and exosomes, which are some of the most commonly used vectors.[85] Recently, chitosan nano/microparticles were used as a novel approach for the transfer of different macromolecules, namely preformed sgRNA:Cas9 RNP complexes, into potato apical meristem cells with the goal of targeting coilin genes or phytoene desaturase (PDS),[86] and the edited lines of a

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minimum of one allele of the coilin gene showed significantly enhanced resistance to potato virus Y infection and osmotic and salt tolerance in potato.[87] The CRISPR-Cas9 gene editing tool was selectively used to develop a targeted disease-resistant trait in a short time in comparison with the conventional breeding approaches. However, the CRISPR-Cas9 tool is still in the early stages; genome-edited crops are limited to field experiments, and researchers need to ensure the robustness of the plant resistance against diseases.[88] The recently developed gene editing tools and their roles, advantages, and limitations are presented in Table 2.

OUTLOOK FOR THE USE OF NANOTECHNOLOGY IN PLANT SCIENCE

The emerging trend in the application of nanotechnology in plant science is significantly important. The use of nanotechnology in agriculture not only improves the overall growth but also helps in generating stress-resilient plants.[89−91] For instance, manipulated nanoparticles were observed in seed germination, plant growth, enhanced resistance against the pathogen, and detection of toxic agrochemicals.[92] Therefore, considering nanotechnology to improve plant health against various environmental stimuli could be useful. Below, we will discuss the role of nanotechnology in plant stress biology.

USE OF NANOTECHNOLOGY AGAINST ABIOTIC STRESSES

Stressors such as drought, heat, cold, salinity, heavy metals, and other stressors continuously cause massive losses in the plants.[93,94] Under abiotic stresses, plants generally generate an immense amount of free radicals, probably due to the ruptured mitochondria. As a result, the levels of antioxidant enzymes plummet many-fold, which eventually causes injury to a plant cell or in some cases cell death.[94] In line with that, the application of Fe_3O_4 NPs, a type of nanoenzyme, displayed activities similar to those of antioxidant enzymes (SOD, POD, and CAT).[95] Thereafter, other different nanoenzymes such as CeO_2, fullerene C_{60}, Au, platinum (Pt), and Mn_3O_4 NPs were used.[95]

Photosynthesis is considered the food factory of the world; however, abiotic stress induced by the ROS level alleviates this process in many ways. The employment of CeO_2 NPs in the *Arabidopsis* plant augments the antioxidant enzyme machinery and confers tolerance to salinity stress.[96] The study also revealed that the improved antioxidant activities after the application of CeO_2 NPs also maintained the normal reactions during the photosynthesis process.[96] Similarly, in sorghum, the foliar application of CeO_2 NPs suppressed the level of the superoxide radical by 41%, that of hydrogen peroxide by 36%, and lipid peroxidation up to 37%. On the contrary, it also boosted the assimilation of carbon in leaves, triggered photosynthesis capacity and pollen germination, and increased the number of seeds per plant under prolonged drought stress.[97] Plants subjected to salinity stress have imbalanced movement of Na^+ and K^+, which is harmful to the cell and affects the normal performance of the plant. In line with that, 50 mg L^{-1} CeO_2 NPs maintained the movement of Na^+ and K^+ ions in the cytosol, which increased the tolerance of the *Arabidopsis* plant to salinity stress. Additionally, the alleviation of hydroxyl radical, which is a harmful ROS, and induction of leaf mesophyll potassium are two of the hallmarks of CeO_2 NP-mediated salinity stress tolerance.[96] The application of

Table 2. Roles, Advantages, and Limitations of Gene Silencing Techniques

gene silencing technique	catalytic components	mechanical functions	advantages	limitations
miRNA-mediated gene silencing, a classic pathway for translation inhibition of mRNA in the cytoplasm and an endogenous regulator of R gene expression in the nucleolus	single-stranded RNA transcripts (MIR genes), resistance genes (R genes), primary miRNA (pri-miRNA), RNAase dicer-like (DCL 1), Argonaute (Ago), RNA-induced silencing complex (RISC), and mRNA	miRNAs inhibit translation by mRNA degradation or destabilization and posttranscriptional cleavage in the cytoplasm. In the nucleus, miRNAs control the transcript stability of R genes by inducing expression or suppression.	Gene expression regulated at post-translational levels by mRNA cleavage. miRNAs can control a wide range of biological and metabolic processes, and less false positive possibilities.	time-consuming process, high cost for application in field experiments, low efficiency, high likelihood of new miRNA, limited availability of R genes within genetic resources, and off-target effects
RANi-mediated gene silencing, an endogenous pathway for negative posttranscriptional regulation of target mRNA	RNA-dependent RNA polymerases (RdRPs), double-stranded RNA (dsRNA), ribonuclease III enzyme Dicer, small interfering RNAs (siRNAs), sRNAs (miRNAs), Argonaute family proteins (AGOs), AGOs/siRNA complex, RNA-induced silencing complex (RISC), and mRNA	gene silencing in a homology-dependent manner, mRNA degradation, modification of mRNA translation or chromatin, posttranscriptional gene silencing (PTGS), translational inhibition, mRNA destabilization, and transcriptional gene silencing (TGs) by DNA methylation	RNAi is a naturally occurring cellular activity in plant−pathogen and −pest interactions, easy transfer from one plant to another plant or from plants to pathogens or pathogens to plants, and easy to establish disease-resistant cops via field experiments	time-consuming process, carriers (pests, insects, and pathogens) that can also spread diseases form one plant to another, and off-target effects
CRISPER-Cas9-mediated gene silencing, a genomic tool for editing of genes or genomic DNA	clustered regularly interspaced short palindromic repeat codes in the DNA (CRISPR), single-guide RNA (sgRNA), CRISPR-associated endonuclease (Cas), and protospacer adjacent motif (PAM) sequences in the genome	The Cas9 system acts as a molecular scissors. Cas9 makes a cut across the double strands of the targeted DNA in the genome that leads to a double-strand break (DSB). DSBs are repaired by nonhomologous end joining or insertion of a known DNA sequence.	faster, more precise, highly efficient in gene editing technology, more versatile and cheaper, user friendly tool for the development of nontransgenic crop plants to counteract, multiplex genome editing with a single molecular construct, existing genome that can be edited by modifying, deleting, or inserting a new sequence, can cure lifelong inherited disease	off-target effects

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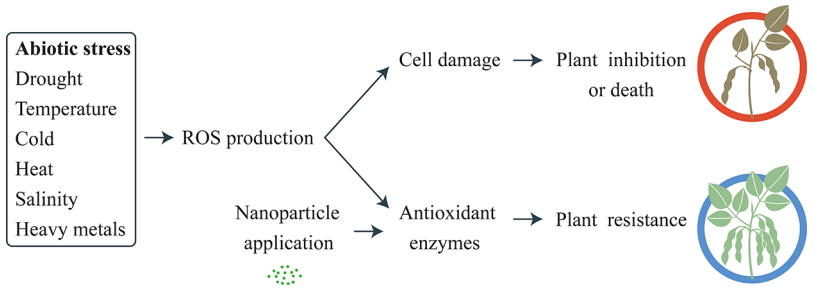

Figure 5. Effects of nanoparticles on plants. The application of nanoparticles can result in the production of antioxidant enzymes by the plants, the reduction of ROS levels, and plant resistance against abiotic stress.

Table 3. Application of Different Nanoparticles to Mitigate the Biotic Stress Factors

nanoparticle	pathogen	defense response	ref
Ag@dsDNA@GO	*X. perforans*	Ag@dsDNA@GO nanoparticles displayed excellent antibacterial activities.	107
Ag	*G. abietinum, G. trabeum, C. globosum*, and *P. sordida*	induced resistance against multiple fungal pathogens	110
TiO_2 NPs	*X. perforans*	enhanced immunity in tomato	111
CeO_2 NPs	*Fusarium oxysporum*	provided protection to tomato plants from *F. wilt*	112
MgO NPs	*R. solanacearum*	upregulated the expression of stress-specific genes that confer tolerance to *R. solanacearum*	113
Si NPs	*Aspergillus* spp.	activated the phenolic compound metabolic pathway	98
MSNs	*F. oxysporum*	modulated stress-related genes that enhanced the disease resistance of watermelon	114

nanoparticles to weaken the damaging effects of ROS could also be related to enhanced antioxidant enzyme activities. For example, the nanoceria nanoparticles (300 μg plant^{-1}) increased the amount of ascorbate peroxidase (APX) gene,[98] which is an important unit of the plant antioxidant enzyme machinery.

Apart from acting as antioxidant enzymes, nanoparticles attenuate the harmful effects of abiotic stresses in several other ways. For example, the fullerol nanoparticles rescued the sugar beet plant from drought stress by inhibiting oxidative stress and also maintained the intercellular water supply.[99] In another report, γ-Fe_2O_3 nanoparticles were applied over *Brassica napus* plants. The results indicated the protective role of γ-Fe_2O_3 as it significantly mitigates drought stress by suppressing the levels of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA).[100] Therefore, γ-Fe_2O_3 nanoparticles could be a useful tool for increasing plant productivity under a variety of different abiotic stresses.

Heavy metal contamination in the soil due to increased industrialization is becoming a serious threat to arable land. A majority of economic crops are extremely sensitive to metal stress, whereas some plants take up metal in massive amounts, which is harmful to human health.[101−103] The application of different nanoparticles was shown to enhance the tolerance of plants to heavy metal stress. For instance, nickel (Ni) and iron (Fe) nanoparticles significantly countered the toxicity of polybrominated diphenyl ethers (PBDEs) along with improved plant development.[104] Also, silicon nanoparticles alleviate the chromium (Cr) toxicity in chickpea plants grown in Cd-contaminated soil.[105] That study showed that the application of Si nanoparticles augmented the growth parameters of chickpea plants such as induced chlorophyll content, total biomass, and accumulation of Cr. The foliar application of titanium dioxide (TiO_2) on maize substantially amended the antioxidant enzyme activities, slowed the accumulation of cadmium (Cd) in shoot tissues, and triggered the response of key metabolic pathways.[106] These lines of evidence suggested that nanoparticles could be used to improve the immune system of plants in a more efficient way (Figure 5). These nanoparticles are generally environmentally friendly and could blunt the hazardous effects of industrial fertilizers and growth regulators.

USE OF NANOTECHNOLOGY AGAINST BIOTIC STRESS

Biotic stress, including chewing insect pests, has drastically impacted food crops in recent times. The increasing use of pesticides, on the contrary, is an alarming threat to our ecosystem. Therefore, nanoparticles could act as a carrier to improve the efficiency of industrial pesticides. Several nanoparticles also serve as a self-biocidal agent. For example, Ag@dsDNA@GO DNA-directed silver (Ag) nanoparticles curb the deleterious effects of *Xanthomonas perforans* in tomatoes.[107] In addition, Ag nanoparticles were found to be lethal against soil-borne pathogens such as nematodes.[108] The *Serratia* sp. of plant growth-promoting rhizobacteria (PGRB) can biosynthesize Ag nanoparticles. The PGRB *Serratia* sp. was used in a greenhouse environment, which reduced the pathogenicity of the spot blotch pathogen named *Bipolaris sorokiniana* in wheat plants.[109] The green syntheses of Ag nanoparticles were found to be effective against an array of fungi such as *Gloeophyllum abietinum*, *Gloeophyllum trabeum*, *Chaetomium globosum*, and *Phanerochaete sordida*.[110] TiO_2 NPs that are photoactive and have a high photocatalytic activity were used under greenhouse conditions in tomato plants. The study addressed the important antibacterial features of TiO_2 NPs against *X. perforans*, a major bacterial spot disease-causing pathogen.[111]

Cerium-based nanoparticles (CeO_2 NPs) have been characterized recently by Adisa et al.[112] They used CeO_2 NPs against *Fusarium wilt* in tomatoes under controlled greenhouse conditions. The application of 250 mg L^{-1} CeO_2 NPs on leaves and also via roots remarkably mitigated the negative effect of *F. wilt* by 57%.[112] This underlies the potency

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Table 4. Nanomaterials Based on Organic, Inorganic, or Hybrid Matrices Used as Carrier Agents for the Application of Genetic Material in Plants

nanomaterial	properties	biological model	objective and results	toxicity	refs
			Inorganic Nanoparticles		
mesoporous silica nanoparticles (MSNs)	pore size of 3 nm	*N. tabacum* plants	Transgenic tobacco plants were obtained by the biolistic method using mesoporous silica nanoparticles (MSNs) with the gene and its inductor in 3 nm pores covered with gold nanoparticles. The expression of fluorescent protein in tobacco plants was induced by the release of β-estradiol.	No toxicity to plant cells was observed.	119
mesoporous silica nanoparticles (MSNs)	pore sizes of 600 and 10 nm and ζ potential of −25 mV	*N. tabacum*, *Z. mays* L., and *Allium cepa* epidermis cells	Delivery of protein to vegetable cells of the onion epidermis and tobacco leaves and teosinte, using a biolistic method, as evidenced by fluorescence microscopy showing the *in vitro* release of bovine serum albumin (BSA) and increased green fluorescent protein (eGFP) 1 day after the bombing. As a platform for the release of proteins and plasmid DNA into plant tissues, MSNs functionalized with gold nanoparticles were synthesized.	not described	120
mesoporous silica nanoparticles (MSNs)	size of 50 nm	*A. thaliana*	Fluorescent and functionalized mesoporous silica nanoparticles with three distinct functional chemical groups were used in the incubation of *Arabidopsis* roots. The nanoparticles are captured by the roots, are distributed in the tissues, and enter the cell plant, reaching several important organelles. Stable MSN nanocomplexes with a plasmid harboring a red fluorescent protein (mCherry) gene driven by a cauliflower mosaic virus 35S promoter were used for plant transformation and promoted transient gene expression of intact roots.	MSNs had no acute toxic effects on *A. thaliana* roots.	121
mesoporous silica nanoparticles (MSNs)	size of 40 nm and ζ potential of 9.8 mV for loaded samples and −10.3 mV for unloaded samples	*S. lycopersicum*	A gene transformation system employing MSNs, to which a recombinant construct (pDNA) containing the *cryIAB* gene under the control of the maize proteinase inhibitor (MPI) promoter was attached, was used in the production of transgenic tomato seeds. Plants with a transformation frequency of 10.71% were obtained. The transformed plants showed an increase in tolerance to *Tuta absoluta*.	They did not show toxicity for tomato plants.	122, 123
layered double hydroxide (LDH) nanoparticles	mean size of 45 nm and polydispersity index of 0.24	*A. thaliana*, *Vigna unguiculata*, and *N. tabacum* plants	Layered double hydroxide clay nanosheets were used as a dsRNA carrier to enhance the protection against viruses in plants. The dsRNA complexed in LDH was detected on the leaf surface even 30 days after spraying. The release of dsRNA has been shown to occur sustainably under environmental conditions and has provided systemic protection based on RNAi, against targeted viruses, for sprayed and newly emerged nonsprayed leaves.	Clay nanosheets have been reported to be nontoxic and biodegradable.	46
layered double hydroxide (LDH) nanoparticles	not described	*N. benthamiana* and *V. unguiculata*	LDH nanoparticles containing dsRNA molecules specific to the bean common mosaic virus (BCMV) were used in spray form and protected the plants of *N. benthamiana* and *V. unguiculata* against inoculation mediated by BCMV aphids. LDH nanoparticles loaded with dsRNA directed to the coding region of the coating protein were demonstrated to be successful against the transmission of BCMV by the aphid *Myzus persicae* in both plants tested.	not described	124
layered double hydroxide lactate nanosheets (LDH-lactate-NS)	≤2 nm thickness and 30−60 nm	*A. thaliana* and *N. tabacum*	Layered double hydroxide lactate nanosheets were used as carrier system for active materials in plant cells. According to the results, inhibitors of endocytosis and low-temperature incubation did not prevent the internalization of the carrier system, indicating nanosheets penetrated the plasma membrane by non-endocytic pathways, which will open perspectives to other applications.	Absence of side effects in cytological studies	125
silicon carbide whiskers	not described	*Gossypium hirsutum* L.	Silicon carbide whiskers were used as a transformation method for the production of transgenic cotton plants, with a transformation efficiency of 94%. Calli cotton treated with silicon carbide whiskers, pGreenoo29, and pRG229 exhibited callis colonies within 6−8 weeks, showing the GUS reporter system (GUS, β-glucuronidase) activity, while the control did not express GUS activity. Transgenic cotton plants developed salt tolerance due to the transgenic overexpression of *AVP1*, maintaining vigorous growth in contrast to the unprocessed cotton plants.	not described	126
silicon carbide whiskers	not described	*Z. mays* L.	Silicon carbide whiskers were used to deliver the bar and *uidA* genes in embryonic corn callus. DNA analyses confirmed the stable integration and inheritance in plants regenerated by callus lines expressed by GUS.	not described	127
silicon carbide whiskers	not described	*Triticum aestivum* L.	Silicon carbide whiskers and microprojectiles coated with cDNA were used for transformation into embryogenic callus lines derived from wheat culture. The type of inoculum has been shown to affect the ability to differentiate. Silicon carbide whiskers proved to be a transformation method that makes genetic delivery less harmful and less compromising in terms of cell survival and enhanced the regeneration potential of treated tissues.	not described	128
silicon carbide whiskers	not described	*Oryza sativa* L.	Fertile transgenic rice plants were produced by DNA delivery mediated with silicon carbide whiskers. The integration and inheritance of the gene in plants were confirmed by the southern analysis of R0 and R1.	not described	129
silicon carbide whiskers	not described	*Lolium multiflorum*, *Lolium perenne*, *Festuca arundinacea*, and *Agrostis stolonifera* plants	Silicon carbide whiskers were used for transformation into plants with a hygromycin resistance gene, which was regenerated after transformation. PCR and Mancha Sul analysis confirmed the transformation.	not described	130
silicon carbide whiskers	not described	*O. sativa* cells	Silicon carbide whiskers were used to deliver a plasmid (p *Act1*-F), containing GUS as a reporter in embryonic rice cells.	not described	131
silicon carbide whiskers	not described	*Z. mays* L.	Fertile transgenic corn plants were produced by embryogenic cell culture transformation mediated by silicon carbide whiskers, which was used to deliver plasmid DNA carrying the bacterial bar and *uidA* (GUS) genes.	not described	132
silicon carbide whiskers	not described	*Arachis hypogaea*	The transformation method mediated by silicon carbide whiskers to deliver the chitinase and hygromycin gene conferred greater resistance to leaf spot in peanut plants. Calli resistance to hygromycin was regenerated, and the plants produced seeds normally.	not described	133

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Table 4. continued

nanomaterial	properties	biological model	objective and results	toxicity	refs
			Inorganic Nanoparticles		
single-walled carbon nanotubes	size of 3−5 nm and ζ potential of 27.2 mV	*N. tabacum* var. Virginia root cells	Functionalization of single-walled carbon nanotubes with arginine improved gene delivery in vegetal cells. The system was able to transfer GFP-expressing plasmids in the plant genome, which has been confirmed by fluorescence microscopy	not described	134
single-walled carbon nanotubes	size between 100 and 120 nm and ζ potential of around 30 mV	*Eruca sativa*, *Nasturtlum officinale*, *N. tabacum*, and *Spinacla oleracea*	The chitosan complex in single-walled carbon nanotubes acts as a stimulus-responsive system for gene delivery in chloroplasts in mature plants. The results showed that the system was able to cross all biological barriers and reach the chloroplast organelles; once, inside the chloroplast, the basic pH allows DNA release and transformation of these specific organelles.	not described	135
single-walled carbon nanotubes	size of 776.6 nm	mGFP5 *N. benthamiana*	Single-walled carbon nanotubes were used as the siRNA carrier system in plants. The system can protect the siRNA from degradation, resulting in mRNA knockdown in 1 day with 95% efficiency.	The delivery platform is nontoxic.	136
single-walled carbon nanotubes	size of 16.3 nm and ζ potential of 31.7 mV	*N. benthamiana*, *E. sativa*, *T. aestivum*, and *G. hirsutum*	Single-walled carbon nanotubes were used for the transformation of plant tissues with intact cell walls. The system was able to deliver the genetic material in the nucleus and result in the reported gene expression. The genetic material was not integrated into the plant genome.	The delivery platform is biocompatible and did not induce toxicity or tissue damage in mature plants.	137
single-walled carbon nanotubes	length and diameter of 533 and 2.9 nm, respectively, and ζ potential of 48.2 mV	*Elaeis guineensis* Jacq	Single-walled carbon nanotubes were functionalized with imidazolium for gene delivery in pollens. The system was able to be taken up by the pollen by the envelope penetration model due to the high material ζ potential. The system was able to deliver genetic material into the pollens and transform the cells.	The delivery platform was reported to exhibit high biocompatibility.	138
			Organic Nanoparticles		
chitosan	600−800 nm	*S. tuberosum*	Chitosan nanoparticles were used for the delivery of CRISPR-Cas9 editing aiming to improve the resistance against abiotic and biotic stress. The system was able to alter colin genes, resulting in the potato plants being more resistant to abiotic stress (salinity) and biotic stress (PVY, Potyviridae virus).	not described	87
chitosan	size of around 185 nm	*S. tuberosum*	Chitosan nanoparticles were used for the transformation of potato plants with thionin genes of *A. thaliana* to improve the resistance against fungal infection. Using tissue culture, the system was able to transform the cells, inserting the thionin genes from *A. thaliana*. The potato plants showed stronger resistance against *Alternaria alternata* and *Rhizoctonia solani* species.	not described	139
peptides	size of 170−200 nm	*A. thaliana*	Peptide nanocarrier systems were glutathione stimulus-responsive. The results demonstrate that the system was taken up by stomata, internalized by vegetal cells, and localized in the cytosol and nucleus. The system responds to an endogenous stimulus, resulting in the delivery of DNA and its expression.	not described	140
polyamidoamine dendrimer	not described	*A. thaliana* cv. Columbi	Polyamidoamine dendrimer nanocarrier systems were used for gene delivery. The results showed that the system was able to insert exogenous genes into the plants to enhance resistance to the herbicide.	not described	141
dimethylaminoethyl methacrylate (DMAEM)	not described	*A. cepa* and *N. tabacum* L.	New DNA carriers as oligoelectrolytes based on polymeric dimethylaminoethyl methacrylate (PDMAEM) have been synthesized, and their physicochemical properties have been presented. The carrier systems were effective in delivering plasmid DNA in plants.	Cytotoxic and genotoxic effects have been reported.	142
peptide	not described	*A. thaliana* and *N. benthamiana*	The authors describe a simple and versatile protocol, which employs peptides as delivery agents for a variety of plant-based nucleic acid and protein charges. The peptides are selected, and the methodology used allows modifications to increase biodegradability, reduce size, and yield diverse and adjustable properties, as well as the ability to gain intracellular access.	not described	143
$(KH)_9$-Bp100 peptide	diameter between 100 and 300 nm and positive ζ potential	*A. thaliana*	A new dsRNA delivery system was prepared on the basis of the ionic complex of dsRNA and a peptide. The results showed that the complex was taken up by leaf cells and induced rapid and efficient regulation of exogenous and endogenous genes.	not described	144

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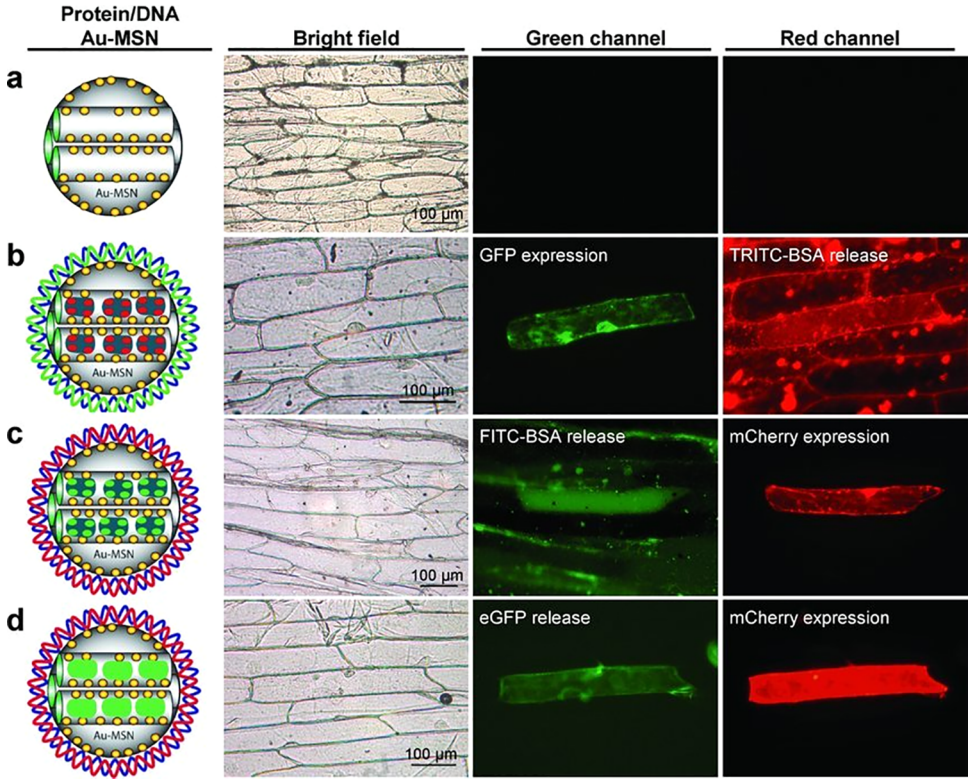

Figure 6. Schematics and microscopy images of (a) onion epidermis cells treated with empty Au-MSNs, (b) TRITC-BSA protein-loaded and GFP-expressing plasmid DNA-coated Au-MSNs, (c) FITC-labeled BSA protein-loaded and mCherry-expressing plasmid DNA-coated loaded Au-MSNs, and (d) eGFP protein-loaded and mCherry-expressing plasmid DNA-coated Au-MSNs. Reprinted with permission from ref 120. Copyright 2012 Wiley-VCH.

of CeO_2 NPs against fungal pathogens, and CeO_2 NPs could be used as an eco-friendly pesticide to minimize the drastic effects of fungal pathogens on tomato and other vegetable crops. The application of MgO NPs to tomato roots induced the expression of pathogenesis-related protein (*PR1*), *LoxA*, *Osm*, and *GluA* genes and thus provided resistance against *Ralstonia solanacearum*.[113] Similarly in cucumber, the use of silicon nanoparticles (Si NPs) at a rate of 15 kg ha^{-1} increased the metabolic activities of various phenolic compounds that confer resistance against *Aspergillus* spp.[98] The mesoporous silicon nanoparticles (MSNs) after being coated with chitosan (CTS-MSNs) were used against *F. wilt* in watermelon grown in soil. The MSNs and CTS-MSNs yielded positive results by suppressing the disease severity index to 40% and 27%, respectively.[114] Table 3 summarizes the studies with regard to the involvement of nanoparticles in mitigating biotic stress.

Overview of How Nanotechnology Can Help to Overcome the Gaps and Pitfalls in the Use of Genetic Material. In the field of genetic engineering, different processes have been developed to improve crops against pests and pathogens. A variety of nanoplatforms, commonly studied for applications in the fields of materials, electronic devices, and medicine, have been attracting interest for applications in agriculture.[115] In addition to their application as nanocarriers for pesticide substances and plant nutrition, nanoparticles can act as vectors of genetic material to cells, overcoming the limitations of conventional transformation methods.[116] The nanomaterials can deliver different biomolecules such as DNA, miRNA, siRNA, ribonucleoproteins, etc. These systems are considered noncytotoxic vectors and present biocompatibility.[117] The great challenge is that these systems are prepared in such a way that the genetic transformations mediated by nanoparticles are stable and can transpose the cell wall present in plant cells.[115] In this case, the nanometric size of these platforms favors the transposition of barriers in plant tissues and can also direct their performance in specific tissues and cells providing security for the desired transformations.[115] Depending on the application, several properties can be adjusted so that an efficient release of the intracellular biomolecule occurs. Thus, these systems can present different sizes and shapes, surface loads, and also functionality with other types of materials.[117] In addition, these systems can also present specific triggers (pH, enzymes, redox, etc.) for the release of these biomolecules.[118] Table 4 shows options of nanoplatforms with genetic material that have already been studied in models with plants; these systems can be of organic, inorganic, or hybrid origin.

TRANSPORT AND DELIVERY SYSTEMS

The transportation of a gene into a plant cell through the multilayered polymeric cell wall often causes challenging scenarios for biotechnology researchers. On the contrary, the low efficacy of the transformation of genetic materials, cellular toxicity, and integration of crippled DNA molecules due to lysis in the host genome substantially impair the efficiency of transgenic techniques.[145] Previously, the viral gene vector was generally used to transport genetic materials into cells. This technique had many drawbacks such as limiting the ranges of hosts, the capacity to carry genetic materials, and transportation and trafficking constraints inside the nucleus.[146] However, the recent advancements in nanotechnology have overcome these gaps and pitfalls.

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Nanomaterials could be the best possible alternative to use in gene delivery systems for crop improvement for several reasons. A nanoparticle-mediated delivery system prevents the DNA from lysis by protecting it from nuclease enzymes, enhances penetration through the plant cell wall, and delivers the active form of genetic materials into the nucleus.[147] For example, the high efficiency of mesoporous nanoparticles in the transportation of genetic materials to plant cells has been reported previously.[148] The following section reviews different works with nanocarriers and genetic materials in plant cells.

NANOPLATFORMS FOR DNA DELIVERY

Martin-Ortigosa et al.[120] synthesized gold-functionalized mesoporous silica nanoparticles (Au-MSNs) for the co-delivery of plasmid protein and DNA into plant tissues using the biological method. The uptake and release of serum albumin protein (BSA) and the increased level of GFP in plant cells were investigated. Au-MSNs loaded with proteins were coated with plasmid DNA, and it was possible to carry out the encoded delivery of plasmid DNA and proteins into onion plant cells, which could be observed by the expression of marker genes 1 day after the bombing (Figure 6). The expression of the red fluorescent protein (mCherry) gene was evident when FITC-BSA or Au-MSNs loaded with GFP were used, and the expression of the GFP gene for Au-MSNs loaded with TRITC-BSA. In the control experiment, the Au-MSNs not coated with DNA were bombarded, which showed no fluorescence in the green or red channels (Figure 6a).[120]

Torney et al.[119] reported that the MSN was capable of transporting DNA and chemicals in plant cells and leaves. After the MSN had been loaded with the gene and its chemical inducer, the pores were covered with gold nanoparticles, which were then used in genetic weapon systems, and when opened, it released the chemicals and triggered the expression of the gene in plants. Compared to systems with the delivery of genetic material with gold nanoparticles, the MSN enables the loading of large portions of biogenic compounds and chemicals that are impermeable to the cell membrane to the target cells along with DNA.

Silica nanoparticles modified with poly-L-lysine (Si NP-PLL) could deliver the plasmid-encoded gene in tobacco plants. The study by Fu et al.[115] showed that the same system was effective when combined with genetic material, also preventing DNA cleavage by DNaseI. The study consisted of bombarding the callus and young tobacco leaves using the gun gene method, and the results showed that the gene can integrate into the DNA of the tobacco genome. In the study, the transformation efficiency was 47.11 ± 0.4% for nanoparticles with a DNA/Si NP ratio of 1/10. The efficiency response also indicates that the penetration of Si NPs is easier in the cellular appearance of the callus than in that of the leaves. Bao et al.[125] synthesized LDH and lactate (LDH-lactate-NS) nanosheets that had a high capacity to adsorb negatively charged biomolecules. The tests carried out on *A. thaliana* seedlings and *N. tabacum* cells demonstrated that the nanosheets could transport fluorescent dyes to the cytosols of plant cells by penetrating the plasma membrane through non-endocytic pathways. LDH-lactate-NS conjugated with DNA could penetrate plant cells and enrich the cell nucleus with ssDNA-FITC. The results achieved thus demonstrate the potential of LDH nanosheets to be used as nanocarriers in plant cells. Golestanipour et al. functionalized single-walled carbon nanotubes with arginine and evaluated the transfections in *N. tabacum* var. Virginia (tobacco) root cells. The system was designed to improve the delivery of a gene containing a peptide fusion with the objectives of (i) keeping the DNA structure condensed, (ii) promoting endosomal escape, and (iii) increasing the rate of release and transfer of the gene into the nucleus cells. The results showed that the GFP expression plasmids in roots cells increase, which demonstrated the capacity of this system for gene delivery.[134] Kwak et al.[135] developed a stimulus-responsive system based on single-walled carbon nanotubes (SWNTs) with chitosan. The system promotes gene delivery without requiring mechanical techniques as biolistic or polyethylene glycol (PEG)-mediated transformation. The system was able to cross the protoplast and cell membrane and to target the chloroplast by lipid exchange envelope penetration mechanisms.[149] The chitosan coating promoted a stimulus-responsive effect, ceasing DNA release in the cytosol vegetal cells (pH 5.5) and promoting the delivery in the chloroplast (pH 8). The methodology was able to deliver genes into the chloroplast for *N. officinale* (watercress), *S. oleracea* (spinach), and *N. tabacum* (tobacco) cells.[135] Demirer et al.[137] used SWNTs to deliver GFP-encoding dicot plasmid DNA into argula, wheat, and cotton leaves. The results showed that the increase in the level of GFP expression and fluorescent protein levels indicated that the system can cross biological barriers, protect the genetic material from degradation, and be delivered into nucleus cells, promoting gene expression. Lew et al.[138] described the use of a SWNT for the delivery of DNA into *E. guineensis* Jacq pollen (palm oil). The SWNT was functionalized with imidazolium that increases the rate of delivery of plasmid DNA into the pollen, resulting in a transformation efficiency of around 35%, which is more efficient than biolistic method (5−10%). Baghdan et al.[150] developed chitosan nanoparticles enveloped in anionic liposomes, which were shown to increase DNA protection, reduce cytotoxic effects, and improve DNA delivery under physiological conditions (in animal cells). In the work of Monash and Smith,[151] the authors describe the preparation of PLGA nanoparticles conjugated with cationic polyethylene finished in methoxy (MePEG-PLGA) as a carrier of DNA. The results showed that the nanoparticles had an average size of 89.7 nm and a ζ potential of 28.3 mV. Transmission electron microscopy (TEM) analyses showed uniform particles, with a smooth surface and a spherical morphology. The release tests showed that 80.2% of the DNA was released and the nanoparticles were able to protect the genetic material from nuclease degradation. Therefore, the nanoparticles proved to be efficient as carriers of genetic material, opening perspectives for their use in different applications.

The mycogenic nanoparticles that are produced by the fungus *Aspergillus ochraceus* are very small (5−25 nm). These nanoparticles upon conjugation with carbon nanoparticles delivered DNA to tobacco cells highly efficiently.[152] The same mycogenic nanoparticles were used in monocot species such as *O. sativa* and resulted in enhanced DNA delivery with minimum damage to the cell.[145] The metallic nanoparticles need less plasmid to carry DNA with low toxicity to plant cells in comparison to those other commercially available nanoparticles.[153] Chuah and Numata[154] developed a nanocarrier system based on stimulus-responsive peptides. The peptides have disulfide bonds between two cysteine groups that allow the capacity to bind the DNA and release it in the presence of endogenous glutathione in plant cells. The nanocarrier systems were applied in *A. thaliana* leaves, taken up by stomata, sprayed

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on the leaf cells, and detected in the cytosol and nucleus cells, resulting in gene expression.

NANOPLATFORMS FOR GENE EDITING AND TRANSFORMATION

Chang et al.[121] demonstrated in a simple co-culture method that MSNs pass through the cell wall and promote the transient gene expression of intact *A. thaliana* roots. MSNs coated with DNA were used to transform the plant, in which *Arabidopsis* seedlings that were 2−3 weeks of age had their roots exposed for 48 h to nanoparticles. MSNs could deliver DNA to deeper tissues, such as the cortex and endoderm, and move to various organelles so that they could function in targeted delivery. The study indicates that MSNs have the potential to operate in transport through vascular bundles and to transport cells to cells of genes and other compounds of interest.

Hajiahmadi and colleagues reported for the first time the stable transformation in tomato plants mediated by MSNs using the injection of functionalized MSNs containing pDNA (pDNA-MSN) into the tomato fruit before the ripening period.[123] The authors reported that pDNA-MSN passes through the nuclear pore of cells and releases pDNA into the nucleus, which is integrated into the genome in the plant, possibly with homologous recombination between pPZP122 and chromosome 11 in tomatoes. The MSN probably accumulates in chloroplasts and remains in plants and cannot be transferred to the next generation. The proposed method is convenient for applications in transformations and provides a viable alternative for similar transformations in other plants, as well as being able to be optimized using vectors or CRISPR-Cas9. The *cryIAB* gene was used under the control of the maize proteinase inhibitor promoter for the production of transgenic tomato seeds, which produced plants with a transformation frequency of 10.71% resistant to gentamicin and greater tolerance to *T. absoluta*.[122]

For transformation into wheat plants (*T. aestrum* L.), silicon carbide whiskers provided a higher frequency of regenerated and transgenic clonal segments when compared to tissues bombarded with microprojectiles coated with cDNA. A higher frequency of transient GUS expression of tissues bombarded with microprojectiles was observed; however, stable gene expression was 6−10 times less effective than treatment with silicon carbide whiskers.[128] Akram et al.[133] used the silicon carbide whisker method to transform the rice chitinase gene to control peanut leaf spot. The RT-PCR analysis indicated a greater number of mRNA transcripts in the labeled plants than in the control, which showed a reduced level of chitinase expression. The transformation provided greater resistance to leaf spots in peanut plants.

Burlaka et al.[155] used single-walled and multiwalled carbon nanotubes for the genetic transformation of *N. tabacum* L. protoplasts, callus cells, and leaf explants. The concentration of carbon nanotubes is one factor in improving the efficiency of gene delivery. The SWNTs can genetically transform protoplasts and walled plant cells, while MWCTs can transform only the protoplast. Makhotenko et al.[87] used a chitosan carrier system to edit the coilin gene in *S. tuberosum* (potato), aiming to improve resistance stress. The target of this study was to alter colin genes that are responsible for the formation of Cajal bodies, proteins that are involved in RNA metabolism, and in some parts of RNA maturation, modification, and transport; moreover, it has a relationship with the plant stress response.[156] The study showed that chitosan nanoparticles inserted into the plants' cells by infiltration could modify the coilin genes and lead to the plants becoming more resistant to potato virus Y (PVY, *Potyviridae*) and abiotic stress (salinity). In another example, chitosan nanoparticles were used to insert two thionin genes from *A. thaliana* into potato plants. The proteins produced by thionin genes have antifungal activity against *A. alternata* and *R. solani* species. The transformation of plants cells was performed in culture plants cells, and the results demonstrate that the transgenic potato plants improve the resistance against the pathogenic fungi, showing that the nanoparticles were efficiently transformed in the plant cells.[139]

Magnetic nanoparticles were successfully used in cotton flowers where a β-glucuronidase (GUS) reporter gene was injected into the pollens through magnetic force, without compromising the viability of pollen grains.[157] These pollen grains carrying magnetic nanoparticles and the GUS gene were shredded over the plant by a process called self-crossing. The stable expression of the gene in the transgenic offspring plants verified the successful transformation. This highlighted the excellent transformation efficiency of magnetic nanoparticles used as carriers. These magnetic nanoparticles could be used in future breeding work as they provide highly efficient virus-free transformation capacity.

NANOPLATFORMS FOR RNA DELIVERY

Mitter et al.[46] demonstrated that dsRNA can be loaded in LDH clays, which is protected from nuclease activity and was made available in a sustained manner for up to 30 days after spraying. The uptake of dsRNA in plant cells was observed by monitoring the fluorophore-labeled dsRNA after topical application onto *A. thaliana* leaves for 48 h using fluorescence microscopy. Absorption via xylem was observed for all treated leaves and also abundant absorption of Cy3 in the spongy mesophyll and systemic movement of flowering Cy3 in untreated apical leaves. The use of a β-glucuronidase (GUS) reporter system in a 6b4 *Arabidopsis* transgenic strain enabled the observation of a significant reduction in GUS activity in seedlings treated with GUS-dsRNA or GUS-dsRNA-LDH. Thus, this demonstrated that the dsRNA released from the clay nanosheets was absorbed by the plant and induced post-transcriptional negative regulation of GUS expression. With regard to crop protection, the loading of dsRNA into the LDH nanofibers was efficient in inducing resistance to viruses of a specific sequence with respect to those challenged in the assays and promoted a reduction in the number of necrotic lesions in leaves of *V. unguiculata* and *N. tabacum*.[46] Worrall et al.[124] used LDH nanoparticles loaded with dsRNA directed to the coating protein-coding region that provided effective plant protection against infection by the bean common mosaic virus (BCMV). According to their results, *N. benthamiana* plants after being infested with aphids for 10 days, submitted to treatment with LDH loaded with dsRNA, were not infected by BCMV, while 13 of 24 plants that did not receive the treatment tested positive for BCMV. The experiments also highlighted the protection of cowpea plants against infection with viruses transmitted by aphids. Demirer et al.[136] reported the use of SWNTs for the delivery of RNAi molecules into intact cells in transgenic mGFP5 *N. benthamiana* plant leaves, intending to silence green fluorescent protein (GFP). The system could be internalized into intact walled plant leaf cells after application

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for 6 h and reduce the GFP transcript levels around 95%, resulting in gene knockdown.

In the literature, several works have demonstrated the effectiveness of PCL nanoparticles as carriers of genetic material. However, there have been no specific studies in plants, and these systems could potentially be used for this purpose.[158−160] Palamà et al.[161] described the preparation and characterization of PCL nanoparticles as carriers of mRNA. The authors obtained nanoparticles (247 nm) in a core−shell structure containing mRNA surrounded by PCL. Furthermore, the release kinetics was pH-dependent (25% of mRNA is released 48 h after incubation at pH 7 and 60% at pH 5.0). For the authors, PCL nanoparticles are a promising system that solves the instability problems for mRNA introduction. In a recent study, Ding et al.[162] reported obtaining a PCL-based nanohydrogel containing Cas9 and single-guide RNA. According to the authors, due to the compact architecture, the nanogel exhibited excellent physiological stability against nuclease digestion and higher cell absorption efficiency. Thus, the system proved to be a promising tool for the delivery of genetic material.

NANOPLATFORMS FOR CRISPR-DCAS9 DELIVERY

In ref 163, Zhang et al. present a promising system based on self-assembly peptide (SAP) and PCL for localized delivery of CRISPR-dCas9. According to the authors, PCL nanofiber coating through strong hydrophobic interactions facilitates encapsulation and assists in the sustained release of pDNA complexes. The results were promising and provide a new, simple, and efficient platform for the delivery of CRISPR-dCas9 systems. Recently, Jo et al.[164] described the preparation and characterization of PLGA nanoparticles with the carrier for delivery of CRISPR-Cas9. The nanoparticles were 160 nm in size with a DNA encapsulation efficiency of 80%. The release studies showed that in the first 24 h most of the DNA was released (approximately two or three copies of the plasmid released by nanoparticles). In this way, the system showed promise for carrying these biomolecules. Alallam et al.[165] described the preparation, optimization, and characterization of alginate nanoparticles, loaded with two CRISPR plasmids, through the electrospray technique. The results showed that the mean size and ζ potential were 228 nm and −4.42 mV, respectively. The nanoparticles presented an encapsulation efficiency of 99.0%, preserving the integrity of the charged material. In addition, the nanoparticles were able to successfully introduce the Cas9 transgene into HepG2 cells. According to the authors, future investigations of these nanoparticles may result in a nanocarrier suitable for *in vivo* delivery of the CRISPR-Cas9 system into plants. The Arg NPs carrying Cas9En (E-tag)-RNP (ribonucleoproteins) improved the efficiency of CRISPR-Cas9 gene editing by ≲30% in cultured cells. This method could be a suitable tool for facilitating target gene editing in crops.[166]

NANOPLATFORMS BASED ON DNA NANOSTRUCTURES AND DNA ORIGAMI

The use of nanocarriers built with DNA is an emerging tool for the delivery of biomolecules into mammalian cells and has great potential for plant cells. These DNA nanostructures can pass through the vegetal plant cell passively for gene delivery. This technology is based on the programmability of DNA Watson−Crick base pairing, which allows one to build and design the shape and mechanical properties of these nanostructures. The DNA origami techniques are also employed for the fabrication of different homogeneous nanostructures, which present different shapes and sizes varying from 50 to 400 nm.[167] They are also used for the delivery of drugs and also for genetic manipulation, the permeability being one of the main advantages of the use of the techniques. These nanostructures of DNA origami can work as a board, where different biomolecules will be projected for a more specific delivery. Moreover, these structures can also present themselves as three-dimensional containers, which have internal cavities for interaction with biomolecules protected from the external environment.[168]

One of the main limitations of these techniques is the stability of these nanostructures, often requiring coating or interaction with other molecules.[169,170] For example, in ref 171, Mikkila et al. coated the DNA origami nanostructures with the capsule of the chlorotic mottled bean virus to increase the structural stability. Another point to be addressed is that many times adaptations and optimizations of these DNA nanostructures are necessary depending on the tissue or species of plants to be used for the application. Variations in the size, shape, stiffness, or compactness of these structures are necessary to achieve maximum internalization because different species of plants and tissues can acquire different morphologies. Also, Zhang et al.[163] describe a protocol for the synthesis of DNA nanostructures. According to the authors, through their protocol, it is possible to obtain nanostructures with different sizes and shapes, with at least one lateral dimension of ≲10 nm and high rigidity. The nanostructures presented a high degree of cellular internalization in cells of the tobacco plant (*N. benthamiana*) and greater efficiency in silencing genes. The authors indicate that the results of gene internalization and silencing were strongly dependent on the geometry of the nanostructures.

In addition, Nguyen et al.[172] developed an approach to coating DNA origami structures with silica. After numerous tests, they were able to achieve reproducibility in the coating of nanostructures with ultrafine silica in solution. Still, according to the authors, the coated structures were stable in water and showed resistance to degradation mediated by nuclease. In addition, the coating with silica also allowed the preservation of structural integrity with polar organic solvents. Therefore, the results are promising for obtaining different nanostructures of DNA origami for plant applications. The work of Miyamoto et al.[173] describes the preparation of a gene delivery system based on the complexation of DNA nanostructures with two peptides for the efficient transfection of plant callus cells. According to the authors, the studies of internalization and cell transfection showed that the system based on the two peptides allowed a more efficient transfection, increasing the level of endocytic uptake.

CHALLENGES, RECOMMENDATIONS, AND FUTURE PERSPECTIVES

Current trends and advancements in the field of genetic engineering, especially plant genetic transformation with a focus on plant protection, have accelerated the process of developing and identifying plant variants that exhibit characteristics such as enhanced yields and resistance to pests, diseases, and other abiotic stresses. In addition, molecular tools such as miRNA, CRISPR-Cas, and RNAi have paved the way for site-specific genome manipulations in plants with enhanced

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precision. However, conventional protocols and techniques are limited in scope for several reasons such as the presence of the cell wall (a hard-to-penetrate barrier for biological molecules), plant-specific protocols (decreasing the application scope of biomolecules), and harm to biomolecules during application due to external environmental conditions and tissue damage to plants resulting in necrosis and browning. Different types of nanoparticles such as metallic, carbon, silica, polymeric, magnetic, and other nanoparticles can efficiently deliver these biomolecules into the plant's cells.

Nanoparticles are important vectors capable of overcoming the barriers of tissues and the cell wall of plants, mediating transformation processes through the transport of exogenous molecules across the plasma membrane. The delivery of miRNA, RNA, and CRISPR into plants, mediated by nanocarriers, has been realized quite recently and must be further developed. Currently, there is still a limited amount of research that has adopted the use of nanocarriers as transport agents for genetic manipulation in plants, which represents a promising strategy for controlling the incidence of pests and pathogens in important cultures around the world. The delivery of genetic material mediated by nanocarriers must target specific problems in plant−pathogen interactions. The use of this type of control can make agriculture less subject to the use of pesticides in large quantities, which should benefit the environment and human health. However, risk studies, both environmental and human and animal health, must be exhaustively carried out with products that are treated with nanoparticulate systems and genetically manipulated.

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Notes

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ABBREVIATIONS USED

MSNs, mesoporous silica nanoparticles; LDH, layered double hydroxide; GUS, β-glucuronidase; GFP, green fluorescent protein; BCMV, bean common mosaic virus; PDMAEM, polymeric dimethylaminoethyl methacrylate; NS, nanosheets; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; SWNTs, single-walled nanotubes; MWNTs, multiwalled nanotubes; PCL, polycaprolactone; PLGA, poly(lactideo-co-glycolide); ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; PAMPs, pathogen-associated molecular patterns; miRNAs, microRNAs; SE, SERRATE; HYL1, HYPONASTIC LEAVES1; AGO1, Argonaute family of proteins; RISC, RNA-induced silencing complex; RDR, RNA-dependent RAN polymerase; CRISPR, cluster regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein 9

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