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A Review of Nanotechnology as a Novel Method of Gene Transfer in Plants

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ABSTRACT

Purpose: *Nanotechnology has evolved as an effective tool in numerous fields including agriculture, medicine and engineering. Recently it's potential as an alternative genetic transformation method has been identified. However, a comprehensive understanding over nanoparticles and their behavior in living cells is important to realize the full potential of this technology in biotechnological applications. Therefore, we review the application potential of widely employed nanoparticles in plant transformation here.*

Literature/Background: *Development of new crop varieties with desirable traits via biotechnological applications is a solution for challenges associated with climate change and higher population growth. In such aspects, transformation of plant cells which is known as the process of changing one's genome by integration exogenous DNA, is an absolute necessity and results far better and improved stable characteristics in original. Rigid and multi layered cell wall impedes penetration of exterior biomolecules and hence causes the transformation process complicated. Even though, numerous conventional methods have been established for plant transformation, lower transformation efficiency, tissue damage and random integration of transgenes warrants the need for novel approaches. In this context, novel techniques have been explored and as a result nanoparticles have been found effective in transformation of protoplasts as well as intact plant cells. Nanoparticles internalized either via endocytosis or direct penetration release transgenes from nanoparticle-DNA complexes and result in transient or stable expression. Nanoparticles ensure higher transformation efficiency, no transgenic silencing and protection of biogenic molecules from degradation by intracellular nucleases.*

Keywords: *biotransformation, nanoparticles, Exogenous DNA*

INTRODUCTION

Food security and food safety are considered as major challenges in the present world attributed mainly to lower supply of food associated with climate change and inadequacy of arable lands to meet the needs of the growing population. Conventional measures taken to address this situation involve the improvement of existing crops and development of new cultivars with desirable traits by breeding strategies. Considering limitations of conventional breeding techniques where hybridization is limited within species or even at wide hybridization of wild relatives and requirement of longer period over several generations to attain desirable traits, the concept of genome modification using transgenic technology and gene editing have become popular. Genetic transformation of crops with the introduction of exogenous genes of interest (GOI) that attribute higher yield, improved nutritional quality, biotic resistance and abiotic tolerance have received attention in the recent past (Rafsanjani *et al.,* 2011; Zhao *et al.,* 2017).

Genome modification is a multistep process which consists of (1) introduction of GOI into cells and tissues (2) integration and expression of inserted DNA into host genome; nucleus (3) selection and culture of transformed cells

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(4) regeneration of entire plant from selected tissues (Rashid and Lateef, 2016). Of these steps, delivery of DNA into plant cells is the crucial step in genetic transformation in which rigid and multi layered cell wall impedes the traverse of biomolecules that are larger in size than size exclusion limit of cell wall (20 nm). From literature, it is known that over ten methods of transformation have been employed to deliver DNA across cell wall, plasma membrane and nuclear membrane in mammalian, animal and plant cells (Fu *et al.,* 2012; Rafsanjani *et al.,* 2012; Burlaka. *et al.,* 2015; Demirer and Landry, 2017; Cunningham *et al.,* 2018). These methods fall into three categories namely, physical, chemical and biological methods (Luo and Salzman,1999; Pasupathy *et al.,* 2008; Akhter *et al.,* 2011; Demirer and Landry, 2017). Each method has their inherent advantages and disadvantages. Major limitations of these methods are limited host range, low transformation efficiency, tissue damages, random integration of GOI into host genome etc.

In order to overcome these limitations, the need for a novel approach that is capable of loading biogenic molecules arises. Recently, nanoparticles of various sizes and shapes have gained potential in delivering DNA into plant cells and stable integration and expression of genes (Rai *et al.,* 2015). Particles range in size from 1 nm to 1000 nm are considered as nanoparticles (Akhter *et al.,* 2011: Chen *et al.,*2011). Specific characteristics of nanoparticle such as size, shape, chemical composition and surface charge make them efficient gene carriers. In this review, we discuss the methods currently utilized in genetic transformation with a special emphasis on nanoparticles as a novel mode of gene delivery into plant cells.

METHODS OF BIOTRANSFORMATION

Conventional methods of biotransformation have been broadly divided into two major groups; direct and indirect methods (Figure 01). Direct methods of transformation do not employ any biological vectors like bacteria, viruses for delivery of DNA into plant cells and it is carried out by means of physical or mechanical forces such as electric or magnetic fields, pressure and temperature. In contrast, indirect method of transformation utilizes biological vectors to deliver the DNA molecules. Common approaches established and utilized for direct method of biotransformation are discussed below.

Particle bombardment

Particle bombardment, commonly known as biolistic particle delivery or gene gun is widely employed from the early history of biotransformation to date. It is a method of gene transformation where desired genes are delivered by means of physical or mechanical force. The principle behind this method is the bombardment of plant cells with gold or tungsten particles loaded with exogenous DNA. These micro-projectiles are accelerated by pressurized helium gas and shot into host plant cells and tissues causing the DNA to enter into cells (Rai *et al.,*2015; Rashid and Lateef, 2016; Cunningham *et al.,* 2018). The ability to be utilized on species with higher level of heterozygosity and over a range of cells and tissues such callus, embryos, leaves make this method successful with a wider application potential (Rai *et al.,*2015). However, this method of delivery has some adverse effects such as non-specific localization, random integration of DNA, short term and low level of expression of genes; low transformation efficiency of about 2-20% (Jun *et al.,* 2008) and shallow penetration depth in plant tissues (Rai *et al.,*2015; Cunningham *et al.,* 2018).

Electroporation

The term "electroporation" itself explains the principle involved in this method of transformation. The short high voltage electric field, in particular effective pulse for plant cells ranges between 500-1000V/cm (Bates, 1995) forms transient pores in cell membrane permitting entry of plasmid DNA into cytoplasm (Rai *et al.,*2015; Rashid and Lateef, 2016; Demirer and Landry, 2017). Earlier, this transformation was successfully applied in protoplasts and later on intact plant cells e.g. meristems, pollen grains

(Demirer and Landry, 2017; Cunningham *et al.,* 2018). Though electroporation seems to be an easy, fast, effective and inexpensive method, its applications are limited to only a few plant species e.g. tobacco, rice, wheat, maize. In addition, mortality of cells is high due to higher voltage exposure, damages to delivered DNA creating inaccurate translational products (Rai *et al.,* 2015; Rashid and Lateef, 2016).

Microinjection

Naked DNA or other biomolecules of interest are directly injected into target tissues (nucleus/ cytoplasm) by means of a glass needle or a micropipette (0.5-1.0 µm diameter). Though this method has high transformation efficiency, usage of this method is limited to transform an individual cell at a time especially large reproductive cells like oocytes (Rakoczy-trojanowska, 2002; Rafsanjani *et al.,* 2012). Further, this method is time consuming, labour intensive and requires sophisticated tools.

Temperature mediated transformation

In this method of transformation, protoplasts in particular are subjected to a higher temperature (about 22 °C) for a shorter period (less than 1 minute) of time. The result is the formation of pores due to lipid fluidization permitting the entry of DNA into the cells (Rafsanjani *et al.,*2012). This method of transformation is economical and easy to adopt. However, low rate and poor efficiency of process, damages to both nucleic acids and cells due to higher temperature may limit the application of this method (Rafsanjani *et al.,* 2012; Burlaka. *et al.,* 2015).

Liposome mediated transformation

Liposomes are relatively small (50 nm in diameter) colloidal vesicular structures that consist of an internal aqueous compartment which can be embedded with desired cargo like plasmid DNA, protein etc. The surrounding hydrophobic phospholipid bilayer has the ability to fuse with cell membrane easily (Chen *et al.,*2011; Shirazi *et al.,* 2011). Unique structure of liposome makes them useful in delivering DNA into plant cells through passive diffusion (endocytosis). This method of gene transfer has several advantages including stability of gene, reduced DNA deletion, controlled release pattern, transformability of wide range of cells and non-toxicity (Rafsanjani *et al.,* 2012).

Silicon carbide mediated transformation

The principle involves in this method of transformation is, holes created upon rigorous and spiral mixing of plant tissue and plasmid DNA or GOI with silicon fibers, allow the entry of DNA into cells resulting stable transformation. Efficiency of transformation depends on fiber size, vortexing time, shape of vessels used and the thickness of plant cell wall (Asad *et al.,* 2008). This method of transformation is simple, less dependent on resources and cost effective (Songstad *et al.,* 1995; Wang *et al.,* 1995; Rashid and Lateef, 2016). However, there are some demerits such as possible health hazards with the use of silicon fibers, low gene transfer efficiency and cell damage (Asad *et al.,* 2008; Sailaja *et al.,* 2008).

Indirect methods of gene transfer

In contrast to direct methods, transformation of cells by indirect methods are carried out with the involvement of biological vectors. Though a number of bacteria are employed for transformation of plant cells, a gram-negative soil bacterium known as *Agrobacterium tumefaciens* has attained much interest in gene transfer because of its self-transferring ability of DNA. This method of transformation has several advantages such as higher transformation efficiency, random integration of DNA into the host genome, low rate of transgenic silencing and ability of transferring long stretches of DNA (Rafsanjani *et al.,* 2012;Cunningham *et al.,* 2018). Although, *Agrobacterium* mediated gene transfer is a commonly used technique, it has several drawbacks including difficulty in isolation and

manipulation of Ti plasmids due to their large size and lower number of copies (Rashid and Lateef, 2016). Furthermore, the narrow range of host plant species and tissue types, inability to perform transgene free editing, unsuitability for high throughput applications (Demirer. *et al.,* 2019) are noteworthy. A very low transformation efficiency (0.01%-20%) in monocotyledons (Jun *et al.,* 2008) is another considerable drawback inherent to this method of transformation.

Besides the bacterial vectors, viral vectors namely, Cauliflower mosaic virus-based vectors (CaMV), Cowpea mosaic virus (CPMV), Bean pod mottle virus (BPMV), Potato virus X (PVX), TMV based vectors, bacteriophage lambda vectors, gemini viruses are few examples that play a key role in DNA delivery into plant cells. Viral vector systems are seen as an effective indirect transformation method because of their higher (>90%) transfection efficiency, non-pathogenic effects of vector viruses, reliable integration of transgenes (Luo and Salzman,1999; Riley and Vermerris, 2017). However, viral vector-based delivery has some drawbacks such as toxicity, restricted targeting of specific cell types, limited DNA carrying capacity and high cost (Luo and Salzman,1999). Therefore, there is a necessity for non-viral delivery of DNA.

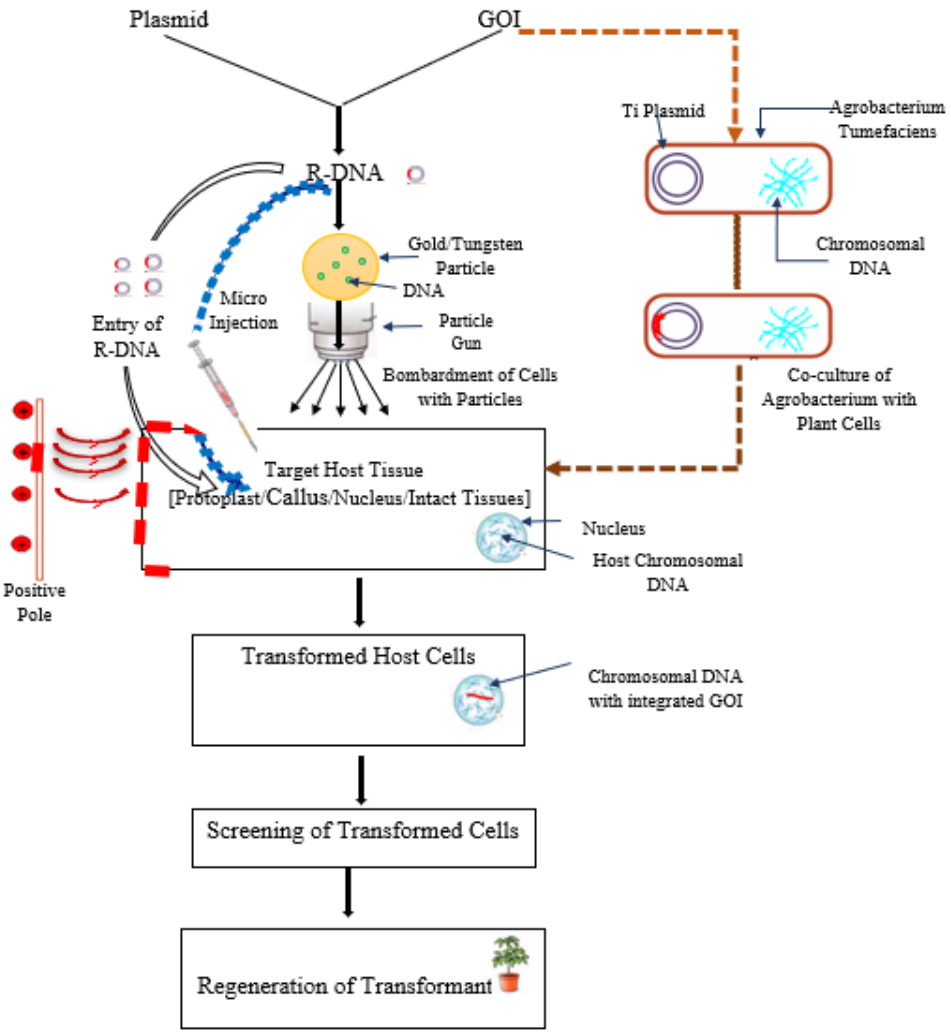


Figure 01: **Overall process of plant biotransformation by various methods; each colour arrow indicates different means; red-electroporation, blue-microinjection, black-particle bombardment and brown-*Agrobacterium* mediated gene transfer. (GOI-Gene of Interest, R-DNA-Recombinant DNA**

Nanoparticles Mediated Gene Transfer

What are nanoparticles?

Nanoparticles are the particles with dimension in nanoscale (Ball 2002; Roco 2003). There are of three types; natural, incidental and engineered (Roberto and Ruffini, 2009). Nanoparticles are synthesized by means of chemical, biological or physical methods. They vary vastly on their physico-chemical properties such as size, shape, surface charge, binding affinity to biomolecules and the surface adsorption capacity (Table 01).

Application of nanoparticles

Nanotechnology as a promising technology has extended its application in several sectors such as health and medicine, industrial (food, material science, electronics), environment and agriculture. Application of nanomaterials in medicine has been exploited in targeted drug delivery in several diseases and genetic disorders (Wang *et al.,* 2016). In the food industry, nanoparticles have been widely employed in various operational units of food processing, preservation, packaging and distribution (Parisi *et al.,* 2015). Further, nanotechnology makes the materials and coatings stronger, lighter, durable, more reactive, better in electrical conductivity and also improves absorption of cosmetics, and resistance to wrinkling and bacterial growth (Kay, 2018). In the field of environmental sciences, nanotechnology is applied in water treatment in which silver nanoparticles are used to filter contaminated water and to improve quality (Kay, 2018). Beyond the above said applications of nanoparticles, applications in agriculture and or plant sciences are extensive. Nanomaterials in particular, nano fertilizers, nano pesticides are aimed at minimizing losses of nutrients, supply of agricultural inputs upon demand by the plants and increase the yield thereby ultimately reduce the adverse effects on plants and environment (Gogos *et al.,* 2012; Parisi *et al.,* 2015; Wang *et al.,* 2016). In addition to these agricultural management activities, nanotechnology aids in addressing challenges in breeding against biotic and abiotic stresses and increasing productivity while ensuring sustainability (Parisi *et al.,* 2015), in which transformation of plant cells by means of nanoparticles has received attention in recent years. Hence, nano particles have found potential in repairing mutation induced diseases via genome editing as well by delivering the genome editing tool along with its components like Cas9 (Lee *et al.,* 2017). Accordingly, this review briefly discusses potential application of numerous engineered nanoparticles that have been widely employed in biotransformation.

Table 01: Physico-chemical characteristics of nanoparticles.

Nanoparticle	Z-average size (nm)	Zeta potential (mV)	Shape	Use	Reference
Calcium phosphate	20-55	-25.6	Spherical	Biomedical	Naqvi *et al.,* 2011; Ardekani *et al.,*2014
Quantum dots (ZnS)	3-5	-32.46		Molecular biology, biomedical	Fu *et al.,* 2012
Magnetic nanoparticle	20-168	+48.2	Spherical	Agriculture	Hao Y *et al.,* 2013; Zhao *et al.,* 2017
Mesoporous nanoparticle	20	-21.4	Hexagonal	Biomedical, plant biology	Hussain *et al.,* 2013
Mesoporous silica nanoparticle (Gold functionalized)	600	-25.5	Hexagonal	Plant biology	Martin-Ortigosa *et al.,* 2012
Mesoporous nanoparticle (Fluorescein isothiocyanate filled)	50	21.7	Hexagonal	Biomedical, plant biology	Chang *et al.,* 2013
Single walled carbon nanotube	Height-1.3 Length 5-20µm Diameter 1-2 nm	-51.9	Cylindrical/ tube	Molecular biology	Burlaka *et al.,* 2015; Demirer *et al.,* 2017
Multi walled carbon nanotube	Length 2.5-20 µm Outer diameter 6-13 nm Inner diameter 2-6 nm			Molecular biology	Burlaka *et al.,* 2015
Starch nanoparticle	50-100			Plant biology	Jun *et al.,* 2008
Synthetic polymer (Dendrimer)	4.5			Molecular biology	Pasupathy *et al.,* 2008

Carbon based nanoparticles

Carbon-based nanoparticles are one of the inorganic forms of nanoparticles (Ghouri *et al.,* 2020) and fullerenes and carbon nanotubes are known as carbon-based nanoparticles (Chandrasekaran *et al.,* 2020). Carbon nanotubes (CNT) are cylindrical structures made of graphene sheets that are rolled into a tube. There are two types; single walled and multi walled CNT. Single walled CNTs (diameter ranges 0.4-3 nm) consist of a single graphene layer while multiwalled CNTs (diameter ranges 4-30 nm), are composed of more than two graphene sheets centrically arranged within each other. The outer diameter of tube ranges from 2 to 100 nm while the inner diameter is 1-3 nm (Burlaka *et al.,* 2015; Riley and Vermerris, 2017). Unique physical and chemical properties such as small size, high aspect ratio, tensile strength, high surface area to volume ratio, biocompatibility and biostability have made these nanotubes efficient gene carriers.

Several researchers studied transformation ability of CNT. Accordingly, Burlaka *et al.* (2015) found that, mesophyll protoplast of tobacco (*Nicotiana tabacum)* was transiently transformed by both single walled CNT (16%) and multi walled CNT (13%) with plasmid DNA pGreen 0029, in contrast, intact plant cells (callus and leaf explants) of *Nicotiana tabacum* were stably transfected by the CNT. The transformation frequency of single walled CNT was higher with callus (8%) and leaf explants (6%) than multi walled CNT (3%, 2% respectively) as larger diameter of multi walled CNTs were least permitted to pass through the pores of cellulose cell wall. The method of binding (direct adsorption or electrostatic adsorption) influences the efficiency of delivery of the biomolecule into plant cells (Demirer *et al.,* 2019). Recently, Demirer et al. (2019) reported localization of single walled CNT-DNA (62%) in mature monocot (wheat) and dicot (*Nicotiana,* argula and cotton) leaves. Further, single walled and oxidized multi walled CNT successfully penetrated into protoplast, mesophyll cells of *Arabidopsis thaliana* (Yuan *et al.,* 2011) and protoplast of *Catharanthus roseus* (Serag *et al.,* 2011) respectively. Though these findings imply the potential ability of CNT in transformation, less solubility and dispersion due to their hydrophobic nature limits their application. However, this can be rectified through functionalization of the nano particle which is known as modification of surface of nanoparticles that improves their properties and enables them to play a major role (Riley and Vermerris, 2017). Functionalized CNTs are less toxic and show higher gene expression (Mohajeri *et al.,* 2019). Carbon nanotubes functionalized with polyethyleneimine (PEI) had potential to traverse cell and transfer the ssDNA-FITC into German chamomile (L *Chamomilla* M), hence, CNTs along with ultrasound showed improved gene transfer as they protect DNA from destructive enzymes and ultra sound waves (Ghaghelestany *et al.,* 2020). Furthermore, Demirer et al. (2019) observed 700 times greater expression of GFP when plasmid DNA is combined electrostatically to single walled CNT functionalized with PEI than plasmid DNA adsorbed to CNT via dialysis. Similarly, single walled carbon nanotubes complexed with chitosan were capable of transferring the DNA into chloroplasts of mature plants (such as *Eruca sativa, Nasturtium officinale, Nicotiana tabacum and Spinacia oleracea*) as well as mesophyll protoplast of *Arabidopsis thaliana* without external forces like physical, chemical and expressed transiently (Kwak *et al.,* 2019). Besides, carbon dots as another carbon based nanomaterial have been found efficient in transformation of plants. Doyle et al. (2019) addressed that plasmids coated with PEG functionalized carbon dots entered the intact cells of cereals like wheat, maize, barely and sorghum upon foliar application and showed transient nuclear targeted expression of GFP. Furthermore, foliar spray of carbon dots- plasmid complex carrying Cas9 and gRNA resulted transient genome editing in *SPO11* genes in wheat. This study implies that application of carbon-based nanoparticles has widened its potential in transformation as well as genome editing where conventional methods are impossible due to their versatility, non-toxicity, no adverse effects on growth and photosynthesis etc.

Silica based nanoparticle

Silica nanoparticles are also known as silicon dioxides nanoparticles. Silica-based nano particles are of either porous or non-porous type. Mesoporous silica nanoparticles (MSNP) that feature like honeycomb have been widely utilized in gene delivery systems in plant cells. Unique characteristics of this nanoparticle such as chemical and thermal stability, mesoporous structures, large surface areas (>800 m^2g^{-1}), tunable pore sizes (2–10 nm in diameter) and well-defined surface properties have made them ideal in the delivery of molecules of interest in plant and animal cells (Hussain *et al.,* 2013; Torney *et al.,* 2007).

Moreover, unlike the other nanoparticles, this type of nanoparticles prevents leaching of molecules as they are entrapped inside the pores and encapsulated with caps. Further, silica materials are safe, biodegradable and biocompatible (Xia *et al.,* 2009). Mesoporous silica nanoparticles have low toxicity and are even more effective in shielding the genetic material within plant tissues from lysis (Li *et al.,* 2018). MSNPs are used to deliver an array of biogenic molecules of various sizes, shapes and functionalities such as protein, ssDNA, dsDNA, RNA in plant (e.g. onion epidermis, tobacco mesophyll protoplasts) and animal cells (Torney *et al.,* 2007; Martin-Ortigosa *et al.,* 2012; Hussain *et al.,* 2013).

Furthermore, surface functionalization of these nanoparticles with several organic and inorganic molecules such as gold, cationic polymers (e.g. polyethyleneimine, polyamidoamine, polylysine) through either covalent or electrostatic interaction enhances their efficiency of delivery (Radu, 2004; Bharali *et al.,* 2005; Xia *et al.,* 2009). Surface functionalized MSNP bound with plasmid DNA (pPZP122:35S: GUS) showed transient transformation in intact tomato cells under *in vivo* conditions and plants transformed with injection of pDNA-MSNP onto lower leaves surface developed more resistance against *Tuta absoluta* (Hajiahmadi *et al.,* 2019). Hence, Torney et al. (2007) reported successful transformation by MSNP functionalized either with TEG (Triethylene glycol) or gold in mesophyll protoplast of tobacco (*Nicotiana tabacum)* and intact plant tissues such as tobacco cotyledon and immature maize embryos (*Zea mays*). Martin-Ortigosa et al. (2014) reported that delivery of Cre recombinase protein by means of gold plated MSNP resulted excision of *lox*P flank genes from maize genome. These findings imply that MSNPs being a carrier material in transformation process, have developed potential to be used in genetically modified crops (Rastogi *et al.,* 2019).

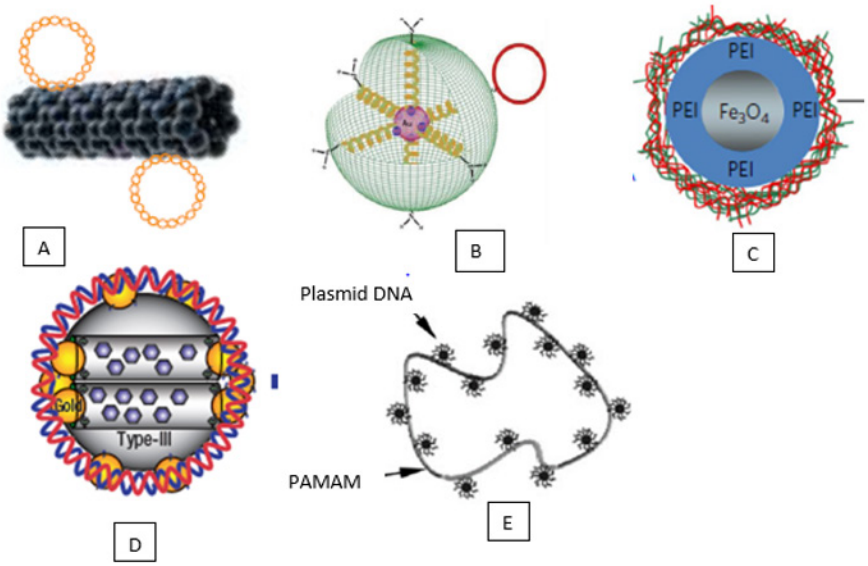
Metal based or magnetic nanoparticles

Metallic nanoparticles (MNP) are of either elemental forms such as gold, silver or oxides such as iron oxide (Figure 02), copper oxide, zinc oxide, magnesium oxides, calcium oxides (Sanzari *et al.,* 2019). Due to their own characteristics of small size and positive surface charge, negatively charged DNA molecules are attracted to MNP and can be used as a biomolecule carrier effectively. Transfer of biomolecules by MNP has been documented in a number of studies. Mortazavi and Zohrabi (2018) stated that gold nanoparticles delivered the plasmids pUBC (carries *cry*IA(c) gene) and pTra132 (carries *hph* gene) into embryogenic rice lines and further, rice genome was successfully integrated. Similarly, Hao et al. (2013) examined the MNP for their latent qualities of delivering plasmid DNA (pBI221 harboring the GUS gene) into canola cells (protoplast and walled cells) in which microscopic images of cells stained in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), confirmed the delivery of plasmid DNA by this nanoparticle. Moreover, MNP have the ability of delivering DNA not only into vegetative tissues of plants but also to transfect reproductive tissues. Zhao et al. (2017) studied the potential of MNP to transfect pollen grains of cotton, in which microscopic images of pollen grains and pollen tubes confirmed temporal and spatial internalization of MNP coated with plasmid DNA (pBI121). Moreover, it was observed that, integration and expression of *Bt* gene in transgenic cotton plants regenerated from transformed seeds produced by artificial pollination with magnetofected pollen (containing pBI35SBTΔα-CPTI plasmid)

showed resistance against insects. Even though, in general, magnetic nanoparticles which have a core of iron oxide are non-biocompatible to cells and modification with PEI, gold nanoparticle makes them biocompatible (Hao *et al.,* 2013; Zhao *et al.,* 2017). AuNP improved with amino acids poly-L-lysine and Arginine respectively showed higher transformation efficiency of 63.3% and 60% than 20% in *Agrobacterium* mediated transformation (Bansod *et al.,* 2019). Use of gold nanoparticles probes for gene transformation is a less time consuming, toxic free, cost effective and less sophisticated method of transformation. Apart from these, gold nanoparticles, as biosensors, have potential to confirm transformation and expression in transformed plants. Accordingly, Ghazi *et al.* (2018) reported that gold nanoparticles probes do not require toxic materials like ethidium bromide and take less than one-hour time and are five times cost effective to confirm the GUS gene transfer in Zabol mildew melon (*Cucumis melo*) compared to PCR, southern blot hybridization and real time PCR which require hazardous substances and take about 2.5 hours, 2-3 days and 3 hours of time respectively.

Calcium phosphate (CaP) is also known as another inorganic metal-based novel, safe, effective non-viral nanoparticulate gene carrier, ranges in size up to 100 nm. Presence of calcium ions potentiate these carriers for safe delivery due to compaction of DNA with Ca ions and their osmotic balance that protects DNA from endosomal digestion (Naqvi *et al.,* 2012). Use of CaP nanoparticles as a non-viral and competent transforming carrier was evident in a number of researches. In particular, with CaP mediated DNA (binary vector pCambia 1301) genetic transformation of hypocotyl of *Brassica juncea* was greater (80.7±6.3) than *Agrobacterium* mediated transfer (54.4±3.5) with respect to the expression of GUS reporter gene (Naqvi *et al.,* 2012). Similarly, Ardekani *et al.* (2014) also utilized CaP nanoparticles to successfully deliver plasmid DNA (PBI120) carrying exogenous GFP into genome of tobacco (*Nicotiana tabacum*) plants.

Quantum dots, often described as artificial atoms that include CdSe, ZnS, CdS are another kind of metal-based nanoparticles (Sanzari *et al.,* 2019) that has been successfully used as gene carriers. For example, ZnS modified with positively charged poly-L-lysine (PLL) to bind plasmid DNA (pBI121) entered young tobacco (*Nicotiana tabacum*) leaves by ultrasound method and resulted effective integration of GOI into the tobacco genome causing stable expression in tobacco (*Nicotiana tabacum*). Moreover, ZnS-PLL gene carrier displayed the ability to protect DNA from ultrasonic damage (Fu *et al.,* 2012).



(Source: Torney et al., 2007; Pasupathy et al., 2008; Hao et al., 2013; Demirer and Landry, 2017; Zhao et al., 2017).

Figure 02: Schematic representation of various nanoparticles loaded with DNA: A-CNT, B and C-MNP, D-FITC-Au-MSNP, E- PAMAM

Organic based nanoparticles

Synthetic polymers

Nanoparticles described above are inorganic forms of nanoparticles. Organic based nanoparticles are the other form of nanoparticles and numbers of organic based nanoparticles are found efficient in delivery of genes as well as genome editing. Polymer based nanoparticles, lipid-based nanoparticles, cell penetrating peptides and cell membrane derived vesicles are a few of examples of bioactive carriers used in gene delivery as well as genome editing (Mashel *et al.,* 2020).

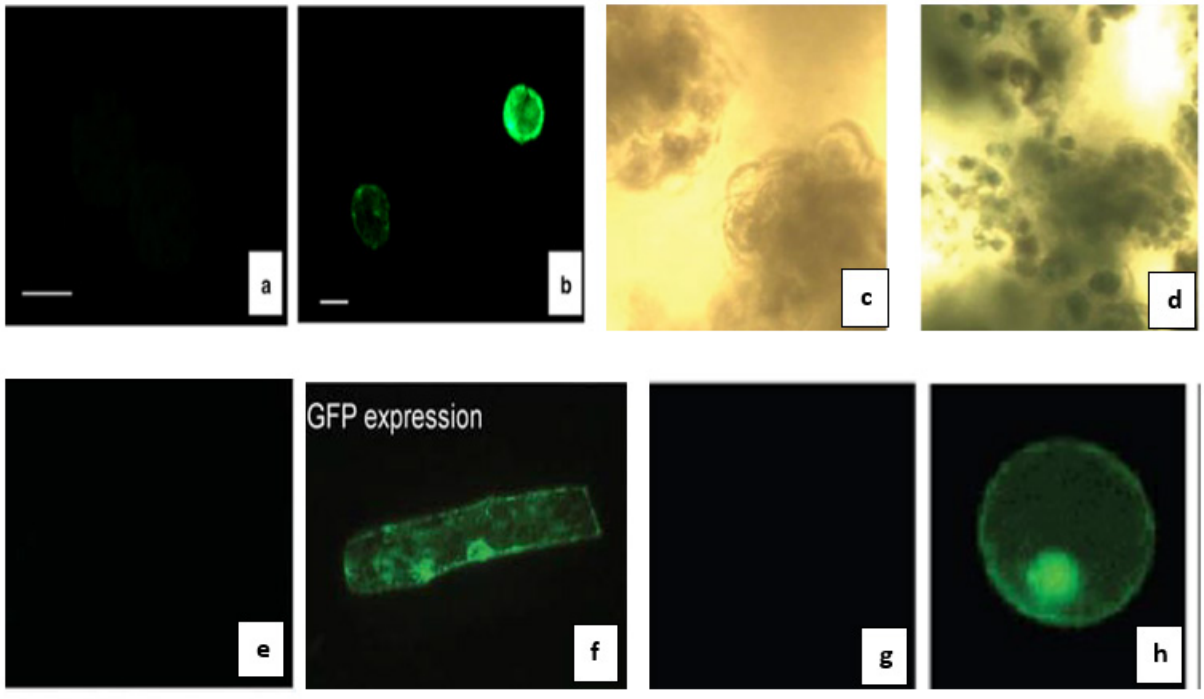
Polymeric nanoparticles are defined as sub-micron (1-1000 nm) colloidal particles comprising active pharmaceutical ingredients encapsulated within or adsorbed to macromolecular substances. Synthetic polymers can be either of oligoelectrolyte containing 2-dimethyl-aminoethyl methacrylate or polyelectrolyte with primary and tertiary amines at interior and exterior surfaces. The linear or branched cationic polymers form supramolecular complexes with exogenous DNA via hydrophobic, hydrophilic and electrostatic interactions (Pasupathy *et al.,* 2008; Li *et al.,* 2012; Samal *et al.,*2012; Finuk *et al.,* 2014; Finuk *et al.,* 2017). Dendrimers (polyamidoamine), one of the synthetic polymers, receive more attention in biotransformation because of their key properties like well-defined molecular shape, controlled chemical structure, high ratio of multivalent surface moieties to molecular volume, high water solubility, prevalence of a large number of chemically versatile surface groups, and unique symmetric architecture (Maiti *et al.,* 2004; Pasupathy *et al.,* 2008;). Jiang et al. (2014) addressed that the cationic G2 dendrimer had the potential to deliver DNA into *Arabidopsis* root cells and furthermore, delivery of dsRNA mediated by dendrimer caused suppression of *STM* and *WER*. Finuk et al. (2017) investigated DNA delivery capacity of oligoelectrolyte polymers in tobacco (*Nicotiana tabacum*) and moss (*Ceratodon purpureus*), in which poly cationic carriers, TN 83/6 and TN 84/5 successfully transformed protoplast of moss. Further, highest frequency of transient YFP gene expression was reported by TN 84/5 while transformation frequency of higher molecular weight polymers was less in tobacco (*N. tabacum*) protoplasts. Similarly, Pasupathy et al. (2008) reported transformation of creeping bent grass (*Agrostis stolonifera*) with plasmid DNA-polymer complex formed between generation 4 polyamidoamine dendrimer (G4 PAMAM) and plasmid DNA containing GFP reporter gene and observed nuclear localized fluorescence upon incubation of callus cells with the DNA-polymer complex. Similarly, lipid-based nanoparticles too have potential to deliver DNA and mRNA and a lipid-based nanoparticle; zwitterionic amino lipids showed improved capacity to deliver Cas9 mRNA and sgRNAs into animal as well as human cells (Miller *et al.,* 2017; Wan *et al.,* 2019).

Starch nanoparticles (StNP), another kind of organic nanoparticles, are defined as particles that have at least one dimension smaller than 1000 nm, but are larger than a single molecule or atom and varies between 10-700 nm (Sun, 2018). These nanoparticles have been suggested as novel biomaterial for transformation due to their smaller size and high surface-to-volume ratio. Surface functionalization of this type of nanoparticles enhances conjugation with plasmid DNA. Jun et al. (2008) studied its ability to transfer the biomolecules into cell suspension of *Dioscrea zigiberensis* G H Wright. The study reports that the ultrasound mediated transfer of surface functionalized with poly L lysine and RuBPY fluorescence material starch nanoparticles combined with pEGAD plasmid DNA effectively traversing cell wall, cell membrane and nucleus membrane of plant cell suspension. Plasmid DNA starch-nanoparticle complexes protected DNA from ultrasound damage as well as from DNase I cleavage.

Delivery of nanoparticles

Biological membranes with different pore size exclusion limits restrict passage of nanoparticle-DNA complexes thus ultimately influencing the integration and expression of genes in the host genome. Unlike animal and mammalian tissues, cell wall with size exclusion limits 5-20 nm (Chang *et al.,* 2013) impedes penetration of

exogenous DNA into plant cells. Irrespective of the tissue type, nanoparticles are taken up by plant cells either via endocytosis or direct penetration (physical or non-physical) methods. For example, nanoparticles such as calcium phosphate, mesoporous silica particles are taken via endocytosis (Torney *et al.,* 2007; Naqvi *et al.,* 2012). Physical methods of delivery include biolistic, electroporation, sonoporation, magnetofection while non-physical methods include co-culture (passive diffusion or incubation), infiltration and cationic transfection (Jun *et al.,* 2008; Pasupathy *et al.,* 2008; Fu *et al.,* 2012; Martin-Ortigosa *et al.,* 2012; Naqvi *et al.,* 2012; Chang *et al.,* 2013; Zhao *et al.,* 2017; Demirer *et al.,* 2019). During delivery, nanoparticles use various mechanisms for the internalization inside the tissues. The possible mechanisms are pore size enlargement, creation of transient pores in biological membranes, loosening of micro-fibril network in cell wall structure during cell wall relaxation, binding to carrier protein, or membrane embedded transporter proteins and induction of transient channels (Jun *et al.,* 2008; Chang *et al.,* 2013; Rai *et al.,* 2015). The uptake and efficiency of delivery of nanoparticles depend on a number of factors such as size, surface charge, ratio between nanoparticle and DNA, surface functionalization, binding affinity of nanoparticles to DNA and interaction between negatively charged cell wall and cationic nanoparticles. For example, in general, nanoparticles smaller than size exclusion limit of cell wall and plasma membrane traverse easily while internalization of cationic nanoparticle is faster than anionic ones. Similarly, functionalized nanoparticles are taken up by the cells easily thus resulting better transformation than the non-functionalized ones. Successful delivery of nanoparticles into plant cells can be detected with expression of fluorescence components via microscope (Figure 03).



Source: Martin-Orgosa et al., 2012; Hao et al., 2013; Finiuk et al., 2017; Demirer et al., 2019

Figure 03: Microscopic images for delivery of plasmid DNA by nanoparticles and subsequent expression: a-YFP gene expression in tobacco protoplasts with delivery of pGreen 0029 plasmid without polymer, b-with polymer, c-GUS gene expression in canola walled cells with delivery of PBI221 plasmid without MNP, d-with MNP, e-green channel fluorescent microscopy images of onion epidermis cells bombarded with empty Au-MSN, f-GFP expressing plasmid DNA coated Au-MSN, g-GFP expression in argula protoplasts incubated with free DNA, h-plasmid DNA carrying CNT.

MERITS OF NANOPARTICLES MEDITAED TRANSFORMATION OVER CONVENTIONAL METHODS

Damage to DNA and plant cells caused by excessive chemicals and energy applied in chemical and physical methods is one of the common drawbacks in conventional method of biotransformation. For successful expression of exogenous genes during development of transgenic plants, the stability of inserted genes is crucial. DNA molecules should be safeguarded from extracellular lysosome fused with endosomes (endosomal escape) and intracellular degradation (cytosolic stability) by nucleases (Roy *et al.,* 2005). Nanoparticles play a key role in maintaining stability *via* several mechanisms. Capability of nanoparticles for DNase I protection was examined by several researchers in which integrity of DNA was assessed by PCR. CaP nanoparticles encapsulated with exogenous plasmid DNAs pCambia 1301 (Naqvi *et al.,* 2012) and pBI121 (Ardekani *et al.,* 2014) were protected from degradation by intracellular nucleases due to compaction of DNA with CaP. Due to smaller size and surface effect of starch nanoparticles, the DNA-nanoparticle complex (pEGAD plasmid DNA-PLL-StNP) was protected from DNase I cleavage (Jun *et al.,* 2008). Similarly, dimethylaminoethyl metacrylate (DMAEM) based cationic polymers protect DNA against cleavage by DNAse I due to repulsion of Mg^{2+} ions by amino groups and hindrance of accessibility of enzymes to DNA (Finiuk *et al.,* 2017). Further, endosomal escape and cytosolic stability were reported by MSNP (Hussain *et al.,* 2013) in which MSNP functionalized with TEG remained stable in endocytotic vesicles in cytoplasm and CNT (Burlaka *et al.,* 2015).

Moreover, nanoparticles are known to be non-toxic and biocompatible with plant tissues. Cytotoxicity of nanoparticles has been evaluated in several studies. Demirer et al. (2019) reported that CNT has no toxicity and causes no tissue damage in tobacco (*Nicotiana tabacum)* leaves as indicated by non-upregulation of NbrbohB gene. Furthermore, similar photosynthesis quantum yield was observed in both infiltrated leaves and non-infiltrated ones. Hussain et al. (2013) witnessed the uptake of MSN by wheat root during post germination in MS liquid medium solution and lupin roots in hydroponic system and did not cause any toxic effect and nanoparticles were found to be internalized near and within the cell wall of wheat root and xylem of lupin roots. Finiuk et al. (2017) studied genotoxic and cytotoxic properties of polymer PDMAEM based nanoparticles in meristem cells of *Allium cepa* roots and protoplasts of tobacco (*Nicotiana tabacum)* based on miotic index and number of viable protoplasts. It was observed that cationic polymers TN 84/5 was least cytotoxic, even at extreme concentration of polymer (5×10^{-2}). This resulted in 3% of live protoplasts and genotoxicity was found to be low with higher mitotic index and low degree of chromosomal aberrations.

CONCLUSION AND FUTURE PROSPECTS

Nanoparticles of varying size, shape, surface charge have been found as an attractive and effective alternative for delivering biomolecules into protoplasts and intact cells as well. Once delivered, successful integration of transgenes into host genome is crucial in the stable expression of transgene which was evident in the nanoparticles. A number of nanoparticles like ZnS, carbon nanotube, CaP, Polymers, Starch nanoparticles have been recognized for their potential to deliver genes into plant cells and for the stable integration of the transgene. However, application of nanoparticles has been studied only in a few plant species including cotton, tobacco, tomato, canola, *Arabidopsis*, wheat, lupin, pumpkin, maize, and potato and studies performed in monocotyledons are very limited to best of authors' knowledge. Further, application of certain types of nanoparticles are not yet studied in plant tissues. With the proven success, there is avenue for testing the capability of nanoparticles in other crop species that are difficult to transform by conventional methods. Thereby, breeding of plant species against biotic and biotic stress would become possible and easy with this novel method of gene transfer rather than relying on conventional method thus in turn would enhance food production in the world to meet the growing demand.

Declaration of conflict of interest

The authors declare that there is no potential conflict of interest.

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