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Original Article

CALCIUM PHOSPHATE NANOPARTICLES A NOVEL NON-VIRAL GENE DELIVERY SYSTEM FOR GENETIC TRANSFORMATION OF TOBACCO

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ABSTRACT

Nanotechnology plays a unique and novel role to develop new methods for genetic engineering. Calcium phosphate (CaP) has various significances in biomedical systems; one of them is plasmid DNA (pDNA) delivery for decades as transfection efficiency relative to the non-viral approaches. In this study, we developed a novel gene transfer carriers, nano-calcium-phosphate (CaP) that provides consistently efficient and satisfactory pDNA delivery in tobacco. Results showed that CaP nanoparticles successfully delivered pBI121 harboring GFP driven by 35S promoter-encoding plasmid DNA into tobacco cells. Characterization of nanoparticles were done using DLS, TEM and found 25-55 nm range. While X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analyses were done to confirm the hydroxyapatite nature of the synthesized CaP nanoparticles. Polymerase chain reaction (PCR) detection, were carried out for the regenerated plants. The established genetically transformed tobacco plants mediated by CaP nanoparticles can be achieved. These studies revealed the significant efficacy of CaP nanoparticles as non-viral gene delivery in tobacco plant transformation and proved a novel system for plant genetic modification.

Keywords: Calcium phosphate nanoparticles, pDNA delivery, Tobacco, PCR, Transformation, pBI 121, GFP.

INTRODUCTION

Calcium phosphate (CaP) plays a key role in several biomedical applications, and it is very important that the interaction of CaP with biological systems should be clearly understood for accomplishing a successful biomedical approach. Viral gene delivery system exposed to several problems like very difficult to production, acute inflammatory response that exhibits the limited prospects for repeated administration and show the low immune responses. Hence, the demand for non-viral gene delivery systems increased. Calcium phosphate was first reported by Graham and Van Der Eb in 1973 for DNA condensation, increased the transfection efficiency and easy preparation [1]. After this report CaP was used in several biomedical applications for gene delivery. Various nanoparticles have been popularized as gene transporter in the mammalian cells [2-4], whereas in plant cells, this non-viral gene delivery system is still very limited [5,6], As, this study revealed approach to apply calcium phosphates as non-viral gene delivery agents for tobacco. Today's era, there is an all accepted requirement for the development of novel, safe and effectual non-viral gene delivery to be used for tissue regeneration, transfection, cancer treatment, DNA vaccines, etc. There are so many approaches are available as non-viral gene delivery which have potential advantages towards tissue-specific targeting [7], production enhancement [8, 9], large DNA inserts potency [4] and also exhibit immunogenicity reduction [10-12] as comparison to other viral approaches. However, these non-viral systems have lower transfection efficiencies as compared to viral approaches [12, 13]. Hence, there is a requirement to develop a new, non-viral carrier which can deliver plasmid DNA (pDNA) in an effectual and efficient manner for plants.

Plant engineering has many problems in transformation of multigenes and towards their safety and so on, where as the nanoparticles has novel approach to promises to solve the gene delivery systems with the potential application in plant transformation [14]. Therefore it is very much needed to new world to explore nanoparticles gene carriers in plant gene engineering. In the gene delivery of plant cells, many factors have to be considered that make it challenging as the presence of cell walls, methodology, the receptor types and so, nanoparticles as plant gene delivery system must be studied extensively [15].

Nanoparticles as gene delivery systems have significant advantages compared with existing viral-gene delivery systems. As already nanoparticles were applied to monocotyledons and dicotyledonous plants [16]. The non-viral-gene delivery systems of nanoparticles successfully controlled the copies of DNA and overcame to transgenic silencing. The nanoparticles can be easily functionalized as per demand of many receptors of plant cell to enhance transformation efficiency.

There are number of methodologies have been used for a long time in traditional plant transgenic methods such as particle bombardment, ultrasound etc. However, these methods have many limitations and disadvantage as most of the times naked DNA is damaged or destroyed. In this new technology era, researches proved that nanoparticles can avoid DNA damage and enhance transformation effectiveness [17, 18]. In present study, we provided a new method to use CaP nanoparticles as a gene delivery system for tobacco plant.

MATERIALS AND METHODS

Materials

Glacial acetic acid, absolute ethanol, di-sodium hydrogen phosphate, Tris–HCl buffer, and n-hexane (AR grade) were obtained from Merck (India) and used without further purification. Ethidium bromide Sodium hypochlorite and calcium phosphate were purchased from Himedia Lab. (India). Agarose gel and Lambda Eco RI/Hind III DNA ladder were procured from Fermentas. The surfactant i.e. sodium bis-(2-ethylhexyl) sulfosuccinate (AR grade) were purchased from Sigma-Aldrich (St Louis, MO).

Plant materials

For transformation studies, seeds of Tobacco plants will be germinated on MS basal medium the tissue culture laboratory of Centre for Transgenic Plant Development (CTPD), Jamia Hamdard, New Delhi, India. The explants from these plants were used in regeneration experiments and MS basal medium with various hormonal combinations were used for transformation. The transformation was confirmed by observing expression of the transgene, GUS.

Plasmid isolation

The vector pBI121 with GFP gene as plant selection marker was isolated from transformed *E. coli* DH5a. The *E. coli* DH5a was

cultured and centrifuged at 10,000 rpm for 10 min at 4°C.The cells were carried out in alkaline medium and treated with potassium acetate solution. Further cells were incubated in ice for 1 hour and the lysed cells were centrifuged at 10,000 rpm for 25 min at 4°C. DNA was precipitated with iso-propanol. The pellet was washed in thrice with 70% ethanol and drying. It was dissolved in Tris–HCl/EDTA buffer. The plasmid, pBI121 with GFP construct, was used analysis of its presence in transformed tobacco.

Methodology

Synthesis of pBI121 harboring GFP -encoding pDNA loaded CaP nanoparticles

The binary vector pBI121 loaded CaP nanoparticles were developed with an AOT (Aerosol-OT)/hexane/water reverse micellar followed by the already described methodology [19] with modifications.

The set A was prepared as:

1. The 0.2 M AOT solution in hexane was prepared. The 10 ml of AOT solution, 40 ml aqueous solution of 2.72 M calcium chloride and 30 ml aqueous solution of pBI121 (300 mg ml^{-1}) were mixed with continuous stirring for 16 h to form reverse micelle A.

The set B was prepared as:

2. The 10 ml of 0.2 M AOT/hexane solution, 40 ml of 0.2 M Tris–HCl buffer (pH 7.4) and 30 ml aqueous solution of 0.35 M Na_2HPO_4 were added with continuous stirring for 16 h to form reverse micelle B.

Water is added in excess volume to make 200 ml for both sets to adjust Wo, i.e. the molar ratio of water to surfactant. Stirring was continued until both the A and B sets became optically clear. The B was, then, added to A set with help of burette at 4-5 drops per min rate with continuous stirring at 4°C. After complete addition of set B to A, stirring was continued for 16 h at 4°C. The translucency was created that indicated the formation of nanoparticles. To separate the nano-particles from the AOT/hexane, the 15 ml absolute ethanol was added at 4°C to break the reverse micelles into oil and aqueous phases and the nanoparticles settled down in round bottom flask. Further particles were separated by centrifugation at 12,000 rpm. The particles were washed with ethanol thrice in order to remove excess surfactant. Now, the nanoparticles were dispersed in 15 ml double distilled water and were dialyzed 16 h using a 12 kD cutoff cellulose membrane. After dialysis, the solution of nanoparticles was lyophilized to get the dry sample.

Physical characterization of CaP nanoparticles

FTIR analysis of the nanoparticles

Fourier transform infrared spectroscopy (FTIR) was conducted on synthesized CaP nanoparticles. Dry powder of nanoparticles 1mg was mixed with KBr powder and a transparent tablet was formed using an agate mortar. All of the spectra were collected in the 4000–400 cm^{-1} wavelength range.

Size and morphology of the nanoparticles

Dynamic light scattering (DLS) and Zeta potential

Particle sizes were determined using dynamic light scattering (DLS) with help of Malvern zeta sizer at Jamia Hamdard, New Delhi, India for CaP–pDNA nanoparticles. For the experiment, 5 ml of the synthesized CaP–pDNA nanoparticles solution were prepared in ddH_2O. The particle sizes were determined from the autocorrelation function using the Stokes–Einstein equation: r ¼ kT/ D6pZ, where r is the particle radius, k is the Boltzmann constant, T is the absolute temperature, D is the diffusion coefficient, and Z is the viscosity of the liquid in which the particles are suspended [20].

Transmission electron microscopy (TEM)

The nanoparticles were suspended in ddH_2O and one drop of sample was put onto a formvar carbon coated grid (Ted Pella, Inc.) and allowed to dry for 5 min. The grid was then stained with 2% uranyl acetate, rinsed in 95% ethanol and allowed to air dry for 10 min. Images were taken using a JEOL 1011 transmission electron microscope (TEM) at JNU, New Delhi, India to an accelerating voltage of 80 kV.

X-ray diffraction of the nanoparticles (XRD)

The CaP nanoparticles developed were determined using X-ray diffraction (XRD). The nanoparticles were determined using a Philips X-ray diffractometer (X'pert Pro), at IIT, Delhi, India. This diffractometer, with a X'celerator detector (Philips), used Cu-Ka radiation (l ¼ 1.5418A°) and was operated at 45 kV and 40 mA.

PCR and Southern Analyses

Genomic DNA was isolated from the leaves of the transformed and non-transformed plants *via* plant DNA extraction kit (Takara, Japan). Primer sequences 5′GGTTTTTCTGCGACGTTCA3′ and 5′TGTGATATGAACCGCATTC3′ for amplifying the 1080 bp GFP gene fragment were used. PCR amplification was done in 25 µl reaction which contained 1 µl of template, 1 U Taq polymerase (Promega), 1×reaction buffer, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L deoxy-ribonucleoside triphosphate (dNTPs) and 2 µmol/L of each primer. Cycling parameters were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 54°C for 30 s, and 72°C for 2 min, with a final extension of 10 min at 72°C [21]. PCR products were visualized on 1.2% agarose gel. Then 10 µg of genomic DNA from the leaves of the PCR-positive transgenic plant and 10 µg of the non-transformed plant were respectively digested with restriction enzyme *BamH*I for 14 h and the products were separated onto a 0.8% agarose gel, and then the gel was transferred to a nylon membrane. The GFP gene labeled with DIG-high prime was used as probe in the hybridization analysis. The hybridization and other steps were performed according to the manufacturer's instructions (Roche, USA).

RESULTS

pBI121 loaded CaP nanoparticles

Preparation, composition and yield of nanoparticles

Reveres micelles of CaP nanoparticles were developed by reaction between the calcium ion and phosphate by controlling size of the aqueous core of the micelles. The yield was obtained 6 mg pBI121 loaded CaP nanoparticles.

Physicochemical characterization of the particles

The physiochemical characteristics like size, zeta potential and aggregation behavior of pBI121 loaded CaP nanoparticles were studied by transmission electron microscopy, zeta sizer and X-ray diffractometry respectively.

FTIR Spectroscopy of CaP nanoparticles

FTIR spectra were used to determine the phase of the nanoparticles developed with Ca/P ratio. All of the FTIR spectra exhibited typical apatitic features (Fig. 1). In particular, all of the spectra contained PO_4^{3-} bands between both 1000–1200, and 470–560 cm^{-1} as well as OH^- bands at around both 3056 and 605 cm^{-1}.

Dynamic light scattering studies of CaP nanoparticles

(i) Particle size and size distribution of CaP nanoparticles. The nm sizes of CaP nanoparticles were confirmed by measuring the size of aqueous dispersed particles using a Malvern zeta-sizer, which revealed the z-average size 25 nm with a maximum population of 20–25 nm size particles (Fig. 2A).

(ii) Surface charge of the CaP nanoparticles. Zeta potential and charge on the particle surface were determined by using the Zeta-Sizer instrument. Fig. 2B showed that the surface-charge of the CaP nanoparticles was negative with a zeta potential of -25.6 mV. In this formulation the negative charge of the nanoparticles was contributed by the phosphate and DNA ions. The particles, therefore, carried the resultant excess negative charge.

TEM Analysis of CaP nanoparticles

The nanoparticles were prepared in the form of reverse micelles and the sizes of nanoparticles were found 55 nm, as shown in Fig. 2C. The TEM picture of pBI121 loaded calcium phosphate nanoparticles revealed that the particles were mostly below 55 nm in diameter and with spherical morphology.

X-Ray diffraction (XRD) study of CaP nanoparticles

X-Ray diffraction was carried out to determine the crystalline characteristics of CaP nanoparticles. Fig. 2D showed the diffractograph of pBI121 loaded CaP nanoparticles which exhibited the crystalline nature of the formulated nanoparticles. The characteristic peaks at 16, 21.3, 25.2, 31.8, 36.5 and 39.4 at 2° theta showed the crystalline nature.

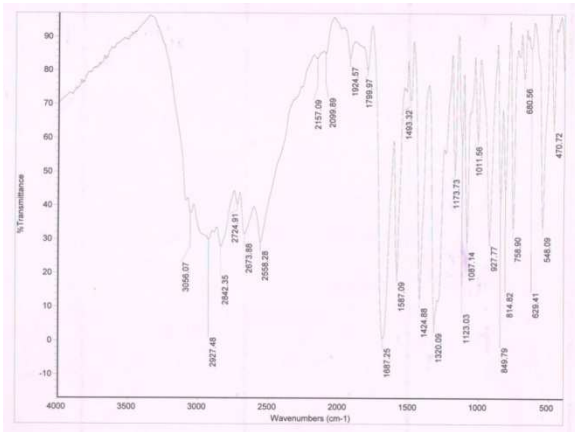

Fig. 1: FTIR spectra of pBI121 loaded CaP nanoparticles

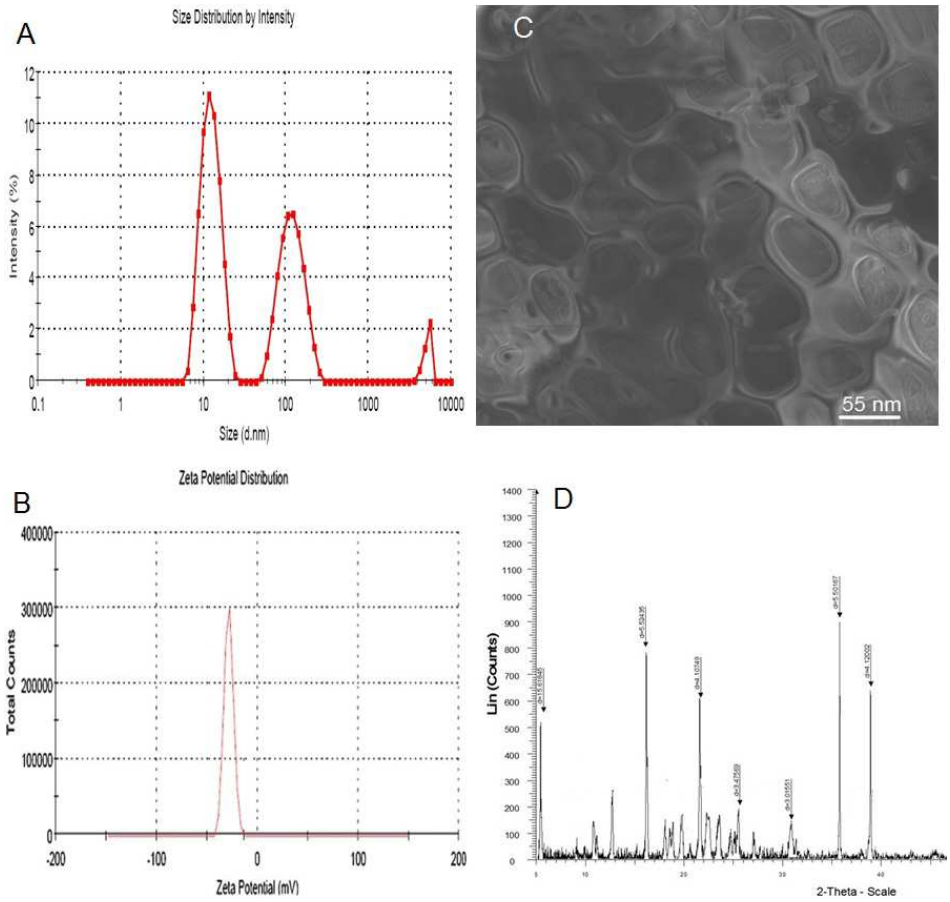

Fig. 2: A. DLS showing the particles size of CaP nanoparticles, B. Zeta potential of CaP nanoparticles, C. Transmission electron micrograph (TEM) showing particles size and morphology, D. XRD pattern on 2 theta scale of CaP nanoparticles.

Stability of released DNA by PCR and Southern Analyses

As already known that the compacted DNA molecules more stable towards DNase1 degradation, pBI121 loaded CaP nanoparticles were digested with DNase1 and the gel was run and found that untreated free plasmid BI121 moved at its usual position in the gel (Fig. 3, Lane 3) while DNA encapsulated nanoparticles remained in the well of the gel and did not move in spite of DNase treatment (Fig. 3, Lane 5). These results confirmed that plasmid DNA loaded in CaP nanoparticles were completely protected from the degradation by these enzymes.

These PCR-positive transgenic plants were further studied for Southern hybridization (Fig.4), and the result revealed that the exogenous GFP gene can be integrated into the genome of transgenic tobacco plants.

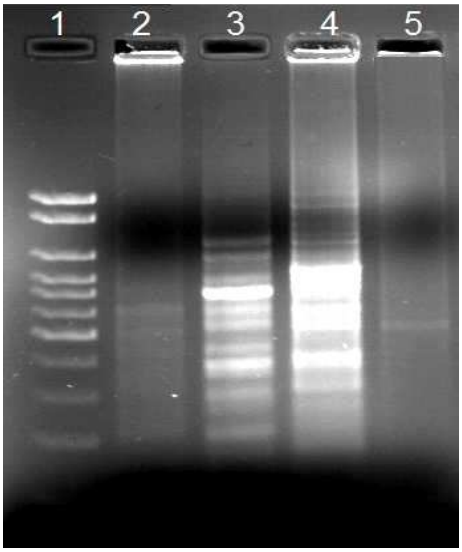

Fig. 3: PCR analysis of GFP gene on Agarose (1%) gel electrophoresis of free, entrapped in CaP nanoparticles by pBI121. Lane 1: marker l DNA (digested with EcoRI/HindIII); Lane 2: free pBI121 DNA; Lane 3: pBI121 treated with DNaseI; Lane 4: pBI121 treated with DNaseI; Lane 5: CaP nanoparticles containing entrapped pBI121 DNA.

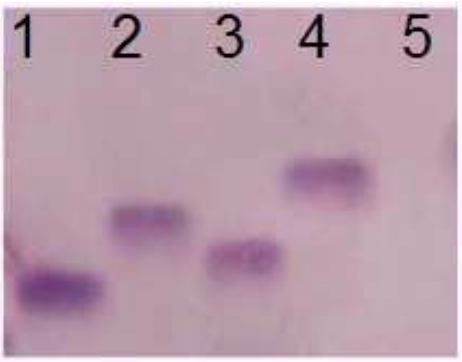

Fig. 4: Southern blot analysis: Lane 1-Plasmid DNA-positive control, lane 2-4 transgenic plant and lane 5- Non transformed plant.

DISCUSSION

The study describes non-viral gene delivery into the tobacco plant cells using CaP nanoparticles. Already many scientists have reported the use of calcium phosphate nanoparticles in non-viral gene delivery as a carrier to the animal cells, but with plant cells are rare [22]. The plant cell has a major difference from animal cell in the form of cell wall, which is the major barricade in plant cell genetic engineering [23,24]. The hypocotyls tissues were chosen for transformation in tobacco plant due to less possibility of gene variation. In the study, CaP nanoparticle's released plasmid DNA has showed significant potential towards *Agrobacterium tumefaciens* as GFP gene put together into genomic DNA by non-homologous recombination.

Due to the anionic properties of pDNA it was easily condensed into CaP nanoparticles ranging between 25-55 nm[25,26]. The pBI121 with GFP loaded CaP nanoparticles were attractive carriers systems to easy delivery and were highly protected from cellular nuclease enzymes.

From our study, it is evidenced that CaP nanoparticles could be used as a non-viral, well-organized and competent transforming carrier in other plants also as compared to viral *Agrobacterium tumefaciens* mediated genetic transformations [27]. This delivery is easy to process, does not have any infections, deliver gene(s) without injury to the cell and also be utilized as a targeted gene delivery medium to nucleus, chloroplast and mitochondria etc[28]. These recourses to plant research will increase the crop varieties having higher yields and better quality.

CONCLUSION

From the above stated results, it is concluded that CaP nanoparticles system in the form of gene carriers for tobacco plant can be valuable to transferring the genes of trade and industry interest into our tobacco plant or other crop plants to improving varieties for higher yield. In both type of cells either in plant cells or animal cells, CaP nanoparticles can be competently used in gene engineering. The CaP nanoparticles easily traverse the plant cell wall and discover a new bright field of transformation. The CaP nanoparticles are cheap, safe and significant as compared to viral gene delivery system.

CONFLICT OF INTERESTS

The authors report no conflicts of interest in this work.

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