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Generation of recombinant protein shells of Johnson grass chlorotic stripe mosaic virus in tobacco plants and their use as drug carrier

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ABSTRACT

The development and use of virus-like particles (VLPs) is a growing field with a powerful potential in generation of nanoparticles. In the present study we have attempted to generate and use empty shells of *Johnson grass chlorotic stripe mosaic virus* (JgCSMV, a member of the genus *Aureusvirus*, family *Tombusviridae*) as VLP nanoparticles for drug loading. In order to successfully produce recombinant JgCSMV-derived VLPs, we followed an approach based on cloning of the JgCSMV CP gene into pBI121 vector and introduction of the latter into *Agrobacterium rhizogenes* and transformation of tobacco cells for coat protein expression. Expression in tobacco tissue was demonstrated in transformed hairy roots as a model system. Recombinant VLPs were purified, analyzed by immune assay and visulalized by electron microscopy. Next, we explored the possibility of using JgCSMV-derived VLPs as a nanocontainer for loading the anticancer drug doxorubicin (DOX), taking advantage of the reversible swelling of VLPs *in vitro*. The results showed that transformed hairy roots produced high levels of the recombinant protein that readily assembled to form empty shells with overall structure similar to native virus particles. In addition, we demonstrated that JgCSMV-VLPs could function as vehicles able to load the chemotherapeutic drug doxorubicin. To our knowledge, this is the first research addressing the question of how this icosahedral virus (JgCSMV) can be used for the production of nanocontainers for biomedical applications.

1. Introduction

Viral capsids are natural protein cages made of hundreds of subunits evolved to assemble with high fidelity in a short period of time. In 1955, Fraenkel-Conrat and Williams (1955) showed that coat protein (CP) subunits of the rod-shaped *Tobacco mosaic virus* (TMV) could self-assemble to form rod-shaped particles *in vitro*. Since then, many structurally simple viruses have been found to be outstanding models of self-assembly of macromolecular structures. Viral "empty shells" known as virus-like particles (VLPs) are multisubunit self-assembled protein structures that lack viral nucleic acid and are noninfectious(Johnson and Chiu, 2000; Pumpens and Grens, 2002). They can be directly purified and used after *in vivo* self-assembly or can be subjected to more efficient reassembly(Arcangeli et al., 2014; Santi et al., 2006). As nanoparticles, viral empty shells are especially valuable because they are biodegradable, biocompatible, and non-infectious in humans and other mammals and can be produced in large quantities(Kaiser et al., 2007; Singh et al., 2007). These particles because of their highly symmetrical structures can be considered as one of the most advanced and versatile nanomaterials produced in nature. The development and use of viral nanoparticles (VNPs) is a growing field with a potential powerful impact because of their regular geometries and uniformity of size, variety of distinct forms (most commonly icosahedrons, spheres, tubes, and helices), high stability toward variations of pH, temperature, salt and solvent and well-characterized surface properties(Steinmetz and Evans, 2007; Wen et al., 2012). Presence of multiple sites on the virus capsid is another significant pro of virus-based nanoplatforms that can be used for the introduction of foreign peptides or ligands. Cell targeting using lower concentrations of targeting molecules is possible by enhancing the targeting properties of the viral nanoparticles through creation of a polyvalent scaffold. Achieving this level of control is not possible by inorganic or lipid materials(Sengupta et al., 2005). Apart from simple disassembly/reassembly of the viral capsid, drug cargo can also be loaded through covalent attachment of drugs or their analogues to certain reactive moieties on the capsid proteins(Ma et al., 2012; Yildiz et al., 2011). Loading the interior cavity of a VLP can be fulfilled by a reversible gating mechanism sensitive to concentration of metal ions and pH. In an acidic environment, many particles adopt a native conformation, but when the pH increases, a structural transition leads to swelling and pore-opening of viral particles. Lowering of the pH leads

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the particles to contract again while the cargo is trapped in their interior(Aniagyei et al., 2008; Ren et al., 2007). Our work is based on generation and use of empty shells of *Johnson grass chlorotic stripe mosaic virus* (JgCSMV), a plant RNA virus belonging to the genus *Aureusvirus* (Family *Tombusviridae*)(Brown et al., 2012; Izadpanah et al., 1993). JgCSMVvirion is a 30 nm icosahedron that is built of 180 identical molecules of CP (41 kDa). Based on comparative amino acid sequence alignments, the CP subunits likely contain the R, S and P domains described for *Tomato bushy stunt virus* and *Red clover necrotic mottle virus*. JgCSMV is a rather stable virus with both "full" and empty particles in the infected plants(Izadpanah, 1988). Although these empty shells are good candidates for use as nanoparticles, their isolation from plants in sufficient quantities does not seem practical. In this paper we explored the possibility of producing recombinant JgCSMV CP and their assembly *in vitro*. We further studied the conditions for reversible swelling of VLPs and possibility of drug loading in the particles.

2. Materials and methods

2.1. Source of the virus

JgCSMV was isolated from naturally infected Johnson grass in Fars province, Iran, and transmitted to maize by embryo puncture (Izadpanah et al., 2002). Infected maize plants were used as the source of the virus for various experiments. Identity of the virus was verified by serology(Izadpanah et al., 1993).

2.2. RNA extraction, cDNA synthesis and RT-PCR

Total RNA was isolated from infected maize tissues as well as from healthy maize leaves using an RNA extraction kit (Denazist, Iran). The RNA samples (13 µl) were reversely transcribed to cDNA using JgCSMV-CP reverse primer and M-MuLV reverse transcriptase (Fermentas) following the manufacturer's instructions. Using available sequence of JgCSMV CP gene (National Center for Biotechnology Information, NC-005287), a pair of forward and reverse primers (5′AGTGGATCCATGCCGCCGCAGGCG3′/5′GTAGAGCTCCTAGGTGAT AGAGATGTCTC3′) containing a *Bam*HI and a *Sac*I restriction sites (underlined), respectively, was designed. These specific primers were used to amplify a fragment of CP gene. One microliter of synthesized cDNA was subjected to a 35 cycle polymerase chain reaction (PCR) program of 30 s at 94 °C, 1 min at 69 °C, and 1 min at 72 °C. The final cycle was followed by 10 min incubation at 72 °C. PCR products were resolved on 1.5% agarose gel, stained by ethidium bromide and visualized under UV light.

2.3. Construction of plant expression vector

CP cDNA was inserted into pTZ57R/T plasmid using the InsT/A clone PCR product Cloning kit from Fermentas. The accuracy of insertion was verified by digestion with *Bam*HI and *Sac*I (Fermentas) and sequencing of recombinant plasmid. The resulting 1095 bp fragment was purified with the QIAquick Gel Extraction Kit (Qiagen) by gel electrophoresis.

A GUS-less pBI121 (pBI121ΔGUS) construct was generated by digesting pBI121 with *Sac*I/*Bam*HI to remove the GUS fragment. The fragment of interest (CP gene) was ligated into pBI121ΔGUS binary vector to make the pBI121-CP construct (Fig. 1). *Escherichia coli* strain DH5α cells were transformed with 3 µl of the pBI121-CP construct via $CaCl_2$ method(Froger and Hall, 2007). One milliliter of pre-warmed LB medium was immediately added, and the cells were grown at 37 °C on a shaker for 1 h and added to plates containing Luria-Bertani (LB) agar medium with kanamycin (50 mg/ml) and incubated overnight at 37 °C. Colonies were picked and cultured in liquid LB medium containing kanamycin (50 mg/ml) and incubated overnight at 37 °C. Plasmid extraction was done using the Accuprep Plasmid Extraction Kit (Bioneer). The engineered pBI121 plasmids were introduced into *Agrobacterium rhizogenes* strain Ar15834 by freeze-thaw method (Sambrook and Russell, 2001) and the bacterium was grown in liquid LB medium containing kanamycin (50 mg/ml) and riphampicin (50 mg/ml) at 28 °C on a shaker. Confirmation of correct clones were done by plasmid extraction and digestion with *Bam*HI and *Sac*I, which gave a 1095 bp band in the electrophoresis gel, colony PCR and sequencing. A GUS-less pBI121 (pBI121ΔGUS) construct and the intact pBI121 vector were used as negative control in colony PCR.

2.4. Generation of transformed hairy roots

Formation of tobacco (*Nicotiana tabacum* cv. Turkish) hairy roots was induced by direct infection of leaves of 3 to 4 week-old seedlings with a 48 h culture of transformed *A. rhizogenes* strain Ar15834. To do so, *A. rhizogenes* containing the construct was cultured in LB medium containing 50 mg/ml kanamycin and 30 mg/ml meropenem, until OD_{600} = 0.6 was reached (ca. 18 h). Cells were pelleted by centrifugation at 3500 rpm for 20 min at 4 °C. The pellets were suspended in MS medium (pH 5.2) with 0.05 mM acetosyringone and infiltrated into tobacco leaves using U-100 insulin syringes. Infiltrated tobacco leaves were placed on MS medium(Murashige and Skoog, 1962) up to the emergence of bacterial colonies around them, and then transferred to MS medium containing 50 mg/ml kanamycin and 30 mg/ml meropenem. Hairy roots were generated in enough quantities within 35 days. Single hairy roots were cut off and transferred to liquid and hormone-free MS medium and further transferred tofresh medium at 2-week intervals.

2.5. Molecular and serological verification of CP gene expression

2.5.1. Verification of hairy root transformation

The Ri plasmid carries approximately 18 potential genes, of which four genes, rol A, B, C and D have a fundamental role in the induction of hairy roots.The presence of rolA and rol B genes confirms the transgenic nature of hairy roots. Molecular verification of induction of hairy roots was achieved by PCR amplification of a 194 bp fragment of the rolB gene. CTAB method was used to isolate DNA from hairy roots. The template DNA was used for PCR amplification of partial rolB gene with specific primer pair, 5′AAGTGCTGAAGGAACAATC3′/5′CAAGTGAATGAACAAGG AAC 3′. PCR cycles comprised of 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 45 s and extension at 72 °C for 1 min. Final extension was done at 72 °C for 10 min.

2.5.2. Examination of transformed hairy roots for the presence of coat protein gene and expressed protein

Presence of coat protein gene in transformed hairy roots was ascertained by PCR using the specific primer pair that amplifies a 1095 bp product. The 35 PCR cycles comprised of denaturation at 94 °C for 30 s, annealing at 69 °C for 45 s and extension at 72 °C for 1 minute. Genomic DNA from untransformed hairy roots was used as negative control. Expression of the heterologous coat protein was confirmed also serologically by indirect-ELISA(Converse and Martin, 1990)using specific polyclonal antibodies against JgCSMV(Izadpanah, 1988).

2.6. Purification of VLPs

Density gradient centrifugation procedure was used to purify VLP empty shells assembled from expressed protein. Hairy roots were homogenized in 0.1 M sodium acetate buffer(pH 5) and the homogenate was clarified by centrifugation at 5000 rpm for 10 min at 4 °C. The supernatant was layered on 10–40% sucrose gradients and centrifuged in a Beckman SW-28 rotor at 23000 rpm for 2 h. The presumed VLP band was removed with a syringe. Alternatively, hairy root sap was centrifuged in Beckman Type 30 rotor at 23800 rpm for 2 h. The pellet was dissolved in 0.05 M sodium acetate buffer, pH 5. Concentration of