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Carbon-nanotube for Transient Expression in Rice Calli

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ABSTRACT

Transient gene expression is an important technique in gene functional analysis, protein production and in plants. However, traditional transient expression methods using *Agrobacterium* are time-consuming with low efficiency. In this study, we demonstrated the use of a single-walled carbon nanotube (SWCNT) to deliver *35S:mCherry:pCXSN* plasmid into rice calli. This transient expression protocol used a plastic medical syringe to create the physical pressure to help the delivery of plasmid DNA into plant cells. This protocol is relatively easy to perform and low cost. The transient expression was observed under fluorescence microscopy, and the mCherry fluorescence signal was quantified. The plasmid DNA was delivered into the rice cell using a 3:1 ratio (plasmid: carbon nanotube). The result showed that the mCherry signal of carbon nanotube + plasmid DNA treatment was the highest signal at 3 days post-transformation and decreased to a lower signal at 6 days post-transformation. Moreover, the quantitative analysis of mCherry mean intensity revealed that the signal intensity of carbon nanotube + plasmid DNA treatment was the highest level, and significantly higher than the control treatments at 3 days post-transformation. Plasmid DNA can be transported easily into plant calli using this carbon nanotube transient expression protocol.

1. Introduction

Plant gene transformation is an important technique for gene functional analysis, protein production, and crop improvement. Beneficial genetic traits for the improvement of crop productivity and the resistance or tolerance to biotic and abiotic stresses were accomplished in the past (Parmar *et al.* 2017). However, the genetic transformation efficiency in plants is challenging due to the plant cell wall, a barrier to exogenous biomolecule delivery. Traditional techniques including *Agrobacterium* transformation and biolistic DNA delivery are used to transform genes in plants for many years (de la Riva *et al.* 1998). However, these methods showed limitations, e.g., low percentage of transformation, requirement of sophisticated equipments and expertise, uncontrolled DNA integration in the plant genome, and time consumption (Cunningham *et al.* 2018).

Carbon nanotube (CNT) is a cylindrical molecule that consists of rolled-up sheets of single-layer carbon atom (graphene). They can be single walled (SWCNT) with a diameter of less than 1 nanometer (nm) or multi-walled (MWCNT), consisting of several concentrically interlinked nanotube, with diameters reaching more than 100 nm (Karousis *et al.* 2010). The CNT can be used as a carrier to deliver biomolecules such as plasmid DNA, small interfering RNA (siRNA), proteins, and drugs into living cells (Iijima 2002). The carboxylated CNT (COOH-CNT) is modified with cationic polymer (PEI) carrying net positive charges form a complex with negatively charged plasmid DNA (Figure 1). The ionic interaction between CNT and plasmid DNA leads to the formation of the plasmid coated CNT, which can deliver the plasmid into plant cells (Figure 1) (Maheshwari *et al.* 2019).

From a previous report, CNT was efficiently used as gene carriers into bacterial cells (Mattos *et al.* 2011) and mammalian cells (Cai *et al.* 2005). CNT was also successfully used for a transiently gene transformation in *Nicotiana benthamiana* (tobacco),

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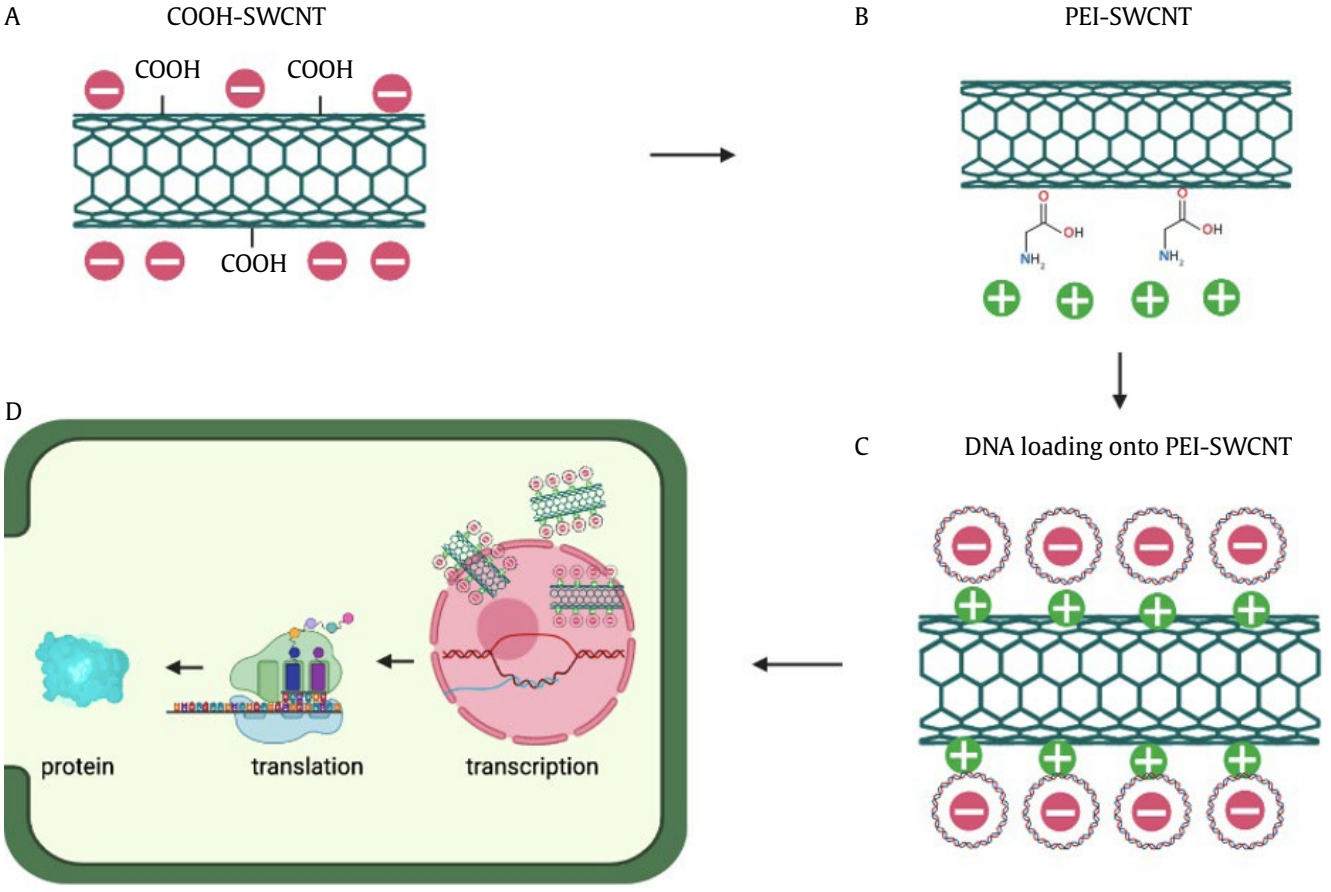

Figure 1. Schematic diagram for CNT-mediated delivery preparation and gene expression. (A) a modified carboxylated SWCNT (COOH-SWCNT) structure, (B) the COOH-SWCNT is modified using cationic polymer (PEI) carrying a net positive charge, (C) a PEI-SWCNT is incubated with negatively charged plasmid DNA to generate DNA-PEI-SWCNT complex, (D) the DNA-PEI-SWCNT complex is delivered into the plant cell, exhibiting the process of transcription and transition

Eruca sativa (arugula), *Triticum aestivum* (wheat), and *Gossypium hirsutum* (cotton) (Demirer *et al.* 2019). The loaded CNT can enter through the plant cell wall and deliver targeted molecules into the plant cells with low cytotoxicity (Burlaka *et al.* 2014). Using CNT to deliver plasmid DNA into plant cells provides advantages over the conventional transformation methods with increasing efficiency, applicability, and reproducibility (Ohadi Rafsanjani *et al.* 2012).

There are several factors affecting the efficiency of CNT transformation protocol including different mass ratio between CNT and plasmid DNA, pH, and physiological or chemical condition of the transformation protocols (Demirer *et al.* 2019; Gou *et al.* 2014). Finding the optimal condition for each plant species remains important. In previous reports, CNT transformation in plants was mainly used for protein production either by leaf infiltration or protoplast transformation (Demirer *et al.* 2018; Demirer *et al.* 2019; Gou *et al.* 2014). However, a protocol for CNT-based DNA delivery into callus has not been reported. In this research, we optimized a CNT-based DNA delivery protocol to transiently express *35S:mCherry:pCXSN* plasmid into rice calli. The combination of the CNT:plasmid DNA ratio and physical force from a plastic medical syringe was used to effectively improve the transient expression in rice callus. This modified protocol can be used for the in-planta protein production for functional characterization.

2. Materials and Methods

2.1. Rice Callus Preparation

Seeds of Thai rice cultivar 'KDML105' were used for callus induction. Seeds were surface sterilized with 70% ethanol for 5 min, followed by 25% (v/v) commercial bleach with shaking rotator at 80 rpm

for 30 min, and washed five times with sterile distilled water. For callus induction, the seeds were cultured on callus induction medium (MCI: MS salts supplemented with 30 g/L maltose, 0.3 g/L casein hydrolysate, 0.6 g/L L-proline, 3.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 mg/L 6-benzylaminopurine (BAP) and 3.0 g/L gellan gum, pH 5.8) at 27±2°C under dark condition (Sahoo *et al.* 2011). After 14 days, embryogenic calli were separated and sub-cultured onto MCI medium for four days before transformation.

2.2. Carbon Nanotube Preparation

2.2.1. COOH-SWCNT Suspension Preparation

The carbon nanotube preparation was performed following a SWCNT preparation procedure (Demirer *et al.* 2019). The carbon nanotube (single-walled, carboxylic acid functionalized; Sigma-Aldrich, cat no. 652490) was used in this experiment. The COOH-SWCNT suspension was prepared by weighing 30 mg of dry COOH-SWCNT into 10 ml of nuclease-free water. This carbon solution was sonicated with an ultrasonic homogenizer at 20% amplitude 120 kHz 120 watt on ice for 30 min followed by rest at room temperature for 10 min. The carbon pellet was precipitated by centrifugation at 12,000 g for 1 hr. The supernatant was collected in a new 50 ml conical tube and measured concentration using absorbance at 632 nm with an extinction coefficient of 0.036 L/mg/cm. Subsequently, zeta potential measurement was performed using NanoPlus (Particle Size and Zeta Potential Analyzer, Micromeritics Instrument, USA) to confirm the surface charge density of COOH-SWCNTs suspension.

2.2.2. PEI Reaction with COOH-SWCNT

PEI reaction with COOH-SWCNT was performed for an activated charge of COOH-SWCNT. Two milligrams of suspended COOH-SWCNT were diluted with 500 mM of MES buffer to obtain the concentration of 100 mM. The EDC-NHS solution was prepared by added 10 mg of EDC and 10 mg of NHS to 2.5 ml of 100 mM MES solution followed by slowly dropwise to the COOH-SWCNT suspension under stirring. COOH-SWCNT was sonicated for 15 min at room temperature, then let the reaction continue for 45 min on an orbital shaker at 180 rpm at room temperature. Two 100,000-MWCO filters were washed with 15 ml of 0.1 × PBS. The activated COOH-SWCNT solution were added with 0.1X PBS buffer until volume up to 50 ml, then wash three times with 100,000-MWCO filter at 300 g for 8 min at 21°C, discard the flow-through and repeat the wash two more times with 0.1 × PBS. PEI was prepared (25,000 MW, branched: Sigma-Aldrich, cat. No. 408727) to 5 ml of 0.1 × PBS (pH 7.5). The activated COOH-SWCNT was added to the PEI solution dropwise followed by run the reaction overnight (~16 h) at room temperature on an orbital shaker at 180 rpm.

2.2.3. Washing and Suspending the PEI-SWCNT Product

PEI-SWCNT reaction solutions were split into 2 tubes. Each tube was filled with nucleus-free water volume up to 50 ml followed by washed with 100,000-MWCO filters at 1,000g for 15 min at 21°C, discard the flow-through, and repeat the wash five more times with nuclease-free water. After the last wash step, bath-sonicate the two 100,000-MWCO filters (top part) for 1 min. The PEI-SWNT reaction solution was centrifuged at 16,000 g for 1 h at room temperature and the supernatant was collected for the measurement of PEI-SWCNT concentration via absorbance at 632 nm with an extinction coefficient of 0.036 L/mg/cm. The sample was also used for the zeta potential measurement to confirm the surface change density.

2.2.4. Plasmid DNA Transformation using Carbon Nanotube

For a transformation, *35S:mCherry:pCXSN* plasmid was used. Three treatments consist of 1) carbon nanotube only; prepared by mixed 500 ng of PEI-SWCNT solution with MES delivery buffer, 2) *35S:mCherry:pCXSN* plasmid only; prepared by mixed 166.67 ng of *35S:mCherry:pCXSN* plasmid with MES delivery buffer, and 3) carbon nanotube + *35S:mCherry:pCXSN* plasmid; prepared by mixed 500 ng of PEI-SWCNTs solution with MES delivery buffer combined with 166.67 ng (3:1 PEI-SWCNTs: DNA mass ratio). Each treatment was incubated at room temperature for 30 min for the formation of the DNA-PEI-SWCNT complex followed by syringe vacuum with rice calli for 5 min. The calli were washed five times with sterilized distilled water and dried on autoclaved tissue paper for 5 min. Subsequently, the calli were cultured on the regeneration medium [MS salts supplemented with 30 g/L maltose, 2 mg/L kinetin, 0.2 mg/L NAA, pH 5.8, and 10 g/L agarose].

2.3. Fluorescence Detection

The fluorescence signal was observed at 3 days and 6 days post-transformation. Ten calli from each treatment were cut into thin slice and observed the mCherry fluorescence signal using fluorescence microscopy (Leica DM6000B, Germany) (excitation wavelength of 540-552 nm, emission wavelength of 565-605 nm). The pictures were taken for quantitative fluorescence intensity analysis by imageJ software.

2.4. Statistical Analysis

The mean value of quantitative fluorescence intensity analysis from all treatments in 3 days and 6 days post-transformation fluorescence pictures were subtracted with mCherry background signal from KDML wild type callus. The mCherry mean intensity was analyzed with one way ANOVA followed by Tukey HSD test at $P<0.001$. The means and standard errors from ten calli of each treatment were calculated ($n = 10$).

3. Results

3.1. Verification of the Surface Charge Density on CNT

To prepare the carbon nanotube for DNA delivery, the surface charge density of CNT was measured through zeta potential measurement. The zeta potential of an initial COOH-SWCNT suspension was -34.17 mV, and the zeta potential of SWCNT modified PEI (PEI-SWCNT) was +34.03 mV (Figure 2). This result indicated an increase in the density charge of CNT. This activated CNT suspension was ready for loading with plasmid DNA for transient expression. Furthermore, the zeta potential of the PEI-SWCNT solutions was stable at +34 mV for more than one month.

3.2. Transient Expression of mCherry Reporter in Rice Callus

To examine the transient expression via the carbon nanotube in rice callus, ten calli of each treatment at 3 days and 6 days post-transformation were observed the mCherry signals using fluorescence microscopy. No or low mCherry signal was observed in neither carbon nanotube nor plasmid DNA treatment (Figure 3). Whereas intense mCherry signals were observed in carbon nanotube + plasmid DNA treatment at 3 days and 6 days post-transformation. The mCherry signal was highest at 3 days post-transformation and decreased to a lower

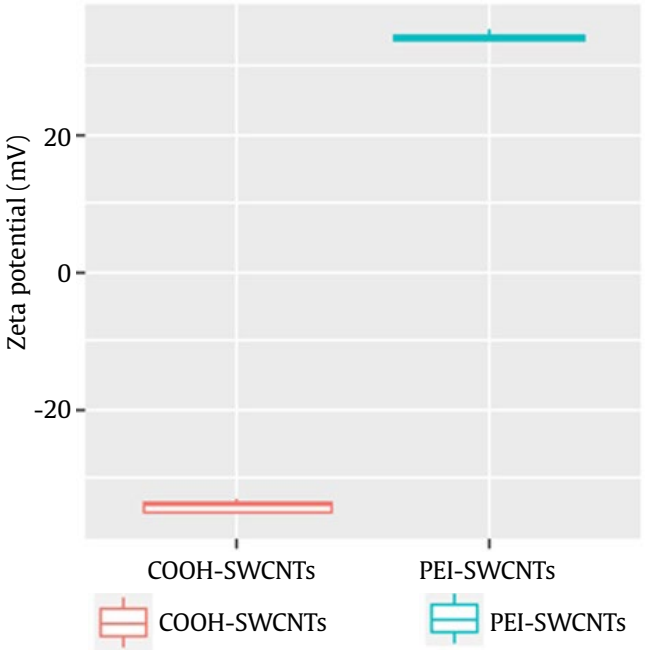

Figure 2. The surface change density of single-walled carbon nanotube (SWCNT) by zeta potential measurement. COOH-SWCNT is the carbon nanotube before activation with cationic polymers, and PEI-SWCNT is the carbon nanotube after activation with cationic polymer. Five measurements were recorded, and standard error was calculated in the R program

level at 6 days post-transformation (Figure 3). This result showed the successful delivery and expression of *35S:mCherry:pCXSN* plasmid into rice calli by carbon nanotube.

3.3. The Quantitative Analysis of mCherry mean Intensity

The mean intensity of mCherry fluorescence signal from ten calli of each treatment was quantified. The mean intensity of mCherry fluorescence signal in carbon nanotube (control treatment), plasmid (control treatment), and carbon nanotube + plasmid treatments at 3 days followed by 6 days post-transformation were 14.07, 14.89, 27.67, 8.1, 7.5, 12.03 respectively. The mCherry signal was high at 3 days post-transformation and lower at 6 days post-transformation in all treatments. The mCherry signal of the carbon nanotube + plasmid treatment was significantly higher than the control treatment at 3 days post-transformation at $P<0.05$. This result confirmed that the CNT-based DNA delivery protocol was able to transiently express the mCherry reporter in rice calli.

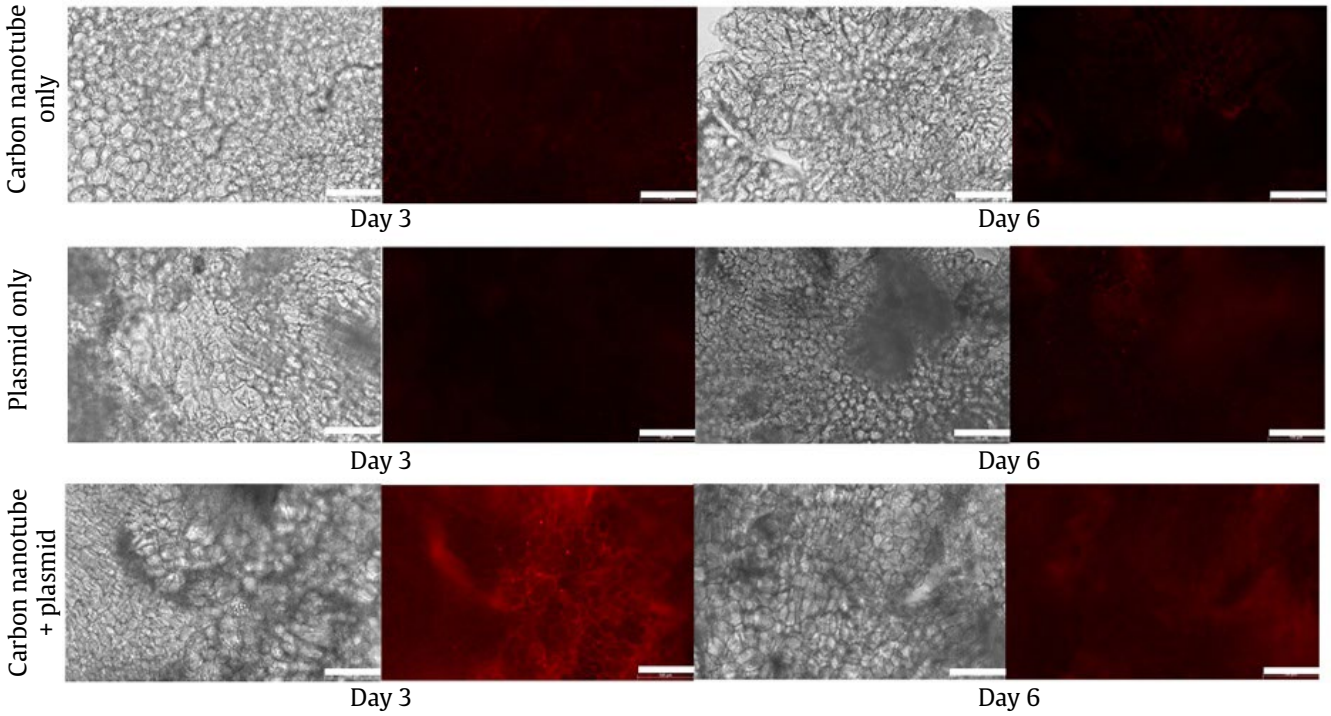

Figure 2. Fluorescence signal detection of Thai jasmine rice cultivar 'KDML105' calli incubated with three different treatments including (1) carbon nanotube, (2) *35S:mCherry:pCXSN* plasmid, and (3) carbon nanotube + *35S:mCherry:pCXSN* plasmid. The images were taken 3 days and 6 days post-transformation with fluorescence microscopy. Scale bar = 100 µm

4. Discussion

Carbon nanotube (CNT) can be used as a carrier to deliver biomolecules such as plasmid DNA, small interfering RNA (siRNA), proteins, and drugs into living cells (Iijima 2002). Previous reports successfully used CNT to deliver plasmid DNA in several plant species, mainly via plant protoplast and leaf infiltration method (Demirer *et al.* 2018; Demirer *et al.* 2019). In this study, we optimized the CNT transformation protocol for rice callus using physical force from a plastic medical syringe. Our optimized protocol is relatively easy to perform and does not require sophisticated equipment. We used 500 ng of CNT for loading with plasmid DNA, which was less than the toxic level (1,000 ng) to plant cell reported by Magnabosco *et al.* (2020).

The CNT preparation for DNA delivery was successful with the initial surface charge density of COOH-SWCNT suspension. The surface charge density of PEI-SWCNT solution was similar with the previous report by Demirer *et al.* (2019). This result confirmed that the density charge of CNT was activated and ready to be loaded with plasmid DNA. We further demonstrated the use of single-walled carbon nanotube (SWCNT) to deliver *35S:mCherry:pCXSN* plasmid vector into rice callus. This transient transformation protocol used a plastic medical syringe to create the physical pressure for the delivery of the DNA plasmid into plant cells. The mCherry fluorescence signal was detected, and the signal level was significantly higher than the control treatments at 3 days post-transformation.

In summary, the optimized transformation method, using syringe vacuum to create the pressure, can successfully drive the carbon nanotube to deliver plasmid DNA into rice calli. This transient transformation protocol is rapid, easy to perform and highly efficient. Our work provides a tool for plant callus transformation, which can be used for diverse biotechnology applications.

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