



An efficient cucumber (*Cucumis sativus* L.) protoplast isolation and transient expression system

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ABSTRACT

The transient gene expression system using plant protoplasts has become widely used for high-throughput analysis and functional characterization of genes. In this work we investigated protoplast isolation and green fluorescent protein (GFP) transient transfection and their main affecting factors, such as mannitol concentration in enzymolysis solution, enzymolysis time, and polyethylene glycol (PEG) concentration and transfection time, on 'xintaimici' cucumber. The results showed that when the enzyme solution had 1.5% cellulase R-10 (w/v), 0.4% macerozyme R-10 (w/v), 0.4M mannitol, 20 mM 2-morpholinoethanesulfonic acid, 10 mM CaCl₂, 0.1% bovine serum albumin, and was at pH 5.8 with an enzymolysis time of 8 h, the protoplast yield was $6\text{--}7 \times 10^6/\text{g}$ fresh weight. Viability was about 90%. GFP was used as the reporter gene to measure protoplast transformation efficiency. When the concentration of PEG4000 was 20% and transfection time was 20 or 30 min, transformation efficiency was greater than 50% and the green fluorescent signal could be detected in the cytoplasm, chloroplasts, and plasma membrane. We show here an efficient PEG-mediated cucumber protoplast transient expression system using GFP reporter gene, laying a technical foundation for future research in cucumber molecular biology.

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1. Introduction

Genetic transformation is an important technique for studying the molecular biology of plants and can be used to understand gene function and genetic improvement in plants. However, the relatively expensive and time-consuming process for stable gene expression in transgenic plants still limits the utilization of this approach for large-scale analyses of plant genes. Transient expression technology is fast, simple, safe, and efficient while resulting in high expression levels comparable to long term stable expression systems (Chen et al., 2006). Accordingly, transient gene expression in a model system has become widely used for characterization of protein function, such as protein subcellular positioning and trafficking, protein–protein interaction, protein stability and degradation, as well as protein activity. Common transient transformation methods include biolistic bombardment, infiltration with *Agrobacterium tumefaciens* (agroinfiltration), and polyethylene glycol (PEG)-mediated transformation of protoplasts. Although the method of microparticle bombardment can be used in a variety of plant species (Taylor and Fauquet, 2002), most experiments

are done on the epidermis of onion (*Allium cepa* L.) scale leaves (Klein et al., 1987). Onion epidermis is easily removed and the large cells have excellent optical properties for in vivo microscopy. However, onion epidermal cells lack normally developed chloroplasts, so it is challenging to use this method to transform many types of chloroplast-targeted proteins (Jaedicke et al., 2011). *Agrobacterium*-mediated transient expression has the advantages of simple operation and low price, but its transformation efficiency is variable and the types of plant materials that can be used are limited (Ueki et al., 2009).

It is now over 50 years since Cocking (1960) published the first paper describing a method for the isolation of plant protoplasts. Protoplasts, which are osmotically fragile membrane-bound cells without cell walls, offer a versatile cell-based experimental system. Macromolecules such as recombinant DNA plasmids can be delivered into protoplasts using a variety of different transfection techniques, e.g., PEG-mediated, electroporation and microinjection. The transfected protoplasts are then used for further investigations. Therein, PEG-mediated protoplast transient expression has high transformation efficiency, so it is widely applied in plant molecular biology studies (Sheen, 2001; Walter et al., 2004). The establishment of new and more economical, convenient and sensitive reporter gene assays for β -glucuronidase (GUS), firefly luciferase (LUC) and later green fluorescent protein (GFP) for plant cells has also facilitated the application of

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protoplast transient expression systems (Leffel et al., 1997; Sheen, 2001). Transient assay utilizing protoplasts has become a powerful tool for rapid gene functional analysis, and has been established in several plant species, including both dicotyledonous and monocotyledonous crops, such as *Arabidopsis* (Yoo et al., 2007; Wu et al., 2009), tobacco (Locatelli et al., 2003), maize (Sheen, 2001) and rice (Chen et al., 2006; Zhang et al., 2011).

Cucumber (*Cucumis sativus* L.) is an important vegetable and a model species for Cucurbitaceae plant. The sequencing of its genome (Huang et al., 2009) has laid the foundation for further study of cucumber molecular biology. However, transformation of cucumber has a low regeneration rate and its transgenic offsprings are unstable. These problems have been limiting the progress of research in this field (Punja et al., 1990). Although there were some early reports of free protoplasts obtained from cucumber cotyledons and true leaves (Orczyk and Malepszy, 1985; Colijn-Hooymans et al., 1988; Kantharajah and Dodd, 1990; Punja et al., 1990), these isolation methods were optimized for plant regeneration from protoplasts. Later, Gradam et al. (1994) employed protoplast transient expression to dissect the functions of the malate synthase (MS) gene promoter in cucumber cell cultures. In this experiment, cucumber protoplasts were transfected with plasmid DNA containing MS promoter-GUS reporter gene via electroporation. Wieczorek and Sanfaçon (1995) described an improved method for the generation and transfection of protoplasts from cucumber cotyledons. In this protocol, PEG and electroporation were compared for their effect on protoplast transfection with the GUS reporter gene.

Green fluorescent protein is increasingly being used in plant biotechnology research and applications. GFP is characterized by low molecular weight, stable structure, easy detection, and non-toxicity. GFP retains fluorescence when fused to another protein, which makes it an attractive fluorescent tag to monitor protein–protein interactions, protein trafficking and subcellular localization (Leffel et al., 1997; Cho et al., 2006; Ehlert et al., 2006). In our laboratory, transient transformation by microparticle bombardments and heterologous expression in chloroplast-free epidermis cells of onion were used to determine the subcellular location of the sucrose transport protein from cucumber (CsSUT4), when tagged with the reporter GFP (Hu et al., 2011). The results suggested that it was targeted to the plasma membrane in plants and may play an important role in the apoplastic phloem unloading in developing cucumber fruits. Until now, however, there have been no reported studies of transient transformation using the protoplast system with GFP reporter in cucumber. In this study, using GFP as a reporter gene, we developed an efficient PEG-mediated cucumber protoplast transient expression system, laying a foundation for future research in cucumber molecular biology. The main affecting factors on the protoplast isolation and transient transfection, such as mannitol concentration in enzymolysis solution, enzymolysis time, PEG concentration and transfection time, were also investigated.

2. Materials and methods

2.1. Materials

'Xintaimici' cucumber (*C. sativus* L.) was used in this study. Full seeds were soaked in tap water at room temperature for 1–2 h, peeled, disinfected with 75% alcohol for 30 s, washed twice with sterile water, disinfected with 3% sodium hypochlorite for 15–25 min, washed 4–5 times with sterile water, and planted on MS media (3% sucrose, 0.7% agar, pH 5.8). The seeds were cultured at $25 \pm 1^\circ\text{C}$, under lighting of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a cycle of 16 h/8 h (light/darkness) for 7–9 d to obtain fully extended

cotyledons, and for 15–17 d to obtain the first true leaf for protoplast isolation (Kantharajah and Dodd, 1990; Wieczorek and Sanfaçon, 1995). The transient expression vector pUC-GFP (35S::GFP, size 4.5 kb) was kindly supplied by Dr. Junping Gao of the College of Agronomy and Biotechnology, China Agricultural University. Plasmid preparation was conducted with the plasmid extraction kit PL1401 (Bomaide biotech Co. Ltd., Beijing).

2.2. Protoplast isolation

Protoplast isolation was performed using the methods described by Guo et al. (2007), Yoo et al. (2007) and Guan et al. (2010), with some modifications. An enzyme solution was prepared containing 1.5% (w/v) cellulase R-10, 0.4% (w/v) macerozyme R-10, 20 mM 2-morpholinoethanesulfonic acid (MES) and mannitol. To determine conditions for maximum protoplast yield, a mannitol concentration gradient experiment (replicated three times) was conducted and varying concentrations of mannitol (0.2, 0.4, 0.6 or 0.8 M, with 0.2 M as control) were compared. The enzyme solution was placed in a water bath at 55°C for 10 min, cooled to room temperature, then bovine serum albumin [BSA, 0.1% (w/v)] and CaCl_2 (10 mM) were added (pH adjusted to 5.8), and the solution was sterilized using a $0.45\text{-}\mu\text{m}$ filter, then stored for later use. Cotyledons or true leaves were cut into fine strip ($\sim 1\text{ mm}$) with a blade along the direction vertical to the main vein. The strips were rapidly transferred to enzymolysis solution (0.3–0.4 g/10 ml), permeated in a vacuum and under weak light for 30 min, and then incubated in enzymolysis solution at $25 \pm 1^\circ\text{C}$ in darkness with rotation (40–50 r/min). To optimize the enzymolysis time, the tissues were digested for 4, 6, 8, 10 or 12 h (with 4 h as control, replicated three times), respectively. After digestion, the enzymolysate was diluted with an equal volume of W5 solution (2 mM MES, 154 mM NaCl, 125 mM CaCl_2 , 5 mM KCl, pH 5.8) (Yoo et al., 2007) and mixed gently, then filtered through nylon membrane (200 mesh). The filtrate was centrifuged at 1000 r/min for 2 min, the supernatant was discarded, and the protoplasts were washed one more time with W5. The supernatant was removed after centrifugation and the protoplasts were resuspended with W5 solution at a final concentration of 2×10^6 protoplasts/ml (Yoo et al., 2007) for the next transient transfection.

The purified protoplasts were diluted appropriately and counted with a hemacytometer under a microscope for protoplast yield statistics. Protoplast yield was calculated as follows: protoplast yield (protoplasts/gFW) = number of the protoplasts yielded in enzymolysis (protoplasts)/fresh weight of the material used in enzymolysis (gFW). Protoplast viability was detected with fluorescein diacetate (FDA) dye (Larkin, 1976). In solution, FDA is very weak fluorescent dye. In a living cell, enzymes called esterases in the cytosol cleave the two acetate molecules from a molecule of FDA, leaving highly fluorescent Fluorescein. Under ultraviolet light, Fluorescein accumulated in viable protoplasts glows with a brilliant green hue. In this experiment, FDA stock solution was added to diluted protoplasts at a final concentration of 0.01%, and were then incubated in darkness for 5 min, washed twice with W5 solution, then resuspended in W1 solution (4 mM MES, 0.5 M mannitol, 20 mM KCl, pH 5.8) (Yoo et al., 2007). Fluorescence microscopy under ultraviolet light (Olympus B $\times$ 51) was used to determine protoplast viability as follows: protoplast viability (%) = (fluorescent protoplast number in view/protoplast total number in view) $\times$ 100%.

2.3. PEG4000- Ca^{2+} -mediated plasmid transfection

PEG4000- Ca^{2+} transfection solutions were prepared by adding PEG4000 (10%, 20%, 30%, 40% and 50%, w/v) in ddH₂O containing 0.2 M mannitol and 100 mM CaCl_2 . Isolated protoplasts were

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