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Protoplast isolation and development of a transient expression system for sweet cherry (*Prunus avium* L.)

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ABSTRACT

Transient gene expression in plant protoplasts is a powerful tool for analyzing gene function and for performing biotechnical manipulations. Here we report the isolation of viable protoplasts from the fruit flesh of sweet cherry (*Prunus avium* L.) cv. Hong Deng and their polyethylene glycol (PEG)-mediated transient transfection using green fluorescent protein (GFP) as a marker gene. We investigated the main factors affecting the efficacy of protoplast isolation and transfection, including the composition of the enzymolysis solution, enzymolysis time, pH of the enzymolysis solution, PEG concentration, and transfection time. Protoplast isolation was optimal when the tissue was incubated in enzymolysis solution composed of 1.0% Cellulase R-10, 0.5% Pectolase Y-23, and 0.6 M mannitol (pH 5.8) for 18 h, resulting in a protoplast yield of 4.3×10^6 protoplasts/g fresh weight [FW] and viability of 84.1%. Protoplast transformation efficiency was measured by transient expression of the GFP reporter gene, and transformation efficiency was highest when protoplasts were incubated in transfection medium containing 40% PEG for 15 min. Collectively, this work describes an efficient protoplast isolation and protoplast transient expression system that can be used to facilitate molecular biology research in sweet cherry.

1. Introduction

Transient gene expression is a powerful tool for studying the functions of exogenous genes, the subcellular localization of proteins, protein–protein interactions, protein complexes, and gene silencing in *vivo*. Three transient transformation strategies have been established in plants, including biolistic bombardment (Ueki et al., 2009), infiltration with *Agrobacterium tumefaciens* (agroinfiltration) (Song and Sink, 2005; Andrieu et al., 2012), and polyethylene glycol (PEG)-mediated transformation of protoplasts (Yoo et al., 2007; Cao et al., 2014). These transient expression methods are simple, fast, safe, and efficient and express the foreign gene but do not integrate it into the genome, typically resulting in high expression levels. Limitations of biolistic bombardment are high cost and low transformation efficiency. Whereas *Agrobacterium*-mediated transient expression is rapid, inexpensive, and easy to perform, it is limited by the physiological responses of the target plant and environmental factors that affect *A. tumefaciens* virulence (Wroblewski et al., 2005).

PEG-mediated protoplast transformation offers a high transformation efficiency and is widely used in somatic hybridization, microprotoplast-mediated chromosome transfer, organelle or DNA microinjection, electroporation, gene transactivation, and nucleocytoplasmic protein trafficking (Lin et al., 2014; Rezazadeh and Niedz, 2015). This approach requires high-quality protoplasts and optimization of transformation methods. Protocols for culturing protoplasts from many fruit species have been established (Ochatt, 1992; Mills and Hammerschlag, 1994; Witjaksono et al., 1998; Ara et al., 2000; Yu et al., 2000; Haicour et al., 2009; Rezazadeh and Niedz, 2015; Wang et al., 2015). Protoplasts have yet to be isolated from the fruit of sweet cherry (*P. avium* L), the major cultivated type of cherry, despite several reports of in vitro regeneration from shoots (Tang et al., 2002; Matt and Jehle, 2005), one report of protoplast isolation from leaf mesophyll tissues and cell suspension cultures (Ochatt et al., 1987) of colt cherry (*P. avium* × *pseudocerasus*). Furthermore, there is no report of the PEG-mediated protoplast transformation of sweet cherry. Sweet cherry (*P. avium* L) is an economically important crop that is widely

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cultivated in temperate regions throughout the world due to its appealing color, delicious taste, and nutritional value (Wei et al., 2015). In addition, cherry fruits are suitable for processing into various candy and milk products, canning, and juice, liqueur, and jam production (Radičević et al., 2011).

Isolating large numbers of viable protoplasts and developing methods for high transformation efficiency are two crucial steps for establishing a reliable protoplast transient expression system. These two steps are affected by the composition of the enzymolysis solution, enzymolysis time, pH of the enzymolysis solution, PEG concentration, and transfection time. The enzymolysis solution consists of a mixture of osmotic stabilizers and lytic enzymes. Mannitol (Hidaka and Omura, 1992; Niedz, 1993; Rezazadeh and Niedz, 2015) and sorbitol (Jumin and Nito, 1996; Ortin-Parraga and Burgos, 2003) or a combination of the two (Myers et al., 1989; Ara et al., 2000) are often used as osmotic stabilizers for the isolation and culture of protoplasts (Rezazadeh and Niedz, 2015), whereas the lytic enzymes cellulase, pectinase 1,3-glucanase, and chitinase are frequently used to digest cell walls during protoplast isolation. Different lytic enzymes have different effects on cell walls degradation, and it is necessary to test each combination of enzyme used. Enzymolysis time affects protoplast isolation; prolonging the period of enzymolysis increases the ratio of protoplasts formed, up to a point. After a certain period, additional enzymolysis results in protoplast breakage. The pH value of the enzymolysis solution affects enzyme activity and thus protoplast isolation. Due to its ability to increase cell wall permeability. PEG is regarded as an effective protoplast transfection agent (Huang et al., 2013). The concentration of PEG affects the transformation efficiency and cell viability. Transfection time affects the transformation efficiency; excessive periods of transfection may damage protoplasts and reduce the transformation efficiency (Shillito et al., 1985). Furthermore, the proportion and concentration of the osmotic stabilizers and lytic enzymes influence the establishment of an efficient protoplast isolation system. Factorial experimental designs have been used to optimize factors for highly efficient protoplast isolation (Ochatt, 1992; Witjaksono et al., 1998; Jiang et al., 2015; Masani et al., 2014); however, a limitation of factorial experimental design is that the effects of the proportion and concentration of compounds are not considered (Anderson and Whitcomb, 2002; Rezazadeh and Niedz, 2015).

In the present study, we set up an efficient protocol to isolate protoplasts from suspension-cultured cells from the fruit flesh of sweet cherry and developed a transient expression system using GFP as a reporter gene. Furthermore, we optimized the factors influence the efficacy of protoplast isolation and transfection, including enzymolysis solution component concentration, enzymolysis time, the pH value of enzymolysis solution, PEG concentration, and transfection time. This is the first report of the transient expression of a gene in sweet cherry protoplasts. Our results lay the foundation for future molecular biology studies in sweet cherry.

2. Materials and methods

2.1. The preparation of cherry berry suspension-cultured cells

Sweet cherry (*P. avium* L.) cv. Hong Deng was used in this study. Ten-year-old trees were maintained in Beijing Institute of Forestry and Pomology, People's Republic of China. Cherry berries were harvested at 34 days after full bloom, washed with tap water for 30 min, sterilized with 5% (v/v) sodium hypochlorite for 13 min, followed by 75% ethanol for 30 s, and then rinsed in sterile distilled water three times. The peel was removed and the fruit flesh was diced into 8 mm^3 cubes and placed in MS medium (with 2.0 mg/l 6-BA, 1.0 mg/l 2, 4-D, 30 g/l sucrose, and 7.5 g/l agar) at 25 ± 1 °C.Callus was produced and subcultured every 2–3 weeks. Cell suspension culture was based on MS medium supplemented with 1.0 mg/l 6-BA, 0.3 mg/l 2, 4-D, and 3.0 mg/l Vitamin C (Vc). The culture was incubated at 80 rpm/min on a rotary shaker, and the culture was subcultured once a week.

Table 1
Combinations of cellulase R-10 and pectolase Y-23 used in enzymolysis solution.

Treatment no.	Cellulase R-10 (%)	Pectolase Y-23 (%)
1	0.5	0.25
2	1.0	0.25
3	1.5	0.25
4	0.5	0.50
5	1.0	0.50
6	1.5	0.50
7	0.5	0.75
8	1.0	0.75
9	1.5	0.75

2.2. Protoplast isolation

Protoplast isolation procedures were carried out as previously described (Ochatt et al., 1987; Yoo et al., 2007; Huang et al., 2013; Wang et al., 2015) with several modifications. Before enzymatic digestion, suspension cells were plasymolyzed in CPW13M [CPW salts with 13% (w/v) mannitol] solution for 1 h at 23 °C, with shaking at 50 rpm/min. The enzyme solution contained bovine serum albumin [BSA, 0.1% (w/v)], MES Free Acid [0.1% (w/v)], polyvinylpyrrolidone [PVP K-30, 1.0% (w/v)], mannitol, and different concentrations of Cellulase R-10 (Japan Yakult) and Pectolase Y-23 (Japan Yakult), as shown in Table 1. To optimize the concentration of mannitol used in the enzyme solution, different concentrations of mannitol were tested (0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M). To optimize enzymolysis time, different digestion times were tested (12, 14, 16, 18, and 20 h). To optimize the pH of the enzymolysis solution, different pH values of the enzymolysis solution were tested (5.2, 5.4, 5.6, 5.8, 6.0, and 6.2). All the experiments were repeated three times. One milliliter of the cell suspension was incubated in 10 ml enzymolysis solution at 23 °C, with rotation (50 rpm/min). After digestion, the mixture was filtered through 74 μm sieves. The filtrate was centrifuged at 900 rpm for 5 min, the supernatant was discarded, and the sediment was resuspended in 3 ml CPW13M [CPW salts with 13% (w/v) mannitol] solution. Then, 6 ml CPW25S [CPW salts with 25% (w/v) sucrose] was added to a new 10 ml centrifuge tube, supplemented with all the protoplast suspensions, and centrifuged at 900 rpm/min for 5 min (Marchant et al., 1997; Hao et al., 2013). Protoplasts were carefully removed from the interface of the solutions, then the protoplasts were washed three times with CPW13M and then centrifuged at 900 rpm for 5 min, the supernatant was discarded, and then the protoplasts were resuspended in MMG (400 mM mannitol, 30 mM $MgCl_2$, 4 mM MES) solution at a final concentration of 4×10^6 protoplasts/ml for transient transfection.

The yield of purified protoplasts was determined using a hematocytometer under a general light microscope (XSZ-G) and their viability was assessed using Trypan blue. For each sample, the count was performed three times. Protoplast viability was tested using 4% Trypan blue (Kamlesh et al., 1984). The Trypan blue solution was prepared by mixing 1 ml Trypan blue with 9 ml PBS at a 1:10 dilution. Briefly, 20 μl Trypan blue solution was added to 180 μl of protoplast suspensions to a final concentration of 0.04%, the samples were incubated in darkness at 25 °C for 5 min, and protoplast viability was assessed using a microscope (Nikon ECLIPSE Ti, Japan), according to the following equation: protoplast viability (%) = number of protoplast not stained blue in view/number of total Protoplast in view × 100%.