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An efficient mesophyll protoplast isolation, purification and PEG-mediated transient gene expression for subcellular localization in Chinese kale

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ABSTRACT

Transient gene expression serves as a valuable tool for gene functional study in plants. Here we reported protoplast isolation and purification from the plantlet young leaves of Chinese kale (*Brassica oleracae* var. *alboglabra* Bailey), and their transient gene expression with polyethylene glycol (PEG)-mediated transformation and subcellular localization of phytoene desaturase (BaPDS1). The procedures of isolation andtransformation ofmesophyll protoplasts derived from Chinese kale were optimized, and the influencing factors were analyzed. The results showed that the optimal protocol of protoplast isolation and purification was initialized by digestion in enzyme solution (2.0% cellulase, 0.1% pectolase, and 0.6 M mannitol) for 9 h. After filtered through 400 mesh and centrifuged at 179 $\times g$ for purification, the total yield of protoplast reached as high as 6.04×10^7 protoplasts g^{-1} fresh weight (FW) and the viability of the protoplasts was up to 95%. A maximum transformation efficiency of approximately 30% measured by using green fluorescent protein (GFP) as a detecting gene was obtained when PEG4000 was at a final concentration of 40% and transformation time was set to 15 min. In addition, the subcellular localization of BaPDS1 in Chinese kale was targeted to the chloroplast, confirming the efficiency and reliability of this transient transformation system. Taken together, an efficient protoplast isolation, purification and transformation system in Chinese kale was established in this study, laying a foundation for future research in molecular biology and gene function in Chinese kale and other *Brassica* vegetables.

1. Introduction

Protoplasts are naked plant cells with totipotency and viability that the cell wall has been enzymatically or mechanically removed, which are suitable materials for the acceptance of exogenous cell organelles and nucleic acids due to the absence of cell wall (Lei et al., 2015). Plant protoplasts provide a unique single cell system for studying most aspects of plant cell physiology and genetics such as cell wall formation, photosynthesis activity, somatic hybridization, and transient gene expression (Davey et al., 2005; Zhang et al., 2011; Eeckhaut et al., 2013; Zhao et al., 2016). With the development of techniques for genome-editing and gene silencing, protoplast systems have found further utility in high-throughput screening applications such as CRISPR/Cas9 technology (Andersson et al., 2017; Tian et al., 2017).

Compared with the stable gene expression in transgenic plants (Sheng et al., 2016; Song and Gao, 2017), transient gene expression offers a fast and efficient technique forcellular, molecular, biochemical and genetic studies, particularly forrapid assessing gene functions such as subcellular protein localization (Wang et al., 2015), protein-protein interactions (Fujikawa et al., 2014), protein activity (Zhao et al., 2016), and signal transduction (Cao et al., 2014). To date, polyethylene glycol (PEG)-mediated protoplast transient expression has become a powerful tool to rapidly characterize gene functions, which is one of the most reliable approaches (Jeon et al., 2007; Lung et al., 2011; Yu et al., 2017). Thus, the establishment of an efficient protoplast isolation and transformation system could be a profitable endeavor to elucidate a candidate gene function. Currently, protocols for protoplasts isolation and protoplast-based transient gene expression have been established in various plant species such as *Arabidopsis thaliana* (Yoo et al., 2007), cucumber (*Cucumis sativus*) (Huang et al., 2013), maize (*Zea mays*) (Chen et al., 2015), sweet cherry (*Prunus avium*) (Yao et al., 2016), cassava (*Manihot esculenta*) (Wu et al., 2017), which have been accelerating high-throughput analysis and rapid functional characterization of genes in these plants.

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Chinese kale belongs to the *Brassica* species of the Brassicaceae family. It is an economically important vegetable crop rich for vitamin C and bioactive compounds like glucosinolates, and widely cultivated in South China and Southeast Asia (Sun et al., 2011). Considering the importance of rapid screening of gene expression, genome-editing and subcellular protein localization, it is necessary to develop an efficient system for protoplast-based transient gene expression in Chinese kale. In *Brassica* species, studies have focused mainly on the generation of protoplast-derived transgenic plants (Mukhopadhyay et al., 1991; Ren et al., 2000; Nugent et al., 2006; Sheng et al., 2011). Protoplast isolation from Chinese kale mesophyll has been previously reported (Pua, 1987). Nonetheless, there is not yet a rapid and effective system for protoplast isolation and purification in Chinese kale. Moreover, there is rare study of transient expression assays utilizing PEG-mediated protoplast in Chinese kale. In the study, we report a simplified and efficient method for protoplast isolation and transient gene expression. Various factors affecting the efficiency of protoplast isolation including enzymatic composition, digestion time, mannitol concentration in the enzyme solution, nylon membrane mesh and centrifugal speed were evaluated to optimize protoplast isolation and purification procedures. Besides, using green fluorescent protein (GFP) as a reporter gene, critical parameters that affect transient transformation efficiency were also investigated such as PEG concentration and transformation time. The feasibility and efficiency of this system in Chinese kale were proved by subcellular localization of phytoene desaturase (BaPDS1), which is a key enzyme involved in carotenoid biosynthesis (Qin et al., 2007).

To our knowledge, this is the first report regarding transient gene expression in Chinese kale protoplasts. This transient gene expression system using leaf mesophyll protoplasts could be applied to analyze the complex regulatory mechanisms and could contribute to study protein subcellular localization, protein-protein interactions and functional gene expression in Chinese kale and related species.

2. Materials and methods

2.1. Plant materials

The cultivar Sijicutiao of white-flowered Chinese kale was used in the study. Mature seeds were surface-sterilized by dipping into 75% (v/v) alcohol for 30 s and disinfected with 0.1% (w/v) mercury chloride for 6 min, respectively, then washed 3–5 times with sterile distilled water for 3 min and dried off with sterilized filter paper. The sterilized seeds were germinated on 1/2 murashige and skoog (MS) medium (Murashige and Skoog, 1962) [3% (w/v) sucrose, 0.8% (w/v) agar, pH 5.8] in 240 ml tissue culture flasks. Seedlings were maintained under a photoperiod of 16/8 h light/dark at 25 °C to obtain fully extended true leaves for protoplast isolation.

2.2. Protoplast isolation and purification

Protoplast isolation was optimized and modified from the protocols provided by Pua (1987) and Yoo et al. (2007). The enzyme solution contained 5 mM 2-(*N*-Morpholino) ethanesulfonic acid (MES) and mannitol that was dissolved in cell protoplast wash medium (CPW) salts (Pua, 1987), then different concentrations of cellulase R-10 (Yakult, Japan) (1.5%, 2.0% and 2.5%) and pectolase Y-23 (Yakult, Japan) (0.05%, 0.10% and 0.20%) were added. To optimize the concentration of mannitol used in the enzyme solution, different concentrations of mannitol (0.4, 0.5, 0.6, 0.7 and 0.8 M) were compared. All enzyme solutions were adjusted to pH 5.8 and filter-sterilized through 0.22 μm syringe filter, and then stored at 4 °C for later use.

Newly expanded young leaves of 3-week-old plants were used as sources of protoplast. Approximately 1 g fresh weight of the plantlet leaves in a similar growth state were cut into thin strips (0.5–1 mm), and treated with 10 ml selected enzymesolution and incubated in the dark at 25 °C with gentle shaking (45 rpm). To optimize digestion time, protoplast yield and viability were checked at different digestion time scales (7, 8, 9, 10, 11 and 12 h).

After digestion, the enzyme mixture was filtered through nylon mesh. To select proper nylon membrane, membrane with different mesh size (300, 400 and 500 mesh) were tested. The filtrate was centrifuged at various speeds for 5 min. The protoplast pellets were resuspended with W5 salt solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, pH 5.7) (Yoo et al., 2007) and mixed gently. To optimize centrifugal speed, different speeds (64, 114, 179, 258 and 351 $\times g$) were set. After washing twice with W5 solution, the collected protoplasts were resuspended in W5 solution for transient transformation.

Protoplast yield was counted using a double-chamber haemocytometer under an Olympus CX21 light microscope (Olympus, Japan). Protoplast viability was determined by staining with 0.01% (w/v) fluorescein diacetate (FDA) (Larkin, 1976). The samples were incubated in darkness for 5 min, then assessed under a Zeiss AxioImager A2 fluorescence microscopy (Carl Zeiss, Germany) with UV excitation light (with a blue filter). Protoplasts were considered viable if they exhibited yellow-green fluorescence. For each sample, the count was performed at least three fields. Protoplasts yield and viability were calculated as follows: protoplast yield [protoplasts g^{-1} fresh weight (FW)] = number of the protoplasts yielded in enzyme solution / fresh weight of the plantlet leaves used in enzyme solution; protoplast viability (%) = (number of the fluorescent protoplast in view / number of the total protoplasts in view) ×100%.

2.3. Protoplast transformation

PEG-mediated transient expression procedures in Chinese kale protoplasts were performed using the methods previously reported (Yoo et al., 2007; Huang et al., 2013) with slight modifications. Isolated protoplasts were kept on ice and precipitated by gravity for 30 min. The upper liquid was discardedand the protoplasts were resuspended at a concentration of 5×10^5 protoplasts ml^{-1} in MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7) at room temperature (Yoo et al., 2007).

The pC2300 vector containing the CaMV 35S promoter was used to test the transformation efficiency of the Chinese kale mesophyll protoplasts. A volume of 10 μl pC2300-35S-GFP plasmid DNA (10 μg) was added to 100 μl of the prepared protoplasts (5×10^4 protoplasts) and mixed gently. An equal volume (110 μl) of freshly prepared PEG4000 solution (PEG4000, 0.2 M mannitol, 100 mM $CaCl_2$) was immediately added and the suspension was carefully mixed by gently inverting the tube. To optimize PEG concentration, different concentrations of PEG4000 (with 0 as control, 10, 20, 30, 40 and 50%, w/v) were tested. To optimize the transformation duration, the mixture was incubated for 5, 10, 15, 20 and 25 min in the dark at room temperature. After incubation, the transformation process was stopped by adding 550 μl W5 solution. The mixture was centrifuged at 179 $\times g$for 2 min and the protoplasts were gently resuspended with 1 ml WI solution (4 mM MES, 0.5 M mannitol, 20 mM KCl, pH 5.7) (Yoo et al., 2007). The transfected protoplasts were incubated at 25 °C in the dark for 20–24 h andcentrifuged at 179 $\times g$ for 2 min, then resuspended with 200 μl WI solution.

The protoplasts expressing GFP-fusion protein were observed and images were captured using a Zeiss AxioImager A2 fluorescence microscope equipped with an Axio MR R3 camera (Carl Zeiss, Germany). For subcellular localization analysis, protoplasts were investigated with a 100× oil objective. The number of green fluorescent protoplasts was used to calculate transformation efficiency, according to the following equation: transformation efficiency (%) = (fluorescent protoplast number in view/total protoplast number in view) ×100%.

2.4. Plasmid construction and subcellular localization of BaPDS1 protein

Total RNA was extracted form true leaves of Chinese kale using an