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ORIGINAL PAPER

Culture and chemical-induced fusion of tobacco mesophyll protoplasts in a microfluidic device

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Abstract Microfluidic systems provide a powerful platform for biological analysis and have been applied in many disciplines. However, few efforts have been devoted to plant cell study. In this article, an optimized culture of tobacco mesophyll protoplasts and their first polyethylene glycol-induced fusion in a microfluidic device are presented. Culture medium optimization and dynamics of protoplast growth including size change, organelle motion, and cell mass formation were also investigated microscopically in real-time. On-chip protoplast culture showed that the first division percentage of tobacco mesophyll protoplasts could be improved as high as up to 85.6% in 5 days using NT1 medium, and the percentage of small cell mass formation was more than 48.0% in 10 days. Meanwhile, chemical-induced fusion of tobacco mesophyll protoplasts was realized in 3–5 min and a 28.8% fusion rate was obtained, which was similar to the conventional fusion in a macro-scale environment. These results will be helpful for the development of microfluidics-based studies on manipulation and analysis of plant cells in a miniaturized environment, including cell growth and differentiation, gene isolation, and cloning.

Keywords Microfluidic device · Tobacco mesophyll protoplast · Cell culture · Chemical-induced fusion

1 Introduction

An organism is any living system, which can either be unicellular (e.g., microorganism) or composed of multiple numbers and types of cells grouped into specialized tissues and organs (e.g., animal, plant) (Woese et al. 1990). Cells, the basic units of life, are subject to multiple cues from their ambient environment that vary in time and space, including soluble biomoleculars from neighboring cells, bio-mechanical signals from the growth scaffold, and direct cell–cell contacts (El-Ali et al. 2006; Bhatia et al. 1999). The biological processes involved are specific, so reproducing and controlling the conditions to the highest possible extent are of great interest. Thus far, many technological approaches have shown potential capabilities in addressing the in vitro maintenance of live cell activities or in simulating an in vivo cellular microenvironment (Du et al. 2008; Alsberg et al. 2002). For example, conventional tissue culture significantly facilitates in vitro research on cell growth, proliferation, metabolism, and responses (Blow 2009), and tissue engineering promotes biomimetic construction of tissue composition and spatial organization (Khetani and Bhatia 2006; Fukuda et al. 2006). Meanwhile, specific biogenesis directs the precise and spatiotemporal principle, which indicates a more efficient governing of the miniature extracellular environment besides cells.

Microfluidics, one of the most novel tools in miniaturization, introduces a new capability into the area of cell

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biology (Whitesides 2006; Psaltis et al. 2006; Regehr et al. 2009), which can keep both cells and their cues in a controllable and reproducible manner. This was not easily achieved in the past just by conventional tissue culture (Ma et al. 2009; Lee et al. 2005). Also, microfluidic connection of cell culture with integrated analytical devices can elucidate the biochemical processes that govern cell behavior (Wang et al. 2006; Kirkness 2009; Park et al. 2010). Recent developments in this field demonstrate that microfluidics can be a versatile platform for mammalian cells and bacteria-related studies (Kirkness 2009; Park et al. 2010; Liu et al. 2010; Yung et al. 2009). However, application of microfluidics in the field of plant cell biology is nearly nil, even those involving the preliminary cultivation of protoplasts and their relation with the high air permeability of polydimethylsiloxane (PDMS) substrates (Ko et al. 2006; Ju et al. 2006). The possible reasons may be that the availability and long-term cultivation of plant protoplast in vitro are very difficult, even using conventional tissue culture methods (Ko et al. 2006; Nagata and Takebe 1971). Being eukaryotes, plant cells and animal cells have many similarities in structure. They all possess nucleus, membrane-bound organelle, ribosome, and cytoskeleton. However, the structures of plant cells and animal cells have clear differences. Chloroplasts and cell wall exist in plant cells, but not in animal cells. Under normal circumstances, plant cells are relatively easy to save. However, protoplasts, plant cells without cell wall, are very difficult to save in vitro because protoplasts lose cell wall protection.

In this article, we present a considerable improvement in the micro-manipulation and micro-analysis of plant cells in a microfluidic device with five pentagon-arranged chambers. Enhanced cultivation of tobacco mesophyll protoplasts with an optimized medium was achieved. Also, chemical fusion of the protoplasts was performed successfully in the microfluidic device using a polyethylene glycol (PEG)-induced method. The results further demonstrated the possibility that microfluidics can serve as a miniaturized version of conventional laboratory techniques applied in botany. Also, this microfluidic approach will facilitate the miniature development of plant cell engineering, such as protoplast culture, transgenesis, and somatic cell hybridization.

2 Experimental

2.1 Materials

RTV 615 poly(dimethylsiloxane) (PDMS) prepolymer and the curing agent were purchased from GE Silicones (Minato-ku, Tokyo); surface-oxidized silicon wafers from Shanghai Xiangjing Electronic Technology Ltd. (Shanghai, China); and SU-8 2025 photoresist and developer from Microchem (Newton, MA, USA). All solvents and other chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. All solutions were prepared using an ultra-purified water supplied by a Milli-Q system (Millipore®).

2.2 Design and fabrication of the microfluidic device

The microfluidic device contains five functional culture chambers arranged in a pentagonal array, to which five channels of 200 μm width extend from a center sample input port (Fig. 1). The size of each functional chamber is 900 μm in width, 3200 μm in length, and 55 μm in height. The overall size of the device is 4 cm × 4 cm. Each input channel was symmetrically bifurcated to permit sufficient dispersion of the sample (i.e., protoplasts) (Carlo et al. 2006; Moraes et al. 2010; Gómez-Sjöberg et al. 2007). Double micro-column (30 μm in length, 20 μm in width, and 55 μm in height) lines with both a column and line space of 20 μm were introduced to realize protoplast trapping. Meanwhile, the 800-μm extended chamber and

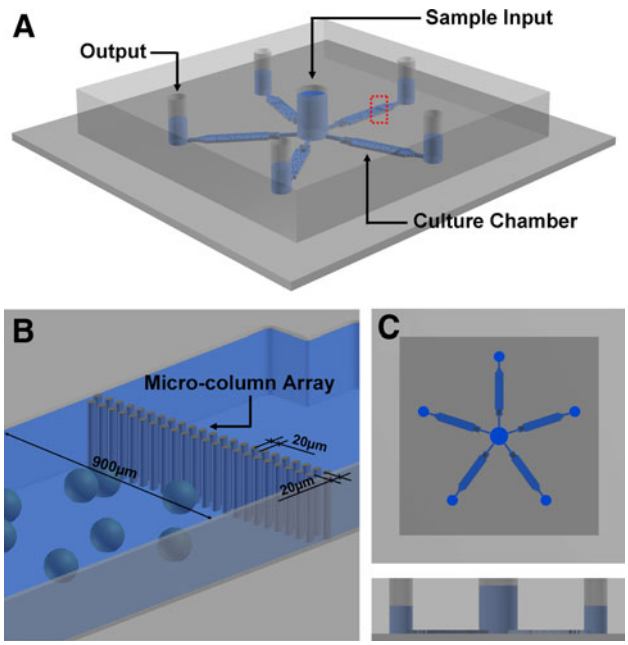

Fig. 1 Configuration and function of the microfluidic device. **a** Schematic representation of the device with five culture chambers arranged in a pentagonal array and applied for the parallel culture of protoplasts. A center sample input was designed for the introduction of various liquids, including protoplast suspension, rinsing solution, culture medium, and dye. The square in the *red dotted line* corresponds to **b**. Double micro-column (30 μm in length, 20 μm in width, and 55 μm in height) lines in each chamber were designed to promote trapping of protoplasts while the seeding process (**b**). The gap between micro-columns was 20 μm. **c** Plan (*up*) and elevation (*down*) of the microfluidic device. Hydrostatic pressure was used to realize material transportation using a small volume difference. (Color figure online)

the stair channels behind the micro-column lines were designed to reduce flow stress gradient influence on the protoplast cultivation as much as possible.

The microfluidic device was fabricated by soft lithography with PDMS (Thorsen et al. 2002; Wang et al. 2006; Unger et al. 2000). First, the prototype mold of the device was fabricated by a lithographic pattern of a 55-μm-thick SU-8 photoresist on a silicon substrate using a BG-401A mask aligner (7 mW/cm^2, CETC, China). Next, the device was fabricated by a PDMS pouring, degassing, and baking process. The sample access port was created by punching a through-hole at the origin of the center circle with a metal pin; the same process was done for the five outports of the device. The punched PDMS was then bonded to a thin glass slide after oxygen plasma treatment. The bonded chips were baked at 85°C overnight. The glass slide was pre-cleaned in a piranha solution (H_2SO_4:H_2O_2 = 3:1, v/v) for 1 h and then rinsed with Millipore ultra-pure water.

2.3 Protoplast preparation

The tobacco mesophyll protoplasts used in this study were isolated according to methods reported previously (Ko et al. 2006; Nagata and Takebe 1971). First, fully expanded leaves of *Nicotiana tabacum* were grown at room temperature for 60–120 days and sterilized by immersing with 75% alcohol for 30 s and 0.1% mercuric chloride for 8–10 min, respectively. The flushing process with Millipore ultrapure water was performed after every immersion. The hypodermal layer was carefully removed with tipped tweezers and cut into small pieces, then the leaf pieces were incubated in an enzyme solution containing 1.0% (w/v) cellulase onozuka R-10, 0.5% (w/v) macerozyme, 0.45 mol/l mannitol, and 5 mmol/l 2 (*N*-morpholino) ethanesulfonic acid (MES) at 25°C for 16 h, followed by filtration using a stainless steel 150 mesh to discard the residues. The enzymatic mixture was centrifuged at 800 rpm for 6 min. Further, the density gradient centrifugation method was used to purify the deposited protoplasts using a cell-protoplast washing (CPW) solution and 21% sucrose solution. The final protoplasts were washed with Nagata–Takebe medium (NT medium) (Nagata and Takebe 1971) thrice and then re-suspended for subsequent experiments. The number of protoplasts was counted using a normal hemacytometer.

2.4 Protoplast seeding and culture in the microfluidic device

For cultivation, tobacco mesophyll protoplasts with a density of 2.0 × 10^5 cells/ml were first transferred into the inlet of the microfluidic device using a pipette. The protoplast suspension flowed through the chamber as an effect of hydrostatic pressure, and the protoplasts were trapped using double micro-column lines. To ensure an uniformity in the input of each microchamber, the microdevice was kept in a level state. After slight rinsing, the fresh culture medium was added into the inlet. We kept the device in a humidified atmosphere with a day/night regime (16 h/8 h) at 27°C under dim fluorescent light and 25°C in the dark. Due to the high air permeability of PDMS substrates, O_2 and CO_2 in ambient environment can enter or discharge the microchambers during protoplast culture (Ko et al. 2006; Ju et al. 2006). Three types of media were applied and tested for the microfluidic culture of the protoplasts, including Murashige–Skoog medium (MS medium) (Murashige and Skoog 1962), NT1 medium (i.e., NT medium modified with 800 mg/l $CaCl_2{\cdot}2H_2O$, 370 mg/l $MgSO_4{\cdot}7H_2O$, 2 mg/l Glycine, 1 mg/l Nicotinic acid, 2 mg/l Thiamine hydrochloride, 0.5 mg/l Pyridoxine hydrochloride, and 0.46 M D-Mannitol), and NT6 medium (i.e., NT medium modified with 1000 mg/l $CaCl_2{\cdot}2H_2O$, 740 mg/l $MgSO_4{\cdot}7H_2O$, 2 mg/l glycine, 5 mg/l nicotinic acid, 10 mg/l thiamine hydrochloride, and 10 mg/l pyridoxine hydrochloride).

2.5 Protoplast viability

The viability of the tobacco mesophyll protoplasts was evaluated using fluorescent staining after the isolation process. The protoplasts were incubated in 0.01% fluorescein diacetate (FDA) acetone solution for 5 min and then rinsed with the culture medium.

2.6 Chemical-induced protoplast fusion

To expand microfluidic application in the field of botany, protoplast fusion was carried out using a chemical-induced method (Ogle et al. 2005; Lentz 2007). A 35% PEG-6000 solution was put into the inlet reservoir and flowed into the chamber, which had loaded tobacco mesophyll protoplasts. A density of 1.0 × 10^5 cells/ml was used here for cell seeding too. This treatment was maintained for 10 min at room temperature and monitored in real-time, followed with a washing process by the culture medium.

2.7 Microscopy and image analysis

We used an inverted microscope (Olympus, CKX41) with a CCD camera (QIMAGING, Micropublisher 5.0 RTV) and a mercury lamp (Olympus, U-RFLT50) to acquire the phase contrast and fluorescent images. The images of the tobacco mesophyll protoplast culture in the device were taken every 24 h. The images were then analyzed using Image-Pro® Plus 6.0 software (Media Cyternetics, Silver Spring, MD). All statistical analyses were performed using Software SPSS 12.0 (SPSS Inc.).

3 Results and discussion

3.1 Culture medium optimization for on-chip protoplast culture

Protoplast culture is indispensable to various in vitro activities of plant cell research (e.g., plant regeneration and genetic manipulation) because of the capability of artificial influence on plant cell activities. It is also vital in the raw material supply for living cell processing and analysis (Davey et al. 2005). Tobacco mesophyll protoplasts are one of the common model organisms used in studies of physiological and cytological processes related to cell wall formation, cell growth, and differentiation (Takahashi et al. 1989; Sheen 2001).

In this work, a PDMS microfluidic device (Fig. 1) with five functional culture chambers arranged in a pentagonal array was fabricated and applied for the parallel culture of tobacco mesophyll protoplasts. The design was based on the prototype designed for protoplast study (Ko et al. 2006). The parallel five function chambers were designed to enhance the consistency and reliability of the experimental data. Also, similar structures utilized for parallel tests were demonstrated previously (Carlo et al. 2006; Moraes et al. 2010; Gómez-Sjöberg et al. 2007). The height (55 μm) of the microchamber was based on our optimization of the device architecture, as well as an analysis of the prototype structure and the experiment results (Ko et al. 2006). If the height was too low (e.g., 30 μm), protoplasts cannot survive. However, the high height caused the waste of expensive photoresist and PDMS. For example, after a microcolony of protoplast was formed in a 100-μm height microchannel, there was still plenty of space available for protoplast growth (Ko et al. 2006). Double micro-column lines in each chamber were designed to promote trapping of the protoplasts while their seeding process. Hydrostatic pressure was used to achieve seeding and feeding of the protoplasts, which represents a general method for microfluidic cell culture, especially for environment-sensitive cells (e.g., neural cells) (Taylor et al. 2005; Wang et al. 2009). In this study, a high viability of over 95% of seeded tobacco mesophyll protoplasts was obtained (Fig. 2c).

To facilitate better growth in the microenvironment of the device, the tobacco mesophyll protoplasts were first cultured using three types of culture media for optimization, including Murashige and Skoog medium (MS medium) and two modified branches based on Nagata–Takebe medium (i.e., NT1 and NT6 medium), respectively (Murashige and Skoog, 1962; Nagata and Takebe 1971). In fact, the other two media, Nagata–Takebe (NT) medium (Nagata and Takebe 1971) and Durand, Potrykus, and Donn (DPD) medium (Durand et al. 1973), were also utilized in our pre-experiments. However, protoplasts cannot grow well on them. Therefore, in the next discussion we only list the three media, MS, NT1, and NT6. The percentage of the first division of cultured protoplasts in 5 days was used to assess the effect of the media quantitatively, as well as the percentage of formation of a small

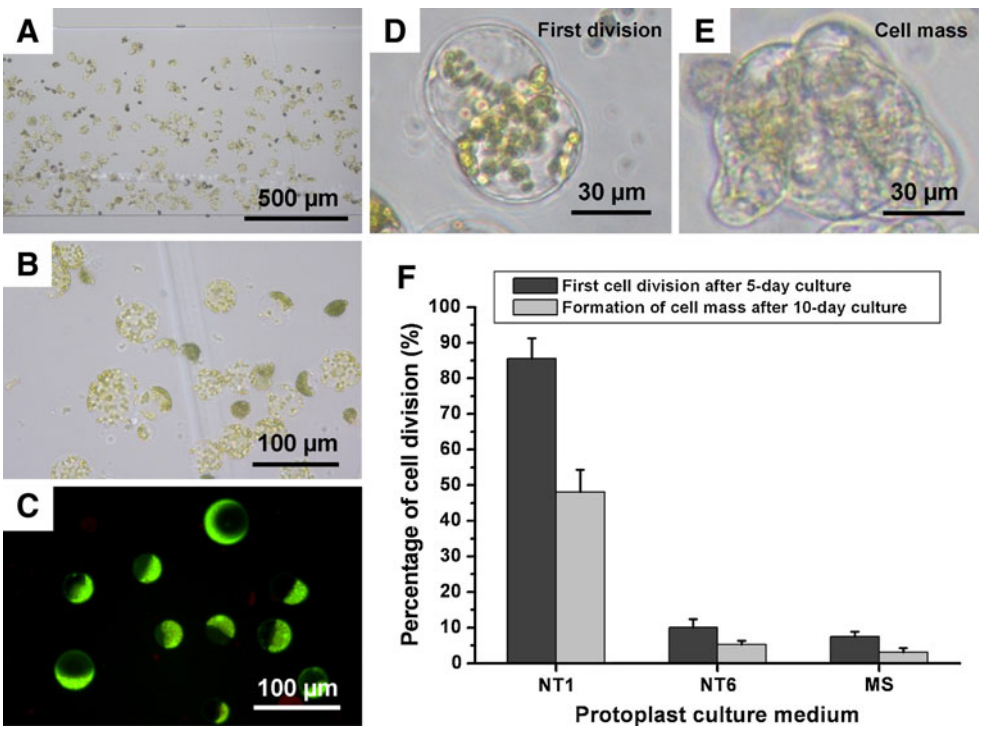

Fig. 2 Seeding and optimized cultivation of tobacco mesophyll protoplasts in the microfluidic device. **a**, **b** Protoplasts introduced into the device. **c** A high viability of protoplasts was achieved for the microfluidic culture. **d** First division. **e** Small cell mass. **f** Protoplasts were cultured using three types of culture media, including Murashige and Skoog medium (MS medium), and two modified branches based on Nagata–Takebe medium (i.e., NT1 and NT6 medium), respectively. Both percentage of first division of the cultured protoplasts in 5 days and that of the formation of a small cell mass in 10 days were used to assess quantitatively the effect of culture media. The NT1 medium can keep the highest division activity of the protoplasts

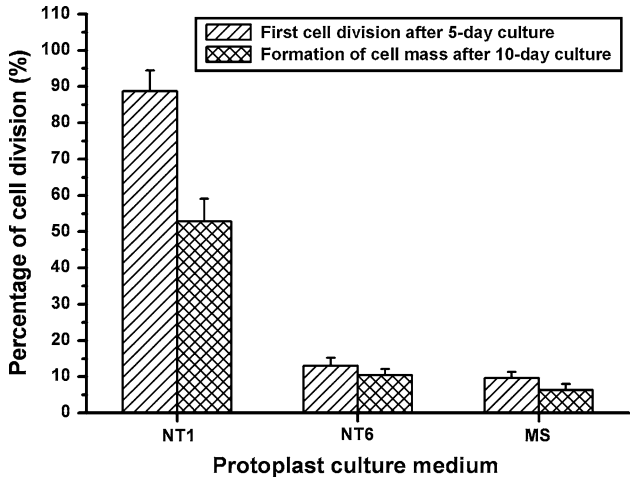

Fig. 3 The tobacco mesophyll protoplasts were cultured in a Petri dish using three types of culture media, including Murashige and Skoog medium (MS medium), and two modified branches based on Nagata–Takebe medium (i.e., NT1 and NT6 medium), respectively. Compared with the MS and NT6 mediums, the NT1 medium kept the highest division activity of the protoplasts. This result was similar to that of the microfluidic protoplast culture

cell mass in 10 days. The results showed that the percentage of the first division of protoplasts cultured in NT1 medium was up to 85.6% in 5 days, and the percentage of formation of a small cell mass was over 48.0% in 10 days (Fig. 2f). These figures are much larger than those of protoplasts cultured using both NT6 and MS media, which were all under 10.0%. All of the surviving protoplasts underwent division and some developed into small but visible colonies. However, some of the initially living protoplasts died without undergoing cell division. The most possible explanation for the superior effect of the NT1 medium (i.e., better cell growth and division) was the appropriate modification of the concentration of inorganic and organic ingredients. These findings are consistent with those of the comparative test using a standard culture in the macro-scale culture environment of a Petri dish (Fig. 3).

3.2 Assay of protoplast growth in the microfluidic device

To further examine cell growth and activities, on-chip cultivation of tobacco mesophyll protoplasts in NT1 medium was carried out. The results show that the chloroplasts dispersed in the cytoplasm after seeding, and they began to expand when cultured in a microchamber for 1 day. Notably, the frequency of this expanding phenomenon in protoplasts increased up to 80% after the 2-day cultivation (Figs. 4, 5a). Also, the chloroplasts in a large number of

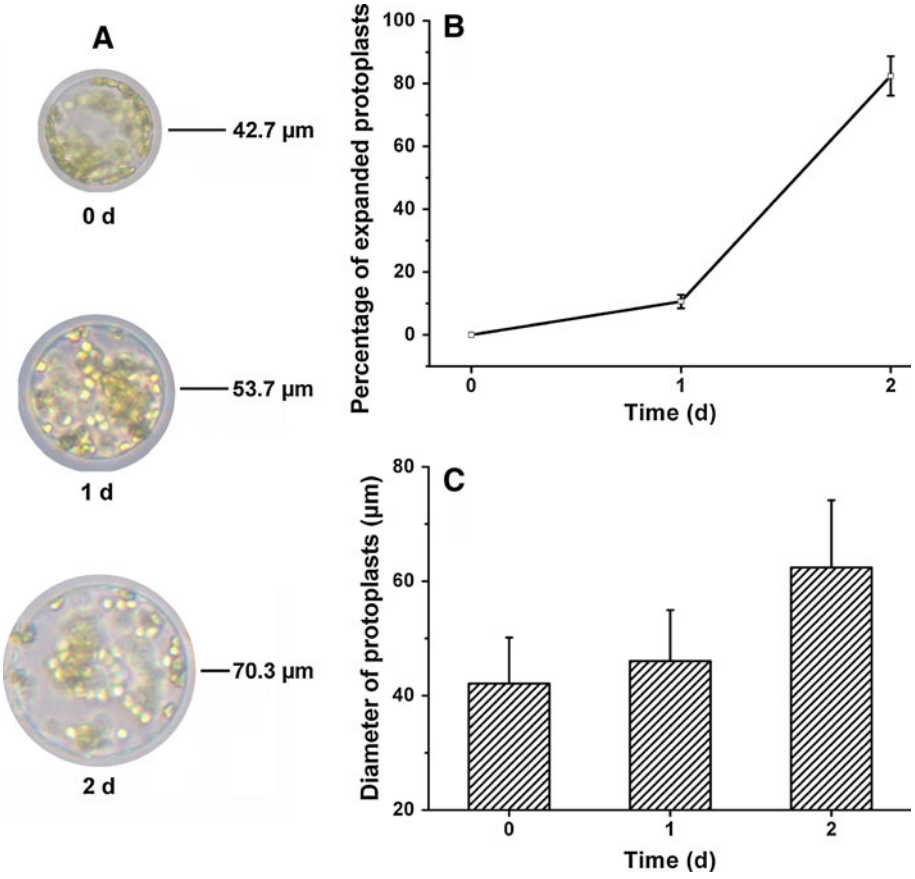

Fig. 4 Volume expansion of the tobacco mesophyll protoplasts before division during the first 2 days of cultivation in the microfluidic device. **a** The typical diametrical variance of the protoplasts during culture and dynamic distribution of the chloroplasts as increasingly central concentrating. **b** Percentage of expanded protoplasts during culture for 0, 1, and 2 days. Obviously, more and more protoplasts expanded along with the cultivation. **c** The mean diameter of all protoplasts after culture for 0, 1, and 2 days in the microchamber. For the statistics of the expanded protoplasts, the mean diameter of the 0-day microfluidic cultured protoplasts (42.1 μm) is the original dimension, and the extent of increase in diameter (i.e., the increased gap ≥10.0 μm) as the expanding

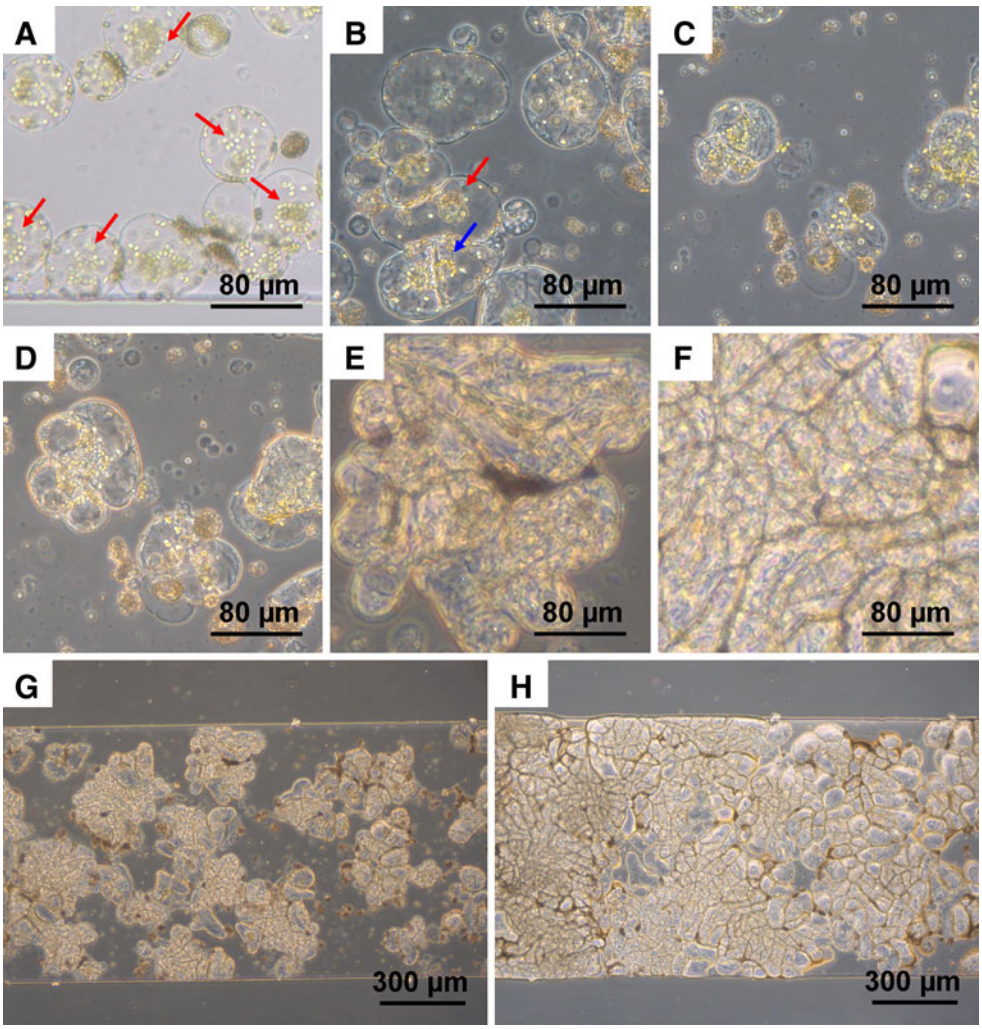

Fig. 5 Growth and division of tobacco mesophyll protoplasts in the microfluidic device. **a** Protoplasts cultured for 2 days. Obviously, the chloroplasts in most protoplasts gathered and concentrated (*red arrows*). **b** The first division of the protoplast appeared after a 3-day culture. The chloroplasts in the parent cell were distributed into two daughter cells (*blue arrow*). **c** The second division of the protoplasts happened while cultured for 5 days. **d** Formation of small cell mass after a 6-day culture. **e** Formation of a big cell mass after an 8-day culture. **f** Tight arrangement of protoplasts cultured for 12 days. **g** Many big cell masses dispersed in the microchamber after an 8-day culture. **h** The microchamber was filled up with cell masses after cell culture for 12 days. (Color figure online)

Table 1 Comparison of tobacco mesophyll protoplast division

Culture model	Occurrence of cell division (days)					Reference
	The 1st division	The 2nd division	Cell mass (6–8 protoplasts)	Cell mass (>10 protoplasts)	Visual cell mass	
Microfluidics	3	5	6	8	12	The current study
Petri dish	4	–	8	–	14	The current study
Petri dish	3	5	–	–	14	Nagata and Takebe (1971)
Petri dish	3	5–7	10	–	–	Nagata and Takebe (1970)
Petri dish	2–4	4–6	6–7	>7	–	Thomas and Rose (1983)
Petri dish	2	–	–	–	14	Ko et al. (2006)

expanded protoplasts (over 90%) gathered and concentrated (Fig. 5a). The increase in cell volume as well as the change in the arrangement of chloroplasts preceded cell division, which corresponded to the previous study on in vitro protoplast culture in a suspension in Petri dishes (Nagata and Takebe 1971; Coutts and Grout 1975). The

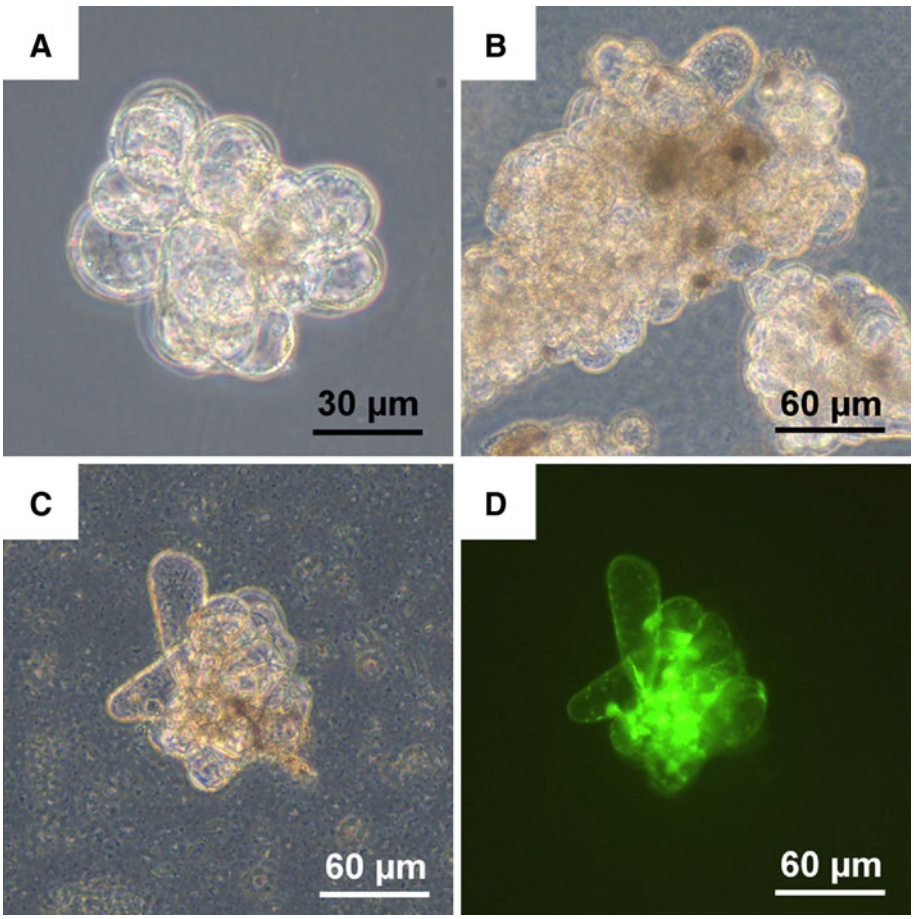

Fig. 6 Growth and division of the tobacco mesophyll protoplasts in a Petri dish. Obviously, the cell colony cultured in this environment was completely three-dimensional in shape. **a** Formation of a big cell mass after the 10-day culture. **b** Formation of a visual cell colony after the 14-day culture. **c** Formation of a big cell mass after the 10-day culture. **d** The high viability of a cell mass after the 10-day culture was retained

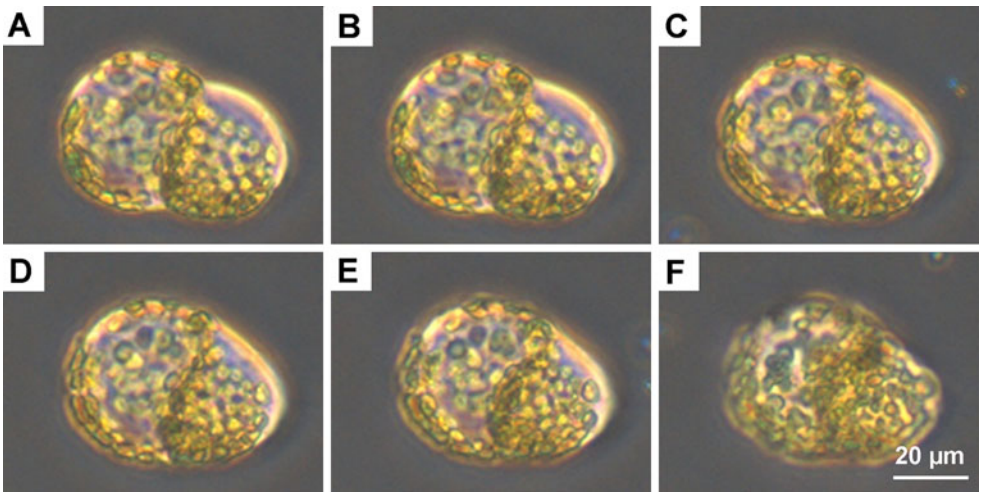

Fig. 7 Chemical fusion of the tobacco mesophyll protoplasts was performed in a microfluidic device using polyethylene glycol (PEG). The time when the two protoplasts contacted is considered as the start of fusion (i.e., 0 s) (**a**), followed by 10 s (**b**), 30 s (**c**), 50 s (**d**), 80 s (**e**), and 190 s (**f**)

results showed that cell division of the protoplasts in the chamber is evident beginning on the third day of culture. The chloroplasts in the parent cell were averagely distributed into two daughter cells (Fig. 5b). The second division was observed during the fifth day of the culture (Fig. 5c). This was followed by successive divisions resulting in the gradual formation of cell masses from small to big (Fig. 5d, e). The cells in this stage appeared to be rich in cytoplasm, and the chloroplasts also became less and less distinct, which indicates a more translucent or colorless

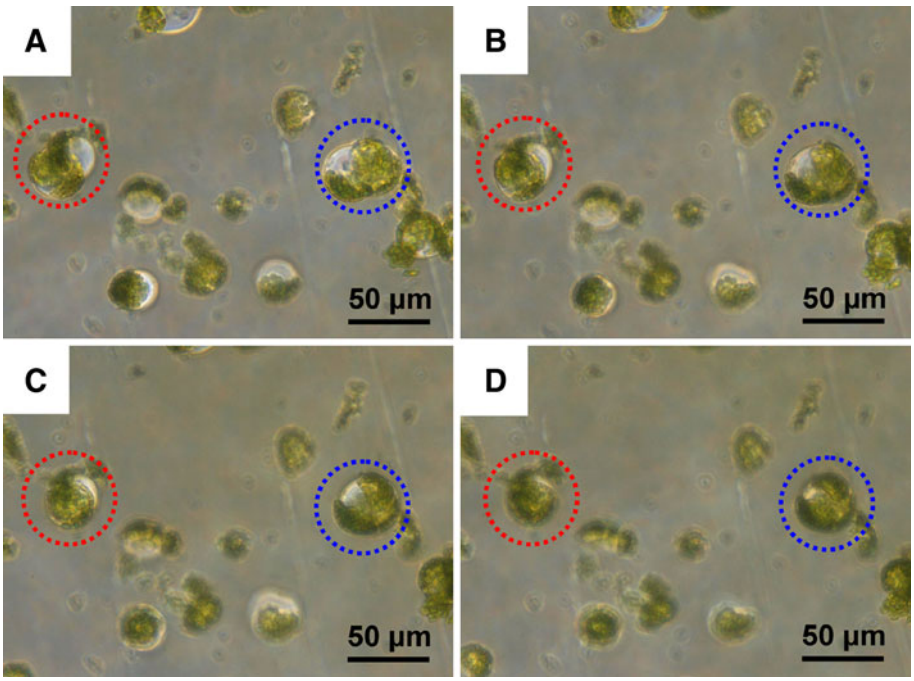

Fig. 8 Chemical fusion of the tobacco mesophyll protoplasts was performed in a Petri dish using polyethylene glycol (PEG). **a–d** The fusion was complete within 3 min

cytoplasm of the protoplasts (Fig. 5e–g). The microchamber was filled up with cell masses after cell culture for 12 days (Fig. 5h). The three-dimensional arrangement of the cells made counting difficult. Meanwhile, this arrangement was also modified by the spatial frame of the chamber and mainly showed horizontal preference as further division took place. However, even with a similar time of cell division (Table 1), this was unlike the conventional suspension culture of protoplasts, of which the colonies are completely three-dimensional in shape (Fig. 6). On the other hand, the optimized cultivation of tobacco mesophyll protoplasts in microfluidics was able to reach the peak, exceeding the values achieved in a previous study (Ko et al. 2006). This protoplast culture and all its growth and division stages can help in further research of plant cells in microfluidics, such as plant gene analysis.

3.3 Chemical-induced protoplast fusion

As a typical genetic manipulation in plant cell engineering, cell fusion (i.e., protoplast fusion) has been used for many different purposes, including generation of hybrids and reprogramming of somatic cells (Ogle et al. 2005). Cell fusion, generally called somatic cell hybridisation, is actually an excellent tool for breeding and genetic analysis of engineered plants (Davey et al. 2005; Takahashi et al. 1989).

To expand microfluidic application in plant cells, we also presented a conceptual attempt of protoplast fusion in the microfluidic device, which was not achieved previously. In this experiment, tobacco mesophyll protoplast fusion was performed through PEG-induced fusion (Ogle et al. 2005; Lentz 2007). The results in Fig. 7 show that adjacent protoplasts came into close contact with one another, and the membrane of the contacted protoplasts fused (Fig. 7a–c). Therefore, a connection was formed between the two cytoplasms. The components of both contacted protoplasts, especially the chloroplasts, mixed and combined with one another (Fig. 7e, f). Finally, the two protoplasts formed a fusion product with an irregular shape, which was different from the common spherical shape (Fig. 8). The possible reason for the deformation can be the spatially mechanical response of the fusion body in

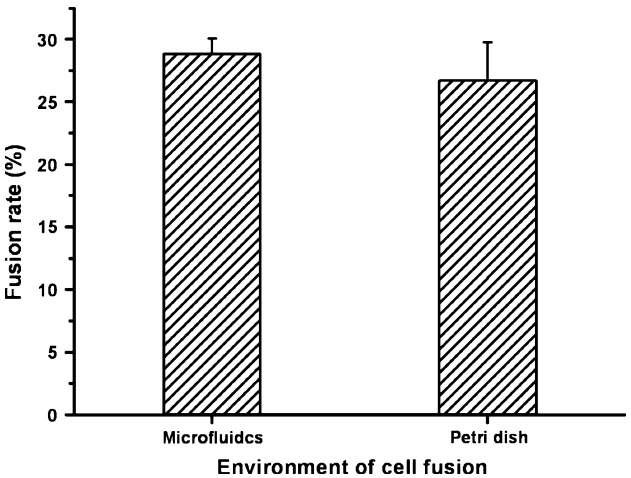

Fig. 9 Fusion rate of the tobacco mesophyll protoplasts using the standard PEG method in both the microfluidic device and a Petri dish. The effect of microfluidic fusion was similar to that of conventional fusion in a macro-scale culture environment

the microfluidic device because of the increased volume. The total duration of this fusion in the microfluidic device only took around 3–5 min, achieving a fusion rate of 28.8%. This result is similar to the conventional fusion in a macro-scale culture environment (Fig. 9).

4 Conclusions

In conclusion, we have demonstrated the feasibility of a microfluidic protoplast culture. We also achieved the chemical fusion of protoplasts in the microfluidic device. These successful microfluidic attempts are expected to facilitate various miniaturized manipulations and analyses of plants at the cellular and molecular levels, such as cell growth and differentiation, gene isolation, and cloning. Further, the approach will facilitate microfluidic development for a wide range of plant-related applications, including cell-based high throughput monitoring and screening, protein purification and analysis, and bio-mimicking. Compared with those designed for mammalian cells, however, the current device structure for protoplast study was relatively simple. More complex microfluidic device integrated multifunctional units should be introduced in the field with the complexity and depth of research problems on plant cells.

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