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# Transient Expression of Minimum Linear Gene Cassettes in Onion Epidermal Cells Via Direct Transformation

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**Abstract** A new method without any special devices for direct transformation of linear gene cassettes was developed. Its feasibility was verified through 5′-fluorescent dye (fluorescein isothiocyanate, FITC)-labeled fluorescent tracing and transient expression of a *gus* reporter gene. Minimal linear gene cassettes, containing necessary regulation elements and a *gus* reporter gene, was prepared by polymerase chain reaction and dissolved in transformation buffer solution to 100 ng/mL. The basic transformation solution used was Murashige and Skoog basal salt mixture (MS) liquid medium. Hypertonic pretreatment of explants and transformation cofactors, including $Ca^{2+}$, surfactant assistants, *Agrobacterium* LBA4404 cell culture on transformation efficiency were evaluated. Prior to the incubation of the explants and target linear cassette in each designed transformation solution for 3 h, the onion low epidermal explants were pre-cultured in darkness at 27 °C for 48 h and then transferred to MS solid media for 72 h. FITC-labeled linear DNA was used to trace the delivery of DNA entry into the cell and the nuclei. By GUS staining and flow-cytometry-mediated fluorescent detection, a significant increase of the ratios of fluorescent nuclei as well as expression of the *gus* reporter gene was observed by each designed transformation solution. This potent and feasible method showed prospective applications in plant transgenic research.

**Keywords** Minimum linear gene cassettes · Onion low epidermal · Transient gene expression · *Gus* gene · Transformation buffer · Cofactors

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## Introduction

Transient gene expression system is a useful and easy method of analyzing the function of a particular gene of interest. *Agrobacteria* infiltration and naked DNA direct transformation are common methods used in transient expression analysis. Usually, *Agrobacteria*-mediated transient expression is commonly used due to its high efficiency and reproducibility, but it is restricted to leaf tissues from dicot, and the necessary binary vector construction is complicated and cockamamie [1–2]. Direct transfer is useful for any plasmid vectors and all plant tissues including monocot and dicot, but special devices are needed, such as electroporator and particle delivery system. One handy direct transformation method which is not mediated by any special devices, but universally valid for both monocot and dicot, is needed in transient expression system.

To avoid undesirable effects on integration of backbone sequences or transgene arrangements, linear DNA fragment lacking vector sequences has been used and affirmed for smaller and easier to handle in transformation system (e.g., may lead to decreased frequency of plasmid ligation and bacterial transformation) [3], and smaller DNA transformation cassettes potentially penetrate into host cell and tissue directly without any assisted devices, and it is suitable for both monocot and dicot. However, there is insufficient information about the ideal conditions for the use of linear DNA cassettes in direct transformation.

A basic requirement for a successful transient expressing system is the power of motivate target gene into cell. T-DNA-mediated gene transformation depends on the *Agrobacteria Vir*A-*Vir*G signal transduction system [4]. $Ca^{2+}$ and heat stimulation can alter the permeability of the cell membrane [5]. The direct plastid transformation, such as polyethylene glycol (PEG)-mediated transformation and vacuum-infiltration transformation of *Arabidopsis*, needs infiltration momentum, and the microinject transformation and particle bombardment were commonly used in plants and some animal transformation for the physical impetus [6]. We thus focused on the development of an alternative device-free direct transformation by employment of motivation actors, including $Ca^{2+}$, *Agrobacterium* cell culture, infiltration, and surfactant, which have shown promising influences on membrane permeability adjustment to improve minimum linear gene cassettes uptake by the host cells and reach an effective transient expression.

Onion epidermal cells, which are easy to obtain, have a single layer, and are very transparent, have long been an object for transient expression system and are particularly excellent specimens in the study of expression and cellular localization of genes of interest [7]. In the present study, onion low epidermal cells were used as host to evaluate the transformation efficiency of designed motivation actors by transient expression of the *gus* reporter gene. Linear DNA fragment labeled with fluorescein isothiocyanate (FITC) were used to trace its delivery procedure, and the nuclei entry quantity was measured by flow cytometry. This study is likely to provide a prospective basis for direct transformation of linear cassettes in a wide range of living cell hosts.

## Materials and Methods

Onion low epidermal explants were surface-sterilized in 70% (*v*/*v*) ethanol for 30 s, followed by 0.1% (*w*/*v*) mercuric chloride for 4 min, and thoroughly washed in sterile distilled water. Before transformation, low epidermal explant sections (2×2 mm) were carried out for 48 h at 27°C in the dark on solid Murashige and Skoog basal salt mixture (MS) media. The pH of the

**Table 1** Treatments of different cofactors in basic transformation buffer solution.

| Treatments | $CaCl_2$ (0.1 mol/L) | Dow Corning Q2-5211 (0.01%) | *Agrobacterium* cell culture (1 OD) |
|---|---|---|---|
| 1 | − | − | − |
| 2 | + | − | − |
| 3 | − | + | − |
| 4 | − | − | + |
| 5 | + | + | − |
| 6 | + | + | + |

+: adding the factor; −: not adding the factor

medium was adjusted to 5.8 with KOH before adding agar (7 g $L^{-1}$). Twelve explants were used in each treatment and each replicated five times.

## Construction of Minimal Linear Gene Cassettes

The liner DNA fragment was produced by polymerase chain reaction (PCR) amplification as described previously [3]. The DNA fragment harboring *gus* gene along with 35S CaMV promoter and nos terminator sequences was amplified by PCR using specific forward primers PT1:5′-*GTTTACCCGCCAATATATCCTGTCA*TGCCTGCAGGTCCCCAGA TTAGCCTT-3′ and reverse primers PT2:5′-*TGGCAGGATATATTGGGTGTAAA*CCCGAT CTAGTAACATAGATGACACCGC-3′; these primers included conserved 25 bp of left and right border sequences of T-DNA shown italics, respectively. The reaction was carried out in 50 μl containing 20 ng PBI121-gus, 10 pmol/μl of specific forward and reverse primers, 1.5 mM $MgCl_2$, 1× PCR buffer, and 1 U LA Taq polymerase. The cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles at 95°C for 30 s, 54°C for 40 s, and 72°C for 90 s, and then a final incubation at 72°C for 10 min. The amplified PCR product was purified by ethanol precipitation method and dissolved in double-distilled water. The linear DNA was finally adjusted to 100 ng/μL with the transformation buffer solution for use. In order to detect the DNA entering efficiency by flow cytometry, the transformation gene was substituted with the forward primer labeled with a 5′-fluorescent dye (FITC), and the concentration was 2 or 20 pmol/μl.

## Direct Transformation Procedure

After onion low epidermal explants were maintained in darkness at 27°C for 48 h, the explants were incubated in basic transformation solution (MS liquid medium, salts

**Table 2** Primers used for RT-PCR amplification.

| Target | Primer | Primer sequence (5′-3′) | Length (bp) | Amplicon length (bp) |
|---|---|---|---|---|
| *Actin* | F | 5′-GGGACAAAAAGATGCTTA-3′ | 18 | 104 |
| | R | 5′-TGTCATCCCAGTTGCTGA-3′ | 18 | |
| *gus* | F | 5′-TAGGACCCTTTTCTCTTTTTATTTTT-3′ | 26 | 146 |
| | R | 5′-TCATCATCATCATAGAACACGAAA-3′ | 25 | |

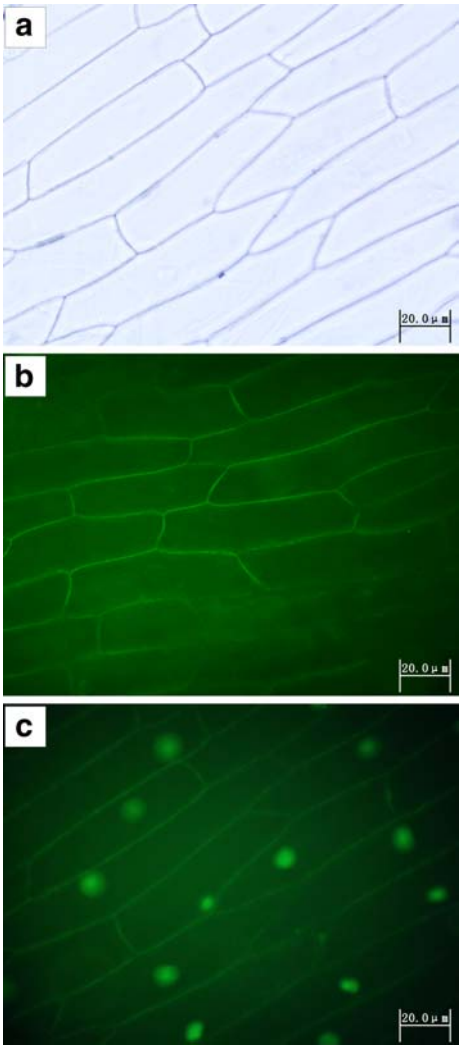



**Fig. 1** Images of onion epidermal cells transformed by FITC-labeled exogenous DNA in basic transformation buffer and the explants were pretreated by hypertonic solution. **a** Onion epidermal under white light. **b** Non-transformation cells under blue light. **c** Transformed cells by linear DNA fragment labeled with FITC under blue light

and vitamins, 3% sucrose, pH=5.7) as a control treatment at 27°C with shaking at speed of 50 rpm. After co-transformation for 3 h in transformation buffer, the explants were washed with MS liquid medium for three times; after dry blotting, the explants were transferred to MS solid media for co-cultivation. The low epidermal explants were assayed for GUS activity after 72 h of the co-cultivation period. The DNA trace and entering efficiency of linear DNA fragment into cell nuclei was analyzed after co-transformation for 3 h.

Hypertonic solution pretreatment of explants and other transformation cofactors were evaluated. The pretreatment hypertonic solution was prepared by MS liquid medium supplemented with 46.67 g/L mannitol and 46.67 g/L sorbinose. Before transformation, the explants were thrown into the hypertonic solution. After penetration for 10 min, the explants were blotted dry on sterile filter paper and then used for transformation. Explants without hypertonic treatment were used as negative control. The cofactors, including

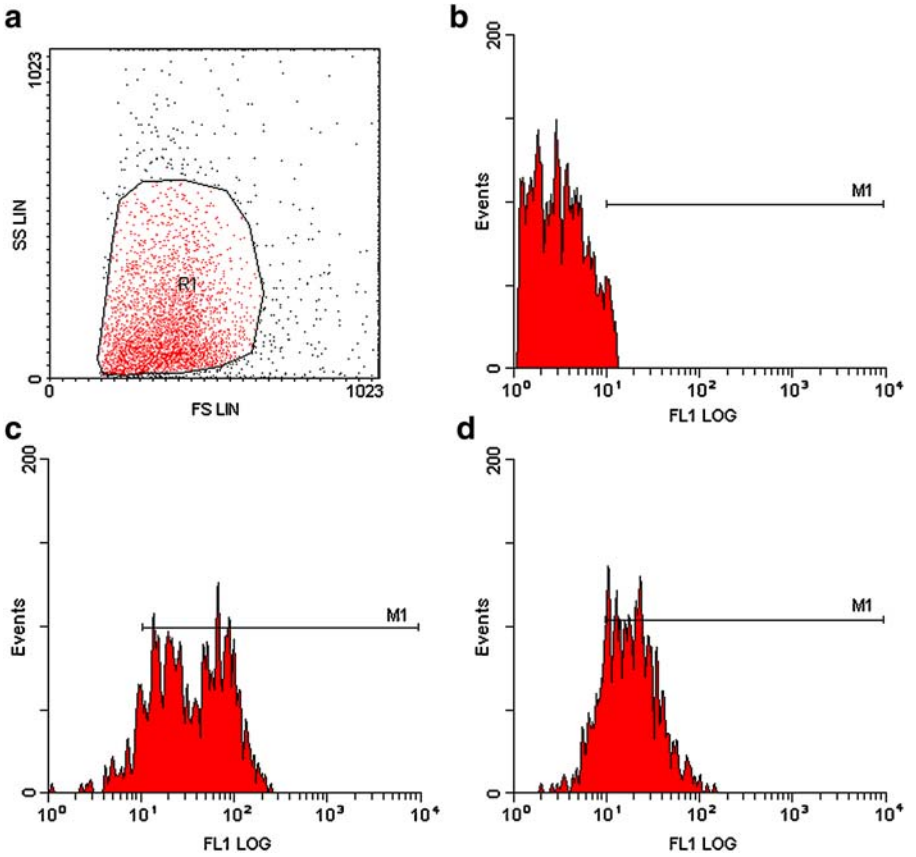
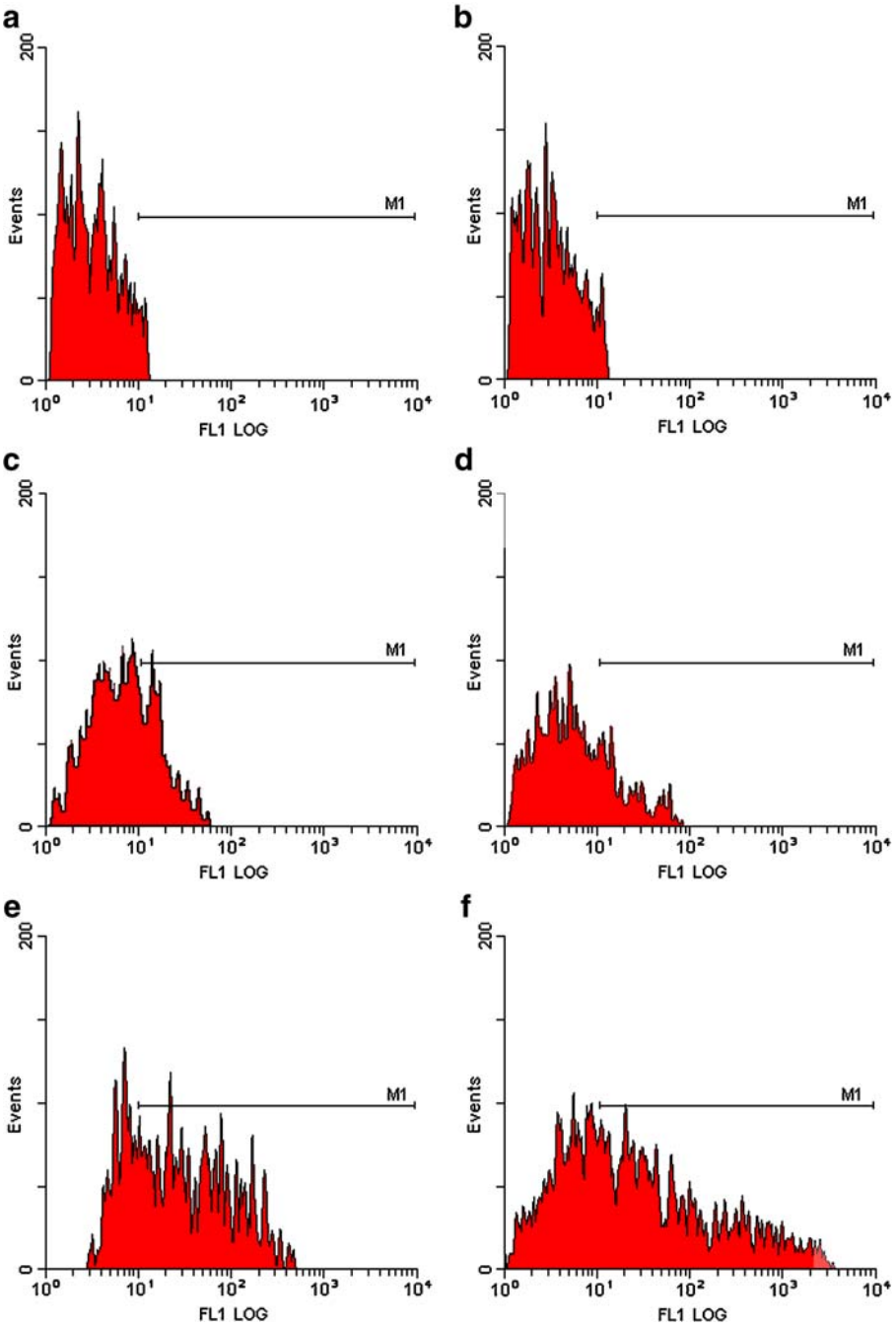



**Fig. 2** Effects of hypertonic pretreatment on the linear DNA entering efficiency into cell nucleus. FITC-labeled linear DNA was transformed into onion low epidermal cells under different pretreatment conditions. After co-cultivation for 3 h in transformation buffer, the cell nuclei were collected and analyzed by flow cytometry. **a** The cell nuclei were gated according to forward/side scatter. The uptake of FITC-labeled 2 pmol/μl linear DNA fragment without pretreatment was shown in **b**; the uptake of FITC-labeled 2 pmol/μl and 20 pmol/μl linear DNA cassettes into cell nucleus with the hypertonic solution pretreatment were shown respectively in **c** and **d**

0.1 mol/L $Ca^{2+}$, 0.01% Dow Corning Q2-5211, and Agrobacteria LBA4404 cell culture, were given in Table 1. The different cofactors were dissolved in the basic transformation buffer solution to the designed concentration. Agrobacteria LBA4404 cell culture were collected as follows: Agrobacteria LBA4404 were incubated in 2 mL of Luria–Bertani medium supplemented with 50 mg/L Rfi antibiotics. Overnight cell cultures were inoculated (1%) and grown at 28°C, centrifuged, and resuspended in relative transformation buffer to a final absorbance at 600 nm of 1.0 OD. Both linear cassettes and circular plasmids were dissolved in the basic transformation buffer solution and used as controls.

The linear DNA fragment entering efficiency was assayed by flow cytometry and exogenous DNA monitoring by a fluorescence microscope.

The quantity of minimum linear length of DNA entering into the cell nuclei was analyzed by flow cytometry (FCM). After co-transformation for 3 h, the explants were




washed six times with TE (0.01 mol/L, pH 7.5) buffer solution, and then the cell nuclei were extracted. To avoid any nuclear disturbances and preserve DNA integrity, operations were carried out on ice. Samples were individually chopped with a razor blade in about 1.5 mL extraction solution (solution A of high-resolution kit for plant DNA, Partec,

◀ **Fig. 3** Effects of different cofactors on the linear DNA entering efficiency into cell nucleus. The linear fragment entering efficiency into the nucleus was analyzed by FCM; DNA entering efficiency corresponded with log fluorescence intensity value. The sensitivity of the flow cytometer was adjusted so that the fluorescence of the stained cell nuclei was larger than scale of $10^1$. **a** Direct transformation with basic buffer solution of Murashige and Skoog basal salt mixture (*MS*) liquid media; **b** with MS liquid media + 0.1 mol/L $CaCl_2$; **c** with MS liquid media + 0.01% Dow Corning Q2-5211; **d** with MS liquid media +0.1 mol/L $CaCl_2$ + 0.01% Dow corning Q2-5211; **e** with MS liquid media + *Agrobacterium* LBA 4404 cell culture (1 OD); **f** with MS liquid media + 0.1 mol/L $CaCl_2$ + 0.01% Dow corning Q2-5211 + *Agro-bacterium* LBA4404 cell culture (1 OD)

Germany). Then they were filtered through a nylon sieve with mesh diameter of 30 μm, centrifuged (1,800 rpm for 14 min) on a 1.5 M sucrose cushion, and the nuclei were resuspended in 1 mL of TE (0.01 mol/L, pH 7.5) buffer solution. Nuclear suspensions were then adjusted with TE buffer solution to obtain $10^4$ nuclei per milliliter per sample to a volume of 2 mL. The nuclei were run in an EPICS ALTRA Flow Cytometer (Bechmann Coulter, Hialeah, FL, USA) equipped with an argon ion laser. Ten thousand events were obtained and analyzed using the Expo 32 software (Beckmann Coulter). The DNA fluorescence intensity ($F_1$) value was related to the DNA content entering into the nucleus, and for each nuclear population, it was thus possible to calculate the DNA entering efficiency. The sensitivity of the flow cytometer was adjusted so that the fluorescence value of the stained cell nuclei was larger than scale of $10^1$.

The monitoring of the FITC-labeled DNA was carried out using fluorescence microscopy. After the explants were transformed by FITC- labeled DNA transformation buffer, the onion epidermal was collected at different periods and then fixed in formalin/acetic acid/alcohol. The FITC fluorescence was monitored at 520 nm with excitation at 495 nm.

## RT-PCR Analysis of *Gus* Gene Transcripts

Total RNA was extracted from 100 mg of onion epidermal after 72 h of the co-cultivation period using Trizol reagent method (Invitrogen), and the first-strand cDNA synthesis and reverse transcription PCR (RT-PCR) were performed using One-Step RNA PCR kit (Takara) using oligo-dT and random oligonucleotide primers. The cDNA was used for further PCR in a reaction mixture containing 5 μL of the RT reaction product. The

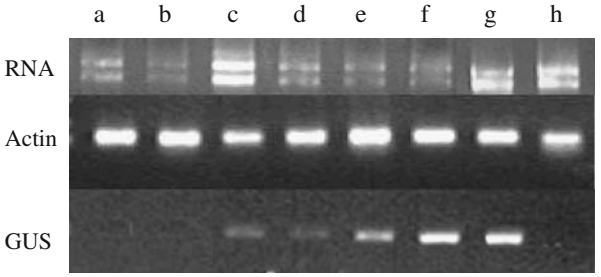



**Fig. 4** RT-PCR analysis of *gus* RNA accumulation in linear gene cassettes transformed onion epidermal. a Transformation buffer solution is the Murashige and Skoog basal salt mixture (*MS*) liquid media; **b** MS liquid media + 0.1 mol/L $CaCl_2$; **c** MS liquid media + 0.01% Dow Corning Q2-5211; **d** MS liquid media + 0.1 mol/L $CaCl_2$ + 0.01% Dow Corning Q2-5211; **e** MS liquid media + the *Agrobacterium* LBA4404 culture (1 OD); **f** MS liquid media + 0.1 mol/L $CaCl_2$+ 0.01% Dow Corning Q2-5211 + *Agrobacterium* LBA4404 culture (1 OD); **g** hypertonic solution pretreatment + MS basic transformation solution; **h** non-transformed explants

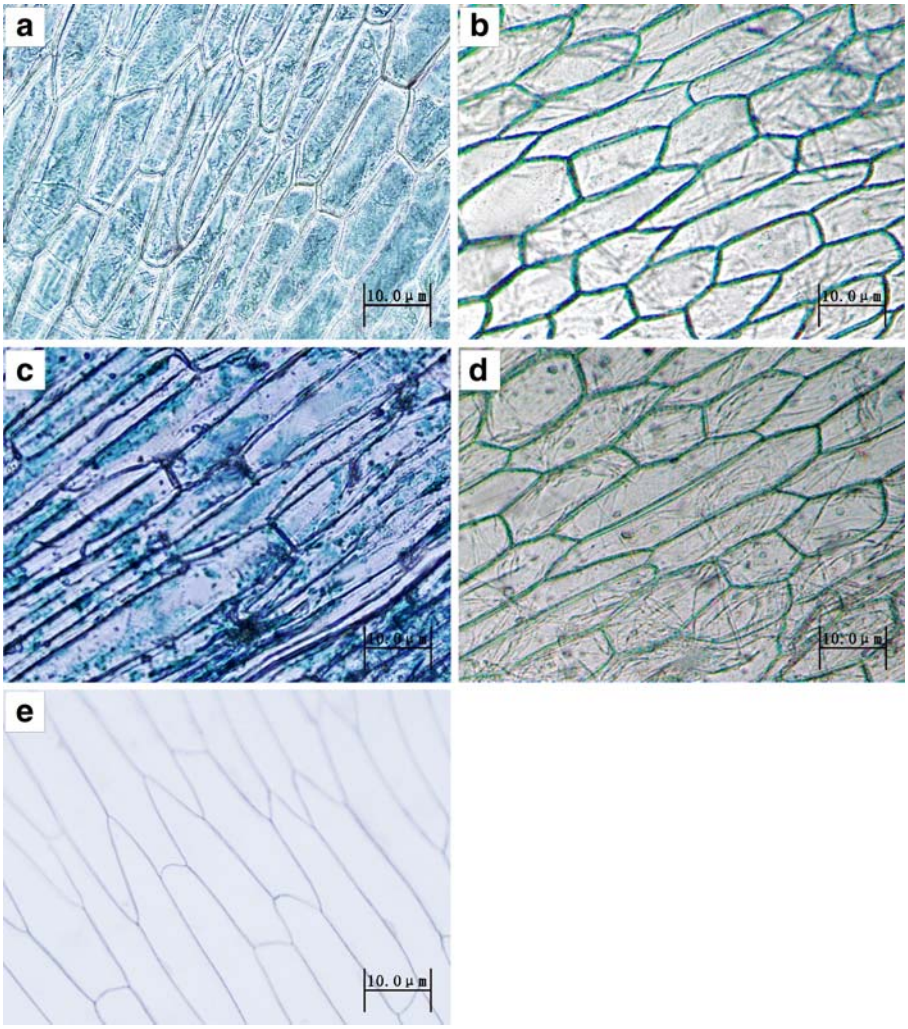



**Fig. 5** Transient *gus* gene expression in onion low epidermal cell: **a** transformation of linear gene cassettes with hypertonic pretreatment; **b** transformation of linear gene cassettes without hypertonic pretreatment; **c** transformation of circular plasmids with hypertonic pretreatment; **d** transformation of circular plasmids without hypertonic pretreatment; **e** non-transformed explants

housekeeping gene, *actin*, was used as an internal control. Primers (10 pmol/μl) used in this procedure are listed in Table 2.

*Gus* Gene Expression Assay and Microscopy

*Gus* gene expression was typically tested after 72 h of the co-cultivation period by histochemical staining using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-Gluc) as the substrate [8]. Digital microscopy was carried out after 2-day sequential dilution of the tissues in 95% ethanol to extract plant pigments. Images were captured by Olympus BX60 (model BX60F5) microscope.

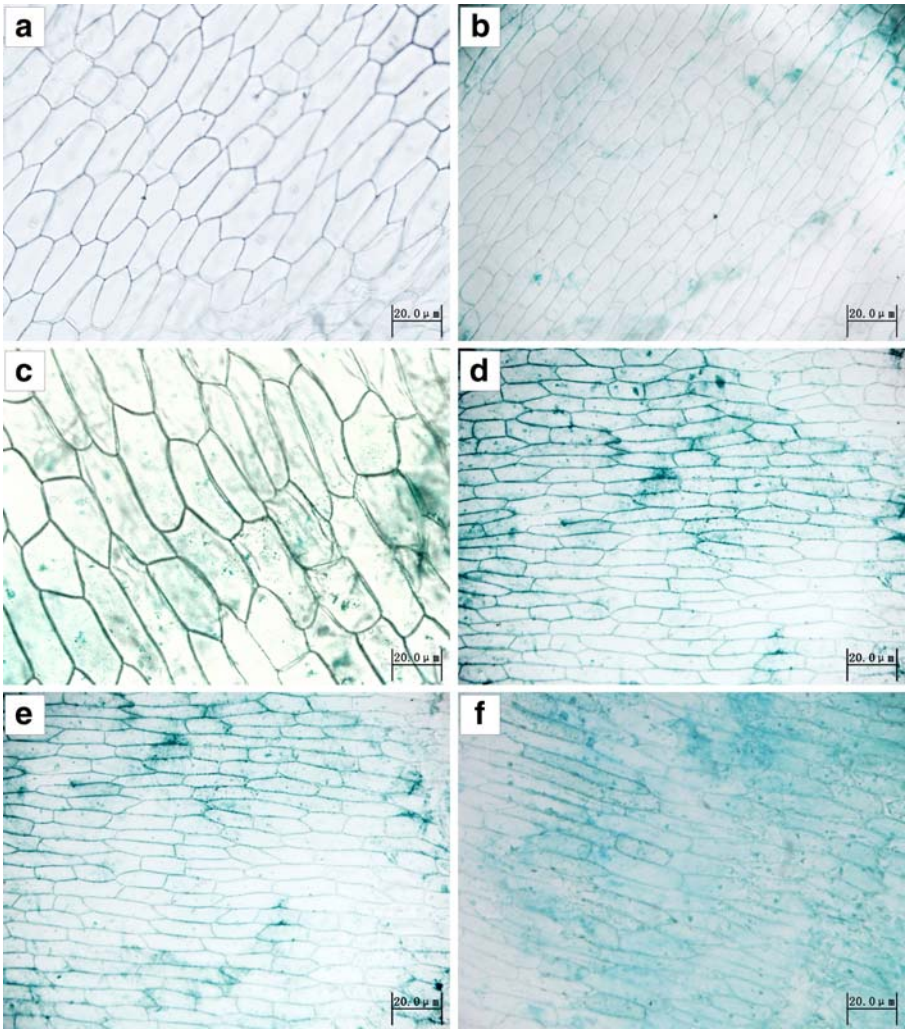



**Fig. 6** Effects of different transformation cofactors on the transient expression of *gus* gene in onion low epidermal cells. **a** Transformation buffer is Murashige and Skoog basal salt mixture (*MS*) liquid media; **b** MS liquid media + 0.1 mol/L $CaCl_2$; **c** MS liquid media + 0.01% Dow Corning Q2-5211; **d** MS liquid media + 0.1 mol/L $CaCl_2$ + 0.01% Dow Corning Q2-5211; **e** MS liquid media + *Agrobacterium* LBA4404 culture (1 OD); **f** MS liquid media + 0.1 mol/L $CaCl_2$+ 0.01% Dow Corning Q2-5211 + *Agrobacterium* LBA4404 culture (1 OD)

## Results

To study the trace of DNA delivery in transient expression system, we transformed onion epidermal cells using FITC-labeled linear DNA. The fluorescence signals were observed in the cell nuclei via basic transformation buffer after the explants were pretreated with hypertonic solution (Fig. 1). The effects of the hypertonic treatment and other motivation cofactors on the entry efficiency of FITC-labeled linear DNA fragment into cell nuclei were shown in Figs. 2 and 3. Hypertonic treatment showed higher delivery efficiency than control (Fig. 2b, c). The increase of linear DNA fragment concentration with the hypertonic solution

reflected higher entering efficiency, and the ratio of positively stained nuclei increased from 62.54% to 92.32% when the DNA concentration increased from 2 to 20 pmol/μl (Fig. 1d). The uptake rate of fluorescent nucleus also showed expected increases of approximately 10.5%, 12.9%, and 36% for solo treatment of the actor $CaCl_2$ (Fig. 3b), Dow Corning Q2-5211 (Fig. 3c), and *Agrobacterium* LBA4404 cell culture (Fig. 3f) supplemented in basic transformation buffer compared to 0.02% uptake rate (Fig. 3a) in control groups. In the presence of both $Ca^{2+}$ and Dow Corning Q2-5211 (Fig. 3d) or presence of $Ca^{2+}$, Dow Corning Q2-5211, and *Agrobacterium* LBA4404, cell culture (Fig. 3f) showed synergistic effects of one another on the linear DNA entering into the cell nuclei with the ratio of fluorescent nuclei attaining 36% and 54.85%.

To analyze the effect of different actors on expression of *gus* gene in the onion epidermal cells, mRNA of explants were subjected to RT-PCR analysis using primers F and R and resulted in the expected 146-bp band (Fig. 4). The results of *gus* gene histochemical assay were shown in Figs. 5 and 6. The effectiveness of hypertonic pretreatment before direct transformation of minimum linear gene cassettes and circle plasmids was confirmed by the dark, uniform indigo blue staining in onion epidermal cells (Fig. 5a, c), which is better than linear cassettes (Fig. 5b) and circle plasmids (Fig. 5d) transformation with basic transformation buffer. The results in Figs. 3 and 6 also presented the effectiveness of other cofactors in assistance of linear cassettes direct transformation. The histochemical staining intensity showed a possible improvement of the uptake of linear cassettes by each solo cofactor (Fig. 5a–d) and significant enhancement by the integrated treatment with multiple actors (Fig. 6e–f).

## Discussion

The optimal transformation method is critical to a successful transformation in plant transgenic research. Currently, the evaluation of transformation methodology is basically focused on the expression detection assay of target or reporter genes [9–10]. However, gene expression is such a complicated process that is difficult to be measured and quantified. The fluorescent labeling method we employed in this study provides a visible way to trace the delivery of target genes and, together with FCM analysis, makes it possible to reflect quantitatively the transformation procedure.

The presence of rigid cell wall and cell membrane limited the free movement of large molecule, such as DNA, entry into the cell. Osmosis across the cell membrane thus plays an important role in the delivery of foreign DNA into the living cells. We confirmed the effectiveness of hypertonic pretreatment in device-free direct transformation by both fluorescent observation and GUS staining.

Other cofactors, which were normally used to alter the permeability of cell membrane, also showed potent applications in improvement of target DNA delivery. $Ca^{2+}$ (0.1 M) contributing to the naked DNA uptake efficiency by cell in PEG-mediated transfection experiments have been studied systematically [11]. Similar to silwet-77 [12], Dow Corning Q2-5211 is a low-molecular-weight nonionic silicone polyether surfactant, 0.01% (*v*/*v*) of which in $H_2O$ can improve the wetting spreading and penetration of agriculture chemicals [13]. The T-DNA of *Agrobacterium* has been firmly demonstrated its capacity to efficiently cargo large DNA molecules into the nucleus [14]. *Agrobacterium* T-DNA transfer is initiated by bacterial virulent proteins encoded by several *vir* genes and followed a complex signal transduction system. Virulent proteins harboring T-DNA may also contribute to the protection and integration of genes of interest when traveling through the host cells [15]. *Agrobacterium* LBA4404 cell cultures we used carry a disarmed Ti plasmid that possesses the *vir* genes

necessary for T-DNA transfer [16]. The results demonstrated that $Ca^{2+}$, surfactant Dow Corning Q2-5211, and the *Agrobacterium* cell cultures could be used as driving force to assist naked linear DNA entry into the cell nucleus and conduct the transient expression, suggesting the feasibility of devices-free direct transformation of linear gene cassettes. The transformation efficiency can be further improved by parameter optimization.

In previous studies, the methods used for gene transient expression in plants were usually particle bombardment [17–18] and electroporation [13]. In the present study, we provide an alternative direct transformation method which is able to motivate linear gene cassettes into the host cell nucleus by agents designed via altering the permeability of cell membrane. Compared to particle bombardment, electro-transformation, and microinjection, this devices-free direct transformation method induces less damage to the host cells and shows promising and wide range of applications in plant transgenic research.

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