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In Vitro Culture of *Petunia hybrida* Microspores and *Agrobacterium*-mediated Transient Expression of *β*-glucuronidase (GUS) Reporter Gene

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Abstract

In this study, we developed two optimal media for *in vitro* culture of microspores and germination of mature pollen in *Petunia hybrida*. The optimal medium for *in vitro* culture of microspores was 15% sucrose + MS salts + White's vitamins, pH = 6.0 with the maturation and germination rate of 41.77% and 5.38%, respectively, and the optimal medium for germination of mature pollen was 15% sucrose + 20 mg/L H_2BO_3 + 10 mg/L $CaCl_2$ +10% PEG_{4000}, pH = 5.8. The germination rates of matured microspores *in vivo* and *in vitro* were 69.82% and 19.87%, respectively. In addition, a system of *Agrobacterium*-mediated transient expression of *β*-glucuronidase (GUS) reporter gene in late unicellular and bicellular pollen was also established. We co-cultivated late unicellular and bicellular pollen of *P. hybrida* with *Agrobacterium tumefaciens* strain EHA105 harboring the plasmid pBI121 with GUS reporter gene under the control of CaMv35S promoter, respectively. The optimal conditions consisted of an OD_{600} of 0.3 and infection time of 10 min, and we obtained 70.82% of GUS transient expression frequency for late unicellular pollen and 79.33% for bicellular pollen.

Keywords: *Petunia hybrida*; Microspores; *In vitro* culture; *Agrobacterium*-mediated; Transformation

Introduction

Petunia hybrida Vilm, Solanaceae perennial herb, is an important ornamental, originating in South America, Argentina, having colorful flowers and a long florescence; it shows high ornamental value and now is widely cultivated throughout the world. Furthermore, it has become an important model plant, because of its short life cycle, clear genetic background, smaller genome and simple cell and tissue operation technique (Drummond *et al.*, 2009).

Microspores at late unicellular or early bicellular stage can be out of the normal process of pollen formation to undergo androgenesis (Heberle-Bors, 1985). This androgenesis of pollen provides an effective pathway for genetic transformation of plants, with its convenient access to homozygous transgenic plants from its haploid nature. Moreover, microspores maturation *in vitro*, avoiding disturbance of somatic tissues surrounding microspores, provides a convenient experimental system for studying the events occurred in the development and biophysiology and biochemistry of male gametophyte. For the first time, Twell *et al.* (1989) introduced exogenous genes into tobacco pollen using high velocity microprojectiles and transiently expressed the genes in leaves and pollen, which is similar to that observed for these genes in stably transformed tobacco plants. Subsequently, Jähne *et al.* (1994) obtained homozygous transgenic doubled haploid by the biolistic transformation of barley using freshly-isolated microspores as the target tissue, and similar work was done in isolated microspores of rapeseed (*Brassica napus* L.) by Fukuoka *et al.* (1998). Although technique of microspores culture is continuously improved in recent years, the transformation efficiency of microspores as receptor materials is still very low.

P. hybrida is attractive targets for genetic modification and has been studied for decades. However, with regard to the pollen-based genetic transformation system of *P. hybrida* in the previous studies were restricted to mature pollen (Sussmuth *et al.*, 1991; Tjokrokusumo *et al.*, 2000). So far, no report showed in *in vitro* maturation and germination of *P. hybrida* microspores and microspore-based genetic transformation system. Therefore, it has an important practical significance to establish *in vitro* microspores development system as well as efficient microspore-based genetic transformation system.

In this study, we took *P. hybrida*, an important ornamental and model plant, as an experimental material, and separated immature microspores culturing *in vitro*. In addition, a system of *Agrobacterium*-mediated transient expression of *β*-glucuronidase (GUS) reporter gene was also established and transgenic research, achieved initial success.

Materials and Methods

Materials

P. hybrida was cultivated in the greenhouse of the

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Laboratory of Bio-resources and Eco-environment, College of Life Science, Sichuan University at 25°C under a 16/8-h-light/dark photoperiod.

Observation of Flower Buds Growth

To observe of pollen development *in vivo*, we marked 50 flower buds when microspores at the tetrad stage and followed their development into mature pollen. Size of flower buds was measured with a caliper and anther appearances were observed at 9:00 a.m. Simultaneously, the stages of pollen development were recorded after DAPI stained (Kapuscinski, 1995).

Detection of Pollen Development Duration and Viability

We removed anthers of *P. hybrida* from flower buds and put them into a drop of 15% sucrose solution on a clean slide, and released pollen grains by crushing with a glass rod, then diameter of microspores were measured using a microscope (Olympus IX71, Japan). The nuclear phase and viability of microspores were observed after stained by DAPI (Kapuscinski, 1995) for 15 min at room temperature and FDA (Heslop-Harrison *et al.*, 1984). Observation was taken under fluorescence microscope (Olympus IX71, Japan).

Isolation and Culture of Microspores

Flower buds were picked at 9 a.m when *P. hybrida* at full-bloom stage, and surface-sterilized with 70% (v/v) ethanol for 30 s, 0.1% HgC1 for 6 min then rinsed 5 times with sterile distilled water. The anthers from flower buds were taken out under sterile condition, and gently pressed with a glass rod in a sterilized glass vial (4 mL) filled 1.5 mL 15% sucrose solution. The suspension was filtered through a 70 μm nylon membrane. The flow-through was collected in a sterile tube on the ice, and then centrifuged at 500 rpm for 3 min. The pellet was washed using 15% sucrose solution and centrifuged for three times.

The isolated microspores were dissolved in sterilized maturation medium to a density of 2–3 × 10^5 mL^{-1} and cultured in 3 cm Petri dish with hanging cell culture insert (20 μm pore size) at 25°C in the dark. New sterilized maturation medium was replaced every other day. At the same time, the developmental phases and viability of pollen cultured *in vitro* were examined under a fluorescence microscope (Olympus IX71, Japan). The experiment each time 300 pollen grains were counted and repeated three times.

***In Vitro* Germination of Mature Microspores**

According to *in vitro* germination of rice (Wang *et al.*, 2000), we designed a basic germination medium (15% sucrose + 20 mg/L H_2BO_3 + 10 mg/L $CaCl_2$, pH = 7.0) for *in vitro* germination of *P. hybrida*. Before anthesis, *in vivo*-matured microspores were collected from anthers, and scattered in germination medium on a clean slide, and then the slide was placed into a moist Petri dish (10 cm diameter) for reducing evaporation and incubated at 25°C in the dark and light, respectively. When the length of pollen tube was greater than its diameter, pollen was considered germinated. About 2 h later, we counted the number of germinated pollen under the microscope. Each time 300 pollen grains were counted and repeated three times.

In vitro germination of matured microspores was the same as the germination of *in vivo*-matured microspores.

***Agrobacterium*-mediated Transformation of *P. hybrida* Microspores**

Agrobacterium tumefaciens strain EHA105 harboring the plasmid pBI121 (Jefferson *et al.*, 1987) with reporter gene GUS under the control of CaMv35S promoter and the selectable marker gene, the neomycin phosphotransferase II (*npt*II), which confers resistance to kanamycin, has been used for this study. *Agrobacterium* was cultured overnight in liquid yeast extract beef medium (YEB medium) (Sambrook *et al*., 1989) added with 50 mg/L kanamycin and 50 mg/L rifampicin at 28°C, while continuously shaking at 200 rpm. *Agrobacterium* culture was centrifuged at 5000 rpm for 3 min and then resuspended with microspore maturation medium to different concentrations (OD_{600} = 0.2, 0.3, 0.4, 0.5, 0.6) prior to infecting pollen.

The unicellular microspores and bicellular pollen were mixed with these bacterial suspensions, respectively, and then slowly shaking cultured for different times (5, 10, 15 and 20 min). Subsequently they were centrifuged at 500 rpm for 3 min and suspended with microspore maturation medium to the density of 2–3 × 10^5 mL^{-1} for co-cultivation experiment. The co-cultivation was conducted in 3 cm diameter glass dishes at 25°C for 20 h in the dark. After co-cultivation, pollen was rinsed several times with microspore maturation medium to eliminate the *Agrobacterium.*

Histochemical Detection of GUS Expression

The GUS histochemical assay was performed referring to the method from Jefferson *et al*. (1987). After co-cultured 20 h in the dark, pollen were collected and incubated in GUS staining solution at 37°C overnight and washed repeatedly in 70% ethanol. The signal of GUS was detected under the inverted microscope IX71 (Olympus, Japan).

Statistical Analysis

In this paper, values were the mean of three replicates per treatments. Data were analyzed at significant levels of $P < 0.05$ using ANOVA followed by the Duncan test to identify significant differences between the means of all treatments (Kleinbaum *et al.*, 1998).

Results

Observation of *P. hybrida* Pollen Development *in Vivo*

Four stages of *P. hybrida* pollen development were observed *in vivo*. Firstly, *P. hybrida* pollen was at the tetrad stage with diameter of 23.8–26.4 μm, meanwhile the size of flower bud was 5–9 mm with green and not full anther. After 1–2 days, *P. hybrida* pollen was developed into unicellular microspores with diameter of 26.4–29.6 μm, and bicellular pollen an additional 1–2 days later, and tricellular pollen a further 1–2 days later. Consequently, the total time from tetrad to tricellular mature pollen was 4–8 days, with morphological characteristics of flower bud and anther appearance changed (Table 1). It shows that the development stage of microspore is intimately related to morphological characteristics of flower bud and anther appearance, despite the fact that the development of some microspores show non-synchronous within the same anther. So the developmental stages of *P. hybrida* microspores can be poorly judged by morphological characteristics of flower bud, which lays a foundation for isolation and purification of the microspores.

In Vitro Mature Culture of *P. hybrida* Microspores

Two different salts, MS salts (Murashige and Skoog, 1962) and BK salts (Brewbaker and Kwack, 1963), were designed to evaluate the effect of salts on the viability of microspores culture. The results showed that the viability of microspores culture in the medium BK salts + White's vitamins + 15% sucrose dropped sharply from 83.05±4% to 11.20±3.58%, while that in the medium containing MS salts still maintained 48.53±3.19% for 72 h of culture (Fig. 1). Therefore, we used MS salts as basic medium to evaluate the effect of different carbohydrates on microspore culture. Four different combinations of maltose and sucrose plus MS salts and White's vitamins were designed and the highest viability (50.32±4.58%) was observed after 4 days of incubation in 15% sucrose plus MS salts and White's vitamins (Table 2). For this reason, the sucrose was used for the further culturing. Subsequently different concentrations of sucrose (10, 15, 20, 25 and 30%) at various pH values (5.5, 6.0, 6.5 and 7.0) were performed to search the optimal pH value and concentration of sucrose for *in vitro* maturation culture of microspores. The results showed that viability of microspores was maximum at 15% concentration of sucrose and gradually decreased with the increase of sucrose concentration. In addition, the viability was maximum at pH 6.0 in each sucrose concentration (Fig. 2). Finally, the optimal medium for *in vitro* mature culture of *P. hybrida* microspores was maturation medium A (15% sucrose + MS salts + White's vitamins, pH = 6.0), yielding 41.77% maturation rate after 5 days of incubation at 25°C in the dark with some small pollen no viability, probably because abortion occurred in the culture lead to pollen stop growing or premature death (Fig. 3A). The

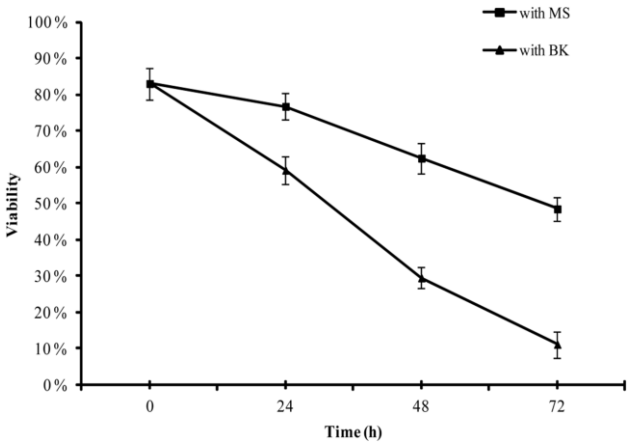

Fig. 1: Viability of *P. hybrida* microspores in the culture medium with MS salts and BK salts

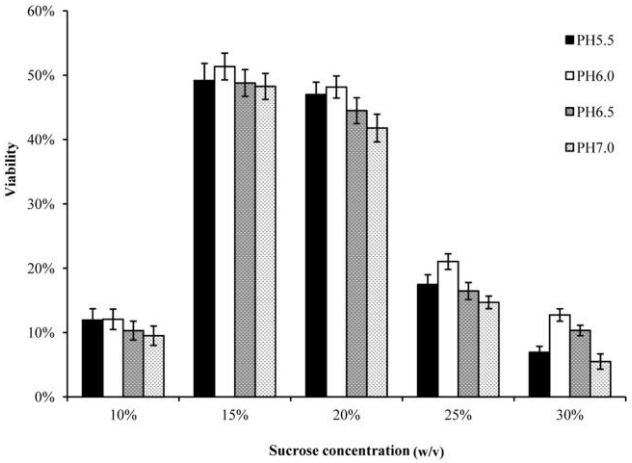

Fig. 2: Viability of *P. hybrida* microspores cultured in medium with different sucrose concentrations and pH values

viability of microspores cultured in maturation medium A declined with the enlargement in microspore size (Table 3). The viability of microspores was 84.23±9.80% at 0 day with diameter of microspores is 28.13±1.42 μm, and then decreased to 77.06±9.02% after 2 days, with diameter up to 30.24±1.61 μm. After 3 days, microspores began to develop into bicellular pollen (diameter = 33.91±1.34 μm) (Fig. 3B). After another 1–2 days of incubation, most bicellular pollen grew into tricellular pollen (diameter = 40.81±1.61 μm) (Fig. 3C) and some mature pollen germinated with 5.38% germination rate.

In Vitro Germination of *P. hybrida* Pollen

We examined different concentrations (0, 5, 10, 15 and 20%) of polyethylene glycol 4000 (PEG_{4000}) plus basic germination medium on the effect of germination rate in the light and in the dark, respectively. The results showed that 10% PEG_{4000} in the light yielded the greatest

Table 1: Correlation between flower bud size and the stage of pollen development in *P. hybrida*

Stage of pollen	Tetrad	Unicellular	Bicellular	Tricellular
Size of flower bud（mm）	5–9	9–15	15–25	≥25
Anther appearance	Green, not full	Yellow-green, slightly plump	Yellow, full	Yellow, full
Diameter of pollen（μm）	23.8–26.4	26.4–29.6	29.6–34.5	34.5–47.2
Progress of development (day）	1–2	2–4	3–6	4–8

Table 2: Viability of *P. hybrida* microspores in different carbohydrates after four days of cultivation

Carbohydrates(w/v)	Other compositions	Viability (%)
Sucrose (15%)	MS salts + White's vitamins, pH = 6.0	50.32±4.58a
Maltose (15%)	MS salts + White's vitamins, pH = 6.0	2.65±0.13c
Maltose (15%) + sucrose (3.4%)	MS salts + White's vitamins, pH = 6.0	5.80±0.45c
Sucrose (15%) + maltose (3.4%)	MS salts + White's vitamins, pH = 6.0	16.47±2.04b

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05)

Table 3: *P. hybrida* microspores' viability, average diameter and developmental stages *in vitro* maturation after different days

Progress of pollen (day)	Viability (%)	Diameter of pollen (μm)	Stage of pollen
0	84.23±9.80a	28.13±1.42c	Unicellular
2	77.06±9.02ab	30.24±1.61c	Unicellular
3	64.53±6.07bc	33.91±1.34b	Unicellular/Bicellular
4	51.49±5.36cd	40.81±1.61a	Tricellular
5	41.77±4.91d	43.25±1.84a	Germinated (Germination rate = 5.38%)

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05)

Table 4: Germination of *P. hybrida* pollen in germination medium with different concentrations of PEG_{4000} in the light and in the dark

Medium components	Germination frequency in the light (%)	Germination frequency in the dark (%)
Basic germination medium[1] + 0% PEG_{4000}	60.08±6.26bc	52.46±5.03bc
Basic germination medium + 5% PEG_{4000}	64.51±4.29ab	60.76±7.17ab
Basic germination medium + 10% PEG_{4000}	69.82±6.11a	62.37±7.93a
Basic germination medium + 15% PEG_{4000}	65.54±5.23ab	60.45±6.34ab
Basic germination medium + 20% PEG_{4000}	51.55±4.30c	46.93±3.81c

[1]Basic germination medium includes: 15% sucrose + 20mg/L H_2BO_3 + 10mg/L $CaCl_2$, pH = 5.8

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05)

germination rate and the rate of germination in the light was higher than that in the dark (Table 4). Therefore, the germination medium B (15% sucrose + 20 mg/L H_2BO_3 + 10 mg/L $CaCl_2$ + 10% PEG_{4000}, pH = 5.8) in the light was suitable for *P. hybrida* pollen *in vitro* germination, with a germination rate of 69.82±6.11%. Moreover, the germination rate of *in vitro* matured pollen grains was 19.87% after incubation in germination medium B. Matured pollen began to germinate after 2 h with twice the pollen diameter of pollen tubes (Fig. 3D).

Agrobacterium-mediated Transformation of *P. hybrida* Microspores

To establish *Agrobacterium*-mediated genetic transformation of *P. hybrida* microspores, concentrations of *Agrobacterium* (OD_{600} = 0.2, 0.3, 0.4, 0.5, 0.6) and infection times (5, 10, 15 and 20 min) were tested in the infection experiments of late-unicellular pollen and bicellular pollen. When the concentration of *Agrobacterium* is OD_{600} = 0.3, GUS transient expression frequency was the highest (Fig. 4A). Similarly when the infection time was 10 min, GUS transient expression frequency was the highest (Fig. 4B). However, too high concentration of *Agrobacterium* or too long infection time would cause damage to microspores because of the excessive breeding of *A. tumefaciens* and GUS transient expression frequency decreased significantly. Therefore, the optimal concentration of *Agrobacterium* and infection time is OD_{600} = 0.3 and 10 min, respectively.

Our study also showed that bicellular pollen gave a higher GUS transient expression frequency than late-unicellular pollen, especially when the concentration of *Agrobacterium* was OD_{600} = 0.3 and infection time was 10 min (Fig. 4). After co-cultured for 20 h in the dark, the late-unicellular pollen and bicellular pollen were stained by GUS staining solution (Fig. 5), with 70.82±2.78% of GUS transient expression frequency and 79.33±2.44% of GUS transient expression frequency, respectively.

Discussion

At present, the technique of microspores matured *in vitro* has been reported in many species, such as *Antirrhinum majus* (Stauffer *et al.*, 1991), *Nicotiana*

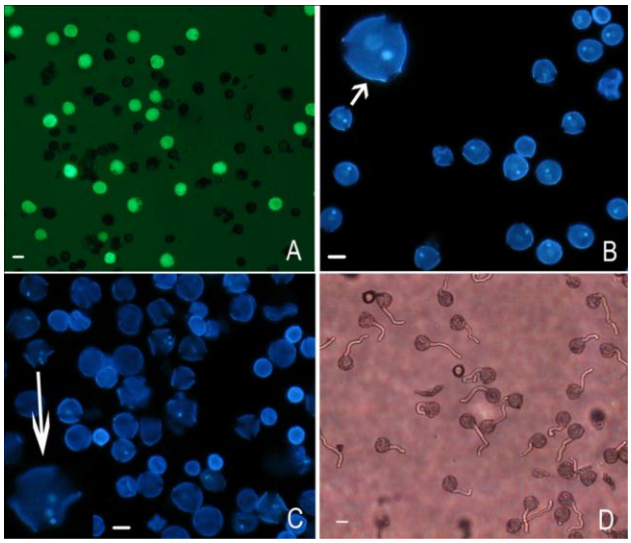

Fig. 3: *In vitro* maturation and germination of *P. hybrida* microspores. (A) Viability of microspores after 5 days of *in vitro* culture; (B) Bicellular pollen derived from microspore after about 3 days of *in vitro* culture, arrow indicates bicellular pollen; (C) Tricellular pollen derived from microspore *in vitro* culture for about 4 days, arrow indicates tricellular pollen; (D) *In vitro* germination of matured pollen after 2 h. Horizontal bars = 30 μm

*tabacu*m (Aziz and Machray, 2003), wheat (Barnabás *et al.*, 1992), *Zea mays* (Pareddy and Petolino, 1992), *Orychophragmus violaceus* (Zhao *et al.*, 2007), *Jatropha curcas* (Li *et al.*, 2010), but no report in *P. hybrida* microspores. Furthermore, the transformation using *in vitro* mature microspores has been reported only in *N. tabacum* (Touraev *et al.*, 1997), *N. glutinosa* (van der Leede-Plegt *et al.*, 1995), *A. majus* (Barinova *et al.*, 2002) and lily (Dong *et al.*, 2007). This paper shows *P. hybrida* microspores can mature *in vitro* successfully with the maturation rate of 41.77%, which was higher than *J. curcas* (39.5%), *O. violaceus* (19.3%), *T. aestivum* (1%) and *Z. mays* (20%). Test of GUS transient expression indicated *A. tumefaciens*-mediated transformation of *P. hybrida* microspores was feasible with the high transformation frequencies up to 70% for late unicellular pollen and 79% for bicellular pollen, which was higher than *A. majus* (Barinova *et al.*, 2002).

The development of *P. hybrida* microspores *in vitro* was the same as that *in vivo*. It takes about four days from unicelluar to tricellular pollen. We have found that the mature pollen of *P. hybrida*, whether matured *in vivo* or *in vitro*, has contained three nuclei before germination (Fig. 3C), contrary to previous reports in which *P. hybrida* pollen was considered as two nuclear pollen (Wei *et al.*, 2007).

In the process of maturation of *P. hybrida* microspores *in vitro*, sucrose not maltose as the optimal carbon source/energy source and an osmotic regulator is a crucial factor contrary to *O. Violaceus* and *J. curcas* (Zhao *et al.*, 2007; Li *et al.*, 2010). This indicated that the developing pollen of *P. hybrida* may be due to low fructose utilization capacity or inhibited by fructose derived from maltose. Concentrations of sucrose either too low or too high significantly decreased the viability of *P. hybrida* microspores. The most appropriate sucrose concentration was 15% for *P. hybrida* microspore mature culture *in vitro*. However, when concentration of sucrose is 15% or 20%, we can get a high viability of *P. hybrida* microspores. It may, because osmosis pressure meets the growth of pollen. PH value is an important factor contributing to pollen germination success (Fan *et al.*, 2001).

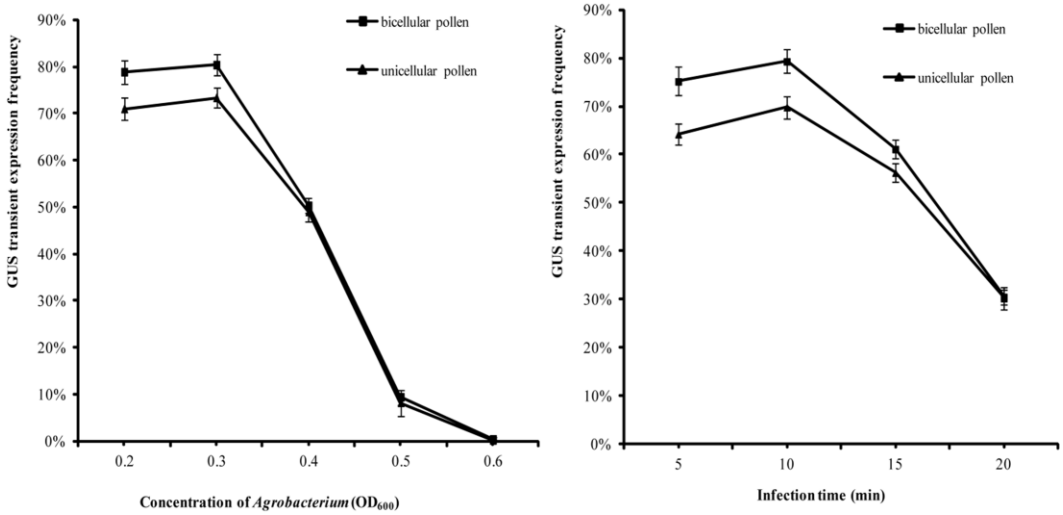

Fig. 4: GUS transient expression frequency in late unicellular pollen and bicellular pollen of *P. hybrida*. (A) Different concentrations of *Agrobacterium* with the same infection time of 10 min; (B) Different infection time with the same concentration of *Agrobacterium* OD_{600} = 0.3

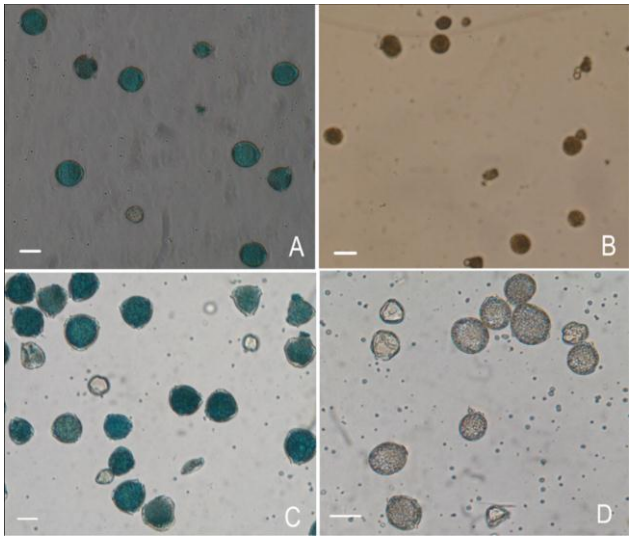

Fig. 5: GUS staining of *Agrobacterium*-transformed *P. hybrida* pollen. (A) GUS transient expression in late unicellular pollen after co-cultured for 20 h in the dark; (B) non-transgenic late unicellular pollen; (C) GUS transient expression in bicellular pollen after co-cultured for 20 h in the dark; (D) non-transgenic bicellular pollen. Horizontal bars = 30 μm

If the pH value is not suit, it will block gene expression and restrict the starch grains accumulation (Barinova *et al.*, 2004). In our study, the optimal pH for culture *P. hybrida* microspore was about 6.0, but not significantly different from pH = 5.5, 6.5, 7.0. This indicated that the sucrose concentration has a greater effect than pH value.

PEG, a kind of macromolecule penetrating agent, has been wildly added to germination medium (Ferrari and Wallace, 1975; Subbaiah, 1984; Golan-Goldhirsh *et al.*, 1991; Read *et al.*, 1993; Shivanna and Sawhney, 1995), although its action mechanism is not well known. PEG_{4000} added to germination medium greatly increased pollen germination of *P. hybrida*. The possible reason is that it can make the pollen endometrial structure and membrane surface charge change and improve the softness and permeability of the membrane, so as to improve the pollen germination and growth of pollen tube (Subbaiah, 1984; Read *et al.*, 1993; Alcaraz *et al.*, 2011).

Microspores provide an effective method for genetic transformation of plants (Touraev *et al.*, 2001). The advantages of using a late-unicellular or bicellular microspores are that (1) microspores are single haploid cells and highly synchronous in development (Aionesei *et al.*, 2006), and (2) the probability of incorporation of introduced DNA into the generative genome is increased (Aziz and Machray, 2003). A variety of methods has been employed in transferring genes into *in vivo* mature pollen grains, including the use of *Agrobacterium* (Hess and Dressler, 1989), electroporation (Matthews *et al.*, 1990; Obermeyer and Weisenseel, 1995; Saunders and Matthews, 1995) and particle bombardment (Twell *et al.*, 1989; van der Leede-Plegt *et al.*, 1995). In comparison with direct gene transfer methods of microspores, in our study we have successfully employed *Agrobacterium*-mediated transformation to transfer *β*-glucuronidase (GUS) reporter gene in *P. hybrida* microspores. It requires no *in vitro* regeneration and hence somaclonal variation may be minimized. Also the procedure is quick and possibly genotype independent (Touraev *et al.*, 1997).

In summary, *P. hybrida* microspores can be cultured into matured pollen and germination, and it is possible for exogenous gene to transfer into microspores. Thus we established *in vitro* microspore development system as well as efficient microspore-based genetic transformation system. This provides an alternative method for microspore development and genetic improvement of *P. hybrida* and other plants and lays a foundation for genetic modification and improvement of *P. hybrida.* Moreover, it also provides an experimental basis for higher plant genetic engineering, crop improvement and breeding.

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