

Full Length Research Paper

Genetic transformation of *Physcomitrella patens* mediated by *Agrobacterium tumefaciens*

Lin-Hui Li^{1*}, Jun Yang², Hui Lan Qiu¹ and Yan Ying Liu¹

¹Key Laboratory of Southwest China Wildlife Resources Conservation, China West Normal University, Nanchong 637009, China.

²Institute of Rare Animals and Plants, China West Normal University, Nanchong 637009, China.

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A simple efficient protocol for introducing exogenous gene from *Agrobacterium tumefaciens* into *Physcomitrella patens* was established. When the gametophores of gametophytes about 12 leaves were inoculated into the wells of PP3 medium at 25°C under the continuous light energy of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool-white fluorescent lamps, they grew up to form many buds at about 11 days, then *A. tumefaciens* at OD₆₀₀ 0.3 was added into the whole wells for 4 times at an interval of 12 h for continuous co-cultivation. After about 17 days of co-culture, the new juvenile gametophytes about 12 leaves grew up and were incubated into PP3 medium containing 50 mg L⁻¹ kanamycin for selection. 100% positive plants could be obtained after 4 generations of selective culture. PCR analysis showed that gene Cameleon YC2.1 in *A. tumefaciens* was transferred into all detected plants of *P. patens* and southern blotting analysis confirmed that exogenous gene was integrated into genomic DNA at a single locus. The results facilitated to analyze genes from more plants *via* genetic transformation by this system.

Key words: *Agrobacterium tumefaciens*, *Physcomitrella patens*, transformation, bud.

INTRODUCTION

As more and more nucleotide sequences have been generated by an increasing number of genomic sequencing projects, the prediction of gene function by comparing sequence with previous reports would not always lead to conclusive results (Holtorf et al., 2002), so the gene function of some new sequences will have to be identified by genetic transformation. However, it is hard to complete because of the extremely low homologous recombination frequency in higher plants. Of interest, *Physcomitrella patens*, a green non-vascular plant, possesses special characteristics: a simple morphogenetic processes containing a dominant haploid gametophyte stage; easy manipulation; small genome size; high degree of sequence conservation at the nucleotide level and no

significant difference in codon usage between it and other seed plants; the ability of integrating transforming DNA at a high frequency by way of homologous recombination (Schaefer and Zrýd, 1997; Reski 1998; Machuka et al., 1999; Cove, 2005; Cove et al., 2006; Quatrano et al., 2007). So it has become the new model system for studying plant morphogenesis and plant developmental evolution (Bhatla et al., 2002; Kramer, 2009), for analyzing plant gene function (Holtorf et al., 2002; Quatrano et al., 2007) and also for producing recombinant protein (Baur et al., 2005; Liénard and Stewart, 2009).

To date, the genetic transformation systems of *P. patens* such as PEG-mediated direct DNA uptake by protoplasts and gene gun transfer of foreign DNA into its protonemal tissue, have been developed and some gene functions were identified (Schaefer et al., 1991; Sawahel et al., 1992; Strepp et al., 1998; Cho et al., 1999; Finka et al., 2008), but all these methods need elaborate process of protoplast isolation and regeneration of constructing recombinant and extracting DNA. Relatively, genetic transformation via *Agrobacterium* is a very simple and convenient technology for operation. Using *Agrobacterium*, exogenous genes have been successfully transferred into

*Corresponding author. E-mail: lilinhui6890@126.com. Tel: +86-13508271859.

Abbreviations: PEG, Polyethylene glycol; OD, optical density; CTAB, cetyl trimethyl ammonium bromide; dCTP, deoxycytidine 5'-triphosphate; SDS, sodium dodecyl sulfate; YFP, your favorite protein.

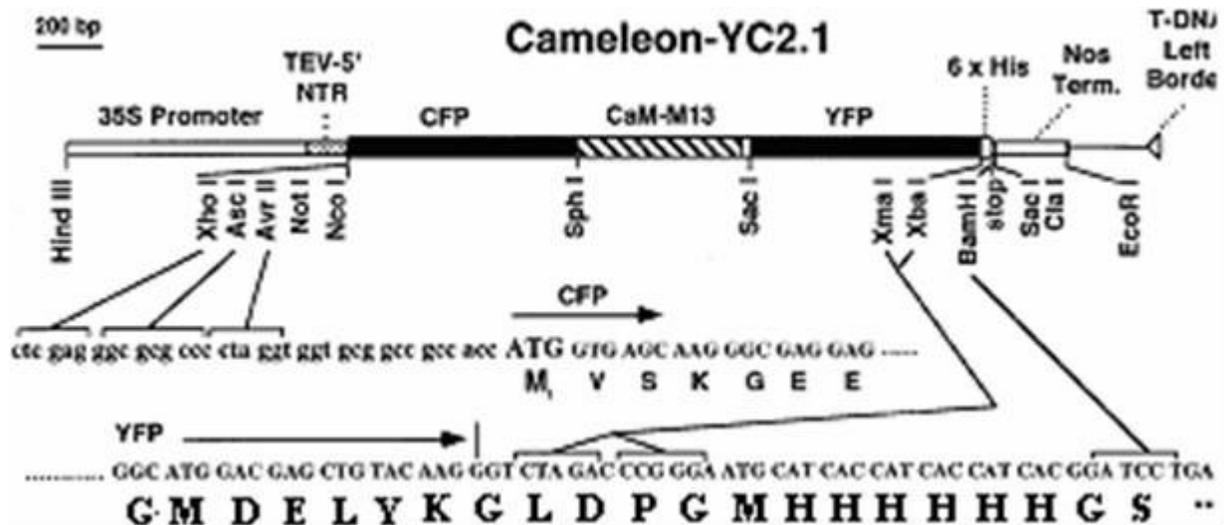


Figure 1. Cameleon-YC2.1 from construct 35S-YC2.1-Kan (Allen et al., 1999).

numerous plants such as *Arabidopsis* (Zhu et al., 2003), *Oryza sativa* (Dai et al., 2001). However, to our best knowledge, no report was found in the literature on genetic transformation of *P. patens* via *Agrobacterium*. Furthermore, Schaefer et al. (1994) and Baur et al. (2005) considered that *P. patens* was not a host of *Agrobacterium*.

Here, we investigated the conditions of genetic transformation in *P. patens* via *Agrobacterium* and a simple efficient protocol for introducing exogenous gene from *A. tumefaciens* into *P. patens* was established.

MATERIALS AND METHODS

Plant material and culture condition

The moss *P. patens* was cultured on PP3 medium in Petri dish at 25°C under continuous light energy of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool-white fluorescent lamps. The medium contained 0.8 g L⁻¹ Ca(NO₃)₂·4H₂O, 0.0125 g L⁻¹ FeSO₄·7H₂O, 0.25 g L⁻¹ MgSO₄·7H₂O, 0.055 mg L⁻¹ CuSO₄·5H₂O, 0.055 mg L⁻¹ ZnSO₄·7H₂O, 0.614 mg L⁻¹ H₃BO₃, 0.389 mg L⁻¹ MnCl₂·4H₂O, 0.055 mg L⁻¹ CoCl₂·6H₂O, 0.028 mg L⁻¹ KI, Na₂MoO₄·2H₂O 0.025 mg L⁻¹, 8 g L⁻¹ agar, pH 7.0, 1 ml L⁻¹ KH₂PO₄ buffer (Ashton et al., 1979).

Agrobacterium infection and co-cultivation

Preparation of *A. tumefaciens*:

A. tumefaciens LBA4404 transformed into plasmid Cameleon-YC2.1 was constructed for plant transformation (Allen et al., 1999) (Figure 1) and the construct contains kanamycin, rifampin and streptomycin resistance marker. The strain LBA4404 was maintained on a Petri dish containing selective YEB medium supplemented with 50 mg l⁻¹ kanamycin at 4°C. When needed *A. tumefaciens* LBA4404 culture for infection, one full loop of culture incubated at 28°C on YEB solid medium for 3 days was inoculated in 10 mL YEB broth and grown to OD₆₀₀ 0.3 for gametophore infection. All YEB medium, regardless of solid or broth for cultivating LBA4404, contained 50 mg L⁻¹ kanamycin, 100 mg L⁻¹ rifampin and 50 mg L⁻¹

streptomycin to ensure that all the *A. tumefaciens* contain plasmid cameleon-YC2.1.

Infection and co-cultivation:

First, sterile PP3 agar medium was poured into 12 cm Petri dishes, 10 wells in every Petri dish were drilled with 12 mm depth and 10 mm diameter at intervals of about 20 mm after the medium cooled down, then the gametophores of gametophytes about 12 leaves were inoculated into the wells with four gametophores in each well (Figure 2B). After the gametophores were incubated for 5, 7, 9, 11 or 13 days, respectively, and developed into protonema at 25°C under continuous light energy of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool-white fluorescent lamps (Figure 2C), *A. tumefaciens* at OD₆₀₀ 0.3 was first added and supplemented into the wells three times at an interval of 12 h for infection and co-cultivation with plants. When the juvenile gametophytes grew up to about 12 leaves in the co-culture wells, their gametophores were transferred into selective medium to select transgenic plants.

Screening of transgenic plants

To select the transgenic plants, the survival gametophores of gametophytes from co-culture wells were picked out and immediately inoculated into wells made up of selective PP3 medium. As the endurable kanamycin concentration for *P. patens* was prior not tested, the first selection was conducted in four selective PP3 media containing 200 mg L⁻¹ carbenicillin and 30, 50, 70 or 90 mg L⁻¹ kanamycin, respectively, and only one gametophore was inoculated into each well.

To ensure the real transgenic plants were picked out, all the survival gametophores of gametophytes from the first selection were transferred to PP3 medium containing 50 mg L⁻¹ kanamycin for three generations of selection, then all the transgenic plants were random picked out for molecular analysis.

Molecular analysis genetically transformed plants

150 clusters of transgenic plants were randomly selected to analyze whether the gene Cameleon-YC2.1 was transferred into *P. patens*,

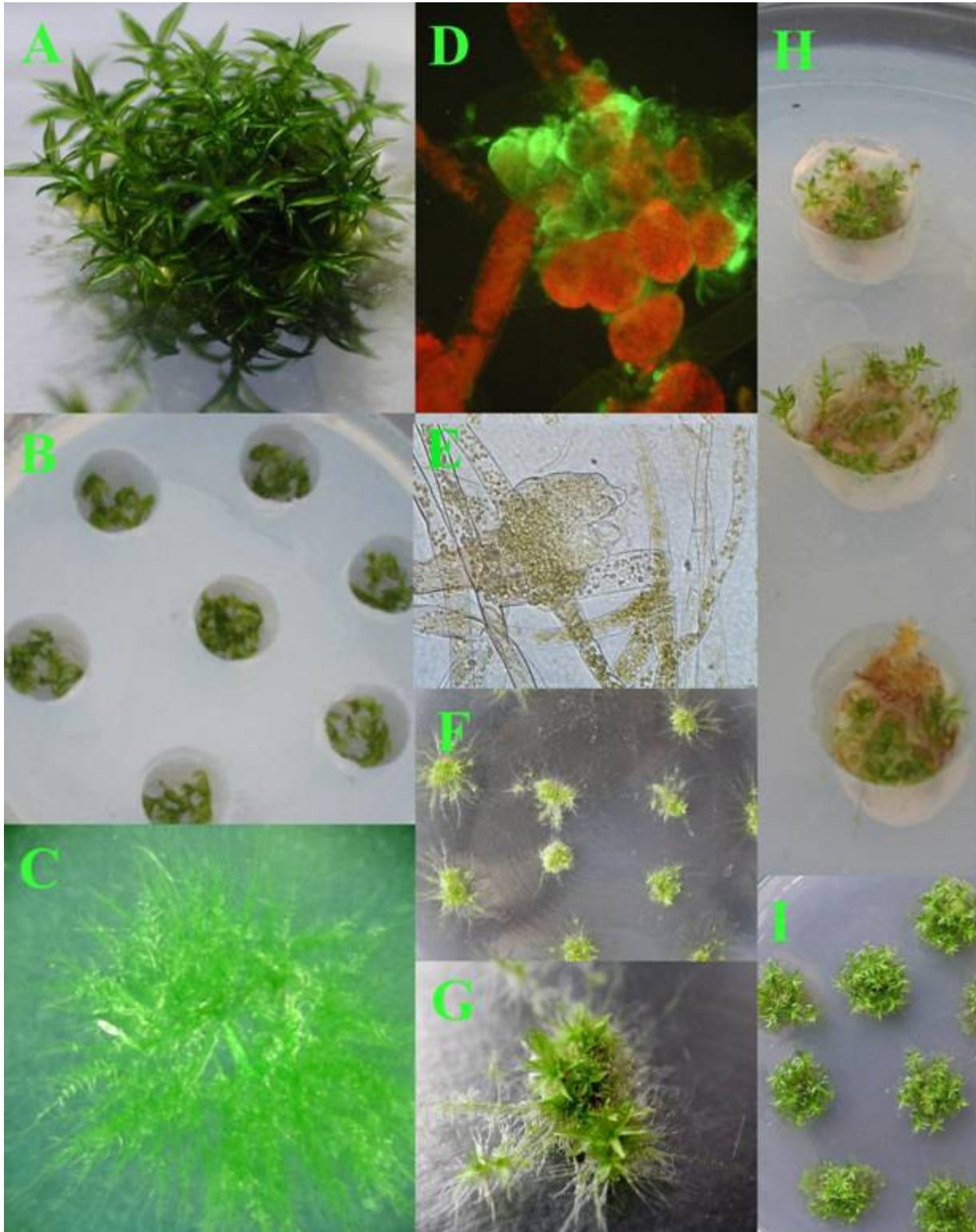


Figure 2. Growth progress of the normal and transformed moss *P. patens*. (A) Normal gametophytes; (B) gametophores inoculated and cultivated in wells; (C) protonema developed from inoculated gametophores; (D) bud in protonema labeled by FITC (a fluorescence stain to study cytoskeleton), showing its multicellular structure; (E) juvenile gametophytes just developed from bud; (F) juvenile leafy gametophytes developed from juvenile gametophytes; (G) mature individual leafy gametophytes showing the elder gametophyte in center and the younger gametophyte in edge; (H) survival putative transgenic gametophytes and albinistic gametophytes on PP3 selective medium containing 50 mg L⁻¹ kanamycin; (I) transgenic gametophytes by 3 times of subculture.

their genomic DNA was extracted individually by CTAB method from about 0.5 g fresh tissue after their free water was partly absorbed by filter paper.

Two analysis methods were employed to test the putative transgenic plants, one was PCR for testing whether the gene was transformed into *P. patens* and the other was southern blotting for detecting whether exogenous genes were stably integrated into the genomic DNA of *P. patens*.

PCR analysis:

Genomic DNA was amplified by specific primers of gene Cameleon YC2.1, the 5' primer was 5'-CAG GAA TTC CTC GAG GGC GCG CCC CTA GGT-3' and the 3' primer was 5'-CAT TCC CGG GTC GAG ACC CTT GTA CAG CTC-3' (Allen et al., 1999). PCR was conducted at 94°C for 3 min, 94°C for 40 s, 64°C for 90 s, 72°C for 3 min, 30 cycles, then extension for 10 min, the PCR reaction system was 2×GC buffer, 25 µl, dNTP 8 µl, the forward primer 1 µl, the reverse primer 1 µl, template DNA 1 µl, DNA *Taq* 0.5 µl, water 13.5 µl, the total volume was 50 µl (*TaKaRa LA Taq*™ with GC buffer, Dalian, Co. Ltd.). PCR products were photographed under UV light by using Gel Doc 2000 (Bio-rad Laboratories-Segrate, Milan, Italy).

Southern blots analysis:

Four restrictions endonucleases, *Bam*H I, *Xho* I, *Hind* and *Sac* I, were selected to digest genomic DNA according to the plasmid construct (Figure 1). DNA was incubated over night at 37°C by 4 U of enzyme per µg DNA (*TaKaRa* Biotechnology (Dalian) Co. Ltd.). One additional unit of enzyme was supplemented into digesting solution per µg DNA the next morning, and the incubation finished till the digestion completed by running aliquots of the digested DNA on a minigel.

Aliquots of digested DNA were separated on 1% agarose gel in 40 mM Tris-acetate 1 mM EDTA, pH 7.4. After electrophoresis, the DNA was stained with ethidium bromide and visualized by UV illumination, then transferred onto a Hybond N⁺ filters (Amersham Biosciences, USA) according to the procedure reported by Southern, (1975). The filters were air dried and baked between 2 sheets of Whatman No. 3 MM paper in oven for 3 h at 80°C. GFP sequence, highly similar to CFP and YFP in sequence, was amplified to act as hybridization probes labeled by ³²P dCTP according to the Prime-a-Gene labeling System (Promega).

The Hybond N⁺ filters were prehybridized with denatured salmon sperm DNA (200 µg/ml) (Sigma) for 4 h at 60°C in a solution containing 6x SSC, 0.5% SDS, 5x Denhardt's solution (Denhardt, 1966). Then the prehybridization buffer was discarded and the ³²P-labeled probes were added to hybridize for 16 h at 65°C in 6x SSC-0.01 MEDTA-5x Denhardt's solution with 0.5% SDS-denatured salmon sperm DNA (100 µg/ml). After hybridization, the filters were washed for many times each for 5 min at 70°C in 2x SSC-0.5% SDS till the control regions did not show radioactivity, followed by once in 2x SSC-0.1 % SDS for 15 min, and then for 30 min at 55°C in 0.2x SSC-0.1% SDS. Blots were scanned by a phosphorimager (Cyclone™ Storage phosphor System, Packard instruments, USA).

RESULTS

The optical stage via *A. tumifaciens* infecting *P. patens* was that more buds could be observed in protonema

The growth process of normal and transgenic plants of *P.*

patens was observed by anatomical microscope. The results showed that, as the tip of normal gametophytes (Figure 2A) was sectioned and inoculated into the wells (Figure 2B), new protonema (Figure 2C) could develop from gametophores after about 2 - 3 days of cultivation on PP3 medium and about 11 days later, much buds generated from caulonemal filaments and every bud was constructed with multiple cells (Figure 2D), this should be the mark of the transition from the filamentous juvenile to the leafy adult gametophyte. No buds could be observed apparently in protonema if the cultivation was short of 9 days, but after 13 days of culture, the bud become juvenile gametophyte (Figure 2E) and then developed to adult leafy gametophytes (Figure 2F). Furthermore, the elder gametophyte and younger gametophyte could be generated from the same well, which represented the different developmental stage of the leafy gametophytes (Figure 2G).

Interestingly, only the gametophores, when they were cultivated in PP3 medium for about 11 days could grow up into new gametophytes after co-culture with *A. tumifaciens*, and these infected gametophores were readily survive in the following selection procedure. More than four new survival gametophytes grew up from every inoculating well. However, when the gametophores were cultivated in PP3 medium less than 9 days, almost no new gametophytes obtained after co-culture with *A. tumifaciens*. If the gametophores were cultivated in PP3 medium more than 13 days, only a few yellow gametophytes could be observed after co-culture with *A. tumifaciens* and they would necrosis finally.

The results showed that the plant *P. patens* could be successfully infected via *A. tumifaciens* when the "budding" stage was clarified.

More generations of screening were essential for obtaining transgenic plants

About 17 days of continual co-cultivation of *P. patens* gametophytes with *A. tumifaciens*, some leafy gametophytes of about 12 leaves began to grow up from the inoculating wells. When their gametophores were incubated into PP3 selective medium containing 200 mg L⁻¹ carbenicillin and 30, 50, 70 or 90 mg L⁻¹ kanamycin for the first selection, only part of plants could survive and others grew to yellow and finally died in selective medium after 35 days of selection during every generation (Figure 2H). The survival rate of the infected gametophores decreased with the increasing initial concentration of Kan⁺ in the first selection and maintained stability to 100% after three generations of selection in selective media containing 50 mg L⁻¹ kanamycin, but no plant was survived in selective medium containing 90 mg L⁻¹ kanamycin, suggesting over high concentration of kanamycin could damage the transgenic plants (Table 1, Figure 2I).

In summary, it was very important to pick out green gametophyte for successive selection to eliminate false

Table 1. The ratio of survival to all gametophores in the fourth generation screening.

Kan (mg L ⁻¹)	Rate (%)			
	First screening	Second screening	Third screening	Fourth screening
30	96	85	98	100
50	87	98	100	100
70	63	100	100	100
90	0	-	-	-

^aThe gametophores of the survival gametophytes in first screening were from infection wells, the gametophores of the survival gametophytes in the following screening were from the former generation. The data of kanamycin concentration in table was omitted in the second, third and fourth screening because all were 50 mg L⁻¹.

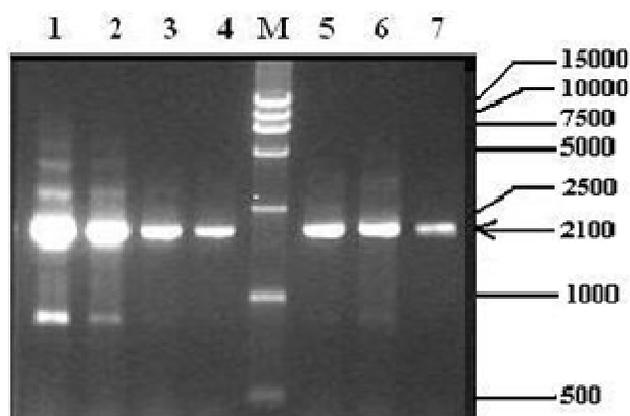


Figure 3. PCR amplification of the transgenic *P. patens*. Lane 1, 2, 3, 4, 5, 6 and 7 represented the individual transgenic plants, lane M, Marker DL15000.

transgenic plants and a higher initial kan⁺ concentration was beneficial of selecting transgenic plants.

Molecular analysis showed that the exogenous gene had been transformed into *P. patens*

150 clusters of transgenic gametophytes were analyzed by PCR, among them, 50 clusters were from the first selective stress 30 mg L⁻¹ kanamycin and then underwent three generations of selection in selective media containing 50 mg L⁻¹ kanamycin, 50 clusters from the first selective stress 50 mg L⁻¹ kanamycin and 50 clusters from the first selective stress 70 mg L⁻¹ kanamycin. Surprisingly, all the transgenic plants showed a PCR band of about 2 kb consistent with the size of the gene Cameleon YC2.1 (Figure 3), the result showed that the gene Cameleon YC2.1 was transferred into *P. patens*.

One cluster of transgenic gametophyte was analyzed by southern blotting. The CFP and YFP sequence in gene Cameleon YC2.1 could not be cut by restricted enzyme *Bam*HI, *Xho* I or *Hind* III into two fragments (Figure 4). If gene Cameleon YC2.1 integrated into the genomic DNA of *P. patens* at a single locus, it should

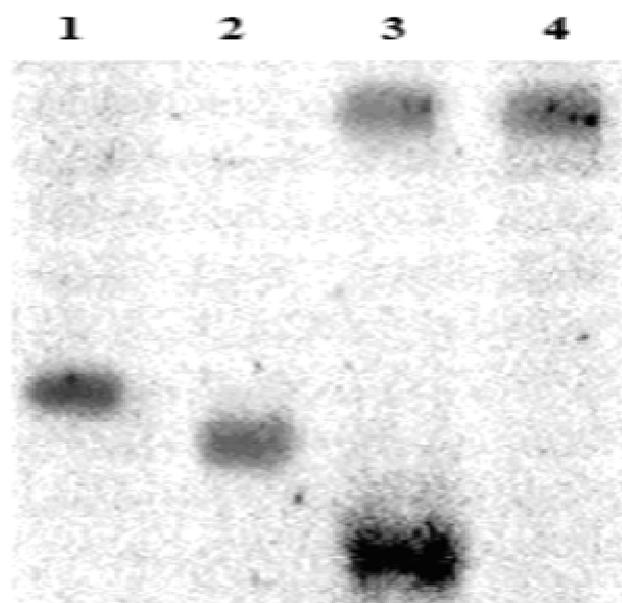


Figure 4. Southern blotting analysis of transformed DNA. Lane 1 Digested with *Bam*HI; Lane 2 Digested with *Xho* I; Lane 3 Digested with *Sac* I; Lane 4 Digested with *Hind* III.

demonstrate only one hybridization band in lanes 1, 2 and 4 after hybridizing with probe GFP, if it was not that, more than one hybridization band would be showed in lanes 1, 2 and 4. The hybridization result in lanes 1, 2 and 4 showed only one hybridization bands, respectively, demonstrating gene Cameleon YC2.1 integrated into the genomic DNA of *P. patens* at a single locus. Whereas The CFP and YFP sequence could be cut into two sequences by restricted enzyme *Sac*I, one contains CFP sequence, another contains YFP sequence (Figure 4), if gene Cameleon YC2.1 integrated into the genomic DNA of *P. patens* at a single locus, it will be cut into two size of DNA sequence and the two DNA sequence contains the CFP or YFP sequence, thus the hybridization band should be two. This is clearly showed in lane 3 and was thoroughly consistent with the predicted result. We concluded that gene Cameleon YC2.1 integrated into genomic DNA of *P. patens* at a single locus.

DISCUSSION

Schaefer et al. (1994) and Baur et al. (2005) reported that *P. patens* was not a host of *Agrobacterium* and so far, there was no report on the transformation of *P. patens* via *A. tumefaciens*. Whether *P. patens* could be transformed via *Agrobacterium* or not? It was investigated in this paper and the results showed that *P. patens* was a host of *Agrobacterium*. We considered the insight into the following knowledge resulted in the successful transformation.

The selection of infection stage was crucial factor for successful transformation. Cheng et al. (1997) indicated the physiological growth state of explants in higher plants affected the transformation rate mediated by *Agrobacterium*, this was confirmed and the result showed only the gametophores cultivated for about 11 days could be successfully transformation after co-culture with *A. tumefaciens* in *P. patens*. We know the life history of *P. patens* has two distinct morphological forms: the juvenile filamentous form and the adult leafy gametophytes one and the transition from filamentous form to gametophytes must undergo "budding" stage (Reski, 1998). Buds are characterized by continuous division into much cells (Figure 2D) and thin cell wall. We considered these bud cells were similar to the protoplasts of *P. patens* because they developed immaturely, in other words, most of these bud cells are characterized by extreme thin cell wall, and it was very successful technology of the protoplasts as transformation materials mediated by PEG in *P. patens* (Schaefer et al., 1991; Strepp et al., 1998; Finka et al., 2008).

The long time of *P. patens* co-cultivating with *Agrobacterium* enhanced the infection efficiencies. Commonly, the co-culture time is controlled in 1 to 3 days in higher plants transformation (Fang et al., 1990; Cheng et al., 1997; Cardoza and Stewart, 2003; Wu et al., 2003; Boyko et al., 2009), but there are reports on increasing transformation efficiencies by prolonging co-cultivation time (Cheng et al., 1997; Zhao et al., 2000). In this experiment, the continuous co-cultivation of gametophytes with *Agrobacterium* was up to 17 days and the next generation leafy gametophytes grew up directly from co-culture wells, while the new plants from explants must commonly grow up in selective media in higher plant transformation. To some extent, the long co-cultivation eliminated the non-infectious gametophytes and only these infected plants could survive whereas the others died slowly (Figure 2H), thus a high transfer efficiency of exogenous T-DNA into buds took place.

Three times of supplementing *A. tumefaciens* ensured its enough concentration and vigor, resulting in a higher transformation frequency. It was reported that a higher density of *A. tumefaciens* usually yielded more efficient delivery of T-DNA into various tissues or cells, but would damage more to plant cells (Cheng et al., 1997). In this experiment, new alive *A. tumefaciens* was supplemented into inoculation wells for 4 times at an interval of 12 h,

this must supply enough quantity and quality of *A. tumefaciens* and its initial concentration was only at OD 0.3, they would further propagate during infecting period, resulting in a much higher of *A. tumefaciens* concentration. But we also found that a too high of *A. tumefaciens* concentration not only resulted in the death of all the protonema but also the bud cell and none gametophytes grew up in co-culture wells.

May be the continuous light illumination increased the infecting stress ability, resulting in a steadily survival of the infected bud and gametophytes. In higher plant transformation, after explants were co-cultivated with *Agrobacterium*, they were transferred to grow under the 16 h light and 8 h dark regime (Boyko et al., 2009; de Oliveira et al., 2009), whereas, continuous light illumination was supplied in this experiment. This was likely one of the critical factors for successful transformation and could explain from two respects, one was to ensure the continuous production of buds, we observed that numerous asynchronous buds existed in protonema, this would provide suitable buds to be infected, another was to ensure the infected buds could continuously be divided and differentiated. In this experiment, its survival and fast division of infected buds were very essential for obtaining transgenic plants, because the infected buds could not transfer to selective media as that in higher plant explant transformation until they developed into visible gametophytes.

More generations of screening ensured that the gametophytes for PCR and southern blotting analysis were transgenic plants. Previous reports showed that unstable transformants could be reduced by a longer selection (McCormick et al., 1986), similarly, in this investigation, eliminating the false positive plants was assured by four generations of selection. It was observed that the unstable transformants (gametophytes) grew to yellow and finally died in selective medium for first and the later selection (Figure 2H). Table 1 showed the rate of transgenic gametophytes improved along with the increased selective stress and screening generations; in the end, all the selective gametophytes were transgenic plants.

In conclusion, a simple efficient protocol for introducing exogenous gene from *A. tumefaciens* into *P. patens* was established, which facilitated to analyze genes from more plants via genetic transformation by this system and the transformation efficiency was higher than those of protoplast transformation mediated by PEG method (Schaefer et al., 1991; Schaefer and Zrýd, 1997). Moreover, this system is more simple, efficient and easy for operation and the integration of exogenous T-DNA to genomic DNA *P. patens* was confirmed also at a single genomic locus.

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