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Optimization of BY2 Cell Suspension as a Stable Transformable System

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Abstract

Tobacco (*Nicotiana tabacum*) cv. 'Bright Yellow 2' (BY2) cell suspension is a useful system to study the structure and function of plant cell. However, low efficiency of *Agrobacterium*-mediated transformation, and transgene silencing during subculture limit its application. Here we present optimization of the traditional protocols of *Agrobacterium*-mediated transformation and genomic DNA extraction. The transforming efficiency and recovery ratio of genomic DNA extraction were substantially increased by these improvements. Southern assay demonstrated that copy number of transgene could be determined unambiguously. Meanwhile by monitoring the GFP fluorescence we found that the *GFP* expression can keep stable in suspension culture cells for at least 20 days in liquid medium. Finally, applicability of constitutive promoters of *Arabidopsis thaliana UBIQUITIN10* (*AtUBQ10*) and *ARABIDOPSIS SKP1 HOMOLOGUE1* (*AtASK1*) also can drive stable *GFP* expression *in vivo* of BY2 cells like CaMV *35S* promoter in this plant system.

Keywords: transgenic cells, transgene expression, promoter, *GFP* gene, *Nicotiana tabacum* BY-2

Abbreviations: BY2 = Bright Yellow 2; GFP = Green Fluorescence Protein; CaMV 35S promoter = Cauliflower mosaic virus promoter; AtUBQ10 promoter = *Arabidopsis thaliana* UBIQUITIN10; AtASK1 promoter = *Arabidopsis thaliana* ARABIDOPSIS SKP1 HOMOLOGUE1; RT-PCR = Reverse transcriotion-polymerase chain reaction; CTAB = Hexadecyltrimethy Ammonium Bromide; DAPI = 4,6-Diamino-2-Phenyl Indole

Introduction

Tobacco (*Nicotiana tabacum* L.) cv. 'Bright Yellow 2' cell suspension (BY2) has been broadly used in plant cell biology (Duby *et al*., 2010; Link and Cosgrove, 1998). However, some problems remain and impede application of this system up to date. One prominent issue is low efficiency of *Agrobacterium*-mediated transformation, and the other problem of transgene silence during subculture makes it worse to use such system to perform experiment in long term (Vaucheret *et al*., 1998; Ito *et al*., 1997). For example, in investigating the function of cell cycle genes in BY2, it would be difficult to obtain precise replicable data if the transgene is silenced during subsequent proliferation cycles without an expectable timing.

In addition, the tremendous large BY2 genome (approximately 4.8 billion base pairs) makes it very hard to determine copy number of transgene using traditional DNA gel blotting assay (Southern assay), even such knowledge is necessary for investigating dose-dependent traits. The requirement of adding antibiotics at each subculture in traditional protocols to prevent growth of untransformed cells also imposes toxic effect, severely affecting the fidelity of treatment experiment using plant growth factors or stress stimulation substances (Miao and Jiang, 2007; Sparkes *et al*., 2006; Cogoni and Macino, 1999).

Though as a broadly applied constitutive promoter in plant, Cauliflower mosaic virus (CaMV) *35S* promoter often fails in driving constitutive expression in early development of male and female reproductive organs and in macro- and microgametophyte (Zhang *et al*., 2008; Nizampatnam *et al*., 2009). Recently maize (*Zea mays*) *UBIQUITIN 1* (*ZmUBQ1*) promoter has been used for constitutive expression of genes of several crops (Cornejo *et al*., 1993). However, this promoter has been found to be temperature-responsive such that it is limited in certain field studies (Chen *et al*., 2007). *Arabidopsis thaliana UBIQUITIN 10* (*AtUBQ10*) might be a good substitute of *ZmUBQ1* promoter, especially in dicots, since its transcript has been used as a constitutive internal control in quantification of some *Arabidopsis* temperature-responsive mRNAs (Grefen *et al*., 2010). Furthermore, the *ARABIDOPSIS SKP1HOMOLOGUE1* (*AtASK1*) gene encodes a homolog of human and yeast Skp1 proteins (Yang *et al*., 1999) and is essential for many aspects of plant development through ubiquitin-mediated degradation of proteins by proteasomes (Zheng *et al*., 2002; Sullivan *et al*., 2003). *AtASK1* also had been reported to be expressed

broadly in male and female reproductive organs (Yang *et al.*, 1999; Liu *et al.*, 2004; Wang *et al.*, 2004; Teixeira *et al.*, 2005). However up to date there are few reports about using of either *AtUBQ10* or *AtASK1* promoter to drive expression of transgene even in *Arabidopsis*.

Here we reported that we had tried to optimize the *Agrobacterium*-mediated BY2 transformation procedure. The adjustment on final liquid medium volume and cell density made it much easier to obtain callus with transgene stably integrated than the traditional method. Meanwhile we optimized the genomic DNA extraction to recover sufficient genomic DNA for performing Southern assay, and characterized *GFP* expression driven by CaMV *35S*, *AtUBQ10* and *AtASK1* promoters. We found the transgene could be stably expressed for at least 3 subculture cycles, approximately 20 days in liquid medium. In later stages of subculture, antibiotics could be dismissed so that some stress simulation experiment could be performed without the interference of the adverse effect of antibiotics. The relative constitutive expression efficiency of CaMV *35S*, *AtUBQ10* and *AtASK1* was quantified and estimated by both fluorescence observation and semi-quantitative RT-PCR.

Method and material

Plant Material

The original non-transformed tobacco (*Nicotiana tabacum* L.) cv. 'Bright Yellow 2' (BY2) suspension was cultivated in a modified liquid Murashige and Skoog (MS) medium (4.3 g/L MSO221 sigma, 3% Sucrose, 0.1mg/ml myo-Inositol, 1 μg/ml Thiamine, 0.2 μg/ml 2,4-D, 0.2 mg/ml KH_2PO_4, PH 5.8) (Nagata *et al.*, 1992; Link and Cosgrove, 1998; Duby *et al.*, 2010), at 28 °C with 120 rpm shaking on an orbital shaker in the dark and maintained by weekly dilution (V/V=1/20) of cell in 100ml liquid medicine.

Plasmid GFP Expression Construct

GFP coding sequence was amplified by PCR using the primers as follow: the forward primer GFPF with CACC at its 5' end (5'-CCACATGGTGAGCAAGGGCGAGGA-3') and the reverse primer GFPR (5'-TTACTTGTACAGCTCGTCCA-3'). Approximately 3000bp *AtUBQ10* and *AtASK1* promoter sequences were amplified using the *AtUBQ10* promoter forward primer UBQ10F with CACC at its 5'end (5'-CCACATGGTGAGCAAGGGCGAG-3') and the reverse primer UBQ10R (5'-TTACTTGTACAGCTCGTC-3'), the *AtASK1* forward primer ASK1F with CACC at its 5' end (5'-CCACAGAGCATCTAGCTGAGCA-3') and reverse primer ASK1R (5'-AGGGAAATTAGGAAA TTGTTTCA-3'). The amplified GFP CDS, *AtUBQ10* and *AtASK1* promoter sequences were integrated into the TOPO ENTRY vector (Invitrogen) and confirmed by DNA sequencing. The verified correct GFP-ENTRY construct was recombined with pGWB2 (Invitrogen) construct to generate *35S:GFP* expression vector, while UBQ10-ENTRY and ASK1-ENTRY constructs were recombined with pGWB4 (Invitrogen) to generate *AtUBQ10:GFP* and *AtASK1:GFP* expression vectors, respectively.

BY2 Cell Transformation

The transformation of BY2 suspension was carried out by a modified method described previously (Nakayama *et al.*, 2000; David and Perrot-Rechenmann, 2001). 5ml 3-d-old, exponentially growing suspension of original non-transformed BY2 cell was transferred into a 60-mm culture dish and blown for 20 times, then incubated at 28 °C with 100 μl *Agrobacterium* GV3101 strain (OD_{600}=0.6) harboring transgene expression construct in dark. After 48 h of co-cultivation, the cells were washed with 25ml modified MS medium only once and plated on a modified MS solid medium containing 25 μg mL^{-1} hygromycin and 100μg mL^{-1} vancomycin. After 3 weeks of incubation, the novel hygromycin-resistant calluses were transferred onto fresh solid medium plates containing 25 μg mL^{-1} hygromycin. 2 weeks later, the calluses could be subjected to genotype and phenotype (including transgenic fluorescence) analysis. As for Southern assay, however, more cell proliferation should be committed through inoculating callus in liquid medium for 10 days.

Southern Hybridization Assay

Exponentially growing transgenic BY2 suspension cells were harvested and extracted by our modified CTAB-based method (Lutz *et al.*, 2011). In detail, BY2 cell was collected from 100ml 10-day liquid culture by centrifuge (5000 rpm, 15 minutes). Then the cell pellet was freeze dried at -40 °C overnight. The dried cell pellet was ground to powder in liquid nitrogen with help of carborundum. Then the DNA was extracted according to the conventional procedure of CTAB method.

Southern blot was performed as modified protocol described (Sambrook and Russell, 2001). In our situation, after digestion by restriction endonuclease EcoRI, the total BY2 genomic DNA (50 μg) was separated in a 0.7% (w/v) agarose gel in 0.5×TBE without ethidium bromide and transferred onto a blotting membrane (Millipore corporation, Billerica, MA). Then the membrane was hybridized with the DIG-tagged DNA probes synthesized by PCR. Hybridizations were carried out at 42 °C. Two rounds of low stringency membrane wash and four rounds of high stringency wash were performed due to the very high BY2 genomic DNA background (50 μg BY2 genomic DNA each sample), and the hybridization signal detection was carried out according to the detection kit manufacturer's instructions (DIG-High Prime DNA labelling and detection starter kit II, Roche).

Analysis of GFP Gene Expression by Fluorescence Microscopy

Fluorescence microscopy was performed using Leica DM2500 fluorescence microscope and DFC420 C camera (Leica, Germany) with the GFP excitation and emission module.

Analysis of GFP Gene Expression by Semi-quantitative RT-PCR

Total RNA was extracted from callus according to Verwoerd *et al.* (1989), with adding carborundum to help grinding. The extracted total RNA was treated by RNase-free DNase I (Takara, Japan). Then the treated RNA was used as template for reverse transcription (RT) reaction according to the manufacturer's instructions of the M-MLV

First strand kit (Invitrogen). The resulted complementary DNA (cDNA) was diluted 10 to 20 folds and then used as the template for PCR reaction. PCR primers for *GFP* gene were PGF (5'-ATGGTGAGCAAGGGCGAGGA-3'), PGR (5'-TTACTTGTACAGCTCGTCCA-3'). In all RT-PCR reactions, the tobacco *UBIQUITIN1* (*NtUBQ1*) gene was used as the internal control for input cDNA amount normalization. To get rid of the interference of residual genomic DNA, the cDNA of *no promoter: GFP,* i.e. BY2 transformed by pGWB4 (Invitrogen) construct, was used as the non-RT control.

Results

An optimized BY2 *Agrobacterium*-mediated transformation protocol was established

When BY2 was transformed according to the traditional procedure (Menges and Murray, 2006), even after three weeks of post-transformation incubation on the selective plates, the cells seemed to have never recovered from the initial growth stasis, which was caused by *Agrobacterium* infection, and exhibited little proliferation. We checked the cells by Evans Blue staining at the end of incubation and found almost all cells (above 85%) was unviable in the groups treated by ditional method (Fig. 1C-E and I). Notably, even on non-selective plate, the transformed cells dispersed by the traditional method also failed in proliferating, suggesting it was not *Agrobacterium* treatment that caused the cell growth failure. However, only a few of cell death (below 15%) occurred in groups treated by modified method (Fig. 1F- I). Comparison between the cells whose proliferation could and could not be efficiently initiated exhibited that the former were exposed in the air during the post-transformation incubation, while the later were soaked in the liquid medium, which was added at the aliquot step to easily disperse the cell on the selective plate. This excess of liquid medium remained at almost all the time of the incubation. Thus we tried to get rid of excess liquid medium covering the cell pellet before cell dispersion, and found that cell growth could be initiated rapidly.

Meanwhile we noticed that the cells in some plates with occasional higher cell density could quickly recover from *Agrobacterium* infection and initiate proliferation more efficiently than those with lower cell density (Fig. 1A and B). We tested several cell density gradients as 1×10^6, 1.5×10^6, 2×10^6 and 3×10^6 cells/ml in the trasformation protocol, and found 2× folds (Fig. 1B) and 3× folds of traditional density worked the best for recovery from *Agrobacterium* infection. If transformation is unsuccessful, the cell density should be optimized to reach at least 2×10^6 cells/ml. Meanwhile the original 9mm diameter plates were replaced by 5mm diameter plates, and the round number of the washing step was changed to one time instead of original three times to save more cells for aliquot.

With all above improvements adopted, separated individual transformed callus could be visible by naked eyes in approximately two weeks after final plating the cells (Fig. 2B-D). Then the new calluses were transferred onto freshly prepared antibiotics plates 3 weeks after aliquot (Fig. 2F-H). The contamination of non-transformed calluses would be inhibited effectively during subculture under selective pressure. Compared with the non-transformant controls

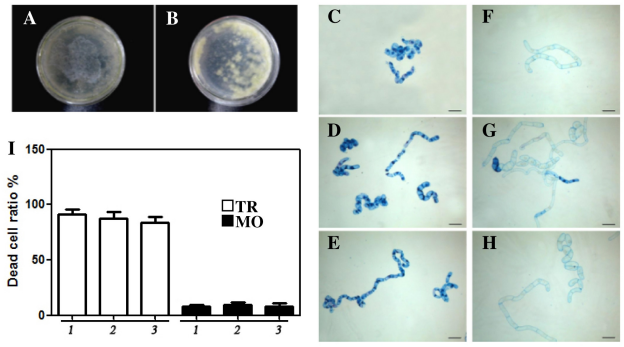

Fig. 1. Cell proliferation and growth compare between traditional and modified mean

(A) 2 weeks-past cells grow slowly and difficultly on plating with lower cell density. (B) 2 weeks-past cells grow and form callus rapidly on plating with higher cell density (2× folds cell density of TR). (C), (D) and (E) Cells treated by traditional mean, were stained with Evans Blue. (F), (G) and (H) Cells treated by modified method, were stained with Evans Blue. (I) Percentage of cell death shown in traditional method (TR) and modified method (MO) (n>30 microscope view areas; $p<0.05$, Student's t test) 1, 2 and 3 means 3 independent transgenic groups. Bar = 10 μm

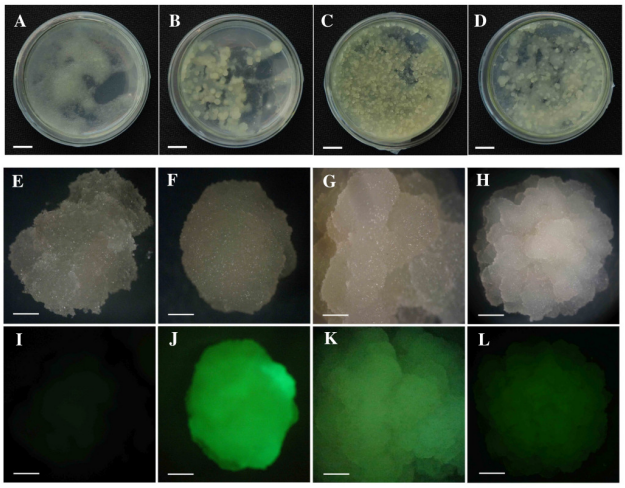

Fig. 2. Calluses of transformed and wild type BY2 cells growing on selective medium

(A), (E) and (I) non-transformed cell cultivated on MS medium. (B), (F) and (J) *35S:GFP* transformed callus on selective medium. (C), (G) and (K) *AtUBQ10:GFP* transformed callus on antibiotic medium. (D), (H) and (L) *AtASK1: GFP* transformed callus on antibiotic medium. (E) and (I), (F) and (J), (G) and (K), (H) and (L) the same views correspondingly, taken by bright field and fluorescence microscopy modules, respectively. Bar = 1cm in A-D and Bar=0.5 mm in E-L

(Fig. 2A, E and I), in the transformed calluses with integrated *GFP* expression construct, green fluorescence respectively driven by CaMV35S, *AtUBQ10* and *AtASK1* promoters could be clearly observed (Fig. 2J-L).

DNA gel blot assay (Southern assay) verified that stable transformed calluses were obtained

Tobacco (*Nicotiana tabacum*) possesses a tremendous large genome with approximately 4.8 billion base pairs overall length, so that huge amount of input of genomic DNA is required for single copy target sequence detection within the whole genome. However, the traditional plant genomic DNA extraction protocol can hardly accomplish sufficient recovery of BY2 genomic DNA (Fig. 3C). We had used DAPI staining to check the homogenized product prepared by the conventional method and found a lot of nuclei had not been broken at all so that the recovery ratio of genomic DNA was very low (Fig. 3A and B). Freeze drying the cell pellet before grinding it in mortars. The resulted stuff was much easier to be ground to powder (Fojtova and Kovarik, 2000), and the breakage ratio of the nuclei was almost 100%, and approximately 0.5-0.6 mg BY2 genomic DNA could be recovered from 100ml 10-day liquid medium culture inoculated from a single callus (Tab. 1).

The Southern assay demonstrated that majority of transformed calluses harbored two copies of integrated transgenes within tobacco genome (Fig. 4). Furthermore, as

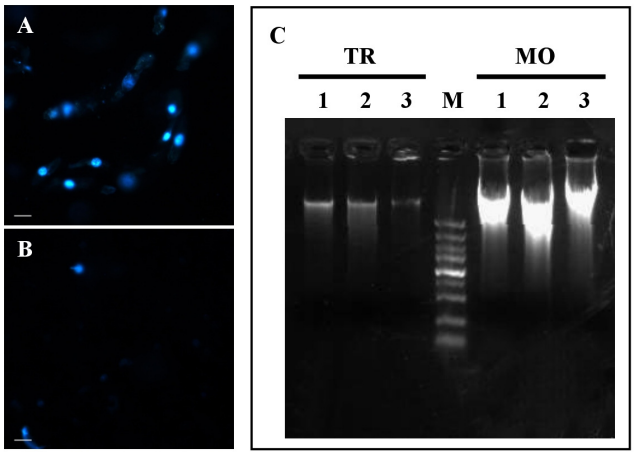

Fig. 3. Total DNA extraction by different methods
(A) and (B) DAPI stained nuclei of cells broken by traditional and modified methods. (C) Detection of total genomic DNA extraction by traditional and modified processing, line 1, 2, 3 are independent sample loaded with 5μl, TR mean traditional method, MO mean modified method. Bar= 10 μm.

for the transformed calluses harboring multiple copies of transgenes, we found the hybridization intensity of the target bands were always the same, as soon as the callus was transferred to a fresh selective plate no later than 3 weeks.

Transgene could be stably expressed at least for 20 days in liquid culture

The GFP fluorescence expression driven by CaMV *35S* promoter (*35S : GFP*) in transformed BY-2 cells, which came from genetically uniform callus, was displayed in Fig. 2B. In most of transformed BY2, GFP fluorescence could be detected for at least 3 cycles of -liquid medium subculture, approximately 20 days (Fig. 5A). According to our observation of cell proliferation rate in liquid medium, approximately 16 generations of cell proliferation were committed during such duration. Notably, we found from the 10th day to the 20th day after inoculation in liquid culture, little difference could be detected in the ratio of GFP fluorescence of the culture under selective pressure compared with those dismissed from selective pressure, i.e. with no antibiotics added (Fig. 5A). The result of semi-quantitative RT-PCR also verified that in later subculture, after 10 days of inoculation in liquid culture, until as late as the 20th days, there was no significant difference between the cultures under selective pressure and non-selective (Fig. 5B).

Tab 1. Comparation between traditional and modified processing of Total DNA extraction

Group	Fresh-weight of sample (g)	Total DNA (μg)	Extraction efficience (μg/g)	Mean (μg/g)
Traditional method	40.51	5.83	0.14	0.14
	47.23	5.19	0.11	
	39.16	2.74	0.07	
	43.52	9.13	0.21	
	44.24	7.52	0.17	
Modified method	42.17	304.81	7.23	6.9
	45.55	310.25	6.81	
	46.05	320.86	6.97	
	41.18	261.66	6.35	
	43.27	308.77	7.14	

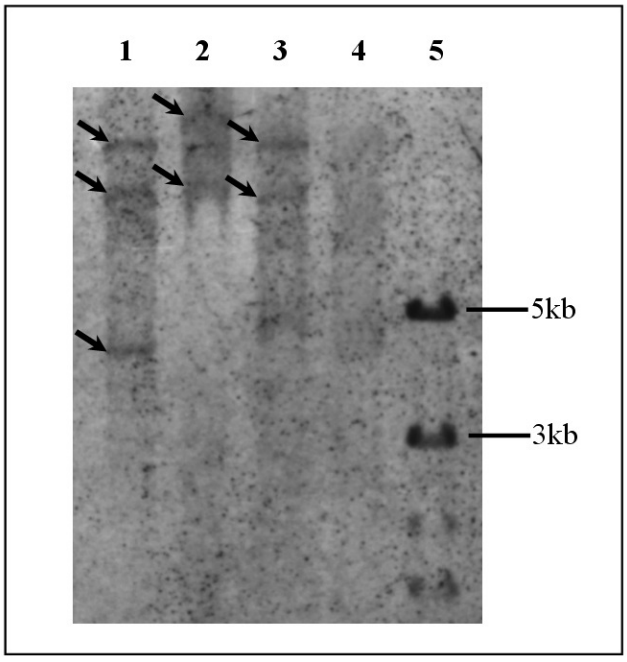

Fig. 4. Estimation of transgene copy number by Southern hybridization
Lane 1 to 3, independent GFP transgenic calluses. Lane 4, non-transformed BY2 control. Lane 5, molecule weight marker. Arrows indicate the integrated transgenes containing GFP sequence were probed

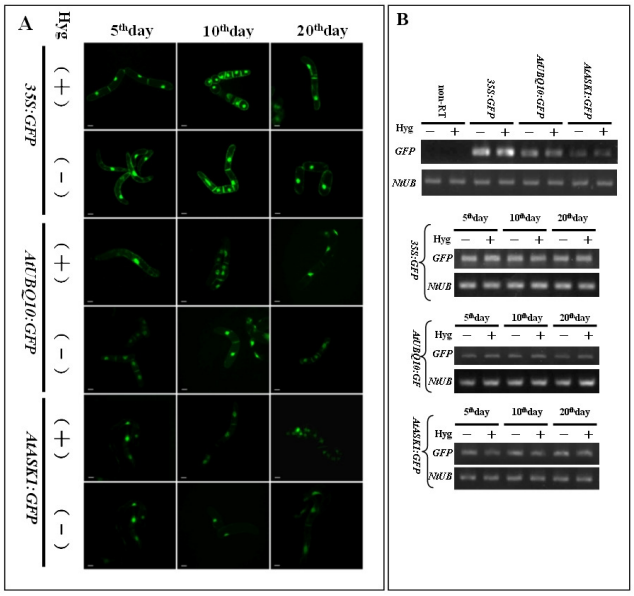

Fig. 5. *GFP* expression in different transgenic BY2 cells

(A) Detection of GFP expression under fluorescence microscope. GFP expression driven by *35S*, *AtUBQ10* and *AtASK1* shown in the BY2 cell cultivated in liquid medium with or without hygromycin (labeled with (+) and (-), respectively) for 5, 10 and 20 days. Bar=10 μm.

(B) Semi-quantitative RT-PCR to quantify GFP expression in the *35S:GFP, AtUBQ10:GFP* and *AtASK1:GFP* transformed cells, respectively. Tobacco *UBIQUITIN 1* (*NtUBQ1*) was used as the internal control for total cDNA amount normalization. *No promoter*: *GFP* was used as the non-RT control. Three independent replicates performed and one representative result illustrated. At least 15 independent calluses all with two copies of transgene integrated for each type transgenic BY2 were used to perform the experiment

Meanwhile *GFP* genes, whose expression were driven by *AtUBQ10 (AtUBQ10:GFP)* and *AtASK1 (AtASK1:GFP)* promoters were introduced into BY2 in parallel with *35S:GFP* construct. Representative features of the cells transformed with *AtUBQ10:GFP*, *AtASK1:GFP* and *35S:GFP*, respectively, are illustrated in Fig. 5. In the prerequisite of harboring the same copy number (here was two) of transgene, the *AtUBQ10* promoting strength was estimated about 50% of CaMV *35S* promoter, and *AtASK1* promoting strength was about 20-30% of *35S* promoter according to fluorescence strength (Fig. 5A), which was further verified by semi-quantitative RT-PCR (Fig. 5B). Thus, it suggested that CaMV *35S*, *AtUBQ10*and *AtASK1* can drive GFP to be expressed *in vivo* no matter when BY2 is at proliferative stage (exponential stage) or stationary growth stage with less mitosis. The BY2 cell suspension is a good platform to test the potentials of constitutive promoters in plant.

Discussion

In the traditional protocol, the final aliquot requires 2 ml liquid medium to disperse the cell pellet transformed by *Agrobacterium* (David and Perrot-Rechenmann, 2001). However, we found the excess liquid medium which was covering the transformed cell during incubation, had impeded severely aerobic respiration of the cells. Meanwhile, the excess liquid medium enforced mobility of the cell such that it was more difficult for the cell to anchor the solid medium and initiate proliferation. According to our experience, almost all aliquots failed to initiate growth with over 0.5 ml liquid medium during the longtime incubation. So an extra centrifuge step was added in the final aliquot step to discard excess liquid medium, i.e. at most 0.5 ml, rather than original 2 ml liquid medium was left for dispersing the cell pellet.

Genetically uniform calluses are ideal for testing the effects of growth factors or stress substances on cell growth. However, according to the traditional protocol antibiotics were kept in the transformed cell suspension during all subsequent subcultures, and the strong adverse effects of antibiotics made the cell grow unhealthy and influenced precise evaluation of effects of growth factors or stress substances. To get rid of such problem, we set up samples of antibiotics-added and no-antibiotics-added. We found in next round of liquid subculture (10 to 20 days after inoculation) no significant difference of transgene abundance could be detected between antibitocis-added and non-antibiotics-added samples (Fig. 5B). That means antibiotics can be dismissed for subculture of transformed callus, only if the initial callus has been well segregated and definitely genotyped. However, caution should be taken to extend our observance to other cases of transgenes, because up to date we have not found any cases that transgenes affect the callus grow significantly compared with non-transformed callus, i.e. even trace of non-transformed cell proliferates, it does not constitute severe problem of genetic impurity during subculture.

Meanwhile we found that the transgenes are stably expressed for at least 20 days in liquid subculture. If it was a little obscure that GFP fluorescence was viewed for evaluation of the expression level of transgene, the semi-quantitative PCR unambiguously exhibited that transgenes are stably expressed. According to the intensity of the fluorescence, the relative potentials of promoters could be estimated and quantified, with assistance of semi-quantitative RT-PCR, in prerequisite of harboring the same copy number of the transgene that was demonstrated by the optimized Southern blot. This advantage would be especially useful to exploit more constitutive promoters in plant, and study *cis*-elements of important signaling pathways. Thus we believed that our optimized protocol has provided a uniform and highly replicable experimental cellular platform to investigate many aspects of plant cell biology.

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