Pak. J. Bot., 49(6): 2227-2233, 2017.

ESTABLISHMENT OF RAPESEED (*BRASSICA NAPUS* L.) COTYLEDON TRANSIENT TRANSFORMATION SYSTEM FOR GENE FUNCTION ANALYSIS

ZHI-YAN ZHANG[1, 2], GUAN-YING LI[1, 2], JIE-LI WANG[1], XIAO-JUAN GUO[1], ZHENG WANG[1] AND XIAO-LI TAN[1 *]

[1]*Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, Jiangsu, People's Republic of China*
[*]*Corresponding author's email: xltan@ujs.edu.cn*
[2]*These authors contributed equally to this work.*

Abstract

Rapeseed *(Brassica napus)* is second leading oil crop worldwide, due to its importance, genomes of its relative *Brassica rapa* (AA genome), *Brassica oleracea* (CC genome), and itself AACC have been sequenced successively. Functional genomic study of rapeseed is urgently needed to elucidate the molecular mechanisms of its yield, quality, and stress resistance. Rapeseed is recalcitrant to genetic transformation compared with other dicots. Therefore, we seek to develop a simple and efficient transient transformation system in rapeseed cotyledons. The beta-glucuronidase (GUS) reporter gene was employed to evaluate the transient expression levels. *Agrobacterium* strains, densities of agrobacteria cell suspension, and the infiltrated time were optimized. To verify the reliability of this system，subcellular localization and bimolecular fluorescence complementation (BiFC) were used. We found that the *A. tumefaciens* strain GV3101 was the suitable strain for transformation of the rapeseed cotyledons. Among the different incubation times, four days after infiltration with *Agrobacterium* GV3101 cells (OD_{600}=0.8) provided the highest frequency of transformation. Subcellular localization and BiFC assays demonstrated a highly efficient transient transformation system in rapeseed cotyledons. The cotyledon transit expression system is efficient and fast and could accelerate functional genomics studies in *B. napus*.

Key words: Rapeseed; Cotyledon; Transient expression; *Agrobacterium*; GUS staining.

Introduction

Rapeseed (*Brassica napus)* is an agriculturally important crop worldwide. It also serves as raw material for a broad range of industrial products, including lubricants, paints, and bio-fuels. Its global production is second only to soybean with ~70.35 million metric tons of oilseed and over 28.12 million metric tons of oil produced in 2016–2017 (https://apps.fas.usda.gov/psdonline/). Because rapeseed oil is a vital edible oil crop, its relative genomes *B. rapa* (AA genome) and *B. oleracea* (CC genome) as well as itself genome (AACC) were sequenced (Chalhoub *et al*., 2014; Liu *et al*., 2014; Wang *et al*., 2011). Thus, functional genomics of *B. napus* will be the research focus soon after.

Transient transformation techniques are powerful tools to study *in vivo* gene functions, such as protein subcellular localization, protein-protein interaction, promoter activity and enzyme activities (Chen *et al*., 2006; Marion *et al*., 2008; Yang *et al*., 2000). However, rapeseed (*B. napus*) is considered relatively recalcitrant to genetic transformation compared with other dicots, such as *Arabidopsis* (Boszoradova & Libantova, 2011).

To date, some transient expression methods have been reported in *B. napus* via protoplast transfection, biolistic bombardment, and *Agrobacterium tumefaciens* co-cultivation (Abdollahi *et al*., 2007; Bhalla & Singh, 2008; Thomzik, 1995). However, these methods have some drawbacks. For example, protoplast transfection is limited due to complicated protoplast preparation procedures, the transformation efficiency is relatively low for biolistic bombardment, which requires expensive equipment and supplies, and the co-cultivation of seedlings with *Agrobacterium* could lead to the necrosis of plant tissues, which would cause low transformation efficiencies (Abdollahi *et al*., 2007; Dan, 2008; Yoo *et al*., 2007). Therefore, there is still a serious need for a quick, efficient, and economical transient transformation system for *B. napus*.

Several factors seriously influence *Agrobacterium*-mediated transient gene expression and stable genetic transformation. LBA4404 is the most effective *Agrobacterium* strain (followed by EHA105 and C58C1) for transient expression in mung bean after 3 days of co-cultivation (Jaiwal *et al*., 2001). For switch grass, the combination of multiple wounding treatments along with the addition of thiol compounds during co-cultivation increased transient expression levels from 6% to 54% (Chen *et al*., 2010). Additionally, the treatment of immature embryos with centrifugation and heat improved the frequency of transformations in rice and maize. Thus, the optimization of experimental conditions is very important for developing an efficient transient transformation system. Transit expression systems established in tobacco, Arabidopsis were widely applied in gene function analysis. Gene function analysis of rapeseed may work in the tobacco, Arabidopsis transit expression system. However, with itself transit expression system to interpret rapeseed genes is superior to heterologous transit expression system. Therefore, there has been an increasing need for the development of a valid and effective transient transformation system in *B. napus.* In this study, we developed a highly efficient and convenient *Agrobacterium*-mediated transient transformation system for *B. napus*. This system minimizes hands-on manipulations and requires no specialized equipment. After investigating several factors that seriously influence transformation efficiency, including *Agrobacterium* strains, bacterial concentrations of *Agrobacterium*, and incubation time, we obtained the optimal conditions for this system. Furthermore, to verify the reliability of this system, we detected the subcellular localization of three *B. napus* proteins in cotyledon cells with our system. The *in vivo* interaction of pathogenesis-related protein and a secreted lipase of *Fusarium graminearum* (FGL) were examined via

bimolecular fluorescence complementation (BiFC). The results demonstrated the high reliability of this system. The transient transformation system developed here provides a high-throughput, efficient, and economical approach to functional genomic researches in *B. napus* and the genus *Brassica*.

Materials and Methods

Plant materials and growth conditions: A rapeseed (*B. napus*) cultivar Zhongshuang9 was used in this study. Plants were grown in a plant growth room. The growth conditions were 20 ± 2°C under 60–90% relative humidity and a photoperiod of 16 h and 8 h at a light intensity of 44 μmol m^{-2} s^{-1} (Wang *et al*., 2012). Seedlings were grown for 4–6 days prior to infiltration. Robust seedlings were used for subsequent procedures.

***A. tumefaciens* strains, plasmids, and bacterial culture:** The plasmids used in this study are listed in Table 1. *A. tumefaciens* cells of strain GV3101 transformed with the various constructs were grown overnight in 5 ml LB liquid medium with the appropriate antibiotics, and used to inoculate a 50 ml culture at 28°C. Two other *A. tumefaciens* strains were tested in this study: (i) LBA4404 and (ii) EHA105. After growing overnight to a stable phase at 28°C, *A. tumefaciens* cells were collected and washed twice using MMA solution (10 mM MES (2-[N-morpholino] ethanesulfonic acid), pH 5.6, 100 μM acetosyringone, 10 mM $MgCl_2$). The *Agrobacterium* was resuspended at the appropriate OD_{600} in 5 ml of MMA, then incubated for 2–4 h at 28°C.

Plant infiltration: Infiltration medium in a needleless syringe was forced into an abaxial epidermis of full-expanded rapeseed cotyledons, which were transferred to a culture room for 4 days. The rapeseed cotyledons after infiltration were kept humid (Fig. 1). Four days after transformation, GUS staining and fluorescence were monitored using a confocal microscope.

GUS assay and imaging: Histochemical GUS staining was performed as previously described (Jefferson *et al*., 1987). The rapeseed cotyledons were thoroughly washed with ddH_2O and immersed in a GUS staining solution, consisting of 10 mM Na_2EDTA, 0.1% (v/v) Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 50 mM phosphate buffer (pH7.0), and 0.6 mg/ml 5-bromo-4-chloro-3-indolyl *β*-D-glucuronide, and then incubated overnight at 37°C. Stained cotyledons were dipped in 70% (v/v) ethanol to remove the chlorophyll, GUS staining was carefully visualized, and images were captured with a camera. The GUS activity was measured according to fluorometric assay using 4-methylumbelliferyl-β -D-glucuronide (Sigma-Aldrich, St Louis, MO, USA) as the GUS substrate (Delaney *et al*., 2007).

Fluorescence was monitored by an inverted Leica TCS-SP5 spectral confocal laser scanning microscope. eGFP and DsRed samples were excited with 488 , 543, or 633 nm argon laser lines, with an emission band of 510–540 nm for eGFP detection, 560–590 nm for DsRed detection, and 675–765 nm for chloroplastid autofluorescence, respectively.

Results

Selection of reporter gene and expression vector: Histochemical GUS staining was widely used in evaluating the transformation efficiency of the transient Agrobacterium-mediated transformation system in plants in previous studies (Li *et al*., 1992; Weaver *et al*., 2014). We first evaluated if the rapeseed cotyledon was stained by GUS, the result showed as Fig. 2a that no background GUS activity in the *B. napus* cotyledons which suggests that GUS staining is a feasible with cotyledon. To select an optimal expression vector, we examined two binary vectors pBI121 and pCAMBIA2301, where GUS expression is driven by the 35S promoter (Hajdukiewicz *et al*., 1994). The plasmid containing a suppressor of gene-silencing gene p19, was co-transformed in our transient expression system (Voinnet *et al*., 2003). The GUS activity of *Agrobacterium* carrying vector pBI121, pCAMBIA2301 and p19 was investigated for its use in this study. Our results showed that no GUS activity could be observed in the *Agrobacterium* carrying pCAMBIA2301and p19, while *Agrobacterium* carrying pBI121 had the GUS activity (Fig. 2b). Therefore, we chose the pCAMBIA2301 and p19 combination to further transform the tobacco leaves to test feasibility of the vectors. As shown in Fig. 2c, the GUS activity was observed after transit transformation with the pCAMBIA2301 and p19 combination. In pBI121vector, the GUS gene without intron inside could express in *Agrobacterium*, and in pCAMBIA2301, the GUS gene contains an intron which led to failure expression in prokaryotic *Agrobacterium* (Fig. 2b). This demonstrated that pCAMBIA2301 was the choice for transit transformation to rapeseed cotyledon.

Table 1. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference or source
pCAMBIA2301	pCAMBIA2301carrying the *gus* and *nptII* gene	CAMBIA, Australia
pBI121	pBI121 carrying the *gus* gene	Huang *et al*., 2012
p35S-p19	p19 carrying the RNA silencing suppressor gene	Voinnet *et al*., 2003
p35S-BnLACS4-EGFP	BnLACS4 localize in endoplasmic reticulum	Tan *et al*., 2014
p35S-BnACS6-EGFP	BnACS6 localize in chloroplast	Laboratory collection
p35S-BnMKS1-EGFP	BnMKS1 localize in nucleus	Wang *et al*., 2014
p35S-DsRed-IND	IND localize in nucleus	Tan *et al*., 2009
p35S-DsRed-CTR3	CTR3 localize in endoplasmic reticulum	Laboratory collection
pFGL-Neyfp	FGL localize in cytoplasm	Niu *et al*., 2013
pPR-cEYFP	PR localize in cytoplasm	Dr. Xin-Yu Wang providing

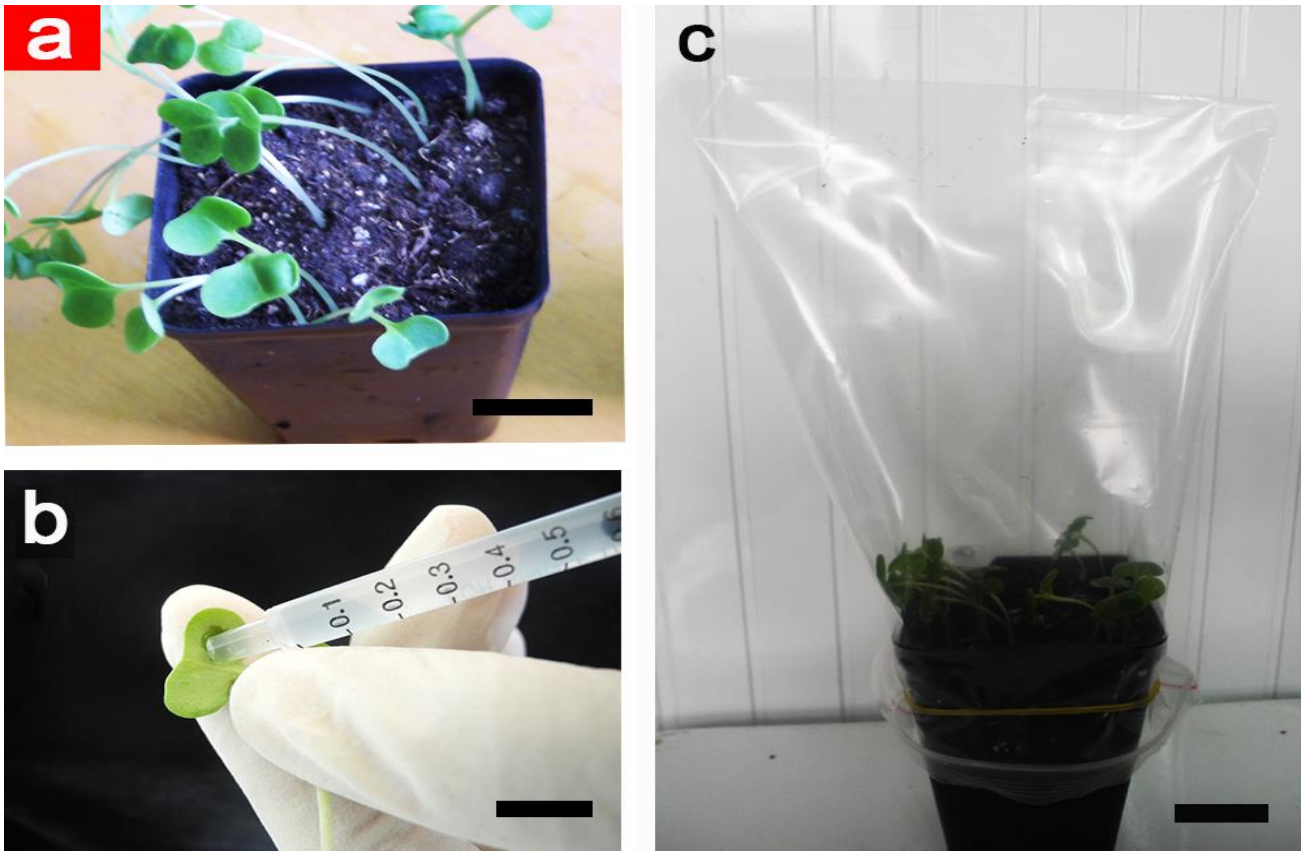

Fig. 1. The process of *Agrobacterium* infiltration into rapeseed (*Brassica napus* L.) cotyledons. (a) The four-day-old rape seedlings after sowing (Scale bar = 1.0 cm). (b) Infiltration into rapeseed cotyledons (Scale bar = 1.5 cm). (c) Moisturizing after injection (Scale bar = 2.0 cm).

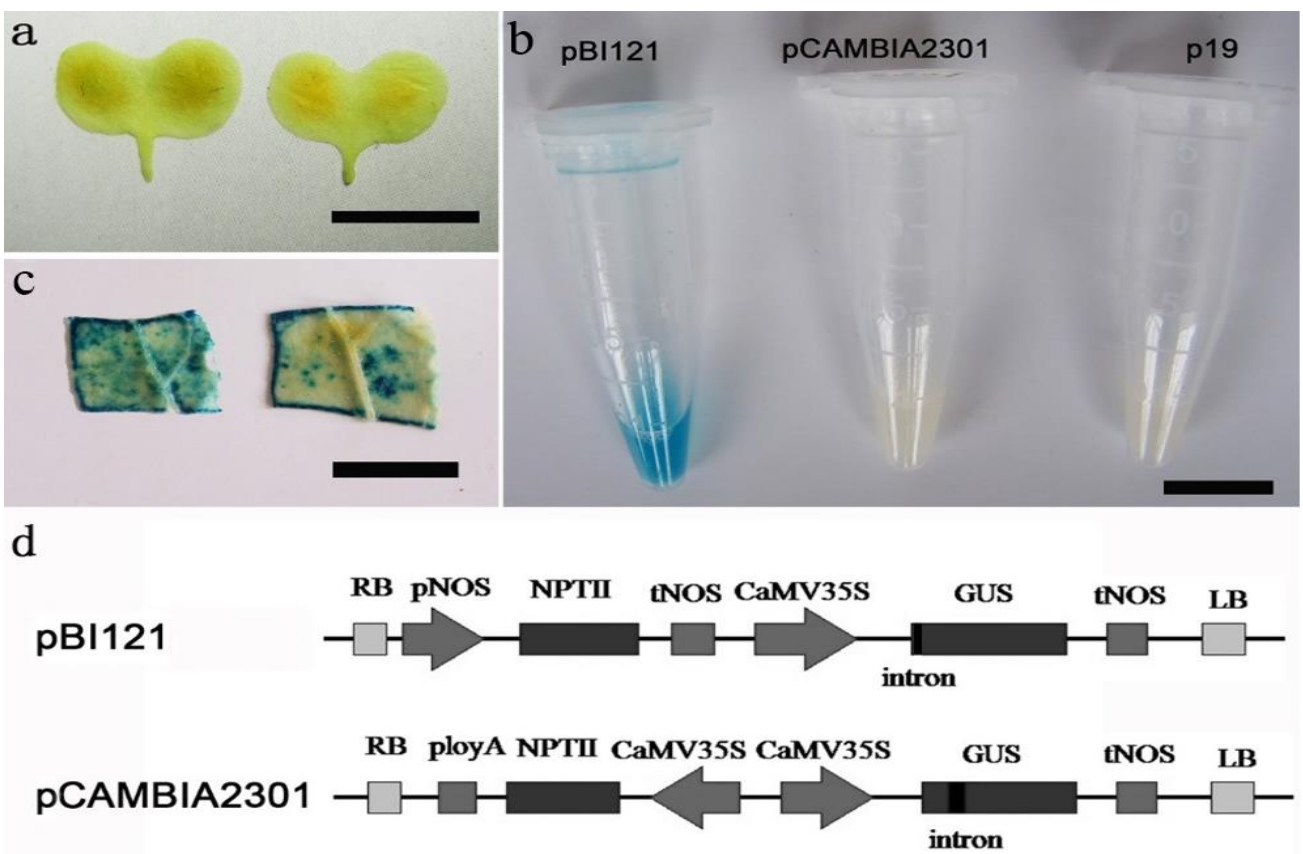

Fig. 2. Selection of reporter gene and expression vector by detecting GUS activity. (a) GUS stainingin rapeseed cotyledons without *Agrobacterium* infiltration. (b) GUS expression in *A. tumefaciens* containing different plasmids. (c) GUS expression in tobacco leaves infiltrated with *A. tumefaciens* containing the pCAMBIA2301 plasmid. (d) Schematic of pBI121 and pCAMBIA2301 plasmids. Scale bar = 1 cm.

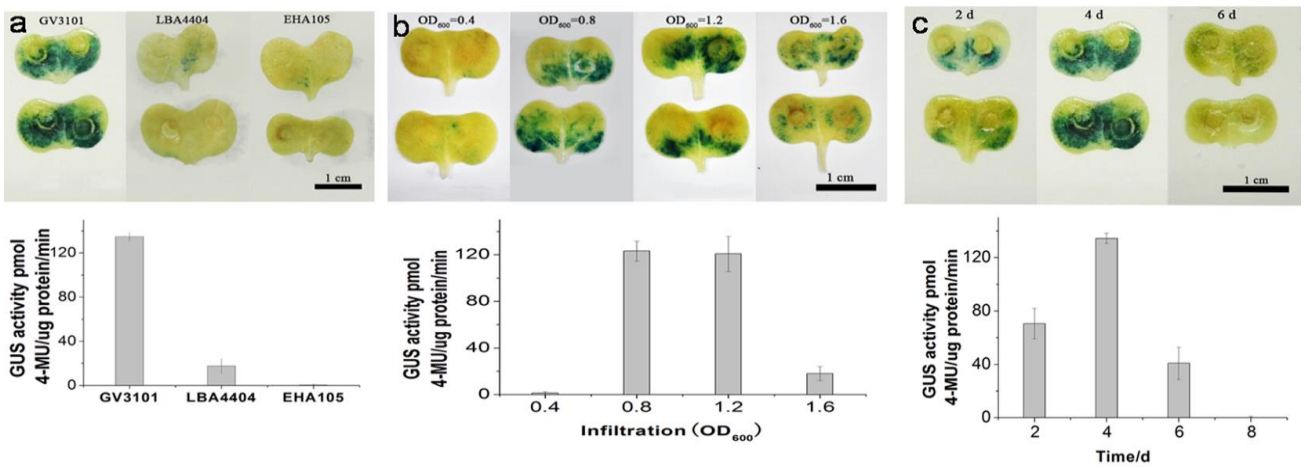

Fig. 3. Transient expression of GUS in rapeseed cotyledons containing the pCAMBIA2301 plasmid. (a) Effect of *Agrobacterium* strains on transient transformation. GUS histochemical and activity analyses in rapeseed cotyledons containing *A. tumefaciens* GV3101, LBA4404, and EHA105. (b) Effect of the bacterial density of the infiltration medium on transient transformation. An overnight culture of *Agrobacterium* GV3101 was re-suspended in the infiltration medium. The final OD_{600} was adjusted to 0.4, 0.8, 1.2, and 1.6. (c) Effect of co-culture time on transient transformation. *B. napus* seedlings (four-day-old) were co-cultivated with GV3101 (OD_{600} = 0.8) by incubation in a culture room for 2, 4, or 6 days. Scale bar = 1 cm. Values are means, with standard errors indicated by bars.

Optimization of the Agrobacterium-mediated transient system

Effect of Agrobacterium strains on transient transformation: To study the ability of different *Agrobacterium* strains to deliver T-DNA to host plants, three frequently-used *Agrobacterium* strains (GV3101, LBA4404, and EHA105) were investigated in four-day-old *B. napus* seedlings. All seedlings were treated with one of the three *Agrobacterium* strains and then transferred to a culture room for 4 days. After cultivation, we assessed the GUS activity in each case. These three strains all harbored the binary vector pCAMBIA2301.

The GUS activity was significantly higher in cotyledon cells after inoculation with the *Agrobacterium* strain GV3101 than in those inoculated with the other two *Agrobacterium* strains, LBA4404 and EHA105 (Fig. 3a), which indicates that GV3101 has the highest transformation efficiency among the three *Agrobacterium* strains. Strains LBA4404 and EHA105 were less efficient in transferring T-DNA to *B. napus* seedlings. Particularly, the GUS activity in cotyledons inoculated with strain EHA105 was close to 0 (Fig. 3a). These results demonstrated that the *A. tumefaciens* strain GV3101 was more applicable than strains LBA4404 and EHA105 to our transient transformation system. Therefore, we employed strain GV3101 to optimize other parameters in subsequent assays.

Effect of bacterial density on transient transformation: Previous studies showed that the *Agrobacterium* concentration could significantly impact plant transformation efficiency (Dai *et al*., 2003; Marion *et al*., 2008; Takata & Eriksson, 2012). To optimize the transformation efficiency in our transient expression system, we examined the bacterial densities of the infiltration medium using bacteria at the stationary phase of growth. Final OD_{600} values of 0.4, 0.8, 1.2, and 1.6 in the infiltration medium were chosen to infiltrate rapeseed cotyledons. The highest transformation efficiency was obtained with a bacterial density of OD_{600} = 0.8 (Fig. 3b). After infiltration for 4 days, the rapeseed cotyledons treated by *Agrobacterium* suspension with OD_{600} = 0.8 displayed the strongest GUS activity (Fig. 3b). When using the lower (OD_{600} = 0.4) and higher (OD_{600} = 1.6) bacterial densities, the GUS activities were very low. These experiments indicated that an optimal *Agrobacterium* density was necessary for a high transformation efficiency in our system, which emphasized the importance of a suitable *Agrobacterium* concentration. Therefore, the concentration of the *Agrobacterium* suspension was set to an OD_{600} value 0.8 in this transient transformation system.

Effect of co-culture time on transient transformation: Finally, we investigated the influence of the incubation time on the transient transformation efficiency (Fig. 3c). We observed the differences among the rapeseed cotyledons harvested at 2, 4, or 6 days after infiltration. As shown in Fig. 3c, the most intense GUS activity occurred in the samples harvested at 4 days after infiltration, which was almost two- or three-folds higher than samples harvested at 2 or 6 days, respectively. Interestingly, the GUS activity of samples harvested at 8 days was close to 0. These results suggest that the incubation time plays an important role in the efficiency of our transient transformation system, and the suitable incubation time is 4 days after infiltration.

Subcellular localization assay: A major purpose of the transient transformation analysis is to determine the subcellular localization of proteins in cells (Takata & Eriksson., 2012). Although several studies have applied transient transformations to monitor the subcellular localization of rapeseed proteins in tobacco or others heterologous plants systems (Tan *et al*., 2011; Tan *et al*., 2009; Yang *et al*., 2009), few localization studies have been tested in homologous rapeseed. Thus, we examined the subcellular localization of rapeseed proteins in a homologous plant system, and then assessed whether our protocol for transient expression could be applied to rapeseed cotyledons cells.

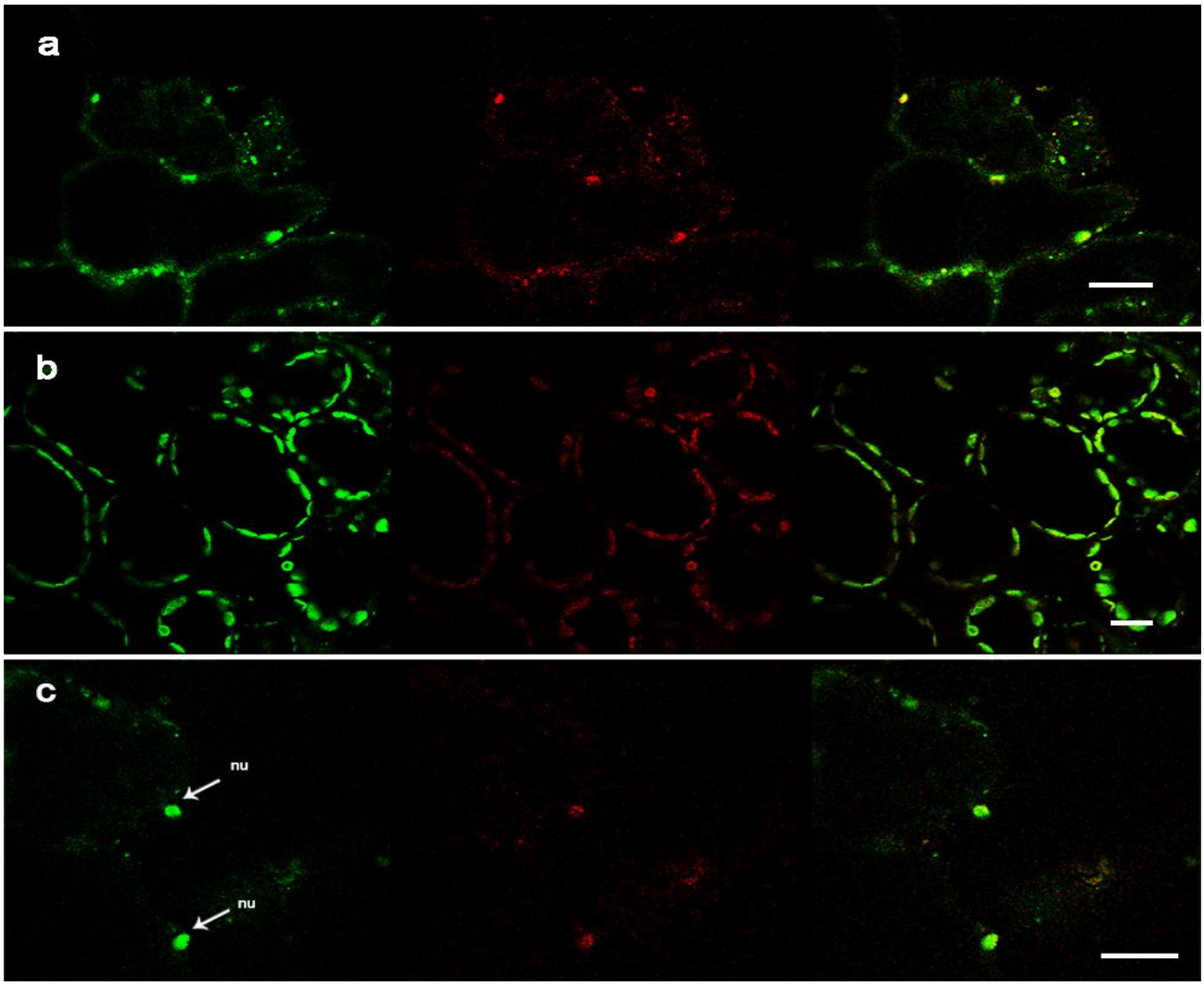

Fig. 4. Subcellular distribution in rapeseed cotyledons. The targeted proteins fused with GFP or RFP were co-expressed in rapeseed cotyledon epidermal cells. (a) The cell nucleus BnMKS1–GFP marker (left), the cell nucleus DsRed-Ind marker (middle), and the merged image (right). Scale bar = 10 μm. (b) BnLACS4–EGFP (left), the ER DsRed-CTR3 marker (middle), and the merged image (right). Scale bar = 10 μm. (c) BnACS6–EGFP (left), chloroplastid autofluorescence (middle), and merged image (right). Scale bar = 20 μm.

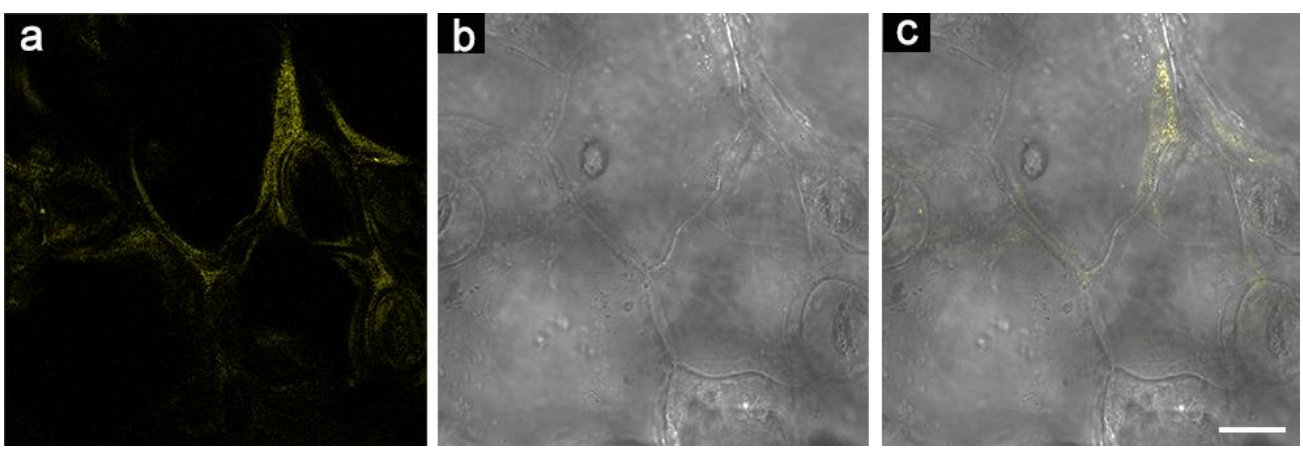

Fig. 5. Protein–protein interactions monitored by BiFC in rapeseed cotyledon epidermal cells using the fluorescence of YFP. (a) Bright field images. (b) Merged image. (c) Image after superimposing. Scale bar = 10 μm.

The transient transformation was monitored using the binary vector pK7FWG2.0 carrying a 35S:: Gene-GFP cassette and the binary vector pCX-DR containing a 35S::DsRed-Marker cassette. The results of subcellular localization are shown in Fig. 4, which were consistent with those of our previous study conducted in *N. tabacum*. BnMKS1, BnLACS4, and BnACS6 were located in the nucleus (Wang *et al*., 2014), endoplasmic reticulum (Tan *et al*., 2014), and chloroplast (data not known), respectively. IND as a marker localized in the nucleus (Tan *et al*., 2009),

while CRT3 is an endoplasmic reticulum marker (Li *et al*., 2009). By co-transforming rapeseed cotyledons cells with vectors 35S-BnMKS1-EGFP and 35S-DsRed-IND, or 35S-BnLACS4-EGFP and 35S-DsRed-CRT3, we found that the green and red fluorescence in cotyledons cells corresponded well. Similarly, the fluorescence from BnACS6 was in accordance with the exciting light of chloroplasts.

BiFC assay: To further confirm the feasibility of our transient transformation system, the BiFC test of FGL and PR were used in this system (Niu *et al*., 2013). The interactions of these two proteins have been demonstrated previously by the yeast-two hybrid technique and BiFC tests in *N. tabacum*. As shown in Fig. 5, the fluorescence signal could be observed in the cytoplasm of cotyledons cells, indicating the interaction between FGL and PR. Therefore, the results indicated the applicability and high efficiency of this transient transformation system in *B. napus* cotyledons cells.

Discussion

Fast and effective transient transformation systems play important roles in plant functional genomic research. Many factors impact the feasibility and efficiency of transient transformation systems. For instance, high proportion of early-stage cell walls, and/ or the stage of the cell cycle affected transformation efficiency (Kirienko & Sylvester, 2012).

Three factors, *Agrobacterium* strains, concentration of *A. tumefaciens* suspension, and incubation time, were investigated using the GUS histochemical assay in this study to optimize the applicability and efficiency of our transient transformation system. The strain GV3101 was the best strain for carrying the genes of interest in our transformation system. The invasiveness of different *Agrobacterium* strains was closely related to target plant species. For instance, LBA4404 is suitable for mungbean (Jaiwal *et al*., 2001), while C58C1 is a better choice for *Arabidopsis*(Marion *et al*., 2008). Different *Agrobacterium* strains are defined by the presence of resident Ti plasmids and chromosomal backgrounds (Kim *et al*., 2009). Previous studies indicated that an *Agrobacterium* strain could be more virulent to several host plants than other strains, which could be explained in several ways, such as the different abilities of various bacterial species to adhere to host plant cells, and the variations in either bacterial-encoded or plant-encoded T-DNA transfer mechanisms (Nam *et al*., 1997). With respect to the concentration of *A. tumefaciens* suspension, the OD_{600} value of 0.8 was found to be the best choice. Additionally, the different incubation times have various effects on the efficiency of this system. The GUS activity reached a peak value in the rapeseed cotyledons harvested at 4 days after infiltration. This might be closely related with the stability of the GUS protein. By optimizing these three factors, we have established a valid transient transformation system for *B. napus* with an increased efficiency.

In this study, the feasibility and efficiency of our protocol were further validated by a subcellular localization investigation of three *B. napus* genes (*BnMKS1*, *BnLACS4*, and *BnACS6*) and the BiFC test of FGL and PR proteins. The results of these two tests were in accordance with previous reports in *N. tabacum* and yeast (Tan *et al*., 2014; Wang *et al*., 2014), which suggests that this transient transformation system in *B. napus* cotyledon cells can serve as a convenient and effective tool for gene studies and protein studies in *B. napus* and the genus *Brassica*.

In the cotyledon stage, the triacylglycerol in seeds is degraded into sucrose with energy released to support the seedling establishment, and in this process, the genes involved in lipid degradation are activated. Therefore, the cotyledon transit expression system is suitable for dissecting the gene functions related to lipid degradation, fatty acid beta oxidation and gluconeogenesis. On the other side, lipid biosynthesis was also approved in cotyledon stage (Hernández *et al.*, 2012), for this reason, the genes related to fatty acids and lipid biosynthesis were proper to study their functions in this system. In brief, the genes involved in the lipid and fatty acid metabolism could be placed into cotyledon to analyze their functions, which are crucial to rapeseed.

Agroinfiltration, an infiltration process of *Agrobacterium* suspensions into plant organs, serves as a fast and highly efficient method for the transient expression of desired gene(s). This study offers the advantages of a rapid procedure (only eight days for an experimental period), less labor, high efficiency, and no need for specialized equipment. Compared with the transient transformation system applied in *N. tabacum*, which generally needs one month for an experimental period, the efficiency of this system has been significantly increased. Furthermore, the features above will contribute to the use of this method as an analytical tool for studying gene and protein functions in *B. napus* and the genus *Brassica*.

In conclusion，we established a fast and efficient Agrobacterium tumefaciens-mediated transient expression system for rapeseed cotyledons. The feasibility and efficiency of this system were proved by subcellular localization and bimolecular fluorescence complementation assays. The developed method will accelerate functional genomics studies in *B. napus*.

Acknowledgment

The authors acknowledge the National Natural Science Foundation of China (31471527，31271760), the Senior Talent Funds of Jiangsu University (05JDG030), and the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions. We thank Dr. Xin-Yu Wang (Nanjing Agricultural University) for providing the FGL-nEYFP and PR-cEYFP plasmids, and Dr. Xin Deng (Nankai University TEDA College) for revising the manuscript.

References

Abdollahi, M.R., A. Moieni, A. Mousavi, A.H. Salmanian, J.M. Jalali and M. Majdi. 2007. Effect of integrated bombardment and *Agrobacterium* transformation system on transient GUS expression in hypocotyls of rapeseed (*Brassica napus* L. cv. PF704) microspore-derived embryos. *Pak. J. Biol. Sci.*, 10: 3141-3145.

Bhalla, P.L. and M.B. Singh. 2008. *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. *Nat. Protoc.*, 3: 181-189.

Boszoradova, E. and J. Libantova. 2011. *Agrobacterium*-mediated genetic transformation of economically important oilseed rape cultivars. *Plant CellTiss. Org.*, 107: 317-323.

Chalhoub, B., F. Denoeud, S. Liu, I.A.P. Parkin, H. Tang, X. Wang, J. Chiquet, H. Belcram, C. Tong, and B. Samans. 2014. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. *Science*, 345: 950-953.

Chen, S., L. Tao, L. Zeng, M.E. Vegasanchez, K. Umemura, and G.L. Wang. 2006. A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. *Mol. Plant Pathol.*, 7: 417-427.

Chen, X., R. Equi, H. Baxter, K. Berk, H. Jin, S. Agarwal and J. Zale. 2010. A high-throughput transient gene expression system for switchgrass (*Panicum virgatum* L.) seedlings. *Biotechnol. Biofuels*, 3: 1-10.

Dai, W., Z.M. Cheng and W. Sargent. 2003. Plant regeneration and *Agrobacterium*-mediated transformation of two elite aspen hybrid clones from in vitro leaf tissues. *In Vitro Cell. Dev. - Pl.*, 39: 6-11.

Dan, Y. 2008. Biological functions of antioxidants in plant transformation. *In Vitro Cell. Dev. - Pl.*, 44: 149-161.

Delaney, S.K., S.J. Orford, M. Martin-Harris and J.N. Timmis. 2007. The Fiber Specificity of the Cotton FSltp4 Gene Promoter is Regulated by an AT-Rich Promoter Region and the AT-Hook Transcription Factor GhAT1. *Plant Cell Physiol.*, 48: 1426-1437.

Hajdukiewicz, P., Z. Svab and P. Maliga. 1994. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. *Plant Mol. Biol.*, 25(6): 989-994.

Hernández, M.L., L. Whitehead, Z. He, V. Gazda, A. Gilday, E. Kozhevnikova, F.E. Vaistij, T.R. Larson, and I.A. Graham. 2012. A cytosolic acyltransferase contributes to triacylglycerol synthesis in sucrose-rescued Arabidopsis seed oil catabolism mutants. *Plant Physiol.*, 160: 215-225.

Jaiwal, P.K., R. Kumari, S. Ignacimuthu, I. Potrykus and C. Sautter. 2001. *Agrobacteriumtumefaciens*-mediated genetic transformation of mungbean (*Vigna radiata* L. Wilczek) - a recalcitrant grain legume. *Plant Sci.*, 161: 239-247.

Jefferson, R.A., T.A. Kavanagh and M.W. Bevan. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.*, 6: 3901-3907.

Kim, M.J., K. Baek and C.M. Park, 2009. Optimization of conditions for transient *Agrobacterium*-mediated gene expression assays in *Arabidopsis*. *Plant Cell Rep.*, 28: 1159-1167.

Kirienko, D.R. and A.W. Sylvester. 2012. Reliable transient transformation of intact maize leaf cells for functional genomics and experimental study. *Plant Physiol.*, 159: 1309-1318.

Li, J., Z.H. Chu., M. Batoux, V. Nekrasov, M. Roux, D. Chinchilla, C. Zipfel and J.D. Jones. 2009. Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR. *P.Natl. Acad. Sci.*, 106: 15973-15978.

Li, X..Q., C.N. Liu, S.W. Ritchie, J.Y. Peng, S.B. Gelvin and T.K. Hodges. 1992. Factors influencing *Agrobacterium*-mediated transient expression of gusA in rice. *Plant Mol. Biol.*, 20: 1037-1048.

Liu, S., Y. Liu, X. Yang, C. Tong, D. Edwards, I.A.P. Parkin, M. Zhao, J. Ma, J. Yu, and S. Huang. 2014. The *Brassica oleracea* genome reveals the asymmetrical evolution of polyploidgenomes. *Nat. Commun.*, 5: 3930.

Marion, J., L. Bach, Y. Bellec, C. Meyer, L. Gissot and J.D. Faure. 2008. Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of *Arabidopsis* seedlings. *Plant J.*, 56: 169-179.

Nam, J., A.G. Matthysse and S.B. Gelvin. 1997. Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell*, 9: 317-333.

Niu, X.W., Z.Y. Zheng, Y.G. Feng, W.Z. Guo and X.Y. Wang. 2013. The *Fusarium Graminearum* virulence factor FGL targets an FKBP12 immunophilin of wheat. *Gene*, 525: 77-83.

Takata, N. and M.E. Eriksson. 2012. A simple and efficient transient transformation for hybrid aspen (*Populus tremula* × *P. tremuloides*).*Plant Methods*, 8: 1-10.

Tan, X., L. Zhang and Z. Xia. 2009. Molecular Cloning and Characterization of a Putative BnHEC3 Gene in Oilseed Rape (*Brassica napus*). *Int. J. Biol.*, 1: 71-77.

Tan, X., Q. Wang, Tian, B. H. Zhang, D. Lu and J. Zhou. 2011. A *Brassica napus* lipase locates at the membrane contact sites involved in chloroplast development. *Plos One*, 6: 552.

Tan, X.L., X.F. Zheng, Z.Y. Zhang, W. Zheng, H.C. Xia, C. M. Lu and S.L. Gu. 2014. Long chain acyl-coenzyme a Synthetase 4 (*BnLACS4*) gene from *Brassica napus* enhances the yeast lipid contents. *J. Integr. Agr.*, 13: 54-62.

Thomzik, J.E. 1995. *Agrobacterium* -mediated transformation of stem disks from oilseed rape (*Brassica napus* L.). *Methods Mol. Biol.*, 44: 79-85.

Voinnet, O., S. Rivas, P. Mestre, and D. Baulcombe. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.*, 33: 949-956.

Wang, X., H. Wang, J. Wang, R. Sun, J. Wu, S. Liu, Y. Bai, J.H. Mun, I. Bancroft. and F. Cheng. 2011. The genome of the mesopolyploid crop species *Brassica rapa*. *Nat. Genet.*, 43: 1035-1039.

Wang, Z., X. Tan, Z. Zhang, S. Gu, G. Li, and H. Shi. 2012. Defense to *Sclerotinia sclerotiorum* in oilseed rape is associated with the sequential activations of salicylic acid signaling and jasmonic acid signaling. *Plant Sci.*, 184: 75-82.

Wang, Z., H. Fang, Y.Chen, K. Chen, G. Li, S. Gu, and X. Tan. 2014. Over expression of BnWRKY33 in oilseed rape enhances resistance to *Sclerotinia sclerotiorum*. *Mol. Plant Pathol.*, 15: 677-689.

Weaver, J., S. Goklany, N. Rizvi, E.J. Cram and C.W.T. Lee-Parsons. 2014. Optimizing the transient fast *agro*-mediated seedling transformation (FAST) method in *Catharanthus roseus* seedlings. *Plant Cell Rep.*, 33: 89-97.

Yang, B., Y. Jiang, M.H. Rahman, M.K. Deyholos and N.N. Kav. 2009. Identification and expression analysis of WRKY transcription factor genes in canola (*Brassica napus* L.) in response to fungal pathogens and hormone treatments. *BMC Plant Biol.*, 9: 261-279.

Yang, Y., R. Li and M. Qi. 2000. In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. *Plant J.*, 22: 543-551.

Yoo, S.D., Y.H. Cho and J. Sheen. 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc*, 2: 1565-1572.

(Received for publication 24 November 2016)