Pak. J. Bot., 45(5): 1761-1766, 2013.

TRANSFER OF THE GAFP AND NPI, TWO DISEASE-RESISTANT GENES, INTO A *PHALAENOPSIS* BY *AGROBACTERIUM TUMEFACIENS*

JIE LI[1*], PING KUANG[1], REN-DAO LIU[1], DAN WANG[1], ZHI-NA WANG[1] AND MIN-REN HUANG[2]

[1]*College of Life Science and Engineering, Southwest University of Science and Technology, Mianyang 621010, China*
[2]*Laboratory of Forest Genetics and Gene Engineering, Nanjing Forestry University, Nanjing 210037, China*
[*]*Corresponding author's: e-mail: jay0224@sina.com*

Abstract

Gastrodia Antifungal Protein (GAFP) and Neutrophils Peptide-I (NPI), two disease-resistant genes were successfully transferred into the *Dtps.Tailin Angel Phalaenopsis* by using *Agrobacterium* strain off gradual methods. Two hundred and eighty kanamycin-resistant plants were regenerated by this method. PCR, Southern blot and RT-PCR confirmed that GAFP and NPI genes have been integrated into the genome of *Phalaenopsis*. *In vitro* antibiosis assay of the *Colletotrichum gloeosporioides Sacc* suggested that the transgenic plants were disease-resistant. Disease-resistance experiments proved that both GAFP and NPI genes were expressed efficiently in *Phalaenopsis*.

Introduction

As an expensive pot-flower, *Phalaenopsis* plays an important role in the global flower trading market. However, *Phalaenopsis* is susceptible to diseases caused by fungi and bacteria, causing great loss to orchid production. Conventional breeding methods to improve the disease resistant of *Phalaenopsis* are limited mainly due to the long reproduction cycles. Genetic modification of *Phalaenopsis* is one of the newly emerged promising methods to enhance the disease resistant of *Phalaenopsis*.

Gastrodia antifungal protein (GAFP) was isolated from the top corm of *Gastrodia elata*. GAFP can inhibit more than 20 fungi strains, including *Armillaria mellea*. GAFP was cloned and the prokaryotic expression of this protein showed bacteriostatic activity (Wang *et al.*, 2003). Neutrophils Peptide-I (NPI) increases permeability of microbeless lipid membrane in the electromagnetic field, which directly or indirectly affect energy metabolism and reduce the Electric Potential on both sides of the membrane. NPI has the widest antibiotic spectrum to bacteria, fungi, viruses, and certain malignant tumor cells (Hammond *et al.*, 1996). Chen *et al.*, (2005) transferred pBin35SGAFP-NPI, a binary resistance gene expression plasmid, into *Populus deltoids*×*P.simonii* and the genes were expressed well in the transgenic plants.

Transformation of plants mediated by *Agrobacterium tumefaciens* has gradually increased its contribution to the production of transgenic plants(Bhatti *et al*., 2013; Shen *et al.*, 2012).Successful transformation of some mark genes like GUS/ HPT and BP/KNAT1 into *Phalaenopsis* by using *Agrobacterium* have been previously reported (Belarmino *et al.*, 2000; Endang *et al.*, 2007; Sreeramanan *et al.*, 2010). However, there is no report on the successful transferring some economically important traits genes into orchids, such as genes associated with flower color and size, and genes resistance to insects and diseases. In order to improve the *Phalaenopsis* disease resistance, we transferred GAFP and NPI genes into the *Dtps.Tailin Angel Phalaenopsis* to investigate the diseases resistance of these transplants.

Materials and Methods

Gene transformation acceptor system: Young leaves of the tissue-cultured plantlets of *Phalaenopsis Dtps.Tailin Angel* (a red-flowered cultivar) were first cut perpendicularly to their rachis into 5~10 mm slices. The slices were put on the inducing medium with their rachis facing the medium. The medium used in this experiment was half-strength MS (½ MS) medium (Chen *et al.*, 2000) with sucrose at 30 mg/L supplemented with N^6-benzyladenin (6-BA) at 0, 5, 10, 15 and 20 mg/L, and α-naphthaleneacetic acid (NAA) at 0, 1.0 and 2.0 mg/L, respectively. After optimization of the medium for stimulating Protocorm-like bodies (*PLB*s) formation, kanamycin (Km) at 0, 5, 10, 15 and 20mg/L and cefotaxime sodium (Cef) at 0, 50,100,200 and 300mg/L, were added respectively to the medium to test the sensitivity of explants to antibiotics.

Eight weeks later, the *PLB*s were subcultured for 12 h on ½ MS medium (pH 5.7) with 7.0 g/L agar, peptone at 2g/L, banana juice at 3%, 2 g/L active carbon, and sucrose at 10, 20, 30, 40 mg/L, respectively. The cultures were incubated for 12 h at 26±2°C under cool-white fluorescent light of 1500 lx and relative humidity at 60%~80%. Km was added at 0, 5, 10, 15 and 20 mg/L respectively to test the effect of antibiotics to the growth of the roots.

Bacterial strain and plasmid vector: *A. tumefaciens* strains LBA4404 was used for the transformation. It harbors a binary vector pBin35SGAFP-NPI (Chen *et al.*, 2005). The T-DNA region of pBin35SGAFP-NPI contains a GAFP gene (380bp) and a NPI gene (170bp) under the control of cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1).

Transformation: As genetic transformation is influenced by acetosyringone in monocotyledons (Rashid *et al.,* 2012), *A.tumefaciens* strains LBA4404 was grown in YEB medium containing 50mg/L kanamycin and also 200μM acetosyringone (AS) overnight at 28°C to OD_{600} 0.5. Prior to transformation, explants were cut into 1×1 cm sections and pre-cultured on ½ MS medium for 0, 1, 2, 3, 4, 5, 6 and 7 days, respectively, and then immersed the explants in the above mentioned cultures of LBA4404 for 5, 15, 25, 35, 45 and 55 min, respectively. Co-cultivation of these explants with 200μM AS-treated *A.tumefaciens* was carried out under a 12-h (light of 400lx) /12-h (dark) photoperiod at 25°C for 1, 2, 3,4,5,10,15 and 20 d respectively.

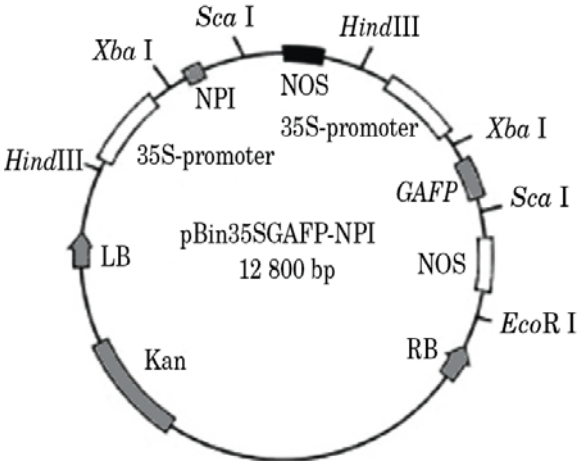

Fig. 1. Construct of pBin35S GAFP-NPI. RB: right border; LB: left border; Kan+: kanamycin resistance gene; NOS: nopaline synthase promoter; 35S-promoter: cauliflower mosaic virus (CaMV) 35S promoter; GAFP: Gastrodia Antifungal Protein gene (380bp); NPI: Neutrophils Peptide-I genes (170bp); multiple cloning sites, including *HindIII*, *XbaI*, *ScaI*, and *EcoRI*.

Regeneration of transformants: The explants were washed thoroughly with sucrose-free ½MS liquid medium containing 500mg/L cefotaxime. The cultures were transferred to a selective medium containing kanamycin and cefotaxime in darkness. When the light yellow granular embryogenic callus was visible on the leaf edge, the cultures were transferred on medium to induce plantlets formation.

Nucleic acid isolation: Genomic DNAs and total RNA of the plantlets were isolated from fresh leaf tissue as described previously (Endang *et al*., 2007).

Molecular detect of the genes in the transformants: PCR amplification was performed to investigate where the plantlets contains the transferred genes. The forward primer for GAFP gene is 5′-ACG TCT AGA AGG GAT CGG TTG AAT-3′and the reverse primer is 5′-GAT CTC GAG GCC AGA AGC CGC CGC TGT-3′,while the forward primer for NPI genes is 5′-GTG TAG GAT CCA TGG TGG TCT GTG GGT GCA GAG-3′and the reverse primer is 5′-GGG AGA GCT CAG TAG TCC AAA CAT GT-3′.

Southern hybridization and detection were performed by using the Dig-dUTP DNA probe, which was prepared from the GAFP cDNA and NPI cDNA by using the Dig DNA Labeling Kit (Boehringer Mannheim).

RT-PCR analysis was carried out by one-step RT-PCR which was used Access RT-PCR kit (Promega) as described previously (Awan *et al*., 2010).

Expression of GAFP-NPI genes: *Colletotrichum gloeosporioides Sacc* isolated from pathologic leaves of *Phalaenopsis* which was incubated on PDA at 30°C by using agarose medium hole diffusion method. When mycelium grew up to 3 cm in diameter, two holes per plate were punched symmetrically to remove the agar located 1 cm away from the mycelium. 200µl of total protein extract from the transgenic plants and non-transgenic plants were added to the holes separately. The plates were incubated at 25~28°C for 24~36 h in darkness.

Inoculation of *Colletotrichum gloeosporioides Sacc* to *Phalaenopsis* transplants: Suspension of *Colletotrichum gloeosporioides Sacc* was prepared as described previously (Zhang *et al*., 2005). 2µl suspension of spores was added to the five needle-wounded holes on the second leaf counted from top of the transformated and non-transformated *Phalaenopsis*. The plants were grown at room temperature. The disease was sored 10 days later as the spot in diameter on the leaves infected by *Colletotrichum gloeosporioides Sacc* as 0:no symptom, 1:less than 5 mm, 2:5~10mm, 3:10mm~15mm and 4: >15mm.

Results and Discussion

Establishment of the leaf dish acceptor system: 6-BA and NAA can stimulate *PLB*s formation. The best results were those grown on ½ MS with 6-BA at 10.0mg/L and NAA at 1.0mg/L (Table 1).

Table 1. Frequency of Protocorm Like Bodies (*PLBs*) induction in *Phalaenopsis.*

Treatments BA×NAA (mg/L)	Mean *PLBs* per explant	*PLBs*-forming explants (%)
0.0 0.0	0.0 ^{e}	0.0% ^{h}
5.0 0.0	0.0 ^{e}	0.0% ^{h}
10.0 0.0	1.0 ^{d}	26.0% ^{f}
15.0 0.0	0.5 ^{de}	20.0% ^{g}
20.0 0.0	0.0 ^{e}	0.0% ^{h}
0.0 1.0	0.0 ^{e}	0.0% ^{h}
5.0 1.0	2.0 ^{cd}	35.0% ^{de}
10.0 1.0	13.0 ^{a}	85.0% ^{a}
15.0 1.0	8.0 ^{b}	80.0% ^{a}
20.0 1.0	3.0 ^{c}	45.0% ^{c}
0.0 2.0	0.0 ^{e}	0.0% ^{h}
5.0 2.0	1.0 ^{d}	30.0% ^{ef}
10.0 2.0	1.5 ^{d}	40.0% ^{cd}
15.0 2.0	3.8 ^{c}	55.0% ^{b}
20.0 2.0	3.3 ^{c}	50.0% ^{bc}
BA	**	**
NAA	**	**
BA×NAA	**	**

Means with the same letter are not significantly different.

The correlations between the concentration of sucrose and plantlets regenerations from *PLB*s was $y=0.01992x^2+0.07751x+0.17000$. Plantlets developed well on ½MS containing 20g/L sucrose without any growth regulators.

Antibiotic sensitivity tests showed no significant difference of Cef concentration on the mean protocorm induction from *Phalaenopsis* between 0 to 300 mg/L (Table 2).

Table 2. Effect of cefotaxime sodium concentration on induction of protocorm from *Phalaenopsis* leaves.

Cefotaxime sodium (mg/L)	*PLBs* forming explant (%)	Number of *PLBs* per explant	*PLBs*-forming capacity
0	85.0	14	11.9
50	83.0	13	10.8
100	83.0	12	10.2
200	87.0	10	8.7
300	84.0	10	8.4

Protocorms (Table 3) and roots (Table 4) were not produced when Km was at 10mg/L. Based on the results, Cef at 50mg/L may be used to eliminate the *Agrobacterium* in selective medium, and Km at 10mg/L may be added to discriminate between transformants and non- transformants.

Tranformation and regeneration of transformants: Using leaf dish of *Phalaenopsis* as initial explants, we tested several factors affecting transformation in *Agrobacterium tumefaciens* mediated system. The optimized system for genetic transformation in *Phalaenopsis* was established. The best condition is as following: pre-culture time to induce kanamycin-resistant (Km^r) *PLBs* 4~5 days, LBA4404 OD_{600} at 0.05, infection time 10 min and pH 5.4. The co-culture time is the most critical on the induction Km^r *PLBs*. We divided this time as two stages, the first stage is 5 days, when the explants were co-cultivated with *Agrobacterium tumefaciens* without adding any antibiotics; the second stage is 2~3weeks, with Cef concentration at 20 mg/L. The Km^r *PLBs* was as high as 16.2%, five times as the first stage of co-cultivation (Table 5).

Table 3. Effect of kanamycin concentration on induction of protocorm from *Phalaenopsis* leaves.

Kanamycin (mg/L)	*PLBs* forming explant (%)	Number of *PLBs* per explant	*PLBs*-forming capacity
0	86.0	13	11.2
5	8.0	4	0.3
10	0.0	0	0.0
15	0.0	0	0.0

Table 4. Effect of kanamycin concentration on induction rooting from protocorm.

Kanamycin (mg/L)	Roots-forming *PLBs* (%)	Number of roots per *PLBs*	Roots-forming capacity
0	100.0	4.1	4.1
5	48.0	1.8	0.9
10	0.0	0.0	0.0
15	0.0	0.0	0.0
20	0.0	0.0	0.0

Table 5. Induction of Km^r protocorm from *Phalaenopsis* leaves in optimum conditions (%).

Treats batch	Frequency of Km^r in CK+	Frequency of Km^r in CK-	Frequency of Km^r in inoculated
1	84	1.0	12.0
2	77	1.5	13.0
3	80	0.5	12.5
4	80	1.0	12.0
5	79	0.5	16.2
6	80	1.0	15.0

CK+: Frequency of protocorm formation without Km and *Agrobacterium tumefaciens* immersed; CK-: Frequency of protocorm formation only with Km.

Km-resistant buds induced from leaf explants were cut for proliferation on selective medium supplemented with Km at 10mg/L with monthly subculture. After three rounds of selection, the putative transformants were transferred to ½MS medium containing Km10mg /L for root development (Fig. 2).The plantlets regenerated from Km-resistant buds were used for further analyses.

Molecular analyses of transformants: The presence of GAFP-NPI genes in eleven randomly chosen Km-resistant plantlets was firstly examined by PCR. As shown in Fig. 3, the fragments showed the expected size of 380bp for GAFP and 170bp for NPI in putatively transformed plantlets. As expected, there were no PCR bands shown with the DNA from non-transgenic plantlets.

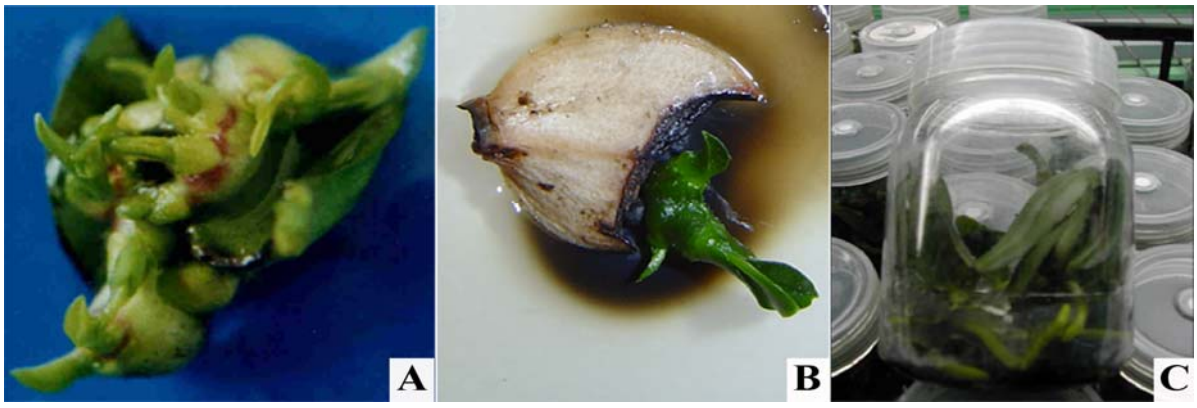

Fig. 2. The kanamycin-resistant buds and regeneration of transgenic plants. (A) protocorm formation from leaves without Km and *Agrobacterium tumefaciens* immersed; (B) Km-resistant buds; (C)Km-resistant plantlets.

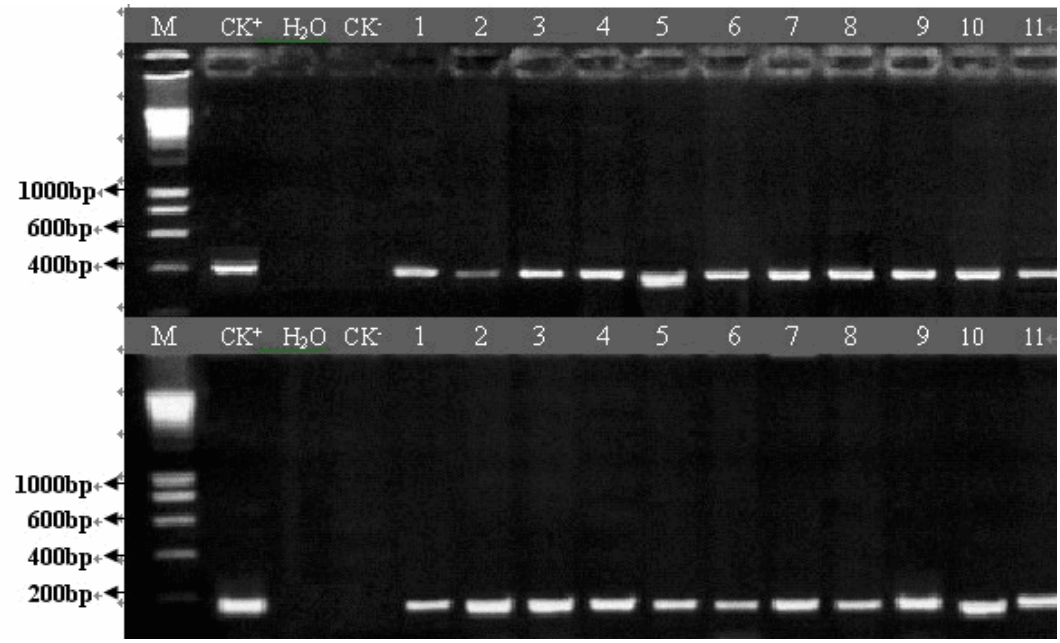

Fig. 3. PCR detection on NPI-GAFP genes in transgenic plantlets. M: 200bp ladder; CK+: pBin35SGAFP-NPI control; CK-: non-transgenic plantlets; 1-11: transgenic plantlets.

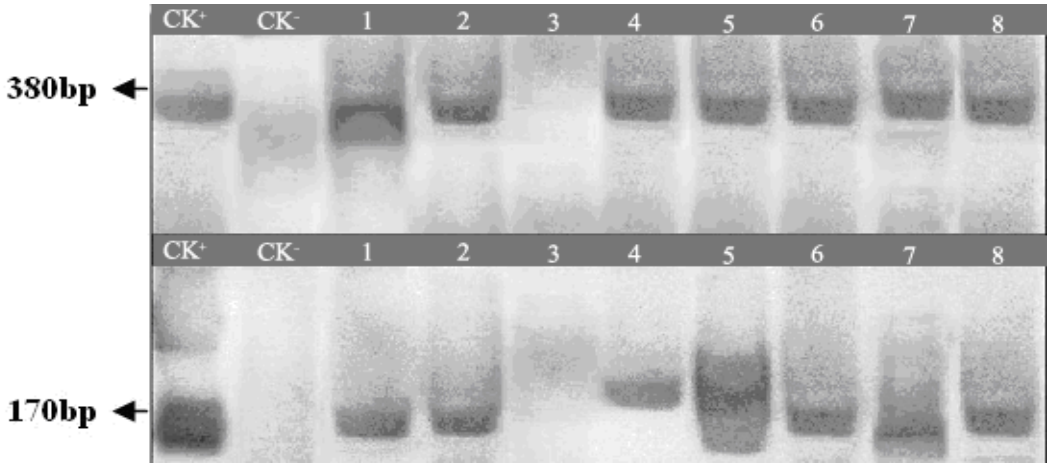

Fig. 4. Southern blot screening for foreign NPI-GAFP genes in transformed plantlets. CK+:pBin35SGAFP-NPI control; CK-: non-transgenic plantlets; 1-8: transformed plantlets.

Southern blot analysis confirmed the presence and integrity of the GAFP gene and NPI gene in transformed plantlets (Fig. 4). To further assess the expression of the GAFP gene and NPI gene driven by the CaMV 35S promoter, we also analyzed the amounts of GAFP mRNA and NPI mRNA in twelve transformed plantlets by RT-PCR. As shown in Fig. 5, the GAFP gene and NPI gene were expressed in ten transgenic lines analyzed. Only two transformed plantlets presented no corresponding fragments.

Pathogen-resistance of the transformants: GAFP and NPI protein were constitutively expressed in plants. The expressed product is fungi-resistant. *Colletotrichum gloeosporioides Sacc* isolated from pathologic leaves of *Phalaenopsis* was incubated at agarose medium by hole diffusion method. The results showed the clear inhibition zones around crude extracts protein of twenty four Km^r plantlets; however, there was no inhibition zone from non-transgenic plantlets (Fig. 6). In the vitro antibiotics assay suggested that the transgenic plantlets were disease-resistant and the two genes can be expressed efficiently in transgenic *Phalaenopsis*.

***In vivo* experiment of disease resistant of transgenic *Phalaenopsis*:** After 10 d's incubation, the disease spots of non-transgenic *Phalaenopsis* reached scale 3-4 while the transgenic *Phalaenopsis* reached only scale 1, which indicated transgenic *Phalaenopsis* was resistant to *C.gloeosporioides Sacc*, even they are not immune to the pathogen (Fig. 7).

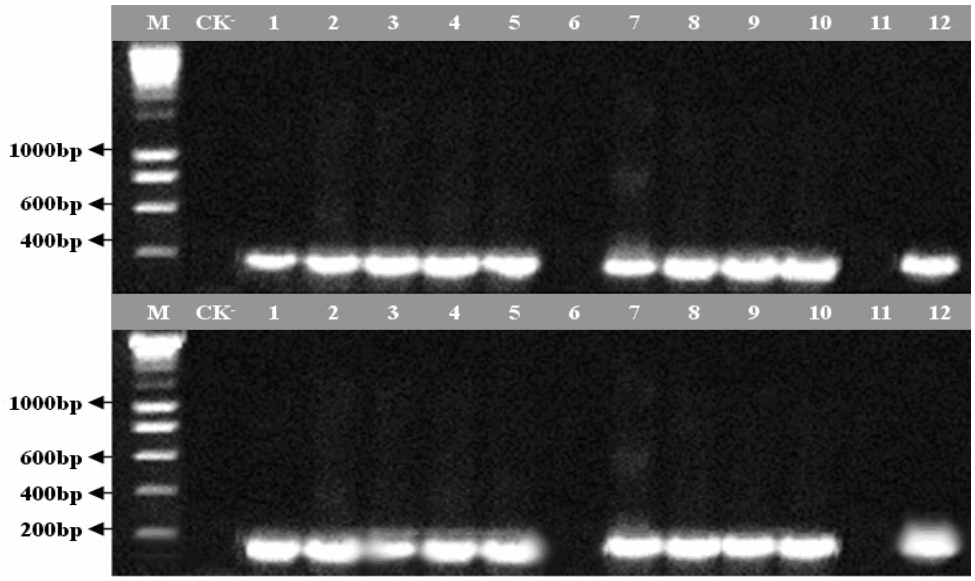

Fig. 5. RT-PCR for foreign NPI-GAFP genes in transformed plantlets. CK-: non-transgenic plants; 1-12: transgenic plants.

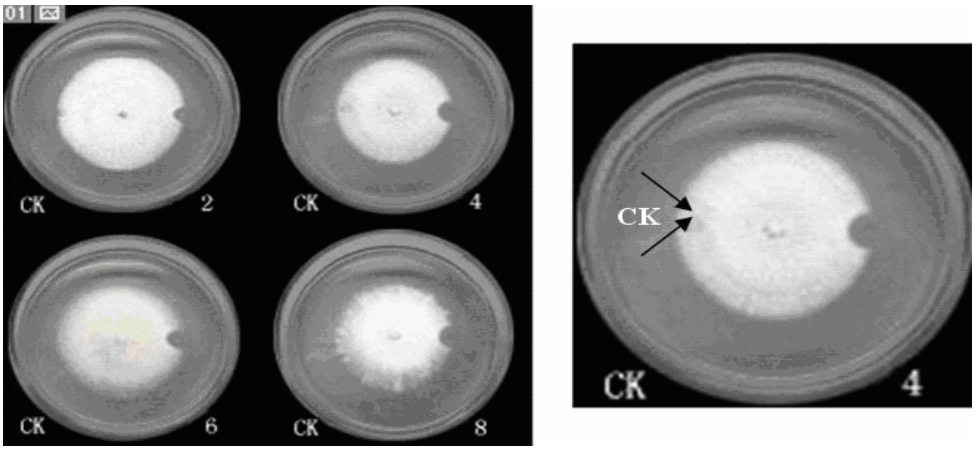

Fig. 6. *In vitro* antifungal assay of transgenic plantlets. CK: no inhibition zone around crude extracts protein of non-transgenic plants; 2, 4, 6,8: inhibition zones around crude extract protein of transgenic plants.

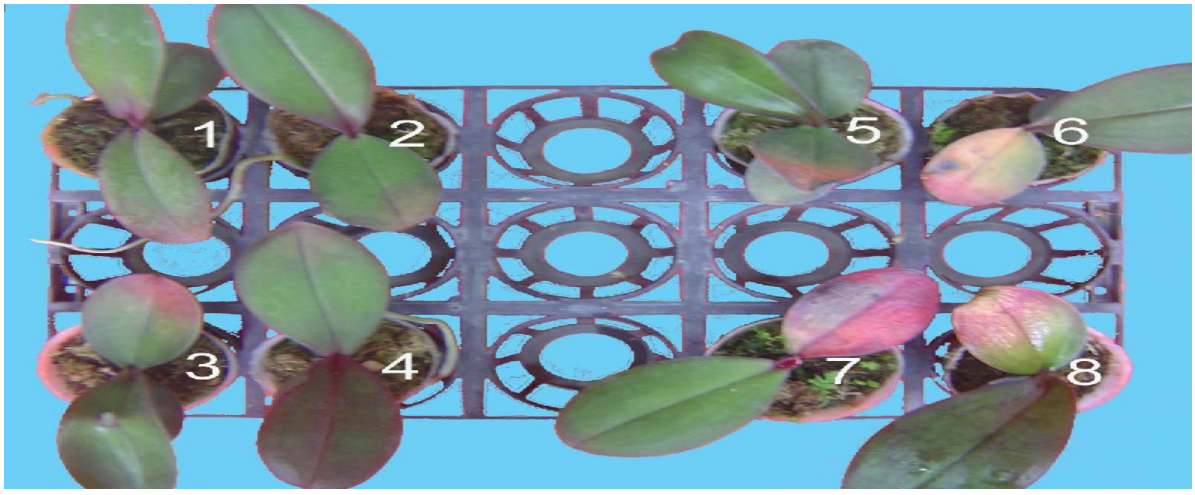

Fig. 7. Antifungal assay on transgenic *Phalaenopsis.* 1~4: transgenic plants; 5~8: non-transgenic plants.

In this study, we showed several factors, e.g., pre-culture time, co-culture time, bacteria concentration infection time, medium pH, which contributed to success of the transformation process. Optimized conditions for co-cultivation were also shown in this study. The Leaf dish acceptor system of gene transformation has been established. In this experiment, leaf dish of *Phalaenopsis* as initial explants can not only regenerate efficiently, but also avoid producing transgenic chimera. Time of co-cultivation is one of the main factors among different factors affecting *Agrobacterium* mediated gene transformation (Raja *et al*., 2010; Narusaka *et al*., 2003).The target explants produced more infecting cells by *Agrobacterium* strain off gradual methods, which improved the frequency of transformation greatly by adjusting the concentration of *Agrobacterium* to balance the maximal frequency of transformation and the minimum frequency of *Agrobacterium* contamination. This method increased the percent of putatively transformed plantlets to 16.2%. These results also indicated that the long co-culture period was essential for successful transformation of plants (Nakashima *et al*., 2000; Yu *et al*., 2001; Liau *et al.,* 2003; Zia *et al.,* 2010).

GAFP-NPI genes were integrated into the genome of *Phalaenopsis* confirmed by PCR, Southern blot and RT-PCR analysis. However, mRNA transcription was not detected in a few of transgenic plants, which suggested that some unknown reasons silenced the transformed foreign genes. *In vitro* antibiotics assay and antifungal assay in greenhouse showed that there was a discrepancy in antibacterial activity in different transgenic plants. The difference between lab results and field application still exists, and much remains to be done.

Acknowledgements

Authors sincerely thank Dr. Chen Ying for providing the plasmid pBin35SGAFP-NPI.Grants from Education Department of Sichuan Province (Grant No.10zd1129), the Doctoral Program of Southwest University of Science and Technology (Grant No. 09zx7107) and Sichuan academic and technical leader training projects (Grant No. 11sd3105).

References

Awan, A.R., I. Ul-Haq, M.E. Babar and I.A. Nasir. 2010. Molecular detection of potato leaf roll polerovirus through reverse transcription polymerase chain reaction in dormant potato tubers. *Pak. J. Bot.,* 42(5): 3299-3306.

Belarmino, M.M. and M. Mii. 2000. *Agrobacterium* –mediated genetic transformation of a *phalaenopsis* orchid. *Plant Cell Rep.*, 19: 435-442.

Bhatti, K.H., A. Shah, K. Hussain, K.N. Amd and J.H. Wu. 2013. Transgenic tobacco with rice fae gene shows enhanced resistance to drought stress. *Pak. J. Bot.,* 45(1): 321-326.

Chen, Y., Y.M. Dai, Y.Q. Wang, Y.R. Sun, M.R. Huang and M.X. Wang. 2005. The construction of two disease-resistance gene NP1 and GAFP vector. *J. Nanjing For. Univ.*, 29: 7-10 (in Chinese).

Chen, Y.C. and C. Chang. 2000. A reliable protocol for plant regeneration from callus culture of *Phalaenopsis*. *In Vitro Cell Dev. Biol. Plant.,* 36: 420-423.

Endang, S., P. Arii, P. Azis, Sulastrii, O.S. Nil, Takaakii, Y. Yasushi, M. Yasunori and M. Chiyoko. 2007. *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis*. *Plant Biotechnol.*, 24: 265-272.

Hammond, K.K.E. and J.D. Jones. 1996. Resistance gene dependent plant defense responses. *Plant Cell*, 8: 1773-1791.

Liau, C.H., S.J. You, V. Prasad, H.H. Hsiao, J.C. Lu, N.S. Yang and M.T. Chan. 2003. *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. *Plant Cell Rep.*, 21: 993-998.

Nakashima, K., Z.K. Shinwari, S. Miura, Y. Sakuma, M. Seki, K.Y. Shinozaki and K. Shinozaki. 2000. Structural organization, expression and promoter activity of an Arabidopsis gene family encoding DRE/CRT binding proteins involved in dehydration- and high salinity-responsive gene expression. *Plant Molecular Biology,* 42(4): 657-665.

Narusaka, Y., K. Nakashima, Z.K. Shinwari, Y. Sakuma, T. Furihata, H. Abe, M. Narusaka, K. Shinozaki and K.Y. Shinozaki. 2003. Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high salinity stresses. *The Plant Journal*, 34(2): 137-149.

Raja, N.I, A. Bano, H. Rashid, Z. Chaudhry and N. Ilyas. 2010. Improving *Agrobacterium* mediated transformation protocol for integration of *XA21* gene in wheat (*Triticum aestivum* L.). *Pak. J. Bot.,* 42(5): 3613-3631.

Rashid, H., M.H. Khan, Z. Chaudhry, R. Bano and N.I. 2012. An improved agrobacterium mediated transformation system in wheat. *Pak. J. Bot.,* 44(1): 297-300.

Shen, J.J., P.Z. Cai, F. Qing, Z.Y. Zhang and G.X. Wang. 2012. A primary study of high performance transgenic rice through maize Ubi-1 promoter fusing selective maker gene. *Pak. J. Bot.,* 44(1): 501-506.

Sreeramanan, S. and R. Xavier. 2010. Emerging factors that Influence efficiency of T-DNA gene transfer into *Phalaenopsis Violacea* Orchid via *Agrobacterium Tumefaciens*–mediated transformation system. *Inter. J. Biol.*, 2: 64-73.

Wang, Y.Q., D.J. Chen, X.W. Wei, M.G. Wu, D.M. Wang, Z.P. Yao, J.S. Huang, F.J. Liu, Meiliguli, R.J. Li and Y.R. Sun. 2003. Study of transferring the gene of *gastrodia* antifungal protein (gafp) into color cotton to enhance wilt resistance. *Chin. Bull. Bot.*, 20: 703-712 (in Chinese).

Yu, H., S.H. Yang and C.J. Goh. 2001. *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class 1 Knox gene DOH1. *Plant Cell Rep.*, 20: 301-305.

Zhang, J.Z. and T. Xu. 2005. Cytological characteristics of the infection in different species, varieties and organs of persimmon by *Colletotrichum Gloeosporioides*. *Mycosystema*, 24: 116~122 (in Chinese).

Zia, M., Z.F. Rizvi, R.U. Rehman and M.F. Chaudhary. 2010. *Agrobacterium* mediated transformation of soybean (*Glycine max L.*): some conditions standardization. *Pak. J. Bot.,* 42(4): 2269-2279.

(Received for publication 18 Marcy 2012)