International Research Journal of Natural Sciences

Vol.4, No.2, pp.1-11, May 2016

Published by European Centre for Research Training and Development UK (www.eajournals.org)

GENETIC TRANSFORMATION OF *PHALAENOPSIS AMABILIS* WITH RESISTANCE TO SOFT ROT DISEASE, HERBICIDE AND GLOWING IN THE DARK BY PARTICLE BOMBARDMENT METHOD

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ABSTRACT: *Genetic transformation of Phalaenopsis amabilis has been performed. We used particle bombardment method. The tungstens were coated with DNA (plasmid containing wasabi and bar gene, and co-plasmid containing luc gene). The purpose of this study is for crop improvement of ornamental plant. We obtained 27 transgenic P. amabilis. It is resistance to soft rot disease, herbicide and glowing in the dark. Interestingly, one of the transgenic plant propagated by itself. To the best of our knowledge, a concurent shot of wasabi and bar gene together with a luciferase gene is reported as a first study in this research.*

KEYWORDS: Genetic Transformation, Bombardment Method, DNA, Soft Rot Disease

INTRODUCTION

Phalaenopsis amabilis orchid is one of the most important orchids grown for commercial production of cut flowers and potted plant, and is becoming an important ornamental plant in the world. This orchid free flowering with inflorescences bearing to more than 30 flowers, flowering 2 to 3 times a year and has a long-lasting vase-life making it ideal for global cut-flower markets [1].

Disease problem can cause a big loose in orchid industry. Bacterial disease such as soft rot disease, caused by Erwinia carotovora commonly cause diseases in all parts of *Phalaenopsis* [2].

One of the alternative approach to avoid pathogen attack in *Phalaenopsis* is by performing genetic transformation for transferring disease resistance gene. Therefore, the plant can protect itself from the bacteria or fungi attack. One of disease resistance gene is wasabi defensin gene. Wasabi defensin gene was isolated from wasabi (*Wasabia japonica*) and showed a strong expression and inhibitory effect on fungal and bacterial growth in transgenic Nicotiana benthamiana [3]

Another problem occuring in horticultural plant is weed disturbance. In orchid, the high quality of flower will be affected by the weed disturbance. Therefore, applying genetic transformation in orchid will be useful in creating herbicide resistance plant (transgenic plant which can control weed). Recently, transgenic crop plants expressing herbicide tolerance have been commercialized, because the use of non-selective herbicides such as glufosinate and glyphosate provides economically superior weed control. The bar gene encoding the enzyme, phosphinothricin acetyltransferase (PAT), that confers resistance to the commercial herbicides, glufosinate and bialaphos, was isolated from the bialaphos biosynthetic pathway of *Streptomyces hygroscopicus*. Otani et al. [4] reported that transgenic herbicide-resistance

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

sweetpotato may enable to control weeds in cultivation more conveniently and more economically.

For increasing aestethics of the flower, we inserted luciferase gene into the genome of Phalaenopsis amabilis as well. The purpose is to anable the plant glowing in the dark. Chia et al. [5] reported the glowing of the dark Dendrobium orchid. He inserted luciferase gene into the genome of Dendrobium orchid.

Based on the background above, in this paper we reported an orchid (Phalaenopsis amabilis) with three important traits, disease resistance, herbicide resistance and glowing in the dark in one plant. The advantage is in this way we can help native orchid (*P. amabilis*) grow more easily in cultivation and hence, prevent further exploitation of wild orchids, thus aiding conservation and germplasm.

One effective alternative procedure is genetic transformation by direct delivery of genes into plant cells by particle bombardment [6]. In this study, we performed transformation by particle bombardment. Genetic transformation by particle bombardment gives a relatively high frequency of transient expression. However, the number of stable transformation events has been estimated to be 1000 fold-less [5]. Because the number and the chromosomal location of introduced gene can not be controlled, the expression of transgene varies among independent transgenic lines. This variation in expression level necessitates the generation of large number of transgenic plants for studies of the introduced gene [5]. Therefore, in this study we will use protocorm-like bodies (plb) at an early stage (embryos) after germination for target plant material, which regenerated large number of transgenic plants.

MATERIAL AND METHODS

Genetic Transformation by particle bombardment

1. Establishment of embryo culture

   Seeds of *P. amabilis* were surface sterilized using 10% chlorox for 10 min. Then the sterilized seeds were washed with distillated water three times. Sterile seeds were cultured in ½ MS liquid medium with 10% coconut water and shaked at 120 rpm for 3 weeks

2. Shooting of embryos

   The tungstens were coated with DNA (plasmid containing wasabi and bar gene, and co-plasmid containing luc gene). Subsequently, the coated DNA was shooted onto embryos . The embryos were then dropped by ½ MS liquid medium. Thereafter, the embryos were put in the dark for 2 weeks for recovery.

3. Shot embryos in selection medium

   The shot embryos were subcultured into 3 µM MSO for 2 weeks. The green and yellow plbs were subcultured into 3 µM MSO every 2 weeks until the green plbs are stabile. The stabile green plbs were subcultured into 4 µM MSO . The green and big plbs were subcultured into plantlet media (1/2 MS with 10% banana) containing 4 µM MSO. The plantlets were subcultured every 3 weeks.

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4. Acclimatization

The survived plantlets were acclimatized on moss. The result was observed after 2 months.

5. Bioassay

Bioassay of rot disease resistance

The leaf of transgenic orchid and control were applied by Erwinia bacteria. Then, applied leaves were observed after 3 to 5 days.

Bioassay of herbicide resistance

The leaf of transgenic orchid and control were sprayed by 4 µM MSO solution. Then, the sprayed leaves were observed after 3 to 5 days.

6. PCR

The presence and integration of wasabi defensin gene and herbicide resistance gene in the plant genome were indicated by the PCR of the extracted genomic DNA from putative transgenic plantlets. Total genomic DNAs were isolated from young leaves of putative transgenic plants and control plant following the CTAB method [7]. PCR reactions was performed using genomic DNA as a target and oligonucleotide primers (Bex Co. Ltd., Tokyo, Japan) for the wasabi defensin gene. A set of primers specific to the regions of the CaMV35S promoter (F) 5'-GAT GTG ATA TCT CCACTG AC-3' and the NOS terminator (R) 5'-CGC AAG ACCGGC AAC AGG AT-3', respectively, were used to amplifyboth 0.7-kb fragment of the wasabi defensin gene and 0.85-kb fragment of the bar gene simultaneously. PCR amplification was carried out using the following conditions: 94^{o} C-4min 1 cycle, 94^{o} C 1min 30 cycles, 59^{o} C-1min 30 cycles, 72^{o} C-1min 30 sec 30cycles, 72^{o}C-5min 1 cycle. The primer pair were design to amplify the entire 1.87 kb luciferase gene; LUC 5: 5'-CAATTACCAACAACAACA and LUC 3:3'-TTTTTAGTTACCTATACC. Amplification condition was 2 min at 94^{o}C, 35 cycles of 30 second at 94^{o}C, 30 second at 50.9^{o}C, 2 min at 72^{o}C and 10 min at 72^{o}C using a DNA thermal cycler.

7. Glowing of leaf

The putative transgenic leaf was put in Petri dish and dropped by luciferin. After 10 min, the leaf was put into DNA illuminator and picture of the glowing of leaf was taken.

RESULT AND DISCUSSION

Genetic Transformation by Particle Bombardment

Initially, each batch of embryos has an average of 1600 embryos in size 100 micrometer. After the first MSO selection, it was found that on average, 10% of the embryos developed into dark green plbs while the rest of embryos appeared yellowish. After the second MSO selection, 5% of the plbs appeared dark green while the rest of plbs appeared yellowish.

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Therefore, 84 of putative transformant plb were obtained. Figure 1 shows plbs of P. amabilis after four weeks of culture on three µM MSO. Green plbs were resistance to MSO and yellowish embryos were not resistance to MSO. Therefore the green plbs are transgenic plb that show herbicide resistance. Figure 2 shows the magnification of green plb (transgenic plb) and yellow plb (non transgenic plb).

Fig. 1. Plbs of *P. amabilis* after 4 weeks of culture on 3 µM MSO. Green plbs are transgenic plbs.

Fig. 2. Green plb (transgenic) and yellow plb (non transgenic)

A new approach of using embryos as target tissue was proven to be highly efficient in producing transformants. Firstly, orchid embryos being small has high surface area-to-volume ratio, which increases the percentage of cells initially transformed within an embryo. This is an important factor in ensuring the high survival rate of the transformed embryo under MSO selection. Secondly, the meristematic cells in embryos are highly embryogenic. This will increase the potential of transformed meristematic cells to divide and differentiated into a plantlet, Thirdly, the meristematic region in an embryo is wide and distinguishable and this increases the chance of the tungsten particles to successfully bombarding the meristematic cells [5]. The combination of these factors contributes to the high transformation efficiency that was achieved when embryos were used as target tissues.

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Being a herbicide that inhibits GS (Glutamine synthetase), MSO causes ammonia accumulation and ammonia toxicity in plants . Ammonia toxicity was shown to cause chlorosis, inhibit growth, inhibits seedling establishment and ultimately causes death of plants. In this study, it is proven that MSO is highly effective in selecting for transformed embryos. Under MSO selection system, the MSO-resistant plb remained dark green and growing due to the embryos were transformed with bar gene that was carried by pEKB-WT plasmid.

The MSO selection system is definitely time-saving. Firstly, this method saved us 2 months to obtain the putative transformant. Secondly, it allow the initial few transformed meristematic cells in an embryo to divide and increase in number before they were subjected to higher concentration of MSO. This may increase the survival rate of these transformed embryos.

After 2 months on MSO selection medium, the green transgenic plbs developed into plantlets. Figure 3 shows the plantlet of transgenic *P. amabilis* on medium containing 3 µM MSO. Figure 4 and Figure 5 shows plantlets of transgenic P. amabilis after 3 months of culture. And 10 months of culture, respectively. The leaves and root have developed. One hundred fifteen transgenic plantlets that show herbicide resistance were obtained.

Fig. 3. Plantlet of transgenic *P. amabilis* on selection medium containing 3 µM MSO

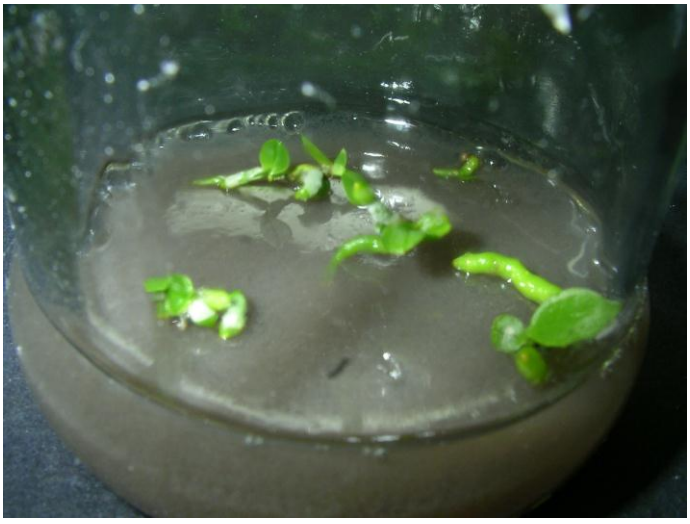

Fig. 4. Plantlet of transgenic *P. amabilis* after 3 months of culture

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Fig. 5. Plantlets that survived on 3 µM MSO (left) and 4 µM MSO (right) after 10 months of culture

Result of Bioassay

Fig. 6 on the left is transgenic P. amabilis With resistance to soft rot disease

Fig. 7 on the right is non transgenic P. amabilis. The leaf was rotten because of Erwinia rhapontini

The leaf of control plant (non transformed plant) was yellowing (rotten) (Fig. 7) whereas the leaf of transgenic plant remain green and healthy (was not infected by Erwinia) (Fig. 6).

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Fig. 8 on the left is transgenic P. amabilis with resistance to herbicide (MSO)

Fig. 9 on the right is non transgenic P. amabilis. The leaf was reddish because of MSO

The leaf of control plant (non transformed plant) was reddish (Fig.9) whereas the leaf of transgenic plant remain green and healthy (was not affected by MSO) (Fig. 8).

Result of PCR

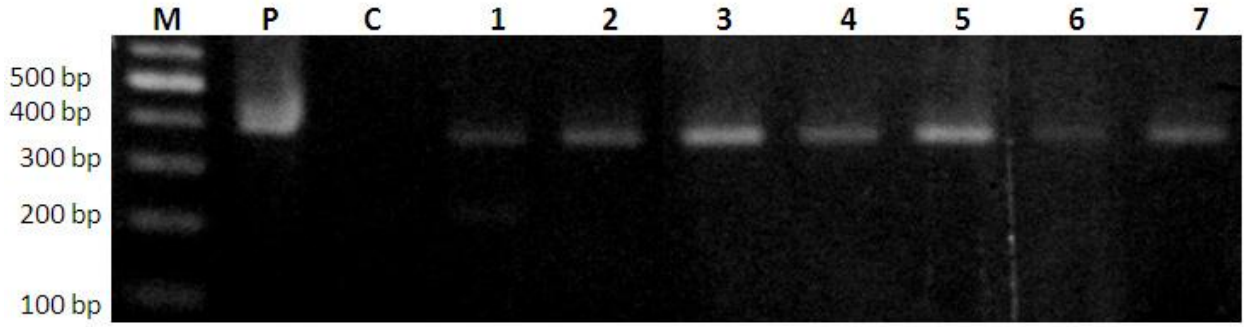

Fig. 10. Detection of wasabi defensin gene by PCR. M is Molecular marker, P is Positive control, C is Control, #1-7 are transgenic P. amabilis plant

When the shooting of co-plasmid luc gene was concurrently shot with DNA containing wasabi and bar gene, the transgenic *P.amabilis* could show the luc gene bands when checked by PCR. Fig. 11 shows electroforegram of luc gene. The transgenic plantlets of *P. amabilis* no 2-22 showed positive bands of luc gene.

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

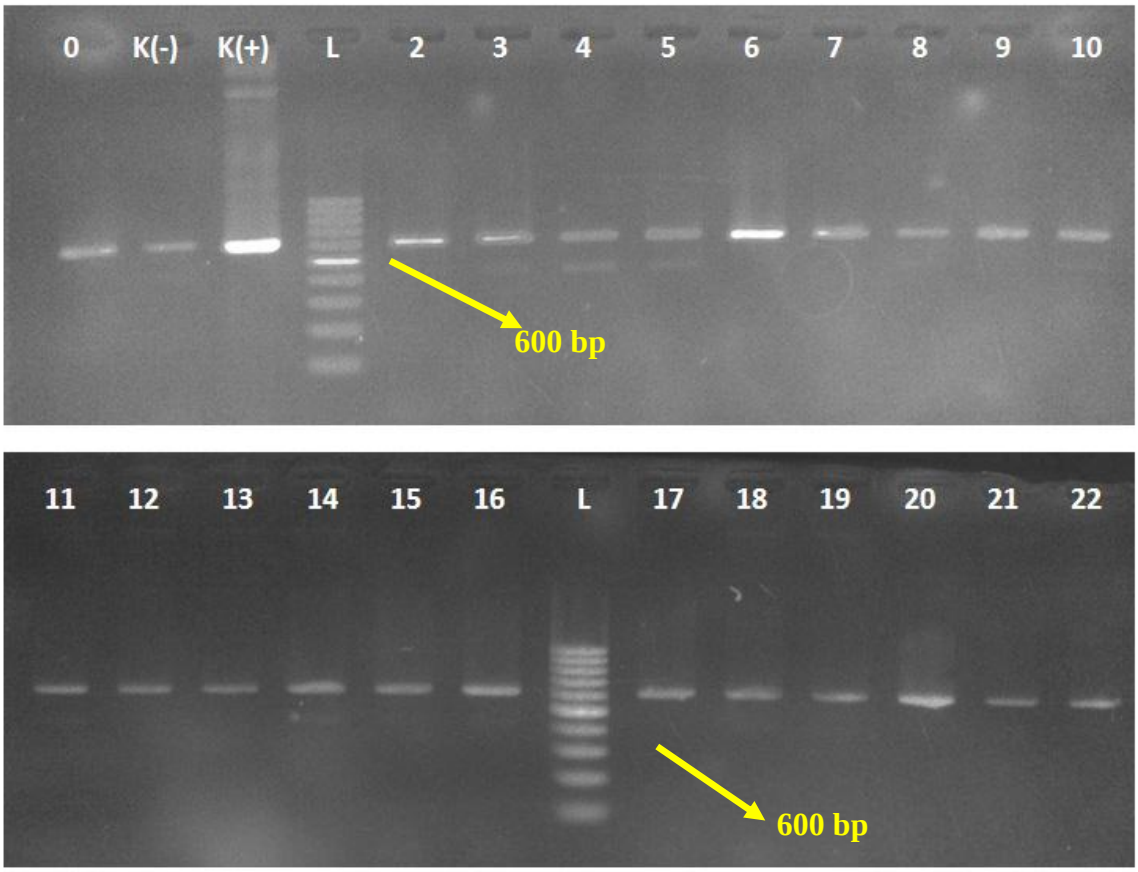

Fig.11. PCR of transgenic *Phalaenopsis amabilis*

Note:

L: 100bp ladder
0: blank (pure water)
(-): negative control (non transgenic)
(+): positive control (luciferase plasmid)
2-22: Transgenic plants no. 2 – 22

Glowing Leaf

Figure 12. Glowing leaf of transgenic Phalaenopsis amabilis

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Fig. 13. Phalaenopsis amabilis plant propagated by itself

Fig. 14. Flower of Phalaenopsis amabilis

The result of PCR shows that wasabi gene could be detected in transgenic orchid (Fig. 10). The bar gene could not be detected by PCR. Therefore, we performed bioassay to prove the activity of bar gene in genome of transgenic orchid. By bioassay, it was proven that the transgenic orchid has the activity of bar gene. The leaf of transgenic orchid is remain green while the leaf of control became reddish.

ISSN 2053-4108(Print), ISSN 2053-4116(Online)

Interestingly, one of transgenic orchid propagated by itself (Fig. 13). To our knowledge, this is the first report that the transgenic orchid could propagated by itself. Fig. 14 shows the flower of Phalaenopsis amabilis orchid.

When the shooting of co-plasmid luc gene was concurently shot with DNA containing wasabi and bar gene, the transgenic *P.amabilis* could show the luc gene bands when checked by PCR. Fig. 11 shows electroforegram of luc gene. The transgenic plantlets of *P. amabilis* no 2-22 showed positive bands of luc gene.

The glowing of transgenic *P. amabilis* leaf was shown in fig. 12. If the luciferin was poured onto the leaf, LUC gene will produce luciferase. After several minutes, ATP will be formed and produced energy and light.

Transgenic cells, tissues, or plants can be readily identified by the luminescence emitted upon addition of a minute amount of luciferin. Successful transformation of plant cells depends critically on two steps: an efficient method for delivering DNA into target cells and the identification of stably transformed cells [5]. In this study, we were successful in performing transformation and identifying the transformed cells or tissues.

CONCLUSION

1. The number of transgenic *Phalaenopsis amabilis is* 27.
2. One of the transgenic *Phalaenopsis amabilis* plant propagated by itself.

Acknowledgement

We thank to Ministry of Research and Technology, Indonesia for the funding support in this study.

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ISSN 2053-4108(Print), ISSN 2053-4116(Online)