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# Female reproductive system of *Amaranthus* as the target for *Agrobacterium*-mediated transformation

**Umaiyal Munusamy[1,2], Siti Nor Akmar Abdullah[1,3*], Maheran Abd Aziz[1,3], Huzwah Khazaai[4]**

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Malaysia
[2]Institute of Biological Science, Faculty of Science, Universiti of Malaya, Kuala Lumpur, Malaysia
[3]Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Malaysia
[4]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia
Email: umaiyal_munusamy@yahoo.com, *sakmar@agri.upm.edu.my, maheran@agri.upm.edu.my, huzwah@medic.upm.edu.my

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## ABSTRACT

***Agrobacterium*-mediated transformation through floral dip and rapid selection process after transgenic event had become a preference as it will overcome the difficulties faced in tissue culturing procedures and lengthy time for screening transformed progenies. Therefore, in this study, three constructs, p5b5 (14,289 bp), p5d9 (15,330 bp) and p5f7 (15,380 bp) in pDRB6b vector which has hygromycin as a selectable marker gene were introduced individually into *Agrobacterium tumefaciens* strain (AGL1). The cell suspension was applied to *Amaranthus* inflorescence by drop-by-drop technique and was left to produce seeds ($T_1$). The $T_1$ seeds were germinated and grown to produce seedlings under non-sterile condition. Hygromycin selection on seedling cotyledon leaves results in identification of 12 putative transformants, three from p5b5, four from p5d9 and five from p5f7. All positive putative transformants that were selected at the first stage through hygromycin spraying showed positive result in leaf disk hygromycin assay and in a construct specific polymerase chain reaction-based assay. A ~750 bp amplified hygromycin gene was further verified through sequencing. Our results suggest that *Amaranthus* inflorescences were able to be transformed and the transformed progenies could be verified through a combination of simple and rapid methods.**

**Keywords:** *Agrobacterium*-Mediated Transformation; *Amaranthus*; Direct PCR; Female Reproductive System; Floral-Dip; Hygromycin

## 1. INTRODUCTION

*Amaranthus* is one of the most important leafy staple crops which can be found throughout the tropics and in many warm temperate regions [1]. Studies have been conducted to develop technologies that aim in exploiting *Amaranthus* due to its high nutritional value [2]. Technological innovation in plant biotechnology is an important catalyst in any crop enrichment [3]. Stable *Agrobacterium*-mediated genetic transformation offers advantages in transferring one or few copies of DNA fragments carrying the genes of interest. It can be carried out on the whole plant by using either tissue infiltration [4] or floral dip [5]. This method is applied in preference to tissue culture-based techniques as it directly produces transformed seed circumventing and avoiding the lengthy tissue culture period and somaclonal variation [6].

The success in the transformation of *Amaranthus* floral would widen the possibilities for the exploitation of the available reported method on many other alternative crops. *Agrobacterium*-mediated floral dip transformation has been developed and successfully applied in the popular model plant such as *Arabidopsis thaliana* [7] and *Medicago truncatula* [8]. Since both *Amaranthus* and *Arabidopsis* are dicot plants with bisexual flowers, it is understandable that they share a common ancestor and similar anatomy and therefore amenable to the same transformation technique.

In addition, successful production of transgenic plants is also heavily dependent on a suitable selection technique to screen for transformants. The effectiveness and ease of use influence the choice of suitable selectable marker gene. A simple leaf spraying method developed for selection using Basta [9] had enabled large scale screening of plantlets, and similarly in the present study, hygromycin was sprayed on the seedlings during the screening process. The homogentisate phytyltransferase gene (*HPT*) was introduced for future manipulation of vitamin E biosynthesis in successfully transformed *Amaranthus*.

*Corresponding author.

## 2. MATERIALS AND METHODS

### 2.1. Vectors

The pDRB6b vector digested with *Hind*III/*Kpn*I served as the backbone vector for p5b5, p5f7 and p5d9 recombinant vectors. The recombinant vector was constructed in between RB2 and LB of pDRB6b. p5b5 contained Ubi1P driving HPT; p5f7 contained Ubi1P, Ubi1-intron and HPT while p5d9 contained LHCB, Ubi1-intron and HPT. The NosT site was located at the 3` end of the HPT gene in all the three constructs. All the recombinant vectors contained hygromycin gene driven by CaMV35S promoter located in between RB1 and RB2 (**Figure 1**). The constructs were transformed separately into AGL1 strain using freeze-thaw method [10].

### 2.2. Floral Transformation in *Amaranthus*

*Amaranthus* seeds (Leckat Corporation Sdn Bhd, Kepong, Malaysia) were sown in a pot containing soil, peatgro (Peatgro™, Batu Caves, Selangor, Malaysia), and sand in a ratio of 1:2:1 in the Transgenic Glasshouse, UPM under natural daylight. The plants were allowed to grow into maturity until the length of the inflorescences was in the range of between 4 and 6 cm.

*A. tumefaciens* strain (AGL1) carrying the recombinant vectors from the glycerol stocks were streaked onto LB agar containing 25 $mg{\cdot}L^{-1}$ rifampicin and 50 $mg{\cdot}L^{-1}$ spectinomycin and was incubated at 28˚C for two to three days in the dark. The bacterial suspension was prepared according to Lee and Yang [11] except Silwet L-77 was replaced with 0.01% Tween 20.

*Amaranthus* inflorescences were inoculated according to Martinez-Trujillo *et al.* [12]. The plants were injected in a shaded area away from direct sunlight and were allowed to remain at similar condition overnight. The treated plants were placed back to the green house and allowed to grow normally [13]. Watering of the plants was continued until the siliques started to lose their green colour and became yellow. Seeds ($T_1$) were harvested and stored according to Curtis [8] and Bent [14]. Seeds were sorted to identify those that are black in colour to determine percentage of seed productivity (w/w) prior to expression analysis.

### 2.3. Expression Studies and Transgene Analysis Using the *hph* Selectable Marker Genes

Seeds from floral dipped plants which were left for maturation until 50% of the plant became yellow were sown into soil and the origin where the seeds were collected was noted. Twenty days after sowing, the young seedlings were sprayed with 150 $mg{\cdot}L^{-1}$ hygromycin containing 0.01% Tween 20 daily for five days. Young seedlings that showed no necrosis on the leaves after the 5th day were considered to contain a functional *hph* gene. Hygromycin resistant plants were transferred to new pots and allowed to grow bigger. To further confirm the transgenicity of the *hph* resistant plants, leaf pieces of one month old plants were excised and analysed by *hph* histochemical assay [9]. Leaf disk assay in hygromycin solution to detect the expression of the *hph* gene was conducted to further distinguish the transformed plants [15] and untransformed control plants.

Fresh plant leaf materials were obtained by using FLOTING PUNCH (adoro™). The leaf sample was crushed with 100 µL pipette tip by pressing against the microcentrifuge wall until there were no large pieces of tissues left. Five hundred microlitre of dilution buffer (0.5 N NaOH) [16] was added to every disk according to $3(\pi/j^2)$; $j^2 = d = 4.5$ mm ($\pi = 22/7$; $j^2 = d$ = diameter). The supernatant was centrifuged using Eppendorf Centrifuge 5810R and 0.5 µL of fresh plant tissue solution was used for preparation of a 20 µL Phire® Plant Direct Polymerase Chain Reaction (PCR) (Finnzymes, Finland). The PCR reaction consist of 7.6 µL of distilled water, 10 µL 2× Phire® Plant PCR buffer, 1 µL of each 0.5 µM primer HyG (F:5`TCCGGATGCCTCCGCTCGAA3`) and primer HyG (R:5`ATGCAGCTCTCGGAGGGCGA3`) and 0.4 µL Phire Hot Start II DNA polymerase (F-130X). The cycling reaction was as follows: Initial denaturation at 98˚C for 5 min, second denaturation at 98˚C for 45 sec, annealing at 72˚C for 45 sec, extension at 72˚C for 40 sec and final extension was at 72˚C for 1 min, the cycle was hold at 4˚C. The PCR product was analysed on 0.8% (w/w) agarose gel and was sequenced. The sequencing

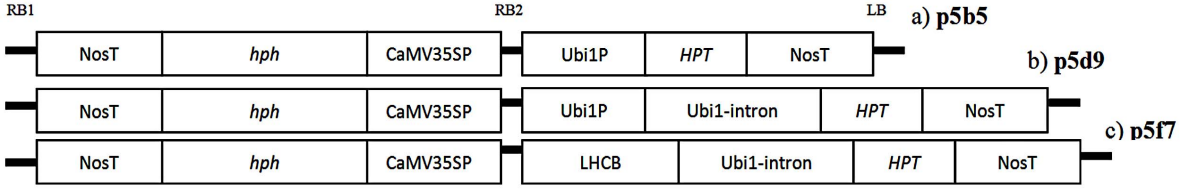



**Figure 1.** Chimeric constructs used in transformation of *Amaranthus*. The constructs were prepared by fusing together short sense sequences of homogentisate phytyltransferase (*HPT*), ubiquitin promoter (Ubi1P) or leaf-specific promoter (LHCB), ubiquitin intron (Ubi1-intron), with cauliflower mosaic virus promoter (CaMV35SP), hygromycin phosphotransferase (*hph*) and both ends terminated with NosT indicates the nos terminator. RB1: first right border, RB2: second right border, LB: left border.

result was analysed using nucleotide blast NCBI.

### 2.4. Statistical Analysis

Bacterial growth, transformation event and percentage of seed productivity value were expressed as mean values accompanied by standard error (SE) from triplicate reading performed repeatedly on the same subjects.

## 3. RESULTS

Based on the condition mentioned **Table 1** shows that AGL1 transformed with p5b5, p5d9 and p5f7 reached the target growth ($OD_{600}$) between 0.7 and 1.4. The results in **Table 2** show that all the inoculated plant with the transformed AGL1 containing p5b5, p5d9 and p5f7 produced more than 95% of seed productivity. This data was better than the value obtained for the non-transformed plant.

The seedlings of non-transformed plants began to show yellow spots (necrosis effect) on the third day after being sprayed, and the effects worsened on the fifth day. While, the transformed plants ($T_1$) remained green and healthy (**Figure 2**). A total of 12 progenies were identified as hygromycin resistant ($hph^R$). Further tests on the $T_1$ progeny seedlings using leaf disk assay and PCR analysis also showed positive results. In the leaf disk assay, all the 12 putative transformants remained green for five days in hygromycin solution. **Figure 3** showed the size (~750 bp) of amplified transgene hygromycin fragments were detected in all the 12 transformant plants ($T_1$). No transgene was detected in the non-transformed plants by PCR.

The identity of the hygromycin gene was confirmed by sequencing analysis which showed more than 95% of maximum identity with pTEXL-Hyg (Accession No: JN596098.1), pTcR-GA-Hyg (Accession No: JN596081.1) and pTcR-HG-Hyg (Accession No: JN596072.1). The transformation efficiency of approximately 1% to 2% was determined for p5b5, p5d9 and p5f7, respectively (**Table 2**).

## 4. DISCUSSION

The rapid ongoing process in engineering metabolites has increased the demands to utilise reported protocols/technology in *in-vivo* plant transformation to plants that are taxonomically related as their anatomical characteristics are similar [17]. A limited number of bisexual plants such as *Arabidopsis thaliana* [18], *Medicago truncatula* [8], *Thellungiella halophila* [19], and *Chenopodium rubrum* [20] have been successfully transformed through floral dip transformation.

*Amaranthus* is a genus in the family of Amaranthaceae. The flowers of most species in the Amaranthaceae are bisexual and it is similar to *Arabidopsis*, where they have both male and female reproductive organs within the same flower [21]. Therefore, a successful attempt was made to utilise standard floral dip procedure which was reported for *Arabidopsis* on *Amaranthus* as both plants fall into the same group (eudicots).

In order to reduce the amount of AGL1 solution needed, infiltration solution containing transformed AGL1 was pipetted rather than dipping the whole plant [22]. This was preferred as flowering *Amaranthus* reaching up to 90 cm tall are difficult to be dipped. The developing female reproductive organ, the ovary, is the place where female genotype cell lineages formed [23]. The stage of open florets during anthesis was selected to inoculate the transformed AGL1. This will subsequently produce transformed ($T_1$) seeds.

The AGL1 inoculums were prepared in infiltration medium containing Tween 20 rather than Silwet L-77. Most of the reports so far used Silwet L-77 as a unique surfactant for transformation procedures [24,25]. However, this can cause some restrictions for researchers in small laboratories with limited source of reagents and budget. In addition, the transformation efficiencies de-

**Table 1.** Growth of AGL1 carrying the recombinant plasmid in LB broth after 48 hours at 28˚C with aeration at 180 rpm.

| Construct | Growth($OD_{600nm}$) ± SE |
|---|---|
| p5b5 | 0.670 ± 0.011 |
| p5d9 | 1.381 ± 0.036 |
| p5f7 | 1.079 ± 0.139 |

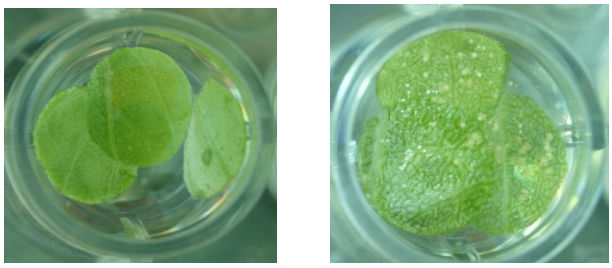
Values are the mean of three replicates followed by standard error (SE).

**Table 2.** Percentage of seed productivity after floral dip transformation compared to non-transformed plant and transformation efficiencies by AGL1 carrying p5b5, p5d9 and p5f7.

| Construct | Seed productivity (% w/w) ± standard error) | No of seeds | No of transformants | Transformation efficiency (% w/w) |
|---|---|---|---|---|
| Non-transformed plant | 90.33 ± 1.57 | | | |
| p5b5 | 95.69 ± 1.30 | 540 | 3 | 0.6 |
| p5d9 | 97.12 ± 2.34 | 2154 | 4 | 0.2 |
| p5f7 | 96.08 ± 0.21 | 285 | 5 | 1.8 |

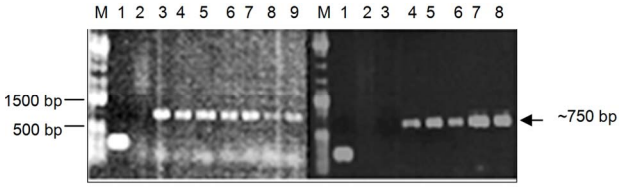
Seed productivity represents as mean replicates ± standard error (SE).



(a) (b)

**Figure 2.** Selection of hygromycin resistant (*hph*$^R$) and hygromycin sensitive (*hph*$^S$) *Amaranthus* seedlings. Seedlings were grown for 20 days and subsequently sprayed with 150 mg·L$^{-1}$ hygromycin containing 0.01% Tween 20 daily for 5 days. (a) Transformed seedlings showed no necrosis effect as the leaves contain a functional *hph* gene (*hph*$^R$), while (b) Untransformed seedling (*hph*$^S$) showed necrosis effect as the leaves did not contain functional *hph* gene (*hph*$^S$).




(a) (b)

**Figure 3.** PCR analysis of genomic DNA to detect the presence of the hygromycin gene (*hph*) in 12 representatives putative transgenic *Amaranthus* plants ($T_1$). The amplified PCR products were visualised in 2 set of 0.8% agarose gels (a and b). Lane M: GeneRuler™ 1 kb Plus DNA Ladder. PCR analysis of genomic DNA from hygromycin resistant seedlings showing the amplification of a ~750 bp fragment of *hph* gene. Lane 1, positive control (297 bp fragment) of highly conserved region of chloroplast DNA; Lane 2, negative controls of non-transformed plant; (a) Lane 3 - 5, putative transformant from p5b5; Lane 6 - 9, putative transformant from p5d9 (b) Lane 3, non-template control from PCR assays; Lane 4 - 8, putative transformant from p5f7.

tected in this study were similar to using Silwet L-77 [18].

Construct specific PCR is being used widely for the detection of specific DNA sequences that is not found in plants [26]. According to Wang *et al*. [16] younger tissues will gave better amplification. Amplification of a specific sequence such as hygromycin (sequence that is not found naturally in the plants) was carried out through construct specific amplification, thus will provide reliable information on a transgenic event [27]. In this study, the first stage of selection contains two steps 1) hygromycin spraying and 2) leaf disk assay. This was highly sensitive and accurate to be used in the selection of transformants as all putative hygromycin positive transformants that were selected through the first stage showed positive result in the construct specific PCR amplification.

Transformation frequency that was determined in this study was similar to the one reported by Clough and Bent [18] for *A. thaliana* which was in the range of 0.5% to 3%. This confirmed the hypothesis of the study that the floral dip transformation method can be applied to any bisexual plants albeit some suitable modifications. In addition, the transformation rate achieved was comparable to that reported by other researchers [23,25]. With this in mind, available technology for transformation can be applied in *Amaranthus*. All transformed *Amaranthus* have to be analysed and expression levels of vitamin E have to be ascertained in order to produce nutritional modified crop.

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## REFERENCES

[1] Makinde, E.A., Ayeni, L.S. and Ojeniyi, S.O. (2010) Morphological characteristics of *Amaranthus cruentus* L. as influenced by kola pod husk, organomineral and NPK fertilizers in Southwestern Nigeria. *New York Science Journal*, **3**, 130-134. http://www.sciencepub.net/newyork

[2] Trucco, F. and Tranel, P.L. (2011) Amaranthus. In: Kole, C., Ed., *Wild Crop Relatives*: *Genomics and Breeding Resources*, *Vegetables*, Springer-Verlag, Berlin Heidelberg, 11-21. doi:10.1007/978-3-642-20450-0_2

[3] Engel, K.H., Frenzel, T.H. and Miller, A. (2002) Current and future benefits from the use of GM technology in food production. *Toxicology Letters*, **127**, 329-336. doi:10.1016/S0378-4274(01)00516-1

[4] Tague, B.W. and Mantis, J. (2006) In planta *Agrobacterium* mediated transformation by vacuum infiltration. *Methods in Molecular Biology*, **323**, 215-223.

[5] Yasmeen, A., Mirza, B., Inayatullah, S., Safdar, N., Jamil, M., Ali, S. and Fayyaz, C.M. (2009) In planta transformation of tomato. *Plant Molecular Biology Reporter*, **27**, 20-28. doi:10.1007/s11105-008-0044-5

[6] Yang, A., Su, Q. and An, L. (2009) Ovary-drip transformation: A simple method for directly generating vector and marker free tranagenic maize (*Zea mays* L.) with linear GFP cassette transformation. *Planta*, **229**, 793-801. doi:10.1007/s00425-008-0871-5

[7] Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C. and Cottage, A. (2006) A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedling following floral dip transformation. *Plant Methods*, **2**, 1-7. doi:10.1186/1746-4811-2-19

[8] Curtis, I.S. (2004) Production of transgenic crops by floral-dip method. *Methods in Molecular Biology*, **286**, 103-109.

 

[9] Curtis, I.S. and Nam, H. (2001) Transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) by floral dip method plant development and surfactant are important in optimizing transformation efficiency. *Transgenic Research*, **10**, 363-371. doi:10.1023/A:1016600517293

[10] Wise, A.A., Liu, Z. and Bins, A.N. (2006) Three methods for the introduction of foreign DNA into *Agrobacterium*. *Methods in Molecular Biology*, **343**, 43-53.

[11] Lee, M.W. and Yang, Y. (2006) Transient expression assay by agroinfiltration of leaves. *Methods in Molecular Biology*, **9**, 323-225.

[12] Martinez-Trujillo, M., Limones-Briones, V., Cabera-Ponce, J.L. and Herrera-Estrella, L. (2004) Improving transformation efficiency of *Arabidopsis thaliana* by modifying the floral dip method. *Plant Molecular Biology Reporter*, **22**, 63-70. doi:10.1007/BF02773350

[13] Zhang, X., Henriques, R., Lin, S.S., Niu, Q. and Chua, N.H. (2006) *Agrobacterium* mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols*, **1**, 1-6. doi:10.1038/nprot.2006.97

[14] Bent, A. (2006) *Arabidopsis thaliana* floral dip transformation method. *Methods in Molecular Biology*, **343**, 87-103.

[15] Ko, M.K., Soh, H., Kim, K.M. and Kim, Y.S. (2007) Stable production of transgenic pepper plants mediated by *Agrobacterium tumefaciens*. *HortScience*, **42**, 1425-1430.

[16] Wang, H., Qi, M. and Cutler, A.J. (1993) A simple method of preparing plant samples for PCR. *Nucleic Acids Research*, **21**, 4153-4154. doi:10.1093/nar/21.17.4153

[17] ISAAA (2010) Agricultural Biotechnology (A Lot More than Just GM Crops). International Service for the Acquisition of Agi-Biotech Application. http://www.isaaa.org/resources/publications/agricultural_biotechnology/download/agricultural_biotechnology.pdf

[18] Clough, S.J. and Bent, A.F. (1998) Floral dip: A simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. *Plant Journal*, **16**, 735-743. doi:10.1046/j.1365-313x.1998.00343.x

[19] Fang, Q., Xu, Z. and Song, R. (2006) Cloning, characterization and genetic engineering of FLC homolog in *Thellungiella halophila*. *Biochemical and Biophysical Research Communications*, **347**, 707-714. doi:10.1016/j.bbrc.2006.06.165

[20] Veit, J., Wagner, E. and Albrechtova, J.T.P. (2004) Isolation of a *FLORICAULA/LEAFY* putative orthologue from *Chenopodium rubrum* and its expression during photoperiodic flower induction. *Plant Physiology and Biochemistry*, **42**, 573-578. doi:10.1016/j.plaphy.2004.06.008

[21] Costea, M., Weaver, S.E. and Tardif, F.J. (2004) The biology of invasive alien plants in Canada 3 *Amaranthus tuberculatus* (Moq.), Sauer var. *rudis* (Sauer), Costea & Tardif. *Canadian Journal of Plant Science*, **85**, 507-522. http://www.wlu.ca/documents/7479/rudis_published_paper.pdf doi:10.4141/P04-101

[22] Liu, M., Yang, J., Cheng, Y. and An, L. (2009) Optimization of soybean (*Glycine max* (L.) Merrill) in planta ovary transformation using a linear minimal *gus* gene cassette. *Journal of Zheijang University Science B*, **10**, 870-876. doi:10.1631/jzus.B0920204

[23] Desfeux, C., Clough, S.J. and Bent, A.F. (2000) Female reproductive tissues are the primary target of *Agrobacterium* mediated transformation by the *Arabidopsis* floral dip method. *Plant Physiology*, **123**, 895-904. doi:10.1104/pp.123.3.895

[24] Logemann, E., Birkenbih, L.R.P., Ulker, B. and Somssich, I.E. (2006) An improved method for preparing *Agrobacterium* cells that simplifies the *Arabidopsis* transformation protocol. *Plant Methods*, **2**, 1-5. doi:10.1186/1746-4811-2-16

[25] Chung, M.H., Chen, M.K. and Pan, S.M. (2000) Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. *Transgenic Research*, **9**, 471-476. doi:10.1023/A:1026522104478

[26] Bai, S.l., Zhong, X., Ma, L., Zheng, W., Fan, L.M., Wei, N. and Deng, X.W. (2007) A simple and reliable assay for detecting specific nucleotide sequences in plants using optical thin-film biosensor chips. *The Plant Journal*, **49**. 354-366. doi:10.1111/j.1365-313X.2006.02951.x

[27] Anklam, E., Gadani, F., Heinze, P., Pijnenburg, H. and Vanden Eede, G. (2002) Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *European Food Research and Technology*, **214**, 3-26. doi:10.1007/s002170100415

 