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Agrobacterium-mediated Genetic Transformation and Plant Regeneration from Cotyledons in Philippine Eggplant (*Solanum melongena* L.) Acc. 'PH 11424'

Mark Gabriel S. Sagarbarria[1,*], John Albert M. Caraan[1], Patrick G. Lipio[1], Ian Bien M. Oloc-oloc[1], Kazuo N. Watanabe[2], and Desiree M. Hautea[1]

[1]Genetics Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, 4031, Philippines

[2]Tsukuba Plant Innovation Research Center, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba City, Ibaraki, 305-8572, Japan

Author for correspondence: Email: mssagarbarria@up.edu.ph

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Eggplant (*Solanum melongena* L.) is one of the most important vegetables grown and consumed in the Philippines hence, continuous breeding programs are vital to maintain the supply of this economically important crop. This study demonstrates the first successful *Agrobacterium*-mediated genetic transformation and plant regeneration of the Philippine eggplant cultivar 'PH 11424', also known as 'Mistisa'. Cotyledons from 2-wk-old seedlings were used as explants, which were transformed with disarmed *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector for CRISPR/Cas9 expression and hygromycin phosphotransferase (*HPT*), an antibiotic selection marker. Growth of shoot primordia from the agro-infected explants was observed during selective culture with 7.5 ppm hygromycin, which indicated an initial success in transformation. The putatively transformed shoot primordia were then transferred to an elongation medium. The elongated and hygromycin-resistant shoots were allowed to develop roots in hormone-free medium supplemented with hygromycin, and subsequently acclimatized under greenhouse conditions. The entire process took at least 5 – 6 mo. Of the total 585 agro-infected explants, the regeneration efficiency of rooted shoots was 4.6%. Successful transformation was confirmed by polymerase chain reaction (PCR) amplification of *Cas9*. Acclimatized plants tested positive for both transgenes. The surviving transgenic eggplants successfully reached maturity, flowered and set seed. These results demonstrate a working *Agrobacterium*-mediated transformation and plant regeneration protocols using cotyledons as explants in a Philippine eggplant genotype. These biotechnology tools are critical for the successful application of genetic engineering (GE) and new breeding techniques (NBTs) in eggplant crop improvement.

Keywords: *Agrobacterium*-mediated transformation, eggplant, plant regeneration, *Solanum melongena*

INTRODUCTION

Eggplant (*Solanum melongena* L.) is one of the most important and popular vegetable crops grown and consumed in the Philippines. It is a high-value vegetable crop, with its value reaching PhP 6 billion in 2020 (PSA 2021). It is widely consumed and prevalent in the Filipino diet. Biotechnological applications such as genetic engineering (GE) and new breeding techniques (NBTs) enable the development of improved eggplant varieties, which promises great economic benefit. To hasten the efficiency and process of plant breeding, transgenic approaches have been employed since the mid-1990s to specifically introduce foreign DNA, producing crops with insect resistance and herbicide tolerance (Hartung and Schiemann 2014). Crops harboring the insecticidal protein *CRY, Bt* corn, and *Bt* eggplant have been developed, with the latter recently approved for commercial propagation in the Philippines on October 18, 2022. While the *Bt* eggplant technology was developed through GE, conventional crossing was performed to produce the Philippine *Bt* eggplant, which was a cross between the original event produced in India by the Maharashtra Hybrid Seed Company (Mahyco) and Philippine cultivated varieties (Shelton 2021). Plant transformation and regeneration are critical steps in the application of GE

and NBTs for crop improvement. However, until now, there is no transformation system reported for Philippine eggplant genotypes.

Agrobacterium-mediated transformation in eggplant has been reported numerous times in previous literature (Rotino and Gleddie 1990; Arpaia et al. 1997; Van Eck and Snyder 2006; Pratap et al. 2011; Subramanyam et al. 2013; Yesmin et al. 2014). However, most investigations used genotypes from India or Europe. Genotype is one of the most important factors for determining the capacity of plant tissue to form somatic embryos and then to regenerate into a whole plant (Sidhu et al. 2014). This study reports a simple and efficient procedure for the *Agrobacterium*-mediated transformation and regeneration of a Philippine eggplant cultivar 'PH 11424' with *A. tumefaciens* harboring a binary vector that confers a *Cas9* expression cassette and resistance to the antibiotic hygromycin.

MATERIALS AND METHODS

Time and Place of the Study

This study was carried out at the Institute of Plant Breeding (IPB) Biosafety Level 2 (BL2) laboratory and greenhouse at the University of the Philippines Los Baños from February 2022 to April 2023.

Permissions

All experiments were conducted in accordance with the Joint Department Circular No. 1 Series of 2016 for contained use. The Department of Science and Technology Biotechnology Committee (DOST-BC) issued the corresponding Biosafety Permit No. 2020-0324. The biosafety permit conditions were complied with throughout the conduct of every experiment and associated greenhouse and laboratory activities under the supervision of the UPLB Institutional Biosafety Committee and the DA-BPI Central Post-Entry Quarantine Station.

Plant Material and Growth Conditions

Eggplant seeds of Philippine variety 'PH 11424 'or 'Mistisa' were obtained from the National Plant Genetic Resources Laboratory, Institute of Plant Breeding, UPLB. This modern-bred cultivar was chosen based on its baseline regeneration efficiency from cotyledon explants (around 30%), which was the highest among the other genotypes screened in previous regeneration experiments done without transformation. Plant materials for transformation were grown in vitro. Seeds were surface sterilized with a 10% commercial bleach and 0.02% Tween 20 solution for 20 min with continuous mixing. Seeds were rinsed thrice with sterile distilled water. Afterwards, seeds were dried on sterile filter paper and placed into sterile bottles containing half-strength Murashige and Skoog (MS) medium with 1.5% sucrose, pH 5.8, and solidified with 0.45% sucrose. The cultures were maintained in a growth room at 25°C and under 16:8 photoperiod.

Bacterial Strain and Growth Conditions

Agrobacterium tumefaciens strain LBA4404, an octopine strain with Ach5 chromosomal background (Ooms et al. 1982), carrying a modified binary vector pZH_PubiMMCas9 (Mikami et al. 2015) was used. pZH_PubiMMCas9 was obtained from the Watanabe Laboratory at the Gene Research Center, University of Tsukuba, Japan. pZH_PubiMMCas9 contains an expression cassette for *Cas9* under the control of the maize ubiquitin promoter, an expression cassette for CRISPR/Cas guide RNA under control of the *Arabidopsis thaliana* U6 promoter (*AtU6*) and an expression cassette for *hygromycin phosphotransferase* (HPT), under control of the 35S promoter from the cauliflower mosaic virus (Fig. 1). The plasmid also contains an expression cassette for CRISPR/Cas guide RNA. *A. tumefaciens* strain 'LBA4404' bacteria were grown at 28°C in YEB medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L $MgCl_2$, pH 7.0) with shaking at 200 rpm until they reached an OD_{600} equal to 1.0 (around 20 – 24 h). Bacteria were pelleted and resuspended in ½-strength MS medium with 1.5% sucrose and 100 μM acetosyringone and was used for co-cultivation.

Stable Transformation and Plant Regeneration

Agrobacterium-mediated transformation of eggplant (*Solanum melongena* L. cv. 'Mistisa') cotyledons was carried out. After agro-infection, an established eggplant

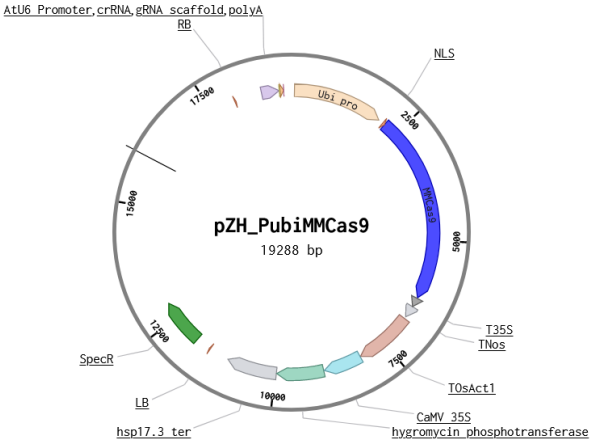

Fig. 1. Plasmid map of pZH_PubiMMCas9.

regeneration protocol using direct organogenesis and zeatin riboside (García-Fortea et al. 2020) was performed with slight modifications to eliminate remaining bacteria and select for transformed cells. All culture conditions were at 25°C and under 16:8 photoperiod. Cotyledons from 2-wk-old seedlings were excised to obtain explants around 0.5 – 1 cm^2 in size; small wounds using the scalpel tip were incised at the adaxial side. Explants were inoculated adaxial-side-down on sterile Petri dishes containing ½-strength MS medium with 1.5% sucrose, 0.45% vitro agar, 2 mg/L zeatin riboside, and 100 µM acetosyringone for 2 d. Afterwards, in a sterile Petri dish, explants were submerged in the *Agrobacterium* solution for 5 min before drying onto a sterile paper towel and transferring back to the same medium again, still adaxial-side-down, for a 3-d co-cultivation period. Explants were washed once with sterile distilled water and then once with a 300 mg/L timentin solution before drying on a sterile paper towel and transferring adaxial-side-up to selective medium (½-strength MS, 1.5% sucrose, 0.45% vitro agar, 2 mg/L zeatin riboside, 100 mg/L timentin, 7.5 mg/L hygromycin, 200 mg/L PVP40). Prior to transformation, a hygromycin sensitivity assay was done to test varying concentrations of hygromycin (0, 5, 7.5, 10, and 15 mg/L), and it was determined that 7.5 mg/L hygromycin was a sufficient concentration that did not allow regeneration of non-transformed explants in a sample size of 30 explants.

Shoot primordia were allowed to develop for 3 – 4 wk. Explants were then transferred to elongation medium (½-strength MS, 1.5% sucrose, 0.45% vitro agar, 0.5 mg/L gibberellic acid (GA_3), 100 mg/L timentin, 7.5 mg/L hygromycin). Shoots were allowed to develop with sub-culturing every 3 wk to fresh elongation medium. Calli that developed were also sub-cultured; calli that already formed shoots were discarded afterwards to avoid propagating multiple shoots from the same cell lineage, for easier management of cultures and subsequent analysis of regenerants. Hygromycin concentration was lowered at this stage to encourage root formation. Shoots around 2 cm in size were transferred to rooting medium (½-strength MS, 1.5% sucrose, 0.45% vitro agar, 100 mg/L timentin, 5 mg/L hygromycin). Shoots were allowed to root twice prior to acclimatization. Culture bottles of rooted shoots were slightly opened and acclimatized in ambient laboratory conditions (approx. 29°C, moderate light) for 1 wk and subsequently in ambient greenhouse conditions for an additional week prior to transplanting. Flowers were self-pollinated by assisted pollination using forceps and then covered with bags. Physiologically mature fruit were harvested 60 d after pollination.

Amplification of Transgenes

Approximately 200 mg of leaf discs was used to extract genomic DNA from transformed and regenerated plants (Paterson et al. 1993). The DNA pellet was resuspended in 40 – 50 µL of TE buffer and stored at -20°C until use. Transgenes were amplified via PCR using the following primers: (MMCas9-F: 5′ – GACCCGCAAGTCTGAAGAAACT – 3′; MMCas9-R: 5′-TCTTGAGGAGATCGTGGTACGT – 3′; HPT-F: 5′ – CGAGAGCCTGACCTATTGCATC–3′; HPT-R: 5′ – GAAATTGCCGTC AACCAAGCTC – 3′). The amplification reaction mixture consisted of 1X buffer (Invitrogen), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 uM of the forward and reverse primers, 1U of Taq polymerase (Invitrogen), and 10 ng of genomic DNA. The thermal profile was: 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min, then 72°C for 7 min. For *HPT* amplification, the annealing temperature was instead set to 56.5°C.

RESULTS AND DISCUSSION

From a total of 585 cotyledon explants, 27 rooted shoots were regenerated resulting in a 4.6% regeneration efficiency ([number of rooted shoots / number of total explants] × 100). Direct shoot organogenesis, indicated by shoot primordia developing directly from the explants, was observed 3 – 4 wk in selective medium (Fig. 2A), even after agro-infection and the presence of 7.5 mg/L hygromycin in the medium. Upon transfer to the elongation medium, shoots elongated and further developed. However, formation of white, friable calli was also observed, which proliferated in the elongation medium. Calli eventually turned brown, but some shoots were also observed to develop from these calli (Fig. 2B). Time for shoot development varied greatly—some shoots that developed directly from the explant tissue started elongating the earliest at 4 wk after transfer to elongation medium. Shoots at least 2 cm in size were transferred to hormone-free medium with 5 mg/L hygromycin, and earliest rooting was observed 8 wk after transfer to this medium (Fig. 2C). For simplicity of discussion, this was noted as a total of 15 wk after transfer to elongation medium. The duration of direct regeneration from explants was estimated to occur between 15 – 21 wk after transfer to elongation medium. This is based on previous regeneration experiments without transformation wherein majority of shoots had rooted within 7 wk after elongation. From plants directly regenerated from explants, it took 5 – 6 mo from agro-infection to obtain fully acclimatized rooted plants. After acclimatization, transformed plants were able to reach maturity, producing flowers and fruit, from which seeds were extracted (Fig. 2D-F).

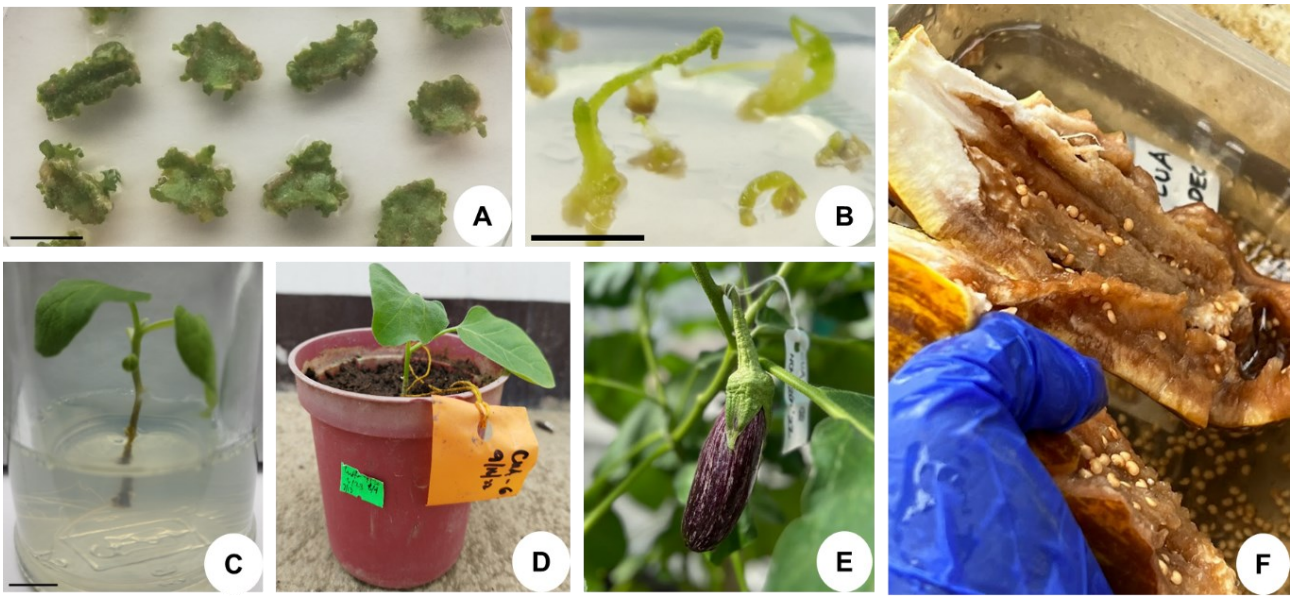

Fig. 2. A) Shoot regeneration from cotyledon explants after *Agrobacterium* co-cultivation, in medium containing 7.5 mg/L hygromycin and 100 mg/L timentin after 3 wk. Scale bar = 1 mm. B) Shoot elongation in elongation medium containing 7.5 mg/L hygromycin and 100 mg/L timentin. Scale bar = 1 mm. C) Rooting of regenerated shoots. Scale bar = 1 mm. D) Acclimatization of rooted shoots in greenhouse conditions and planting in soil. E) Fruit production of a self-pollinated flower in the transformed plants. F) Extraction of seeds from physiologically mature self-pollinated fruits.

In contrast, emergence of new shoots and elongation of extant shoots derived from calli started around 8 wk after transfer to elongation medium. Since the calli were kept in culture, continuous shoot regeneration from calli was observed. Regeneration of call-derived shoots occurred 27 – 40 wk after transfer to elongation medium. The long regeneration time from calli could be attributed to reduced organogenic potential because only gibberellic acid (GA_3) was the hormone present in the elongation medium, and the added stress or selection pressure from the presence of hygromycin (Tran and Sanan-Mishra 2015).

Among the total of 27 rooted plants, 8 or 29.60% were expected to have regenerated directly from explants since they had rooted after a total of 15 – 21 wk after transfer to elongation medium (Fig. 3). The remaining 19 or 70.40% were expected to be calli-derived since they regenerated 22 – 40 wk after transfer to elongation medium.

Since regenerants were directly obtained from explants and indirectly from calli, the performance of the two sources after acclimatization was compared (Table 1).

While more plants were calli-derived, most did not survive the acclimatization process with only 26.30% acclimatization rate. Furthermore, only one out of the five acclimatized calli-derived plants successfully produced fruit. On the other hand, all except one plant derived from direct organogenesis had survived the acclimatization process. From the surviving plants, all but one was able to produce seeded fruit. It is also worth noting that the plant that did not produce fruit had died due to *Phomopsis* rot disease.

Low acclimatization and fruiting percentages from calli-derived plants may be due to tissue culture-induced variation or somaclonal variation from their relatively longer total culture periods. While this was not experimentally verified, tissue culture conditions such as wounding, media, and environment cause oxidative stress and have been known to lead to the accumulation of mutations that ultimately result in somaclonal variation (Cassells and Curry 2001; Gaspar et al. 2002; Ghosh et al. 2021).

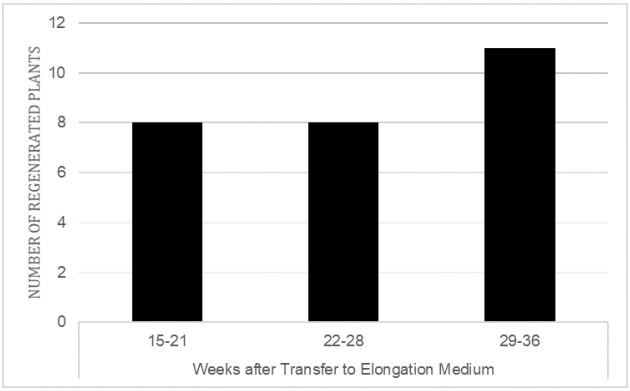

Fig. 3. Number of rooted plants generated over the total time cultures were maintained after transfer to elongation medium. Weeks 15 – 21 represent number of plants obtained from direct organogenesis. Weeks 22 – 28 and 29 – 36 represent number of plants obtained from calli.

Table 1. Number of rooted plants, acclimatized plants, and fruiting plants from explant and calli-derived plants.

Source	Rooted Plants (n)	Acclimatized Plants		Fruiting Plants	
		n	%	n	%
Explant-derived (direct organogenesis)	8	7	87.50	6	85.70
Calli-derived (indirect organogenesis)	19	5	26.30	1	20.00
TOTAL	27	12	44.40	7	58.30

Results of the PCR test detected positive amplification products of *Cas9* and *HPT* from DNA extracted from nine acclimatized plants, which includes the seven fruiting plants, while the wild-type (WT), non-transformed control of 'PH 11424' tested negative for *Cas9* and *HPT* (Fig. 4).

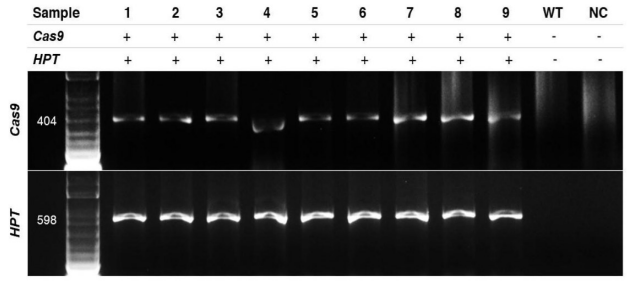

Fig. 4. Amplification of *Cas9* and *HPT* in from nine acclimatized plants. WT = negative control using wild-type PH11424 DNA as template during PCR. NC = PCR mix with no added DNA template.

CONCLUSION

These results describe a successful *Agrobacterium*-mediated transformation and regeneration protocol for a Philippine eggplant genotype. *Agrobacterium*-transformed plants were able to regenerate shoots through both direct organogenesis and an intermediary callus step. While more plants were obtained from calli, these plants had low survival rates during acclimatization. Furthermore, due to the increased likelihood of obtaining somaclonal variations from plants the longer they are cultured in vitro, it is recommended that only shoots from direct organogenesis be kept in future transformation experiments.

While the obtained regeneration efficiency from this study (4.6%) may be improved upon as compared to other protocols (reviewed by Saini and Kaushik 2019), this study reports a working protocol for a Philippine eggplant genotype, with high acclimatization rate and reproductive capability of plants from direct organogenesis and consistent transgene presence in surviving plants. These results will provide a useful platform for future eggplant genetic engineering and genome editing studies, which can ultimately pave the way towards a more robust Philippine Agricultural Biotechnology sector.

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Author Contributions

MGSSagarbarria wrote the first draft of the manuscript. MGSSagarbarria, JAMCaraan, IBMOloc-oloc, and PGLipio performed tissue culture and transformation experiments. KNWatanabe and DMHautea supervised the early stages of this work. MGSSagarbarria, KNWatanabe and DMHautea contributed to study conception. All authors contributed to the revision of the manuscript and approved the final version.

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