Comparison of Particle Bombardment-transformation Parameters Between Stem and Calli Explants of *Aglaonema simplex*

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ABSTRACT

Aquatic plant possess a very interesting stress tolerance mechanism compared to terrestrial plants. However, lack of aquatic mutants available is a hindrance to explore the cellular mechanism that governs various adaptations between these two types of plants. Therefore, the aim of this study is to provide the optimum parameters for the genetic transformation of native aquatic plant in tropical country, *Aglaonema simplex*, by using stem and callus tissue. Explants were bombarded with various sizes of gold particles coated with 35 sec promoter constructed into 35 sec-sGFP-TYG-nos (puc18) plasmid harbouring green fluorescent protein. High transient expression was obtained when the tissues were bombarded twice by gold particles coated with spermidine under acceleration pressure of 1100-1350 psi and with the vacuum pressure set to 24 Hg. The calli were found susceptible to high pressure impact, showing better result at a longer bombardment distance (12 cm) of stopping screen to explants by the use of gold particle of smaller size (1 μm). In contrast, the optimal bombardment distance shown by stem tissues was achieved at a 6 cm distance by the use of gold particle of 1.6 μm. PCR and PCR-southern blot analyses exhibited that the gene was integrated into the genome of transformants and this indicates that the protocol can be used for genetic engineering purpose in the future.

Key words: Green fluorescent protein, tissue culture, bombardment

INTRODUCTION

Aquatic plant occupies almost half of the earth area that is submerged in water. The aquatic plants are able to ‘clean’ up the pollutant in water and are commonly known as important bio indicator (Ferrat et al., 2003). In addition, the difference in stress adaptation ability of aquatic plants (Lacoul and Freedman, 2006) compared to that of terrestrial plants makes aquatic plant a good candidate to study the different stress adaptation mechanisms in plant. Stress mutant can be used to study the stress adaptation pathway in order to improve the stress adaptation ability of an aquatic plant (Mirouze and Paszkowski, 2011). However, the lack of suitable mutant has caused the stagnant development in this area. Hence, the development of an efficient transformation protocol to deliver foreign gene into a typical aquatic plant, e.g., *Aglonema simplex*, provides a useful platform for research into stress signalling mechanism in aquatic plant. The use of conventional plant breeding methods for plant improvement is tedious and time consuming. The particle gun bombardment has been reported to overcome the inherent problems associated with
the use of *Agrobacterium* and the direct transfer of foreign DNA into monocots plant cells. However, to perfect the particle gun bombardment as a powerful transformation tool, the optimization of the DNA delivery conditions is necessary to obtain the stable transformants (Rochange *et al.*, 1995). The variation in the optimized parameters shows that the bombardment transformation parameters are species and tissue specific; hence it is essential to optimize the parameters according to the nature of the specific explants (Tadesse *et al.*, 2003). The physical and biological parameters of particle bombardment have major impacts upon the efficacy of the system (Puddephat, 2003).

Establishment of the optimal parameters for plant transformation by particle bombardment is important, especially for plant tissue that is used for the first time in this operation. There have been no reports on the bombardment transformation method for *A. simplex*. In this study, the optimum physical and biological particle-bombardment transformation parameters on stem and calli of *A. simplex* (Araceae) (or commonly known as ‘Borneo sward’) were investigated. The efficiency of bombardment was measured based on the expression of the GFP expression and the confirmation of DNA integration was proven by southern blotting.

**MATERIALS AND METHODS**

**Plant tissue preparation before transformation:** Two types of explants were obtained from the *in vitro* culture of *A. simplex* plantlets (Aziz Bin Ahmad *et al.*, 2008): (1) Stem tissues (2-3 mm diameter in size) and (2) Calli were subjected to bombardment. Nine pieces of sample (i.e., stem tissue or calli clump) were arranged in each Petri dish containing solid MS (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 30 g L⁻¹ of sucrose, 2.5 g L⁻¹ of phytogel and 3 mg L⁻¹ of benzylaminopurine (BAP). The pH of the media was adjusted to 5.8 before autoclave.

**Plasmid construct and coating onto micro-carrier:** Thirty five second-sGFP-TYG-nos (puc18) plasmid was used in this experiment; the plasmid was provided by Dr Jen Sheen (Harvard Medical School, Boston). The 4.23 kb-plasmid is driven by 35 sec promoter and contains the gfp reporter gene encoding a synthetic GFP (sGFP-TYG) that terminates with 3′ nos terminator (Ponappa *et al.*, 1999). Prior to bombardment, DNA was precipitated onto gold micro-carriers according to the Biolistic PDS-1000/He device instruction manual (BioRad, USA). Gold particles with size ranging from 0.6-1.6 μm were prepared separately by suspension in absolute ethanol (60 mg mL⁻¹) and vigorous vortex for 1-2 min in order to remove the aggregated lumps. The suspension was spun at 10,000 rpm for 1 min and the gold particle pellet was washed twice in 1 mL of distilled water. This was followed by re-suspending the gold particle in 1 mL of distilled water prior to use. An aliquot of 50 μL of suspension was transferred into micro-tubes containing a known volume of DNA solution (2, 4, 6 or 8 μg). Plasmid was coated onto the gold particle by 2.5 M of CaCl₂, 0.1 M of spermidine, or combination of both. The mixture was then vortexed for 3 min and spun for 10 sec at 10,000 rpm and the supernatant was then discarded. The DNA-gold micro-carrier pellet was then rinsed in 250 μL of absolute ethanol followed by re-suspending the pellet in 60 μL of absolute ethanol. A total of 10 μL of DNA-gold micro-carriers aliquot was loaded onto the centre of macro-carrier and the aliquot was air dried prior to bombardment.

**Bombardment parameters:** The physical parameters selected for optimization were includes rapture disc pressure (450, 650, 900, 1100, 1350 and 1800 psi), vacuum pressure (24, 25, 26 and 27 mmHg), distance of target tissue from macrocarrier (3, 6, 9 and 12 cm) and number of
bombardment per tissue (1, 2 and 3 times). The experiments were repeated for three times with three replicates of nine explants in each replicate and the explants bombarded with DNA-free micro-carrier were used as control. Following bombardment, plant samples were incubated under 16 h day night−1 photoperiod at 28°C. GFP expression was observed on day 6 of post-bombardment using a fluorescence microscope (Leica MZFL III) equipped with GFP2 filter (Excitation filter: 480/40 nm) at 25× magnification. GFP score were calculated as the average number of spots observed from 27 replicates of explants.

**Molecular analysis:** DNA was isolated from bombarded tissues expressing GFP using modified CTAB method (Doyle and Doyle, 1990). Then PCR and Southern Blotting were performed to determine the integration of marker gene. The gfp gene was amplified using specific primer, forward 5′ATG GTG AGC AAG GGC GAG GAG 3′ and reverse 5′ TTA CTT GTA CAG CTC GTC CAT 3′. The reaction mixture (25 μL) contained 10 ng μL−1 of plasmid DNA, 2 mM of MgCl₂, 200 μM of dNTP mix, 1X PCR buffer (750 mM of Tris-HCl, pH 8.8, 500 mM of KCl, 0.8% Nonidet P40) and 0.2 Unit Taq DNA polymerase. PCR was performed using the DNA Thermal Cycle (Eppendorf) with initial denaturation steps performed at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing step at 60°C for 35 sec and extension step at 72°C for 40 sec and a final run of cycle at 72°C for 5 min. Electrophoresis was performed using 1% agarose gel in TAE buffer at 56 V for 1 h.

The Southern Blotting and hybridization were performed according to the methods of Sambrook et al. (1989). A 10 μg of genomic DNA was digested with EcoR1 and separated on 1% of agarose gel. DNA fragments were transferred to Zeta-probe blotting membrane, dried at room temperature, then cross-linked with UV. DNA probe specific to gfp gene were labelled with dioxigenin using DIG labelling kit and hybridization was performed for at least 16 h at 68°C in standard hybridization buffer consists of 5×SSC pH 7.0 (0.75 M of NaCl, 75 mM of Na-Citrate dihydrate, 0.1% (w/v) N-lauroylsarcosine salt, 0.01% (w/v) SDS and 1% (w/v) blocking reagent). The membrane was thoroughly washed and processed as described in DIG labelling kit (Bohringer Mannheim, Germany).

**Statistical analysis:** Data analyses were performed using the SPSS ver. 13.0 statistical package (SPSS Inc. USA). Two tailed Student's t-test was performed assuming statistical differences between input variables (callus and stem tissue) if p<0.05. Data are presented as Mean±SD and were subjected to one-way analysis of variance (ANOVA). The mean differences represent the value from three repeated experiments and each experiment was performed with three biological replicates.

**RESULTS AND DISCUSSION**

**Optimization of bombardment conditions**

**Distance of explants to stopping screen:** In stem explants, the highest GFP expression was observed at 6 cm and the expression was found to decrease as the distant increased. In embryogenic calli, there were no significant difference (p>0.05) in GFP expression between the bombardment distance of 6, 9 and 12 cm (Fig. 1a).

**Acceleration pressure:** Generally, the GFP spots were found to increase as the acceleration pressure increased, but they showed a drastic reduction at an acceleration pressure of 1800 psi.
High GFP expression was obtained at 1100 and 1350 psi for both embryogenic calli and stem tissues (Fig. 1b). Moreover, both 1100 and 1350 psi contributed to the similar expression in calli and stem tissues. However, low pressure of 450 psi and high pressure of 1800 psi contributed to very low expression.

**Number of bombardment:** The number of bombardment in a transformation experiment contributes to a similar response in stem tissue and calli (Fig. 1c). Transformation was achieved by one or two times of repeated bombardment but the efficiency dropped almost two fold in triple bombardments (Fig. 1c).

**Vacuum pressure:** The highest number of GFP spot was observed in calli when bombardment was operated at a vacuum pressure of 27 mmHg, whereas low expression was observed at a lower vacuum pressure ranging from 24-26 mmHg (Fig. 1d). This indicates that a vacuum pressure of 27 mm Hg or above is required to achieve better transformation.

**Size of gold particle:** Highest GFP expression observed at gold particle having a size of 1 μm (280 GFP spots) which was approximately 4-fold higher than that of 0.6 μm (74 GFP spots) and 2-fold higher than that of 1.6 μm (135 GFP spots) (Fig. 1e). Interestingly, different expression patterns were observed in stem tissue and the increase in GFP expression was found positively correlated to the increase in the size of the gold particle used (Fig. 1e).

**Coating chemicals:** The result revealed the effectiveness of different coating agent to wrap the DNA onto the microcarrier and the similar response observed in calli and stem tissues suggests that coating agent is not tissue specific (Fig. 1f). The results also showed the importance of coating agent for a successful transformation as shown by low expression level observed in control which no coating agent was used to prepare the microcarries.

**Pre-bombardment incubation period:** Pre-bombardment incubation period is not crucial for different tissue types but the developmental stage are crucial for bombarment as explants exhibit the same amount of expression in both tissues at the same period of pre-bombardment incubation (Fig. 1g). Four days of pre-bombardment incubation period recorded the highest GFP expression.

**Quantity of plasmid DNA:** Figure 1h indicates that the success of bombardment depended on amount of plasmid that was able to adhere to microcarrier while the different types of tissue contributed only to little variation. The GFP expression was found to increase as the quantity of plasmid DNA used increased for both tissue types and at least 8 μg of plasmid is required to achieve the highest GFP expression (Fig. 1h). It was thus speculated that the lower expression observed through the use of little amount of plasmid is due to the loss in DNA over the processing process and only little DNA plasmid that was left coated onto the microcarrier were delivered to the plant tissue. In this study, the number of GFP spot in calli was found higher than that in stem tissue, indicating that calli is more suitable candidate for particle bombardment transformation compared to stem tissue. Due to the high tolerant ability of this plant to ampicillin selection (survive under 500 mg mL⁻¹ ampicillin), the regeneration of transformation tissue were selected only based on green fluorescent expression under microscope and the tissue were extracted and analysed by Southern blot analysis (Fig. 2).
Fig. 1(a-h): GFP expression on *A. simplex* using different bombardment parameters (a) Distance of sample from stopping screen, (b) Acceleration pressure, (c) Number of bombardment, (d) Vacuum pressure, (e) Size of gold particle, (f) Effect of gold particle, (g) Pre-bombardment incubation time and (h) Quantity of plasmid DNA.
Fig. 2(a-b): Molecular analysis of transformant (a) PCR analysis of GFP gene. PCR amplification of the specific 750 bp GFP gene fragment from the putative transformants; lane 1 and 2 from transformed plant, Lane 3 is GFP gene from GFP plasmid and lane 4 is genomic DNA from untransformed plant and (b) Southern blot analysis showing the integration of GFP elements into transgenic plant. Lane 1: Molecular marker, Lane 2-5: Independent transgenic lines from embryogenic calli bombardment, Lane 6-10: Independent transgenic lines from rhizome bombardment and Lane 11: Untransformed embryogenic calli.

The integration of GFP in tissue was further confirmed through standard southern blotting (Sambrook et al., 1989) by using PCR GFP fragment from the plasmid as the probe (Belarmino and Mii, 2000). However, only 4 explants out of 25 GFP expression embryogenic calli examined showed positive result (with single or two hybridizing bands; Fig. 2). One copy of insertion was observed in lane 4 and 5 with approximately 5.5 kbp while two copies of insertion were observed in lane 2 and 3 with approximately 5.5 and 10 kbp, respectively. This indicated that not all the explants were transformed, or in other words, some of the explants only proved transient transformation with the GFP plasmid bombarded into genomic but failed to integrate with the genome.

Meanwhile, only 5 out of 25 positive GFP expression rhizomes examined showed integration into genome. Those integrations, detected in transformed rhizome, were shown in single and multiple gene insertion events of GFP gene (Fig. 2). The single insertion was recorded in lane 6, 9 and 10. Interestingly, the size of single insertion (in between 4-6 kbp) seemed to be different. While two copies of insertion were shown in lane 7 with approximately 5.5 and 10 kbp.

DISCUSSION

It is the foremost requirement to carry out parameter optimization in order to achieve successful and high gene expression level in transgenic plants. This report shows that some of the parameters are tissue specific for gene bombardment of A. simplex which could be a useful piece of information for transformation of aquatic plant of the same family. One of the crucial parameter is alteration of distance from stopping screen to sample resulted to contradict expression level in different tissue. This is most likely due to the different adaptation of the tissue on particle pressure (Rasco-Gaunt et al., 1999). Longer distance could weaken the acceleration power even with the use of same acceleration pressure but short distance caused high acceleration pressure and damage the
tissue when penetrating fragile and soft tissue. This parameter works closely with acceleration pressure; therefore it is not surprise to observe very low expression level when low acceleration pressure was applied. In contrast, the high acceleration pressure is likely to have caused deep penetration and poor distribution of particle on target tissues which may increase the rate of tissue damage and consequently affect the gene expression (Rasco-Gaunt et al., 1999). Considering that the explants used in this study is not a single layer but a clump of cell, a high acceleration pressure coupled with a long target tissue distance could be employed to avoid tissue damage. The combination of 1350 psi of acceleration pressure with a long target tissue distance of 12 cm was found to exhibit the highest transient GUS expression in Coffea arabica (Rosillo et al., 2003).

In this study, increasing the number of bombardment per transformation cause mechanical injuries to the target tissue, thereby decreasing the cells survival with GFP expression. Similar results were reported on buds of banana (Sreeramanan et al., 2005) and embryogenic suspension cells of cassava (Schopke et al., 1997). Nonetheless, the parameter seemed to be species and tissues specific as triple bombardment in cotton transformation was reported to enhance almost 5-fold of gene expression (Rajasekaran et al., 2000) while repeating bombardment in one transformation were found to have no effect in transformation of wheat (Rasco-Gaunt et al., 1999) and oil palm (Parveez et al., 1998).

Microcarriers are very small and light, so the presence of gas could lead to rapid deceleration of microcarriers and therefore the increment of vacuum pressure could enhances their acceleration to the cell (Sanford et al., 1993). However, high vacuum pressure could cause a rapid reduction in the moisture content of the tissue, thereby contributing to the reduction of cell viability (McCabe and Christou, 1993). In this study, the highest vacuum pressure that the stem tissue could tolerate is recorded at only 24 mmHg (Fig. 1d), suggesting that this tissue dried up rapidly with the presence of vacuum pressure and this caused the tissue to be harder for penetration. This result is correlated with the size of gold particle used; to avoid the dried up of the stem tissue, larger gold particle size was found more suitable for stem tissue bombardment. These results reveal that the influence of gold particle size are tissue and species specific; this was also reported in the studies of transformation in embryogenic suspension cultures of cassava (Schopke et al., 1997), oil palm calli (Parveez et al., 1998) and banana (Sreeramanan et al., 2005). The results also suggest that big-sized microcarrier particle is needed to be used for penetration into a hard tissue while small-sized particle could be used to easily penetrate into softer cell without damaging the cell (Schopke et al., 1997). Nevertheless, Hansen and Wright (1999) and Polling and Olesen (2001) have reported that the size of microcarrier particle contributed to no changes in GUS expression in conifer and wheat.

Spermidine seemed to be an effective coating agent for either stem or calli, as the addition of spermidine, either on its own or by combination with CaCl₂, contributed to the highest gene expression level (Fig. 1f). This indicates that spermidine is a suitable precipitation agent to coat DNA onto gold particle in order to avoid the loss of DNA during washing process (Rasco-Gaunt et al., 1999). The desired quantity of plasmid used can then be prepared with spermidine. This study agrees with Hansen and Wright (1999) that increasing the quantity of plasmid DNA resulted in increase efficiency of DNA transfer. Similar results have been reported in oil palm (Parveez et al., 1998) and wheat (Rasco-Gaunt et al., 1999). However, it should be noted that the use of high quantities of DNA could result in aggregation of gold particle, causing uneven distribution of microcarriers on the macrocarrier. This could eventually decrease the efficiency of microcarriers to penetrate into tissue and cause damage to the cells (Humara et al., 1999).
Freshly micropropagated or dissected tissue showed very low expression level. The pre-bombardment incubation is thought to provide the competency and readiness of cells or tissues in accepting and expressing the foreign genes (Rochange et al., 1995). The active dividing cells are the most receptive targets in transformation and the developmental stage of the culture was reported to strongly influence the gene expression (Folling and Olesen, 2001).

CONCLUSIONS

Optimised bombardment conditions for stem disc and calli explant of A. simplex were successfully established. The conditions can be categorised into two distinct groups: tissue specific and non-tissue specific parameters. The non-tissue specific parameters, comprising the use of suitable coating agent, the times of bombardment and the quantity of plasmid DNA, can be applied on both tissues and these parameters were found influenced by the quantity and availability of DNA delivered into plant cell. The mechanical force of bombardment was found crucial for distinct tissue type. This study have shown that vacuum pressure, distance of stopping screen to sample, size of microcarrier and acceleration pressure influenced the stress level to push the microcarrier into different tissue types and hence the yield of different expression levels. The protocol developed for A. simplex was found to successfully produce a stable transgenic plant and the optimized parameters can potentially be used to produce mutant plant for biomolecule study.

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