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EFFECTIVE HYGROMYCIN CONCENTRATION FOR SELECTION OF *Agrobacterium*-MEDIATED TRANSGENIC *Arabidopsis thaliana*

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ABSTRACT

The floral-dip transformation method for *Arabidopsis thaliana* has long been established. Following transformation, an important step is involving the selection for transgenic plants through antibiotics or herbicides. Hygromycin has been widely applied for transgenic plants selection. However, the concentrations used were varied between laboratories mostly in a range of 10 mg/L up to 200 mg/L. In the present study, the hygromycin sensitivity test was performed on wild-type seeds to obtain the most effective hygromycin concentration for selection of the transgenic. A standard curve on average mean of hypocotyls lengths against hygromycin concentrations was constructed. Based on hypocotyls length and leaf colour, the most effective concentration of hygromycin was determined in the range of 20 to 30 mg/L. For screening the transformed seeds, hygromycin concentration at 25 mg/L was used. Non-transgenic plants demonstrated a clear decrease in hypocotyls lengths and no root elongation, as compared to transgenic plants. The identified transgenic seedlings were further verified through polymerase chain reaction (PCR), giving fragment with an expected size of 365 bp.

Key words: *Arabidopsis thaliana*, Hygromycin, *Agrobacterium*-mediated, floral-dip transformation

INTRODUCTION

Arabidopsis thaliana being the first plant to complete its genome sequences, has been a model plant for gene expression study. Its well established *Agrobacterium*-mediated floral-dip transformation method allows the development of transgenic plants without plant tissue culture or regeneration (Clough & Bent, 1998). This technique involves merely a simple immersion of floral inflorescences into *Agrobacterium* inoculation medium. The used of *Arabidopsis* and its transformation method is indeed very useful for gene functional study in plants, particularly in local plants, which many of the species have yet to develop transformation and regeneration systems. For example the *Polygonum minus*, a local aromatic plant, which recently gained huge amount of transcriptomic data from the suppression subtractive hybridization (SSH) library, the expressed sequence tags (EST) library and the cDNA-AFLP transcriptome profiling (Gor *et al.*, 2011; Roslan *et al.*, 2012; Ee *et al*., 2013).

Developing a transgenic plant that harbour the gene of interest allows us to study in depth of a gene's function. The selection step comes after transformation is the most crucial step to identify the transgenic *Arabidopsis* from the non-transgenic. Seeds were cultured on selection plates containing antibiotics or herbicides. Selection is usually based on the plant selectable marker, which is present on the T-DNA, integrated together with the gene of interest into the genome of these transgenic plants. Hygromycin was found in many of the readily available vectors, for example the widely used pCAMBIA series vectors. Even though hygromycin has long been applied for screening of transgenic plants, however, there is no report on single optimum concentration of hygromycin so far. Based on literature searches, the concentrations of hygromycin used range from 10 mg/L up to 200 mg/L (Cho & Cosgrove, 2000; Rivarola *et al*., 2009).

Therefore, in this study, we aimed to determine the most effective hygromycin concentration for the selection of transgenic *Arabidopsis*. Firstly, the effect of different hygromycin concentrations on wild-type seeds, through the hygromycin sensitivity

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test was tested. Subsequently, selection of transgenic plants on the most effective concentration of hygromycin was performed. This optimum concentration allows a more cost-effective, time-saving and less laborious selection method, which also clearly distinguish between the transgenic and the non-transgenic plants.

MATERIALS AND METHODS

Plant Materials

Wild-type *Arabidopsis thaliana* Columbia-0 seeds were used in the hygromycin sensitivity test. For screening, transformed seeds were collected from *Arabidopsis* plants transformed with *Agrobacterium tumefaciens* harbouring pCAMSS vector (Fig. 1). The transformation was done using the well established floral-dip method by Clough & Bent (1998). The floral inflorescences were immersed into the *Agrobacterium* innoculation medium, which consisted of the harvested *Agrobacterium* cells strain GV3101, 5% sucrose and 0.05% silwet.

Hygromycin Sensitivity Test

Hygromycin sensitivity test was done at concentration of 0, 10, 20, 30 and 40 mg/L on the wild-type seeds. The effective concentration was when more than 90% of the wild-type seedlings were affected. Surface sterilization of seeds was performed with the addition of 50% Clorox and a drop of Tween-20 for 10 min, followed by 80% ethanol for 2 min and continued with several rinsing of sterile distilled water before culturing the seeds on each selection plate. Approximately 100 seeds were screened on each 10 cm (in diameter) plate of MS media supplemented with hygromycin. The seeds were spread evenly on selection plate and left to dry in the laminar air flow.

Following the rapid and robust selection method designed by Harrison *et al.* (2006), the seeds were left on selection medium in 4ºC for 2 days and continued with placing the plates under light for 6 h at normal growth conditions. Then, plates were kept in dark for another 2 days before growing in the normal conditions with 16 h day/8 h night. The seedlings grown were observed for hygromycin-sensitive and hygromycin-resistant phenotypes after a week to a month period in normal growth conditions.

Screening on Transformed Seeds

Transformed seeds were screened on MS media supplemented with the selected most effective concentration of hygromycin determined from the hygromycin sensitivity test. These transformed seeds were obtained from the *Agrobacterium*-mediated floral-dip transformation of pCAMSS vector done earlier. Similar surface sterilization and plating method were applied. The phenotypes of these seedlings grown were observed. To further confirm the presence of transgene, standard PCR was done on all four identified transgenic plants. Genomic DNA from leaves was extracted using CTAB DNA extraction method (Clarke 2009). The primers used were designed from the CaMV 35S promoter region (5'-TCCCACTATCCTTCGCAAGACCC-3') and the gene-specific region (5'-AGTGATAGGCAACTCC AAGC-3').

RESULTS AND DISCUSSION

Results showed hygromycin had no effect on the seeds germination. Almost 100% of the seeds were germinated. After one week on selection medium, the wild-type seedlings demonstrated differences in hypocotyls lengths, which is due to the effect of

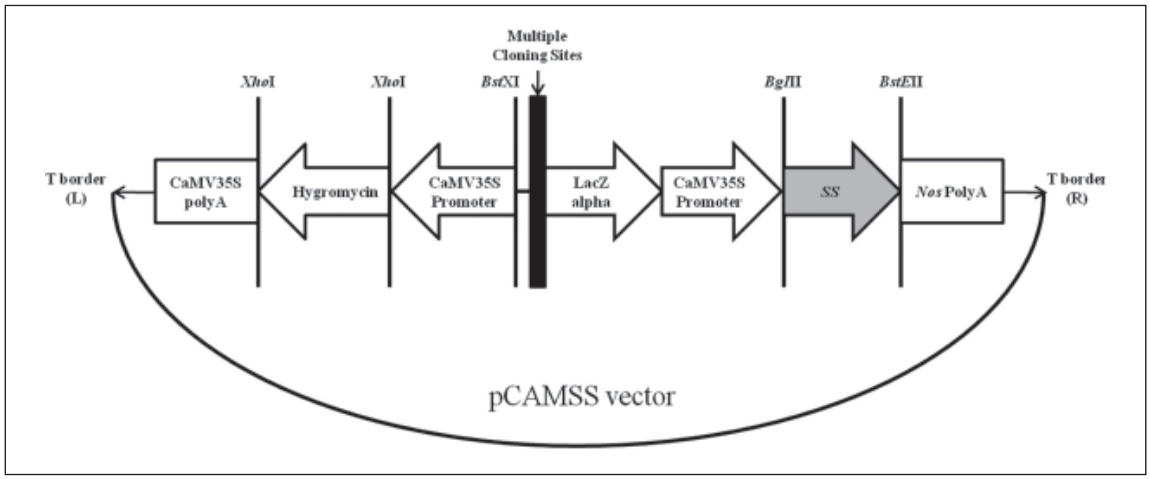

Fig. 1. Schematic diagram of the pCAMSS plant transformation vector. *SS* is the full open reading frame of sesquiterpene synthase gene from *Polygonum minus*.

hygromycin to the hypocotyls growth (Harrison *et al*., 2006). The seedlings on hygromycin showed a significant decreased in hypocotyls lengths to 0.20±0.02 cm, compared to those that were grown on MS basal media (MS0) with 3.21±0.20 cm (Table 1).

The seedlings grew on 20 to 40 mg/L hygromycin selection medium showed similar decrease in hypocotyls length to approximately 0.3 cm. Green leaves were observed at lower concentration of hygromycin. However, as the concentration of hygromycin increase to 20 mg/L, the leaves started to turn lighter green. While in 40 mg/L, the leaves were completely bleached out at 40 mg/L (Fig. 2). This observation allows the researchers to better differentiate transgenic seedlings from the non-transgenic, based on hypocotyls length and also on the difference in leaf colour. Taken into consideration of both hygromycin effects on hypocotyls length and leaf colour, the most effective concentration of hygromycin determined was in the range between 20 – 30 mg/L. It was suggested that 40 mg/L hygromycin is too high for selection process, based on the bleached out phenotype observed.

The concentration of hygromycin at 25 mg/L was tested on the transformed *Arabidopsis* seeds harbouring pCAMSS vector. Differences in phenotypes can be observed after a week on selection plate (Fig. 3a). Hygromycin-resistant seedlings or transgenic seedlings demonstrated longer hypocotyls growth, compared to the non-

Table 1. Effect of hygromycin concentration on the hypocotyls lengths of the *Arabidopsis* seedlings

Hygromycin concentration (mg/L)	Hypocotyls length (cm)			Average mean (cm)
	Replicate 1	Replicate 2	Replicate 3	
0	2.50	3.03	3.43	3.21±0.20[a]
10	0.60	0.77	0.70	0.75±0.04[b]
20	0.50	0.40	0.35	0.41±0.07[c]
30	0.20	0.33	0.22	0.30±0.07[cd]
40	0.30	0.20	0.18	0.20±0.02[d]

*Each replicate represents the mean of the seedlings hypocotyls lengths from 3 selected plates of the same batch.
*Average mean±SE, followed by different small alphabet are significantly different according to Duncan's multiple range test at $P \leq 0.05$.

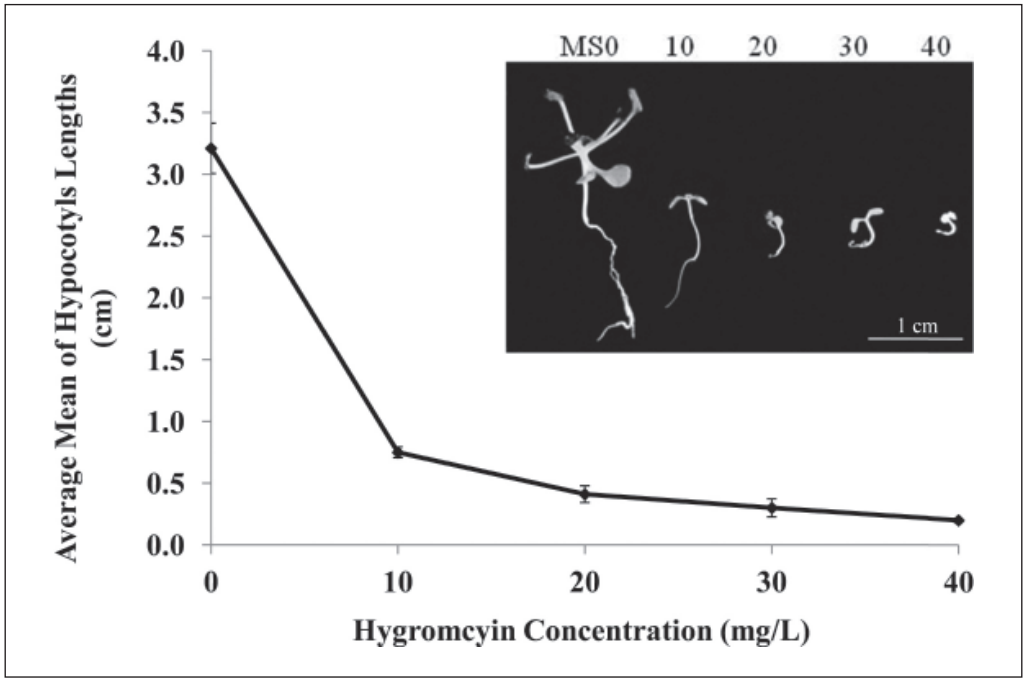

Fig. 2. The average means length of *Arabidopsis* seedlings hypocotyls (cm) in 0, 10, 20, 30, 40 mg/L of hygromycin (Table 1). Embedded image shows the different lengths of hypocotyls obtained under the different concentrations of hygromycin selection.

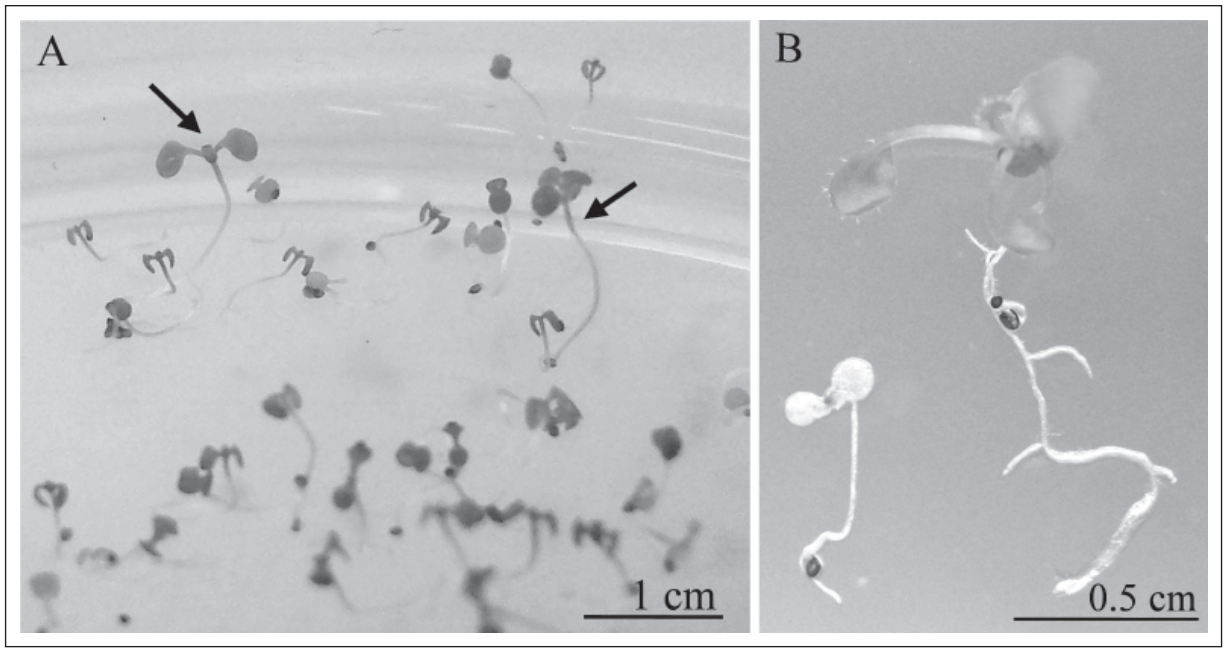

Fig. 3. Comparison between hygromycin-sensitive and hygromycin-resistant seedlings on selection plate containing MS media with 25 mg/L of hygromycin. (A) Seedlings after 7 days of selection. Arrows indicate transgenic seedlings that show longer hypocotyls. (B) The seedlings after 20 days of selection. Left seedling indicates the transgenic seedling with expanded rosette leaves and long roots. Right seedling is the non-transgenic seedling that is sensitive to hygromycin, showing bleached out effect and stopped growing.

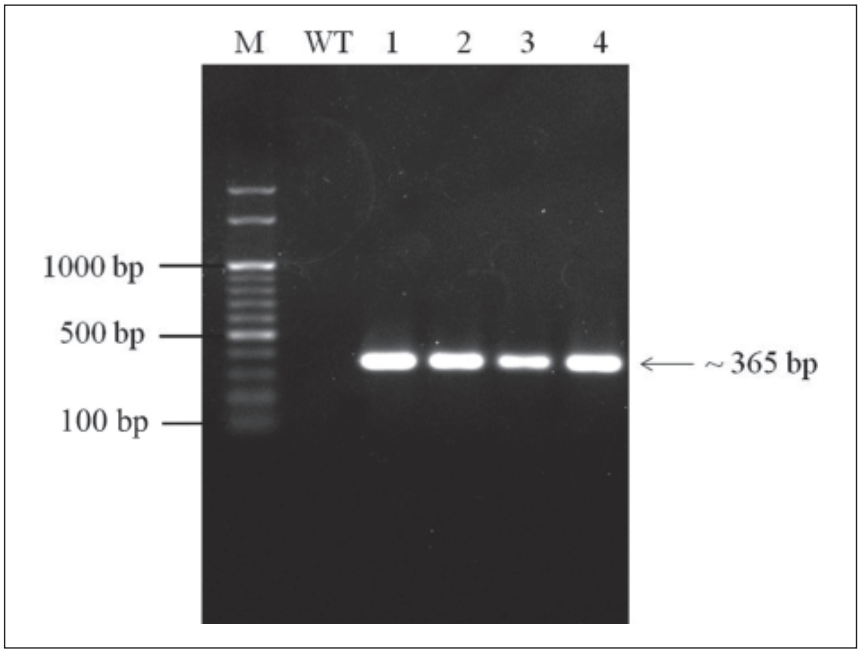

Fig. 4. PCR confirmation of transgene integration in four transgenic plants identified from the selection plates. M: 100 bp DNA ladder; WT: wild-type *Arabidopsis* plant as control; Lane 1 – 4: four different lines of transgenic *Arabidopsis* plants.

transgenic. Formation of expanded rosette leaves and established long roots can be seen when these transgenic seedlings were continued growing on selection plates for a month (Fig. 3b). Meanwhile, all the non-transgenic seedlings turned bleach and stopped growing after a month on selection plates (Fig. 3b). The PCR verification, with the expected product size of 365 bp, further confirmed the presence of transgene in all four transgenic plants identified from the 25 mg/L hygromycin selection plates (Fig. 4).

CONCLUSION

The most effective concentration of hygromycin for selection of transgenic was in the range from 20 to 30 mg/L. The concentration within this range (25 mg/L) was manageable to distinguish transgenic *Arabidopsis* plants from the non-transgenic, which showed a significant decreased in hypocotyls length and bleached out after one month on selection medium.

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