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Hygromycin as selective marker in *Agrobacterium*-mediated genetic transformation of *indica* rice MR 219

(Hygromycin sebagai penanda terpilih dalam transformasi genetik berperantaraan *Agrobacterium* pada padi *indica* MR 219)

A.R. Zuraida*, K. Rahiniza***, A.S. Zulkifli*, Z. Alizah*, Z. Zamri** and A. Aziz****

Keywords: *Oryza sativa* L., somatic embryo, phosphotransferase, plant regeneration, tissue culture

Abstract

The success of *Agrobacterium*-mediated genetic transformation depends on the efficiency of gene delivery, plant regeneration systems and selection agents. The effects of hygromycin B (hyg B), a selection agent that is commonly used in plant genetic transformation, on embryogenic calli and somatic embryos of indica rice *var* MR 219 was evaluated. Embryogenic calli and embryos were cultured on pre-regeneration medium containing hygromycin concentrations from 0 to 200 mg/litre for 12 weeks to determine the lethal dose. The potential of the hygromycin phosphotransferase (*hpt*) gene as the selective marker was also evaluated. The embryogenic calli was co-cultivated with the *Agrobacterium* strain LBA 4404 that harbours the binary vector pCAMBIA1305.2 with the *hpt* gene and subsequently cultured on regeneration medium with added hygromycin concentrations at 0 to 50 mg/litre. Results showed that hygromycin concentrations above 30 mg/litre strongly inhibited growth and development of non-transformed embryogenic calli and somatic embryos. Ten to 20 mg/litre hygromycin concentrations were the most suitable concentrations used for selection of transgenic embryos or plantlets of rice MR 219 with 2.4% efficiency. Hygromycin B is a suitable selection agent and selective marker for genetic transformation of rice MR 219.

Introduction

Rice is a staple food consumed in many countries worldwide. It has been estimated that rice production must be increased by 60% of the present production to fulfil the projected demand in 2020 (Anil and Deepika 2000). To meet the growing demand for rice, biotechnological intervention for its improvement using genetic engineering is becoming increasingly important. Such interventions hinge on the development of efficient and reproducible transformation protocols for agronomically superior and popular rice varieties grown

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
**Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
***Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
****Department of Biological Sciences, Faculty of Science and Technology, Universiti Malaysia Terengganu. 21030 Kuala Terengganu, Malaysia
Authors' full names: Zuraida Ab. Rahman, Rahiniza Kamaruzama, Zulkifli Ahmad Seman, Alizah Zainal, Zamri Zainal and Aziz Ahmad
E-mail: azuraida@mardi.gov.my

in rice-consuming countries (Tyagi et al. 2007). Genetic transformation technology in combination with conventional breeding methods is a powerful tool for improvement of agronomic traits and economic characters in crop plants by incorporating exogenous genes encoding the desired transgenic traits (Sawahel 1997). Two transformation protocols are generally available for rice, namely, *Agrobacterium*-mediated and biolistics.

Agrobacterium-mediated transformation has several advantages, such as higher transformation efficiency, minimal re-arrangement of transferred DNA, integration in low copy numbers, the ability to transfer large pieces of DNA and low cost. *Agrobacterium tumefaciens*-mediated method is commonly used in rice genetic transformations (Lin et al. 2009; Xiao 2009; Lin and Zhang 2005). This method has a remarkable advantage over other transformation methods as it allows preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Olhoft et al. 2004). The transfer and integration of foreign DNA into plant genome are influenced by various factors including plant genotype, type of explants, vector-plasmid design, bacterial strain, phenolic compounds, culture medium composition, damaged tissues and chemicals used in eliminating the *Agrobacterium* after co-cultivation (Klee 2000). The rate of transformed plants is also dependent on the selection system used. The effectiveness of the selection is dependent on tissue types, size of explants, chemical properties and concentration of the selective agents (Bowen 1993). In addition, an efficient and reproducible selection system for transformed cells and tissues plays a key role in ensuring a good transformation system (Meng et al. 2007). A selectable marker gene that confers antibiotic or herbicide resistance is generally incorporated together with the gene of interest in the transformation process to assist the selection of transformants (Sujatha and Sailaja 2005). The two most popular aminoglycoside antibiotic resistance marker genes in plant transformation are neomycin phosphotransferase II for resistance to kanamycin (Flavell et al. 1992) and hygromycin phosphotransferase (*hpt*) for resistance to hygromycin B (hyg B) (Ishida et al. 1996).

Hygromycin B has been successfully applied as a selection agent in a number of crop plants including sorghum (Hagio et al. 1991), *Dendrobium* orchid (Kuehnle and Sugii 1992), maize (Weymann et al. 1993), bent grass, *Agrostis mongolica* (Vanjildorj et al. 2006), a wetland monocot, *Thypa latifolia* L. (Nandakumar et al. 2005), grass, *Brachypodium distachyon* (Vogel et al. 2006) and turf-type perennial ryegrass (Cao et al. 2006). It was also reported that the incorporation of hyg B resulted in good growth and plant regeneration with no albino plants produced in transgenic barley (Hagio et al. 1995). However, the optimal concentration for selection varies between plant species. For example, in the genetic transformations of tomato and cotton, 25 mg/litre (Zubeda and Hamid 2010) and 20 mg/litre of hygromycin (Meng et al. 2007) were used respectively. In the present study, the effects of hyg B concentrations on calli development and plant regeneration of *indica* rice were investigated to determine the suitability of the *hpt* gene as a selective marker for *Agrobacterium*-mediated genetic transformation of rice MR 219.

Materials and methods

Plant materials

Embryogenic calli of *indica* rice (*Oryza sativa* L.) MR 219 was established from matured embryos according to Zuraida et al. (2010). Calli were maintained and grown on MS (Murashige and Skoog 1962) basic salt medium containing B5 vitamins, 1 mg/litre 2,4-D, 10 mg/litre NAA, 30 mg/litre sucrose and 3.5 mg/litre agar. The medium was adjusted to pH 5.7 with 1 M KOH prior to autoclaving. Throughout the experiment, all

cultures were incubated at 25 ± 2 °C in the dark or otherwise mentioned.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens, strain LBA 4404, harbouring the plasmid pCAMBIA 1305.2 (http://www.cambia.org.au/) which carries the *hpt* gene (*Figure 1*) was used in this study. This vector has the *hpt* gene in the T-DNA region which is driven by the CaMV35S promoter and CaMV35S polyA terminator and confers resistance to the antibiotic hygromycin as a plant selection marker.

Incubation of cultures in media containing hyg B

Ten clumps of embryogenic calli (1 cm diameter each) were transferred and arranged on each of the embryogenic calli (EC) induction medium and pre-regeneration (PR) medium (Zuraida et al. 2010) in Petri dishes respectively. The media contained filtered sterilised hyg B at concentrations of 5, 10, 20, 30, 50, 100, 150 or 200 mg/litre respectively. Three replicates for each hyg B concentration were used with 10 petri dishes per replicate. Cultures were incubated at 25 °C ± 2 °C in the dark and sub-cultured onto the same medium at 2-week intervals for 16 weeks. Embryogenic calli growth and browning on EC medium were counted and scored accordingly after 4 and 12 weeks of culture. Meanwhile, somatic embryos produced on PR medium were transferred to a shoot regeneration (SR) medium (Zuraida et al. 2010) for further development. The percentage of embryogenic calli clumps producing somatic embryos and number of shoots regenerated as normal and albino plants were recorded.

Agrobacterium-mediated genetic transformation

Agrobacterium-mediated transformation was carried out by co-cultivation of embryogenic calli with bacteria broth (OD_{600} at 0.2) for 30 min with continuous shaking. Subsequently, the explants were dried on a sterilised filter paper for 1 h, transferred onto co-cultivation medium (Zuraida et al. 2010) and incubated in the dark for 5 days prior to transfer onto selection medium containing hyg B of concentrations 0 to 50 mg/litre. Sub-culture was done on the same medium at 2-week intervals until somatic embryos developed. The somatic embryos were then transferred onto the regeneration medium (Zuraida et al. 2010) and allowed to grow until 1 or 2 plants regenerated. Leaves of

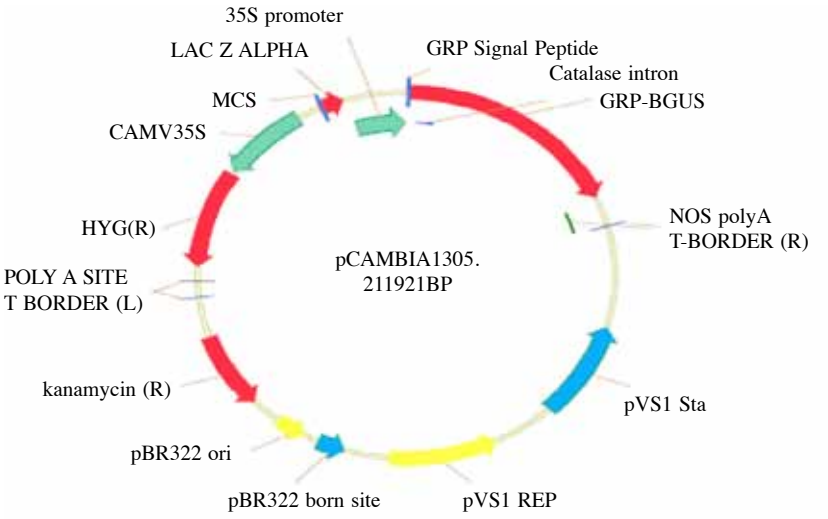

Figure 1. Schematic diagram of the plasmid pCAMBIA 1305.2

regenerated plantlets were sampled and DNA analysis carried out for the presence of the *hpt* gene using PCR.

PCR analysis of transgenic plants

Leaf genomic DNA was extracted using the CTAB method according to Sambrook et al. (1989). At least 2 replicates were used for each sample. PCR was carried out in 25 µl reaction mixtures containing 200 ng genomic DNA, 1.0 µl of 10 µM forward and reverse primers, 1µl of 10 mM dNTP mixtures, 2.5 µl of 10X reaction buffer and 0.5 µl DyNAzyme™ DNA Polymerase (Finnzyme, Finland). For positive controls, 5 ng plasmid DNA was added to the reaction mix.

The primers used were 5'-CGCATAACA GCGGTCATTGACTGGAGC-3' (forward) and 5'-GCTGGGGCGTCGG TTTCCACTATCCG-3^ (reverse). Amplification of the *hpt* gene was carried out in a thermal cycler (Eppendorf) with an initial denaturation at 95 °C for 2 min and 30 cycles of denaturation at 94 °C for 30 s, 72 °C for 30 s (annealing and extension) followed by a final extension at 72 °C for 5 min. The amplified products were separated on a 1% agarose gel in 1X TAE buffer.

Results and discussion

Effect of hyg B on embryogenic calli, somatic embryos and regenerated plantlets

Hygromycin B exhibited negative effect on non-transformed embryogenic calli of rice *var.* MR 219 (*Figure 2* and *Table 1*) after 4 and 12 weeks of culture. The percentage of calli which showed development, drastically declined as the hyg B concentration increased. No calli development was observed at hyg B concentration 100 mg/litre and above (*Figure 2*) (*Plate 1a* and *1b*). Most of the calli turned brown and completely necrotic after a few weeks of culture in hyg B concentrations above 50 mg/litre. At these concentrations, hyg B became toxic to rice calli and necrosis symptoms were observed. The fresh weight of brown calli was 4.3-fold higher than the control after 16 weeks of culture (*Table 1*). Results also showed that hyg B concentration at 10 mg/litre was enough to suppress the growth and development of non-transformed MR 219 embryogenic calli. Hyg B at concentrations higher than 50 mg/litre caused transformed rice callus to turn brown and necrotic (Sujatha and Sailaja 2005). A similar result was reported by Kim et al. (2002) on transformation of *Alstroemeria* where all explants turn brown and necrotic in 20 mg/litre hyg B.

Table 1. Effects of hygromycin on level of browning and fresh weight of embryogenic calli

Hygromycin (mg/litre)	Level of browning*	Fresh weight of browned embryogenic calli (g)**
0 (control)	–	0.90
5	-	1.21
10	+	2.12
20	+	2.67
30	++	2.91
50	+++	3.10
75	++++	3.87
100	++++ (Dormant)	NA
150	++++ (Dormant)	NA
200	++++ (Dormant)	NA

*Data collected after 2 months on PR medium
**Data collected after 4 months on PR medium
NA = no embryogenic calli developed

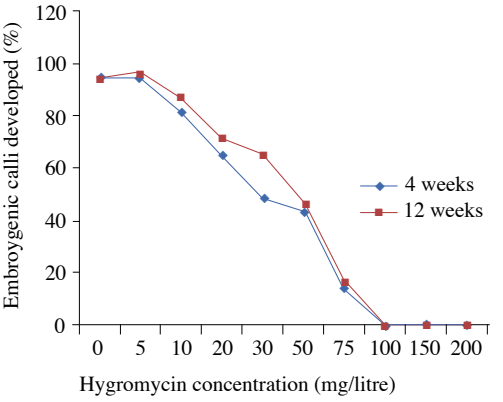

Figure 2. The effect of hygromycin on the percentage of embryogenic calli developed after 4 and 12 weeks culture on callus induction medium

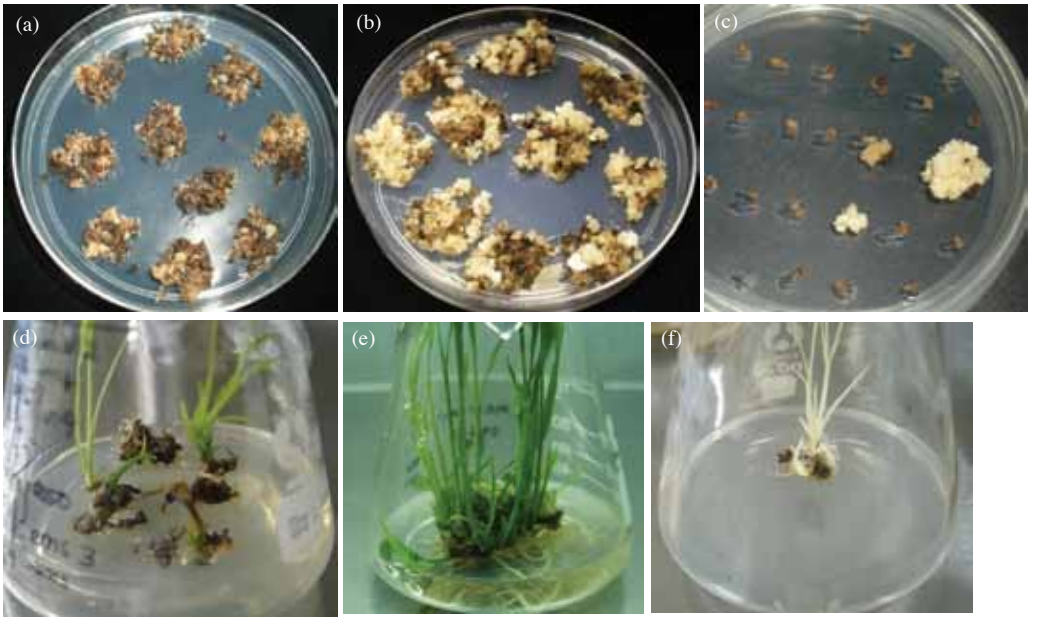

Plate 1. Effect of hygromycin on embryogenic calli, somatic embryos and regenerated plantlets. Brown embryogenic calli on 75 mg/litre (a) and 30 mg/litre hygromycin (b), somatic embryos developed on hygromycin B containing medium (c), regenerated plantlets after co-cultivation with Agrobacterium (d), regenerated control plants on hygromycin-free medium (e) and regenerated albino plant on selection medium (f)

In transformation of tomato (Paramesh et al. 2010; Roy et al. 2006), it was found that hyg B concentration at 25 mg/litre was the optimal lethal dose for selection and less escaped shoots compared to control.

Figure 3 shows the effect of hyg B on somatic embryogenesis of rice MR 219. The percentage of somatic embryos decreased as hyg B concentration increased. In general, somatic embryogenesis was not inhibited by hyg B concentrations below 10 mg/litre but it was strongly inhibited at concentrations above 50 mg/litre. No somatic embryos were obtained at hyg B concentrations above 75 mg/litre (*Figure 3*). Rice plantlets were successfully regenerated at concentrations lower than 50 mg/litre (*Table 2* and *Plate 1d*), but less plants were generated compared to the control (*Plate 1e*). No plantlets were regenerated at hyg B concentrations of 75 mg/litre and above. Hyg B concentrations between 20 and 50 mg/litre resulted in plantlet regeneration with albino characteristics (*Table 2*). It is known that hyg B is one of the aminoglycoside antibiotics which kills plant cells by inhibiting gene transcription and translation (Pipatpanukul et al. 2004). According to Visarada and Sarma (2004), the optimal concentrations for selection varied with plant species where 50 and 20 mg/litre hyg B were efficient for the selection of transformed rice and soybean cells expressing the *hpt* gene respectively.

Transformation and selection of putative transgenic plants

Results showed that the number of somatic embryos, surviving-explants regenerated and transformed plantlets decreased as hyg B concentration increased (*Table 3*). In the control, 213 somatic embryos were produced with no plant regeneration. Interestingly, it was observed that the presence of hyg B assisted plant regeneration. In 5 mg/litre hyg B, 78 somatic embryos were produced

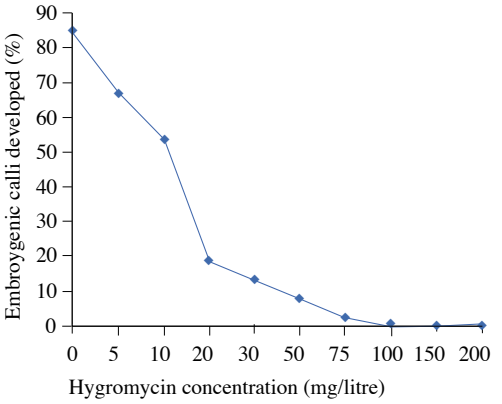

Figure 3.The effect of hygromycin on percentage of somatic embryo produced after 16 weeks culture on pre-regeneration medium

Table 2. Effects of hygromycin on number of regenerated plantlets and albino plantlets

Hygromycin (mg/litre)	Number of regenerated plantlets	Number of regenerated albino-plantlets
0 (control)	59	0
5	36	0
10	18	0
20	16	1
30	11	3
50	8	5
75	0	0
100	0	0
150	0	0
200	0	0

Table 3. Effects of hygromycin on transformation efficiency of indica rice callus

Hygromycin (mg/litre)	Total embryogenic calli co-cultivated with *Agrobacterium*	Number of somatic embryos developed	Number of regenerated plantlets on hyg-selection medium	Number of regenerated plantlets showing presence of *hpt* gene	Transformation efficiency (%) (Based on total calli used)
0	500	213	0	0	0
5	500	78	29	13	2.6
10	500	68	16	12	2.4
20	500	41	11	11	2.2
30	500	21	9	6	1.2
50	500	10	7	3	0.6

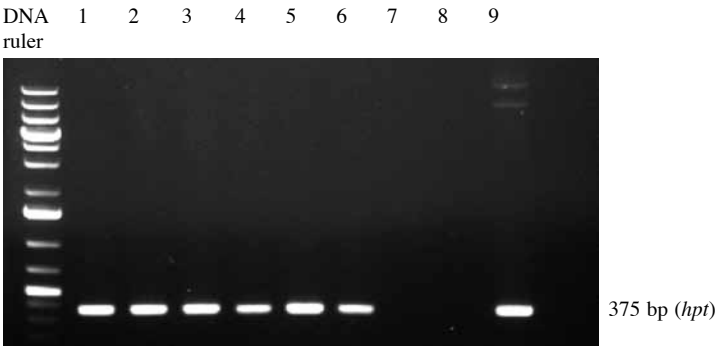

Plate 2. Polymerase chain reaction (PCR) analysis of DNA isolated from regenerated rice plantlets. Lanes 1 to 6 shows the presence of the hpt gene in transgenic plants. A 100 bp DNA ladder served as molecular markers. Non-transformed plants served as negative controls (lanes 7 and 8) while lane 9 (the plasmid pCAMBIA1305.2) served as the positive control

with 37 % plant regeneration, 45% of these regenerated plants showed the presence of the *hpt* gene (*Table 3* and *Plate 2*). Antibiotics at high levels not only killed the non-transformed cells but also inhibited growth of the transformed cells and plants, which delayed plant regeneration (Wilmink and Dons 1993). Generally, low numbers of regenerated plantlets were related to low numbers of somatic embryos produced and low numbers of transgenic plants with increased hyg B concentrations. This finding revealed that escapism of shoot development without carrying the *hpt* gene was not eliminated in the presence of hyg B. The transformation efficiency was found to fluctuate between 0.6 to 2.6% (*Table 3*). Thus, selective agents applied at appropriate concentrations are important in avoiding an undesirable number of untransformed or escape shoots. Cheng et al. (1998) used 50 mg/litre hyg B in the media throughout the callusing stage as well as during the regeneration period to avoid development of the escapes in transformation of the *hptII* gene in indica rice.

Conclusion

Genetic transformation of indica rice MR 219 through calli transformation using *Agrobacterium tumefaciens* was accomplished in this current study. The optimal concentration of hyg B used for transgenic rice selection was defined by looking at different effects of hyg B on regeneration and necrotic events. The number of somatic embryos and regenerated plantlets were strongly influenced by the hyg B concentrations in the culture medium. Moderate hyg B concentrations at 10 to 20 mg/litre were found to be the most suitable concentrations for the selection process of the local rice variety, MR 219. Further work is necessary to optimize the transformation and regeneration protocols in some other cultivars. The protocols will be used for potential genetic modification of this important crop and facilitate the future goal of producing transgenic *indica* rice with specific agronomical traits.

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Abstrak

Kejayaan transformasi genetik berperantaraan *Agrobacterium* bergantung kepada kecekapan penghantaran gen, sistem pertumbuhan semula tumbuhan dan agen-agen pemilihan. Kesan higromisin B (hyg B), suatu agen pemilihan yang biasa digunakan dalam transformasi gen tumbuhan telah diuji ke atas kalus embriogenik dan embrio somatik padi MR 219. Kalus embriogenik dan embrio somatik telah dikultur di atas media pre-pertumbuhan semula yang mengandungi higromisin pada kepekatan 0 ke 200 mg/liter selama dua belas minggu untuk menentukan dos maut. Potensi gen higromisin fosfotransferase (*hpt*) sebagai penanda terpilih juga telah dinilai. Kalus embryogenik telah dikultur bersama dengan *Agrobacterium* strain LBA 4404 yang mengandungi vektor dedua pCAMBIA 1305.2 dengan gen *hpt* dan kemudiannya dikultur di atas medium pertumbuhan semula yang ditambah dengan higromisin pada kepekatan 0 ke 50 mg/liter. Hasil menunjukkan kepekatan higromisin melebihi 30 mg/liter merencat pertumbuhan dan perkembangan kalus embriogenik dan embrio somatik yang tidak ditransformasi. Kepekatan higromisin pada 10 – 20 mg/liter adalah kepekatan paling sesuai digunakan untuk pemilihan embrio atau anak benih transgenik padi MR 219 dengan 2.4% kecekapan. Hyb B adalah sesuai sebagai agen pemilihan dan penanda terpilih bagi transformasi genetik padi MR 219.

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