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Agrobacterium tumefaciens-mediated transformation of the isopentenyltransferase gene in japonica rice suspension cell culture

^{1,3}Alina Wagiran *, ^{1,2}Ismanizan Ismail*, ¹Che Radziah Che Mohd Zain, and ⁴Ruslan Abdullah

¹School of Bioscience and Biotechnology, Faculty Science and Technology, Universiti Kebangsaan Malaysia, Bangi, 43600, Selangor, Malaysia

²Center for Plant Biotechnology, Institute of System Biology (INBIOSIS), Universiti Kebangsaan Malaysia, Bangi, 43600, Selangor, Malaysia

³Department of Biotechnology Industry, Faculty Bioscience and Bioengineering, UTM Skudai, 81310, Johor Bahru, Johor, Malaysia

⁴Plantation Research, Sime Darby R & D Centre, 42960 Selangor, Malaysia

*Corresponding authors: Alina Wagiran: alina@fbb.utm.my; Ismanizan Ismail: maniz@ukm.my

Abstract

The global population is expected to grow from 6 to 8 billion people and rice consumers are projected to increase by 1.8% annually until 2020. Hence, rice production must be increased between 25-45% to fulfill the growing need. Efforts to genetically improve rice for high quality grains are extensively being carried out. The cloning vectors containing the *ipt* gene driven by the glutenin high molecular weight promoter were successfully constructed in pCAMBIA1305.2 and transformed into *A. tumefaciens* LBA4404, which were then used in the genetic transformation of a japonica suspension cell culture. The highest percentage of transformation frequency based on GUS activity was 93% in the variety *Hayahishiki* and 77% in *Nippon Bare* when 200 μ M AS was included in the inoculation media. The highest percentage of GUS activity was 30% in the variety *Fujisaka 5* in the presence of 100 μ M AS. There was no difference in terms of GUS expression when different inoculation times were tested. A twenty minute post-dehydration treatment led to the highest GUS activity in all varieties tested. The inclusion of AS is critical and very important to obtain successful transformation. The sensitivity and response of suspension cells to different hygromycin proved to be easy and facilitated the removal of non-transformed cells. PCR analysis has shown that 2.3% of the putatively transformed rice variety *Nippon Bare* contained the *ipt* gene, while only 2.0% for the *Hayahishiki* variety. The finding of this research shows the potential for rice suspension cells in regeneration and genetic transformation systems by providing continuous explants and could be used as tools to obtain large scale transformation of rice plants via *Agrobacterium tumefaciens*.

Keywords: suspension cells culture, Oryza sativa L., AS (acetosyiringone), Agrobacterium-mediated transformation, isopentenyltransferase gene

Abbreviations : BAP-Benzylaminopurine; NAA-Naphthalene acetic acid; .2,4-D-2,4-dichloroacetic acids; MS media- Murashige and Skoog; HMW-High Molecular Weight

Introduction

Rice is the second most widely grown cereal crop and the staple food for more than half of the world's population. Worldwide, rice provides 27% of the dietary energy supply and 20% of dietary protein. The world's population consumed more rice than wheat or maize in 2001. In the same year, more than 3.1 billion people consumed 100 kg or more of rice, while the world's rice growth rate declined and has also been less than the world rice consumption (Vguyen and Ferrero, 2006). Several factors may contribute to the decline of the growth yield, such as declining productivities in rice production systems, pressures from abiotic and biotic stresses, and increasing production costs and low returns in developing countries. One of the most effective means of addressing the issues in rice cultivation and raising the average yields is through genetic transformation methods. A number of genes whose expression results in cytokinin (CK) production have been isolated and sequenced from various strains of the plant pathogen Agrobacterium tumefaciens that are located on Ti plasmid (tmr, tzs) (Barry et al., 1984), from Pseudomonas syringae pv savastanoi (ptz) (Powell and Morris, 1986) and Rhodococcus fascians (fas1) (Crespi et al., 1992). Each of these genes encodes the isopentenyl transferase enzyme (Akiyoshi et al., 1983) that catalyzes the rate limiting step of the CK biosynthesis pathway, which is the condensation of isopentenyl pyrophosphate and adenosine monophosphate (AMP) to form isopentenyladenosine monophosphate. It has been shown that CKs are involved in aspects of plant growth and development. The effect of the ipt gene in seed development of Nicotiana tabaccum driven by the high molecular weight (HMW) glutenin promoter showed no morphological abnormalities but increased seed weight and protein content (Daskalova et al., (2007). Ma and

Liu (2009) also reported no abnormalities in root development and photosynthetic rates of tobacco transformed with the ipt gene. Cao et al. (2004) also reported significant increases in plant height, panicle length, and total grains per panicle in transgenic rice plants containing the tzs gene (homologous to ipt gene). On the basis of these observations, it has been proposed that an engineered increase in CK concentration at the beginning of seed development would stimulate cell division, enhance seed size, grain filling and ultimately yield. High yield under stress conditions in rice was also recently reported using the OsNHX1 gene driven by actin1D promoter (Touhidul Islam et al., 2009). Agrobacterium-mediated transformation is now a standard technique for genetic modification in diverse plant species (Komari & Kubo, 1999). In the recent years, rice transformation mediated by Agrobacterium has become the most used method for the introduction of foreign genes. Agrobacterium tumefaciens can efficiently transfer a small number of copies of relatively large segments of DNA with defined ends to plant cells with minimal rearrangement and result in stable integration of the transferred DNA into the plant genome (Zupan et al., 2000). The range of host plant species, subspecies, varieties and cultivars has been extended tremendously, and even major cereals, which have been considered outside the host range of bacteria, have been transformed routinely since the mid 1990s (Hiei et al., 1994; Ishida et al., 1996; Cheng et al., 1997). Genetic transformation of rice via Agrobacterium has been widely used following the reports of Hiei et al., (1994) and has been reported successfully in several japonica species and some indica rice (Toki et al., 1997; Yara et al., 2001; Rashid et al., 1996; Aldemita & Hodges, 1996). The frequency of transformation varies with the plant genotype and with the bacterial strains and vectors, since rice was not originally infected by Agrobacterium in outfield conditions (Hiei et al., 1997). Therefore, it is premature to conclude that all cultivars of rice are transformable. Suspension cell culture may be one of the best explant sources to produce a large number of transgenic rice plants, although long term culture can lead to somaclonal variation (Peschke and Philips, 1992). Advantages of using embryogenic cell suspension cultures are that chimeric cells can be avoided if used for transformation, constant availability of competent embryogenic cells and ease for scaling up. Mature seed-derived calli can be used as starting materials for the induction of embryogenic calli and suspension cell cultures. Embryogenic callus suspensions have been used to generate transgenic indica rice by biolistic transfection (Zhang et al., 1996) as well as transgenic japonica rice (Urushibara et al., 2001). However, the transient and stable transformation efficiency was low. The watery condition of suspension cell culture might cause low transformation frequency as reported by Urushibara et al. (2001). Therefore, callus cell suspensions need to be precultured on solid media for several days (Hiei et al., 1997), placed on osmoticum media (Jain et al., 1997) or exposed to air-drying treatments (Urushibara et al., 2001) to obtain a high frequency of transformation. In the present study, we investigate the effect of several factors on the efficiency of transformation using suspension cell cultures.

Methods and materials

Plant Sources

Mature seeds of japonica rice *var Nippon Bare* (10010), *Hayahishiki* (02883) and *Fujisaka 5* (00443) were from MARDI, Penang and stored at 4°C prior to use. The embryogenic callus induction and development of suspension cell cultures were conducted according to Alina et al. (2008). The suspension cells were later used as explants for *A. tumefaciens* transformation.

Construction of Plasmids for Transformation

The glutenin HMW promoter (1358 bp) was amplified by PCR from pHG (Vickers et al., 2006) (Accession No: AY 795083.1) while ipt gene (723 bp) from Ti plasmid Agrobacterium tumefaciens LBA4404 (Accession No: AF242881.1) using Mastermix PCR from Takara, Japan and conducted using Mastercycler Personal PCR (Eppendorf). The Gtn HMW promoter fragments were PCR amplified using the Gtn forward primer (5' CGC AAG CTT CTG CCC AGC AAA 3') and the Gtn reverse primer (5' GCG TCT AGA GAT CTG TAG ATT GGG G3') then digested with HindIII and XbaI (1358 bp). The cycling parameters were 95°C for 5 min and further 1 min for denaturation, annealing at 64°C for 30 sec, extension at 72°C for 1 min 45 sec and final extension at 72°C for 5 min. The oligonucleotides 5' GCG TCT AGA ATG GAC CTG CAT CTA A 3' (forward ipt) and 5' GCG AGA TCTCTA GCT TCA CCT C 3' (reverse ipt) with addition of XbaI and BglII sites at 5' and 3' ends were use for PCR amplification of a 723-bp fragment encoding the ipt gene. The PCR conditions were 95°C, 5 min for the initial denaturation, 95°C, 1 min, annealing at 57°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 5 min. The cycle number for both Glutenin HMW and ipt primers was 30 cycles. Each PCR reaction mixture contained 200 ng of template, 10X PCR buffer, 20 mM dNTP, 1.5 mM MgCl₂, 0.25 U Taq DNA polymerase and 20 pmol of each primer. All the fragments and recombinant plasmid were confirmed by restriction enzyme digestion, PCR and sequencing. Ligation of Gtn HMW promoter into pMR104a was achieved by ligating pMR104a digested at Hind III and Xba I (3595 bp) (pMR104a H-X) with the Hind III and XbaI digested fragment of Gtn HMW (Gtn HMW H-X). The new recombinant plasmid was called pMR104aGtn. The fragment of the *ipt* gene containing XbaI and Bgl II sites at the 5' and 3' ends was ligated into digested pMR104a at the XbaI and BamHI sites to obtain the recombinant plasmid, pMR104aGtnipt. The recombinant plasmid, pMR104aGtnipt was then digested with HindIII and ligated into HindIII digested pCAMBIA1305.2 to obtain pCAMBIA1305.2Gtnipt and finally transferred into A. tumefaciens (Institute Norman Borlaug, UK). The hpt gene was used as selection and GUS gene as reporter gene, while VirC primers were used for amplification of the vir gene of A. tumefaciens. Fig 1 shows the construction of plasmids used in the present study.#

Inoculation and co-cultivation

A single colony of Agrobacterium tumefaciens containing pCAMBIA 1305.2Gtnipt was inoculated in LBG liquid media supplemented with 100 µg/ml streptomycin, 50 µg/mL kanamycin and 25 µg/L ampicillin and shaken at 28°C for 48 h. A 1 mL aliquot of overnight culture was added to 50 mL of LBG medium with the appropriate antibiotics and shaken at 28°C until the OD₆₀₀ reached 0.8. The suspension cells were plated for 3 weeks on N₆3K solidified media before cocultivation with Agrobacterium. An aliquot of 10 mL of suspension bacteria was mixed with 5 mL of rice suspension cell culture supplemented with 200 µM acetosyringone (AS) and shaken at 28°C at 40 rpm for 20 min. The mixture was then left for 5 min before the supernatant was discarded and washed with sterile water containing 250 mg/L carbenicillin for 30 min. Then, the infected explants were blot dried on sterile filter paper and cultured on two layers of sterile filter



pMR104aGtnipt

Fig 1. Schematic diagram for pMR104aGtnipt construction.

paper on top of N₆2.5GAS (N6 basal media, 10 g/L glucose, 150 µM AS and 2.5 mg/L 2.4-D) media and later incubated at 26°C in the dark for 7 days before GUS assays (5-bromo-4-chloro-3-indolyl glucuronide, X-gluc) were conducted. The infected explants were visually examined for the percentage of the blue spots (GUS activity). The procedures were repeated for optimization of transformation parameters. For Agrobacterium transformation, half of the infected explants were subjected to GUS assays and another half were immersed in N₆3K liquid media containing 25 mg/L hygromycin for 2 weeks and later plated on N62.5-50Hg solidified media for 6 weeks. The plates were washed with sterile water containing 250 mg/L carbenicillin if there was over growth of bacteria. The proliferating callus cells on hygromycin plates were transferred onto shoot regeneration media containing 40 mg/L hygromycin for shoot proliferation for 2 months. The putative plantlet with good rooting was later transferred in vermiculate for acclimatization. Each of the experiments contained 3 replicates and was repeated twice.

Optimization of parameters for Agrobacterium-mediated transformation

Three concentrations of AS were included (100, 200 and 300 μ M) in bacterial suspensions during the infection time of 20 min while including no AS for a control to determine the optimal parameter. Infection time (20, 40, and 60 min) was studied with an optimal concentration of AS (200 μ M) for

variety *Nippon Bare* and *Hayahishiki* or 100 μ M for *Fujiska* 5. Desiccation of the explant post-*Agrobacterium* mediated transformation was investigated in the present study where a 10, 20 and 30 minute incubation for post-transformation desiccation was included.

Selection of transformed cells and regeneration of transgenic putative plants

The explants were subcultured in new N₆3K liquid media containing 25 mg/L hygromycin for 2 weeks and later on N62.5P (N₆ media containing 2.5 mg/L 2,4-D) (Chu et al. 1975) plates containing 50 mg/L hygromycin for 3 weeks and cultured in the dark at 26°C. This process was repeated for 2 cycles. The resistant white calli that proliferated from selection media were carefully isolated and transferred to SRM4 medium (Murashige and Skoog (1968) media, 10 g/L sorbitol, 3 mg/L kinetin, 1 mg/L BAP, 0.5 mg/L NAA) for shoot formation for 1 month. Proliferating calli with green spots were cultured on SRM4 for another 2 months and plantlets with good rooting were transferred into pots for acclimatization in a glass house. Histochemical detection of GUS activity was performed according to Jefferson et al. (1987) with 2 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-gluc) as a substrate after 7 days of transformation. Transformed suspension cell cultures, hygromycin resistant calli and plantlets were immersed in an X-gluc solution and incubated for 48 h at 37°C.



Fig 2. The mortality percentage determined nine weeks after the addition of different hygromycin concentrations to rice suspension cells

Standard kill curve for hygromycin

To investigate concentrations of antibiotic suitable for use in the rice transformation method, a kill curve for hygromycin was evaluated. Five mL of two-month-old suspension cells (non-transformed) of variety *Hayahishiki*, *Nippon Bare* and *Fujisaka 5* were plated on N₆ 2.5P media containing different hygromycin concentrations (10, 25, 50, 60 and 80 mg/L) for 3 weeks. After three weeks, the plate was subcultured in new media for another 2 cycles. The percentage of cell mortality was observed at the end of the study.

DNA isolation and PCR analysis of putatively transformed plants

To confirm the existence of transgenes in the putative transformed plants, we performed polymerase chain reaction (PCR). Total DNA from leaves of plants putatively transformed was extracted using Qiagen DNeasy [®] Plant Mini Kit. The sequences of primers used to amplify the fragment of the gene for *hpt* were *HPT* F 5' ACA GCG TCT CCG ACC TGA TGC A 3' and *HPT* R 5' AGT CAA TGA CCG TGT TAT GCG 3', *gus GUS* F 5' CGC CGA TGC AGA TAT TCG TA 3' and *GUS* R 5' ATT AAT GCG TGG TCG TGC AC 3'and *ipt* with Ipt 1 F 5' GCG TCT AGA ATG GAC CTG CAT CTA A3'Ipt 1 R 5' GCG and AGA TCTCTA GCT TCA CCT C 3'. The primers for the Vir gene are VirC F 5' ATC ATT TGT AGC GAC T 3', VirC R 5' AGC TCA AAC CTG CTT C 3'. GoTaq (M8295: Promega) DNA Polymerase was used for the PCR analyses.

Results and discussion

Endogenous hygromycin selection study

Primary studies to find out the lethal doses of hygromycin revealed that calli proliferated on 0, 10, 25, and 50 mg/L hygromycin in all varieties, but complete necrosis was observed at the 60 mg/L hygromycin concentration and higher after 9 weeks of culture. Fig 2 shows that each variety of rice differs in terms of sensitivity to hygromycin doses. Suspension cells were starting to necrose at 10 mg/L hygromycin for *Hayahishiki* (5%) but *Nippon Bare* and *Fujisaka 5* necrosed at 25 mg/L. The mortality percentage of explants was 10% for *Hayahishiki*, 35% for *Nippon Bare*, while for *Fujisaka 5*, 50% on solidified media containing 25

mg/L hygromycin. The calli started to necrose at 50 mg/L hygromycin where the mortality percentage was 50% for the variety Hayahishiki, 62% for Nippon Bare and 90% for Fujisaka 5. However, the calli cultured in media containing 60 mg/L and 80 mg/L hygromycin showed complete necrosis and died after 6 weeks. Since the use of suspension cells in the present study required intensive selection methods, the transformed suspension cells were immersed in liquid N₆3KP medium containing 25 mg/L hygromycin to facilitate the removal of non transformed cells. The study showed that necrotic cells changed from light brown to dark brown after 2 weeks compared to the cells on plates, which took about 9 weeks. The finding shows that this method proved easier and facilitated the removal of non transformed cells early because the hygromycin completely surrounded the cells. Therefore, the transformed cells were cultured on N₆3KP liquid medium for 2 weeks containing 25 mg/L hygromycin and later plated on 50 mg/L hygromycin in the subsequent experiments. In many protocols for Agrobacterium-mediated genetic transformation of rice using hpt as a selectable marker gene, 30-50 mg/L hygromycin is used for selection of transformants (Hiei and Komari, 2006; Toki et al., 2006; Rachmawati et al., 2004) The optimal concentrations for selection varied with rice plant species and were determined empirically. For example, 50 mg/L hygromycin was efficient for the selection of transformed rice cells (Visarada and Sarma 2004; Hamid et al., 2001). The present study examined a lethal dose for suspension cells, which were exposed to different levels of hygromycin. The concentration of 50 mg/L hygromycin was used in the present study as a threshold for selection. In agreement with this, 50 mg/L hygromycin was also reported to be lethal for the callus of *indica* rice var Super Basmati, Basmati 370, 385 and 6129 (Rashid et al., 2001; 1996). However, NERICA cultivars appear to be more susceptible to hygromycin than O. sativa cultivars where 20 mg/L is suitable for selection of transformants, as reported by Ishizaki and Kumashiro (2008). This suggests that the sensitivity of the explants exposed to hygromycin were different depending on cell type and genotype. Therefore, the present study will use liquid media N₆3KP containing 25 mg/L hygromycin for 2 weeks before transferring the infected explants to solidified N62.5P media containing 50 mg/L hygromycin for 9 weeks.

Parameters affecting Agrobacterium-mediated transformation of rice using suspension cell culture

Acetosyringone concentrations

The addition of AS during transformation of suspension cells in the present study varied among rice variety. Inclusion of 200 µM AS during infection results in the highest GUS activity for the variety Hayahishiki (93%) and Nippon bare (77%), while 100 µM for Fujisaka 5 (30%) (Fig. 3). In all varieties tested, the percentage of GUS activity was low for Hayahishiki (4%) and Nippon Bare (2%) while no GUS activity was detected in Fujisaka 5 when no AS was included during infection. Increase of the AS concentration to 300 µM caused the percentage of GUS activity to decline in all varieties tested. The percentage of GUS activity was decreased by 15% (93% to 78%) for variety Hayahishiki, while Nippon Bare GUS activity decreased by 32% (77% to 45%). Variety Fujisaka 5 shows a decline in GUS activity percentage from 30%, 10% and 7% when AS was increased from 100 µM, 200 µM to 300 µM, respectively. Therefore, based on the GUS activity results, in the subsequent experiment AS will be included in the inoculation media at a concentration of 200 µM for variety Hayahishiki and Nippon Bare and 100 µM for Fujisaka 5. In the virulence system of



Fig 3. The percentage of rice suspension cells with GUS activity after infection with inoculation media containing different concentrations of acetosyringone



Fig 4. The percentage of rice suspension cells with GUS activity after different times of infection.

Agrobacterium, unit VirA acted as a sensor of phenolic compounds like AS. Wounded dicot tissues are known to exude phenolic compounds, such as 4-acetyl-2,6-dimethoxyphenol (AS), which activate vir genes present in Ti plasmid (Stachel et al., 1985). However, monocots either do not produce these compounds or if they do, the levels are insufficient to serve as a signal for vir gene induction (Smith et al., 1995). Other than AS, potato suspension cell cultures (psc) are a rich source of phenolic compounds and sinapic acid, which can activate the vir gene and can be used in the transformation protocol (Chan et al., 1993). Several critical factors including Agrobacterium strain and vectors, use of AS for induction of vir genes, competence of the rice genotype, co-cultivation period and conditions, and tissue culture media have been reported to affect transient GUS expression and transformation efficiency (Hiei et al., 2006; Khanna and Raina, 1999). The present studies show that AS played a crucial role for improvement and efficient transformation in all varieties tested. The present study shows that the level of transient GUS activity after co-cultivation with Agrobacterium in all varieties tested varied among genotype (data not shown). Addition of AS in the transformation of Fujisaka 5 variety showed a very low level of GUS activity and no expression observed when no AS was added. The present study shows that GUS activity was different between genotypes. Hayahishiki variety showed highest GUS activity (90%) followed by Nippon Bare (77%), while Fujisaka 5 had the lowest. The present study shows that the inclusion of AS in the inoculation media was critical and important to obtain successful transformation. The addition of AS in co-cultivation medium has been reported to induce vir genes, extend the host range of some Agrobacterium strains, found to be essential for rice transformation, and recommended in most of the monocot transformation protocols (Saharan et al., 2004; Zhao et al., 2000; Hiei et al., 1994). Previous reports show that when AS was omitted, the level of GUS expression was low and stably transformed plants could not be generated in rice (Hiei et al., 1997; Rashid et al., 1996). The same results were observed in the present study for Fujisaka 5 where no regeneration occurred. The present study shows that addition of AS was essential for successful and higher frequency of transformation, but the concentration of AS in co-cultivation medium may vary between varieties of rice as also reported by Saharan et al. (2004). However, Ali et al. (2007) reported that 100-150 µM AS was the optimal range of concentrations giving maximal transformation efficiency using strain EHA 105 irrespective of japonica rice genotype tested. This may be because of the different sensitivity of VirA genes to AS concentrations with respect to plasmid type and bacterial strain (Aldemita and Hodges 1996).

Inoculation time

We investigated different inoculation times (20, 40 and 60 minutes) in the present study. No difference was seen in terms of GUS expression in all varieties tested. The range of GUS expression was between 80 to 90% in all varieties (Fig. 4). A 60 minute inoculation showed the highest percentage of GUS activity (90%) for Hayahishiki and Nippon Bare while 85% for Fujisaka 5. A 40 minute inoculation showed GUS activity at 85%, 88% and 80% for Hayahishiki, Nippon Bare and Fujisaka 5, respectively. Even though 40 minute and 60 minute inoculations slightly increased GUS expression, the cells were prone to bacterial overgrowth. Therefore, subse quent experiments will use 20 minute inoculations as prolonged exposure of cells to Agrobacterium may cause cell death or overgrowth of bacteria. The efficiency of Agrobacterium infection differed by explant type and inoculation. For example, optimal inoculation time for banana suspension cell cultures was 7 days (Huang et al., 2007), 3 days for embryogenic rice suspension cells (Urushibara et al., 2001), and 3 to 4 days for rice callus (Hoque et al. 2005; Chan et al. 1992). The present study showed that a 20 minute infection time resulted in 82% of GUS activity in Hayahishiki, 90% in Nippon Bare and 78% in Fujiska 5 without overgrowth of Agrobacterium. The present study showed that 20 minutes is suitable for infection of Agrobacterium as longer time will cause bacterial overgrowth. In contrast, Ali et al. (2007) reported that using a bacterial density between 0.8-1.0 with a 20 minute infection time affected the proliferation of the callus and transformation efficiency. The same finding was also reported by Kumria et al. (2001) where infection times of more than 15 minutes affected the growth of indica callus, even though the GUS percentage was high (81% to 85%). Previous work shows that inoculation time was different in different plant species and type of explants used. This may be

 Table 1. Efficiency of transformation of A. tumefacies LBA4404 and regeneration of putative transformed plants in three japonica cultivars

Rice Variety	No of explants (A)	Hyg ® (%)	Hyg ®showing regeneration (%)	Plantlet regenerated	GUS +ve plantlet (B)	Efficiency (%) B/A
NipponBare	300	(23.1±1.3) 70/300	(10±0.9) 30/300	30	30	10
Hayahishiki	300	(13.0 ±0.5) 40/300	(8±1.4) 25/300	20	15	5
Fujisaka5	300	(10±1.0) 30/300	(0±0) 0/300	0	0	0



Fig 5. The percentage of rice suspension cells with GUS activity after different dehydration treatment.

because of susceptibility of explants was different to *Agrobacterium* infection.

Post dehydration of explants after infection of Agrobacterium

Variability of GUS expression was observed in all varieties when different dehydration treatments were conducted. Twenty minutes post dehydration results in the highest percentage of GUS activity in Hayahishiki (77%), Nippon Bare (70%) and Fujisaka 5 (30%) (Fig 5). However, long exposure to dehydration treatment decreased the GUS activity and the suspension cells necrosed and finally died. The GUS activity percentage was decreased by 27% for Hayahishiki (77% to 50%), 25% for Nippon Bare (70% to 45%) and 15% for Fujisaka 5 (30% to 15%). These data show that post dehydration treatment after infection had negative effects on the cells based on GUS activity. A significant factor that enhances transformation of monocot species is desiccation of explants prior to or post Agrobacterium infection. It is unclear to researchers what factors were affected by air-drying, but it is possible that plasmolysis or wounding may be important. Post-dehydration treatment for 2 to 3 days after Agrobacterium infection increased GUS activity in mature wheat embryo as reported by Ding et al. (2009). Urushibara et al., (2001) reported that air-drying calluses derived from rice suspension cultures prior to infection for 10-15 min increased the transformation efficiency 10-fold or more as compared to the control. However, air-drying pre-cultured immature embryos of wheat prior to inoculation did not have the same effect as in sugarcane and rice. Desiccation of pre-cultured immature embryos, suspension culture cells, embryogenic calluses of wheat and embryogenic calluses of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, leading to increased stable transformation frequency (Cheng et al.,



Fig 6. (A) GUS expression (blue color) on in vitro putative transformed plantlets (B). Shoot proliferated on SRM4 containing 40 mg/L hygromycin (C). Putatively transformed rice plants in polybag.

2003). Although the molecular mechanism of desiccation during co-culture remains unclear, it is known that desiccation significantly suppresses the growth of *Agrobacterium* (Cheng et al., 2004). The same results were observed in the present study. The present study shows that the post-dehydration treatment did not benefit the explants and shows that this factor was not critical, compared to inclusion of AS. Based on the present study, it can be concluded that dehydration treatment after infection of *Agrobacterium* depends on species, type of explants and variety.



Fig 7. PCR analysis of the presence of the genes ipt gene in plant regenerated from rice suspension cells inoculated with *A. tumefaciens*. (A) Variety Nippon Bare. Lane 1: negative control, lane 2: untransformed, lanes: 3,4,6,7,8,9,10: Positive ipt; lane 11: pCAMBIA1305.2Gtnipt, lane 12: pMR104Gtnipt. (B) Hayahishiki. lane 1: untransformed, lanes: 3,5,7,10: Positive ipt; lane 12: pCAMBIA1305.2Gtnipt M: Molecular marker, 1kb

Regeneration of putative transformants and PCR analysis

Resistant suspension cells that proliferated to white calluses were isolated carefully and transferred to SRM4 containing 40 mg/L hygromycin, while untransformed calli were discarded. GUS assay for in vitro plantlets shows blue (Fig. 6A) and later developed into shoot (Fig 6B) and plantlets were transferred into polybag (Fig 6C). Hygromycin-resistant calli were obtained in all three varieties examined, but genotype differences were found in the frequencies of both hygromycin resistance and GUS positive calli formation (Table 1). The frequency of calli resistant to hygromycin that developed into shoots was different among varieties tested where Nippon Bare showed 10%, 8% for Hayahishiki and 0% for Fujiska 5. Transformation efficiency in the present study was 10% (30/300) in Nippon Bare, 5% (15/300) in Hayahishiki and 0% in Fujisaka 5. The overall transformation efficiency of the variety used revealed that Nippon Bare had a high transformation efficiency compared to others. Similar results were also reported where Rachmawati et al. (2004) showed that the range of transformation efficiency was 10% to 16% when Nippon Bare calli were infected with A. tumefaciens strain LBA4404. The differences in the transformation efficiency among the cultivars are likely due to differences in the sensitivities of the genotypes to Agrobacterium infection and in the regeneration frequency. As reported earlier, Agrobacterium-mediated transformation of higher plants is highly dependent upon species, genotypes and competency of the target plant tissue, host recognition and other factors (Hiei et

al., 1994; Toki et al., 1997). Presence of Ti plasmid in the genomic DNA of putatively transformed plants will lead to false positive results thereby making conventional PCR unconvincing unless stable incorporation of the transgene in the plant genome is verified. To overcome these problems, in the present study the DNA genome was isolated from Agrobacterium-free suspension cells after being maintained on antibiotic-free medium (except hygromycin) for more than 3 months, while the suspension cells were washed with carbenicillin to remove the excess Agrobacterium. PCR amplification of the virC gene from the genomic DNA of putatively transformed plants did not result in a positive band. The presence of the transgene encoding *ipt* in the genome of these plants was confirmed by PCR analysis (Fig. 7). Of 15 putative plants from the Nippon Bare variety tested for ipt gene, only 7 showed a positive band while Hayahishiki showed only 4 putative plants. This shows that the presence of the *ipt* gene in the genome of putative plants was low, which was 2.3% for Nippon Bare and 2.0% for Hayahishiki. The percentage of transformation based on GUS activities in regenerated plantlets was 10% or less and may be because interaction of factors even the optimization of parameters has been included. In addition, the size of explants was so small and isolation of transformed cells was extremely difficult. The use of suitable Agrobacterium strains may improve the transformation efficiency as reported in earlier studies where the percentages were double when strain EHA105 was used in the callus of Nippon Bare (Rachmawati et al., 2004). Direct use of cells in suspension cultures for

co-cultivation gave low frequencies of transient expression of GUS activity and therefore stable transformation, even though such cells are actively proliferating (Hiei et al., 1996). In contrast, the transient expression of gus gene was high in the varieties tested, although the stable expression study was not included in the present study to compare to Urushibara et al. (2001). Urushibara et al. (2001) reported GUS activity up to 20%. In the present study, the incubation of suspension cells on the N₆3K solidified medium for three weeks before Agrobacterium co-cultivation caused the cells to proliferate rapidly and adapt to new conditions. The present study also showed that the choice of rice variety used in transformation was important where Fujisaka 5 showed a poor response compared to Nippon Bare and Hayahishiki. This study emphasizes the importance of the tissue culture conditions for transformation efficiency and demonstrates that the success in genetic transformation studies depends on the genotypes and tissue culture conditions. To validate the finding in the present study, Southern blot analysis of DNA from the endosperm needs to be included. Therefore, the Agrobacterium-mediated transformation system might be used in japonica rice from suspension cells culture and could be used as a platform to generate large scale production of transgenic plants. However, before the system could be used widely, some of the criteria affecting transformation and tissue culture conditions should be investigated. The effect of ipt gene on plant growth and development, such as plant height, panicle length, ripening date, total grains per panicle, seedsetting rate and 1000 grain filled per panicle should be investigated in the future study. More detailed studies involving prolonged expression and inheritance of the transgenes, along with the breeding progress, must be done to obtain a new plant type with increased yield potential.

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