Plant Biotechnology 27, 47–58 (2010)

Original Paper

A high-throughput *Agrobacterium tumefaciens*-mediated transformation system for molecular breeding and functional genomics of rice (*Oryza sativa* L.)

Ashok K. Shrawat*, Allen G. Good

Department of Biological Sciences, G-425, Biological Sciences Centre, University of Alberta, Edmonton, Alberta T6G 2E9, Canada
* E-mail: ashrawat@ualberta.ca Tel: +1-780-492-5992 Fax: +1-780-492-9234

Received August 27, 2009; accepted November 6, 2009 (Edited by K.Hiratsuka)

Abstract *Agrobacterium tumefaciens*-mediated transformation is the preferred method for genetic transformation of rice. Here we provide a comprehensive and high-through-put protocol for *Agrobacterium*-mediated transformation of rice for generating the large numbers of transgenic plants that are required for functional analysis and for evaluating traits of agronomic importance. In a project designed to produce nitrogen use efficient rice plants, we refined/improved several factors including optimization of conditions for inducing *vir* genes prior to transformation, infection and co-cultivation medium for better interaction of target tissues with *Agrobacterium*. These optimizations supported not only the survival of co-cultured calli in high frequency, but also the production of multiple resistant cell lines per co-cultured embryogenic and nodular callus. In addition, improvements in plant tissue culture, selection and regeneration media has enabled us to produce large numbers of transgenic rice plants containing genes of agronomical importance. Partial desiccation and ABA treatment to hygromycin-resistant callus tissue significantly ($P<0.05$) enhanced both regeneration frequency and regeneration of transgenic plantlets per callus tissue. Regeneration frequency was further improved by optimizing the concentration of copper sulphate in the regeneration medium. The majority of the transgenic plants obtained using this improved protocol displayed a normal phenotype and the gene of interest was inherited by the progeny in a Mendelian fashion. Homozygous plants of selected lines showed stable phenotypes both in soil and hydroponic conditions even after five generations. Availability of such a high-throughput *Agrobacterium*-mediated transformation system will improve future opportunities for rice genetics and functional genomics study.

Key words: *Agrobacterium tumefaciens*, genetic transformation, rice, transgenic plants.

Reliable and highly efficient technique to genetically transform important crop plants including rice is required for comprehensive functional gene analyses and molecular breeding programs. *Agrobacterium*-based systems and biolistic transformation have both been successfully used in genetic transformation of cereals (reviewed by Altpeter et al. 2005, reviewed by Shrawat and Lörz 2006). Although the method of introducing DNA into cells using microprojectile bombardment has revolutionized the field of genetic transformation of crop plants, a major drawback of this system is the considerable variation seen in stability, integration and expression of the introduced transgene (Kohli et al. 1999). The *Agrobacterium*-mediated transformation system, on the other hand, facilitates the integration of a small number of gene copies into the plant genome and shows a greater degree of stability for the transgene (Dai et al. 2001, reviewed by Shrawat and Lörz 2006). Therefore this method has become more popular since the first production of transgenic rice plants in the mid 1990s (Hiei et al. 1994).

In rice, Hiei et al. (1994) provided unequivocal evidence for stable transformation of a Japonica cultivar with *Agrobacterium* after molecular and genetic analysis of large numbers of R_0, R_1 and R_2 progeny of transgenic plants. Using the same strategy or with modifications, in recent years, a number of laboratories have successfully produced transgenic rice plants (Bajaj and Mohanty 2005). Several factors influencing *Agrobacterium*-mediated transformation of monocotyledonous plants including cereals have been elucidated (reviewed by Cheng et al. 2004; Shrawat and Lörz 2006). Although high transformation efficiency has been obtained by using immature embryos for co-cultivation, this technique has many limitations, such as non-availability of immature embryos throughout the year and laboriousness. While transformation of immature embryos gives excellent results, it is more expensive than transformation of calli induced from mature seeds and therefore, *Agrobacterium*-mediated transformation of

This article can be found at http://www.jspcmb.jp/

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

rice using mature embryo-derived calli is considered the method of choice for most laboratories (Hiei et al. 1994, Sallaud et al. 2003, Yang et al. 2004, Shrawat et al. 2008).

The most critical step in using mature seeds as the starting material for genetic transformation is the induction of high quality embryogenic calli suitable for transformation. The response of rice seeds to callus induction varies from genotype to genotype. Another drawback to this system is that a large number of highly embryogenic calli is required to produce large numbers of independent transgenic plants. Therefore, with the aim of producing large numbers of transgenic rice plants for our nitrogen use efficiency project, we have developed a system that not only supports the production of highly embryogenic nodular calli and a better interaction of *Agrobacterium* with target tissue but also promotes a high frequency of plant regeneration. In cereal tissue culture, including rice, a number of factors, such as the effect of ABA on callus induction and plant regeneration, optimization of micronutrients in tissue culture and regeneration medium and the effect of partial dehydration of calli on plant regeneration have been studied (Rancé et al. 1994, Sahrawat and Chand 1999, Dahleen and Bregitzer 2002, Wagiran et al. 2008, Suprasanna et al. 2008). However, these parameters have not been tested to increase the regeneration frequency from transformed calli, which is considered crucial for the success of genetic improvement of a crop plants through transgenic studies. Here, by optimizing the factors considered important for high frequency plant regeneration, we demonstrate that a large number of transgenic rice plants can be obtained within a short period of time for functional genome analysis and molecular breeding purposes.

Materials and methods

Plant material, growth conditions and plasmid constructs

Mature seeds of rice (*Oryza sativa* L. cv. Nipponbare) were used in transformation experiments. Seeds were sterilized in 70% ethanol for 1 min and 5–10 min in 50% (v/v) bleach (5.25% sodium hypochlorite) and finally rinsed 4–5 times with sterile distilled water. Following sterilization, mature seeds were cultured either on MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968), N6 (Chu et al. 1975), NB (a combination of N6 major salts and iron source + B5 minor salts and vitamins) or MNB callus induction medium (a combination of N6 major salts and iron source + MS iron source + B5 minor salts and vitamins) (Table 1) and incubated for three weeks in the dark at 28°C. After three weeks, embryonic scutellum developed a clump of compact primary callus, 0.5 to 1 cm in size. Some of these primary calli formed several small pieces of highly embryogenic spherical nodules at the explant/medium interface. These spherical nodules, which were 1 to 1.5 mm in size, converted into 3–5 mm long embryogenic nodular units when transferred onto fresh MNB medium. Primary calli were kept in the original plates and used to 'harvest' more embryogenic units. These 3–5 mm spherical units, with a rough and yellowish surface were finally harvested and transformed using an *Agrobacterium* strain, EHA105 (Hood et al. 1993), harboring the binary vector pCAMBIA1300-*OsAnt1*/*AlaAT* (T-DNA containing the *AlaAT* (alanine aminotransferase) gene under the control of a tissue specific promoter *OsAnt1* (*Oryza sativa* antiquitin 1 promoter) and *hpt* (hygromycin phosphotransferase) gene under the control of *CaMV 35S* (cauliflower mosaic virus) promoter, or pCAMBIA1305.1-*OsAnt1*/*gus*^{Plus} (T-DNA containing the *gus*^{Plus} (β-glucuronidase) and *hpt* genes under the control of *OsAnt1* and *CaMV 35S* promoters, respectively. These constructs were made as described previously (Shrawat et al. 2008). Transformation vectors, *OsAnt1*/*AlaAT* and *OsAnt1*/*gus*^{plus}, were introduced into *Agrobacterium tumefaciens* strain, EHA105, separately by triparental mating.

Preparation of Agrobacterium inoculum for rice transformation

Four days prior to co-culture, a piece of *Agrobacterium* glycerol stock containing the transformation vector was used to inoculate 50 ml of liquid LB medium containing appropriate antibiotics. *Agrobacterium* cultures were grown overnight at 28°C in a shaker at 220 rpm. The next day, 200 μl of each bacterial culture was spread onto an AB medium plate amended with the appropriate antibiotics (Chilton et al. 1974). The plate was incubated at 28°C for three days, during which a thick layer of *Agrobacterium* was formed. After 3 days, *Agrobacterium* cells were collected using a sterilized microspatula and suspended in *vir* genes induction medium (1×AB salts, 2 mM phosphate buffer (pH 5.6), 50 mM 2-(4-morpholino)-ethane sulfonic acid (MES), 0.5% glucose and 100 μM acetosyringone (Gelvin 2006). *Agrobacterium* cultures in induction medium were incubated at room temperature on a shaker (~50 rpm) for 12–14 hrs. After pre-induction, the *Agrobacterium cells* were pelleted by centrifugation at 9000g for 5 min and suspended in liquid co-culture medium (AAR2B-CL, Table 1) at a density of 0.5×10^9 colony forming units (OD = 1.0 at 600 nm) for transformation.

Agrobacterium-mediated transformation of embryogenic spherical nodules

For transformation, embryogenic spherical nodules were immersed into 25 ml of AAR2B-CL medium containing *Agrobacterium* cells at the density of 0.5×10^9 cells ml^{-1} (OD_{600} = 1) in a 100 mm-diameter Petri dish for 10–15 min. Embryogenic spherical nodules were then blotted dry on sterilized filter paper, transferred to a Petri dish containing solid co-culture medium (AAR2B-CS, Table 1) and incubated for three days at 25°C in the dark. AAR2B-CS medium was used in experiments after optimization of the co-culture medium by calculating the frequency of spherical nodules showing GUS expression, and browning and necrosis after co-cultivation on N6, B5, AA (Toriyama and Hinata 1985), R2 (Ohira et al. 1973) or NB medium. Co-cultured embryogenic calli were then transferred to resting medium (R2S-I, Table 1) and incubated at 28°C in the dark for a week. After a week, uncontaminated embryogenic units were individually transferred to selection

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Table 1. Medium used for callus induction, inoculation, co-culture, resting phase, selection, regeneration and rooting.

Medium[a]	Composition
MNB Rice Callus Induction Medium (filter sterilize)	N6 macrolements (Chu 1975)+MS iron source (Murashige and Skoog 1962)+B5 microelements and vitamins (Gamborg et al. 1968) +3AA (100 mg l^{-1} L-tryptophan +500 mg l^{-1} L-proline +500 mg l^{-1} L-glutamine) +500 mg l^{-1} casein hydrolysate +2.0 mg l^{-1} 2,4-D +0.5 mg l^{-1} picloram +0.1 mg l^{-1} BAP +2.0 mg l^{-1} ABA +30.0 g l^{-1} sucrose, pH 5.8, 0.3% gelrite
AAR2B-CL Rice Liquid Co-Culture Medium (filter sterilize)	R2 macroelements, microelements and iron source (Ohira et al. 1973)+AA amino acids (Toriyama and Hinata 1985)+B5 vitamins +0.25 M glucose/l +200 μM acetosyringone +0.5 g l^{-1} casamino acids +10 mM MES buffer, pH 5.2 +50 mM KHP buffer, pH 5.2 +2.0 mg l^{-1} 2,4-D +0.5 mg l^{-1} picloram +0.1 mg l^{-1} BAP, pH 5.2
AAR2B-CS Rice Solid Co-Culture Medium (filter sterilize)	AAR2B-CL medium except 0.25 M glucose/l was replaced with 20.0 g l^{-1} sucrose and 10.0 g l^{-1} glucose and medium was solidified with 0.4% gelrite
R2S-I Resting Phase Medium (filter sterilize)	R2 major and minor salts, vitamins and iron source +0.25 M sucrose +0.5 mM acetosyringone +10.0 mM MES buffer, pH 5.0 +50.0 mM KHP buffer, pH 5.0 +10.0 mM $CaCl_2$ +3AA +2.0 mg l^{-1} 2,4-D +0.5 mg l^{-1} picloram +0.25 mg l^{-1} BAP +250 mg l^{-1} cefotaxime +250 mg l^{-1} amoxicillin +30.0 g l^{-1} sucrose, pH 5.0, 0.3% gelrite
AAR2BS Rice-Selection Medium-I (filter sterilize)	R2 macro and microelements and iron source+AA amino acids+B5 vitamins+3AA +30.0 mg l^{-1} sucrose +3.0 mg l^{-1} 2,4-D +0.5 mg l^{-1} picloram +0.1 mg l^{-1} BAP +50.0 mg l^{-1} hygromycin +250 mg l^{-1} cefotaxime +250 mg l^{-1} amoxicillin +30.0 g l^{-1} sucrose, pH 5.8, 0.3% gelrite
MNBS Rice-Selection Medium-II (filter sterilize)	MNB medium without ABA+50.0 mg l^{-1} hygromycin +250 mg l^{-1} cefotaxime +100 mg l^{-1} amoxicillin +30.0 g l^{-1} sucrose, pH 5.8, 0.3% gelrite
PRN Rice-Pre-Regeneration Medium (filter sterilize)	MNB medium without 2,4-D and picloram +5.0 mg l^{-1} ABA +2.0 mg l^{-1} BAP +1.0 mg l^{-1} NAA +50.0 mg l^{-1} hygromycin +100 mg l^{-1} cefotaxime +50.0 mg l^{-1} amoxicillin +30.0 g l^{-1} sucrose, pH 5.8, 0.4% gelrite
RN Rice-Regeneration Medium (filter sterilize)	PRN medium without ABA+concentration of BAP was increased to 3.0 mg l^{-1} and concentration of NAA was decreased to 0.5 mg l^{-1} +30.0 g l^{-1} sucrose, pH 5.8, 0.4% gelrite
R Rice-Rooting Medium (autoclave/filter sterilize)	$^1/_2$MS +100 mg l^{-1} cefotaxime +50 mg l^{-1} amoxicillin +30.0 g l^{-1} sucrose, pH 5.8, 0.3% gelrite

[a] All Microelements in all the medium contains 18.6 mg l^{-1} H_3BO_3.

medium-I (AAR2BS, Table 1) containing hygromycin (used for selection of transformed tissue) and incubated at 28°C in the dark. Following 2 weeks of selection on AAR2BS medium, the embryogenic units that were dark brown with whitish protuberances arising throughout the callus surface were transferred to selection medium II (MNBS, Table 1). After two week of selection on MNBS medium, the protuberances developed into white globular structures that were gently teased apart from the callus and incubated for another one weeks in the same Petri dish. During subculture, these globular structures converted into compact and yellowish calli.

ABA treatment and plant regeneration

ABA treatment was performed by adding different concentrations of ABA (0, 1, 3, 5, 8 or 10 mg l^{-1}) into the PRN medium (pre-regeneration medium, Table 1). After ABA treatment, the calli were transferred onto RN medium (pre-regeneration medium, Table 1) where regeneration frequency and regeneration of transgenic plants per resistant callus tissue were recorded two and three weeks after transfer of ABA treated calli to the regeneration medium, respectively. The experiment was repeated three times and data were analyzed using Student's *t*-test.

Desiccation treatment and plant regeneration

Desiccation treatment was performed by transferring hygromycin resistant callus tissues to sterile Petri dishes containing two sterile Whatmann filter papers. The Petri dishes were sealed with parafilm and incubated at 25°C in the dark for 0, 12, 24, 48 or 72 hrs to obtain desiccated calli. After desiccation treatment, the calli were directly transferred to RN medium where regeneration frequency and regeneration of transgenic plants per resistant callus tissues were recorded two and three weeks after transfer of desiccated calli to the regeneration medium, respectively. The experiment was repeated three times and data were analyzed using Student's *t*-test.

Copper sulphate ($CuSO_4$) treatment and plant regeneration

Copper sulphate treatment was performed by adding 0.025 (original concentration), 0.5, 1.0, 1.25, 1.5 or 1.75 mg l^{-1} $CuSO_4$ to PRN medium and RN medium. Regeneration frequency and regeneration of transgenic plants per resistant callus were recorded two and three weeks after transfer of calli to the regeneration medium, respectively. The experiment was repeated three times and data were analyzed using Student's *t*-test.

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Regeneration of transgenic rice plants

The putatively transgenic, hygromycin-resistant calli were gently picked out and cultured on PRN medium for a week. All of the resistant calli originating from a single co-cultured embryogenic nodular unit were grouped in a sector on the PRN dish. PRN medium containing 5 mg l^{-1} ABA and 1.25 mg l^{-1} $CuSO_4$ and regeneration medium containing 1.25 mg l^{-1} $CuSO_4$ were used throughout the study unless otherwise stated. Creamy-white, lobed calli with a smooth and dry appearance were individually transferred to RN medium, incubated for 2 days in the dark, and then maintained for three weeks at 28°C under a 14/10-hrs light/dark photoperiod with light provided at an intensity of 55 μmol/m per sec. Green shoots regenerated from a resistant callus were dissected and sub-cultured in a Magenta jars containing rooting medium (R, Table 1) for 1–2 weeks to promote vigorous roots and tillers before transferring to 16-inch pots containing a soil-less potting mixture (Metromix 220™) in the green house. The plants were fertilized twice a week with a water soluble fertilizer, Plant-Prod® 20-20-20 (Plant Products Co., Ltd., Ontario Canada).

Molecular analysis of transgenic plants

Expression of *AlaAT* was assessed in shoots and roots of control and three independent homozygous T2 transgenic rice lines by Western blot and enzyme activity analysis. Western blot and enzyme activity was studied as described previously (Shrawat et al. 2008).

Histochemical staining of GUS activity in transgenic calli

Histochemical assay, to assess the expression of the reporter gene, β-gucurondise (*uid*A), was carried out by staining a transformed calli or roots of T2 homozygous transgenic plants with 5-bromo-4-chloro-3-indolyl-b-D-glucuronide acid (X-Gluc) as a substrate (Jefferson 1987). Stained calli were examined using a dissection microscope (Nikon). Plants used for GUS expression studies were grown on sterile plastic mesh in Magenta jars. For cross sections, stained roots were fixed, dehydrated, embedded in paraffin and cut into sections 5 µm thick sections using rotary microtome.

Results

Production of high quality nodular embryogenic callus

High frequency of embryogenic callus induction was obtained from mature seeds of rice (*Oryza sativa* L. cv. Nipponbare) on MNB callus induction medium (Table 1). Callus induction started after 4 days of culture initiation, which further proliferated and converted into compact and nodular callus within two weeks. On the contact surface with the culture medium, some of the individual primary callus (mother callus) produced small, embryogenic and compact callus, referred to as 'embryogenic spherical nodules'. When these spherical nodules reached 1 to 1.5 mm in size and were highly embryogenic in nature, they were isolated from the mother callus and maintained on fresh MNB medium until they reached 3–5 mm in size and convert into yellowish and rough surfaced nodular calli (Figure 1A). Medium composition had a highly significant effect ($P<0.05$) on the production of embryogenic spherical

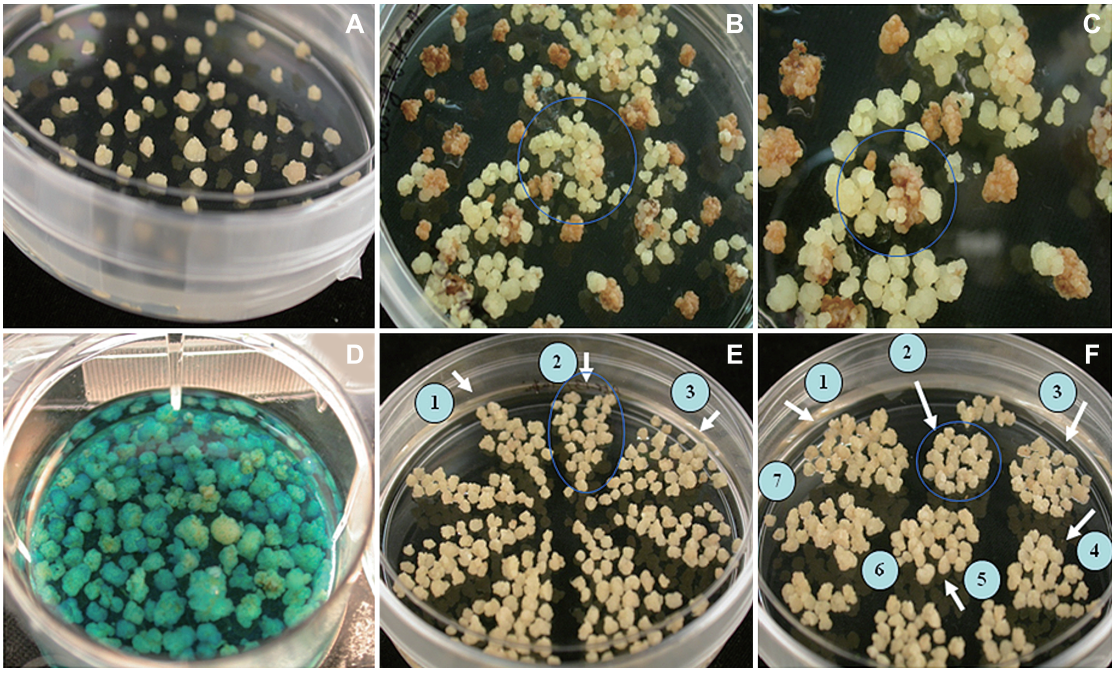

Figure 1. *Agrobacterium*-mediated transformation of rice cv. Nipponbare. (A) Seed embryo-derived embryogenic calli selected after reaching an optimal size for co-culture with *Agrobacterium*. (B, C) Development of hygromycin-resistant cell lines from co-cultured calli. (D) GUS expression in hygromycin-resistant cell lines. (E) Hygromycin-resistant cell lines originated from independent co-cultured calli on PRN medium. (F) Matured hygromycin-resistant cell lines on PRN medium.

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

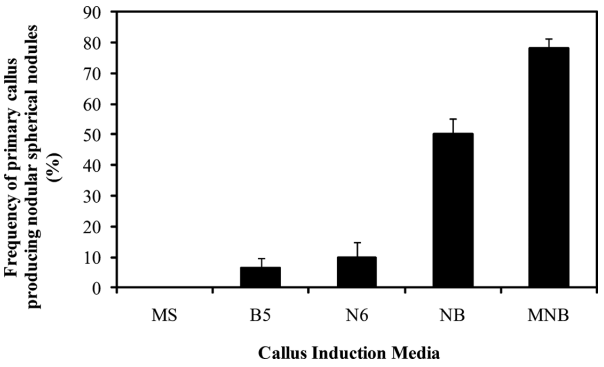

Figure 2. Frequency of primary spherical nodules originating from seed embryo on different callus induction medium.

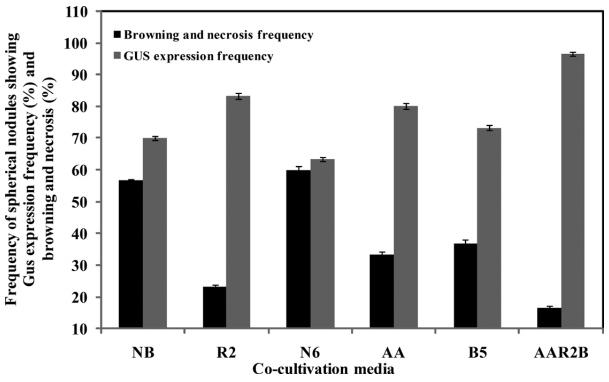

Figure 3. Frequency of browning/necrosis and GUS expression in co-cultured calli on different co-cultivation medium.

units from the mother callus (Figure 2). The highest frequency (78%) of primary callus released spherical units at the contact surface of MNB medium. In comparison to B5, N6, NB or MNB medium, none of the primary compact calli obtained on MS medium released spherical nodules. In order to minimize potential somaclonal variation, three weeks old (2 weeks+1 week, referred to as the 2+1 subculture regime) shperical nodules were used for transformation.

Agrobacterium-mediated transformation of embryogenic spherical nodules

Three weeks old highly embryogenic spherical units released from the scutellum-derived calli at the explant/medium interface were used for co-cultivation with an *Agrobacterium* strain harboring either a binary vector pCAMBIA1 305.1/*OsAnt1-Gus*^{Plus} or pCAMBIA1300/*OsAnt1-AlaAT* (Shrawat et al. 2008). To optimize the medium for better interaction and growth of co-cultured spherical nodules, co-cultivation was carried out on several medium (Figure 3). All the components in the co-culture media were kept similar as described in Table 1. Interestingly, AAR2B-CS medium supported not only the highest frequency of GUS expression in spherical nodules (96.6% ± 0.443), but also showed a very low frequency of browning and necrosis (16.7% ± 0.667) in the nodules after co-cultivation with *Agrobacterium* (Figure 3). After co-cultivation, the explants were allowed to grow for a week on R2S-I medium without hygromycin (Resting phase medium, Table 1) but with cefotaxime and amoxicillin to kill the *Agrobacterium*. This period without selection seemed to help the explants to recover from infection and apparently did not affect embryo selection in later steps with hygromycin. GUS staining of proliferating sectors of transformed calli on selection medium enabled an early identification of transformation events and drastically reduced the quantity of tissue handled during the selection process. Seven days following the transfer of co-cultured embryogenic spherical units to the AAR2BS medium (first selection medium, Table 1), we observed a few developing brown sectors at the surface of developing callus. Fourteen days after transfer to AAR2BS medium, growth of the co-cultured units was inhibited by hygromycin and they turned brown. Twenty days following the co-culture, small white proliferation had developed from the co-cultured spherical embryogenic nodules that had by now turned completely dark brown (Figure 1B, C).

Following 2 weeks of selection on AAR2BS medium, the co-cultured shperical units that had turned dark brown and had white protuberances arising throughout the callus surface were transferred to MNBS selection medium (second selection medium, Table 1). After 2 weeks on MNBS, the protuberances developed into small white globular structures were gently teased apart from dark brown callus and spread over the medium surface (Figure 1B, C). The resistant cell lines appeared to develop from structurally independent regions of the callus, and most of them exhibited GUS expression at this time. At this stage, more than 98% of the resistant nodular calli expressed the GUS gene (Figure 1D). Independent proliferating sectors isolated from individual co-cultured calli were kept as independent resistant cell lines to maximize the recovery of transformation events (Figure 1E, F). In comparison to NBS medium where 92.5% (± 0.577) of co-cultured produced resistant cell lines, 98.2% (± 0.334) of co-cultured calli yielded resistant cell lines on MNBS medium (Figure 4A). Moreover, while NBS medium produced hygromycin resistant spherical nodules with an average of 12.2 (± 0.825) per co-cultured callus, the MNBS medium produced hygromycin resistant spherical nodules with an average of 16.9 (± 0.389) per co-cultured callus (Figure 4B). Modification in NB medium significantly ($P<0.05$) enhanced the number of spherical nodules harvested per co-cultured callus tissues. The second round of harvesting of spherical nodules from resistant co-cultured callus pieces significantly increased the total number of resistant nodules per co-cultured

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

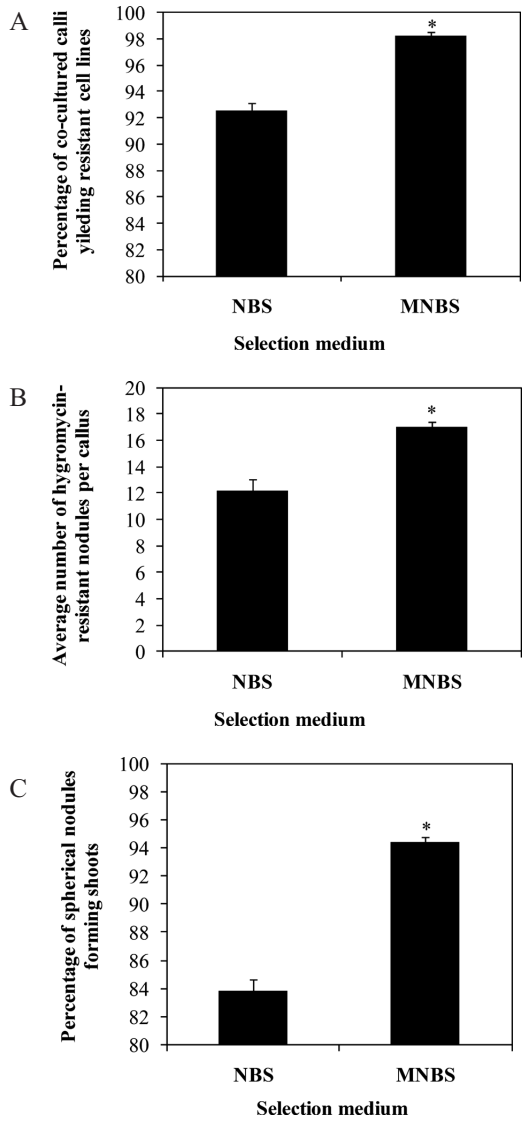

Figure 4. Co-cultured calli derived multiple resistant cell lines on selection medium. (A) Frequency co-cultured calli yielding resistant cell lines on NBS or MNBS selection medium. (B) Average number of NBS or MNBS derived resistant cell lines per co-cultured calli. (C) Frequency of NBS or MNBS derived resistant cell lines able to regenerate shoots on RN medium. Results shown are the mean of triplicate samples. Vertical bars represent standard error and asterisks indicate significant differences between NBS and MNBS medium using Student's *t*-test ($P<0.05$).

callus.

Regeneration of transgenic rice plants

A subset of the hygromycin-resistant embryogenic spherical nodules were gently picked out and cultured on PRN medium (pre-regeneration medium, Table 1). In order to keep track of the origin of the callus, these highly embryogenic spherical nodules originating from a single co-cultured embryogenic nodular callus were grouped together on PRN medium (Figure 1E). After maturation on PRN medium for a week, individual spherical nodules, which turned into creamy-white, lobed calli with a smooth and dry appearance (Figure 1F) were individually transferred to RN medium (regeneration medium, Table 1). Shoot bud regeneration started 4 days after transfer of hygromycin resistant spherical nodules onto RN medium (Figure 5A). These shoot buds further proliferated and converted into green shoots within 2 weeks after transfer onto RN medium (Figure 5B). After three weeks of regeneration, calli derived from NBS and MNBS medium were compared. While the regeneration frequency of NBS medium-derived calli reached 83.8% ($\pm$ 1.12), the regeneration frequency of MNBS medium-derived calli significantly ($P<0.05$) increased to 94.4% ($\pm$ 0.887) (Figure 4C). Green shoots were dissected from calli and sub-cultured onto rooting medium (R, Table 1) for 1–2 weeks to promote vigorous roots and tillers before transfer to soil pots in the greenhouse (Figure 5C). For screening the desired phenotypes, a large number of transgenic rice plants with strong root systems were transferred to the greenhouse (Figure 5D), where they attained maturity and produced healthy seeds (Figure 5E). The majority of transgenic plants segregated in a Mendelian fashion (data not shown). Strong Gus expression was detected in the roots of T2 progeny of *OsAnt1/gus* transgenic plants. After germinating the seeds in Magenta jars for 4 days, strong GUS expression was detected in primary and lateral roots when stained with X-Gluc buffer (Jefferson 1987) (Figure 5F). Cross sections of primary roots of the transgenic plants also showed strong GUS expression in epidermal cell layers and root hairs (Figure 5G). No GUS expression was detected in cross sections of the roots of wild type plants (Figure 5H). Homozygous transgenic plants screened from T2 seed on MS medium containing 50 mg l^{-1} hygromycin.

In order to study the effect of ABA on plantlet regeneration frequency, different concentrations of ABA (1, 3, 5, 8 or 10 mg l^{-1}) were added to PRN medium. Plant regeneration frequency of embryogenic calli increased significantly with ABA concentration between 3 mg l^{-1} to 8 mg l^{-1} (Figure 6A). In comparison to the control (calli without ABA treatment), plantlet regeneration frequency and the average number of regenerated plantlets were increased significantly ($P<0.05$) from 3, 5 and 8 mg l^{-1} ABA treated calli. Of the five levels of ABA tested, the highest frequency (95.6% $\pm$ 0.8) of plant regeneration and maximum number of plantlets (6.8 $\pm$ 0.8) per callus was obtained when 5 mg l^{-1} ABA treated calli were transferred from PRN to RN medium. Plant regeneration frequency was reduced significantly ($P<0.05$) when ABA concentration increased from 8 to 10 mg l^{-1}. The higher concentration of ABA (10 mg l^{-1}) suppressed shoot bud induction and therefore, may be reasons for significant reduction in regeneration frequency. Since high regeneration frequency was obtained from 5 mg l^{-1} ABA treated calli,

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

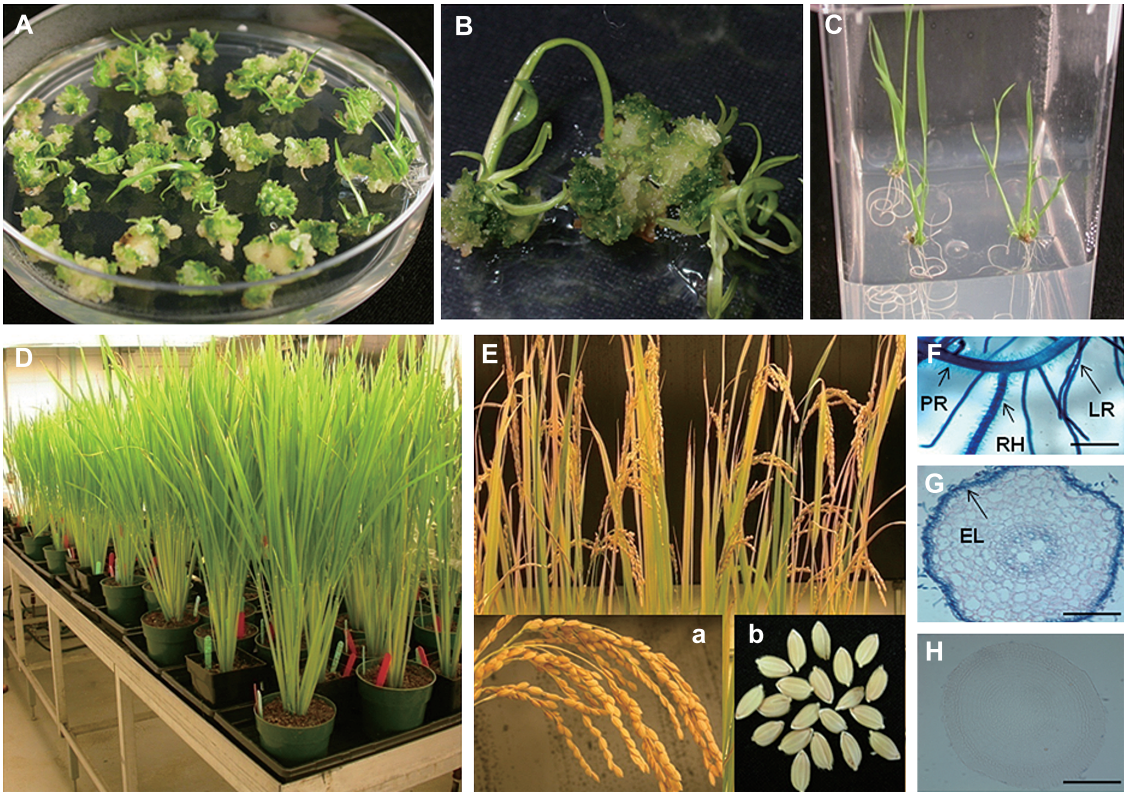

Figure 5. Regeneration of transgenic rice plants from hygromycin-resistant calli. (A) Shoot buds inductions from hygromycin-resistant cell lines on RN medium. (B) Young plantlets proliferating from shoot buds on RN medium. (C) Young plantlets showing strong root initiation on rooting medium. (D) Primary transgenic plants in a controlled growth chamber. (E) Primary transgenic plants at maturity. Inset (a) shows fully fertile spikes from transgenic plants, and inset (b) shows fully matured fertile seeds. (F) Strong GUS expression was detected in primary root, lateral root and root hairs of transgenic plants. (G) Cross section of primary roots of transgenic plants showed strong GUS expression in epidermal cell layers and root hairs. (H) No GUS expression was detected in the cross section of the roots of controlled plants. Bars: 50 μm. PR, primary root; RH, root hairs; LR, lateral root; EL, epidermal layer.

this ABA concentration was adopted in later experiments.

In the present study, callus dehydration tremendously enhanced plantlet regeneration frequency (Figure 6B). Desiccated resistant calli initiated shoot bud formation four days after their transfer to regeneration medium. In non-desiccated calli, shoot bud regeneration started nine days after their transfer to the regeneration medium. During subculture, these shoot buds further elongated and multiplied vigorously. Shoot multiplication rate was comparatively low in non-dehydrated calli. Hygromycin resistant calli showed the highest frequency (93.3% $\pm$ 0.771) of plantlet regeneration with 24-h partial desiccation treatment. In comparison, shoot regeneration frequency was significantly lower ($P<0.05$) in non-dehydrated calli. Also non-desiccated calli produced transgenic plants with an average of 3.18 ($\pm$ 0.231), while 24-h and 48-h desiccated calli regenerated plantlets with an average of 6.5 ($\pm$ 0.763) and 5.4 ($\pm$ 0.648), respectively. However, further dehydration of callus tissues to 72-h resulted in a decrease of both regeneration frequency and plantlet regeneration.

Plant regeneration from hygromycin-resistant embryogenic calli was also increased significantly ($P<0.05$) by supplementing medium with 1.0 or 1.25 mg l^{-1} $CuSO_4$ (Figure 6C). After transfer of hygromycin-resistant calli onto regeneration medium containing 1.0 mg l^{-1} or 1.25 mg l^{-1} copper sulphate, dense green sectors appeared on the surface of the calli within 5 days, while medium containing 0.025, 0.5, 1.5 or 1.75 mg l^{-1} copper sulphate produced green sectors after 10 days. The highest frequency of plant regeneration (91.7% $\pm$ 0.577) and average number of plantlets (7.13 $\pm$ 0.324) per callus cultures were obtained on regeneration medium containing 1.25 mg l^{-1} copper sulphate. However, both plant regeneration frequency and the average number of plantlets decreased when concentration of copper sulphate was increased from 1.25 mg l^{-1} to 1.5 and 1.75 mg l^{-1}. These results show that regeneration frequency is dependent on the level of copper sulphate incorporated into the regeneration medium.

Molecular analysis of transgenic plants

Expression of *AlaAT* was assessed in shoots and roots of the control and the three independent homozygous

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

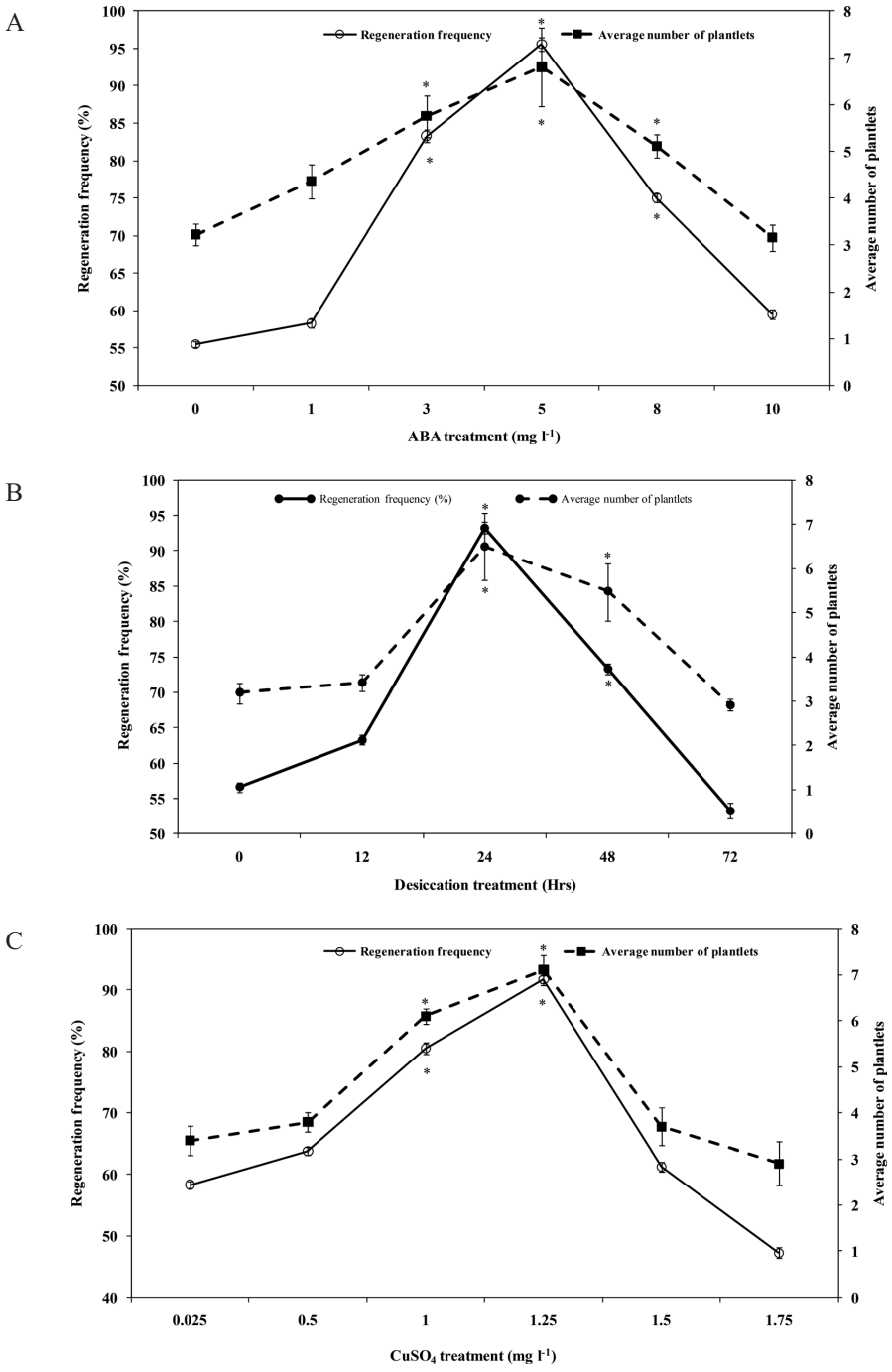

Figure 6. (A) Regeneration frequency and average number of transgenic plants from pre-treated calli on PRN medium containing 0, 1, 3, 5, 8 or 10 mg l^{-1} ABA. (B) Regeneration frequency and average number of transgenic plants from 0, 12, 24, 48 or 72-hrs partially desiccated calli on RN medium. Hygromycin-resistant calli were partially desiccated before being transferred to regeneration medium. (C) Regeneration frequency and average number of transgenic plants on RN medium containing 0.025 (original concentration in the medium), 0.5, 1.0, 1.25, 1.5 or 1.75 mg l^{-1} $CuSO_4$. Regeneration frequency and average number of transgenic plants per co-cultured callus were calculated two and three weeks after transfer of hygromycin resistant calli onto RN medium, respectively. Results shown are the mean of triplicate samples. Vertical bars represent standard error and asterisks indicate significant differences between non-treated calli (0 mg ABA, 0 hrs of partial desiccation or 0.025 mg l^{-1} $CuSO_4$) and calli treated with ABA (1, 3, 5, 8 or 10 mg l^{-1} ABA), partial desiccation (12, 24, 48 or 72-hrs) or $CuSO_4$ (0.5, 1.0, 1.25, 1.5 or 1.75 mg l^{-1} $CuSO_4$) using Student's *t*-test ($P<0.05$).

transgenic rice lines by Western blot and enzyme activity analysis (data not shown). AlaAT enzyme activity in the shoots and roots of the transgenics was significantly higher than in the controls. As well, compared to control plants, Western blot analysis detected a higher level of AlaAT protein in the shoots and roots of the transgenic plants (Shrawat et al. 2008).

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Discussion

Here we report a reliable and high-throughput *Agrobacterium*-mediated transformation procedure for producing large numbers of transgenic rice plants carrying agronomically important genes. Transformation frequency of a rice japonica cultivar Nipponbare was improved when mature seeds were cultured on MNB medium (Table 1). The beneficial effect of MNB medium was associated with increased and rapid production of spherical nodules from the mother callus and improved recovery of shperical nodules after co-culture with *Agrobacterium*. MNB medium supported not only the production of high quality nodular embryogenic mother calli from cultured seeds, but also released highly embryogenic nodular embryogenic spherical nodules from mother calli at a higher frequency. The combination of 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} picloram, 0.01 mg l^{-1} BAP and 2 mg l^{-1} ABA into the MNB medium induced high frequency of somatic embryos all over the surface of the embryogenic calli. Medium composition had a highly significant effect ($P<0.05$) on the production of embryogenic spherical units from the mother callus. On NB callus induction medium, the frequency of primary callus producing these small embryogenic spherical units was around 40%. Modification in the NB callus medium, designated as MNB, resulted in 78% of primary calli producing small spherical nodules at the contact surface of the medium and the primary callus (Figure 2). Interestingly, although MS medium produced embryogenic and compact calli from mature seeds, none of the primary compact calli released spherical nodules. Addition of 100 mg l^{-1} L-tryptophan, 500 mg l^{-1} L-proline and 500 mg l^{-1} L-glutamine, designated as 3AA, into NB medium may have also triggered the production of embryogenic and nodular spherical units at the interface of explants and the medium. L-tryptophan, L-glutamine and L-proline have been shown to induce high frequency of embryogenic callus induction in cereals (Chowdhry et al. 1993, Peterson and Smith 1991). The effect of L-proline on the frequency of overall callus production has been reported for maize (Suprasanna et al. 1994) and wheat (Vasil and Vasil 1986). The ability of nodular embryogenic calli to multiply during subcultures is considered critical in genetic transformation experiments, as it proportionally influences the number of transgenic plants that can be subsequently produced. Our transformation system enabled a routine 10-fold multiplication between the number of explants (embryogenic calli) and high embryogenic spherical nodules used as target tissue for transformation.

In order to provide the better interaction of *Agrobacterium* with target tissues and minimize the browning and necrosis after infection, co-cultivation was carried out on several medium (Figure 3). In comparison to NB, R2, N6, AA or B5 co-cultivation medium, AAR2B co-cultivation medium supported the highest frequency of embryogenic nodular calli showing GUS expression. Also, in comparison to other co-cultivation media, the lowest frequency of embryogenic spherical nodules with necrosis and browning was recorded after three days of co-cultivation duration. The high frequency of spherical nodules showing GUS expression may be due to the fact that spherical units on AAR2B-CS medium remained free of browning and necrosis after co-cultivation with *Agrobacterium*, which is considered one of the critical factors in routine application of *Agrobacterium* for genetic transformation of crop plants. Induced cellular necrosis is typical of the resistance mechanism, termed the hypersensitive response, and it is associated with limiting the spread of pathogens (Lamb and Dixon 1997, Parrott et al. 2002). Immature embryos of wheat inoculated with *Agrobacterium,* browned and displayed altered cell wall composition, increased production of H_2O_2 and higher level of cellular necrosis leading to cell death (Parrott et al. 2002).

After two weeks of selection on AAR2BS selection medium, only spherical units that were yellowish in color with a rough surface were carried further in the transformation experiment. The use of MNBS medium and spreading of resistant globular structures on the surface of the medium considerably enhanced the growth of resistant cell lines, which developed into yellowish compact calli within two weeks. Spherical nodules that were whitish in color with a smooth surface were discarded as these units tend to become necrotic and eventually die on selection medium. In comparison to NBS medium, addition of 3AA into MNBS medium without ABA significantly ($P<0.05$) enhanced the number of spherical nodules harvested per co-cultured callus tissues (Figure 4B). MNBS selection medium not only supported the production of resistant spherical nodule in higher frequency, but also maintained the proliferation of resistant white patches overall the surface of resistant spherical units. A combination of L-tyrptophan, L-proline and L-glutamine (Table 1) might have induced the production of more spherical nodules from the co-cultured calli. Addition of these amino acids in the medium gave a rapid increase in the production of highly embryogenic spherical nodules. The combination of these amino acids has been reported in promoting the production of somatic embryos and green regenerants in wheat (Afshar-Sterle 1996) and barley (Shrawat et al. 2007). The synergistic effects of 2,4-D and dicamba might have also contributed to the production of high quality embryogenic spherical nodular calli from co-cultured calli. The effect of a combination of two auxins on production of high quality embryogenic callus induction has also been reported in wheat (Afshar-Sterle 1996) and barley (Shrawat et al. 2007). In addition, early

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

differentiation of these embryogenic nodular calli was overcome by adding ABA and a combination of Dicamba and 2,4-D. Embryogenic units between 3-5 mm in size, which were compact and spherically shaped with a yellowish rough surface, produced the maximum numbers of resistant cell lines after co-cultivation with *Agrobacterium* and consequently the highest frequency of transgenic events. It was observed that the co-cultivation of embryogenic units, which were larger than 5 mm and smaller than 3 mm, produced very low numbers of resistant cell lines. Smaller spherical units tended to become necrotic during co-culture with *Agrobacterium*. In addition, more complex shaped calli with a smoother surface, an indication of differentiation, did not produce any transformation events.

With the aim of regenerating transgenic plantlets, resistant spherical nodules were first transferred to PRN medium for a week and then onto RN medium. Interestingly, we found that the composition of selection media strongly influenced the morphogenic ability of the embryogenic callus as was evident with the regeneration frequency from NBS and MNBS-derived cultures (Figure 4C). In comparison to NBS medium-derived calli, the regeneration frequency of MNBS medium-derived calli increased significantly ($P<0.05$) (Figure 4C). The difference in the regeneration frequency between spherical nodules derived from NB and MNB medium may be due to the embryogenic nature of spherical nodules. Interestingly, it was observed that the maturation of resistant calli on PRN medium significantly enhanced the regeneration frequency in resistant calli after transfer to regeneration medium (Figure 6A). The effect of maturation of calli in plant regeneration may be due to fact that ABA treatment converted the hygromycin resistant calli to a state more responsive to subsequent culture conditions for plant regeneration. Although the calli could grow on pre-regeneration medium without ABA and retained the ability to regenerate plants, addition of ABA increased the growth rate and number of regenerated plants. It was observed that the supplementation of ABA at all levels suppressed proliferation of the calli and delayed the appearance of green sectors, particularly when ABA was applied at higher levels. Supplementation of ABA into the PRN medium also increased the compact dry areas on the surface of the calli and inhibited the elongation of green shoot buds. We also observed that the plantlet regeneration potential of the ABA-treated calli was greatly enhanced as was evident by the presence of green sectors on the surfaces of the calli. These ABA pre-treated calli fully converted into green plantlets upon transfer to the RN medium. Although the degree of suppression by ABA on callus growth differed noticeably among the five levels tested, ABA at the higher concentration (8 and 10 mg l^{-1}) prolonged the appearance of shoot buds after transfer of ABA treated calli from PRN to RN medium. These results suggest that ABA might stimulate the processes of callus differentiation and promote plant regeneration. We also observed that ABA treatment not only inhibited callus growth, but also stimulated the formation of white compact regions on the callus. Moreover, these white regions of callus showed higher potential to regenerate plantlets after transfer of the ABA pre-treated resistant calli to regeneration medium. However, the frequency of the appearance of such white regions differed with each ABA treatment. In our study, we have found that only an optimal concentration of ABA stimulates the formation of white regenerative regions on the calli, and consequently, produce more transgenic plants. The effect of ABA treatment on plant regeneration may be correlated to the fact that exogenous ABA promotes somatic embryo maturation and therefore may have enhanced regeneration rate (Kong and Aderkus 2007). The stimulatory effect of ABA on embryogenic callus induction and plant regeneration has also been reported in rice (Higuchi and Maeda 1990, Peterson and Smith 1991). Peterson and Smith (1991) observed that addition of ABA to the medium shortened the plant regeneration period by about 1 week and increased the percent of plants regenerated from each callus. This indicates that ABA can promote the differentiation process of cells.

In the present study, the effect of callus dehydration on plant regeneration was also studied. Interestingly, hygromycin resistant calli showed both the highest frequency (93.3% $\pm$ 0.771) of plantlet regeneration and the highest average number of transgenic plants (6.5 ($\pm$ 0.763) with 24-h partial desiccation treatment. In comparison, shoot regeneration frequency was significantly lower ($P<0.05$) in non-dehydrated calli (Figure 6B). The present study shows that 24-h and 48-h partial desiccation resulted in an up to two fold increase in transgenic plantlet regeneration. 24-h desiccation treatment has been reported to enhance plant regeneration both in Japonica and Indica varieties of rice (Jain et al. 1996, Chand and Sahrawat 2001). Recently, Wagiran et al. (2008) demonstrated that 48-h partial dehydration treatment dramatically enhanced regeneration frequency both in Indica and Japonica rice cultivars. Desiccation of plant tissues post-*Agrobacterium* infection was also found to enhance T-DNA delivery and subsequent stable transformation efficiency in wheat (Cheng et al. 2003). The dramatic increase in both regeneration frequency and plantlet regeneration from partially dehydrated calli has been suggested to be due to decreased water content in the dehydrated callus leading to an increase in endogenous ABA levels (Yang et al. 1999) and improvement of oxygen supply to the callus (Jain et al. 1996). Rance et

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

al. (1994) observed that partial desiccation of mature embryo derived calli led to differences in the soluble protein pattern as early as one day after desiccation. They suggested that partial desiccation treatment might trigger rapid biochemical changes in the calli.

We also observed that both regeneration frequency and the average number of transgenic plantlets per callus were highly correlated to the concentration of copper sulphate added to the regeneration medium (Figure 6C). The highest frequency of plant regeneration and average number of plantlets per callus cultures were obtained on regeneration medium containing $1.25\,mg\,l^{-1}$ copper sulphate. In barley, Dahleen (1995) achieved three to five times higher regeneration frequencies by optimizing the concentration of copper sulphate in MS medium. Purnhauser (1991) and Sahrawat and Chand (1999) reported the beneficial effect of copper ions on plant regeneration in wheat and rice, respectively. Dahleen and Bregitzer (2002) investigated the effect of eight micronutrients on plant regeneration in barley and found that green plant regeneration can be increased by increasing the concentration of H_3BO_3 to 0.75 mM and decreasing the concentration of $FeSO_4$ to 0.05 mM. They also reported enhanced regeneration frequency by optimizing the concentration of $CuSO_4$ to $5\,\mu$M. In comparison to macronutrients, relatively little is known about the basic mechanism of micronutrient function in higher plant growth and development. The appropriate level of micronutrients is essential for the optimal functioning and growth of plants (Welch 1995). Copper has been considered one of the essential micronutrients for plant regeneration in the majority of crop plants (Murashige and Skoog 1962). It has also been reported that copper plays a role in several metabolic activities such as protein and carbohydrate metabolism (Delhaize et al. 1985). The stimulatory effect of copper may be due to the fact that copper is an important part of several enzymes and hence may play a key role in morphogenesis when it is used at an optimum concentration.

In conclusion, the transformation procedure reported in the present study allows the production of large numbers of transgenic rice plants. The transgenic plants obtained are of high quality, are highly fertile and show stable expression and inheritance of the transgene. This, together with the fast and simple procedure for selection of transformed tissue and a high frequency of regeneration of transgenic plants, makes this an attractive method to be adopted in any industrial and academic research setting.

Acknowledgements

We would like to thank Rebecka Carroll for critical reviews of the manuscript and Jayne D'Entremont for helping in making graphs.

References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, et al. (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. *Mol Breed* 15: 305–327

Afshar-Sterle S, Peng ECK, Brown JS, Kollmorgen JF (1996) Embryogenic callus induction and plant regeneration in *Triticum tauschii*, the diploid D-genome donor for bread wheat. *Aust J Bot* 44: 489–497

Bajaj S, Mohanty A (2005) Recent advances in rice biotechnology—towards genetically superior transgenic rice. *Plant Biotechnol J* 3: 275–307

Chand S, Sahrawat AK (2001) Stimulatory effect of partial desiccation on plant regeneration in Indica rice (*Oryza sativa* L.). *J Biochem Biotechnol* 10: 43–47

Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev Biol Plant* 40: 31–45

Cheng M, Hu T, Layton J, Liu CN, Fry J (2003) Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. *In Vitro Cell Dev Biol Plant* 39: 595–604

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci USA* 71: 3672–3676

Chowdhry CN, Tyagi AK, Maheshwari N, Maheshwari SC (1993) Effect of L-proline and L-tryptophan on somatic embryogenesis and plantlet regeneration of rice (*Oryza sativa* L. cv. Pusa 169). *Plant Cell Tissue Organ Cult* 32: 357–361

Chu CC, Wang CC, Sun CS, Hsu C, Kin KC, Yin C, Chy Y, Fy B (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen source. *Sci Sin* 5: 659–668

Dahleen LS, Bregitzer P (2002) An Improved Media System for High Regeneration Rates from Barley Immature Embryo-Derived Callus Cultures of Commercial Cultivars. *Crop Sci* 42: 934–938

Dahleen LS (1995) Improved plant regeneration from barley callus cultures by increased copper levels. *Plant Cell Tissue Organ Cult* 43: 267–269

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacteirum*-mediated transformation and particle bombardment. *Mol Breed* 7: 25–33

Delhaize E, Loneragan JF, Webb J (1985) Development of three copper metalloenzyme in clover leaves. *Plant Physiol* 78: 4–7

Gamborg OL, Miller RA, Ojima K (1968) Plant cells cultures. 1. Nutrient requirements of suspension cultures of soybean root cells. *Exp Cell Res* 50: 151–158

Gelvin SB (2006) *Agrobacterium* virulence gene induction. In: Wang K (ed) *Methods in Molecular Biology Series, vol. 343: Agrobacterium Protocols*. Humana Press Inc., Totowa, NJ, USA, pp 77–84

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the DNA. *Plant J* 6: 271–282

Higuchi N, Maeda E (1990) Enhanced plant regeneration in rice callus cultures following abscisic acid treatment. *Jpn J Crop Sci* 59: 359–368

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* 2: 208–218

Jain RK, Jain S, Wu R (1996) Stimulatory effect of water stress on plant regeneration in aromatic indica rice varieties. *Plant Cell Rep* 15: 449–454

Jefferson RA (1987) Assaying chimeric genes in plants: the genes fusion system. *Plant Mol Biol Rep* 5: 387–405

Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999) Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. *Planta* 208: 88–97

Kong, L, Aderkas PV (2007) Genotype effects on ABA consumption and somatic embryo maturation in interior spruce (*Picea glauca* x *engelmanni*). *J Exp Bot* 58: 1525–1531

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 48: 251–275

Murashige F, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473–497

Ohira K, Ojima K, Fujiwara A (1973) Studies on the nutrition of rice cell culture. I. A simple defined medium for rapid growth in suspension culture. *Plant Cell Physiol* 14: 1113–1121

Parrott DL, Anderson AJ, Carman JG (2002) *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.). *Physiol Mol Plant Pathol* 60: 59–69

Peterson G, Smith R (1991) Effect of abscicic acid and callus size on regeneration of American and International rice varieties. *Plant Cell Rep* 10: 35–38

Purnhauser L (1991) Stimulation of shoot and root regeneration in wheat *Triticum aestivum* callus cultures by copper. *Cereal Res Commun* 19: 419–423

Rancé IM, Tian W, Mathews H, Kochko AD, Beachy RN, Fauquet C (1994) Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. *Plant Cell Rep* 13: 647–651

Sahrawat AK, Chand S (1999) Stimulatory effect of copper on plant regeneration in indica rice (*Oryza sativa* L.). *J Plant Physiol* 154: 517–522

Sallaud C, Meynard D, van Boxtel J, Gay C, Bès M, et al. (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. *Theor Appl Genet* 106: 1396–1408

Shrawat AK, Lörz H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnol J* 4: 575–603

Shrawat AK, Becker D, Lörz H (2007) *Agrobacterium-tumefaciens*-mediated genetic transformation of barley (*Hordeum vulgare* L.). *Plant Sci* 172: 281–290

Shrawat AK, Carroll RT, DePauw M, Taylor GJ, Good AG (2008) Genetic engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of *alanine aminotransferase*. *Plant Biotechnol J* 6: 722–732

Suprasanna P, Rao KV, Reddy GM (1994) Embryogenic callus in maize: genotypic and amino acid effects. *Cereal Res Commun* 22: 79–82

Suprasanna P, Rupali C, Desai NS, Bapat VA (2008) Partial desiccation augments plant regeneration from irradiated embryogenic cultures of sugarcane. *Plant Cell Tissue Organ Cult* 92: 101–105

Toriyama K, Hinata K (1985) Cell suspension and protoplast culture in rice. *Plant Sci* 41: 179–183

Vasil V, Vasil IK (1986) Plant regeneration from friable embryogenic callus and cell suspension cultures of *Zea mays* L. *J Plant Physiol* 124: 399–408

Wagiran A, Ismail I, Radziah C, Zain M, Abdullah R (2008) Improvement of plant regeneration from embryogenic suspension cell cultures of Japonica rice. *J Biol Sci* 48: 570–576

Welch RM (1995) Micronutrient nutrition of plants. *Critl Rev Plant Sci* 14: 49–82

Yang YS, Zheng YD, Chen YL, Jian YY (1999) Improvement of plant regeneration from long-term cultured calluses of Taipei 309, a model rice variety in *in vitro* studies. *Plant Cell Tissue Organ Cult* 57: 199–206

Yang Y, Peng H, Huang H, Wu J, Jia S, Huang D, Lu T (2004) Large-scale production of enhancer trapping lines for rice functional genomics. *Plant Sci* 167: 281–288

Copyright © 2010 The Japanese Society for Plant Cell and Molecular Biology