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Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics

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Abstract We investigated the potential of an improved *Agrobacterium tumefaciens*-mediated transformation procedure of *japonica* rice (*Oryza sativa* L.) for generating large numbers of T-DNA plants that are required for functional analysis of this model genome. Using a T-DNA construct bearing the hygromycin resistance (*hpt*), green fluorescent protein (*gfp*) and *β*-glucuronidase (*gusA*) genes, each individually driven by a CaMV 35S promoter, we established a highly efficient seed-embryo callus transformation procedure that results both in a high frequency (75–95%) of co-cultured calli yielding resistant cell lines and the generation of multiple (10 to more than 20) resistant cell lines per co-cultured callus. Efficiencies ranged from four to ten independent transformants per co-cultivated callus in various *japonica* cultivars. We further analysed the T-DNA integration patterns within a population of more than 200 transgenic plants. In the three cultivars studied, 30–40% of the T_0 plants were found to have integrated a single T-DNA copy. Analyses of segregation for hygromycin resistance in T_1 progenies showed that 30–50% of the lines harbouring multiple T-DNA insertions exhibited *hpt* gene silencing, whereas only 10% of lines harbouring a single T-DNA insertion was prone to silencing. Most of the lines silenced for *hpt* also exhibited apparent silencing of the *gus* and *gfp* genes borne by the T-DNA. The genomic regions flanking the left border of T-DNA insertion points were recovered in 477 plants and sequenced. Adapter-ligation Polymerase chain reaction analysis proved to be an efficient and reliable method to identify these sequences. By homology search, 77 T-DNA insertion sites were localized on BAC/PAC rice Nipponbare sequences. The influence of the organization of T-DNA integration on subsequent identification of T-DNA insertion sites and gene expression detection systems is discussed.

Keywords Functional genomics · Gene transfer · Insertional mutagenesis · *Oryza sativa L.* · T-DNA

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Introduction

Cultivated rice of Asian origin, *Oryza sativa* L., has the smallest genome among graminaceous crops (2n = 24, n = 0.45 pg and 430 Mb) and has become over the last decade a model species for investigating the molecular genetics and genomics of cereals and grasses (Delseny et al. 2001). In the last few years, major advances in the constitution of publicly available rice genomic resources have included the massive sequencing of more than 100,000 expressed sequenced tags (ESTs) from which more than 6,000 have been used to establish a comprehensive Yeast artificial chromosome-based EST map (Wu et al. 2002), the TIGR rice gene index (Quackenbush et al. 2000) and the integration of the physical and genetic maps (Chen et al. 2002; Saji et al. 2001). The subsequent on-going sequencing effort conducted by the International Rice Genome Sequencing Project (IRGSP) (Sasaki and Burr 2000) using a clone by clone sequencing strategy should release a complete and public genome sequence of

japonica rice cv. Nipponbare within a 1- to 2-year time frame. Following the reporting of two independent 5–6x shot-gun company sequencings of the genome of cv. Nipponbare (Davenport 2001; Goff et al. 2002), a first draft sequence covering more than 97% of the genome of an *indica* rice variety has been recently published (Yu et al. 2002)

Faced with this enormous wealth of sequence information, the next challenge for the scientific community is clearly to assign a biochemical, cellular, developmental or adaptive function to the majority of the rice genes. This will be accomplished through the development of efficient tools for discovering and validating gene function in rice such as targeted/random gene disruption, gene detection, gene activation and/or gene silencing methods. As previously shown in *Arabidopsis,* the routine integration of these technologies will be facilitated by the establishment of a highly efficient transformation procedure in rice. For example, down-regulation resulting from RNA interference (Fire et al. 1998) is an elegant way to inactivate the expression of a target gene or a gene family. Nevertheless, expanding RNAi-mediated gene silencing on a systematic and large-scale basis requires both a convenient shuttle recombinational cloning system and an efficient transformation procedure allowing high-throughput analysis of cDNA clones in the host genome.

The development of homologous recombination (HR) in model higher plants would also be of considerable value for targeted gene disruption or gene replacement. In contrast to *Drosophila*, yeast and moss, where HR has proved to be highly efficient (Mengiste and Paszkowski 1999), HR was until recently thought to occur at a very low frequency in higher plants, as exemplified in *Arabidopsis* (Kempin et al. 1997). However, a very recent breakthrough report unambiguously demonstrated *Agrobacterium*-mediated targeted disruption of the *waxy* gene through homologous recombination in rice, with an efficiency representing one HR event every 1,500 potential illegitimate T-DNA integration events (Terada et al. 2002). This success also stressed the need for further simultaneous improvements of the procedure of T-DNA delivery and of current methods of enrichment of HR versus illegitimate recombination events (reviewed in Mengiste and Paszkowski 1999).

Random disruption through insertional mutagenesis, which is based on the insertion of a foreign DNA into the genome, remains to date the most suitable method for inactivating plant gene function on a large-scale basis. Insertional mutagenesis involves the use of transposable elements – such as transposons or retrotransposons – or T-DNA (Hirochika 2001; Krysan et al. 1999; Parinov et al. 1999; Speulman et al. 1999; Tissier et al. 1999). T-DNA offers the advantage of exhibiting a low number of inserts per line. It allows the use of specialized T-DNA harbouring trap- or activation tag-systems (Springer 2000), thereby increasing the frequency of gene identification. Moreover, the inserted DNA is chemically and physically stable over generations and is phenotypically tagged via the selectable marker, which facilitates genetic analysis. T-DNA has emerged as the preferred insertional mutagen in *Arabidopsis*, as evidenced by the increasing number of large T-DNA populations which have been produced recently (Bechtold and Pelletier 1998; Feldmann 1991; Krysan et al. 1999; McElver et al. 2001). Although T-DNA insertional mutagenesis has been used in rice by G. An and collaborators to generate more than 22,000 T-DNA lines (Jeon et al. 2000), the transformation efficiencies so far reported in rice make the effort of generating a specialized collection of insertion lines unaffordable by most laboratories.

With respect to transformation procedures in rice, Hiei and co-workers were the first to demonstrate that gene delivery mediated by *Agrobacterium* could be achieved in an efficient way (Hiei et al. 1994). In particular, these authors found that calli induced from the scutellum of mature seeds was the best starting material, in comparison to many different tissues, for its response to *Agrobacterium* co-cultivation. The composition of the media for co-culture as well as for cell selection was also an important factor in obtaining a high transformation efficiency. Many subsequent studies have used this method or its derivatives as a standard rice transformation procedure to engineer genes of interest or to investigate the function of selected genes through down/up regulation in rice. However, the 10–50% range of transformation efficiencies – defined as the number of transgenic lines obtained per co-cultivated callus – reported so far in the rice literature appears to be too limiting for a high-throughput analysis of gene function in rice.

We investigated here the potential of an improved *Agrobacterium tumefaciens*-mediated transformation of *japonica* rice (*Oryza sativa* L.) for generating large populations of transgenic plants for the functional analysis of this model genome. The protocol enabled the generation of a large number of T-DNA lines in several cultivars and within a short period, thereby reducing time and labour input. The method relies both on the careful choice of the target callus source and a modified co-culture and selection procedure, resulting in the generation of four to ten independent transformants per co-cultured callus. The molecular and genetic characterization of several hundred transformation events determined the T-DNA integration patterns as well as the number of integration loci resulting from this high-throughput transformation procedure. We further identified a large number of T-DNA flanking sites to confirm that T-DNA can be used as an efficient insertional mutagen in rice. With the aim of using specialized T-DNA for in vivo gene detection, we also discuss the correlation between gene expression of the β-glucuronidase (*gusA*) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and T-DNA integration pattern.

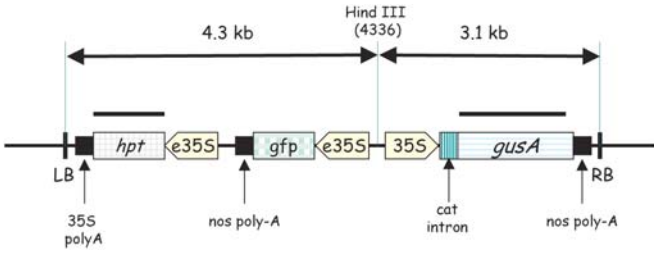

Fig. 1 Schematic representation of the T-DNA of the binary vector pC30063. *Black horizontal bars* indicate the *hpt* and *gusA* probe used for molecular analysis, *35S* cauliflower mosaic virus (CaMV) 35S promotor; *e35S* CaMV 35S promotor with a duplicated enhancer sequence, *hpt* hygromycin resistance gene, *gfp* green fluorescent protein gene, *gusA* β-glucuronidase gene, *35S-polyA* terminator sequence of the CaMV 35S gene, *nos poly-A* nopaline synthase terminator, *cat intron* castor bean catalase intron. *LB*, *RB* Left and right border, respectively

Materials and methods

Plasmid construct

The binary plasmid pC30063 was constructed by inserting the 1.915-bp *Bam*HI/*Pst*I fragment of pMON30063 (Pang et al. 1996) (kindly provided by K.L. Fincher, Monsanto Co, St Louis, Mo.) containing the S65Tgfp coding sequence controlled by the CaMV 35S promoter with a duplicated enhancer sequence (Kay et al. 1987) and the nos3′ terminator, in the multiple cloning site of the pCAMBIA 1301 binary vector (R. Jefferson, CAMBIA, Australia) between the *gusA* and the hydromycin resistance (*hpt*) gene cassettes (Fig. 1). The green fluorescent protein (*gfp*) and *gusA* sequences contain the ST-LS1 intron and the castor bean catalase intron, thereby preventing their expression in*Agrobacterium*. The 13.8 kb resulting pC30063 plasmid was transferred into *Agrobacterium* strain EHA105, an EHA101 derivative (Hood et al. 1993) by electroporation (Sambrook et al. 1989). *Agrobacterium* cells were plated on solid AB medium (Chilton et al. 1974) containing 50 mg/l kanamycin sulfate and incubated at 28 °C for 3 days. The bacteria were then collected with a flat spatula and resuspended in liquid co-cultivation medium (CCL) by gentle vortexing prior to transforming the rice tissues.

Plant materials and induction of embryogenic tissues

Mature seeds of the *japonica* rice (*Oryza sativa* L.) cvs. Taipei309, Nipponbare, Zhongzuo321 and Azucena – all belonging to group VI in a varietal classification based on isozyme polymorphism (Glazmann 1987) – were used in this study. Dehulled seeds were sterilized, inoculated on NB medium and incubated for 18–21 days in the dark as described in Chen et al. (1998). Embryogenic nodular units (0.5–1 mm long), released from the primary embryo scutellum-derived callus at the explant/medium interface, were transferred onto fresh NB medium and incubated for an additional 10–15 days depending on the variety.

Transformation procedure

Between 50 and 100 3- to 5-mm-long embryogenic nodular units were immersed into 25 ml of liquid co-culture medium (CCL) containing *Agrobacterium* cells at a density of 3–5×10^9 cells/ml (OD_{600} = 1) in a 100-mm-diameter petri dish for 10–15 min. Ten callus pieces were then blotted dry on sterilized filter paper, transferred to a petri dish containing solid co-culture medium (CCS) and incubated for 3 days at 25 °C in the dark. Five to seven uncontaminated co-cultured calli were then individually transferred to one dish of R2S (Ohira et al. 1973) selection medium, which contained hygromycin for selection of transformed tissues and cefotaxime and vancomycin for eliminating *Agrobacterium*, and incubated at 27 °C in the dark. Following 2 weeks of selection on R2S medium, the calli – which have turned dark brown with tiny brownish translucent protuberances arising throughout the callus surface – were transferred to NBS medium. After 1 week of incubation, the protuberances developed into brownish globular structures which were gently teased apart with forceps on the medium around the original callus and incubated for 10–15 days in the resealed petri dish. Five weeks after co-culture, the globular structures had evolved into round shaped, compact, opaque and yellowish calli. The putatively transgenic, hygromycin-resistant calli were gently picked out, placed on the PRAG pre-regeneration medium and incubated for a further week. All of the resistant calli originating from a single co-cultured embryogenic nodular unit were grouped in a sector of the PRAG dish which can accomodate 40–50 resistant calli. Four to five, creamy-white, lobed calli with a smooth and dry appearance were individually transferred to one dish of RN regeneration medium, kept for 2 days in the dark, then maintained for 3 weeks under a 12/12-h (day/night) photoperiod with light provided at an intensity of 55 μmol/m per sec. Shoots regenerating from a resistant callus were dissected and sub-cultured in test tubes containing P medium for a further 3-week growth period to promote vigorous tiller and root development before being transferred to Jiffy peat pellets in the containment greenhouse for acclimatization. At 15 days, the plants were transferred to soil pots. Composition of culture media is detailed in Table 1.

Table 1 Media used for the *Agrobacterium*-mediated transformation procedure

Medium	Composition
NB Basic	N6 major salts and iron source (Chu 1975), B5 minor salts and vitamins (Gamborg et al. 1968), 30 g/l sucrose
NB	NB Basic + 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein hydrolysate, 2.6 g/l Phytagel, pH 5.8 (Li et al. 1993)
R2 Basic	R2 major and minor salts, vitamins and iron source (Ohira et al. 1973), 2.5 mg/l 2,4-D
CCL	R2 Basic + 10 g/l glucose, 100 μ*M* acetosyringone, pH 5.2
CCS	CCL + 7 g/l agarose
R2S	R2 Basic + 30 g/l sucrose, 50 mg/l hygromycin, 400 mg/l cefotaxime, 100 mg/l vancomycine, 7 g/l agarose, pH 6.0
NBS	NB Basic + 2.5 mg/l 2,4-D, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein hydrolysate, 50 mg/l hygromycin, 400 mg/l cefotaxime, 100 mg/l vancomycine, 7 g/l agarose, pH 6.0
PRAG	NB Basic + 2 mg/l BAP, 1 mg/l NAA, 5 mg/l ABA, 500 mg/l proline, 500 mg/l glutamine, 300 mg/l casein hydrolysate, 50 mg/l hygromycin, 100 mg/l cefotaxime, 100 mg/l vancomycine, 7 g/l agarose, pH 5.8
RN	NB Basic + 3 mg/l benzylaminopurine, 0.5 mg/l α-naphthaleneacetic acid, 30 g/l sucrose, 50 mg/l hygromycin[a], 4.5 g/l Phytagel, pH 5.8
P	MS major and minor salts, vitamins and iron source (Murashige and Skoog 1962), 50 g/l sucrose, 2.6 g/l Phytagel, pH 5.8

[a] Optional

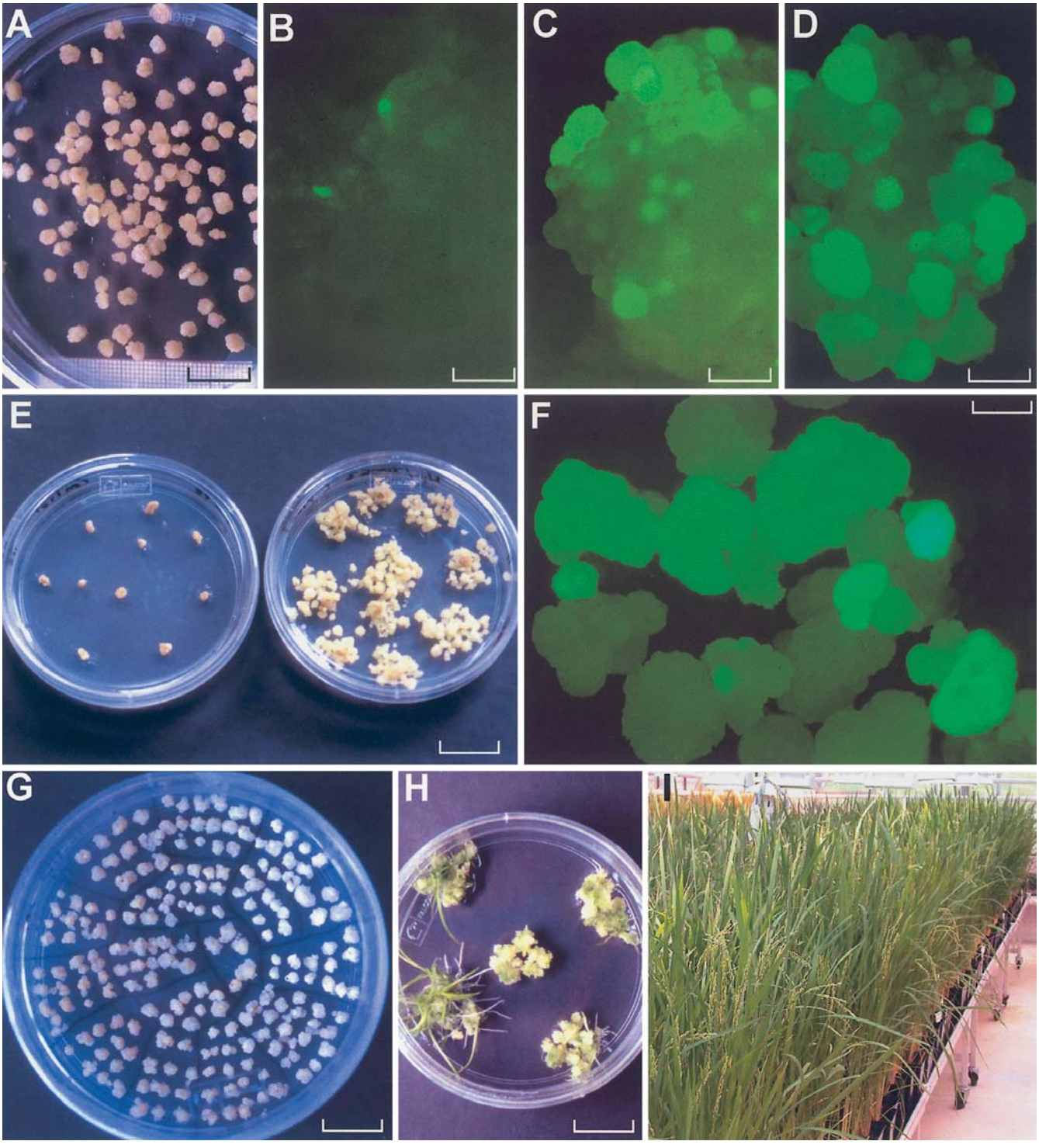

Fig. 2A–I Outlines of the *Agrobacterium*-mediated transformation procedure in rice cv. Nipponbare. **A** Seed embryo-derived embryogenic calli selected after reaching an optimal size for co-culture with *Agrobacterium* cells. *Bar*: 1 cm **B** GFP-positive sectors at the surface of an embryogenic callus, 4 days following transfer to R2S selection medium. **C, D** Independent development of hygromycin-resistant cell lines at the surface of an embryogenic callus, 12 (**C**) and 14 (**D**) days following transfer to R2S selection medium. *Bar*: 0.5 mm. **E** Development of hygromycin-resistant cell lines from ten calli, 5 weeks following their contact with a liquid co-culture medium containing (*right dish*) or not (*left dish*; negative control for testing stringency of selection) *Agrobacterium* strain EHA105 bearing the pC30063 plasmid. *Bar*: 2 cm. **F** GFP-positive and -negative, hygromycin-resistant calli proliferating from a single co-cultured callus. *Bar*: 0.5 mm. **G** Hygromycin-resistant cell lines originating from 15 co-cultured calli maturating on the preregeneration medium. *Bar*: 1.5 cm. **H** Young plants regenerating from five hygromycin-resistant calli. *Bar*: 2 cm. **I** Primary transformants flowering in the greenhouse

Detection of activity of gusA and gfp reporter genes

Histochemical assays to assess the expression of the *gusA* gene in rice tissues were carried out by staining with 5-bromo-4chloro-3-indolyl-β-D-glucuronid acid as described by Jefferson (1987). GFP activity was observed using a Leica MZFLIII fluorescence stereomicroscope. For the detection of GUS activity in leaves of T_1 progeny plants, three to five plants per line were tested.

T1 segregation analysis for hygromycin resistance

Thirty to fifty dehusked T_1 seeds were surface-sterilized, placed on a petri dish containing 50 mg/ml hygromycin and allowed to germinate under light at 25 °C. After 5–7 days, seeds were scored for germination. Segregation for hygromycin resistance (3:1 or 15:1) was calculated by a statistical method using chi_2-test with a probability of $P < 0.05$.

Southern blot analysis

Genomic DNA was extracted from 250 mg of fresh 4-week-old rice leaves using the MATAB/CETAB method. Aliquots of DNA (2 μg) were then digested with the *Hin*dIII restriction enzyme, incubated at 37 °C for 12 h and samples loaded onto a 0.8% agarose gel for electrophoresis in TAE buffer. After alkaline transfer of the DNA to Hybond-N_+ membrane, hybridization with the *gusA* or *hpt* probes labelled with α-[^{32}p]-dCTP was performed as indicated by the manufacturer (Amersham, UK). Autoradiography of the membrane was done using X-Ray film at –80 °C.

Amplification and sequencing of T-DNA left border flanking regions

The protocol is a modification of that of Siebert et al. (1995) and Devic et al. (1997) and consists of three steps: ligation, polymerase chain reaction (PCR)1 and PCR2. Each DNA sample (25 ng) was digested separately with the *Dra*I, *Nae*I or *Ssp*I restriction enzymes (10 u/ug) and ligated with the ADPR1/ADPR2 adaptor using T4 DNA ligase at 25 °C in 10-μl volume. The adaptor was prepared by annealing the complementary oligonucleotides, ADPR1 (5′-CTAATACGACTCACTATAGGGCTCGAGCGCCGCCCGGGG-AGGT-3′) and ADPR2 (5′-P-ACCTCCCC-NH2-3′) by incubation at 94 °C for 5 min in restriction enzyme buffer no. 4 (Gibco-BRL, Gathersburg, Md.) followed by gradual cooling to 28 °C. PCR1 was performed with a specific adaptor primer, AP1 (5′-GGATCC-TAATACGACTCACTATAGGGC-3′), and a specific T-DNA left border primer, HYG1 (5′-ATCAGAGCTTGGTTGACGGCAA-TTT-3′). PCR reactions contained 2 μl of the ligation/digestion, 1× PCR buffer (Eurobio), 0.25 u *Taq* DNA polymerase (Eurobio), 50 μM dNTP, 1.5 mM $MgCl_2$, 200 nM AP1 and HYG1 primers in a 20-μl volume reaction. Cycle conditions were performed in a MJR thermo-cycler (MJR Research, Mass.) as follows: 5 min at 94 °C followed by 29 cycles of 94 °C 30 s, 67 °C for 45 s, and 72 °C for 2 min and 30 s. A last cycle–the elongation step – was carried out at 72 °C for 5 min. PCR2 was performed with a nested specific adaptor primer, AP2 (5′-CTATAGGGCTCGAGCGGC-3′), and a nested specific T-DNA left border primer, CAMB3 (5′-AGATGC-CGACCGGATCTGTC-3′). PCR2 was performed with a 1/50 dilution of the PCR1 product using the same conditions as for PCR1, except for the final volume of the reaction which was adjusted to 100 μl. A 4-μl aliquot of the PCR2 reaction was loaded on a 1.2% agarose gel for electrophoresis. After gel staining with ethidium bromide, PCR products showing a unique band were then directly sequenced with a third nested specific T-DNA primer, CAMB6 (5′-CGCTCATGTGTTGAGCATAT-3′), either by M. Delseny's laboratory (University of Perpignan, France) or by Genome Express (Grenoble, France).

Homology searches

Identification of the T-DNA left border sequence (T-DNA footprint) was performed locally with BLASTN and a programme developed under the UNIX system for systematic identification (Brunaud et al., unpublished; INRA-Evry, France). It has been adapted by P. Larmande for this specific purpose (BIOTROP programme, CIRAD). After removing the T-DNA sequence, homology searches for remaining T-DNA or binary vector were performed with BLASTN. Sequences with no homology to the T-DNA or the binary vector (pC30063) were assigned to the rice genome if the sequence size was greater than 30 bp. Assignment to the rice Nipponbare genome sequence was done by homology search with BLASTN on the BAC/PAC sequence of the IRGSP consortium. The size of the query sequence influences the score obtained. To be accurate for assigning T-DNA insertions on the rice genome based on sequence homology, the following criteria were used. First, using BLASTN, homology of the T-DNA flanking sequence (FST) against itself gave the maximum score for the FST (S-FST max). Second, a score was obtained for the FST homology on the BAC/PAC sequence (S-FST). Third, the ratio between S-FST and S-FST max was calculated. An FST was assigned to the rice genome sequence when this ratio was greater than 0.95.

Results

Time course of production of HygR/GFP+ cell lines from co-cultured calli

Seed-derived embryogenic calli of cvs. Taipei 309, Nipponbare, Zhongzuo321 and Azucena were co-cultured with *Agrobacterium* strain EHA105 harbouring the pC30063 binary plasmid (Fig. 1) according to the procedure described in the Materials and methods. GFP expression was followed from 4 days after co-culture of the embryogenic callus pieces until plant regeneration (Fig. 2). Four days following the transfer of co-cultured callus pieces to the R2S first selection medium, we observed few developing green sectors at the surface of the callus (Fig. 2). Twelve days after transfer to selective medium, growth of the co-cultured callus preces was completely inhibited by hygromycin and they turned brown, whereas numerous translucent globules appearing as fluorescent sectors under UV light excitation arose from the callus surface. Fifteen days following the co-culture, whitish resistant cell lines had developed from the co-cultured callus that had by now turned dark brown. The resistant cell lines appeared to develop from structurally independent regions of the callus, and most of them exhibited GFP activity at this stage. Occasional bacterial outgrowth did occur. Uncontaminated calli were then transferred onto the second selection medium (NBS), and 1 week later resistant cell lines were gently spread over the medium surface. The use of the NBS medium as well as spreading considerably enhanced the growth of resistant cell lines, which developed into round, compact and opaque nodular calli within an additional 10–15 days. At this stage, approximately 70% of the resistant calli expressed either one or both reporter genes (Table 2).

Table 2 Frequency of hygromycin-resistant cell lines exhibiting either or both *gusA* and *gfp* reporter gene activity, 5 weeks after co-culture of Zhongzuo321 (top) and Nipponbare (bottom) calli

	GUS++	GUS+	GUS-	Total
GFP[a]++	32.6	3.1	2.7	38.5
GFP+	10.6	11.3	8.1	30.0
GFP–	4.5	10.4	20.7	31.5
Total	43.7	24.8	31.5	100 (444 calli)
GFP ++	25.9	7.2	17.7	50.8
GFP+	12.7	9.4	5.5	27.6
GFP–	1.1	7.2	13.3	21.6
Total	39.7	23.7	36.6	100 (181 calli)

[a] Calli exhibiting: ++, bright fluorescence/deep-blue staining; +, faint or sectorial fluorescence/staining; –, no fluroscence/staining

Transformation potential of four japonica rice cultivars

The results of the co-culture experiments of embryogenic calli are summarized in Table 3. Between 75.4% and 98.4% of the calli yielded at least one hygromycin-resistant and compact cell line by 5 weeks of co-culture. The mean number of hygromycin-resistant cell lines unambiguously exhibiting GFP activity produced per co-cultured callus ranged from 4.9 to 9.5. When those calli displaying no or low GFP activity – which accounted for more than one-half of the hygromycin-resistant cell lines irrespective of the cultivar (Table 2 and data not shown) – were included in the calculation, that mean number actually ranged from 10 to more than 20. For instance, in the most responsive cultivar, Zhongzuo 321, the mean number (± Standard deviation) of cell lines obtained per co-cultured callus reached 23.3 ± 10.4 when the hygromycin-resistant calli exhibiting faint and no GFP activity were taken into account. As many as 1,235 hygromycin-resistant calli were derived from 53 initial co-cultured callus pieces for this cultivar.

A subset of hygromycin-resistant, compact embryogenic calli that had not been selected on the basis of GFP activity was placed onto pre-regeneration (PRAG) and then regeneration (RN) medium. For each co-cultivated callus, several hygromycin-resistant cell lines were transferred while keeping track of the origin of the callus. Following 3 weeks of regeneration, the frequency of shoot-forming calli ranged from 23.0% to 61.6%. Upon monitoring the GFP activity of calli on RN medium, we also found that there was no significant difference between GFP-positive and GFP-negative calli in terms of regeneration frequency (data not shown). The average number of structurally independent, shoot-forming, GFP-positive, hygromycin-resistant cell lines ranged from 1.95 to 4.76 per co-cultured callus depending on the cultivar (Table 3). Should all the hygromycin-resistant cell lines formed on the NBS medium have been transferred to regeneration medium, these efficiencies would actually ranged from four to ten putative independent transformed plants per co-cultured callus. Plantlets developed a vigorous shoot and root system and were transferred to the greenhouse.

Independent versus clonal origin of the hygromycin-resistant plants regenerated from the same co-cultured callus

To ascertain that hygromycin-resistant plants regenerated from the same co-cultured callus were, as suggested by fluorescence observations, independent transformation events, we performed molecular analysis of the regenerated plants. We selected 120 regenerated plants from 17 independent co-cultivated calli of cv. Taipei 309, each of which generated 4–23 hygromycin-resistant plants (Table 4). Genomic DNA was isolated from each plant, digested by a restriction enzyme that cut once in the middle of the T-DNA (Fig. 1) and hybridized with the *hpt* and *gusA* probes localized at both T-DNA ends. As illustrated in Fig. 3 for callus no. 86, all but one of the 22 plants exhibited a distinct hybridization pattern, indicating that 21 plants were independent events, whereas plants 8 and 10 were of clonal origin. Overall, 114 out 119 plants (96%) exhibited a unique integration pattern of the T-DNA leading to the conclusion that most of the regenerated plants from the same co-cultured callus were independent events. The same results were obtained with other *japonica* rice cultivars, i.e *Nipponbare, Zhongzuo321, Azucena,* the only difference occurring in the average number of plants that can be regenerated per co-cultivated callus (data not shown).

Table 3 Summary of transformation experiments using four rice cultivars and *Agrobacterium* strain EHA105 bearing the pC30063 binary plasmid (*SD* standard deviation)

Genotype	Number of co-cultured calli (A)	Percentage of co-cultured calli yielding resistant cell lines (B)	Mean (±SD) number of GFP++ cell lines per co-cultured callus[a] (C)	Number of shoot-forming resistant cell lines[b] (frequency of regeneration (%) (D))	Number of shoot-forming, GFP++ calli per co-cultured callus (A × B × C × D)/A
Taipei309	110	75.4	4.9±3.0	264 (52.8)	1.95
Nipponbare	100	96.0	7.3±4.2	116 (49.8)	3.49
Zhongzuo321	53	94.3	8.2±4.1	909 (61.6)	4.76
Azucena	63	98.4	9.5±8.9	83 (48.4)	4.52

[a] The GFP++ cell lines represent 40–50% of the hygromycin-resistant cell lines

[b] A random subset of hygromycin-resistant calli was placed under regeneration conditions irrespective of whether or not the calli exhibited GFP activity

Table 4 Summary of the number of independent transgenic Taipei 309 plants obtained per callus transformed with pC30063

Callus number	Number of plants regenerated	Plants analysed[a]	Independent events[b]
11	10	8	8
14	5	5	4
16	10	11	11
21	5	5	5
26	5	5	5
27	5	4	4
33	5	4	4
45	8	8	7
48	6	6	6
60	12	9	8
63	5	5	5
68	5	5	5
70	5	5	5
79	8	7	7
81	5	4	4
83	7	6	5
86	24	22	21
Total	130	119	114
Percentage			96[c]

[a] Number of plants that have been analysed by Southern blot
[b] Number of independent events identified by Southern analysis using hybridisation with the *hpt* and *gusA* probes on genomic DNA extracted for each transgenic plant and digested by *Hin*dIII (see Fig. 3 for detailed analysis of callus no. 86)
[c] Percentage of independent events from the total number of transgenic plants analysed (n = 119)

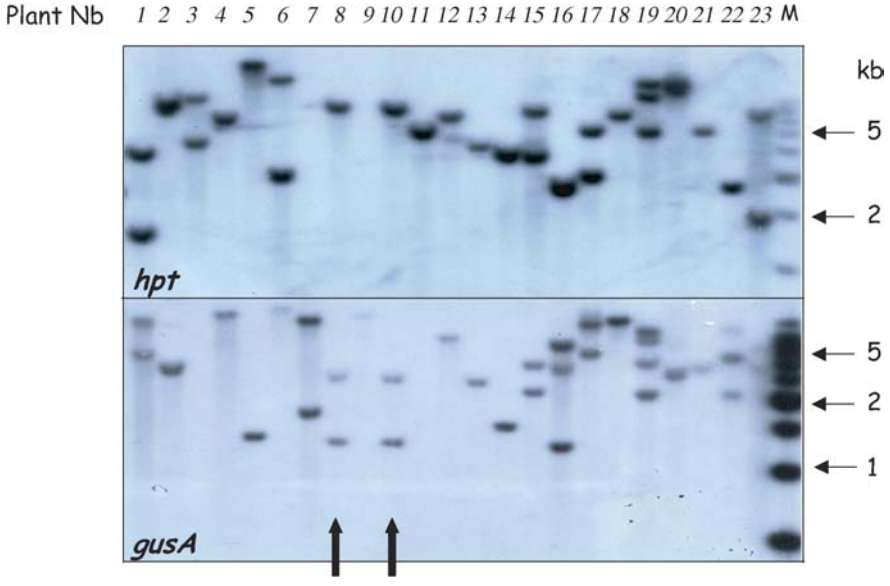

Fig. 3 Twenty-one independent transformation events obtained from one Taipei 309 callus (no. 86) transformed with the binary vector pC30063. Genomic DNA from *lines 1 to 23* except for *line 9* (*empty lane*) was digested with *Hin*dIII, and hybridization was performed with the *hpt* or *gusA* probe. The sizes of the bands visualized for each plant with the *hpt* or *gusA* probe were different, indicating that T-DNA integrated at different locations in the rice genome and that 21 out of 22 transgenic plants represent independent transformation events. *Black arrows* indicate the two primary transformants showing the same pattern of hybridization with *hpt* and *gusA* probes

T-DNA integration pattern and gene expression

The molecular analysis described above also allowed us to distinguish integration of single and multiple T-DNA copies among a large number of independent plants of the three rice cultivars studied here. We observed that a large number of plants with multiple T-DNA copies showed a putative tandem structure. For example, in Taipei309 plants numbered 3, 6, 8, 16, 19, 22, the number of bands hybridizing to the *gusA* (RB) and the *hpt* (LB) probes were different (Fig. 3). This could be interpreted as a head-to-head tandem repeat or as truncated T-DNA copies. In this case, the number of T-DNA copies was calculated as the highest number of bands observed either

Table 5 Distribution of the number of T-DNA copies in three different *japonica* cultivars transformed with pC30063. T-DNA copies were estimated by Southern blot analysis with the *hpt* and *gusA* probes as described in Fig. 3 and in the text

Estimated number of T-DNA copies	Number of plants[a] (%)		
	Taipei309	Nipponbare	Zhongzuo 321
1	57 (46.72)	36 (43.90)	14 (36.84)
2	40 (32.79)	31 (37.80)	12 (31.58)
3	17 (13.93)	12 (14.63)	9 (23.68)
4	8 (6.56)	3 (3.66)	2 (5.26)
5	0	0	1 (2.63)
ND	0	10	0
Total number of plants analysed	122	82	38
Total number of estimated T-DNA copies	220	146	78
Average number of T-DNA copies per plant	1.80	1.78	2.05

[a] Six and four plants from Taipei309 and Zhongzuo 321, respectively, contained a single truncated T-DNA with a *gusA* gene missing. Numbers in brackets indicate the percentage based on the total number of plants analysed for each cultivar

Table 6 Segregation analysis for hygromycin resistance and GUS activity in T_1 progenies of Taipei309 and Nipponbare transgenic lines

	Single T-DNA copy lines[a]		Multiple T-DNA copy lines[a]	
	Taipei 309	Nipponbare	Taipei 309	Nipponbare
hpt segregation 3:1	19/20 (95.00)	15/18 (83.33)	8/21 (38.09)	3/20 (15.00)
hpt segregation 15:1	0/20 (0.00)	0/20 (0.00)	4/21 (19.04)	0/20 (0.00)
GUS-positive lines	18[b]/20 (90.00)	14/18 (77.77)	8[c]/21 (38.09)	4/20 (20.00)

[a] Values represent the number of positive lines for the biological assay (segregation for hygromycin resistance or GUS assay for *gusA* expression) over the total number of lines tested. Numbers in brackets refer to the percentage obtained based on the total number of lines analysed per category, i.e. single or multiple copy

[b] The line which does not show *gusA* expression but segregates for *hpt* resistance has a truncated *gusA* gene

[c] All GUS-positive lines show *hpt* expression and segregate for one locus (five lines) or two loci (three lines)

with the *hpt* or the *gusA* probe. The presence of one or two T-DNA copies is the most prevalent situation in these populations, with a single T-DNA copy representing 47%, 44% and 37% of the regenerated plants in Taipei 309, Nipponbare, and Zhongzuo 321 cultivars, respectively (Table 5). Overall, the number of T-DNA inserts averaged 1.8–2.0. To estimate the average locus number, we conducted segregation analysis for hygromycin resistance in T_1 progenies of 40 Taipei 309 and Nipponbare primary regenerants. About 30–50 T_1 seeds were germinated on hygromycin (50 mg/ml), and resistant seedling development was scored after 5–7 days. Expression of the *gusA* gene was also tested using the GUS histochemical assay on young leaves of three to five hygromycin-resistant plants per line. To analyse the results, we divided the lines in two groups based on their T-DNA integration pattern. The first group represents lines with a single T-DNA copy, whereas the second group represents lines with multiple T-DNA copies. In the single-copy group, 80–95% of the T_1 lines were found to exhibit a 3:1 segregation ratio for hygromycin resistance, thereby confirming the presence of only one locus (Table 6). In the multiple-copy group, unusual segregation was observed in many lines showing a percentage of hygromycin-resistant plants much lower than 75%, indicating that the expression of *hpt* gene was silenced. In some lines, *hpt* gene expression was only partially silenced because plants germinated but displayed reduced growth compared to hygromycin-resistant plants (data not shown). Nipponbare pC30063 multiple-copy lines appeared to be more prone to silencing than Taipei 309 lines because only 15% of their T_1 progenies segregated according to a 3:1 or 15:1 ratio (the expected ratios for one or two independent integration loci, respectively). These results were confirmed by the detection of GUS activity using a histochemical assay on leaves: all but one line silenced for *hpt* did not exhibit any GUS activity in both cultivars, whereas most of the progeny lines segregating in a Mendelian fashion for hygromycin resistance did express the *gusA* gene. As a consequence, it was not possible to deduce from phenotypic assays an average number of T-DNA integration loci in multiple T-DNA copy primary regenerants. Therefore, the frequency of lines which segregated for two or more locus (9.5%) in Taipei 309 was probably underestimated.

Estimation of the frequency of binary vector backbone integration in T-DNA plants is another essential feature of T-DNA integration when the aim is to recover genomic regions flanking insertion points. We used two probes (LBout and Rbout) located just outside of the right and left T-DNA border ends (Fig. 1) to detect the presence of such sequences. By doing so, more than 30% of the plants in the three cultivars tested were found to have integrated the binary vector backbone. Nevertheless, plants with a

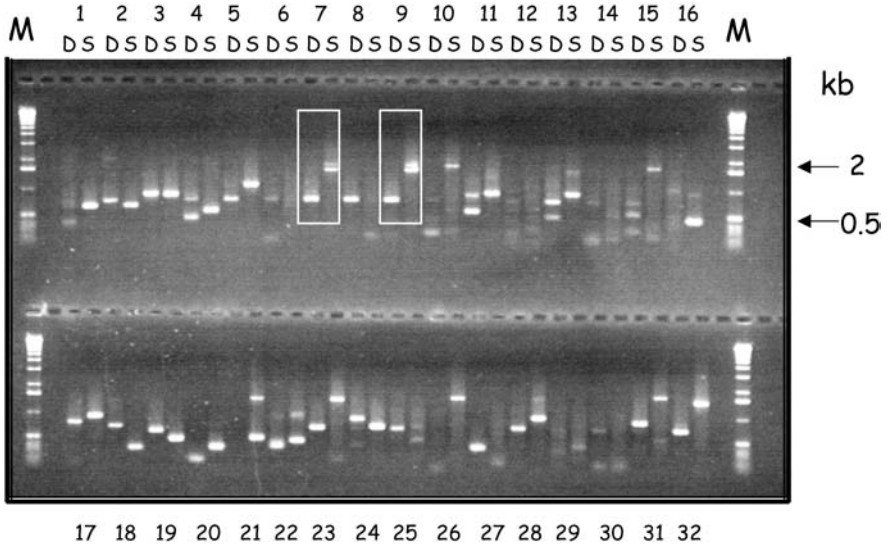

Fig. 4 Isolation of FSTs from DNA of 32 transgenic plants by the adapter-ligation PCR method. Visualization of the PCR2 reaction product was performed by electrophoresis on a 1.2% agarose gel. Transgenic plants are numbered from *1 to 32*. For each plant, genomic DNA was digested independently with the *Dra*I and *Ssp*I restriction enzymes and ligated with the ADPR1/ADPR2 adaptor. Two-step PCR using the combination of primers HYG1/AP1 and CAMB3/AP2 at the T-DNA left border was then performed. *Squares* highlight identical pattern obtained for two different plants, indicating that they likely arise from a unique transformation event. *D Dra*I, *S Ssp*I, *M* 1-kb ladder marker (Gibco BRL)

single T-DNA copy had integrated the binary vector backbone at a lower frequency than plants with multiple T-DNA copies. For example, in Zhongzuo 321, 12 out of 37 plants had integrated the binary vector, but none of them harboured a single T-DNA copy.

Isolation and sequencing of T-DNA flanking regions

Our next objective was the characterization of a large number of T-DNA flanking sequences (FSTs). For this purpose, we generated additional 477 Nipponbare T-DNA plants from 98 calli co-cultured with the EHA105 strain bearing the pC30063 vector. Efficiency was comparable to that reported in Table 3. Amplification of the region flanking the T-DNA left border was performed using an adaptor-ligation PCR method (Siebert et al. 1995) and adapted to plants by Devic et al. (1997) (see Materials and methods). Amplification from the left border site was preferred because molecular characterization of T-DNA rice plants also showed that the right border is more often associated with head-to-head tandem repeats, thereby reducing the efficiency of FST isolation. This three-step method based on PCR amplification with specific nested T-DNA primers was later found to be amenable to automatization, allowing high-throughput recovery and sequencing of T-DNA flanking regions in rice (Sallaud et al., in preparation). Using genomic DNA of pC30063 primary regenerants, the procedure proved to be highly reproducible and gave satisfactory results with more than 60% of the lines showing a unique PCR2 product. A typical agarose gel showing the PCR2 product corresponding to FST amplification from DNA isolated from 32 T-DNA lines and digested with *Dra*I and *Ssp*I restriction enzymes is shown in Fig. 4.

The use of both enzymes for DNA restriction allowed unambiguous detection of rare clones resulting from callus fragmentation that could have occurred through the transformation procedure (see Fig. 3). From 477 plants, 356 unique PCR2 products with either *Dra*I or *Ssp*I were obtained and directly sequenced with a T-DNA nested primer (CAMB6). Only 42 PCR2 products did not show readable sequences (11%). Sequence analysis allowed the identification of the T-DNA footprint for 296 sequences (94.3%) (Table 6), indicating that the process was reliable. After the T-DNA footprint sequence was removed, the size of the genomic DNA sequence averaged 250 bp. As expected, we observed a deletion of the T-DNA left border ranging from a few to up to 200 bp with most deletions found between –1 to –50 bp from the end of the 25-bp direct repeat of the left border sequence (data not shown). FST sequence homology with the T-DNA or the binary vector was detected using the BLASTN programme with approximately 35% of the FSTs falling into this category (Table 7). Detailed analysis of these sequences indicated that tandem repeats, filler DNA, T-DNA deletion or inversion due to T-DNA rearrangement (13%) as well as binary vector sequence (22%) were common. Due to the presence of a *Dra*I or *Ssp*I site close to the T-DNA insertion and used for FST isolation, some (11%) sequences were too short (<30 bp) for the homology search and were discarded. To localize the T-DNA insertion sites to the rice genome, we used the remaining sequences for the homology search with the BLASTN programme on the BAC/PAC Nipponbare sequences from the IRGSP sequencing project. To assign a

Table 7 Sequence characteristics of PCR2 products

Sequence characteristics	T-DNA missing	Binary vector	Tandem	FST <30 bp	FST >30 bp	Total
Number of sequences	18	69	43	34	151	315
Percentage	5.73	21.97	13.38	10.83	48.09	

position of the T-DNA insertion to the rice genome, we used high-stringency criteria based on the BLASTN score obtained when significant homology is detected (see Materials and methods for more details). By doing so, 77 of 151 FST sequences (50%) were assigned to a rice chromosome coinciding with the percentage of the genome which was available at the time of the study (February 2002, 57%).

Discussion

A highly efficient transformation procedure for japonica rice

We report here the application of an *Agrobacterium*-mediated transformation procedure which allows the generation of large populations of independent transformation events in rice. A wide range of *japonica* cultivars appeared amenable to this method, and repeated experiments subsequently performed in our laboratory yielded comparable results demonstrating that the procedure is reliable.

The efficiency of this transformation procedure results from a high frequency (75–98%) of co-cultured calli forming hygromycin-resistant cell lines. This is likely due to the precise timing in sub-culturing and careful selection of seed embryo derived-callus tissue before co-culture. This procedure essentially followed that detailed by L. Chen and collaborators for producing target callus tissues for particle bombardment, a method which yielded an average of 22 transgenic plants per 100 calli, in 42 independent experiments (Chen et al. 1998). Previously reported efficiencies of *Agrobacterium*-mediated transformation of rice callus tissue typically ranged from 10% to 50% (Datta et al. 2000; Dong et al. 1996; Hiei et al. 1994; Nakagawa et al. 2000; Rashid et al. 1996) and were considered satisfactory to engineer genes of interest into rice.

Secondly, the fact that each responsive co-cultured callus may generate a large number of independent hygromycin-resistant cell lines increased the number of transgenic plants generated in a given experiment by five to ten fold. The several resistant cell lines arising from a single immature embryo scutellum or callus piece subjected to microprojectile-bombardment have so far been considered to be mostly clonal in nature, resulting from resistant callus fragmentation (Chen et al. 1998) and, consequently, only one also resistant callus is generally selected for regeneration. However, it has recently been reported that the plants regenerated from the two to four resistant callus pieces which sometimes arise from a single bombarded callus may represent independent transformation events (Bec et al. 1998). This point has not been further investigated in *Agrobacterium*-mediated transformation even though the number of cells equally accessible to a safe gene transfer could be assumed to be greater during immersion in liquid co-culture with *Agrobacterium* suspension than following an exposure to a microprojectile bombardment. It is likely again that the careful choice of rice tissue and the conditions of transfection and selection of transformed cells used in our transformation method may favour the generation of multiple independent events compared to those used in other laboratories. We are currently investigating the influence of each transformation step to identify which one is critical in achieving such a high transformation efficiency. The choice of the bacterial strain EHA105, a kanamycin-sensitive derivative of EHA101 (Hood et al. 1993), bearing the so-called "super-virulent" Ti plasmid pTiBo542 might, for instance, ascribe for a high transfer efficiency even though the latter has been shown to be less efficient than the "ordinary" LBA4404 strain (Hoekema et al. 1983) for transforming rice (Hiei et al. 1994). EHA105 has also been used in parallel with LBA4404 in both a recent study (Yin and Wang 2000) and in further experiments in our laboratory, and no striking difference of efficiency of transformation has been noticed.

Transgene expression is influenced by T-DNA integration patterns

It was important to investigate whether the increased efficiency of T-DNA transfer in our procedure would also result in a change in the distribution of integration patterns of T-DNA copies with respect to results previously reported on the molecular characterization of T-DNA rice plants. Our molecular analysis performed on a very large number of plants showed that the number of T-DNA copies averaged 1.8–2, including a 30–45% range of plants harbouring a unique T-DNA copy depending on the cultivar tested. In two independent studies involving the analysis of 30 plants by Southern hybridization, the integration of a similar mean number (2.1 and 2.3, respectively) of *hpt* or *gusA* copies was observed (Hiei et al. 1994; Jeon et al. 2000). In our study, the average number of integration loci was difficult to estimate in multiple-copy plants due to gene silencing. T_1 segregation analysis for *hpt* and *gusA* gene expression showed an unexpected ratio with a higher than anticipated number of sensitive plants. This has been previously described by Hiei and collaborators (1994) but was not mentioned by

Jeon and collaborators (2000). Moreover, we clearly found that plants with multiple T-DNA copies were more affected by gene silencing than plants with a unique T-DNA. Tandem structures such as inverted repeats have shown to be responsible for gene silencing by a mechanism known as post transcriptional gene silencing (PTGS), also termed RNAi (Metzlaff et al. 1997). Establishing the correlation between T-DNA integration pattern and transgene expression in a significant number of lines has not been previously reported in rice. T-DNA inverted repeats are most likely responsible for the gene silencing which occurred in our transgenic lines, as shown by experiments of Wang and Waterhouse (2000). Strategies to create large T-DNA insertion line populations often incorporate the use of enhancer or gene trap systems to allow in vivo gene detection (Springer 2000). Forward genetic screening to identify tissue specific- or induced gene expression is also a classical approach. If silencing occurred in plants with multiple insertions of the T-DNA bearing a gene detector, which may account for more than half of the rice T-DNA population, a large proportion of these plants would be consequently useless to screen. Moreover, multiple T-DNA insertions are often located at the same locus and subject to T-DNA rearrangement. The T-DNA carries at least one promoter to direct the expression of the gene coding for antibiotic resistance to select the transformed cells. Promoters such as CaMV 35S often carry enhancer elements which then could interact positively or negatively with the gene detection system. This silencing phenomenon, the fundamental mechanism of which has been extensively studied in the past few years, is not often taken into account when a gene detection system is used in a functional genomics project.

Identification of genomic regions flanking T-DNA inserts depends on the T-DNA integration pattern

When large insertional mutant populations are available, PCR screening of detection for insertion within a gene using gene- and insertion-specific primers is commonly used. Recently, insertions within genes coding for a rice phytochrome A (OsphyA) and an homeobox (Osh15) protein have been identified in the *tos17* rice mutant population (Sato et al. 1999; Takano et al. 2001). When the genome sequence of a plant species is available, systematic characterization of the insertion from each mutant is an alternative approach for reverse genetic studies. The insertion can be easily localized in the genome and large sequence information surrounding the insertion point is accessible. Annotation of the surrounding sequence is then accurate and allows *in silico* database searching to identify the mutant in a gene of interest. This strategy has been recently developed for *Arabidopsis* T-DNA mutant populations (Samson et al. 2002: FlagDB http://genoplante-info.infobiogen.fr). A rice T-DNA insertion line population of more than 25,000 individuals has been recently produced in our laboratory, and we are currently working to systematically identify sequences of T-DNA insertion sites (Sallaud et al., in preparation).

A prerequisite for this reverse genetics approach is to investigate the amenability of T-DNA inserts for the isolation and further sequencing of T-DNA flanking sequences. We have demonstrated here that the adaptor-ligation PCR method is highly efficient and reliable for rapidly isolating a large number of FSTs in rice. From 477 mutant lines, 150 FST with an average size of 250 bp were rapidly identified, half of which were assigned to rice bacterial artificial chromosome clones, and several genes were tagged (data not shown). The size of the FST obtained is clearly sufficient to identify the T-DNA insertion within the rice genome as the rice sequence becomes available. The fact that the percentage of insertions which have been assigned on the genome corresponds to the actual percentage of the genome sequence available indicates that the strategy is reliable. However, this preliminary experience in T-DNA FST recovery and analysis leads to some important observations. As previously described in several species, T-DNA rearrangement and the presence of the binary vector sequence occurred frequently (35%) in plants harbouring multiple T-DNA insertions. It is clearly a drawback for the identification of the T-DNA insertion site within this subset of plants, and it would be too tedious of a task to circumvent it. In a large-scale programme, removal of these sequences prior to the costly sequencing step is necessary. Identification of flanking regions containing vector sequences could be achieved through high-density spotting of amplification products on nylon filters and subsequent hybridization with a binary vector probe. Such filters would also be appropriate substrates for identifying a line containing an insertion within a specific gene, or differential screening of cDNA populations.

The transformation efficiency reported here allows the production of several thousand transgenic rice plants from a few hundred callus pieces in a single co-culture experiment. Such a highly efficient T-DNA delivery procedure should further assist the development of large-scale RNAi and HR studies in this model genome species. The T-DNA integration patterns in the primary transformants are consistent with results reported in the literature, and automated recovery of genomic regions flanking insertion points appears efficient. The availability of high-throughput transformation and characterization procedures of T-DNA plants allows rapid creation of large populations of T-DNA insertion lines, including specialized libraries with T-DNAs carrying *Ds* elements or activation tags. Detailed analysis and annotation of insertion points in a large library of lines will also provide more precise information on the preference of integration of the T-DNA in the rice genome.

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