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ORIGINAL PAPER

Agrobacterium-mediated transformation of Australian rice varieties and promoter analysis of major pollen allergen gene, *Ory s 1*

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Abstract A simple protocol for *Agrobacterium*-mediated transformation of Australian rice using mature embryos is described. Transgenic plants of two commercial genotypes of Australian rice, Amaroo and Millin, were produced. Transgenic plants were obtained by applying selection pressure to callus and to the regenerated shoots. Exclusion of the selective agent (hygromycin) during plant regeneration was found to be critical for recovery of transgenic plants from these commercial varieties. Transgenic plants were produced after 3 months. The developed system was also used to study spatial and temporal expression of a rice pollen-specific gene, *Ory s 1*. Expression of p*Ory s 1::uidA* in transgenic rice demonstrated GUS expression in mature pollen, hence indicating potential use of this promoter to direct pollen-specific gene expression. Further *Ory s 1* 5′ deletion study indicated that the pollen-specificity element may reside within −405 bp to the start of the transcription, while the region upstream of −405 contained a cis-acting regulatory element(s) responsible for quantitative expression of this gene.

Keywords *Agrobacterium*-mediated transformation · Pollen-specific gene · Australian rice · *Ory s 1* · Pollen allergen

Abbreviations
BAP 6-Benzylaminopurine
2,4-D 2,4-Dichlorophenoxyacetic acid
NAA 1-Naphthaleneacetic acid
ABA Abscisic acid

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Introduction

Pollen grains of grasses are predominate source of outdoor allergen afflicting up to 25% of the population in sub-temperate climates. Inhalation of likely allergen induces production of allergen-specific IgE antibodies in sensitive individuals. IgE-mediated symptoms, rhinitis and bronchial asthma are triggered by the release of proteins from pollen following contact with the moist surface of the human respiratory tract. Grass genera pollen has been implicated as the main cause of respiratory allergy than any other flowering plant family in different parts of the world. Allergenic proteins of pollen that provoke allergic reactions have been identified and classified into groups. The major and most abundant allergen belongs to group 1 and constitute up to 5% of the total soluble protein of pollen. Group 1 allergens are present in pollen cytoplasm and are discharged swiftly upon hydration. Ory s 1 (group 1) has been identified as a major allergen in rice pollen (Xu et al. 1995a, b, 1999). We previously reported cloning and immunological characterization of Ory s 1 and showed that the *Ory s1* gene is expressed in mature rice anthers, but not in other floral and vegetative tissues (Xu et al. 1995a).

Rice represents one of the major cereals consumed by more than half of the world's population. Australia has been one of the highest rice yield producing countries of the world. Popular Australian commercial rice includes Amaroo, Jarrah, Pelde, Langi and Millin varieties. However, the main rice growing areas in southern New South Wales suffer from environmental factors such as low

temperature during flowering season, adversely reducing the yield up to 25% (Williams and Wensing 1998). A narrow genetic base of Australian rice (Ko et al. 1994) impedes the development of high-yielding and low temperature-tolerant rice varieties through conventional breeding methods. Genetic manipulation and transformation technology provide an opportunity to express desirable genes in crop plants. *Agrobacterium*-mediated transformation has been used successfully in transformation of dicotyledonous plants; however, this bacterium was initially thought not to be suitable for transformation of monocotyledonous plants, including rice. However, several successful attempts have been made to develop protocols for *Agrobacterium*-mediated transformation of monocots such as barley (Tingay et al. 1997), maize (Ishida et al. 1996) and rice (reviewed by Hiei et al. 1997; Giri and Laxmi 2000). Some examples have been the production of breeding lines conferring resistance against disease (Nishizawa et al. 1999), insect (Cheng et al. 1998) and herbicide (Toki 1997), tolerance against salinity (Mohanty et al. 2002) and chilling (Yokoi et al. 1998), and also improvement of the nutritional quality of rice including production of ferritin (Goto et al. 1999). These studies mostly used highly regenerable model rice variety. However, the development of transformation technology for commercial varieties of rice still needs attention.

Protocols for *Agrobacterium*-mediated transformation have been developed for *japonica* rice (Hiei et al. 1994, Toki 1997), *javanica* variety, Gulfmont, Jefferson (Dong et al. 1996), *indica* variety, Basmati 370, Pusa Basmati 1 (Rashid et al. 1996 and Mohanty et al. 2002) and Australian rice, Jarrah and Amaroo (Upadhyaya et al. 2000). One of the major problems facing *Agrobacterium*-mediated transformation has been the development of methods to produce a high proportion of plants showing predictable transgene expression without collateral genetic damage (Birch 1997).

This paper discusses the development of an efficient and simple protocol for *Agrobacterium*-mediated transformation of commercial Australian rice varieties, Amaroo and Millin. The developed protocol was then used to study temporal and spatial expression of *uidA* gene driven by *Ory s 1* promoter in transgenic rice, Amaroo. Furthermore, expression analysis of GFP reporter gene driven by a set of *Ory s 1* 5′ deletion promoters is also reported.

Materials and methods

Bacterial strains and transformation vectors

Agrobacterium tumefaciens EHA 101 and plasmid pIG121Hm were obtained from Prof K Toriyama (Laboratory of Plant Breeding, Faculty of Agriculture, Tohoku University, Seandai, Japan). p*Ory s 1*::*uidA* construct was prepared by fusion of 1,524 bp of *Ory s 1* promoter with *uidA* reporter gene and cloned into *Hind*III site of pIG121Hm.

Plant material

The seeds of two commercial varieties of Australian rice, Amaroo and Millin, were supplied by the Yanco Agricultural Institute, New South Wales, Australia. The seeds were stored at 4°C until use.

Explant preparation

Callus induction was essentially the same as described by Azria and Bhalla (2000). Scutellum-derived calli were initiated on callus induction (CI) medium for 2–3 weeks and subcultured on fresh CI media for 1, 2 and 3 days before use for transformation experiments.

All the cultures were incubated at 25°C under light 55 μEm^{-2} s^{-1} at the Petri dish level.

Plant transformation and regeneration of transgenic plants

The scultellum-derived calli were immersed in the bacterial suspension (OD600 nm of 0.2; 0.3; and 0.4) for 2, 4, and 6 min as described by Yokoi et al. (1997) and subsequently transferred to co-cultivation media either (MSCO) (CI with proline and casamino acids, 10 g/L glucose, 100 μM acetosyringone, 2 g/L gelrite, pH 5. 2) or N6CO (N6 salts and vitamins, 30 g/L sucrose, 10 g/L glucose, 2 mg/L 2,4-D, 100 μM acetosyringone, 2 g/L gelrite, pH 5.2, Chu et al. 1975). The infected calli were co-cultivated at either 25°C or 28°C in dark for 2, 3, 4 and 5 days, and then washed three times with sterile water supplemented with 500 mg/L carbenicillin. The calli were selected on MSE medium (MSCO without acetosyringone and glucose, 500 mg/L carbenicillin, 30 mg/L hygromycin and 3 g/L gelrite at pH 5.8).

After 3 weeks on selection, the calli were transferred to regeneration (MSRE) medium (MSE, 30 g/L sorbitol, 2 g/L casamino acids, 1 mg/L NAA, 3 mg/L BAP, 250 mg/L carbenicillin and 30 mg/L hygromycin). After 2–3 weeks on this medium, cultures were transferred to fresh MSRE medium with or without hygromycin. This was repeated twice. Regenerated shoots were then transferred to root induction (RI) medium (MSE with or without 30 mg/L hygromycin, pH 5.8). Hygromycin-resistant plants were transferred to pots containing pasteurized mixture of soil, sand, bark and peat moss (1:2:2:2) and grown under glasshouse conditions.

DNA blot analysis

Genomic DNA was isolated from leaf tissues using the method of Doyle and Doyle (1990). Genomic DNA (10 μg) from each sample was digested with *Bam*HI and resolved on 0.7% (w/v) agarose gel. The DNA blot was probed with ^{32}P*hph* gene (*Bam*HI/*Eco*RI fragment) of pIG121Hm. Oligolabelling and hybridization procedures were conducted following standard protocols (Bresatec, Australia).

Polymerase chain reaction (PCR)

The integration of T-DNA into rice chromosome was also confirmed by PCR analysis using rice genomic DNA according to Spertini et al. (1999). Two successive PCR amplifications were conducted using specific primers, right border (RB) and adaptor primers, amplifying genomic plant DNA flanking the right border of T-DNA. The PCR products were separated on 1.5% (w/v) agarose gel. The DNA blot of the PCR products was probed using the right border probe prepared by PCR amplification of the right border fragment of T-DNA region of pBI101 using specific primers (5′CATGAGCGGAGAATTAAGGG3′ and 5′TCTTGACAAAAAGAACCGGG3′).

GUS and DAPI staining

Histochemical detection of GUS activity in pollen of transgenic rice was performed according to Jefferson (1987). The stages of pollen development were determined by DAPI (4,6-diamidino-2-phenylindole) staining followed by viewing using a fluorescent microscope (Olympus BX60) at 490 nm excitation.

Progeny analysis

Segregation analysis of the transgene (*hph* gene) was carried out on T1 seeds from each transgenic plant. After surface sterilization, a total of 40–50 mature seeds from untransformed plant and each of transgenic plant were germinated on MS media (Murashige and Skoog 1962) for 3 days followed by transfer to MS media containing 30 mg/L hygromycin. The number of seedlings producing shoots and roots was scored 7 days after the transfer (Rashid et al. 1996).

Ory s 1 promoter characterization

Full-length promoter of 1,507 bp isolated previously in our laboratory (Xu et al. 1999) was ligated with the plant optimized GFP coding region of 1,032 bp in pBlue Skript vector (SK+) (Stratagene), resulting in the p*Ory–GFP* construct. To characterize *Ory s 1* promoter deletion constructs were generated by PCR amplification using the p*Ory s 1*–GFP vector as template. Full-length promoter used for stable transformation experiments and template for deletion constructs was −1,524 bp. For amplification of the 405-bp deletion fragment, the T3 promoter primer (Promega) was used together with primer D-4 (GCTAGT ATATAATTGCG), and for amplification of the 812-bp deletion fragment the T3 promoter primer in combination with D-8 (CGATGTCATAGAGGTAC) primer was used. The PCR-generated fragments were purified through Qiagen PCR columns and cloned into pBlueScript (SK+). Purified plasmid DNAs were used for transformation of lily pollen and onion peels by microprojectile bombardment.

Transient expression analysis of GFP reporter gene driven by a set of *Ory s 1* 5′ deletion promoters was performed by particle bombardment using a helium-driven DuPont Biolistic Deliver System PDS-1000 (BioRad) at 1,100 psi by using 1.0um gold particles following themanufacturer's instructions. GFP expression in lily pollen and onion peel (vegetative cells) was observed after 20 h under UV excitation with a fluorescent microscope (Olympus BX60) and imaged with a digital camera (Olympus).

Results

Optimization of *Agrobacterium* infection

Agrobacterium infection protocol was optimized using Amaroo callus. *Agrobacterium tumefaciens* EHA 101 harboring pIG121Hm containing *uidA* as reporter and *hph* as selection marker genes was used. Co-cultivation media were either MSCO or N6CO (Azria and Bhalla 2000). The efficiency of *Agrobacterium* infection was calculated as the number of callus showing GUS expression divided by the total number of callus used. A series of experiments with EHA 101 showed that the density of bacterial suspension was critical (data not shown). Blue spots as a result of GUS expression were observed as early as 2 days of co-cultivation, with 25.0–33.3% calli showing GUS expression (data not shown). In general, the number of calli showing GUS expression increased with prolonged co-cultivation period and higher density of *Agrobacterium* while keeping infection time (4 min) constant. However, severe callus necrosis was observed when *Agrobacterium* at density higher than 0.2 and co-cultivation period of longer than 3 days was used. Hence, co-cultivation of calli for 3 days with *Agrobacterium* at OD 0.2 and infection time of 2 min resulted in minimum callus necrosis. Therefore, this combination was used in subsequent experiments.

The effect of pre-culture of callus prior to *Agrobacterium* infection was also assessed. Pre-culture the calli for 1

and 2 days improved the percentage of calli showing GUS expression up to 41.1 and 52.9%, respectively. The results indicated that actively growing or dividing callus cells were the best explant for *Agrobacterium* infection.

Selection and regeneration of transgenic rice plants

Following co-cultivation, the explants were transferred to selection medium. Preliminary experiments showed that hygromycin at a concentration of 30 mg/L completely inhibited growth and further regeneration of untransformed Amaroo calli (data not shown). After 3 weeks on selection medium, the resistant calli continued to grow and remained yellowish, while the untransformed cells turned brown.

Resistant calli were subsequently transferred to fresh shoot regeneration (MSRE) medium for further selection. After 2–3 weeks on this media, cultures were transferred to fresh MSRE without hygromycin for shoot initiation. An outline of our *Agrobacterium tumefaciens*-mediated transformation approach is shown in Fig. 1. Our earlier experiments failed to regenerate shoots if hygromycin was included in the medium. On hygromycin-free shoot initiation medium, a total of 1,450 shoots were regenerated from Amaroo and 516 shoots were regenerated from the Millin variety (Table 1). The regenerated shoots were further selected on root induction medium containing 30 mg/L hygromycin. After the first round (2–3 weeks) on root induction media, most of the shoots turned brown and died. After the second round of selection or about 4–5 weeks on this medium, a total of 23 putative transgenic plants in experiment I, 26 putative transformants in experiment II and 106 putative transgenic plants in experiment III were recovered from both Amaroo and Millin varieties, giving an average transformation efficiency of 8.8% for Amaroo and 1.8% for Millin (Table 1). Twenty putative transgenic Amaroo plants from each experiment and all the putative transgenic Millin plants were transferred to a glasshouse. The plants grew normally and set seed.

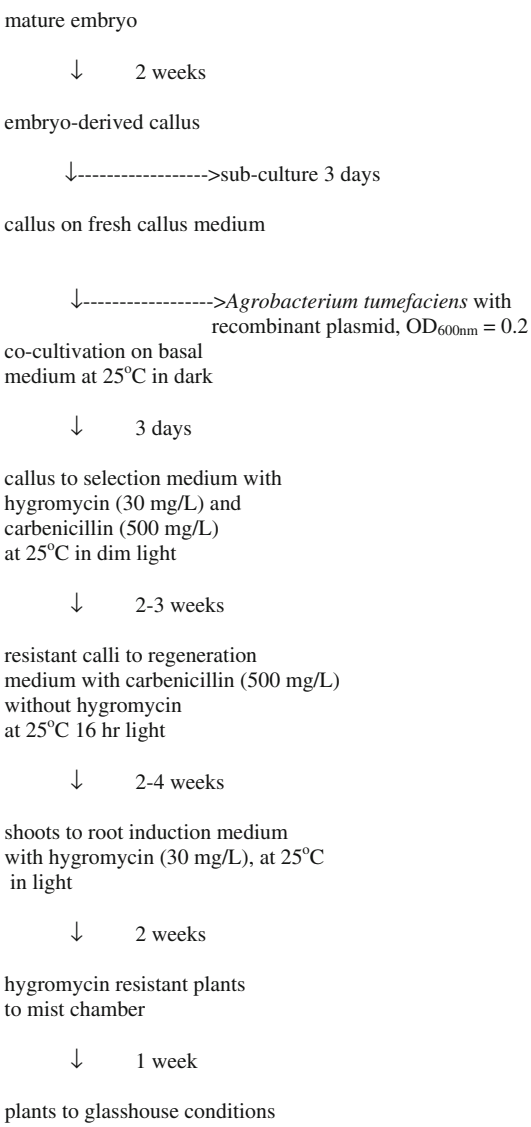

Fig. 1 Outline of *Agrobacterium tumefaciens*-mediated transformation of Australian rice

DNA blot and PCR analyses

Genomic DNA from 40 putative transgenic rice plants carrying pIG121Hm were digested with restriction enzyme *Bam*HI, which cuts twice in the integrated T-DNA resulting in 3.5 kb size fragments. Hybridization with the *hph* probe detected a fragment of 3.5 kb in 23 of the 40 putative transgenic plants (data not shown).

PCR analysis amplifying the right border of the T-DNA region of pIG 121Hm flanking plant genome was further carried out to determine the copy number of the transgene in several of the transgenic rice plants. Gel electrophoresis of PCR products showed various patterns of amplification, indicating various numbers of T-DNA inserts in each samples. Southern analysis of the PCR product probed with the RB-specific probe revealed that most transgenic plants carried one or two inserts of DNA (data not shown).

Expression of the p*Ory s 1::uidA* chimeric gene in transgenic rice plants

Using the optimized protocol as described above, chimeric construct obtained by fusion of *Ory s 1* promoter and the *uidA* reporter gene was introduced into Amaroo rice via *Agrobacterium*-mediated transformation. Fourteen putative transgenic rice plants were obtained after selection on hygromycin. Nine primary transformants with stable integration of the p*Ory s 1::uidA* gene were transferred to the glasshouse. The plants grew normally to maturity under

Table 1 *Agrobacterium*-mediated transformation of Australian rice varieties, Amaroo and Millin

Rice variety	No. of callus infected	No. of shoots regenerated	No. of shoots survived on selection	Transformation efficiency (%)
Amaroo				
Experiment I	959	704	20	2
Experiment II	259	564	20	7.7
Experiment III	414	182	104	25
Total	1,632	1,450	144	8.8
Millin				
Experiment I	236	175	3	1.2
Experiment II	295	280	6	2.0
Experiment III	69	61	2	2.9
Total	600	516	11	1.8

Transformation efficiency was calculated as the number of shoots survived on selection media containing 30 mg/L hygromycin divided by the number of calli infected and multiplied by 100

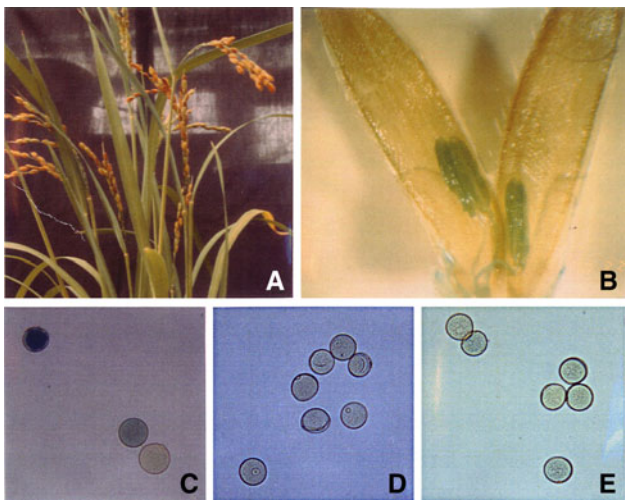

Fig. 2 GUS expression in mature flowers of transgenic rice plants. **a** Mature transgenic rice plant carrying p*Ory s 1::uidA* gene at the seed set stage. **b** Mature flowers of transgenic plants showing detection of GUS expression in anthers only, ×10. **c** Mature pollen of the transgenic plant showing GUS expression in the pollen grain, ×200. **d** Mature pollen of control plant. No GUS expression was detected, ×200. **e** Immature (bicellular) pollen of the transgenic plants showing no GUS expression, ×200

glasshouse conditions. The flowers appeared normal and seed set was found to be in a similar abundance to those of control plants (Fig. 2a; Table 2).

Flowers from untransformed plants and transgenic rice plants carrying the p*Ory s 1::uidA* gene were collected for GUS expression analysis. GUS activity, as shown by blue stain, was observed only in whole mature pollen of the transgenic plants (Fig. 2c, 2d). No GUS activity was detected in other floral organs such as stigma or style of the transgenic plants (Fig. 2b). The number of blue-stained mature pollen showing GUS expression (Fig. 2c) in the transgenic plants ranged from 45 to 57% (Table 3). GUS staining was not detected in immature pollen of the transgenic plants (Figs. 2e, 3). This expression pattern confirmed earlier observations (Xu et al. 1995a; Xu et al. 1999) that *Ory s 1* is expressed at the mature pollen stage.

Intensity of GUS expression in pollen varied among the transgenic plants. Strong GUS expression was observed in the pollen of plant I33 that had a single copy of the transgene. However, weaker GUS expression was observed in the pollen of plants I24 and I31, carrying two copies of the transgene (Table 3).

Table 2 Seed set of transgenic rice plants carrying *pOry s 1::uidA* transgene

Plant number	Number of seeds/plant
Control[a]	215
121	143
33	346
142	116
133	134
203	154

[a] Control plant was regenerated from tissue culture

Based on paired *t* test at a significance level of 0.05, the transgenic plants did not produce significantly different number of seeds from control plant (*P* value is 0.4267)

Table 3 Expression of p*Ory s 1::uidA* transgene in transgenic Amaroo rice

Plant number	Number of GUS (+) pollen/extent of GUS stain	Number of GUS (−) pollen	GUS (+) to GUS (−) ratio	*P* value
I21	101/++	102	1:1	0.994
I24	130/++	155	1:1.2	0.138
I25	261/++	223	1:0.85	0.449
I31	412/++	402	1:0.97	0.553
I33	280/++++	258	1:0.92	0.343
I4	129/++	138	1:1.07	0.582
I41	427/++	463	1:1.08	0.227
34	160/+	167	1:1.04	0.699
I42	244/+++	179	1:0.73	0.002

Mature pollen was stained for GUS expression. Percentage of blue pollen was calculated as the number of GUS-positive pollen divided by the total number of pollen counted × 100%. The extent of GUS staining is shown as very weak (+) to very strong (++++)

GUS (+), GUS positive; GUS (−), GUS negative

Based on χ^2 test at a significance level of 0.05, the transgenic plants showed GUS-positive and GUS-negative segregation ratio of 1:1

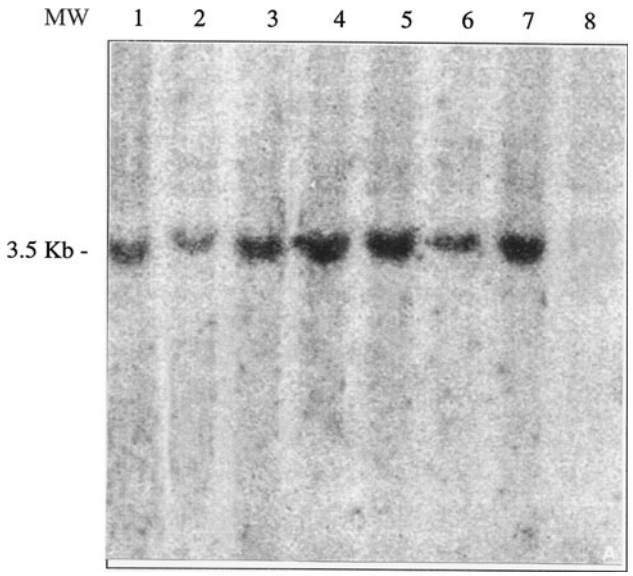

Fig. 3 DNA blot analysis of transgenic rice plants. The total DNA from transgenic plants was digested with *Bam*HI releasing a 3.5-Kb fragment. The blot was probed with *hph* gene fragment as described in "Materials and methods". *Lane* 1–7, transgenic plants; *lane* 8, control plant; MW, molecular weight marker (kb)

Progeny analysis

Progeny analysis was performed using seeds from primary transgenic plant numbers 133, 203 and 33 (carrying p*Ory s 1::uidA*). After 7 days on media containing 30 mg/L hygromycin, germinated seeds were scored as being resistant if a green shoot and root system had emerged. No germination was observed from control seeds on this medium (Fig. 4). The results showed that T1 seedlings of plants 133, 203, and 33 conform to a 1:1 segregation for resistance: sensitivity to hygromycin (Table 4).

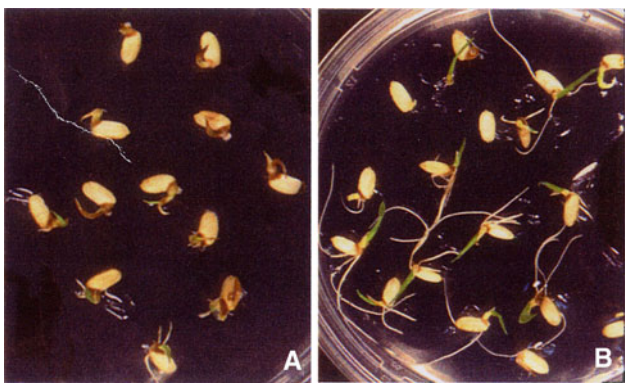

Fig. 4 Progeny analysis of the transgenic rice plants. Seeds from primary transgenic plant number 133 and control plant were germinated on medium containing 30 mg/L hygromycin. Germination was scored as positive if green shoots and root system emerged after 7 days on the medium. **a** Control plant; **b** Transgenic plant number 133

Table 4 Segregation of *hph* gene in T1 progeny of the transgenic Amaroo rice

Plant	hph^+: hph^-number of plants	Ratio	χ^2
Control	0:25	0:1	25
I33	19:21	1:1	0.1
203	21:24	1:1	0.2
33	25:25	1:1	0

Based on χ^2 test at a significance level of 0.05, T1 progeny of transgenic Amaroo rice showed *hph*-positive and *hph*-negative segregation ratio of 1:1

Deletion analysis of the *Ory s 1* promoter

To study 5′ sequence elements necessary for Ory s 1 promoter activity, a GFP reporter gene was fused to deletion fragments of *Ory s 1* promoter and bombarded into mature lily pollen and onion peels (Fig. 5). There was no significant difference observed in GFP expression pattern between full-length, i.e., 1,524-bp and 812-bp promoter constructs, as florescence was observed in whole pollen grain (Fig. 5a, b). However, the shortest fragment of the promoter, i.e., 405 bp, directed weaker GFP expression in the pollen (Fig. 5c). No expression was observed in onion peel cells transformed with GFP driven by either full-length or both deletion fragments of Ory s 1 (Fig. 5d). Our results indicate that the pollen-specificity element may reside within −405 bp at the start of the transcription, while the region upstream of −405 contains a cis-acting regulatory element(s) responsible for quantitative expression of this gene.

Discussion

Optimization of *Agrobacterium*-mediated transformation

Several factors have been reported to affect *Agrobacterium*-mediated transformation. These include genotype of plants, types and ages of tissues inoculated, vectors, strains of *Agrobacterium*, selection marker genes and selective agents, and various conditions of tissue culture (reviewed by Hiei et al. 1997). In the present study using mature embryo-derived callus of Australian rice, Amaroo, transient expression of GUS after co-cultivation was used as parameter of infection efficiency. Observations on co-cultivation regimes revealed that a co-culture period of 3 days with *Agrobacterium tumefaciens* EHA 101 at a density of 0.2 and infection time of 2 min resulted in minimum callus necrosis of Amaroo rice, whereas co-cultivation period of more than 3 days reduced survival of the explants. Similar observations were also reported for *Agrobacterium*-mediated

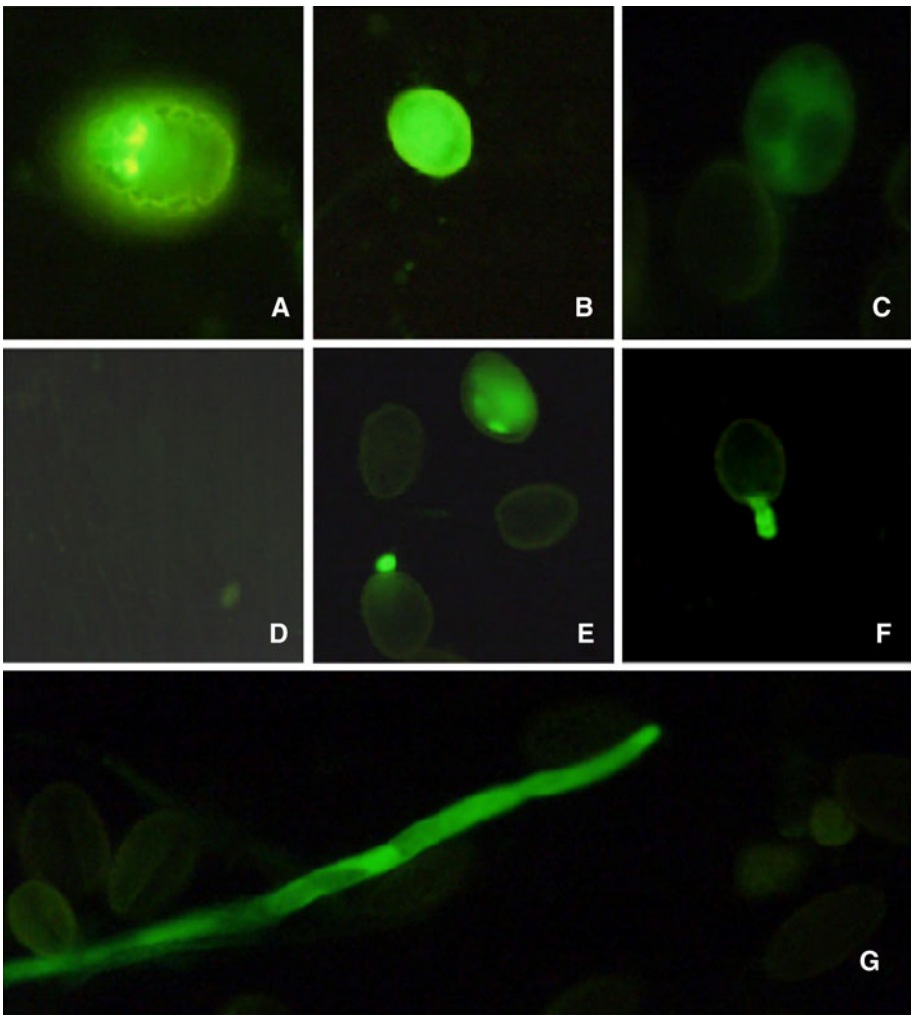

Fig. 5 Analysis of p*Ory s 1*–*GFP* expression in mature lily pollen and onion peel cells. Full-length and deletion fragments of *Ory s 1* promoter fused with *GFP* coding region was introduced into mature lily pollen and onion peel cell by particle bombardment. **a** Full-length (1,524 bp) *Ory s 1* promoter drove strong GFP expression in whole pollen. **b** A 812-bp fragment of *Ory s 1* promoter drove strong GFP expression in whole pollen of lily. **c** The shortest fragment (405 bp) of *Ory s 1* promoter drove weak GFP expression in the cytoplasm of lily pollen. **d** No GFP expression was observed in onion peel cells transformed with 405-bp fragment of *Ory s 1* promoter. **e–g** Expression of p*Ory s 1*–GFP chimeric gene was observed during pollen germination. *Ory s 1* was expressed in the whole pollen cytoplasm (**e**, *arrowhead*), growing pollen tube (**e**, *arrow*; **f**)

transformation of soybean (Yan et al. 2000). Plasmid pIG121Hm and *Agrobacterium* EHA 101 were used in the present study, as this combination has been reported to result in higher transformation efficiency than the use of most commonly available *Agrobacterium* strain, LBA4404 (Hiei et al. 1997).

Mature embryo-derived callus was used in this study, thus avoiding the use of developing embryos for transformation. Our previous study showed that it was possible to regenerate viable plants efficiently from these explants (Azria and Bhalla 2000). Pre-culture of calli on fresh medium was found to be necessary, as this process allows the formation of actively growing cells critical for *Agrobacterium* infection. Inclusion of acetosyringone (100 μM) in bacterial suspension and co-cultivation medium was also found to be critical for Amaroo and Millin varieties. A similar effect of acetosyringone was also observed by Hiei et al. (1994) using *japonica* rice varieties, namely, Tsukinohikari, Asanohikari and Koshihikari. Regeneration of cultures on MS-based media was preferred to N6-based media for the Australian rice (Azria and Bhalla, 2000). An incubation temperature of 25°C was found to be suitable for Amaroo transformation. This result is in accordance with earlier findings using *japonica* rice that co-cultivation at temperature between 22 and 28°C resulted in strongest transient GUS expression (Hiei et al. 1994).

Inclusion of hygromycin at a concentration of 30 mg/L was effective to inhibit the growth of untransformed calli and shoots during the selection process, while 50 mg/L hygromycin was found to be detrimental to the callus. Sensitivity to hygromycin is probably genotype dependent, as hygromycin at a concentration of 50 mg/L was found to be effective for other *japonica* varieties such as Tsukinohikari and Yamahoushi (Hiei et al. 1994; Yokoi et al. 1997). In contrast to published reports on *Agrobacterium*-mediated rice transformation that included hygromycin throughout the selection, regeneration and root induction (Hiei et al. 1994; Yokoi et al. 1997), exclusion of hygromycin during regeneration was found to be necessary to allow shoot regeneration in the present study. Shoots could not be initiated from these varieties if hygromycin was included in the regeneration medium. Longer exposure to

hygromycin has been suggested to impair plant regeneration (Raineri et al. 1990; Peng et al. 1992). Exclusion of hygromycin in the shoot regeneration medium, however, resulted in the initiation of transgenic as well as non-transgenic shoots. Nevertheless, putative transgenic shoots were later selected on root induction media containing 30 mg/L hygromycin. Using this protocol, transgenic plants from both Amaroo and Millin varieties were obtained. The developed protocol produced transgenic Amaroo rice after 3 months; a shorter tissue culture period has been suggested to decrease the possibility of somaclonal variation (Toki 1997).

Expression of chimeric p*Ory s 1::uidA* construct in transgenic rice plants

Pollen development requires a coordinate expression of a large number of genes in both tapetum (diploid) and pollen (haploid) cells. Out of 26,000 genes expressed in anthers, about 10–15% are estimated to be pollen specific (Mascarenhas 1990). Isolation and characterization of anthers and pollen-specific genes is important to understand gene regulation during sexual reproduction of flowering plants, as the discovery of novel genes has potential application in agriculture.

Our laboratory previously reported isolation of a rice pollen-specific gene, *Ory s 1*, and its expression in a heterologous system, tobacco (Xu et al. 1995b; 1999). In the present study, we studied *Ory s 1* expression in a more suitable system, rice. Expression of the p*Ory s 1::uidA* fusion in transgenic plants allowed us to examine the spatial and temporal expression directed by the 5′ flanking region of the *Ory s 1* gene. Expression of the *uidA* reporter gene was observed in pollen, but not in other floral tissues of the transgenic plants. No GUS expression was observed in the pollen of control plants (Fig. 2d). In addition, GUS expression was detected only in mature pollen (Fig. 2c, e). The number of pollen expressing GUS in each independent primary transgenic plant tested ranged from 45 to 57%, as expected for single locus integration of gametophytic expressed genes. Thus, our study shows a potential use of *Ory s 1* promoter to direct pollen-specific expression of chimeric genes in rice indicating that this promoter could be used in both monocots and dicots plants to direct pollen-specific gene expression.

In the present study, a variable level of GUS expression was detected in different transgenic plants. Plant 133 containing a single copy of the *uidA* gene showed stronger GUS activity than plants 124 and 131 carrying two copies of the reporter gene. Possible explanations for this variable expression could include interaction between the introduced genes that might have caused suppression of the promoter activity, which resulted in variation in GUS expression levels. It is unlikely that the position effect of the transgene in the rice chromosome is responsible for variable GUS expression, as a similar level of GUS expression was detected in plants 124 and 131, while transgene copies were found to be at different sites of the genome. Such an explanation is also in accordance with results obtained by Hobbs et al. (1993) who reported that transgene copy number could be positively or negatively correlated with the level of transgene expression.

Deletion analysis of the *Ory s 1* promoter

Expression of GFP driven by 5′ deletions of *Ory s 1* promoter in transient expression assay using lily pollen revealed that pollen-specificity elements reside within the −405-bp region and the region upstream of −405 contained regulatory element(s) responsible for quantitative expression of *Ory s 1* gene. Pollen specificity of full length and deletions was observed by a lack of expression in onion peel cells following bombardment. Two pollen- and/or anther-specific motifs, TGTGG and TGTGA, were found in PS1, a rice pollen-specific gene, at positions −261 to −267 and −327 to −323 of the PS1 5′ upstream region (Zou et al. 1994). These motifs are also present in pollen-specific gene isolated from tomato, *Lat 52*. Sequence search for *Ory s 1* promoter revealed the TGTGG motif at −234 to −240 only. The presence of this motif is in agreement with previous studies that the cis-active sequence elements responsible for pollen specificity appear to reside in the 5′ flanking region near the start of transcription (Eyal et al. 1995; Xu et al. 1993; Weterings et al. 1995). Several quantitative elements enhancing gene expression level have been reported in 5′ flaking region of pollen-specific genes such as *Lat 52* (TGGTTA), *Zm13* (AGGTCA) and *Ntp303* (AAATGA). Searching for these sequencing in *Ory s 1* promoter found a single TGGTTA motif at position −454 to −449 as well as single motif of AAATGA at position −474 to −469. As these elements reside upstream of −405 bp, it is more likely that the low expression on the shortest promoter fragment tested resulted from a limited number of transcription factor binding sites.

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