

***Agrobacterium*-mediated co-transformation of rice using two selectable marker genes derived from rice genome components**

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Abstract A method for *Agrobacterium*-mediated co-transformation of rice (*Oryza sativa* L.) was developed using rice-derived selection markers. Two T-DNAs were efficiently introduced into separate loci using selectable marker gene cassettes consisting of the mutated acetolactate synthase gene (*mALS*) under the control of the callus-specific promoter (CSP) (CSP:*mALS*) and the ferredoxin nitrite reductase gene (*NiR*) under the control of its own promoter (NiR P:*NiR*). The CSP:*mALS* gene cassette confers sulfonylurea herbicide resistance to transgenic rice callus. The NiR P:*NiR* construct complements NiR-deficient mutant cultivars such as ‘Koshihikari’, which are defective in the regulation of nitrogen metabolism. In the present study, the CaMV35S:*GUS* and CaMV35S:*GFP* gene cassettes were co-introduced into the ‘Koshihikari’ genome using our system. Approximately 5–10 independent transgenic lines expressing both the *GUS* and *GFP* reporters were obtained from 100 *Agrobacterium* co-inoculated calli. Furthermore, transgenic ‘Koshihikari’ rice lines with reduced content of two major seed allergen proteins, the 33 and 14–16 kDa allergens, were generated by this co-transformation system. The present results indicate that the generation of selectable antibiotic resistance marker gene-free transgenic rice is possible using our rice-derived selection marker co-transformation system.

Key message An improved rice transformation method was developed based on *Agrobacterium*-mediated co-transformation using two rice genome-derived selectable marker gene cassettes.

Keywords Acetolactate synthase · *Agrobacterium* · Co-transformation · Ferredoxin nitrite reductase · GM crop · Transgenic rice

Abbreviations

CSP	Callus-specific promoter
2,4-D	2,4-Dichlorophenoxyacetic acid
GFP	Green fluorescent protein
GM crop	Genetically modified crop
GUS	β -Glucuronidase
HPT	Hygromycin phosphotransferase
Mals	Mutated acetolactate synthase
MCS	Multi-cloning site
NAA	Naphthaleneacetic acid
NiR	Ferredoxin nitrite reductase
NPT	Neomycin phosphotransferase

Introduction

Genetically modified crops (GM crops) are at present commercially cultivated in 29 countries worldwide. By 2011, it is estimated that the total global cultivated area of transgenic crops will reach approximately 160 million hectares (James 2011). The number of species and cultivated area of GM crops will certainly increase in the future.

Transformation of crop plants has been achieved by both direct (e.g., electroporation, particle-bombardment) and indirect (*Agrobacterium*-mediated) methods. The selection

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of transformants, which is critical in this process, is mostly based on the use of antibiotic-resistant genes. However, the presence of such selection markers in commercialized GM crops is not desired, which underscores the importance of developing marker-free transformation systems to eliminate selectable markers and generate a product that is acceptable by consumers. Various marker-free plant transformation systems have been developed including the multi-auto-transformation (MAT) vector system and the Cre-lox recombination system (Ebinuma et al. 1997; Endo et al. 2002; Sugita et al. 2000; Zhang et al. 2003). Although these systems are available and have been tested using various strategies, they have not yet been established as convenient tools for the production of marker-free transgenic plants. An *Agrobacterium*-mediated co-transformation system has been developed to produce marker-free transgenic plants (Baisakh et al. 2006; Depicker et al. 1985; Komari et al. 1996; Parkhi et al. 2005; Tu et al. 2003). In this system, transgenic plants are generated by introducing two T-DNAs containing a selectable marker gene and the target gene. The regenerated plants are selected for the presence of both T-DNAs and the selectable marker gene is removed by segregation in the seed progeny. Recently, the efficiency of co-transformation systems has been considerably improved. Sripriya et al. (2011) described a highly efficient *Agrobacterium*-mediated co-transformation method based on the use of high copy number binary vectors in *indica* rice. *Agrobacterium*-mediated co-transformation systems are simple in principle and are applicable to various plant species when compared with MAT vector and Cre-lox systems.

In a previous report, we described a marker-free like transformation system based on the use of native mutated-acetolactate synthase (*mALS*) under the control of a callus-specific promoter as rice genome-derived selectable marker gene (Wakasa et al. 2006). ALS is the first common enzyme in the biosynthetic pathway of the branched-chain amino acids leucine, isoleucine, and valine. Sulfonylurea, imidazolinone and pyrimidinyl carboxy herbicides are competitive inhibitors of ALS (Chaleff and Mauvais 1984; Shimizu et al. 2002). Mutated-ALS (*mALS*) has significantly lower affinity for these herbicides, and has been used to develop transformation systems. Certain *mALS*s were discovered as products of somaclonal variation during tissue culture of various plant species (Shimizu et al. 2002; Okuzaki et al. 2007).

'Koshihikari' is the most popular cultivated variety in Japan based on its superior eating quality. 'Koshihikari' is cultured in not only Japan but also China, Thailand, Philippine and the USA (Ito et al. 1995). However, 'Koshihikari' has not been used as a transgenic host because of its low regeneration and callus propagation abilities, which have been attributed to reduced expression of the ferredoxin

nitrite reductase gene (*NiR*) in the callus. Nishimura and co-authors reported that not only lower expression of *NiR* mRNA but also insufficient unspliced *NiR* mRNA retaining the third intron were related to the low nitrite reductase (*NiR*) activity in 'Koshihikari' cells. Furthermore, rice varieties that can easily regenerate plantlets from callus have higher *NiR* activity than 'Koshihikari' (Nishimura et al. 2005). A specific DNA deletion (31 bp) in the 5' untranslated region (5'UTR) of the *NiR* gene was detected in 'Koshihikari' (Ozawa and Kawahigashi 2006). These findings suggest that specific culture media and culture conditions are required for 'Koshihikari' transformation (Ozawa and Kawahigashi 2006; Wakasa et al. 2007). The *NiR* gene derived from rice cultivars such as 'Kasarath' and 'Nipponbare' was therefore used as a selection marker to overcome the transformation difficulties in the low-regeneration rice 'Koshihikari' (Nishimura et al. 2005; Ozawa and Kawahigashi 2006).

In the present study, we developed a high-frequency co-transformation system for 'Koshihikari' rice using the *mALS* gene driven by a rice callus-specific promoter (*CSP:mALS*) and the rice cultivar 'Nipponbare' *NiR* gene under the control of its own promoter (*NiR P:NiR*) as selectable marker genes. Approximately 8 % co-transformation efficiency (number of plants integrating two T-DNAs per callus used for *Agrobacterium* inoculation) was achieved by this method.

The proposed system has at least two important advantages. The use of two selectable marker genes derived from the rice genome is expected to increase public acceptance regarding food safety when compared with transgenic rice seeds produced using conventional antibiotic marker selection systems. In addition, multiple desired genes can be co-introduced into the rice genome, enabling the molecular breeding of transgenic rice plants carrying desirable traits.

Materials and methods

Plant materials

Rice (*Oryza sativa* L. cv. 'Koshihikari') was used for transformation.

Vector construction

Binary vectors were constructed by using restriction enzymes, nucleotide ligase and Gateway LR clonase (Invitrogen). Two kinds of binary vectors harboring *CSP:mALS* (Kumiai Chemical Industry, Tokyo, Japan) or *NiR P:NiR* were prepared as Gateway destination binary vectors (Fig. 1a). Both binary vectors had Multi-site

Gateway LR reaction attachment sequences [att R4–chloramphenicol resistance gene (Cm R)–lethal gene targeting DNA gyrase (*ccdB*)–att R3]. Three types of Gateway entry clones were constructed (pKS4-1 MCS II, pKS221 MCS II and pKS2-3 MCS II; Fig. 1b). Because the gene cassette of interest can be inserted into each multi-cloning site (MCS) (Fig. 1c), it is possible to introduce three independent gene cassettes into one destination binary vector using the Multi-site Gateway LR clonase reaction (Fig. 1d). In the present study, one gene cassette was inserted into each binary vector (CaMV 35S promoter: *GUS* (35S P:*GUS*) was inserted into the CSP:*mALS* destination binary vector and CaMV 35S promoter: *GFP* (35S P:*GFP*) was inserted into the NiR P:*NiR* destination binary vector) (Fig. 1e). Furthermore, a seed-specific 33 kDa seed allergen RNA interference (RNAi) gene cassette and a seed-specific 14–16 kDa seed allergen RNAi gene cassette were also inserted into the CSP:*mALS* and NiR P:*NiR* destination binary vectors, respectively (Fig. 6a).

Callus induction

Sterilized mature ‘Koshihikari’ seeds were sown on N6D solid medium [4 g/L CHU salt mixture (Wako Pure Chemical Industries, Tokyo, Japan), 30 g/L sucrose, 2.78 g/L proline, 100 mg/L myo-inositol, 300 mg/L casamino acids, 1 mL/L 1,000× N6-vitamin, 2 mg/L 2, 4-D, 0.4 % gelrite (Wako Pure Chemical Industries, Tokyo, Japan), pH 5.8] and cultured at 32 °C for 1 week. Small calli (2–5 mm) induced from seed scutellum were transferred to modified-DKN solid medium [30 g/L sucrose, 1.15 g/L proline, 100 mg/L myo-inositol, 300 mg/L casamino acids, 50 mL/L 20× DKN-macro stock (5.46 g/L NaH_2PO_4 , 16 g/L KNO_3 , 1.34 g/L $(\text{NH}_4)_2\text{SO}_4$, 5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), 1 mL/L 1,000× DKN micro stock (1.6 g/L $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.2 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L H_3BO_3 , 125 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 125 mg/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), 1 mL/L 1,000× R2-Iron stock (7.5 g/L Na_2EDTA , 5.5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 1 mL/L 1,000× modified B5-vitamin (1 g/L nicotinic acid, 10 g/L thiamin HCl, 1 g/L pyridoxine HCl, 2 g/L glycine), 660 mg/L L-aspartic acid, 731 mg/L L-glutamine, 2 mg/L 2, 4-D, pH 5.8] and cultured for 3–4 weeks at 32 °C (16 h light/8 h dark).

Agrobacterium inoculation

Agrobacterium harboring binary vectors containing either the CaMV 35S promoter:*GUS* and CSP:*mALS* gene cassettes, or the CaMV 35S promoter:*GFP* and NiR P:*NiR* gene cassettes were precultured in AB solid medium (3 g/L K_2HPO_4 , 1.3 g/L $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/L NH_4Cl , 150 mg/L KCl, 10 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.5 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L glucose, 500 mg/L MgSO_4 , 1.5 % agarose, pH 7.2) for

3 days at 28 °C. Subsequently, *Agrobacterium* colonies were suspended in DKN-AS liquid medium (30 g/L sucrose, 1.15 g/L proline, 100 mg/L myo-inositol, 300 mg/L casamino acids, 50 mL/L 20× DKN-macro stock, 1 mL/L 1,000× DKN micro stock, 1 mL/L 1,000× R2-Iron stock, 1 mL/L 1,000× modified B5-vitamin, 660 mg/L L-aspartic acid, 731 mg/L L-glutamine, 2 mg/L 2, 4-D, 20 mg/L acetosyringone, pH 5.2) and the concentration was adjusted to OD600 = 0.05–0.1.

Two kinds of *Agrobacterium* suspension (15 mL each) were mixed in a 50 mL tube (total, 30 mL *Agrobacterium* suspension). A total of 100 calli (2–4 mm diameter) were inoculated for 90 s with gentle shaking. Inoculated calli were transferred onto filter paper to remove the *Agrobacterium* suspension. Calli were then transferred to sterilized plates (9 cm diameter), attached to two-ply filter paper moisturized with 3.5 mL of liquid DKN-AS medium (Fig. 2a) and incubated for 3 days at 25 °C in the dark.

Callus selection

After co-cultivation, *Agrobacterium*-inoculated calli were collected into a 50-mL tube and washed with sterilized water three times. Washed calli were transferred onto modified-DKN solid medium containing 400 mg/L carbenicillin and cultured at 32 °C for 3 days as acclimation step (16 h light/8 h dark).

Calli were collected again onto a nylon mesh (pore size 400 µm) as shown in Fig. 2b, c. Calli were passed through the nylon mesh and crushed into small pieces with a spatula. Calli pieces were collected into a conical beaker filled with 1/2 N6D liquid medium (Fig. 2d). Small calli that sunk to the bottom of the conical beaker were collected by filtration, transferred into a 50-mL tube, and then 10 mL of calli suspension were prepared by removal or addition of 1/2 N6D liquid medium (Fig. 2e).

Ten milliliters of calli suspension were spread onto 6–8 plates of N6D solid medium containing 1.0 µM pyriminobac and 400 mg/L carbenicillin (Fig. 2f). After the excess 1/2 N6D liquid medium was removed from the N6D solid medium, calli were incubated at 32 °C for 4–6 weeks (16 h light/8 h dark).

Regeneration of transformed calli

After selection, growing calli (Fig. 2g, arrowhead) were transferred onto MS regeneration medium [4 g/L MS salt mixture (Wako Pure Chemical Industries, Tokyo, Japan), 30 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.2 mg/L NAA, 2 mg/L kinetin, 1 mL/L 1,000× B5-vitamin, 0.4 % gelrite, pH 5.8] containing 400 mg/L carbenicillin and incubated at 28 °C for shoot regeneration (16 h light/8 h dark). Regenerated shoots were transferred onto

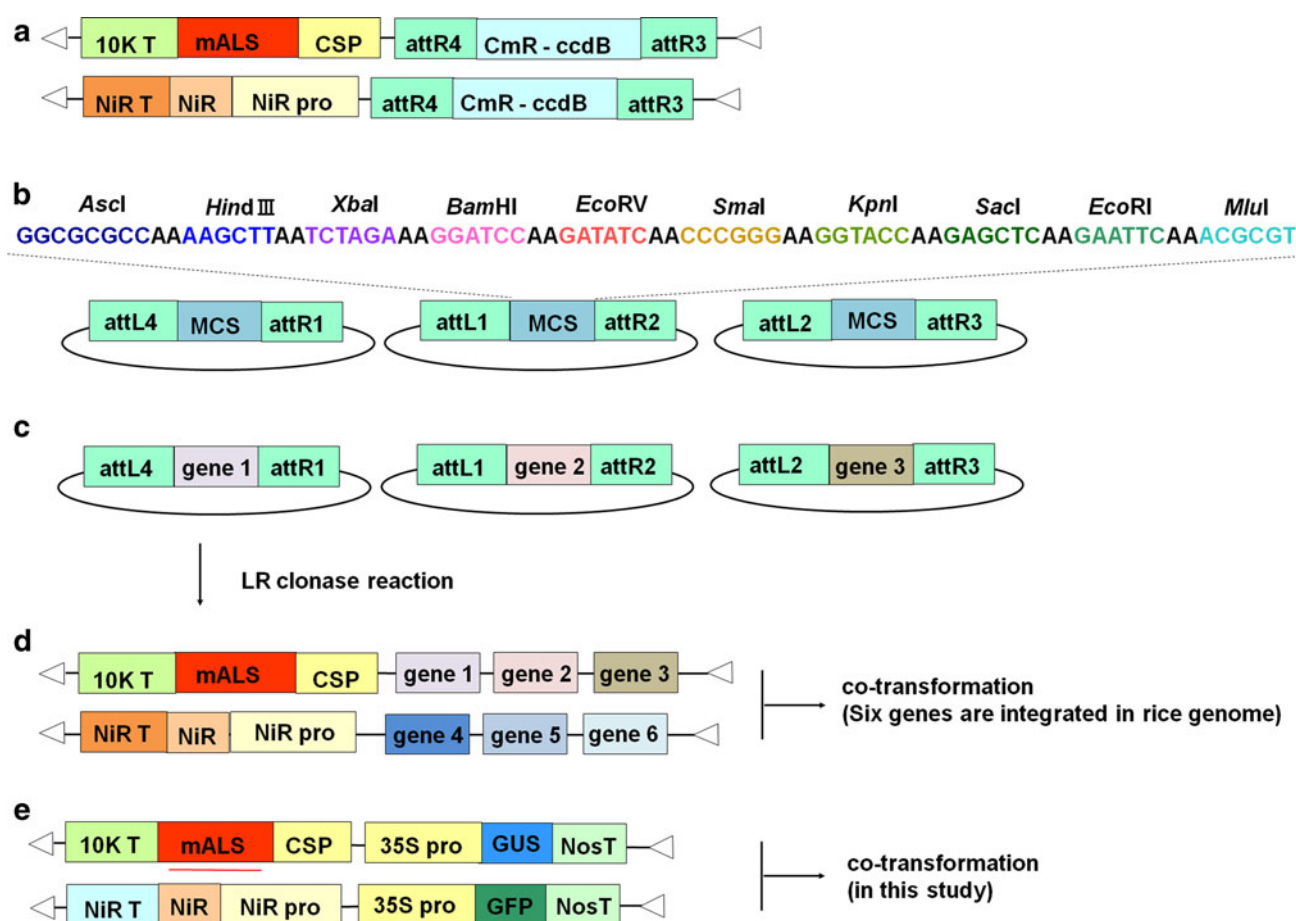


Fig. 1 Schematic representation of the rice-derived double selectable marker genes used for co-transformation. **a** Destination binary vectors, CSP:*mALS* 43GW (upper construct) and *NiR* P:*NiR* 43GW (lower construct). The backbone of these vectors is pPZP200. **b** Entry clones containing the multiple cloning sites pKS 4-1 MCS (left plasmid), pKS221 MCS (middle plasmid) and pKS2-3 MCS (right plasmid). The backbone of these plasmids is pBlueScript KS+. **c** and **d** Use of the destination binary vector and entry clones for the construction of a binary vector containing multiple gene cassettes.

e Constitutive expression of GUS or GFP in transgenic rice plants. The area underlined in red was used for Southern hybridization (Fig. 4). CSP callus-specific promoter, *mALS* mutated *acetolactate synthase* cDNA, *10K T* 10 kDa prolamine gene terminator, *NiR pro* *Nipponbare ferredoxin nitrite reductase* gene promoter derived from cv. Nipponbare, *NiR* *Nipponbare ferredoxin nitrite reductase* gene cDNA, *NiR T* *Nipponbare ferredoxin nitrite reductase* gene terminator, MCS multi-cloning site, att Gateway attachment regions, 35S pro CaMV 35S promoter, Nos T nopaline synthase gene terminator

MS hormone-free medium (4 g/L MS salt mixture, 30 g/L sucrose, 1 mL/L 1,000× B5-vitamin, 0.25 % gelrite, pH 5.8) containing 400 mg/L carbenicillin and cultured at 28 °C for root regeneration (16 h light/8 h dark). The regenerated plantlets were grown in a closed glasshouse.

DNA extraction and Southern blot analysis

Genomic DNA was extracted from young leaves derived from wild type and 8 randomly selected transgenic rice lines by using the CTAB method (Murray and Thompson 1980). A total of 5 µg of genomic DNA were digested with *Hind*III, fractionated by electrophoresis on a 0.8 % agarose gel, and transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Probes were prepared from purified PCR products of the

mALS coding region or the *NiR* terminator region. Southern hybridization was carried out with a Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare, Buckinghamshire, UK).

Protein extraction and immunoblot analysis

Mature seeds were separately ground into a fine powder. For total protein extraction, 500 µL of extraction buffer [50 mM Tris-HCl (pH 6.8), 8 M urea, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol, 0.01 % bromophenol blue (BPB)] was added to the seed powder and vortexed for more than 1 h at room temperature. The mixture was centrifuged at 12,000×g for 20 min at room temperature, and the crude soluble protein sample was decanted into a new tube. An aliquot of 2 µL of total protein was subjected

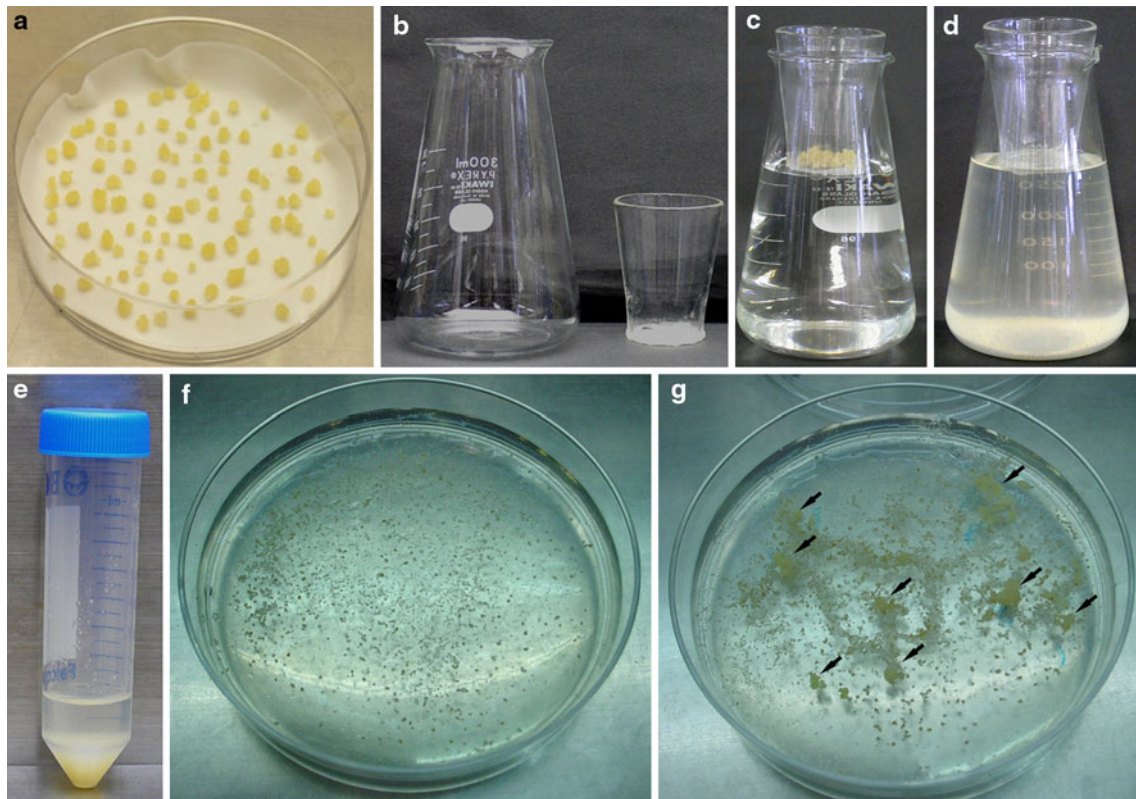


Fig. 2 Co-transformation process. **a** Calli growing on filter paper with DKN-AS liquid medium before selection. **b** 300 mL conical beaker and glass cylinder with attached nylon mesh. **c** *Agrobacterium*-inoculated calli on the nylon mesh. **d** Small calli after filtration

through the nylon mesh (bottom of conical beaker). **e** Crushed calli are collected again into 50-mL tubes. **f** Calli are spread onto selection medium. **g** Growing calli (arrowheads) 4 weeks after selection

to immunoblotting against polyclonal anti-33 kDa seed allergen or anti-14–16 kDa seed allergen antibodies after electrophoresis on 12 % SDS-PAGE gels as described by Yasuda et al. (2005). Signals were detected by the ECL detection system (GE Healthcare, Buckinghamshire, UK).

Results and discussion

Evaluation of a novel co-transformation system

Stable transgenic rice plants were generated in which two T-DNAs were introduced by co-transformation using the rice-derived CSP:*mALS* and NiR P:*NiR* selection markers. The CSP:*mALS* gene cassette confers resistance to specific herbicides such as pyriminobac and bysphybac, whereas the NiR P:*NiR* gene cassette enables the growth of ‘Koshihikari’ callus and its varieties on N6D solid medium. Transformation efficiency was evaluated by the expression of the GFP and GUS reporter proteins in the callus (Fig. 3). Four rounds of inoculation experiments were performed. Approximately 60 % of growing calli on the selection medium were positive for GFP fluorescence and GUS

staining, as shown in Fig. 3a–e. The average transformation efficiency was 8.1 %, indicating that 6–10 transgenic rice plants harboring T-DNAs derived from both binary vectors (referred to as ‘duplicate transgenic plants’) were obtained from 100 *Agrobacterium*-inoculated calli (Table 1). On the other hand, there were a few escape calli, which were transgenic calli expressing either the GFP or GUS protein, or chimera calli, composed of escape and transgenic calli growing on the selection medium (Fig. 3f–j; Table 1).

The key steps in this co-transformation method were the co-cultivation using moisturized filter paper and the callus crushing steps. Although in vitro co-cultivation is usually performed on a solid medium, we used filter paper moisturized with liquid medium for co-cultivation (refer to “Materials and methods”) to prevent excess growth of *Agrobacterium*, thus increasing the transformation efficiency of the calli (Ozawa 2009). The callus crushing step enhanced the efficacy of the NiR P:*NiR* selectable marker gene. Low levels of expression of the *NiR* gene, which does not have a null mutation in this cultivar, and decreased enzyme activity were detected in the callus (Nishimura et al. 2005). Callus selection without the callus crushing

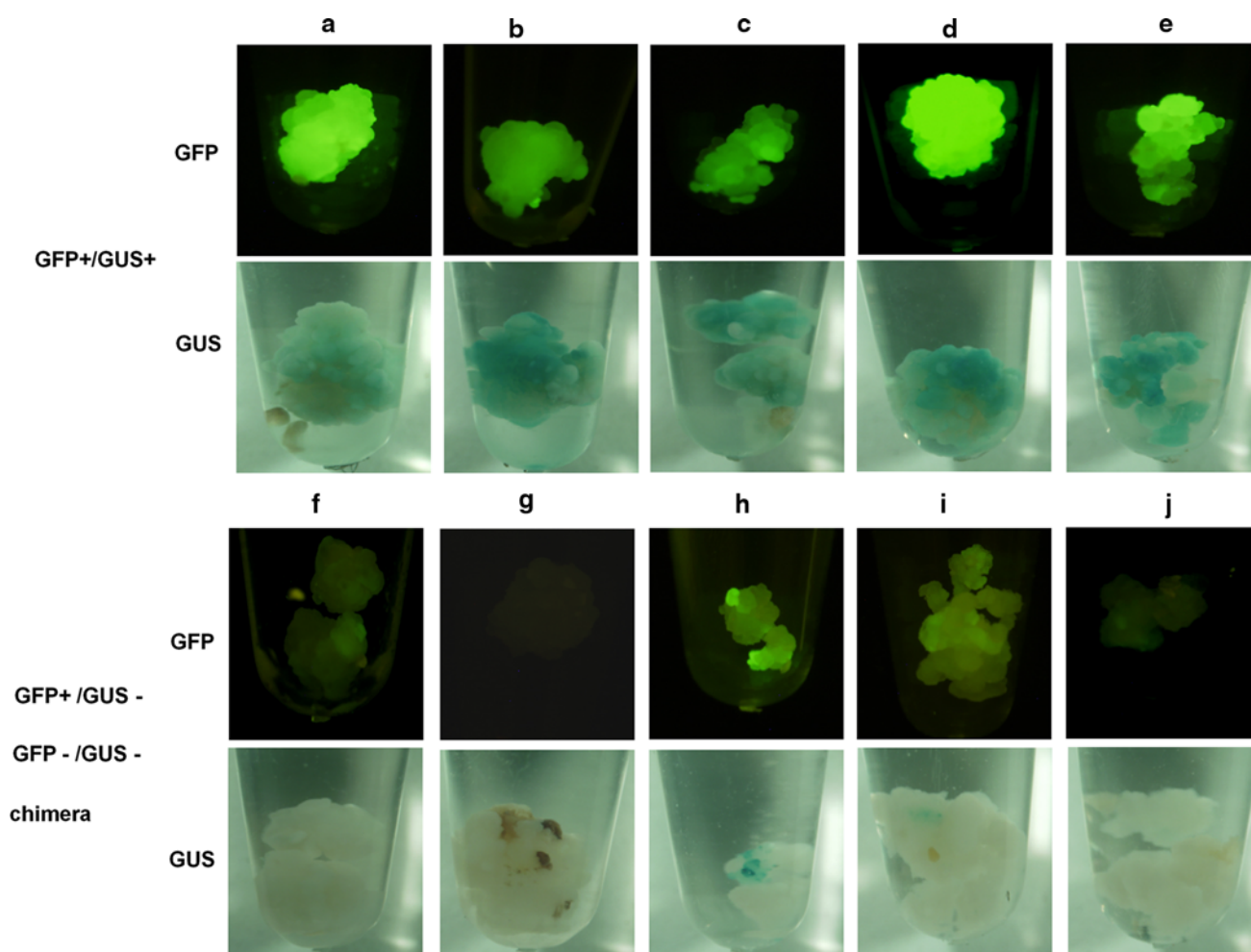


Fig. 3 Expression of GFP and GUS in transgenic rice calli. **a–e** Transgenic calli expressing GFP and GUS (duplicate transgenic calli). **f–j** Calli lacking expression of reporter proteins, transgenic

calli expressing GFP only, and chimera calli are shown. GFP fluorescence and X-Gluc staining were used for detection of transgenic calli

Table 1 Transformation efficiency

	Inoculated calli	Growth calli on selection medium	Growth calli				Transformation efficiency
			GFP+/GUS+ calli	GFP+/GUS– calli	GFP–/GUS+ calli	GFP–/GUS– calli	
Exp. 1	200	23/200 (11.5)	13/23 (56.5)	5/23 (21.7)	0/23 (0)	5/23 (21.7)	13/200 (6.5)
Exp. 2	100	11/100 (11.0)	6/11 (54.5)	4/11 (36.3)	0/11 (0)	1/11 (9.1)	6/100 (6.0)
Exp. 3	100	16/100 (16.0)	10/16 (62.5)	2/16 (12.5)	1/16 (6.3)	3/16 (18.8)	10/100 (10.0)
Exp. 4	100	16/100 (16.0)	10/16 (62.5)	4/16 (25.0)	0/16 (0)	2/16 (12.5)	10/100 (10.0)

Values in parentheses are in percent

step would reduce the selection efficiency of NiR P:NiR and increase escape callus formation.

Transgene copy number was investigated by Southern blot analysis in eight duplicate transgenic rice plants. Approximately 1–4 copies of T-DNA were integrated into the rice genome with the CSP:*mALS*–35S P:*GUS* binary

vector, whereas 1–5 copies of T-DNA were integrated with the NiR P:NiR–35S P:*GFP* binary vector (Fig. 4), and the difference in copy numbers between the two selectable marker genes was not significant. Because *Agrobacterium*-inoculated calli were cultured for 3 days without selection pressure for acclimation and then crushed into small pieces,

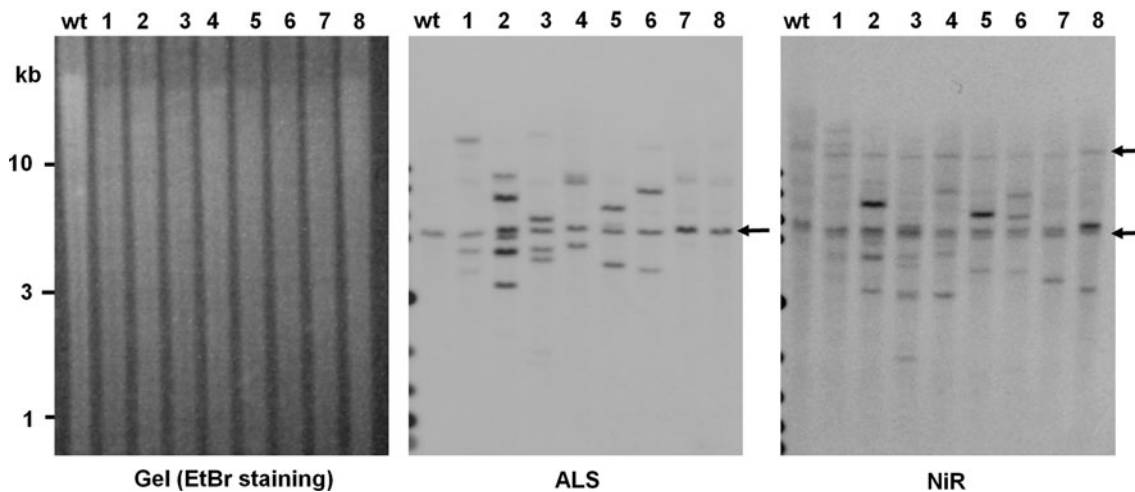


Fig. 4 Southern blot analysis of transgenic rice plants. Eight transgenic plants are randomly selected for this analysis. The *mALS* cDNA or *NiR* terminator regions were used as probes. Arrows indicate signals derived from endogenous genes

transgenic lines could have been generated from identical cells of regenerated rice plants. However, Southern blot analysis revealed different restriction patterns among the eight duplicate rice transgenic lines, indicating that they were the product of independent transgenic events.

Segregation in T1 progenies was assessed by observing the pattern of GFP fluorescence and GUS staining in T1 seeds derived from one transgenic line. Figure 5 shows that GFP and GUS were segregated, suggesting that these gene cassettes were integrated into different chromosomes.

Production of transgenic rice seeds with reduced content of major rice seed allergens

The co-transformation method was used to generate transgenic rice lines with reduced contents of two seed allergens, a 33 kDa seed allergen (β -glyoxarase) and the 14–16 kDa seed allergens (α -amylase inhibitor), which react with IgE antibodies in the sera of rice-sensitive patients. The 33 kDa seed allergen is encoded by a single gene, while the 14–16 kDa seed allergens are encoded by at least nine genes in rice (Matsuda et al. 2006; Wakasa et al. 2011).

A novel gene suppression system based on an RNA silencing inducible sequence (RSIS) in rice (Kawakatsu et al. 2010) was used for this experiment. Two gene cassettes were constructed by linking the 5' and 3' highly conserved regions of the mRNA of the 14–16 kDa seed allergens to the upstream and downstream sequences of the RSIS under the control of the endosperm-specific *RISBZ1* promoter (Onodera et al. 2001), and the 5' and 3' untranslated regions (UTR) of the 33 kDa seed allergen mRNA to the upstream and downstream sequences of the RSIS under the control of the endosperm-specific *GluB1*

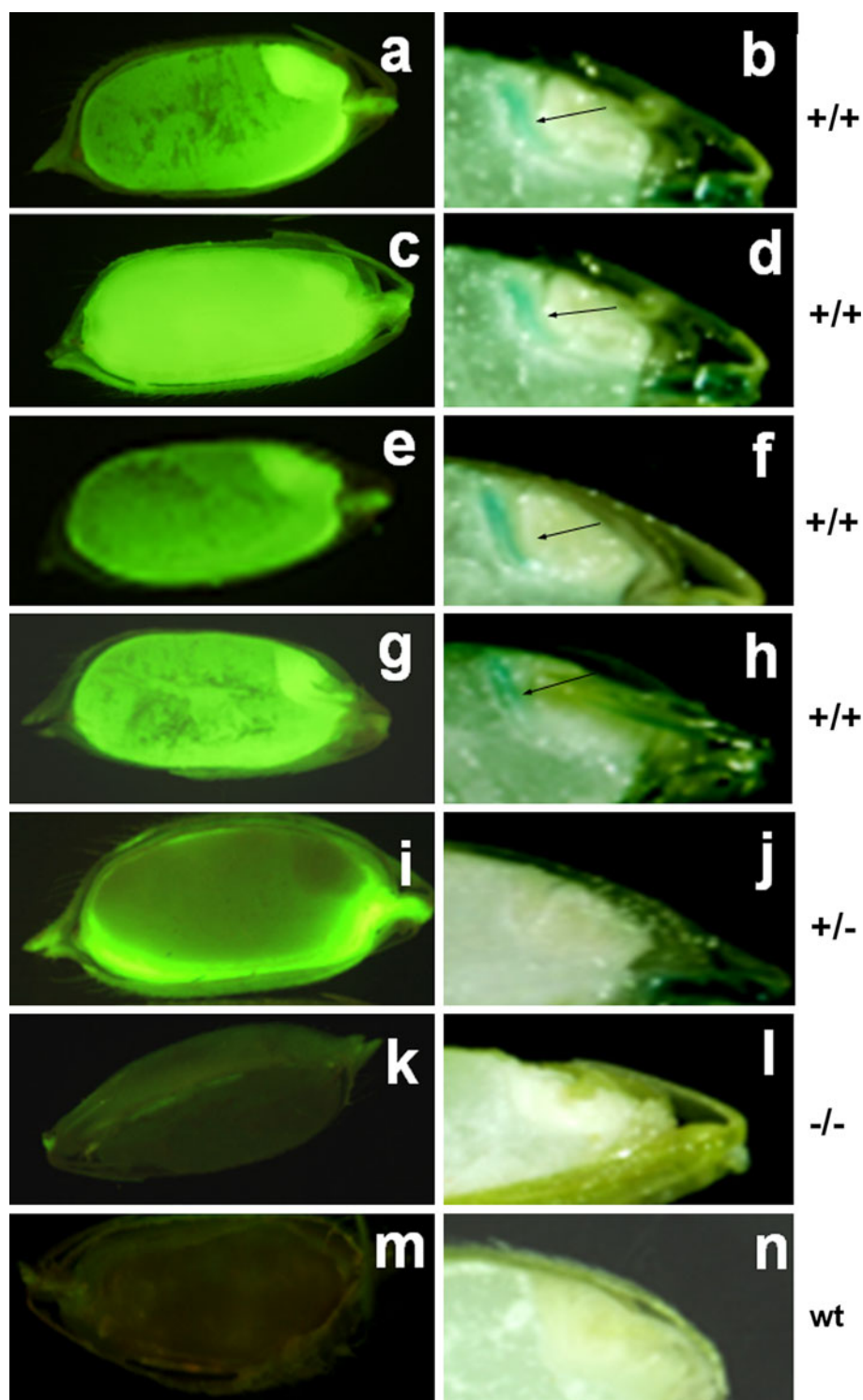
promoter (Qu and Takaiwa 2004). The cassettes were integrated by co-transformation (Fig. 6a).

Gene integration in regenerated plants was confirmed by genomic PCR (Fig. 5b). PCR products derived from both T-DNAs were detected in seven transgenic rice lines from 13 regenerated rice plants. Total proteins from seeds derived from duplicate transgenic rice lines were analyzed by immunoblotting against anti-33 kDa seed allergen and anti-14–16 kDa seed allergen antibodies. As shown in Fig. 6c, the expression of the 33 and 14–16 kDa seed allergens was strongly suppressed in these transgenic rice lines (Fig. 6c, first row, lanes 2–5). The seeds used for the immunoblot analysis were T1 seeds, and a few segregation patterns were observed (Fig. 6c).

Conclusions and perspectives

A novel co-transformation method was used to generate stable transgenic rice plants with two traits introduced by *Agrobacterium* inoculation. The effectiveness of our co-transformation system was demonstrated with reporter proteins (GFP and GUS) and the down-regulation of the expression of the rice 33 kDa and 14–16 kDa seed allergens. The use of selectable marker genes derived from rice genome is of particular value for the commercial production of genetically modified rice. Furthermore, the combination of our co-transformation method and the Multi-Site Gateway system allows the introduction of multiple gene cassettes (2–6 genes) into rice. Although the present study was restricted to 'Koshihikari' and its varieties, co-transformation using CSP:*mALS* and *NiR* P:*NiR* could be applied to many *japonica* rice cultivars that also show low regeneration efficiency and low *NiR* activity such as 'Akitakomachi', 'Milky-queen',

Fig. 5 Segregation of GFP and GUS gene cassettes in the T1 seed endosperm. Transgenic plant derived from callus shown in Fig. 3a is used for this observation. The *left images* show GFP fluorescence and the *right images* show GUS staining. $+/+$ GFP-positive and GUS-positive seed, $+/-$ GFP-positive and GUS-negative seed, $-/-$ GFP-negative and GUS-negative seed, *wt* wild-type seed. Arrows indicate the blue signal of GUS staining

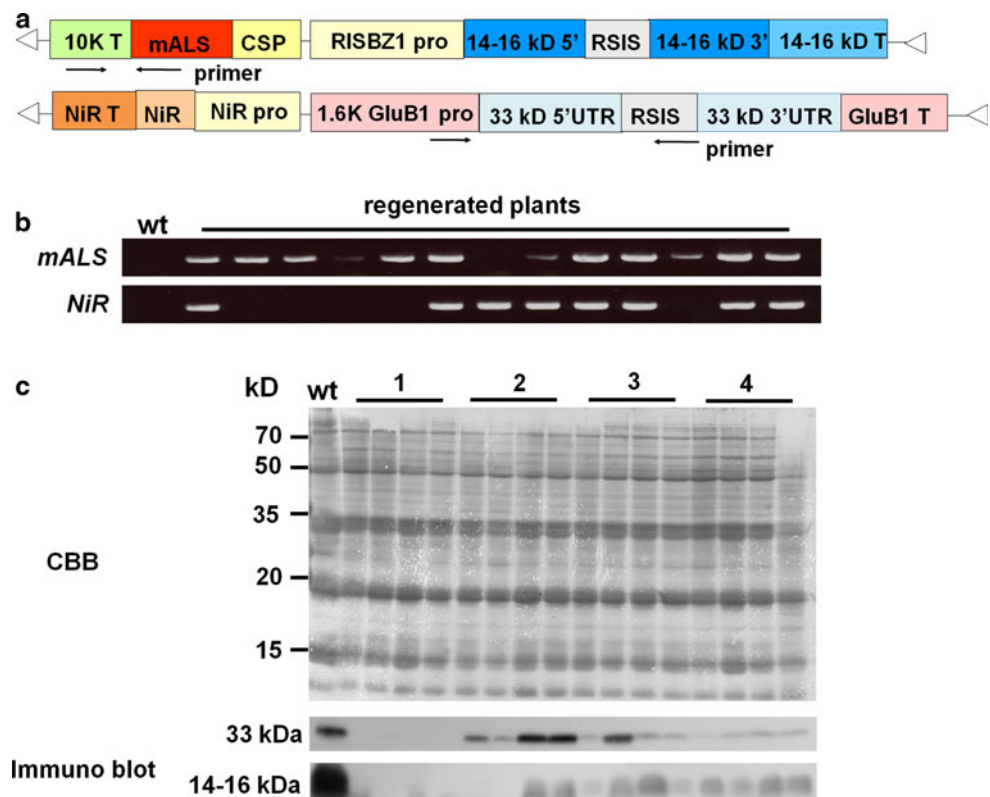


‘Hitomebore’, ‘Norin 1’, ‘Moritawase’ and ‘Ginbouzu’ (Nishimura et al. 2005).

The introduction of antibiotic resistance genes such as *NPT II* and *HPT* using our co-transformation system should be simpler than that of the *CSP:mALS* and *NiR P:NiR* described in the present study. Furthermore, the

introduction of antibiotic resistance genes enables the elimination of the callus crushing step by using a nylon mesh. In addition, there are fewer restrictions in the rice cultivars that can be used as hosts, and transformation efficiency is improved. However, the aim of the present study was to develop transgenic rice plants as GM crops.

Fig. 6 Production of transgenic rice seeds with reduced contents of the 33 and 14–16 kDa seed allergens. **a** Binary vector constructs used to suppress the expression of the 33 and 14–16 kDa allergens. Arrows in each construct indicate the primer region used for PCR. **b** Detection of the transgene in genomic DNA derived from regenerated plants by PCR. **c** Immunoblot analysis of total seed proteins against anti-33 kDa seed allergen and anti-14–16 kDa seed allergen antibodies



Future studies will be aimed at generating transgenic rice expressing multiple cassettes carrying proteins related to human health or secondary metabolism.

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