Characterization of Agrobacterium-mediated co-transformation events in rice using green and red fluorescent proteins

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Abstract

Background

Biotechnologists seeking to develop marker-free transgenic plants have established co-transformation methods. For co-transformation using mixed *Agrobacterium* strains, the mix ratio of *Agrobacterium* strains and selection scheme may influence co-transformation frequency. This study used fluorescent GFP and RFP markers to compose different selection schemes for observation of the selective dynamics of transformed rice cells and to investigate the factors affecting co-transformation efficiency.

Methods and Results

We utilized GFP and RFP markers in co-transformation and tested the combinations of an antibiotic-selectable vector (pGFP-HPT) and a single RFP vector (pRFP) and of two antibiotic-selectable vectors (pGFP-HPT and pRFP-HPT) in rice. The pGFP-HPT/pRFP combination resulted in 70.9 % to 81.2 % of co-transformation frequencies while lower frequencies (56.6 % on average) were obtained with the pGFP-HPT/pRFP-HPT combination. Based on GFP/RFP segregation patterns, 55 % of the pGFP-HPT/pRFP co-transformants contained unlinked T-DNAs and segregated single RFP progeny, which simulated the selection process of marker-free transgenic plants that carry an actual gene of interest. Transgene expression levels in the rice lines varied as revealed by RT-PCR, and tandem-linked T-DNAs were detected in co-transformants, suggesting that transgene expression might be affected by duplicated T-DNA structures.

Conclusion

Co-transformation via mixed *Agrobacterium* strains is feasible, and approximately 55 % of the pGFP-HPT/pRFP co-transformants contained unlinked T-DNAs and segregated single RFP progeny. The pGFP-HPT/pRFP and the pGFP-HPT/pRFP-HPT vector combinations showed distinctive selective dynamics of transformed rice cells, suggesting that co-transformation efficiency depends on both vector system and selection scheme.

Introduction

Co-transformation is an important approach for generation of marker-free transgenic plants (Liu et al., 2020). Co-transformation of plants can be achieved by both *Agrobacterium*-mediated transformation (Kapusi et al. 2013) and particle bombardment (Chen et al. 1998a). The *Agrobacterium* approach possesses the advantages of lower transgene copy number and fewer events of transgene silencing, and is widely used for co-transformation of multiple genes. Genetically, two different T-DNAs can be integrated into separate genome sites in a fraction of co-transformants, and the T-DNAs can be inherited by different transgenic progeny. Marker-free transgenic plants are generated by screening transgenic offspring harboring a single T-DNA. Different T-DNAs can be delivered into plant cells either from one *Agrobacterium* strain (single-strain method; Sripriya et al. 2008) or from mixed strains (mixed strain method; Kapusi et al. 2013; Komari et al. 1996). A single strain harboring one vector that carried two T-DNA regions was also used for co-transformation (twin T-DNA method; Breitler et al. 2004).

Co-transformation frequency is defined as the frequency with which transformants with a first T-DNA contained a second unselected T-DNA (De Buck et al. 1998). For the method using mixed *Agrobacterium* strains, the mix ratio may influence co-transformation efficiency (Park et al. 2004). High-frequency co-transformation was achieved at the 1:2 and 1:1 ratios of strains harboring both antibiotic and visually selectable vectors, and the co-transformation frequency decreased dramatically as the ratio increased (Park et al. 2004). In rice, the frequency of co-transformed plants for 1:3 ratio of the strain harboring hygromycin-selectable T-DNA and the strain of visually selectable *GUS* T-DNA was higher than that of the 1:1 ratio (Komari et al. 1996).

Selectable marker and selection scheme are also important factors affecting co-transformation efficiency. Generally, one vector for co-transformation contains an antibiotic-selectable marker and the other vector carries a visually selectable marker such as *GUS* (Miller et al. 2002; Komari et al. 1996), *GFP* (Dutt et al. 2008), or an actual gene encoding an agronomic trait (Singh et al. 2011; Sripriya et al. 2008). When seven to ten T-DNA vectors, each of which contained an *NPT* marker, were co-transformed into *Arabidopsis*, 6–19% of the transformed plants were co-transformants and most of the co-transformants contained just two types of T-DNAs (De Buck et al. 2009).

Genetic analysis of co-transformed plants showed that the co-transformed T-DNAs were integrated into both linked and unlinked loci (De Neve et al. 1997; Komari et al. 1996). Segregants with linked T-DNAs were identified from T₁ progeny of *Arabidopsis* and tobacco co-transformants at the frequencies of 68.2% (15/22) and 78.6% (11/14), respectively (De Neve et al. 1997). Unlinked T-DNA integration events were identified from more than half of the co-transformed tobacco and rice lines (Komari et al. 1996). The unlinked T-DNA loci

can be utilized for segregating the gene of interest (GOI) from the selectable marker gene and generation of marker-free transgenic plants.

The objective of this study is to investigate the factors affecting co-transformation in rice using the mixed strain method. We used both antibiotic and fluorescent protein markers to compose various selective schemes so that the dynamics of transformed cell screening can be investigated. The combination of an antibiotic-selectable *GFP* vector and a single *RFP* vector and that of two distinguishable antibiotic-GFP/RFP vectors were tested. Single RFP-fluorescent progeny were screened to simulate the process of selecting for marker-free transgenic plants that carry an actual gene of interest.

Materials And Methods

Plant material

The rice (*Oryza sativa* L.) cultivar Nipponbare was used for plant transformation.

Vector construction

Hygromycin (Hyg)-selectable vectors pGFP-HPT and pRFP-HPT (Fig. 1) were constructed by inserting the *GFP* and *RFP* cassettes, respectively, between the *EcoRI* and *HindIII* sites in pCAMBIA1300 (Genbank AF234296). To construct the visually selectable vector pRFP, pCAMBIA0380 (Genbank AF234290) was modified by removing the Nos polyA sequence to generate pCAMBIA0380* and the *RFP* cassette was subsequently inserted between the *EcoRI* and *HindIII* sites in pCAMBIA0380*.

Agrobacterium-mediated co-transformation of rice

Co-transformation was performed via *Agrobacterium* utilizing scutellum-derived embryogenic callus (Hiei et al. 1994). *Agrobacterium* strains were independently grown in YEP liquid medium at 28°C to OD₆₀₀ between 1.4 and 1.8, and the strain with higher OD₆₀₀ was diluted with YEP to adjust the bacterial concentration approximate to the OD₆₀₀ of the other strain. Subsequently, the pGFP-HPT and pRFP cultures were mixed by 1:1 and 1:4 ratios, centrifuged and re-suspended in 30ml AAM (Toki et al. 2006). The final OD₆₀₀ of the infection mixture was brought to the range of 0.25–0.7 (Table.1). For pGFP-HPT and pRFP-HPT co-transformation, the *Agrobacterium* strains were mixed by 1:1 and the final OD₆₀₀ was brought to 0.098–0.145 (Table 1). For co-cultivation, 100 to 300 rice calli were immersed in *Agrobacterium* mixture for 10 min, dried on sterilized filter paper for 15 min, transferred onto co-cultivation medium that was covered by a sterilized filter paper, and incubated at 20°C (in dark) for 3 days. Co-cultivated calli were thoroughly washed with sterilized water and 300mg/L carbenicillin solution, dried briefly on filter paper and cultured on selection medium (NB, 50mg/L Hyg and 300mg/L Cefotaxime) at 26°C (light 12h/dark 12h) for 3 weeks. The calli were then transferred to fresh selection medium and cultured for 3 more weeks. And third-round selection was performed depending on the condition of co-transformed calli. Calli expressing both GFP and RFP were cultured on regeneration medium (NB, 3mg/L 6-benzyl aminopurine and 0.5mg/L naphthalene acetic acid) at 26°C (light 12h/dark 12h) for 1 month. Shoots were transferred to half-strength NB medium for root growth.

Table 1

Frequencies of *Agrobacterium*-infected calli producing single- and double-fluorescent microcalli in the first- and second-round selection of co-transformation

Vector combination	OD ₆₀₀ of mixture	GFP ⁺ freq (%) ^{a, c}	RFP ⁺ freq (%) ^{a, c}	GFP ⁺ RFP ⁺ freq (%) ^{b, c}
pGFP-HPT/pRFP	0.688	92.4 (122/132)	78.0 (103/132)	84.4 (103/122)
(1 : 1 mix ratio) ^d	0.585	95.5 (213/223)	70.9 (158/223)	89.2 (140/157)
	0.327	97.1 (268/276)	81.2 (224/276)	84.3 (145/172)
	0.258	93.5 (187/200)	81.0 (162/200)	92.4 (146/158)
Average		94.6	77.8	87.6
pGFP-HPT/pRFP	0.696	93.3 (279/299)	78.9 (236/299)	89.0 (234/263)
(1 : 4 mix ratio) ^d	0.570	96.8 (92/95)	72.6 (69/95)	88.0 (81/92)
	0.325	94.1 (287/305)	78.0 (238/305)	86.8 (204/235)
	0.252	89.1 (179/201)	77.6 (156/201)	94.8 (146/154)
Average		93.3	76.8	89.7
pGFP-HPT/pRFP-HPT	0.145	97.0 (325/335)	95.2 (319/335)	68.4 (137/201)
(1 : 1 mix ratio)	0.098	90.5 (199/220)	92.3 (203/220)	44.8 (82/183)
Average		93.8	93.8	56.6

^a GFP⁺ (or RFP⁺) freq, percentage of *Agrobacterium*-infected calli producing GFP⁺ (or RFP⁺) spots in the first-round selection. For the pGFP-HPT/pRFP vector combination, the GFP⁺ freq and RFP⁺ freq at each OD₆₀₀ of 1:1 or 1:4 ratio were paired, and the data were analyzed using a paired t-test (<http://udel.edu/~mcdonald/statpaired.html>). The RFP⁺ freq was significantly lower than GFP⁺ freq ($P < 0.05$). ^b GFP⁺RFP⁺ freq, percentage of the infected-calli with GFP⁺ spots producing GFP⁺RFP⁺ microcalli after subculture on fresh Hyg medium for another three weeks. ^c Callus numbers were given in the brackets. ^d For the pGFP-HPT/pRFP vector combination, the same type of frequencies (GFP⁺ freq, RFP⁺ freq or GFP⁺RFP⁺ freq) for the 1:1 and 1:4 ratios at each OD₆₀₀ were paired and analyzed using a paired t-test. No significant difference was detected between the 1:1 and 1:4 ratios ($P > 0.05$).

GFP and RFP assays of transgenic rice

GFP and RFP fluorescence expressed from transformed rice calli or plants was assayed under a fluorescent lamp (BLS, Biological Laboratory Equipment Maintenance and Service, Hungary) with the excitation light sources FHS/LS-1B (460-495nm) and FHS/LS-1G (515-588nm), respectively. GFP/RFP fluorescence was observed using glasses with GFP filter FHS/EF-2G2 (505-515nm) or RFP filter FHS/EF-4R2 (590-660nm). For pGFP-HPT/pRFP co-transformed rice lines, 50 to 100 T₁ seeds per line were germinated for 7 days and each seedling was assayed under fluorescent lamp to determine GFP/RFP pattern (GFP⁺/RFP⁺, GFP⁺/RFP⁻, GFP⁻/RFP⁺ or GFP⁻/RFP⁻). Chi-square analyses were performed to predict *GFP/RFP* segregation pattern (see Table 2). GFP/RFP images of calli and seedlings were taken under fluorescent lamp and using digital camera (SONY, α550, Japan) equipped with GFP or RFP filter. GFP/RFP images of single callus were taken under fluorescent stereomicroscope (Leica M165FC, Germany) with color CCD camera (Leica DFC425C, Germany) or cold light fluorescence CCD camera (HAMAMATSU, C4742-80-12AG, Japan).

Table 2
Genetic analysis of *GFP* and *RFP* segregation in T₁ plants of pGFP-HPT/pRFP co-transformation (1:4 mix ratio, OD600 0.696)

Co-transformants	No of T ₁ plants	Fluorescence patterns of T ₁ plants				Predicted GFP/RFP loci			Segregation pattern ^a	<i>P</i> value ^b
		GFP ⁺ /RFP ⁺	GFP ⁺ /RFP ⁻	GFP ⁻ /RFP ⁻	GFP ⁻ /RFP ⁺	<i>GFP</i>	<i>RFP</i>	<i>GFP</i> + <i>RFP</i>		
1	38	29	0	0	9	1	2	0	45:1:3:15	0.463
2	42	31	10	0	1				NT	-
3	41	32	4	0	5	0	1	1	12:1:0:3	0.407
4	45	35	2	1	7	1	2	0	45:1:3:15	0.210
5	42	27	3	0	12	0	1	1	12:1:0:3	0.240
6	41	22	2	11	6	1	1	0	9:1:3:3	0.579
7	36	27	3	5	1				NT	-
8	45	40	2	0	3	0	1	1	12:1:0:3	0.086
9	36	28	0	2	6	1	2	0	45:1:3:15	0.657
10	43	26	16	1	0				NT	-
11	46	33	7	0	6				NT	-
12	42	35	2	0	5	0	1	1	12:1:0:3	0.452
13	29	25	1	0	3	0	1	1	12:1:0:3	0.378
14	39	28	3	7	1	1	1	0	9:1:3:3	0.064
15	40	26	3	9	2	1	1	0	9:1:3:3	0.173
16	43	38	1	1	3	1	2	0	45:1:3:15	0.054
17	38	27	2	9	0	1	0	1	12:1:3:0	0.729
18	37	25	1	10	1	2	1	0	45:1:15:3	0.835
19	33	20	2	1	10	1	1	0	9:1:3:3	0.078
20	41	39	1	1	0				NT	-
21	80	58	5	0	17	0	1	1	12:1:0:3	0.846
22	56	38	1	0	17	0	2	1	48:1:0:15	0.462
23	24	15	9	0	0	0	0	1	3:1	0.157
24	48	37	11	0	0	0	0	1	3:1	0.739
25	44	40	4	0	0	0	0	2	15:1	0.436
26	47	38	9	0	0	0	0	1	3:1	0.354
27	49	29	20	0	0				NT	-
28	37	27	10	0	0	0	0	1	3:1	0.776

NT, not tested: segregation pattern was not given due to the limited number of T₁ plants and difficulty to perform Chi-square test (indicated by – in the *P* value column). ^a Segregation ratio was calculated based on the predicted T-DNA loci. The 3:1 ratio represents one *GFP* + *RFP* locus where the pGFP-HPT and pRFP T-DNAs were linked; 15:1, two *GFP* + *RFP* loci; 63:1, three *GFP* + *RFP* loci. The 12:1:0:3 ratio represents one *GFP* + *RFP* locus and one *RFP* locus (single pRFP locus); 12:1:3:0, one *GFP* + *RFP* locus and one *GFP* locus (single pGFP-HPT locus); 48:1:0:15, one *GFP* + *RFP* locus and two *RFP* loci; 9:1:3:3, one *GFP* locus and one *RFP* locus; 45:1:3:15, one *GFP* locus and two *RFP* loci; 45:1:15:3, two *GFP* loci and one *RFP* locus. ^b All the predicted patterns of T-DNA loci were analyzed by Chi-square test, and significant difference was rejected as *P* value > 0.05. Abbreviations: GFP⁺, expressing GFP; GFP⁻, not expressing GFP; RFP⁺, expressing RFP; RFP⁻, not expressing RFP; *GFP* + *RFP*, locus where the pGFP-HPT and pRFP T-DNAs were linked; *P*, probability that the observed segregation deviates from the expected by chance.

Co-transformants	No of T ₁ plants	Fluorescence patterns of T ₁ plants				Predicted GFP/RFP loci			Segregation pattern ^a	P value ^b
		GFP ⁺ /RFP ⁺	GFP ⁻ /RFP ⁻	GFP ⁺ /RFP ⁻	GFP ⁻ /RFP ⁺	GFP	RFP	GFP + RFP		
29	46	33	13	0	0	0	0	1	3:1	0.610
30	42	28	14	0	0	0	0	1	3:1	0.212
31	38	31	7	0	0	0	0	1	3:1	0.349
32	41	30	11	0	0	0	0	1	3:1	0.787
33	41	31	10	0	0	0	0	1	3:1	0.928
34	45	35	10	0	0	0	0	1	3:1	0.667
35	71	48	23	0	0	0	0	1	3:1	0.150
36	57	41	16	0	0	0	0	1	3:1	0.592
37	72	58	14	0	0	0	0	1	3:1	0.276
38	67	51	16	0	0	0	0	1	3:1	0.832
39	84	70	14	0	0	0	0	1	3:1	0.078
40	73	72	1	0	0	0	0	3	63:1	0.894
NT, not tested: segregation pattern was not given due to the limited number of T ₁ plants and difficulty to perform Chi-square test (indicated by – in the P value column). ^a Segregation ratio was calculated based on the predicted T-DNA loci. The 3:1 ratio represents one <i>GFP</i> + <i>RFP</i> locus where the pGFP-HPT and pRFP T-DNAs were linked; 15:1, two <i>GFP</i> + <i>RFP</i> loci; 63:1, three <i>GFP</i> + <i>RFP</i> loci. The 12:1:0:3 ratio represents one <i>GFP</i> + <i>RFP</i> locus and one <i>RFP</i> locus (single pRFP locus); 12:1:3:0, one <i>GFP</i> + <i>RFP</i> locus and one <i>GFP</i> locus (single pGFP-HPT locus); 48:1:0:15, one <i>GFP</i> + <i>RFP</i> locus and two <i>RFP</i> loci; 9:1:3:3, one <i>GFP</i> locus and one <i>RFP</i> locus; 45:1:3:15, one <i>GFP</i> locus and two <i>RFP</i> loci; 45:1:15:3, two <i>GFP</i> loci and one <i>RFP</i> locus. ^b All the predicted patterns of T-DNA loci were analyzed by Chi-square test, and significant difference was rejected as P value > 0.05. Abbreviations: GFP ⁺ , expressing GFP; GFP ⁻ , not expressing GFP; RFP ⁺ , expressing RFP; RFP ⁻ , not expressing RFP; <i>GFP</i> + <i>RFP</i> , locus where the pGFP-HPT and pRFP T-DNAs were linked; P, probability that the observed segregation deviates from the expected by chance.										

Rice DNA isolation and PCR amplification

Rice genomic DNA was isolated from leaf tissue using a small-scale procedure (Komari et al. 1997). *GFP* and *RFP* genes were amplified in PCR using rTaq DNA polymerase (TaKaRa, Dalian, China) and primers as follows: *GFP*-forward (5'-CGACCACATGAAGCAGCAGAC-3'), *GFP*-reverse (5'-TCCTCGATGTTGTGGCGATCT-3'), *RFP*-forward (5'-GGGCTTCAAGTGGGAGCGCGTG-3'), and *RFP*-reverse (5'-CCGCCGTCCTTCAGCTTCAGGG-3'). Primers for amplification of the junctions between tandem-linked pGFP-HPT and pRFP T-DNAs in co-transformants were P1 (5'-ATCTGTCGATCGACAAGCTCGA-3'), P2 (5'-ACAACGTCGTGACTGGGAAAAC-3'), P3 (5'-CCGCCACCACTGTTCTCTGT-3'), and P4 (5'-TAAGAGGAGTCCACCATGGTAG-3'). The cycling conditions were 95°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min; 72°C for 7 min.

Rice RNA isolation and Reverse Transcriptase (RT)-PCR

Rice RNA was extracted using TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's protocol. The complementary DNA (cDNA) was synthesized by reverse-transcribing 1 µg of rice RNA using a ReverTra Ace® qPCR RT Kit (Toyobo, Japan). The *GFP* and *RFP* cDNAs were amplified using primers the same as in PCR of rice genomic DNA. Primers for amplification of *HPT* cDNA were: *HPT* forward (5'-CGCCGATGGTTTCTACAAAG-3') and *HPT* reverse (5'-ACACATGGGGATCAGCAATC-3'). Primers for amplification of rice actin cDNA were: Actin forward, 5'-GGAAGTGGTATGGTCAAGGC-3'; Actin reverse, 5'-AGTCTCATGGATACCCGAG-3'. The cycling condition for semi-quantitative RT-PCR was 95°C for 5 min; 28 or 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 50 s; 72°C for 7 min. qRT-PCR was conducted using the CFX96™ Real-Time System (Bio-Rad, USA) and in the conditions as follows: 95°C for 2 min; 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s. The resulting data were analyzed using the CFX Manager™ software (Version 1.0) (Bio-Rad, USA).

Results

Co-transformation using different *GFP/RFP* vector combinations

Hygromycin (Hyg) selectable vector pGFP-HPT and visually selectable vector pRFP (Fig. 1) were constructed for *Agrobacterium*-mediated co-transformation of rice. The hygromycin phosphotransferase (*HPT*) marker in pGFP-HPT is antibiotic-selectable, and *GFP* in the same T-DNA functions as a visual and visually selectable marker for transformed cells and plants. The pRFP T-DNA contains *RFP* only, which was used as a model gene simulating the gene of interest (GOI) in marker-free transformation. The visually selectable pRFP T-DNA can be retrieved only when it is co-transformed with the pGFP-HPT T-DNA that confers Hyg resistance for the transformed cells to survive on selection medium.

Agrobacterium strains harboring each of the pGFP-HPT and pRFP vectors were grown independently, and the cultures were brought to equal concentration (OD600) and mixed by 1:1 and 1:4 ratios. The mixtures were diluted to four concentrations, and the 1:1 and 1:4 mixtures of each dilution were adjusted to approximately equal concentration (Table 1). For the pGFP-HPT/pRFP vector combination, eight co-transformation experiments were conducted using two mix ratios and four bacterial concentrations.

Rice calli infected with each mixture were cultured on Hyg medium, and after three weeks, Hyg-resistant (Hyg⁺) cells growing from the infected calli exhibited fluorescence when assayed under GFP/RFP macro detector. One to several GFP-positive (GFP⁺) or RFP-positive (RFP⁺) spots were observed from each infected callus. The average frequencies of GFP⁺ calli (GFP⁺ freq, Table 1) for the 1:1 and 1:4 ratios of mixtures were 94.6% and 93.3%, respectively. The RFP⁺ frequencies (RFP⁺ freq), ranging from 70.9–81.2%, were significantly lower than the GFP⁺ frequencies ($P < 0.05$). Since the RFP⁺ cells observed were Hyg resistant and the visually selectable pRFP vector did not contain *HPT*, the RFP⁺ cells were double-fluorescent (GFP⁺RFP⁺) co-transformants of the pGFP-HPT and pRFP vectors.

During the first-round selection, the single-fluorescent (GFP⁺RFP⁻) and double-fluorescent cell groups growing from the infected calli were too small to be distinguished from each other. The primary calli with GFP⁺ spots were thus transferred to fresh Hyg medium for second-round selection. After three weeks, fluorescent microcalli with larger sizes were identified under GFP/RFP macro detector, and the number of GFP⁺ microcalli derived from each primary callus was more than that of the RFP⁺ microcalli (Fig. 1a-b). Under fluorescent stereo microscope, one to several GFP⁺RFP⁺ microcalli were distributed around the cultures of each primary callus, and in most cases were easy to be distinguished from the other cell groups (Fig. 2a).

To explore whether the mix ratios of pGFP-HPT and pRFP strains affected co-transformation, the same frequencies of GFP⁺ freq, RFP⁺ freq or GFP⁺RFP⁺ freq for the 1:1 and 1:4 ratios at each OD600 (Table 1) were paired, and the data were analyzed using a paired t-test. No significant difference was detected between the 1:1 and 1:4 ratios ($P > 0.05$). Therefore, the 1:1 and 1:4 ratios resulted in comparable frequencies of co-transformed GFP⁺RFP⁺ calli in the described conditions.

To investigate the effect of the antibiotic-selectable *HPT* marker on co-transformation efficiency, we constructed another antibiotic-selectable vector (pRFP-HPT, Fig. 1) and conducted co-transformation using the pGFP-HPT and pRFP-HPT vectors. The pGFP-HPT/pRFP-HPT combination provides an experimental condition in which the co-transformed GFP⁺RFP⁺ cells and the single-vector transformed GFP⁺RFP⁻ or GFP⁻RFP⁺ cells can proliferate independently on Hyg medium.

Two pGFP-HPT/pRFP-HPT co-transformation experiments (1:1 mix ratio, Table 1) were performed together with the pGFP-HPT/pRFP co-transformations in the same tissue culture conditions. At the end of the first-round selection, the percentage of primary calli producing GFP⁺ spots was 93.8% on average (Table 1). The RFP⁺ frequencies were comparable to GFP⁺ frequencies, which were different from the results with the pGFP-HPT/pRFP vector combination and revealed that the GFP⁺ or RFP⁺ cells derived from pGFP-HPT or pRFP-HPT transformation can grow independently in Hyg selection medium. The *Agrobacterium*-infected calli with GFP⁺ or RFP⁺ spots were subcultured. At the end of the second-round selection, the frequency of the primary calli with GFP⁺ spots producing GFP⁺RFP⁺ microcalli was 56.6% on average, which was significantly lower than the GFP⁺RFP⁺ freq in pGFP-HPT/pRFP co-transformations (Table 1). The GFP⁺RFP⁻, GFP⁻RFP⁺ and GFP⁺RFP⁺ microcalli were independently distributed around the cultures derived from each primary callus (Fig. 1c, Fig. 2b-d). GFP⁺RFP⁺ calli with large sizes were observed (Fig. 1b-d), but in most cases the GFP⁺RFP⁺ calli were small (Fig. 1c) and difficult to be distinguished from the single-fluorescent calli. Therefore, the dynamics of selection for rice cells transformed with the pGFP-HPT/pRFP-HPT vector combination was different from that with the pGFP-HPT/pRFP combination, and co-transformation efficiency depends on the antibiotic-selectable *HPT* marker in both vectors and individual selection schemes.

Genetic analysis of co-transformed T-DNA loci in rice

The double-fluorescent microcalli of pGFP-HPT/pRFP co-transformation (1:4 ratio, OD600 0.696, Table 1) were subcultured for the third-round selection (Fig. 2e), and the GFP⁺RFP⁺ calli were regenerated into plants. At T₁ generation, forty independent co-transformation lines were randomly selected for genetic analysis of *GFP* and *RFP* segregation. The T₁ seeds were germinated and individual seedlings were examined under GFP/RFP macro detector. Among the forty T₁ lines (Table 2), 18 (45%) lines segregated double-fluorescent plants but not any single-fluorescent (GFP⁺RFP⁻ or GFP⁻RFP⁺) progeny, suggesting that the pGFP-HPT and pRFP T-DNAs were genetically linked in the co-transformants. Single-fluorescent GFP⁻RFP⁺ plants were recovered from 47.5% (19/40) of the T₁ lines, which simulated the recovery of *HPT* marker-free transgenic progeny from the co-transformation.

The linkage between the pGFP-HPT and pRFP T-DNAs was predicted according to the *GFP/RFP* segregation patterns and examined by Chi-square test (Table 2). For the co-transformants with unlinked T-DNAs, 63.6% (14/22) of them segregated GFP⁻RFP⁺ progeny with frequencies higher than those of the GFP⁺RFP⁻ progeny and the predicted *RFP* loci were more than *GFP* loci. Taken together, about one half of the pGFP-HPT/pRFP co-transformants contained unlinked *pGFP-HPT* and *pRFP* loci, and the average locus number of pRFP T-DNA was higher than that of pGFP-HPT T-DNA.

A pGFP-HPT/pRFP co-transformant containing unlinked *GFP* and *RFP* loci (#6 co-transformant, Table 2) was chosen for illustration of the *GFP* and *RFP* segregation patterns in T₁ generation (Fig. 3a-c). Rice genomic DNA was purified from the T₁ plants with different fluorescence patterns, and the DNA samples were amplified in PCR reactions using *GFP* and *RFP* specific primers (Fig. 3d). The results of PCR amplification were well correlated with the GFP and RFP fluorescence observation.

Molecular characterization of co-transformed T-DNA loci in rice

The expression levels of *RFP*, *GFP* and *HPT* in six pGFP-HPT/pRFP co-transformed lines were determined by semi-quantitative reverse-transcriptase (RT)-PCR (Fig. 4a) and real-time RT-PCR (Fig. 4b). Appropriate transcript levels were detected in the T₂ plants 7 – 1 (GFP⁻RFP⁺), 10-1S (GFP⁺RFP⁻) and 10-1D (GFP⁺RFP⁺) from two unlinked co-transformation lines. The *RFP*, *GFP* and *HPT* transcripts were also detected in the #29 and #36 linked lines based on the results of the double-fluorescent T₂ plants 29 – 4 and 36 – 1 (Fig. 4). However, transgene expression in the #28 and #34 linked lines varied dramatically. The double-fluorescent T₂ plant 28 – 1 showed low level of *GFP* transcription. And the T₂ plant 34 – 1 exhibited no apparent GPT and HPT expression but contained pGFP-HPT T-DNA based on the PCR results (see below), suggesting that *GFP* and *HPT* in the plant were silenced. These data indicated that the co-transformed rice lines had different levels of transgene expression.

The T-DNA loci in eight linked lines were investigated by PCR amplification and sequence analysis (Table 3, Fig. 5). For each rice line, five progeny (T₂) from a double-fluorescent T₁ plant were analyzed and the junctions between pGFP-HPT and pRFP T-DNAs were amplified using the primer pairs P1/P3, P1/P4, P2/P3 and P2/P4 (Fig. 1, Table 3). Physically-linked pGFP-HPT and pRFP T-DNAs were detected in six (75%) of the rice lines, and the T-DNAs were linked into tandem configurations (LRRL, RLLR and LRLR) as revealed by the junction sequences (Fig. 5a-d). Deletion of RB/LB borders and inward T-DNA sequences was also detected in the T-DNA junctions.

Table 3

PCR amplification of the junctions between linked pGFP-HPT and pRFP T-DNAs in the *GFP/RFP* co-segregating T₂ progeny*

T ₂ lines	PCR primers					
	P1/P3	P1/P4	P2/P3	P2/P4	P1/P2	P3/P4
23 - 1	-	-	-	-	-	+
24 - 1	+	+	-	+	-	+
26 - 1	+	-	-	+	+	-
28 - 1	-	+	-	-	-	+
29 - 4	-	+	-	-	-	-
34 - 1	-	-	-	-	+	-
35 - 1	-	+	+	+	+	+
36 - 1	-	+	-	-	+	-

* For each independent co-transformation line, five progeny (T₂) from a double-fluorescent T₁ plant were analyzed using primers specific to the pGFP-HPT and pRFP T-DNAs (Fig. 1)

In addition, the linkage between two copies of pGFP-HPT or pRFP T-DNA was examined by PCR reactions using the primer pairs P1/P2 and P3/P4. Among the six rice lines with linked pGFP-HPT and pRFP T-DNAs, five (83.3%) lines contained tandem repeats of one T-DNA type based on the PCR and sequencing results (Table 3, Fig. 5e and f). Because the five rice lines showed single-locus inheritance in T₁ generation (Table 2), the physically-linked pGFP-HPT/pRFP T-DNAs and the tandem repeats of single T-DNA types were clustered into the same locus in the rice genome. Therefore, most of the *GFP/RFP* co-segregating lines contained physically-linked pGFP-HPT and pRFP T-DNAs, and the tandem configurations were formed by the linkage between different T-DNAs as well as two identical T-DNA copies.

Discussion

In this study, we used GFP and RFP to track co-transformation events in rice and investigated the factors affecting co-transformation efficiency. The co-transformation frequencies of Hyg-selectable pGFP-HPT vector and single pRFP vector ranged from 70.9–81.2%, as revealed by the percentages of *Agrobacterium*-infected calli growing Hyg⁺RFP⁺ cells. Komari et al. (1996) co-transformed a Hyg-selectable T-DNA vector and visually selectable *GUS* vector into rice via mixed strains and produced only small portion (2%-17%) of co-transformants. The higher co-transformation efficiency in this study can be explained as follows. First, with the high-efficient *GFP/RFP* markers, a single GFP⁺RFP⁺ spot representing co-transformation can be identified from an *Agrobacterium*-infected callus. In the experiments by Komari et al. (1996), the co-transformed cells of many primary calli might have been unselected or unavailable for plant regeneration, because GUS assay is destructive to plant tissues and they were not able to examine the GUS activity in all the co-transformed cells. Also, our co-transformation frequencies were based on the percentage of primary calli growing GFP⁺RFP⁺ cells, but the frequencies by Komari et al. (1996) were calculated as the percentage of plants transformed with the *HPT* and *GUS* T-DNAs. For the independent GFP⁺RFP⁺ callus lines, just a portion of them can be regenerated into rice plants.

The mix ratio of *Agrobacterium* strains may influence co-transformation frequency (Park et al. 2004; Komari et al. 1996). In our experiments, 1:1 and 1:4 ratios of pGFP-HPT/pRFP strains produced comparable frequencies of co-transformed GFP⁺RFP⁺ calli. We cannot compare our results directly to those of other reports because different plant materials and selection conditions were used. If the 1:1 and 1:4 ratios just result in different numbers of GFP⁺RFP⁺ spots in single primary calli, comparable co-transformation frequencies can be obtained using the efficient GFP/RFP selection scheme.

We compared the effects of the pGFP-HPT/pRFP-HPT and pGFP-HPT/pRFP vector combinations on transformation selection. Under similar tissue culture conditions, the pGFP-HPT/pRFP-HPT combination resulted in lower frequencies of co-transformed GFP⁺RFP⁺ microcalli. Theoretically, pGFP-HPT/pRFP combination produced three types of fluorescent cells that contained the T-DNA of pGFP-HPT or pRFP or both of the T-DNAs. Because the pRFP transformed rice cells were eliminated under Hyg selection, the GFP⁺RFP⁺ cells were co-transformants and the GFP⁺/RFP⁻ cells carried the pGFP-HPT T-DNA only. For the pGFP-HPT/pRFP-HPT combination, the rice cells transformed with a single vector or both vectors can survive Hyg selection and independently grow into GFP⁺RFP⁻, GFP⁻RFP⁺, and

GFP⁺RFP⁺ microcalli. It is believed that the probability of which a rice cell is transformed with a single vector is much higher than that of co-transformation. Also, because a single GFP⁺RFP⁺ spot can be distinguished from the other rice cells, the visually selectable T-DNAs can be efficiently selected. Therefore, our data with the pGFP-HPT/pRFP-HPT vector combination suggested selective dynamics distinctive from that with the pGFP-HPT/pRFP vector combination. The antibiotic-selectable and visually-selectable T-DNAs functioned differently, and the marker genes and selection schemes affected the efficiency of co-transformation.

Komari et al. (1996) co-transformed *HPT* and *GUS* T-DNAs into tobacco by both twin T-DNA and mixed strain methods, and obtained GUS positive, antibiotic-sensitive progeny from more than half of the co-transformants. They also tested the mixed strain method in rice but analyzed limited number of rice co-transformants. In this study, we performed genetic analysis of forty co-transformed rice plants and showed that the co-transformants produced by mixed strains contained linked and unlinked T-DNAs at the frequencies of 45% and 55%, respectively, which is similar to the results of the twin T-DNA method (Komari et al. 1996).

The efficiency of the mixed strain method is affected by the plant materials and starter explants for co-transformation. Relatively higher co-transformation frequencies (ranged from 30%-50%) were obtained when tobacco was transformed via mixed strains (Park et al. 2004; Komari et al. 1996). For marker-free transformation in rice, it is not suitable to use fluorescent protein to select the T-DNA containing the gene of interest, but the mixed strain method can be improved by increasing the efficiency of screening co-transformed cells. The selection scheme by Komari et al. (1996) was to obtain Hyg⁺ rice plants and then to screen co-transformed plants by PCR detection of the gene of interest. We suggest that PCR assay is performed initially with independent Hyg⁺ calli and then the co-transformed calli are regenerated into plants.

We predicted the *GFP/RFP* inheritance patterns of the pGFP-HPT/pRFP co-transformants that carried unlinked T-DNAs. Interestingly, in 63.6% of the unlinked co-transformants, the frequencies of GFP⁺RFP⁺ progeny were higher than those of the GFP⁺RFP⁻ progeny and the predicted *RFP* loci were more than *GFP* loci. Breitler et al. (2004) obtained a number of rice co-transformants using a twin T-DNA vector containing the *HPT* and *Bar* genes in separate T-DNAs and by Hyg selection. For most of the co-transformants containing unlinked *HPT* and *Bar* T-DNAs, the Bar⁺/HPT⁻ segregants were more than the Bar⁻/HPT⁺ plants. The molecular mechanism for more visually selectable T-DNA loci in the co-transformants needs to be addressed in further studies.

The pGFP-HPT/pRFP co-transformants showed various transgene expression levels or even transgene silencing based on the quantitative RT-PCR results of *RFP*, *GFP* and *HPT* genes. And tandem-linked T-DNAs were detected in most of the rice lines. The variation of transgene expression can be explained by the T-DNA structures in the co-transformed lines. It was reported that co-integrated T-DNAs are usually linked into tandem configurations (De Buck et al. 2009; De Neve et al. 1997) and the T-DNAs in inverted repeats may result in convergent transcription and trigger transgene silencing (De Buck et al. 2001). It is necessary to screen co-transformation events by evaluation of their T-DNA structures and transgene expression levels.

In this study, we used GFP and RFP fluorescent proteins as visual markers in co-transformation and were able to observe distinctive selection dynamics of the rice cells transformed with different vector combinations. We demonstrated that rice calli can be efficiently co-transformed with both antibiotic- and GFP/RFP-selectable T-DNA derived from mixed *A. tumefaciens* strains. The mixed strains method has the advantages of simple T-DNA vectors and ease to handle, and with appropriate selectable markers and vector systems, it can be efficiently utilized in marker-free transformation.

Abbreviations

GFP Green fluorescent protein

RFP Red fluorescent protein

HPT Hygromycin phosphotransferase

Hyg Hygromycin

Declarations

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Figures

Fig 1

Figure 1

Binary vectors for *Agrobacterium*-mediated co-transformation of rice. pGFP-HPT, Hgy-selectable *GFP* vector; pRFP-HPT, single *RFP* marker vector; pRFP-HPT, Hgy-selectable *RFP* vector. PUBi, maize ubiquitin 1 promoter; *GFP*, green fluorescent protein; *RFP*, red fluorescent protein; Tnos, Nos polyA signal sequence; P35S, CaMV 35S promoter; *HPT*, hygromycin phosphotransferase; T35S, CaMV 35S polyA signal sequence; L, T-DNA left border; R, T-DNA right border. P1, P2, P3 and P4, PCR primers for amplification of the junctions

between tandem-linked pGFP-HPT and pRFP T-DNAs. **a-b** Fluorescent Hyg⁺ microcalli growing from the primary calli infected with 1:1 and 1:4 ratios of pGFP-HPT and pRFP strains, respectively. **c** Fluorescent Hyg⁺ microcalli growing from the primary calli infected with 1:1 ratio of pGFP-HPT and pRFP-HPT strains. *Left panel*, under white light; *middle panel*, GFP images under blue light; *right panel*, RFP images under green light.

Fig. 2

Figure 2

Double-fluorescent Hyg⁺ calli from the second- and third-round selection under stereo fluorescence microscope. **a** Double-fluorescent (GFP⁺RFP⁺) Hyg⁺ calli derived from two rounds of selection of the pGFP-HPT/pRFP co-transformation (1:1 ratio). **b-d** Randomly distributed GFP⁺RFP⁺, GFP⁺RFP⁻ and GFP⁻RFP⁺ calli derived from the second-round selection of pGFP-HPT/pRFP-HPT co-transformation. *Left panel*, under white light; *middle panel*, GFP images; *right panel*, RFP images. Scale bar=2mm. **e** GFP⁺RFP⁺ calli derived from the third-round selection of pGFP-HPT/pRFP co-transformation (1:4 ratio). *Upper left panel*, under white light; *upper right panel*, GFP image; *lower left panel*, RFP image; *lower right panel*, merged image of white, GFP and RFP signals by Image J software. Scale bar=1mm.

Fig. 3

Figure 3

Segregation of the *pGFP-HPT* and *pRFP* loci in a co-transformed rice line revealed by GFP and RFP fluorescence assay and PCR amplification. T₁ seedlings of the #6 co-transformed rice line (Table 2) with different *GFP* and *RFP* genotypes were selected to illustrate the fluorescence patterns of GFP⁺RFP⁺, GFP⁺RFP⁻, GFP⁺RFP⁺ and GFP⁻RFP⁻ from left to right successively. **a** Image of the rice seedlings under white light. **b** GFP image under blue excitation light. **c** RFP image under green excitation light. **d** Rice genomic DNA was amplified in PCR reactions using *GFP* and *RFP* specific primers.

Fig. 4

Figure 4

Reverse-transcriptase (RT)-PCR of the *RFP*, *GFP* and *HPT* genes in T_2 plants of pGFP-HPT/pRFP co-transformed rice lines. **a** Semi-quantitative RT-PCR. The cDNA fragments of *RFP*, *GFP*, *HPT* and *Actin* (rice *actin 1* gene) was amplified for 28 and 30 cycles, respectively. **b** Real-time RT-PCR. The vertical axes represent the relative expression levels of *RFP*, *GFP* and *HPT*, respectively. 7-1 (GFP^-RFP^+), T_2 plant segregated from the #7 unlinked co-transformant (Table 2); 10-1S (GFP^+RFP^-) and 10-1D (GFP^+RFP^+), T_2 plants from the #10 unlinked co-transformant; 34-1 (GFP^+RFP^+), T_2 plant from the #34 linked co-transformant; 28-1, 29-4 and 36-1, GFP^+RFP^+ T_2 plants derived from the #28, #29 and #36 linked co-transformants, respectively.

Fig. 5

Figure 5

Nucleotide variations in the junctions between tandem-linked pGFP-HPT and pRFP T-DNAs in the *GFP/RFP* co-segregating co-transformants. **a** Junction sequences between tandem-linked pGFP-HPT and pRFP T-DNAs in the T₂ plants 24-1 and 26-1, segregated from the #24 and #26 linked co-transformants (Table 2), respectively. On the top line are the reference sequences of pGFP-HPT and pRFP vectors. The T-DNAs were linked into RB-LB-LB-RB (RLLR) configurations. Deletion of LB and its inward sequences is marked with stars. **b-c** Junctions between the tandem-linked pGFP-HPT and pRFP T-DNAs in T₂ plants segregated from the #24, #28, #29, #35 and #36 co-transformants (Table 2). The T-DNAs were linked into RB-LB-RB-LB (RLRL) configurations, where RB and its 19-bp inward sequence were deleted and the LB sequence was intact except for a C→T transversion (marked with a caret). **d** Junctions between the linked pGFP-HPT and pRFP T-DNAs (LRLR configurations) in T₂ plants from the #24 and #26 co-transformants. Deletion of RB and its inward sequences was shown. Nucleotide variations in the junctions between two linked copies of pGFP-HPT or pRFP T-DNA in the *GFP/RFP* co-segregating co-transformants: **e** Junction sequences between two linked copies of pGFP-HPT T-DNA in T₂ plants segregated from the #26, #34, #35 and #36 co-transformants (Table 2). The T-DNAs were linked into LRLR configurations. On the top line are the pGFP-HPT reference sequences. Deletion of RB and its 19-bp inward sequence is marked with stars, and C→T transversion in LB is marked with a caret. **f** Junction sequences between two linked copies of pRFP T-DNA in T₂ plants from the #23, #24, #28 and #35 co-transformants. Deletion of RB and its 18-bp inward sequence is marked with stars.