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

Lihua Li1 · Xudan Tian2,3 · Lanlan Wang2 · Jianhua Zhao2,3 · Jie Zhou2 · Haiyan He2 · Liangying Dai1 · Shaohong Qu2

Received: 5 April 2022 / Accepted: 11 August 2022 / Published online: 30 August 2022 © The Author(s), under exclusive licence to Springer Nature B.V. 2022

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 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.

Keywords Agrobacterium · Co-transformation · GFP · RFP · Selectable marker · Rice

Introduction

Co-transformation is an important approach for generation of marker-free transgenic plants [1]. Co-transformation of plants can be achieved by both Agrobacterium-mediated transformation [2] and particle bombardment [3]. 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) [4] or from mixed strains (mixed strain method) [2, 5]. A single strain harboring one vector that carried two
T-DNA regions was also used for co-transformation (twin T-DNA method) [6].

Co-transformation frequency is defined as the frequency with which transformants with a first T-DNA contained a second unselected T-DNA [7]. For the method using mixed Agrobacterium strains, the mix ratio may influence co-transformation efficiency [8]. 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 [8]. 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 [5].

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 [5], GFP, or an actual gene encoding an agronomic trait [4]. When seven to ten T-DNA vectors, each of which contained an NPT marker, were co-transformed into Arabidopsis, 6% to 19% of the transformed plants were co-transformants and most of the co-transformants contained just two types of T-DNAs [9].

Genetic analysis of co-transformed plants showed that the co-transformed T-DNAs were integrated into both linked and unlinked loci [5, 10]. 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 [10]. Unlinked T-DNA integration events were identified from more than half of the co-transformed tobacco and rice lines [5]. 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*.

**Fig. 1** Binary vectors for *Agrobacterium*-mediated co-transformation of rice. pGFP-HPT, Hgy-selectable *GFP* vector; pRFP, single *RFP* marker vector; pRFP-HPT, Hgy-selectable *RFP* vector. PUbi, maize ubiquitin one 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 Hgy* microcalli growing from the primary calli infected with 1:1 and 1:4 ratios of pGFP-HPT and pRFP strains, respectively. c Fluorescent Hgy* 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.
Agrobacterium-mediated co-transformation of rice

Co-transformation was performed via Agrobacterium utilizing scutellum-derived embryogenic callus [11]. 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 resuspended in 30 ml AAM [12]. 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 300 mg/L carbenicillin solution, dried briefly on filter paper and cultured on selection medium (NB, 50 mg/L Hyg and 300 mg/L Cefotaxime) at 26 °C (light 12 h/dark 12 h) for 3 weeks. The calli were then transferred to fresh selection medium and cultured for three 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, 3 mg/L 6-benzyl aminopurine and 0.5 mg/L naphthalene acetic acid) at 26 °C (light 12 h/dark 12 h) for 1 month. Shoots were transferred to half-strength NB medium for root growth.

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 (460–495 nm) and FHS/LS-1G (515–588 nm), respectively. GFP/RFP fluorescence was observed using glasses with GFP filter FHS/EF-2G2 (460–495 nm) or RFP filter FHS/EF-4R2 (590-660 nm). 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 stereomicroscope (Leica M165FC, Germany) with color CCD camera (Leica DFC425C, Germany) or cold

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 | OD600 of mixture | GFP+ freq (%) | RFP+ freq (%) | GFP+RFP+ freq (%) |
|--------------------|-----------------|---------------|---------------|-------------------|
| pGFP-HPT/pRFP (1: 1 mix ratio) | 0.688 | 92.4 (12/2/32) | 78.0 (103/132) | 84.4 (103/122) |
|                   | 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 (1: 4 mix ratio) | 0.696 | 93.3 (27/299) | 78.9 (236/299) | 89.0 (234/263) |
|                   | 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 (1: 1 mix ratio) | 0.145 | 97.0 (325/335) | 95.2 (319/335) | 68.4 (137/201) |
|                   | 0.098 | 90.5 (199/220) | 92.3 (203/220) | 44.8 (82/183)    |
| Average            |       | 93.8            | 93.8          | 56.6              |

*aGFP+ (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/statspaired.html). The RFP+ freq was significantly lower than GFP+ freq (P < 0.05)

*bGFP+RFP+ freq, frequency of the infected-calli with GFP+ spots producing GFP+/RFP+ microcalli after subculture on fresh Hyg medium for another three weeks

*cCallus numbers were given in the brackets

*dFor 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)
light fluorescence CCD camera (HAMAMATSU, C4742-80-12AG, Japan).

**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'-CGACCACATGAGCAGCACGAC-3'), GFP-reverse (5'-TCCTCAGTGTGCGGCGATCT-3'), RFP-forward (5'-GGGCTTCAAGTTGGAGCCTGT-3'), and RFP-reverse (5'-CCGGCTTCCTGCTCAGGAGG-3'). Primers for amplification of the junctions between tandem-linked pGFP-HPT and pRFP T-DNAs in co-transformants were P1 (5'-ATCTGTCGATCGCAAGCTCGA-3'), P2 (5'-ACACGTGACTGGGAAAC-3'), P3 (5'-CGCCCAACCTGTTCTCGT-3'), and P4 (5'-TAAGAGGAGTCCACCAGTAG-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 transcription (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'-CGGCCATGCTTCTACAAAG-3') and HPT reverse (5'-ACACGTGAGCCTGCTATCAC-3'). Primers for amplification of rice actin cDNA were: Actin forward, 5'-GGAACCTGATGTTGCAAGGC-3'; Actin reverse, 5'-AGCTACGTAGACACCGACG-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 GFP+ frequencies (RFP+ freq), ranging from 70.9% to 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 (GFP+RFP+) co-transformants of the pGFP-HPT and pRFP vectors growing from the infected calli were too small to be distinguished from each other. The primary callus 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
### 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 T1 plants | Fluorescence patterns of T1 plants | Predicted GFP/RFP loci | Segregation patterna | P valueb |
|-----------------|-----------------|----------------------------------|------------------------|----------------------|----------|
|                 |                 | GFP+/RFP+ | GFP+/RFP− | GFP−/RFP+ | GFP−/RFP− | GFP+ | RFP | GFP+ + RFP |
| 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    |
| 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 T1 plants and difficulty to perform Chi-square test (indicated by – in the P value column)

*aSegregation 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.

bAll 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.
to several GFP<sup>+</sup>RFP<sup>+</sup> 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<sup>+</sup> freq, RFP<sup>+</sup> freq or GFP<sup>+</sup>RFP<sup>+</sup> 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<sup>+</sup>RFP<sup>+</sup> 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<sup>+</sup>RFP<sup>+</sup> cells and the single-vector transformed GFP<sup>+</sup>RFP<sup>−</sup> or GFP<sup>−</sup>RFP<sup>+</sup> 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<sup>+</sup> spots was 93.8% on average (Table 1). The RFP<sup>+</sup> frequencies were comparable to GFP<sup>+</sup> frequencies, which were different from the results with the pGFP-HPT/pRFP vector combination and revealed that the GFP<sup>+</sup> or RFP<sup>+</sup> cells derived from pGFP-HPT or pRFP-HPT transformation can grow independently in Hyg selection medium. The Agrobacterium-infected calli with GFP<sup>+</sup> or RFP<sup>+</sup> spots were subcultured. At the end of the second-round selection, the frequency of the primary calli with GFP<sup>+</sup> spots producing GFP<sup>+</sup>RFP<sup>+</sup> microcalli was 56.6% on average, which was significantly lower than the GFP<sup>+</sup>RFP<sup>+</sup> freq in pGFP-HPT/pRFP co-transformations (Table 1). The GFP<sup>+</sup>RFP<sup>−</sup>, GFP<sup>−</sup>RFP<sup>+</sup> and GFP<sup>+</sup>RFP<sup>+</sup> microcalli were independently distributed around the cultures derived from each primary callus (Fig. 1c, Fig. 2b–d). GFP<sup>+</sup>RFP<sup>+</sup> calli with large sizes were observed (Fig. 2b–d), but in most cases the GFP<sup>+</sup>RFP<sup>+</sup> 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.

![Fig. 2](image-url) Double-fluorescent Hyg<sup>+</sup> calli from the second- and third-round selection under stereo fluorescence microscope. a Double-fluorescent (GFP<sup>+</sup>RFP<sup>+</sup>) Hyg<sup>+</sup> calli derived from two rounds of selection of the pGFP-HPT/pRFP co-transformation (1:1 ratio). b-d Randomly distributed GFP<sup>+</sup>RFP<sup>−</sup>, GFP<sup>−</sup>RFP<sup>+</sup> and GFP<sup>+</sup>RFP<sup>+</sup> 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 = 2 mm. e GFP<sup>+</sup>RFP<sup>+</sup> calli derived from the third-round selection of pGFP-HPT/pRFP-HPT 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 = 1 mm.
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-transcription (RT)-PCR (Fig. S1a) and real-time RT-PCR (Fig. S1b). 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. S1). 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 S1, Fig. S2). 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 S1). 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. S2a-d). Deletion of RB/LB borders and inward T-DNA sequences was also detected in the T-DNA junctions.

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 S1, Fig. S2e 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% to 81.2%, as revealed by the percentages of Agrobacterium-infected calli growing Hyg+RFP+ cells. Komari et al. [5] 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. [5], 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 the authors 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. [5] 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 [5, 8]. 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. [5] 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 [5].
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 to 50%) were obtained when tobacco was transformed via mixed strains [5, 8]. 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. [5] 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. [6] 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 [7, 10] and the T-DNAs in inverted repeats may result in convergent transcription and trigger transgene silencing [13]. 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.

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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-022-07864-6.

Acknowledgements The authors are grateful to Xiaowei Zou, Xiaqing Gao, Haihe Yang, Haiyan Qu, and Suqin Zheng for their experimental assistance.

Author contributions LL, XT, LW, JZ, JZ and HH conducted the experiments, collected and analyzed the data. LL and SQ wrote the manuscript. SQ and DL designed the research schemes and supervised the whole process. All authors read and approved the final manuscript.

Funding This work was financially supported by the State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-products in Zhejiang Academy of Agricultural Sciences, and by the National Natural Science Foundation of China (Grant No. 31672016).

Declarations

Competing interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to publication The authors consent to publish all the data in this study.

References

1. Liu F, Wang P, Xiong X, Fu P, Gao H, Ding X, Wu G (2020) Comparison of three Agrobacterium-mediated co-transformation methods for generating marker-free transgenic Brassica napus plants. Plant Methods 16:81. https://doi.org/10.1186/s13007-020-00628-y
2. Kapusi E, Hensel G, Coronado MJ, Broeders S, Marthe C, Otto I, Kumlehn J (2013) The elimination of a selectable marker gene in the doubled haploid progeny of co-transformed barley plants. Plant Mol Biol 81:149–160
3. Chen L, Marmey P, Taylor NJ, Brizard JP, Espinoza C, D’curz P, Huet H, Zhang S, De Kochko A, Beachy RN, Fauquet CM (1998) Expression and inheritance of multiple transgenes in rice plants. Nat Biotechnol 16:1060–1064
4. Sripriya R, Raghupathy V, Veluthambi K (2008) Generation of selectable marker-free sheath blight resistant transgenic rice plants
by efficient co-transformation of a cointegrate vector T-DNA and a binary vector T-DNA in one *Agrobacterium tumefaciens* strain. Plant Cell Rep 27:1635–1644
5. Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J 10:165–174
6. Breitler JC, Meynard D, Van Boxtel J, Royer M, Bonnot F, Cambillau L, Guideroni E (2004) A novel two T-DNA binary vector allows efficient generation of marker-free transgenic plants in three elite cultivars of rice (*Oryza sativa* L.). Transgenic Res 13:271–287
7. De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. Mol Plant Microbe Interact 11:449–457
8. Park J, Lee YK, Kang BK, Chung WI (2004) Co-transformation using a negative selectable marker gene for the production of selectable marker gene-free transgenic plants. Theor Appl Genet 109:1562–1567
9. De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in Arabidopsis transformants is highly determined by the transformed target cell. Plant J 60:134–145
10. De Neve M, De Buck S, Jacobs A, Van Montagu M, Depicker A (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J 11:15–29
11. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
12. Toki S, Hara N, Ono K, Onodera H, Tagiri A, Oka S, Tanaka H (2006) Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. Plant J 47:969–976
13. De Buck S, Van Montagu M, Depicker A (2001) Transgene silencing of inversely repeated transgenes is released upon deletion of one of the transgenes involved. Plant Mol Biol 46:433–445

**Publisher’s Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.