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SHORT COMMUNICATION

Strong activity of FLPe recombinase in rice plants does not correlate with the transmission of the recombined locus to the progeny

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Abstract Efficient methods for DNA excision are needed for removing selectable marker genes from transgenic plants. The present work evaluated the enhanced FLP recombinase, FLPe, for excising FLP recombination target (*FRT*)-flanked marker genes, and generating marker-free rice lines. Previously, the transient FLPe activity was found to be at least threefold higher on the transgene locus compared to that of FLPwt, the wild-type FLP recombinase. In this study, transgenic plants expressing *FLPe* were cross-pollinated with the plants harboring *FRT* site to analyze marker excision in F1 plants, and the transmission of marker-free locus to F2 progeny. The FLPe activity, expressed by the strong promoter (maize ubiquitin-1 gene), efficiently excised *FRT*-flanked marker gene in rice plants. However, marker excision in F2 progeny was tightly linked with the presence of *FLPe* gene, suggesting insufficient recombination in the gametophyte. The maize ubiquitin-1 promoter is reportedly active in gametophytic tissue and effective in meiotic transmission of the marker-free locus generated by Cre–*lox* recombination. Therefore, the observed lack of meiotic transmission in this study is possibly due to the limited efficiency of FLPe recombinase. While the reason for the FLPe inefficiency in the gametophyte is not clear, this work highlights the constraints of FLPe recombinase in generating stable marker-free plant lines through cross-pollination or gene induction methods.

Keywords FLPe · FLPo · FLP–*FRT* · Marker-gene removal · Marker-free plants · Site-specific recombination

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Introduction

The presence of selection marker genes (SMG), especially antibiotic-resistant genes, in transgenic crops has raised biosafety concerns (Lemaux 2008); therefore, SMG removal from transgenic lines is highly desirable (Darbani et al. 2007; Srivastava et al. 2011; Wang et al. 2011; Yau and Stewart 2013). A number of methods have been described for removing SMG, including the use of site-specific recombination systems that carry out precise recombination on specific DNA sequences leading to the excision of the selected DNA fragment (Gilbertson 2003; Ow 2002). SMG removal by Cre–*lox*, a prominent site-specific recombination system, is highly successful in the generation of 'stable' marker-free lines that faithfully transmit the marker-free locus to the progeny and no longer need Cre activity to maintain it (Gidoni et al. 2008; Srivastava et al. 2011). Several site-specific recombination systems have been identified, one of which is FLP–*FRT* that is functional in many plant species (Lloyd and Davis 1994; Lyznik et al. 1993; Sonti et al. 1995). Although the effective use of FLP–*FRT* in marker excision in rice and maize has been reported (Hu et al. 2008; Li et al. 2010), several studies found FLP–*FRT* to be somewhat inefficient

in plant cells (Bar et al. 1996; Davies et al. 1999; Gidoni et al. 2001; Kerbach et al. 2005; Kilby et al. 1995). Since then, an improved FLP recombinase, FLPe, has been developed that shows higher activity in plant and human cells (Akbudak and Srivastava 2011; Takata et al. 2011). However, its efficacy in marker removal and generation of stable marker-free plant lines has not been studied.

This study analyzed the utility of FLPe, a thermostable FLP recombinase (Buchholz et al. 1998), in generating marker-free transgenic rice lines. A number of studies have shown the development of marker-free transgenic rice lines by Cre–*lox* system using cross-pollination method (Hoa et al. 2002; Moore and Srivastava 2006; Sengupta et al. 2010). The marker-excision process is initiated in the resulting F1 hybrids, and stable marker-free lines are identified among F2 progeny that segregate the marker-free locus from the *cre* gene. Other approaches, involving inducible *cre* gene or tissue-specific *cre* expression, have also been successful in isolating 'stable' marker-free plant lines (Chong-Pérez et al. 2013; Khattri et al. 2011; Sreekala et al. 2005). FLP–*FRT* is analogous to Cre–*lox* consisting of FLP recombinase and a 34-bp FLP-recombination target (*FRT*). High efficiency and efficacy of the Cre–*lox* system in marker excision is well established; however, only fragmented information is available for FLP–*FRT*, and it is still not clear whether FLP–*FRT* is a reliable tool for marker-gene excision from transgenic plants.

Most of the studies have utilized a modified version of the native yeast *FLP* gene that carries silent mutations to remove three canonical polyadenylation AATAAA signals and cryptic splice acceptor sites (O'Gorman et al. 1991). This FLP, referred to as FLPwt in the present study, is functional in plant cells; however, FLPwt-mediated excisions were mostly observed in clonal sectors giving rise to chimeric plants that failed to transmit the marker-free locus to the progeny (Bar et al. 1996; Kilby et al. 1995). Development of FLPe and its codon-optimized derivative, FLPo (Raymond and Soriano 2007), renewed the potential of FLP–*FRT* in plant biotechnology. In previous studies, we showed that FLPe and FLPo show much higher recombination efficiency, and are efficient in driving site-specific gene integration in the rice genome (Akbudak and Srivastava 2011; Nandy and Srivastava 2011, 2012).

In this study, the efficiency of FLPe for marker-excision application was tested in rice by crossing FLPe-expressing lines with the line harboring the *FRT* target site. Consistent with the previous study, FLPe was highly efficient in marker excision in F1 and F2 plants as indicated by the activation of the *GUS* reporter gene, a product of marker excision. However, marker excision in the F2 population was tightly linked with the presence of the *FLPe* gene, and, as a result, only ~55 % of F2 progeny showed GUS activity. These observations indicate high FLPe activity in somatic tissues of rice, and raise the question of its activity in the gametophytic tissue. For the isolation of stable marker-free lines, it is critical for marker excision to occur in the germline or gametophyte. Therefore, FLPe appears to be unsuitable for marker excision from transgenic plants, and may require strong gametophytic promoters to optimize marker excision in the germline.

Materials and methods

Plasmid constructs and plant lines

The FLP constructs have been described earlier (Akbudak and Srivastava 2011), consisting of FLPwt, FLPe or FLPo coding sequence transcriptionally fused with maize ubiquitin-1 promoter (*ZmUBI1*). The transcription termination sequence of the nopaline synthase gene (*nos 3′*) is present in each construct. The nucleotide sequences of *FLPe* and *FLPo* are available on http://www.addgene.org, and that of *FLPwt* at NCBI (accession no. I59684). The *FRT* target line, 17D, was developed by Khattri (2006), it contains a single copy of the pRP9 construct (Radhakrishnan and Srivastava 2005) consisting of the *FRT*-flanked neomycin phosphotransferase II gene (*NPT*) between the *ZmUBI1* promoter and the β-glucuronidase (*GUS*) gene (Fig. 1a). FLP lines were generated by the co-bombardment of FLP plasmids with p35S:HPT, which contains the hygromycin phosphotransferase (*HPT*) gene controlled by the 35S promoter and the *nos 3′* terminator. All rice lines are in Nipponbare background, and developed by particle bombardment (PDS-1000/He; Bio-Rad, Hercules, CA, USA). FLP plants were crossed with 17D plants to collect F1 hybrids, and the F1 plants were self-fertilized to generate F2 seeds. GUS staining was done according to the protocol described by Jefferson (1987).

Molecular analysis

Polymerase chain reaction (PCR) on genomic DNA was done using *Taq* Polymerase (Promega, Madison, WI, USA) following the manufacturer's recommendations. All PCR reactions consisted of 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 56 °C, and 1 min extension at 72 °C, followed by a final elongation step at 72 °C for 15 min. Primers '*a*' (5′-TCTACTTCTGTTCATGTTTGTG-3′) and '*b*' (5′-AATTACGAATATCTCGATCGG-3′) were used to detect *17D* non-recombined locus or the recombined locus (recombination footprint). FLPe-F (5′-CGCGCCACCATGAGCCAATTT-3′) and FLPe-R (5′-ATGCGGGGTATCGTATGCTTCC-3′) detected the *FLPe* gene in genomic DNA. *FLP* gene expression analysis was done using SuperScript III Platinum SYBR Green One-

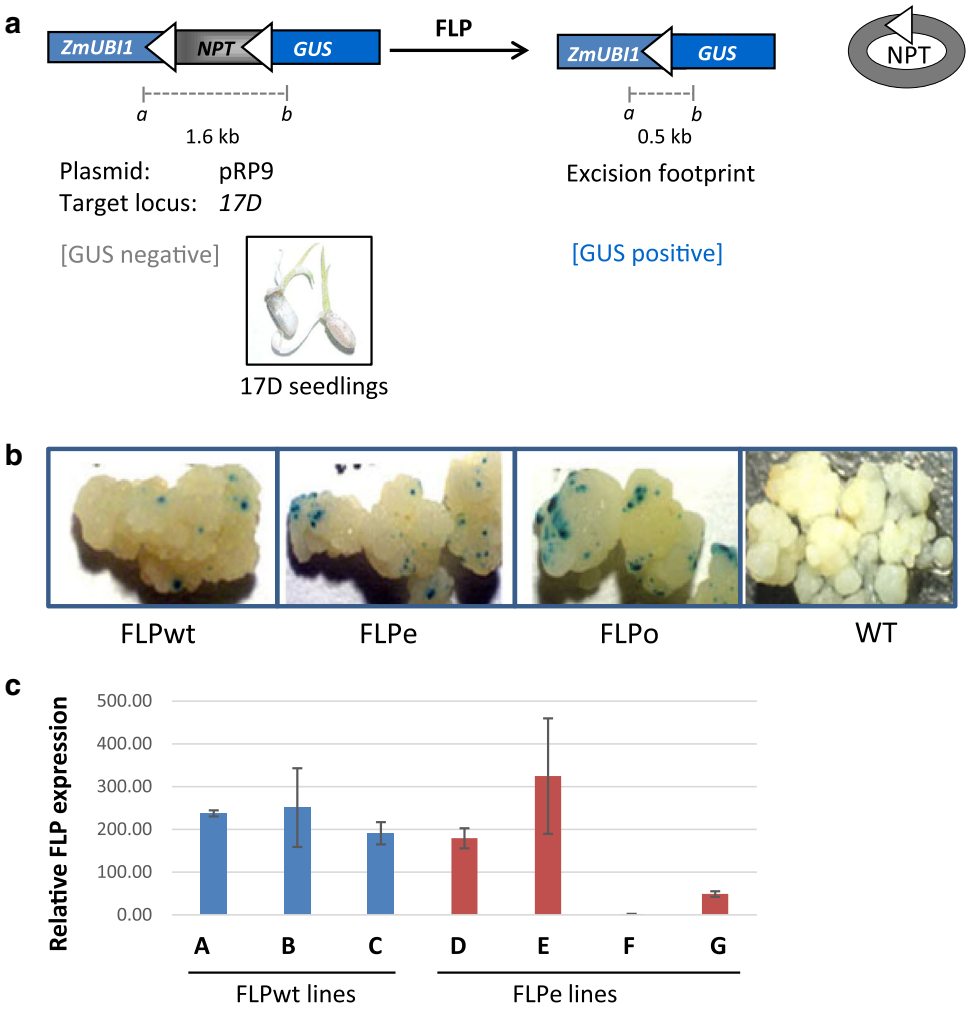

Fig. 1 Analysis of FLP-expressing rice lines. **a** FLP recombination assay utilizes plasmid pRP9 or the transgene locus *17D* that contains a single-copy of neomycin phosphotransferase II (*NPT*) gene flanked by *FRT* sites (*white triangles*) and expressed by maize ubiquitin-1 promoter (*ZmUBI1*). Downstream is a promoter-less β-glucuronidase (*GUS*) gene that is silent; however, upon excision of *NPT* fragment by FLP-mediated *FRT* × *FRT* recombination, the GUS gene would gain a promoter and become active. Priming sites of primers *a* and *b* used for analyzing the resulting recombination footprint are shown *below the map*. Also shown are 17D seedlings exposed to GUS stain for 3 days. Note the lack of any staining in the seedlings. **b** Relative activity of FLPwt, FLPe and FLPo in the respective transgenic callus lines determined by particle bombardment of pRP9 on callus followed by overnight GUS staining. Representative samples are shown. Bombardment of pRP9 on wild-type (*WT*) Nipponbare callus serves as the negative control. **c** Quantitative reverse-transcriptase PCR analysis to determine *FLP* gene expression in the primary transgenic plants of FLPwt and FLPe lines. *Error bars* SD

Step quantitative reverse transcriptase-PCR (qRT-PCR) kit on Bio-Rad CFX96 Touch™ real-time detection system. Total RNA was isolated from young leaves of 1-month-old plants using the RNeasy kit (Qiagen), and treated with DNase I to be used as a template in the qRT-PCR reaction using FLPwt primers (5′-GCATCTGGGAGATCACTGAG-3′ and 5′-CTGTCACTAAACACTGGATTA-3′) or FLPe primers (5′-CCGGCAATTCTTCAAGCAAC-3′ and 5′-CAACTCCGTTAGGCCCTTCA-3′), and phytoene desaturase (*PDS*) primers (5′-GCAGAGGAATGGGTTGGAC-3′ and 5′-AGAGGTCGGCAAGGTTCAC-3′). FLP expression was determined against *PDS* as a reference gene using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Results

Experimental design

The efficiency of the FLP–*FRT* recombination was tested on plasmid pRP9, and additionally on the genomic target locus, *17D*, containing a single-copy insertion of pRP9 (Fig. 1a). pRP9 contains a *FRT*-flanked selectable marker gene (SMG), *NPT*, between the *ZmUBI1* promoter and the *GUS* reporter gene. The *17D* locus expresses *NPT* but not the *GUS* gene (see 17D seedlings in Fig. 1a); however, the excision of the *NPT* gene via *FRT* × *FRT* recombination activates the *GUS* gene by fusing it with the *ZmUBI1* promoter (Fig. 1a). To initiate FLP–*FRT* recombination,

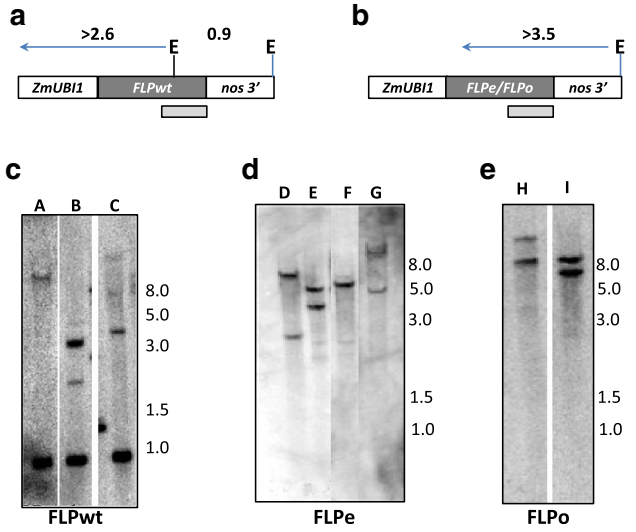

Fig. 2 Southern analysis of FLP-expressing rice lines. **a**, **b** Constructs of *FLPwt, FLPe* and *FLPo* genes used for rice transformations. *Eco*RI (*E*) site, the expected fragment sizes, and the position of probe DNA (*gray bar*) are indicated. *ZmUBI1*, maize ubiquitin-1 promoter, *nos 3′*, nopaline synthase gene transcription terminator. **c**–**e** Southern hybridization of *Eco*RI-digested genomic DNA of FLPwt lines (*A*–*C*), FLPe lines (*D*–*G*), and FLPo lines (*H*, *I*) with the respective probes. The presence of the internal 0.9-kb band is observed in each FLPwt line, with an additional band that indicates the gene copy number. The number of hybridizing bands in FLPe and FLPo lines represent gene copy numbers, as only one E site is present in the respective constructs

17D plants were crossed with FLP-expressing plants, and the resulting F1 and F2 progeny were analyzed by GUS staining and PCR to determine marker excision and meiotic transmission of the marker-free locus.

Development of FLP lines

Through co-transformation with pUbiFLP (containing either FLPwt, FLPe or FLPo genes; Fig. 2a, b) and p35S:HPT, three FLPwt (A–C), four FLPe (D–G), and two FLPo (H, I) lines were developed (Table 1). Each of these lines was verified by Southern analysis, and found to contain 1–3 copies of the respective *FLP* gene (Fig. 2c–e; Table 1). A transient expression assay, based on the bombardment of pRP9 was used to carry out a preliminary screening of FLP activity in these lines. Callus of each line was bombarded with the reporter construct, pRP9, and stained for GUS activity, the direct readout of FLP–*FRT* recombination. A much greater GUS activity was observed on the FLPe and FLPo lines (>200 dots per plate) than on the FLPwt lines (≤10 blue dots per plate) (Fig. 1b; Table 1). Only one FLPe line (F), which was later found to be transcriptionally inactive, showed no GUS activity. While callus could consist of a mixture of transformed and untransformed cell lines, the consistency of data between

Table 1 Analysis of rice FLP lines

Line	FLP variant	Copy no.	FLP activity[a]	Regenerated plants[b]
A	FLPwt	1	9 ± 5	√
B	FLPwt	2	10.3 ± 5	√
C	FLPwt	3	2.3 ± 2.5	√
D	FLPe	2	326 ± 54	√
E	FLPe	2	396 ± 60	√
F	FLPe	1	0	√
G	FLPe	2	228 ± 82	√
H	FLPo	2	340 ± 57	X
I	FLPo	2	280 ± 64	X
Nipponbare	–	–	0	–

[a] Based on average number of GUS dots on callus lines ($n = 3$)

[b] Regenerated plants fertile (√) or not fertile or not available (*X*)

replicates and between the lines suggests that a lower GUS activity observed in the FLPwt lines is not due to their chimeric nature. Therefore, the recombination efficiency of FLPe and FLPo is much higher than that of FLPwt. This observation is consistent with the previous study (Akbudak and Srivastava 2011), in which transient expression of *FLPe* and *FLPo* genes was found to induce a much higher rate of recombination on the *17D* locus.

The regenerated plants were obtained from FLPwt and FLPe lines; however, the two FLPo lines failed to regenerate (Table 1). These plant lines were grown in the greenhouse, and subjected to transgene expression analysis by qRT-PCR, which showed that the A–C lines abundantly expressed the *FLPwt* gene, and the D–G lines showed variable expression of the *FLPe* gene (Fig. 1c). Line F did not express *FLPe*, and line G expressed *FLPe* at a much lower level than lines D and E, although only a minor difference in FLPe recombinase activity was detected in this line upon bombardment of pRP9 (Table 1).

FLPe generates efficient excisions in the somatic tissues

To study FLP-mediated marker excision from the transgene locus, four FLP lines, two each of FLPwt (A and B) and FLPe (D and E), were selected (Tables 1, 2). These lines were crossed with the FLP-target line, 17D, which contains a single copy of the pRP9 construct. 17D plants did not show GUS activity as indicated by the lack of GUS staining in the whole seedling as well as in different tissues (leaves, endosperm, and roots) of the mature plant (Figs. 1a, 3a). A number of F1 hybrids were obtained by the reciprocal crosses of FLP plants with 17D plants. Since T_0 (hemizygous) FLP plants were used, PCR was done to isolate F1 plants positive for the FLP gene and *17D* locus. Through PCR screening, 2–4 double-positive F1 plants representing each FLP line were identified (Table 2).

Table 2 GUS activity in F1 plants

FLP line	FLP gene	F1 plants	Crosses	GUS activity[a]
A	FLPwt	A1	17D × A	N
		A2	A × 17D	N
		A3	A × 17D	N
		A4	A × 17D	N
B	FLPwt	B1	B × 17D	N
		B2	17D × B	N
D	FLPe	D1	D × 17D	Y
		D2	D × 17D	Y
		D3	D × 17D	Y
		D4	17D × D	Y
E	FLPe	E1	17D × E	Y
		E2	E × 17D	Y

N Not detected, *Y* abundantly detected

[a] GUS staining in leaf cuttings recorded after overnight staining

To detect FLP–*FRT* recombination in F1 plants, several leaf cuttings from each F1 plant were stained for GUS activity. All samples derived from D and E lines showed strong GUS activity after overnight staining, while those of A and B were negative (Fig. 3a; Table 2). However, weak GUS staining in two F1 seedlings, A1 and A2, was visible after about a week of staining at room temperature (Fig. 3a). The presence or absence of strong GUS activity (overnight staining) in F1 plants tightly correlated with the presence or absence of the characteristic 0.5-kb excision 'footprint' (Zm*UBI1:FRT:GUS*) in the PCR analysis (Fig. 3b). The 0.5-kb PCR fragment obtained from D4 and E1 plants was sequenced and found to contain the expected 34-bp *FRT* sequence between the *ZmUBI1* promoter and the *GUS* gene (data not shown). Since A and B lines abundantly express the *FLPwt* gene, the lack of detectable GUS activity or weak staining in F1 plants expressing the *FLPwt* gene was surprising; however, this observation indicates poor recombination efficiency of FLPwt on the transgene locus. Both FLPe lines, on the other hand, efficiently excised the *NPT* gene from the *17D* locus as indicated by the uniform staining of the leaf cuttings, and the amplification of the excision 'footprint' (Fig. 3a, b; Table 2). The weak GUS activity observed in A1 samples after 1 week of staining could be due to fungal or bacterial contaminations on plants growing in the greenhouse, as the characteristic 0.5-kb recombination footprint was not amplified from these samples. Such intrinsic GUS activity has been reported in many eukaryotes, and is particularly abundant in bacteria and fungi (Eudes et al. 2008).

Next, F2 progeny derived from self-fertilized F1 plants was analyzed by GUS staining to determine the segregation ratio of the marker-free *17D* locus. F2 seedlings of the A1, A2, B1 and B2 lines (FLPwt lines) did not show any detectable GUS activity after 2 days of staining, whereas strong staining was observed in the F2 seeds and seedlings derived from D1 to D4, E1, and E2 plants (FLPe lines) (Fig. 3c; Table 3). Only a small number of F2 progeny of D1–D3 plants were tested, while a much larger number of D4, E1, and E2 progeny were analyzed for determining segregation ratio of GUS-positive *17D* locus (excision locus). The percent of GUS-positive seedlings in each F2 family ranged from 55 to 57 %, suggesting that GUS staining was associated with the inheritance of *FLPe* gene.

Undetectable germinal transmission

The development of 'stable' marker-free plants depends on meiotic transmission of the marker-free locus, which in turn depends on efficient recombination in the germline. Since ~56 % of F2 plants are expected to contain both the *17D* locus and the *FLPe* gene, and only 55–57 % F2 plants in this study showed GUS activity, a simple explanation is that de novo excisions in F2 seedlings contributed to the observed GUS activity. To determine if indeed all GUS-positive seedlings contain the *FLPe* gene, PCR was done on the genomic DNA isolated from 110 and 129 F2 seedlings of D4 and E1, respectively. All GUS-positive F2 plants of these two families amplified the characteristic 0.5-kb fragment indicating the presence of the excision 'footprint', and also amplified the *FLPe* gene fragment (Fig. 3d). Therefore, the presence of GUS activity in F2 seedlings tightly correlated with the presence of the *FLPe* gene, and the meiotic transmission of the marker-free locus remained undetectable in these populations (Table 3). While the possibility of finding a 'stable' marker-free F2 line in a larger population cannot be ruled out, these data indicate that the efficiency of the process is likely to be low and unpredictable. The segregation of *17D* and *FLPe* loci was observed in GUS-negative F2 plants, none of which showed the presence of the excision 'footprint' (Fig. 3d). If FLPe-mediated recombination had occurred in germ cells (pollen mother cells or egg cells) of the F1 plants, transmission of the marker-free excision footprint would have been independent of FLPe activity. This analysis provided strong evidence for insufficient FLPe recombinase activity in the germline.

Discussion

DNA recombination-based technologies including site-specific recombinases, rare-cutting nucleases, and transposases have been proposed for excising SMG from transgenic plants (Liu et al. 2013). Each of these technologies involve flanking SMG with specific DNA sequences, and introducing the corresponding enzyme activity

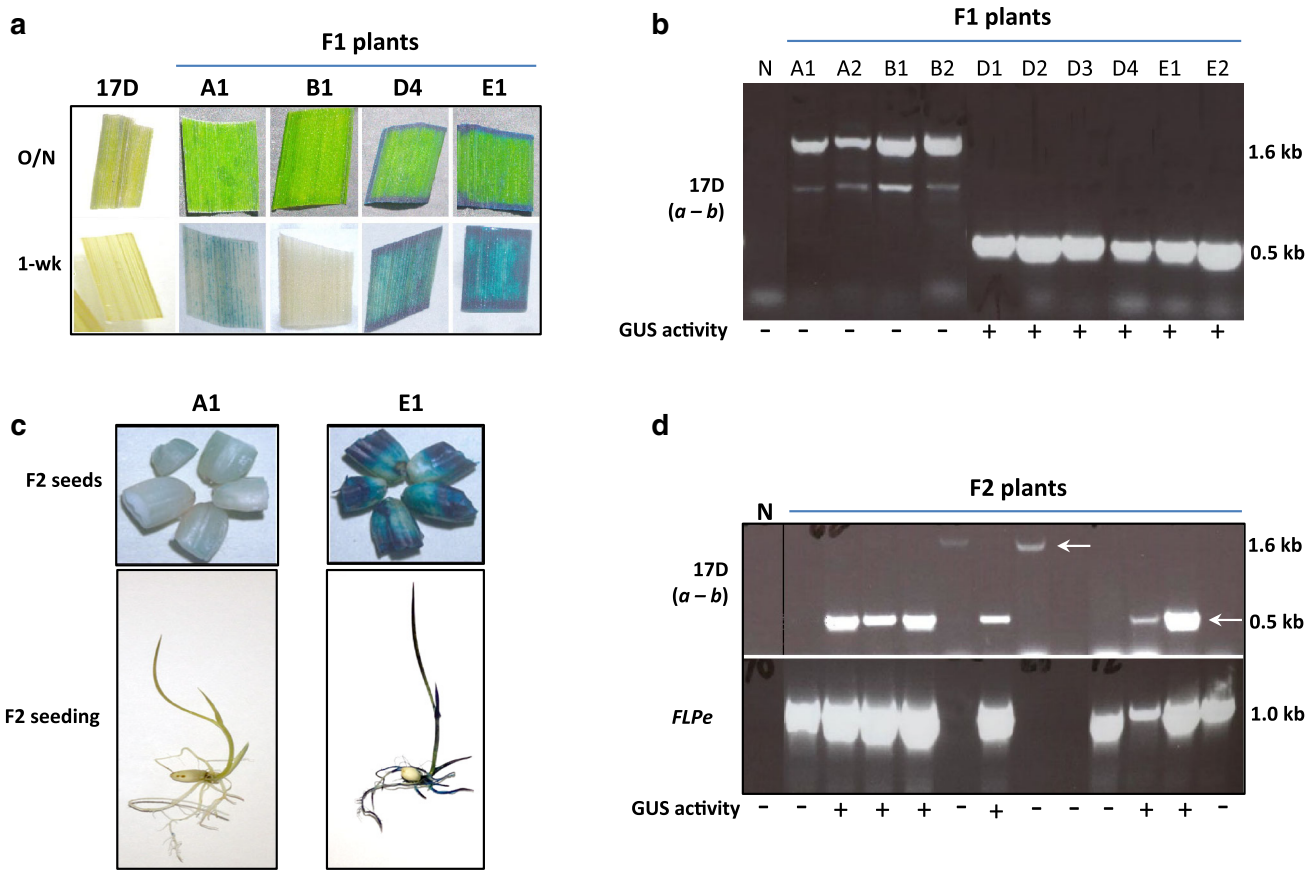

Fig. 3 FLP–*FRT* recombination efficiency in rice plants. **a** Detection of FLP–*FRT* recombination by GUS staining of the leaves of F1 plants derived from cross-pollination of FLP plants with the 17D plants (see Fig. 1a). Representative F1 samples derived from crosses with FLPwt (*A1*, *B1*) and FLPe (*D4*, *E1*) are shown. Note the lack of GUS staining in A1 and B1 leaves, and staining on the edges of D4 and E1 leaves after overnight (*O/N*) staining. After 1 week, D4 and E1 samples are strongly stained while only weak staining is visible in A1 line. B1 failed to show any staining. 17D leaves that lack GUS activity are shown as the negative control. **b** PCR analysis of F1 plants to detect the recombination footprint. Primers *a* and *b* (shown in Fig. 1a) that amplify the 1.6-kb fragment from the non-recombined 17D locus and 0.5 kb from the recombination footprint. The presence or absence of GUS activity in the F1 plants is indicated as −/+ below each lane. **c** Detection of FLP–*FRT* recombination in F2 progeny (derived from selfed A1and E1 plants) by GUS staining of seeds and seedlings. **d** A representative PCR analysis on F2 seedlings derived from selfed C or D F1 plants with primers for recombination footprint (0.5 kb, *upper panel*) or *FLPe* gene (*lower panel*). The *arrow* points at the 1.6-kb fragment that is expected from the non-recombined 17D locus. *N* Non-transgenic Nipponbare rice DNA

Table 3 Determination of germinal transfer of the excision locus to F2 progeny

Line	FLP	F1 plant	No. of F2 seedlings analyzed	No. of GUS+ F2 seedlings (%)	Excision efficiency[a]	Transmission efficiency[b]
A	FLPwt	A1	40	0	–	–
		A2	20	0	–	–
B	FLPwt	B1	40	0	–	–
		B2	20	0	–	–
D	FLPe	D1–D3	5–10	3–6	ND	–
		D4	110	60 (54.5)	72.6	0
E	FLPe	E1	129	72 (55.8)	74.4	0
		E2	35	20 (57.1)	76	ND

ND Not determined

[a] Percent GUS-positive F2 plants/75 (since only 75 % of F2 progeny will inherit 17D locus)

[b] Meiotic transmission as determined by the number of GUS-positive, FLPe-negative F2 plants

(recombinase/nuclease/transposase) to initiate the excision process. The enzyme activity can be introduced by cross-pollination, tissue-specific expression, or gene induction by external stimuli. Regardless of which method is used, it is imperative for marker excision to occur in the germline to transmit the marker-free locus to the progeny. The progeny

plants that inherit the marker-free locus serve as the stable marker-free lines, as they faithfully transmit the (marker-free) locus through generations. With the exception of one report (Hu et al. 2008), the site-specific recombination system, FLP–*FRT*, has often been reported to be inefficient in transmitting the marker-free locus to the next generation (Bar et al. 1996; Davies et al.1999; Gidoni et al. 2001; Kerbach et al. 2005; Kilby et al. 1995). The basis of its poor efficiency could be attributed to the instability of the FLP protein at 37 °C (Buchholz et al. 1996). Subsequently, thermo-stable FLPe recombinase was developed, which showed a much higher activity in plant and animal cells (Akbudak and Srivastava 2011; Takata et al. 2011).

This study assessed the utility of FLPe recombinase in excising marker genes from rice plants, and generating stable marker-free lines. A standard assay based on the activation of the *GUS* gene upon marker excision was used to monitor the FLPe activity on the transgene locus. As expected, FLPe was found to be highly efficient in excising SMG from the rice genome. However, surprisingly, the marker-free locus was not found to segregate from *FLPe* in the progeny, suggesting insufficient FLPe recombinase activity in the germline. Tissue-specific gene expression is determined by the promoter elements. The maize ubiquitin-1 promoter (*ZmUBI1*), used for driving *FLPe* expression in this study, is reportedly active in male and female gametophyted (Krohn et al. 2012; Schreiber and Dresselhaus 2003; Srilunchang et al. 2010; Xu et al. 2002). Consistent with its activity in the germline, the *ZmUBI1:Cre* construct successfully generated stable marker-free lines of rice and wheat (Moore and Srivastava 2006; Srivastava et al. 1999). Similarly, other promoters, including the 35S promoter, heat-inducible, and chemical-inducible promoters, have also been successful in generating stable marker-free lines when the Cre–*lox* system was used for marker excision (Bala et al. 2013; Dale and Ow 1991; Nandy and Srivastava 2012; Russell et al. 1992; Sreekala et al. 2005; Zhang et al. 2003). Thus, gametophytic promoters have mostly been used for pollen-specific excision of the transgene for bio-containment applications (Luo et al. 2007; Mlynárová et al. 2006). Further, since strong gametophytic expression of the Cre recombinase (using the AtDMC1 promoter) has been found to induce male sterility in *Arabidopsis* (unpublished data), marker excision by constitutive Cre expression is easier to practice.

The FLPe-expressing rice plants appear normal, and no sterility was detected. Fertility of the FLPe plants was also evident from the successful reciprocal crosses obtained with the two FLPe lines. Hence, no obvious explanation for the possible exclusion of FLPe activity in the gametophyte has been found in the present work. Nevertheless, *ZmUBI1:FLPe* expression in the gametophyte is clearly not sufficient for marker excision, and the successful use of FLPe would require improving its efficiency or using proper gametophytic promoters. A quantitative comparison of FLPwt and FLPe activities in mammalian cells found that the recombination efficiency of FLPe on a molar basis was not higher than that of FLPwt, even at 37 °C (Kondo et al. 2009). Therefore, higher efficiency of FLPe in plant or animal cells is due not to higher enzyme activity but to the higher steady-state level of the FLPe protein, owing to its thermo-stability. The rapidly dividing germline possibly requires higher recombinase efficiency to excise transgene fragments, in addition to stabilizing the steady-state level of the FLPe protein. More research is needed to understand the inefficiency of FLPe in the germline; however, this study has unveiled the limitation of FLPe in marker-excision applications, and suggests retransformation as an alternative approach for introducing FLPe activity to obtain marker-free plants through regeneration of marker-free tissue cultures.

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