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Note

Rapid evaluation of the frequency of gene targeting in rice via a convenient positive-negative selection method

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Abstract Although gene targeting (GT) is a useful technology for precise mutagenesis of target sequences, its frequency is quite low. Establishing experimental procedures using a model system will enable us to improve this frequency and apply to GT as a universal system. Here, we propose a convenient system with which to evaluate the frequency of site-directed mutagenesis via GT using a positive-negative selection method. We constructed a GT vector harboring a partial rice *acetolactate synthase* gene with mutations conferring bispyribac sodium (BS) tolerance and a gene conferring blasticidin-S tolerance as a positive selection marker. In addition, *diphtheria toxin A subunit* gene was used as a negative selection marker to enrich GT cells. We regenerated GT candidate plants successfully at a frequency of 2.1 putative GT events/gram *Agrobacterium*-infected callus following dual selection on BS and blasticidin-S. Moreover, molecular analyses confirmed that GT events occurred in >80% of regenerated plants. Existing GT methods using positive-negative selection require that true putative GT events be verified by molecular analysis because of the growth of large numbers of cells in which partial GT vectors containing positive selection marker cassettes, but lacking the negative selection marker, have inserted at random loci. In contrast, the present method with dual selection on both BS and blasticidin-S allows direct enrichment of GT cells at high frequency without the need for further extensive molecular screening.

Key words: Acetolactate synthase, gene targeting, positive-negative selection, rice, site-directed mutagenesis.

A number of studies using forward genetics approaches and protein engineering have revealed that small mutations such as single or several point mutations can dramatically alter plant phenotypes, and various means of introducing such mutations into target plants have been developed. Gene targeting (GT) is a precise mutagenesis technology enabling the introduction of desired mutations into endogenous target sequences via homologous recombination (HR). GT can be used not only for basic research but also for practical applications such as the molecular breeding of crops. In rice, beginning with a study in 2002, successful GT of many genes using positive-negative selection methods has been reported (Shimatani et al. 2015). In such systems, positive and negative selection markers are used to select cells in which stable transformation has occurred successfully and to kill cells in which a GT vector has integrated into a random locus, respectively. Small mutations can be introduced precisely into the target sequence of interest accompanied by integration of the positive selection marker. Just recently, using the insect-derived transposon *piggyBac*, we succeeded in the complete elimination of the positive selection marker from a targeted locus without leaving a footprint (Nishizawa-Yokoi et al. 2015). Currently, site-directed mutagenesis via GT combined with subsequent complete marker elimination enables the production of desired rice mutants that are exactly equivalent at the sequence level to those produced by conventional mutagenesis approaches (Endo et al. 2015; Shimatani et al. 2015).

However, the frequency of GT in flowering plants, including rice, is 10^{-3} to 10^{-6} compared to the random integration of donor DNAs, due primarily to the very low frequency of HR (Paszkowski et al. 1988). Several approaches aimed at addressing this serious limitation have been taken, e.g., enhancement of HR activity using site-directed nucleases and/or the modification of DNA repair pathways (D'Halluin et al. 2013; Qi et al. 2013; Shaked et al. 2005; Shukla et al. 2009; Townsend et al. 2009; Zhang et al. 2013). However, it is difficult to make any direct comparison of the effects on GT frequency of those factors because different experimental conditions

Abbreviations: ALS, acetolactate synthase; BS, bispyribac sodium; Bla-S, blasticidin-S; CAPS, cleaved amplified polymorphic sequences; DT-A, diphtheria toxin A subunit; gfbsd2, green fluorescent protein fused to blasticidin-S deaminase; GT, gene targeting; HR, homologous recombination; T-DNA, transferred DNA.

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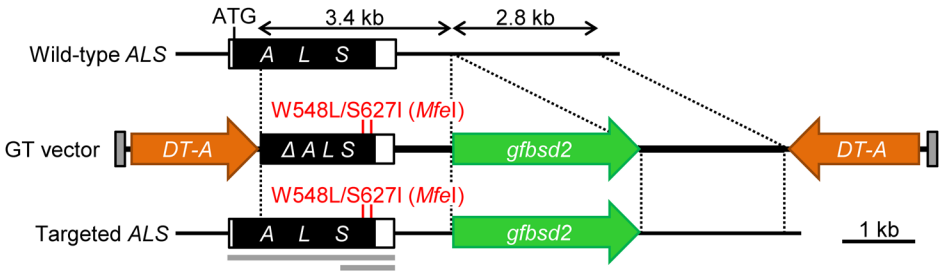

Figure 1. Schematic representation of GT vector for the rice *ALS* gene. A 6.2-kb fragment containing a partial *ALS* gene with W548L/S627I mutations was used to construct the GT vector. The W548L and S627I mutations (W548L; W [TGG] to L [TTG] at amino acid 548 and S627I; S [AGT] to I [ATT] at amino acid 627) create novel *Mfe*I sites (CAATGG to CAATTG, and CAAGTG to CAATTG, respectively). The white and black boxes show the untranslated and coding regions of ALS, respectively. The *gfbsd2* and *DT-A* expression cassettes are as described in previous reports (Saika et al. 2012; Nishizawa-Yokoi et al. 2015). Grey horizontal bars show PCR products for CAPS analysis. A schematic representation of vector construction is shown in Supplemental Figure S2.

and materials were used in all these various studies. Thus, a convenient model GT system is needed to allow evaluation and screening of previously reported and novel factors to enhance GT frequency under standardized conditions.

As shown in Supplemental Figure S1, in rice, secondary calli derived from mature seeds are infected with *Agrobacterium* harboring the GT vector and are selected with appropriate antibiotics. The ratio of the number of cells in which GT has occurred successfully to those that survive under antibiotic pressure has been estimated at ca. 1 : 100 in many organisms, including rice (Nishizawa-Yokoi et al. 2015; Shimatani et al. 2015). The growth of false positive cells is thought to be due to the introduction into random loci of transferred DNA (T-DNA) in which negative selection marker cassettes are partially or entirely lacking (Terada et al. 2007). Thus, selection of the 1% of GT cells among the 99% false positive cells requires molecular analyses such as PCR and Southern blotting. However, these additional steps are time-consuming and labor- and cost-intensive. Here, we propose a model positive-negative-selection-mediated GT system that allows the estimation of GT frequency easily and rapidly by direct selection of GT cells using herbicide without the need for further molecular analyses.

Acetolactate synthase (ALS)—a key enzyme in the branched-chain amino acid biosynthesis pathway—is the primary target site for many herbicides, including Bispyribac sodium (BS) (Shimizu et al. 2002). Previously, we succeeded in the pin-point mutagenesis of the *ALS* gene of rice by the introduction of W548L/S627I mutations to confer BS tolerance via GT without any exogenous positive selection markers (Endo et al. 2007). Based on those GT experiments, we produced a GT vector for the *ALS* gene using a gene encoding green fluorescent protein fused to blasticidin-S deaminase (*gfbsd2*) conferring blasticidin-S (Bla-S) tolerance and a green fluorescent signal (Kimura et al. 1994; Ochiai-Fukuda et al. 2006), and the *diphtheria toxin A subunit* (*DT-A*) gene (Terada et al. 2002; Terada et al. 2004) as positive and negative selection markers, respectively (Figure 1). Using this GT vector, it was expected that GT cells could be selected directly in the presence of both BS and Bla-S, without the need for extensive molecular screening, due to the simultaneous introduction of (1) W548L/S627I mutations into the wild-type *ALS* gene and (2) the *gfbsd2* gene downstream of the *ALS* gene.

First, to check whether GT cells could be obtained successfully using this vector, we performed the following experiment using a transformation procedure we reported previously in rice (*Oryza sativa* L. cv. Nipponbare; (Toki 1997; Toki et al. 2006): nine grams of 3-week-old rice secondary calli were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring a GT vector (OD_{600}=0.1) at 24°C for 3 days. *Agrobacterium*-infected calli were cultured on medium containing 10 mg l^{-1} Bla-S (Nakalai tesque, Kyoto, Japan) and 25 mg l^{-1} meropenem (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 31–33°C for 1 month. We obtained 1020 lines of Bla-S resistant and green-fluorescence-emitting calli that were thought to be a mixture of true GT cells and false positive cells (Supplemental Table S1). Subsequently, these calli were cultured on medium containing 0.75 μM BS (Kumiai Chemical Industry, Tokyo, Japan) and 25 mg l^{-1} meropenem at 31–33°C for 1 month. Vigorously growing calli were cultured on regeneration medium containing 0.75 μM BS and 12.5 mg l^{-1} meropenem at 30°C for 1 month for regeneration. Finally, 6 lines of BS-tolerant calli were regenerated successfully from 1020 lines of Bla-S resistant and green-fluorescence-emitting calli (Supplemental Table S1). Thus, we confirmed that Bla-S- and BS-tolerant GT cells and plants can be obtained by GT using this vector. Next, for dual selection with Bla-S and BS, we selected calli infected with *Agrobacterium* harboring the GT vector on medium containing 10 mg l^{-1} Bla-S, 0.75 μM BS and 25 mg l^{-1} meropenem at 31–33°C for 1.5–2 months. Vigorously growing calli were cultured on regeneration medium containing

Table 1. Frequency of selection and regeneration of GT cells under selection with dual Bla-S and BS selection.

	Agrobacterium-infected calli (A)	No. of lines regenerated successfully under Bla-S and BS selection (B)	GT frequency (B/A)
Experiment 1	1.8 g	3 lines (W548L/S627I)	1.7 (events/g)
Experiment 2	6.8 g	13 lines (W548L/S627I), 2 lines (W548L)	2.2 (events/g)

10 mg l^{-1} Bla-S, 0.75 μM BS and 12.5 mg l^{-1} meropenem at 30°C for 1 month. We obtained a total of 18 lines of regenerated plants from 8.6 g *Agrobacterium*-infected callus (Table 1). In the site-directed mutagenesis system via GT using positive-negative selection, desired point mutations are not always introduced into the targeted gene, despite true GT events having occurred successfully (Nishizawa-Yokoi et al. 2015). This situation could arise if HR occurs between point mutations of the targeted gene and the positive selection marker, or if point mutations are corrected back to wild-type by a mismatch repair system. In this GT system, dual pressure with Bla-S and BS enables direct selection of cells in which W548L/S627I mutations and the *gfbsd2* gene are introduced simultaneously into the *ALS* locus. The GT frequency using selection with both Bla-S and BS is ca. 3-fold higher than that with initial selection on Bla-S and subsequent selection on BS (Table 1, Supplemental Table S1). This might be because the single selection pressure of Bla-S and subsequent BS to select GT cells is rather mild compared to the double selection pressure of Bla-S and BS together.

To confirm whether true GT events in which the wild-type gene was modified as expected had occurred successfully in regenerated plants following selection with both Bla-S and BS, molecular analyses were performed. In this GT system, W548L/S627I mutations generate novel recognition sites for the restriction enzyme *Mfe*I (Figure 1). For cleaved amplified polymorphic sequence (CAPS) analysis, a 2.3-kb fragment was amplified by PCR from genomic DNA of regenerated plants using DNA polymerase KOD dash (TOYOBO) with the primer set shown in Supplemental Table S2, and the resulting product was digested with *Mfe*I (Figure 1). CAPS analysis and sequencing revealed that mutations were introduced successfully into the endogenous *ALS* gene in all lines of regenerated plants (Table 1, and data not shown). Both mutations (W548L/S627I) appeared to have been introduced into the endogenous *ALS* gene in 16 lines of regenerated plants, although a single mutation only (W548L) was introduced in 2 lines. Southern blot analysis of *Mfe*I-digested genomic DNA from 17 lines of regenerated plants showed that a true GT event had occurred without any additional genomic rearrangements in the *ALS* locus in 14 lines (except for #2, #7 and #8, Figure 2 and data not shown), suggesting that true GT events occurred in 82% of regenerated plants. It is suggested that both

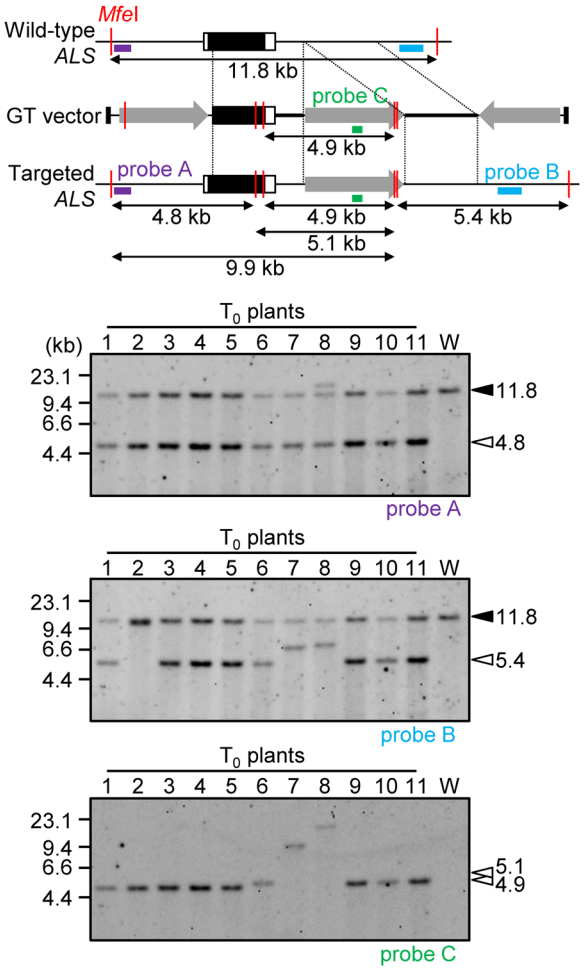

Figure 2. Molecular analysis of GT plants. Southern blot analysis of *Mfe*I-digested DNA of lines #1–11 in the T_0 generation using probes A, B and C. A single mutation W548L was introduced into the endogenous *ALS* gene in #6 and 8, while both mutations W548L/S627I were introduced in other lines. Specific DNA probes were prepared using a PCR digoxigenin probe synthesis kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol with the primer set shown in Supplemental Table 2. The wild-type (11.8 kb) and targeted (4.8 kb and 5.4 kb) bands were detected using probes A and B, respectively. Targeted bands (4.9 kb and 5.1 kb in plants harboring ALS with W548L/S627I and W548L mutations, respectively) were detected using probe C. This probe also recognizes randomly integrated T-DNA. W, wild-type.

random integration of T-DNA and an ectopic GT event, in which the *ALS* gene harboring W548L/S627I and its upstream sequences seemed to be integrated elsewhere in the genome, had occurred simultaneously in line #2 (Figure 2). Similarly, in lines #7 and #8, unexpected bands thought to be derived from randomly integrated

T-DNA and/or ectopic GT were observed, confirming that complicated genome rearrangements have occurred. However, it was not possible to unravel the details further in these lines. Furthermore, segregation analysis of line #4 was performed in the T_1 generation. For CAPS analysis, a 0.7-kb fragment was amplified by PCR from genomic DNA using DNA polymerase KOD dash (TOYOBO) with the primer set shown in Supplemental Table S2, and the resulting product was digested with *Mfe*I (Figure 1). CAPS analysis for the *ALS* gene showed that the ratio of wild-type, heterozygous and homozygous alleles for the modified *ALS* locus was 6 : 9 : 4, which fits a 1 : 2 : 1 ratio (χ^2=0.47; p=0.79, data not shown), suggesting that the introduced mutations were inherited stably in a Mendelian manner. These results support the conclusion that true GT had occurred successfully in this line.

Here, we demonstrated that GT calli can be selected directly under the pressure of both BS and Bla-S simultaneously in our novel GT system. This new system is very convenient since PCR analysis is not required to select GT cells, and true GT events had occurred in almost all regenerated plants. We believe this model GT system is useful both to compare the effects on GT frequency of factors already reported in previous studies and to screen novel factors enhancing HR activity and GT frequency. Also, it can be applied to the optimization of GT experimental procedures by modification of tissue culture conditions and selection of rice varieties and *Agrobacterium* strains. Transformation efficiency is a critical factor in determining GT efficiency because the delivery of donor DNA into rice cells is the crucial step in achieving GT (Voytas 2013). We showed that the *indica* rice variety Kasalath is more competent than Nipponbare in *Agrobacterium*-mediated transformation (Saika and Toki 2010). Moreover, we have already prepared rice mutants that show higher HR activity and lower frequency of random T-DNA integration (Nishizawa-Yokoi et al. 2012). These are expected to be useful materials in our continued efforts to improve GT frequency.

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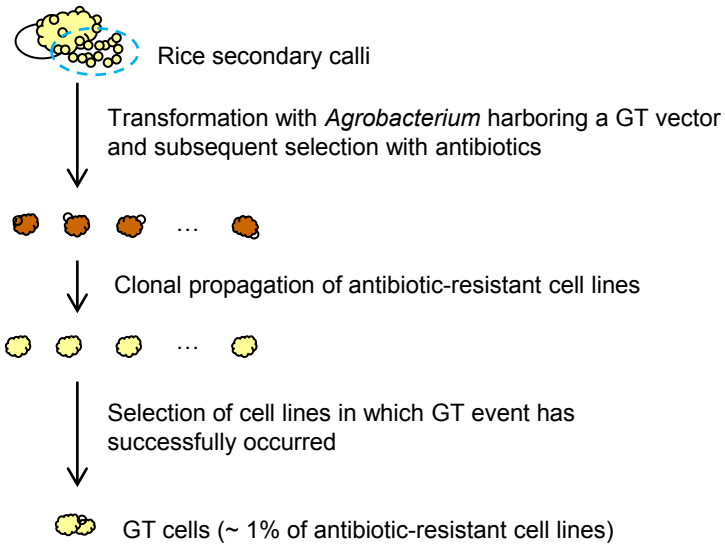
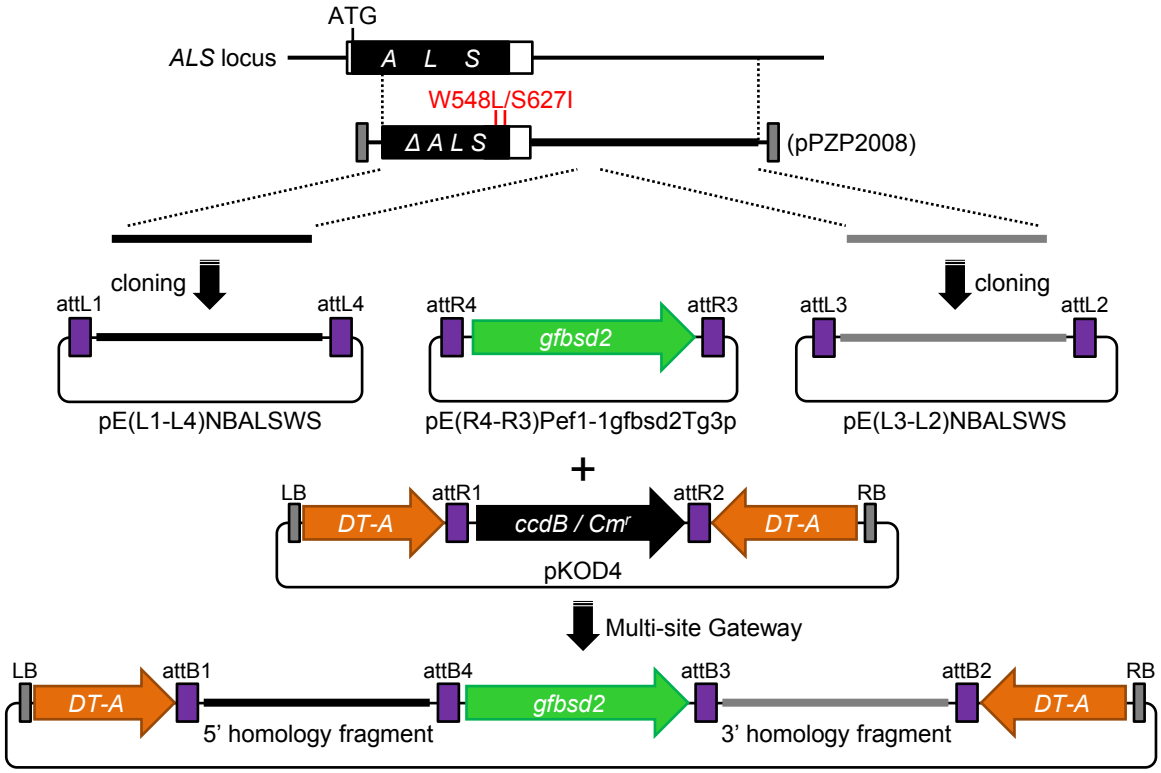


Supplemental Figure S1. Schematic representation of GT experiment in rice



Supplemental Figure S2. Vector construction of a GT vector used in this study
To clone the *ALS* gene with W548L/S627I mutations, a 6.2-kb fragment was amplified by PCR from the GT vector for the *ALS* gene used in a previous study (Endo et al. 2007) with the primer set described in Supplemental Table S2, and the resulting product was cloned into the vector pPZP2028 (Kuroda et al. 2010) using *Asc*I/*Pac*I. To construct pE(L1-L4)NBALSWS harboring the 5' homology sequence for *ALS* locus, the latter vector was digested with *Asc*I and *Bam*HI (blunt-ended with T4 DNA polymerase), and inserted into an *Asc*I and *Pac*I (blunt-ended with T4 DNA polymerase)-digested entry vector with attL1/L4 sites. Similarly, for the construction of pE(L3-L2)NBALSWS harboring the 3' homology sequence for the *ALS* locus, the vector was digested with *Bam*HI (blunt-ended with T4 DNA polymerase) and *Pac*I, and inserted into an *Asc*I (blunt-ended with T4 DNA polymerase) and *Pac*I-digested entry vector with attL3/L2 sites. The LR reaction for the introduction of three entry clones into the destination vector, pKOD4 (Nishizawa-Yokoi et al., 2015) was performed using LR clonase II (Life Technologies, Carlsbad, CA). In our system, the positive selection marker cassette in the entry vector can be modified easily as required.

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Supplemental Table S1. Frequency of selection and regeneration of GT cells under sequential selection using Bla-S then BS

Agrobacterium-infected calli (A)	No. of green-fluorescence-emitting cell lines growing under Bla-S selection (B)	No. of lines regenerated successfully under BS selection (C)	Transformation frequency with negative selection marker (B/A)	Ratio of putative GT events to transformation events (C/B)	GT frequency (events/g of inoculated calli, C/A)
1803 cell lines (equivalent in 9.0 g)[1]	1020 cell lines	6 cell lines	56.6 %	0.59 %	0.67

[1]calculated as 200 pieces/g calli

Supplemental Table S2. Primer sets used in this study

	Sequences (5' - 3')
For GT vector construction	GGCGCGCCGCCGGCCACGCCGCTCCGGCCGT
	TATTAATTAAGCTTTGCCGCTGCCGGTTGGTGGAG
For CAPS analysis (T_0 plants)	CGTCACCGCGCGCGGACAAAACACCCAC
	ACATGATATCTTGTGATGCATATGCCTAC
For CAPS analysis (T_1 plants)	TGTCTTCGGCTGGTCTGGGCGCAA
	ACATGATATCTTGTGATGCATATGCCTAC
For probe A	TTCTTTTTCAATACTTTCCTCGCTTGCTCT
	ATTCAGCCACTTATCTTGACACAACCATTT
For probe B	ACATTCATGACCCGTGAGGGCAATATGAGAT
	AGTGGTCTCCCAGCAGACAGGCTG
For probe C	GACCACATGAAGCAGCACGACTTCT
	TGTACTCCAGCTTGTGCCCCAGGAT