# Efficient gene targeting by homologous recombination in rice

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Modification of genes through homologous recombination, termed gene targeting, is the most direct method to characterize gene function. In higher plants, however, the method is far from a common practice. Here we describe an efficient and reproducible procedure with a strong positive/negative selection for gene targeting in rice, which feeds more than half of the world's population and is an important model plant. About 1% of selected calli and their regenerated fertile plants were heterozygous at the targeted locus, and only one copy of the selective marker used was found at the targeted site in their genomes. The procedure's applicability to other genes will make it feasible to obtain various gene-targeted lines of rice.

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Rice (*Oryza sativa* L.) is an important staple food for more than half of the world's population and is a model plant for other cereal species[1–3]. With the elucidation of the entire genome sequence of rice[3–6], it is becoming clear that a large proportion of predicted rice genes have no recognizable homologs in *Arabidopsis*, in which <10% of putative genes have been studied experimentally[7]. Under these circumstances, the development of a method to study gene function by modifying genomic sequences precisely is extremely important. Gene targeting is a powerful tool of reverse genetics[8]. In higher plants, however, a transgene is usually integrated randomly into the genome by illegitimate recombination even if the introduced sequence has homology with a gene to be targeted[9–11]. Most of the targeted genes used in transgenic plants that have been successfully modified by homologous recombination were artificially modified exotic genes introduced before gene targeting, and only dicotyledonous plants such as *Arabidopsis* and tobacco were used in these experiments[9–13]. There are only two reports of successful targeting of endogenous natural genes in *Arabidopsis*. Only one plant with a targeted regulatory gene was obtained by Kempin *et al.*[14], and no one has yet repeated the experiments. Out of five experiments, Hanin *et al.*[15] identified three targeted plants that acquired herbicide resistance through homologous recombination of the endogenous gene chosen. However, the latter event was gene-specific, and these researchers also detected the occurrence of undesirable events, including ectopic recombination and/or simultaneous ectopic integration of the transgene used[15].

We have developed an efficient and reproducible procedure for targeted gene disruption by homologous recombination in the monocot rice. All of the six independently obtained fertile transgenic plants from six experiments were heterozygous at a chosen targeted locus, a wild-type allele and a targeted recombinant allele, and we observed neither ectopic recombination nor ectopic integration of the transgene. Because our strategy is independent of a gene-specific selection, the procedure should be generally applicable for other rice genes.

## Results and discussion

**Experimental design for gene targeting.** The *japonica* subspecies of rice, cv. Nipponbare, was used for gene targeting because its genomic sequence is well characterized[3,4,6]. As a model gene to be targeted, we chose the gene *Waxy*, which encodes granule-bound starch synthase, a key enzyme in amylose synthesis (Fig. 1)[16,17]. We selected *Waxy* because its mutations affect the quality and quantity of rice grain and because the associated phenotype in pollen and in endosperm can easily be assessed by simple iodine staining[18]. The rice *Waxy* gene comprises 14 exons. Exon 2, which carries the ATG initiation codon, is preceded by the 1.1 kilobase intron 1 (Fig. 1B). Our targeting strategy assumes that the *Waxy* gene will be inactivated when the *hpt* gene[19] for hygromycin B resistance ($Hm^r$), together with the 3′ part of the maize *En* element (*ΔEn*) carrying a transcriptional terminator[20], is inserted into the middle of intron 1.

To achieve efficient isolation of rarely occurring recombinants with the targeted *waxy* gene, we employed strong selection using a modified *hpt* gene[19] and a diphtheria toxin (*DT-A*) gene[21] driven by highly active promoters[22–24] as positive and negative markers, respectively (see Experimental Protocol). Because the reported frequencies of somatic homologous recombination for gene targeting were low[8–15], we first optimized the efficiency of *Agrobacterium*-mediated transformation[25] using pRIT1 (Fig. 1A) and succeeded in obtaining ~1,500 independent $Hm^r$ calli from ~350 rice seeds in each experiment (Table 1). pRIT1 also carries the *gus* gene for β-glucuronidase[24], and most of the $Hm^r$ calli showed the $GUS^+$ phenotype. For strong positive/negative selection, we constructed pINA134, in which the *hpt-ΔEn* sequence is flanked by two copies of the *DT-A* gene (Fig. 1A). An average of 65 $Hm^r$ transformants survived the positive/negative selection with pINA134, and the physiological state of calli appeared to affect the efficiency of the positive/negative selection (Table 1). We detected no PCR-amplified fragments corresponding to the *DT-A* coding region among the calli that survived (data not shown).

Our targeting vector, pRW1, is a pINA134 derivative, in which *hpt-ΔEn* is flanked by the 6.3 kilobase *Waxy* promoter and 6.8 kilobase *Waxy* coding regions (Fig. 1C). Homologous recombination between the *Waxy* sequences should result in the substitution of *hpt-ΔEn* for the 146 base pair *Xba*I-*Xba*I sequence in the *Waxy* intron 1

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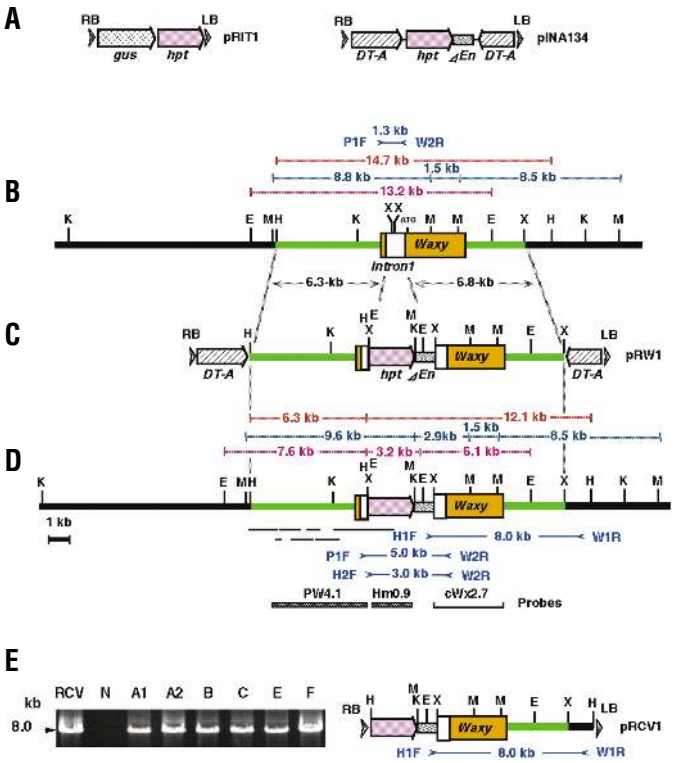


**Figure 1**. Strategy for the targeted disruption of the *Waxy* locus and analysis of homologous recombination events. (A) The structures of pRIT1 and pINA134. Only the structures between the left and right borders (LB and RB) of the vectors are shown. (B) The genomic structure of the wild-type *Waxy* gene region. The white rectangle in the orange *Waxy* box indicates the *Waxy* intron 1 sequence. The green bars represent the sequences corresponding to the *Waxy* flanking regions carried by pRW1. The primers P1F and W2R contain *Waxy* promoter and coding sequences, respectively. (C) The structure of pRW1. (D) The structure of the *waxy* gene disrupted by homologous recombination. The primer H1F contains the junction sequence between *ΔEn* and its adjacent *Xba*I sequence on pRW1. The primers W1R and H2F contain rice genomic and *hpt* sequences, respectively. The overlapping horizontal bars indicate the PCR fragments used for sequencing the recombinant junction. (E) PCR analysis of six calli with the primers H1F and W1R, and the structure of pRCV1 carrying the positive control marker for the PCR analysis. RCV, an equimolar mixture of pRCV1 and rice DNA, mimicking the heterozygous state; N, rice cv. Nipponbare. Restriction sites abbreviations: E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; M, *Mun*I; X, *Xba*I.

without the *DT-A* gene (Fig. 1D). When pRW1 was used for transformation, an average of 106 Hm$^r$ calli were obtained from 32 g of calli (Table 1). To examine whether homologous recombination had occurred, we employed PCR amplification with the primers H1F and W1R for the 8.0 kilobase junction fragment (Fig. 1D) that should also be produced from pRCV1 carrying the anticipated junction structure (Fig. 1E). Of ~600 Hm$^r$ surviving calli from six experiments (Table 1), six independent callus lines designated A1, A2, B, C, E, and F produced the expected 8.0 kilobase band (Fig. 1E). As described subsequently, precise gene-targeting events occurred in these six lines. Therefore, ~1% of Hm$^r$ transformants that survived the positive/negative selection were the result of precise homologous recombination between pRW1 and the *Waxy* gene (Table 1).

**Characterization of the targeted locus.** We regenerated fertile plants from multiple shoots[25] produced in the six callus lines and subjected DNA prepared from leaves to Southern blot analyses. In the *Hin*dIII digests (Fig. 2A), all the selected lines contained the identical 6.3- and 12.1-kilobase *Waxy* flanking segments (Fig. 1D) and the 14.7 kilobase wild-type *Waxy* band (Fig. 1B). We detected two other bands of 12.0 and 4.4 kilobases that hybridized with the probe PW4.1 in both the control and the transformed plants; hence they represent nonspecific hybridization. In *Mun*I digests (Fig. 2B), all the six lines produced the same 9.6- and 2.9-kilobase bands derived from the recombinant allele (Fig. 1D) as well as the 8.8 kilobase fragment from the wild-type *Waxy* allele (Fig. 1B), in addition to the common 1.5- and 8.5-kilobase *Waxy* bands. The results suggest that the six $T_0$ plants were all heterozygotes with only one copy of *hpt-ΔEn* integrated into the *Waxy* locus. This notion was also supported by hybridization patterns of *Eco*RV digests (see $T_1$ data below).

**Table 1. Numbers of Hm$^r$ calli obtained by positive/negative selection and targeted events at the *Waxy* locus[a]**

| Experiment | Numbers of transformants with: pRIT1 | pINA134 | pRW1 | Number of targeted calli |
|---|---|---|---|---|
| A | 1,950 | 21 | 86 | 2 |
| B | 1,600 | 83 | 158 | 1 |
| C | 2,154 | 210 | 136 | 1 |
| D | 1,280 | 11 | 67 | 0 |
| E | 443 | 0 | 76 | 1 |
| F | 1,842 | 64 | 115 | 1 |
| Total | 9,269 | 389 | 638 | 6 |

[a]The numbers of Hm$^r$ calli are shown as though they were obtained from the 32 g calli (see Experimental Protocol). The targeted calli contain the disrupted *waxy* gene by homologous recombination.

The heterozygosity of the *Waxy* locus in these $T_0$ plants was confirmed by the mendelian segregation of the *Waxy* and Hm$^r$ phenotypes (Fig. 3). Pollen and endosperm tissues of self-pollinated $T_0$ plants took up iodine stain[18], and we germinated the remaining $T_1$ embryos. Using a portion of the roots from $T_1$ plants, we assessed the Hm$^r$ trait by the ability to form callus after incubating the roots for two to three weeks in callus induction media[25] with or without hygromycin B. Simultaneously, we subjected DNAs prepared from leaves of the same $T_1$ plants to PCR and Southern blot analyses to characterize their *Waxy* gene structures (Figs 3 and 4). Homozygous *waxy* mutants and wild-type *Waxy* plants produce opaque white and translucent seeds, respectively. Pollen and seeds of the *waxy* mutants become reddish brown upon iodine staining, whereas those of the *Waxy* plants are dark blue. In iodine-stained pollen of all six $T_0$ plants, the ratio of reddish-brown to dark blue was ~1:1 (Fig. 3A). All the opaque white seeds stained reddish brown, and the $T_0$ plants produced reddish-brown as compared to dark blue seeds at the ratio of 1:3 (Fig. 3B): A1, 13:40; A2, 17:60; B, 28:100; C, 58:158; E, 17:52; F, 1:6. Although plant F was less fertile and set few seeds, the mendelian segregation of the *Waxy* and Hm$^r$ traits was confirmed in the $T_2$ plants of line F. The results clearly indicated that the six $T_0$ plants were all heterozygotes at the *Waxy* locus. This conclusion was further supported by the Hm$^r$ trait and PCR analyses of their $T_1$ progeny. Out of 53 seeds of the A1 plant tested, 13 *waxy* $T_1$ plants exhibited Hm$^r$ phenotype (Fig. 3C). All of these *waxy*$^{-/-}$ Hm$^r$ plants produced the 8.0- and 5.0-kilobase fragments (Fig. 1D) but not the 1.3 kilobase band (Fig. 1B) on PCR analysis (Fig. 3D), confirming that they are all homozygotes carrying the *hpt-ΔEn* sequence in the *Waxy* intron 1. Among the remaining 40 *Waxy* plants, 27 and 13 were Hm$^r$ and Hm$^s$ (hygromycin B sensitive), respectively (Fig. 3C). The latter 13 *Waxy*$^{+/+}$ Hm$^s$ gave only the 1.3 kilobase fragment (Fig. 3D), while the former 27 *Waxy*$^{+/-}$ Hm$^r$ showed band patterns identical to the heterozygous parental $T_0$ plant A1: they gave the 8.0- and 1.3-kilobase bands. Although the 5.0 kilobase band was often too weak to

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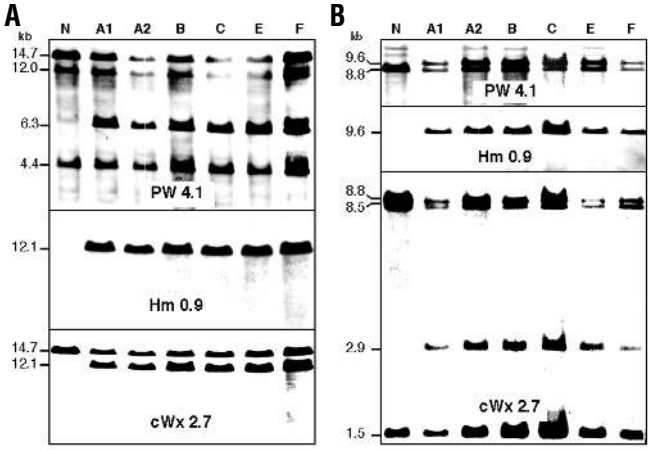


**Figure 2.** Southern blot analysis of the *Waxy* region and integrated *hpt* sequence in the six $T_0$ plants. (A) *Hin*dIII digests. The 12.0 kb and 4.4 kb bands are nonspecific. (B) *Mun*I digests. The probes used and the genomic segments corresponding to the bands detected are shown in Figure 1.

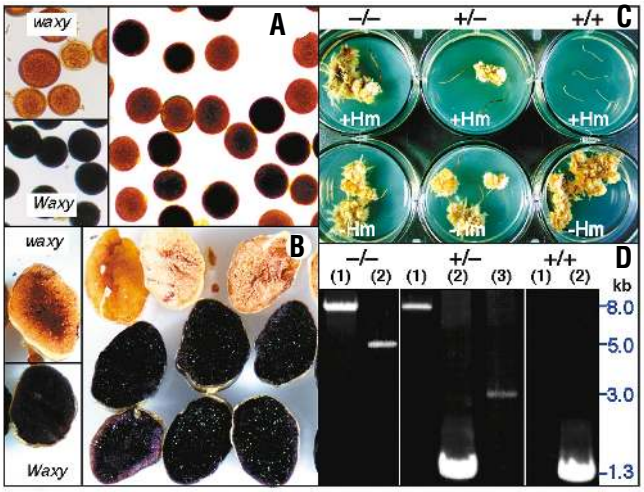


**Figure 3**. Segregation of the targeted *waxy* $Hm^r$ allele revealed by phenotypic and PCR analyses. (A, B) The *Waxy* phenotype in pollen (A) and endosperm (B) of the $T_0$ plant. The authentic *Waxy* and *waxy* rice varieties in the left inset are cv. Nipponbare and cv. Iwaimochi, respectively. (C) The $Hm^r$ phenotype in roots of $T_1$ plants. The *waxy*/*waxy*, *Waxy*/*waxy*, and *Waxy*/*Waxy* plants are indicated by –/–, +/–, and +/+, respectively. The media with and without hygromycin B are indicated by +Hm and –Hm, respectively. (D) PCR analysis of $T_1$ plants. The symbols for the *Waxy* genotypes are as in (C). The primers used for PCR analysis are (1) H1F and W1R; (2) P1F and W2R; (3) nested PCR with H2F and W2R. The positions of the primers are shown in Figure 1.

be clearly visible, probably because the 1.3 kilobase fragment tends to be amplified more efficiently than the longer 5.0 kilobase fragment, its presence was confirmed by detection of the 3.0 kilobase band by subsequent nested PCR amplification with the primers H2F and W2R (Figs 1D and 3D). We obtained similar results in the $T_1$ plants of the other targeted lines displaying *waxy* $Hm^r$, *Waxy* $Hm^r$, and *Waxy* $Hm^s$ phenotypes (data not shown).

Southern blot analyses of $T_1$ plants of the six targeted lines further confirmed the conclusion that the *waxy* mutations are caused by targeted gene disruption resulting from homologous recombination and that neither an ectopic recombination nor an additional ectopic integration[10,11,13,15] of *hpt-ΔEn* occurred. In *Hin*dIII digests (Fig. 4A), the *waxy*$^{-/-}$ $Hm^r$ allele produced the 6.3- and 12.1-kilobase *Waxy* flanking bands but no 14.7 kilobase *Waxy* fragment (Fig. 1). The 14.7 kilobase *Waxy* band appeared only in the wild-type *Waxy*$^{+/+}$ $Hm^s$ and the heterozygous *Waxy*$^{+/-}$ plants. In *Mun*I digests (Fig. 4B), the 9.6- and 2.9-kilobase bands were produced from the *waxy*$^{-/-}$ $Hm^r$ allele, and the 8.8 kilobase fragment was from the wild-type *Waxy*$^{+/+}$ $Hm^s$ allele (Fig. 1). Likewise, we observed the expected 7.6-, 6.1-, and 3.2-kilobase *Eco*RV bands in the *waxy*$^{-/-}$ $Hm^r$ homozygotes, whereas the *Waxy*$^{+/+}$ $Hm^s$ plants gave a 13.2 kilobase *Eco*RV band (Figs 1 and 4C). Southern analyses of these plants after *Kpn*I digestion (data not shown) verified the conclusion: the *Kpn*I-*Mun*I *waxy* region of 35 kilobases (Fig. 1D) in the $Hm^r$ plants conformed to the anticipated structure. Furthermore, on sequencing both junction fragments flanking *hpt-ΔEn* in the three different *waxy* $Hm^r$ homozygous $T_1$ plants of the A1, B, and C lines, we found no sequence aberrations in the sequenced regions. From these results, we conclude that the rice *Waxy* gene can be specifically disrupted by insertion of the *hpt-ΔEn* sequence into the *Waxy* intron 1 through homologous recombination (Fig. 1D).

Neither the *Waxy* promoter nor the *Actin 1* promoter for the *hpt* gene appeared to activate the interrupted *Waxy* gene, presumably because of transcriptional termination and/or degradation of aberrant transcripts due to the presence of the *ΔEn* sequence[20,26]. Indeed, preliminary northern blot analysis indicated the presence of abundant *hpt* transcripts, but not *Waxy* transcripts, in the endosperm (data not shown). The inserted *hpt-ΔEn* sequence could be eliminated by employing site-specific recombination systems[27], and the resulting *Waxy* gene, containing only a small site-specific recombination sequence in the middle of intron 1, would be reactivated. Moreover, the remaining recombination sequence in the *Waxy* intron 1 can serve as a genomic targeting site for integration of foreign DNA mediated by the site-specific recombination system employed. There is a recent report of such site-specific integration of DNA into the rice genome using the Cre-*lox* recombination system[28].

**Gene targeting by homologous recombination in rice.** Using an efficient and straightforward positive/negative selection method, we repeatedly obtained specific substitution mutants of the targeted rice *Waxy* gene by precise homologous recombination in about 1% of the survivors. Such a targeted integration of an introduced transgene by somatic homologous recombination occurs $10^{-3}$–$10^{-6}$ as frequently as random integration of the transgene by illegitimate recombination[8–15]. The observed targeting frequency as determined from the ratio of the number of targeted calli resulting from somatic homologous recombination to the number of calli resulting from integration of the $Hm^r$ marker of pRIT1 by illegitimate recombination was estimated to be $6.5 \times 10^{-4}$ (Table 1). It should be noted that the number of the $Hm^r$ calli containing pRIT1 (Table 1) appeared to be underestimated as compared with actual illegitimate recombination events. It is difficult to obtain an exact count of the transformed $Hm^r$ calli because scattered pieces of rice calli tend to break off easily. We therefore counted it as one $Hm^r$ callus when an inoculated callus became $Hm^r$, even though we often observed more than one independent illegitimate recombination event in an inoculated callus. In addition, the number of targeted $Hm^r$ *waxy* mutant calli observed may have been smaller than the number of actual homologous recombination events because some of the recombinants might have been killed by the DT-A protein. Nevertheless, the observed frequency of somatic homologous recombination for gene targeting is comparable to the frequencies previously reported[8–15].

All of the six independently isolated fertile transgenic plants were heterozygous at the *Waxy* locus: a wild-type *Waxy* $Hm^s$ allele and a recombinant *waxy* $Hm^r$ allele, with one copy of the *hpt-ΔEn*

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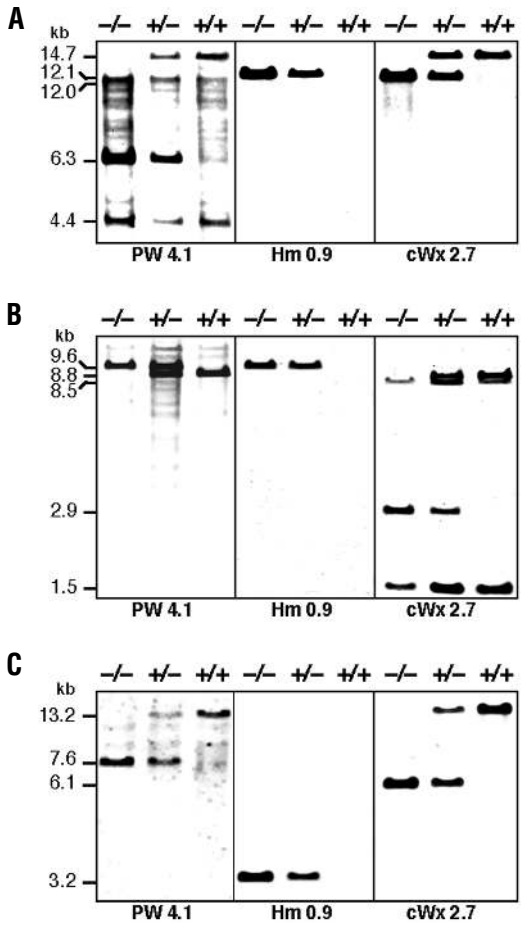


**Figure 4.** Southern blot analysis of the *Waxy* region in the $T_1$ segregants from the targeted $T_0$ heterozygous plant. (A) *Hin*dIII digests. (B) *Mun*I digests. (C) *Eco*RV digests. The symbols are as in Figure 2.

sequence integrated at the *waxy* locus, were found in their genomes. We detected neither ectopic recombination nor ectopic integration of the introduced transgene. The rice embryogenic callus used for *Agrobacterium*-mediated transformation might be especially recombination-proficient for gene targeting. Alternatively, the strong positive/negative selection vector, with two copies of the negative selection marker next to each border sequence, together with a powerful PCR screen for detecting the targeted allele, might efficiently select against calli in which undesirable ectopic events had occurred. Because the strategy we adapted is independent of gene-specific selection as demonstrated in *Arabidopsis*[15], it is, in principle, applicable to other genes, including essential genes, because all the recombinant $T_0$ plants obtained were heterozygous. The method, therefore, should be useful for obtaining various gene-targeted or knockout lines of rice and, presumably, other plants.

## Experimental protocol

**Vector construction.** Vectors pRIT1 and pINA134 are pGSGLUC1 (ref. 29) derivatives having the entire 4.6 kb *Sal*I segment in the T-DNA region (except for the border sequences) substituted (Fig. 1A). pRIT1 carries the highly active rice *Actin 1* promoter with its intron 1 (ref. 22) fused with an *hpt* gene modified at its 3′ coding region[19] and the cauliflower mosaic virus (CaMV) 35S promoter with the intron 1 of the *catalase* gene from castor bean fused with the *gus* gene[24]. pINA134 contains the same *hpt* gene flanked by two copies of the *DT-A* gene[21], with highly active promoters (the maize *Ubiquitin 1* promoter with its intron 1 (ref. 23) residing near the right border of the T-DNA and the CaMV 35S promoter with intron 1 of the *catalase* gene[24] near the left border) in the inverted orientation (Fig. 1A). Both *DT-A* and *hpt* genes were fused with the CaMV 35S terminator, and the *gus* gene was fused with the *Nos* terminator[19,24]. To minimize read-through transcription from the *hpt* gene into the right *DT-A* gene, the 1.0 kb segment at the 3′ end of the maize *En* element[20] (*ΔEn*) was placed between the *hpt* and right *DT-A* genes (Fig. 1A). pRW1 is a pINA134 derivative carrying the 6.3 kb *Hin*dIII-*Xba*I fragment containing the *Waxy* promoter between the *DT-A* and *hpt* genes as well as the 6.8 kb *Xba*I-*Xba*I segment carrying the *Waxy* coding region between the *hpt* and *DT-A* genes (Fig. 1C). Thus pRW1 contains the 13.1 kb *Waxy* region of rice *japonica* cv. Shimokita[17], the sequence of which was found to be identical to that of cv. Nipponbare. pRCV1, used for a positive marker in the PCR analysis, is a pINA134 derivative carrying the 12.1 kb *Xba*I-*Hin*dIII segment containing the *Waxy* coding region (Fig. 1E).

**Rice transformation.** We modified the published *Agrobacterium*-mediated transformation[25] using small and vigorously dividing calli from mature seeds of rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) with 400 μM acetosyringone. In each experiment, ~36 g (fresh weight: 34.8–38.5 g in individual experiments) of vigorously growing calli were obtained from 400 mature rice seeds. A portion of the calli (0.94–1.06 g) was inoculated with *A. tumefaciens* EHA101 (ref. 25) harboring pRIT1, and another portion (3.0–3.07 g) was inoculated with EHA101 containing pINA134. The remaining average 32 g (30.7–34.4 g) of calli were used for targeting with pRW1. Through multiple shoots[25], >100 transgenic plants can be obtained from a single targeted callus.

**Nucleic acid procedures.** General nucleic acid procedures have been described[26,30]. PCR amplification was conducted with LA *Taq* polymerase (TaKaRa Biomedicals, Otsu, Japan): initial denaturation was at 94°C for 1 min, 32 cycles of denaturation at 94°C for 1 min, annealing and extension at 60°C for 20 min, and final extension at 72°C for 10 min. The primers were as follows: H1F, 5′-GTATAATGTATGCTATACGAAGTTATGTTT-3′; W1R, 5′-GTTTGGTCATATTATGTACTTAAGCTAAGT-3′; P1F, 5′-ACACAAATAACTGCAGTCTC-3′; W2R, 5′-CCGACATGGTGGTTGTCTAG-3′; H2F, 5′-GTAGGTAGACCGGGGGCAATGAG-3′; H2R, 5′-ACGCCCGACAGTCCCGGCTCCGG-3′. For Southern blot analysis, digoxygenin-labeled probes (Roche Diagnostics Corp., Basel, Switzerland) were used. The probes PW4.1 and Hm0.9 are the 4.1 kb *Xho*I-*Xho*I fragment obtained from pRW1 and the 0.9 kb PCR fragment amplified with primers H2F and H2R, respectively. The probe cWx2.7 contains 2.3 kb *Waxy* cDNA and ~0.4 kb intron 1 sequences[18]. The 8.0 kb PCR fragment amplified with the primers H1F and W1R, and short overlapping PCR fragments (Fig. 1D) were used for sequencing the recombinant junctions containing the *Waxy* coding and promoter regions, respectively.

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*Competing interests statement*

*The authors declare that they have no competing interests.*

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