Plant Molecular Biology (2005) 59:205–219
DOI 10.1007/s11103-005-2162-x

Modification of endogenous natural genes by gene targeting in rice and other higher plants

Shigeru Iida* and Rie Terada
[1]*Division of Molecular Genetics, National Institute for Basic Biology, National Institutes of Natural Sciences, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan (*author for correspondence; e-mail shigiida@nibb.ac.jp);* [2]*Department of Molecular Biomechanics, The Graduate University for Advanced Studies, 444-8585, Japan*

Received 10 May 2004; accepted in revised form 11 February 2005

Key words: Agrobacterium-mediated transformation; chimeric RNA/DNA oligonucleotides, *DT-A (diptheria toxin A fragment)* gene, gene targeting, homologous recombination, positive–negative selection

Abstract

The capability to modify a genomic sequence into a designed sequence is a powerful tool for biologists and breeders to elucidate the function of an individual gene and its *cis*-acting elements of multigene families in the genome. Gene targeting refers to the alteration of a specific DNA sequence in an endogenous gene at its original locus in the genome. In higher plants, however, the overwhelming occurrence of the random integration of transgenes by non-homologous end-joining is the main obstacle to develop efficient gene targeting. Two approaches have been undertaken to modify a genomic sequence in higher plants: chimeric RNA/DNA oligonucleotide-directed gene targeting to generate a site-specific base conversion, and homologous recombination-dependent gene targeting to produce either a base change or a gene replacement in a sequence-specific manner. The successful and reproducible targeting of an endogenous gene by homologous recombination, independently of gene-specific selection by employing a strong positive-negative selection, has been demonstrated for the first time in rice, an important staple food and a model plant for other cereal species. This review addresses the current status of targeting of an endogenous natural gene in rice and other higher plants and discusses possible models for *Agrobacterium*-mediated gene targeting by homologous recombination using a strong positive–negative selection.

Introduction

Gene targeting refers to the alteration of a specific DNA sequence in an endogenous gene at its original locus in the genome. To modify an endogenous gene in higher plants, two approaches are generally taken: chimeric RNA/DNA oligonucleotide-directed gene targeting and homologous recombination-dependent gene targeting (Hohn and Puchta, 1999; Kumar and Fladung, 2001; Britt and May, 2003). The chimeric RNA/DNA oligonucleotide-directed gene targeting generates site-specific base changes, whereas the homologous recombination-dependent gene targeting can lead to both base changes and gene replacement events in a sequence-specific manner (Figure 1). It should also be emphasized that the application of gene targeting to rice (*Oryza sativa* L.) is particularly important, since rice is an important staple food for more than half of the world's population and a model plant for other cereal species because of its small genome of 430 Mb and the availability of draft sequences for *japonica* and *indica* subspecies together with the high-quality sequences of three out of 12 chromosomes of the *japonica* cultivar Nipponbare (Feng *et al.,* 2002; Goff *et al*., 2002;

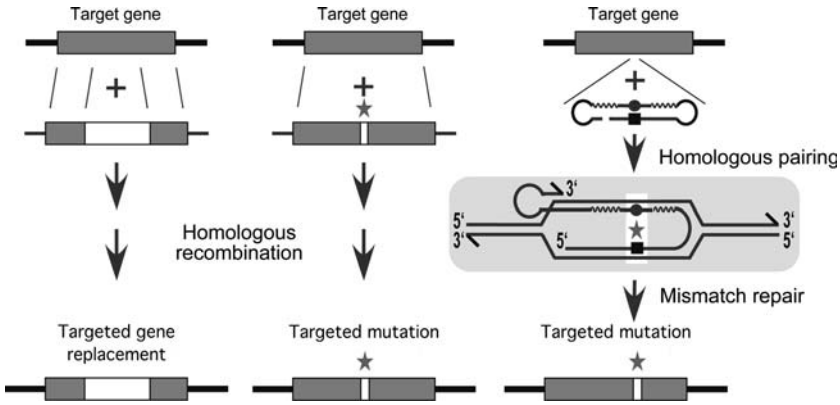

Figure 1. Homologous recombination-dependent gene targeting and chimeric RNA/DNA oligonucleotide-directed targeted point mutations. Homologous recombination-dependent gene targeting can lead to both targeted gene replacements and targeted point mutations. Targeted gene replacement is generally regarded to occur via double crossover events at the flanking homologous regions. Targeted mutation can also be explained by double crossover events. Alternatively, mismatch repair of a hetroduplex intermediate produced by single crossover and subsequent branch migration, which is followed by resolution, can result in the targeted mutation. For chimeric RNA/DNA oligonucleotide-directed targeted point mutations, the chimeric oligonucleotide is thought to interact with the target sequence by homologous base-pairing and to form a double D-loop juncture, which is subjected to mismatch repair and resolution processes. DNA sequence is represented by smooth lines, and 2′-*O*-methyl RNA, by wavy lines. The filled circles and squares in the chimeric oligonucleotides indicate the base-pair of the nucleotide alteration to be introduced. The narrow open boxes with ⋆ represent single-base conversions.

Sasaki *et al.,* 2002; Yu *et al.,* 2002; Delseny, 2003; The rice chromosome 10 sequencing consortium, 2003). Moreover, the International Rice Genome Sequencing Project (IRGSP) plans to complete the high-quality sequencing of the rice genome by December 2004 (http://rgp.dna.affrc.go.jp/IRGSP/). Even though various rice mutant resources have been established (Hirochika *et al.,* 2004) and RNA interference (RNAi) is applicable to modification of gene expression in rice (Hayama *et al.,* 2003; Kusaba, 2004; Miki and Shimamoto, 2004), the capability to modify the genomic sequences into designed sequences by gene targeting provides a powerful tool for plant biologists and breeders to elucidate the function of individual genes of multigene families and their *cis*-acting elements in the genome. Therefore, successful and reproducible gene targeting by homologous recombination without concomitant occurrence of ectopic events (Terada *et al.,* 2002) is the important first step for developing a precise modification system of the genomic sequences in rice.

Various aspects of gene targeting in higher plants have been discussed recently (Hohn and Puchta, 1999; Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999; Kumar and Fladung, 2001; Puchta, 2002; Ray and Langer, 2002; Britt and May, 2003; Gong and Rong, 2003; Hanin and Paszkowski, 2003; Hohn and Puchta, 2003; Reiss, 2003; Iida and Terada, 2004). In this review, we focus on the current status of modifying endogenous genes in higher plants, including rice. We also discuss certain aspects of models for the generation of gene replacement by homologous recombination.

Chimeric RNA/DNA oligonucleotide-directed gene targeting

Chimeric oligonucleotide-directed gene targeting could allow generating site-specific point mutations in individual genes of multigene families. Self-complementary chimeric oligonucleotides contain a DNA 'mutator' region of 5-bp nucleotides complementary to the target site flanked by 2'-*O*-methyl RNA segments, and the 'mutator' region carries a mutation to be introduced into the endogenous target gene (Figure 1; Beetham *et al.,* 1999; Zhu *et al.,* 1999; Oh and May, 2001; Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004). A proposed mechanism involves the formation of a complement-stabilized D-loop as a key intermediate in chimeric oligonucleotide-directed gene targeting (Figure 1). The RNA and DNA strands in the chimeric oligonucleotides are suggested to facilitate complex D-loop formation

with the target sequence and to act as a template for the mismatch repair of the heteroduplex intermediate, respectively (Gamper *et al.*, 2000; Rice *et al.,* 2000; Andersen *et al.,* 2002). Although three plant species, tobacco, maize, and rice, were employed for chimeric RNA/DNA oligonucleotide-directed gene targeting (Table 1), their endogenous genes to be targeted encode the same enzyme catalyzing the first common step in the biosynthesis of the branched amino acids, i.e., valine, leucine, and isoleucine, which are generally named as acetolactate synthase (ALS) and as acetohydroxy acid synthase (AHAS) in maize (Lee *et al.,* 1988; Sathasivan *et al.,* 1991; Fang *et al.,* 1992). Since mutations of certain amino acids in these enzymes confer resistance to herbicides, chimeric RNA/DNA oligonucleotides containing such mutated sequences were introduced by bombardment to plant cells and immature embryos or by electroporation of protoplasts, and herbicide-resistant transformants carrying the targeted *ALS*/*AHAS* genes were selected. Not only the precisely anticipated base conversion but also non-specific adjacent nucleotide alterations were observed at the targeted sites (Beetham *et al.,*1999; Zhu *et al.,* 1999; 2000; Kochevenko and Willmitzer, 2003). In two cases using maize and tobacco plants, fertile transgenic plants were subsequently obtained and transmission of the modified *ALS*/*AHAS* genes into their progeny was demonstrated (Zhu *et al.,* 2000; Kochevenko and Willmitzer, 2003). Because the published strategy is specific for the particular herbicide-resistance gene, it remains to be seen whether this strategy with appropriate modifications can be applicable to genes other than directly selectable genes such as the *ALS*/*AHAS* genes.

Table 1. Chimeric RNA/DNA oligonucleotide-directed gene targeting.

Plant (genome size)	Gene	Gene delivery system	Frequency of predicted conversion	Regenerants*[a]	Transmission	Reference
Maize (2600 Mb)	*AHAS* (*ALS*)	Particle bombardment (embryogenic calli)	$\sim 1 \times 10^{-4}$	9/12 plant lines	Mendelian inheritance demonstrated	Zhu *et al.* (1999, 2000)
Tobacco (4000 Mb)	*ALS*	Particle bombardment (suspension calli)	Only semi-targeted calli*[b]	–	–	Beetham *et al.* (1999)
		Electroporation (protoplasts)*[c]	$\sim 1 \times 10^{-6}$	3/6 plant lines	Mendelian inheritance demonstrated	Kochevenko and Willmitzer (2003)
		Particle bombardment (calli)				
Rice (430 Mb)	*ALS*	Particle bombardment (embryogenic calli)	$\sim 1 \times 10^{-4}$	5/5 plantlets (1 albino)	–	Okuzaki and Toriyama (2004)

The *ALS* gene in maize is traditionally named as the *AHAS* gene (see text).
*[a] Regenerated plants or plant lines with the predicted conversion / Regenerated plants with per Regenerated plants examined.
*[b] The site of the all detected mutations was shifted to the 5′ side from the expected site by one base (semitargeting).
*[c] Both wild-type and haploid protoplasts were used.

Homologous recombination-dependent gene targeting

Homologous recombination-dependent gene targeting in higher plants has a longer history than chimeric RNA/DNA oligonucleotide-directed gene targeting. Since the first report of successful gene targeting of an artificially truncated and integrated drug-resistance gene in the tobacco genome (Paszkowski *et al.,* 1988), various approaches for gene targeting based upon homologous recombination in higher plants have revealed that the integration of a transgene by somatic homologous recombination occurs in the

order of 10^{-3} to 10^{-6} compared with random integration by non-homologous end-joining (Hohn and Puchta, 1999; Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999; Kumar and Fladung, 2001; Puchta, 2002; Ray and Langer, 2002; Britt and May, 2003; Gong and Rong, 2003; Hanin and Paszkowski, 2003; Hohn and Puchta, 2003; Reiss, 2003; Iida and Terada, 2004). The overwhelming occurrence of random integration of transgenes by non-homologous end-joining relative to targeted homologous recombination is the main obstacle to the development of an efficient system for homologous recombination-dependent gene targeting. In addition to random integration mediated by non-homologous end-joining, the occurrence of aberrant recombination events associated with homologous gene targeting, called one-sided invasion and ectopic targeting, has also been reported (Figure 2; Risseeuw *et al.,* 1995; Puchta, 1998; Hanin *et al.,* 2001; Hohn and Puchta, 2003; Iida and Terada, 2004). One-sided invasion results from one homologous recombination event and another non-homologous end-joining event at the target locus, whereas ectopic targeting is thought to be generated by ectopic integration (integration elsewhere in the genome without altering the target gene) of a recombinant molecule produced by homologous recombination between the introduced transgene and a copy of the target sequence.

Targeting of an endogenous gene in tobacco and Arabidopsis

Table 2 and Figure 3 show the several attempts to target endogenous genes including the *ALS* gene in tobacco (Lee *et al.,* 1990) and the *TGA3*, *AGL5, CHS, ADH,* and *PPO* loci in *Arabidopsis thaliana* (Miao and Lam, 1995; Kempin *et al.*, 1997; Gallego *et al.,* 1999; Hanin *et al.,* 2001; Wang *et al.,* 2001). The targeting of these endogenous genes was conducted by *Agrobacterium*-mediated transformation, presumably because longer DNA segments can be more easily transformed by *Agrobacterium*-mediated transformation, which gives rise to lower copy numbers of a transgene, compared with transgene sequences obtained by direct transformation methods (Kohli *et al.,* 2003). Of these endogenous genes, the strategy employed

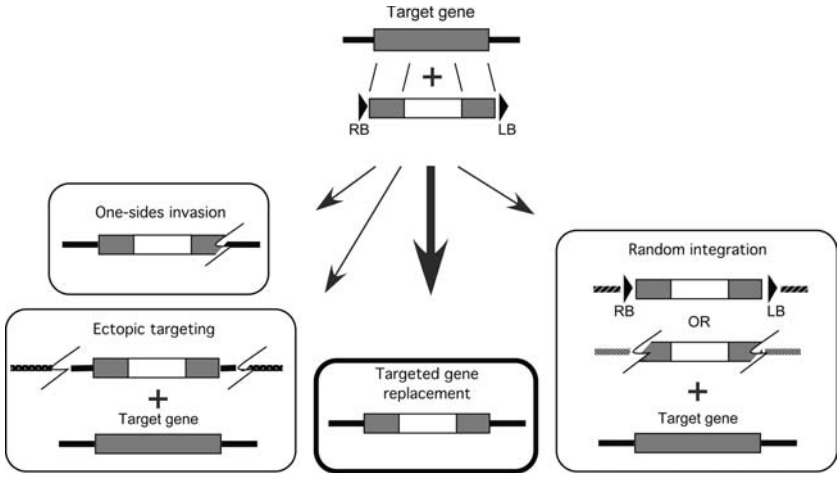

Figure 2. Integration events of a transgene associated with homologous recombination-dependent gene targeting. The thick arrow indicates the homologous recombination pathway leading to the targeted gene replacement generated by double crossover events at the flanking homologous regions. Most of the T-DNA integrations are random integration mediated by non-homologous end-joining and are often flanked by the right and left border sequences of T-DNA. The filled arrowheads with RB and LB represent the right and left border sequences of T-DNA, respectively. One-sided invasion results from one homologous and another non-homologous events at the target locus. In ectopic targeting, a recombinant molecule produced by homologous recombination between the introduced transgene and a copy of the target sequence is inserted in a genome other than the target locus. The target gene remains intact in both random integration and ectopic targeting. Non-homologous end-joining events take place within the sequences carried by the introduced T-DNA segment in the random integration, whereas non-homologous crossovers can occur at the flanking target sequences outside of the introduced T-DNA segment in ectopic targeting.

Table 2. Experiments for endogenous gene targeting by homologous recombination.

Plant (geneome size)	Gene	Gene delivery system	Selection and screening	Regenerants	Frequency (EGT or TGT)	Transmission	Vector structures and reference
Tobacco (4000 Mb)	*ALS*	*Agrobacterium* (protoprasts)	GSS	2 EGT(?) plant lines*[a]	(8.4×10^{-5})*[b]	Mendelian inheritance	(A) Lee *et al.* (1990)
Arbidopsis (125 Mb)	*TGA3*	*Agrobacterium* (root explants)	PSNS-PCR [*NPTII* (Km^r) /*GUS*$^-$]	1–2 calli *[c]	1–2 TGT or EGT/2580 Hm^r calli $(4–8 \times 10^{-4})$	–	(B) Miao and Lam (1995)
	CHS	*Agrobacterium* (suspension calli)	PNS [*NPTII* (Km^r) /*codA*]	None	$<1/109\ 475$ km^r calli $(<1 \times 10^{-6})$	–	(F) Gallego *et al.* (1999)
	AGL5	*Agrobacterium* (intact plants)	PS-PCR [*NPTII* (Km^r)]	1 TGT plant	1TGT/750 T1 plants (1×10^{-3})	–	(C) Kempin *et al.* (1997)
	ADH	*Agrobacterium* (root explants)	PNS [*NPTII* (Km^r) /*codA*]	1 callus 1 plant line	1 EGT (No TGT)*[d] /6250 km^r calli (1.6×10^{-4})	1 EGT line studied	(G) Wang *et al.* (2001)
	PPO	*Agrobacterium* (intact plants)	GSS-PCR	3 TGT plants*[e] 6 EGT plantsx	3 TGT/1410 T1 plants (7.2×10^{-4}) 6 EGT /1410 T1 plants	Medelian inheritance	(H) Hanin *et al.* (2001)
Lotus japonicus (450 Mb)	*Glul, Pzf*	*Agrobacterium* (hypocotyl segments)	PNS-PCR *[NPTII* ($G418^r$)/*codA*]	None	$<1/18\ 974$ $G418^r$ calli $(<5.3 \times 10^{-5})$	–	(D,E) Thykjaer *et al.* (1997)
Rice (430 Mb)	*Waxy*	*Agrobacterium* (embryogenic calli)	PNS-PCR *[HPT* (Hm^r)/*DT-A]*	6 independent TGT plants	6 TGT /9269 Hm^r calli (6.5×10^{-4})	Medelian inheritance	(I) Terada *et al.* (2002)

Abbreviations are. GSS, gene-specific selection; PSNS-PCR, positive selection and negative screening followed by PCR screening; PNS, positive-negative selection; PS-PCR, positive selection and PCR screening; GSS-PCR, gene-specific selection followed by PCR screening; PNS-PCR, positive–negative selection followed by PCR screening; EGT, ectopic gene targeting; TGT, true gene targeting. For the designation of the genes used and the structures of the vectors employed, see Figure 3.

*[a] Available data indicate that they are probably EGT plants.

*[b] The frequency was determined by comparing ETG calli obtained by the targeting construct with transformants obtained using an appropriate control construct.

*[c] One callus showed no regeneration, and another ill-characterized callus was lost.

*[d] One additional candidate callus was lost.

*[e] All three TGT plants also carried additional unlinked copies of the transgene by random integration.

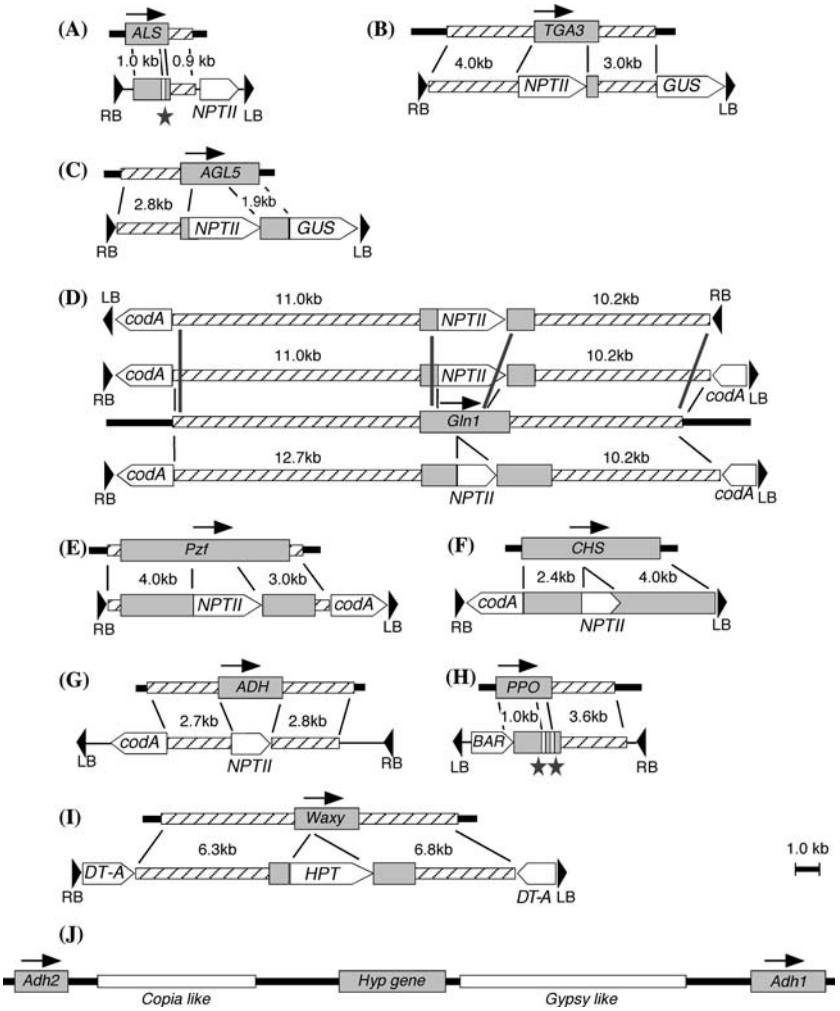

Figure 3. Schematic representations of targeted replacement of endogenous genes by homologous recombination in higher plants. (A) The *ALS* gene for acetolactate synthase in *Nicotiana tabacum* (Lee *et al*., 1990). (B) The *TGA3* gene for a transcriptional factor containing the bZIP domain in *Arabidopsis thaliana* (Miao and Lam, 1995). (C) The *AGL5* gene encoding a MADS box transcriptional factor in *Arabidopsis thaliana* (Kempin *et al.,* 1997). (D) The *Gln1* gene for cytosolic glutamine synthase in *Lotus japonicus* (Thykjaer *et al.,* 1997). (E) The *Pzf* gene for plant zinc finger protein oxidase in *Lotus japonicus* (Thykjaer *et al.,* 1997). (F) The *CHS* gene for chalcone synthase in *Arabidopsis thaliana* (Gallego *et al.,* 1999). (G) The *ADH* gene for alcohol dehydrogenase in *Arabidopsis thaliana* (Wang *et al.,* 2001). (H) The *PPO* gene encoding protoporhyrinogen oxidase in *Arabidopsis thaliana* (Hanin *et al*., 2001). (I) The *Waxy* gene encoding granule-bound starch synthase in rice (Terada *et al.,* 2002). (J) The genomic *Adh* gene region in rice (Tarchini *et al.,* 2000). The *Adh1* and *Adh2* encode alcohol dehydrogenase, and the *Hyp* gene is a hypothetical gene. The *Copia-like* and *Gypsy-like* are retroelements. The genes *NPTII, HPT, codA*, and *DT-A* encode neomycin phosphotransferase, hygromycin phosphotransferase, cytosine deaminase, and diphtheria toxin A-fragment, respectively. The stippled and hatched boxes indicate the target genes and their flanking homologous sequences carried by the introduced T-DNA segments, respectively, and the filled arrowheads represent the border sequences of T-DNA. The orientations of the target genes are indicated by the arrows above the genes. The narrow open boxes with ⋆ on the T-DNA segments represent single base alterations.

for targeting the *ALS* and *PPO* genes was gene-specific selection based on the fact that certain mutations could confer herbicide resistance (Lee *et al.,* 1990; Hanin *et al.,* 2001). In the first attempt to target the endogenous *ALS* gene in tobacco, Lee *et al.* (1990) obtained seven herbicide-resistant calli out of about 130 000 transformants and found that only three of them to be recombinants between the endogenous *ALS* gene and an introduced segment whereas the remaining four calli

were spontaneous mutants for herbicide resistance. Extensive analysis of two out of three recombinants revealed that ectopic targeting occurred in their genome and that the endogenous *ALS* gene appeared to be intact.

Only three *Arabidopsis* genes, *TGA3*, *AGL5*, and *PPO*, were successfully targeted by homologous recombination (Table 2). A root explant-transformation method was first used for targeting the non-selectable *TGA3* gene (Valvekens *et al*., 1988; Miao and Lam, 1995), while an intact plant-transformation procedure was later employed for targeting the non-selectable *AGL5* and selectable *PPO* genes (Bechtold *et al.,* 1993; Kempin *et al*., 1997; Hanin *et al.,* 2001). For the targeted replacement of the *TGA3* gene (Miao and Lam, 1995), the *NPTII* gene for kanamycin resistance (Km^r) was placed in the middle of the *TGA3* gene region, and the *GUS* gene as a cell-autonomous and easily screenable marker (Jefferson *et al.,* 1987) was inserted between the *TGA3* cording region and the left T-DNA border sequence (Figure 3B). Out of 2580 calli examined, one Km^rGUS^- transformed callus that could not be regenerated was confirmed to contain a successfully targeted *TGA3* gene, and another possibly targeted callus was unfortunately lost (Miao and Lam, 1995). Subsequently, a single *Arabidopsis* plant having the *AGL5* MADS-box regulatory gene modified was isolated among 750 transformants as the first successful targeting event of an endogenous gene in a transgenic plant (Kempin *et al.,* 1997). Although the introduced vector contained not only the positively selectable *NPTII* gene flanked by slightly less than 2- and 3-kb *AGL5* gene sequences but also a screenable *GUS* gene originated from the vector pZM104A (Figure 3C; Miao and Lam, 1995), the authors appeared to rely more on polymerase chain reaction (PCR)-based screening for the presence of an approximately 3-kb junction fragment than on screening against the expressed *GUS* gene (Kempin *et al.,* 1997; Liljegren and Yanofsky, 1998). They have succeeded with only one targeted transgenic plant, and no one has yet repeated the experiments. Choosing the *PPO* gene as a gene-specific selectable target (see Figure 3H), Hanin *et al*., (2001) successfully identified three fertile transgenic *Arabidopsis* plants carrying the targeted *PPO* gene from five experiments at a frequency of approximately 7.2×10^{-4} targeted events per transformant. All three targeted plants also had additional ectopically inserted *PPO* sequences, which could segregate easily because they were not linked to the endogenous *PPO* locus. The introduction of the expected sequence alterations in the targeted genomic *PPO* locus by precise homologous recombination was confirmed by DNA sequencing analysis. In addition to these truly targeted plants, Hanin *et al.* (2001) found six ectopically targeted plants. It should be pointed out that the targeting strategy in the *PPO* gene was based upon gene-specific selection and its design for the targeting configuration could be interpreted as targeted mutation; on the other hand, the strategy in the *AGL5* gene was independent of gene-specific selection, and its targeting configuration was a typical targeted gene replacement (Figure 1).

Positive–negative selection

To overcome the difficulties to perform efficient gene targeting by homologous recombination in higher plants, various approaches have been attempted to enhance homologous recombination processes and to suppress non-homologous end-joining. Such attempts include engineering of the host recombination and/or repair systems in tobacco and *Arabidopsis* (Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999; Kumar and Fladung, 2001; Puchta, 2002; Ray and Langer, 2002; Britt and May, 2003; Gong and Rong, 2003; Hanin and Paszkowski, 2003; Reiss, 2003). Since alteration of the recombination and/or repair systems often results in conferring pleiotropic phenotypes, however, the occurrence of such undesirable phenotypic changes might hamper the functional characterization of an endogenous gene by targeted modification (Iida and Terada, 2004).

One approach to enrich transformants carrying a targeted replacement of endogenous genes of wild-type plants is to employ positive–negative selection in the introduced vectors. This approach was originally developed to enrich rare 'knock-out' mutants in mouse (Mansour *et al.,* 1988). In the positive–negative selection vectors, positive selection markers are located within the segments homologous to the target genes, and negative selection markers, which act for counter selection against random or non-targeted integration

events, flank the targeting homologous sequence (Figure 3). In higher plants, only two positive selection markers, the *NPTII* gene for resistance to kanamycin or genticin (G418) and the *HPT* gene for hygromycin resistance, have been used for such positive–negative selections (Thykjaer *et al.,* 1997; Gallego *et al.,* 1999; Wang *et al.,* 2001; Terada *et al*., 2002). Likewise, only two negative selection markers, the *codA* and *DT-A* genes, were used for a positive–negative selection in these experiments.

The *codA* gene of *Escherichia coli* encoding cytosine deaminase catalyzes the conversion of non-toxic 5-fluorocytosine to toxic 5-fluorouracil and has been developed as a substrate-dependent negative selection marker for *Arabidopsis thaliana*, *Lotus japonicus*, and tobacco (Perera *et al.,* 1993; Stougaard, 1993). The *DT-A* gene, derived from the *tox* gene carried by temperate corynephages of *Corynebacterium diphtheriae,* encodes the diphtheria toxin A fragment, causes the NAD^+-dependent ADP-ribosylation of elongation factor 2, and inhibits protein biosynthesis (Pappenheimer, 1977). Although the DT-A protein was once reported to bear nuclease activity (Chang *et al*., 1989), which might affect homologous recombination, the association of the nuclease activity later became questionable (Bodley *et al.,* 1990). The DT-A protein was shown to be highly toxic to tobacco cells, and even transient expression of the *DT-A* gene in the plant cells causes cell death (Koltunow *et al.,* 1990; Czako and An, 1991; Thorsness *et al.,* 1991). While one DT-A molecule introduced into a mammalian cell is sufficient to kill the cell (Yamaizumi *et al.,* 1978), its toxicity to a plant cell appears to be slightly lower (Nilsson *et al.,* 1998). Since the *DT-A* gene lacks the region encoding the diphtheria toxin B fragment, which is required for the recognition of membrane receptors and for the import of the toxic A fragment into the cell, the DT-A protein cannot be taken up by adjacent cells, and this cell-autonomous and non-conditional mode of killing is suitable for genetic ablation (Day and Irish, 1997). After arresting translation, DNA fragmentation associated with cell death was detectable (Day and Irish, 1997). Because the transient expression of the introduced *DT-A* gene before integration into the genome was thought to kill potential targeted transformants (Morton and Hooykaas, 1995), the substrate-dependent negative selection gene *codA* was employed as a first choice (Table 2; Thykjaer *et al.,* 1997; Gallego *et al.,* 1999; Wang *et al.,* 2001). As described below, however, only the cell-autonomous and non-conditional negative selection gene *DT-A* has led to the isolation of successful and reproducible gene targeting by homologous recombination (Terada *et al*., 2002).

Attempts to target endogenous genes by homologous recombination using positive–negative selection in Lotus *and* Arabidopsis

The first attempt to target endogenous genes by homologous recombination using positive-negative selection was to modify the *Gln1* and *Pzf* loci in *Lotus japonicus* by employing the *NPTII* and *codA* genes as positive and negative markers, respectively (Thykjaer *et al.,* 1997). Three out of four different vectors used for the replacement of the *Gln1* locus carried two *codA* genes at both ends of the T-DNA segments, whereas the remaining one vector for the *Gln1* locus and a vector for the *Pzf* gene contained only one *codA* gene (Figure 3D, E). None of the anticipated targeted survivors could be obtained out of 185 survivors of the positive-negative selection among an estimated total of about 19 000 transformation events. Subsequently, the *CHS* locus in a cell suspension of *Arabidopsis thaliana* was chosen for targeting using the positive–negative selection with the *NPTII* gene and one copy of the *codA* gene inserted next to the right border sequence (Figure 3F; Gallego *et al.,* 1999). None of the targeted replacements of the *CHS* gene could be found out of about 3455 survivors of the positive-negative selection, which corresponded to less than about 4% of the primary transformed cells. The same *NPTII* and *codA* genes were also used to target the *Arabidopsis ADH* gene as the positive and negative markers, respectively, and only one transgenic plant carrying the ectopically targeted *ADH* gene was obtained out of 39 surviving calli among 6250 transformants derived from root explants (Figure 3G; Wang *et al.,* 2001). Available data suggested that further improvements would be necessary to utilize the *codA* gene as a substrate-dependent negative selection marker for positive–negative selection for the enrichment of homologous recombination-dependent targeted gene replacement.

Homologous recombination-dependent targeting of an endogenous gene using strong positive–negative selection in rice

Somatic homologous recombination is a major pathway for DNA repair in vegetative growing cells, and transcription is implicated in stimulating such somatic homologous recombination, which is referred to as transcription-associated recombination (Aguilera, 2002). The most commonly used *Agrobacterium*-mediated transformation procedures in rice employ embryo scutellum-derived calli generated from mature rice seeds (Hiei *et al.,* 1994, 1997). Since many tissue-specific genes are known to be expressed in callus tissue, it is conceivable that vigorously dividing calli are proficient and suitable tissues for gene targeting through transcription-associated somatic recombination. To obtain a rare homologous recombination-dependent callus containing a targeted replacement of an endogenous gene, Terada *et al.* (2004) first developed a large-scale transformation protocol using vigorously growing calli derived from the wild-type *japonica* rice cultivar Nipponbare and examined the killing effects of vectors containing the *DT-A* gene on the introduced rice calli. Another large-scale *Agrobacterium*-mediated transformation protocol for several *japonica* varieties has recently been reported (Sallaud *et al.,* 2003). As an endogenous model gene to be targeted, Terada *et al.* (2002) chose the *Waxy* gene present as a single copy on rice chromosome 6 and succeeded in the first reproducible gene targeting by homologous recombination without the occurrence of ectopic events. In their introduced vector for targeting of the *Waxy* gene, the strongly expressed *HPT* gene used as a positive selection marker was flanked by targeting *Waxy* homologous sequences of 6.3–6.8 kb, and two inversely oriented *DT-A* genes fused with strongly and constitutively expressing promoters were placed at both ends of the T-DNA segment adjacent to its border sequences (Figure 3I). Out of 638 survivors of the positive–negative selection, six independent callus lines could be isolated by PCR-based screening for the appearance of an 8-kb junction fragment generated by homologous recombination. Six fertile transgenic plants having the *Waxy* gene disrupted by homologous recombination were obtained from six experiments with an estimated frequency of around 6.5×10^{-4} per random integration of the transgene used (Terada *et al.*, 2002). All of the six-targeted plants obtained were heterozygous at the *Waxy* locus (one wild-type *Waxy* allele and a targeted *waxy* allele), and neither random integration of additional transgene sequences nor ectopic events could be detected. The occurrence of precise homologous recombination at the *Waxy* locus was confirmed by DNA sequencing. Because this strategy is independent of gene-specific selection, it is, in principle, applicable to any other gene. Indeed, we are currently applying the same strategy to disrupt either one of the two directly repeated *Adh1* and *Adh2* genes on rice chromosome 11, where repetitive *Gypsy*-like and *Copia*-like retroelements can be found next to the *Adh1* and *Adh2* genes, respectively (Figure 3J; Tarchini *et al.,* 2000). Preliminary results indicated that we were indeed able to repeatedly obtain transgenic plants having the *Adh2* gene disrupted with frequency comparable to the published targeting frequency of the *Waxy* gene (Terada *et al.*, 2002; R. Terada, M. Saito, Y. Johzuka-Hisatomi, and S. Iida, unpublished). The results indicate that the strategy can be applied to loci other than that of the *Waxy* gene. Therefore, the homologous recombination-dependent targeting of an endogenous gene is now feasible, at least in rice.

Models for homologous recombination-dependent gene targeting using positive-negative selection with the DT-A *gene as a negative selection marker*

Since homologous recombination is thought to be mainly initiated with a double-strand break (Gorbunova and Levy, 1999; Paques and Haber, 1999; Prado and Aguilera, 2003), gene targeting by homologous recombination is also regarded to be associated with the repair of double-strand breaks (Paques and Haber, 1999; Vergunst and Hooykaas, 1999; Ray and Langer, 2002; Britt and May, 2003). The most generally accepted models to explain gene targeting in plants are the double-strand break repair and synthesis-dependent strand-annealing models (Vergunst and Hooykaas, 1999; Puchta, 2002; Ray and Langer, 2002; Britt and May, 2003; Iida and Terada, 2004), which were originally developed for explaining recombination and repair events mainly studied in yeast (Paques and Haber, 1999; Prado and Aguilera, 2003). Although targeted gene replacement by a linear double-stranded segment in the

ends-out configuration, in which the two double-stranded DNA ends are oriented outward (Ray and Langer, 2002; Gong and Rong, 2003), is usually described via two independent crossover events at the flanking homologous segments (Figures 2 and 3), it can be explained not only by the conventional model of double-strand break repair but also by two alternative models based on synthesis-dependent strand-annealing or break-induced replication (Paques and Haber, 1999; Britt and May, 2003; Iida and Terada, 2004). Furthermore, the invading 3′ ends can be either from the introduced DNA segment or from the host chromosomal DNA generated by a double-stranded break (see Figure 4B, C; Britt and May, 2003; Iida and Terada, 2004).

Since all the experiments to target endogenous genes were conducted by *Agrobacterium*-mediated transformation (Table 2), we summarize briefly its characteristic features. The single-stranded T-DNA that is covalently linked to the VirD2 protein at its 5′ end, corresponding to the right border of T-DNA, was imported into the plant nucleus in *Agrobacterium*-mediated transformation, although the precise mechanism of T-DNA integration into the plant genome remains largely unknown (Gelvin, 2000, 2003; Zupan *et al*., 2000; Tzfira *et al*., 2000). The fast kinetics of the transient transcription of a T-DNA-encoded *GUS* gene indicated that the imported single-stranded T-DNA in the nucleus rapidly becomes double-stranded before integration of T-DNA into the

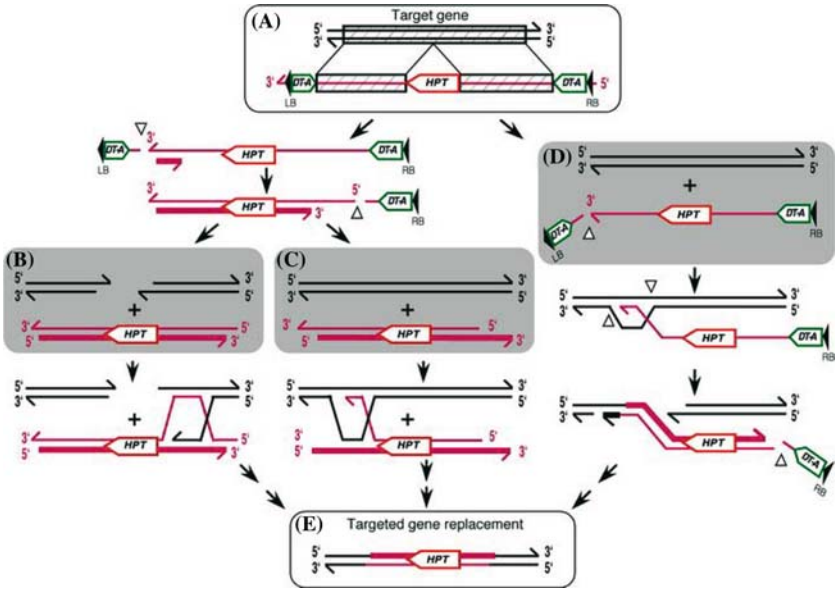

Figure 4. Possible models for targeted gene replacement using the *DT-A* gene as a negative selection marker. (A) The single-stranded T-DNA, which contains the *HPT* gene as a positive selection marker and two copies of the *DT-A* genes at both ends, was imported into the plant nucleus. The black and red lines represent the sequence information on the plant genome and introduced T-DNA, respectively, and the hatched boxes indicate homologous regions for recombination. The internal part of the imported single-stranded T-DNA may become double-stranded by random priming, and the resultant molecule can undergo an alternative homologous recombination pathway (B) or (C). The thick lines indicate newly synthesized segments and the open triangle points to an introduced nick on the single-stranded region of the T-DNA segment. The other symbols are as in Figure 3. Alternatively, the internal part of the imported single-stranded T-DNA may be directly involved in recombination pathway (D). See text for further details. (B) A double-strand break is introduced at the target plant DNA, which is followed by either double-strand break repair or synthesis-dependent strand-annealing pathways. (C) The 5′-ends of the double-stranded T-DNA in the ends-out configuration are resected, and the resulting 3′-ended single strand can subsequently invade homologous double-stranded regions of plant DNA to initiate the double-strand break repair pathway. (D) The single-stranded nick is first introduced to remove the *DT-A* sequence, and the broken 3′ end of single-stranded T-DNA subsequently invades and hybridizes with the homologous plant DNA to initiate homologous recombination. Only the initiation of one of two crossover events was shown in each recombination pathway (B–D), because the subsequent recombination processes were well-documented (Puchta, 1998; Vergunst and Hooykaas, 1999; Ray and Langer, 2002; Reiss, 2003; Iida and Terada, 2004). (E) Successful targeted gene replacement is completed.

plant genome (Narasimhulu *et al.,* 1996), although the mechanism to convert the imported T-DNA into double-stranded T-DNA remains to be established. Two alternative models have been proposed to explain the integration processes, in which the intermediate molecules are different: single-stranded T-DNA that invades the plant DNA or double-stranded T-DNA that undergoes non-homologous end-joining processes associated with double-strand breaks (Tinland, 1996; Brunaud, *et al*., 2002; Chilton and Que, 2003; Tzfira *et al.,* 2003). It is conceivable that single-stranded T-DNA can serve as an intermediate under certain physiological conditions, even though double-stranded intermediates may represent one of the major processes of T-DNA integration. In this respect, Hanin *et al*. (2001) argued that the targeted recombinants of the *PPO* gene (Figure 3H) were formed via two crossover events. Alternatively, the successful gene targeting can also be explained by the assimilation of the imported single-stranded and mutated T-DNA directly into the homologous target gene through the mismatch repair of an intermediate heteroduplex (Paques and Haber, 1999).

Figure 4 shows possible models of successful targeted gene replacement through ends-out recombination with two cell-copies of the autonomous and non-conditional negative selective *DT-A* gene at both ends. The consequences of the introduction of the *DT-A* gene fused with a strong constitutive promoter as a non-conditional negative selection marker in the somatic recombination pathways for successful gene targeting might be different from those of the introduction of the *codA* gene, a substrate-dependent negative selection marker. This was because a few toxin molecules produced by transient expression of the *DT-A* gene on the double-stranded T-DNA intermediate would probably be sufficient to kill the host cell instantly (Czako and An, 1990; Morton and Hooykaas, 1995). The following models are based upon the presumption that in successfully targeted rice cells the two *DT-A* gene regions flanking the *Waxy* sequence have not been converted into a double-stranded intermediate after the introduction into the plant nucleus as a single-stranded molecule, because the *DT-A* gene on the double-stranded T-DNA intermediate can be transiently expressed and a few *DT-A* molecules produced are sufficient to kill the host cell. If the DT-A gene products were ever produced from the single-stranded T-DNA intermediate, such calli would be killed even successful gene targeting had occurred.

One model predicts that the imported single-stranded T-DNA in the nucleus becomes double-stranded probably by random priming initiated at a homologous segment between the positive and negative selection markers, and the *DT-A* gene region at the 3′ terminus of the imported single-stranded T-DNA remains single-stranded and is eventually removed by introducing a nick at the homologous single-stranded part. Alternatively, the single-stranded nick is introduced first, and then random priming starts at the remaining homologous segment. Before the *DT-A* gene at the 5′ terminus of the imported single-stranded T-DNA becomes double-stranded, another nick is introduced to remove the 5′ *DT-A* gene, which results in the double-stranded T-DNA without containing the *DT-A* sequence. Subsequently, the 5′ ends of the resultant double-stranded T-DNA intermediate in the ends-out configuration are resected, and the resulting 3′-ended single strands can invade the homologous double-stranded region of plant DNA to initiate the previously described double-stranded break repair processes (Figure 4C; Britt and May, 2003; Iida and Terada, 2004). Alternatively, a double stranded break is introduced into the target plant DNA and is followed by the well documented processes: double-strand break repair or synthesis-dependent strand-annealing (Figure 4B; Puchta, 1998; Vergunst and Hooykaas, 1999; Ray and Langer, 2002; Reiss, 2003; Iida and Terada, 2004). After the occurrence of the first single crossover, several alternative pathways (double-strand break repair or synthesis-dependent strand-annealing) can lead to successful targeted gene replacement (Figure 4E; Iida and Terada, 2004).

Another model postulates that the single-stranded nick is first introduced at the homologous segment of the imported single-stranded T-DNA in the nucleus, and the resulting 3′-ended single strand without carrying the *DT-A* gene at the 3′ terminus directly invades the homologous double-stranded target region of plant DNA without becoming double-stranded (Figure 4D; Iida and Terada, 2004). Although the presence of the *DT-A* gene at the 3′ terminus in the imported single-stranded T-DNA may hinder the processes of the

single strand invasion and the annealing to the target region in the homologous double-stranded molecule, we believe that the *DT-A* genes at both ends of T-DNA is necessary to remove efficiently a large number of undesirable calli that have the introduced transgene integrated randomly. Subsequently, new DNA synthesis is initiated from the nicked plant DNA to copy the T-DNA template. Before the *DT-A* gene at the 5′ terminus of the single-stranded T-DNA intermediate becomes double-stranded, another nick is introduced to remove the 5′ *DT-A* gene, and the resulting partially double-stranded molecule undergoes either double-strand break repair or synthesis-dependent strand-annealing processes to complete targeted gene replacement (Figure 4E). In addition, the occurrence of non-homologous end-joining at the second crossover instead of homologous recombination would result in one-sided invasion (Figure 2). In either model, the longer homologous segments flanking the positive selection marker in the T-DNA appear to provide not only more homology for homologous recombination but also a greater chance to introduce a nick in the imported single-stranded T-DNA between the *HPT* and *DT-A* genes in order to prevent the *DT-A* gene from being expressed transiently. We believe that these models would facilitate to improve further homologous recombination mediated gene targeting of endogenous genes using positive–negative section.

Conclusions

Rice, one of the most important crops for human consumption, has become a model plant for monocotyledonous cereals, as high-quality sequencing of its genome will soon be completed. There has been a single report describing the successful and reproducible targeting of the endogenous *Waxy* gene by homologous recombination without concomitant occurrence of ectopic events is an important first step for developing a precise modification system of the genomic sequences in rice. Since the strategy used is independent of gene-specific selection, it should be applicable to any other gene, including essential ones, because all the primary transgenic plants obtained were heterozygous. However, no article on targeting of another natural endogenous gene in rice has been published in the past 2 years since the first report, indicating that the progress of gene targeting in rice is apparently not so fast and that we still need to accumulate more knowledge and know-how to improve the procedures. In the future, homologous recombination-dependent gene targeting will hopefully become a routine method for precisely modifying genomic sequences into designed sequences to elucidate the function of individual genes of multigene families and their *cis*-acting elements in rice and, presumably, other higher plants.

Acknowledgements

We thank Yasuyo Johzuka-Hisatomi and Miho Saitoh for discussions, Hirohiko Hirochika for providing a manuscript prior to publication, and Atsushi Hoshino and Kohji Kusano for comments on the manuscript. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

References

Aguilera, A. 2002. The connection between transcription and genomic instability. EMBO J. 21: 195–201.

Andersen, M.S., Sorensen, C.B., Bolund, L. and Jensen, T.G. 2002. Mechanisms underlying targeted gene correction using chimeric RNA/DNA and single-stranded DNA oligonucleotides. J. Mol. Med. 80: 770–781.

Bechtold, N., Ellis, J. and Pelletier, G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris Life Sci. 316: 1194–1199.

Beetham, P.R., Kipp, P.B., Sawycky, X.L., Arntzen, C.J. and May, G.D. 1999. A tool for functional plant genomics: chimeric RNA/DNA oligonucleotides cause *in vivo* gene-specific mutations. Proc. Natl. Acad. Sci. USA 96: 8774–8778.

Bodley, J.W., Johnson, V.G., Wilson, B.A., Blanke, S.R., Murphy, J.R., Pappenheimer, Jr., A.M., Collier, R.J., Lessnick, S.L., Bruce, C., Baldwin, R.L., Chang, M.P., Nakanura, L.T. and Wisnieski, B.J. 1990. Does diphtheria toxin have nuclease activity? Science 250: 832–838.

Britt, A.B. and May, G.D. 2003. Re-engineering plant gene targeting. Trends Plant Sci. 8: 90–95.

Brunaud, V., Balzergue, S., Dubreucq, B., Aubourg, S., Samson, F., Chauvin, S., Bechtold, N., Cruaud, C., DeRose, R., Pelletier, G., Lepiniec, L., Caboche, M. and Lecharny, A. 2002. T-DNA integration into the *Arabidopsis* genome depends on sequences of pre-insertion sites. EMBO Rep. 3: 1152–1157.

Chang, M.P., Baldwin, R.L., Bruce, C. and Wisnieski, B.J. 1989. Second cytotoxic pathway of diphtheria toxin suggested by nuclease activity. Science 246: 1165–1168.

Chilton, M.-D.M. and Que, Q. 2003. Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. Plant Physiol. 133: 956–965.

Czako, M. and An, G. 1991. Expression of DNA coding for diphtheria toxin chain A is toxic to plant cells. Plant Physiol. 95: 687–692.

Day, C.D. and Irish, V.F. 1997. Cell ablation and the analysis of plant development. Trends Plant Sci. 2: 106–111.

Delseny, M. 2003. Towards an accurate sequence of the rice genome. Curr. Opin. Plant Biol. 6: 101–105.

Fang, L.Y., Gross, P.R., Chen, C.-H. and Lillis, M. 1992. Sequence of two acetohydroxyacid synthase genes from *Zea mays*. Plant Mol. Biol. 18:1185–1187.

Feng, Q., Zhang, Y., Hao, P., Wang, S., Fu, G., Huang, Y., Li, Y., Zhu, J., Liu, Y., Hu, X., Jia, P., Zhang, Y., Zhao, Q., Ying, K., Yu, S., Tang, Y., Weng, Q., Zhang, L., Lu, Y., Mu, J., Lu, Y., Zhang, L.S., Yu, Z., Fan, D., Liu, X., Lu, T., Li, C., Wu, Y., Sun, T., Lei, H., Li, T., Hu, H., Guan, J., Wu, M., Zhang, R., Zhou, B., Chen, Z., Chen, L., Jin, Z., Wang, R., Yin, H., Cai, Z., Ren, S., Lv, G., Gu, W., Zhu, G., Tu, Y., Jia, J., Zhang, Y., Chen, J., Kang, H., Chen, X., Shao, C., Sun, Y., Hu, Q., Zhang, X., Zhang, W., Wang, L., Ding, C., Sheng, H., Gu, J., Chen, S., Ni, L., Zhu, F., Chen, W., Lan, L., Lai, Y., Cheng, Z., Gu, M., Jiang, J., Li, J., Hong, G., Xue, Y. and Han, B. 2002. Sequence and analysis of rice chromosome 4. Nature 420: 316–320.

Gallego, M.E., Sirand-Pugnet, P. and White, C.I. 1999. Positive–negative selection and T-DNA stability in *Arabidopsis* transformation. Plant Mol. Biol. 39: 83–93.

Gamper, H.B., Cole-Strauss, A., Metz, R., Parekh, H., Kumar, R. and Kmiec, E.B. 2000. A plausible mechanism for gene correction by chimeric oligonucleotides. Biochemistry 39: 5808–5816.

Gelvin, S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Annu. Rev. Plant Physiol. Plant Mol. Biol. 51: 223–256.

Gelvin, S.B. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. 67: 16–37.

Goff, S.A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B.M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W.-L., Chen, L., Cooper, B., Park, S., Wood, T.C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R.M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A. and Briggs, S. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). Science 296: 92–100.

Gong, M. and Rong, Y.S. 2003. Targeting multi-cellular organisms. Curr. Opin. Genet. Dev. 13: 215–220.

Gorbunova, V. and Levy, A.A. 1999. How plants make ends meet: DNA double-strand break repair. Trends Plant Sci. 4: 263–269.

Hanin, M. and Paszkowski, J. 2003. Plant genome modification by homologous recombination. Curr. Opin. Plant Biol. 6: 157–162.

Hanin, M., Volrath, S., Bogucki, A., Briker, M., Ward, E. and Paszkowski, J. 2001. Gene targeting in *Arabidopsis*. Plant J. 28: 671–677.

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. and Shimamoto, K. 2003. Adaptation of photoperiodic control pathways produces short-day flowering in rice. Nature 422: 719–722.

Hiei, Y., Komari, T. and Kubo, T. 1997. Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. 35: 205–218.

Hiei, Y., Ohta, S., Komari, T. and 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.

Hirochika, H., Guiderdoni, E. An, G., Hsing, Y.-I., Eun, M.Y., Han, C.-D,, Upadhyaya, N., Ramachandran, S., Zhang, Q., Pereira, A., Sundaresan, V. and Leung, H. 2004. Rice mutant resources for gene discovery. Plant Mol. Biol. 54: 325–334.

Hohn, B. and Puchta, H. 1999. Gene therapy in plants. Proc. Natl. Acad. Sci. USA 96: 8321–8323.

Hohn, B. and Puchta, H. 2003. Some like it sticky: targeting of the rice gene *Waxy*. Trends Plant Sci. 8: 51–53.

Iida, S. and Terada, R. 2004. A tale of two integrations, transgene and T-DNA: gene targeting by homologous recombination in rice. Curr. Opin. Biotechnol. 15: 132–138.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Kempin, S.A., Liljegren, S.J., Block, L.M., Rounsley, S.D., Yanofsky, M.F. and Lam, E. 1997. Targeted disruption in *Arabidopsis*. Nature 389: 802–803.

Kochevenko, A. and Willmitzer, L. 2003. Chimeric RNA/DNA oligonucleotide-based site-specific modification of the tobacco acetolactate syntase gene. Plant Physiol. 132: 174–184.

Kohli, A., Twyman, R.M., Abranches, R., Wegel, E., Stoger, E. and Christou, P. 2003. Transgene integration, organization and interaction in plants. Plant Mol. Biol. 52: 247–258.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M. and Goldberg, R.B. 1990. Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224.

Kumar, S. and Fladung, M. 2001. Controlling transgene integration in plants. Trends Plant Sci. 6: 155–159.

Kusaba, M. 2004. RNA interference in crop plants. Curr. Opin. Biotechnol. 15: 139–143.

Lee, K.Y., Lund, P., Lowe, K. and Dunsmuir, P. 1990. Homologous recombination in plant cells after *Agrobacterium*-mediated transformation. Plant Cell 2: 415–425.

Lee, K.Y., Townsend, J., Tepperman, J., Black, M., Chui, C.F., Mazur, B., Dunsmuir, P. and Bedbrook, J. 1988. The molecular basis of sulfonylurea herbicide resistance in tobacco. EMBO J. 7: 1241–1248.

Liljegren, S.J. and Yanofsky, M.F. 1998. …response: Targeting *Arabidopsis*. Trends Plant Sci. 3: 79–80.

Mansour, S.L., Thomas, K.R. and Capecchi, M.R. 1988. Disruption of the proto-oncogene *int*-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336: 348–352.

Mengiste, T. and Paszkowski, J. 1999. Prospects for the precise engineering of plant genomes by homologous recombination. Biol. Chem. 380: 749–758.

Miao, Z.-H. and Lam, E. 1995. Targeted disruption of the TGA3 locus in *Arabidopsis thaliana*. Plant J. 7: 359–365.

Miki, D. and Shimamoto, K. 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiol. 45: 490–495.

Morton, R. and Hooykaas, P.J.J. 1995. Gene replacement. Mol. Breed. 1: 123–132.

Narasimhulu, S.B., Deng, X.-B., Sarria, R. and Gelvin, S.B. 1996. Early transcription of Agrobactertium T-DNA genes in tobacco and maize. Plant Cell 8: 873–886.

Nilsson, O., Wu, E., Wolfe, D.S. and Weigel, D. 1998. Genetic ablation of flowers in transgenic *Arabidopsis*. Plant J. 15: 799–804.

Oh, T.J. and May, G.D. 2001. Oligonucleotide-directed plant gene targeting. Curr. Opin. Biotechnol. 12: 169–172.

Okuzaki, A. and Toriyama, K. 2004. Chimeric RNA/DNA oligonucleotide-directed gene targeting in rice. Plant Cell Rep. 22: 509–512.

Pappenheimer, Jr., A.M. 1977. Diphtheria toxin. Annu. Rev. Biochem. 46: 69–94.

Paques, F. and Haber, J.E. 1999. Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 63: 349–404.

Paszkowski, J., Baur, M., Bogucki, A. and Potrykus, I. 1988. Gene targeting in plants. EMBO J. 7: 4021–4026.

Perera, R.J., Linard, C.G. and Signer, E.R. 1993. Cytosine deaminase as a negative selective marker for *Arabidopsis*. Plant Mol. Biol. 23: 793–799.

Prado, F. and Aguilera, A. 2003. Control of cross-over by single-strand DNA resection. Trends Genet. 19: 428–431.

Puchta, H. 1998. Repair of genomic double-strand breaks in somatic plant cells by one-sided invasion of homologous sequences. Plant J. 13: 331–339.

Puchta, H. 2002. Gene replacement by homologous recombination in plants. Plant Mol. Biol. 48: 173–182.

Ray, A. and Langer, M. 2002. Homologous recombination: ends as the means. Trends Plant Sci. 7: 435–440.

Reiss, B. 2003. Homologous recombination and gene targeting in plant cells. Int. Rev. Cytol. 228: 85–139.

Rice, M.C., May, G.D., Kipp, P.B., Parekh, H. and Kmiec, E.B. 2000. Genetic repair of mutations in plant cell-free extracts directed by specific chimeric oligonucleotides. Plant Physiol. 123: 427–437.

Risseeuw, E., Offringa, R., Franke-van-Dijk, M.E.I. and Hooykaas, P.J.J. 1995. Targeted recombination in plants using *Agrobacterium* coincides with additional rearrangements at the target locus. Plant J. 7: 109–119.

Sallaud, C., Meynard, D., van Boxtel, J., Gay, C., Bes, M., Brizard, J.P., Larmande, P., Ortega, D., Raynal, M., Portefaix, M., Ouwerkerk, P.B.F., Rueb, S., Delseny, M. and Guiderdoni, E. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theor. Appl. Genet. 106: 1396–1408.

Sasaki, T., Matsumoto, T., Yamamoto, K., Sakata, K., Baba, T., Katayose, Y., Wu, J., Niimura, Y., Cheng, Z., Nagamura, Y., Antonio, B.A., Kanamori, H., Hosokawa, S., Masukawa, M., Arikawa, K., Chiden, Y., Hayashi, M., Okamoto, M., Ando, T., Aoki, H., Arita, K., Hamada, M., Harada, C., Hijishita, S., Honda, M., Ichikawa, Y., Idonuma, A., Iijima, M., Ikeda, M., Ikeno, M., Ito, S., Ito, T., Ito, Y., Ito, Y., Iwabuchi, A., Kamiya, K., Karasawa, W., Katagiri, S., Kikuta, A., Kobayashi, N., Kono, I., Machita, K., Maehara, T., Mizuno, H., Mizubayashi, T., Mukai, Y., Nagasaki, H., Nakashima, M., Nakama, Y., Nakamichi, Y., Nakamura, M., Namiki, N., Negishi, M., Ohta, I., Ono, N., Saji, S., Sakai, K., Shibata, M., Shimokawa, T., Shomura, A., Song, J., Takazaki, Y., Terasawa, K., Tsuji, K., Waki, K., Yamagata, H., Yamane, H., Yoshiki, S., Yoshihara, R., Yukawa, K., Zhong, H., Iwama, H., Endo, T., Ito, H., Hahn, J.H., Kim, H.-I., Eun, M.-Y., Yano, M., Jiang, J. and Gojobori, T. 2002. The genome sequence and structure of rice chromosome 1. Nature 420: 312–316.

Sathasivan, K., Haughn, G.W. and Murai, N. 1991. Molecular basis of imidazolinone herbicide resistance in *Arabidopsis thaliana* var Columbia. Plant Physiol. 97: 1044–1050.

Stougaard, J. 1993. Substrate-dependent negative selection in plants using a bacterial cytosine deaminase gene. Plant J. 3: 755–761.

Tarchini, R., Biddle, P., Wineland, R., Tingey, S. and Rafalski, A. 2000. The complete sequence of 340 kb DNA around the rice *Adh1–Adh2* region reveals interrupted colinearity with maize chromosome 4. Plant Cell 12: 381–391.

Terada, R., Asao, H. and Iida, S. 2004. A large-scale *Agrobacterium*-mediated transformation procedure with a strong positive-negative selection for gene targeting in rice (*Oryza sativa* L.). Plant Cell Rep. 22: 653–659.

Terada, R., Urawa, H., Inagaki, Y., Tsugane, K. and Iida, S. 2002. Efficient gene targeting by homologous recombination in rice. Nat. Biotechnol. 20: 1030–1034.

The rice chromosome 10 sequencing consortium. 2003. In-depth view of structure, activity, and evolution of rice chromosome 10. Science 300: 1566–1569.

Thorsness, M.K., Kandasamy, M.K., Nasrallah, M.E. and Nasrallah, J.B. 1991. A *Brassica* S-locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic *Nicotiana*. Dev. Biol. 143: 173–184.

Thykjaer, T., Finnemann, J., Schauser, L., Christensen, L., Poulsen, C., Stougaard, J. 1997. Gene targeting approaches using positive–negative selection and large flanking regions. Plant Mol. Biol. 35: 523–530.

Tinland, B. 1996. The integration of T-DNA into plant genomes. Trends Plant Sci. 1: 178–184.

Tzfira, T., Frankman, L.R., Vaidya, M. and Citovsky, V. 2003. Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. Plant Physiol. 133: 1011–1023.

Tzfira, T., Rhee, Y., Chen, M.-H., Kunik, T. and Citovsky, V. 2000. Nucleic acid transport in plant-microbe interactions: the molecules that walk through the walls. Annu. Rev. Microbiol. 54: 187–219.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85: 5536–5540.

Vergunst, A.C. and Hooykaas, P.J.J. 1999. Recombination in the plant genome and its application in biotechnology. Crit. Rev. Plant Sci. 18: 1–31.

Wang, H.X., Viret, J.-F., Eldridge, A., Perera, R., Signer, E.R. and Chiurazzi, M. 2001. Positive–Negative selection for homologous recombination in *Arabidopsis*. Gene 272: 249–255.

Yamaizumi, M., Mekada, E., Uchida, T. and Okada, Y. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 15: 245–250.

Yu, J., Hu, S., Wang, J., Wong, G.K.-S., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han,

H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang, J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., Guo, W., Li, G., Liu, S., Tao, M., Wang, J., Zhu, L., Yuan, L. and Yang, H. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). Science 296: 79–92.

Zhu, T., Mettenburg, K., Peterson, D.J., Tagliani, L. and Baszczynski, C.L. 2000. Engineering herbicide-resistant maize using chimeric RNA/DNA oligonucleotides. Nature Biotechnol. 18: 555–558.

Zhu, T., Peterson, D.J., Tagliani, L., St. Clair, G., Baszczynski, C.L. and Bowen, B. 1999. Targeted manipulation of maize genes *in vivo* using chimeric RNA/DNA oligonucleotides. Proc. Natl. Acad. Sci. USA 96: 8768–8773.

Zupan, J., Muth, T.R., Draper, O. and Zambryski, P. 2000. The transfer of DNA from *Agrobacterium tumefaciences* into plants: a feast of fundamental insights. Plant J. 23: 11–28.