High-frequency gene targeting in *Arabidopsis* plants expressing the yeast *RAD54* gene

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Gene targeting, which is homologous recombination-mediated integration of an extra-chromosomal DNA segment into a chromosomal target sequence, enables the precise disruption or replacement of any gene. Despite its value as a molecular genetic tool, gene targeting remains an inefficient technology in most species. We report that expression of the yeast *RAD54* gene, a member of the *SWI2/SNF2* chromatin remodeling gene family, enhances gene targeting in *Arabidopsis* by one to two orders of magnitude, from 10^{-4} to 10^{-3} in WT plants to 10^{-2} to 10^{-1}. We show that integration events, detected with an assay based on the use of a fluorescent seed marker, are precise and germinally transmitted. These findings suggest that chromatin remodeling is rate-limiting for gene targeting in plants and improves the prospects for using gene targeting for the precise modification of plant genomes.

DNA integration | genetically modified organisms | homologous recombination

Gene targeting is inefficient in most higher eukaryotes, including plants, because the frequency of random DNA integration exceeds that of homologous integration by three to four orders of magnitude (1, 2). The number of gene-targeting events detected in early studies in plants was too small to permit a statistically reliable estimate of gene-targeting efficiency (3–8). The recent report of several additional gene-targeting events in two studies has made it possible to estimate the gene-targeting frequency with greater accuracy. The first study described a gene-targeting assay in which the endogenous *Arabidopsis protoporphyrinogen oxidase* gene was replaced by a modified gene coding for a variant of the enzyme that confers resistance to the herbicide butafenacil (9). The second study used a positive-negative selection strategy in rice tissue culture cells to select for homologous integration and against nonhomologous insertions (10). Based on the results of these and earlier studies, the targeting frequency, defined as the ratio of homologous integration to nonhomologous insertion, is between 10^{-4} and 10^{-3}. Because the transformation frequency is only 1–5%, the total number of seeds or calli that must be generated and screened is one to two orders of magnitude larger than the 10^3 to 10^4 that must be screened to detect a single homologous integration event. Thus gene targeting in plants is extremely labor intensive, requiring the screening of hundreds of thousands of seedlings (9) or large-scale tissue culture and PCR screening to identify even a small number of homologous integration events (10). More efficient gene-targeting methods must be developed if gene targeting is to become a routine genetic approach in plant biology for genetic modification of plants and analysis of the homologous recombination mechanism.

It has been reported that gene-targeting efficiency can be increased in plants by using I-SceI, a site-specific endonuclease that mediates DNA double-strand breaks, and its recognition site, which creates a recombination hotspot in the chromosome (11). However, this approach requires the prior insertion of an I-SceI recognition site into the genome. The enhancement of homologous integration is limited to the insertion site and has no effect on gene targeting elsewhere in the genome. Expression of the *Escherichia coli RecA* gene in transgenic tobacco plants stimulates homologous recombination between sister chromatids, but has no effect on gene-targeting frequency (12). Similarly, *Hyrec*, a tobacco mutation that enhances the rate of somatic crossing over between homologous chromosomes (13), does not affect gene-targeting efficiency (unpublished data). The above studies suggest that recombination pathways differ depending on the chromosomal partners involved. To enhance gene targeting, it is therefore important to identify genes that are specifically involved in recombination between extra-chromosomal DNA and a chromosomal target.

In this study, we address both of the major obstacles to developing a practical plant gene-targeting system: laborious screening procedures and the low inherent homologous recombination frequency. We show that expression of the *Saccharomyces cerevisiae RAD54* gene increases the gene-targeting frequency by one to two orders of magnitude (≈27-fold on average) in *Arabidopsis*. Moreover, we have developed an efficient, high-throughput assay to detect gene-targeting events. The assay is based on visual screening of GFP in seeds, which makes it possible to screen hundreds of thousands of seeds rapidly and therefore to easily identify a large number of gene-targeting events.

RAD54 is a member of the *SWI2/SNF2* superfamily of chromatin remodeling genes defined by the presence of conserved ATPase/helicase motifs in the proteins (14). We chose this gene because it promotes strand invasion, an essential step in recombination between homologous DNA segments in yeast (15). Moreover, it is known that disruption of the *RAD54* gene in yeast and of its homologs in mouse embryonic stem cells and chicken DT40 cells leads to reduced rates of gene targeting and increased radiation sensitivity (16, 17). We surmised that *RAD54*-like activity may be a limiting factor in gene targeting and tested this hypothesis by expressing the yeast *RAD54* gene in *Arabidopsis* plants. We report here that expression of the yeast *RAD54* gene enhances the gene-targeting frequency in *Arabidopsis* by an average of 27-fold, suggesting that chromatin remodeling is a bottleneck in homologous integration.

Materials and Methods

Plasmids. Plasmid pHS-35SRAD54 (Fig. 1*a*) was constructed to express the full-length *S. cerevisiae RAD54* gene in plants. The *RAD54* ORF was amplified with primers containing EcoRI and XbaI sites, cloned into the same sites of the pArt7 vector (18), isolated as a NotI fragment, and cloned into the same site of the pMBLArt binary vector that contains a gene that confers glufosinate (BASTA) resistance in plants (Fig. 1*a*). The gene-targeting vector, pHS-GT1 (Fig. 1*b*), was constructed to monitor gene-targeting events through visual screen of seed-expressed *GFP*. For this purpose the *GFP* was cloned in-frame with the *Arabidopsis* CRUCIFERIN gene. The N-terminal fragment

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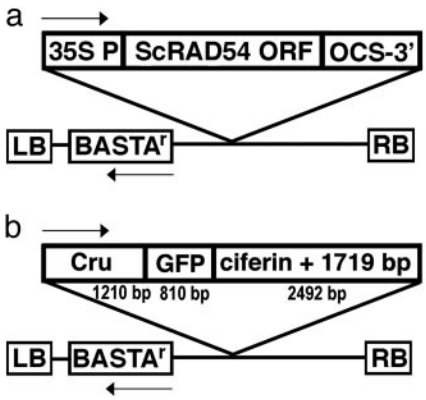

Fig. 1. Constructs used in gene-targeting experiments in *Arabidopsis*. (*a*) Construct pHS-35SRAD54 contains the ORF of the *S. cerevisiae RAD54* gene (*ScRAD54*-ORF) under the control of the cauliflower mosaic virus 35S promoter (35S PRO) and the transcription termination signals of the *Octopine synthase* gene (OCS-3′). LB, left border; RB, right border. (*b*) Construct pHS-GT1, the gene-targeting vector, contains the Cru box corresponding to the 1,210-bp genomic sequence in the 5′ end of the *Cruciferin* gene. The Cru box lacks the ATG initiation codon and 36 bp downstream of the ATG. Upstream of the Cru box is the glufosinate (BASTA) resistance gene transcribed in opposite orientation to the *Cruciferin* gene (arrows). The *GFP* reporter is fused in-frame downstream to the Cru box and is followed by the transcription termination sequence of the *Nopaline synthase* gene (NOS). A 2,492-bp fragment identical to the genomic region downstream of the Cru box flanks the GFP reporter. This fragment contains 773 bp corresponding to the 3′ end of the *Cruciferin* gene (ciferin) followed by 1,719 bp of downstream noncoding DNA. Constructs pHS-35SRAD54 and pHS-GT1 were cloned in the backbone of the T-DNA binary vector pMBLArt in between the left and right borders of the vectors (LB and RB, respectively).

lacking the ATG initiation codon (Cru) was amplified with primers containing NsiI and PstI sites. The *GFP-NOS* gene was cloned in-frame with the Cru fragment by using primers containing PstI and HindIII sites. The C-terminal fragment of the CRUCIFERIN gene (ciferin) was amplified with primers containing HindIII and SpeI sites and cloned in the same sites. All fragments were ligated into the pArt7 vector (18), isolated as a NotI fragment, and cloned into the same site of the pMBLArt binary vector that contains a gene conferring glufosinate (BASTA) resistance in plants (Fig. 1*b*).

Plant Growth and Transformation. Plants were grown in a growth chamber with a controlled environment at 19°C and a 16-hr day. The binary plasmids pHS-35SRAD54 and pHS-GT1 were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformation of *Arabidopsis* (ecotype Columbia) was carried out by the floral dip method as described (19). *Agrobacterium* cultures were grown overnight to stationary phase in LB medium at 28°C. In each T-DNA transformation experiment, the cells were concentrated to an OD_{600} of ≈1.8 in 5% sucrose and 0.5 MS salts and Silwett L-77 was added to a final concentration of 0.02% before dipping.

Seed Selection. Transformed seeds corresponding to homologous integration events were selected for fluorescence with an Olympus SZX12 stereomicroscope with a reflected light fluorescence unit URF-LT and filter sets SZX-MG for GFP. This setup is similar to that described for visualization of fluorescent seeds expressing GFP under the NAPINE promoter (20). To determine the frequency of nonhomologous integration a sample of a few thousand seeds was set apart for germination under herbicide or antibiotic selection. In the WT background, nonhomologous integration was estimated by the number of seedlings resistant to glufosinate. Six thousand seeds were germinated, and the seedlings were selected for resistance to glufosinate. Of those, 91 (1.5%) were glufosinate-resistant. A control transformation was done by using a vector (pArt27) containing a gene conferring kanamycin resistance in both the WT and *RAD54* backgrounds to test the effect of *RAD54* expression on transformation efficiency. Six thousand seeds were germinated for each genotype, and seedlings were selected for resistance to kanamycin. Of those, 105 seedlings (1.75%) were resistant in the WT background, compared with 109 (1.8%) in the *RAD54* background. Thus there was no significant difference in rates of nonhomologous integration between WT and *RAD54* backgrounds, indicating that *RAD54* does not affect the transformation efficiency.

DNA Isolation. DNA extraction was done by grinding three to four flower buds in the presence of liquid nitrogen. A volume of 0.7 ml of preheated isolation buffer (0.8 M NaCl/0.12 M Tris·HCl, pH 7.5/25 mM EDTA/0.8% cetyltrimethylammonium bromide/0.8% sarcosyl) was added to each tube. An equal volume (0.7 ml) of chloroform/octanol (24:1) was added to each tube, mixed, and centrifuged at 20,000 × *g* for 5 min in a microfuge. This step was repeated twice. Two volumes of storage buffer (75% ethanol/0.2 M sodium acetate) were added to the supernatant, and DNA was recovered by centrifugation at 20,000 × *g* for 20 min. Finally, the pellet was resuspended in 50 μl of water.

PCR Analysis. Two PCRs were performed to confirm integration of the T-DNA targeting vector (pHS-GT1) into the CRUCIFERIN locus. In both cases, one primer corresponded to the vector sequence and the other primer was from the targeted genomic locus beyond the region homologous between vector and target, as indicated in *Results*. To check integration from the 5′ end, we used the following primers: 5′-GATTCAGCACACAAAGCC, corresponding to the cruciferin promoter and 5′-GACCGGCAACAGGATTCA (with HindIII tail), corresponding to the *GFP-NOS* sequence in the pHS-GT1 vector. To check the integration from the 3′ end, we used the following primers: 5′-ATGGTGAGCAAGGGCGA (with PstI tail), corresponding to the *GFP-NOS* sequence in the pHS-GT1 vector and 5′-GATAAAACAAAAAAACAAAATAAAA, corresponding to the sequence beyond the region of homology between vector and target.

Southern Blot Analysis. Genomic DNA was extracted as described above from plants containing the pHS-GT1 vector in both WT and pHS-35SRAD54 backgrounds and digested with HindIII. The restricted DNA was fractionated on a 1.0% agarose gel, transferred to a nylon membrane (Hybond N+, Amersham Pharmacia), and hybridized overnight at 65°C by using the ^{32}P-labeled Cru and *GFP* probes as described in *Results*. The hybridization signal was obtained by using a Fuji Phosphoimager BAS2500.

Results

The yeast *RAD54* gene was expressed under the control of the strong constitutive cauliflower mosaic virus 35S promoter as described in Fig. 1*a*. Transgenic *Arabidopsis* plants expressing *RAD54*, as determined by RT-PCR (data not shown), were tested for resistance to γ-irradiation as an indicator of recombination repair efficiency. All transformants were significantly more resistant than WT plants, even when exposed to doses of irradiation (60 kRad) that are lethal to WT plants (data not shown). The most resistant transformant was selected as the line in which subsequent gene-targeting experiments were performed. Neither this nor any other transformant expressing the yeast *RAD54* gene exhibited an altered phenotypes; all grew at the same rate as their WT progenitors and were equally fertile.

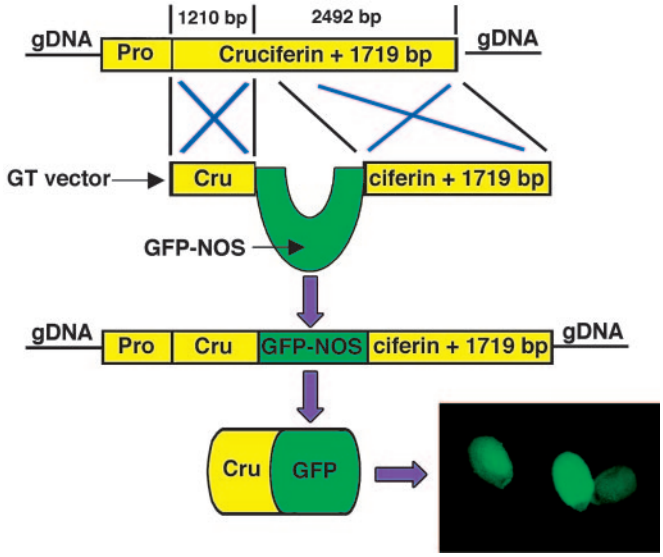

Fig. 2. The seed-based gene-targeting assay. The assay is based on the activation of GFP upon homologous integration of the gene-targeting vector (pHS-GT1, described in Fig. 1) into the genomic DNA (gDNA) of the *Arabidopsis Cruciferin* gene. Such events are identified by visualization of green fluorescent seeds under a fluorescent microscope (see black box with green seeds on the bottom right). The vector is a linear T-DNA sequence replacement vector with homology to the target *Cruciferin* gene on both sides of the vector (1,210 and 2,492 bp, respectively). The gene-targeting product gives rise to a chimeric Cru-GFP fusion protein expressed in the seed under the control of the *Cruciferin* promoter. *NOS*, Nopaline synthase.

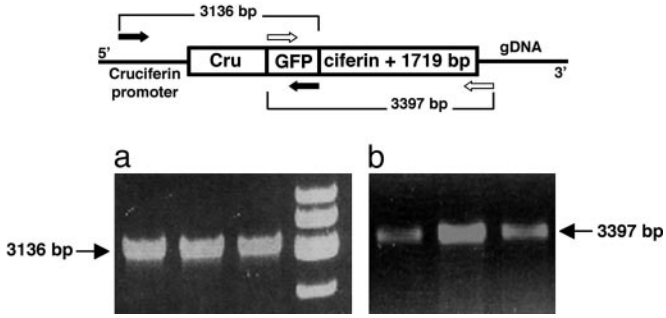

Fig. 3. Validation of gene-targeting events by PCR. To confirm the precise integration of pHS-GT1 into the *Arabidopsis* genome, we used two primer pairs: one pair (black arrows) for the 5′ border and the other for the 3′ border (white arrows) on genomic DNA template from plants derived from green fluorescent seeds. In both cases, one primer anneals to the genomic DNA beyond the gene-targeting vector, and the other primer is homologous to the GFP sequence. (*a*) The 5′ PCR product yielded a 3,136-bp fragment for a sample of 3 of the 15 plants analyzed (the right lane is a molecular weight marker). (*b*) Amplification of the 3′ integration junction of the same three plants, yielded the expected 3,397-bp fragment. The PCR fragments were sequenced, indicating precise integration from both sides of the vector.

An assay was developed to facilitate detection of gene targeting and assess the effect of *RAD54* on gene-targeting frequency. The assay is based on homologous recombination between the endogenous CRUCIFERIN gene (At4g28520) and a gene-targeting vector, pHS-GT1 (Fig. 1*b*), that contains a promoterless *GFP* ORF flanked on one side by 1,210 bp and on the other side by 2,492 bp identical to the CRUCIFERIN genomic target (Fig. 2). The outcome of homologous recombination should result in expression of *GFP* fused to the N-terminal part of the CRUCIFERIN ORF from the endogenous CRUCIFERIN promoter (Fig. 2). Cruciferin is a seed-specific storage protein, so homologous integration events should give rise to fluorescent seeds, whereas random insertions should not. To evaluate the efficiency of gene targeting, four experiments were carried out at different times, but under the same growth conditions (16-h day length, 19°C day and night). A total of 60 *RAD54*-expressing plants and 60 WT plants were transformed with the T-DNA gene targeting vector (pHS-GT1). The 1,210 bp of homologous sequence at the 5′ end of the CRUCIFERIN gene (represented by Cru in all figures) does not contain an ATG initiation codon. Nonhomologous integration of the targeting vector should not give rise to fluorescent seeds unless the T-DNA is truncated and inserted in-frame under an endogenous strong seed-specific promoter. A total of 655,000 seeds from the 60 pHS-GT1-transformed WT plants and 610,500 seeds from the 60 pHS-GT1-transformed *RAD54*-expressing plants were screened for green fluorescent seeds (Table 1). The overall transformation frequencies were the same for WT and RAD54-expressing plants, varying between 1.5% and 2% in the different experiments (see *Materials and Methods*). This finding (see *Materials* and *Methods*) indicates that *RAD54* does not affect the rate of nonhomologous DNA insertion. The gene-targeting frequency is expressed as the ratio of homologous integration (estimated by the number of green fluorescent seeds) to nonhomologous DNA insertion (estimated by transformation frequencies). The estimated gene targeting frequencies were 8.8×10^{-4} to 5.6×10^{-3} for WT plants and 5.5×10^{-2} to 1.4×10^{-1} for *RAD54*-expressing plants (Table 1). Thus the occurrence of fluorescent seeds representing putative gene-targeting events was 5- to 62-fold higher in plants expressing the yeast *RAD54* gene than in WT plants (Table 1).

The structure of the *cruciferin* allele in fluorescing seeds was determined by amplifying the DNA at the 5′ and 3′ borders of the integration site (Fig. 3). DNA was extracted from leaves of 3- to 4-week-old plants grown from these seeds. One primer corresponded to the vector sequence and the other to the

Table 1. Summary of gene-targeting experiments with the seed assay in *RAD54*-expressing and WT plants

Exp.	Plant type	No. of seeds: Total scored	No. of seeds: Transformed	No. of seeds: Fluorescent (putative targeting events)	Gene-targeting frequency (fluorescent/transformed)	Fold gene-targeting enhancement (*RAD54*/WT)
I	WT	300,000	4,500	29	6.4×10^{-3}	5
	RAD54	415,000	6,225	197	3.1×10^{-2}	
II	WT	95,000	1,425	8	5.6×10^{-3}	25
	RAD54	42,000	630	88	1.4×10^{-1}	
III	WT	225,000	4,500	4	8.8×10^{-4}	62
	RAD54	132,000	2,640	146	5.5×10^{-2}	
IV	WT	35,000	735	7	9.5×10^{-3}	18
	RAD54	21,500	451	77	1.7×10^{-1}	

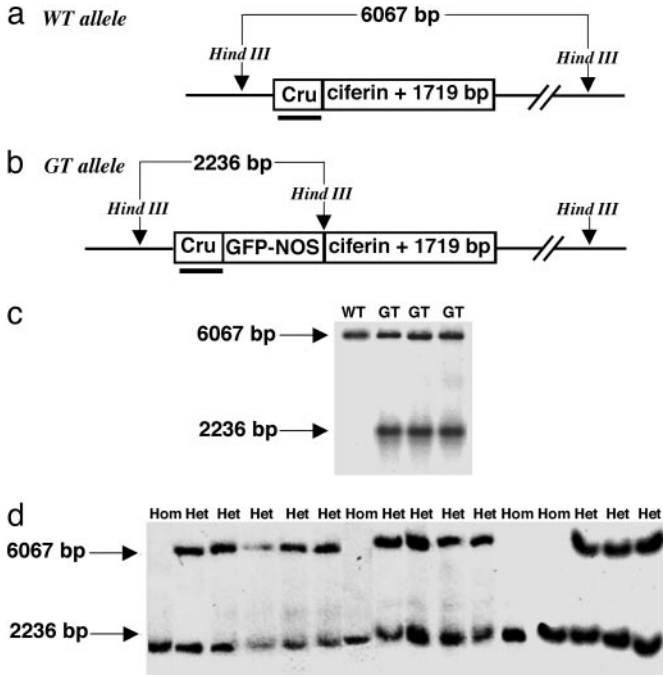

Fig. 4. Validation of gene-targeting events by Southern blot analysis. A Southern blot analysis was performed by using the Cru box as a probe (black rectangle) on genomic DNA from plants derived from green fluorescent seeds (GT) and WT seeds (WT) digested by HindIII. This analysis was done to confirm homologous integration of pHS-GT1 into the *Arabidopsis* genome from both 5′ and 3′ ends and to estimate the copy number of pHS-GT1 insertions into the genome. (*a* and *b*) The structure of the WT allele (*a*) and the targeted allele (*b*) is shown. (*c*) As expected for precise gene-targeting events, fluorescent seeds were heterozygous, showing one band (2,236 bp) corresponding to the gene-targeting allele (lane GT) and another band (6,067 bp) to the WT allele. DNA from WT seeds (lane WT) showed only the 6,067-bp band. These data suggest that plants shown in the GT lanes are heterozygote and contain a single copy of the vector at the CRUCIFERIN locus. (*d*) Germinal transmission of the targeted allele was tested by Southern blot analysis in the progeny of these heterozygous plants. The Cru probe was hybridized to HindIII-digested genomic DNA from self-pollinated progeny of the targeted plants shown in *c*. A sample of 16 plants of the 27 tested is shown. Of the 16 lanes shown, the first 12 (from the left) are from one gel and the last four are from another gel. Five of the 27 tested plants showed a single band of ≈2,236 bp, as expected for the homozygote-targeted allele (Hom lanes). The remaining 22 plants showed two bands as expected for heterozygotes (Het lanes).

targeted genomic locus, beyond the region of homology between vector and target (Fig. 3). No PCR product was expected in the WT allele, whereas products of 3,136 and 3,397 bp were expected to amplify from the 5′ and 3′ borders, respectively, for a homologous integration event in the CRUCIFERIN gene. We have analyzed four putative gene-targeting events that occurred in the WT background and 15 in the *RAD54*-expressing plants. The fragment obtained was of the expected size for a gene-targeting event in all of the plants analyzed. Eight PCR fragments, all from *RAD54*-expressing plants, were sequenced for each integration border, and all 16 junctions had the expected sequence for a precise homologous recombination event. Southern blots of genomic DNA digested with HindIII were probed with a fragment of the CRUCIFERIN gene (Fig. 4). The gene-targeting event is expected to yield two fragments, one corresponding to the size of the WT HindIII fragment (6,067 bp, Fig. 4*a*) and the other corresponding to the *GFP*-containing HindIII fragment (2,236 bp, Fig. 4*b*). Both fragments were observed in DNA samples from all of the plants grown from fluorescent seeds (Fig. 4*c*), whereas DNA from WT plants gave a single band of the expected size (Fig. 4*c*). A single band of ≈2,236 bp was observed when a labeled fragment of the *GFP* gene was used as a probe on the same blot (data not shown). Thus the results of Southern blot analysis using CRUCIFERIN (the Cru region shown in Fig. 4) and *GFP* probes, as well as the PCR and sequencing data, are those expected for the precise integration of the gene-targeting vector at the CRUCIFERIN locus to yield plants that are heterozygous for the WT and the *GFP* insertion alleles.

To determine whether the targeted allele is transmitted to the next generation, seeds were harvested from 10 self-pollinated plants grown from fluorescent seeds, and in all cases ≈75% of the seeds were fluorescent, indicating normal transmission of the GFP-containing allele through gametes. Three seeds were grown from each of the above 10 progenitor plants. Of the 30 progeny plants grown from these seeds, 27 were analyzed by Southern blot hybridization using the CRUCIFERIN probe. Five of the 27 plants, each originating from a different progenitor plant, were homozygous for the GFP-containing allele (Fig. 4*d*), further supporting the normal germinal transmission and the replacement of the WT allele by the targeted allele. The viability of the homozygote mutant might be explained by gene redundancy, because there are three cruciferin-like proteins in *Arabidopsis* and they are encoded by genes that are highly divergent at the nucleotide level (data not shown).

Discussion

This study reports an average 27-fold enhancement in the rate of gene targeting resulting from the expression of the yeast *RAD54* gene in *Arabidopsis*. An increase of this magnitude in the homologous integration frequency has not previously been reported to result from up-regulation of a component of the recombination machinery either in a plant or any other higher eukaryote. In total, 556 fluorescent seeds, representing putative gene-targeting events, were identified (Table 1). Fifteen plants grown from fluorescent seeds were analyzed by Southern blot hybridization, and all 15 gave fragments of the expected size for precise gene-targeting events. Moreover, plants homozygous for the targeted allele were derived from these plants (Fig. 4*d*). This result suggests that the most or all of the 556 fluorescent seeds contain true gene-targeting events. In addition, fragments from both sides of the integration borders were amplified from 8 of the 15 insertions and sequenced. All junctions analyzed showed precise insertion at the nucleotide level. These data contrast with those in previous reports showing that most putative targeting events are in fact classified as ectopic gene-targeting events (see ref. 9 and references therein). Precise integration of the 5′ end of the vector is expected with our assay, because seed fluorescence selected for an accurate Cruciferin-GFP protein fusion under the native genomic *CRUCIFERIN* promoter. Precise integration at the 3′ end of the vector is more surprising because it was not selected for and may result from the extended region of homology at this end.

Previous studies showing the importance of *RAD54*-like activity for gene targeting were based on knockout experiments (16, 17). The reduced rates found in knockout experiments, together with the enhancement detected in the present study by expression under a strong plant promoter, suggests that *RAD54*-like activity is a major rate-limiting factor in gene targeting in plants and also possibly in other eukaryotes. In addition, the normal growth and reproduction of transgenic plants that express *RAD54* suggests that the plant is not under a stress that activates recombination (21), suggesting that Rad54 exerts a direct, rather than an indirect, pleiotropic effect, on homologous recombination. Future experiments on the *Arabidopsis RAD54* ortholog should enable further analysis of the link between Rad54 function and gene targeting. However, given what is known about the mode of action of Rad54, these results raise a number of questions. It has been reported that the Rad54 protein

alone can mediate nucleosome movement along the DNA *in vitro* and thus might facilitate strand invasion during homologous recombination (22). The yeast Rad54 protein may itself promote gene targeting in *Arabidopsis*, either because its concentration is higher than that of the endogenous *Arabidopsis* ortholog At3g19210 (unpublished data) or its enzymatic activity differs from that of the plant's *RAD54*-like genes. On the other hand, the effect of Rad54 on homologous recombination is often attributed to its interaction with Rad51 at sites of double-strand breaks in the DNA. For example, it was shown recently that Rad54's chromatin remodeling activity is enhanced by interaction with the Rad51–ssDNA complex (22). Rad54 might therefore interact with a plant AtRad51 ortholog to stimulate homologous recombination, a possibility that seems *a priori* unlikely given the phylogenetic divergence of plants and fungi. Another possibility is that Rad54 recognizes the filament structure of the plant AtRad51–ssDNA complex. With the tools currently available, providing direct *in vivo* evidence that the reported effect of Rad54 is through chromatin remodeling may be a difficult task because the Rad54 activity is expected to be localized along the chromosome at sites of DNA double-strand breaks during strand invasion and within the egg cells where T-DNA transformation is thought to occur (23, 24). Despite the impact of gene targeting on biological research in the moss *Physcomitrella* (25), *Drosophila* (26), chicken (17), and mouse (27), very little is known about the underlying mechanisms (see refs. 28 and 29 and references therein). Results reported here suggest the possibility that Rad54 activity is a limiting factor in the gene-targeting process.

Assuming that marked enhancement of gene targeting observed in *RAD54*-expressing plants for the CRUCIFERIN gene is similar for other target loci, the present results markedly improve the prospects for the use of gene targeting as a routine analytical tool. The observed increase in gene-targeting frequency from 10^{-4} to 10^{-3} to between 10^{-2} and 10^{-1} makes it feasible to screen individual transformants for homologous insertion by using PCR. It might also be possible to further enhance gene-targeting frequency by stacking *RAD54* with additional genes and mutations that enhance homologous recombination (30–33). Gene targeting may be further enhanced by combining Rad54 expression with the induction of double-strand breaks in DNA with zinc finger nucleases (34–36). *RAD54* expression might also improve gene targeting with special types of vectors and vector deliveries, such as the ends-in vectors used in *Drosophila* (26), and facilitate the use of the type of positive-negative selection strategy recently reported for cultured rice cells (10).

In conclusion, the seed-based assay described here makes it possible to test a broad range of factors that affect gene targeting and thus to analyze the gene-targeting mechanism in plants and other eukaryotes with naturally low gene-targeting frequencies. The stimulating effect of *RAD54* reported here, together with evidence from knockout studies in other species, suggests that chromatin remodeling might be a limiting factor for efficient gene targeting. If repeated at additional loci and in different species, the present results will be directly applicable to the development of gene targeting in plants, a topic that is of great importance for both plant research and the implementation of precise genetic engineering technologies in agriculture.

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