COMMENTARY

Green light for gene targeting in plants

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Precision engineering of eukaryotic organisms requires efficient techniques for homology-based replacement of an endogenous gene by an introduced gene, a process termed gene targeting (GT). Because foreign DNA is preferentially integrated by nonhomologous recombination in plants and animals, special strategies are needed to increase the frequency and facilitate the detection of GT. Developing these strategies has proved particularly difficult in plants, for which GT efficiencies are on the order of 10^{-3} to 10^{-4} targeted events per transformed plant (1, 2). This low frequency seems to be the result of a natural barrier to integration of homologous sequences in higher plants. By contrast, efficient GT has been demonstrated in lower plants, such as the moss *Physcomitrella patens* (3).

The time appears ripe for a breakthrough in this field, and one has now been reported in this issue of PNAS. Avraham Levy and his group (4) describe a jump of between one and two orders of magnitude in GT frequency in *Arabidopsis thaliana* plants overexpressing the yeast chromatin remodeling protein Rad54. Their innovative approach was facilitated by the development of an ingenious assay that uses fluorescent seeds to identify targeted insertions.

Targeting by Breaking

Previous attempts to improve GT efficiency include the introduction of double-stranded breaks (DSBs) at the target site and modification of proteins involved in homologous recombination (HR). DSBs are obligatory recombination intermediates, and dedicated endonucleases are recruited to introduce them during meiosis. The introduction of a target site for a rare cutter restriction enzyme increased the targeting efficiency to such a site by two orders of magnitude in the somatic tissues in which GT normally takes place (5). However, the DSB has to be introduced at or close to the gene to be changed, which has been achieved in mammalian cells by using reprogrammed endonucleases whose catalytic domain was fused to a sequence-specific DNA-binding domain (ref. 6 and references therein). A site-specific zinc-finger endonuclease has been successfully employed to induce site-specific mutations by nonhomologous end-joining in *Arabidopsis* (7).

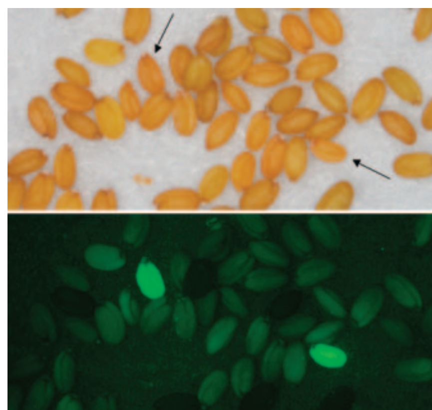

Fig. 1. Glowing seeds: The cruciferin promoter–GFP-based GT technology. (Image courtesy of A. Levy, Weizmann Institute, Rehovot, Israel.)

Most important in the context of GT is the recent achievement in tobacco of a targeting efficiency of 10% using a zinc-finger endonuclease to introduce breaks in a model target gene (D. Voytas, personal communication).

Targeting by Modifications of Proteins Involved in the Mechanics of HR

The molecular machinery involved in HR and nonhomologous recombination is largely conserved in eukaryotes and, to some extent, in prokaryotes. An obvious approach to improving the efficiency of GT in plants is to overexpress HR or heterologous recombination proteins. However, overexpression of neither the bacterial RecA protein, the molecule central to strand exchange, nor RuvC, the Holliday junction resolvase, improved GT efficiency (8, 9). Screening of *Arabidopsis* mutants for plants with increased levels of somatic HR or of radiation sensitivity for use in GT applications remains an option (discussed in ref. 10).

Now Shaked *et al*. (4) report that the *Saccharomyces cerevisiae* Rad54 protein substantially boosts GT frequency. These authors devised an ingenious GT assay by using a promoterless GFP gene inserted in an *Arabidopsis* cruciferin gene. Cruciferin is a seed storage protein; hence, integration of the GFP-containing gene into the one of the genomic genes should (and does) produce fluorescent seeds (Fig. 1). Because *Arabidopsis* plants produce many thousands of seeds, this convenient and nondestructive assay allowed the authors to identify, recover, and analyze many putative GT events. Surprisingly, Shaked *et al*. report that the GFP–cruciferin construct was correctly integrated into a resident cruciferin gene in all of the 19 plants grown from fluorescent seeds and subjected to molecular analysis. This precision is particularly remarkable because expression of the GFP gene required a single crossover to capture the cruciferin promoter, yet all analyzed insertions had the genomic sequence downstream of the GFP gene as well. Ectopic targeting, or integration by a combination of HR and nonhomologous recombination, had been reported in earlier GT experiments (11,12). Moreover, the frequency of GT increased from 0.1–1% in control plants to 3–17% in transgenic Rad54 plants, a frequency sufficiently high to form the basis of a routine genetic modification procedure. Thus, Rad54 might not only enhance targeting efficiency *per se* but might also suppress nonhomologous recombination. Either alternatively or in addition, the relatively long stretch of homology on the 3′ side of the GFP gene may lead to preferential use of the HR pathway, given that the assay does not select for restoration of the sequence downstream of the marker gene.

GT by Chromatin Remodeling?

HR, like many other DNA-related functions, depends on sequence recognition, necessitating the temporary removal of nucleosomes. The *Arabidopsis* INO80 protein is, like Rad54, a member of the Swi2/Snf2 ATPase superfamily and has been shown to be important for efficient somatic HR (13). Its yeast ortholog is part of a chromatin remodeling complex (14). Because *Arabidopsis* orthologs of the other components of the complex have been identified in the plant genome (O. Fritsch, personal communication), INO80 may have a function similar to that of the Rad54 protein. Rad54 orthologs in other organisms have also been implicated in GT. A knockout of the chicken *RAD54* ortholog substantially decreased GT in the highly recombinogenic chicken DT40 cell line (15); likewise, disruption of the

See companion article on page 12265.

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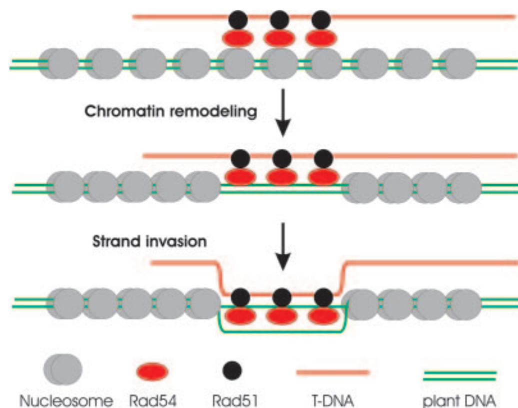

Fig. 2. Hypothetical model for Rad54-aided chromatin remodeling and GT. Shown is the chromatin remodeling activity of Rad54 stimulated by the presence of a filament consisting of single-stranded DNA and the single-stranded DNA-binding protein RAD51 (20, 21). *In vitro* data suggest the following hypothetical scenario for GT in *Arabidopsis*: T-DNA, coated by the plant ortholog RAD51, recruits yeast RAD54, which, by its capacity to interact with nucleosomes, promotes remodeling of chromatin until homology is encountered. This homology would lead to strand invasion and subsequent formation and resolution of Holliday junctions. It is not known, however, whether the yeast protein would interact with the endogenous RAD51 protein, and species specificity in this interaction has indeed been demonstrated (22). The activity of the yeast Rad54 protein in dislocating Rad51 from double-stranded DNA (22) may be essential for the later stages of the recombination reaction. (Image courtesy of T. Hohn, Botanical Institute, Basel.)

mouse *RAD54* ortholog suppresses GT (16). RAD54 proteins, especially the well characterized yeast protein, have been shown to participate in several aspects of HR (reviewed in ref. 17). Therefore, several scenarios can be envisaged for how the yeast protein might enhance GT in plants. Fig. 2 shows a model based on the assumption that the yeast Rad54 protein interacts with the Rad51 strand-exchange protein of *Arabidopsis*. An alternative explanation favored by Shaked *et al.* (4) suggests that the chromatin remodeling activity of Rad54 is permitting invasion of the incoming DNA strand without involvement of other plant proteins. Analysis of *Arabidopsis* plants overexpressing other Rad54 proteins, especially plant orthologs, will help explore this mechanism.

Challenges for the Future

The results reported by Shaked *et al.* (4) represent a major advance toward the goal of precision gene modification in plants. If increased levels of either the yeast Rad54 protein or its plant orthologs confer similar increases in GT frequency in crop plants, it will become feasible to modify gene sequences *in vitro* and replace the resident gene with the modified gene, something that is not currently possible. However, there are likely to be major hurdles ahead.

Will the results reported with the *Arabidopsis* cruciferin gene be generalizable to other *Arabidopsis* genes? It is generally believed, at least in animal systems, that the acceptor gene must be highly expressed at the moment of the targeting event. Yet egg cells, the putative targets of T-DNA-mediated transformation (18), probably do not express the seed-specific cruciferin gene. Because chromatin structures differ between active and inactive genes, it will be interesting to see whether GT efficiencies depend on the gene expression levels of Rad54-overexpressing plants. Will such techniques work in crop plants whose genomes are very much larger than that of *Arabidopsis*? Comparisons of GT efficiencies obtained in previous work in *Arabidopsis* and rice rather suggest that genome size is not limiting and therefore that enhancing chromating remodeling activity may work (1, 2).

A more serious limitation may be the difficulty of transforming crop plants. *Arabidopsis* plants are readily transformed by dipping or infiltrating plants with *Agrobacterium* strains carrying the transgene (19). This simple procedure has so far not been successful in major crop species, and the heroic efforts required to transform rice calli and screen them for GT events are far from routine (2). The choice of GT vector may also be important. In the experiments described in this Commentary, the organism used for transformation in most cases was *Agrobacterium tumefaciens*, the transforming entity of which is single-stranded DNA. Thus far, differences have not been observed in GT efficiencies between experiments using T-DNA and those using DNA transformation. It is to be hoped that the technique described here will be independent of the transformation procedure used.

Despite the many questions to be answered, the development of this new GT technology represents a crucial step in improving GT for experimental purposes. Moreover, it has the potential to increase public acceptance of plant gene modification by molecular techniques.

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