



The right chemistry for marker gene removal?

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Marker genes are indispensable for identifying rare plants that have taken up foreign DNA. Unfortunately, their presence is also often problematic for commercial biotechnology products because of consumer concerns and regulatory requirements over the presence of “excess” exogenous DNA. Over the past decade, researchers have developed several approaches for excising marker DNA¹ from transgenic plants and crops, including several site-specific recombination systems (e.g., *lox*/Cre recombinase, FLP recombination target (FRT)/Flp recombinase, or *R*s/*R* recombinase). In this issue, Zuo et al.² present a system that makes the site-specific recombination approach more convenient and applicable for a wider range of plants.

Previous approaches that exploit site-specific recombination have flanked reporter DNA with recombination sites, such as *lox*, FRT, or *R*s, which specifically interact with a recombinase protein (e.g., Cre, Flp, or *R*, respectively). This interaction promotes recombination between the sites and deletes the marker DNA from the host genome. The gene for producing the recombinase can be introduced by crossing to another plant that harbors the recombinase gene, or through a second round of gene transfer. The resulting plant then loses the marker when the recombination sites are cut by the recombinase. Thus, Cre/*lox*-mediated recombination has been applied to the removal of marker genes from nuclear DNA and more recently from plant chloroplasts (Larry Gilbertson, personal communication; Pal Maliga, personal communication).

Building on this strategy, Zuo et al.² describe a transformation strategy whereby the recombinase gene is placed under a regulated transcription system (see also ref. 3). Both the marker gene and the recombinase gene are co-introduced into the genome as a single unit flanked by recombination sites. Induction by a specific chemical brings about production of the recombinase protein, which then excises its own coding sequence and that of the linked marker, thereby producing plants free of both the marker gene and the recombinase gene. Compared with a previous report³, the induction system described in this issue shows remarkably tight

control and high recombination efficiency².

Whether or not to remove marker genes from the plant genome has been a controversial topic, but the benefits are worth considering. Removing the marker gene eliminates the safety concerns caused by the excess DNA, particularly if the DNA is derived from an unusual source. This saves costly and time-consuming risk assessment studies. It also averts consumer concerns on genes that do not offer apparent value-added properties. For example, many of the most effective marker genes confer resistance to antibiotics, and whether these genes may transmit to pathogenic microbes has already provoked a decade-long debate. Because only a small fraction of microbes can be cultured for analysis, the possibility, probability, and predicted consequences of transmission to unintended hosts will likely remain an open question.

Even more important for commercial practice, the removal of a marker gene after successful gene transfer allows its appropriate re-use in future transformation. For plants that are propagated by vegetative cuttings, introducing a second trait to an already transformed plant would require the use of a new marker gene, and yet for any particular plant, only a few markers are available. To develop a new marker gene for each new trait appended to the existing stock is not practical, not to mention the costs involved for safety evaluations of each new gene.

For sexually propagated plants, different traits can be introduced with the same marker into separately transformed lines, and then the traits combined through genetic crosses. However, the progeny with the multiple traits would end up with multiple copies of the same marker DNA, and homologous sequences can lead to gene silencing. If only the marker genes were silenced, it would be inconsequential. But should gene silencing spread to the closely linked trait gene, the whole exercise of engineering the value-added trait could be undermined. Given that future commercial products will most likely be engineered through the stacking of multiple traits, recycling the use of a marker gene,

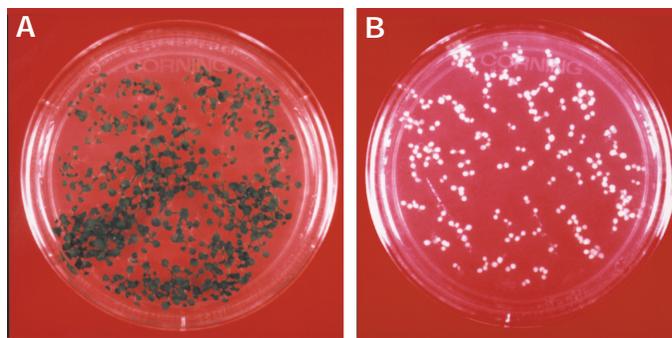


Figure 1. Chemical cutout. β -Estradiol-treated plants on (A) nonselective medium or (B) kanamycin-selective medium, where they show a complete loss of the antibiotic-selectable marker.

which necessitates its removal after each use, would be a most appropriate solution.

Besides site-specific recombination, several other marker removal methods have been employed, including homologous recombination, transposition, and co-transformation¹. Similar to site-specific recombination, spontaneous recombination between directly repeated sequences can be used to excise the intervening DNA. Alternatively, the transposition method introduces either the marker or the trait gene on a transposon. Subsequent relocation of the transposon separates the two genes. A final method, co-transformation, can also unlink the marker gene. When the two genes are introduced on separate *Agrobacterium* vectors, as many as a quarter of the transformants can be found with the marker gene at an unlinked location. Once separated from the trait gene, the marker gene can be segregated away.

Despite the various options for marker removal, each method is not without its limitations. For example, although homologous recombination operates efficiently in plant chloroplasts—and marker gene removal through this approach has recently been achieved⁴—this process is much less predictable and efficient when it comes to nuclear DNA. In the sole report for its use in deleting a nuclear marker⁵, two successive rounds of screening were needed, with an overall efficiency of less than a percent.

The transposition method can also be unreliable, in that transposition activity is highly variable among plants. When it occurs at low frequency, extensive screening would be needed to find the rare events. Moreover, the excision of a transposon from the genome can alter adjacent DNA sequences.

The co-transformation method is technically simple and has an efficiency that appears

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promising; however, when a developer typically has to screen hundreds to thousand of independent transformation events to find the rare clone with suitable field performance, there is very little incentive to quadruple that effort.

All marker excision methods (at least in their original forms), with the exception of homologous recombination, require a genetic segregation step to remove either the marker or the recombinase gene. This confines their use to plants that are propagated by sexual crosses. Even for these plants, such methods are not convenient for species with long generation times, such as trees.

Attempts have been made to adapt the transposon and the recombinase methods for asexually propagated crops¹. The occasional failure of an excised transposon to reinsert back into the genome can produce marker-free plants without a marker segregation step. Likewise, the recombinase gene can be transiently introduced to delete marker DNA without requiring its incorporation into the host genome. However, these variant methods lack adequate efficiency. Moreover, they may require the selected plant clone to undergo another round of tissue culture-induced regeneration, a step that may cause further genetic or epigenetic changes known as somaclonal variation.

In the present paper, Zuo et al. use an effective induction system. The recombinase gene is transcriptionally repressed when co-introduced with the marker gene, and subsequently activated to remove itself as well as other unneeded DNA. This system not only extends the technology to vegetatively propagated plants, but also makes the marker removal process much more convenient for sexually propagated species. Moreover, the tightly controlled induction system has the virtue that it can restrict the expression of the recombinase gene.

In petunia and tomato, the presence of a constitutive and highly expressed *cre* gene has been associated with crinkled leaves and/or reduced fertility (Mark van Haaren, personal communication)⁶. A similar, though less drastic, effect can be seen in some tobacco and *Arabidopsis* lines (Mark van Haaren, personal communication). Fortunately, the abnormal phenotype cosegregates away with *cre* DNA, so the recombinase is not likely to have caused permanent genetic changes. Because recombinases are DNA-binding proteins, it is possible that their hyperaccumulation may interfere with normal DNA activities. A recent report described infertility resulting from high *cre* expression in the spermatids of transgenic

mice⁷. Their data are compatible with an interaction between the Cre protein and host DNA, whether resulting in actual recombination, or just the formation of protein-DNA intermediates. Unfortunately, the animals are infertile, so it is not possible to determine if heritable genetic changes have been made.

Nonetheless, these findings suggest that it might be best to limit the expression of a recombinase gene to the designated recombination event. In this regard, the use of tightly regulated recombinase expression systems such as that described by Zuo et al. provides this option and advances the marker removal concept another step closer to commercial implementation.

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