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Topic 12: Marker Genes

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INTRODUCTION

Genetic engineering offers tremendous potential for creating plants with improved characteristics. Through gene transfer, novel traits can be introduced into plants that are not possible through conventional breeding. There are certain technical limitations, however, in current gene transfer methods. One is the low frequency of uptake and integration of the introduced DNA into the plant genome. Successful incorporation of this foreign DNA occurs at a rate from one in a thousand to one in a million. For this reason, the desired gene¹ is invariably co-introduced along with a marker gene. Marker genes are needed to identify genetically engineered -- referred to as transformed -- plants by conferring a new trait that is usually selectable. With selectable markers, only the plant cells that incorporate the marker-encoded trait thrive under appropriate growth conditions.

The most commonly used selectable marker genes have been those that code for resistance to herbicides or antibiotics. The antibiotic-resistance genes have been the most controversial, especially if a gene can be readily expressed and replicated in bacteria. The concern is that genes engineered into crop plants might find their way to pathogenic microbes. If so, this raises the possibility of compromising the clinical effectiveness of the drugs. Already, there has been growing medical concern about the rise in drug-resistant bacteria and the diminishing number of effective antibiotics.

Marker genes are like any other novel genes. They should be evaluated with the same safety criteria. The marker gene's product should be examined for possible toxicity and allergenicity. The potential impact from horizontal transmission of the marker gene to unintended hosts should be assessed. As each marker gene has distinct properties, each should be evaluated independently. Since toxicity, allergenicity and horizontal gene transfer considerations are addressed in topics 6, 13 and 11, respectively, they will not be a primary focus here. Instead, this article will discuss the alternatives available to limit or even eliminate the dissemination of marker genes, particularly antibiotic-resistance genes, in genetically modified plants.

OPTION FOR MARKER GENE REMOVAL

Although marker genes are necessary for the gene transfer process, most often they are not the intended traits to be introduced into the final product. In some instances, additional marker genes that were not used to detect transformed plants, but were used as part of the cloning process in bacteria, have also been introduced into the plant genome. As a by-product of the engineering process, marker genes can be considered DNA that is not essential for the final product. It may be prudent, therefore, to remove these genes after the intended traits have been engineered into the plant.

The removal of marker genes can alleviate two potential problems. First, it would eliminate the safety concerns caused by the excess DNA, particularly if the DNA is derived from a novel source. This would reduce the need for costly and time-consuming risk-assessment studies. It would also avert consumer concerns on novel genes that do not have apparent value-added properties. Second, removal of marker genes after successful gene transfer allows for the appropriate re-use of that marker gene in future work. For plants that are propagated by vegetative cuttings, such as potato, introducing a second trait to an already transformed plant would require the use of a new marker gene. For most plant species, there are only a few effective marker genes available. It would not be practical to develop a new marker gene for each new trait appended to the existing stock. For sexually propagated plants, different traits can be introduced into separate transformed lines using the same marker gene. The combination of traits can then be grouped together through genetic crosses. However, the progeny with the multiple traits would end up with multiple copies of the same marker gene. The higher gene copy number may warrant a reassessment of safety risks. In addition, multiple copies of the same DNA could lead to a gene-inactivation phenomenon known as gene silencing². If only the marker genes were silenced, it would be

¹ In this paper, the term "desired gene" refers to the intended gene(s) to be engineered into the plant.

² Wolffe, AP, Matzke MA, 1999. Epigenetic regulation through repression. *Science* 286: 481-486.

inconsequential. However, a silenced gene may also affect the expression of nearby genes³. Should this affect a desired gene, the whole exercise of engineering that value-added trait could be undermined.

Methods on marker gene removal

Recombination-based deletion of marker gene. The recombination between two identical or homologous DNA causes a deletion of the intervening DNA segment. This can occur through homologous recombination that uses host proteins, or through a much more efficient process known as site-specific recombination. The first described method for marker gene removal is based on site-specific recombination^{4,5}. Several site-specific recombination systems, known as Cre-*lox*, FLP-*FRT*, and R-*Rs*, have worked well in plants⁶. The general method consists of flanking the marker gene with recombination sites (*lox*, *FRT*, or *Rs*). A recombination site is a short stretch of DNA (typically 34 nucleotides long) that specifically interacts with a protein known as a recombinase (Cre, FLP, or R). This interaction promotes recombination between the recombination-sites and causes the marker gene to be deleted from the host genome. The gene for producing the recombinase can be introduced by crossing to another plant that harbors the recombinase gene. Alternatively, it can be introduced through a second round of gene transfer.

Once the marker gene is deleted, the recombinase gene itself can be segregated away in the next generation through conventional breeding. For plants propagated by vegetative cuttings, genetic segregation is not practical. Therefore, the recombinase gene must be introduced transiently into the transformed cell⁷, long enough to synthesize the recombinase to delete the marker gene, but without integrating into the plant genome. One strategy incorporates a second marker gene to select for the loss of both itself and the linked antibiotic-resistance gene⁸. Homologous recombination has also been successful. Unlike site-specific recombination, the introduction of a separate recombinase gene is not necessary since the recombination reaction occurs with host proteins. This makes the method suitable for plants maintained by vegetative propagation. Homologous recombination reactions occur at high efficiency with DNA in the plant chloroplast, but at low efficiency with DNA in the plant nucleus. Nonetheless, a recent advance has shown the removal of a selectable marker gene from the plant nucleus when placed between two identical DNA fragments⁹. After an initial screening procedure that picked out the transformed cells likely to lose the marker gene, about 5% of the plants regenerated from those cells indeed were marker gene-free.

Transposon-mediated relocation of marker gene. A second approach for removing marker genes relies on repositioning the marker gene from the desired gene such that it can be segregated away in the next generation¹⁰. This approach makes use of genetic elements that can excise from its original location and reinsert itself into a new location. These elements are known as transposons. Gene transfer can be conducted with the marker gene situated within a transposon. Once transformed plants are obtained, activation of the

³ Dorer DR, Henikoff S, 1997. Transgene repeat arrays interact with distant heterochromatin and cause silencing in *cis* and *trans*. *Genetics* 147: 1181-1190.

⁴ Dale EC, Ow DW, 1991. Gene transfer with subsequent removal of the selection gene from the host genome. *Proceedings of the National Academy Sciences USA*, 88: 10558-10562 (The method described in this paper is not patented. Other parties, however, may have patent rights to the site-specific recombination systems described above).

⁵ Russell SH, Hoopes JL, Odell JT, 1992. Directed excision of a transgene from the plant genome. *Molecular & General Genetics* 234: 49-59.

⁶ Reviewed in Ow DW, Medberry SL, 1995. Genome manipulation through site-specific recombination. *Critical Reviews in Plant Sciences* 14: 239-261.

⁷ Lyznik LA, Rao KV, Hodges TK, 1996. FLP-mediated recombination of *FRT* sites in the maize genome. *Nucleic Acids Research* 24: 3784-3789.

⁸ Gleave, AP, Mitra DS, Mudge SR, Morris BAM, 1999. Selectable marker-free transgenic plants without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. *Plant Molecular Biology* 40: 223-235.

⁹ Zubko E, Scutt C, Meyer P, 2000. Intrachromosomal recombination between *attP* regions as a tool to remove selectable marker genes from tobacco transgenes. *Nature Biotechnology* 18: 442-445.

¹⁰ Goldsbrough AP, Lastrella CN, Yoder JI, 1993. Transposition mediated repositioning and subsequent elimination of marker genes from transgenic tomato. *Bio/Technology* 11: 1286-1292.

transposon can relocate the marker gene to a distant position in the genome. The desired gene can then be segregated away from the marker gene in the next generation. For plants that are propagated as vegetative cuttings, a variation of this scheme has been described that enriches for the number of incomplete transposition events in which the transposon excises but fails to reinsert back into the genome¹¹. When this happens, a segregation step to remove the marker gene would not be needed.

Co-transfer with marker gene on separate vector. A third method for generating marker-free transgenic plants is to use separate gene transfer vectors for the desired gene and the marker gene¹². Upon selecting for the marker-encoded trait, a certain percentage of the transformed cells would have also taken up the desired gene. Among those transformed with both the desired gene and the marker gene, some could have the two genes on separate locations in the genome. In these clones, the marker gene can be segregated away in the next generation. This is probably the simplest method to obtain the desired product, and should be more convenient as improvements are made in transformation efficiency. With a higher efficiency of DNA delivery and integration, the probability of finding multiple gene transfer events in the same cell becomes more likely.

ALTERNATIVES TO ANTIBIOTIC-RESISTANCE GENES

Although antibiotic-resistance genes have been among the most effective markers for genetic transformation, other genes have also been used. Herbicide resistance genes have served the same purpose in many instances, not simply when the plants were specifically engineered for herbicide tolerance. In recent years, a number of new marker genes have been developed. One selectable marker gene, the tryptophan decarboxylase gene, enables the transformed cell to survive by converting a toxic tryptophan analog to a non-toxic form¹³. Another selectable marker, the β glucuronidase gene, converts an inactive cytokinin derivative to an active cytokinin¹⁴. Only the transformed cell will have sufficient cytokinin hormone for cell division. Other selection schemes are based on the use of special carbon sources. Cells transformed with a xylulose isomerase gene or a phosphomannose isomerase gene, for instance, can metabolize xylose or mannose, respectively^{14,15}. The new trait permits them to grow on carbon sources while other cells cannot. A recent strategy is based on the inducible expression of the isopentenyl transferase gene that leads to higher cytokinin levels¹⁶. Under appropriate culture conditions, only transformed cells regenerate shoots. Finally, two visual markers have been successful for wheat¹⁷ and sugarcane¹⁸. Cells harboring either the firefly luciferase gene or the jellyfish green fluorescent-protein gene can emit light. The transformed cells were monitored for light output over a period of weeks. The cells that continued to emit light were regenerated into plants and were shown to have incorporated the introduced DNA.

¹¹ Ebinuma H, Sugita K, Matsunaga, Yamakado M, 1997. Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proceedings of the National Academy Sciences USA*, 94: 2117-2121.

¹² Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T, 1996. Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of the transformants free from selection markers. *Plant Journal* 10: 165-174.

¹³ Goddijn OJ, van der Duyn Schouten PM, Schilperoort RA, Hoge JH, 1993. A chimaeric tryptophan decarboxylase gene as a novel selectable marker gene in plant cells. *Plant Molecular Biology* 22: 907-912.

¹⁴ Joersbo M, 1997. New selection systems for plant transformation using cytokinin glucuronides, xylose or mannose. In: *Proceeding of the Nordic Seminar on antibiotic resistance marker genes and transgenic plants*. June 12-13, 1997, Oslo, Norway, pp. 69-75.

¹⁵ Haldrup A, Peterson SG, Okkels FT, 1998. The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. *Plant Molecular Biology* 37: 287-296.

¹⁶ Kunkel T, Niu Q-W, Chan Y-S, Chua N-H. 1999. Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. *Nature Biotechnology* 17: 916-919.

¹⁷ Lonsdale DM, Lindup S, Moisan LJ, Harvey AJ, 1998. Using firefly luciferase to identify the transition from transient to stable expression in bombarded wheat scutellar tissue. *Physiologia Plantarum* 102: 447-453.

¹⁸ Elliott AR, Campbell JA, Brettell RIS, Grof CPL, 1998. *Agrobacterium*-mediated transformation of sugarcane using GFP as a screenable marker. *Aust. J. Plant Physiol.* 25: 739-743.

LIMITATIONS OF EXISTING METHODS

Despite the various options available to construct transgenic plants without the use of antibiotic-resistance genes, it is important to note that the process of genetic transformation differs among different plant species. A given method may be applicable for some plants, may require substantial modifications for others, or may not be applicable at all in certain species. This section discusses some of the limitations of the available methods.

The site-specific recombination approach is most likely to be widely applicable. Collectively, the three known site-specific recombination systems, *Cre-lox*, *FLP-FRT*, and *R-Rs* have been shown to operate in a wide range of plant species including the major cereals^{6,7,19}. New recombination systems are also being developed. The known recombination systems all operate at efficiencies that are practical. When the recombinase gene is introduced into a plant, the efficiency of marker gene removal is usually between 10% to 100%. For plants that must be maintained as vegetative cuttings, however, the strategy of transiently expressing the recombinase gene must be followed by moderate to extensive screening of the treated cells or plants. With some plants, the frequency may be too low for this technique to be practical. On the positive note, there are inducible expression systems, such as the heat-induced expression of the recombinase gene²⁰, that could be developed further to direct the deletion of both the marker gene and the recombinase gene from the host genome. This would bypass the otherwise obligate step of genetic segregation.

Besides site-specific recombination, homologous recombination has also shown promise. With DNA located in the plant nucleus, however, the low recombination frequency makes this approach less practical. The potential use could lie in the removal of marker genes from the chloroplast genome, where the efficiency of homologous recombination is comparable to recombinase-mediated reactions. As more plants are being engineered to express the desired genes from the chloroplasts²¹, both types of recombination reactions could play prominent roles in the management of chloroplast transgenes.

The transposon approach also has some limitations. The excision of the transposon from its original location is imprecise and may alter the structure of nearby genes. The transposition efficiency is also highly variable among plants species. If the transposon relocates at a low frequency, as in the case with lettuce²², the extensive screening needed to find the rare events would make the technique impractical.

The successful co-transformation by separate vectors is dependent on the overall transformation efficiency. In plants that are relatively easy to transform, the probability that two separate vectors delivering DNA into the same cell--but in different locations--may be sufficiently high to serve the purpose of removing marker DNA. In plants that are relatively difficult to transform, this occurrence may not be as prevalent. A second limitation is that the marker gene must be removed by genetic segregation. Finally, the co-transformation procedure is effective only with *Agrobacterium* mediated gene transfer. With direct DNA uptake methods, the separation of the desired gene from the marker gene in different vectors does not produce independent insertions of each DNA type. More often than not, the separate molecules link up efficiently prior to integration into the genome. This results in a complex integration structure with long stretches of linked molecules. In such a situation, it would not be possible to segregate the desired gene from the marker gene. It is of interest to note that for these complex integration structures, site-specific recombination not only removes the marker genes, but will also reduce transgene copies to a single unit¹⁹.

As for the non-antibiotic resistance markers, herbicide resistance genes are useful only if that trait is desired in the final product. Other selectable markers that rely on differences in hormone concentrations or nutritional requirements may require adaptation for different plant species, as hormone and nutrient requirements in cell growth and regeneration vary among different plants. Should the non-antibiotic

¹⁹ Srivastava V, Anderson OD, Ow DW, 1999. Single-copy transgenic wheat generated through the resolution of complex integration patterns. *Proceedings of the National Academy of Sciences USA* 96: 11117-11121.

²⁰ Lyznik LA, Hirayama L, Rao KV, Abad A, Hodges TK, 1995. Heat-inducible expression of *FLP* gene in maize cells. *Plant Journal* 8: 177-186.

²¹ Maliga P, 1993. Towards plasmid transformation in flowering plants. *Trends in Biotechnology* 11: 101-107.

²² Yang CH, Ellis JG, Michelmore RW, 1993. Infrequent transposition of *Ac* in lettuce, *Lactuca sativa*. *Plant Molecular Biology* 22: 793-805.

resistance markers be used without provisions for their subsequent removal, the markers would remain in the host genome. Although they may not create the concerns associated with antibiotic-resistance genes, future complications could arise when successive experiments combine multiple traits into the same plant. As described above, this would lead to a genome with multiple marker genes or multiple copies of the same marker gene.

THE ALARA CONCEPT

The issue of antibiotic-resistance genes in plants has been contentious in scientific circles^{23,24,25}. Some concerns expressed about the dissemination of antibiotic-resistance genes are difficult to address, given that our knowledge of the science is not fixed in time, but is an evolutionary process in itself. Our evaluation of acceptable risk could also change. In addition, depending on one's point of view, there will always be a certain degree of subjective interpretation to risk-benefit arguments. Likewise, transformation technology will continue to evolve, bringing improved efficiency and precision. The advancement of new science and technology directs us to the concept often applied in risk management -- to reduce the risk of concern to **As Low As Reasonably Achievable**, or the ALARA concept.

The key issue that needs addressing is what is considered "reasonably achievable". A decade ago, when technology became available to remove marker genes from the final product, industry already had a number of transgenic plants in their developmental pipeline. There was no way the antibiotic-resistance genes could be removed from those plants because they were engineered prior to the availability of the technology. Since then, additional alternatives have emerged. Reducing the dissemination of antibiotic-resistance genes can now be considered "reasonably achievable" for many crop plants. For other plants, additional research is still needed to make these methods more practical. The comforting thought for this particular issue is that in the not too distant future, it should be possible to reduce the risk, however insignificant, down to zero.

To conclude, this author's views have not changed since the initial article on this subject⁴. Our society has limited resources for scientific research, risk management, public relations, litigation, and legislation. Those resources should be devoted to the trait genes that impart greater benefits to our society, not to leftover marker genes that are merely unneeded byproducts of yesteryear's genetic transformation technology.

²³ Flavell RB, Dart E, Fuchs RL, Fraley RT, 1992. Selectable marker genes: Safe for plants? *Bio/Technology* 10: 141-144.

²⁴ Bryant J, Leather S, 1992. Removal of selectable marker genes from transgenic plants: needless sophistication or social necessity? *Trends in Biotechnology* 10: 274-275.

²⁵ Goldsbrough A, 1992. Marker gene removal: A practical necessity? *Trends in Biotechnology* 10: 417.