Nontransgenic crops from transgenic plants

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It is possible to produce nontransgenic crops from transgenic plants. The transgenic sequence is deleted by tissue-specific or chemically induced excision from plants in the field. An inducible promoter drives expression of a gene for a site-specific recombinase, such as Cre, which excises a transgenic cassette located between two recombinase sites (*loxP*). Inducible promoters and recombinases that work well in plants have already been developed. While retaining the benefits of transgenic technology, the removal of transgenes from specific tissues in crop plants may be a pragmatic way to address certain public concerns regarding transgenic technology, such as food safety, gene dispersal (via pollen or seed), seed replanting, and identity preservation.

In the current generation of transgenic plants, transgenes are typically expressed throughout the plant. Such continuous and ubiquitous expression has several drawbacks (e.g., yield drag) and is not always necessary; often the transgene is required only in a subset of tissues within the plant. In nematode control, for example, expression of the resistance trait is required only in the roots. Thus in soybeans engineered for nematode resistance, with the root as the target tissue, the transgene need not be present in the edible portion of the plant. Similarly, plants containing transgenes encoding input traits, such as resistance to insects, fungi, nematodes, herbicides, and certain output traits may not have to contain these genes in the pollen, seed, or fruit.

Although tissue-specific promoters are increasingly applied to regulate the expression levels and tissue distribution of a transgene in the plant, the transgenic DNA remains present throughout the plant. Current UK GMO policy[1] favors the absence of transgenic material—in particular from the edible parts of a crop, from pollen, and from seed. We propose to conform to this policy by using gene deletion technology, excising transgenes when and where desired.

Several groups have published strategies for removing marker genes (e.g., antibiotic resistance genes) from crops using recombinases, but the possibility of creating nontransgenic plants has surprisingly not been addressed[2,3]. Zuo *et al.*[3] have demonstrated the efficient removal of marker genes from plants using the Cre/*loxP* site-specific recombinase system. We propose that such a system can be generalized to remove all transgenic DNA to generate nontransgenic plant tissues that otherwise retain all of the benefits of transgenic plants.

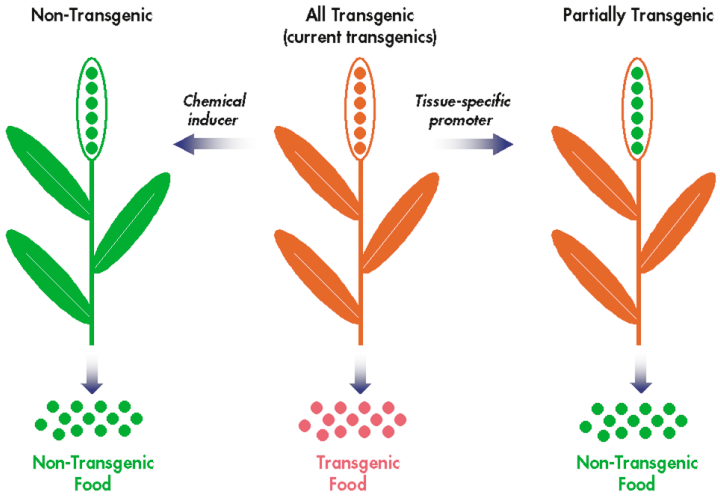

Figure 1. In the current generation of transgenic crops, every tissue of the plants is transgenic, including the fruit. In the proposed approach, transgenic plants are used to grow nontransgenic fruit by excising the transgenes from the fruit with a site-specific recombinase. Expression of the recombinase is controlled using a chemically inducible promoter or a fruit-specific promoter.

The basic design of a general gene deletion cassette comprises three elements (see Fig. 1). First, a tissue-specific or chemically inducible promoter drives the expression of a site-specific recombinase. Second, a separate promoter drives expression of the desired trait. Third, two site-specific recombinase sites flank the entire cassette. Induction of the tissue-specific or chemically inducible promoter drives expression of the recombinase, resulting in excision of the transgenic cassette from the genome. Additional levels of complexity,

Potential benefits of transgene deletion

Perceived food safety. The presence of transgenes and their encoded proteins in food products is perceived as a potential safety concern for GM foods. Transgene deletion would mitigate these concerns and increase consumer confidence.

Gene dispersal. As in some crops pollen drifts over long distances to cross-fertilize closely related wild or cultivated nontransgenic plants, the transmission of traits such as herbicide resistance to create "superweeds" has been a concern with regard to GM plants. The use of a pollen- or seed-specific promoter to control transgene excision could reduce the possibility of unwanted gene dispersal.

Replanting. Gene deletion provides farmers with the ability to replant nontransgenic seeds that they harvest from their transgenic plants. For situations in which it is desirable to retain the transgene (e.g., seed production), chemically induced excision could simply be avoided by not applying the inducer. Alternatively, excision induced by seed- or pollen-specific promoters could be prevented by placing them under the control of a chemically induced inhibitory sequence (applicable at any appropriate stage in plant development) or by producing transgenic seed from parental lines in which parent A contains recombinase B and parent B contains recombinase A, allowing gene excision only in the hybrid.

Identity preservation. The need to separate transgenic from nontransgenic crops after harvest to satisfy consumer choice is time-consuming, costly in itself, and a source of expensive food recalls when mix-ups occur (e.g., StarLink corn). Transgene deletion strategies would eliminate the need for identity preservation.

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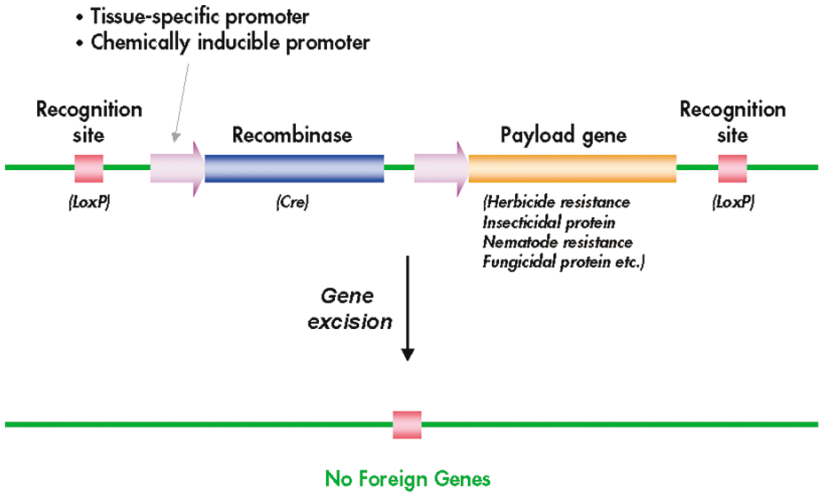

Figure 2. The transgenic cassette contains a tissue-specific or chemically inducible promoter driving a recombinase gene (such as Cre) and a second promoter driving a payload gene encoding the trait of interest. This cassette is flanked by recognition sites for the recombinase (i.e., *loxP* sites). Most excision systems leave behind a single recognition site. Removal of all foreign DNA sequences would require the recognition site to consist of native DNA sequence or the cut-site to be outside of the recognition sequence.

such as multiple traits, stacked promoters, and multistep regulated switches, could be added as needed.

In the field, an environmentally friendly chemical could be delivered to the plant to induce expression of the recombinase, leading to excision of the transgenic cassette throughout the entire plant (see Fig. 2). Alternatively, a fruit-specific promoter could be used to eliminate the transgene selectively from the food portion (corn, soybean, potato, etc.), while allowing the trait to be present and expressed in the remainder of the plant (Fig. 2). Similarly, a pollen-specific promoter driving the recombinase could be used to excise transgenes selectively from pollen, thereby reducing the potential spread of transgenes through cross-pollination of nontransgenic plants.

After gene excision, three potential sources of transgenic residue remain. First, excision may not be completely effective. While the excision described by Zuo *et al.*[3] is highly efficient, for other crops the recombinase system may have to be re-optimized, such as by DNA shuffling. It is also possible that a chemical inducer would not uniformly penetrate the plant tissue upon treatment, or that a fruit-specific promoter would not be induced in all tissues of the fruit, resulting in a low level of transgenic DNA remaining in the product.

Second, even if excision is 100% efficient, a small amount of nonnative DNA—comprising the recombinase recognition site (32 base pairs for *loxP*)—will remain. Because even a few bases of nonnative sequence would be detectable by PCR, methods need to be developed that result in the removal of the entire foreign sequence, including the recognition sequence. One approach is to use recombinases that recognize related native plant sequences ("pseudo-sites"). Two groups have evolved recombinases by DNA shuffling to recognize native sequences[4–6]. Alternatively, one could use or evolve enzymes that cut outside of their recognition sequence.

Third, while excision of transgenic DNA stops the expression of transgenic protein, any previously expressed protein may degrade so slowly that it remains detectable in the product after harvest and may still pose a safety issue. Current regulatory protocols favor transgenic proteins (including the recombinase itself) that are rapidly degraded, and place an emphasis on engineering proteins that are destabilized so that they do not linger in the plant.

By analogy to the current situation with pesticides, some level of transgenic DNA "residue" might be acceptable in a transgenic food. A critical question is what an acceptable level would be.

The current concerns regarding transgenic plants create barriers to their widespread acceptance. Gene deletion effectively addresses many of the primary concerns in a way that allows access to the tremendous benefits of transgenic technology.

1. http://www.environment.detr.gov.uk/acre/bestprac/index.html.
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3. Zuo, J., Niu, Q.W., Moller, S.G. & Chua, N.H. *Nat. Biotechnol.* **19**, 157–161 (2001).
4. Thyagarajan, B., Guimaraes, M.J., Groth, A.C. & Calos, M.P. *Gene* **244**, 47–54 (2000).
5. Buchholz, F. & Stewart, A.F. *Nat. Biotechnol.* **19,** 1047–1052 (2001) .
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