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GM maize from site-specific recombination technology, what next?

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The term plant genetic engineering has long conveyed a highly efficient and precise process for the manipulation of plant genomes. For nearly two decades, research on recombinase-based applications has steadily advanced the surgical capabilities of plant genome rearrangements. Once considered interesting laboratory exercises, a first crop plant derived from this type of DNA acrobatics is heading to market. Originally configured for a specific application, to remove a selectable marker, it could be the first of more to come — and not just market-free plants.

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Introduction

Just last year, Renessen, a joint venture between Cargill and Monsanto, received US regulatory approval for LY038 [1]. Marketed under the trade name Mavera™ High Value Corn with Lysine, LY038 is targeted for the poultry feed industry [2]. LY038 is derived from the biolistic transformation of maize to incorporate a *cordapA* gene that directs the seed-specific production of a lysine-insensitive dihydrodipicolinate synthase enzyme. The kanamycin resistance marker used for transformation, *nptII*, was subsequently removed by site-specific recombination in a process described in model plants some 15 years earlier [3,4]. In the transformation vector, *nptII* was flanked by directly oriented *lox* sites from the Cre-*lox* recombination system (Figure 1a). Upon recovery of the desired transformant, a *cre* gene was introduced into the genome from a genetic cross. Cre recombinase excised away the *lox*-flanked DNA, and the *cre* gene was then removed by genetic segregation. With a decade-long development timeline for genetically modified (GM) maize, the decision to incorporate a recombinase-mediated marker removal step must have been made in the mid-1990s. Since that time, new advances and concepts have emerged in recombinase-based applications. As most commercial products improve over time through new innovations, this article describes how additional features can be incorporated into this first crop product derived from site-specific recombination.

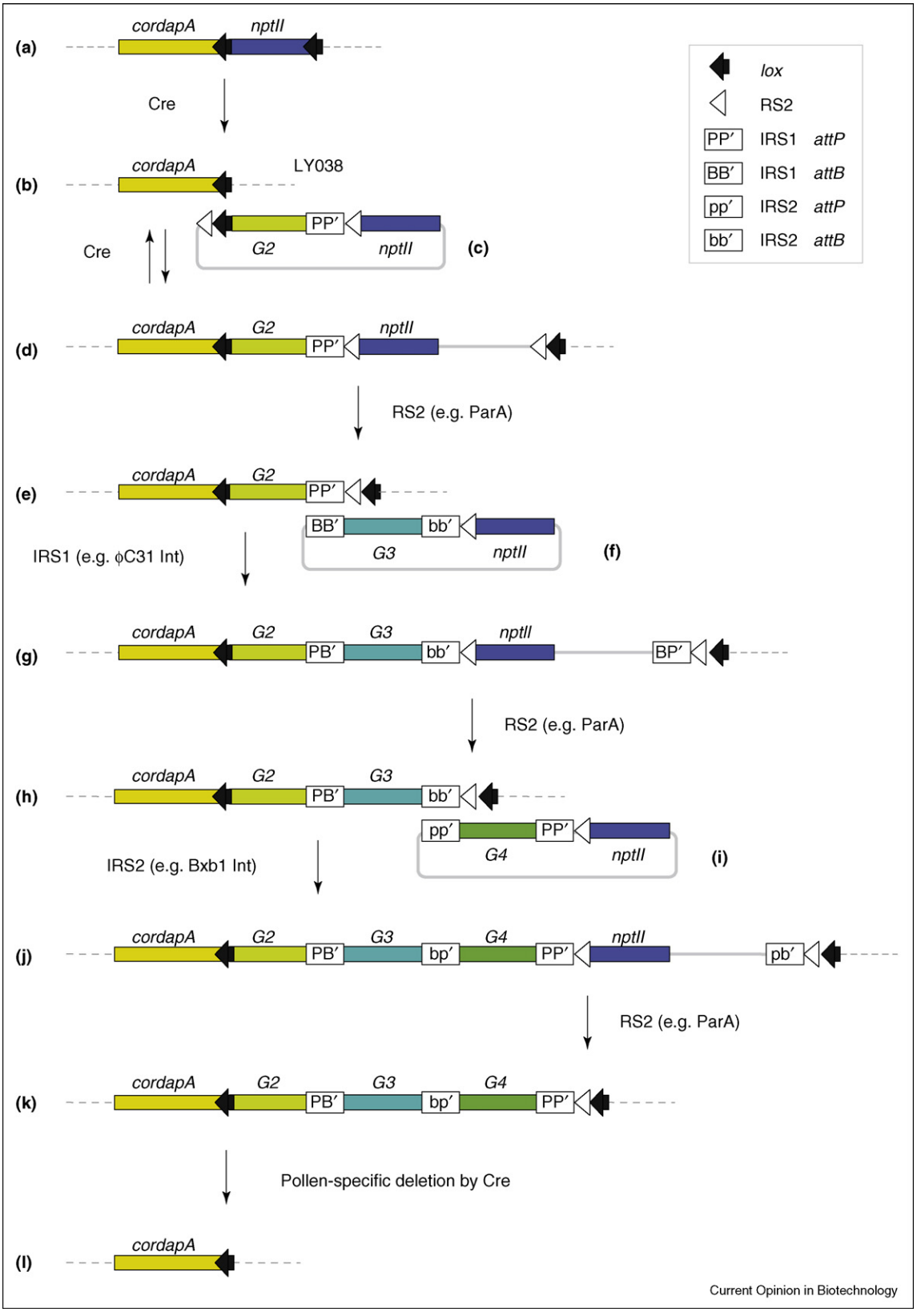
Not just marker removal

The recombinase-based marker removal strategy has received a resurgence of interest in recent years [5–31]. One noteworthy development is the conditional expression of recombinase genes that can induce deletion of not just the marker, but also of the recombinase gene itself [9,10,14,15]. For plants that are propagated sexually, the crossing in and out of the recombinase gene is not a problem, as transformants go through some back-crossing anyhow to test heritable expression as well as to 'clean up' the genetic background. But, for plants that are propagated asexually, or for those with long generation times, genetic segregation is impractical.

In product development, the foremost objective is to obtain effective and stable transgene expression or 'trait efficacy'. Variable levels of expression among individual transformation events have been widely observed. Although integration locations, or 'position effects', have received much of the blame, one wonders whether a far greater share might be attributed to differences in the transgene integration structure. Many transformation methods yield a high percentage, as high as 90% in biolistic transformation, of multicopy insertions that are also often associated with reduced or unstable transgene expression. The industrial solution to this problem has been the brute-force screening of a sufficiently large pool of independent transformants (up to thousands) for the few with suitable expression levels along with a single-copy structure.

In the development of LY038, one can only surmise that the selected transformation event was obtained through such a brute-force screen before the marker removal step. If the same engineering feat were to be conducted today, an inducible *cre* gene could be considered. Co-introduced along with the trait gene, with both *cre* and *nptII* flanked by directly oriented *lox* sites, site-specific recombination induced after the selection of transformed plants would remove the marker and *cre*. Additionally, the same recombination process would remove excess transgene copies [32–34] that not only would reduce the high background of undesired multicopy insertions, but through conversion should also increase by a corresponding amount the plants that harbor simple integration patterns. This would

Figure 1


(a)
cordapA
nptII
Cre
(b)
cordapA
LY038
Cre
PP′
G2
nptII
(c)
(d)
cordapA
G2
PP′
nptII
RS2 (e.g. ParA)
(e)
cordapA
G2
PP′
BB′
bb′
G3
nptII
(f)
IRS1 (e.g. ϕC31 Int)
(g)
cordapA
G2
PB′
G3
bb′
nptII
BP′
RS2 (e.g. ParA)
(h)
cordapA
G2
PB′
G3
bb′
pp′
PP′
G4
nptII
(i)
IRS2 (e.g. Bxb1 Int)
(j)
cordapA
G2
PB′
G3
bp′
G4
PP′
nptII
pb′
RS2 (e.g. ParA)
(k)
cordapA
G2
PB′
G3
bp′
G4
PP′
Pollen-specific deletion by Cre
(l)
cordapA
lox
RS2
PP′ IRS1 attP
BB′ IRS1 attB
pp′ IRS2 attP
bb′ IRS2 attB
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be similar to the reactivation of transgene expression upon genetic segregation of unlinked extra copies [35•]. An expectation would be that far fewer individual transformants would be needed to find the 'good expressor' lines, and expression differences would be more likely to result from genome location than from transgene structure. This strategy could be particularly useful for transformation-recalcitrant plants, which include many field cultivars of otherwise transformation-competent species. Obtaining thousands of independent transformants in these plants would require substantial advances in tissue culture methodology.

LY038 as a target line

Whether or not it was their intention, the developers of LY038 have created in this transformation event a target line for the stacking of additional traits through site-specific integration. The excision of *nptII* left behind a single *lox* site that can serve as a target for an integrating molecule (Figure 1b); circular DNA harboring a *lox* site can integrate into a genomic *lox* target. The use of *Agrobacterium* to integrate a circular molecule in *Arabidopsis* was successful but inefficient: about 1% of the recovery rate for random integration [36,37]. With free DNA delivery in tobacco protoplasts and in rice biolistic transformations, the recovery rates of selected events were often comparable to conventional transformation [38–40,41•]. About one sixth to a half of the recovered clones were found to be site-specific single-copy precise insertions that also yielded predictable and heritable levels of expression [35•,39,41•]. Granted the strategies used in most studies were tipped in their favour; most experiments were conducted using mutant *lox* sites which, when recombined, are less likely to excise the integrated molecule. Moreover, a promoter-exchange scheme that disconnects a promoter from the *cre* gene and fuses it instead to a selectable marker in the integrating DNA was frequently used, which eliminates most random insertions that fail to capture a genomic promoter.

The single *lox* site in LY038 is of the wild-type variety (*loxP*). Had *nptII* been flanked by one *loxP* and one mutant *lox* site, such as *lox75* or *lox76* [38], the excision of *nptII* could have left behind a mutant *lox* site for more efficient site-specific integration [40]. Nonetheless, the genomic *loxP* target is still usable. The insertion of circular DNA into a genomic *loxP* target in tobacco and *Arabidopsis* was successful when *cre* was introduced as a co-transforming molecule [37,38]. Because *cre* is available transiently, the loss of Cre activity allows integrated molecules to remain in place. With the use of *loxP* as a target, and without a selection scheme that deselects most random insertion events, site-specific integration into the LY038 locus would occur with a lower efficiency than with using mutant *lox* sites in a promoter-exchange scheme. Nonetheless, for several reasons this approach may still be preferable to inserting new DNA at a random location. First, it can be done. Despite lower efficiency, the event can be found by PCR screening for predicted integration junctions. Should some of the site-specific integrants yield good expression, it would save an otherwise enormous effort to search for another chromosome location that provides effective expression without a penalty in yield. Second, the LY038 integration locus has already gone through regulatory review, implying that the genetic disruption does not cause undesirable side effects. Stacking a new trait to this locus should simplify both the characterization of the insertion by developers and the review of its environmental safety by regulators. Third, stacking genes next to each other should expedite the breeding program. If 10 stack traits were unlinked, the breeding of the collection to new germplasm would require the improbable co-assortment of all 10 independent loci into the same genome. By contrast, if 10 traits were stacked into a single locus, the transmission of all 10 traits in an outcross is at worst 50% (from a hemizygous donor).

LY038 can be retrofitted

To transform new DNA into the LY308 locus would need selection and, if the marker DNA were again to be removed, a different recombination system would be needed, such as FLP-*FRT* [5] or R-*RS* [9] (where FLP and R are the recombinases and *FRT* and *RS* the respective recombination sites). The excision process would leave behind a *FRT* or *RS* site for another round of integration. The problem with this type of gene stacking strategy is the continual need to develop new recombination systems, a taxing endeavor for the engineering process as well as for the regulatory review. A practical gene-stacking strategy must limit the number of recombination systems by recycling their use [42].

The LY308 locus can be retrofitted to make use of an Int-*att* type of irreversible recombination system (IRS). In these systems, a recombinase, generally known as an integrase (Int), acts on two recombination sites that differ in sequence, typically known as attachment sites *attB* (Figure 1 depicts BB′ or bb′) and *attP* (PP′ or pp′), to yield

(Figure 1 Legend) Engineering prospects for a crop derived from marker removal. **(a,b)** Schematic representation of marker removal to yield LY038. **(c–e)** Proposed retrofitting of LY038 for gene stacking. **(e–k)** Proposed gene stacking via site-specific recombination. **(k,l)** Proposed selective deletion of stacked traits. New traits (shown as *G2, G3, G4*) are appended sequentially into the target locus as they become available. Recombination sites for the Cre-*lox* system (*lox*), the second recombination system (RS2), and the *attP* and *attB* sites of the first and second irreversible recombination systems IRS1 and IRS2 are depicted by the symbols shown in the inset drawing. The use of IRS2 is optional and can be substituted by the corresponding recombination sites from IRS1. Examples given for RS2, IRS1 and IRS2 systems are ParA, ϕC31 and Bxb1, respectively. The insertion of the *G2* molecule (b–d), which is easily reversed, relies on the loss of transient Cre activity to prevent its excision.

product sites known as *attL* (BP′ or bp′) and *attR* (PB′ or pb′). The reaction can be excision, inversion or integration, and is not reversible unless an additional protein (an excisionase) is provided. Functional recombination in plants reported in the refereed literature includes the ϕC31 [43•,44•] and the HK022 [45] recombination systems. The Bxb1 system that is active in other eukaryotic cells [46,47] is also active in plants (JG Thomson, DW Ow, unpublished data).

Figures 1c–k depict one retrofit scheme for the LY038 locus. The circular DNA bearing the second trait gene (*G2*) is configured with a *lox* site for recombination with the genomic *loxP* target (Figure 1c). It also harbors a pair of recombination sites from a second recombination system (RS2) so that again the unnecessary DNA can be deleted following successful integration. RS2 can represent FLP-*FRT*, R-*RS* or any of the new deletion systems, such as ParA [48•], in which site-specific recombination leaves behind a site of the same sequence that can be reused for subsequent excision events. To permit the next round of site-specific recombination, the molecule must also bring along a new target, as in this example, a ϕC31 *attP*. Upon integration of the *G2* molecule, facilitated by a transiently introduced *cre* gene, the expected structure is shown in Figure 1d. The DNA that is no longer needed, *nptII* and the plasmid backbone, is flanked by directly oriented RS2 sites and can be removed by the RS2 recombinase to yield the structure shown in Figure 1e. As in the Cre-mediated excision of *nptII* (Figures 1a,b), this step can be conducted by the familiar crossing in and out of the recombinase gene.

The single *attP* site in Figure 1e serves as the target for integration of the gene 3 (*G3*) molecule (Figure 1f), which harbors a corresponding *attB* site for integration into the locus. In addition, the *G3* molecule brings a second *attB* site as a target for the next round of insertion. The second *attB* can be from the same Int-*att* recombination system, in which case site-specific integration can proceed via either *attB* site to yield two possible structures. Alternatively, the second *attB* site can be derived from IRS2, a second Int-*att* system such as the Bxb1 recombination system [46,47,48•]. Figure 1f shows the optional use of an IRS2 *attB* site, in which recombination by IRS1 *attP* and IRS1 *attB* yields the structure shown in Figure 1g. Analogous to Cre-mediated integration, the integration of the *G3* molecule can be conducted by the transient expression of an IRS1 integrase gene. As the *G3* molecule also carries an RS2 site, the unneeded DNA will be flanked by directly oriented RS2 sites, and can be subsequently removed by RS2 recombinase. The resulting structure (Figure 1h) contains an *attR* site (PB′) derived from the previous *attP* × *attB* recombination, but it is not reactive with either *attP* or *attB*. The integrated structure also contains a new *attB* target (bb′) for the stacking of the *G4* molecule (Figure 1i), which is similar to the *G3* molecule but contains *attP* sites. In this case, the non-recombining *attP* (PP′) can be derived from IRS1. Integration of the *G4* molecule into the locus will yield the structure shown in Figure 1j, which upon removal of the DNA that is no longer needed by RS2 recombinase will yield the structure shown in Figure 1k. This structure contains an *attP* target for the insertion of a *G5* molecule that is analogous to the *G3* molecule, in a process that begins once again as depicted in Figures 1e,f.

Control of trait dispersal

An interesting aspect of the proposed scheme is that the new DNA appended next to *cordapA* is confined within a set of directly oriented *lox* sites (Figures 1e,h,k) and can therefore be removed by Cre recombinase (Figures 1k,l). Selective removal of certain types of transgenic traits might have practical use as well as public appeal. For example, if corn hybrid seeds were produced from crossing the LY038-stacked line to a self-excizing, sperm-specific *cre* expression line, the resulting progeny plants would produce pollen with both the stacked traits and *cre* removed. The trick, however, is in figuring out a way to keep Cre activity repressed so that homozygous *cre* breeding lines can be maintained. Pollen-specific excision of DNA has been reported [49•,50•], and use of this feature could help minimize pollen-dispersal of certain transgenic traits, providing that they are not needed in pollen. The excision of a sizable percentage of the transgenes in the next generation would also hinder the unauthorized reproduction of transgenic materials.

Conclusions

The public perception of antibiotic resistance genes in GMOs has prompted the research community to develop various ways to remove them from commercial products (reviewed in [51–53]). Marker removal methods include the use of homologous recombination to excise the DNA and the placement of marker genes in different transformation cassettes or in transposable elements to promote the genetic segregation of the marker from the trait of interest. Alternative marker genes that are not based on bacteria-derived antibiotic resistance genes have also been described [54] (reviewed in [55]), and the conceptually simplest of all methods is the brute force screening for transformation events without prior selection [56]. Whether or not these alternative transformation schemes will be adopted by industry will depend on many factors. Aside from a freedom-to-operate consideration, an alternative method must be effective in generating the large number of transformation events needed for commercial product development. Given the now proven efficacy of using site-specific recombination for crop engineering, at least with the Cre-*lox* system in maize, the regulatory acceptance of this method, at least in the

US, and the potential derivation of more advanced products, as described above, use of this technology could become more prevalent in future GMOs.

LY038 is the first of its kind. For now, it might represent an example where the marker gene was intentionally removed. Should the high lysine trait in LY038 bear market durability, LY038's claim to fame in the years to come could be that of a first platform upon which site-specific recombination technology takes hold. Now that industry has planted the seed, perhaps the next product to emerge from this technology will not take as long as a decade and a half.

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Similar to [49•], but also demonstrated seed-specific excision of transgenic DNA and the FLP-*FRT* recombination system. A surprising finding was that excision was more effective with Cre or FLP on a pair of fused *loxP-FRT* sites than with *loxP* or *FRT*, respectively. The use of both Cre and FLP at the same time, however, led to poor excision rates. As with [49•], this method will not be suitable for use with non-vegetatively propagated crops until homozygous transgenic lines can be maintained.

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