In Vitro Cell. Dev. Biol.—Plant 41:213–219, May–June 2005
DOI: 10.1079/IVP2004610

1054-5476/05 $18.00+0.00

2004 SIVB CONGRESS SYMPOSIUM PROCEEDING: TRANSGENE MANAGEMENT VIA MULTIPLE SITE-SPECIFIC RECOMBINATION SYSTEMS

DAVID W. OW*

Plant Gene Expression Center, United States Department of Agriculture, Agricultural Research Service, Albany, CA 94 710 and Department of Plant and Microbial Biology, University of California at Berkeley, Berkeley, CA

(Received 25 June 2004; accepted 15 October 2004; G. C. Phillips editor)

Summary

Current methods for creating transgenic varieties are labor and time intensive, comprised of the generation of hundreds of plants with random DNA insertions, screening for the few individuals with appropriate transgene expression and simple integration structure, and followed by a lengthy breeding process to introgress the engineered trait into cultivated varieties. Various modifications of existing methods have been proposed to speed up the different steps involved in plant transformation, as well as a few add-on technologies that seek to address issues related to biosafety or intellectual property. The problem with an assortment of independently developed improvements is that they do not integrate seamlessly into a single transformation system. This paper presents an integrated strategy for plant transformation, where the introduced DNA will be inserted precisely into the genome, the transgenic locus will be introgressed rapidly into field varieties, the extraneous transgenic DNA will be removed, the transgenic plants will be molecularly tagged, and the transgenic locus may be excised from pollen and/or seed.

Key words: DNA integration; gene stacking; line conversion; transgene expression; transgene removal.

Introduction

Plant transformation has become a routine procedure in mainstream research. Many model plants can be transformed with relative ease. The random integration of complex transgene structures is generally not a concern in research, so long as expression of the introduced DNA is stable within the few generations needed to assess its biological effect. For commercial development of transgenic plants, however, plant transformation is still a tedious process. Many important crop plants, including those of resource-poor countries, are difficult to transform. Moreover, for adequate field performance and long-term stability of gene expression, a much larger number of independent integration events, often in the hundreds, are needed to screen for the few that have appropriate and stable expression, and consist of a single or a near-single copy of the introduced DNA. The latter feature is thought to confer greater structural and functional stability, as well as deemed acceptable for regulatory approval.

The ability to introduce DNA into a known chromosome location and with a precise structure offers an attractive alternative to the random integration methods in current use. Recombinase-mediated gene targeting has been achieved in tobacco, *Arabidopsis*, rice and maize (for review, see Ow, 2002). In tobacco, recombinase-directed site-specific integration places a precise single-copy DNA fragment into the target site in about a third of the selected events (Albert et al., 1995; Day et al., 2000). In rice, nearly half of the selected events consist of a single precise copy at the target site (Srivastava and Ow, 2001b; Srivastava et al., 2004). These rates are significantly higher than those reported for homology-dependent insertions (Terada et al., 2002). Moreover, half of the precise single-copy insertions in tobacco, and nearly all of those in rice, express the transgene within a range that is predictable and reproducible (Day et al., 2000; Srivastava et al., 2004). This indicates that once a suitable target site is found, the plant line can be used for the predictable insertion and expression of trait genes.

The ability to use the same target site for reproducible transgene expression could find immediate applications, for example, in the expression of different antibodies from an allelic series of transgenic lines. Transgenic plants destined for agricultural uses, however, would most likely be improved over time, through a series of transformation steps, to incorporate new traits as they become available. For this reason, a method is needed to permit the sequential insertion of trangenes to the same target locus. Appending DNA onto existing target sites justifies the initial investment in screening for suitable chromosome locations, since clustering new transgenes into a previously defined site would more likely permit predictable expression of the new DNA. Additionally, a site previously approved through the regulatory process should more readily pass regulatory approval. More importantly, by clustering the transgenic DNA, the entire transgenic locus can be

*Author to whom correspondence should be addressed: Email david_ow@berkeley.edu

more conveniently managed, for its introgression to elite field varieties, as well as its possible deletion from the genome should that become necessary.

Genomic Target

Recombinase-mediated site-specific integration requires a first recombination site to be introduced into the genome to serve as the target site for the subsequent insertion or replacement of a new DNA molecule. In theory, the first recombination site may be placed into the genome by homologous recombination. However, the targeting of DNA by homologous recombination in higher plants has had limited success (for review, see Puchta, 2002). Only a single promising case has been reported, where about 1% of the rice transformants harbored the introduced DNA at the designated genomic target (Terada et al., 2002). Despite this achievement, a 1% frequency would still require generating 100 transgenic lines to recover a site-directed event, which may not be more attractive than screening random insertions. More importantly, the precise placement of a transgene in itself does not guarantee suitable transgene expression, as current knowledge of the plant genome cannot predict how a chromosome location affects newly introduced DNA. Hence, even if homologous recombination were practical, the screening of a collection of random integration events may still be a preferred option. Given that a 'favorable' integration site is found empirically, the construct used as the genomic target will most likely be introduced through conventional transformation. Random DNA insertions will be screened for appropriate and stable expression, as well as for a single copy of the genomic target. This latter requirement can be expedited through the use of site-specific recombination to resolve complex integration patterns.

Transformation Vector

Figure 1*a* shows a generic construct for the integrated transformation system, where the first gene to be introduced (*G1*) is linked to a selectable marker (*M1*). Unlike conventional transformation, recombination sites from three different recombination systems are incorporated into the construct. Each of the recombination sites is a unique DNA sequence that is typically ~30 to ~200 bp. Reversible recombination system #1 (RRS1), with recombination sites indicated by filled arrowheads, permits the resolution of multiple insertions into a single copy, as well as removes the DNA that is no longer needed after successful DNA integration. For sexually propagated plants, RRS1 also permits the translocation of DNA between plant chromosomes to facilitate the introgression of traits to elite varieties. Recombination system #2 (RS2), which may or may not be a reversible system, with recombination sites indicated by open arrowheads, brackets the transgenic locus to permit the optional excision of the transgenic DNA from pollen or seed. This will help prevent the spread of the transgenic trait to unintended hosts. A third recombination system, in which recombination reactions are not freely reversible, will permit the repeated insertion of new transgenes into the same integration locus, in a process referred to as gene stacking. The sites of this irreversible recombination system (IRS) are depicted as BB′ or PP′ to represent *attB* and *attP* sites, respectively.

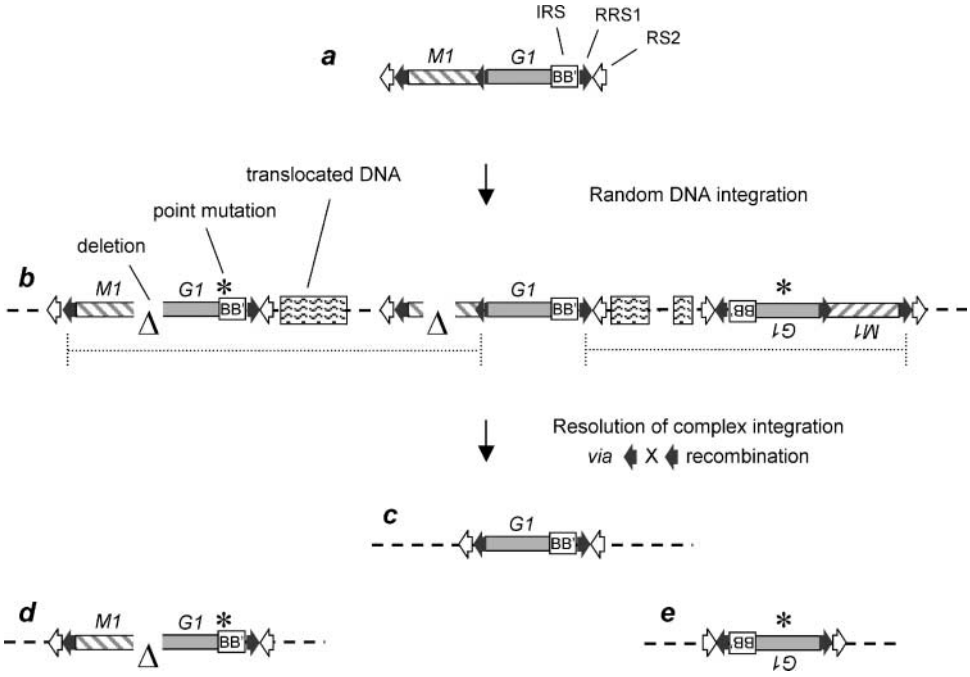

Fig. 1. Resolution of complex integration pattern through site-specific recombination. Transformation construct (*a*) integrates in multiple copies (*b*), but resolved by recombination of the RRS1 sites into lowest recombinational units shown in (*c*–*e*). Abbreviations: *M1*, marker gene 1; *G1*, gene 1; RRS1, reversible recombination system 1; RS2, recombination system 2; IRS, irreversible recombination system. Symbols for deletion, point mutation, translocated DNA, and recombination sites of RRS1, RS2, and IRS *attB* (BB′) and *attP* (PP′) are as indicated.

Resolution of Complex Integration Structures

Figure 1*b* exemplifies a typical integration locus obtained by conventional transformation methods. Some of the multiple DNA copies may be arranged in opposite orientations and may harbor various point mutations, deletions and inversions. DNA delivered by biolistic may even contain DNA translocated elsewhere in the genome interspersed with the introduced DNA (Kohli et al., 1998; Pawlowski and Somers, 1998). However, since the construct shown in Fig. 1*a* contains a set of RRS1 sites, intramolecular recombination among these sites should reduce the complex to the lowest recombinational unit, as exemplified by the structures shown in Fig. 1*c*–*e*. Structural and functional characterization can then select out the truly single functional copy of the introduced DNA. As shown in Fig. 1*c*, the single functional copy would be devoid of the *M1* fragment that should also be deleted through this process.

The above scenario assumes that outermost RRS1 sites are functional. If not, the resolved structure would be a near single copy, along with remnants of the outermost copy. Hence, the recombination process does not guarantee a single copy in all instances, but it would at least generate a near single copy that may be acceptable for commercial use. Note also that the recombination between oppositely situated sites can invert the intervening DNA, yielding a single-copy transgene in either chromosome orientation. This should not pose a problem and may even be desirable, as a given integration locus might offer two distinct patterns of transgene expression depending on its chromosome orientation. The feasibility of the resolution strategy has been tested in wheat and maize (Srivastava et al., 1999; Srivastava and Ow, 2001a).

Gene Stacking

The idea of gene stacking rests on a concept that the integrating DNA brings along an extra recombination site, such that after insertion of the new recombination site into the genome, the extra recombination site then becomes the new target for the next round of integration. While some recombination systems catalyze freely reversible reactions, others do not. Instead, the substrate sites, typically known as *attB* and *attP*, are not identical. This necessitates that the product sites generated from an *attB* × *attP* reaction, *attL* and *attR*, are dissimilar in sequence to *attB* and *attP*. The recombination enzyme that promotes the *attB* × *attP* reaction, often referred to as the integrase, by itself does not recombine *attL* × *attR*. The lack of a readily reversible reaction gives a distinct advantage for employing such a system in DNA integration since integrated molecules are stable. Most importantly, an irreversible system permits a novel gene-stacking strategy that is not achievable using only freely reversible systems.

The gene-stacking process begins with a single copy of the target construct. If *M1* has been previously removed, the structure would be as shown in Fig. 1*c*. For illustration, Fig. 2*a* shows the target construct without prior removal of *M1*. To append the second gene of interest (*G2*) to the *G1* locus, Fig. 2*b* shows the integrating plasmid with the PP′-*G2*-PP′-RSS1 > -*M2* configuration (RRS1 orientation indicated by > or <) which can recombine with the genomic BB′ target (Fig. 2*a*). The integrase can be provided, for example, by transient expression from a cotransformed plasmid (not shown). Since either of the two PP′ sequences can recombine with the single BB′ sequence, two different integration structures would arise that are distinguishable by molecular analysis. Fig. 2*c* shows only the structure useful for further stacking, consisting of RS2-RRS1 < -*M1*-RRS1 < -*G1*-BP′-*G2*-PP′-RRS1 > -*M2*-plasmid backbone-PB′-RRS1 > -RS2. The RRS1 recombinase is introduced into the system to remove the unneeded DNA (indicated by dotted lines). The resulting structure becomes RS2-RRS1 < -*G1*-BP′-*G2*-PP′-RRS1 > -RS2 (Fig. 2*d*). To stack the third gene of interest (*G3*), the construct BB′-*G3*-BB′-RRS1 > -*M2* is introduced (Fig. 2*e*). Analogous to the previous steps, the genome has only a single PP′ site to recombine with either of the BB′ sites on the plasmid. Recombination with the *G3* upstream site produces the structure shown in Fig. 2*f*. After removing the unneeded DNA, the locus containing *G1*, *G2*, and *G3* is ready for the stacking of *G4* (Fig. 2*g*, *h*). In another variation, sets of inverted *attB* and *attP* sites, rather than sets of directly oriented sites, can also be used. The sequence of events is analogous to those described for Fig. 2.

There are several features worth noting. First, the vector for delivery of the fourth gene of interest (*G4*) is the same as the vector for delivery of *G2*. Likewise, the vector for delivery of the fifth gene of interest (*G5*; Fig. 2*k*) is the same as the vector for delivery of *G3*. In principle, the stacking process can be repeated indefinitely, alternating between the uses of two simple vectors. Second, the stacking of *G2* onward requires only a single marker gene, and if *M1* is first removed, a single marker can be used throughout. This bypasses the need to continually develop new selectable markers. Third, the trait genes, such as *G1*, *G2* and so on, should not be narrowly interpreted as a single promoter-coding region-terminator fragment. Not only could each DNA fragment be composed of multiple transgenes, but could also include border DNA that insulate its (their) expression from surrounding regulatory elements. This may be useful when clustering transgenes that bring along dominant *cis*-regulatory sequences.

Molecular Tags

The stacking strategy embeds a unique DNA sequence that can be used to track the transgenic locus. As shown in Fig. 2*a*, *d*, *g*, *j*, the transgenic locus ends with either an *attB* or an *attP* sequence. This means that the transgenic locus is automatically tagged, and that this tag quantifies the number of transgenic loci, not the number of transgenes within a locus. For instance, for plants with transgenes clustered to a single locus, the abundance of PP′ and BB′ sequences relative to the genome size of the particular plant can be used to estimate the proportion of tagged over non-tagged products. Quantitative PCR or chip-based hybridization may be convenient methods to assess the amount of the tagged sequence. Having the ability to track products may find uses in the management of genetically engineered plants, such as distinguishing the crops for food or non-food uses.

The number of hybrid sites formed by the *attB* × *attP* reaction can also be used to estimate the number of stacked insertions placed into the locus. For instance, if a DNA chip can hybridize individually to PP′, BB′, BP′, and PB′, then the locus shown in Fig. 2*j* should show a relative signal of 1, 0, 2, and 1, respectively, indicating that the product contains three DNA fragments stacked into the initial transgenic BB′-containing locus.

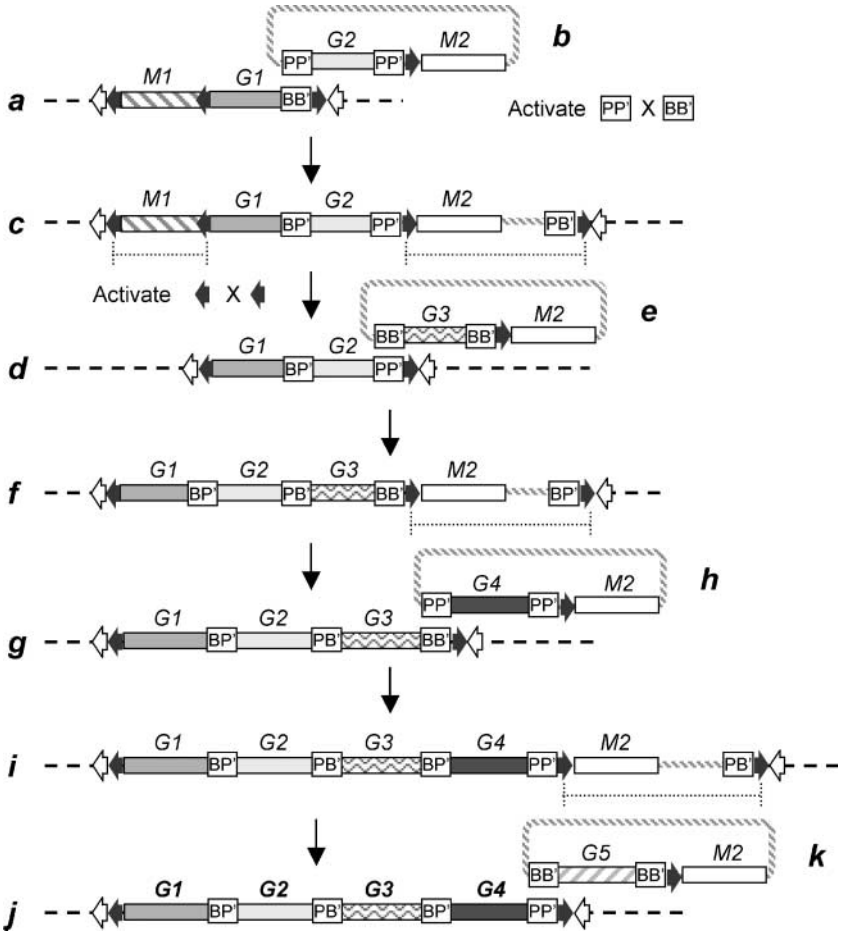

FIG. 2. Transgene stacking via site-specific recombination. New DNA (*b*) inserts into target site (*a*) through one of two possible *attB* × *attP* recombination events. Shown in (*c*) is the configuration that permits removal of excess DNA by RRS1 recombination (*dotted line*), and subsequent stacking of new DNA (*d*, *e*). Subsequent stacking steps are analogous, with each transgenic locus ending with either a unique BB′ or PP′ molecular tag. Abbreviations: *M1*, marker gene 1; *M2*, marker gene 2; *G1*, *G2*, *G3*, *G4*, *G5* represent genes 1–5. Symbols are defined in Fig. 1.

TRANSGENE TRANSLOCATION

Crop improvement through genetic engineering requires that the transgene be introduced into cultivated varieties, also referred to as elite lines. In principle, this can be accomplished through direct gene transfer into the cultivated lines. However, this may not be an option as transformation protocols, especially those involving the tissue-cultured regeneration of plants, are often specific for a plant variety where DNA uptake and cell regeneration procedures have been worked out. Therefore, in many instances, the transgene is first introduced into a transformable laboratory line and subsequently converted into cultivated lines through backcrosses to cultivated varieties. This may seem less efficient, but it does offer one advantage in that the steps involved in the gene transfer and selection for transgene expression are conducted once, rather than repeatedly with each and every locale-specific plant variety.

The major drawback to a line conversion approach is the length of the backcrossing program. Segregating away the DNA closely linked to the transgene is time consuming. Only a small fraction of the progeny would have a recombination event between the transgene and a tightly linked marker, a phenomenom known as 'linkage drag'. Take for example a transgene, *G1*, situated between two undesirable genetic traits y' and z'. If it were 0.1 genetic map units from the transgene to either y' or z', a progeny pool size of 1 million would be needed to find a recombinant with both y' and z' segregated away, or in other words, with the desired *Y-G1-Z* genotype. Consequently, linkage drag can make line conversion a rate-limiting step for crop improvement, with 6–10 backcross generations to produce commercially acceptable varieties.

The gene-stacking strategy presented above incorporates a feature that permits the use of site-specific recombination to unlink a transgenic locus from its closely flanked DNA. Removing linkage drag of adjacent DNA would reduce the number of backcrosses since both the transgenic and the adjacent non-transgenic DNA would segregate as unlinked entities. In the example described above, if y', *G1*, and z' were to assort independently, a *Y-G1-Z* genotype would arise with a probability of 0.5^3, or one in eight individuals. In theory, an entire collection of desirable elite traits could be recovered in an individual from a single backcross.

Figure 3*a* depicts the genomic target line harboring *G1* and *M1*. This target line is introgressed by conventional backcrosses to an

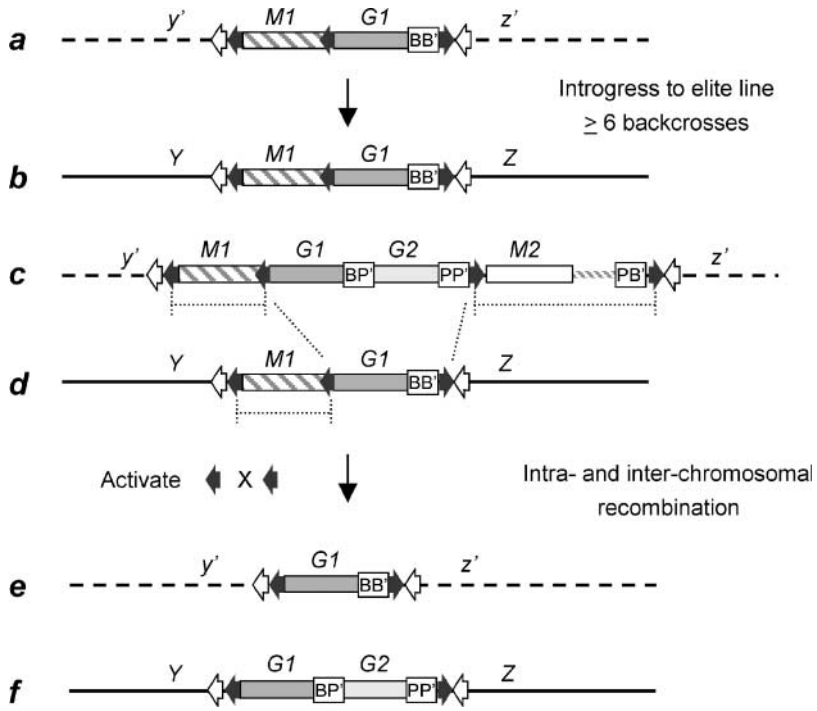

FIG. 3. Transgene introgression via chromosome translocation. First transgene (*a*) is introgressed by conventional breeding into first product elite line (*b*). Second product line (*c*) is crossed to first product elite line (*d*) for intramolecular and intermolecular recombination of RRS1 sites, deleting unneeded DNA (*dotted lines*) and breaking linkage drag to flanking genes y' and z' to yield products shown in (*e*) and (*f*). Abbreviations: *M1*, marker gene 1; *M2*, marker gene 2; *G1*, *G2*, represent genes 1 and 2, respectively. *Y*, *Z*, and alleles y', z' are flanking genes. Symbols are defined in Fig. 1.

elite line to establish a target line in an elite genetic background (Fig. 3*b*). Some of the DNA adjacent to the transgenic locus may still be derived from the laboratory line, but as long as the linked undesirable traits (y' and z') are segregated out, the remaining laboratory line DNA is inconsequential. Note also that the *M1* transformation marker can be removed by site-specific recombination to generate a selectable marker-free *G1* elite line, which could be a more consumer-friendly product (Hohn et al., 2001; Ow, 2001).

As before (Fig. 2*a*, *b*), the stacking of new transgenes is conducted using the laboratory line where genetic transformation is practical (Fig. 3*c*). To convert the product shown in Fig. 3*c* to the elite genetic background, it is crossed to the elite target line shown in Fig. 3*b*. The progeny from this cross contains both the *G1*,*G2*/lab line chromosome (Fig. 3*c*) and its homologous *G1*/elite line chromosome (Fig. 3*d*). Activation of the RRS1 recombinase, by constitutive or transient means, will promote the recombination between RRS1 sites. Most likely, the sequence of events will begin with the intramolecular deletion of unneeded DNA (Fig. 3*c*, *d*) since closely linked recombination sites are most efficiently recombined. This removal of unneeded DNA is not an extra step, but is a part of the line conversion strategy.

Intermolecular site-specific recombination should follow, resulting in a reversible reciprocal translocation of the transgenic DNA (Fig. 3*e*, *f*). This latter event breaks the linkage drag of nearby undesirable genetic entities. Without linkage drag, a much smaller progeny pool would be needed to find the recombinant with the desired set of relevant elite traits, in this example, the *Y-G1-G2-Z* combination. Therefore, even though the initial construction of the elite target line requires six or more backcrosses, subsequent introduction of newly stacked transgenes should require substantially fewer generations. For instance, if each of 10 elite traits segregates without linkage drag, the cosegregation of all 10 traits would be 0.5^{10}, or one in 1024 individuals, a population size readily obtained in a single progeny generation.

The conversion from a laboratory line to multiple independent elite lines can be conducted in parallel, provided that each independent elite line is first introduced with an appropriate target construct through the introgression process shown in Fig. 3*a*, *b*. In this fashion, once a useful trait is engineered into a laboratory variety, a multitude of elite cultivars can rapidly be developed to host the new transgene. This should speed up the introduction of new traits in crops grown in different parts of the world, and in a much more precise and predictable fashion.

Deletion of the Transgenic Locus

The set of directly oriented RS2 sites that flanks the transgenic locus provides an option to remove the transgenic DNA from the plant genome. This may be desirable at a specific developmental stage, and/or in specific tissues of the plant, and may be achieved by expressing the RS2 recombinase through developmental stage- and/or tissue-specific promoters. For instance, the outflow of the transgenic DNA via pollen transmission may be prevented by the pollen-specific expression of the RS2 recombinase to delete away the transgenic DNA from male gametes (Fig. 4*a*, *b*). For controlling pollen transmission of transgenes, this is an alternative to engineering male-sterility, where seed production would be severely curtailed in self-pollinating crops. For these crop plants,

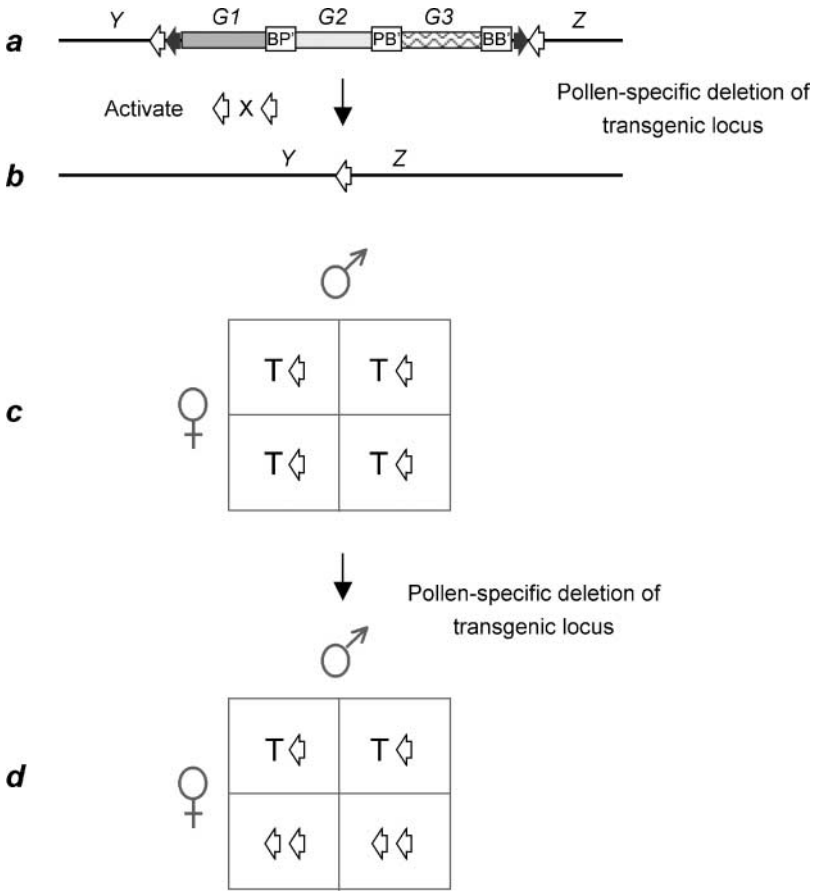

FIG. 4. Recombinase-controlled deletion of the transgenic locus. Depicted is pollen-specific removal of the transgenic DNA (*a*) from pollen (*b*). Seed genotype expected from pollen-specific deletion of transgenic DNA (T) derived from a homozygous parent (*c*), and in the following generation derived from a heterozygous parent (*d*). Strategy requires a method to repress pollen-specific deletion during commercial seed production. Abbreviations: *G1*, *G2*, *G3* represent genes 1, 2 and 3, respectively. *Y* and *Z* are flanking genes. Symbols are defined in Fig. 1.

pollen-specific deletion of the transgenic DNA would still permit seed formation, and through maternal inheritance the expression of an engineered trait, such as a seed trait (Fig. 4*c*). Should pollen-specific deletion of transgenic DNA continue to the next generation, however, half of the seed pool would be devoid of the engineered trait (Fig. 4*d*). Hence, this transgene removal feature could also discourage unauthorized dissemination of transgenic materials.

A key requirement to implementing this strategy, however, is an efficient method for commercial seed production. With seeds derived by hybridization, it may be possible to use the transgenic plants as the female recipient, although half of the seeds produced by the hybridization will not be transgenic. A preferred generic strategy would be to engineer repressed pollen-specific expression or activity of the RS2 recombinase during commercial seed production.

Concluding Remarks

With an ever-growing wealth of genomic data, it will not be long before crop plants will be engineered with multitudes of useful traits. Without doubt, plants will continue to be improved over time through the sequential addition of new transgenes. How the many different fragments of DNA are integrated and introgressed into cultivated varieties can expedite or impede the growth of the transgenic era. This paper describes an integrated strategy to improve the integration and introgression efficiencies of the introduced DNA. Moreover, the system accommodates the repeated delivery of new traits to a designated chromosome location.

Efficiency aside, precise delivery of DNA, with minimal incorporation of excess DNA, will also help alleviate public and regulatory concerns. Only short recombination sequences are necessarily co-introduced; and these sequences can serve as a molecular tag that may be used for management purposes, such as for the tracking of plants destined for food versus non-food uses. Of particular significance is that the various features to improve the precision and efficiency of trangene integration, introgression, and containment are not separate stand-alone technologies, but are incorporated seamlessly into a single transformation system.

The strategy as outlined in this paper can be implemented with recombination systems known to function in plants. The first and second recombination systems can be a combination of the Cre-*lox*, FLP-*FRT*, and R-*RS* systems (for review see Ow, 2002), where the recombinases Cre, FLP, and R catalyze recombination, respectively, on recombination sites *lox*, *FRT*, and *RS*. The irreversible recombination system can be ϕC31 (Thomason et al., 2001) or lambda (Suttie et al., 2003), with integrases that catalyze recombination between system-specific *attB* and *attP* sites.

Unfortunately, most of these systems are not generally available for commercial use. Hence, development of alternative recombination systems is necessary to make this technology usable. For the past 2 years, this laboratory has invested in this direction, and several new systems should be available soon. With a common set of background tools, crop plants may be engineered with common elements that can be shared among research and commercial communities.

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