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INVITED REVIEW

Site-specific gene integration technologies for crop improvement

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Abstract Targeted integration of foreign genes into plant genomes is a much sought-after technology for engineering precise integration structures. Homologous recombination-mediated targeted integration into native genomic sites remained somewhat elusive until made possible by zinc finger nuclease-mediated double-stranded breaks. In the meantime, an alternative approach based on the use of site-specific recombination systems has been developed which enables integration into previously engineered genomic sites (site-specific integration). Follow-up studies have validated the efficacy of the site-specific integration technology in generating transgenic events with a predictable range and stability of expression through successive generations, which are critical features of reliable and practically useful transgenic lines. Any DNA delivery methods can be used for site-specific integration; however, best efficiency is mostly obtained with direct DNA delivery methods such as particle bombardment. Although site-specific integration approach provides unique advantages for producing transgenic plants, it is still not a commonly used method. The present article discusses barriers and solutions for making it readily available to both academic research and applicative use.

Keywords Gene targeting · Site-specific integration · Site-specific recombination · Transgenic crops · Cre-lox · FLP-FRT · R-RS · phiC31

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Introduction

Numerous advances have been made in the field of plant genetic transformation since its advent 25 yr ago. Transformation of a wide range of plant species, improvement in transformation efficiency and technologies, and *Agrobacterium*-mediated transformation of monocotyledonous plants have been accomplished. As the foreign gene integration into the plant genome is predominantly mediated by a process called "illegitimate recombination" that utilizes DNA repair enzymes and micro-homologies between the introduced DNA and plant genome (Gheysen et al. 1991; Mayerhofer et al. 1991; Somers and Makarevitch 2004), homology-mediated integration is very rare. As a result, foreign DNA integrates randomly into the genome, frequently generating undesirable complex integration structures (Jorgensen et al. 1987; Takano et al. 1997; Pawlowski and Somers 1998; Kohli et al. 2003). As complex structures produce highly variable and unstable expression patterns (Breyne et al. 1992; Kumpatla and Hall 1998; Pawlowski et al. 1998), isolation of single-copy integrations is an important step in developing stable and commercially useful transgenic lines. Generally, *Agrobacterium*-mediated transformation yields a higher percentage of single-copy plants compared to direct DNA delivery methods (Hansen and Chilton 1996; Cheng et al. 2001; Dai et al. 2001; Travella et al. 2005), making it a preferred method. However, these single-copy clones may contain vector backbone at the rate of 20–75% (Kononov et al. 1997; De Buck et al. 2000; Olhoft et al. 2004) and even large fragments of *Agrobacterium* chromosomal DNA, albeit at much lower rate (0.4%) (Ulker et al. 2008). Since most studies rely on a single Southern analysis to isolate single-copy lines, the number of "clean" single-copy clones often remains unknown in the reports available in the literature.

Despite many advances, transformation of most crop species is generally an inefficient process, with highly variable transformation frequencies between species and even between varieties (Birch 1997; Bhalla 2006). The transformation process can be divided into two steps—DNA delivery followed by DNA integration. Most of the optimizations, so far, have been done at the DNA delivery step; however, the majority of transformed clones produced in the process are eliminated because they contain complex integration structures. Therefore, further optimization can be achieved by controlling DNA integration mechanism and ensuring production of single-copy lines. Targeted integration approach would allow precise engineering of single-copy locus, which is important for ensuring stable gene expression through successive generations. Numerous attempts have been made to harness homologous recombination (HR) for plant transformation, yielding only poor efficiencies and limited success (Lee et al. 1990; Halfter et al. 1992; Offringa et al. 1993). Subsequently, several studies developed strategies for optimizing HR-mediated genome manipulation in plants leading to significant improvement in gene targeting frequency (Shalev et al. 1999; Zhu et al. 1999; Gherbi et al. 2001; Terada et al. 2002, 2007; Shaked et al. 2005; D'Halluin et al. 2008; Johzuka-Hisatomi et al. 2008); however, their improved efficiencies were too low to develop a robust plant transformation technology. Recently, zinc finger (ZF) nucleases, which generate target site-specific double-stranded breaks at significantly higher frequencies, have been demonstrated to efficiently induce gene targeting in plants genome (Lloyd et al. 2005; Tzfira and White 2005; Wright et al. 2005; Shukla et al. 2009; Townsend et al. 2009). However, the need to further enhance ZF target site recognition specificities, to broaden genome coverage for new sites, and to prevent potential off-target nuclease activity requires further development of the application of ZF nucleases in plant transformation. While HR-mediated gene targeting is now feasible, plant biotechnology requires additional precise gene targeting technologies. One of the alternatives is based on utilizing site-specific recombination (SSR) systems such as Cre-*lox*, FLP-*FRT*, R-*RS*, ΦC31, and λ-att. These systems are functional in a variety of plant species and serve as versatile tools for two important applications in genetic engineering: (a) marker gene removal and (b) precise integration of foreign gene via site-specific integration. A recent review article summarized research on the use of SSR systems for marker excision (Gidoni et al. 2008). This article will focus on the site-specific integration application.

Site-Specific Recombination Systems

SSR systems were initially developed for the removal of marker genes, DNA that are essential for the isolation of transgenic clones, but may not be desirable in the transgenic plants. The three SSR systems described in the early 1990s that are still being used today are known as the Cre-*lox* from *Escherichia coli* bacteriophage P1, the FLP-*FRT* from *Saccharomyces cerevisiae*, and the R-*RS* from *Zygosaccharomyces rouxii*. These recombination systems consist of a single recombinase enzyme, Cre, FLP, or R, that catalyzes recombination between its corresponding recombination sites, *lox*, *FRT*, or *RS*, respectively. Each minimal recombination target site (*RTS*) is 34 bp or less and consists of an asymmetric core or spacer region and flanking inverted repeats (RE and LE) that serve as recombinase-binding sites (Fig. 1*a*). The core (spacer) region provides the site of crossover, and its asymmetry confers orientation on the recombination site. Recombination between two directly oriented sites *in cis* results in a deletion, while that of oppositely oriented sites *in cis* results in an inversion of the intervening DNA (Fig. 1*b*). Likewise, a recombination between two sites *in trans* results in co-integration if at least one of them is a circular molecule (Fig. 1*b*). Since the product sites are identical to the substrate sites, the reaction is freely reversible. However, in practice, the rate of reversibility is limited by the probability of interaction between the recombination substrates. Therefore, while the integration reaction is readily reversed, the excision reaction is practically unidirectional.

In recent years, a few other SSR systems have been developed for plant transformation; for example, the ΦC31-*att* and the λ-*att* systems that comprise a recombinase protein, ΦC31 or λ integrase (Int), catalyzing recombination between non-identical recombination sites known as *attB* and *attP* to generate hybrid sites *attL* and *attR*. Depending on the placement of *attB* and *attP*, deletion, inversion, co-integration, and translocation may occur. However, the reverse reaction, regenerating *attB* and *attP* from *attL* and *attR*, requires an additional excisionase/resolvase protein; therefore, in its absence, the integration reaction is unidirectional.

Site-Specific Gene Integration

To ensure faithful expression through successive generations and to generate reproducible expression among independent transgenic lines, it is necessary to direct integration of a single copy of transgene into a selected, pre-characterized genomic site. For crop transformation, it is further important to prevent disruption of endogenous genes and to ensure integration into a "safe" genomic site. Thus far, the most reliable method to achieve these objectives in plants is the SSR-mediated site-specific gene integration. Since the structure of site-specific integration locus is predictable, such precise single-copy events would

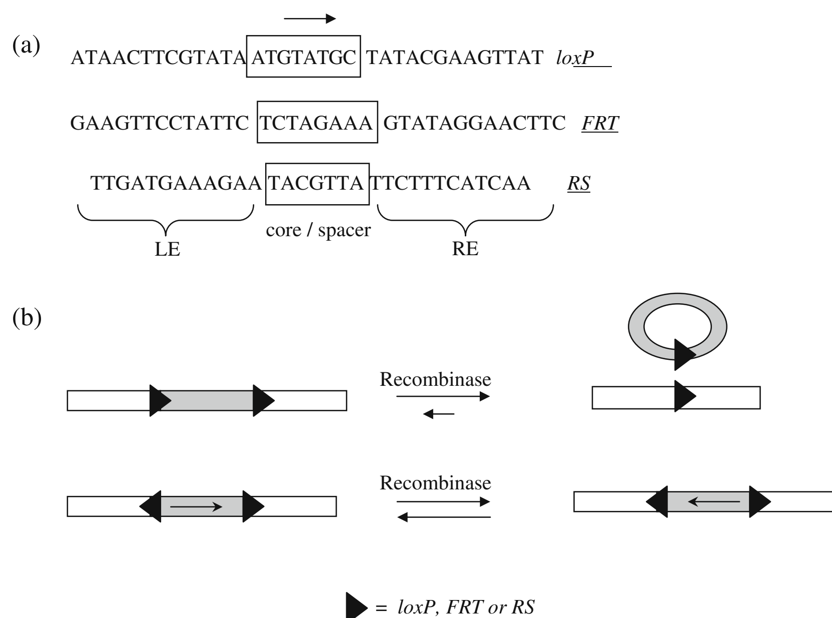

Figure 1. Site-specific recombination systems. (*a*) Recombination sites of Cre-*lox*, FLP-*FRT*, and R-*RS* systems. Each site consists of left and right inverted repeat elements (*LE* and *RE*) which serve as the recombinase binding sites flanking the core/spacer sequence (*boxed*), (the nicking/strand exchange site), which gives directionality to the site (*arrow*). (*b*) Recombinase-mediated interaction between cognate recombination sites (*arrowheads*) generates distinct products depending on the location and relative orientation of the two sites. Recombination between two directly oriented sites on a single DNA molecule results in the deletion of the intervening fragment in the form of a circular molecule. On the other hand, a similar reaction between oppositely oriented sites would result in inversion of the intervening fragment. Since substrates are identical to the products, these recombination reactions are freely reversible. However, a deletion reaction is less likely to reverse because of the formation of two separate molecules.

be more readily characterized for subsequent regulatory approval processes and serve as pre-characterized sites for additional allelic integrations.

In the first demonstration of site-specific integration in plant genome, a "co-integration" strategy was utilized that involves integration of a circular DNA molecule into a genomic target site *via* a single crossover. Later, an alternative strategy (discussed in the subsequent section), called as the "recombinase-mediated cassette exchange" was developed that involves two crossover events to exchange genomic fragment with a defined cassette resident to the introduced DNA. A summary of site-specific integration studies is presented in Table 1.

Co-integration strategy. A prerequisite of the site-specific integration strategy is the availability of "target lines" which contain a single copy of a target site construct. For example, the Cre-*lox*-based "target" constructs consist of a *lox* site that serves as the site of transgene insertion. Two versions of co-integration strategy were initially developed which differ in the way Cre activity was supplied, whether from stable expression of a *cre* gene from the target locus or via transient expression from a *cre* gene vector (Fig. 2*a*, *b*). Since integration efficiency mediated by transiently expressed *cre* gene was relatively lower (see Table 1) and also because random *cre* insertions could occur, the subsequent studies mostly utilized target constructs consisting of *cre* expression cassette (Fig. 2*a*). Although a non-targeted transformation approach is utilized for developing target lines, in practice, more than 50% of transformants with Cre-*lox* target construct represent single-copy events because multimers of the construct recombine out to generate single-copy integrations (Srivastava and Ow 2002). Once the "target line" is available, it is transformed with a circular integration (incoming donor) construct containing a *lox* site and the gene of interest (GOI). Cre-mediated recombination between the previously inserted *lox* in the "target" locus and the *lox* resident to the incoming donor construct results in the formation of a defined single-copy co-integration locus (Fig. 2).

Selection of clones is an integral process of transformation procedures, and SSR-mediated plant transformation is no exception. To allow selection of the site-specific integration events, a marker gene, which otherwise lies split between target site and integration construct, is reconstructed upon site-specific integration. For example, a promoterless marker gene located in the integration construct becomes active upon fusing with the target site promoter or *vice versa* (Fig. 2*a*, *b*). Most site-specific integration strategies utilize this split-gene approach for selection of clones. For the Cre-*lox* system, the recombination sites being relatively short (≤34 bp) can be incorporat-

Table 1. Summary of site-specific gene integration studies

Plant	SSR system	Integration strategy	Efficiency[z]	Precise/Total[y]	SC/Total[x]	Transformation method	Reference
Tobacco	Cre-*lox*	Co-I (S) (T)	(1.6–96) (6.5–2,270)	25/31 19/29	6/9 4/5	PEG/protoplast	Albert et al. (1995)
Arabidopsis	Cre-*lox*	Co-I (T)	($1–9\times10^{-3}$)	3/15	4/6	*Agrobacterium*/ T-DNA	Vergunst and Hooykaas (1998)
Arabidopsis	Cre-*lox*	Co-I (S)	($2.3–2.5\times10^{-2}$)	39/44	~20/44	-do-	Vergunst et al. (1998)
Tobacco	Cre-*lox*	Co-I (S)	Not reported	73/81	35/81	PEG/protoplast	Day et al. (2000)
Rice	Cre-*lox*	Co-I (S)	Not reported	33/36	1/3	Particle bombardment	Srivastava and Ow (2002)
Rice	Cre-*lox*	Co-I (S)	1–4/plate	68/79	40/79	Particle bombardment	Srivastava et al. (2004)
Rice	Cre-*lox*	Co-I (S)	Not reported	18/20	11/20	Particle bombardment	Chawla et al. (2006)
Tobacco plastid	ΦC31	Co-I (S)	~5.2/leaf	0–100	Not applicable	Particle bombardment	Lutz et al. (2004)
Maize	Cre-*lox*	Transient Co-I assay	0.15–0.72	Not applicable	Not applicable	Particle bombardment	Kerbach et al. (2005)
Arabid-opsis	Cre-*lox*	RMCE	49/13,584	22/49	1/5	*Agrobacterium*/T-DNA	Louwerse et al. (2007)
Tobacco	R-*RS*	RMCE	60/64	6/120	3/120	*Agrobacterium*/T-DNA	Nanto et al. (2005)
Tobacco	R-*RS*	RMCE	20/60	3/22	2/22	T-DNA	Nanto and Ebinuma (2008)
Maize	FLP-*FRT*	RMCE	Not reported	5/5	3/5	Particle bombardment	Baszczynski et al. (2003)

[z] *Numbers in parentheses* represent efficiency relative to that of random integration of a plasmid or T-DNA. Ratios represent efficiency of the site-specific integration process by displaying number of site-specific integration lines/total number of explants or plates

[y] Number of precise integration lines among total transformants

[x] Number of single-copy lines among total lines recovered or analyzed

ed readily into a transcript untranslated region without a significant reduction in transcriptional activity.

Critical parameters of efficient Cre-lox-mediated gene integration. Although Cre-mediated site-specific gene integration has been experimentally demonstrated in only a few different plant models, it is likely to work in most plant systems. A strategically important component of these experiments is the use of mutant *lox* sites. Since a *loxP* X *loxP* reaction is freely reversible, integration generated by a recombination between two *loxP* sites would be unstable (Fig. 1). Therefore, to stabilize the integration locus, Albert et al. (1995) developed mutant *lox* sites that contain four to seven base alterations in either the right or left inverted repeat elements (RE and LE mutants, respectively; Fig. 3). For example, a LE mutant *lox* site, *lox76*, and a RE mutant *lox* site, *lox75*, contain seven base alterations in their respective left element or right element (Fig. 3*a*). A reaction between *lox75* and *lox76* produces a *loxP* and a LE::RE site (double mutant), the latter being far less reactive than *lox75*, *lox76*, or *loxP* sites (Albert et al. 1995), thus favoring the forward reaction over the reverse reaction (Fig. 3*b*). Additional available LE and RE mutant *lox* sites interact at an equal or better efficiency with each other compared to *lox75* X *lox76* (Albert et al. 1995). Similarly, *FRT* mutants, bearing a single base alteration in the left (FRT_L) or the right (FRT_R) inverted-repeat sequence, have been identified (Fig. 3; Senecoff et al. 1988; Huang et al. 1991). *In vitro* and bacterial assays indicated that while each of these mutants is active, a recombination between the two generates a double mutant (FRT_{L+R}) that is nearly inactive; however, FRT_L X FRT_R recombination efficiency was low. Accordingly, efficient FLP-*FRT*-based, site-specific co-integration strategy for plants is yet to be developed.

The site-specific co-integration strategy has been further modified to prevent integration of plasmid backbone by incorporating two *lox* sites within a DNA construct (Fig. 4; Srivastava and Ow 2002). Backbone is not only an unnecessary element of the introduced plasmid; its presence in the locus is suspected to induce gene silencing (Iglesias et al. 1997; Francis and Spiker 2005). Therefore, this “two-*lox* strategy” is a significant improvement of the original site-specific co-integration strategy.

Cre-*lox*-mediated site-specific gene integration is remarkably precise, whether DNA is delivered by *Agrobacterium*, particle bombardment, or polyethylene glycol treatment of protoplasts. However, its efficiency appears to be significantly higher when direct DNA is used, such as in particle bombardment and protoplast transformation, than when *Agrobacterium* is employed to deliver T-DNA (Table 1). Efficiencies close to that of random integration were observed when direct DNA delivery methods were utilized (Albert et al. 1995; Srivastava et al., unpublished data). However, lower efficiency of site-specific integra-

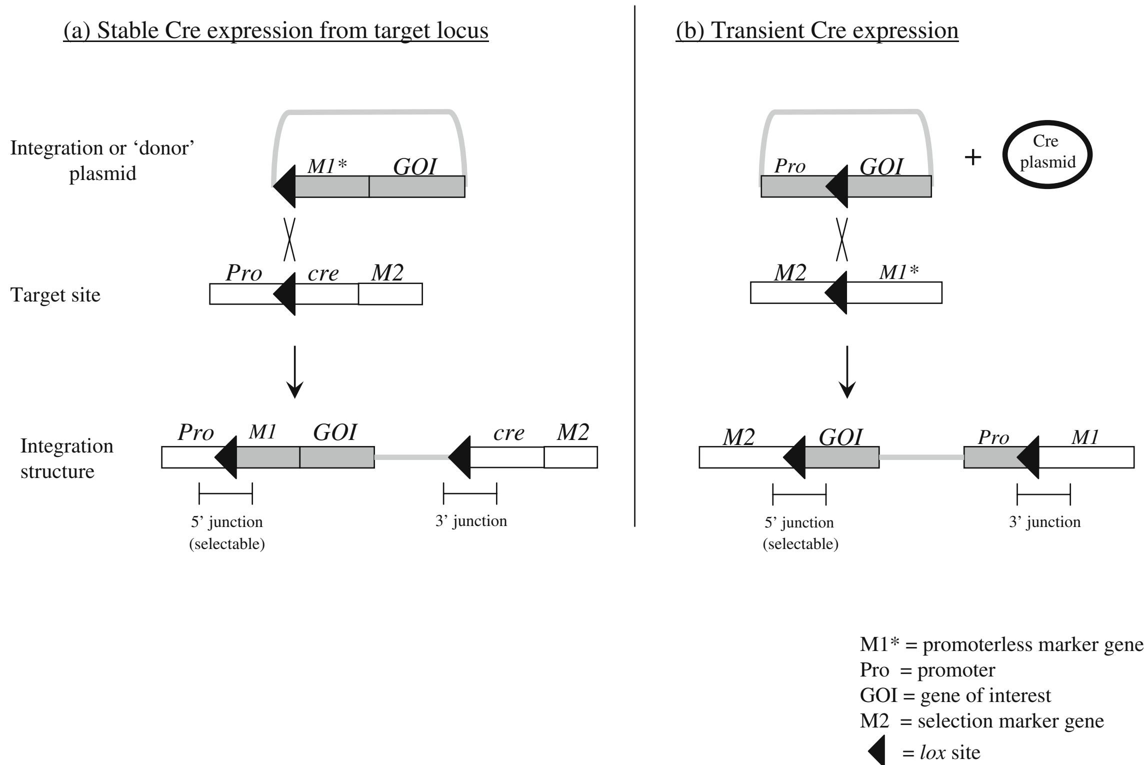

Figure 2. Co-integration using Cre-*lox* system. Co-integration strategy results in the integration of the whole plasmid into the target site. Target site design can vary depending on the way Cre activity is to be supplied (*a*) through stable expression of *cre* gene located in the target site locus or (*b*) through transient expression from a co-introduced plasmid. A target site contains a single *lox* site either (*a*) embedded in the *cre* leader sequence or (*b*) upstream of a promoterless marker gene ($M1^*$). *LoxP* X *LoxP* recombination between the integration (incoming donor) plasmid and the target site results in the activation of the marker gene and formation of distinct upstream and downstream junctions (5′ and 3′ junctions).

tions was reported with the *Agrobacterium*-mediated T-DNA delivery method (Vergunst and Hooykaas 1998; Vergunst et al. 1998). Three factors may be responsible for this difference: (a) lower amount of DNA introduced per cell, (b) inefficient conversion of single-stranded T-DNA to double-stranded molecule, and (c) circularization of the double-stranded T-DNA donor molecule mediated either by self-ligation or excision recombination processes.

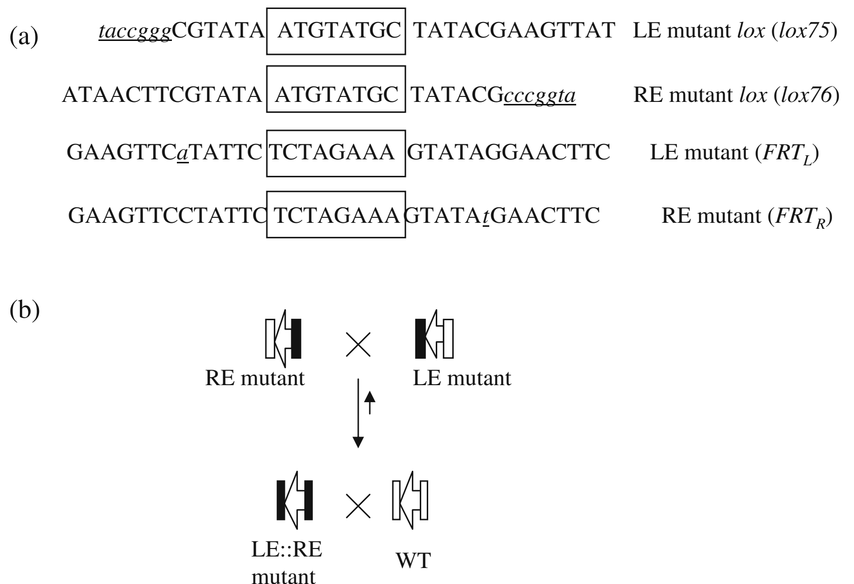

Figure 3. Co-integration of mutant *lox* or *FRT* sites. (*a*) Four to seven base alteration in left or right inverted repeat elements (*LE* or *RE*) of *lox* sites and a single-based mutation in the LE and RE of *FRT* sites generates the LE or RE mutants, e.g., *lox75*, *lox76*, and FRT_L, FRT_R. (*b*) A reaction between LE and RE mutants is practically unidirectional because one of the products is a double mutant (LE::RE) site, a poor substrate of Cre recombinase.

During site-specific integration process, random integrations may also occur in the genome. As a result, two types of integrant lines are generally produced: (a) without random integrations representing single-copy lines (SC) and (b) with random integrations representing multi-copy lines. Southern analysis of integrant lines is usually required to clearly identify SC lines. The percentage of SC lines among primary integrants varies between different experiments and protocols, with an average of 50% SC lines among integrants obtained by polyethylene glycol-mediated tobacco protoplast transformation (Day et al. 2000) and particle bombardment-mediated rice transformation (Table 1; Srivastava et al. 2004).

The RMCE strategies. Alternative to the co-integration approach based on the use of inverted repeat-mutants *lox* (LE/RE) sites described above, an additional stabilization strategy of the integrated state is based on recombinase-mediated cassette exchange (RMCE; Fig. 5). Here, the recombinase directs double reciprocal crossover between two pairs of compatible (incoming donor and genomic target) *RTSs* exchanging an incoming DNA (donor) fragment with a previously introduced chromosomal (target) segment, each flanked by a pair of incompatible *RTS*s, thus preventing self-excision or inversion in the presence of the cognate recombinase. The exchange results from replacement of the genomic target gene/marker cassette with the incoming gene/marker cassette (Bode et al. 2000; Baer and Bode 2001). With symmetrical cognate sites, e.g., *lox*, *FRT*, and *RS*, incompatibility between two *RTS*s may derive from sequence alterations in the spacer region (see boxed sequence in Fig. 1). Two distinct spacer mutants are mostly incompatible, whereas efficient recombination between two identical sites may occur (Hoess et al. 1986; Schlake and Bode 1994; Seibler and Bode 1997; Lee and Saito 1998; Siegel et al. 2001; Langer et al. 2002; Sheren et al. 2007). Based on this principle, pairs of *RTS*s with various extents of incompatibility (e.g., *FRT*-*FRT*3, *FRT*-*FRT*5, *lox*P-*lox*511, *lox*P-*lox*257, *lox*P-*m*2, *lox*P-*lox*5171, *lox*P-*lox*2272, and LE/RE-*lox*2272 in either direct or inverted orientations with respect to each other) have been applied in RMCE in different cell cultures and whole organism systems, including bacteria, flies, mice (Waterhouse et al. 1993; Schlake and Bode 1994; Bethke and Sauer 1997; Seibler and Bode 1997; reviewed in Branda and Dymecki 2004; Horn and Handler 2005; Sorrell and Kolb 2005; Oumard et al. 2006; Wirth et al. 2007), and in plants (Baszczynski et al. 2003; Nanto et al. 2005; Louwerse et al. 2007; Nanto and Ebinuma 2008). Alternative approaches for preventing self-excision of the exchanging cassettes were designed by positioning of two identical *RTSs* in opposite orientation (Saveliev et al. 1993; Feng et al. 1999; Nanto et al. 2005) or by combined use of *RTSs* derived from two different site-specific recombination systems (Lauth et al. 2002). Implementation of the former approach, however (using either identical or partially compatible *RTSs*), did not prevent inversion of exchange cassette in the presence of the cognate recombinase; as a result, two types of exchange products were recovered that differ in their relative orientation. This outcome might not be desirable, especially if the orientation of the integrated incoming gene/cassette is conditional to expression of its own or of a target gene. In addition to the bidirectionally active tyrosine recombinases Cre, FLP, and R systems described above, the *Streptomyces* phage ΦC31 integrase (Int) system, a member of the serine family of recombinases, has also been applied in the RMCE integration strategy. This integrase was previously shown to catalyze, in the absence of a bacterial excisionase/resolvase gene, unidirectional site-specific integration between its cognate non-identical *attB* and *attP* sites in various organisms, including plant plastids (Groth et al. 2000, 2004; Thomason et al. 2001; Thyagarajan et al. 2001; Belteki et al. 2003; Hollis et al. 2003; Lutz et al. 2004). Taking advantage of its unidirectional reaction, Int-mediated cassette exchange was successfully applied using cassettes that were flanked by either *attP* or *attB* sites (in either direct or invert orientations) in various cells and organisms (Thomason et al. 2001; Belteki et al. 2003; Bateman et al. 2006). Similar to the ΦC31 system, integrase-mediated RMCE between a pair of cognate *attB*- with a corresponding pair of the heterogenous *attP* site-flanking cassettes have been recently shown using the coliphage HB022 integrase system in *E. coli* (Malchin et al. 2008). Additionally, based on atomic force microscopic visualization of *in vitro* reaction integrase-synapse intermediates, this report suggests that the process of crossovers between the two pairs of *RTS* counterparts in RMCE is sequential rather than simultaneous.

In RMCE, stability of the integration product depends predominantly on the incompatibility between the two *RTS*s employed. Additionally, re-integration of the excised DNA (target) is at low likelihood, presumably due to separation of the excised DNA fragment from the integrated genomic product, followed by its degradation in the nuclear environment. In both co-integration and RMCE strategies, selection of cell clones involves reconstruction of a functional marker gene (as described in "Co-integration strategy" and shown in Figs. 2 and 5). Nevertheless, consistent with higher recombinase activity and/or sufficient incompatibility between flanking sites, RMCE was demonstrated in mammalian cultured cells and *Drosophila* to be efficient enough to allow generation of transformed clones without the use of a selectable marker gene. These reports further demonstrated enrichment of the expected exchanged events via employment of a negative selection marker in the target cassette (Seibler et al. 1998; Feng et al. 1999; Soukharev et al. 1999; Kolb 2001; Lauth et al. 2002; Horn and Handler 2005;

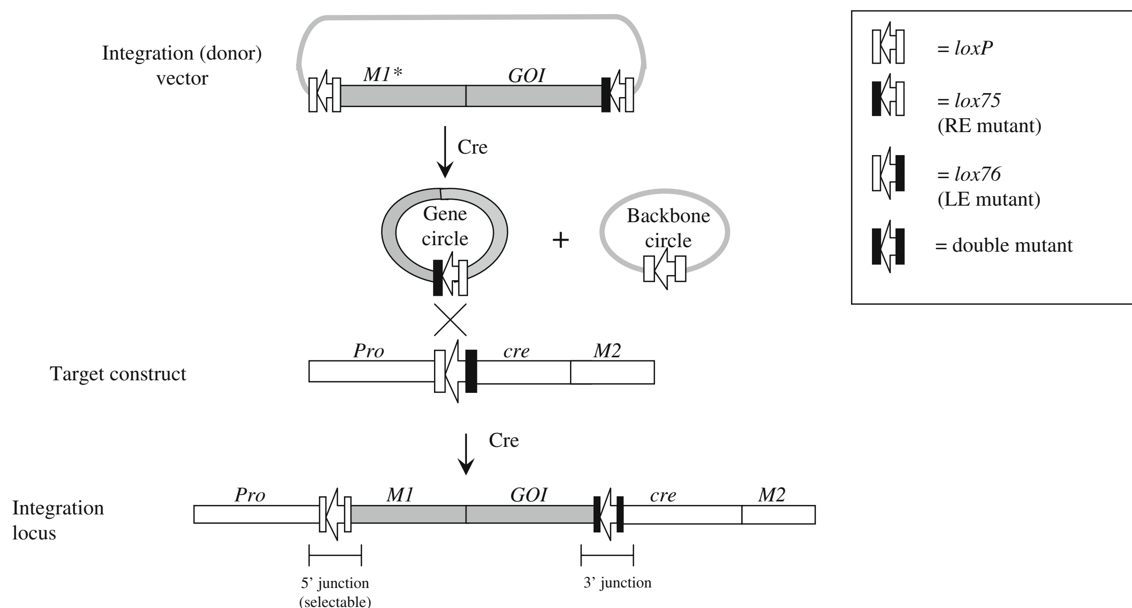

Figure 4. Use of two *lox* sites in the integration vector to generate backbone-free site-specific integration. Most of the features of this strategy are similar to Fig. 2*a*. Introduction of the integration construct containing two *lox* sites, *loxP* and *lox75*, flanking the genes results in the separation of vector backbone from the gene circle. A *lox75* X *lox76* recombination results in the incorporation of gene circle into the target site. The resulting co-integration structure does not contain the vector backbone.

Bateman et al. 2006). In this regard, in plants, while selectable marker-free incoming cassette exchange has not been demonstrated, other strategies for obtaining marker-free integration lines were developed (described in "'Clean' Site-Specific Integration" below). In a different aspect, *Agrobacterium*-mediated delivery of T-DNA harboring the incoming donor cassette in a linear form is consistent with implementation of the RMCE strategy and is further advantageous by its relative ease and efficiency in the formation of simple and precise integration exchange products, as was shown with both FLP-*FRT* (Baszczynski et al. 2003), R-*RS* (Nanto et al. 2005; Nanto and Ebinuma 2008), and Cre-*lox* (Louwerse et al. 2007) systems (Table 1; Lyznik et al. 2007).

Both the co-integration and RMCE strategies are highly advantageous biotechnological tools in their ability to facilitate integration of a single-copy GOI into specific, predetermined genomic target loci. Like co-integration, the future application of RMCE in crop plants requires the pre-characterization and definition of "safe" and appropriate chromosomal target sites to function as genomic "entrance" loci for re-integration of different gene cassettes, thus providing tools toward facilitation of precision, predictability, and stability of expression of transgenes through successive generations.

Gene Expression from Site-Specific Integration Locus

Only two studies have so far analyzed expression of site-specifically integrated genes in primary integration plant lines and their progeny. In the first study, Day et al. (2000) developed tobacco lines using the polyethylene glycol-mediated protoplast transformation method. They placed a *GUS* reporter gene driven by Commelina yellow mottle virus promoter (*Cp-GUS*) into four different target sites and addressed the following questions: (a) whether independently

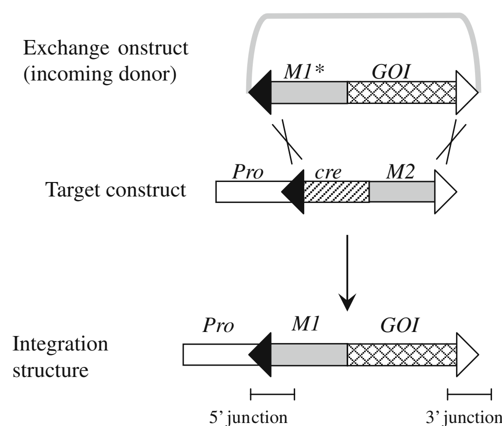

Figure 5. Recombinase-mediated cassette exchange (*RMCE*). A dual recombination reaction between incompatible sites (e.g., *lox511* and *loxP*) results in reciprocal exchange of the intervening (incoming donor) gene cassette with the target site cassette. Fusion of the promoterless marker gene ($M1^*$) with the target site promoter generates a selectable integration structure and defined 5′ and 3′ junctions. The *dark* and *light arrows* indicate a pair of incompatible recombination target sites (*RTS*s) derived from distinct spacer mutations or from two different site-specific recombination (*SSR*) systems (e.g., *lox* and *FRT*). Alternatively, incompatibility within the donor and target cassettes may be obtained through employment of a pair of the ΦC31-*attB* sites present in one cassette and *attP* sites present in the other.

transformed allelic lines express *Cp-GUS* gene at similar levels and (b) whether *Cp-GUS* gene expression would differ according to the genomic position of each integration. To their surprise, Day et al. (2000) found that *Cp-GUS* gene is expressed in only half of the allelic integrant lines derived from independent transformations of each of the four target sites, while the remaining half showed a variable degree of silencing. As different integrant lines derived from a single target site contain structurally identical isogenic locus, identical pattern of GUS expression was expected in each. The expression patterns, both full spatial expression or partially silenced, were stable throughout plant development and through generations. Molecular analysis revealed that DNA methylation in *Cp-GUS* gene was correlative with gene silencing. Since the silenced *Cp-GUS* gene in tobacco lines was hypermethylated, an imprinting phenomenon was implicated in gene silencing. Therefore, it is likely that integration of an imprinted copy of *Cp-GUS* led to the development of silenced tobacco lines. To address the second question, Day et al. (2000) studied fully expressing integrant lines derived from four different target sites. The expression level in different integration lines derived from a single target site was similar; however, expression differed between different target sites. Thus, a site-specific integration method was successful in developing stable transgenic lines, although it required gene expression analysis to eliminate unstable lines from the allelic pool of the primary transformants.

In the second study, Srivastava and colleagues (Srivastava et al. 2004; Chawla et al. 2006) addressed the same two questions using rice integrant lines developed by particle bombardment. Srivastava et al. (2004) employed the two *lox* strategy (Fig. 4) which prevented integration of plasmid backbone into the site-specific integration locus. They placed a *GUS* gene driven by maize ubiquitin-1 promoter (*Ubi-GUS*) integrated into three different target sites. When examined for *Ubi-GUS* expression, different lines derived from the same target site displayed expression levels (within two to threefold variation). However, contrary to the findings of Day et al. (2000), all single-copy integrant lines developed in these studies displayed the expected full spatial expression pattern. In other words, no gene silencing was observed in the site-specific integration allelic lines of rice produced by particle bombardment. The most important question emerging from these studies is why *Cp-GUS* gene was methylated at high rate in tobacco transformation experiments while *Ubi-GUS* gene in rice was not.

A careful comparison of the two studies suggests that one or more of the following could be involved in methylation-based silencing: (a) excessive amounts of DNA introduced by PEG-mediated protoplast transformation generated robust transient expression, initiating RNA-mediated DNA methylation; (b) the allotetraploid tobacco genome may be more proficient in gene imprinting compared to diploid rice genome; (c) presence of plasmid backbone in tobacco integration sites (strategy shown in Fig. 2 was utilized) may have initiated the chromatin modification process; or (d) the viral promoter in *Cp-GUS* gene may be more prone to gene silencing as are other viral promoters, such as cauliflower mosaic virus *35S* promoter (Elmayan and Vaucheret 1996; Mishiba et al. 2005). Particle bombardment, on the other hand, probably delivers a much lower amount of DNA (limited by the particle size). Furthermore, use of two *lox* sites in the transformation vector developed by Srivastava and Ow (2002) generated backbone-free integration locus. Thus, the site-specific integration method developed by Srivastava and Ow (2002) appears to be more suitable for optimizing transgenic plant production as all single-copy integrants express transgene at predictable expression levels. Another important finding of studies conducted by Chawla et al. (2006) is that *Ubi-GUS* gene is expressed stably through successive generations and that its expression doubles in the homozygous state. Thus, gene expression from site-specific integration loci correlates directly with allelic gene dosage

“Clean” Site-Specific Integration

The site-specific integration strategies described above have no provision for marker gene removal; hence, they are not suitable for commercial use. Srivastava and Ow (2002, 2004) developed a new strategy, referred to as marker-free site-specific integration (MFSSI), for commercial applications (Fig. 6). This strategy has recently been demonstrated in tobacco by Nanto and Ebinuma (2008). MFSSI strategy mandates the use of two separate recombination systems, one for each step: gene integration followed by marker excision. Nanto and Ebinuma (2008) used R-*RS* system for site-specific integration and Cre-*lox* for marker excision. Cre-*lox* has displayed exceptional efficiency in a variety of plant cells; therefore, it is utilized for excision, a non-selectable step. Of the other known systems, FLP-*FRT* and ΦC31 are also promising for MFSSI technology. While different studies suggest that they have much lower recombination efficiency than Cre-*lox*, they may function at sufficient level to recover integration lines as shown for R-*RS* system in tobacco genome (Nanto et al. 2005; Nanto and Ebinuma 2008), FLP-*FRT* in maize (Baszczynski et al. 2003), and ΦC31-*att* system in tobacco plastid genome (Lutz et al. 2004). The report of R-*RS* mediated integration followed by Cre-*lox*-mediated marker excision serves as an important feasibility study; however, their method involved a tedious retransformation step to introduce Cre activity. In contrast, Srivastava and Ow (2004) have proposed a streamlined integration and excision strategy based on the use of inducible Cre-*lox* system, eliminating the need of

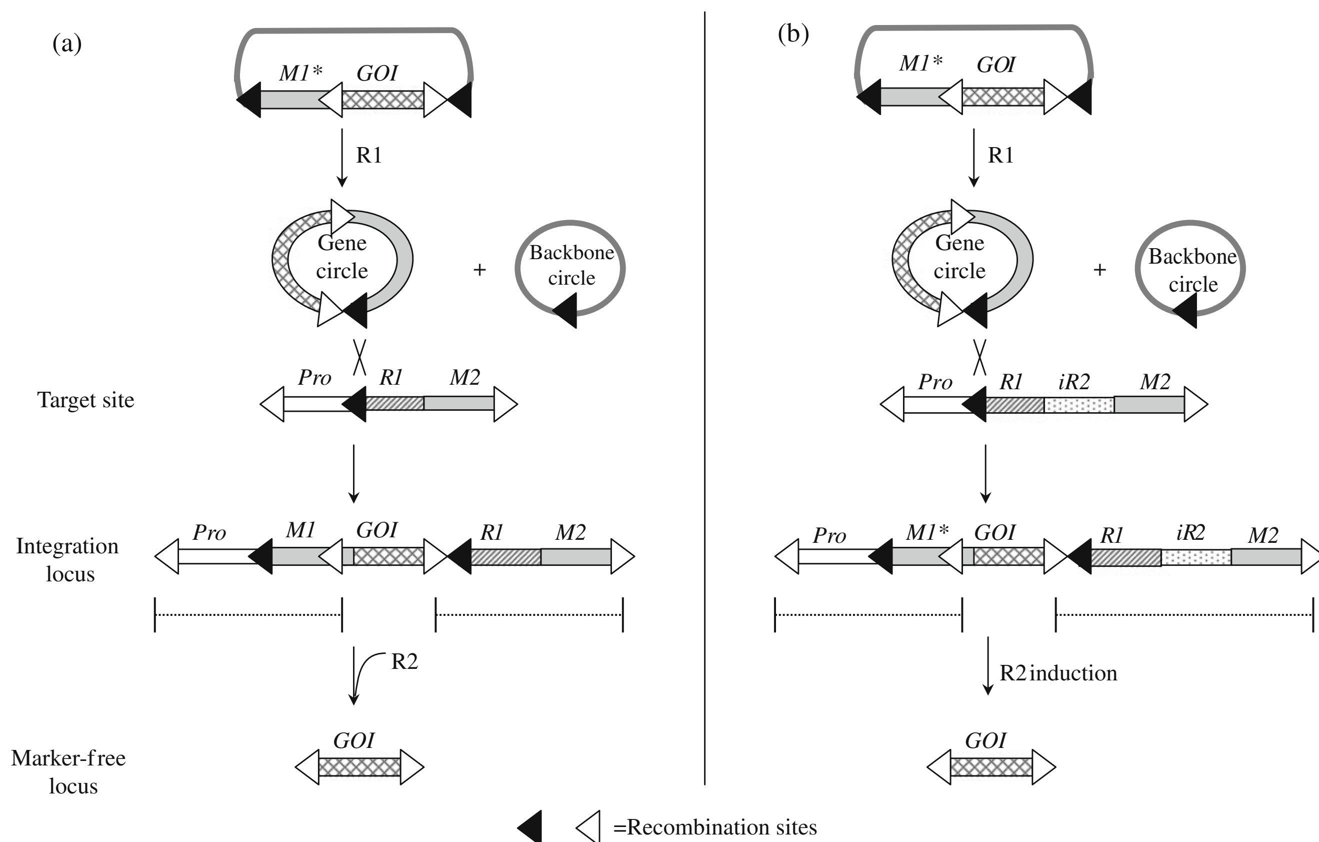

Figure 6. Marker-free, site-specific integration system. The strategy is based on the use of two separate recombination systems—one for integration (R1, e.g., FLP-*FRT*) and a second for excision (R2, e.g., Cre-*lox*). R2 activity can be supplied by (*a*) crossing in a constitutively expressed *R2* gene or (*b*) inducing an R2 activity from an inducible gene (*iR2*).

retransformation (Fig. 6*b*). Thus, MFSSI locus can be produced by inducing Cre activity, eliminating the need of retransformation. Heat-shock promoter (*HSP*) is particularly attractive for this purpose as it is easily induced in callus, regenerated plants, and seedlings. The most important criterion in promoter selection is that the inducible promoter should not have detectable activity during the selection phase. To test the utility of *HSP* for MFSSI technology, a *HSP-cre* gene was introduced along with a *lox*-target vector into rice cells (Khattri and Srivastava, unpublished data). The *lox*-target vector contained a *lox*-flanked neomycin phosphotransferase (*npt*) gene between *Ubi* promoter and β-glucoronidase (*GUS*) coding sequence, thereby blocking the transcription of *GUS* gene. Cre-mediated excision of *npt* fragment fuses *Ubi* promoter with *GUS* codons, allowing its transcription. Co-transformation of *HSP-cre* and *lox*-target vector generated a high number of kanamycin-resistant clones. As all of these clones contained *HSP-cre* gene, it was inferred that *HSP* promoter does not have excessively leaky activity during the selection phase. These data were further substantiated by regenerating some of these clones and studying *HSP-cre* activity in callus, leaves, and seedlings. While a number of them displayed GUS activity without heat treatment in at least one of the developmental phases, indicative of leaky promoter activity, clones containing negligible activity were also isolated (Khattri and Srivastava, unpublished data).

Recombinase Toxicity

Genomes of prokaryotic and eukaryotic organisms contain cryptic or pseudo-recombination target sites (ps-*RTS*s) that share partial identity with their corresponding wild-type *RTS* (wt-*RTS*), e.g., *lox*, *FRT*, and the phage ΦC31 integrase recognition sites *attB* and *attP*. These sites exhibit various degrees of recombination activity mediated by each corresponding wild-type recombinase protein (Sternberg et al. 1981; Hoess et al. 1982; Sauer 1992; Thyagarajan et al. 2000; Combes et al. 2002; Bolusani et al. 2006; Chalberg et al. 2006). With respect to the Cre-*lox* and ΦC31 integrase systems, their expression in the absence of transgenic *lox* or *att* constructs, respectively, has been associated with genetic instabilities reflected by inhibitory growth effects which were attributed to recombinase-mediated chromosomal rearrangements between endogenous cognate ps-*RTS* sites in cultured

mammalian cells and mice (Schmidt et al. 2000; Adams and van der Weyden 2001; Loonstra et al. 2001; Silver and Livingston 2001; Baba et al. 2005; Liu et al. 2006; Hameyer et al. 2007; Schmidt-Supprian and Rajewsky 2007). It is possible that plant genomes also contain fully or partially functional ps-*RTS* sequences which can bind to their cognate recombinase and even recombine with each other in the presence of recombinase activity. For example, if multiple pseudo-*lox* sites occur in a single genome, deletions, inversions, or translocations of chromosomes could be induced upon Cre expression. Such Cre "toxicity" is undesirable in transgenic plants. Coppoolse et al. (2003) demonstrated the generation of stunted phenotypes in several actively Cre-expressing lines covering four plant species. However, they observed that the *cre*-negative progenies of aberrant plants displayed a normal phenotype; therefore, the Cre activity did not induce any heritable changes, suggesting that aberrant phenotypes were not related to any chromosomal rearrangements. It is possible that specific or non-specific association of Cre protein with tomato chromosomal DNA induced epigenetic changes leading to establishment of aberrant phenotype. Similarly, phenotypic aberrations were observed in relation to FLP-*FRT* in *Arabidopsis* and Cre-*lox* in petunia by Sonti et al. (1995) and Que et al. (1998), respectively. In contrast, Ream et al. (2005) found no phenotypic aberration or chromosomal translocations in Cre-expressing maize plants, and similarly, constitutive expression of the ΦC31 integrase protein in transgenic wheat seems to bear no aberrant phenotypic effects (Rubtsova et al. 2008). While no direct evidence of chromosomal rearrangements in Cre-expressing plants has so far been presented, ectopic deletions in tobacco plastid genome were observed originating from a recombination between *loxP* and a pseudo-*lox* site (Corneille et al. 2003). Therefore, chromosomal rearrangement in plant genomes as a result of recombination between pseudo-*lox* sites is quite feasible. By transforming a range of species, Coppoolse et al. (2003) also show that certain plants, e.g., petunia and tobacco, may be more susceptible to Cre toxicity than others as they observed the "Cre phenotype" at a low frequency in tomato and only once in *Arabidopsis*. Furthermore, Coppoolse et al. (2003) speculate that the use of strong promoters is more likely to induce a "Cre phenotype" than the use of native plant or inducible promoters. This hypothesis is supported by Ream et al. who drove *cre* expression using an artificially weakened promoter derived from rice Actin-1 gene, in contrast to the very strong CaMV35S and plastocyanin promoters utilized in the Coppoolse et al. (2003) study. Therefore, use of moderately strong *cre* genes should be able to avoid the potential chromosomal rearrangements. On one hand, the presence of pseudo-*lox* sites in plant genome is a matter of concern, and on the other hand, such sites could be used to develop novel methods for biotechnology (discussed below).

Direct Integration into Endogenous Target-Like Genomic Loci

Cryptic ps-*RTS*s, when located in inert genomic regions, combined with highly specific recombinase interaction, can be potentially useful as safe entrance loci for site-specific gene insertions and thus avoid the need to generate target lines by a previous transformation step. Toward this end, wild-type recombinase-mediated integration of exogenous DNA into recognizable genomic *RTS*-like target elements has been demonstrated in various prokaryotic and eukaryotic cells and organisms, including mammalian cells, yeast, *Drosophila*, *Xenopus laevis*, and mice (Sauer 1996; Thyagarajan et al. 2001; Olivares et al. 2002; Ortiz-Urda et al. 2002; Groth et al. 2004; Allen and Weeks 2005; Held et al. 2005; Ou et al. 2008). Additionally, direct molecular evolution technologies based on random and directed mutations coupled with selection techniques have been applied, facilitating isolation of recombinase proteins with relaxed or altered recognition specificities and improved efficiencies (Buchholz and Stewart 2001; Sclimenti et al. 2001; Rufer and Sauer 2002; Santoro and Schultz 2002; Voziyanov et al. 2002, 2003). With regard to *lox* and *FRT*, since their corresponding endogenous genomic ps-*RTS* sequences are most likely asymmetric, two alternative strategies have been proposed to allow recombination between asymmetric recombination target sites. One is based on employment of variant-2 recombinase specificities where each binds its cognate half-site of the asymmetric palindrome element, thus forming a functional hetero-tetrameric complex (Konieczka et al. 2004; Saraf-Levy et al. 2006; Gelato et al. 2008). The other strategy is based on direct evolution of a recombinase variant with relaxed or altered target specificity adapted for each new *RTS*-like element candidate to form a homo-tetrameric complex that allows recombination of its cognate two asymmetric sites (Konieczka et al. 2004; Bolusani et al. 2006). Application of the latter strategy was reported with the phiC31 integrase (Int) for insertion of foreign DNA into genomic cognate ps-*RTS* loci, thus broadening the range of active genomic ps-*RTS*s integration potential in mammalian cultured cells and whole organism systems for molecular genetics and gene therapy studies (Sclimenti et al. 2001; reviewed in Groth and Calos 2004; Palazzoli et al. 2008).

In contrast to the progress made in mammalian systems, the potential for interaction of altered specificity recombinases with their specific cognate endogenous pseudo-target sites for gene targeting in plants has not been explored yet. The encouraging progress made in mammalian systems raise the feasibility for its application in the plant genome toward widening the prospects of genome manipulations in plants in a more direct and safe manner.

Is Recombinase Technology Too Complicated for Routine Plant Transformation?

More than 10 yr ago, site-specific gene integration in tobacco was obtained using the polyethylene glycol-mediated protoplast transformation (Albert et al. 1995). While this study elegantly demonstrated efficiency of the process and fidelity of the integration structure, it utilized a tedious and unpopular transformation protocol. A few years later, Cre-*lox* mediated site-specific gene integration was demonstrated in *Arabidopsis* using *Agrobacterium*-mediated transformation (Vergunst and Hooykaas 1998; Vergunst et al. 1998), a popular method for plant transformation. However, relative efficiency of site-specific integration process compared to the random integration in these studies was estimated to be 40–600 times lower. Thus, initial protocols of site-specific integration did not seem practical for routine use. A few years later, both T-DNA-mediated RMCE integration and particle bombardment-mediated site-specific co-integration were demonstrated (Srivastava and Ow 2002; Baszczynski et al. 2003; Srivastava et al. 2004; Nanto et al. 2005; Louwerse et al. 2007; Nanto and Ebinuma 2008), which are commonly used transformation methods. In the latter studies (Srivastava and Ow 2002; Srivastava et al. 2004), Cre-*lox*-mediated integrations of bombarded DNA were obtained at a rate similar to that of random integrations (one to four events per bombarded plate of callus). Although particle bombardment-mediated transformations generally produce undesirable complex integration structures, site-specific integration of DNA delivered by particle bombardment is mostly of precise single-copy structure (Srivastava and Ow 2002; Srivastava et al. 2004; Chawla et al. 2006). Therefore, T-DNA-mediated RMCE and particle bombardment-mediated site-specific co-integration together provide a unique opportunity to optimize plant transformation protocols.

Quite often, the site-specific gene integration approach is perceived as a two-step process, probably because all of the above studies described development of target lines prior to the generation of the integrant lines. While availability of target lines is a prerequisite for the site-specific integration process, it is not entirely a two-step process. A careful glance at the protocol shows that the site-specific integration process is not much different from the random integration process except that it utilizes a previously engineered “target line” instead of a wild-type variety. Similarly, the integration vectors are not different from other *E. coli* or T-DNA vectors, except that they contain Cre-*lox* or other SSR components to facilitate site-specific integration. Such vectors with multi-cloning sites can easily be developed for public use. However, the important questions to address are: (a) is this approach suitable for routine use? (b) If so, what will it take to develop it into a routinely used technique?

To address these questions, advantages and disadvantages of the site-specific gene integration approach should be considered. The advantages of this approach in commercial production of transgenic plants are discussed in “Site-Specific Gene Integration.” However, routine laboratory experiments rarely involve production of transgenic plants of any commercial value. On the other hand, scores of transgenic plants are produced in several academic laboratories to study the function of a newly isolated gene or compare the function of different promoters by their expression in model or crop plants. If the experiment involves crop transformation, such as in wheat, rice, corn, tomato, or soybean, usually, several lines are developed through a long tissue culture process and analyzed by Southern blot to isolate single-copy lines. Site-specific integration approach provides a clear advantage over the conventional approach as it avoids both mutating an endogenous gene or control element and alleviates expression variations originating from genomic position and locus structure. In addition, it ensures isolation of stably expressing lines within a small population of primary transgenic lines. These factors can also be disadvantageous if variation in expression is desired. Therefore, a site-specific gene integration method can serve as a powerful tool for expressing foreign genes in crop species.

For routine use, however, target lines should be readily available. Therefore, it is necessary to develop five to ten pre-characterized “target” lines of selected crop species—wheat, corn, rice, tomato, and soybean. These target lines could serve as freely available community resources just as mutant lines of *Arabidopsis* are for functional genomics projects. Also, according to experimental study and requirements, users may use *Agrobacterium* or adopt the particle bombardment method for delivering DNA, which will ensure efficient production of site-specific lines by either RMCE or co-integration approach, respectively. These are fairly easy transitions for optimizing plants transformation procedures. In conclusion, the site-specific gene integration method is valuable not only for commercial production of transgenic plants but also for functional genomics projects. However, as long as the target lines are not readily available, this method will not be adopted in academic laboratories.

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