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Application of Site-Specific Recombination Systems for Targeted Modification of Plant Genomes

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ABSTRACT

Biotechnology has already become a major driving force in the creation of new agricultural products. Transgenic plants bring benefits to farmers, consumers, and reduce a heavy toll inflicted on the environment by conventional agricultural practices. Yet debate continues around the issues related to the production of transgenic organisms, including plants. New transformation technologies being developed, such as site-specific recombination systems, may address some concerns and, at the same time, streamline procedures utilized in the production of transgenic crops. Here, we review progress in the implementation of emerging technologies based on site-specific recombination for plant transgenic research. We also comment on potential improvements that can make such technologies increasingly applicable for transgenic plant production.

Keywords: Cre, FLP, GMO, genetic engineering, marker excision, recombinase, transformation
Abbreviations: Cre, site-specific recombinase from bacteriophage P1; **DsRed2**, red fluorescence protein; **FLP**, site-specific recombinase from yeast; ***FRT***, FLP target site; **GFP**, green fluorescent protein; ***loxP***, locus of crossing over; **MAT**, multi-auto-transformation; **R**, site-specific recombinase from yeast; **RMCE**, recombinase-mediated cassette exchange; **YFP**, yellow fluorescent protein

CONTENTS

SITE-SPECIFIC RECOMBINATION SYSTEMS AND THEIR SELECTION FOR TRANSFORMATION EXPERIMENTS

Site-specific recombination systems are common in bacteria and lower eukaryotes. One broad group of recombinases, also refered to as integrases, can be divided into two families based on the structure of the active sites. Site-specific recombinases within the tyrosine family (e.g. Cre, FLP, R, SSV1, λ integrase) use the catalytic tyrosine's hydroxyl group for a nucleophilic attack on a phosphodiester bond of the target DNA site, while a conserved serine residue of the serine family (to which the ϕC31, Hin, Gin recombinases belong) is used to create the covalent link between recombinases and their DNA target sites (Grindley *et al*. 2006). The reaction outcome is a conservative recombinant product between two target sites: excision, integration, or inversion. The bacteriophage λ integrase is probably the best characterized member of the tyrosine family, still its biotechnological applications have been limited due to the complexity of factors involved in the recombination reaction (van Duyne 2005). The λ integrase enzyme and its close relative from coliphage HK022, can work without any of the accessory factors required by λ, but their performance, under such conditions, has been impacted, e.g., no products of recombination were found in the T2 generation of *Arabidopsis* (Gottfried *et al*. 2005). Unlike the λ recombinase, the ϕC31 recombinase and the phage R4 integrase (members of the serine family) do not require host factors to perform either inter- or intramolecular recombination reactions (Groth *et al*. 2000; Olivares *et al*. 2001). The recombinant products (*attL* and *attR*) are different from substrates (*attB* and *attP*), thus the reaction is unidirectional and the sites may be described as irreversible. The system is very promising, however, it seems that the enzyme has a relatively low level of activity compared to other recombinases (Andreas *et al*. 2002). The R/RS recombination system of yeast or the Gin/*gix* system from bacteriophage Mu have also found applications in plant biotechnology (Maeser and Kahmann 1991; Sugita *et al*. 2000; Ebinuma *et al*. 2005). The later one is a highly specific recombinase that requires three accessory factors and a supercoiled DNA substrate to invert the G-element, although mutant versions of Gin that require neither additional factors nor DNA supercoiling have been obtained (Crisona *et al*. 1994). There is ongoing research to identify more site-specific recombination systems for potential future applications, in particular, from the serine family of recombinases (Keravala *et al*. 2006; Thomson and Ow 2006).

The most popular systems for genetic manipulations in eukaryotic cells are the bacteriophage P1 Cre/*lox*P system and the yeast FLP/*FRT* system, both members of the tyro-

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Special Feature

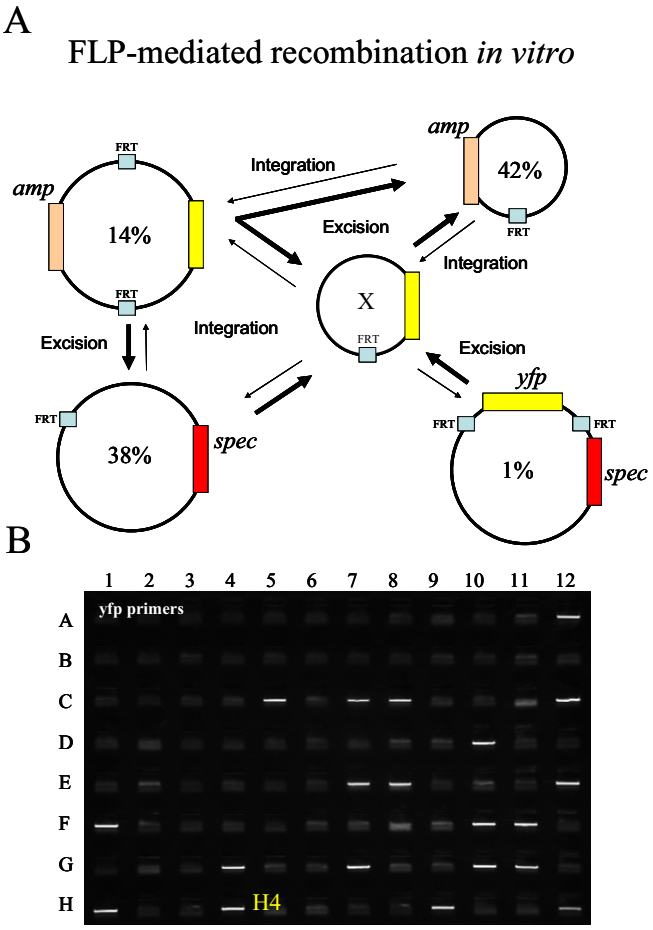

Fig. 1 Two plasmids, one containing the *YFP* gene flanked by two *FRT* sites in the same orientation and the other containing just one *FRT* site are incubated with FLP protein (**A**). Selective isolation of intermediates and products can be accomplished by transforming *E. coli* with the reaction mixture and then testing *E. coli* colonies by PCR for the anticipated arrangement of genetic elements. In this case, a number of *E. coli* colonies resistant to spectinomycin were also positive for the YFP coding region (**B**). One of them was found to be the expected product of FLP-mediated excision and subsequent integration of YFP (marked as H4). The rough estimate of the percentage of some intermediates and products in the final incubation mixture are shown associated with each structure.

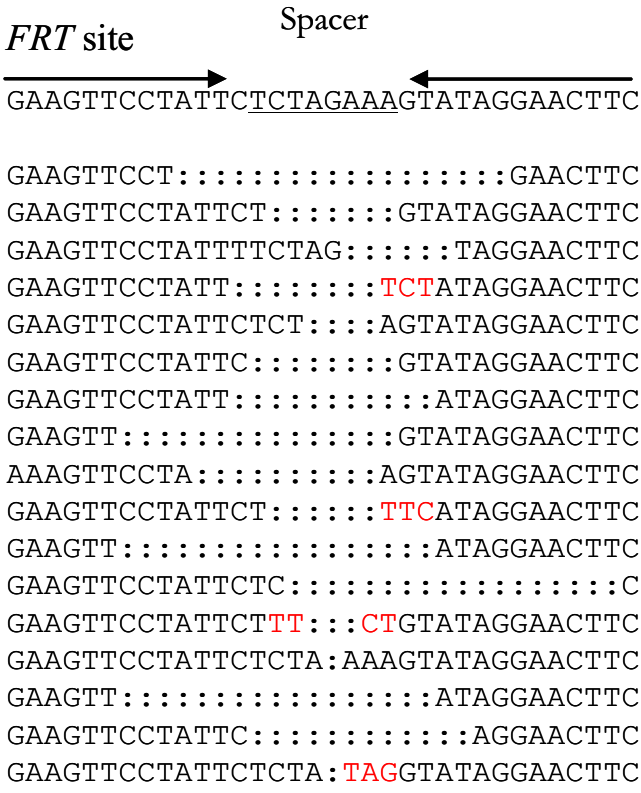

Fig. 2 Examples of mutations identified at the *FRT* site in the transgenic maize plants expressing FLP. Most mutations are relatively short deletions centered around the spacer region of the original *FRT* site. Some mutations also involve insertions of a few nucleotides (marked in red).

sine family of site-specific recombinases (Lyznik *et al*. 2003; Sorrell and Kolb 2005). Cre and FLP are popular because they are simple and unrestrictive. No auxiliary factors are needed to execute recombination reactions, requiring only the recombinase monomers binding to their cognate DNA targets. The original FLP target site is 599 bp long (the inverted repeat of the 2 μm yeast plasmid) but it can be trimmed to 30 bp and still be recombined by FLP. The products and substrates (the *FRT* sites) are the same, the recombination reactions are reversible, and it makes little difference whether they are on the same or different DNA molecules, or whether they are on supercoiled, linear, or relaxed molecules. The target sites are asymmetrical, as determined by sequences of the spacer regions, and they are recombined in direct or inverted orientation. The flexibility of the FLP/*FRT* system may be illustrated by recombination of plasmid DNA molecules containing *FRT* sites *in vitro* (**Fig. 1**). A wide variety of intermediates and products can be formed by FLP-mediated recombination of *FRT* sites with the excision products being predominant. The overall efficiency of the anticipated recombination reaction (e.g., transfer of *YFP* from one plasmid DNA molecule to the other) catalyzed by FLP is highly compromised by a lack of directionality in the reaction. In addition, because covalent intermediates are formed between enzymes and substrates, the recombination reactions require stoichiometric, rather than catalytic, amounts of the enzymatic protein. It is not surprising then that other site-specific recombination systems capable of catalyzing irreversible reactions (for example, λ integrase with its auxiliary factors), have been utilized for vector construction to assure greater specificity and efficiency (Chen *et al*. 2006).

The *in vivo* cellular environment of site-specific recombination reactions catalyzed by Cre or FLP may impose additional limitations. Once the target sites are incorporated into chromatin structures, they become a part of genomic DNA subjected to natural processes of DNA maintenance and repair. It has been shown that the formation of synaptic complexes of the recombinase monomers with two target sites to be recombined are not required for the initial nicking of the target site's DNA strands (Prado *et al*. 2000). As such, the nicked sites are prone to DNA repairs that, in turn, may lead to their modification and inactivation. In particular, it is established that double-strand breaks can be formed when replication forks face a nick at the replicating template DNA strand (Kuzminov 2001; Cortes-Ledesma and Aguilera 2006). Frequently, they are repaired by non-homologous end joining leading to mutations. In the case of recombination target sites, this process may lead to the inactivation of the entire site-specific recombination system. In this context, it is important to mention that members of the serine family of recombinases actually produce double-strand breaks at the recombination sites as a part of their catalytic activities (Grindley *et al*. 2006).

Such natural DNA repair processes may be detrimental to site specific recombination when a chosen strategy involves prolonged or constitutive expression of a recombinase gene in the presence of its cognate recombination target sites. For example, transgenic maize plants of the T_1 generation with constitutive FLP expression were analyzed for *FRT* site integrity by PCR amplification and sequence analysis (*FRT* site, **Fig. 2**). Five transgenic lines were tested and multiple clones were sequenced for each line. Four out of the five lines showed deletions and/or mutations in the *FRT* site (**Fig. 2**). Only one transgenic line showed all ori-

ginal sequence in 10 *FRT* site PCR clones isolated. We are not certain of the extent of these mutations on a cellular basis relative to the whole plant, although they must be abundant in most of these lines. Interestingly, such mutations (mostly deletions) resemble those found at the double-strand breaks generated by endonucleases (Lloyd *et al*. 2005). In a manner similar to the FLP/*FRT* system in maize, the R/RS system in sweet orange also appeared to induce mutations of RS sites leading to chromosomal deletions not associated with site-specific recombination reactions *per se* (Ballester *et al*. 2006). If the excisional activation of gene expression (e.g., marker gene activation) is not required by a protocol, such non-specific excisions may still be tolerated.

Strong expression of recombinase proteins in plant cells seems to be essential for successful experimentation. Basically, there are two strategies to express recombinases in plant cells: either to sequentially introduce them to the substrate-containing cells (e.g., by re-transformation or genetic crosses) or to regulate their expression using chemicals (synthetic steroids), tissue-specific promoters, or environmental factors such as heat shock (Lyznik *et al*. 1995; Zuo *et al*. 2001; Zhang *et al*. 2003, 2006). Re-transformation seems to be the most popular method of choice, since it delivers the strongest recombinase activity. In addition, it was recently shown that Cre recombinase can also be delivered to transgenic tobacco plants containing the *lox*P sites through agroinfection using the potato virus X (PVX) and the tobacco mosaic virus (TMV) (Kopertekh and Schiemann 2005; Jia *et al*. 2006), or just by systemic infection of tobacco plants with PVX (Kopertekh *et al*. 2004). The agroinfection experiments demonstrated that transient expression of Cre from the viral vectors was the major factor contributing to recombination reactions, since the majority of the recovered recombinant products did not contain an integrated Cre gene. This observation was consistent with a high percentage (40-80%) of recombinant, regenerated plants in the PVX/systemic infection experiments (Kopertekh *et al*. 2004). Transient expression of Cre has also proven sufficient to induce recombinations after delivering Cre to cells in the form of mRNA or protein (de Wit *et al*. 1998; Jo *et al*. 2001; Cao *et al*. 2006).

CHROMOSOMAL DELETIONS INDUCED BY SITE-SPECIFIC RECOMBINATION

Currently, the use of site-specific recombinases provides the most straightforward method for chromosomal excision. The elimination of marker genes from commercial, genetically modified plants is of particular interest, since it may deliver a new generation of transgenic plant products (Hare and Chua 2002). There is an ongoing effort to extend earlier observations in plant model systems to plant species of agronomic importance, and to use this technology to assist plant genomic projects (Ow 2002; Miki *et al*. 2004).

Both Cre and FLP recombinases have been evaluated in crops. Zhang *et al*. (2003) tested the Cre/*loxP* system for marker gene excision in maize using either genetic crossing or an autoexcision strategy. The crossing strategy is an attractive option for the production of transgenic plants since it can be incorporated into transformation and transgenic breeding protocols without additional production steps. For experimental research, the autoexcision process may be more efficient, since it does not require segregation of recombinant events and recombinase genes in subsequent generations. The possibility of producing large quantities of potential recombination events (every kernel is a potential site for excision) and the ease of handling such material may compensate for any potential reduction in expression of the recombinase genes due to chromosomal location (as opposed to transient expression of the recombinase). Nevertheless, the Cre/*lox*P system performed well in both the zygote and developing embryo of maize producing complete, stable, and heritable excision events in the F_1 and F_2 progeny of genetic crosses (Zhang *et al*. 2003). Ectopic recombinations (chromosomal rearrangements) have not been observed in transgenic maize plants expressing Cre (Ream *et al*. 2005), however, other plant species may be susceptible to such modifications of genomic DNA (Coppoolse *et al*. 2003).

Although maize cells were first used to show functionality of the yeast FLP/*FRT* system in plants (Lyznik *et al*. 1993), its application for maize chromosomal rearrangements still needs a comprehensive evaluation. Kerbach *et al*. (2005) compared two site-specific recombination systems (Cre/*loxP* and FLP/*FRT*) for marker excision in maize plants by genetic crosses. While the Cre/*loxP* system produced recombination events in the F_1 progeny, FLP-mediated excisions were not detected except in one F_1 plant identified by PCR. In contrast to these results, earlier work has demonstrated that FLP recombinase can find and recombine *FRT* sites embedded in maize chromatin structures following re-transformation (Lyznik *et al*. 1996; Baszczynski *et al*. 2002). In addition, more recent crossing experiments performed in our lab corroborate the efficacy of FLP in maize plants. **Fig. 3** illustrates FLP-mediated excisional activation of the DsRed2 marker gene in progeny of crosses between two transgenic maize parental plants containing *FRT* sites and FLP expression cassettes. These excision products were faithfully transmitted to the F_2 generation.

Rice is another important crop that has been used to test excisions catalyzed by Cre and FLP. In one of the original works on the Cre/*loxP* system in rice, transgenic plants carrying the recombination substrate were crossed to Cre-expressing plants, thus producing the Cre/*loxP* hybrids (Hoa *et al*. 2002). The recombination products were identified by activation of *gusA* expression in the F_1 progeny and they were also found in a number of F_2 plants. Unlike maize, rice is difficult to cross pollinate so the crossing strategy, although feasible, is quite cumbersome. Sreekala *et al*. (2005) reported successful production of marker-free transgenic rice plants by applying a chemically-regulated Cre/*loxP* autoexcision system developed by Zuo *et al*. (2001). Induction of Cre expression in the selected transformed calli by 10 μM β-estradiol for 2 weeks led to the activation of GFP fluorescence and regeneration of T_0 plants without hygromycin resistance or Cre genes. Site-specific DNA excision was also accomplished in transgenic rice plants by delivering a cell-permeable Cre recombinase (the membrane translocation sequence from the Kaposi fibroblast growth factor attached to the Cre protein) to the rice cell culture (Cao *et al*. 2006).

There is not yet much information available on transgenic rice plants expressing FLP. In one study, T_0 rice plants

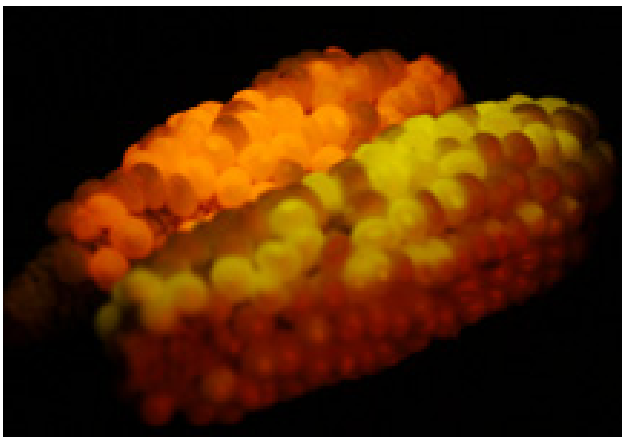

Fig. 3 FLP-mediated excision of YFP leads to the activation of DsRed2 in the progeny of maize crosses. The excision product identified in the F_1 kernels segregates in the F_2 generation of a testcross (the ear showing red fluorescent kernels). If excision does not occur, the F_2 testcross kernels display yellow fluorescence originating from the original excision substrate (the ear closer to the camera, the blue light illuminates ears from the top).

expressing FLP and T0 plants containing an excision substrate (the *npt* selectable marker flanked by *FRT* sites and inserted between a promoter and gusA coding sequence) were produced (Radhakrishnan and Srivastava 2004). The selected calli and leaf segments of the regenerated T_0 plants (FLP-expressing or substrate-containing) were bombarded with either FLP expression cassettes or a plasmid containing the recombination substrate. GUS activity in the bombarded tissues provided an assessment of the FLP/*FRT* performance. The authors concluded that the FLP/*FRT* system operated in rice at a level comparable to the Cre/*loxP* system. The transformation experiments failed to produce transgenic rice plants with strong expression of FLP, which appears to be a prerequisite for genetic crossing experiments.

Cre/*loxP* has also been successfully used in wheat. Transgenic wheat plants containing a foreign DNA fragment flanked by recombination sites (*lox511*) in the opposite orientation and the *loxP* sites (in the same orientation) flanking the *bar* gene were crossed to *cre*-expressing transgenic parental plants (Srivastava *et al*. 1999). The F_2 progeny plants were found to contain the product of *cre*-mediated excision of *bar* and a simplified integration pattern (resolving the original multi-copy integrant patterns down to lower copy numbers). A similar goal was accomplished in maize by co-bombardment of maize embryos with both a *cre*-expressing vector and a *lox511*-flanked *bar* cassette (Srivastava and Ow 2001b). Interestingly, 23% of single-copy events did not contain *cre* indicating that transient cre expression was sufficient to recombine *lox* sites. Thus, reduction of copy number is yet another potential application of site-specific recombination in plants as also shown for the R/RS system (Sugita *et al*. 2000).

An autoexcision strategy has been used in a variety of plant systems. In potato, the autoexcision strategy has been adopted for removal of the *npt*II antibiotic resistance gene and *cre* (Cuellar *et al*. 2006). It seems that this approach has been gaining popularity since its early application for transgenic *Arabidopsis* (Hoff *et al*. 2001; Zuo *et al*. 2001). The key element is to keep a recombinase gene under stringent control until transformation events are selected. This was originally accomplished by using a synthetic steroid (β-estradiol) acting on an estrogen receptor, however, the use of heat-shock treatments in conjunction with the heat-shock responsive promoters have also been reported (Hoff *et al*. 2001). In maize, the soybean HSP17.5E promoter was used to switch on *cre* gene expression in selected calli by 5 hrs incubation at 42°C (Zhang *et al*. 2003). Accordingly, the 3 hrs treatment at 42°C of potato regenerated plantlets, or a more extensive heat-shock regime for mini-tubers produced from the transgenic lines, resulted in excision of the *nptII* and *cre* genes when the *cre* gene was controlled by the *Drosophila hsp70* promoter (Cuellar *et al*. 2006). This study underscores the importance of selecting balanced conditions for the heat-shock treatment by inducing strong *cre* expression for clean, complete excision while keeping plant growth as unimpaired as possible (incubations at 37°C did not produce excisions but incubations at 45°C did not produce regenerated plants). The original autoexcision system based on the synthetic steroid (β-estradiol) activation of *cre* has been successfully implemented in the production of marker-free tomato lines transgenic for the insect endotoxin gene *cryIAc* (Zhang *et al*. 2006).

Recently, marker-free transgenic citrus plants were produced using the MAT system (Multi-Auto-Transformation) originally developed for making marker-free transgenic tobacco and aspen (Ballester *et al*. 2006). In the original work, a chimeric isopentyl transferase gene (*ipt*) was used for the initial selection of transgenic events based on a distinctive growth response of transformed cells followed by Ac transposon-mediated excision. The persistent overexpression of plant oncogenes, such as *ipt*, is not compatible with somatic embryogenesis of many crop plant species, thus the *ipt* gene was flanked by the *RS* sites of the R/*RS* site-specific recombination system to facilitate early excisions. As a result, transgenic marker-free rice plants were produced in a single-step transformation protocol (Endo *et al*. 2002). The system does not rely on inducible expression of either *ipt* or *R*, so careful selection of promoters driving their expression is required (Ebinuma *et al*. 2005).

The self-excised version of an inducible R/RS system was applied for the production of marker-free transgenic strawberries (Schaart *et al*. 2004). These experiments contained a number of noteworthy enhancements: the *R* gene was re-synthesized to accommodate a preferred codon usage in plants, a hybrid positive/negative selection marker consisting of the *codA* gene fused to *npt*II was integrated between two RS sites in order to allow for an initial selection of transformation events followed by negative selection for the self-excision events (a forced self-excision scheme, **Fig. 4**), and the rat glucocorticoid receptor was fused in frame with the R coding sequence to keep the R protein inactive unless activated by binding the dexamethasone ligand. While marker-free transgenic strawberries were identified, more experiments will help to unequivocally demonstrate the value of this system.

In the fast advancing field of site-specific recombination, there is a constant re-evaluation of parameters that may affect these reactions in plants. Many site-specific recombinases are small proteins that can penetrate a nuclear membrane without any additional modifications. Still, adding a nuclear localization signal (e.g., from the maize opaque-2 gene) to the Cre-coding region apparently improves Cre performance in *Arabidopsis* (Hoff *et al*. 2001). Introduction of a plant

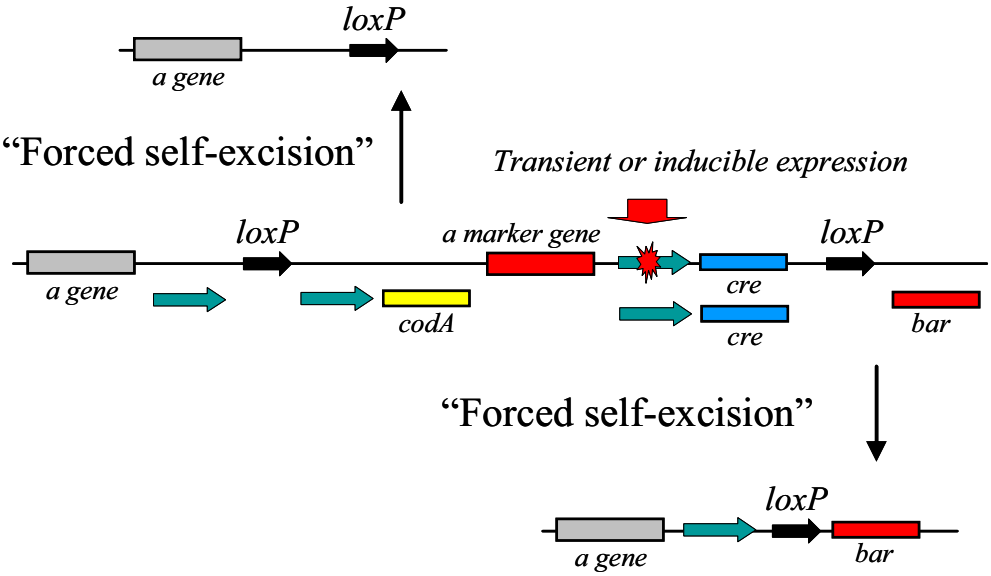

Fig. 4 A hypothetical vector illustrating the concept of forced excisions. A marker gene is flanked by two recombination target sites in the same orientation, while a gene of interest is located outside of the region. Transient, or inducible, expression a recombinase gene generates a product that can be identified either by a direct selection for *bar* gene activity or by a counter-selection against *codA* gene expression (forced self-excision). In the latter case, the *codA* (cytosine deaminase) gene needs to be included in the *loxP* flanked cassette.

intron into the *cre* recombines gene may, or may not, increase *cre* expression, but it is essential to keep structural integrity of vectors in *E. coli* when both *cre* and *lox* sites are to be delivered on the same plasmid DNA. This is particularly important for the modified autoexcision strategy that does not include the recombinase induction step (Mlynarova and Nap 2003).

It was shown that transient expression of Cre was sufficient to self-excise the *cre* gene and to recombine *lox* sites (either the original *loxP* sites or its spacer variants such as *lox511* and *lox2272*) integrated into ectopic loci in tobacco (Mlynarova and Nap 2003). This was called a "forced" self-excision because the activation of a marker gene was required to identify autoexcision events. By analogy, the use of a conditional lethal dominant gene (a negative selection marker such as the cytosine deaminase gene) to select for excision events may be called a forced excision. Such a strategy was used to produce marker-free transgenic tobacco plants by transient re-transformation with *cre* (Gleave *et al*. 1999). No selection for *cre* retransformation events was applied and still two plants were identified as marker-free, Cre-mediated excisions out of 773 shoots regenerated in the absence of selection for *cre* integration. Although feasible, such an approach requires an efficient transformation method, and repeated transformation is probably not the most attractive alternative for the production of transgenic crop plants.

Finally, there are questions regarding the effectiveness of site-specific recombinases over long chromosomal distances and the fate of the excised chromosomal fragments. Coppoolse *et al*. (2005) tested the size of chromosomal deletions in the progeny of transgenic tobacco plants expressing Cre and having the *loxP* sites dispersed by *Ds* transpositions. The authors concluded that the efficiency of somatic deletion decreased with increasing deletion size and large somatic deletions (up to 200 kb) are rarely transmitted to the next generation. The largest germinally transmitted deletion was only 55 kb in size. Also, the products of Cre-mediated chromosomal deletions were found to be unstable, so the finding of Cre-produced circles in wheat plants appears to be the exception rather than the rule (Srivastava and Ow 2003).

USING RECOMBINASES FOR SITE-SPECIFIC INTEGRATION

There are two types of recombinase systems that have been used for integration into the genome of higher eukaryotes. These include those that normally catalyze recombination between identical target sites (such as Cre, FLP, or R) and those that recombine two dissimilar sites such as the λ, ϕC31, and HK022 integrases.

Cre and FLP Recombinases

In plants, Cre and FLP have received the most attention for integration, and in the simplest scenario the recombinase would recombine a single target site in an introduced plasmid with an identical target site located in the genome. Such a strategy has been successfully employed, using Cre/*lox* after *Agrobacterium*-mediated transformation of *Arabidopsis* to facilitate T-DNA integration (Vergunst and Hooykaas 1998; Vergunst *et al*. 1998). This was accomplished in two sequential recombinations, the first occurring between two directly-repeated *loxP* sites in the T-DNA to form a circularized molecule with a single target site, and the second between the single *loxP* site in the circular molecule and a pre-integrated target site in the genome. While precise site-specific integration was demonstrated, the frequency was quite low. In part, this may have been due the problem of reversibility in Cre-mediated recombination (this is also true for FLP). Due to substrate proximity, the reversibility of these reactions will favor intra- over intermolecular recombination (i.e. excision is favored over integration). Fortunately, there are potential ways to overcome these unfavorable kinetics, or stabilize the recombined genomic locus after integration.

To address kinetics, the first method relies on temporally limiting either expression or activity of the recombinase. A number of such strategies have been reported, including transient expression of the recombinase (Gagneten *et al*. 1997; Vergunst and Hooykaas 1998), introducing a recombinase mRNA (de Wit *et al*. 1998), introducing the recombinase protein along with the DNA substrates (Baubonis and Sauer 1993; Jo *et al*. 2001), or using inducible expression of the recombinase (Lyznik *et al*. 1995; Kilby *et al*. 2000; Zuo *et al*. 2001; Kopertekh *et al*. 2004; Sreekala *et al*. 2005; Wang *et al*. 2005). Another strategy for limiting recombinase activity is to inactivate recombinase expression as a result of integration, as demonstrated by Day *et al*. (2000) and Choi *et al*. (2000) in tobacco and *Arabidopsis*, respectively. In both reports, the genomic target site contained 35S-*loxP*-Cre, and recombinase expression was inactivated when either a *loxP*-GUS cassette (Day *et al*. 2000) or a *loxP*-BAC (Choi *et al*. 2000) were integrated in front of Cre.

Another method to decrease the reversibility after integration is to modify the target sites acted upon by either Cre or FLP so that the enzyme will catalyze recombination between specific pairs of non-identical sites. Such a strategy was developed by Albert *et al*. (1995) to improve site-specific integration in plant cells. This method revolved around using two recombinase target sites with altered recombinase protein binding sites that i) contain distinct single mutations, and ii) will still recombine with each other unless double-mutant sites are produced as a result of recombination (Albert *et al*. 1995). One target site contains the mutation in the left repeat element, and the second target site contains the mutation in the right repeat element. One of these target sites is pre-integrated into the genome and the other is placed within the incoming donor plasmid. Upon Cre-mediated recombination of two such mutant *lox* sites (i.e. *lox66* and *lox72*), the integrated plasmid sequence is now flanked by a wild-type *loxP* site and a newly formed double-mutant *lox* site (*lox71*). Because the resultant double-mutant target site (*lox71*) is less efficiently used by Cre for recombination, the reversal of the integration should be much reduced. Using such a system, Albert *et al*. (1995) demonstrated that integration was indeed favored over the subsequent excision in tobacco cells. This single site integration system has continued to be developed, in tobacco (Day *et al*. 2000), and has been extended to rice and also to other eukaryotic organisms (Araki *et al*. 1997; Srivastava and Ow 2001a, 2004).

While the above methods can increase integration efficiency by reducing subsequent excisions, the RMCE (recombination-mediated cassette exchange) strategy has been developed that has the potential to make the integration step irreversible (Baer and Bode 2001). It was first pioneered in mammalian cells (Schlake and Bode 1994; Seibler and Bode 1997). The method takes advantage of the fact that small changes in the 8 bp spacer region of recombinase target sites could produce functional but mutually non-interactive mutant sites (Lee and Saito 1998). In this method, a donor DNA cassette flanked by two incompatible recombination sites (i.e. *FRT1* and *FRT5* or *loxP* and *lox511*) is integrated into a pre-existing genomic site containing the same two sites (see **Fig. 5E**). This procedure, for example, found practical application in gene therapy for the production of high-titer retroviral vector producer cell lines (Coroadinha *et al*. 2006), gene targeting in *Drosophila* (Horn and Handler 2005), gene knockouts in mice (Cesari *et al*. 2004), or other transformation applications (Lauth *et al*. 2000). Its utility has since been documented in tobacco and maize (Baszczynski *et al*. 2002; Nanto *et al*. 2005).

There are several potential benefits to using a RMCE approach: 1) the efficiency of site specific integration improves by "locking in" the integrated cassette (Feng *et al*. 1999), 2) efficiencies can become high enough that selection markers are not required to recovered recombined ge-

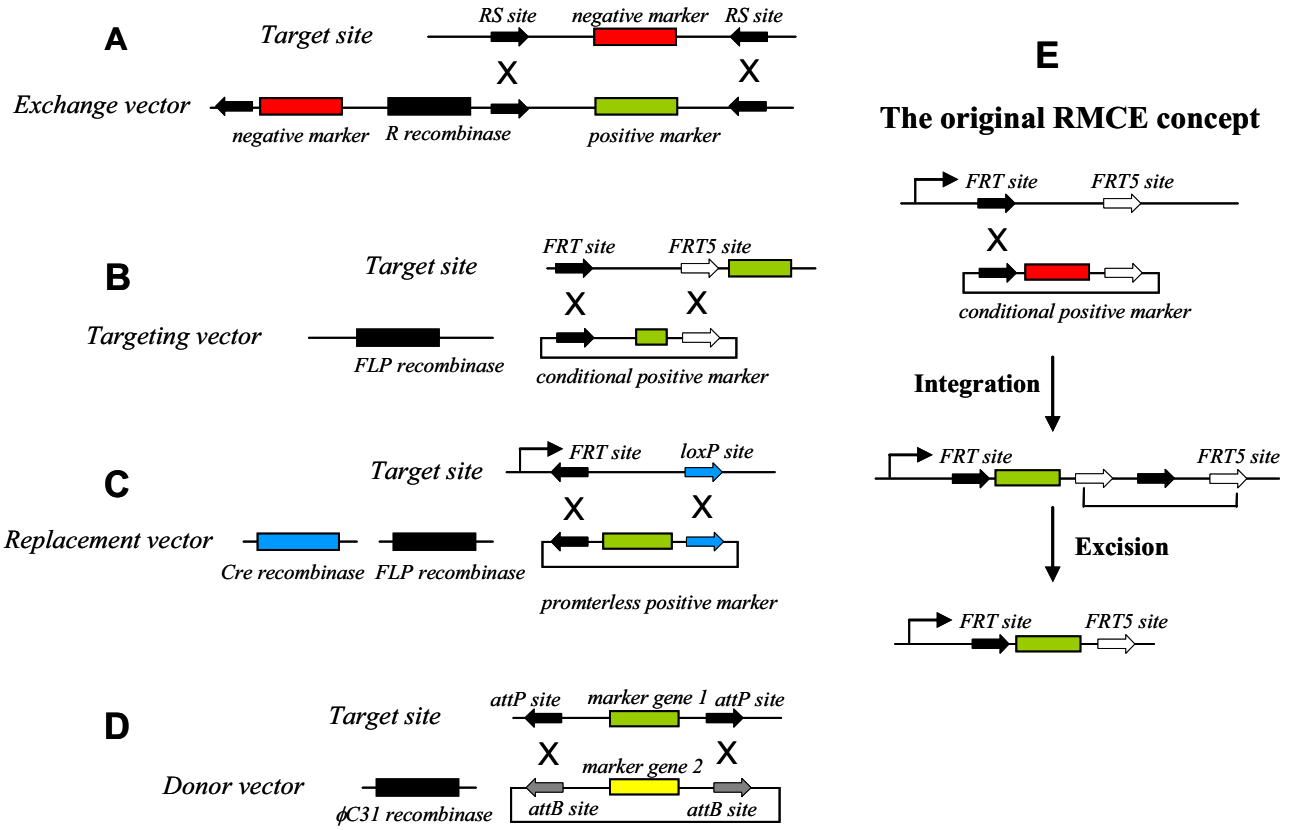

Fig. 5 Design of RMCE experiments. (**A**) T-DNA exchange as described in Nanto *et al.* 2005. Wild-type RS sites in inverted orientation; T-DNA R-mediated excisions generate circular RMCE substrates. (**B**) Classical RMCE using mutated *FRT* sites (open arrows) as shown in Coroadinha *et al.* (2006). (**C**) Two recombinases (FLP and Cre) working on their cognate sites as used in Lauth *et al.* (2002). (**D**) Two independent integrations catalyzed by ϕC31. Recombination sites in inverted orientation as designed by Bateman *et al.* (2006). (**E**) Please note that the original RMCE concept calls for two sequential recombination reactions, first integration and then excision to stabilize the exchange product. The substrate vectors need to be in a circular form and there is no restriction on expression of a recombinase, whether transient or permanent.

nomic loci (Kolb 2001; Lauth *et al*. 2002), and 3) similar to homologous recombination strategies, RMCE can be combined with negative selection markers to reduce the recovery of random integration events (Terada *et al*. 2002).

Originally developed for the FLP/*FRT* system, the RMCE methodology has been extended to other recombination systems and organisms. In *Drosophila*, the ϕC31 integrase system utilizing two *attP* and *attB* (target site and replacement vector, respectively) in inverted orientation permitted the integration of foreign genes at a single genomic position (Bateman *et al*. 2006). In this study, an RMCE efficiency of up to 24% was achieved by injecting embryos with the RMCE replacement substrate and the ϕC31 expression cassette. Similarly, two *RS* recombination sites in inverted orientation were used for the integration of a single-copy foreign gene into a predefined target locus in tobacco (Nanto *et al*. 2005). In this report, *Agrobacterium*-mediated delivery of exchange vectors together with the R recombinase expression unit resulted in about 9% of selected transformation events showing products of the R recombinase-mediated exchange. Interestingly, the exchange vectors contained one more R site in direct orientation flanking the *ipt* gene and the *R* gene. Thus, the "forced self-excision" procedure helped to eliminate random integrations of the exchange vectors, eliminated expression of the *R* gene and allowed for the selected enrichment for the re-transformation events without expression of *ipt* (abnormal phenotypes related to the aberrant cytokinin synthesis).

Yet another variant of the RMCE strategy calls for using two independent recombination systems instead of pairs of mutated recombination sites (Lauth *et al*. 2002). A trangene to be replaced is flanked by *loxP* on one and *FRT* on the other side. Two consecutive recombination events catalyzed by FLP and Cre generate a replacement product between the target transgene and plasmid DNA containing similarly arranged *loxP* and *FRT* sites. Fourteen percent of the mouse cells that received the targeting plasmid DNA and the FLP/Cre expression cassette underwent site-specific replacement of DNA fragments (Lauth *et al*. 2002). It is likely that such observations can also be extended to other plant species in the future.

Integrases

As described above, Cre and FLP catalyze freely reversible recombination, which has necessitated modifications to these systems to reduce or obviate re-excision. In contrast, integrases control the reversibility of the reaction by recombining dissimilar target sites. For example, lambda integrase, working in conjunction with specific cofactors, catalyzes recombination between an *attB* and *attP*. Because these target sites are dissimilar, recombination forms two new products referred to as *attL* and *attR*. Working with different cofactors, lambda can catalyze the reverse reaction, but by choosing only one set of cofactors, this can be turned into a non-reversible system. To date, the only report in the journal literature on the use of such an irreversible integrase in plants is by Gottfried *et al*. (2005). Using the Int gene from the coliphage HK022, these workers demonstrated that the encoded protein catalyzed *attB* × *attP* integration in *Arabidopsis*. In addition, when supplied with the *attL* and *attR* substrates, the reverse excision was performed. On one hand, these results are encouraging, because they demonstrate that HK022 Int works in plant cells. Unfortunately, they did not provide the specific cofactors that would eliminate the reverse reaction. Further work with this system will hopefully demonstrate that this can be accomplished.

Other integrase systems such as λ and ϕC31 have been investigated for use in plants. Of the two systems, λ is me-

chanistically most similar to HK022, requiring cofactors to control directionality, and successful λ-mediated integration has been reported in human cells (Lorbach *et al*. 2000). ϕC31 requires no cofactor for integration, and precise integration has been demonstrated in the fission yeast *Schizosaccharomyces pombe* (Thomason *et al*. 2001), and in insect, mouse or human cells (Groth *et al*. 2000; Thygarajan *et al*. 2001; Nakayama *et al*. 2006). Similar work in plants has not yet appeared in refereed journals, but encouraging information can be found in the patent literature. Based on recently published patent applications, either λ integrase (Suttie *et al*. 2004) or ϕC31 (Ow *et al*. 2001, and described in review by Ow 2002) have been tested for site-specific integration in plants. Further work will undoubtedly provide a clearer understanding of the utility of these tools in plant genetic modification. Particular attention needs to be dedicated to the evaluation of chromosomal rearrangements and integrations into chromosomal pseudo *attP* sites that may be catalyzed by integrases, both events were commonly found in mammalian cells (Chalberg *et al*. 2006; Liu *et al*. 2006).

"Good" sites for reproducible gene expression

Random integration after DNA introduction produces variable results, being impacted by transgene copy number, integrity and location. The result is a range, which can sometimes be substantial, both in terms of the desired transgenic phenotype and overall agronomic performance. Accordingly, a major impetus for developing site-specific integration for plants has been to demonstrate that independent integrations into the same genomic target site will produce consistent and reproducible transgene expression. Finding target sites that support the desired type of transgene expression while imposing no agronomic penalties will be valuable tools for subsequent re-introduction of traits or trait stacks.

Despite this underlying objective, evidence surrounding gene expression after site-specific integration has accumulated slowly. Early studies using Cre/*lox* to integrate a marker gene into mammalian cell lines indicated that integration into a number of distinct genomic loci produced reproducible expression at any given site (Fukashige and Sauer 1992; Feng *et al*. 1999). Such conclusions have been supported by more recent results, again using mammalian cell lines. In both Hela and Chinese hamster ovary cell lines, Cre-mediated RMCE of a doxycycline-inducible transgene into different pre-determined chromosomal locations produced reproducible gene expression (Wong *et al*. 2005). The first report in plants, characterizing expression after site-specific integration, was conducted at the level of the whole organism (Day *et al*. 2000). In this study, Day *et al*. (2000) succeeded in inserting a promoterless hygromycin phosphotransferase (*hpt*) gene plus a GUS expression cassette between the 35S promoter and the Cre gene, creating the expected $HPT^+/GUS^+/Cre^-$ phenotype. While some variability was observed in both the spatial pattern and overall activity of the GUS enzyme, and some silencing was also noted, these workers were able to find specific genomic target sites that produced stable, reproducible expression in the recovered recombinant lines. Subsequent work in plants has continued to support the initial hypothesis that genomic target sites can support consistent levels of high transgene expression. Working in rice, Srivastava and coworkers (Srivastava *et al*. 2004; Chawla *et al*. 2006), have demonstrated that consistent gene expression can be obtained by Cre-mediated site-specific integration. In addition, careful analysis of the recovered recombinant lines may have shed some light on why variability was observed earlier by Day *et al*. (2000). The authors observed two categories of recovered events; single copy lines that contained the GUS gene integrated in the target site without any additional randomly integrated copies, and multicopy events that contained both the site-specific and randomly integrated copies of the transgene (Srivastava and Ow 2004). Interestingly, the single copy lines displayed much lower expression variability. Further, the single copy lines lines exhibited constant, heritiable expression over multiple generations, while the multi-copy events were prone to variation and/or silencing of transgene expression (Chawla *et al*. 2006).

FINAL NOTE

Site-specific recombination systems have been developed, tested, and implemented into transformation protocols of crop plant species. Whether elimination of unnecessary foreign DNA fragments from chromosomes of transgenic plants (making marker-free transgenic plants), placing foreign genes into well-defined chromosomal loci (regulatory-friendly transgenic plants that do not affect/change the overall agronomic performance of elite lines), side-by-side integration of a number of genes (breeding-friendly transgenic plants with exchangeable multiple traits), reducing the copy number of transgenic events, or just assuring strong and reliable expression of newly introduced foreign genes, site-specific recombination systems have become powerful adjuncts to our crop transformation methods. Using such methods, the next generation of transgenic plants may truly be described as genetically engineered organisms (GEO) as opposed to genetically modified organisms (GMO).

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