In Vitro Cell.Dev.Biol.-Plant (2008) 44:457–467
DOI 10.1007/s11627-008-9140-3

INVITED REVIEW

Site-specific excisional recombination strategies for elimination of undesirable transgenes from crop plants

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Received: 12 December 2007 / Accepted: 30 April 2008 / Published online: 14 June 2008 / Editor: Prakash Lakshmanan

Abstract A major limitation of crop biotechnology and breeding is the lack of efficient molecular technologies for precise engineering of target genomic loci. While transformation procedures have become routine for a growing number of plant species, the random introduction of complex transgenenic DNA into the plant genome by current methods generates unpredictable effects on both transgene and homologous native gene expression. The risk of transgene transfer into related plant species and consumers is another concern associated with the conventional transformation technologies. Various approaches to avoid or eliminate undesirable transgenes, most notably selectable marker genes used in plant transformation, have recently been developed. These approaches include cotransformation with two independent T-DNAs or plasmid DNAs followed by their subsequent segregation, transposon-mediated DNA elimination, and most recently, attempts to replace bacterial T-DNA borders and selectable marker genes with functional equivalents of plant origin. The use of site-specific recombination to remove undesired DNA from the plant genome and concomitantly, via excision-mediated DNA rearrangement, switch-activate by choice transgenes of agronomical, food or feed quality traits provides a versatile "transgene maintenance and control" strategy that can significantly contribute to the transfer of transgenic laboratory developments into farming practice. This review focuses on recent reports demonstrating the elimination of undesirable transgenes (essentially selectable marker and recombinase genes) from the plant genome and concomitant activation of a silent transgene (e.g., a reporter gene) mediated by different site-specific recombinases driven by constitutive or chemically, environmentally or developmentally regulated promoters. These reports indicate major progress in excision strategies which extends application of the technology from annual, sexually propagated plants towards perennial, woody and vegetatively propagated plants. Current trends and future prospects for optimization of excision-activation machinery and its practical implementation for the generation of transgenic plants and plant products free of undesired genes are discussed.

Keywords Transgenic plants · Site-specific recombination · Inducible promoters · Auto-excision · Marker-free

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Introduction

The future potential of transgenic technologies for crop improvement depends greatly on our abilities to engineer stable expression of multiple transgenic traits in a predictable fashion and to prevent the transfer of undesirable transgenic material to nontransgenic crops and related species and to the marketplace. These considerations underline the development of various approaches designed to facilitate timely elimination of transgenes when their function is no longer needed. While the focus has been most notably on removal of selectable marker genes used during plant transformation, other transgenes and transgenic elements could similarly be eliminated. There is an additional advantage of saving the cell the burden of maintaining unwanted transgene and, additionally, allowing gene stacking through reuse of the same marker gene in subsequent transformations. Although currently commercial-

ly used genetically engineered traits are of agronomic nature (e.g., insect or herbicide resistance), the introduction of other traits of similar category, including pathogen resistance and environmental stress tolerance (e.g., to drought, heat, salinity) is expected in the near future. Additionally, use of transgenic plants for producing consumer-directed products (e.g., vitamins, antioxidants, and nutrients-enriched food), pharmaceutical and industrial materials, and animal feed derived from either single or multiple transgenes is next in line. In view of these expectations, and with continuous implementation of regulatory restrictions, there is a growing need for technologies with improved precision and efficiency to create and commercially exploit stable transgenic complexes in the plant genome. In this respect, site-specific recombination systems have been proven to be highly versatile for removing unwanted foreign DNA as well as controlling gene integration and activation of silent genes when required.

Site-specific recombination systems, in their natural prokaryote and lower eukaryote hosts, mediate control of a large variety of biological functions by carrying out precise excision, inversion, or integration of defined DNA units. These functions include: control of transcription, control of plasmid copy number, resolution of phage genome concatamers into monomeric units, and integration or excision of phage genomes in and out of the bacterial host chromosome in the lysogenic or lytic growth phases, respectively.

Due to their accuracy and relative simplicity, the site-specific recombination systems which have been the focus of most intensive studies in plants and other organisms are Cre/*lox* of bacteriophage P1 of *Escherichia coli*, R/*RS* from the SR1 plasmid of *Zygosaccharomyces rouxii*, and FLP/*FRT* from the 2-μm plasmid of *Saccharomyces cerevisiae*. These systems are characterized by their ability to function independently of DNA topology, through the interaction of a single recombinase (e.g., Cre, FLP, R) with a pair of identical recognition target sites. Each corresponding target site (34 bp *lox* and *FRT*; 31 bp *RS*) is palindromic, comprising of 12–13 bp inverted repeats surrounding a 7–8 bp asymmetric spacer region that confers directionality to the site and, hence, to the recombination reaction ((Fig. 1*A*); Sternberg and Hamilton 1981; Broach 1982; Vetter et al. 1983; Hoess and Abremski 1984, 1985; Araki et al. 1985; Hoess et al. 1986; Matsuzaki et al. 1990; Huang et al. 1991; Lee and Saito 1998).

The Cre, R, and FLP recombinases are derived from the tyrosine family of recombinases, which are defined by a catalytic tyrosine residue used to attack and cleave the target DNA during a "cut and paste" recombination process.

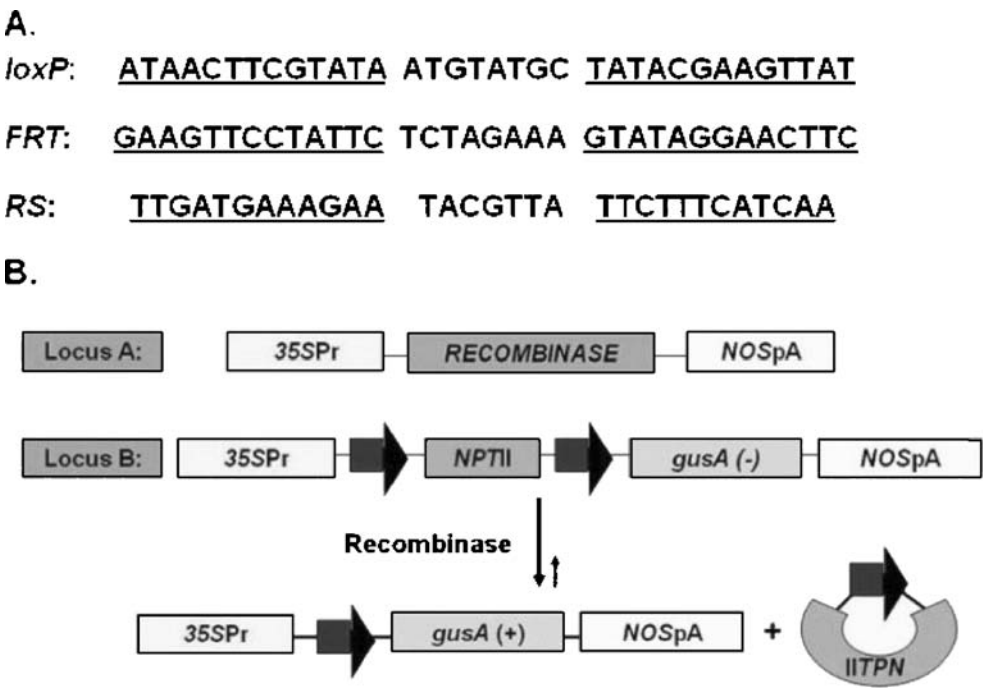

Figure 1. Sequence and excision recombination presentations of *loxP*, *FRT*, *RS* recombination target sites. **a**, *loxP*, *FRT*, and *RS* are composed each of a palindrome of 12–13 bp inverted symmetrical repeat (*underlined*), flanking a 7–8 bp asymmetric spacer, which is the sequence where strand exchange occurs. **b**, Site-specific recombination/excision of the *NPT*II coding region between directly oriented, identical recombination target sites (*loxP*, *FRT* or *RS*; *RTS*s, *wide arrows*) and concomitant activation of *gusA* in the recombination target substrate [locus B] in hybrid plants, harboring a recombinase (Cre, FLP or R, respectively; [locus A]) under the constitutive CaMV *35S* gene promoter (*35S*Pr). In this bidirectional reaction process, intramolecular excision is kinetically favored over the reverse intermolecular integration. The excised circular molecule contains an *RTS* site and the *NPT*II selectable marker gene (in a *left to right direction*). *NOS*pA indicates a *NOS* gene transcription termination-polyadenylation signal.

Protein–DNA interaction studies indicate that each identical 12 or 13 bp palindrome half-site (Fig. 1*A*) is a binding site for a monomeric recombinase protein, and recombination occurs within the spacer region. Accordingly, recombination is catalyzed between two target sites brought together in an antiparallel fashion through protein–protein interactions between four recombinase monomers in a four stranded DNA synapse configuration (*see* reviews and references in Gopaul and Van Duyne 1999; Chen and Rice 2003; Grindley et al. 2006). Whether intermolecular (integration) or intramolecular (excision, inversion) recombination occurs depends on the position and orientation of the recombination target sites relative to each other. Intermolecular recombination between target sites placed on two (e.g., circular, or circular and linear) DNA molecules produces cointegrates. However, intramolecular recombination between inversely or directly oriented sites results respectively in inversion of the intervening DNA or its excision as a circular molecule. All three functions (integration, inversion, excision) have been found to operate faithfully, under different expression control systems, in various heterologous organisms, including plants (*see* reviews of Ow 2001, 2002; Hare and Chua 2002; Luo and Kausch 2002; Sauer 2002; Gilbertson 2003; Lyznik et al. 2003; Goldstein et al. 2005). It is important to note that the recombination reactions with *lox*, *RS*, and *FRT* sites are bidirectional. However, because intramolecular excision recombination is kinetically favored over the intermolecular reintegration, the excised circular DNA is expected to undergo cellular degradation, leaving behind one genome target site (Fig. 1*B*). Yet, a rare possibility of illegitimate reintegration of the excised DNA into random, nontarget chromosomal sites or persistence as an extra-chromosomal circle should be considered (Golic and Lindquist 1989; Srivastava and Ow 2003).

In the initial characterization studies in plants of excisional recombination, two main strategies, retransformation and cross-fertilization, were used to introduce recombinase expression to target-site transformants and, thereby, activate site-specific recombination for excision of DNA from the target plant chromosome (Cre/*lox* (Dale and Ow 1990, 1991; Odell et al. 1990; Bayley et al. 1992; Russell et al. 1992), R/*RS* (Onouchi et al. 1991, 1995; Ebinuma et al. 1997) and FLP/*FRT* (Lyznik et al. 1993, 1995, 1996; Lloyd and Davis 1994; Kilby et al. 1995; Bar et al. 1996)). In these reports, a constitutively expressed or induced recombinase and target-site-bounded DNA loci were brought together in the same plant and the recombinase locus removed by genetic segregation (Fig. 1). Uses of these strategies include the elimination of selectable marker genes (Dale and Ow 1991; Russell et al. 1992; Gleave et al. 1999; McCormac et al. 1999; Ebinuma et al. 2001; Hoa et al. 2002; Endo et al. 2002; Kerbach et al. 2005), fertility restoration by excision of a sterility-inducing transgene (Luo et al. 2000; Gidoni et al. 2001a; Bayer and Hess 2005), and conversion of complex transgene integration structures to single-copy units (Srivastava et al. 1999; Srivastava and Ow 2001; Moore and Srivastava 2006; De Buck et al. 2007). However, retransformation is lengthy and cross-fertilization-dependent strategies are applicable only for sexually propagated species with relatively short reproduction cycles. Additionally, constitutive overexpression of recombinase proteins may cause aberrant developmental phenotypes in plants (Sonti et al. 1995; Que et al. 1998; Coppoolse et al. 2003). Therefore, alternative approaches based on transient gene expression have been developed.

Plant virus vectors and *Agrobacterium* T-DNA vectors have been recently reported as alternative vehicles to deliver transient high level expression of recombinase proteins in transgenic plants harboring a selectable marker gene bounded by recombination target sites. Vegetative parts of the plant may be cocultivated or infiltrated with *Agrobacterium* containing the T-DNA binary vector bearing the recombinase. Alternatively, systemic infection with a virus vector bearng the recombinase is possible. Both techniques are followed by regeneration of recombined somatic cells into whole plants. Marker gene excision was demonstrated with both T-DNA-based Cre expression vectors (Gleave et al. 1999; Kopertekh and Schiemann 2005) and with Potato Virus X-based and Tobacco Mosaic Virus-based Cre expression vectors in *Nicotiana* (Kopertekh et al. 2004; Jia et al. 2006). This strategy could be extended to a wide range of plant species dependent upon virus vectors host range.

Site-specific recombination has been utilized for marker elimination from genetically engineered plastids. Expression of foreign genes in the plastome is much less subject to gene silencing and position effects than the nuclear transformation. Additionally, plastome transformation has gene containment advantages due to maternal plastid inheritance in most plants. Therefore, development of marker removal applications from transplastomes is a correct step towards environmental safety (reviewed in Lutz and Maliga 2007). However, documented cases of transfer and subsequent integration of plastid DNA into nuclear genome, presence of plastids in pollen grains, albeit at low levels, and additionally, lack of efficient chloroplasts transformation procedures for a large number of plant species, provide subjects for further study and consideration toward widespread use of plastid-based transgene elimination procedures.

Induced Recombination—More Flexible and Versatile Gene Excision–Activation Strategies

In order to provide further control on the excision process in terms of timing flexibility and tissue-specificity and widen its applicability toward perennial woody plants and vegeta-

tively propagated plant species, the recombinase gene driven by various inducible promoters has been developed. This strategy allows cointroduction of both the marker gene and the recombinase gene, alongside the gene(s) of interest, in a single construct flanked by recombination sites. Thus, induced expression of the recombinase gene by either external or intrinsic signals results in auto-excision of both the recombinase and marker genes placed within the excision site boundaries after their function is no longer needed (Fig. 2). The timing of excision is facilitated by the regulatory promoter used to control the recombinase gene. This approach was described (Table 1) with heat-shock-induced promoter-recombinase expression cassettes in *Arabidopsis* (Hoff et al. 2001), tobacco (Liu et al. 2005; Wang et al. 2005), potato (Cuellar et al. 2006), and maize (Zhang et al. 2003) for chemically regulated promoters, employing a herbicide antidote compound to control the R/*RS* system in tobacco (Sugita et al. 2000) and aspen (Ebinuma and Komamine 2001; Matsunaga et al. 2002), with β-estradiol transinduction of Cre/*lox* in *Arabidopsis* (Zuo at al. 2001), rice (Sreekala et al. 2005), and tomato (Zhang et al. 2006) and with a dexamethasone-glucocorticoid receptor ligand binding domain activated R/*RS* system in strawberry (Schaart et al. 2004). In the latter case, while control of the induction system was less stringent, the use of a combined positive–negative selection scheme enabled selection of a completely excised, marker- and recombinase-free genotype (Schaart et al. 2004). These results are consistent with Gleave et al. (1999) and Kondrak et al. (2006) with similar products from a similar combination of positive–negative selection approach (see Hohn et al. 2001; Darbani et al. 2007).

Compared to the heat shock and chemical induction systems, developmentally and spatially regulated stimuli are less dependent on penetration of external factors into plant cells. Taking advantage of the somatic embryogenesis developmental stage required in soybean transformation, activation of Cre from an embryo-specific gene promoter successfully led to production of marker- and recombinase-free soybean through a one step self-excision event (Li et al. 2007). In a different approach, to examine pollen-directed auto-excision of transgene, Mlynarova et al. (2006) employed an early microsporogenesis-specific promoter from tobacco fused to an intron-containing *Cre* alongside the selectable marker transcription unit, bounded by two *lox* sites. Activation of *Cre* during pollen formation in tobacco and *Arabidopsis* resulted in a highly efficient excision rate (nearly 100%) of all *lox*-bounded transgenic components, giving rise to "transgene-free" pollen harboring one *lox* target site, bounded by T-DNA left border (LB) and right border (RB) elements. Luo et al. (2007) took this approach a step further by flanking the excision target unit with two different fused target sites (*lox-FRT*), instead of the traditional use of one recombination site (either *lox* or *FRT*) at each side. Activation of either recombinase by a pollen or pollen- and seed-specific promoter, expressed from a multiple gene construct unit flanked by combined *lox-FRT* sites, gave up to 100% excision efficiency of *lox-FRT* fusion-bounded transgenes, leaving residual LB and RB elements flanking a *lox-FRT* site, in both pollen and/or seed. These findings, particularly the surprising enhancement of recombination efficiency stemming from fusion of different recombination sites, increase the prospects of field application of site-specific recombination technologies in plants. In another germline-specific auto-excision experimental approach, where the recombinase is fused to germline-specific promoters derived from the *Arabidopsis APETALA1* and *SOLO DANCERS* genes, and combined with a positive–negative selection strategy, Verweire et al. (2007) were able to produce completely marker- and recombinase-free *Arabidopsis* plants.

Inherent to the flexibility of the timing of recombinase induction, external–chemical- and intrinsic–germline-gamete-activated recombinase enable delay of the excision until late developmental stages and, thus, can potentially be used for regulating function of transgenic agronomic traits during vegetative plant growth. These approaches extend the applicability of marker–recombinase–trait gene excision tech-

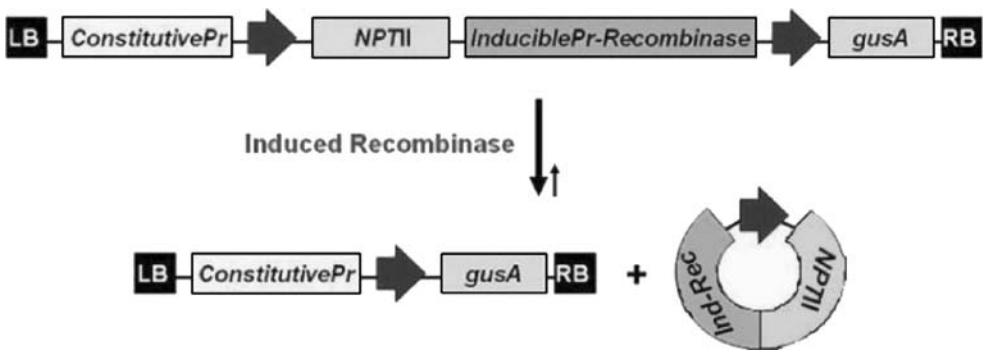

Figure 2. Induced recombination; auto-excision of the recombination target sites (*wide arrows*)—bounded selectable marker and recombinase genes (as a circular DNA molecule) via recombinase induction by external (heat shock, chemical) or intrinsic (developmental) signals and concomitant activation of the *gusA* reporter gene (representing any gene of interest) under a constitutively expressed promoter. LB and RB, T-DNA left and right border sequence elements, respectively.

nologies to perennial woody trees and vegetatively propagated plant species. Additionally, in externally-activated recombination strategies, the undesirable transgenes can potentially be deleted from both gametes, and importantly, all necessary transgenic components can be maintained in a diploid state for future use. This can be achieved by propagating uninduced "transgene-maintainer" lines kept under containment conditions. An open dilemma with regard to these strategies, however, is the efficiency and effectiveness of externally applied signals in different plants and under changing physiological and field conditions. Comparatively, in "pollen-directed excision" approaches, the female gametes stay transgenic and thus produce hemizygous seed and fruit upon self-hybridization with its own (recombined) pollen or outcross with nontransgenic pollen. Therefore, while hemizygous transgenes can be maintained through female linage, a potential escape of transgenic seed or fruit to the environment and food chain, would still be a concern. In pollen or seed-specific and flower meristem or germline-specific recombination strategies, elimination of transgenes from both gametes and seed provides greater environmental safety. However, transgene maintenance with all currently proposed germline–microspore–seed-specific auto-excision strategies becomes practically difficult, and is essentially limited to plant species vegetatively propagated. In these regards, further improvements of these strategies are needed to realize their full commercial potential.

Newly Introduced Site-specific Recombination System for Plant Genetic Engineering: The *Streptomyces* Phage PhiC31 Integrase System

Certain transgenic practices may require a concomitant use of more than one recombination system and strategy (Srivastava and Ow 2004; Ow 2005, 2007). Moreover, different recombination systems and strategies may function in various efficiencies under different environmental conditions and plant growth and physiological stages. Therefore, the development of novel strategies to provide further versatility in the control of transgenes and genome manipulations through excision-activation technologies is needed. In this respect, the use of multiple site-specific recombination systems for genome manipulation in plants has been suggested (van Haaren and Ow 1993; Ow 2002, 2005, 2007; Gidoni et al. 2001b, 2003; Srivastava and Ow 2004; Djukanovic et al. 2006). A large number of site-specific recombination systems from prokaryotes and lower eukaryotes have already been identified and characterized, and more are likely to be discovered. New site-specific recombination systems for potential use in eukaryotic systems, including plants, have recently been described (Groth and Calos 2004; Gottfried et al. 2005; Russell et al. 2006; Keravala et al. 2006; Thomson and Ow 2006; Gronlund et al. 2007). In general, recombinase systems have been classified into two major families based on amino acid sequence homology and the recombinase catalytic amino acid residue (tyrosine or serine). Basic requirements for successful utilization of both classes in heterologous cell environments include autonomy of the recombinase activity and template independence. Among currently studied recombinases that fulfill these requirements, the phiC31 integrase (Int) system, a member of the serine family of recombinases, is most advanced. Although its mechanism of action is not fully resolved yet, the phiC31 integrase resembles other members of these families and binds its two short cognate DNA substrates, *attB* and *attP*, together into a synapse. Recombination is, then, initiated by cleavage at the dinucleotide (TT) core of each substrate, resulting in staggered end formation followed by strand exchange and reciprocal rejoining of the two alternative half sites of each substrate to form recombination products (Fig. 3).

The PhiC31 Int was shown to deliver an autonomous and unidirectional intermolecular integration reaction between its two nonidentical *attP* and *attB* cognate attachment sites *in vitro* (Thorpe and Smith 1998; Thorpe et al. 2000) and *in vivo*, in *E. coli* (Thorpe and Smith 1998), mammalian (Groth et al. 2000; Thyagarajan et al. 2001; Hollis et al. 2003), *Drosophila* (Groth et al. 2004), in plant (tobacco) extra-chromosomally (Marillonnet et al. 2004), and plastid (Lutz et al. 2004). Int was similarly demonstrated to enable integration-mediated cassette exchange between *attB*-flanked DNA and *attP*-flanked chromosomal targets in yeast (*Schizosaccharomyces pombe*; Thomason et al. 2001) and mouse cells (Belteki et al. 2003). Additionally, employment of Int in intramolecular excision of DNA segments bordered by an *attB* and an *attP* site was demonstrated *in vitro* (Thorpe and Smith 1998), in *E. coli* (Thorpe and Smith 1998; Groth et al. 2000), cultured human cells (Groth et al. 2000), and tobacco plastids (Kittiwongwattana et al. 2007). Minimal *attB* and *attP* sizes of 34 and 39 bp, respectively (derived from approximate full length 280 bp *attB* and 84 bp *attP* sequences), were defined on the ability to maintain 100% recombination frequency in *E. coli* (Groth et al. 2000). Although these minimal sites are largely competent for Int binding (Thorpe et al. 2000), it is worth noting, however, that the extent to which these sites maintain full recombination efficiency in other heterologous environments has yet to be experimentally tested (Groth et al. 2000).

In contrast to the freely reversible recombination characteristic of the two identical recombination target substrates (of the Cre, FLP, and R recombination systems), the nonidentical *attB* and *attP* sequences of PhiC31 generate two new sites upon recombination, namely *attL* and *attR*.

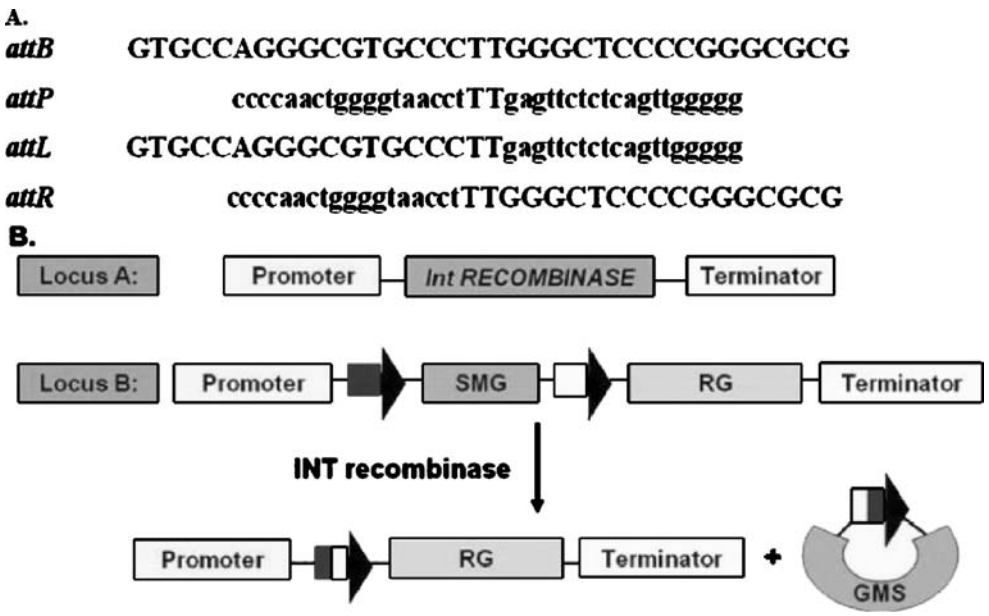

Figure 3. Sequence and schematic presentation of excision recombination between the phage PhiC31 integrase (Int) recombinase minimal *attB* and *attP* attachment sites. **a**, The nonidentical *attB* (34 bp; *upper case*) and *attP* (39 bp; *lower case*) sites, are composed of imperfect inverted repeat sequences comprising binding sites for Int, flanking the dinucleotide core sequence TT where recombination occurs (Combes et al. 2002). The recombination products *attL* and *attR* constitute chimeric compositions of *attB* and *attP* sites. **b**, Interaction of the phage PhiC31 Int recombinase (from locus A) with intramolecularly positioned *attB* (*dark arrow*) and *attP* (*light arrow*) attachment sites (in locus B) have been demonstrated to direct efficient and unidirectional excision of the intermediate DNA, *in vitro* (Thorpe and Smith 1998), and extra-chromosomally in tobacco (Marillonnet et al. 2004) and plastids (Kittiwongwattana et al. 2007), giving rise to *attL* (dark–light arrow) and *attR* (light–dark arrow) recombination products. SMG and GMS, selectable marker gene coding region from right to left and left to right, respectively; RG, reporter gene.

The newly generated sites are chimeric compositions of *attP* and *attB* sites (Fig. 3) and, in the absence of an (as yet unidentified) excisionase protein, provide noncompatible substrates for Int (Thorpe and Smith 1998; Thorpe et al. 2000), thus, confer unidirectionality to the recombination reaction. These features, alongside successful employment of variant Int specificities derived from direct protein evolution methods (Sclimenti et al. 2001), encourage the employment of the PhiC31 system as a tool for site-specific gene and genome manipulation studies and applications in genetic engineering of plants.

Conclusion and Future Perspectives

Transgene excision-activation via site-specific recombination in extra- and intra-chromosomal environments has been so far demonstrated in a growing number of plant species including tobacco (Dale and Ow 1990, 1991; Odell et al. 1990; Onouchi et al. 1991; Bayley et al. 1992; Russell et al. 1992; Lloyd and Davis 1994; Kilby et al. 1995: Bar et al. 1996; Gidoni et al. 2001b), *Arabidopsis* (Russell et al. 1992; Kilby et al. 1995; Onouchi et al. 1995; Sonti et al. 1995; Luo et al. 2000; Hoff et al. 2001; Marjanac et al. 2008), petunia (Que et al. 1998; Coppoolse et al. 2003), tomato (Stuurman et al. 1996; Coppoolse et al. 2003; Zhang et al. 2006, Gidoni et al. unpublished results), potato (Cuellar et al. 2006; Kondrak et al. 2006), soybean (Li et al. 2007), strawberry (Schaart et al. 2004), wheat (Srivastava et al. 1999), rice (Endo et al. 2002; Hoa et al. 2002; Toriyama et al. 2003; Radhakrishnan and Srivastava 2005; Sreekala et al. 2005; Hu et al. 2008), maize (Lyznik et al. 1996; Srivastava and Ow 2001; Zhang et al. 2003; Kerbach et al. 2005), turfgrass (Hu et al. 2006), *Brassica juncea* (Indian mustard; Arumugam et al. 2007), citrus (Ballester et al. 2007), aspen, and snapdragon (Ebinuma and Komamine 2001). This progress, along with progress made in development of trans- and auto-excision recombination strategies (Table 1), indicates a significant step toward incorporation of site-specific directed excision-activation of transgenes as an integral part of current plant transformation technologies. Indeed implementation of the technology for agricultural practice has been recently materialized with the first US regulatory approval given to Renessen company for their recombinase-mediated marker-free transgenic maize line LY038 (reviewed in Ow 2007).

Incorporation of site-specific recombination technologies in plant transformation allows a variety of gene excision-activation control strategies. This can be achieved by the construct design in which different transgene expression units might be placed either within or outside the recombination boundaries. The choice of strategy depends basically on the desirable time of deletion which is dictated by the appropriate

Table 1. Induced recombination-mediated auto-excision of marker and recombinase genes

Induction signal/ promoter system	Recombination system/plant tested	Stage of induction/ treatment	Excision rate	Additional notes	References
Chemical/ herbicide antidote-inducible maize *GST*	R-*RS*/tobacco	Shoot explants/inducer in the medium	14–58%[a]	Low background (leakiness) in tobacco and wound-induction of promoter activity in aspen	Sugita et al. 2000; Ebinuma and Komamine 2001
Chemical/β-estradiol inducible transactivator *XVE*	Cre-*lox*/ *Arabidopsis*, rice, tomato	Shoot explants, callus-germinating seeds/ inducer in the medium	15–66% [a,b,c]	Tight control of the β-estradiol-*XVE* induction system in transgenic plants	Zuo et al. 2001; Sreekala et al. 2005; Zhang et al. 2006
Chemical/ dexamethasone-activated R-LBD	R-*RS*/strawberry	Regenerating leaf explants/inducer in the medium	N.D.	Leakiness in recombinase-LBD activity among transgenic lines/ positive–negative selection for completely marker-free plants	Schaart et al. 2004
Heat-shock/ *Arabidopsis HSP81–1*	Cre-*lox*/ *Arabidopsis*	Whole seedlings/×2 alternate 37° C-16 h and recovery	N.D.	Potential leakiness in promoter activity among transgenic lines	Hoff et al. 2001
Heat-shock/ *Arabidopsis HSP81–1*	Cre-*lox*/tobacco	Whole seedlings/×2 alternate 37° C-16 h and recovery	<100%[b]	Potential leakiness in promoter activity among transgenic lines	Liu et al. 2005
Heat-shock/ soybean *HSP17.5E*	Cre-*lox*/maize	Callus and immature embryos/42° C for 3–5 h	<100%[b,c]	Potential leakiness in promoter activity among transgenic lines	Zhang et al. 2003
Heat-shock/ soybean *HSP17.5E*	Cre-*lox*/tobacco	Seed, leaf/×3 alternate 42° C-2 h and recovery	40–80%[a,b,c]	Potential leakiness in promoter activity among transgenic lines	Wang et al. 2005
Heat-shock/ *Drosophila hsp70*	Cre-*lox*/potato	Shoot internodes and mini-tubers/42° C for 2–3 h	5–14%[a]	Rare cases of low leakiness in promoter activity among transgenic lines	Cuellar et al. 2006
Embryo-specific/ *Arabidopsis app1*	Cre-*lox*/soybean	Somatic embryogenesis	5–59%[a,b,c]	Stringent regulation of activation of *app1* promoter during the maturation of somatic embryos	Li et al. 2007
Microspore-specific/tobacco *NTM19*	Cre-*lox*/ *Arabidopsis*, tobacco	Early pollen development	<99.98%[a,c]	Stringent promoter control/ generation of marker-free pollen	Mlynárová et al. 2006
Pollen-specific/*B. campestris BGP1* and tomato *LAT5*	Cre- or FLP-*lox*-*FRT* fusion/ tobacco	Mature pollen development	<100%[a,c]	Stringent promoter control/ generation of marker-free pollen or pollen and seed/use of *lox*P-*FRT* fusion enhanced recombination efficiency by either recombinase	Luo et al. 2007
Pollen and seed-specific/*Arabidopsis PAB5*		Pollen and seed development			
Male germline-specific/*Arabidopsis SDS*	Cre-*lox*/ *Arabidopsis*	Male gamete development	83–100%[a,c]	Stringent promoters control/ generation of marker-free male or male and female gametes/ positive–negative selection for completely marker-free plants	Verweire et al. 2007
Floral meristem-specific/*Arabidopsis AP1*		Flower meristem-male and female germlines			

Summary of recent reports utilizing a single recombination construct where a marker gene and a recombinase gene co-reside between the recombination target sites under control of an inducible promoter
GST Glutathione-S-transferase (GST-II-27) promoter-MAT vector, *R-LBD* glucocorticoid receptor ligand binding domain fused to the C-terminus of R recombinase gene, *AP1 Apetala1*, *SDS* solo dancers *N.D.* not determined
[a] Complete plant gene excision events
[b] Incomplete plant (chimeric) gene excision events
[c] Provided indications for germline excision events

stage of gene function through plant development. As an example, in one strategy, a recombinase gene might be triggered immediately after plant transformation [for removal of, e.g., recombination target sites (*RTS*)-bounded selectable marker and recombinase genes] leaving other transgenes in functionally regulated forms to be activated through later or appropriate stages. In a second strategy, where genes of agronomic interest are required to function during vegetative growth, appropriate excision timing for these genes (in addition to the selectable marker and recombinase genes) would be during germline developmental stages. In this strategy, inclusion of other genes (e.g., consumer-oriented traits such as flower pigmentation or nutritional traits) outside the recombination boundaries would allow their expression through later stages (e.g., flowering, fruit, seed). Alternatively, upon fusion of directly oriented *RTS* boundaries to the T-DNA LB and RB, recombination will result with excision of "all" transgenes from the plant genome, giving rise to "nontransgenic" plants. The latter approach, as proposed by Keenan and Stemmer (2002) and demonstrated by Mlynarova et al. (2006) and Luo et al. (2007), may narrow the debate on transgene acceptability down to a short (approximately 100–300 bp) transgenic plant sequence, composed of the T-DNA LB and RB elements (or an RB element only, Kondrak et al. 2006) flanking a recombination target site of bacterial or yeast origin.

A speculative approach to address this issue might emerge from the recent progress made in "intragenic" plant transformation based on replacement of bacterial T-DNA borders and selectable marker genes with functional sequence equivalents native to plants (reviewed in Rommens 2007; Rommens et al. 2007). Here, pseudo LB and RB elements and selectable marker gene sequences derived from plants could successfully substitute for the traditionally used bacterial T-DNA borders and selectable marker genes for plant transformation. Accordingly, studies of functional site-specific excision or activation of plant pseudo-*RTS* sequences which contain partial homology to a corresponding wild-type *RTS*, in conjunction with recombinase variants of altered specificities, might be considered. Using direct molecular evolution techniques, tailoring of recombinase specificities to exploit variant pseudo-recombination target sites (of either symmetric or asymmetric structure) have been previously demonstrated (Buchholz and Stewart 2001; Sclimenti et al. 2001; Thyagarajan et al. 2001; Santoro and Schultz 2002; Rufer and Sauer 2002; Voziyanov et al. 2003; Konieczka et al. 2004; Saraf-Levy et al. 2006; Bolusani et al. 2006; Sarkar et al. 2007). Implementation of the "intragenic" approach in excision recombination using plant-derived pseudo-*RTS* elements should be restricted to heterologous plants where the particular *RTS* sequences in use are absent. One would predict that the remaining pseudo "LB-*RTS*-RB" footprint of plant origin in the genome, consequent to the excision event, may improve the prospects of environmental safety, market acceptance, and regulatory approval of transgenic crops.

Acknowledgments We thank Victor Gaba, The Volcani Center, Israel, and Michele P. Calos, Stanford University, Stanford, CA, USA, for fruitful comments on the manuscript, with additional thanks to Victor Gaba for his encouragement and to Ms. Cecilia Cohen for creating the figures. This paper is a contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 136/2007.

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