Marker-free transgenic plants

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Abstract

Selectable marker genes are widely used for the efficient transformation of crop plants. In most cases, selection is based on antibiotic or herbicide resistance. Due mainly to consumer concerns, a suite of strategies (site-specific recombination, homologous recombination, transposition and co-transformation) have been developed to eliminate the marker gene from the nuclear or chloroplast genome after selection. Current efforts concentrate on systems where marker genes are eliminated efficiently soon after transformation. Alternatively, transgenic plants are produced by the use of marker genes that do not rely on antibiotic or herbicide resistance but instead promote regeneration after transformation. Here, the merits and shortcomings of different approaches and possible directions for their future development are discussed.

Abbreviations: DSB – double-strand break; HR – homologous recombination; NHEJ – non-homologous end joining

Introduction

The genetic modification of crop plants offers substantial improvements to agricultural practices, food quality and human health. A major focus of plant biotechnology over the last years is the development of improved tools for these genetic modifications. Two main goals are: the integration of sequences at any possible site of interest into the plant genome ('gene targeting') and the elimination of specific sequences from the plant genome that, similar to selectable markers, are dispensable for further use. Although various attempts have been made to establish general and efficient gene targeting strategies in plants, this has not yet been achieved (Kumar and Fladung, 2001; Puchta, 2002, 2003; Hohn and Puchta, 2003). In contrast, several techniques have been successfully established for the elimination of selectable marker genes.

Plant transformation is based on the ability to integrate foreign DNA into host plant genomes and on the efficiency of regeneration of transformed cells usually into shoots or embryos. Presently, the low transformation efficiency for many crops necessitates the use of selectable marker genes to identify transgenic plants. These dominant genes confer resistance to an antibiotic or herbicide that kills non-transformed cells. Thus, single cells with an integrated transgene within a bulk of non-transformed cells can often be identified.

During recent years concerns were raised – mainly by environmentalists and consumer organizations – that the presence of such genes within the environment or the food supply might be an unpredictable hazard to the ecosystem or to human health. Herbicide resistance genes might be transferred by outcrossing into weeds (see Dale et al., 2002). The presence of resistance genes against antibiotics in food products might theoretically lead to the spread of these resistances via intestinal bacteria in human populations,

although there is no evidence supporting this proposition. The absence of resistance genes in transgenic plants could also lower the costs for developing and marketing of genetically modified products and might speed up the commercial release of new products (Kuiper et al., 2001; Daniell, 2002; Smyth et al., 2002). Moreover, current transformation protocols severely limit the number of genes that can be introduced simultaneously. Therefore, re-transformation of a single line is a feasible and important approach towards selective introduction of multiple genes for complex traits such as broad pathogen resistance or tolerance to abiotic stress. Co-incorporation of different markers with each transgene or set of transgenes increases safety concerns and it is expensive and time-consuming. Tissue culture regimes for transformant selection would have to be repeatedly optimised, and the food safety and environmental impact of different markers would have to be assessed on a case-by-case basis, particularly difficult for combinations of resistance genes. Only a limited number of constitutive promoters are commonly used to express marker genes, and their repeated introduction could activate gene silencing mechanisms with negative effects on the expression of one or more transgenes of interest. Transgene elimination mechanisms permit the recycling of a single marker by its removal after each transformation step. If suitable technology becomes available in the foreseeable future, it is likely that regulatory legislation will strongly favour the absence of dispensable transgenic material in GM crops. The recent UK guidelines on 'Best Practices for the Design of GM crops' recommends minimizing the 'foreign' genetic material in GM crops, and the European Council Directive 2001/18/EC on 'the Deliberate Release into the Environment of Genetically Modified Organisms' requests a 'phase out' of the use of antibiotic resistance markers that confer resistance to 'clinically used' antibiotics by 2004. Therefore, studies to avoid marker genes or to eliminate them after use, have been conducted, and a growing number of methods are under development for the elimination of these genes. The topic has increasing interest and a number of reviews have been published recently (Puchta, 2000, 2003; Ebinuma et al., 2001; Hohn et al., 2001; Ow, 2001, 2002; Hare and Chua, 2002; Zuo et al., 2002). This review will focus on recent improvements of strategies that use recombination systems for the elimination of marker genes.

In principle, there are four ways to either avoid or get rid of 'problematic' selectable marker genes before transgenic plants are introduced into the field:

- Totally avoiding the use selectable marker genes. Theoretically, it should be possible to identify among a large number of cells the ones that carry a transgene directly by molecular methods particularly if transformation efficiencies can be improved. However, even in the days of automated analysis and polymerase chain reaction such a project is still highly demanding. A first report published recently indicates that feasible techniques might be indeed set up in the near future (Aziz and Machray, 2003).
- Use of marker genes ('screenable markers') that have no potentially 'harmful' biological activities.
- Co-transformation of two transgenes, one carrying the desired trait and the other the selection marker, followed by the segregation of the two.
- Excision of the selectable marker gene out of the integrated transgene after successful selection by using site-specific recombination, transposition or homologous recombination (HR). In the following discussion, these strategies are described in detail.

Replacing selectable with screenable markers

In parallel to or in combination with marker elimination, a new set of markers is being developed. The rationale behind this system is that non transformed cells are not killed as in the procedures using antibiotic or herbicide resistance genes; rather, the transformed cells experience a metabolic or developmental advantage. This might even increase the efficiency of regeneration of transformed plants. Genes that permit identification of transgenic plants in the absence of a selective agent are known as screenable markers. Non-toxic selective chemicals, as opposed to antibiotics and herbicides have been used successfully, e.g. the bacterial β-glucuronidase (Joersbo and Okkels, 1996), xylose isomerase (Haldrup et al., 1998) and phosphomannose isomerase genes (Joersbo et al., 1998; Negrotto et al., 2000) as well as the yeast 2-desoxyglucose-6-phosphate phosphatase (Kunze et al., 2001). Also, genes encoding enzymes playing a role in phytohormone metabolism such as the isopentenyl transferase (*ipt*) gene from the T-DNA of *Agrobacterium* were successfully used for the selec-

tion of transformants (Ebinuma et al., 1997a). *Rol A,B,C* genes, which increase the sensitivity of transgenic cells to plant hormones, were used to select visually transgenic plants as hairy roots (Ebinuma et al., 1997b). The use of a dexamethasone-inducible promoter driving the *ipt* gene led to the recovery of lettuce and tobacco transformants under inducing conditions (Kunkel et al., 1999). Recently, more approaches for the isolation of screenable markers for species that can be regenerated by organogenesis or somatic embryogenesis were undertaken (see Zuo et al., 2002). With the development of these new markers, concerns about the spread of herbicide or antibiotic resistance into the environment become irrelevant; especially if the marker originates from the respective crop plant itself and therefore is not 'foreign' DNA. However, considering reduction of transgene sequences to an absolute minimum as a final aim, the complete elimination of transformation markers seems to be more favorable in the long run.

Elimination of marker genes by co-transformation

One way to separate selectable marker genes from the transgene of interest is to separate them at the stage of transformation. Usually *Agrobacterium*-mediated transformation is used for this purpose, because separate integration events occur more regularly using this method than with direct gene delivery methods. In principle, in co-transformation experiments the desired gene and the transformation marker can be supplied on two T-DNAs within the same binary vector (Depicker et al., 1985; Komari et al., 1996; Lu et al., 2001) or on two binary vectors within the same *Agrobacterium* (Daley et al., 1998) or with two different *Agrobacterium* strains (Depicker et al., 1985; McKnight et al., 1987; De Block and Debrouwer, 1991; Komari et al., 1996; De Neve et al., 1997). A wide range of variations have been reported, and it is difficult to give a comprehensive or final evaluation on the general applicability of the different methods. However, co-transformation frequencies obtained are much higher than expected for independent events. A significant fraction of transformants will carry both transgenes as unlinked copies. For example, a high proportion of both tobacco and rice transformants carrying unlinked transgenes can be obtained routinely (Komari et al., 1996). An inherent limitation for the further optimization of this strategy: non-linked transgene loci have to be separated by crossing. Therefore, the procedure not only requires fertile plants, but also it is very time consuming. It is also not applicable to transgenic trees with long generation times.

Site-specific recombination

The ability of microbial site-specific recombinases to cleave DNA at specific sites and ligate it to the cleaved DNA at a second target sequence has led to their widespread use in manipulating DNA in higher eukaryotes. The first demonstration that a selectable marker gene can be removed from the genome of a transgenic plant – a milestone in plant biotechnology – was achieved more than a decade ago. A kanamycin gene placed between two *lox* sites was excised from the tobacco genome by the expression of the Cre recombinase (Dale and Ow, 1991). Besides Cre other single chain recombinases were used for removal of transgene sequences namely the FLP/*ftr* system of the 2 μ plasmid of *S. cerevisiae* (Kilby et al., 1995; Lyznik et al., 1996; Davies et al., 1999; Luo et al., 2000; Gidoni et al., 2001) or the R-*RS* system of the pSR1 plasmid of *Zygosaccharomyces rouxii* (Onouchi et al., 1995; Sugita et al., 1999, 2000; Ebinuma and Komamine, 2001). Unlike most recombinases, Cre, Flp and R require neither modification nor host-specific factors to function in plants. A common feature of all these systems is that after a first round of transformation, transgenic plants are produced that contain the respective recombinase and the sequence to be eliminated between two directly oriented recognition sites. After expression of the single-chain recombinase, the recombination reaction is initiated resulting in transgenic plants devoid of the selectable marker (Figure 1). Cre, Flp and R are members of the integrase family.

Crystal structures of the complexes formed for several site-specific recombinases with their DNA targets have revealed conservation within their catalytic regions and fundamental similarities in their modes of action (van Duyne, 2001). Recognition sites for recombinases comprise palindromes which flank 6–8 innermost base pairs. Each recombinase binding element is bound by a single recombinase subunit. Cleavage of the sites occurs at the borders between the recombinase binding elements and the core se-

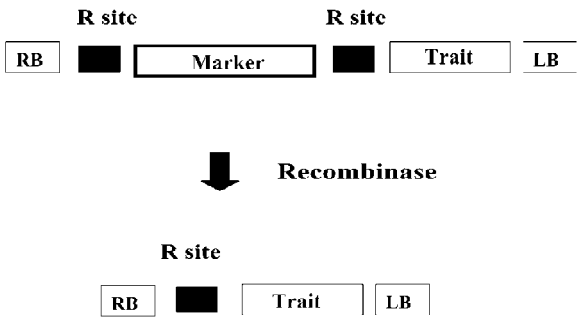

Figure 1. General strategy for the excision of selectable marker genes by site-specific recombinases (Dale and Ow, 1991). The selectable marker gene is inserted into the transformation vector between two directly repeated recombination (R) sites that are recognized by a site-specific recombinase and used for the selection of transgenic plant cells. After expression of the respective recombinase, the marker gene is excised from the plant genome and the trait gene is left behind. RB – right border; LB – left border of T-DNA.

quence. The core element is the site of strand exchange and confers directionality on the recombination site. Recombination requires two recombinase recognition sites bound by four identical recombinase subunits. The Cre recombinase of bacteriophage P1 converts dimeric phage P1 plasmids into their monomeric constituents through recombination between two directly repeated *lox* sites in the genome of *Escherichia coli*. The yeast Flp and R recombinases enable efficient replication of plasmids bearing the *FRT* and *RS* sites. Inversion of a segment of the plasmid which is flanked by two recombination sites in opposite orientation promotes replication by switching the relative orientations of the replication forks.

By controlled expression of the respective recombinase and specific allocation of the recombination sites within transgenic constructs, the system can be applied to a set of different genome manipulations. Most studies were performed with the Cre-System (for review see Vergunst and Hooykaas, 1999, Ow, 2002). In general, two *lox*-sites in direct orientation are required for excision of the intervening sequences (e.g. Russell et al., 1992; Gleave et al., 1999; Hoff et al., 2001; Zuo et al., 2001). If the *lox* sites have been moved apart via transposon jumping (Osborne et al., 1995), then larger genomic sequences can be excised via expression of Cre.

The Cre/*lox* system can also be used as a precaution to avoid transgene silencing. Direct transformation protocols tend to incorporate multiple copies of the same transgene at a single locus. These complex integration patterns can be resolved to a single transgene copy by flanking the transgene of interest with inverted recombination sites or only a single site. Flanking the cassette containing the selectable marker and recombinase gene with direct repeats ensures simultaneous elimination of the selectable marker (Srivastava et al., 1999; Srivastava and Ow, 2001). Inversely a transgene can be integrated site-specifically into a *lox* site (Albert et al., 1995; Vergunst et al., 1998; Srivastava and Ow, 2002). Because this reaction is reversible with a bias towards excision, specific *lox* sites were developed in which the newly combined half sites were no longer functional after integration (Albert et al., 1995). Two *lox*-sites in inverted orientation are necessary for inversion of the intervening sequence (Medberry et al., 1995). Even the exchange of chromosome arms (Qin et al., 1994; Koshinsky et al., 2000; Vergunst et al., 2000a) was achieved with the Cre-*lox* system in plants.

Transposition

Besides site-specific recombination, transposable elements can be used to obtain marker-free transgenic plants. The strategy is to connect either the transgene or the selectable marker with transposable sequences in such a way that the two entities can be separated from each other in a controlled reaction after transformation and selection. Both approaches have been applied successfully. In the first one, the marker gene is placed on a mobile element which is lost after transposition (Gorbunova and Levy, 2000). Marker-free transgenic tobacco and aspen plants have been generated at low frequencies by inserting the selectable *ipt* gene into the transposable element *Ac* (Ebinuma et al., 1997a, b). The second possibility for transposon-induced dissociation of the marker and the desired gene consists in relocation of the desired gene away from the original transgene locus. The feasibili-

ty of this approach was demonstrated in tomato (Goldsbrough et al., 1993; Yoder and Goldsbrough, 1994). The advantage of this system is not only to unlink the marker-gene, but also to create a series of plants with transgene loci different from one original transformant, which is especially appreciated if recalcitrant plants have to be transformed. This re-positioning allows expression of the transgene at different genomic positions and consequently at different levels of expression. However, as segregation of transgene and marker are required and transposons tend to jump into linked positions, this approach is very time consuming.

Homologous recombination

Deletion of sequences positioned between direct repeats in the genome via homologous recombination (HR) occurs at low frequencies in somatic cells (Puchta et al., 1995). Therefore for a long time HR was regarded as not feasible for the removal of marker genes. Only recently this picture changed due to an observation by the group of Peter Meyer. In transgenic tobacco calli carrying between two, 352 bp long *attP*-sites, a kanamycin gene and a negative selectable marker, both genes were lost at high frequency during growth (Zubko et al., 2000). The regeneration protocol produced marker-free plants more quickly than procedures involving re-transformation or cross-pollination and also avoided potential problems associated with expression of a site-specific recombinase. This was surprising since *attP*-sites are used by the bacteriophage lambda for integration at the *attB*-site into the *E. coli* genome, a reaction for which two proteins are needed, the phage-encoded integrase (int) and the bacterial integration host factor (IHF). However, none of the proteins was expressed in plants. As we know from various experiments, intrachromosomal HR (ICR) between closely linked repeated sequences in tobacco occurs at frequencies of about 10^{-6} (e.g. Puchta et al., 1995). Therefore, what could cause the much higher rates in the reported experiments? Interestingly, only two out of 11 lines tested showed high deletion frequencies. They were in three out of 23 cases associated with HR between the 352 bp long repeats. Perhaps the formation of a recombination hot spot due to the induction of double-strand breaks (DSBs) at the respective transgene locus might be responsible for the phenomenon (Puchta, 2000). DSBs enhance HR as well as non-homologous end joining (NHEJ) (Gorbunova and Levy, 1999). NHEJ could not only result in preferential integration of transgenes in these loci (Salomon and Puchta, 1998), but also in a decreased stability of the transgene sequences later on. Further experiments on the general applicability of the technique and the stability of the resulting transgenes have to be performed.

Recombination is inducible by DSBs and all current recombination models are based on the repair of such breaks (Rubin and Levy, 1997; Puchta, 1998; for a general overview see Paques and Haber, 1999). Therefore, it is indeed possible to enhance recombination in plant cells by 'activating' specific sites in the genome via induction of double-strand breaks (DSB). Transient expression of the restriction enzyme I-*Sce*I can induce DSBs at transgenic restriction sites within the plant genome *in vivo*, resulting in an enhancement of HR as well as of NHEJ by several orders of magnitude (Puchta et al., 1996; Salomon and Puchta, 1998; Puchta, 1999; Kirik et al., 2000). Although this strategy is not applicable for targeting genes at will as such a break can only be induced at the transgenic recognition sites of the rare cutting restriction enzyme, it can be used to excise transgenic sequences from the genome. Recently, a marker gene flanked by I-*Sce*I sites and homologous sequences was excised from transgenic tobacco plants (Figure 2). After I-*Sce*I expression, the marker gene was removed in a third of the cases by HR, demonstrating that DSB-mediated recombination induced by highly specific endonucleases is a feasible alternative to site-specific recombinases for marker elimination (Siebert and Puchta, 2002). Moreover in another third of the cases, the break was rejoined via NHEJ after excision of the marker with the molecular scissors I-*Sce*I. The marker loss was in most cases accompanied by small deletions at the break site by which the recognition site of the enzyme was destroyed (Siebert and Puchta, 2002). Thus, DSB-induced NHEJ is also an attractive alternative technique for the elimination of marker genes (Figure 3). DSB-induced techniques leave no 'active' footprint behind in the genome. This is in contrast to the use of site-specific recombinases to excise DNA. Here every elimination event leaves a residual recognition sequence at the recombination site. Removal of these elements might be prudent in instances where transgene 'stacking' and several marker elimination steps have resulted in multiple copies of the same recombination site distributed throughout the genome. Activation of gene silencing mechanisms by multiple copies of the same element might counteract the

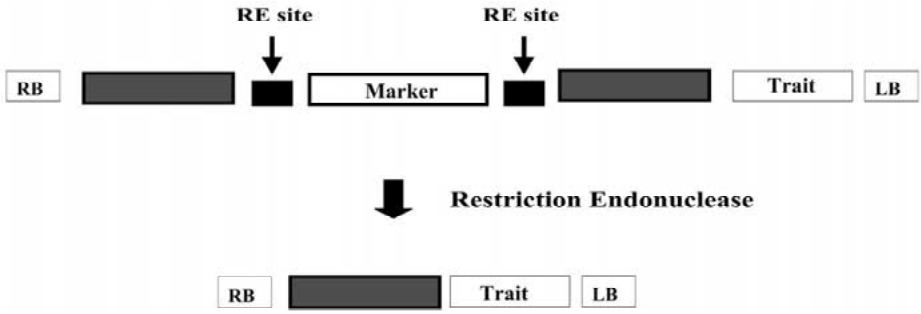

Figure 2. Excision of a marker gene by homologous recombination (HR) after induction of DSBs with a rare cutting restriction endonuclease (Siebert and Puchta, 2002). The selectable marker gene is inserted into the transformation vector between two homologous sequences (grey boxes) and two restriction sites (RE) and used for the selection of transgenic plant cells. After expression of the restriction enzyme HR between the overlapping sequences takes place, the marker gene and the restriction sites are deleted from the construct. The homologous sequence supplied can be part of the trait gene so that no extra transgenic sequences are left in the genome after recombination. RB – right border; LB – left border of T-DNA.

engineering of the desired trait. As the recognition sites of the restriction enzyme are destroyed during DSB-repair, this kind of drawback can be avoided by the use of HR.

Recent experiments indicate that HR can also be enhanced in somatic plant cells by the expression of bacterial proteins involved in recombination such as RecA and RuvC (Reiss et al., 1996, 2000; Shalev et al., 1999). However, in these cases and in contrast to site-specific DSB induction, all sequences in the genome carrying homologies will be 'activated'. The resulting enhanced recombination frequencies will lead to a general destabilization of the plant genome. Therefore, these kinds of strategies are not a useful alternative to DSB induction for marker gene excision by HR.

Elimination of marker genes from chloroplast DNA: site-specific and homologous recombination

In recent years, chloroplast transformation has gained more and more interest because outcrossing of transgenic material into the environment by pollen flow can be avoided for many angiosperms (see also recent reviews: Bock, 2001; Daniell et al., 2002; Maliga, 2002). However, the current state of the art excludes alternatives to the use of selectable markers to ensure homoplasmic transformation. The plastidic presence of tens of thousands of copies of a marker gene in each cell, of a transplastomic line, resulting also in larger amount of marker protein in theses cells, is a strong argument for the development of effective

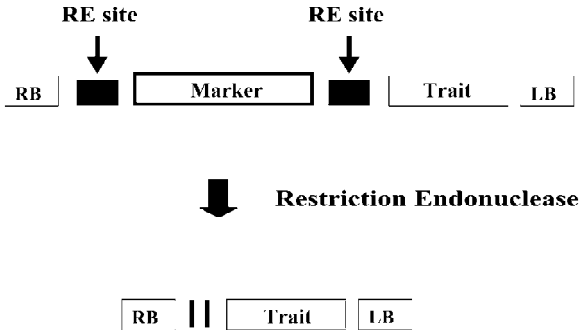

Figure 3. Excision of a marker gene by non-homologous end joining (NHEJ) after induction of DSBs with a rare cutting restriction endonuclease (Siebert and Puchta, 2002). The selectable marker gene is inserted into the transformation vector between two restriction sites (RE) and used for the selection of transgenic plant cells. After expression of the restriction enzyme NHEJ takes place between the break sites, the marker gene and is deleted from the construct. In most cases the restriction site is not restored. RB – right border; LB – left border of T-DNA.

strategies to eliminate chloroplastic selectable markers once they have served their function in identifying transplastomic lines. Two elegant studies demonstrated that the introduction of plastid-targeted Cre into the nuclear genome by either *Agrobacterium*--mediated transformation or pollination can also eliminate selectable markers from the plastome (Corneille et al., 2001; Hajdukiewicz et al., 2001). The elimination of the marker was efficient, however, beside the intended elimination events, DNA deletions in plastidic DNA from clones transformed with *Agrobacterium*, and to a lesser extent from those derived from crossing with a Cre-expressing line were also reported. However, this is not necessarily a major limitation of the approach. Due to the reported high frequencies, the identification of plants with the envisaged elimination is feasible. This also indicates that Cre is capable of catalysing recombination between certain naturally-occurring 'pseudo-*lox* sites' sequences that can be highly divergent from the *lox* consensus (Thyagarajan et al., 2000). Thus, unwanted and even unrecognised by-products can be produced. Indeed, unwanted chromosome rearrangements in mammalian cells due to Cre expression have been described (Schmidt et al., 2000; Loonstra et al., 2001). Crinkled leaves and reduced fertility have been observed in certain plant lines expressing high levels of Cre, indicating that the expression of the enzyme under certain circumstances might also be deleterious for plants (Coppoolse et al., 2003).

In contrast to the nuclei of higher eukaryotes, HR is for chloroplasts as well as for bacteria the main mode of DNA recombination (Heifetz, 2000; Bock, 2001). If no DSBs are supplied (as discussed in detail above, Siebert and Puchta, 2002), flanking of a selectable marker with direct repeats in the nuclear plant genome leads only in rare cases to elimination of the marker. Therefore, HR seems to be a more suitable alternative for removing unwanted transgene sequences from the chloroplast genome. Recently this was demonstrated by an elegant study in tobacco showing removal of different marker genes by the use of flanking homologies of 174 or 418 bps (Iamtham and Day, 2001).

Improving the applicability of the technique: new enzymes for excision

Normally a specific site-specific recombinase can be used only for a single round of genome manipulation as functional sites are left behind in the genome. The most obvious solution to this problem is the sequential use of different recombinases. Therefore, the interest on 'new' site-specific integration systems has risen over the years. Mutant Int proteins have been developed that no longer require supplementary factors to perform excessive recombination in human cells (Lorbach et al., 2000), but their efficacy in plants remains to be tested. Similarly, the highly efficient site-specific recombination system of the *Streptomyces* bacteriophage phiC31 seems to be attractive for use in plants (Thomason et al., 2001; Ow, 2002). The recent demonstration that directed evolution strategies can be used to modify recombinase substrate specificities (Buchholz and Stewart, 2001) extends the potential of this approach.

Another sophisticated approach is to use tailor-made endonucleases (Bibikova et al., 2001) designed to induce DSBs at unique sites within the genome. Placing different restriction endonucleases under the regulation of chemical-inducible promoters (see below) will further extend the range and flexibility of different approaches for transgene elimination.

Improving the efficiency of the technique: the concept of the inducible excision

Another economically important goal is the reduction of time required to obtain marker-free transgenic plants. Cross-pollination is not compatible with breeding programs for crops that are vegetatively propagated or have long generation times, and repeated passage through tissue culture increases the incidence of somaclonal variation. In early experiments, transgenic plants containing the marker were regenerated first; then they were crossed to plants expressing the recombinase, and finally the progeny of this cross were checked for marker-free seedlings (Dale and Ow, 1991). At present, the marker is mostly eliminated soon after transformation within a single tissue culture phase. One approach to prevent excessive exposure to recombinase is the transient exposure of plants to *Agrobacteria* which express the respective recombinase. Transcription of unintegrated T-DNA molecules seems to be sufficient to eliminate the selectable marker without integration of the recombinase gene into the genome. A negative selectable marker gene can be included as well as the selection marker in the sequence that is to be deleted from the transforming DNA. After transient expression of the

recombinase, transgenic plant cells without the marker genes can then be selected (Gleave et al., 1999). Drawbacks of this approach are: the lower efficiency in comparison to other techniques (see below); and in a significant percentage of the lines besides recombinase-mediated excision, a co-integration of the T-DNA harbouring the recombinase takes place. The use of another negative selectable marker directly linked to the recombinase gene might help to identify plants without co-integrated T-DNA. A very promising new approach to circumvent this problem seems to be fusions between Cre and the *Agrobacterium* VirE2 and VirF proteins. The fused proteins retain recombinase activity and can be transported into plant cells independently of T-DNA transfer (Vergunst et al., 2000b). The main limitation of all transient expression approaches is that an additional regeneration step is required after transformation to obtain the desired plant lines.

Alternatively, inducible marker elimination strategies that provide the trait gene, selectable marker, recombinase gene and its recognition sites within a single vector can be used to simplify the multi-step procedures described above. Prevention of recombinase expression in the absence of its inducer is also likely to limit unintentional damage to plant DNA sequences and to ensure that the selectable markers are eliminated only after transformants have been identified. Flanking the recombinase gene with its own recognition sites ensures that the recombinase will excise the gene directing its own synthesis as soon as the critical level of expression required for excision is reached, thereby avoiding excessive recombinase activity (Figure 4). Due to the loss of the marker gene, an unintended basal rate of recombinase gene expression will prevent regeneration of transgenic plants Two independent systems have been developed recently.

In the CLX (Cre/*lox* DNA excision) system shown to function in *Arabidopsis*, Cre expression was placed under the control of the β-estradiol-inducible XVE hybrid transactivator (Ow, 2001; Zuo et al., 2001). Inclusion of both transcription units required for recombinase activity and antibiotic resistance within *lox* sites ensured that a negative feedback loop restricted Cre expression to the level required for excision. β-estradiol-induced excision of the transcriptional units encoding Cre, the XVE transactivator and kanamycin resistance marker activated a downstream GFP gene by bringing it into proximity with the G10–90 promoter. An intron within the Cre gene prevented its expression in *E. coli* during cloning, thereby preventing excision of genetic material contained between the *lox* sites. Using this system, excision occurred in all 19 primary transformants. However, by far the most significant feature of the CLX system is that this system ensures inheritance of the excision event in a significant percentage of the progeny of lines exposed to the inducer. Indeed the properties of the inducible promoter used are of utmost importance. Although a series of inducible promoters have been described for plants (for review see Zuo and Chua, 2000), the XVE system seems to be most suited (Zuo et al., 2000), whereas other promoters do not fulfil the high potential originally placed with them (e.g. Aoyama and Chua, 1997; Kang et al., 1999).

Using the GST-MAT (Multi-Auto-Transformation) vector system (Sugita et al., 2000), tobacco trans-

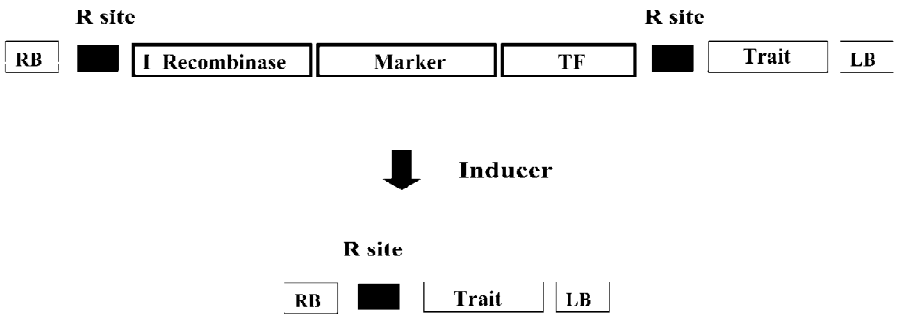

Figure 4. General strategy for the inducible one step excision of selectable marker genes by site-specific recombinases (Sugita et al., 2000; Zuo et al., 2001). The coding sequence for a chemically-inducible transactivator (TF) and transcriptional unit for the chemically-inducible recombinase (I Recombinase) is inserted into the transformation vector between two directly repeated recombination (R) sites that are recognized by a site-specific recombinase a cassette consisting of a selectable marker gene. It is then used for the selection of transgenic plant cells. After chemical induction of the expression of the recombinase, the cassette is excised from the plant genome RB – right border; LB – left border of T-DNA.

formants were selected by screening that involved *ipt*-mediated regeneration in the absence of exogenous cytokinin. Excision of both the *R* and the *ipt* genes was regulated by placing *R* recombinase under control of the maize glutathione-*S*-transferase (GST-II-27) promoter, which is induced by the herbicide antidote 'Safener'. Marker-free plants were generated at a frequency of approximately 14%. A similar strategy was applied to rice. Scutellum tissues of 5-day pre-cultured rice seeds were transformed, and marker-free transgenic rice plants could be regenerated directly from 25% of the infected scutella (Endo et al., 2002). The system needs no selective agent and no crossing for identification of transgenic plants that do not contain a selectable marker gene (Ebinuma and Komamine, 2001).

While applying the strategy of inducible elimination, even 'non-transgenic' plants can be produced out of transgenic crops (Keenan and Stemmer, 2002). Today plant transgenes are often expressed abundantly in all tissues. However, a transgene is often required to function only in a subset of cells, e.g. nematode resistance genes need only to be expressed in roots. Transgenic sequences are thus not required to be present in the edible fruits of these plants. This strategy relies on the inclusion of all transgenes including the gene of interest between the recognition sites for the respective recombinase. After expression of the recombinase all transgenic sequences beside the one restored recognition site are eliminated from the plant genome (Figure 5). For the recombinase expression, localized induction of tightly-regulated chemically-induced and/or stringently-regulated tissue-specific promoters can be used.

Conclusions

A major goal of plant biotechnology is to improve existing cultivars and to develop new and elite cultivars. For this purpose, the improvement of existing strategies and the development of novel approaches for plant genome manipulation are desirable. Different approaches for elimination of selectable marker genes have been developed over the last several years, and further improvements are now underway. These techniques are in the process of being transferred to many crop plants of interest. Thus, there is no need anymore for planting transgenic plants of a new generation out in the field that contain genes conferring antibiotic or herbicide resistance that served only in the transformation process. Concerns about an uncontrolled spread of these genes in ecosystems will become irrelevant the in near future.

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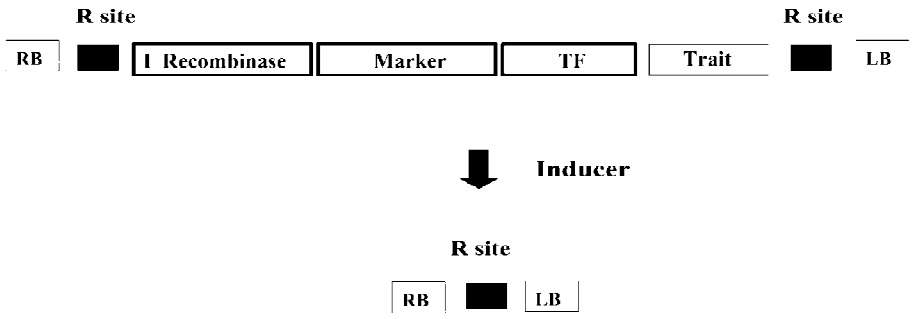

Figure 5. General strategy for transgene elimination after use (Keenan and Stemmer, 2002). The transcriptional unit for a tissue-specific or chemically-inducible recombinase (I Recombinase) and the trait genes is inserted into the transformation vector between two directly repeated recombination (R) sites that are recognized by a site-specific recombinase a cassette consiting of all transgenes including a selectable marker gene, the coding sequence for a chemically-inducible transactivator (TF). After expression of the recombinase in organs where the trait genes is not required for the biotechnological application (e.g. reproductive organs or eatable parts of the plant), only one recognition site of the recombinase is left behind in the genome after the excision of the transgene cassette RB – right border; LB – left border of T-DNA.

quences with optimal function' which is part of a cluster of biosafety research of the Bundesministerium für Bildung und Forschung, Bonn, Germany.

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