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Chapter 2

Strategies for Generating Marker-Free Transgenic Plants

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Why marker-free transgenic plants?

Selectable marker genes (SMGs), such as antibiotic or herbicide resistance genes, are used in nearly every plant transformation protocol to efficiently distinguish transformed from non-transformed cells. However, once a transgenic event has been selected, marker genes are generally of no use. On the contrary, the continued presence of marker genes in transgenic plants may raise public and regulatory concerns and may have technological disadvantages.

The main perceived risk is horizontal gene transfer of antibiotic resistance genes to pathogenic organisms or the transfer of herbicide resistance genes to weeds. Regulatory agencies may thus advice or require the absence of certain marker genes in commercialized transgenic plants [1].

Fears concerning SMGs center around the presence of antibiotic resistance genes in transgenic crops or its products that might reduce the efficacy of a clinically important antibiotic. A lot of attention has been spent on risk assessment concerning the transfer of antibiotic resistance genes from genetically modified (GM) plants to soil- and plant-related micro-organisms by horizontal gene transfer. For example, the transformation of bacteria in the food chain where free DNA persists in some materials for weeks, and moreover, some bacteria develop natural/chemical competence to take up DNA from the environment. In addition, in the gastrointestinal tract of humans and farm animals, DNA may remain stable for some time, particularly in the colon. However, degradation already begins before the DNA or the material containing the DNA arrives at the critical sites for horizontal gene transfer, which are generally believed to be the lower part of the small intestine, caecum, and the colon. In the case that DNA can arrive to this part, it will be mostly fractionated in pieces smaller than a gene sequence. Thus, breakdown of DNA in the gut, combined with the breakdown of the DNA due to food processing, strongly reduces the risk of dissemination [2]. Moreover, the antibiotic resistance genes that are commonly used as selectable marker genes in transgenic plants actually have a

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bacterial origin [3]. Indeed bacteria have developed very sophisticated mechanisms to eliminate competitors and guarantee their own survival producing antibiotics and genes to confer resistance to these antibiotics. Thus, the contribution of horizontal transfer of antibiotic resistance genes between transgenic plants and microorganism is most likely insignificant compared to the existing exchange of such genes between bacteria [3-6].

On the other hand the escape of herbicide resistance genes to wild relatives is also a concern. Many crops are sexually compatible with wild and/or weedy relatives, then if the plants grow close one to another, crop-to-weed or crop-to-wild relative gene flow could result (reviewed by [6, 7]. The success of the introgression of a transgene in a wild relative has many barriers. Firstly, both have to grow in close proximity; secondly, both have to be flowering in overlapping time frames; thirdly, the progeny must be sufficiently fertile to propagate; and fourthly, a selective pressure should be applied (herbicide) [8]. There will only be a selective advantage for the wild relative if the herbicide is used in the habitat where the relative grows. For example, it is well known that cultivated rice is sexually compatible with perennial wild red rice (*Oryza rufipogon* Griff.), considered a harmful weed. It grows in many of the same regions, often has overlapping flowering times, and thus is a prime candidate for gene flow with cultivated rice. Indeed, Chen et al. [9] showed that the gene flow rate was 0.01% under natural conditions. This and other studies showed the risk of the transfer of transgene(s) to the wild relative or weeds. Thus precautions should be taken into account to prevent gene flow and introgression. A possible way consists in containing transgenic pollen by growing barrier crops in adjacent areas or by alternating transgenic cultivars carrying different herbicide resistance genes [10]. Other strategies consists in the creation of biological containment, to limit the transfer of pollen to plants in the surrounding area, e.g. by engineering male sterility or by delaying and/or decreasing flowering [11, 12]. Alternatively, complete removal of the marker gene should alleviate concerns regarding effects on human health and the environment.

In some specific cases, selectable marker genes are needed after selection, for example in propagation of lines with nuclear male sterility [13]. However, generally SMGs are not needed after the selection of the transgene event. On the contrary, their presence may have some technological drawbacks. It has been reported that some genes (selectable markers included) may induce pleiotropic effects under certain conditions [14, 15]. In fact, a transcriptome analysis of three *Arabidopsis* transgenic lines containing pCAMBIA3300 vector (*35S-bar-35S*) showed that they differ from their WT counterparts by expression of 7, 18 and 32 genes respectively. However, only four genes were found to be significantly different in all three lines compared with the wild type plant in glufosinate untreated plants [14]. Thereafter, 81 genes were found to be differentially expressed in the presence of glufosinate in transgenic plants, in contrast to the 3762 differentially expressed genes in WT plants. From these 81 genes 29 were specific to transgenic plants [14]. These results suggested to the authors that glufosinate or a metabolic derivative of glufosinate activates unique detoxification pathways to offset any effects on plant growth and development. Nevertheless, in the above mentioned work, no indication or study of the position effect and/or effect of transgene regulatory sequences was reported. Indeed the regulatory sequences (promoters and terminators) can influence the activity of some genes in the same T-DNA or even endogenous genes that are close to the

insertion site [16-18]. Furthermore, in systems where the number of efficient SMGs is limited, the re-transformation with the same SMG is precluded by its presence. This is problematic as most transformation protocols are indeed based on one or a few selectable marker genes only. Miki and McHugh [3] reported that more than 90 % of the scientific publications that use transgenic plants were based on three selection systems: the antibiotics kanamycin or hygromycin and the herbicide phosphinothricin. These outcomes provide an extra motivation to remove SMGs and other unnecessary sequences as soon as possible after selection of transgenic plants.

2. Strategies to obtain marker free transgenic plants

2.1. Transformation without selection

The most straightforward method to obtain marker-free plants is to transform without any selectable marker gene. However, most of the transformation protocols described are inefficient and just few cells integrate the foreign DNA. Nonetheless, some groups have studied the feasibility to obtain transgenic plants omitting selection. De Buck et al. [19] failed to obtain any transgenic plants when *Arabidopsis* roots were transformed via *A. tumefaciens* and shoots regenerated on non-selective media. However, in tobacco protoplast transformation, these authors obtained a total transformation frequency of 18%. Transformation protocols have important influence on these and other results. For example, in a study where *Arabidopsis* was transformed by the floral dip protocol and seedlings were grown on non-selective media, transgenic plants could be obtained with an efficiency of 3.5% [20]. In citrus, 35 plants out of 620 analyzed were transgenic in the absence of selection [21]. The main objective of the experiments mentioned until here was not to obtain marker-free plants, but they showed the possibility or not to do so. Other experiments have as a goal to obtain marker free transgenic plants. For example, in wheat transformation via micro-projectile bombardment, 23 out of 191 regenerated plants in non-selective media were transgenic (12%) [22]. Also in potato and cassava transformation *via A. tumefaciens*, without selection pressure, resulted in transformed shoots at an efficiency of 1–5% of the harvested shoots [23]. In this case the presence of chimeric plants was less than 2% of transgenic plants. Other authors that mention the possibility to obtain chimeric plants were Doshi et al.[24], which obtained a transformation frequency of 0.93% and 1.55% in triticale and wheat, respectively, without selection. These authors suggest circumventing the chimera problem with two embryogenesis cycles, where the plantlets can be regenerated from secondary embryos formed from transformed sectors within a primary somatic embryo. A report of a non-selection approach for tobacco transformation showed a transformation efficiency of 2.2-2.8% for the most effective binary vector; the authors found that the number of chimeric plants was 28-56%, which is expected taking into account the regeneration system applied [25]. In another interesting report the direct production of marker-free citrus plants under non-selective conditions was assessed [26]. In two genotypes evaluated, only one produced transgenic plants with an efficiency of 1.7%. Remarkably, the expression of the gene of interest (*sgfp*) was very low in transgenic plants. This phenomenon has been reported before in citrus [21], *Arabidopsis thaliana* [20, 27] and white pine (*Pinus*

strobus L.) [28] transformed with *A. tumefaciens* and regenerated without selection. These results are in line with the fact that T-DNA integrates randomly with respect to particular DNA sequences in the genome, and that target sites include transcriptionally 'silent' regions, like telomeres [29]. Cells with insertion events in such silent regions likely do not survive when selection is applied.

2.2. Co-transformation of a marker gene and the gene-of-interest followed by segregation and selection of marker free progeny plants

Many approaches have been reported to remove selectable marker genes since the transformation technology was developed in the 80s. One of the earliest methods was based on co-transformation of a transgene and a selectable marker delivered by two separate DNA molecules and thereafter, segregation of both in the progeny (reviewed in [3, 30-32]). This strategy is based on the fact that cells selected for the presence of the marker gene, often contain the non-selected gene of interest as well. The SMG and the gene of interest can be delivered by: (i) two different *Agrobacterium* strains each containing a binary plasmid carrying a single T-DNA region (Fig. 1A) [33-37]; (ii) a single *Agrobacterium* strain, either containing one plasmid with two separate T-DNAs (Fig. 1C) [33, 36, 38-41] or (iii) containing two separate plasmids each containing a T-DNA (Fig. 1B) [42,43]. Alternatively, co-transformation can be achieved by particle co-bombardment (Fig. 1D) [44, 45]. The co-transformation strategy is limited because co-integration of both T-DNAs at the same genomic locus is frequently observed leading to linkage between the marker and the transgene, which makes their segregation impossible. This phenomenon has even more frequently been observed with particle bombardment-mediated transformation. Moreover, these methods cannot be applied to sterile plants and vegetatively propagated species, and are not practical in plants with a long life cycle such as trees [46]. This approach requires the generation of many transformants (to find unlinked marker genes and genes-of-interest) and further crossing steps (to remove the marker gene) making it a labor intensive work.

2.3. Placing the selectable marker gene or the gene-of-interest on a transposable element

Transposable elements (e.g. *Ac*/*Ds* from maize) can mediate repositioning of genetic material in the plant genome. The *Ac*/*Ds* transposable element system has been used for relocation and elimination of a selectable marker in tomato [47, 48] and rice [49]. Transposable elements can be excised from the genome after the expression of the transposase; they can either re-insert or not (Fig. 2). Taking into account these options, two approaches can be followed. In the first one, if one counts on re-insertion of the transposon, the gene of interest (GOI) is placed on the transposable element. Thus, the GOI will be excised and can be reinserted in a locus that is not linked to the locus in which the selectable marker gene is located; they can be segregated in the next generation [47, 49]. In a second approach, one relies on the fact that the transposon will not be re-inserted [50]. An example of such a system is the one described by Ebinuma et al. [51], in which the *ipt* selectable marker gene was inserted in an *Ac* derivative. However, marker-free transgenic plants were obtained only with a very low efficiency (5%) as a result of a high rate of re-insertion.

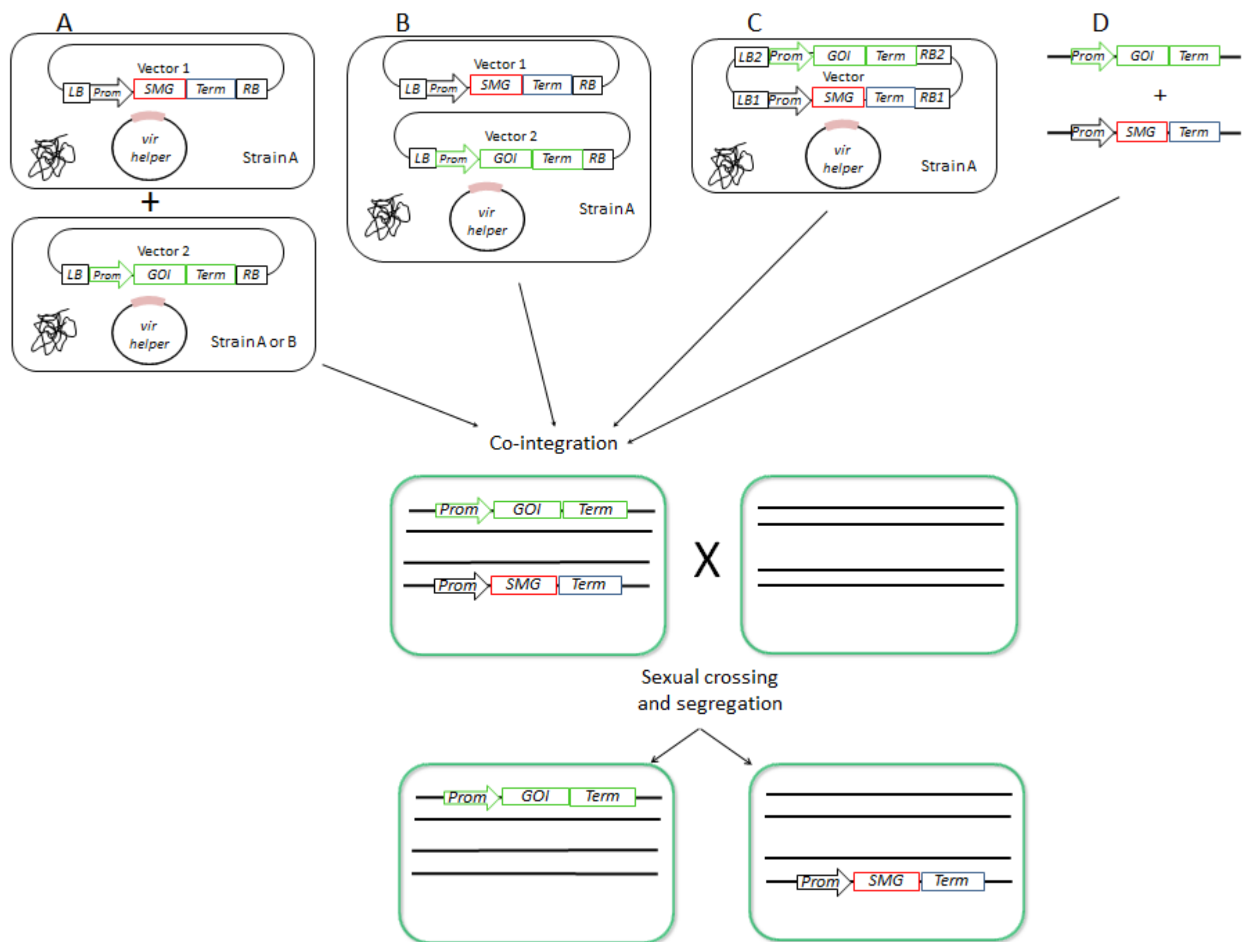

Figure 1. Co-transformation / segregation strategy to obtain marker-free transgenic plants. The SMG and the gene of interest (GOI) are introduced on separate T-DNAs present in two different *Agrobacterium strains* (A), on separate vectors in the same *Agrobacterium* strain (B), or on the same vector (C); the two genes can also be delivered by a direct gene transfer method such as particle bombardment (D). If the GOI and the SMG are integrated at unlinked positions, progeny plants with only the GOI can be obtained after sexual propagation. LB: T-DNA left border, RB: T-DNA right border, Prom: promoter; Term: terminator

This system has some advantages associated with the relocation of the gene of interest. For example, it permits to study a large range of position effects thereby generating an extensive qualitative and quantitative variation in expression levels from a single transpositionally active transformant line [49]. Moreover, relocation allows elimination through recombination in the progeny of all sequences co-integrated at the original integration site. Thus the integration pattern is simplified and the relocated transposon-borne transgene may be less susceptible to gene silencing than at the original integration [52].

On the other hand, this system has several drawbacks. First, the transposition efficiency is variable in different species. Second, the method is labor intensive and time consuming because it requires crossing transgenic plants and the selection of the progeny [53, 54]. The method shows low efficiency of marker gene elimination because of the tendency of transposable elements to reinsert in positions genetically linked to the original position. Other disadvantages of this system are the genomic instability of transgenic plants because of the

continuous presence of heterologous transposons and the generation of mutations because of insertion and excision cycles. Transposition can induce genome rearrangements, including deletions, inverted duplications, inversions, and translocations [55]. Additionally, this system cannot be used for sterile plants and vegetatively propagated species and is impractical for plants with a long life cycle.

2.4. Homologous recombination

Another method developed for marker gene removal takes advantage of the DNA repair machinery of plant cells. Indeed, efficient repair of double-strand breaks (DSBs) is important for survival of all organisms. DSBs can be repaired via homologous recombination (HR) or non-homologous end-joining (NHEJ) [56]. The ratio of HR to NHEJ events increases if homologous sequences near the brake are available [57]. During the repair process the gene can be converted or deleted [58]. Orel et al. [56] showed that deletion-associated pathway was about five times more frequent than the pathway resulting in gene conversion. These findings were exploited by Zubko et al. [59], who placed the selectable marker genes between two directly repeated 352 bp *attP* regions of bacteriophage λ. This sequence is rich in A+T nucleotides that is supposed to have a stimulatory effect on recombination [60]. Moreover, these elements were situated adjacent to a copy of the transformation booster sequence (TBS) from *Petunia hybrida*, which was shown to increase both HR and NHEJ in *Petunia*, *Nicotiana* and maize [61]. After selection on antibiotic (kanamycin) containing media, tobacco callus was

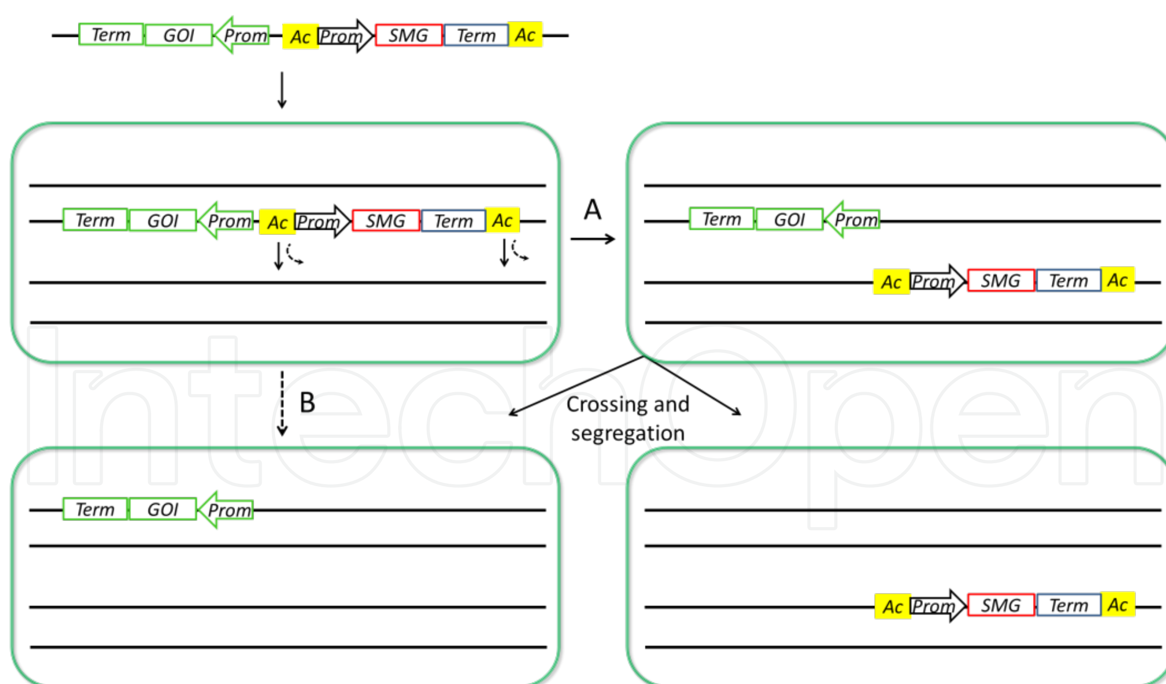

Figure 2. Transposon-mediated repositioning of the SMG. The SMG is cloned as part of a modified transposable element, e.g. the maize transposable element *Ac*, and linked to the gene of interest (GOI). Transposition may result in re-insertion of the modified element with the SMG (A); if the re-insertion occurs in an unlinked position, marker-free progeny may be obtained after crossing. Alternatively, no re-insertion occurs after excision of the modified transposable element (B), also resulting in the loss of the SMG.

placed on antibiotic-free media to allow for the loss of SMG by homologous recombination. Thereafter, plants were regenerated from callus and selection of marker free plants was based on sensitivity to the antibiotic. Two clones showed sensitivity to the antibiotic and formed green and white shoots. From these clones the authors regenerated 23 marker free plants. However, from these marker-free plants, 20 lost the gene of interest that was outside of the *attP* sites, probably because the NHEJ mechanism [59]. This protocol should produce marker-free plants faster than do procedures involving re-transformation or cross-pollination, and also avoid potential problems related with expression of recombinases (discussed below). Nonetheless, the method has some major disadvantages, like low efficiency, deletions of non-target genes, the recombination cannot be controlled and many transgenic events can be lost during the selection process. The mechanistic basis of the phenomenon is not yet understood and it is not yet known how the system could be applied in other crops.

2.5. Removal of the selectable marker gene after the selection procedure via site-specific recombinases or zinc finger nucleases

Another system to remove selectable marker genes is based on site-specific recombinases and was first reported about 20 years ago [62, 63]. Microbial site-specific recombinases have the ability to cleave DNA at specific sites and ligate it to the cleaved DNA at a second target sequence. The excision of foreign DNA that is placed in between recognition sites in a direct repeat orientation has been used to eliminate unwanted transgenic material from the nuclear genome of plants (Fig. 3). The most used recombination systems are Cre/*lox* from bacteriophage P1 [64, 65], FLP/*FRT* from *Saccharomyces cerevisiae* [66, 67] and R/*RS* from *Zygosaccharomyces rouxii* [68]. These systems are belonging to the tyrosine recombinase family [69, 70]. After the reaction, a recombination site (*lox*, *FRT* or *RS*) is remaining in the genome and it could potentially serve as a site for integrative recombination. However, re-insertion of the elimination fragment has not been detected [53, 71], probably because excision is an intramolecular event, whereas integration needs interaction between unlinked sites; and second, the excised circle cannot replicate autonomously and is probably rapidly lost *in vivo* [30].

The site-specific recombination systems can be divided in two categories according to the position of the recombinase gene. In a first category of strategies, the recombinase gene and the selectable marker are on a different vector and the recombinase gene is delivered to the plant containing the SMG by re-transformation [62, 72, 73] or by sexual crosses [63, 74-77].

A main limitation of both systems is that they require a time-consuming and labor-intensive breeding step, and that they are only applicable to sexually reproducing species or some species where the retransformation is available. An alternative approach depends on the expression of the recombinase transiently [78]. Marker-free plants were also obtained after infection of PPT resistant *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves with a modified plant virus carrying the *cre* gene (PVX-Cre) [79-81]; as well as in kanamycin resistant tobacco with a TMV-Cre [82]. This method can be applied to vegetatively propagated and long life cycle plants, but the lack of virus-based transformation system in these species is a drawback that should be improved in the future.

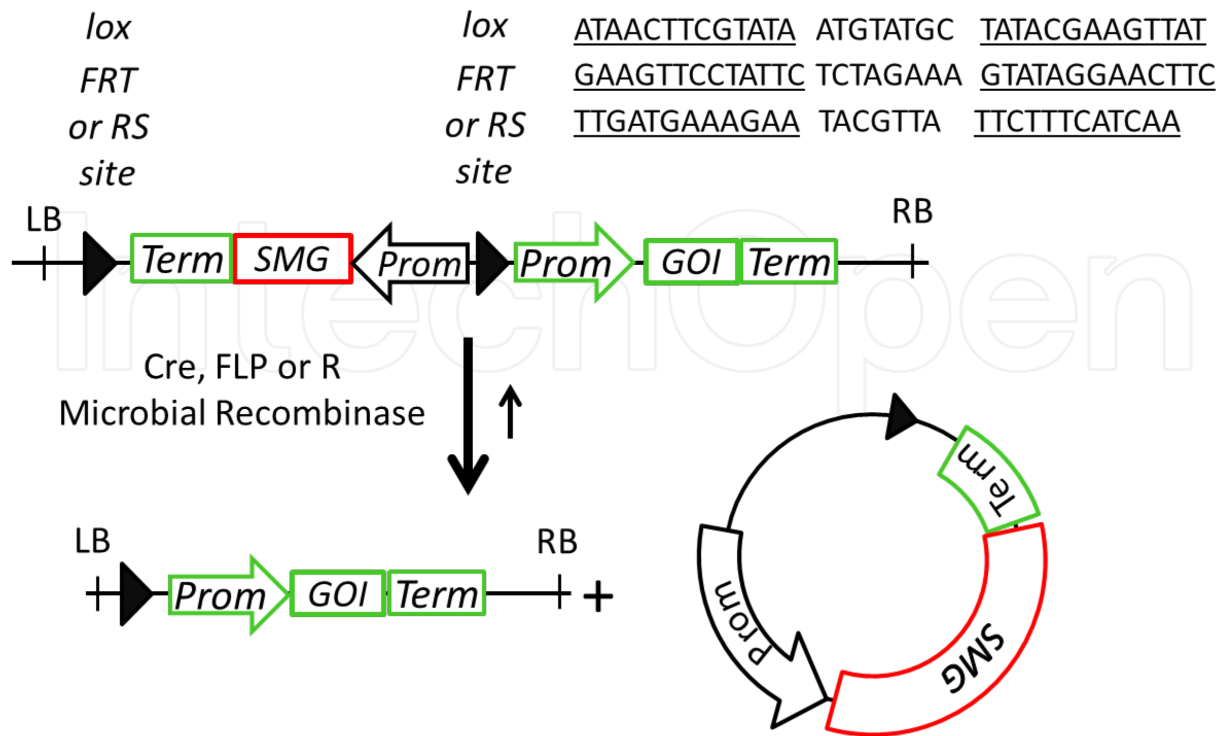

Figure 3. Removal of selectable marker genes through site specific recombinases. The SMG is flanked by directly repeated recombinase recognition sites, most often the *lox*, *FRT* or *RS* sites (black triangles; sequence of the sites is shown in the upper right corner). The presence of the cognate recombinase enzyme, Cre, FLP or R respectively, directs excision of the SMG. Re-insertion of the SMG occurs with low frequency if at all.

Nevertheless, as all technologies also the site-specific recombination systems have some drawbacks. *In vitro* studies suggest that Cre can catalyze recombination between certain naturally occurring "pseudo-*lox* sites" that can be highly divergent from the *lox* consensus sequence [83]. It was also shown that constitutive expression of *cre* can lead in animal cells to growth-inhibitory and genotoxic effects as a result of the endonuclease activity of Cre [84, 85]. This toxic effect was also investigated in *cre* expressing transgenic plants where a correlation was found between aberrant phenotypes and constitutive *cre* expression [86]. Data regarding the presence of cryptic *FRT* or *RS* sites or the infidelity of FLP- or R-recombinase activities in higher eukaryotes do not appear to be available [30]. These findings suggest it may be useful to limit *cre* expression both temporally and spatially, by placing it under the control of regulated promoters.

In a second category of methods using site-specific recombination, the selectable marker and the recombinase genes are on the same vector between the recombination sites (Fig. 4). This system is often referred to as "auto-excision" [87] or self-excision [88]. The auto-excision strategy is a versatile system that could be applied in every species and that shows flexibility in spatial and temporal control. The expression of the recombinase gene can be induced by either external or intrinsic signals resulting in auto-excision of both the recombinase and marker genes placed within the excision site boundaries after their function is no longer needed. The control of excision is enabled by the regulated promoter used to control the

recombinase gene. This approach was described with heat-shock inducible promoter-recombinase expression cassettes in *Arabidopsis* [89, 90], tobacco [91, 92], potato [93], maize [94], Chinese white poplar (*Populus tomentosa* Carr.) [95], hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) [96] and rice [97, 98]. In the latter experiment, the selectable marker gene and *cre* gene were co-bombarded, but probably the efficiency could be higher if both were on the same vector [98]. We have recently obtained transgenic banana plants devoid of the marker gene using a Cre-*lox* auto-excision strategy induced by heat shock [99].

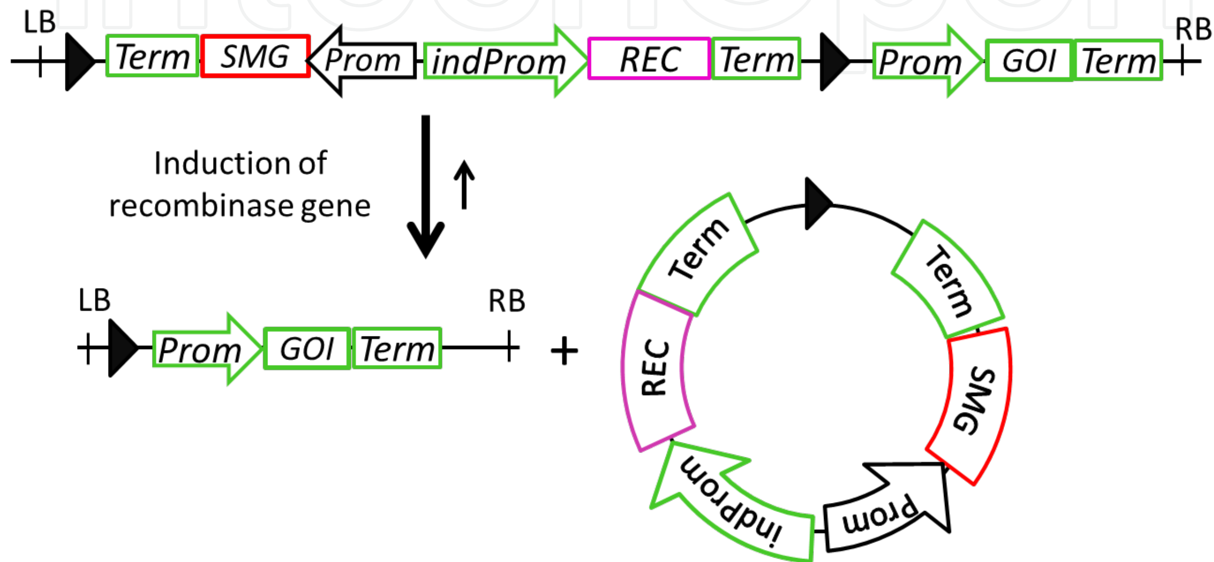

Figure 4. Site-specific recombinase based auto-excision systems. The site specific recombinase gene (*REC*) is under control of an inducible promoter (*indProm*) and is placed together with the SMG between directly repeated recombinase recognition sites (black triangles). Induction of recombinase gene expression leads to excision of the SMG and the recombinase gene.

The recombinase can also be driven by chemically regulated promoters, like the GST-II-27 promoter from maize which is induced by an herbicide antidote Safener, to control the *R/RS* system in tobacco [100] and aspen [46, 101], with β-estradiol trans-induction of *Cre/lox* in *Arabidopsis* [102], rice [103], and tomato [104] and with a dexamethasone-glucocorticoid receptor ligand binding domain activated *R/RS* system in strawberry [105] and potato [106]. In the latter cases, the authors used a combined positive–negative selection scheme to obtain marker- and recombinase-free genotypes [105, 106].

A more refined approach comprises self-excision controlled by an endogenous stimulus that is a part of the plant life cycle. For example, Mlynárová et al. [107] reported the use of a microspore-specific NTM19 promoter from tobacco to drive the expression of the *cre* gene. Thus the excision of the marker gene is taking place during the microsporogenesis where the efficiency was close to 100% in tobacco seeds. An improvement of this system was reported by Luo et al. [108] and was called 'GM-gene-deletor'. In this, the excision target unit is flanked with two different fused target sites (*lox-FRT*), as an alternative to the use of one recombination

site (either *lox* or *FRT*) at each side. Activation of either recombinase (Cre or FLP) by a pollen or pollen- and seed-specific promoter PAB5, gave up to 100% excision efficiency of *lox-FRT* fusion-bounded transgenes in some transformation events, leaving residual LB and RB elements flanking a *lox-FRT* site, in both pollen and/or seed. The use of germline-specific promoters derived from the *Arabidopsis APETALA1* and *SOLO DANCERS* genes, and combined with a positive-negative selection strategy, allowed Verweire et al. [87] to produce completely marker- and recombinase-free *Arabidopsis* plants. Similarly, the expression of Cre driven by the rice floral specific *OsMADS45* gene promoter, excised the *nptII* gene flanked by *lox* recombination sites in T1 rice generation [109]. In another approach, Li et al. [110] took advantage of the somatic embryogenesis developmental stage required in soybean transformation. In this report, the activation of *cre* gene was driven by the *Arabidopsis app1* embryo-specific gene promoter and successfully directed the production of marker- and recombinase-free soybean; in 13% of the events complete excision was noted, whereas 31% yielded chimeras and in 56% of the events the excision failed [110]. Excision systems have also been developed based on seed inducible promoters. Indeed, the cruciferin C promoter from *Arabidopsis* was used to control the expression of *cre* gene in tobacco seeds [88] but the excision efficiency was low (10.2%). Additionally, in a similar strategy *Brassica napus* and tobacco marker free plants were obtained when *cre* gene was driven by a seed-specific-napin promoter form *Brassica napus* [111, 112]. In *B. napus* the efficiency ranged from 13 to 81% and in tobacco from 55 to 100% [111, 112].

The auto-excision strategy is very flexible in timing enabling the excision to take place in late (e.g. flowering or seedling) or early (e.g. somatic embryos) developmental stages. In addition many of these approaches are applicable to vegetatively propagated plant species and and long life cycle plants like perennial trees.

An additional feature of recombination based systems is the capability to resolve complex insertion sites containing multiple tandem insertions of the T-DNA down to more simple or even single copy structures. In wheat, maize and Arabidopsis it was demonstrated that complex integration patterns can be resolved by Cre-mediated recombination, thereby generating single copy transformants [87, 113, 114].

Additionally, the apparent disadvantage of the remaining presence of one *lox* site after the excision is, in some cases, an important advantage. Indeed, the marker-free transgenic line containing one *lox* site can be used as a target line for gene stacking. It has been reported that the Cre/*lox* system can be used to introduce DNA via site-specific integration [115-120]. This has three major advantages. First, the stacked trait is integrated in the genome in a genomic locus giving predictable transgene expression. Second, the trait is introduced in a locus which is already approved by the regulating authorities. Third, by stacking the traits in this way they are linked to one another facilitating breeding programs [121].

A number of novel recombinase systems have been identified that also show the ability to excise DNA in eukaryotic cells [90, 122-126]. So far, only ParA [90] and ΦC31 [126] have been effectively used in plant.

An alternative to the site-specific recombination system would be to construct restriction endonucleases that recognize specific T-DNA sequences. Zinc finger nucleases (ZFNs) could for example be used to eliminate selectable marker genes or other unnecessary DNA sequences from the plant genome. ZFNs are artificial restriction enzymes that consist of a synthetic C_2H_2 zinc finger DNA-binding domain fused to the DNA cleavage domain of the restriction enzyme *Fok*I [127, 128]. ZFNs are capable of inducing targeted double strand breaks[129]. Until now, a unique approach for ZFN-mediated transgene deletion was reported by Petolino et al. [130]. These authors crossed ZFN-overexpressing plants with target transgenic plants, which were engineered to carry a GUS expression cassette that was flanked by recognition sites for the ZFN. Both types of transgenic plants were homozygous for the transgene. The higher frequency of GUS negative hybrid plants was 35 % for one particular cross. PCR and sequencing analyses confirmed that the GUS cassette had indeed been removed.

However, many ZFNs have been reported to be toxic [131-133] presumably as a result of the creation of non-target DSBs [134]. Thus, the strategy to address this problem would be the regulation of ZFN expression by the use of inducible promoters or the use of transient expression systems like the plant virus systems mentioned above. Another approach could be the redesigning of the *Fok*I cleavage domain to create obligate heterodimers [134].

2.6. Removal of transplastome marker gene

In the last decade plastid genome (plastome or ptDNA) has become a popular target for engineering, as this has several advantages like potentially high level protein expression, maternal inheritance and non-dissemination of transgenes through pollen, high transgene copy number per cell and no detected gene silencing [135]. However, selectable marker genes are unnecessary once transplastomic plant has been obtained. Moreover high levels of marker gene expression can cause metabolic problems. Additionally, for selection only spectinomycin and streptomycin (*aadA*) or kanamycin (*nptII* or *kan* and *aphA-6*) genes have been used. Then, four strategies to produce marker-free transplastomic plant have been developed: (i) homology-based excision via directly repeated sequences, (ii) excision by phage site-specific recombinanses, (iii) transient cointegration of the marker gene, and (iv) co-transformation-segregation approach.

2.6.1. Homology based SMG excision via directly repeated sequences

This approach is based on the efficient native homologous recombination apparatus of the plastid. This system relies on the presence of directly repeated identical sequences of plastid DNA. Then, any sequence between them could be excised [136, 137]. The first indication of this phenomenon was observed in the unicellular alga, *Chlamydomonas reinhardtii* where homologous recombination between two direct repeats allowed marker removal under non-selective growth conditions [136]. Later experiments demonstrate marker excision in tobacco chloroplasts after transformation with a construct carrying three transgenes (*uidA*, *aadA* and *bar* genes) [135]. In the transformation vector, the authors placed two of the three genes under the same promoter (*Prrn* promoter of the rRNA operon, and all the genes with the same transcription terminator (*TpsbA*) (Fig. 5). Initial heteroplastomic clones were obtained by

selection for spectinomycin and streptomycin resistance conferred by *aadA*. Thereafter, herbicide-resistant and -sensitive derivatives were identified in the absence of antibiotic selection [135]. Finally the recombination between repeated sequences rendered two types of stable marker-free plants: recombination R1 via the *Prrn* repeat produced herbicide resistant clones and by recombination R2 via *TpsbA*, Gus expressing clones were obtained (Fig. 5). Neither type of marker-free transgenic plants has repeated sequences. As herbicide resistance genes could not be used to directly select plastid transformants [138], this strategy is very useful to obtain marker-free highly herbicide resistant plants. Actually there are two versions of this strategy. The first one allowed visual tracking of the SMG excision by creation of a pigment-deficient zone due to the loss of a plastid photosynthetic gene *rbc*L [139]. The authors placed the *uidA* gene under control of the *PatpB* promoter. The recombination between this sequence with the native *PatpB*, which is located closed to the *rbc*L gene, allowed the deletion of a large DNA segment that comprises the *uidA-aadA-rbc*L genes. The cells lacking *rbc*L could be visually identified by their pale green color; these cells also lack the *uidA* and *aadA* genes. This approach could facilitate advanced studies that require the isolation of double mutants in distant plastid genes and the replacement of the deleted locus with site-directed mutant alleles and is not easily achieved using other methods [139].

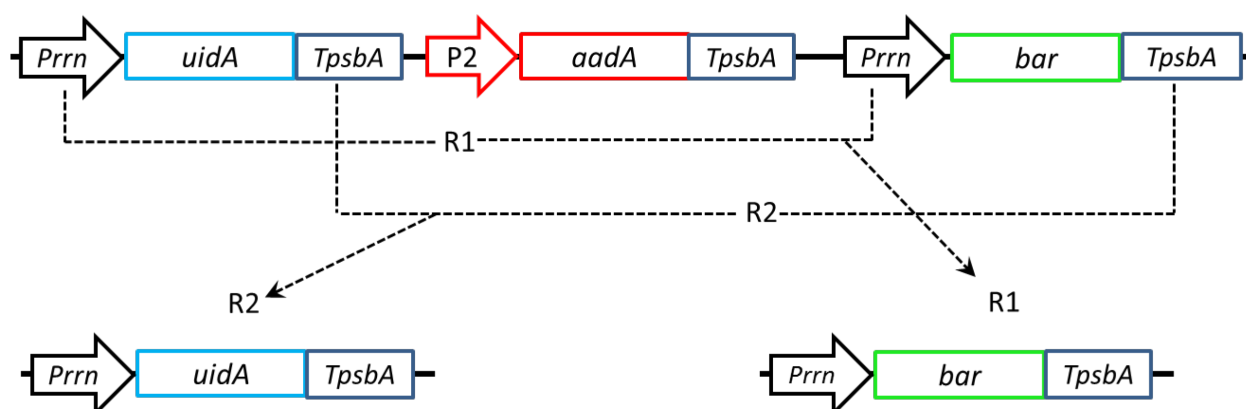

Figure 5. Homology-based marker gene excision via directly repeated sequences [135]. The repeats were the promoters (*Prrn*) and transcription terminators (*TpsbA*). Recombination via the *Prrn* promoter or *TpsbA* repeats yielded the two stable marker-free ptDNA carrying only the *uidA* (recombination R2) or only the *bar* (recombination R1) gene. No sequence is repeated in the final product. *uidA*: reporter gene encoding β-glucuronidase; *aadA*: spectinomycin resistance marker gene; *bar*: herbicide resistance gene.

In the second version [140] marker-free tobacco plants were generated by the use of a vector that harboured an *aadA* gene disrupting the herbicide resistance gene *hppd* (4-*hydroxyphenyl-pyruvate dioxygenase* from *Pseudomonas fluorescens* (HPPD) enzyme that confers resistance to sulcotrione and isoxaflutole). Initially, antibiotic-resistant clones were obtained. Marker-free herbicide-resistant plants were identified after excision of the *aadA* marker gene by homologous recombination within the overlapping region (403 bp) of the 5′ and 3′ halves of the herbicide resistance gene. Excision of *aadA* led to reconstitution of a complete herbicide resistance gene and expression of the HPPD (Fig. 6).

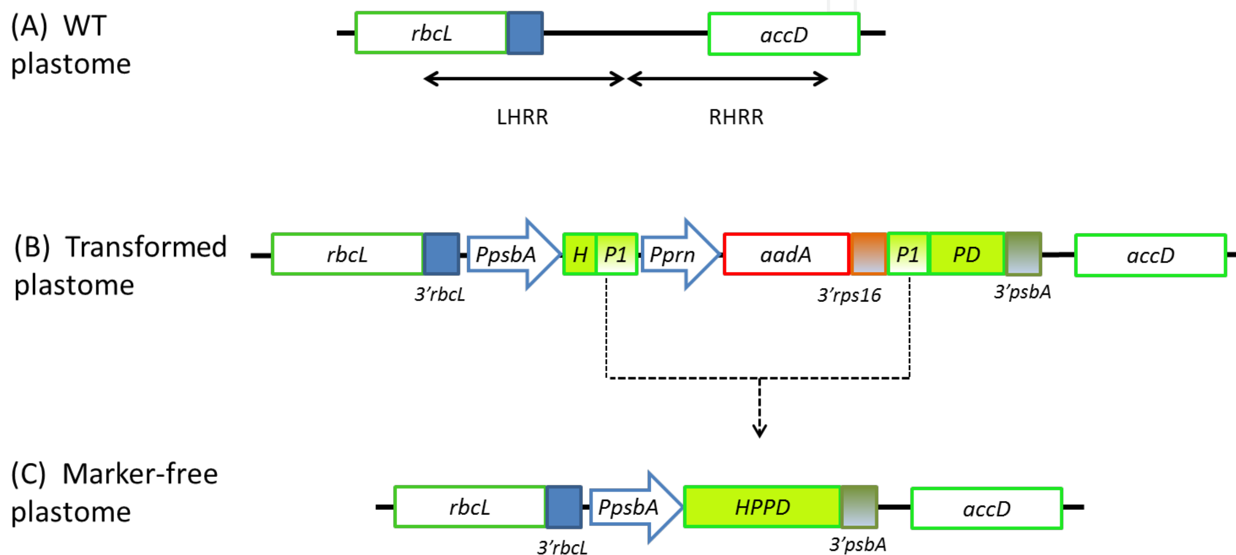

Figure 6. Homology-based marker gene excision via directly repeated sequences [140]. Integration of transgenes into the wild-type (WT) tobacco plastid genome (A) after transformation with the designed vector, giving a transformed plastome (B). After the recombination between the two P1 repeats, a marker-free plastome was obtained (C). HP1: 5′ fragment of the *4-hydroxyphenylpyruvate dioxygenase* gene (*hppd*) coding region; LHRR and RHRR, left and right homologous recombination regions; P1, repeat segment overlapping the 5′ and 3′ fragments; P1PD, 3′ fragment of the *hppd* coding region.

2.6.2. Excision by phage site-specific recombinases

Site-specific recombinases have also been used to produce marker-free transplastomic plants. This approach exploits a two-step protocol. Step one is the production of transplastomic plants, which carry a SMG flanked by two directly oriented recombinase target sites (Fig. 7). Afterward, marker-free plants could be obtained when the recombinase activity is introduced by nuclear transformation of a gene encoding a plastid-targeted recombinase[141, 142].

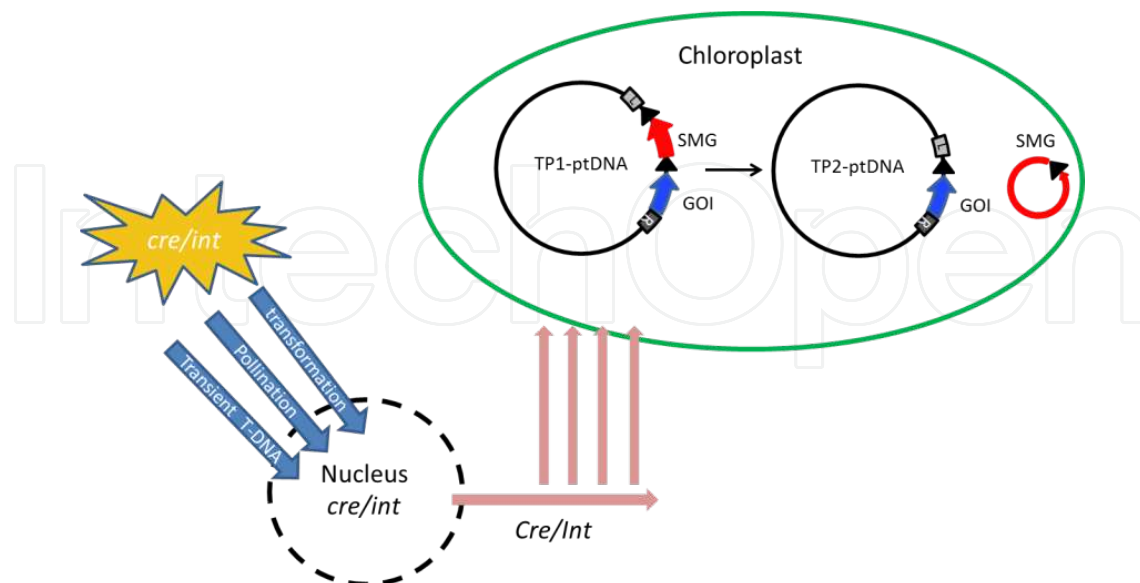

Figure 7. Marker gene excision from the plastid genome by Cre or Int site-specific recombinases [137]. A site-specific recombinase gene (*cre*/*int*) introduced into the nucleus by transformation, pollination or transient Agroinfiltration, encodes a plastid-targeted recombinase that excises selectable marker gene (SMG) from TP1-ptDNA after import into plastids. Excision of the marker gene by phage recombinases via the target sites (black triangles) yields marker-free TP2-ptDNA carrying only the gene of interest (GOI) and one recombinase recognition sequence [141-143].

Cre/*lox* was the first site-specific recombination system used to excise the SMG from the plastid genome [141, 142]. In these works Cre activity was introduced by nuclear transformation and marker-free transplastomic plants were obtained in tissue culture. However, these plants still contain *cre* and *nptII* genes in their genome that had to be segregated away in the seed progeny [141, 142]. Another way to introduce Cre activity is by pollination, in which apparently non-specific Cre-induced re-arrangements between homologous ptDNA sequences were absent or occurred significantly less often than in directly transformed plants [141]. On the other hand, Lutz et al. [144] took into account the fact that not every T-DNA delivery results in stable integration and expressed Cre transiently from T-DNA introduced by Agroinfiltration. As a result in this experiment approximately 10% of the regenerated plants did not carry either a plastid selectable marker or a nuclear *cre* gene. Nevertheless, Cre-mediated marker excision can cause the deletion of ptDNA sequences by recombination via directly repeated non-*lox* sequences that result in mutation of target plant [141, 142].

As an alternative, the ΦC31 phage site-specific integrase (Int) that mediates recombination between bacterial (*attB*) and phage (*attP*) attachment sites was tested to excise the SMG [143]. The authors tested marker gene excision in a two-step process. In the first step, tobacco chloroplast were transformed with a vector that contains the SMG (*aadA* gene) flanked with directly oriented non-identical phage *attP* (215 bp) and bacterial *attB* (54 bp) recombination sites, which are recognised by Int recombinase. The *bar* gene was used as gene of interest and it was placed outside of the excision cassette. Spectinomycin-resistant clones were obtained

and these were stable in the absence of Int. In the second step, a plastid-targeted Int was introduced by *Agrobacterium*-mediated nuclear transformation that directed efficient marker gene excision. No fortuitous sequences appear to be present in the plastid genome that would be recognized by the Int [143]. In the homology-mediated marker excision the frequency of deletion is proportional to the length of the repeat. As the *lox* sequences are short (34 bp in length), the probability to cause loss of the marker gene in the absence of Cre is not completely absent. However the *attB* and *attP* sequences are not homologous, therefore plastid genomes carrying *att*-flanked marker genes are predicted to be more stable than those with marker genes flanked by identical *lox* sequences. The absence of homology between the *attB* and *attP* sites and the absence of pseudo-*att* sites in ptDNA would make Int a preferred alternative to Cre for plastid marker excision [137].

2.6.3. Transient cointegration of the marker gene

Based on the mechanism of integration of the foreign DNA in the plastid genome, Klaus et al. [145] designed a system to excise the SMG. Indeed, two homologous recombination events (Left and Rigth) are needed for DNA integration. However, considering that cointegrate formation is a common phenomenon that takes place in bacterial plasmid recombination, the authors assumed that in the chloroplast a transformation vector first forms a cointegrate following recombination between a single region of homology in the transformation vector and the plastome (Fig. 8A). Cointegrates are naturally unstable due to the presence of direct repeats in these molecules. Subsequent homology recombination events (between duplicated sequences) lead either to stable integration of both the GOI and SMG gene or to loss of the integrated vector, yielding a wild-type plastome (Fig. 8A) [145]. In this work the authors used a vector where the marker gene (*aphA-6*) was located outside of the recombination region. This strategy allowed the selection for a cointegrate structure that forms by recombination via only one of the targeting sequences (Fig. 8B). When selection for kanamycin resistance was withdrawn, the second recombination event can take place and the marker gene is lost.

2.6.4. Co-transformation-segregation

The co-transformation-segregation method in plastid transformation technology is based on the same principle that has been applied in nuclear transformation. Indeed, the SMG and the gene of interest are inserted in two different plasmids and introduced into two locations (Fig. 9A) of the same plastid by biolistic transformation to generate heteroplastomic cells with both or either of the genes (Fig. 9B) [137, 146]. After segregation, a marker-free transplastomic plant could be obtained (Fig. 9C). The approach was developed to obtain antibiotic resistance gene-free plants with resistance to herbicides (glyphosate or phosphinothricin) due to the impossibility to obtain such plants directly. Indeed, transplastomic plants cannot be obtained directly by selection with herbicides after transformation with the resistance genes (*CP4* or *bar*) because cells harboring only a few copies of the transgene die [147, 148]. Nonetheless, when these genes were co-transformed with a plasmid carrying the spectinomycin resistance (*aadA*) gene and most ptDNA copies carry the genes, the cells and regenerated plants showed resistance to high levels of the herbicides [147, 148].

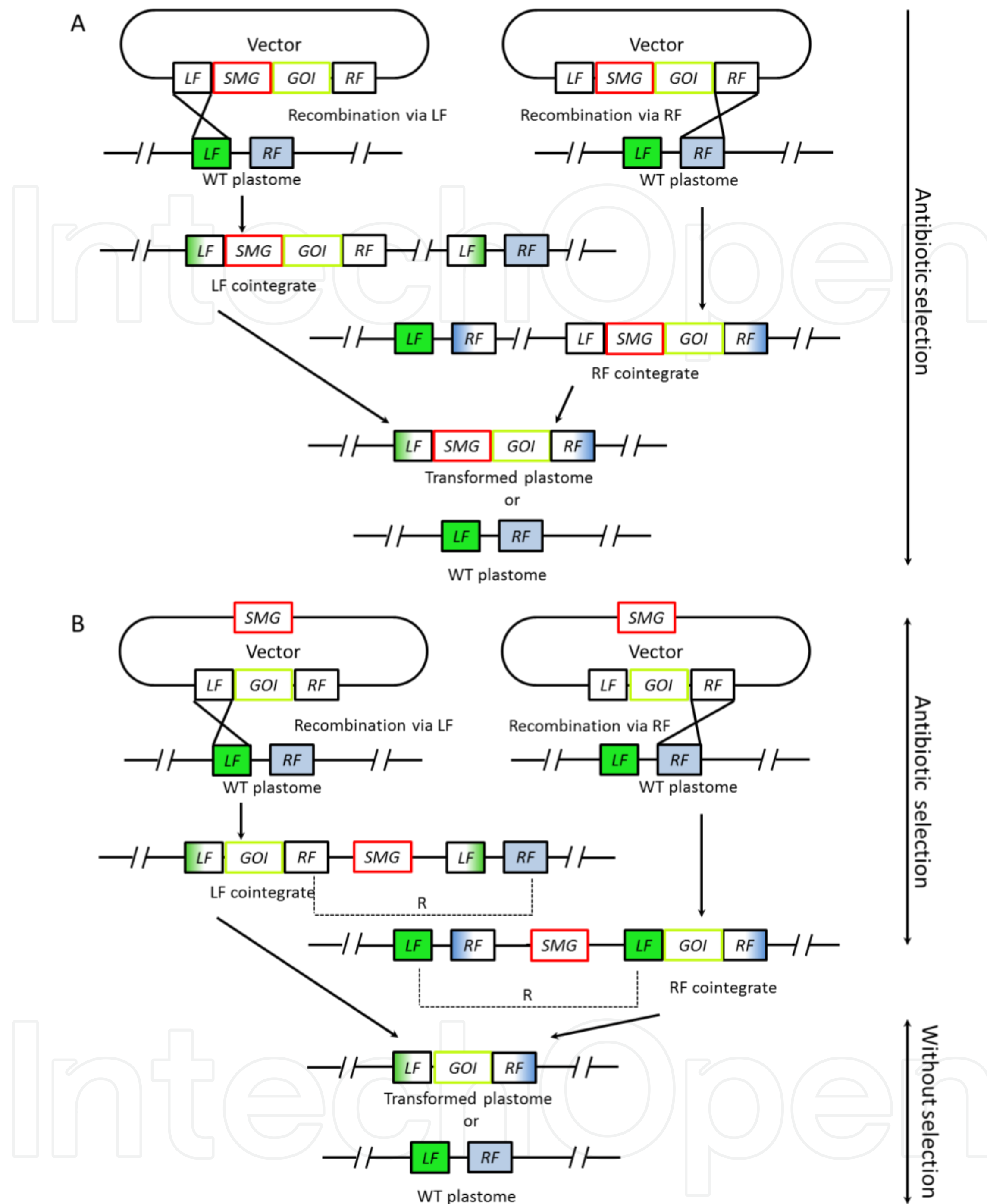

Figure 8. Transient cointegration of the marker gene to obtain marker-free transplastomic plants [145]. Cointegrate formation and subsequent recombination events with conventional and alternative plastid transformation vectors. (A) Standard plastid transformation using a vector in which the SMG is cloned between the homologous flanks. Recombination via a single flank (left or right) results in cointegration of the vector; subsequent loop-out recombination events between direct repeats lead either to a stably transformed plastome containing the sequence of interest and marker or wild-type plastome. (B) Plastid transformation using a vector in which the selection marker is cloned outside of the homologous flanks. Again recombination via either left or right flanks results in cointegration of the vector; however, following additional recombination events only the GOI is stably integrated and the marker gene is lost.

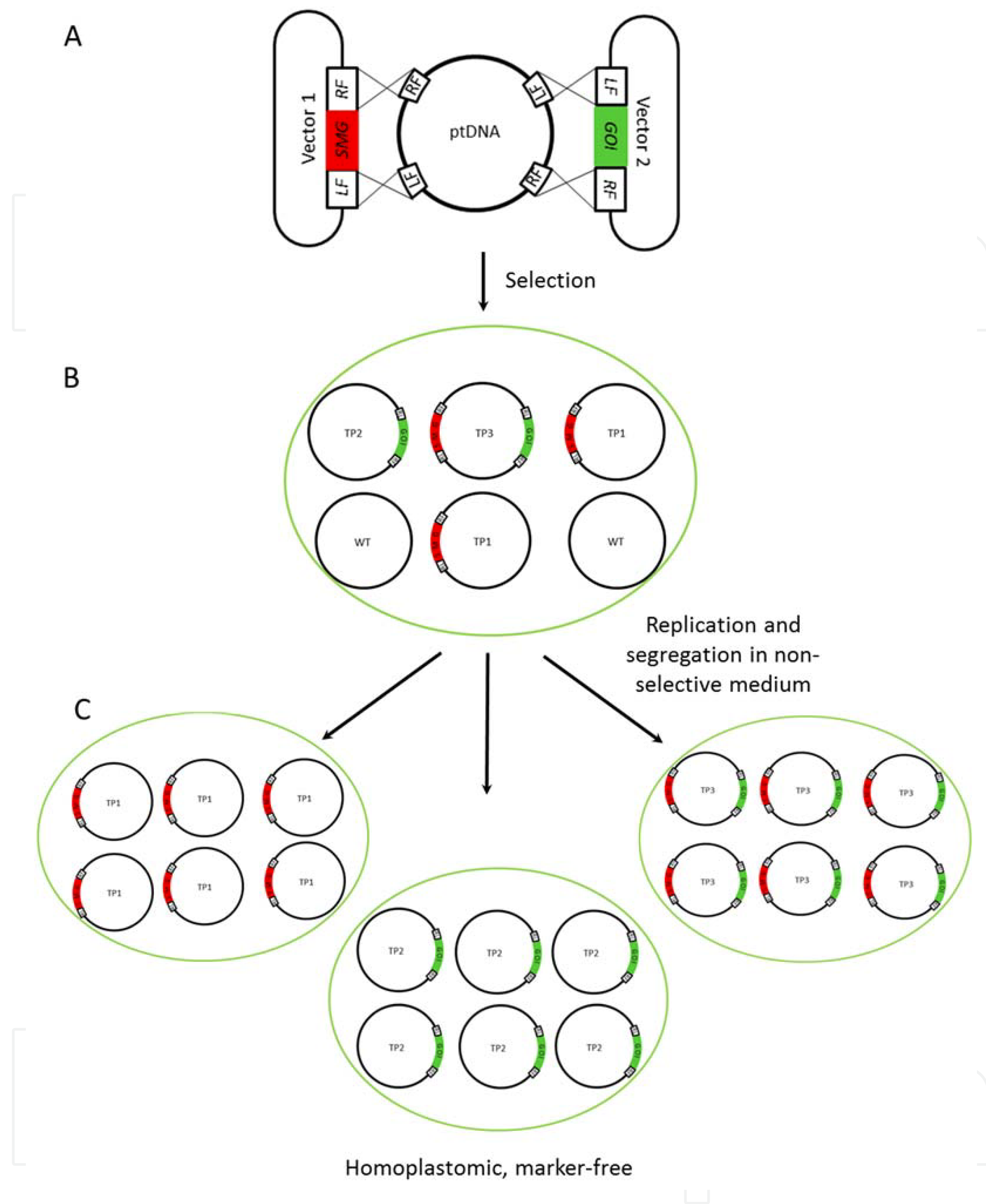

Figure 9. The cotransformation-segregation method to remove selectable marker genes from transplastomic plants [149]. (A) Transformation of the plastid genome (ptDNA) with two vectors. Vector 1 containing the selectable marker gene (SMG) and Vector 2 the gen of interest (GOI). (B) Transplastomic clones are identified by selection for antibiotic resistance. The heteroplastomic cell carries wild-type ptDNA (wt), TP1-ptDNA obtained by transformation with Vector 1; TP2-ptDNA transformed with Vector 2; and TP3-ptDNA transformed with both vectors. (C) Replication and segregation of ptDNA on non-selective medium eventually yields homoplastomic cells with TP1-ptDNA, TP2-ptDNA and TP3-ptDNA. Desired marker-free transplastomic plants carry TP2-ptDNA and lack the antibiotic resistance marker (adapted from [137]).

3. Marker free transgenic plants with agronomically useful genes

The various methods to obtain marker-free transgenic plants have proven their utility and are increasingly being deployed to obtain crop plants with agronomically useful genes. One of the crops that have received more attention is rice. Indeed some papers have described the production of marker-free transgenic plants with different genes of interest. Applying the co-transformation / segregation strategy with the use of 'double right border' twin T-DNA vectors Lu et al. [39] obtained marker-free transgenic rice plants harboring a *Rice ragged stunt virus* (RRSV) derived synthetic resistance gene. Another group obtained transgenic rice devoid of selectable marker genes that produce high levels of carotenoids using the same strategy but with two binary vectors in one *Agrobacterium* strain [150]. One of the vectors contained in the T-DNA the phytoene synthase (*psy*) and phytoene desaturase (*crt*I) expression cassettes whereas the other vector contained the *hph, nptII* and *gus* genes. Marker-free rice plants, with improved resistance to *Magnaporthe grisea* were obtained by the expression of the rice pistil-predominant chitinase gene using a vector system with two T-DNAs [151].

Sripriya et al. [152] generated marker-free transgenic plants with improved resistance to sheath blight. A single *A. tumefaciens* strain harbored a cointegrate vector with the *hph* and *gus* genes and a binary vector with the rice chitinase (*chi1*) gene. The elimination of SMG was accomplished by segregation in T_1 progeny. Some of the lines showed an enhanced resistance to *Rhizoctonia solani*. Thereafter, the same group sequentially retransformed one of the *chi1* lines with the tobacco osmotin *ap24* gene by co-transformation using an *Agrobacterium* strain harboring a single-copy cointegrate vector pGV2260::pSSJ1 (*hph* and *gus*) and a multi-copy binary vector pBin19ΔnptII-ap24 in the same cell [153]. They obtained one line in the T_1 progeny where the SMG was absent and *chi1* and *ap24* genes were integrated. Homozygous plants with both genes were obtained and some of those showed enhanced resistance to *R. solani*. Selectable marker-free rice plants expressing the *Bacillus thuringiensis* synthetic *cry1B* gene were obtained by transposon-mediated repositioning of the GOI [49]. The Cry1B expression cassette was flanked by the inverted terminal repeats of the maize *Ac* transposon that permit the repositioning of this cassette in the rice genome. Preliminary bioassays suggested that the T-DNA free relocation events exhibit a level of resistance to a major rice insect pest, *Chilo suppressalis*.

On the other hand, Sengupta et al. [154] have exploited the Cre/*lox* site-specific recombination system to produce selectable marker-free transgenic rice plants with improved resistance to green leafhoppers (*Nephotettix virescens*) and brown planthopper (*Nilaparvata lugens*). In this work two independent vectors were used, one having the *ASAL* (*Allium sativum* leaf agglutinin) gene and the *hpt* gene flanked by *lox* sites, and the other with the *cre* and *bar* genes. Cre activity was introduced by crossing single copy T_0 plants and marker excision was detected in T_1 hybrids. T_2 progeny showed the segregation of the *cre-bar* T-DNA and improved insect resistance.

The first commercially available marker-free transgenic plant that was obtained through this system was developed by the company Renessen. They generated the transgenic corn line LY038, from which the *nptII* selectable marker gene, originally present between tandemly oriented *lox* sites, was removed through introduction of the *cre* gene by a sexual cross [121]. For the market, this corn line has the name Mavera™ High Value Corn with Lysine, and was developed for the feed industry. This line was obtained from a biolistic transformation event

where a *cordapA* gene encoding a seed-specifically expressed lysine insensitive dihydrodipicolinate synthase enzyme. This approach was also applied to obtain marker-free salt tolerant maize plants by the expression *AtNHX1*, a Na^+ /H^+ antiporter gene from *Arabidopsis*, but using the FLP/*FRT* system [155].

In another report, the production of marker-free transgenic soybean [*Glycine max* (L.) Merr.] is described which produces γ-linolenic acid and stearidonic acid that are important for pharmaceutical and nutraceutical industries [41]. The authors applied the co-transformation / segregation strategy, using a vector with two T-DNAs: the first harbored a cDNA of the *Borago officinalis* L. Δ^6 desaturase gene driven by the embryo- specific β-conglycinin promoter, whereas the second T-DNA contained the selectable marker gene *bar*. In this work ~7% of the transgenic lines were marker-free.

The expression of a chitinase gene, *ChiC*, on an *ipt*-type MAT (isopentenyl transferase-type multi-auto-transformation) vector, allowed the production of marker-free disease-resistant transgenic potato plants [156]. Based on transformation without selectable marker gene Stiller et al. [157] obtained two potato lines with increased levels of the semi-essential amino acid cysteine by expression of the serine acetyltransferase encoding *cys*E gene. This system was also used by Ahmad et al. [158] to produce marker-free potato plants with enhanced tolerance to oxidative stress by the expression of the superoxide dismutase and ascorbate peroxidase genes. The same strategy was used in Chinese cabbage (*Brassica campestris* ssp. *pekinensis* (Lour) Olsson) to produce Turnip mosaic virus (TuMV) resistant marker-free transgenic plants [159]. This approach was also applied in melon (*Cucumis melo*), where ripening was delayed by the introduction of marker-free and vector-free antisense 1-aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase) construct [160].

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