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Production of the Marker free transgenic plants – An update Review

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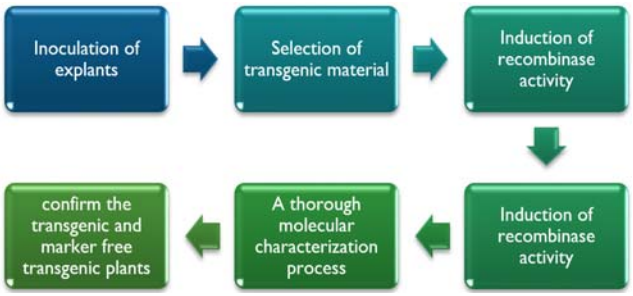
Abstract
Selectable marker genes are pivotal for the development of plant transformation technologies because marker genes allow researchers to identify or isolate the cells that are expressing the cloned DNA, to monitor and select the transformed progeny. As only a very small portion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Since the selectable marker gene is expected to function in a range of cell types it is usually constructed as a chimeric gene using regulatory sequences that ensure constitutive expression throughout the plant. Transgenic crop or plants are very decisive for the growing population to maintain their necessary food, medicine, protect the environment and other things. But the transgenic crop or plants have some risk or controversy in human health, agriculture, and environment. 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. Recently, scientists are producing several transgenic plants without selectable marker gene. This review article describes the developments made in the recent past on plant transformation systems using different selection methods adding a note on their importance as marker genes in transgenic crop plants.

Keywords: Plant transformation, Selectable marker, Site-specific recombination, Transgenic plants.

1. Introduction
The genetic modification of crop plants offers substantial improvements to agricultural practices, food quality and human health. A main focus of plant biotechnology over the last few 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. 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 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 [1]. Transgenic plants are plants that have been genetically engineered, a breeding approach that uses recombinant DNA techniques to create plants with new characteristics. They are identified as a class of genetically modified organism (GMO) [2]. A marker gene must be on the same chromosome as the target gene and near enough to it so that the two genes (the marker gene and the target gene) are genetically linked and are usually inherited together. [3] Recently marker free transgenic potato plants resistant to oxidative stress and to potato virus X and Y were developed using this approach. In both cases, the transgenic event was confirmed by PCR and by challenging the putative transformants to oxidative stress and also resistant to potato virus X and Y, respectively. Similarly, constructed a novel plant transformation vector by removing the nptII marker gene conferring resistance to kanamycin from the vector together with its promoter polyadenylation signal of the Agrobacterial nopaline synthase gene [4-9].

Steps involving production of marker free transgenic plants [10].



2. Molecular mechanism of marker free transgenic process

2.1 Replacing selectable marker with screenable markers

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- and phosphomannose isomerase genes as well as the yeast, 2-desoxyglucose-6-phosphate phosphatase. 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 selection of transformants. 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. The use of a dexamet has one-inducible promoter driving the ipt gene led to the recovery of lettuce and tobacco transformants under inducing conditions. Recently, more approaches for the isolation of screenable markers for species that can be regenerated by organogenesis or somatic embryogenesis were undertaken. 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 [11-13].

2.2 Elimination of marker genes by co-transformation

Most common way to separate 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 or on two binary vectors within the same *Agrobacterium* or with two different *Agrobacterium* strains. 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. 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 [14].

2.3 Site-specific recombination

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. In general, two lox-sites in direct orientation are required for excision of the intervening sequences. If the lox sites have been moved apart via transposon jumping, 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. Inversely a transgene can be integrated site-specifically into a lox site. 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. Two lox sites in inverted orientation are necessary for inversion of the intervening sequence. Even the exchange of chromosome arms was achieved with the Cre-lox system in plants [15-19].

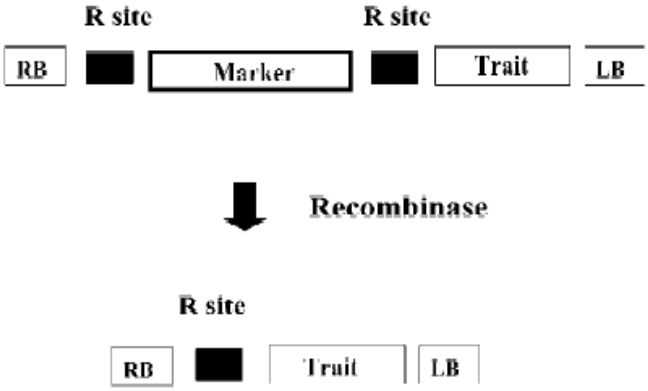


Fig 1: General strategy for the excision of selectable marker genes by site-specific recombinases (Dale and Ow, 1991). The selectable marker gene is inserted into the transformation vector between two directly repeated recombination (R) sites that are recognized by a site-specific recombinase and used 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.

2.4 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. Marker-free transgenic tobacco and aspen plants have been generated at low frequencies by inserting the selectable ipt gene into the transposable element Ac. 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 feasibility of this approach was demonstrated in tomato. 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 repositioning 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 [19-21].

2.5 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, 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. The recent demonstration that directed evolution strategies can be used to modify recombinase substrate specificities extends the potential of this approach. Another sophisticated approach is to use tail or made endonucleases designed to induce DSBs at unique sites within the genome. Placing different restriction endonucleases under the regulation of chemical-inducible promoters will further extend the range and flexibility of different approaches for transgene elimination.

3. Selection of marker-free transgenic plants using the isopentenyl transferase gene:

Dominant genes encoding either antibiotic or herbicide resistance are widely used as selectable markers in plant transformation. The antibiotics and herbicides that select rare transgenic cells from nontransgenic cells generally have negative effects on proliferation and differentiation. These agents may retard differentiation of adventitious shoots during the transformation process. Some plant species are insensitive to or tolerant of the selective agents, and therefore, it is difficult to separate the transformed and untransformed cells or tissues. Therefore, it is difficult to find appropriate selectable markers and to establish optimal conditions for transformation of such difficult species. Selectable marker genes remain in transgenic plants, and their gene products need to be assessed for safety and environmental impact [22-24].

3.1 Mechanism of action

Construction of MAT Vector Plasmids (pNPI106). The 20.4-kb PstI fragment from the T-DNA (portion of the Ti plasmid that is transferred to plant cells) of *A. tumefaciens* P022 was cloned into an unique PstI site of pUC 7. From this plasmid, the 1.9-kb BamHI–PstI fragment containing the entire ipt gene was excised and cloned into the BamHI–PstI site of pUC119. From this plasmid, the 1.3-kb RsaI fragment containing the coding sequence and terminator of the ipt gene was excised and cloned into the unique SmaI site of pUC119. The BamHI–SacI fragment was inserted to the BamHI–SacI site of pBI121 (CLONTECH), downstream of the CaMV 35S promoter. The HindIII–SacI fragment containing the chimeric ipt gene with the CaMV35S promoter was converted to a blunt end fragment with T4 polymerase I and inserted to the unique blunted BamHI site ofAcin pCKR97. Finally, PstI

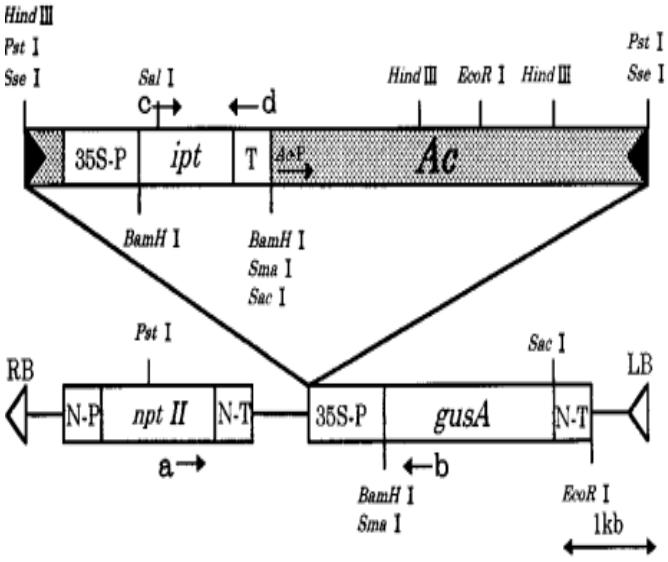


Fig 2: Diagram of MAT vector pNPI106. Plasmid pNPI106 has a 'hit and run'' cassette in which the chimeric ipt gene with 35S promoter is inserted into Ac as a selectable marker. The gusA and nptII genes are unselected markers in these experiments. Arrows, PCR primers, 35S-P, CaMV35 S promoter;ipt, isopentenyl transferase gene; T, isopentenyl transferase terminator; N-P, nopaline synthase pro-moter; N-T, nopaline synthase terminator;nptII, neomycin phospho-transferase gene; gusA, β-glucuronidase gene. Fragments containing the Ac and the inserted ipt gene with the CaMV35 S promoter were cut from this plasmid and ligated to the unique SseI site of the binary vector pBI121. This plasmid was called MAT vector pNPI106 (Fig. 2) [24-26].

3.2 Plant Transformation

Plasmid pNPI106 was transformed into *A. tumefaciens* LBA4404 using a freeze–thaw method. Leaves of Nicotiana tabacum *cv. Xanthi* were sterilized with 1% sodium hypochlorite and cut into leaf segments of 0.8 × o.8 cm. These leaf segments were inoculated for 1 min with an overnight culture of bacteria diluted to OD_{630} = 0.25 and put on a sterilized filter paper to remove the bacteria in suspension. The infected leaf segments were cocultivated for 3 days on hormone-free Murashige-Skoog (MS) medium containing 2% sucrose, 0.8% agar, and 50 mg/liter acetosyringone and then transferred to hormone-free MS medium containing 500 mg/liter carbenicillin but no kanamycin (nonselective medium). When adventitious shoots were regenerated, they were separated from the leaf segments, transferred to fresh nonselective medium (with carbenicillin), and cultured under 3000 lux at 25 °C. A stem of an aseptically flask-grown hybrid aspen ''Kitakami Hakuyo'' was cut to obtain an internodal stem segment 5 mm in length, further cut lengthwise in two, and then inoculated with the same strain of *A. tumefaciens* (LBA4404 containing pNPI106) used for tobacco plants. These infected stem segments were transferred to nonselective medium for hybrid aspen (hormone-free modified MS medium with 800 mg/liter ammonium nitrate and 2 g/liter potassium nitrate). Regenerated shoots were cultured in the same medium. The normal shoots were transferred to root-inducing

medium (2/3 MS medium, 2% sucrose, 0.25% Gelrite and 0.05 mg/liter 3-indolebutyric acid). Kanamycin and Histochemical assays. Leaf segments were placed on MS agar medium containing l mg/liter benzyladenine, 0.2 mg/liter 1-napthalene, and 200 mg/liter kanamycin. After 1 month in culture, the formation of callus and adventitious shoots was observed on the leaf segments. Histochemical assays for β-glucuronidase (GUS) activity were performed as described [27].

3.3 DNA Analysis

Genomic DNA samples were prepared from transgenic tobacco shoots and plants using cetyltrimethylam-monium bromide, and used as templates for PCR amplification and Southern blot analysis. The PCR mixture contained 1mg of genomic DNA, each primer at a concentration of 0.5 mM, 10 mM Tris HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 1% Triton X-100, 0.1 mM dNTP, and 1.25 units ofTaq DNA polymerase (TAKARA shuzo Otsu, Shiga, Japan) in a total volume of 50 ml, overlaid with 40 ml of mineral oil. After the mixtures were heated at 94 °C for 1.5 min, the amplification occurred during 30 cycles of 1 min at 94 °C, 2 min at 60 °C, and 3 min extension at 72 °C. Reaction products were resolved by electrophoresis through 1.8% agarose gel. The sequences of the two primers used to detect excision of the modified Ac element were 5′-TTGTCAAGACCGACCTGTCC-3′(Fig.3, primer a) and 5′-TGCATCGGCGAACTGATCGT-3′(Fig.3, primer b). The expected fragment size of the empty donor is approximately 3 kb. The sequences of the two primers to detect the ipt gene in were 5′-CTTGCACAGGAAAGACGT-CG-3′ (Fig.3, primer c) and 5′-AATGAAGACAGGTGT-GAC-GC-3′ (Fig.3, primer d). The expected fragment size of the amplified DNA segment is 800 bp. Genomic DNA (10 mg) was digested with HindIII and separated by 0.8% agarose gel electrophoresis, and then analyzed on Southern blots. The DNA probe, a fragment of the nptII gene, was labeled by PCR using DIG-DUTP (Boehringer Mannheim). Southern blots were carried out using DIG Easy Hyb (hybridization solution) and DIGWash and Block Buffer Set (Boehringer Mannheim).

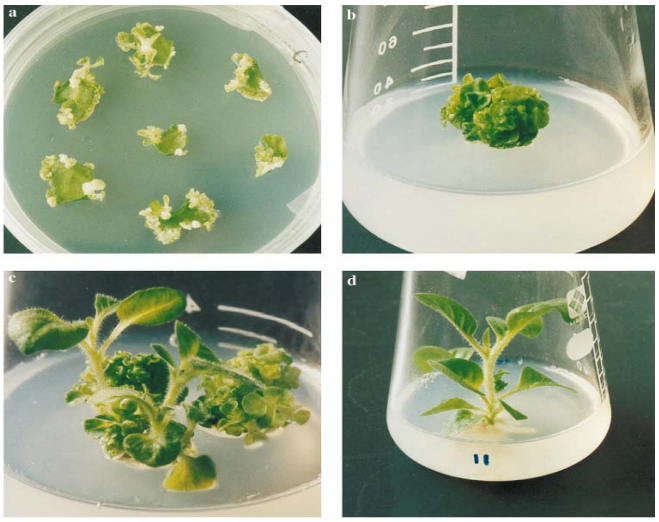


Fig 3: Visible selection of marker-free transgenic tobacco plants. (a) Regeneration of adventitious shoots from leaf segments on nonselective medium. (b) Differentiation of ESP from adventitious shoots. (c) Appearance of ‘‘normal’’ morphological shoots from ESP. (d) Normal rooted plant.

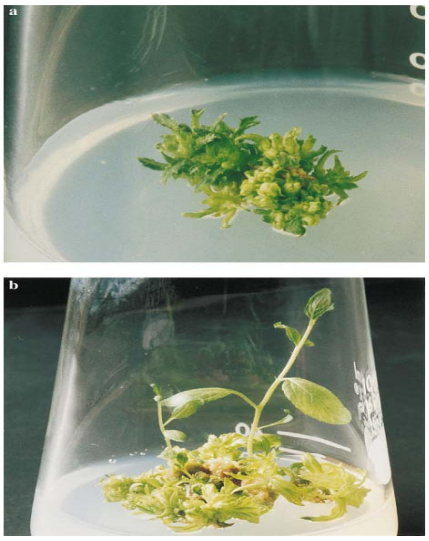


Fig 4: Transformation of hybrid aspens by the MAT vector system. (a) Differentiation of the ESP from adventitious shoots. (b) Appearance of morphologically normal shoots from ESP [28-37].

4. The elimination of a selectable marker gene in the doubled haploid progeny of co-transformed barley plants:

Barley is one of our major arable crop species, and may well have been the first species to have been domesticated. Among the temperate cereals it is one of the best adapted to low rainfall and poor soil conditions. Its grain is used both for animal feed and malting, with minor usage in the health food and bio-ethanol sectors [38].

4.1 Construction of binary transformation vectors:

The binary vectors used were p6U (DNA Cloning Service, Hamburg, Germany) based plasmids, constructed using standard DNA cloning methods in the *E. coli* strains DH5α and DH10B. The pSB227 plasmid (designated later as phpt::gfpto highlight its relevant elements) incorporates hygromycin phosphotransferase (hpt) as a selectable marker gene driven by the maizeubiquitin1 promoter, fused to the gfpS65T coding sequence driven by the rice actin1promoter (McElroy *et al.*1990) (Fig.5a). The second binary vector, pgus, was obtained by replacing the hptexpression cassette in p6U with the *E. coli* ß-glucuronidase gene (gus) including the StLS1 intron driven by the cauliflower mosaic virus (CaMV) doubled enhanced 35S (d35S) promoter. The Twin binary vectors harbour both T-DNAs separated by left and right border sequences (Fig.5b). These vectors were generated by modifying pSB227 (phpt::gfp) via digestion with SpeI and StuI, followed by a 5–3′ exonuclease treatment and religation. This step also eliminated the SfiI restriction site adjacent to the rice actin1promoter sequence, because it overlaps with the StuI site. The second SfiI restriction site between the 35S and nos terminator sequences was then removed by SfiI digestion, followed by a 3–5′ exonuclease treatment and religation. The Left Border-Multicloning Site-Right Border (LB-MCS-RB) fragment was PCR amplified by primers which incorporated flanking EcoRV restriction sites (5′- AGATATCTGCAA-GCTCCACCGG GTGCAAAGCGGCAGC and 5-CCGATA TCATATCC GATTATTCTAATAAACGCTC) using the hpt-free p6U vector as template. The LB-MCS-RB fragment was then inserted into the modified pSB227 plasmid at the EcoRV site with the help of a TOPO-Cloning kit (Invitrogen) in both possible orientations. The d35S::gus sequence was released

from the hpt-free p6U vector containing the d35S: gusi: Tnos cassette by restriction with SfiI and inserted into the pSB227 vector containing the multiple cloning site fragment flanked by the border sequences. This resulted in the two binary vectors pTwin T and pTwin I (Fig.5a), differing in their orientation of gus in relation to gfp, with T standing for tandem and I for inverted.

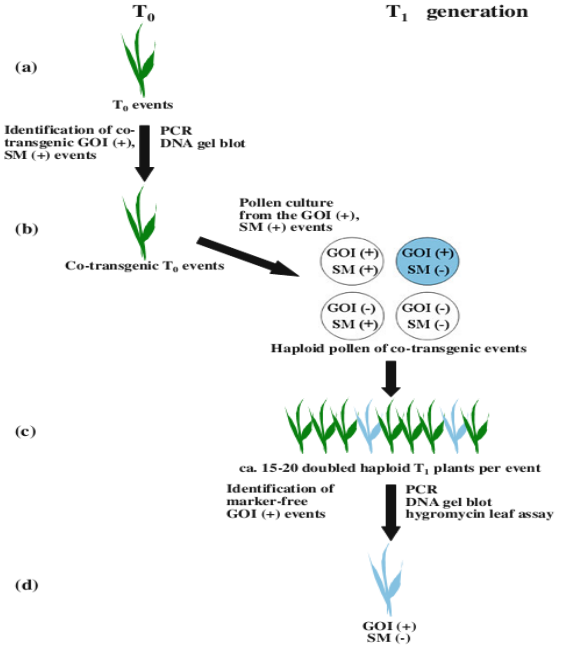


Fig 5: Schema for the production of selectable marker-free transgenic barley. (a) Immature embryos were used as the explant subjected to inoculation with *Agrobacterium.*(b) The selectable marker (SM, hpt::gfp) and the model gene-of-interest (GOI, gus) were co-transformed using separate T-DNAs. (c)Homozygous selectable marker-free GOI positive doubled haploid barley plants were regenerated from embryogenic pollen cultures. (d) If the two T-DNAs are inserted in different chromosomal locations, selectable marker-free GOI positive derivatives can be identified within the doubled haploid progeny [39-42].

4.2 Barley genetic transformation

The transformation protocol applied to immature embryos and the generation of primary transgenic plants followed that of Hensel and Kumlehn (2004). Two *Agrobacterium tumefaciens* strains were used: a hypervirulent derivative LBA4404 and AGL-1. Genetic transformation of barley (*Hordeum vulgare* L.) was carried out using 14 different *Agrobacterium*/vector combinations, which are specified in Table 1. Each of the three replicates making up the entire experiment consisted of the inoculation of 90 immature embryos of the cultivar 'Golden Promise' with each of the 14 combinations. Because it was technically impossible to compare all 14 combinations in a single experimental run, combination 7 (a 1:1 mixture of LBA4404/phpt: gfpand AGL-1/pgus) was included as an internal control in each transformation experiment. This 'control' was thus applied in a total of six replicates using 270 embryos each [43].

5. Disease-resistant marker-free tomato by R/RS site-specific recombination

The selection marker genes, imparting antibiotic or herbicide resistance, in the final transgenics have been criticize by the public and considered a hindrance in their commercialization. Multi auto-transformation (MAT) vector system has been one of the strategies to produce marker-free transgenic plants without using selective chemicals and plant growth regulators (PGRs). In the study reported here, isopentenyltransferase (ipt) gene was used as a selection marker and wasabi defensin (WD) gene, isolated from *Wasabia japonica* as a target gene. WD was cloned from the binary vector, pEKH-WD to an ipt-type MAT vector, pMAT21 by gateway cloning and transferred to *Agrobacterium tumefaciens* strain EHA105. Infected cotyledons of tomato cv. Reiyo were cultured on PGR- and antibiotic-free MS medium. Adventitious shoots were developed by the explants infected with the pMAT21/wasabi defensin [44-45]. The same PGR- and antibiotic-free MS medium was used in subclutures of the adventitious shoot lines (ASLs) to produce ipt and normal shoots. Approximately, 6 months after infection morphologically normal shoots were produced. Molecular analyses of the developed shoots confirmed the integration of gene of interest (WD) and excision of the selection marker (ipt). Expression of WD was confirmed by Northern bolt and Western blot analyses. The marker-free transgenic plants exhibited enhanced resistance against Botrytis cinerea (gray mold), Alternaria solani (early blight), Fusarium oxysporum (Fusarium wilt) and Erysiphe Lycopersici (powdery mildew).

5.1 Site-specific recombination-mediated marker deletion:

Recombination is a universal phenomenon that can occur at any place along two homologous DNA molecules. In temperate bacteriophages, there is a second type of re-combination called site-specific recombination, which takes place only between defined excision sites in the phage and in the bacterial chromosome. Positions of the site-specific recombination in the bacterial and phage DNA are called the bacterial and phage attachment sites, respectively. Each attachment site consists of three segments. The central segment has the conserved nucleotide sequence that sites the recombination event. A phage protein, an integrase, catalyzes the site-specific recombination events, which lead to physical exchange of DNA. Excision requires the phage enzyme integrase plus an additional phage protein called excisionase. There are three well-described site-specific recombination systems that might be useful for the production of marker-free transgenic plants: Cre/loxP system from bacteriophage P1, where the Cre enzyme recognizes its specific target sites, FLP/FRT recombination system from Saccharomyces cerevisiae, where the FLP recombinase acts on the FRT sites and R/RS recombination system from Zygosaccharomyces rouxii, where R and RS are the recombinase and recombination site, respectively. Recognition sites for recombinases consist of palindromes, which are flanked with 7–12-bp core sequences. Cleavage of the sites occurs at the borders between the recombinase binding elements and the core sequence (Fig. 6). In these systems, elimination of SMG would require recombinase expression in transgenic plants. The recombinase gene cassette can be introduced into trans-formed plants that contain the SMG between two recognition sites. Alternatively, a transgenic plant of interest can be crossed with a plant that expresses a recombinase gene. After segregation, marker-free transgenic progeny plants can be identified. To eliminate the breeding step, a co-transformation based on transient expression of the site-specific recombination system in combination with a conditional lethal dominant gene, coda was proposed. Furthermore, the characterization and use of inducible promoters, CLX vector

system, and GST-MAT vector system (multi-auto-transformation) including oncogenes for cell proliferation and regeneration of transgenic plants (see Fig. 7), to express of recombinase genes would be useful [60]. After applying the induction agent, the recombinase would be expressed with induced excision of SMGs and all sequences between the two recombination site. Also, tissue-specific promoters for producing marker-free transformants could be useful for fine-tuning the excision patterns. Recently, plant Cre virus vectors (TMV-Cre and PVX-Cre) for transient expression of cre recombinase has been developed as an alternative method for the production of marker-free transgenic *N. benthamiana* plants. In this method, transgenic plants containing lox sites and the bar SMG are inoculated with PVX-Cre and TMV-Cre recombinant viruses. PVX-Cre and TMV-Cre systemically infect leaves and allow regeneration without selection pressure. This strategy can be applied to plant species that depend on organogenesis or somatic embryogenesis for regeneration, particularly, soybean, potato and a number of woody plant species. Also, a tightly controlled microspore-specific promoter and a site-specific recombination system was recently employed in an efficient marker gene removal in tobacco pollen. Another strategy was proposed employing two site-specific recombination systems: one for integrating the DNA in a recombination site into the host genome at the designated genomic target site, and a second for removing sequences that are not needed after DNA transfer. This strategy is based on the tandem use of the Cre/lox, FLP/FRT and R/RS inducible systems (Fig. 8). In this method it is feasible to achieve site-specific integrations at an efficient rate with predictable transgene expression.

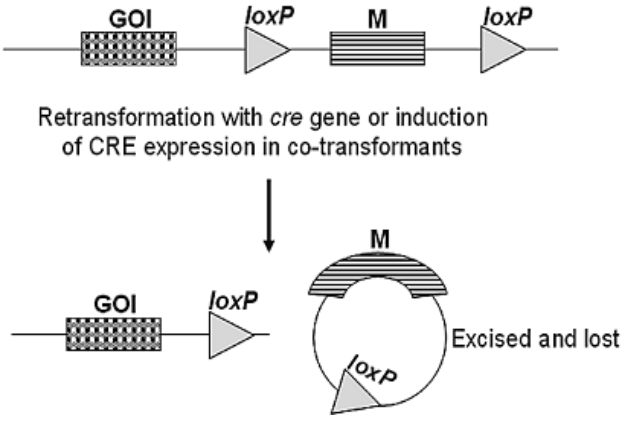
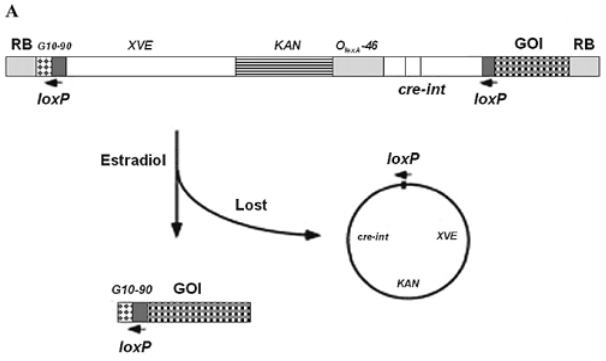


Fig 6: Cre/loxP recombination system (M: marker gene, and GOI: gene of interest.



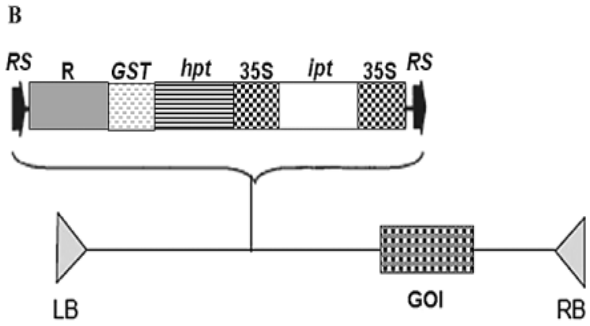


Fig 7: (A) Structure of the CLX vector system. See [44]. Cre-intincludes eight copies of the LexA operator sequence fused to the -46 CaMV35Spromoter. Sequence of Cre is interrupted by an intron. Excision is produced via the ,-estradiol-induced site-specific DNA recombination. (B) MAT Vector System. See. Recombinase genes (R) promoting with GST promoter and hpt marker gene flanked by two directly oriented RS sites. XVE G10-90and GOI are hybrid trans-activator constitutive promoter and gene of interest, respectively.

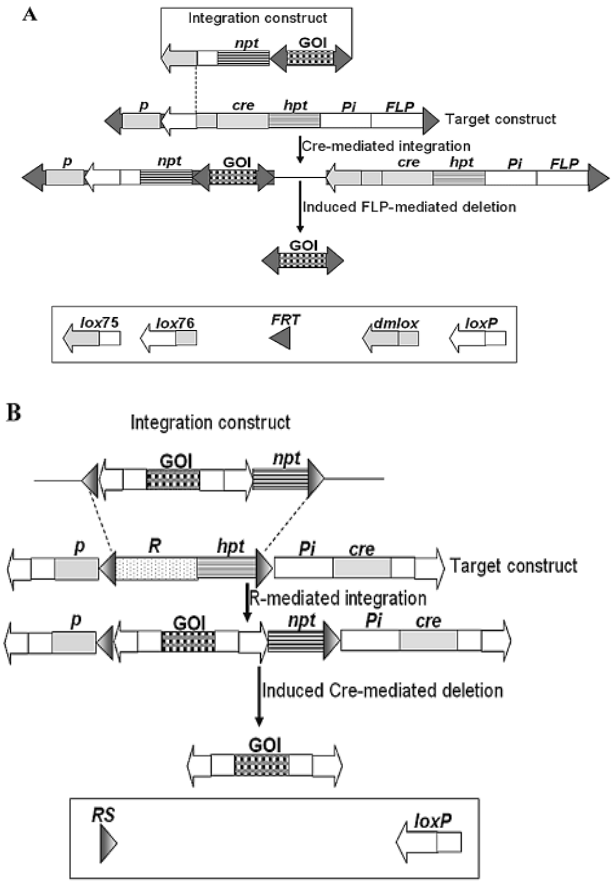


Fig 8: **'**C'mbined step' strategy based on two site-specific recombination systems to remove excess DNA after site-specific integration. (A) Use of Cre/lox and inducible FLP/FRT system to integrate a circular DNA and deleting of excess DNA from the integration locus, respectively. Introduction of circular DNA containing a lox75 (left arm mutant) site into cells containing a lox76 (right arm mutant) locus, site-specific integration of the gene of interest (GOI), and formation of a double mutant lox (dmlox) site, which stabilizes the integration locus. (B) Use of R/RS system to integrate a linear T-DNA, delivered by Agrobacterium, into target RS sites, followed by use of the inducible Cre/loxP system to remove excess DNA from the integration locus. hpt, hygromycin phosphotransferase gene; npt, neomycin phosphotransferase; P, promoter; Pi, inducible promoter.

5.2 Transposon-based marker methods

In the 1940s, Barbara McClintock made an astonishing discovery. She detected two factors of DNA transposition in maize: a Ds (disassociation) element that was located at a chromosome break site and an unlinked genetic factor (Ac) that was required to activate the breakage of Chromosome 9. McClintock concluded that such an un-stable phenotype resulted from the movement or transposition of Ds. These came to be known as transposons. Transposons are DNA sequences between hundreds to thousands of bases long. They code at least one protein, which enables them to replicate. The most widely studied transposon is the P element from the fruitfly (*Drosophila melanogaster*). Transposable elements can also be used to produce marker-free transgenic plants (Fig. 9). Use of transposable elements for marker gene removal involves several steps:

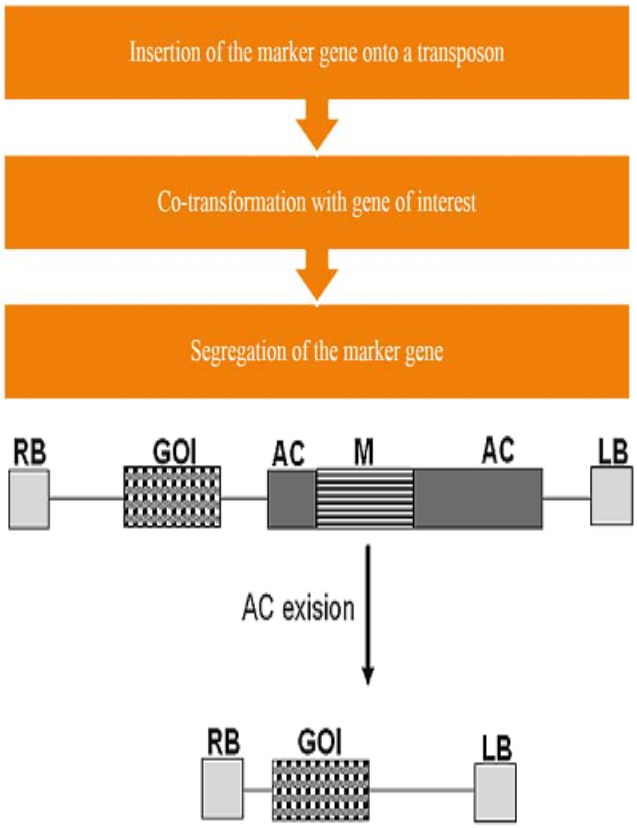


Fig 9: Ac transposon-based expelling of nuclear marker genes (M: marker gene, and GOI: gene of interest).

5.3 Intrachromosomal recombination system

A variant of site-specific recombination systems de-scribed above employs an intra- chromosomal recombination system. As above, recombination sites are engineered into the plant, but no recombinase is expressed. The attachment site from phage origin is denoted POP' (P for phage) or attP, and the attachment site from bacterial origin is denoted BOB' (B for bacteria) or attB. Intra-chromosomal recombination in plants is obtained by insertion of SMG between two direct repeats of attP that facilitates spontaneous excision. Base composition of the attP site sequence is A + T rich, which is conjectured to play a recombination-stimulating role.

Possibly, the formation of a recombination hot spot is caused via the induction of double-strand breaks (DSBs), but may also reduce of the stability of transgene sequences later on. Thirty three percent efficiency of marker gene by ISceI expression, a site-specific homing endonuclease encoded by a mitochondrial intron of Saccharomyces cerevisiae, demonstrated that induced DSB-mediated recombination by highly specific endonucleases could be a feasible alternative to site-specific recombinases for marker elimination. In addition, the inclusion of a transformation booster sequence (TBS) from Petunia hybrid inserted into the construct adjacent to the attachment sequences increased the frequency of intra-chromosomal recombination and illegitimate recombination events in Petunia, Nicotiana and maize.

The potential advantages are: (i) expression of a heterologous recombinase and sexual reproduction are not necessary; (ii) there is a one-step selection procedure for transgenic calli (lengthy propagation two-step time as above might increase the risk of somaclonal variation); (iii) it utilizes a natural nuclear recombination systems present in plants; (iv) the frequency of intra-chromosomal recombination between two homologous sequences in plants might be increased by stimulation of repair systems; and (v) the efficiency of homologous recombination is directly correlated with the size of the homologous regions.

5.4 Removal of chloroplast marker genes

Mitochondria and chloroplasts have independent genomes in plants that have been the target (especially chloroplasts) of genetic transformation. Biosafety might be facilitated by maternal inheritance, which is the case in most plant species, in which transgenes in plastids would not be disseminated via pollen. Chloroplast transformation vectors are designed with homologous flanking sequences on either side of the transgene. In addition, chloroplast engineering overcomes the challenges of gene silencing, position effects, and multistep engineering of multiple genes, which are current limitations of nuclear transformation [46]. Homologous recombination (the use of identical sequences for example in promoters and terminators between genes) (Fig. 10) and site-specific recombination (for example Cre/lox recombination based systems) or transient expression of recombinase are all potentially suitable for producing marker-free engineered chloroplast of plants. Retransformation using the same marker gene has been recently demonstrated, and provides first rigorous proof that despite the high copy number of chloroplast genes, all copies of a marker gene can be removed by homologous recombination.

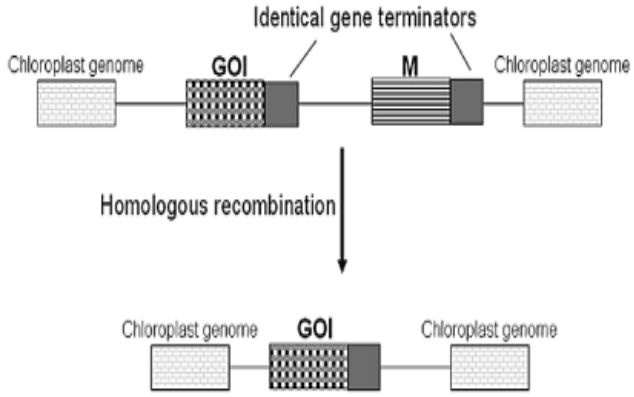


Fig 10: Homologous recombination based removal chloroplast marker gene (M: for marker gene and GOI: gene of interest)

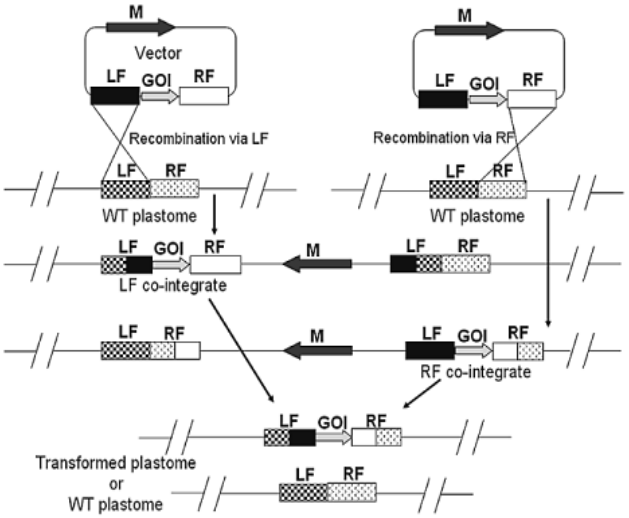


Fig 11: Co-integrated based marker excision method for generation of marker-free plastid transformants (M: marker gene, LF: left and RF: right homologous fragments, GOI: gene of interest, and WT: wild type).

6. Markers not based on antibiotic or herbicide resistance

Recently, researchers have described substitute marker genes of nonbacterial origin that could have inherently increased biosafety. Of most interest are marker genes from plants themselves. One potential alternative method to produce transformants without any antibiotic/herbicide marker gene is so called positive selection systems. Recently, an *Escherichia coli* derived phosphomannose isomerase (PMI) was used to convert mannose-6-phosphate to fructose-6 phosphate for positive selectable marker in plant transformation. Only transformed cells are capable of utilizing mannose as a carbon source. PMI has been used as a selectable marker for transformation of many plant species, such as sugar beet, maize, wheat, rice, pearl millet, and canola. However, this system may not be as effective in plant species that contain endogenous PMI [54]. As a possible solution, the xylose isomerase (xylA) gene of *Streptomyces rubiginosus* can be used as the selectable marker and xylose as the selective agent. The enzyme from S. rubiginosus catalyses the isomerization of D-xylose to D-xylulose. The non-transformed plant cells cannot utilize the D-xylose as a sole carbon source, but xylA transformed cells with are capable of growing on xylose. The xylose isomerase selection system was tested in potato, leading to a 10 fold transformation frequency compared with kanamycin selection. The levels of enzyme activity in transgenic plants selected on xylose were 5–25 fold higher than the enzyme activity in control plants. Most recently, a notable replacement of the bacterial kanamycin-resistant nptII gene is an Arabidopsis thaliana ATP binding cassette (ABC) transporter (Atwbc19) gene. When over expressed in transgenic tobacco, it yielded roughly equivalent degrees of kanamycin resistance in plants; however, because of cellular targeting to the tonoplast, it is not expected to confer kanamycin resistance in bacteria if horizontal gene flow were to occur. Other plant based markers are plant counterparts of aspartate kinase (AK), and dihydrodipicolinate synthase (DHPS) genes for lysine inhibition [47-48].

7. Contributions of recombinant DNA technology

Biotechnology is offering innovative possibilities for increasing crop and livestock production and for the protection of the environment by the reduced use of chemicals. The major thrust is presently directed towards medicine, industry, and agriculture in the industrialized countries, with significant investments by trans-national companies. The specificity with which genes can be examined and manipulated opens up real opportunities to tailor made new plants, and new livestock for specific environment. While traditional plant breeding involves the transfer of large numbers of new genes into a crop followed by cycles of backcrossing and de-selection of undesired genes and traits, genetic manipulation can now be done in the laboratory. Biotechnology is thus comprised of a continuum of technologies, ranging from the long-established and widely used technologies, which are based on the commercial use of microbes and other living organisms, through to the more strategic research on genetic engineering of plants and animals. Genetic engineering evolved from an understanding of how cells function naturally, particularly how the genetic material (DNA) codes for the production of proteins essential for the life of the cell. Based on this understanding, other scientists then devised a series of new techniques, collectively called recombinant DNA technology, to allow the manipulation of these processes in the cell. The major limitation is to identify genes which, when transferred with appropriate molecular controls, will confer agriculturally useful traits on the recipient microorganism, plant or animal. Biotechnology is said to be "the first business with enough glamour to persuade eminent scientists that the entrepreneurial spirit and academic respectability are not mutually exclusive. It has been used in a number of crops for several years, and more genetically enhanced products are expected to be on the market in the coming years. By increasing a crop's ability to withstand environmental factors, growers will be able to farm in parts of the world currently unsuitable for crop production. Along with additional food, this could also provide the economies of developing nations with much-needed jobs and greater productivity. Biotechnology will also enable growers to produce further enhancements in plant varieties. This would allow for the possibility of increasing the agricultural gene pool that billions of people rely on for basic foodstuffs [49-51].

8. Plants on soil and microbial communities' impact of transgenic

Transgenic plants are those plants whose heredity DNA has been augmented by the addition of DNA from a source other than the parental germplasm, using recombinant DNA techniques. They possess novel genes that impart beneficial characteristics such as increased nutritive value, improved flavour, prolonged freshness and even disease fighting properties. The debate surrounding the use and commercialization of genetically modified crops is emotive and presently un abating. The "perceived" risks include plant invasiveness or dispersal of the plant itself into the native ecosystem causing indirect impacts on the diversity of crops, gene flow through pollen transfer or through horizontal gene transfer with associated microorganisms, development of resistance in target organisms, and non-target effects on native flora and fauna including effects on the biodiversity of beneficial and antagonistic micro-organisms. One of the primary concerns about genetically modified crops is the presence of clinically important antibiotic resistance gene products in transgenic plants that could inactivate oral doses of the antibiotic. Another concern is that the antibiotic resistance genes could be transferred to pathogenic microbes in the gastrointestinal tract or soil, rendering them resistant to treatment with such

antibiotics. Though evidence for the persistence of transgenic plant DNA exists, the transformation of plant DNA to native soil microorganisms has not been found. Several studies attempted to assess natural transformation from plant DNA to soil microorganisms under field conditions and determined that while free DNA persisted in the soil, no proof of a plant gene being transferred to soil bacteria was found demonstrated that genetically engineered plants might alter their biological environment, more precisely the root-associated bac-terial populations. A response in the composition of the microbial population was observed after the introduction of a single genetic trait into the plant genome. According to Dunfield and Germida in 2004, the effect of plant variety on the microbial community at one field site was sometimes entirely different at another field site, suggesting that the environment will play a major role in determining the potential ecological significance of growing genetically modified plants. Furthermore, a time course study examining genetically modified plants over an entire field season suggests that changes to the microbial community structure associated with genetically modified plants are not permanent. Collectively, these results seem to indicate that microbial diversity can sometimes be altered when associated with transgenic plants; however, these effects are minor in comparison with environmental factors such as sampling date and field site [52-54].

9. Future prospect

Number of approaches to eliminate antibiotic and herbicide markers has been developed over the last several years and further improvements are now underway. Recently, researchers have described procedures to eliminate residual recognition sequences at recombination sites. The production of marker-free transgenic crops eliminates the risk of HGT and could mitigate vertical gene transfer. Transfer of plant DNA into microbial or mammalian cells under normal conditions of dietary exposure would require all of the following events to occur: (i) removal of the relevant genes from the plant genome, probably as linear fragments; (ii) protection of the genes from nuclease degradation in the plant as well as animal gastrointestinal tract; (iii) uptake of the genes with dietary DNA; (iv) transformation of bacteria or competent mammalian cells; (v) insertion of the gene(s) into the host DNA transgenics would further strengthen the crop improvement programme with widespread applications in both fundamental research and biotechnology.

10. Conclusion

A crucial 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 any more 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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