Review

Recent advances in development of marker-free transgenic plants: Regulation and biosafety concern

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During the efficient genetic transformation of plants with the gene of interest, some selectable marker genes are also used in order to identify the transgenic plant cells or tissues. Usually, antibiotic- or herbicide-selective agents and their corresponding resistance genes are used to introduce economically valuable genes into crop plants. From the biosafety authority and consumer viewpoints, the presence of selectable marker genes in released transgenic crops may be transferred to weeds or pathogenic microorganisms in the gastrointestinal tract or soil, making them resistant to treatment with herbicides or antibiotics, respectively. Sexual crossing also raises the problem of transgene expression because redundancy of transgenes in the genome may trigger homology-dependent gene silencing. The future potential of transgenic technologies for crop improvement depends greatly on our abilities to engineer stable expression of multiple transgenic traits in a predictable fashion and to prevent the transfer of undesirable transgenic material to non-transgenic crops and related species. Therefore, it is now essential to develop an efficient marker-free transgenic system. These considerations underline the development of various approaches designed to facilitate timely elimination of transgenes when their function is no longer needed. Due to the limiting number of available selectable marker genes, in future the stacking of transgenes will be increasingly desirable. The production of marker-free transgenic plants is now a critical requisite for their commercial deployment and also for engineering multiple and complex trait. Here we describe the current technologies to eliminate the selectable marker genes (SMG) in order to develop marker-free transgenic plants and also discuss the regulation and biosafety concern of genetically modified (GM) crops.

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1. Introduction

Genetic engineering of plants mostly involves the addition of genetic material (single or multiple genes) that is integrated into a recipient plant, leading to the modification of the plant's genome. The plants with modified genome are known as transgenic plants or genetically modified (GM) plants. The first successful genetic engineering of a plant was reported in 1983. Broad-leafed plants such as tobacco and tomato were easiest to transform, and reliable transformation of cereals such as rice and maize were not reported until the late 1980s. In 2008, 13.3 million farmers worldwide grew GM crops. Of these, 12.3 million, or 90%, were smallholder, resource-poor farmers in developing countries.

In 1996, the transgenic GM crops were released and commercialized in the US, China, Canada, Argentina, Australia and Mexico. The estimated area of the commercial production of GM crops was approximately 52.6 million ha. GM crop was legally accepted and commercialized in only 13 countries by that time (the US, Argentina, Canada,

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China, South Africa, Australia, Mexico, Bulgaria, Uruguay, Romania, Spain, Indonesia and Germany). The commercial production of GM crops in India and Brazil began in 2002. The increase between 2000 and 2001, in 1 year alone, was 9.4 million ha and represented a 19% increase (*http://www.agnet.org/library/eb/526/*). By the end of 2001, 52.6 million ha was allotted to transgenic or GM crops, and the number of countries growing these crops has since increased dramatically to almost more than double (James 2001). The US and Argentina account for 83.5% of all commercial GM crops planted to date. Together with Canada and China, these four countries accounted for almost 99% of the global GM crop area in 2001 (James 2001).

The GM crop area in developing countries increased from 14% in 1997 to 26% in 2001 (James 2001), which represents a higher percentage of growth than in industrial countries. Over 98% of all GM crops in developing countries are grown in Argentina and China. China has approved 31 applications for commercialization of GM crops (Huang *et al.* 2002). In the Latin American continent, Mexico banned GM maize in 1998. Brazil has a moratorium on growing commercial GM crops and is sometimes presented as the country that will supply the world its non-GM soybean (Campolina de Oliveira Soares 2001). However, especially in regions close to Argentina and Paraguay, GM soybean is estimated to occupy 35% of the Brazilian total soybean growing area, albeit illegally (Schuhmacher 2002). In Asia, India has approved the commercial application of GM cotton in 2002 (James 2002a, b). In the African continent, South Africa is so far the only country growing commercialized GM crops. Europe and Australasia are not growing substantial number of GM crops.

On a global basis in 2010, a record 15.4 million farmers grew biotech crops in about 1 billion ha. It is interesting to note that over 90% of these, about 14.4 million, were small resource-poor farmers in developing countries (James 2010). The number of countries growing GM crops has increased to 29 in recent years. This suggests that the GM crops are the fastest adopted technology in the field of agriculture (James 2010). The benefits and dangers of GM crops are the subject of intense debate. The five key areas of political controversy related to GM crops are food safety, the effect on natural ecosystems, gene flow into non-GM crops, moral/religious concerns and corporate control of the food supply. Horizontal transfer of antibiotic-resistance genes to animal and human gut bacteria and vertical transfer of herbicide-resistance genes to weedy relatives are perceived as major biosafety concerns in genetically engineered crops (Dale *et al.* 2002). Selectable marker gene (SMG) elimination is very important for transgene stacking (Francois *et al.* 2002; Halpin 2005; Manimaran *et al.* 2011). SMG elimination enables engineering of a transgenic plant with additional transgenic traits by sequential transformations using the same SMG (Hohn *et al.* 2001). Only a few SMGs are available for transforming a crop plant. In addition, repeated use of the same promoter and a polyadenylation signal for different SMGs could cause gene silencing in transgene-stacked lines (Matzke *et al.* 1989). Although the advantage of SMG elimination in transgene stacking by sequential transformation was recognized very early (Yoder and Goldsbrough 1994), very little progress has been made in this direction. Here we discuss the regulation of GM crops and also describe the recent advances and current technologies to eliminate the SMG from the transgenic plant genome. This article will be very useful for a better understanding of developing marker-free transgenics plants keeping in mind that this is the major biosafety public concern.

2. Genetic transformation

Transgenic technologies have enormous potential to improve crops of interest in a relatively precise way (Barampuram and Zhang 2011). Genes of interest are introduced, often by *Agrobacterium*-mediated transformation, and become integrated at random positions in the genome. Initial experiments involved gene transfer by using *Agrobacterium tumefaciens* (Herrera-Estrella 1983). The development of sophisticated methods has opened the way for an alternative procedure for engineering plants using direct DNA transfer. The protocols for this transfer include particle bombardment (Gan 1989), chemical treatments and electroporation (Bates 1994). Following the development of particle bombardment methods, the transformation of most crop species has been rapidly achieved using various modifications of the technique. However, the methods to introduce foreign DNA in a plant cell, either by *Agrobacterium tumefaciens*, microinjection, particle gun or protoplast transformation, are relatively inefficient (Rakoczy-Trojanowska 2002). The addition of gene to the desired trait of the plant also requires the use of selection marker genes to select the transformed cells and tissues.

The genetic markers developed for use in plant cells in general have been derived from either bacterial or plant sources and can be divided into two types: selectable and screenable markers.

- Selectable markers are those which allow the selection of transformed cells, or tissue explants, by their ability to grow in the presence of an antibiotic or a herbicide, such as Hygromycin, Kanamycin and Glyphosate. In addition to selecting for transformants, such markers can be used to follow the inheritance of a foreign gene in a segregating population of plants.

- Screenable markers encode gene products whose enzyme activity can be easily assayed, allowing not only the detection of transformants but also an estimation of the levels of foreign gene expression in transgenic tissue. Markers such as β-glucuronidase (GUS), luciferase or β-galactosidase allow screening for enzyme activity by histochemical staining or fluorimetric assay of individual cells and can be used to study cell-specific as well as developmentally regulated gene expression.

The co-introduction of selectable marker genes, especially antibiotic-resistance genes, is required for the initial selection of plant cells that are complemented with a new trait.

3. Selectable marker genes

SMG systems and reporter genes are essential to plant genetic engineering and for the development of transgenic crops. These are almost always present in engineered DNA plasmids used for genetic transformation of plant tissue (Lee and Gelvin 2008). Without them, creation of transgenic crops is not feasible on purely economic and practical terms. These systems allow the relatively straightforward identification and selection of plants that have stably incorporated the marker genes along with the genes of interest. Selectable markers allow the transformed tissue to tolerate an otherwise lethal exposure of an antibiotic or herbicide. Untransformed cells and tissues are killed while the cells carrying the desired gene grow and regenerate into plants. A visible marker gene will display a colour characteristic when the transformed tissue is exposed in certain assays. Selectable markers and visible marker reporter genes rarely affect the studied trait of interest, but provide a powerful tool in determining the success of the transformation events or identification of transformation events before the gene of interest (GOI) can be identified in the culture (Sheen *et al.* 1995).

Two main aspects of the marker gene have to be considered. Firstly, its structure (nucleic acid sequence), which will determine factors such as regulation of transcription (constitutive, environmental or developmental expression), rate of transcription, transcript stability and efficiency of translation. Secondly, the gene product is responsible for the dominant expression of a suitable selective phenotype. The selectable functions on most general transformation vectors are prokaryotic antibiotic-resistance enzymes that have been engineered to be expressed constitutively in plant cells.

3.1 *Commonly used selectable marker genes*

Table 1 describes a list of different marker genes, mostly conferring resistance to antibiotics or herbicides, which have been used previously for plant transformation studies. However, the most commonly used selectable markers are:

- *npt*II and *hpt* genes (for resistance to the aminoglycoside antibiotics, kanamycin and hygromycin)
- *bar* gene (for resistance to herbicide phosphinothricin)

Commercialization of products from plant biotechnology is hampered largely by (public) concerns about possible risks related to the introduction of genetically modified (GM) plants. An unprecedented debate has accompanied the development and commercialization of transgenic crops. The presence of selectable marker genes, which include genes coding for antibiotic resistance that are essential for the initial selection of transgenic plants, is considered undesirable by regulatory agencies in Europe. Divergent policies and their implementation in the European Union on one hand and the rest of the world have resulted in disputes with serious consequences on agricultural policy, world trade and food security. The possible ecological risks formed by the spread of these markers in the natural environment represent a major issue of debate. A major issue of concern relates to the fact that transgenes integrate at random positions in the genome leading to possible unwanted side effects (position effect) and unpredictable expression patterns (Prols and Meyer 1992).

Approximately 50 different selection systems have been developed over the past several years (Sundar and Sakthivel 2008; Miki and McHugh 2004). In addition, the existence of marker genes in transgenic crops could evoke additional, lengthy risk assessments for release of crops that contain useful novel traits. Horizontal gene transfer (HGT) is the transfer of genetic material directly to a living cell or an organism followed by its expression. HGT occurs only among unrelated species, such as between plants and microbes, as well as between microorganisms (Thomson 2001). HGT has been shown to engage members of the same species, of different species, or even of different domains of life. HGT as a biosafety issue has been addressed in several studies and a number of potential hazards have received much attention and have been debated intensively in the scientific and popular press (Ho *et al.* 1999).

Markers that used in genetic transformation and plant regeneration have been described recently. However, continuous expression of these markers may interfere with normal plant growth and development (Ebinuma *et al.* 1997). Gene stacking by using different selectable marker genes will lead to duplication of promoters and polyA signals that may cause un-anticipated gene silencing. Removal of this type of marker from plant tissues is necessary unless expression is under stringent control. Furthermore, current transformation technologies permit only the introduction of a very limited number of genes

Table 1. Selectable marker gene used in plant transformation

Gene	Sources	Gene product	Selective agent	References
aadA	*Shigella flexneri*	Aminoglycoside-3-adenyltransferase	Streptomycin, spectinomycin	Hare and Chua (2002)
neo nptII	*Escherichia coli* Tn5	Neomycin phosphtransferase II	Kanamycin, neomycin, geneticin (G418), paromommycin, amikacin	Fraley *et al*. (1983)
nptIII	*Streptococcus faecalis* R plasmid	Neomycin phosphotransferase III	Kanamycin, neomycin, geneticin (G418), paromommycin, amikacin	Hare and Chua (2002)
nptI, (aphA1) aaC3	*Serratia marcesens*	Aminoglycoside-*N*-acetyltransferase	kanamycin, neomycin, geneticin, paramomycin, gentamycin, tobramycin, apramycin	Hayford *et al*. (1988)
AK	*Escherichia coli*	Aspartate kinase	High concentration lysine and threonine	Yoder and Goldsbrough (1994)
SPT	*Escherichia coli* Tn5	Streptomycin phosphotransferase	Streptomycin	Maliga *et al*. (1988)
TUAm	*Eleusine indica*	a-Tubulin	Trifluralin	Yemets *et al*. (2008)
hph, (aphIV)	*Escherichia coli*	Hygromycin phosphotransferase	Hygromycin B	Waldron *et al*. (1985)
gox	*Ochrobactrum anthropi*	Glyphosate oxidoreductase	Glyphosate	Barry *et al*. (1992)
Ble	*Escherichia coli Tn5*	Bleomycin resistance	Bleomycin	Hille *et al*. (1986)
bnx	*Klebsiella pneumoniae* sub sp. Ozanaenae	Bromoxynil nitrilase	Oxynils	Freyssinet *et al*. (1996)
ilvA or ilvA- 466	*Escherichia coli*	Threonine deaminase	L-O-Methylthreonine	Ebmeier *et al*. (2004)
sulI	*Escherichia coli* pR46	Dihydropteroate synthase	Sulphonamides	Guerineau *et al*. (1990)
DHFR	*Escherichia coli*, mouse, *Candida albicans*	Dihydrofolate reductase	Methotrexate	Herrera-Estrella *et al*. (1983)
sat3	Streptomyces sp.	Acetyl transferase	Streptothricin	Jelenska *et al*. (2000)
ASA2	Tobacco	Anthranilate synthase	5-Methyltryptophan	Cho *et al*. (2004)
EPSP synthase	*Petunia hybrida*	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate	Zhou *et al*. (1995)
DHPS	*Escherichia coli*	Dihydropicolinate synthase	*S*-Aminoethyl	Perl *et al*. (1993)
cat	*Escherichia coli* Tn5, Phagep1cm	Chloramphenicol acetyl transferase	Chloramphenicol	De Block *et al*. (1984a, 1984b)
cah	*Myrothecium verrucaria*	Cyanamide hydratase	Cyanamide	Weeks *et al*. (2000)
OASA1D	Rice	Mutant anthranilate synthase	5-Methyltryptophan (5MT)	Kobayashi *et al*. (2005)
pat, bar	*Streptomyces hygroscopicus*	Phosphinothricin acetyl transferase	Phosphinothricin	De Block *et al*. (1989)
csr1-1	*Arabidopsis thaliana*	Acetolactate synthase	Sulfonylueras	Olszewski *et al*. (1988)
csr1-2	*Arabidopsis thaliana*	Acetolactate synthase	Imidazolinones	Aragao *et al*. (2000)
BADH	*Spinacea oleracea*	Betaine aldehyde dehydrogenase	Betaine aldehyde	Hare and Chua (2002)
dhfr	Plasmid R67	Dihydrofolate reductase	Methotrexate	Yoder and Goldsbrough (1994)
DOG 1	Saccharomyces cerevisiae	2-Deoxyglucose-6-phosphate phosphatase	2-Deoxyglucose	Kunze *et al*. (2001)
ocs	*Agrobacterium tumefaciens*	Octopine synthase	L-Cysteine (AEC)	Koziel *et al*. (1984)
hemL	*Synechococcus* PCC6301	Glutamate-1- semialdehyde aminotransferase	Gabaculine	Gough *et al*. (2001)
als	*Arabidopsis thaliana*, *Nicotiana tabacum*	Acetolactate synthase	Sulfonyl ureas, imidazolinones, thiazolopyrimidines	Hare and Chua (2002)
sul	Plasmid R46	Dihydropteroate synthase	Sulfonamide	Yoder and Goldsbrough (1994)
TDC	*Catharanthus roseus*	Tryptophan decarboxylase	4-Methyltryptophan (4- mT)	Goddijn *et al*. (1993)
TSB1	*Arabidopsis thaliana*	Tryptophan synthase	5MT/Cadmium cholride	Hsiao *et al*. (2007)
pds	*Hydrilla verticillata*	Phytoene desaturase	Norflurazon and fluridone	Arias *et al*. (2006)

into plants (Francois *et al.* 2002). Re-transformation of the same line is needed for multiple trait modifications and new selectable markers are thus needed with each transformation to pyramid the same crop variety with different desirable traits (Hohn *et al.* 2001). The number of selectable marker genes that are suitable for each crop species is usually very limited, and this is true for transformation of recalcitrant species. Marker excision can allow reuse of a marker after each transformation step. Marker elimination will not only appease some potential environmental and consumer concerns, it will also remove technical barriers for plant genetic transformation (Herrera-Estrella *et al.* 1983; Bevan *et al.* 1983). In the recent years, concerns have been raised that the presence of such genes might be an unpredictable hazard to the ecosystem as well as to human health. For example, some of the genes like *Bt* genes and herbicide-resistant gene might be transferred by out-crossing into weeds; and the presence of resistance genes against antibiotic in food products might theoretically lead to the spread of these resistances via gut bacteria in humans (Dale *et al.* 2002). However, there is as yet no scientific evidence to support these statements.

The successful use of antibiotics in medicine has now become a problem. Many bacteria, including pathogens of infectious diseases, are already resistant and can no longer be controlled with the particular antibiotic (Goossens *et al.* 2005). These concerns have been taken seriously and various governments have initiated studies in which such scenarios are now under investigation. However, the most elegant way to overcome all the concerns is to just remove the cause of concern – the selectable marker gene itself.

The drawbacks of traditional markers are becoming apparent even in practical research:

- Different marker gene systems are required for the retransformation of plants that have already been genetically modified. However, there are only a few available for each crop species.
- If several marker genes left over from various developmental phases accumulate in a plant, the stability of the genetically engineered trait can be impaired.
- The probability of unforeseen effects (pleiotropic effect) occurring in the plants increases with the number of transferred genes and marker genes because the role of one gene is affected by the other.

Therefore, there is a need for the development of techniques for the efficient production of 'clean' marker-free transgenic plants. Thus, the development of efficient techniques for the removal of selection markers, as well as the directed integration of transgenes at safe locations in the genome, is of great interest to biotech companies. Furthermore, the removal of selectable marker genes will also have a technical advantage, since the number of available selectable marker genes is limiting, and stacking of transgenes will become more and more desirable in the near future. In the next generation of transgenic plants, antibiotic-resistance markers will be the exception rather than the rule. However, there is still a long way to go before sufficient new procedures and strategies.

4. Methods to eliminate marker genes from nuclear genome

Table 2 describes an up to date progress, in chronological order from 1985 to 2011, of development of marker-free crops of various kinds by using different methods and marker genes. There are several strategies to exclude the selection gene for marker-free plants in transgenic generations, such as co-transformation (Depicker *et al.* 1985; McKnight *et al.* 1987; De Block and Debrouwer 1991), site-specific recombination (Dale and Ow 1991; Gleave *et al.* 1999), multi-autotransformation vector (Ebinuma *et al.* 1997), transposition system (Goldsbrough *et al.* 1993) and homologous recombination (Puchta 2000; Zubko *et al.* 2000), among which co-transformation has been widely used. The first reported example of selectable marker elimination in plants employed the bacteriophage P1 Cre–lox system, comprising Cre-catalysed recombination between Lox sites (Dale and Ow 1991). In this study, a lox-flanked *hpt* gene was removed from transgenic plants upon re-transformation with a construct expressing the Cre-recombinase gene. An important improvement of this early technique was reported by Zuo *et al.* (2001), who used a chemically inducible artificial transcription factor for indirect transcriptional regulation of Cre-recombinase gene expression. Thus, the recombinase gene and the *lox* recombination sites could coexist without leading to premature recombination. Following the selection of transgenic tissue, chemical induction of the recombinase gene produced the desired excision events. A major drawback of this method was the formation of genetic chimeras due to incomplete DNA excision. A particle gun can deliver a mixture of DNA of two plasmids carrying a target gene and a selection gene into plant cells, but the efficiency of marker-free plants obtained was very low in T_1 or T_2 progeny (Yohichi *et al.* 1998). Two *Agrobacterium* strains implementing two binary vectors (Depicker *et al.* 1985; McKnight *et al.* 1987; De Block and Debrouwer 1991) and one *Agrobacterium* strain harbouring two binary vectors (Daley *et al.* 1998) or one binary vector with two T-DNAs containing target gene and selection gene (Depicker *et al.* 1985; Komari *et al.* 1996; Xing *et al.* 2000; Shirley *et al.* 2004) can also be used to get marker-free plants, but in all these cases the efficiency of the marker-free plants was also very low. Moreover, Rommens *et al.* (2004) demonstrated that a plant-derived (P-) DNA fragment can be used to replace the universally employed *Agrobacterium* transfer (T-) DNA. Marker-free P-DNAs are transferred to plant cell nuclei together with conventional T-DNAs carrying a selectable marker gene.

Table 2. Development of marker free plants

Method	Marker gene	Crop plants	Reference
Co-transformation	Neomycin phosphotransferase gene (npt)	Tobacco (*Nicotiana tabacum)*	Depicker *et al.* (1985)
Co-transformation	-	Tomato (*Lycopersicon esculentum*)	McKnight *et al.* (1987)
Cre/lox site-specific recombination	Hygromycin phosphotransferase (hpt)	Tobacco (*Nicotiana tabacum)*	(Dale and David 1991)
Cre/lox site-specific recombination	Acetolactate synthase (ALS) and β-glucuronidase (GUS)	*Arabidopsis thaliana* and Tobacco (*Nicotiana tabacum)*	Russell *et al.* (1992)
Ac-Ds transposon system	NPT-II, neomycin phosphotransferase II and β-glucuronidase (GUS)	Tomato (*Lycopersicon esculentum*)	Goldsbrough *et al.* (1993)
Co-transformation	Hygromycin phosphotransferase (*hpt*), NPT-II, neomycin phosphotransferase II and β-glucuronidase (GUS)	Tobacco (*Nicotiana tabacum* L.) and rice (*Oryza sativa* L.)	Komari *et al.* (1996)
MAT system (Multi-Auto-Transformation	Isopentenyl transferase (*ipt)* , Neomycin phosphotransferase gene (npt) and β-glucuronidase (GUS)	Hybrid aspen (*Populus sieboldii* 3 *Populus grandidentata*)	Ebinuma *et al.* (1997)
Co-transformation	Neomycin phosphotransferase gene (npt) and β-glucuronidase (GUS)	Rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*)	Daley *et al.* (1998)
MAT system (Multi-Auto-Transformation	*npt, ipt and Gus*	Tobacco (*Nicotiana tabacum)*	Sugita *et al.* (1999)
TREGED (transposon-and recombinase-mediated genome deletion)	*tetR* gene and two visible markers, *GUS* and *Lc*	Petunia	Liu *et al.* (2000)
Ac-Ds transposon system	Neomycin phosphotransferase gene (nptII)	Tobacco (*Nicotiana tabacum)*	Zubko *et al.* (2000)
Particle-bombardment	*aadA* and *bar* gene	Tobacco (*Nicotiana tabacum)*	Iamtham and Day (2000)
Double T-DNA binary vector system	Gus and bar gene	Soybean	Xing *et al.* (2000)
R/RS site-specific recombination and the Ac transposon	β-glucuronidase (GUS)	Rice (*Oryza sativa* L.)	Nakagawa *et al.* (2001)
MAT (Multi-Auto-Transformation)	ipt gene and the R gene	Tobacco, Aspen, Rice and Snapdragon.	Ebinuma and Komamine (2001)
Positive selection	pmi	Rice (*Oryza sativa* L.)	Lucca *et al.* (2001)
Co-transformation	Hpt, low-pI *α*-amylase, and *α*-glucosidase	Barley	Matthews *et al.* (2001)
Chloroplast transformation	BADH gene	Tobacco (*Nicotiana tabacum)*	Daniell *et al.* (2001)
GST-MAT vector system	*Ipt* gene combined with *iaaM/H* genes	Tobacco *Nicotiana tabacum*	Endo *et al.* (2002)
GST-MAT vector	*ipt gene*	Hybrid aspen (*Populus Sieboldii* X *Populus grandidentata)*	Matsunaga *et al.* (2002)
Co-transformation	Gus, hph and npt II	Tobacco (*Nicotiana tabacum)*	Jacob and Veluthambi (2002)
Ac-Ds transposon system	synthetic *cry1B* gene, gfp and hph gene	Rice (*Oryza sativa* L.)	Cotsaftis *et al.* (2002)
Co-transformation	hygromycin phosphotransferase (*hpt*)	Rice (*Oryza sativa* L.)	Tu *et al.* (2003)
Cre/lox Site-Specific Recombination	hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (npt)	Tobacco (*Nicotiana tabacum)*	Mlynarova and Nap (2003)
Co-transformation	*hpt* gene and *pmi* gene	Rice (*Oryza sativa* L.)	Datta *et al.* (2003)
Marker-free binary vector	-	Potato (*Solanum tuberosum* L.)	Nick de Vetten *et al.* (2003)
Co-transformation	*hpt* gene and *uidA* gene	Wheat (*Triticum aestivum* L.)	Permingeat *et al.* (2003)
Inducible site-specific recombination system	R recombinase gene, HPT, hygromycin resistance gene; hybrid gene for positive (*npt*II) and negative (*cod*A) selection; GUS, β-glucuronidase reporter gene	Strawberry	Schaart *et al.* (2004)

Table 2 (continued)

Method	Marker gene	Crop plants	Reference
Co-transformation	codA and nptII gene	Tobacco *Nicotiana tabacum*	Park *et al.* (2004)
Potato Virus X (PVX) with Cre recombinase based vector	*Pat* for phosphinothricin selection and the *gfp* reporter	*N. benthamiana*	Kopertekh *et al.* (2004a)
2 T-DNA system	hygromycin phosphotransferase (*hpt*) selectable gene and the green fluorescent protein (*gfp*) reporter gene phosphinothricin acetyl transferase (*bar)* gene	Rice (*Oryza sativa* L.)	Breitler *et al.* (2004)
PVX-Cre-mediated marker gene elimination	Gfp and bar gene	*Nicotiana benthamiana*	Kopertekh *et al.* (2004b)
Two-border binary vector	epsps-cp4, EPSP synthase gene	Maize (*Zea mays* L.)	Huang *et al.* (2004)
Double T-DNA binary vector system	mgfp5 and neomycin phosphotransferase gene (nptII)	Tobacco (*Nicotiana tabacum)*	Chen *et al.* (2005)
Co-transformation	phytoene synthase (psy) and phytoene desaturase (crtI) gene	Rice (*Oryza sativa* L.)	Parkhi *et al.* (2005)
Cre/lox recombination system	Gus and nptII	Tobacco (*Nicotiana tabacum)*	Wang *et al.* (2005)
Cre/loxP recombination system	Gus and stilbene synthase (sts)	Tobacco (*Nicotiana tabacum)*	Bayer and Hess (2005)
Positive selection	Mutated acetolactate synthase (ALS) gene from rice and gus	Rice (*Oryza sativa* L.)	Osakabe *et al.* (2005)
Chemically regulated Cre/loxP Site-Specific Recombination	hpt and gfp	Rice (*Oryza sativa* L.)	Sreekala *et al.* (2005)
Cre/loxP recombination system	Gus, bar and npt II	Tobacco (*Nicotiana tabacum)*	Liu *et al.* (2005)
Positive selection with Marker-free binary vector	*AtTPS1* gene	Tobacco (*Nicotiana tabacum)*	Leyman *et al.* (2006)
Co-transformation	*Hpt gene*	Rice (*Oryza sativa* L.)	Baisakh *et al.* (2006)
MAT (multi-auto-transformation)	Gus, npt II and ipt gene	Nierembergia caerulea	Khan *et al.* (2006)
Cre/lox site-specific recombination	Gus, npt II and gfp gene	Tobacco (*Nicotiana tabacum)*	Jia *et al.* (2006)
Heat inducible Cre-loxP system	Npt II	Potato (*Solanum tuberosum* L.)	Cuellar *et al.* (2006)
R/Rs recombination system	codA-nptII bi-functional, positive/negative selectable marker gene	Potato (*Solanum tuberosum* L.)	Kondrak *et al.* (2006)
Minimal gene cassettes	phosphomannose isomerase (pmi) gene	Durum wheat	Gadaleta *et al.* (2006)
2 T-DNA system	AP1 gene of *Capsicum annuurn* and hygromycin-resistance gene (HPT)	Rice (*Oryza sativa* L.)	Yu *et al.* (2006)
Chemically regulated Cre/lox Site-Specific Recombination	neomycin phosphotransferase gene (npt)	Tomato (*Lycopersicon esculentum*)	Zhang *et al.* (2006)
Co-transformation	Gfp, ACC synthase 1 and ACC oxidase 1 and 2	Broccoli (*Brassica oleracea*)	Higgins *et al.* (2006)
Positive selection	*knotted1* (*kn1*)gene, ipt, Gus and npt II	Tobacco (*Nicotiana tabacum)*	Luo *et al.* (2006)
MAT system (multi-auto-transformation)	ipt and nptII genes	White poplar (*Populus alba* L.)	Zelasco *et al.* (2007)
Cre/loxP	Hygromycin phosphotransferase (hpt), β-glucuronidase (Gus) and glyphosate acetyltransferase (Gat).	Soybean	Li *et al.* (2007)
Cre–loxP system	nptII or gfp genes	Indian mustard. *(Brassica juncea)*	Arumugam *et al.* (2007)
Marker-free binary vector	ipt gene	Hungarian potato (*Solanum tuberosum)*	Bukovinszki *et al.* (2007)

Table 2 (continued)

Method	Marker gene	Crop plants	Reference
MAT system (multi-auto-transformation)	ipt and npt gene	Pineapple sweet orange (*Citrus sinensis* L. Osb.) and Carrizo citrange (*C. sinensis* L. Osb. × *Poncirus trifoliate* L. Raf.).	Ballester *et al.* (2007)
b/six recombination system	neomycin phosphotransferase gene (npt) and β-glucuronidase (GUS)	Tobacco (*Nicotiana tabacum)* and Arabidopsis	Gronlund *et al.* (2007)
Co-bombardment	Bar gene and non-selected cecropinB gene	Rice (*Oryza sativa* L.)	Zhao *et al.* (2007)
Marker-free binary vector	TuMV-NIb gene	Chinese cabbage (*Brassica campestris*)	Zhandong *et al.* (2007)
Co-Transformation	*hpt* and three target (*hLF*, *SB401*, *RZ10*) genes	Rice (*Oryza sativa* L.)	Li *et al.* (2007)
Positive selection	tryptophan synthase beta 1 (AtTSB1)	*Arabidopsis thaliana*	Hsiao *et al.* (2007)
Marker-free transformation	*SAT, trfA* and *nptIII* genes	Potato (*Solanum tuberosum* L.)	Stiller *et al.* (2007)
Cre-loxP system	Npt II and glyoxalase I (glyI) gene	*Arabidopsis thaliana*	Deb Roy *et al.* (2008)
Marker-free binary vector with salt stress	OsDREB2A and AtSOS1	Rrice (*Oryza sativa* L.)	Zhu and Wu (2008)
Marker-free and backbone-free transformation	ipt gene	Various Solanaceous plant species as well as canola (*Brassica napus*).	Richael *et al.* (2008)
Cre/lox Site-Specific Recombination	neomycin phosphotransferase gene (npt) and β-glucuronidase (GUS)	Tobacco (*Nicotiana tabacum)*	Moravcıkova *et al.* (2008)
Cre/lox Site-Specific Recombination	neomycin phosphotransferase gene (npt)	Tobacco (*Nicotiana tabacum)*	Chakraborti *et al.* (2008)
Co-transformation	nptII and codA gene	grapevines	Dutt *et al.* (2008)
Transposon	5-enolpyruvylshikimate- 3-phosphate synthase (epsps) and hpt	Rice (*Oryza sativa* L.)	Charng *et al.* (2008)
Vector backbone-free and selectable marker-free linear GFP cassette	Gfp	Maize (*Zea mays* L.)	Yang *et al.* (2009a)
Co-transformation	Hygromycin phosphotransferase (hph), β-glucuronidase (gus) and rice chitinase (chi11) gene	Rice (*Oryza sativa* L.)	Sripriya *et al.* (2008)
Marker-free binary vector	superoxide dismutase (SOD)	Potato (*Solanum tuberosum* L.)	Ahmad *et al.* (2008)
Heat inducible FLP/FRT recombination system	ipt, npt and Gus	Tobacco (*Nicotiana tabacum)*	Luo *et al.* (2008)
Cre/loxP recombination system	OsMADS45 and Gus	Rice (*Oryza sativa* L.)	Bai *et al.* (2008)
Three T-DNA Binary Vectors	BAR gene and two VIP1 gene	Soybean (*Glycine max*)	Xingguo and Qin (2008)
Marker-free transformation	TPS1 gene	Potato (*Solanum tuberosum* L.)	Stiller *et al.* (2008)
Site-specific integration	luc, hpt, and cre genes	Tobacco (*Nicotiana tabacum)*	Nanto and Ebinuma (2008)
Minimal linear gene cassette	Soluble modified green fluorescent protein (smGFP)	Soybean	Liu *et al.* (2009)
Vector backbone-free and selectable marker-free linear GFP cassette	Gfp	Maize (*Zea mays* L.)	Yang *et al.* (2009b)
Co-bombardment	Gus and nptII	Maize (*Zea mays* L.)	Shiva Prakash *et al.* (2009)
Salicylic acid-inducible Cre/loxP recombination system	Gus and neomycin phosphotransferase gene (npt)	Tomato (*Lycopersicon esculentum*)	Ma *et al.* (2009)
Twin T-DNA Binary Vectors	Gus and hpt	Chrysanthemum	Sun *et al.* (2009)
MAT with site-specific recombination	isopentenyl transferase (ipt) and Gus gene	*Kalanchoe blossfeldiana*	Thirukkumaran *et al.* (2009)
Co-transformation	*Hpt* and *Cry1Ab* genes	Rice (*Oryza sativa* L.*)*	Qi *et al.* (2009)

Table 2 (continued)

Method	Marker gene	Crop plants	Reference
MAT system (multi-auto-transformation)	ipt and nptII genes	Cassava	Saelim *et al.* (2009)
Marker-free and backbone-free transformation	gfp	Maize (*Zea mays* L.)	Yang *et al.* (2009c)
Cre/lox site-specific recombination	vstI	Oilseed rape (*Brassica napus*)	Kopertekh *et al.* (2009)
Two-strain/ two-vector system	*Wx* gene and the other carrying a *HPT* gene	Rice (*Oryza sativa* L.)	Yu *et al.* (2009)
Marker-free binary vector	Gus and pmi gene	Chickpea (*Cicer arietinum* L.)	Patil *et al.* (2009)
Marker-free binary vector with Salt stress	rstB, gfp and hpt	Tobacco (*Nicotiana tabacum)*	Zhang *et al.* (2009)
Stress inducible FLP/FRT site-specific recombination system	Hygromycin-resistance gene (*hpt)*	Tobacco *Nicotiana tabacum*	Woo *et al.* (2009)
Cre/loxP DNA excision system	npt and AtIpk2b, an inositol polyphosphate 6-/3-kinase gene	Tomato (*Lycopersicon esculentum*)	Zhang *et al.* (2009)
MAT (multi-auto-transformation)	ipt gene	Barrel medic (*Medicago truncatula* Gaertn.)	Scaramelli *et al.* (2009)
Marker-free binary vector	Hepatitis B virus surface antigen (HBsAg)	Tobacco (*Nicotiana tabacum),* Tomato (*Lycopersicon esculentum*)	Rukavtsova *et al.* (2009)
Marker-free transformation	Gus, hpt and npt II	Tobacco (*Nicotiana tabacum)*	Li *et al.* (2009)
Twin T-DNA Binary Vectors	Hygromycin-resistance gene (*hpt)*	Rice (*Oryza sativa* L.)	Xia *et al.* (2009)
Positive selection	Phosphomannose-isomerase (pmi) gene	Rapeseed (*Brassica napus*)	Wallbraun *et al.* (2009)
Marker-free binary vector	cecropin P1 (*cecP1*)	Tobacco (*Nicotiana tabacum)*	Zakharchenko *et al.* (2009)
Co-Transformation	antisense Wx gene and (HPT)	Rice (*Oryza sativa* L.)	Yu *et al.* (2009)
MAT (multi-auto-transformation)	Gus and ipt	*Petunia hybrida*	Khan *et al.* (2010)
Cre/lox site-specific recombination	Hpt, Allium sativum leaf agglutinin, ASAL	Rice (*Oryza sativa* L.)	Sengupta *et al.* (2010)
MAT (multi-auto-transformation)	Rol gene	*Petunia hybrida*	Khan *et al.* (2010)
Cre/loxP recombination system	Hpt, Cry1Ab/Ac gene	Rice (*Oryza sativa* L.)	Qiu *et al.* (2010)
Positive selection	lysine racemase (lyr)	Tobacco (*Nicotiana tabacum)* and Arabidopsis	Chen *et al.* (2010)
Marker-free binary vector	Sgfp gene	Carrizo citrange and Pineapple sweet orange (*C. sinensis* L. Osb.),	Ballester *et al.* (2010)
Co-transformation	Mungbean yellow mosaic virus (MYMV) transcriptional activator protein gene (TrAP)	Tobacco (*Nicotiana tabacum)*	Ramana Rao & Veluthambi (2010)
Markerless DNA transformation technology (MDTT)	Attacin E and *MpNPR1*	Apple	Malnoy *et al.* (2010)
FLP/FRT recombination system	AtNHX1, a Na+/H+ antiporter gene	Maize (Zea *mays* L.)	Li *et al.* (2010)
Marker-free binary vectors	phytoene synthase gene (Zmpsy1) or chitinase gene (Rchit)	Peanut (*Arachis hypogaea* L.)	Bhatnagar *et al.* (2010)
Marker-free binary vector with Salt stress	Monodehydroascorbate reductase (Am-MDAR).	Tobacco (*Nicotiana tabacum)*	Kavitha *et al.* (2010)
Co-bombardment	*cry1B-1Aa* and hpt	Rice (*Oryza sativa* L.)	Kumar *et al.* (2010)
Heat inducible Cre/lox and FLP/FRT site-specific recombination	Bar and hpt	Hybrid aspen (Populus tremula x Populus tremuloides)	Fladung and Becker (2010)
Cre/lox site-specific recombination	Bar and vstI gene	*N. benthamiana*	Kopertekh *et al.* (2010)
Positive selection	Mannose-6-phosphate reductase gene (M6PR)	Arabidopsis and tobacco *Nicotiana tabacum*	Song *et al.* (2010)

Table 2 (continued)

Method	Marker gene	Crop plants	Reference
Heat inducible FLP/FRT site-specific recombination system	nptII and a promoterless uidA gene	Hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.)	Fladung *et al.* (2010)
MAT System (Multi-auto-transformation)	isopentenyltransferase (ipt) gene and wasabi defensin (WD) gene	Tomato (*Lycopersicon esculentum*)	Khan *et al.* (2011)
Co-transformation	Rice chitinase (chi11), tobacco osmotin (ap24) genes, Gus and hpt genes	Rice (*Oryza sativa* L.)	Ramana Rao *et al.* (2011)
Marker-free binary vector	Arabidopsis C-repeat binding factor 1 (At-CBF1) and npt II genes	Tomato (*Solanum lycopersicum*)	Shweta Singh *et al.* (2011)
Cre/lox site-specific recombination	Hygromycin phosphotransferase (hph), β-glucuronidase (gus) and npt gene	Rice (*Oryza sativa* L.)	Akbudak and Srivastava (2011)
Heat inducible Cre/lox site-specific recombination system	neomycin phosphotransferase gene (npt) and β-glucuronidase (GUS)	Rice (*Oryza sativa* L.)	Khattri *et al.* (2011)
Ipt-type MAT vector	Gus, ipt and ChiC gene	Potato (*Solanum tuberosum* L.)	Khan *et al.* (2011)
Marker-free and vector-free cassette	ACO1 gene	Melon (*Cucumis melo*)	Hao *et al.* (2011)
Ipt-type MAT vector	isopentenyltransferase (ipt) and wasabi defensin (WD) gene	Tomato (*Solanum lycopersicum*)	Khan *et al.* (2011)

4.1 *Co-transformation*

The co-transformation method is a very simple method to eliminate the marker gene from the nuclear genome. Co-transformation involves transformation with two plasmids that target insertion at two different plant genome loci. One plasmid carries a selective marker gene and the other carries the GOI (figure 1). The following three methods are used in the co-transformation system: (i) Two different vectors carried by different *Agrobacterium* strains (McKnight *et al.* 1987; De Block and Debrouwer 1991; De Neve *et al.* 1997) and biolistic introduction of two plasmids in the same tissue (Shiva Prakash *et al.* 2009; Kumar *et al.* 2010); (ii) two different vectors in the same *Agrobacterium* cell (De Framond *et al.* 1986; Daley *et al.* 1998; Sripriya *et al.* 2008); and (iii) two T-DNAs can be borne by a single binary vector (2 T-DNA system) (Komari *et al.* 1996; Xing *et al.* 2000; Matthews *et al.* 2001; McCormac *et al.* 2001; Miller *et al.* 2002).

In these co-transformation systems, selectable marker genes and target genes are not loaded between the same pair of T-DNA borders. Instead, they are loaded into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion (Framond *et al.* 1986; McKnight *et al.* 1987; Daley *et al.* 1998; Matthews *et al.* 2001; Jacob & Veluthambi 2002; Vain *et al.* 2003; Permingeat *et al.* 2003; Park *et al.* 2004; Parkhi *et al.* 2005; Higgins *et al.* 2006; Li *et al.* 2007a, b; Zhao *et al.* 2007; Sripriya *et al.* 2008; Qi *et al.* 2009; Yu *et al.* 2009; Ramana Rao and Veluthambi 2010; Ramana Rao *et al.* 2011). The advantages of co-transformation methods include the high adaptability of conventional, unmodified *Agrobacterium*-mediated gene transfer methods and easier handling of the binary vectors because the two T-DNA are separated and, hence, target gene T-DNA can be handled independently of selectable marker gene T-DNA. This method depends on the co-transformation efficiency and the independent integration of T-DNA into the plant genome. Generally, the co-transformation efficiency is in the range of 30–50%, which is acceptable for practical applications (Depicker *et al.* 1985; McCormac *et al.* 2001; Komari *et al.* 1996).

In this method SMG can be eliminated from the plant genome at the time of segregation and recombination that occurs during sexual reproduction by selecting on the transgene of interest and not the SMG in progeny. In spite of all these, there are several inevitable limitations. The methods described above are very time consuming and compatible only for fertile plants. The tight linkage between co-integrated DNAs limits the efficiency of co-transformation. Indeed, integration of SMG and the transgene is at indiscriminate event: both the SMG and transgene may integrate

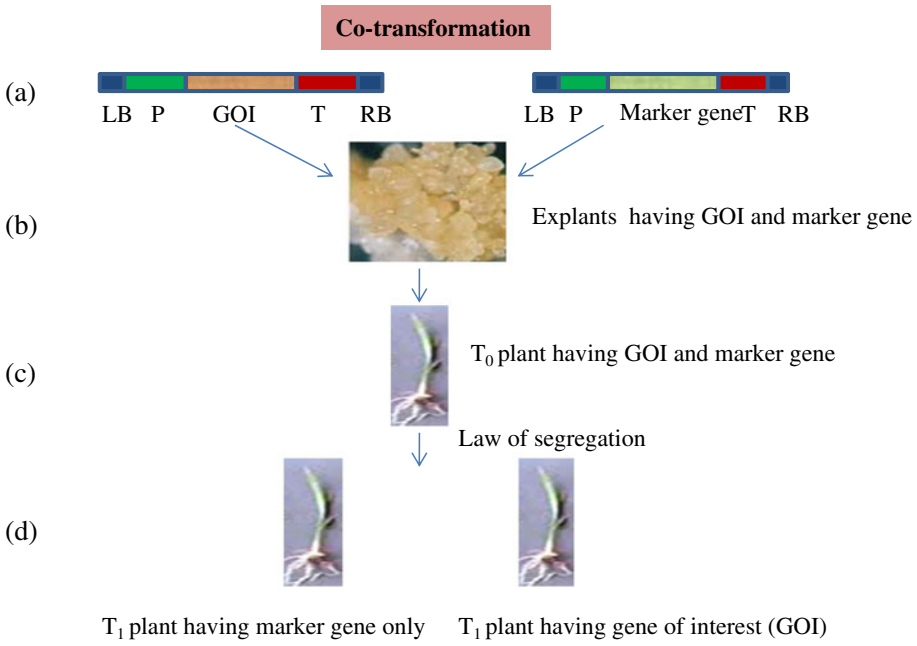

Figure 1. Schematic diagram of Co-transformation method for making marker free transgenic plants. (**a**) Physical diagram of two T-DNA region showing gene of interest (GOI) and marker gene. (**b**) Transformed calli having GOI and marker gene. (**c**) T_0 plant having GOI and marker gene. (**d**) Two T_1 plants one with GOI and another with marker gene.

in the same loci and that is not feasible for co-transformation. However, the overall advantages of these methods remain unclear. Most of the research paper documented the limitations of the co-transformation methods that are limited and useful only for flowering plants but de Vetten *et al.* (2003) developed a silencing construct (pKGBA50mf-IR 1.1) and transformed in to potato (karnico) via highly virulent LBA4404 or AGL *Agrobacterium*-mediated transformation without the use of selection marker gene. They have developed a PCR-based detection method, and >2% of the recovered shoots showed a complete gene silencing of the granule-bound starch synthase 1 (*GBSS1*) gene resulting in an amylase-free phenotype. They have successfully developed a protocol that is useful for vegetative as well as flowering plants. Recently, in rice, high transformation frequency (86%) was achieved through genetic separation in 4 out of 10 primary co-transformants that were forwarded to the T_1 generation (Sripriya *et al.* 2011). A majority of marker elimination strategies involve elimination of marker genes and genes encoding recombinases (or transposons) by segregation in the T_1 generation or T_2 generation. This would not be feasible in vegetatively propagated plants and in plants that take many years to flower (e.g. rubber). Ramana Rao and Veluthambi (2010) reported efficient strategies to employ marker elimination and achieved marker-free transgenic tobacco in the T_0 generation itself.

4.2 *Multi-autotransformation*

The multi-autotransformation (MAT) vector system represents a highly sophisticated approach for the removal of nuclear marker genes (Ebinuma *et al.* 1997). The MAT vector system is a positive selection system that gives the advantage of regeneration to the transgenic cells without killing the non-transgenic cells. It is a unique transformation system that uses morphological changes caused by oncogene [the isopentenyltransferase (*ipt*) gene] or rhizogene (the *rol* gene) of *A. tumefaciens* which control the endogenous levels of plant hormones and the cell responses to plant growth regulators as the selection marker. Expression of the *ipt* gene causes abnormal shoot morphology called extreme shooty phenotype (ESP), which subsequently reverts into normal shoots with objective transgenes due to the excision of *ipt* gene by the function of 'hit-and-run' cassette system (Ebinuma and Komamine 2001).

In this MAT system, a chosen GOI is placed adjacent to a multigenic element flanked by *RS* recombination sites (figure 2). A copy of the selectable *ipt* gene from *A. tumefaciens* is inserted between these recombinase sites, together with the yeast *R* recombinase gene and this entire assembly is situated within a T-DNA element for the *Agrobacterium*-mediated transformation of plant tissues. In this plant transformation system, neither antibiotic- nor herbicide-resistance genes are

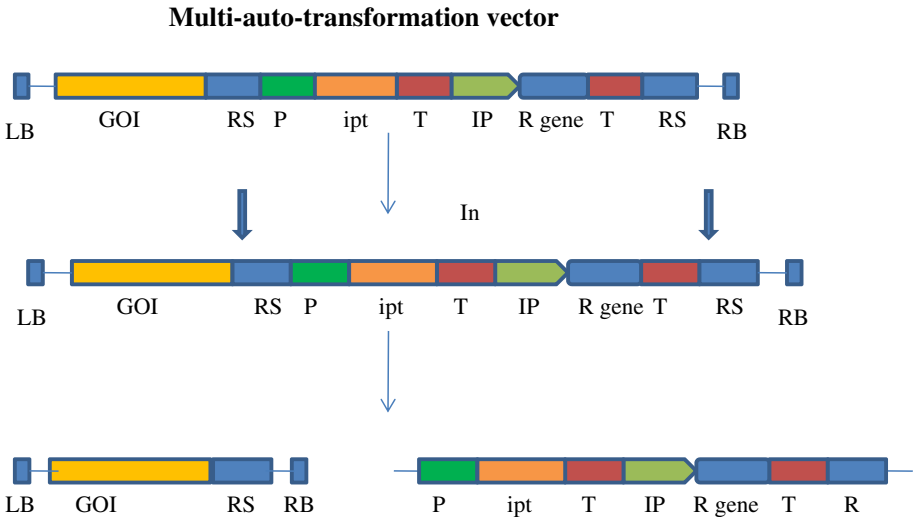

Figure 2. Principle of MAT (multi-autotransformation) uses oncogene (ipt) for selection of transgenic plants and a site-specific recombination system (*R*/*Rs*). Recombinase (R) catalyses recombination between two directly oriented recognition sites (Rs) and removes a 'hit and run' cassette from a plant genome. Recombinase (R) gene expression is under the chemically inducible promoter (IP) in order to avoid early removal of ipt gene. P; promoter, T; terminator, GOI; gene of interest, LB; left border, RB; right border.

necessary as a selection marker. In addition, this system of transformation allows for repeated transformation of genes of interest in a plant (Sugita *et al.* 2000).

In an earlier version of the MAT vector, R recombinase activity was constitutively up-regulated by the action of the CaMV 35S promoter. This system was found to incur a risk of marker gene excision before the selection of transformed plant tissues could take place. To circumvent this problem, a more recent version of the MAT vector (Matsunaga *et al.* 2002) allows for a delay in the excision of the *ipt* and *R* recombinase genes. This is made possible by the use of a chemically inducible glutathione S-transferase promoter from maize to drive *R* recombinase gene expression. Once the positive selection of transformed plant tissues showing an 'extreme shooty phenotype' has occurred, the excisive recombination of RS sites, leading to a loss of the recombinase and marker genes, is induced by treatment with the herbicide. Kunkel *et al.* (1999) have shown that with a dexamethasone-inducible promoter to control the expression of the *ipt* gene, transgenic calli and shoots can be produced in the presence of dexamethasone. Once the inducer is removed, the transgenic shoots/plants will be morphologically or developmentally normal. Angela *et al.* (2003) have reported that the KN1 activity can be controlled by a steroid induction system in *Arabidopsis thaliana*. The steroid inducible *kn1* fusion gene described by Angela *et al.* (2003) may be a suitable system for production of transgenic plants if the steroid induction system is tightly regulated.

Recent reports on the Ipt-type MAT system have shown that it is a better system to produce marker-free transgenics. The Ipt-type MAT vector system has been successfully used to generate marker-free transgenic cassava plants (Saelim *et al.* 2009). This system utilizes the *ipt* gene as morphological marker for visual selection of transgenic lines. The ESP of transgenic lines is lost due to the removal of *ipt* gene mediated by the yeast R/RS system. As a result, phenotypically normal shoots, considered marker-free transgenic plants, could be obtained. This is the first demonstration of the efficacy of Rint/RS system in promoting excision of ipt marker gene in cassava species. Expression of the uidA reporter gene was tested in transformation experiments of barrel medic (*Medicago truncatula* Gaertn.) with the Ipt-type control vectors pIPT5, pIPT10 and pIPT20 and distinct *in vitro* culture conditions (Scaramelli *et al.* 2009). The same strategy was used for producing marker-free transgenic *Kalanchoe blossfeldiana* Poelln. In this study, we used *A. tumefaciens* strain EHA105 harbouring an ipt-type MAT vector, pMAT21, containing *lacZ* and *gus* genes and the removable cassette in the T-DNA region, employing *ipt* gene as the selectable marker gene (Thirukkumaran *et al.* 2009). Rol-type MAT vector (pMAT101) containing *lacZ* gene as a model gene and the removable cassette with *gus* gene in the T-DNA region were used to produce morphologically normal transgenic *Kalanchoe blossfeldiana* Poelln, employing *rol* gene as the selectable marker gene and *gus* gene as a reporter gene (Thirukkumarana *et al.* 2010). In an attempt to produce transgenic marker-free *Petunia hybrida* plants resistant to *Botrytis cinerea* (gray mold), *ipt* gene was used as a selectable marker gene (Khan *et al.* 2010a). There is a report of *ipt* gene being used as a selection marker gene to produce marker-free disease-resistant potato (Khan *et al.* 2010b). A

chitinase gene, *ChiC* (isolated from *Streptomyces griseus* strain HUT 6037) was used as a gene of interest.

The Rol-type MAT vector has been tried in *Antirrhinum majus* (Cui *et al.* 2000, 2001), tobacco (Ebinuma and Komamine 2001), white poplar (Zelasco *et al.* 2007), *Petunia hybrida* (Khan *et al.* 2010c), etc. A total of 11 independent β-glucuronidase (GUS)-positive hairy roots were induced following co-cultivation of leaf explants of *Antirrhinum majus* L. with *A. tumefaciens* strain GV2260 containing Rol-type MAT vector pNPI702 (Cui *et al.* 2000, 2001). The chimeric *ipt* gene or the *rol* genes are combined with the site-specific recombination *R*/RS system to remove the oncogenes from the transgenic cells after transformation in case of tobacco (Ebinuma and Komamine 2001). Genetic transformation of an elite white poplar genotype (*Populus alba* L., cv. 'Villafranca') was performed with MAT vectors carrying the *ipt* and *rol* genes from *A. tumefaciens* spp. as morphological markers. The occurrence of abnormal ipt and rol phenotypes allowed the visual selection of transformants (Zelasco *et al.* 2007). *A. tumefaciens* strain EHA105 harbouring a Rol-type MAT vector, pMAT101, was used to produce morphologically normal transgenic *Petunia hybrida* 'Dainty Lady' employing *rol* gene as the selection marker gene. The *lacZ* gene was used as a model GOI (Khan *et al.* 2010c).

4.3 *Site-specific recombination*

Recombination is very clear phenomenon in biological systems: it occurs between two homologous DNA molecules. In bacteriophage, site-specific recombination takes place between defined excision sites in the phage and in the bacterial chromosome. In site-specific recombination, DNA strand exchange takes place between segments possessing only a limited degree of sequence homology (Kolb 2002; Coates *et al.* 2005). The site-specific recombination methods in plants have been developed to delete selection markers to produce marker-free transgenic plants or to integrate the transgene into a predetermined genomic location to produce site-specific transgenic plants (Nanto and Ebinuma 2008). Basically three site-specific recombination systems are well known and are described in the following sections for the elimination of selection marker gene.

4.3.1 *Cre/lox site-specific recombination system:* The Cre/loxP system consists of two components: (a) two loxP sites each consisting of 34 bp inverted repeats cloned in direct orientation flanking a DNA sequence and (b) the *cre* gene encoding a 38 kDa recombinase protein that specifically binds to the loxP sites and excises the intervening sequence along with one of the loxP sites (figure 3A). The Cre/loxP system has been tested in several plants including *Arabidopsis* (Zuo *et al.* 2001), *Nicotiana* (Odell *et al.* 1990; Dale and Ow 1991; Gleave *et al.* 1999), *Zea mays* (Zhang *et al.* 2003) and *Oryza sativa* (Hoa *et al.* 2002; Sreekala *et al.* 2005).

One of the greatest advantages of the Cre/lox system is the specificity of the enzyme for its 34 bp recognition sequence. With a few exceptions, it is difficult to insert and to excise genes with precision in the plant genome without a site-specific recombination system. Marker gene removal from transgenic plants using the Cre/lox recombination system of bacteriophage P1 requires re-transformation and out-crossing approaches that are laborious and time consuming (Dale and Ow 1991). In order to initiate the Cre/lox recombination for removal of the marker gene, other novel inducible site-specific recombination systems have been used (figure 3B).

However, several approaches were developed to overcome these shortcomings, including the use of some chemical inducers (Schaart *et al.* 2004; Yuan *et al.* 2004; Zhang *et al.* 2006) and heat shock (Wang *et al.* 2005; Cuellar *et al.* 2006). Marker-free transgenic tomato plants expressing *cry1Ac* were obtained by using a chemically regulated Cre/lox-mediated site specific recombination system (Zuo *et al.* 2001; Zhang *et al.* 2006). Lin *et al.* (2008) reported a chemical induction method for creating selectively terminable transgenic rice using benzothiadiazole (Bentazon), a herbicide used for weed control in major crops like rice, maize, wheat, cotton and soybean. Similarly, Ma *et al.* (2009) reported a marker-free transgenic tomato using a salicyclic acid-inducible Cre–loxP recombination system. Through this system they have developed 41% transgenic tomato that are marker free (*npt*II gene) in the F_1 generation.

A Cre/loxP recombination system was used for elimination of a caseinolytic protease P1 (*clpP1*) in tobacco (Kuroda and Maliga 2003). Deb Roy *et al.* (2008) reported a heat inducible Cre/loxP site-specific recombination system to remove *npt*II gene from *A. thaliana* transgenic plants transformed with *glyI* gene. The *cre* gene was driven by the heat-inducible promoter (*hsp*), and the *npt*II gene is flanked by *lox* sequences. These inducible site-specific recombination systems can also be applied in vegetatively propagated crop plants for marker gene excision.

4.3.2 *FLP/FRT recombination system:* In the FLP/frt site-specific system of the 2 μm plasmid of *Saccharomyces cerevisiae*, the FLP enzyme efficiently catalyses recombination between two directly repeated FLP recombination target (frt) sites, eliminating the sequence between them. By controlled expression of the FLP recombinase and specific allocation of the frt sites within transgenic constructs, the system can be applied to eliminate the marker genes after selection (Lyznik *et al.* 1996; Cho 2009).

Shan *et al.* (2006) used the heat-inducible system in a FLP/frt site-specific recombinase system. Under this, the

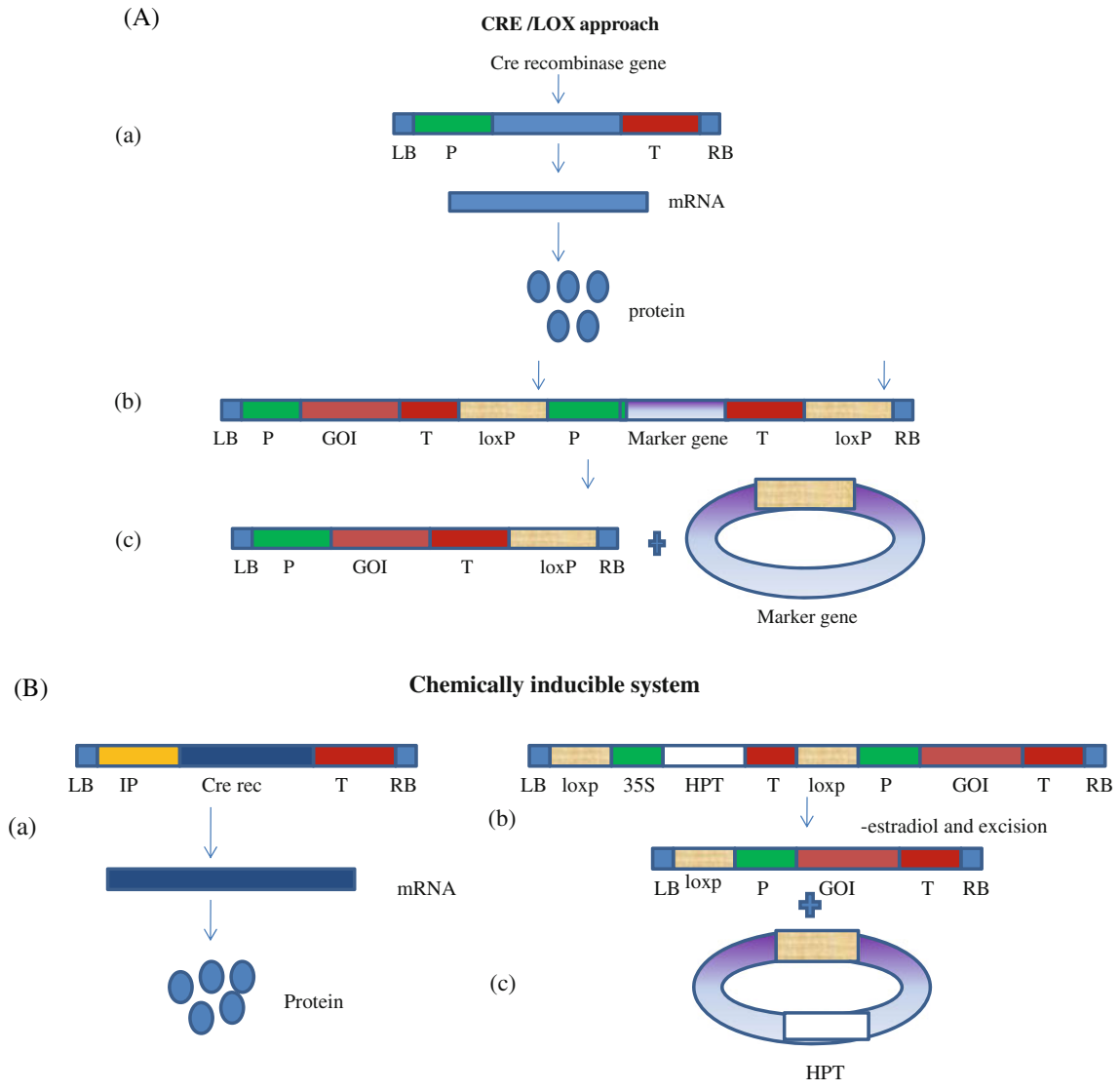

Figure 3. (**A**) Hypothetical diagram of Cre–lox approach used in developing marker free transgenic plants. (**a**) The T-DNA region showing Cre gene followed by the transcribed mRNA and Cre protein expression. (**b**) T-DNA region showing GOI and marker gene merged between loxP sites. (**c**) Resulting transgenic plants showing excision of marker gene. (**B**) Hypothetical diagram of chemically inducible Cre/lox approach used in developing marker free transgenic plants. (**a**) The T-DNA region showing Cre gene controlled by an inducible promoter followed by the transcribed mRNA and Cre protein expression. (**b**) T-DNA region showing GOI and marker gene merged between loxP sites. (**c**) Resulting transgenic plants showing excision of marker gene when induced chemically.

expression of *FLP* was tightly under the control of the heat shock protein, hsp (figure 4). Two different constructs were used, the *frt*-containing vector (pCAMBIA1300-betA-frt-als-*frt*) and the *FLP* expression vector (pCAMBIA1300-hsp-*FLP*hpt). Through the process of re-transformation, the *FLP* recombinase gene was introduced into transgenic (betAfrt-als-*frt*) tobacco. In the re-transgenic plants after heat shock treatment, the marker gene *als*, flanked by two *frt* sites, could be excised by the inducible expression of *FLP* recombinase under the control of *hsp* promoter. A heat-inducible strategy for the elimination of selection marker genes was also reported in vegetatively propagated plants like potato (Cuellar *et al.* 2006).

Recent report describes the generation of marker-free transgenic maize plants constitutively expressing *AtNHX1*, a Na(+)/H(+) antiporter gene from *A. thaliana* that conferred salt tolerance on plants, using the FLP/frt site-specific recombination system (Li *et al.* (2010)). Transgenic plant expressing a modified *FLP* recombinase gene was crossed with transgenic plant harbouring *AtNHX1* and mutant *als*, a

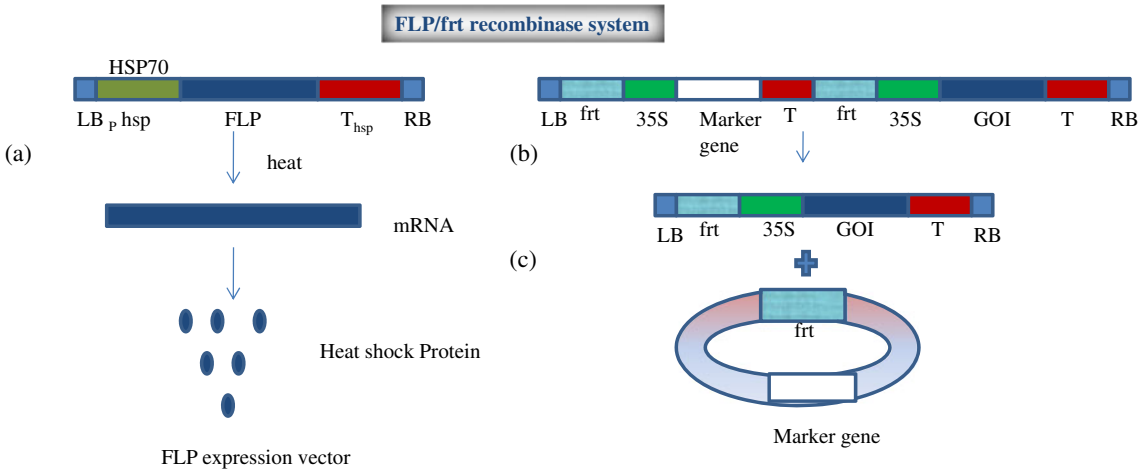

Figure 4. *FLB/frt* site-specific recombination system. (**a**) The T-DNA region showing *FLP* gene controlled by heat inducible promoter (hsp70) followed by the transcribed mRNA and FLP protein expression. (**b**) T-DNA region showing GOI and marker gene merged between frt sites followed by resulting transgenic plants showing excision of marker gene.

selectable marker gene flanked by two directed FRT sites. The sexual crossing led to precise and complete excision of the FRT-surrounding the *als* marker gene in the F_1 progenies. Further, salt tolerance examinations indicated that marker-free AtNHX1 transgenic plants accumulated more Na(+) and K(+), and produced greater biomass and yields than did the wild-type plants when grown in high-saline fields. These results demonstrate the feasibility of using this FLP/frt-based marker elimination system to generate marker-free transgenic important cereal crops with improved salt tolerance.

Woo *et al.* (2009) described the successful excision of antibiotic-resistance genes from transgenic plants via the use of an oxidative stress-inducible *FLP* gene. FLP encodes a recombinase that can eliminate *FLP* and *hpt* selection genes flanked by two FRT sites. During a transformation procedure in tobacco, transformants were obtained by selection on hygromycin media. Regenerants of the initial transformants were screened for selective marker excision in hydrogen-peroxide-supplemented media and both the *FLP* and *hpt* genes were found to be eliminated.

Two site-specific recombination systems, Cre/lox and FLP/frt, were tested for marker gene removal and targeted gene transfer in a *Populus* (Fladung *et al.* 2010). A hybrid aspen clone (*Populus tremula* x *Populus tremuloides*) was co-transformed with plasmids containing either the FLP or the Cre recombinase, both under control of a heat-inducible promoter (HSP, Gmhsp17.5-E from soybean) flanked by the two recognition sites (FRT or lox). Molecular investigations of heat-shock-treated Cre or FLP transgenic lines indicate excision of inserts between the two recognition sites. Further, a site-specific recombination at the FRT sites leading to targeted integration of a fragment could be demonstrated for the FLP/frt system. Transgenic aspen carrying two constructs (each with different genes between the FRT sites) revealed the excision of both fragments between the FRT sites as well as targeted integration of the fragment from the second construct exactly at the former position of the fragment in the first construct. Combining both site-specific recombination systems, this strategy suggested the targeted transgene transfer and removal of antibiotic marker genes.

Nandy and Srivastava (2011) reported the use of FLP/frt system for efficient targeting of foreign gene into the engineered genomic site in rice. The transgene vector containing a pair of directly oriented FRT sites was introduced by particle bombardment into the cells containing the target locus. FLP activity generated by the co-bombarded FLP gene efficiently separated the transgene construct from the vector-backbone and integrated the backbone-free construct into the target site. Strong FLP activity, derived from the enhanced FLP protein, FLPe, was important for the successful site-specific integration (SSI). The majority of the transgenic events contained a precise integration and expressed the transgene. Progeny of the precise transgenic lines inherited the stable SSI locus and expressed the transgene.

4.3.3 *R/RS recombination system:* The MAT vectors consist of yeast site-specific recombination R/RS system to excise the DNA fragment and the *ipt* gene positioned between two directly oriented recombination sites (Araki *et al.* 1987). The *ipt* gene encodes isopentenyltransferase, which catalyses the formation of isopentenyl AMP, a precursor of several cytokinins. Following gene transfer, overexpression of the *ipt* gene leads to an increase in endogenous

cytokinins and, subsequently, the production of ESP (named as ipt shoots). Ipt shoots are characterized by reduced apical dominance, abnormal morphological changes, short internodes and lack of rooting ability (Smigocki and Hammerschlag 1991; Hewelt *et al.* 1994). Therefore, the Ipt shoots are visually selected and subcultured to develop normal looking shoots. Site-specific recombination mediated by recombinase of the R/RS system during subculturing produces morphologically normal marker-free transgenic plants. However, one of the major limitations of using this system is the low frequency of marker-free transgenic plants, as most of the modified transposable elements (containing *ipt* gene) reinsert elsewhere in the genome shortly after their excision, and thus only cells with transposition errors would generate phenotypically normal plants.

A new MAT vector has been created in which the maize transposable element Ac for removing the *ipt* gene is exchanged with a site-specific recombination system R/rs isolated from *Zygosaccharomyces rouxii* (Sugita *et al.* 1999) The R/rs system comprises a *R* recombinase gene and two *rs* recombination site sequences. The *ipt* combined with the (*R*) gene was placed within two directly-oriented recognition sites to remove it from transgenic cells after transformation. The improved MAT vector is used to generate marker-free transgenic plants efficiently. Such a system can be applied to woody plants or vegetatively propagated species to produce marker-free transgenic plants as well as providing the basis for the development of an inducible plant transformation system. Expression of *ipt* gene under dexamethasone-inducible promoter led to the recovery of lettuce and tobacco transformants under inducing conditions (Kunkel *et al.* 1999).

The recombination sites are typically between 30 and 200 nucleotide in length and consist of two motifs with a partial inverted repeat symmetry, to which the recombinase binds and which flank a central crossover sequence at which the recombination take place (figure 2). The unique ability of *Cre* to catalyse a crossover between directly repeated lox sites flanking any fragment of DNA has been exploited to remove selectable marker genes from transgenic plants. The pairs of sites between which the recombination occurs are usually identical, but there are exceptions (e.g. *attP* and *attB* of λ integrase (Landy 1989)). The simplest approach is to generate plants that express the *cre* gene and to cross them with plants in which the selectable marker gene is flanked by lox sites. The marker gene is excised in the F_1 generation and the *cre* gene is segregated away in the subsequent generation. The selection marker gene can be eliminated either by re-transformation (Odell *et al.* 1990; Dale and Ow 1991; Russell *et al.* 1992) or by crossing over (Bayley *et al.* 1992; Russell *et al.* 1992; Chakraborti *et al.* 2008). The re-transformation and crossingover strategy was very labour intensive and time consuming, and in both the approaches the selection marker gene is eliminated at F_1 generation. The answer to the above problem was an autoexcision system controlled by inducible promoter, and with this system the F1 progengy is free of the selection marker gene. This is very well studied in most of the agronomical important crops, and marker-free transgenic plants were successfully generated in *Arabiodopsis*, maize, tobacco and rice (Hoff *et al.* 2001; Zuo *et al.* 2001; Zhang *et al.* 2003; Yuan *et al.* 2004; Sreekala *et al.* 2005). In a recent report for the development of disease-resistant marker-free tomato, *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 (Khan *et al.* 2011).

4.4 *Transposon-based marker methods*

Transposon-mediated repositioning of a transgene of interest has been proposed as an alternative for generating a wide range of expression levels in selectable marker-gene-free transgenic plants (Yoder and Goldsbrough 1994). The functionality of the Maize Activator/Dissociation (Ac/Ds) elements system as a gene tagging tool has been being successfully demonstrated since the early 1990s (Izawa *et al.* 1991, 1997; Chin *et al.* 1999; Enoki *et al.* 1999). In general, all Ac elements are identical, 4563 bp in length. Ds elements are Ac elements that have undergone deletions. Transposase are the proteins that stimulates the movement of Ac. Deletions of Ac elements created Ds elements in which all or part of this transposase was eliminated (figure 5). This lack of transposase activity accounts for the inability of Ds elements to move in the absence of Ac. The transposase that is encoded by Ac elements can move throughout the cell and excise any Ds or Ac element. Because of this ability, the Ac/Ds transposase is said to be transacting (Kunze and Starlinger 1989). Two transposon-mediated strategies have been developed to generate marker -free transgenic plants. The first strategy involves *Agrobacterium*-mediated transformation followed by intragenomic relocation of transgene of interest, and its subsequent segregation form the selectable marker in the progeny (Goldsbrough *et al.* 1993). The second involves excision of the marker gene from the genome (Ebinuma *et al*. 1997). Both strategies were developed using the maize Ac/Ds transposable element but can be adapted to use similar autonomous transposable element.

Ebinuma *et al.* (1997) proved the feasibility of this strategy by eliminating the *ipt* marker gene from transgenic tobacco plants. Transgenic plants constitutively expressing the *ipt* gene have elevated cytokinin-to-auxin ratios, resulting in loss of apical dominace, suppression of root formation and what is referred to as shooty phenotype. In

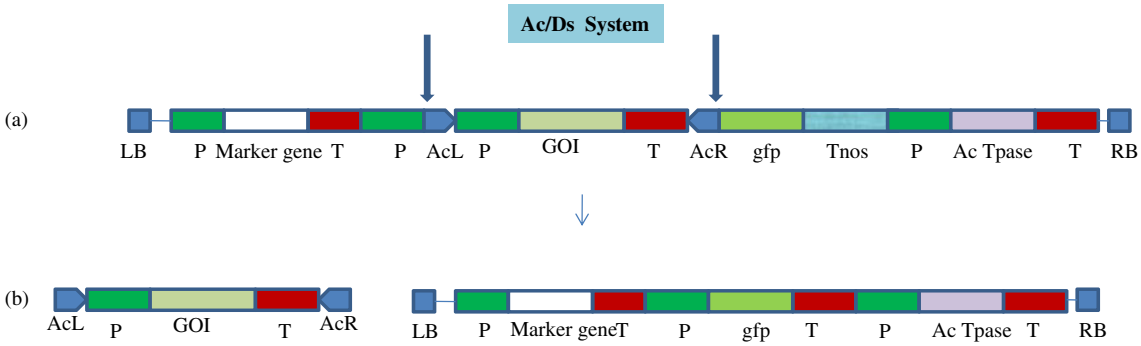

Figure 5. Schematic diagram of minimal terminal inverted repeats of the *Ac-Ds* transposon system. (**a**) T-DNA region showing GOI merged between Ac sites and marker gene, reporter gene and AcTpase region is outside the Ac sites. (**b**) Diagram showing the T-DNA region having GOI merged in Ac region excised out from marker and reporter gene.

transformed tobacco leaf disc with a T-DNA containing *npt*II and *gus* genes and a chimeric Ac element with a *35S-ipt* gene, two-thirds of this differentiated adventitious shoots showed extremely shooty phenotype. Upon subculturing these phenotypic distinct shoots, normal shoots were developed, which indicated the removal of *ipt* gene expression. The basic advantage of this strategy is that marker-free transgenic plants can easily be screened at the T_0 generation, avoiding the need for sexual cross plants and thereby making the strategy applicable to the vegetatively propogated crops like banana, potato, grapes and so on. In spite of all the advantages, the main limitation for this strategy is that the generation of marker-free transgenic plants is very low. The transgenic plants are genomically instable due to the continuous presence of heterlogous transposons (Scutt *et al.* 2002). Besides marker elimination, Cotsaftis *et al.* (2002) also developed an approach to generate 'new events' by relocating the 'gene of interest' by transposing to new locations on the genome.

4.5 *Chemical-inducible system*

For the past several years, this recombination system was very often used in plant transformation to eliminate selection marker gene. Cre/lox recombination system of Bacteiophage P1 is one of the systems developed in the context of marker removal in transgenic plants (Dale and Ow 1991). In order to remove the *cre* gene from the transgenic plants, re-transformation and out-crossing approaches have been used, which enables the loss of *cre* gene in subsequent generations, but this process is very laborious and time consuming (Dale and Ow 1991). In order to initiate the Cre/lox recombination for removal of the marker gene, other novel inducible site-specific recombination systems have been developed (figure 3B). Now several approaches are used to overcome these shortcomings by using some chemical inducers (Yuan *et al.* 2004; Zhang *et al.* 2006) or by heat shock (Wang *et al.* 2005; Cuellar *et al.* 2006). The chemical-inducible Cre/loxP (CLX) vector system benefits also from a particularly regulated system of chemical induction (Sreekala *et al.* 2005). The procedure could be used for vegetatively propagated species and may be particularly well adapted to crop species requiring transformation by the regeneration of embryo cultures.

Marker-free transgenic tomato expressing *cry1AC* were obtained by using chemically regulated Cre/lox-mediated site-specific recombination system. The marker gene *npt*II was eliminated by two directly oriented and loxP sites were located between the CaMV35S promoter and a promoterless *cry1AC*. Upon induction by 2μM β-estradiol, sequence encoded the selectable marker and two *loxP* sites were excised from the tomato gemone (Zhang *et al.* 2006). Using the Cre/loxP recomnbination system the expression of Cre recombinase was under the control of estrogen receptor-based transactivator XVE. Upon induction by β-estradiaol, the selection marker gene fused with Cre recombinase, flanked by two lox sites, was autoexcised from the *Arbidopsis* genome, and thus the chemical-inducible system is reliable method for generating marker-free transgenic plants (Zuo *et al.* 2001). Recently Lin *et al.* (2008) have reported a chemical-induced method for creating selectively terminable transgenic rice. They have used benzothiadiazole herbicide (Bentazon), which has been used for weed control of several major crops, such as rice, corn wheat, cotton and soyabean. These crops express cytochrome P450 for detoxifying the herbicide benzaton. They generate benzaton-sensitive rice plants, by suppressing the expression of this detoxification gene through antisense RNA, or benzaton-sensitive transgenic rice with high glyphosphate tolerance.

4.6 *Heat-inducible system*

This site-specific recombination system is used widely in the applied biotechnology for generating marker-free transgenic

plants. Cre/loxP and FLP/frt recombination systems and the knowledge of promoters give researchers an upper hand for generating marker-free transgenics. Shan *et al.* (2006) have developed the transgenic tobacco using FLP/frt recombinase system in which the expression of *FLP* was tightly under the control of *hsp* (heat shock protein) (figure 4). Two different constructs were used in this approach (*frt*-containing vector pCAMBIA1300-betA-frt-als-frt and the *FLP* expression vector pCAMBIA1300-hsp-FLP-hpt), and through the process of re-transformation, the *FLP* recombinase gene was introduced into transgenic (betA-frt-als-frt) tobacco. In re-transgenic plants, after heat-shock treatment, the marker gene *als* flanked by two identical orientation frt sites could be excised by the inducible expression of *FLP* recombinase under the control of hsp promoter. Excision of all the genes was found in 41% re-transgenic tobacco plants. Heat-inducible strategy for the elimination of selection marker gene was also used in vegetatively propogated plants like potato (Cuellar *et al.* 2006) and seed-producing plants like tobacco (Wang *et al.* 2005). In this strategy *HSP*70 was used as heat-inducible promoter in Cre/lox recombination system. A new binary expression vector based on the 'genetically modified-gene-deletor' system was constructed. In this vector, the gene coding for FLP site-specific recombinase under the control of a heat shock-inducible promoter *HSP*18.2 from *A. thaliana* and *ipt* gene as a selectable marker gene under the control of the cauliflower mosaic virus 35S (*CaMV* 35S) promoter was flanked by two loxP/FRT fusion sequences as recombination sites in direct orientation. Further characterization of the transgenic tobacco plants confirmed the elimination of the *ipt* gene along with *gusA* in the primary stage. Heat-inducible approach provides a reliable strategy for autoexcising a selectable marker gene from calli, shoots or other tissues of transgenic plants after transformation and producing marker-free transgenic plants. The disadvantage of this method is not negotiable. When autoexcision constructs are used, the recombinase can be activated by a chemical compound or by a heat shock in the shoots and seeds or during a subculture step and an extra regeneration step. The latter possibility lengthens the time to obtain marker-free transgenic plants and can introduce (additional) somaclonal variation.

4.7 *Positive selection system*

Some marker genes for positive selection (table 3) enable the identification and selection of genetically modified cells without injury or death of the non-transformed cell population (negative selection). In this case, the selection marker genes should give the transformed cell the capacity to metabolize some compounds that are not usually metabolized. This fact will give the transformed cells an advantage over the non-transformed ones. The addition of this new compound in the culture medium, as nutrient source during the regeneration process, allows normal growth and differentiation of transformed cells, while non-transformed cells will not be able to grow and regenerate *de novo* plants.

4.7.1 *The gus gene:* The *gus* gene codes for the β-glucuronidase enzyme (GUS; EC 3.2.1.31) and was isolated from *Escherichia coli*. This gene is widely used as a reporter gene in transgenic plants. In this system, the selective agent is a glucuronide derivative of benzyladenine (benzyladenine *N*-3-glucuronide), an inactive form of the plant hormone cytokinin. This glucuronide present in the selection medium can be hydrolysed by the GUS enzyme produced in the transformed cells, releasing active cytokinin (benzyladenine) in the medium. This cytokinin will be a stimulator for transformed cell regeneration while the non-transformed cell development is arrested. The selective agent (benzyladenine *N*-3-glucuronide) does not have any effect on the non-transformed cells because the cytokinin is in its inactive form. There are only few reports concerning the successful use of this system in the effective recovery of transgenic plants (Joersbo and Okkels 1996; Okkels *et al.* 1997).

4.7.2 *The manA gene:* The *man* gene codes for the phosphomannose isomerase enzyme (PMI; EC 5.3.1.8) isolated from *Escherichia coli*. In the presence of mannose, the PMI converts mannose-6-phosphate into fructose-6-phosphate in transformed cells that can be immediately incorporated in the plant metabolic pathway. Thus, mannose can be used as the sole carbohydrate source for the transformed cells. This selection system is immediate and extremely efficient (Joersbo *et al.* 1998).

Mannose cannot be usually metabolized by non-transformed cells and is converted into mannose-6-phosphate by endogenous hexokinase. Therefore, when mannose is added to the culture medium, plant growth may be minimized due to mannose-6-phosphate accumulation. The mannose-6-phosphate toxicity in plant cells was shown to be responsible for apoptosis, or programmed cell death, through induction of an endonuclease, responsible for DNA laddering (Stein and Hansen 1999). Mannose-6-phosphate accumulation also causes phosphate and ATP starvation that deplete cells of energy for critical functions such as cell division and elongation, giving rise to growth inhibition. Therefore, mannose is a hexose that fills the desirable requirements for a good selection agent: it is (a) soluble in plant culture media, (b) absorbed by plant cells, (c) inexpensive, (d) easily available and (e) safe.

Although most plant species are sensitive to mannose, some species, especially dicotyledonous, have shown a considerable

Table 3. Positive selection used in plant transformation

Gene	Sources	Gene product	Selective agent	References
ipt	*Agrobacterium tumefaciens*	Isopentyl transferase	None	Endo *et al.* (2001)
rolC	*Agrobacterium rhizogenes*	'Hairy root' phenotype	None	Ebinuma and Komamine (2001)
iaaM, iaaH	*Agrobacterium tumefaciens*	Indole acetic acid	None	Tuominen *et al.* (1995)
dsdA	*Escherichia coli*	D-Serine ammonialyase	None	Erikson *et al.* (2005)
manA (pmi)	*Escherichia coli*	Phosphomannose isomerase	D-Mannose	Joersbo *et al.* (1998)
uidA (gusA)	*Escherichia coli*	b-Glucuronidase	Benzyladenine-*N*-3-glucuronide	Joersbo and Okkels (1996)
codA	*Escherichia coli*	Cytosine deaminase	5-Flurocytosine (5-FC)	Kobayashi *et al.* (1995)
atlD	*Escherichia coli* strain C	Arabitol dehydrogenase	Arabitol	LaFayette *et al.* (2005)
xylA	*Thermoanaerobacterium sulfurogenes*	Xylose isomerase	D-Xylose	Haldrup *et al.* (1998a, b)
AtTPS1	*Arabidopsis thaliana*	Trehalose-6-phosphate synthase	Glucose	Leyman *et al.* (2006)
dao1	*Rhodotorula gracilis*	D-Amino acidoxidase	D-Amino acids(D-alanine and D-serine)	Erikson *et al.* (2004)
OsDREB2A and AtSOS1	Rice and *Arabidopsis thaliana*	Salt resistant phenotype	High concentration of Nacl	Zhu and Wu (2008)

insensitivity to this sugar, including carrot, tobacco, sweet potato and legumes. Other species are extremely sensitive and have been successfully transformed by the use of mannose as selective agent, such as sugar beet, maize, wheat, oat, barley, tomato, potato, sunflower, oilseed rape and pea (Joersbo *et al.* 1998; 2000; Negrotto *et al.* 2000; Wang *et al.* 2000). Some plant transformation protocols that use the positive selection system with PMI were at least 10 times more efficient than the traditional protocols based on the use of kanamycin as selection agent (Wright *et al.* 2001).

4.7.3 *The xylA and DOG^{R}1 genes:* A similar positive selection system has been developed using the xylose isomerase gene (*xyl*A) isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus*, as selection marker gene (Haldrup *et al.* 1998a, b). Transgenic plants of potato, tobacco and tomato were successfully selected in xylose-containing media. Recently, the *DOG^{R}1* gene encoding 2-deoxyglucose-6-phosphate phosphatase (2-DOG-6-P) was used to develop a positive selection system for tobacco and potato plants (Kunze *et al.* 2001). *DOG^{R}1* gene, which has been isolated from yeast, gives resistance to 2-deoxyglucose (2-DOG) when overexpressed in transgenic plants.

4.8 *Negative selection system*

An alternate and potentially more efficient strategy is based on the incorporation of a negative selection step. Negative selectable markers are of two types: (a) conditional negative selectable marker (e.g. *cod*A) and (b) non-conditional negative selectable marker (e.g. Diphtheria toxin). Recently, Ramana Rao and K Veluthambi (2010) reported that MYMV TrAP is a good non-conditional negative selectable marker for developing marker-free transgenic plants. Finally, the combination of using a mixture of mechanisms, transient selection, sequential transformation, negative marker genes, P-DNA and a mutated *virD2* gene together should be capable of producing high-frequency marker-free transgenic plants by co-transformation methods. Recently, a novel marker gene *dao1*, encoding D-amino acid oxidase, has been characterized. It can be used as positive or negative marker, depending on the substrate (Erikson *et al.* 2004). Therefore, it is possible to apply the negative selection after a positive selection using one marker gene, *dao1*, via changing D-alanine or D-serine to D-isoleucine or D-valine for the substrates. Conversion of an externally provided specific substrate into its phytotoxic derivative by the marker gene encoded enzyme enables this counter selection. The *tms2* gene was the first conditional selective marker gene to be used in tobacco (Depicker *et al.* 1988) and in *Arabidopsis* (Karlin-Neumann *et al.* 1991). Indole acetic acid hydrolase (IAAH) encoded by the *tms2* gene confers sensitivity of plants to naphthalene acetamide (NAM) because IAAH converts NAM to the potent auxin naphthalene acetic acid (NAA), which inhibits seedling growth. Other conditional markers proven to be effective in dicots are *aux2* in cabbage (Beclin *et al.* 1993), the HSV-tk gene in tobacco (Czako and Marton 1994), a bacterial cytochrome *P450* mono-oxygenase gene in tobacco (O'Keefe *et al.* 1994) and *Arabidopsis* (Tissier *et al.* 1999) and *codA* in *Arabidopsis* (Kobayashi *et al.* 1995) and tobacco (Schlaman and Hooykaas 1997). So far, the cytochrome *P450* (the product of which catalyses the dealkylation of a sulfonylurea compound, R7402, into its

cytotoxic metabolite) and *codA* (whose product cytosine deaminase converts the non-toxic 5-fluorocytosine into phytotoxic 5-fluorouracil) are the only genes to have been used as conditional negative selectors in monocots. Both have been proven to be effective in barley (Koprek *et al.* 1999). The only gene used in rice so far is the cytochrome *P450* (Chin *et al.* 1999). Moreover a selection system based on a mutant rice gene for a feedback-insensitive α- subunit of anthranilate synthase (*OASA1D)* was developed for the transformation of rice and potato (Yamada *et al.* 2004). Expression of *OASA1D* conferred resistance to the tryptophan analog 5-methyltryptophan (5MT). The selection system based on *OASA1D* and 5MT was as effective as hygromycin B selection in rice (monocotyledon) and kanamycin selection in potato (dicotyledon) (Yamada *et al.* 2004).

Osakabe *et al.* (2005) reported the coding sequences of acetolactate synthase (*ALS*) gene from rice, and mutagenized the *ALS* gene into a herbicide-resistant form. After transfer of this construct to the rice genome, transgenic plants were efficiently selected with a herbicide, bispyribac-sodium salt, which inhibits the activity of wild-type ALS. The marker system consisted exclusively of host plant DNA and enabled efficient selection in a monocot crop plant, rice. The selection system can potentially be applied to generate transgenic plants of other crop species and can be expected to be publicly acceptable.

4.9 *Autoexcision strategy*

A number of methods to eliminate the selection marker gene form the plant genome are now known. The earlier methods of autoexcision such as the heat-inducible system and chemical-inducible system are time consuming and the marker gene is eliminated in the next generation after segregation. For the development of marker-free transgenic plants, scientists have developed a novel and ideal method, which eliminates the selection marker gene in a single generation. This method is known as 'autoexcision strategy', in which the marker is easily eliminated in the T_1 seeds of the transgenic plants (the seeds which is collected from the T_0 plants) (Mlynarova *et al.* 2006). The next generation of the transgenic plants will be marker free.

Autoexcision strategy is very recently introduced and used in the plant biological system to eliminate selection marker gene from the plant genome. Autoexcision system is controlled by pollen- and /or seed-specific promoters and it was reported that the highly-efficient autoexcision of selective markers is successfully achieved in tobacco (Mlynarova *et al.* 2006; Luo *et al.* 2007). Autoexcision strategy relies on floral-specific promoters to regulate the expression of *cre* recombinase to generate marker-free transgenic plants. The functionally characterized promoters were used in the strategy and the system is successfully demonstrated in rice (Bai *et al.* 2008). The novel marker-free approach mediated by the Cre–loxp recombination system and the Cre gene was under the control of floral-specific promoter *OsMADS45*. The marker gene *npt*II was completely removed from the T_1 progenny of the rice with 37.5% efficiency.

Verweire *et al.* (2007) have developed marker-free transgenic plants of *A. thaliana* introducing a germline-specific autoexcision vector containing a *cre* recombinase gene under the control of a germline-specific promoter (*APETALA1* and *SOLO DANCERS* genes from Arabidopsis (*A.thaliana*) Columbia-0). Transgenic plants become genetically programmed to lose the marker when its presence is no longer required. Using this method the frequency of regenerating marker-free transgenic lines in *Arabidopsis* is 83–100%. In spite of all the above, autoexcision strategy has its limitations: it is successful only in flowering plants. It will not be useful for the vegetatively propogated plants like grapes, potato and banana.

4.10 *Abiotic stress-related gene as selection marker*

In all these methods such as co-transformation, site-specific integration, chemical induced and heat-induced marker gene elimination, the marker gene is eliminated in the second generation, but in only the autoexcision strategy method the marker gene is eliminated in the F_1 generation. Here we discuss the novel approaches for the development of marker-free transgenic. It is a well-known fact that various genes encode proteins that protect the plants at the time of several environmental stresses like drought stress, salt stress and oxidative stress. Till date so many genes that are well characterized in *A. thaliana* or in several agronomically important crops can be used for the development of marker-free transgenic plants. Incorporation of such well-characterized genes in to those plants that are salt sensitive, including rice (*Oryza sativa*), maize (*Zea mays*), soybean (*Glycine max*), beans (*Phaseolus vulgaris*) and tomato (*Lycopersicum esculantum*), is a contribution to the agriculture sector for developing transgenic plants (Munns 2005).

The basic ideas behind this strategy is that plant tissue or the plant senses high Na^+ concentration in the soil/media and initiates signal transduction to activate a set of stress-responsive genes for salt tolerance. The gene is incorporated into the plant tissue or explants without the selection marker gene. After transformation the tissue will grow under the pressure of salt stress, and explants that grow well without any deformities are selected and grown further in the salt stress medium until the vegetative proliferation of the explants. In the whole experiment there is no need to use the selection

marker. The gene itself can be used as selection marker to select the transformed tissue (Zhang *et al.* 2009a, b).

ESKIMO1 gene is reported to be involved in plant water economy as well as cold and salt tolerance (Bouchabke-Coussa *et al.* 2008). Yoshida and Shinmyo (2000) attempted to make the yeast Na^+-ATPase function in plant cells. The *ENA1* (i.e. *exitus natru*, for the latin words meaning 'exit sodium') gene that encodes the *Saccharomyces cerevisiae* Na^+-ATPase was placed under the control of the CaMV35S promoter and introduced into BY2 cells. Transgenie BY2 cells that produced Enal protein were able to grow in modified LS medium containing 120 mM of LiCl, conditions which markedly inhibited the growth of untransformed cells. Sanan-Mishra *et al.* (2005) explored the potential role of PDH45 (pea DNA helicase 45) in overcoming salinity stress. PDH45 mRNA is induced in pea seedlings in response to high salt, and its overexpression driven by a constitutive cauliflower mosaic virus-35S promoter in tobacco plants confers salinity tolerance. The overexpression of barley group 3 LEA gene *HVA1* in leaves and roots of rice and wheat lead to improved tolerance against osmotic stress as well as improved recovery after drought and salinity stress (Sivamani *et al.* 2000; Rohila *et al.* 2002). There are following limitations:

i. In case of an unknown gene it will be difficult to use the system.
ii. Screening will be tedious because there will be chances of escape of untransformed calli.

5. Regulation and biosafety concern of GM crops

The great success of GM crops has had an enormous impact on world crop production and cultivation pattern of agricultural species (James 2006). The extensive environmental release and cultivation of GM crop varieties have aroused tremendous biosafety concerns and debates worldwide (Stewart *et al.* 2000). Biosafety issue has already become a crucial factor in constraining the further development of transgenic biotechnology and wider application of GM products in agriculture. There are quite number of biosafety related concerns in general, but the most important ones can be summarized as follows. (1) direct and indirect effects of toxic transgenes (e.g. the Bt insect-resistance gene) to non-target organisms (O'Callaghan *et al.* 2005; Oliveira *et al.* 2007); (2) influences of transgenes and GM plants on biodiversity, ecosystem functions and soil microbes (Giovannetti *et al.* 2005; Oliveira *et al.* 2007); (3) transgene escape to crop landraces and wild relatives through gene flow and its potential ecological consequences (Wilkinson *et al.* 2000; Snow *et al.* 2003; Lu and Snow 2005; Mercer *et al.* 2007); and (4) potential risks associated with the development of resistance to biotic-resistance transgenes in the target organisms (Dalecky *et al.* 2007; Li *et al.* 2007a, b; Wu 2007). In addition, there are still some unknown involvements in potentially significant interactions between transgenic traits and the environments. Among the above environmental biosafety issues, transgene escape from a GM crop variety to its non-GM crop counterparts or wild relatives has aroused tremendous debates worldwide (Ellstrand *et al.* 1999; Ellstrand 2001, 2003; Lu and Snow 2005). This is because transgene escape can easily occur via gene flow that may result in potential ecological consequences if significant amounts of transgenes constantly move to non-GM crops and wild relative species. This is particularly true when these transgenes can bring evolutionary selective advantages or disadvantages to crop varieties or wild populations. It is therefore essential to properly address the most relevant questions relating to the transgene outflow and its potential environmental consequences on a science-based altitude.

6. Conclusion and future prospects

The improvement of agricultural production and productivity as well as the future versatility of agricultural production are dependent on the rational utilization of technologies. We stand at the convergence of an incredible array of new technologies, such as recombinant DNA technology, information technology and high-throughput genomics, to enhance our understanding of the structure and function of the genomes and to apply this information for plant and animal improvement. Products arising from modern biotechnology such as GM or transgenic crops are providing new opportunities to achieve sustainable productivity gains in agriculture.

The presence of selectable marker genes, especially those which include genes coding for antibiotic resistance and which are essential for the initial selection of transgenic plants, is seen by European regulatory agencies as undesirable. An issue of concern relates to the fact that transgenes integrate at random positions in the genome leading to possible unwanted side effects (mutation) and unpredictable expression patterns. In addition to the risk of HGT, there is also a 'vertical cross-species' transfer risk that could potentially create enhanced weediness problems (Dale *et al.* 2002). 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 gene(s) from the plant genome, probably as linear fragments; (ii) protection of the gene(s) from nuclease degradation in the plant as well as animal gastrointestinal tract; (iii) uptake of the gene(s) with dietary DNA; (iv) transformation of bacteria or competent mammalian cells; (v) insertion of the gene(s) into the host DNA

by rare repair or recombination events into a transcribable unit; and finally (vi) continuous stabilization of the inserted gene (FAO/WHO 2000). Thus, the development of efficient techniques for the removal of selection markers, as well as the directed integration of transgenes at safe locations in the genome, is of great interest to biotech companies. Furthermore, removal of selectable marker genes will also have a technical advantage, since the number of available selectable marker genes is limited, and stacking of transgenes will become more and more desirable in the near future.

Generally, selectable marker genes are not required once the transgenic plants are regenerated and the genetic analyses completed. The presence of a particular marker gene in a transgenic plant necessarily precludes the use of the same marker in subsequent transformation and the use of a different marker system is required for each transformation round or event. Thus, any technique that can remove or eliminate a selection marker gene in transgenic crops is highly desirable if for no other reason than that the same procedure can be used in subsequent transformations. For transgene technology to be commercially successful, multiple independent transgenes available need to be added in existing sequence.

Therefore, there is need for the development of techniques for the efficient production of 'clean' marker-free transgenic plants. Among the several technologies described, two have emerged with significant potential. The simplest is the co-transformation of genes of interest with selectable marker genes followed by the segregation of the separate genes through conventional genetics. The more complicated strategy is the use of site-specific recombinases, under the control of inducible promoters, to excise the marker genes and excision machinery from the transgenic plant after selection has been achieved. The field of marker gene removal continues to produce new innovations. For example, the possibilities of increasing the number of different heterologous recombinase systems available by molecular evolution approaches have been discussed, and new marker gene and marker-free strategies are under development (Schubbert *et al.* 1998). The removal of marker gene and backbone from the transgenic plants supports multiple transformation cycles for transgene pyramiding. Although research continues, it is clear that several viable methods for the removal of unwanted marker genes already exist. It seems highly likely that continued work in this area will soon remove the question of unwanted marker genes from the debate concerning the public acceptability of transgenic crop plants. At present there is no commercialization of marker-free transgenic crop as it is still in the stage of proof-of-concept (Manimaran *et al.* 2011). Development for production of marker-free transgenics would further strengthen the crop improvement programme with widespread applications in both fundamental research and biotechnology. Overall, the GM crops are expected to contribute globally to the food security.

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