TRENDS TOWARDS THE PRODUCTION OF BIOLOGICALLY SAFE MARKER FREE TRANSGENIC PLANTS

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Abstract. In the majority of plant biotechnology laboratories throughout the world, plant transformation is a common practice to improve several traits of plants, particularly grain yield. During the experiments, only a small percentage of cells transform in the targeted population. For selection of transformed cells, it is necessary to use the selectable markers such as Neomycin phosphotransferase (*nptII*), Chloramphenicolacetyle transferase (*Cat*), Hygromycin phosphotransferase (*hph*), Streptomycin phosphotransferase (*spt*), Phosphinothrycin acetyletransferase (*pat*) and Dihydrofolate reductase (*dhf*). The majority of these and other markers detoxify different antibiotics including paromomycin, kanamycin, hygromycin, neomycin, and streptomycin etc. But as these antibiotics become resistant, most of the markers raise serious safety concerns to human health. Additionally, in case of multiple genes transfer, it also limits the availability of these selectable markers. Keeping in view the limitations of selectable markers, production of marker-free transgenic plants is becoming the global trend. Current review explored the probabilities and prospects to cope with these issues. This review describes in detail the various plant transformation systems for the development of selectable marker gene (SMG) free transgenic plants. Replacement of selectable marker with screenable marker and some worldwide examples of SMG free crop plants produced by these strategies are also discussed.
Keywords: *antibiotics, biosafety, gene of interest, plant transformation, selectable markers, screenable marker*

Introduction

Genetic engineering generally involves the introduction of foreign material (either single or multiple genes) into host plant which ultimately modifies the host plant genome. Transferring genes for desired features to improve agricultural plants from completely other species of plants and animals is a common practice in the majority of plant transformation labs across the world. A number of economically significant genes are currently being worked on for transfer to other desirable crop species. Since tobacco was the first genetically transformed plant (Horsch et al., 1985), several transgenic have been developed in economically important crops which exhibit resistance to herbicides,

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diseases and insects while some offer better nutritional and post-harvest qualities (Pattanayak and Kumar, 2000). According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA), it is clear how much more land is being used to cultivate transgenic plants from practically nothing in 1996 to 2.7 billion hectares in 2019 (James, 2019). Agrobacterium-mediated transformation, electroporation, polyethylene glycol-mediated transformation, protoplast-mediated transformation, and others are some of the several techniques used to perform the transformation experiments. Transformation, of various crop species, has been achieved rapidly through advancement and modification of particle bombardment or biolistic technique. However, all these methods either transformation through particle bombardment or through *Agrobacterium tumefaciens* are inefficient (Rakoczy-Trojanowska, 2002). Separation of transformed cells or tissues from non-transformants requires a selectable marker gene linked with gene of interest. Two possible barriers to improve crop plants through transformation are the limited availability of selectable markers and the antibiotic resistance of the majority of selectable markers (Yoder and Goldsbrough, 1994). Additionally, the proliferation and differentiation mechanisms of transgenic cells are typically negatively affected by the antibiotics that distinguish the transformants from non-transgenic cells (Puchta, 2003). Differentiation of adventitious shoots may be retarded due to these agents during transformation process (Ebinuma et al., 1997b). It can also be challenging or even impossible to distinguish between transformed and untransformed cells or tissues in some plant species because they are resistant to or insensitive to these selection agents. As a result, finding a good selectable marker and creating the ideal circumstances for transformation of such challenging species becomes difficult (Vasil et al., 1991; Perl et al., 1993; Hanover and Keathley, 2012). Environmentalists have recently expressed concerns about the biosafety of transgenic organisms since the presence of selectable markers in the environment or in the food supply chain may pose an unpredictably high risk to the ecosystem or to human health. An important example to back up this assertion is the way in which the gene for herbicide resistance is passed down to weeds, which are related species (Dale et al., 2002). Important gene that are involved for herbicide resistance in weeds are Glyphosate Resistance (*EPSPS* Gene) produces resistance against herbicides by causing mutation in *EPSPS* gene, ALS Inhibitor Resistance (*ALS* Gene) that is involved in the synthesis of branched-chain amino acids, PPO Inhibitor Resistance (*PPO* Gene) that produces resistance by causing mutation in *PPO* gene, ACCase Inhibitor Resistance (*ACCase* Gene) that causes rsistance by mutating *ACCase* gene. The presence of antibiotic resistance genes might conceivably result in the spread of these resistances among humans via intestinal microbes. Additionally, due to the limitation of selectable markers, current transformation protocols severely restrict the pyramiding of several genes of interest, such as those involved in abiotic stress tolerance and broad-spectrum disease, into a single line.

Keeping in mind the limitations of using selectable markers for crop improvement through plant transformation, numerous methods for successfully removing the selectable markers from the transgenic plants were developed (McKnight et al., 1987; Dale and Ow, 1991; Goldsbrough et al., 1993; Gleave et al., 1999; Zuo et al., 2001; Lu et al., 2001; Endo et al., 2002; Cotsaftis et al., 2002). The effectiveness of these methods was still debatable, therefore improving them was made the primary objective for integrating these selectable markers at particular locations in the genome of plants and then removing them from the target site to add more genes of interest (Puchta, 2003). This review describes in detail the numerous plant transformation systems such as direct transformation, co-

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transformation, site-specific recombination systems, intra-genomic relocation of trans-genes via transposable elements and SMG editing by genomic tools for producing transgenics without marker. Replacement of selectable marker with screenable marker and some worldwide examples of SMG free crop plants produced by these strategies are also discussed.

Production of SMG free transgenic plants

Approximately 48 selectable marker genes from different sources, which primarily give resistance to herbicides and antibiotics, have been used successfully in plant transformation (*Table 1*). So far, most generally used gene in plant transformation is *ipt* (Ti plasmid of *Agrobacterium tumefaciens*), that encode isopentyl transferase enzyme (Ebinuma et al., 1997b) *Ipt*, *nptII*, *hpt* and *bar* contribute to production of over 95% transgenic plants. Additionally, several markers gene-free strategies for plant transformation have been created (Zuo et al., 2002). In these systems, the selection of transformed tissues is based on genes that give the capacity to multiply or differentiate in the absence of some other critical component, such as an external plant hormone required for tissue culture. Two main solutions to produce SMG free plants have been developed. The first one is to eliminate selectable marker by following different transformation systems and genome editing tools and the second one is to replace selectable marker with screenable marker. All possible strategies are presented in *Figure 1*. From last two decades, scientists are working on to produce SMG free plants and at present commercial products are available in different crops. *Table 2* summarizes the worldwide examples of SMG free transgenic plants for all potential techniques of producing selectable marker free plants. De Vetten et al. (2003) selected SMG free direct transformants in potato through PCR analysis. Similarly, SMG free direct transformants were detected by gene of interest (GOI) expression product in tobacco (Zakharchenko et al., 2009). Holme et al. (2012) and Shiva Prakash et al. (2009) reported the removal of *nptII* gene from barley and maize through co-transformation and commercial product was named PAPhy07 and H99 respectively. *Hpt*, *nptII* and *ipt* marker genes were also removed from tobacco, maize, tomato through site specific recombination FLP/FRT, Cre/*lox* and R/RS system of transformation, respectively (Ow, 2007; Woo et al., 2009; Khan et al., 2011). Ebinuma et al. (1997b) used the Multi-Auto-Transformation system (MAT) for the removing ipt marker gene from transgenic tobacco and commercial product was Xanthi with tolerance against abiotic stresses (hydrogen peroxide/reactive oxygen species). Similarly, different genome editing tools were also used for the removal of SMG after selection of transformants. Smith and Jantz (2010) used Meganucleases to remove bar gene from *Arabidopsis thaliana*. Svitashev et al. (2016) used the CRISPR-Cas9 to edit *MOPAT-DSRED* for herbicide resistance in maize. Cermak et al. (2011) used TALENs to knock out *ADHI* from *Arabidopsis thaliana*. Examples of transformants selection by using screenable markers i.e. green fluorescent proteins, GUS Assay (using beta glucuronidase) and blue white screen using beta galactosidase enzyme were also reported in different crops like Papaya, tobacco, maize and wheat, respectively (Jefferson et al., 1987; Zhu et al., 2004; Gholizadeh, 2012; Richardson et al., 2014).

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Table 1. *Selectable marker genes for plant transformation*

Gene	Gene product	Source	Selection	Reference
nptII	Neomycin	*E. coli* Tn5	Kanamycin, Neomycin	Bevan et al. (1983)
Ble	Bleomycin resistance	*E. coli* Tn5 and *Streptoalloteichus hindustanus*	Bleomycin, Phleomycin	Perez et al. (1989)
Dhfr	Dihydrofolate reductase	E. coli, mouse, *Candida albicans*	Methotrexate	Herrera-Estrella et al. (1983)
Cat	Chloramphenicolacetyle transferase	E. coli Tn5, Phage P1Cm	Chloramphenicol	De Block et al. (1985)
hph (aphIV)	Hygromycin phosphotransferase	*E. coli*	Hygromycin B	Waldron et al. (1985)
SPT	Streptomycin phosphotransferase	Tn5	Streptomycin	Jones et al. (1987)
aaC3 *aaC4*	Gentamycin-3-N-acetyltransferase	*Serratia marcescens Klebsiella pneumonia*	Aminoglycosides	Hayford et al. (1988)
npt1(aphA1)	ATP- binding cassette	*Arabidopsis thaliana*	Paramomycin	Mentewab and Stewart Jr (2005)
aphA2,Atwbc19	Phosphotransferase	*E. coli* Tn601	Kanamycin	Carrer et al. (1993)
aadA	Aminoglycoside-3”-adenyl transferase	*Shigella* sp.	Spectinomycin	Svab and Maliga (1993)
Sul1	Dihydropteroate synthase	*E. coli* pR46	Sulphonamides	Guerineau et al. (1990)
sat3	Acetyl transferase	Streptomyces sp.	Streptothricin	Jelenska et al. (2000)
pat, bar	Phosphinothrycin acetyletransferase	Streptomyces hygroscopicus	Phosphinothrycin,bialophos	De Block et al. (1989)
EPSP	5-enolpyruvnylshikimate-3 phosphate synthase	Petunia hybrida	Glyphosate	Shah et al. (1986)
aroA	5-enolpyruvnylshikimate-3 phosphate synthase	*Salmonella typhimurium*	Glyphosate	Comai et al. (1988)
cp4 epsps	5-enolpyruvnylshikimate-3 phosphate synthase	*Agrobacterium tumefaciens*	Glyphosate	Barry (1992)
Gox	Glyphosate oxidoreductase	*Ochrobactrum anthropi*	Glyphosate	Barry (1992)
csr1-1	Acetolactate synthase	*Arabidopsis thaliana*	Sulfonylureas	Olszewski et al. (1988)
csr1-2	Acetolactate synthase	*Arabidopsis thaliana*	Imidazolinone	Aragão et al. (2000)
Bnx	Bomoxynil nitrilase	Klebsiella pneumoniae sub sp. Ozanaenae	Oxynils	Freyssinet et al. (1996)
hemL	Glutamate-1- semialdehyde aminotransferase	Synechococcus PCC6301	Gabaculine	Gough et al. (2001)
Cah	Cyanamide hydratase	Myrothecium verrucaria	Cyanamide	Weeks et al. (2000)

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Gene	Gene product	Source	Selection	Reference
P450	Cytochrome P450	Human	Acetochlor	Inui et al. (2005)
Pds	Phytoene desaturase	*Hydrilla verticillata*	Norflurazon and fluridone	Arias et al. (2006)
TUAm	a-Tubulin	*Eleusine indica*	Trifluralin	Yemets et al. (2008)
psbA	Qb protein	Amaranthus hybrids	Atrazine	Cheung et al. (1988)
rfdA	2-4-D monooxygenase	Alcaligeneseutrophus	2-4-D	Lyon et al. (1989)
BADH	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	Betaine Aldehyde	Daniell et al. (2001)
Ocs	Octopine synthase	Agrobacterium tumefaciens	L-Cysteine (AEC)	Koziel et al. (1984)
TDC	Tryptophan decarboxylase	*Catharanthus roseus*	4-Methyltryptophan (4- mT)	Goddijn et al. (1993)
ASA2	Anthranilate synthase	Tobacco	5-Methyltryptophan	Cho et al. (2004)
OASA1D	Mutant anthranilate synthase	Rice	5-Methyltryptophan (5MT)	Kobayashi et al. (1995)
TSB1	Tryptophan synthase beta	*Arabidopsis thaliana*	5MT/Cadmium cholride	Hsiao et al. (2007)
ilvA or ilvA- 466	Threonine deaminase	*Escherichia coli*	L-O-Methylthreonine	Ebmeier et al. (2004)
Coda	Cytosine deaminase	*Escherichia coli*	5-Flurocytosine (5-FC)	Kobayashi et al. (1995)
xylA	Xylose isomerase	*Streptomyces rubignosus*	D-Xylose	Haldrup et al. (1998)
manA (pmi)	Phosphomannose isomerase	*Escherichia coli*	D-Mannose	Joersbo et al. (1998)
uidA (gusA)	b-Glucuronidase	*Escherichia coli*	Benzyladenine-N-3- glucuronide	Joersbo and Okkels (1996)
iaaM, iaaH	Indole acetic acid	*Agrobacterium tumefaciens*	None	Tuominen et al. (1995)
rolC	'Hairy root' phenotype	*Agrobacterium rhizogenes*	None	Ebinuma and Komamine (2001)
dao1	D-Amino acid oxidase	Rhodotorula gracilis	D-Amino acids (D-alanine and D-serine)	Erikson et al. (2004)
atlD	Arabitol dehydrogenase	*Escherichia coli* strain C	Arabitol	LaFayette et al. (2005)
dsdA	D-Serine ammonia lyase	*Escherichia coli*	D-Serine	Erikson et al. (2005)
AtTPS1	Trehalose-6-phosphate synthase	*Arabidopsis thaliana*	Glucose	Leyman et al. (2006)
psbA	Qb protein	Amaranthus hybrids	Atrazine	Cheung et al. (1988)
rfdA	2-4-D monooxygenase	Alcaligeneseutrophus	2-4-D	Lyon et al. (1989)
DHPS	Dihydropicolinate synthase	*E.coli*	S aminoethyle	Perl et al. (1993)
AK	Aspartate kinase	E.coli	High concentration of lycine and threonine	Perl et al. (1993)

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Table 2. *Selectable marker gene free transgenic crop plants*

Crop	Line Name/No.	GM Trait	Removed SMG	Strategy	References
Potato	AV43-6-G7	Amylopectin starch	No SMG	SMG free direct transformation (screening by PCR analysis)	De Vetten et al. (2003)
Tobacco	-	Resistance against fungal diseases	No SMG	SMG free direct transformation (Detection of GOI expression product)	Zakharchenko et al. (2009)
Barley	PAPhy07	Improved phytase activity in the grain	nptII	Co-Transformation (Single plasmid carrying T-DNA borders)	Holme et al. (2012)
Rice	Japonica	Chewing insect resistant	Hpt	Co-Transformation (using single mini-twin DNA binary vector and two separate binary vectors)	(Yu et al., 2009)
Maize	H99	Herbicide resistance	nptII	Co-Transformation (Biolistic-using linear DNA fragment)	Shiva Prakash et al. (2009)
Maize	LY038	Increased free lysine in the germ portion	nptII	Site-specific recombination Cre/lox system	Ow (2007)
Tobacco	-	Herbicide resistance	Hpt	Site-specific recombination FLP/FRT system	Woo et al. (2009)
Tomato	-	Resistance against fungal diseases	Ipt	Site-specific recombination R/RS system	Khan et al. (2011)
Rice	Ariete	Insect resistance (striped stem borer)	Hph	Transposition Ac/Ds (Transfer of GOI)	Cotsaftis et al. (2002)
Tobacco	Xanthi	Abiotic stress tolerance (hydrogen peroxide/ROS)	Ipt	Transposition Ac/Ds (Multi-Auto-Transformation system)	Ebinuma et al. (1997b)
Arabidopsis thaliana	-	Basta resistance	bar	SMG Editing (Meganucleases)	Smith and Jantz (2010)
Maize	Hi-II	Herbicide resistance	MOPAT-DSRED	SMG Editing (CRISPR-Cas9)	Svitashev et al. (2016)
Tobacco	-	Basta resistance	Pat	SMG Editing (Zinc Finger Nucleases)	Petolino et al. (2010)
Arabidopsis Thaliana	-	-	ADH1 (knockout)	SMG Editing (Transcription activator-like effector nucleases)	Cermak et al. (2011)
Papaya	Kapoho	-	No SMG	Green Florescent Protein	Zhu et al. (2004)
Wheat	Kronos 51 and Stewart	Herbicide resistance	No SMG	GUS Assay (using beta glucoronidase)	Richardson et al. (2014)
Maize	-	-	No SMG	Blue White Screen (using beta galactosidase enzyme)	Gholizadeh (2012)

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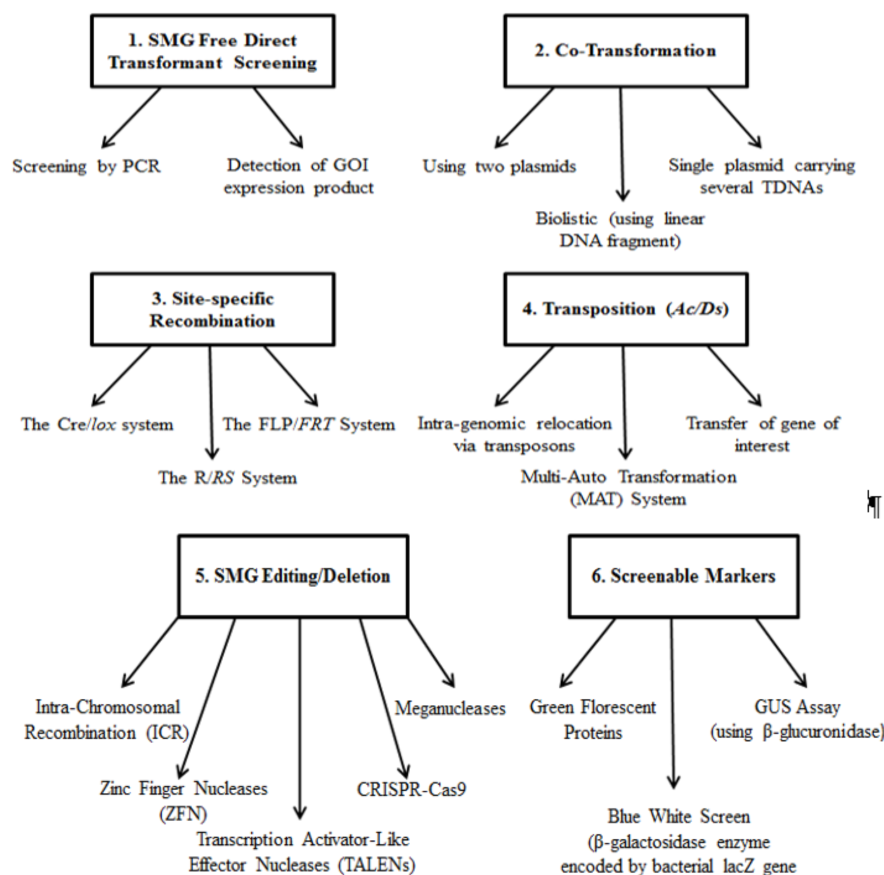

Figure 1. *Strategies for the production of selectable marker gene free transgenic plants*

Selectable marker gene elimination strategies

SMG free direct transformation

Screening through PCR

The transformed plant cells could be distinguished from non-transformed cells using PCR, because the T-DNA contains the known fragments which are separated by designing specific primers. PCR based selection offers an alternate to selectable marker gene free transformation (Breyer et al., 2014). Many successful examples of SMG transformed plants used PCR based selection approach i.e., tobacco, potato, peanut, Arabidopsis, lime, cassava, triticale, and barley (Bhatnagar et al., 2010; Manimaran et al., 2011). Similarly, photooxidation resistance “Brookfield Gala” apple was engineered using astaxanthin biosynthetic genes crtR-B and bkt. The selection of transformed experiments was done using RT-PCR and qPCR (Jia et al., 2019).

SMG free cis-genic rice plants were developed to express blast resistance rice gene and putative transgenic plants were screened using PCR dependent selection approach (Tamang et al., 2018). PCR screening method was used to select SMG free *Brassica napus* transgenic lines showing enhanced phytate utilization ability (Xu et al., 2020). Processing quality of the Xindong No. 26 a wheat variety was enhanced by transformation of HMW-GS 1Dx5 gene using SMG free transformation system. The screening of potential transformed plants were performed by PCR dichotomy analysis of 343-bp product amplification (Qin et al., 2014).

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Detection of GoI expression product

Screening of transgenic plants should also be done by quantifying the expression product of GOI. The transgene may be quantified using the enzyme-linked immunosorbent assay (ELISA). Immunoassays have been always preferred to detect transgenic plants because of its ruggedness, inexpensive and sensitivity characteristics. It has become a choice of breeders for determining GM content and testing of unapproved events. The Immunoassay is dependent on antibody antigen assay for the detection of protein (Kamle et al., 2019). The detection based on expression product of GOI was used for selection of Vip3Aa harboring transgenic cotton plants (Liu et al., 2020). European corn borer resistant and herbicide resistant soybean plants were selected using ELISA and expression product of GOI (Ma et al., 2005). Similarly ELISA protocols are available for detection of transgenic plants carrying *Cry1Ac* (that produces resistance against certain insect pests) (Estrada et al., 2007), *Cry2A* (that confer resistance against certain insect pests) (Kamle et al., 2011), Vip3Aa (that confer resistance against specific insect pests) (Liu et al., 2020), EPSPS (that produces resistance in weeds due to mutations in the EPSPS gene) (Deng et al., 2014), Liberty Link and other important genes (Liu et al., 2020).

Co-transformation

Using two plasmids

An (1985) demonstrated that a single Agrobacterium strain carrying both a Ti plasmid (phytohormones independent growth) and a T-DNA binary vector (kanamycin-resistant growth) could co-transform tobacco cells to two distinct phenotypes. Extending these studies, De Framond et al. (1986) demonstrated that the one-strain, two-replicon method could produce fertile transgenic plants from cloned tobacco tissue that had been simultaneously transformed by T-DNA from a Ti plasmid and from a micro-Ti. In progeny plants, the segregation of two T-DNAs, shows that they had assimilated into genetically distinct loci. Other research teams have created transgenic plants using the one-strain, two-replicon method. Both T-DNA markers were initially expressed by these plants, but they later developed the ability to separate the markers (Daley et al., 1998). Numerous studies looked into the delivery of different T-DNAs to the same plant cells using two Agrobacterium strains (McKnight et al., 1987; De Buck et al., 1998; De Buck et al., 2000).

Using single plasmid carrying several T-DNAs

Early studies characterized the T-DNA integration pattern in crown gall tumors and found that each of the two T-DNAs encoded by an octopine-type Ti plasmid cold individually incorporate into the genome of plant, occasionally in multiple copies (Chilton et al., 1977; Thomashow et al., 1980; De Beuckeleer et al., 1981). According to molecular study, these T-DNAs might be integrated into locations that are unlinked. These findings indicated that (i) two T-DNAs from different bacteria could be introduced; (ii) two T-DNAs from various replicons within the same bacteria could be introduced; and (iii) a possible integration of transgenes carried by two separate T-DNAs during transformation, and these T-DNAs may segregate in following generations. In a study by Depicker et al. (1985), markers that were selected were nopaline synthesis (encoded by a Ti plasmid), phytohormones independent growth, and kanamycin-resistant growth (encoded by a T-DNA binary vector). The results showed that co-transfer of T-DNAs

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from the single strain's identical plasmid was substantially more successful than was co-transfer from two distinct strains. The co-transformation of plants with two T-DNAs from the same replicon using a single *Agrobacterium* strain, followed by segregation of the selection gene to create marker-free transgenic plants, has been described by Komari et al. (1996) and Xing et al. (2000).

Biolistic co-transformation using linear DNA fragments

Agrobacterium mediated co-transformation in combination with biolistic transformation has been studied in a number of species, particularly in those that are challenging to transform (Breyer et al., 2014). Both plasmids with the GOI or SMG are coated onto the gold particles using biolistic co-transformation before being bombarding into plant tissue cells. According to some research, this strategy can be utilized to create transgenic plants without markers with efficiencies on par with those of other co-transformation techniques by bombarding the plants with little amounts of DNA rather than the entire plasmid (Altpeter et al., 2005; Shiva Prakash et al., 2009; Kumar et al., 2010; Elghabi et al., 2011). In comparison to other co-transformation methods involving *Agrobacterium*, using a minimal cassette with only the promoter, coding region, and terminator gives biolistic transformation methods a clear biosafety advantage. With this technique, Shiva Prakash et al. (2009) has recovered several maize plants without SMG from the progeny of 103 plants and developed a marker free herbicide resistant line (H99). The resistance mechanism in the H99 maize line likely involved the incorporation of genes that encode for enzymes or proteins capable of detoxifying or inhibiting the action of specific herbicides. These genes might have been sourced from naturally occurring organisms with inherent resistance to the targeted herbicides. When the maize plants express these introduced genes, they can produce the corresponding proteins that counteract the herbicides' effects, allowing the maize to withstand herbicide applications intended to control weeds.

Site-specific recombination

A site-specific recombinase can remove the selectable marker gene from plant's genome by means of enzyme-mediated site-specific recombination if the selectable marker gene is present in flanking site of direct repeats of recognition sites for the enzyme.

The Cre/lox system

Bacteriophage PI *Cre/lox* is one of the many characterized site-specific recombination systems. It is a combination of 38-kDa product of Cre recombinase (*cre* gene) and 34 bp asymmetric lox sites, that is compose of asymmetric core region of 8 bp and two pairs of 13 bp inverted repeats that directs the site (Hoess and Abremski, 1985). Cre-catalyzed recombination between the *lox* sites doesn't need any other components. According to Albert et al. (1995) and Vergunst and Hooykaas (1998) the *Cre/lox* system has been utilized to direct the site-specific integration of incoming plasmids at the *lox* sites previously implanted in the genome by direct gene transfer or Agrobacterium-mediated transfer. Thus, the Cre-lox system provides a technique for finely inserting single copy DNA into genomic targets. The integrated DNA has two flanking recombination *lox* sites with similar orientation. If the *lox* repetitions are oriented directly, Cre produces excision of the internal sequence (insert). By transformation or sexual crossing, the *cre* gene can

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be inserted into the lox-containing plant. About 95% of the secondary transformants lose a marker gene that was cloned between two *lox* sites during transformation. Using the *cre/lox* recombination technique, transgenic plants of the *Arabidopsis* and Tobacco species were recovered that do not have that selectable marker gene (Dale and Ow, 1991; Russell et al., 1992). The excision events in the plant genome that are catalyzed by Cre are relatively precise and conservative, meaning that no nucleotides are lost or altered at the recombinant site. On the other hand, if the *lox* sites are positioned in the opposite orientation, inversion of internal sequences will be catalyzed by Cre, Generating inversion has been suggested as a potential method for transforming functioning genes into their anti-sense derivatives.

The FLP/FRT system

The FLP/frt system of Saccharomyces cerevisiae’s 2mm plasmid is the other single chain recombinase that has been discovered to be beneficial for removing flag genes (Kilby et al., 1995; Lyznik et al., 1996). In this technique, first round of transformation often results in transgenic plants that have selection marker positioned between two recognition sites that are directly orientated for corresponding recombinase. Once the single-chain recombinase is expressed, recombination reaction will start, either by crossing in plants that already express the enzyme, by transitory expression via second transformation, or by using an inducible promoter, resulting in marker-free transgenic plants.

The R/RS system

The R/*rs* system of the pSR1 plasmid of *Zygosaccharomyces rouxii* is another single chain recombinase (Onouchi et al., 1995; Sugita et al., 2000). The selection of transformed plants is done on the base of ipt shoots which exhibit a typical morphological alteration, short internodes, reduced apical dominance, and lack of rooting capacity. During sub-culturing in the tissue culture, the Ipt shoots are removed by site-specific recombination, which is mediated by recombinase of the R/RS system (Khan et al., 2011). Salient feature of this recombination system is that the recombinase (R) gene of R/RS site specific recombination system is fused with chemical inducible promoter of *GST-II-27* gene of *Zea mays*. *Ipt* gene (selectable marker gene) is removed upon excision and *CaMV35S* promotor expresses R gene. *CaMV35S* promoter efficiently and quickly excise ipt gene during callogenesis (Sugita et al., 2000; Khan et al., 2011). The R/RS system has been utilized for generating SMG free transgenic eggplants (Darwish et al., 2014), rice (Nakagawa et al., 2001), tomato (Khan et al., 2011) and potato (Kondrák et al., 2006).

Transposition

Intra-genomic relocation of transgenes via transposable elements

When inserted into different plant species, many maize transposable elements continue to function as transposons (Baker et al., 1986; Yoder et al., 1988). The removal of components from one locus before reinsert it into a second is the outcome of transposition in Ac/Ds and the Spm/dSpm families that are two best characterized families (Fedoroff, 1989). In general, selectable marker genes and other auxiliary sequences are eliminated when transposition occurs at linked or unlinked locations in dicots and maize (Greenblatt, 1984; Jones et al., 1990). In order to separate marker and desired genes, these elements have been used in two different ways: (i) a mobile element is used for carrying mobile

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element, which lost following transposition (Yoder and Goldsbrough, 1994), (ii) The intended transgene can be moved to a different chromosomal place when transposase is activated; the transgene is movable on its own. Marker-free transgenic aspen and tobacco plants have been created by insertion of selectable ipt gene in Ac 35 transposable element. Two transgenes will be separated by genetic crosses and/or segregation. This method's viability was shown in tomato (Goldsbrough et al., 1993). Different degrees of expression are the result of moving the transgene to different chromosomal locations. Additionally, a single transformant can produce a variety of plants with various loci, and the one that have best transgene expression will be helpful for species that are challenging to transform.

Transfer of GOI

Transposable elements are involved in repositioning of genetic material in genomes. The Ac/Ds transposons of maize are extensively used for elimination and repositioning of SMG in various crops (Chong-Pérez and Angenon, 2013). The Ds and Ac are two correlated elements, Ac is acronym for activator which encodes transposase enzyme, while Ds is short form of dissociation which take part in deletion of Ac element. These two elements are both 11bp terminal inverted repeats. The Ac produce 102 KD functional enzyme transposase for its moving within the genome hence it is called as autonomous whereas Ds is dependent on Ac hence it is consider non-autonomous (Yau and Stewart, 2013). A marker gene is placed onto a transposable element during transposons-mediated transformation. This transposable element with a marker has been co-transformed with the relevant gene. SMG-free plants can be created by segregating transgenic plants in later generations. This is a very useful method for producing SMG free plants and insertion of GOI at various places in the genome. This method also allows for the transformation of plant species that are resistant to change, which is a constant issue for biotechnologists. However, transposable elements based transformation is not equally effective in all plants species. The second major limitation of this system is a tedious selection process for tagging of SMG free transgenic plants in the segregation generations (Singh et al., 2019).

Multi-auto transformation (MAT) system

In addition to GOI transfer using DNA transposition, after producing transgenic plants there is a possibility of removing the SMG. The solution to this task is called the MAT vector system that was particularly explained for production of SMG free tobacco and vegetative propagated plants and plants with the long reproduction cycles as it does not require genetic crosses (Ebinuma et al., 1997a). Some of the challenges of the present transformation techniques can be overcome by MAT vector system with chimeric iptas SMG insertion into the maize transposable element Accan. When a chimeric *ipt* gene controlled by (CaMV) 35S promoter inserted into cucumber cells (Smigocki and Owens, 1989) transgenic cells proliferate and adventitious shoots differentiate in hormone free medium. These transgenic plants show loss of apical dominance and therefore plants that have functional ipt gene can be detected visually. In transgenic cells, the chimeric ipt gene may disappear or transpose along with the Ac that was put into the MAT vector. As a result, it is possible to create transgenic plants without the *ipt* gene that are phenotypically normal (Belzile et al., 1989).

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SMG editing

Intra-chromosomal recombination

Meyer (2000) came up with a novel method for producing transgenic tobacco after just one round of transformation that lacks a selectable marker gene. In this method, two homologous sequences undergo intra-chromosomal homologous recombination (ICR), which results in DNA deletion. Two 352 bp attachment P (attP) sections of bacteriophage-l were placed on either side of the selectable marker gene *nptII* and the negative selectable marker gene *tms2* in a binary vector (pattP-ICR). The bacteriophage-l uses the attP sites to integrate the genome of E. coli at the attB-site, a process that requires the cooperation of two proteins, the integrase (int) encoded by the phage and the bacterial integration host factor (IHF). A. tumefaciens was used to introduce pattP-ICR construct in leaves of tobacco, and kanamycin was used to select resistant calli. These calli were transplanted to a medium for shoot regeneration that contained kanamycin. Two of the 11 calli generated shoots that were a mixture of white and green, shows *nptII* gene deletion. For additional research, kanamycin-free medium was used for growing white leaves, and sprouted shoots were then placed on naphthalene acetamide (NAM) containing medium, while *tms2* gene product transforms into phytohormone auxin (NAA), which prevents the growth of roots. Molecular analysis verified that not only *nptII* and *tms2* genes were lost, but also in three out of 23 cases, this reaction was induced by homologous recombination between the attP-sites. Only shoots that lost *tms2* gene were predicted to generate shoots. The attP system offers a helpful tool to eliminate undesired trans-gene regions, especially in species that are vegetatively propagated, because it does not need a genetic segregation phase to eliminate recombinase genes or the production of helper proteins to cause deletion events.

Meganucleases

Meganucleases are homing endonucleases, found in bacteria and eukaryotes (Chevalier and Stoddard, 2001). One of the often-utilized homing endonucleases, I-Scel, can recognize and cleave a recognition sequence of 18bp. Meganucleases engineering was reported to be used for transgene deletions as well as gene targeting. Smith and Jantz (2010) reported the removal of *bar* SMG from Basta resistant *Arabidopsis thaliana* transgenic plants. This can be accomplished by placing two restriction sites flanking an SMG and employing two I-Scel cutting sites to release the SMG upon the expression of I-Scel. Through the non-homologous end joining (NHEJ) repair pathway, the cut ends can reattach (Siebert and Puchta, 2002).

Zinc finger nucleases (ZFNs)

ZFNs could be used to eliminate unnecessary DNA sequences or selectable marker genes from the genome of plants. For removal of SMG, ZFN-overexpressing plants are crossed with transgenic plants harboring SMG. The recognition sites for ZFNs are attached on flanking sites of SMG expression cassette in transgenic plants; as a result SMG is edited/removed (Chong-Pérez and Angenon, 2013). Another approach for SMG editing includes building of SMG, GOI, and site specific recombination systems on the same plasmid which is inserted at targeted locus using customize ZFNs initially, later on site specific recombination systems are used to edit/remove SMG (Chong-Pérez and Angenon, 2013). SMG was removed from a stable transformed tobacco plants expressing GUS reporter gene flanked by ZFNs cleavage site by crossing them with second plant

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expressing ZFNs gene (Petolino et al., 2010). ZFNs were also used for removal of SMG from rice plants (Nandy et al., 2015).

Transcription activator-like effector nucleases (TALENs)

TALENs produce double stranded break (DSB) at specified regions in the genome just like ZFNs hence these also have the potential for SMG removal. For removal of transgene using TALENs two identical sets of TALENs binding sequences are designed in the flanking region of SMG. As a result after expression of TALENs, DSB occur and remove the SMG and broken DNA is repaired by homologous recombination NHEJ repairing pathways (Chong-Pérez and Angenon, 2013; Chen and Gao, 2013). TALENs were used for transgene removal in rice (Li et al., 2012), *Arabidopsis thaliana* (Cermak et al., 2011), and Tobbaco (Petolino et al., 2010).

CRISPR-Cas9

Similarly clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) protein systems has the capability of genome editing at specified locations in the genome identified by guide RNA (gRNA). gRNA may be designed from the SMG genomic regions for its removal and production of transgene free plants. SMG *MOPAT-DSRED* was removed from herbicide resistant maize plants using CRISPR (Srivastava et al., 1999). However CRISPR/Cas9 itself has the capability of insertion and deletion of genomic regions hence scientists prefer it for development of SMG free gene edited crops rather developing transgenic and using CRISPR for removing of SMG (Ahmad et al., 2020).

Replacing the selectable markers with screenable markers

Screenable markers are genes that can be used to identify transgenic plants without the need of a selective agent. These markers include regeneration-promoting genes like *ipt* (Ebinuma et al., 1997a; Kunkel et al., 1999). It is only recently that this set of markers has been discovered. The basic idea behind this set of markers is that transformed cells have particular benefits in their growth and function while untransformed cells are not eliminated. Screenable markers contain gene products that can be easily identified by their enzyme activity. Mostly three types of screenable are used in crop transformation. There have recently been more methods used to isolate screenable markers for species that can regenerate through organogenesis or somatic embryogenesis (Zuo et al., 2002). By discovering these novel markers, worries regarding the spread of herbicide or antibiotic resistance into the environment are rendered irrelevant, specifically if the marker itself is derived from the relevant crop plant and is thus not 'foreign' DNA.

Green fluorescent proteins

An appropriate screenable marker and reporter for analyzing the expression of gene and transforming plants is the green fluorescent protein (*gfp*) gene. The *gfp* gene was discovered in the jellyfish *Aequorea victoria*, and it produces a little protein with a barrel structure that surrounds a fluorescent chromophore and produces green fluorescent light right away in the blue to ultraviolet spectrum. Living cells can be visually detected at any time without being damaged, adding a cofactor, or using an external substrate. Additionally, the *gfp* gene product has no negative effects on the cell growth,

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regeneration, or fertility of modified plants. Using the *gfp* to choose Papaya transformants, Zhu et al. (2004) created the Kapoho SMG free line.

GUS assay (using β glucuronidase)

For the selection of transformants, non-toxic chemicals such as bacterial β glucuronidase (Joersbo and Okkels, 1996), Xylose isomerase (Haldrup et al., 1998) and Phosphomannose isomerase genes (Joersbo et al., 1998; Negrotto et al., 2000) as well as the yeast 2-deoxyglucose-6-phosphate phosphatase (Kunze et al., 2001) are used. Beta-glucuronidase, which is produced by the *E. coli gusA* gene, catalysis the breakdown of a wide range of beta-glucuronides to enable spectrophotometric or fluorometric measurement. This process can be histologically localized and measured using a non-destructive fluorescence-based technique. The disadvantage is that it kills the converted cell, and quantitative assays are time-consuming and not ideal for screening large populations of cells. Nevertheless, it is a great approach for identifying single cell transformation.

Blue-white screen

Beta-galactosidase enzyme is encoded by bacterial *Lac Z* gene. When such galactosidase enzymes (like X gal) are given to medium, the cells that are expressing the gene change X gal into a blue substance that can be seen with the naked eye. The existence of an insert is shown by colorless colonies while the lack of an insert is indicated by blue-colored colonies. Besides instability to temperature and light, this technique may lead to false positive unless pick up strictly white colonies.

Enhancing the effectiveness and usability of transgene elimination techniques

Every elimination event left behind a remnant recognition sequence at the recombination site, which is a drawback when using site-specific recombinases to remove DNA. In cases when gene "stacking" and repeated marker elimination procedures have led to several copies of the same recombination site dispersed throughout the genome, it could be wise to get remove these components. After being exposed to the recombinase again, residual recombinase recognition sequences may serve as the location of chromosomal rearrangements. In rare cases, (such as the generic vector system shown in the illustration, where transcription of recognition sequence may occur), several copies of the same element can activate gene-silencing processes, preventing the engineering of the trait of interest. When an excision event activates a gene of interest by positioning it close to a promoter, one strategy to reduce the chance of gene silence is to place the recombination site precisely adjacent to the TATA box of the promoter (Zuo et al., 2001). It seems improbable that transcription of just a few nucleotides at the distal end of the recombinase recognition site will activate gene-silencing mechanisms because transcription typically starts about 30 nucleotides downstream of TATA sequences. This strategy would not address the potential for chromosome deletions, inversions, or translocations to result via intermolecular or intramolecular recombination between residual sites. The successive application of various recombinases is the most obvious remedy for this issue.

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Table 3. *Comparison of SMG free technologies for their positive and negative aspects*

Countering Technique	Positive Aspects	Doubts	References
SMG Free Direct Transformation			
Screening through PCR	• Simple and require less time • Does not require numerous crosses for segregation of GOI and marker gene	• High probability of false positive • Suitable only for limited number of plant species with high potential of regeneration and transformation	Rukavtsova et al. (2013)
Detection of GOI Expression Product	•The time of plant production harboring GOI shortens •Permits a reduction of long antibiotic or herbicide stress load on plants during transformants screening on selective media •Plant lines with maximal synthesis can be revealed •It can be used for vegetatively propagated crops	• Suitable for plant species with high potential of regeneration and transformation	Rukavtsova et al. (2013)
Co-Transformation Using Two Plasmids Using Single Plasmid carrying several TDNAs Using Linear DNA Fragments	•Simplest method •Simultaneous delivery and integration into the plant genome of GOI and marker gene within genetically unlinked DNA fragments •Allows obtaining independent GOI insertion into a great number of unlinked genome loci using a single selectable marker •Insertion of multiple genes up to 13 is possible using biolistic transformation	• Need high frequency of transformation and the insertion of GOI and marker genes into different loci • Sometimes after biolistic transformation GOI turned out to be linked with marker gene which substantially hampered the removal of marker gene • Requires the large-scale crosses between regenerants, so not applicable to vegetatively propagated plants and to woody plants with long reproductive cycle	Hadi et al. (1996), Chen et al. (1998) and Rukavtsova et al. (2013
Site Specific Recombination The Cre/*lox* System The FLP/*FRT* System The R/*RS* System	•Widely applicable •Exploits a transformation cassette designed to eliminate multiple tandem insertions of transgenes and to remove marker genes in one step.	• All of these systems require sexual crosses for the removal of recombinase genes and so cannot be used with vegetatively propagated plants. • Causes plant cell toxicity	Scut et al. (2002), Srivastava et al. (1999) Yau and Stewart (2013)

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		• The expression of microbial recombinases for prolonged periods in plant cells may result in unwanted changes to the genome • The fate of the excised SMG cassette needs to be checked in progeny	
Transposition Intra-Genomic Relocation Intra-Chromosomal Recombination Transfer of GOI	• Transposition may take place at linked or unlinked sites leading to the elimination of SMG and other ancillary sequences. • Useful for species difficult to transform	• System must exist in the laboratory for the species of interest • Typically, not very precise and can take a long time for the repeated insertion and excision cycles to delete the SMG • Process itself can lead to mutation and increase the genomic instability • Requires crosses between regenerants	Jones et al. (1990), Ebinuma et al. (1997a) Miura et al. (2001) Rukavtsova et al. (2013)
Multi-Auto Transformation System	• Select transformants using the markers of plant growth regulation	• Does not require genetic crosses and can be used for vegetatively propagated plants	
SMG Editing			
Meganucleases	• Achievable in plants • Fast and direct method • It can be used for all kind of plants including vegetatively propagated and woody plants with long life cycles	• They have not yet been widely explored • Recognize specific DNA sequence which needs to be pre-inserted • Genome fractionation • Product is not conserved need non-homologous end joining (NHEJ) repair which may truncate neighboring genes	Salomon and Puchta (1998), Yau and Stewart (2013)
ZFN	• Fast and direct method • It can be used for all kind of plants including vegetatively propagated and woody plants with long life cycles • This method can be used for SMG deletion in plant pollen	• Low affinity to target DNA • Complicated design and intensive testing might limit the scope • Non-specific double strand breaks (DSB) induction at the non-specific sites • Product is not conserved	Moon et al. (2010), Yau and Stewart (2013)
TALENs	• Achievable in plants • Used to knockout *ADH1* genes in *Arabidopsis thaliana*	• Product is not conserved need homologous recombination (HR) or NHEJ repairing pathway which may truncate neighboring genes	Cermak et al. (2011), Yau and Stewart (2013)

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CRISPR-Cas9	• Powerful DNA DSB technology • Used as a completely DNA and selectable marker free method for the recovery of plants with mutated alleles at high frequency in *Zea Mays*	• Potentially lead to gene disruption, plant mosaicism and potential off-site cutting • Undesired secondary changes can be segregated away by backcrossing to the wild type parent but this can be time consuming especially for crops with complex polyploidy genomes	Svitashev et al. (2016)
Screenable Markers			
Green Florescent Protein	• Makes cell glow under UV light • Direct visualization of GFP in living cells in real time without invasive procedures • Can be introduced and maintained in the genome through breeding or local injection with viral vector	• Requires specific and costly equipment for detection	Zhu et al. (2004) Breyer et al. (2014)
GUS Assay	• Excellent method for detecting single cell transformation	• It kills the transformed cells during the process • Laborious and not suitable to large populations	Jefferson et al. (1987)
Blue White Screen	• The lacZ gene makes cell turn blue in special media (e.g., X-gal) • Colony of cells with the gene can be seen with the naked eye	• Instability to temperature and light • Not working for small fragments • May lead to false positive unless pick up strictly white colonies	Gholizadeh (2012)

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The promise of this method is increased by the recent evidence that directed evolution approaches can be employed to change recombinase substrate specificities (Buchholz and Stewart, 2001; Sclimenti et al., 2001; Santoro and Schultz, 2002). Crops should be able to use an auto-regulatory chemically inducible Flp/*FRT* system, and recent developments in plant Flp expression optimization may benefit this system (Baszcynski et al., 1999; Luo et al., 2000; Gidoni et al., 2001). The effectiveness of Int protein mutants in plants has yet to be determined, even though they no longer accomplish excessive recombination in human cells with the use of auxiliary components from λ phage (Lorbach et al., 2000). The range and versatility of various techniques to gene excision will be further increased by putting various recombinases under the control of a variety of chemically induce systems now in use (Zuo and Chua, 2000). Inducible DNA excision cassettes for agricultural application should be approved more quickly and at lower cost if registered agrochemicals are used as inducers. In scaled-up operations to select for effective excision, it would be beneficial to include a negative selectable marker in the elimination cassette depending on the system's effectiveness in various species. The CLX system might be able to activate several transgenes once the elimination cassette is removed and there were one or more internal ribosome entrance site(s) (Kumar and Fladung, 2001). Using specialized recombinases for recognizing specific places within the crop genome to be changed is a more complex method for preventing residual recombination sites (Buchholz and Stewart, 2001; Sclimenti et al., 2001; Santoro and Schultz, 2002). If there are effective genes targeting methods available in higher plants, this strategy will work. The fact that excessive recombination may be sensitive to position effects if target genes are located in a chromatin configuration inaccessible to the recombinase is another advantage of combining recombinase-based gene excision strategies with novel methods to ensure the precise integration of foreign DNA. Genomic double strand breaks can only currently be induced in order to significantly boost homologous recombination (Kumar and Fladung, 2001). In order to ensure the simultaneous removal of "used" components, it is possible to modify a recent proposal by Kumar and Fladung (2001) for achieving gene targeting in plants with the use of both a site-specific recombinase and an endonuclease, leaving only a residual recombinase recognition site with the target gene. The next generation of transformation vectors may also benefit from the use of *FokI* zinc finger chimeric nucleases (Bibikova et al., 2001) and group II introns modified to insert into particular loci (Guo et al., 2000). These tools are capable of both gene targeting and the subsequent removal of extra foreign DNA.

Conclusion

Though such ideas are still very speculative, it is important to keep in mind that the pace of gene discovery will probably not have as much of an impact on improvement of crop in post-genomic era as it will on the availability of appropriate transformation technologies. Whether or not transformation proficiencies can be increased to the point where selectable markers are no longer required, the ability to exactly remove foreign genetic material will remain a critical component of approaches to increase transformation rates and prevent the unintended spread of genes encoding novel traits in the ecosystem. Despite the safety issues raised by environmentalists and consumer advocacy groups, plant breeding for desired traits has a significant impact on the development and commercialization of existing cultivars. With advancements in plant transformation technology, it won't be necessary to put transgenic plants of the latest

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generation in the fields that include antibiotic or herbicide resistance genes that were only used during the transformation process.

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