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Alternatives to Antibiotic Resistance Marker Genes for In Vitro Selection of Genetically Modified Plants - Scientific Developments, Current Use, Operational Access and Biosafety Considerations

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Alternatives to Antibiotic Resistance Marker Genes for *In Vitro* Selection of Genetically Modified Plants – Scientific Developments, Current Use, Operational Access and Biosafety Considerations

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Genes conferring resistance to antibiotics have been widely used as markers for the selection of transformed cells in the development of genetically modified (GM) plants. Their presence in GM plants released in the environment or used as food or feed has raised concerns over the past years regarding possible risks for human health and the environment. Although these concerns have not been supported so far by scientific evidence, the implementation of selection approaches avoiding the presence of antibiotic resistance marker genes (ARMGs) in the final GM plant is increasingly considered by GM plant developers, not only to alleviate the above-mentioned concerns, but also to circumvent technical limitations associated with the use of ARMGs. In the current paper, we present the results of a three-step analysis of selectable markers and reporter genes as well as methods aiming at developing marker-free GM plants. First, based on a comprehensive review of the scientific literature, technical developments in this domain are presented. Second, a state-of-the-art of the current use of selection approaches is provided based on publicly available information on GM plants tested in the field or authorized for commercialization. Third, in order to get more insight in the underlying practical, scientific and/or regulatory arguments supporting the choice of selection approaches, we present the results of a survey directed at relevant developers and users of GM plants. The applicability, efficiency, operational access and biosafety of the various selection approaches is discussed and considered in light of their current use, and in perspective to the long history of use of ARMGs in plant biotechnology.

Keywords marker-free transgenic plants, plant transformation, risk assessment, selectable marker genes, selection approaches

I. INTRODUCTION

Efficient selection of transformation events (the plant cells having stably integrated the exogenous DNA) amongst the large number of non-transformed plant cells is a critical step in the production of genetically modified (GM) plants. Plant transformation methods used so far show very variable efficiencies (see, e.g., Barampuram and Zhang, 2011; Rosellini, 2012) and are characterized by highly variable levels and patterns of expression of the gene of interest (GOI). Therefore, many independent transformants must be screened to identify the few GM events with useful profile.

In GM plants of the first generation, genes encoding resistance to specific antibiotics have been largely used for selection and rapid identification of transformed cells. The use of antibiotic resistance marker genes (ARMGs) has been demonstrated to be very effective for selection, cost-efficient and applicable to a large number of plant species. In this selection approach, the ARMG under control of plant regulatory sequences is transferred and expressed in the plant together with a GOI. The selectable marker gene (SMG) enables the transformed cells to survive on growth medium supplemented with the corresponding antibiotic, whereas non-transformed cells die, or are unable to regenerate. In most of the cases, once the GM cells have been selected and the GM plant generated, the ARMG remains in the plant although it serves no purpose anymore. The most commonly used ARMGs have been two *Escherichia coli* genes: *nptII* that encodes the neomycin phosphotransferase enzyme conferring resistance to the aminoglycoside antibiotics neomycin and kanamycin, and *hpt* that encodes the hygromycin phosphotransferase enzyme conferring resistance to hygromycin. Many GM plants containing these SMGs have been approved for field trials and for marketing in several countries.

Other ARMGs are used in GM plants development to select recombinant vectors in intermediate hosts such as *E. coli* or *Agrobacterium tumefaciens*. In this case, the ARMG is under control of its native bacterial promoter. Since this gene is inserted in the "vector backbone" it is generally not transferred into the plant genome. Nevertheless in some cases it can remain in the GM plant. The most commonly used ARMGs in this context are the *bla* gene that encodes the beta-lactamase enzyme degrading ampicillin, and the *aad* gene that encodes the aminoglycoside adenyl transferase enzyme inactivating spectinomycin and streptomycin (Miki and McHugh, 2004; Goldstein *et al.*, 2005; Sundar and Sakthivel, 2008).

Selection of GM plants using ARMGs has raised some concerns over the past years regarding the potential risks posed by such markers. This relates in particular to risks associated with horizontal gene transfer from plants to bacteria in soil, or from plant products consumed as food to intestinal microorganisms, which could possibly result in reduced effectiveness of antimicrobial therapy. These hazards and their potential consequences have been the subject of many scientific papers and opinions (for a comprehensive list of references, see, e.g., Wogerbauer, 2007; Ramessar *et al.*, 2007; EFSA, 2009) and will not be discussed in this review. The major trend coming out of these papers is that there is no scientific evidence to date suggesting that either ARMGs currently used in GM plants have been harmful for human or animal health or have significantly contributed to the problem of clinical antibiotic resistance.

Nevertheless, the use of ARMGs in GM plants, in particular those approved for commercialization, remains a contentious issue. Several groups, including official bodies, regulators, NGOs and the industry have indicated that the use of GM plants devoid of any ARMGs (or at least those raising safety issues) should be envisaged or strongly recommended (BMA, 1999; AMA, 2000; FAO/WHO, 2000; NRC, 2000; EFB, 2001; Caplan, 2002; Royal Society, 2002; Wogerbauer, 2007). Concerns over the use of ARMGs in GM plants are particularly present in the European Union (EU), where the debate began in 1996 during the approval process of the GM maize Bt176, containing the *bla* gene. In line with the Community Strategy against antimicrobial resistance (EC, 2001), the current GMO legislation in the EU imposes that "GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment, with a view to identifying and phasing out antibiotic resistance markers in GMOs which may have adverse effects on human health and the environment" (EU, 2001). In light of these provisions, the European Food Safety Authority (EFSA) issued opinions (EFSA, 2004; EFSA, 2009) suggesting a careful approach to the use of ARMGs in GM plants on an individual basis: some should not be used at all, others should be used on a limited basis (field trials) whereas for others (such as the *nptII* gene) EFSA was of the opinion that there is no rationale for inhibiting or restricting their use, neither for field experimentation nor for placing on the market. Moving beyond the provisions of Directive 2001/18/EC (where the phasing-out applies only to ARMGs which may have adverse effects on human health and the environment), the Commission Implementing Regulation (EU) No 503/2013 (EC, 2013) states that "the applicant shall therefore aim to develop GMOs without the use of antibiotic resistance marker genes" arguing that "it is now possible to develop GMOs without the use of antibiotic resistance marker genes."

Various selection approaches have been developed in the lab (some of them many years ago) that may be used to avoid the presence of ARMGs in GM plants. They include the use of alternative selectable markers or reporter genes, methods aiming at removing selectable markers from the genome of the GM plant following the initial transformation process, and methods not using SMGs. This article provides an up-to-date overview of the latest technical developments regarding these selection approaches, extends and complements previous reviews on this topic (Hare and Chua, 2002; Puchta, 2003; Miki and McHugh, 2004; Darbani *et al.*, 2007; Sundar and Sakthivel, 2008; Kraus, 2010; Upadhyaya *et al.*, 2010; Barampuram and Zhang, 2011; Manimaran *et al.*, 2011; Tuteja *et al.*, 2012; Rosellini, 2012; Yau and Stewart, 2013), and addresses in particular practical and safety aspects. We present also an analysis of publicly available information on GM plants tested in the field or authorized for commercialization, focusing on the situation in the EU and the United States. Finally, in order to get more insight in the underlying practical, scientific and regulatory arguments supporting the choice of selection approaches, we present the results of a survey directed at relevant developers and users of GM plants.

II. ALTERNATIVE SELECTION APPROACHES

A. Plant-derived Marker Genes Conferring Antibiotic Resistance

All antibiotic resistance genes currently used as selectable markers in GM plants grown in open field are of microbial origin. A few plant genes can also be associated with antibiotic resistance (Rosellini, 2011). For instance, overexpression of an *Arabidopsis thaliana* ATP binding cassette (ABC) transporter (At-*WBC19*) gene confers high level resistance to kanamycin (and in some cases to other aminoglycoside antibiotics) in GM tobacco, hybrid aspen (*Populus canescens x P. grandidentata*) and muskmelon. The *A. thaliana DEF2* (peptide deformylase) and *GPT* (UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase) genes can confer resistance to the antibiotics actinonin and tunicamycin respectively when overexpressed in tobacco and *Arabidopsis*.

The plant origin of these genes has been presented as a way to bypass some of the biosafety concerns associated with the use of ARMGs of microbial origin, since they would not be expected to confer antibiotic resistance in bacteria if horizontal gene flow was to occur (Burris *et al.*, 2008). The use of plant-derived ARMGs remains however restricted to a very limited number of genes. The potential phenotypic effects and safety issues associated with their overexpression in plants remain to be assessed. Safety issues associated with the inherent characteristics of the At-*WBC19* gene have already been raised jeopardizing its use as SMG in GM plants. Indeed the WBC19 protein is hypothesized to prevent ribosome inactivation by translocating kanamycin into the vacuole. Because such transporters often recognize multiple exogenous substrates, overexpression of At-*WBC19* could result in the vacuolar accumulation of unexpected (and possibly toxic) compounds in GM plants (Rommens, 2006).

B. Marker Genes Conferring Herbicide Resistance

Herbicide resistant crops represent the overwhelming majority of GM plants currently tested in the field or approved for commercialization. Genes conferring herbicide resistance inserted into plants may also be used as SMGs and provide therefore for a potential alternative to the use of ARMGs. Several of them have already gone through a complete biosafety assessment in the frame of the regulatory approval of the corresponding GM plants (see section IV).

The *epsps* genes, conferring resistance to glyphosate (the active ingredient of the commercial Roundup® formulations) are by far the most commonly used. The *cp4 epsps* gene from *A. tumefaciens* is found mainly in GM plants developed by Monsanto, whereas other *epsps* genes from bacterial or plant origin (e.g., a mutated maize gene) are also used. They are used sometimes in association with the *gox* (glyphosate

oxydoreductase) gene from the bacterium *Ochrobactrum antrophi*, encoding an enzyme that accelerates the physiological degradation of glyphosate. Genes from the soil bacterium *Bacillus licheniformis* encoding GAT proteins also confer resistance to glyphosate-containing herbicides by detoxifying glyphosate to the non-herbicidal form N-acetylglyphosate. *gat4601* and *gat4621* have been used as SMG in commercially approved GM plants (maize and soybean).

The *bar* gene from *Streptomyces hygroscopicus* and the *pat* gene from *S. viridochromogenes* confer resistance to phosphinothricin (PPT; glufosinate ammonium), the active ingredient of several commercial broad spectrum herbicide formulations (e.g., Basta™, Ignite™, Liberty™). These genes have been used as selectable marker in many crops, and also in plants like orchids that are naturally resistant to antibiotics (Knapp *et al.*, 2000). Mutant forms of the gene encoding acetolactate (or acetohydroxyacid) synthase (ALS or AHAS) have been used as SMGs by conferring resistance in plants to sulfonylurea or imidazolinone herbicides (Rosellini, 2011). For instance, mutant genes from *Arabidopsis* or tobacco (like *CSR* or *SurB*) are commonly used as selectable markers in commercial lines of GM carnation. Other herbicide resistance genes that have been used as SMGs in commercialized GM plants include the *bxn* gene from the bacterium *Klebsiella pneumoniae* conferring resistance to bromoxynil and a modified form of the *aad-1* gene from the bacterium *Sphingobium herbicidovorans*, conferring resistance to 2,4-D (see Section IV.1).

The scientific literature provides some more examples of herbicide resistance genes from bacterial, fungal, plant or even mammalian origin, employed as SMG for transformation in GM plants (Miki and McHugh, 2004; Sundar and Sakthivel, 2008). Some of these marker genes have been used in GM plants tested in the field but not in commercialized GM plants. Therefore, their safety for human health and the environment has still to be fully assessed. These examples include the following:

- The cyanamide hydratase (*cah*) gene from the soil fungus *Myrothecium verrucaria* that confers resistance to cyanamide, a fertilizer that also has herbicide activity (Weeks *et al.*, 2000).
- The *deh1* gene from *Pseudomonas putida* coding for a dehalogenase capable of degrading 2,2-dichloropropionic acid (2,2-DCPA), the active ingredient of the herbicide dalapon (Buchanan-Wollaston *et al.*, 1992).
- The organophosphorus hydrolase (*oph*) gene from *Pseudomonas diminuta* that is capable of degrading organophosphorus pesticides and has been successfully tested in maize (Pinkerton *et al.*, 2012).
- Several human *P450* genes that have been shown to act as a selectable marker by conferring herbicide resistance (herbicides are metabolized by P450 monooxygenases) when transformed into *Arabidopsis*, tobacco, potato and rice plants (Inui *et al.*, 2005).
- Mutated versions of the phytoene desaturase (*Pds*) plant gene. Plants expressing them have the particularity to show increased resistance to a limited spectrum of herbicides (fluridone, norflurazon and flurtamone), while at the same time, being more susceptible than the wild-type to other PDS inhibitors, such as diflufenican, picolinafen and beflubutamid. This feature has been presented as a potential risk management tool to eliminate undesirable GM plants that would escape in the wild (Arias *et al.*, 2006).
- Plant and bacterial mutants or native forms of the *ppo* (protoporphyrinogen oxidase) gene that encodes a key enzyme in the chlorophyll/heme biosynthetic pathway, and is a good target of various peroxidizing herbicides. They have been successfully used as SMG in *Arabidopsis*, maize and rice, in combination with the herbicide butafenacil as selection agent (Sundar and Sakthivel, 2008).
- The *sul*I gene from *Escherichia coli* conferring resistance to sulfadiazine and other sulfonamide chemicals (Rosellini, 2012).
- A mutant alpha-tubulin *Tub1* gene from goose grass that confers resistance to herbicides of the dinitroaniline (e.g., trifluralin) and phosphoroamidate families. It was employed as SMG for transformation of finger millet, soybean, flax, tobacco and more recently barley (Yemets *et al.*, 2009; Tanasienko *et al.*, 2011).

Using genes conferring herbicide resistance as selectable markers in GM plants is a mature technology applicable across a wide range of plant species. It is therefore not surprising that this selection approach has been used in many GM plants currently tested in the field or commercialized. However, herbicide resistance-mediated selection can raise environmental concerns and suffer some limitations. First, the presence of such genes in GM plants could contribute to herbicide resistance in other cultivars or sexually compatible wild relatives as a result of gene flow and introgression (Kwit *et al.*, 2011). This concern is not specifically related to their use as SMGs, but has to be considered in the frame of a risk assessment. Second, in a case where herbicide resistance in plants is not a trait of interest and is used for selection only, it has to be carefully chosen in function of the plant species transformed. Indeed its presence in the GM plant could limit control options for volunteers emerging in following crop cultivation (Goldstein *et al.*, 2005). For instance, using glyphosate resistance marker genes in potato plants would exclude glyphosate to control potato volunteers developing after mild winters. Third, commercializing GM plants containing herbicide resistance genes used for selection only, might raise some regulatory and enforcement issues (EFB, 2001). Because such plants might not be authorized as herbicide resistant crop it will be important to ensure that the plant and the corresponding herbicide are not misused. Fourth, the use of the most efficient herbicide resistance genes (such as *epsps* and *bar*) is patented

by the companies which produce the corresponding herbicide. This makes accessibility to such selection systems limited or expensive for non-research purposes (see Section III).

C. Other Marker Genes

To overcome the concerns associated with the use of antibiotic or herbicide resistance genes into GM plants, alternative SMGs have been developed. Theoretically, a large number of marker genes exist for plants. In practice however, due to specific limitations in the efficiency or applicability of many of these genes, only a few of them are used for GM plant development and even fewer for commercialization. Since plant SMGs have been the subject of several reviews (see in particular Jaiwal *et al.*, 2002; Miki and McHugh, 2004; Darbani *et al.*, 2007; Sundar and Sakthivel, 2008; Penna and Ganapathi, 2010; Manimaran *et al.*, 2011; Rosellini, 2011; Wei *et al.*, 2012; Rosellini, 2012), this paper will provide a general overview of them and will address in particular technical and safety aspects associated with their use. SMGs have been classified into different categories depending either on the selective chemical (Miki and McHugh, 2004) or on their mechanism of action (Rosellini, 2012). The latter classification scheme is used in this paper. Genes that can serve as markers in chloroplast transformation are reviewed in Section II.E.

1. Positive SMGs

Positive SMGs selectively promote the growth of the transformed cells. The antibiotic and herbicide resistance marker genes mentioned before belong to this category, but many other positive SMGs have been developed in the laboratory. A comprehensive list of genes allowing positive selection is provided in Table 1, including some of the most recent references from the peer-reviewed literature. SMGs described in patent applications only are not listed inTable 1. General considerations relating to patenting are presented in Section III.

Amongst the SMGs listed in Table 1, those belonging to type 6 have received much attention. They confer to the plant cells the ability to utilize as carbon sources carbohydrates that otherwise cannot be metabolized. In these cases the growth and development of non-transformed cells is prevented by starvation without killing them. The most remarkable example is the PMI system. It is based on the *manA* gene encoding an *E. coli*–derived phosphomannose isomerase (PMI). The expression of this gene allows transformed plants to grow on medium supplied with mannose as a carbon source, whereas growth of the non-transformed cells is arrested or limited due to the toxic effect associated with the accumulation of mannose-6-phosphate in the plant tissues. *manA* has been used as SMG in *Arabidopsis*, potato, sugar beet, maize, wheat, onion, pearl millet, barley, papaya, sorghum, sugarcane, cucumber, watermelon, oilseed rape, pepper, sweet orange, bentgrass, cabbage, flax, sunflower, apple, citrus, potato, pea, cassava, oil palm, cowpea or tomato (see references cited in Miki and McHugh, 2004; Sonntag *et al.*, 2004; Darbani *et al.*, 2007; Ballester *et al.*, 2008; Sundar and Sakthivel, 2008; Wallbraun *et al.*, 2009; Kraus, 2010; Penna and Ganapathi, 2010; Song *et al.*, 2010; Manimaran *et al.*, 2011; Stoykova and Stoeva-Popova, 2011). It was also used in rice to develop the most recent generation of Golden Rice cultivars with expression of high-carotenoid levels (Paine *et al.*, 2005; Datta *et al.*, 2007).

Mannose-based selection has also been reported using the *M6PR* gene from celery. Due to its plant origin, this SMG could be a potential alternative to the *E. coli* PMI system. However, more studies are still required to determine its broad effectiveness in the selection of GM plants. For plant species that have endogenous PMI activity (like soybean) or that are mannose-tolerant (like carrot), a potential alternative consists in using the bacterial xylose isomerase (*xylA*) genes as SMG and xylose as the selective agent (Haldrup *et al.*, 1998a; Haldrup *et al.*, 1998b). This system has been tested successfully in potato, tobacco, tomato, maize and recently patented for use in sunflower and oilseeds (Morawala and Rajyashri, 2007).

Most of the positive SMGs listed in Table 1 allow plants to survive in media supplied with substances exerting a toxic effect and killing non-transformed plant cells. Resistance to the phytotoxin is mediated either through its detoxification (type 1), through its removal or exclusion from the affected cell compartment (type 2), or through the expression of an insensitive mutated form of the target molecule or a naturally resistant form from a different organism (type 3). Amongst these genes, *gsa*, allowing selection in the presence of gabaculine, has been presented as a powerful, safe and versatile selection system from plant origin that could even be used for plastid transformation. Genes belonging to type 2 are particularly applicable when tolerance to abiotic stress is also the trait of interest. This reasoning also applies to the *Hsp101* gene, the only gene conferring heat tolerance successfully tested as SMG (type 5).

Selection based on pathogen resistance (type 4) has only been reported once in the scientific literature (using the *Pflp* gene). A similar approach has been recently patented for potato species. It involves the use of SMGs conferring late blight resistance. GM plants expressing such genes do not display disease symptoms when challenged with the pathogen *Phytophthora infestans* (de Vetten *et al.*, 2008).

Type 7 SMGs differ from the previous types because they promote plant growth and/or differentiation of transformed cells without the use of selective substances, by modifying their morphology, development and/or physiology. These genes encode transcription factors, cell cycle proteins, or components of hormone biosynthesis and signaling pathways. They confer for instance to the plant cells the ability to autonomously produce plant hormones (such as cytokinin) necessary for regeneration. Transformed cells containing such genes can regenerate in the absence of key hormones whereas non-transformed cells are unable to regenerate. The most commonly used is the *ipt* gene. It encodes the enzyme isopentenyl transferase involved in the cytokinin biosynthesis and is located in the T-DNA (transfer DNA) region of the Ti-plasmids of *A. tumefaciens*. GM plants carrying a functional *ipt* gene overproduce cytokinin and exhibit

TABLE 1
Positive SMGs usable in GM plants

Gene	Protein	Origin	Compound or condition used for selection	GM Plant	References
Type 1. Selection based on the chemical detoxification of a phytotoxin					
alr	Alanine racemase	Bacterium (*Corynebacterium glutamicum*)	D-alanine	*Arabidopsis*	Thiruvengadam *et al.*, 2010
dao1	D-amino acid oxidase	Yeast (*Rhodotorula gracilis*)	D-amino acids	*Arabidopsis*, apple, tobacco	Hättasch *et al.*, 2009; Garcia-Almodovar *et al.*, 2012
dogR1	2-deoxyglucose-6-phosphate phosphatase	Yeast (*Saccharomyces cerevisiae*)	2-deoxyglucose	Tobacco, potato, pea	Kunze *et al.*, 2001
dsdA	D-serine ammonia lyase	Bacterium (*E. coli*)	D-serine	*Arabidopsis*, maize, tobacco, poplar	Lai *et al.*, 2011
galT	UDP-glucose:galactose-1-phosphate uridyltransferase	Bacterium (*E. coli*)	Galactose	Potato, oilseed rape	Joersbo *et al.*, 2003
HOL1	Methyl halide	Plant (*A. thaliana*)	Potassium thiocyanate	*Arabidopsis*	Midorikawa *et al.*, 2009
ilvA (mutant)	Threonine deaminase	Bacterium (*E. coli*)	O-methylthreonine (structural analog of isoleucine)	Tobacco	Ebmeier *et al.*, 2004
D-*LDH*	D-lactate dehydrogenase	Plant (*A. thaliana*)	D-Lactate	*Arabidopsis*	Wienstroer *et al.*, 2012
lyr	Lysine racemase	Bacterium (Soil metagenome)	L-lysine	*Arabidopsis*, tobacco	Chen *et al.*, 2010
merA or *merB*	Organomercurial lyase	Bacterium (*E.coli*, *Streptococcus aureus*)	Mercury	Poplar, peanut	Choi *et al.*, 2005
mpr1	N-acetyltransferase	Yeast (*S. cerevisiae*)	Azetidine-2-carboxylic acid (L-proline analog)	Tobacco	Tsai *et al.*, 2010
NiR	Ferredoxin nitrite reductase	Plant (Rice)	High nitrite conditions	Rice	Ozawa and Kawahigashi, 2006
Tdc	Tryptophan decarboxylase	Plant (*Catharanthus roseus*)	4-methyl tryptophan (tryptophan analog)	Tobacco	Goddijn *et al.*, 1993
tflA	Toxoflavin-degrading enzyme	Bacterium (*Paenicillum polymixa*)	Toxoflavin	*Arabidopsis*, rice	Koh *et al.*, 2011
TPS1	Trehalose 6 phosphate synthase	Plant (*A. thaliana*)	Reduced glucose sensitivity of transformed plants	*Arabidopsis*, tobacco	Leyman *et al.*, 2006

(Continued on next page)

TABLE 1
Positive SMGs usable in GM plants *(Continued)*

Gene	Protein	Origin	Compound or condition used for selection	GM Plant	References
Type 2. Selection based on the removal or exclusion of a phytotoxin from the cell					
Dreb2A	Transcription activator	Plant (Rice)	NaCl	Rice	Zhu and Wu, 2008
rstB	*Rhizobium* salt tolerance B	Bacterium (*Sinorhizobium fredii*)	NaCl	Tobacco	Zhang *et al.*, 2009a
SOS1	Ion antiporter	Plant (*A. thaliana*)	NaCl	Rice	Zhu and Wu, 2008
Type 3. Selection based on over-expression of a phytotoxin-sensitive, or expression of an insensitive target molecule					
ak (wild-type) or *lysC* (mutant)	Aspartate kinase	Bacterium (*E. coli*)	Lysine + threonine	Potato, barley, chickpea	Perl *et al.*, 1993; Tewari-Singh *et al.*, 2004
ASA (mutant)	Anthranilate synthase	Plant (*A. thaliana, tobacco, rice*)	Tryptophan analogs, e.g., 5-methyl tryptophan	*Arabidopsis*, potato, tobacco, rice, soybean	Kawagishi-Kobayashi *et al.*, 2005; Barone and Widholm, 2008
dhps or *dapA*	Dihydrodipicolinate synthase	Bacterium (*E. coli*)	S-aminoethyl L-cysteine (toxic lysin analog)	Soybean, potato	Perl *et al.*, 1993; Rao *et al.*, 2009
dhfr	Dihydrofolate reductase	Bacterium (*E. coli*), Yeast (*Candida albicans)*, mouse	Methotrexate	*Arabidopsis*, tobacco, petunia	Aionesei *et al.*, 2006
Gsa (mutant)	Glutamate 1-semialdehyde aminotransferase	Plant (*Alfalfa*)	Gabaculine	Tobacco, alfalfa, durum wheat	Ferradini *et al.*, 2011a; Giancaspro *et al.*, 2012
*hem*L (mutant)	Glutamate 1-semialdehyde aminotransferase	Bacterium (*Synechococcus*)	Gabaculine	Tobacco, alfalfa, durum wheat	Gough *et al.*, 2001 ; Rosellini *et al.*, 2007; Giancaspro *et al.*, 2012
MSRB	Methionine sulfoxide reductase B	Plant (*A. thaliana*)	Methyl viologen	*Arabidopsis*, tomato	Li *et al.*, 2013a
TSB1 (mutant)	Tryptophan synthase beta 1	Plant (*A. thaliana*)	5-methyl tryptophan or Cadmium chloride	*Arabidopsis*	Hsiao *et al.*, 2007
Type 4. Selection based on pathogen resistance					
Pflp	Pepper ferridoxin-like protein	Plant (Sweet pepper)	Resistance to *Erwinia carotovora*	Orchids	Hood, 2003; You *et al.*, 2003
Type 5. Selection based on heat tolerance					
Hsp101	Heat shock protein	Plant (Rice)	Heat	Tobacco	Chang *et al.*, 2007
Type 6. Selection based on the ability of plant cells to utilize non-metabolized carbohydrates					
atlD	Arabitol dehydrogenase	Bacterium (*E. coli*)	Arabitol	Rice	LaFayette *et al.*, 2005
man A (pmi)	Phosphomannose isomerase	Bacterium (*E. coli*)	Mannose	Many species	Stoykova and Stoeva-Popova, 2011

(Continued on next page)

TABLE 1
Positive SMGs usable in GM plants *(Continued)*

Gene	Protein	Origin	Compound or condition used for selection	GM Plant	References
M6PR	Mannose-6-phosphate reductase	Plant (celery)	Mannose	*Arabidopsis*	Song *et al.*, 2010
ptxd	Phosphite oxydoreductase	Bacterium (*Pseudomonas stutzeri*)	Phosphite	*Arabidopsis*, tobacco	Lopez-Arredondo and Herrera-Estrella, 2013
xylA	Xylose isomerase	Bacterium (*Streptomyces rubiginosus, Thermoanaerobacterium thermosulfurogenes*)	Xylose	Potato, tobacco, tomato, maize, sunflower, oilseed rape	Guo *et al.*, 2007
Type 7. Selection based on the ability of plant cells to autonomously produce growth regulators					
BBM	Baby Boom Transcription Factor	Plant (oilseed rape)	—	Poplar	Deng *et al.*, 2009;Heidmann *et al.*, 2011
ipt	Isopentenyl transferase	Bacterium (*E. coli*)	Hormone-free medium	Many species	see references listed in Rosellini, 2012
Kn1	Homeobox	Plant (maize)	Hormone-free medium	Tobacco	Luo *et al.*, 2006
uidA (gusA)	β-glucuronidase	Bacterium (*E. coli*)	Benzyladenine N-3-glucuronide	Tobacco	Joersbo and Okkels, 1996
rol	"Root loci"	Bacterium (*A. rhizogenes*)	Hormone-free medium	Tobacco	Ebinuma *et al.*, 1997a

morphological abnormalities (in particular shoots with abnormal morphology and loss of apical dominance) in hormone-free medium, thereby making them easy to detect visually. The efficient use of *ipt* as SMG in plant transformation has been reported for the first time in tobacco and hybrid aspens (Ebinuma *et al.*, 1997a; Ebinuma *et al.*, 1997b) and afterwards in many other plant species. The *rol* genes from *A. rhizogenes* and the maize homeobox gene *Kn1* have been used in the same way as the *ipt* gene. The former are responsible for the proliferation of "hairy roots" by increasing auxin sensitivity. GM plants regenerated from "hairy roots" exhibit abnormal phenotypes such as wrinkled leaves, shortened internodes or reduced apical dominance. The latter allows selecting transformed plants based on changes in leaf shape, loss of apical dominance, and production of ectopic meristems on leaves. Several plant genes also have the potential to be used as type 7 SMGs (Zuo *et al.*, 2002; Miki and McHugh, 2004). The gene encoding the *BABY BOOM (BBM)* transcription factor can induce formation of somatic embryos from calli when expressed in Chinese white poplar (Deng *et al.*, 2009) and sweet pepper (Heidmann *et al.*, 2011). Other putative candidates include genes involved in cytokinin biosynthesis and signaling as well as genes that increase the embryogenic potential or enhance the vegetative-to-embryogenic transition. The latter would be particularly useful in plant species that are regenerated through somatic embryogenesis in contrast to the bacterial SMGs described above, which can be used only in species that are regenerated through the organogenesis pathway. However, the functioning of these putative candidates as efficient SMGs remains to be demonstrated (Rosellini, 2012).

The performances of positive SMGs in terms of transformation and selection efficiency vary widely and in most cases do not reach the efficiency of antibiotic- or herbicide-based selection systems (Rosellini, 2012). As pointed out by Miki and McHugh (2004), one cannot assume that plant resistance to a selective agent conferred by a specific gene will result in a good selection system just because highly-resistant cells do survive. To be effective, a SMG must encourage the selective growth and differentiation of the transformed tissues in addition to providing resistance to a substrate. Long-time exposure to

stringent selection conditions may result in growth retardation of selected transformed cells and consequently low regeneration efficiency of the GM plants. It has been observed that in selection systems where non-transformed cells are killed, dying cells can inhibit the supply of nutrients to the transformed cells or excrete toxic compounds thereby negatively affecting the ability of transformed cells to proliferate and differentiate into GM plants (Joersbo and Okkels, 1996; Ebinuma *et al.*, 2001). This problem might potentially be circumvented by using SMGs like the sugar-based systems described above, where non-transformed cells are not killed but rather stopped in their development. However higher transformation efficiency are not systematically observed. The use of these SMGs is sometimes accompanied by a toxicity effect of the non-conventional carbohydrate, requiring an optimization of the concentration of the selective compound in the medium (Rosellini, 2012). Potential deleterious effects associated with dying cells can also be avoided when type 7 SMGs are used since they only impair regeneration of non-transformed cells. The problem of escapes (i.e., the regeneration of non-transformed cells contiguous to transformed cells in inoculated explants) is also well known with positive SMGs using selective substances and is an important factor influencing selection efficiency.

PMI is the only positive selection system for which key parameters affecting transformation and selection efficiency have been studied and optimized in many plant species. In some species, the *pmi* gene was more efficient for the production of transformed plants compared to the *nptII* gene (Stoykova and Stoeva-Popova, 2011). The PMI system has been used to develop several GM maize events approved for commercialization (see Section IV.A). By contrast, with the exception of the *ipt* gene, the applicability of the great majority of the other above-mentioned approaches has been tested in a limited number of basic research studies involving very often experimental models only (such as *Arabidopsis* or tobacco) or at the best very few plant species. In some cases, experimental results have been published many years ago without any recent application described in the scientific literature. It remains therefore to be demonstrated whether these approaches are applicable to a significant range of plant species and culture procedures, and more generally whether they are really efficient tools for the development or commercialization of GM plants.

Biosafety considerations are a crucial factor influencing the applicability of a selection system. Amongst the selection systems listed above, the PMI system is the only one that has undergone a full risk assessment. Safety assessments have been performed in the frame of approval procedures for commercialization, including allergenicity and toxicity studies, and no evidence of adverse health or environmental effects has been found associated with PMI (see Section IV.A). The *dsdA* gene has been subjected to preliminary safety assessments (by the developers) when used as selectable marker in maize. Results suggest that no adverse effects are to be expected if humans were exposed to the corresponding protein in the diet from an allergenicity or toxicity perspective (Lai *et al.*, 2011). The *xylA* system depends on an enzyme that is generally recognized as safe for use in the starch industry and which is already being widely used in specific food processes (Haldrup *et al.*, 2001). However, its safety has not been assessed yet in the context of its use as SMG in GM plants.

Many of the SMGs described in Table 1 participate in or can interfere with endogenous metabolic pathways. Most of them must be overexpressed to confer the expected selective effect. Marker gene expression in the GM plant might therefore interfere with plant metabolism during further growth and/or lead to potential unintended effects on other genes. Detrimental effects on plant metabolism and/or phenotype have been reported in several cases, for example, with the *ak*, *alr*, *ilvA, Tdc* and *xylA* genes (Perl *et al.*, 1993; Ebmeier *et al.*, 2004; Rosellini, 2011; Rosellini, 2012). Such potential negative effects can be limited by expressing the SMG transiently under the control of an inducible or developmentally-regulated promoter.

Temporal expression is also a prerequisite for using type 7 SMGs. Since these genes induce alterations in the morphology, development and/or physiology of the GM plant, their presence or activity has to be eliminated or turned down for instance through their excision after initial selection or through their transient expression. The MAT (Multi-Auto-Transformation) vectors have been designed to remove the *ipt* or *rol* genes from GM plants after transformants have been selected, through the use of site-specific recombination systems or transposase (Ebinuma *et al.*, 1997a; Ebinuma and Komamine, 2001; see Sections II.D.2 and II.D.4 for further details). Transient expression of the *ipt* gene has been achieved by placing the gene under the control of an inducible promoter (Kunkel *et al.*, 1999; Lu *et al.*, 2010b). Transformed cells are first regenerated in a hormone-free medium with inducer, and subsequently transferred onto an inducer-free medium to allow normal plant growth and development. Alternatively the SMG can be transiently present in the transformed plant cells. This has been achieved by positioning the *ipt* gene within the backbone (non-transferable part) of the vector, leaving the GOI in the T-DNA. After transformation, temporary expression of the SMG allows selection of transformed plant cells on hormone-free medium. Subsequently, shoots displaying a wild-type appearance, in which backbone sequences (including the SMG) have been lost, can be selected (Rommens *et al.*, 2004; Richael *et al.*, 2008; Rommens, 2010b; Richael and Rommens, 2012). A transient effect of the *ipt* gene has also been reported in tobacco (Mihalka *et al.*, 2003) and potato (Bukovinszki *et al.*, 2007) using "shooter" mutant *Agrobacterium* strains. These strains carry mutant Ti plasmids deficient in the auxin biosynthesis genes but still containing an intact *ipt* gene. When plant cells are transformed with "shooter" strains harboring a second plasmid containing a GOI (inserted in the T-DNA), phenotypically normal shoots containing the GOI can be regenerated on growth hormone-free medium. It is assumed that the *ipt* gene is either transiently expressed in the cells of these regenerants, with *ipt* sequences being lost during

subsequent cell divisions, or these cells were fed with exogenous cytokinins synthesized by neighboring *ipt* transformed cells.

2. Negative SMGs

Negative selection functions in such a way that the transformed cells containing the SMG experience a growth disadvantage (death or impaired growth), either due to the conversion of a non-toxic substrate into a toxic agent (conditional negative selection), or due to the direct intracellular production of a toxic agent (non-conditional negative selection). Negative selection is generally used in combination with positive selection and/or in concert with methods for marker removal to optimize the selection of marker-free plants (see Section II.D). Gene targeting is another application of negative selection. In combination with positive selection, it allows to counter-select illegitimate recombination events and isolate the rare gene targeting events occurring through homologous recombination (Rosellini, 2012).

The *codA* gene from *E. coli* is the most frequently used marker gene for negative selection. It encodes cytosine deaminase, an enzyme that converts the non-toxic 5-fluorocytosine to cytotoxic 5-fluorouracil. Its potential for negative selection has been reported many years ago (Stougaard, 1993). It has been used in positive/negative SMG combinations in different transformation protocols (see Section II.D).

The *Agrobacterium tms2* and *aux2* genes have been identified many years ago as potential candidates for negative selection. Both genes encode proteins that catalyze the conversion of biologically inactive auxin amides into active auxins, resulting in GM plants exhibiting a variety of auxin toxicity effects. They were successfully used in *Arabidopsis* (Karlin-Neumann *et al.*, 1991) and cabbage (Béclin *et al.*, 1993) respectively. The *tms2* gene has further been used as a negative SMG in tobacco (Zubko *et al.*, 2000) and rice (Upadhyaya *et al.*, 2000).

The bacterial haloalkane dehalogenase (*dhla*) gene that mediates the conversion of a non-toxic substrate into cytotoxic chlorinated alcohol and chlorinated aldehyde has been used for negative selection in *Arabidopsis* (Naested *et al.*, 1999) and more recently in rice (Moore and Srivastava, 2008). Other conditional negative SMGs that have been described in the literature include alcohol dehydrogenase (*adh*) and human herpes simplex virus thymidine kinase (*HSVtk*) requiring respectively the substrates allyl alcohol and Ganciclovir (reviewed in Rosellini, 2012), and the *E. coli* ornithine deacetylase gene *argE* that converts N-acetyl phosphinothricin to the phosphinothricin herbicide (Chen *et al.*, 2005).

Some of the positive SMGs mentioned in Section II.C.1 can be used as negative SMGs. In some cases, this may depend on the plant species. The *M6PR* gene encoding mannose-6-phosphate reductase allows a positive selection of GM mannose-sensitive species (such as *Arabidopsis*) and a negative selection of mannose-tolerant species (such as tobacco) (Song *et al.*, 2010). In other cases, the bifunctional property of the SMG depends on the substrate used for selection. The *dao1* gene mediates the metabolization of D-alanine and D-serine, which are toxic to plants, into non-toxic products, whereas D-isoleucine and D-valine, which have low toxicity, are metabolized into more toxic products (Erikson *et al.*, 2004). This double selection property has recently been combined with site-specific recombination (see Section II.D.2) to allow the production of marker-free GM tobacco (Garcia-Almodovar *et al.*, 2012).

In addition to their possible use as positive SMGs (see Section II.B), genes encoding cytochrome P450 monooxygenases can be effective for negative selection, due to their ability to metabolize sulfonyl urea pro-herbicide into a highly phytotoxic product. Reported examples include the human *CYP2B6* gene in rice (Kawahigashi *et al.*, 2002) and the bacterial *P450* gene in *Arabidopsis*, tobacco, rice (Upadhyaya *et al.*, 2010) and barley (Koprek *et al.*, 1999). Although it was suggested that the use of the *P450* gene was more amenable to large-scale screening under greenhouse or field conditions as compared with the use of the *codA* gene, aberrant growth phenotypes have been observed in *Arabidopsis* plants transformed with the bacterial *P450* gene (Dasgupta *et al.*, 2011).

BASF patented in 2010 a selection method consisting in a refinement of the use of negative SMGs (Kock *et al.*, 2010). Plant cells already containing a negative SMG are transformed with a "cassette" containing the GOI and a nucleic acid sequence ("anti-marker") leading to reduction or inhibition of the expression of the SMG or of the activity and/or function of the corresponding protein. This reduction or inhibition hampers the conversion of a non-toxic substrate into a toxic agent, thereby giving the transformed plant cells a growth advantage over non-transformed cells. This method can be used potentially with most of the negative SMGs described above and with different types of "anti-markers" including the action of a double-stranded or antisense RNA, the partial or complete deletion of the SMG (e.g., using site-specific recombinases), or the inactivation of the SMG by targeted insertion (e.g., using enzymes inducing DNA double-strand breaks). Since the negative SMG is unlinked to the GOI in the plant genome, it can be removed by segregation during subsequent breeding.

In contrast to the use of conditional negative SMGs, very few examples of selection approaches involving a non-conditional negative SMG have been reported. RamanaRao and Veluthambi (2010) have shown their potential using the *Mungbean yellow mosaic virus* transcriptional activator protein (*MYMV TrAP*) gene as negative selectable marker in co-transformation experiments. Transient positive selection with phosphinothricin and the negative selection exerted by *TrAP* expression made it possible to produce marker-free plants, although with low frequency. The genes encoding barnase (used to engineer male sterility), carnation ribosome-inactivating protein (RIP), *Pseudomonas aeruginosa* exotoxin A, yeast RAS2, or diphtheria toxin A chain (DT-A) are other examples of potential non-conditional negative SMGs (Rosellini, 2012). From the safety viewpoint the use of such selectable markers directly encoding a toxic agent raises

questions as regards their acceptability in GM plants destined for human or animal consumption.

3. Reporter genes

Reporter genes are markers that make GM plants visually recognizable. They have become integral tools in studying plant cell biology (see e.g., Berg and Beachy, 2008) and can also be used in association with SMGs to improve the efficiency of recovering GM plants. This is particularly useful in selection approaches where "escapes" (i.e., non-transformed cells growing in the vicinity of transformed cells) are common. In other cases, they allow the selection of transformed tissues without or prior to the application of selective agents. Reporter genes can be vital (visualization can be monitored in living cells) or non-vital (visualization requires killing the cells), and conditional (requiring an exogenous substrate) or non-conditional. The use of reporter genes in plant selection has been reviewed in particular by Miki and McHugh (2004), Wei *et al.* (2012) and Rosellini (2012).

The *uidA* (*gusA)* gene from *E. coli* has been very popular as a reporter gene in plant genetic engineering. It encodes beta-glucuronidase (GUS) that catalyzes the cleavage of a wide variety of beta-glucuronides allowing spectrophotometric or fluorometric analyzes. Activity can be localized histochemically and quantified by a non-destructive fluorescence-based assay, but these quantitative assays are laborious and not well suited for screening of large populations of cells. The *uidA* gene is present in several plants approved for commercialization (see Section IV.A) and has therefore undergone a full assessment of the potential risks for human health and the environment.

The *gfp* gene from jellyfish, that directly emits fluorescent light when excited by UV light, is another commonly used reporter gene (Finer, 2011). It has been used recently for genetic transformation of *Hevea brasiliensis* (Leclercq *et al.*, 2010), pepper (Jung *et al.*, 2011) and rice (Saika *et al.*, 2011). Several GM plants containing the *gfp* gene have been tested in the field. Preliminary studies suggest that its expression in GM plants is not likely to represent a health risk (Richards *et al.*, 2003). There are several advantages of using GFP as visual tool in selection, including direct visualization in living tissue in real time, no cytotoxic effect on plant cells, and no substrate required for detection (Miki and McHugh, 2004; Manimaran *et al.*, 2011). Moreover, a wide range of spectral variants has been generated making them useful in studies requiring more than one marker (Rosellini, 2012). A potential limitation of this system is the need to use specific and costly equipment for detection.

Several other genes encoding Fluorescent Proteins (FPs) have been added to the color palette during the past years. Amongst them FPs (yellow through far red) derived from coral reef organisms have gained wider use in the context of GM plant selection (Shaner *et al.*, 2007). For example, the *DsRed2* gene (Jach *et al.*, 2001) was expressed in the GM maize event DP-32138-1, which was granted in 2011 non-regulated status in the U.S. DP-32138-1 maize was not developed for food or feed use or large-scale cultivation but for facilitating production of non-transgenic seeds. Its assessment indicated that the DsRed2 protein is unlikely to have adverse allergenic or toxic effects (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p.pdf). The red FPs are of special interest in GM plants because there is little natural reflectance in green tissue in the red wavelengths, thus various red FPs should be easier to visualize compared with GFP (Stewart, 2006).

More recently, plant genes coding for transcription factors of the MYB family involved in anthocyanin synthesis have been proposed as promising reporter gene candidates for plant transformation (Kim *et al.*, 2010; Gao *et al.*, 2011; Kortstee *et al.*, 2011; Li *et al.*, 2011b). They have been successfully tested in tobacco, wheat, apple and potatoes. They make the GM plants visible by the human eye (the plants totally or partly harbors red or purple colors), do not require addition of substrate, are not essential for development and (at least for some of them) have been shown to produce non-toxic proteins. Recent reports have even shown that consumption of anthocyanins can be beneficial for health (Butelli *et al.*, 2008). It has been suggested that the possibility of having plants colored, including eventually edible parts, would not be a concern from a consumer's point of view (Espley *et al.*, 2013).

Other reporter genes that have been tested *in planta* include the following (see above-mentioned reviews for details):

- The *lacZ* gene from *E. coli* encoding beta-galactosidase that catalyzes color development in the presence of X-Gal.
- The *luc* gene from firefly that catalyzes the conversion of luciferin to emit fluorescence.
- The *bgl1* gene from *Aspergillus niger* encoding beta-glucosidase that catalyzes the conversion of a corresponding substrate to emit fluorescence.
- The wheat germin *OxO* gene, with oxalate oxidase activity, that allows histochemical detection of H_2O_2 generated from oxidation of oxalate.
- The organophosphorus hydrolase (*oph*) gene which, besides its role as herbicide resistance marker gene (see Section II.B), has the ability to act as a screenable marker by producing fluorescence under UV illumination as a result of the hydrolyzation of specific substrates.
- The *ipt* gene mentioned above that has also served as a visual marker of backbone integration within the plant genome (Bukovinszki *et al.*, 2007).

D. Removal of Marker Genes

As mentioned in the introduction, removing SMGs or reporter genes from the GM plant after initial selection of the transformants can be envisaged to address regulatory and public concerns. Their elimination also prevents any possible position or pleiotropic effects leading to unintended changes thereby

addressing corresponding safety concerns (Cellini *et al.*, 2004). Position effects result from interactions between the transgene and processes occurring at the site of insertion, together with its downstream effects. Pleiotropic effects are attributable to transgene insertion and/or expression but are transgene-locus independent (Miki *et al.*, 2009). These effects may be associated not only with the inherent properties of the marker genes, but also with the diverse genetic elements used to control its expression. Recent data obtained from biochemical profiling technologies (genomics, transcriptomics, proteomics and metabolomics) suggest that the insertion of transgenes induces very few changes on the plant transcriptome, proteome or metabolome as compared with changes linked to natural plant variability or environmental and agronomical factors (see e.g., Batista *et al.*, 2008; Coll *et al.*, 2010; Davies *et al.*, 2010). With regards to SMGs specifically, studies have shown that the transcriptome of GM plants was not affected by the expression of genes such as *nptII*, *uidA* or *CSR1-2* (El Ouakfaoui and Miki, 2005; Manabe *et al.*, 2007). The existence of pleiotropic effects has been postulated in GM plants containing the *bar* gene, but these effects were associated with herbicide application rather than expression of the *bar* gene itself (Abdeen and Miki, 2009) and were not observed in another proteomics study (Ren *et al.*, 2009).

A marker-free approach can be useful to generate GM plants carrying multiple GOIs. There has been a rapid increase in the development of such plants. Stacking multiple genes can be done for example by sexual crossings between GM plants, by co-transformation or by sequential transformation (François *et al.*, 2002; Que *et al.*, 2010). In cases trait stacking requires repeated transformation rounds of an initial event (e.g., for species which must be vegetatively propagated), re-using SMGs would be greatly facilitated if the GM plants are freed from the marker genes after each round of transformation (see e.g., Sugita *et al.*, 2000b; Ramana Rao *et al.*, 2011). Moreover, gene silencing associated with sequence homologies has been reported (Meyer and Saedler, 1996; Cogoni and Macino, 1999). Since a limited number of promoters are currently used to drive transgene expression in GM plants, the repeated introduction of these promoters may negatively affect transgene expression.

There are situations where the elimination of non-native DNA sequences is really necessary, for instance in the development of cisgenic or intragenic plants. These plants contain only genetic material coming from within their own genome, from the same species or from a closely related species capable of sexual hybridization (Rommens *et al.*, 2004; Schouten and Jacobsen, 2008; Rommens *et al.*, 2011).

Different approaches for removing SMGs (including ARMG) from the nuclear genome to develop marker-free GM plants are available. They have been reviewed by several authors (Yoder and Goldsbrough, 1994; Hohn *et al.*, 2001; Scutt *et al.*, 2002; Hare and Chua, 2002; Puchta, 2003; Miki and McHugh, 2004; Goldstein *et al.*, 2005; Afolabi, 2007; Darbani *et al.*, 2007; Upadhyaya *et al.*, 2010; Manimaran *et al.*, 2011; Woo *et al.*, 2011; Tuteja *et al.*, 2012; Chong-Pérez and Angenon, 2013; Rukavtsova *et al.*, 2013; Yau and Stewart, 2013). In the following sections, these approaches are presented with up-to-date information and discussed with regards to their biosafety implications.

1. Co-transformation

Co-transformation consists in delivering simultaneously two or more foreign genes into a plant genome. The use of two T-DNAs containing the GOI and the SMG respectively is the basis for generating marker-free plants by *Agrobacterium*-mediated co-transformation. If the GOI and the SMG integrate at independent loci in the plant genome, it is possible to eliminate the SMG by simple selection in subsequent generations. *Agrobacterium*-mediated co-transformation has been applied in the early days of plant biotechnology (Depicker *et al.*, 1985; DeFramond *et al.*, 1986; McKnight *et al.*, 1987). It can be achieved following three alternative approaches:

- The use of two *Agrobacterium* strains, each with a single T-DNA.
- The use of two plasmids, each with a single T-DNA region, in a single *Agrobacterium* strain.
- The use of a single plasmid carrying two independent T-DNA regions, in a single *Agrobacterium* strain.

The development of marker-free GM plants through *Agrobacterium*-mediated co-transformation is well documented (see list of references in Miki and McHugh, 2004; Goldstein *et al.*, 2005; Darbani *et al.*, 2007; Manimaran *et al.*, 2011; Tuteja *et al.*, 2012). It has been successful in many monocots and dicots, including maize, rice, soybean and oilseed rape. This approach has been used for the development of the first generation of Golden Rice devoid of the hygromycin resistance marker gene (Al-Babili and Beyer, 2005). Recently, Holme *et al.* (2012) reported the use of co-transformation to develop cisgenic marker-free barley. In theory, this is a simple and safe approach for producing marker-free GM plants. As compared to other marker removal approaches described in the next sections, it does not leave behind residual DNA sequences, therefore avoiding biosafety concerns associated with the presence of functionally unnecessary sequences in the GM plant.

Co-transformation is generally considered to be time consuming. It has been estimated that four times more plants should be generated and screened in order to produce a marker-free GM plant by co-transformation in comparison to a single T-DNA transformation (Daley *et al.*, 1998). Since most GM plants obtained in the lab have no direct agricultural value, they need to be crossed to transfer the transgene into breeding germplasm, and segregation of the GOI from the SMG can be accomplished in the subsequent generations. Importantly, co-transformation followed by SMG elimination is relevant in sexual reproducing species but is much less practical in vegetatively propagated species like potato and in species with long life cycles such as trees (Scutt *et al.*, 2002; Darbani *et al.*, 2007; Manimaran *et al.*, 2011).

The overall applicability and efficiency of the approach is influenced by several factors. It has been suggested that expression of the genes cloned into one of the T-DNAs could be affected by the presence of the second unlinked T-DNA (Matzke *et al.*, 1989). Limitations in the efficiency of co-transformation using two T-DNAs in one single vector have be associated with high frequency of "linked co-delivery" of the GOI and the SMG along with intervening non-T-DNA sequences (McCormac *et al.*, 2001). Even when located on different plasmids or independent regions, the two T-DNAs used in co-transformation are frequently integrated at the same locus—i.e., no breakage of the genetic linkage is achievable—independent of the plant species or co-transformation method used (De Neve *et al.*, 1997; De Buck *et al.*, 1999). Factors involved in regulating these frequencies are not yet fully understood but are intimately linked with the bacterial and plant host-driven molecular mechanisms that underlie the different steps in the *Agrobacterium*-mediated plant cell transformation process (Windels *et al.*, 2008; Dafny-Yelin *et al.*, 2009).

To overcome these drawbacks, many variations of the co-transformation approach have been tested (Yau and Stewart, 2013). They aim in particular at improving the co-transformation frequency and the percentage of unlinked integration of the two T-DNAs containing the GOI and the SMG respectively. These two parameters seem to vary considerably from case to case and are influenced by many factors such as the *Agrobacterium* strain (octopine-type strains might be more favorable for unlinked integration as compared with nopaline-type strains; De Block and Debrouwer, 1991), the size and complexity of the vector, or the physiology and competence of the plant tissue (Ebinuma *et al.*, 2001; Miki and McHugh, 2004; Woo *et al.*, 2011).

In tobacco, high co-transformation frequencies, ranging from 10 to 100%, have been reported (Miki and McHugh, 2004). Such high frequencies are far to be achieved in other species (see e.g., Ferradini *et al.*, 2011b). It has been shown that approaches using two T-DNAs from a single strain of *Agrobacterium* (using one single or two separate vectors) yield higher co-transformation efficiency than using two *Agrobacterium* strains (Komari *et al.*, 1996; Daley *et al.*, 1998; Miller *et al.*, 2002; Yu *et al.*, 2009b). Increasing the molar ratio of the T-DNA with the GOI *vs.* the T-DNA with the SMG seems also to positively influence the co-transformation frequency. This can be achieved more easily in *Agrobacterium*-mediated co-transformation involving two T-DNAs on different plasmids (Sripriya *et al.*, 2011). In the case of co-transformation by two T-DNAs on a single plasmid, increasing the relative size of the T-DNA with the SMG over that of the T-DNA with the GOI seems to positively influence the capacity for segregational separation of co-transformed T-DNA regions (McCormac *et al.*, 2001). Matthews *et al.* (2001) reported high levels of co-transformation and further segregation when the two T-DNAs on a single plasmid were located adjacent to each other with no intervening region. However, a factor positively influencing the co-transformation frequency can be detrimental to the segregation process. Yu *et al.* (2009a) showed that the co-transformation frequency in rice was six times higher when the GOI and SMG were located in a single vector than in two separate vectors. But the frequency of marker-free GM rice plants obtained from the offspring of co-transformed plants was lower for the single vector system than that for the latter.

In most of the co-transformation methods using a single binary vector, each T-DNA is flanked by a left and a right border. An alternative approach involving a double right-border (RB) binary vector has been tested successfully in rice (Lu *et al.*, 2001; Xia *et al.*, 2006). This vector carries two copies of T-DNA RB sequences flanking a SMG, followed by the GOI and one copy of the left border sequence. Two types of T-DNA inserts, one initiated from the first RB containing both the SMG and the GOI, and the other from the second RB containing only the GOI, can be produced and integrated into the plant genome. In the subsequent generation, these inserts can segregate separately, allowing the selection of the progeny with only the GOI. Another approach exploits the observation that vector backbone sequences are frequently transferred along with T-DNA into plant cells. By positioning the SMG in the backbone of a binary vector and leaving only the GOI in the T-DNA region, marker-free GM plants can be recovered in the segregating progeny (when T-DNA and backbone fragments integrate at different loci). This approach has been materialized for instance in the pCLEAN constructs and has been applied to maize, tobacco and rice (Huang *et al.*, 2004; Vain, 2011).

The incorporation of a negative selection step has been proposed as a possible way to speed up the recovery of marker-free GM plants. It involves using two independent T-DNAs, one harboring the GOI and the second harboring a positive SMG like *nptII*, coupled to a negative SMG like *codA*. This selection method has been successfully tested in tobacco (Park *et al.*, 2004). Co-transformed T0 plants are first selected on antibiotic-containing medium. T1 seeds from co-transformants are then germinated on medium containing 5-fluorocytosine leading to the elimination of plants having stably integrated the T-DNA harboring the positive and negative SMGs. A similar approach has been applied to develop marker-free GM potatoes containing native DNA only (Rommens *et al.*, 2004). RamanaRao and Veluthambi (2010) reported a variant of this method allowing the elimination of the selectable markers in the T0 generation. It involves the use of a non-conditional negative SMG (*MYMV TrAP* – see Section II.C.2) and exploits the fact that in rare cases, the T-DNA harboring the GOI stably integrates in the plant genome whereas the T-DNA harboring the SMG is present only transiently in the same cell. The transient expression of the positive SMG allows the corresponding cell to survive on a medium containing the selection agent. When the agent is removed and due to the expression of the *TrAP* gene, the rare events containing only the GOI survive. Transient positive-negative selection has also been used to develop GM grapevine from T0 generation (Dutt *et al.*, 2012).

Androgenetic segregation has been applied in barley to increase the production of marker-free GM plants following co-transformation (Kapusi *et al.*, 2013). If the GOI and SMG are integrated on different chromosomes after co-transformation, meiosis in T0 plants will produce some pollen grains carrying only the GOI. Androgenesis with this pollen enables to produce GM homozygous T1 plants. A similar approach involving anther culture has been applied in rice (Zhu *et al.*, 2007).

The use of co-transformation in association with biolistic transformation methods has been explored in several species. Initial results showed that integration of the SMG and the GOI at two different loci followed by segregation in subsequent generations was in many cases very difficult to achieve (Goldstein *et al.*, 2005). Recent experiments suggest that by using cassette DNA (in place of whole plasmid) and low quantities of DNA for bombardment, this method may be applicable for producing marker-free GM plants at efficiencies comparable to that of *Agrobacterium*-based co-transformation methods (Zhao *et al.*, 2007; Shiva Prakash *et al.*, 2009; Kumar *et al.*, 2010). Elghabi *et al.* (2011) even showed that biolistic transformation with particles coated with DNA constructs for the plastid and nuclear genomes can occasionally lead to co-transformation of the two genomes in one step. If the SMG is placed in the plastid genome, marker-free nuclear-GM plants can be generated by a simple cross using the co-transformed plant as pollen donor since plastids are maternally inherited in most plant species. These results pave the way for using the co-transformation approach in plant species that are difficult to transform via *Agrobacterium*. As noticed by some of these authors (see also Altpeter *et al.*, 2005), the possibility of using a minimal gene expression cassette (containing only the promoter, coding region, and terminator) gives biolistic transformation methods a distinct biosafety advantage over *Agrobacterium* methods for production of plants devoid of any non-essential sequences, including vector backbone sequences.

2. *Site-specific recombination*

Site-specific recombination is a type of genetic recombination occurring between two defined sites in the DNA with sequence homology and involving a recombinase protein. This recombinase protein recognizes and binds to short DNA sequences containing palindromes, at which it cleaves the DNA. Depending on the relative orientation and location of the recombination target sites, excision, inversion, or integration of DNA fragments occur (for a recent review, see Hirano *et al.*, 2011). Site-specific recombination systems have been tested and implemented into transformation protocols of several plant species for different purposes. These include conditional recombinase-mediated gene regulation (Joubès *et al.*, 2004; Tremblay *et al.*, 2007), site-specific integration of a single copy of foreign genes into well-defined chromosomal loci aiming at more stable and reliable gene expression (Wang *et al.*, 2011), side-by-side integration of a number of genes leading to plants with exchangeable multiple traits (Gidoni *et al.*, 2008), transgene confinement (Mlynarova *et al.*, 2006; Luo *et al.*, 2007), and marker gene elimination (Ow, 2002; Lyznik *et al.*, 2007; Wang *et al.*, 2011). In the specific context of the production of marker-free GM plants, directly oriented recognition sequences are present on both sides of the SMG. Once the transformed plant cells have been selected through the corresponding marker, the recombinase protein is expressed in the cells, allowing excision of the SMG which then undergoes cellular degradation. Although several site-specific recombination systems are available, three of them have been used more extensively:

- The Cre-*lox* system from bacteriophage P1. It is the most extensively used in eukaryotes, including plants. It has been developed many years ago as a genetic tool to control site-specific recombination events in genomic DNA (Sternberg and Hamilton, 1981; Sauer, 1987; Sauer and Henderson, 1988). The Cre recombinase catalyzes the recombination of DNA between specific *lox*P sequences. In the context of the generation of marker-free GM plants, it has been successfully deployed in both model (tobacco, *Arabidopsis*) and crop plant species such as wheat, maize, sugar beet, potato, oilseed rape, soybean, tomato, rice, and banana.
- The FLP/*FRT* system from *S. cerevisiae*. It involves the recombination of sequences between short Flippase Recognition Target (*FRT*) sites mediated by the Flippase recombination enzyme (FLP). Recent examples have demonstrated the efficiency of this system in maize (Li *et al.*, 2010), poplar (Fladung *et al.*, 2010) and rice (Nandy and Srivastava, 2011).
- The R/*Rs* system from *Zygosaccharomyces rouxii*. A recombinase encoded by the *R* gene mediates recombination between two specific *R*s sites. The R/*Rs* system has been used for example for generating marker-free GM plants with the MAT vector system (see text box below).

In the constitutive expression approach, a GM plant containing a SMG (flanked by recognition sequences) and the GOI is generated and the gene expressing the recombinase protein is introduced subsequently, either via a second round of transformation (Dale and Ow, 1991; Russell *et al.*, 1992; Lyznik *et al.*, 1996; Shan *et al.*, 2006) or by sexual crosses with a line expressing the recombinase protein (Chakraborti *et al.*, 2008; Li *et al.*, 2010; Sengupta *et al.*, 2010). The SMG will be excised and the recombinase gene can be eliminated by selection in the segregating subsequent generations. Excision of the SMG seems to occur less efficiently and later in development when the recombinase gene is introduced by cross-breeding than by re-transformation (Russell *et al.*, 1992). The GM maize event LY038 is an example of a GM plant approved for commercialization from which the SMG (*nptII*), originally present between tandemly oriented *lox* sites, was removed through introduction of the *cre* gene by a sexual cross (Ow, 2007; Glenn, 2007).

Elimination of the recombinase gene by segregation is problematic in vegetatively propagated plants and in plants with long generation times. Furthermore genetic and phenotypic changes can be associated with the prolonged persistence of the recombinase protein in plant cells (see below). Site specific recombination through constitutive expression of the recombinase gene is therefore less appealing than co-transformation. To circumvent these problems, transient and temporal expression approaches have been developed (reviewed in Ebinuma *et al.*, 2001; Goldstein *et al.*, 2005; Darbani *et al.*, 2007; Wogerbauer, 2007; Gidoni *et al.*, 2008).

During transient expression, the gene encoding the recombinase protein is not integrated into the plant genome. Three approaches have been described in the literature:

i. Microinjection of m-RNA or addition of purified recombinase protein, as demonstrated by Wang *et al.* (2011) and Cao *et al.* (2006), respectively.
ii. *A. tumefaciens*-mediated delivery: *Agrobacterium* can be used as a recombinase expression vector in T-DNA-dependent and T-DNA-independent manner. In the first case, the recombinase gene is placed between left and right T-DNA borders and delivered into plant cells by agroinoculation (Gleave *et al.*, 1999; Kopertekh and Schiemann, 2005). In the second case, the Cre protein may be delivered via a fusion with Vir proteins (virulence proteins of the Ti plasmid of *Agrobacterium*) that are translocated to the plant cell (Vergunst *et al.*, 2000). In both cases, the recombinase gene should not pass on to progeny; however, a significant percentage of the infected lines that undergo Cre-mediated excision may show stably integrated *cre* T-DNA (Hare and Chua, 2002).
iii. Virus-mediated delivery: more efficient SMG excision was demonstrated for virus vectors expressing recombinase (Kopertekh *et al.*, 2004; Jia *et al.*, 2007; Kopertekh *et al.*, 2012b). Viruses are eliminated during seed formation hence preventing the recombinase gene to be transmitted to the progeny.

Although transient recombinase expression allows circumventing the segregation process for the *cre* gene, one main limitation of all transient expression approaches is that an additional regeneration step is required after initial transformation to obtain the desired marker-free plant lines. This may result in increased somaclonal variation (Kopertekh *et al.*, 2012b). The necessity to create efficient agroinfiltration procedure or infectious virus vectors expressing recombinase for each plant species might also hamper an easy application of these approaches.

Temporal expression approaches provide other possibilities to reduce the duration of recombinase expression. The recombinase gene is regulated by inducible or tissue-specific promoters to allow a controlled excision of the SMG after transformed plant tissues have been selected. Several systems involving chemically inducible promoters have been developed (see e.g., Zhang *et al.*, 2006; Lin *et al.*, 2008; Ma *et al.*, 2008; Zhang *et al.*, 2009b). The most extensively described is the MAT vector developed by the team of Ebinuma in Japan (see textbox). Recently the Recombination-assisted Multifunctional DNA Assembly Platform (RMDAP) has been presented as a versatile, ready-to-use platform allowing multigene cloning and marker-free transformation (Ma *et al.*, 2011). A more sophisticated approach involves the use of a transactivator that, upon chemical induction, binds to the promoter of the recombinase gene and initiates its expression (Zuo *et al.*, 2001). Heat-shock induced expression of the recombinase gene has been applied to potato, aspen (see e.g., Cuellar *et al.*, 2006; Fladung *et al.*, 2010), and recently rice (Khattri *et al.*, 2011), apple (Herzog *et al.*, 2012) and banana (Chong-Pérez *et al.*, 2012). Cold treatment (Lu *et al.*, 2010a) and oxidative stress (Woo *et al.*, 2009) are other examples of external stimuli that have been used to induce excision of the SMG.

The expression of the recombinase gene can also be controlled by development. This can be achieved through the use of promoters that are germline-specific (Van Ex *et al.*, 2009), flower-specific (Bai *et al.*, 2008), pollen-specific (Mlynarova *et al.*, 2006; Luo *et al.*, 2007), embryo-specific (Li *et al.*, 2007), seed-specific (Kopertekh *et al.*, 2010; Kopertekh *et al.*, 2012a), or growth-phase dependent. Verweire *et al.* (2007) reported the use of promoters conferring germline functionality (male, female, or both) allowing easier selection of marker-free plants that are homozygous for the GOI. Application of germline-specific promoters allows more efficient inheritance of the recombined loci. Schaart *et al.* (2011) also developed a vector system where the recombinase activity is regulated at the level of its subcellular location rather than expression (the recombinase protein being able to enter the nucleus only after addition of a chemical).

Temporal expression is usually combined with autoexcision, in such a way that both the SMG and the recombinase gene are flanked by the recombination sites. The recombinase will excise the gene directing its own synthesis as soon as the critical level of expression required for excision is reached. Since the SMG and recombinase gene are removed in the primary transformant, the development of a marker-free plant is faster as compared with the other above-mentioned approaches, since at least one generation can be saved. The autoexcision approach offers several benefits including introduction of GOI and recombinase genes in one transformation step and possible application to both sexually and vegetatively propagated species.

Using these approaches, achieving 100% excision efficiency can be challenging, in particular when high copy numbers of GOI and SMG are inserted (Kopertekh *et al.*, 2009). Refinements of the autoexcision approach have therefore been developed. A first one involves the combination of a positive and a negative SMG (such as the *codA* gene) in the cassette surrounded by the recombination sites. This allows the initial positive selection of GM cells followed by subsequent negative selection against GM cells that have kept the marker and recombinase genes despite the action of the recombinase (Gleave *et al.*, 1999; Schaart *et al.*, 2004; Verweire *et al.*, 2007). This approach has been used for instance to develop the PROGMO vector (Kondrak

et al., 2006). It has been applied recently to produce cisgenic apples (Vanblaere *et al.*, 2011). Using another approach, Luo *et al.* (2007) showed that the use of fused *loxP-FRT* recognition sequences had the potential to dramatically improve the efficiency of FLP or Cre recombinase on gene excision (up to 100% excision under glasshouse conditions).

The MAT vector system

The MAT (multi-auto-transformation) vector system combines the use of the *ipt* or *rol* SMG (see Section II.C.2) with a marker removal system based on R/*Rs* site-specific recombination (Ebinuma *et al.*, 1997a; Ebinuma *et al.*, 1997b; Ebinuma *et al.*, 2001; Endo *et al.*, 2001; Endo *et al.*, 2002a; Ebinuma *et al.*, 2005). The *ipt* or *rol* gene is inserted between the recombination sites, together with the recombinase gene. This assembly is placed adjacent to the GOI within a T-DNA element. After *Agrobacterium*-mediated transformation, plant tissues displaying abnormal phenotype (due to the expression of the *ipt* or *rol* gene) are first selected on hormone-free medium. In a second step, normally growing shoots corresponding to marker-free plants tissues are derived from these abnormal tissues without sexual crossings. Since the recombinase gene is removed along with the *ipt* gene, recombinase expression in plant tissues is limited to a minimal period of time, thereby reducing the possibility of unwanted recombination effects. This vector system also supports recurrent transformation for the pyramiding of genes into plants.

MAT vectors have been refined over time, for instance by optimizing the expression of the *ipt* or *rol* genes, or by placing recombinase gene expression under the control of a glutathione-S-transferase promoter that is inducible by an herbicide (Sugita *et al.*, 2000a; Ebinuma and Komamine, 2001).

The MAT vector system has been successfully tested in many plant species including tobacco (Ebinuma and Komamine, 2001), hybrid aspen (Matsunaga *et al.*, 2002), rice (Sugita *et al.*, 2005), apricot (Lopez-Noguera *et al.*, 2006; Lopez-Noguera *et al.*, 2009), citrus (Ballester *et al.*, 2008), cassava (Saelim *et al.*, 2009), white poplar (Balestrazzi *et al.*, 2009), *Kalanchoe blossfeldiana* (Thirukkumaran *et al.*, 2010), tomato (Khan *et al.*, 2011b), potato (Khan *et al.*, 2011b; Khan *et al.*, 2011c) and *Petunia hybrida* (Khan *et al.*, 2011a). It shows very different levels of efficiency, and the use of the *ipt* SMG may require the optimization of transformation protocols due to changes in tissue culture conditions. This selection system appears to be more efficient in species that rely on organogenesis for *in vitro* plant regeneration than in plants that rely on embryogenesis (Scaramelli *et al.*, 2009). Nevertheless, transformed rice plants have been regenerated from embryogenic cultures using the MAT vector system (Endo *et al.*, 2002b).

Site-specific recombination approaches for generating marker-free GM plants are continuously evolving. From the technical point of view, the major issues remain the large variability in excision efficiency and the choice of the appropriate time for induction of marker removal during the transformation/regeneration procedure. This is particularly critical when elite events intended for commercialization are developed. Schaart *et al.* (2011) observed that late induction was more efficient in recovery of marker-free GM strawberries using the Cre-*lox* system. However, it is highly probable that the most suitable strategy will have to be determined on a case-by-case basis. In general, site-specific recombination is laborious and time consuming. Complete excision of the undesired sequences is influenced by several factors and has to be demonstrated case by case. The approaches relying on developmentally regulated excision might be more efficient. They allow production of marker-free plants in the progeny without the need for extra handling to activate the recombinase and to eliminate the SMG. They provide also high reliability since the efficiency with which the marker-free transgene locus is transmitted to the next generation can reach (nearly) 100% (Mlynarova *et al.*, 2006; Verweire *et al.*, 2007). However their applicability in a wide range of plant species has still to be demonstrated.

Site-specific recombination has been applied recently to combine marker excision and site-specific integration (SSI) of the GOI (Ebinuma and Nanto, 2010). The feasibility of this combined approach has been demonstrated using a combination of two site-specific recombination systems such as R/*Rs* and Cre-*lox* (Nanto and Ebinuma, 2008; Ebinuma *et al.*, 2012), or Cre-*lox* and FLP/*FRT* (Akbudak and Srivastava, 2011), or one recombination system only, e.g., R/*Rs* (Ebinuma *et al.*, 2012) or FLP/*FRT* (Fladung *et al.*, 2010). In these studies however, marker-free GM plants were obtained only after a second round of transformation. Recently, first-generation marker-free GM rice has been produced using heat-inducible Cre-*lox*-mediated marker gene excision and an improved FLP/*FRT* recombination system for SSI of the transgene (Nandy and Srivastava, 2012). Nevertheless this combined approach remains complex and requires the use of at least two different vectors and a specific line containing a single target site for transgene integration (for example, the Cre-*lox*-based "target" constructs consist of a *lox* site that serves as the site of transgene insertion). Its routine use for the development of GM plants is not yet possible.

From the biosafety viewpoint, the case-by-case assessment of marker-free GM plants developed through site-specific recombination should focus on issues related to molecular characterization. Indeed the recombination reactions with *lox*, *Rs*, and *FRT* sites are bidirectional. Because intramolecular excision recombination is kinetically favored over the intermolecular reintegration, the excised DNA is expected to undergo cellular degradation (Ow, 2002). Yet, a rare possibility of illegitimate reintegration of the excised DNA into random, non-target chromosomal sites or persistence as an extra-chromosomal circle cannot be excluded (Srivastava and Ow, 2003). It has also been suggested that recombinase expression may result in

rearrangements using cryptic-target sites in the plant genome (Miki and McHugh, 2004; Scaramelli *et al.*, 2009; Srivastava and Gidoni, 2010). Cre-mediated chromosomal rearrangements have indeed been observed in mammalian and yeast cells and could be associated with pseudo-*lox* regions showing varying degrees of sequence similarity to the *lox*P sequences (Gilbertson, 2003). In plants, cryptic *lox* sites have been described and potentially associated with rearrangements in the plastid genome when the Cre recombinase was expressed in chloroplasts (Corneille *et al.*, 2003; see also Section II.E). DNA sequences within the maize genome with limited homology to *lox*P sites have also been detected. However Ream *et al.* (2005) did not observe chromosomal aberrations in maize lines expressing Cre recombinase. Although high levels and early onset of Cre recombinase nuclear expression have shown to result in aberrant phenotypes in petunia, tomato, and tobacco, the relationship with chromosomal rearrangements has not been demonstrated (Coppoolse *et al.*, 2003). Moreover, it is known that the Cre recombinase has a very high specificity for the *lox*P sequence and it has been reported that the frequency of Cre-mediated DNA recombination is significantly reduced when only a few nucleotides are changed in specific regions of the *lox*P sequence (Lucas *et al.*, 2004). Since potential unexpected DNA rearrangements associated with site-specific recombination sites seem to occur only when high levels of recombinase are produced during prolonged period, temporal control of recombinase expression might provide a good mean to address this issue.

A related potential biosafety issue concerns the residual single *lox*, *Rs*, or *FRT* recombination site left behind in the plant genome after recombination and excision. It is unclear to what extent such site(s) can contribute to genetic instability in the plant genome. On the one hand it has been observed that prolonged or constitutive expression of the FLP or R recombinases in the presence of a corresponding target site may lead to chromosomal deletions at the level of the target site, not associated with the site-specific recombination reaction *per se* but rather with the natural DNA repair processes (Lyznik *et al.*, 2007). Recombination can also potentially occur between several residual recombination sites or between a residual recombination site and an endogenous plant sequence homologous to a recombinase site. This is the case in particular if multiple copies of the same recombination site are distributed throughout the genome as a result of transgene stacking through several marker elimination steps (Ebinuma *et al.*, 2001; Puchta, 2003; Ow, 2007). Cre-mediated site-specific recombination leading to chromosome translocation has already been observed in the plant nuclear genome (Qin *et al.*, 1994; Koshinsky *et al.*, 2000). On the other hand, the safety assessment of the lysine-fortified maize LY038 (in which the *nptII* gene was removed by Cre-mediated recombination) did not reveal any unexpected rearrangement at the molecular level across breeding generations, nor at the level of the compositional, agronomic and phenotypic properties of the GM plant (Glenn, 2007). Other available data indicate that the repeated use of the MAT vector does not cause DNA rearrangement between the first and subsequently introduced *Rs* sequences (Sugita *et al.*, 2000a). The potential for recombination involving *lox* recombination sites is also markedly diminished as the physical distance that separates them on a chromosome increases. Additionally, *lox*P sites recombine much less efficiently when they are located on unlinked chromosomal locations than when they are closely linked (Lucas *et al.*, 2004).

A solution to limit potential negative side-effects of site-specific recombination marker removal is the sequential use of different recombinases. In addition to the three site-specific recombination systems mentioned above, other systems are functional in plants and have been proposed as potential candidates for gene excision and/or integration (Ow, 2007; Wang *et al.*, 2011). These include irreversible recombination systems where the recombinase protein, also called integrase, catalyzes recombination between non-identical recognition sites. An interesting feature of these systems is that, following action of the recombinase, the recognition sites are changed. As a result, the reverse reaction cannot occur (unless a helper protein called excisionase is provided), which makes each recombination reaction unidirectional. The *Streptomyces* phiC31 site-specific integrase has been tested successfully for chromosomal DNA excision in *Arabidopsis* (Thomson *et al.*, 2010), wheat (Kempe *et al.*, 2010) and barley (Kapusi *et al.*, 2012), and for deletion of plastid marker genes in tobacco (see Section II.E). Site-specific excision can also be mediated by the integrase protein of coliphage HK022 in *Arabidopsis* (Gottfried *et al.*, 2005), and by the bacteriophage *Bxb1* recombinase in *Arabidopsis* (Thomson *et al.*, 2012) and wheat (Blechl *et al.*, 2012). Other recombination systems that may be further developed for the site-specific rearrangement of plant genomes include the recombinases CinH (Moon *et al.*, 2011), ParA (Thomson *et al.*, 2009; Zhou *et al.*, 2012), beta/six (Gronlund *et al.*, 2007), and gamma delta (Reichmann M., 2005). Although these systems belong to a family of recombinases utilizing identical recognition sites, they appear only capable of excision due to topological constraints. Therefore, an excision event mediated by these recombinases is considered irreversible. Recently, Wang *et al.* (2011) proposed a new model involving two unidirectional recombinase systems and positive and negative SMGs, allowing precise insertion of the GOI coupled with excision of unwanted DNA (including marker genes). The use of strategies aiming at modifying recombinase substrate specificities (so that recombinases recognize a new DNA target sequence) is also investigated to develop new recombinases (Hare and Chua, 2002). The utilization of these recombination systems in plants is still at an experimental stage. To our knowledge, no applications for marker gene removal in commercial GM plants have been reported yet.

3. Intrachromosomal homologous recombination

Removing SMGs can potentially be achieved by exploiting the natural nuclear recombination systems present in plants. It is based on intrachromosomal homologous recombination (HR) between two directly repeated sequences flanking the

gene to be excised. The effectiveness of this approach has been demonstrated in tobacco with the spontaneous excision of an ARMG and a negative SMG flanked by two recombination sites (*attP*) of bacteriophage λ (Zubko *et al.*, 2000; Puchta, 2000). Intrachromosomal HR is a relatively simple one-step selection procedure which does not require the expression of a heterologous recombinase, nor any sexual reproduction steps. It can therefore be used for vegetatively propagated plants. In contrast to the use of site-specific recombinases, intrachromosomal HR does not leave any "active" residual recognition sequence behind in the genome. Therefore, this approach has been proposed as an attractive alternative to site-specific recombination for marker gene removal (Scutt *et al.*, 2002; Puchta, 2003; Darbani *et al.*, 2007).

A main limitation in this approach is the low frequency of perfect re-ligation by HR relative to the frequency of mutagenic repair by non-homologous end joining (NHEJ). It is well known that HR is a minor recombination pathway in somatic plant cells and proceeds at frequencies that are several orders of magnitude lower than NHEJ (Siebert and Puchta, 2002). However these authors have shown that HR can be enhanced by the induction of double-strand breaks (DSBs) in the genome. Creation of targeted DSBs to stimulate HR can be achieved using sequence-specific nucleases (see Section II.G).

Despite these methodological improvements, the frequency at which HR occurs in somatic plant cells remains low, varies according to plant species, and the whole process is difficult to control. The mechanism by which HR occurs at target sequences in plants is not yet fully understood. The activity of such sequences as recombination sites has yet to be demonstrated in a large range of plant species (Scutt *et al.*, 2002; Goldstein *et al.*, 2005). In addition, the possibility of larger fragment deletion due to illegitimate recombination has also been reported (Zubko *et al.*, 2000; Miki and McHugh, 2004). Finally, this approach generally involves a lengthy propagation procedure to select GM cells, which may increase the risk of somaclonal mutations (Ow, 2001).

4. Transposon-based methods

Transposition is catalyzed by specific enzymes, called transposases, which recognize certain DNA sequences (in most cases inverted repeats). A DNA fragment flanked by these sequences can be "cut and pasted" in another genomic target site. The most characterized transposons belong to the *Ac/Ds* family: the *Ac* (activator) transposase catalyzes the transposition of DNA sequences flanked by the *Ds* (dissociator) elements. Transposition has three interesting features: (i) sequences inserted between the *Ds* elements are mobilized to new genomic locations; (ii) reinsertion can occur at a varying frequency in a distinct chromatid or chromosome; and (iii) transposition can be activated in *trans* by the transposase, which can therefore be introduced into the plant by a secondary transformation or by crossing with a plant expressing transposase. These features have been exploited to use the maize *Ac/Ds* transposon system in the generation of marker-free GM plants following transformation with *Agrobacterium tumefaciens*. This can be done in two different ways:

i. The GOI is inserted between the *Ds* elements. After initial transformation and in the presence of an active transposase, the gene can be repositioned to another chromosomal location, resulting in the physical separation of the GOI from the T-DNA and SMG (Goldsbrough *et al.*, 1993; Cotsaftis *et al.*, 2002). The GOI can be segregated from the SMG and the gene encoding the transposase through subsequent phenotypic and genetic selection. Since the GOI is transposed internally to the *Ds* sequences, this might lead to relocation of the transgene into another chromosomal location if the transposase is expressed in subsequent generations.
ii. The SMG is inserted between the *Ds* elements. This will lead to transposition of the SMG (and possibly the transposase) leaving only the GOI inserted into the T-DNA. This approach has the advantage that in addition to the SMG, all transposon elements are eliminated from the GM plant containing the GOI after segregation. It has been implemented in the development of the first versions of the MAT vector system (Ebinuma *et al.*, 1997a; Ebinuma *et al.*, 1997b) but was rapidly replaced by the use of the site-specific recombination R/*Rs* system (see Section II.D.2).

Elimination of the SMG can even be achieved at the T0 generation as the *Ds* elements fail to re-integrate in a small percentage of somatic tissues, therefore obviating the need for segregation and making this approach applicable to vegetatively propagated plants (Yoder and Goldsbrough, 1994). This occurs however at a very low frequency, as most of the transposable elements reinsert elsewhere in the genome shortly after their excision (Tuteja *et al.*, 2012).

Only a few other examples of the use of transposon-based methods to generate marker-free GM plants have been reported. Jin *et al.* (2003) have applied this approach in GM rice. The use of an inducible transposon system has also been described to truncate a SMG in GM rice and tobacco (Li and Charng, 2012). The limited use of this approach can be explained by a number of disadvantages, which makes it less suitable than other marker removal approaches (Chong-Pérez and Angenon, 2013). From the technical viewpoint, it requires increased labor and time when selection in segregating generations is needed to separate the GOI and the SMG. As all the methods requiring sexual segregation of SMG and GOI, it cannot be used in plants that are vegetatively propagated and is of limited use for those having a long reproductive cycle. In addition, variable rates in transposition efficiency have been observed, in particular in unlinked loci, depending on the plant species involved. Potential biosafety issues should also be considered. Mutations (sometimes at unknown loci) can be generated as a result of imprecise excision of the transposons. The continuous presence of heterologous transposons may also lead to genomic instability of GM plants. In summary, this approach appears of limited practical interest.

E. Chloroplast Transformation: Selectable Markers and Marker Removal Approaches

Chloroplast transformation is considered an interesting alternative to nuclear transformation for expressing transgenes into plants. Chloroplasts offer indeed unique advantages like high copy numbers of genomes per cell, high rates of transgene expression and protein accumulation, or no observed gene silencing. Integration of the transgene in the chloroplast genome (leading to "transplastomic" plants) occurs spontaneously by homologous recombination through homologous flanking sequences on either side of the transgene cassette, allowing a more precise control of the site of gene insertion as compared with nuclear transformation. In most flowering plants, chloroplast transformation also facilitates transgene confinement due to the lack of transmission of transgenes via pollen (plastids are maternally inherited in most plant species) (Verma and Daniell, 2007; Meyers *et al.*, 2010; Day and Goldschmidt-Clermont, 2011). More than 50 different recombinant proteins have been produced in plastids, in particular in tobacco (Scotti *et al.*, 2012; Ahmad and Mukhtar, 2013). Although reproducible protocols for plastid transformation have been described for a few species other than tobacco, including soybean, rice and cotton (Maliga and Bock, 2011), a main limitation of this approach is associated with the absence of routine plastid transformation in other major field crops.

Fewer efficient selectable markers are available for chloroplast transformation as compared with nuclear transformation. The transformation of the plastid genome was originally accomplished by using mutated versions of the plastid *16S* or *23S* rRNA genes and the plastid ribosomal protein *rps12* gene, mutations conferring spectinomycin and/or streptomycin resistance (Rosellini, 2012). With these markers, the native ribosomal gene is replaced by the antibiotic resistant variant by homologous recombination. Limitations and low transformation efficiency associated with these markers were subsequently bypassed with the use of a chimeric *aadA* gene encoding resistance to streptomycin and spectinomycin. This gene is now commonly used as selectable marker in plastid transformation (Day and Goldschmidt-Clermont, 2011). However, it is ineffective for the production of stable transplastomic plants in cereal species since cereals are endogenously resistant to spectinomycin. As alternative, genes conferring resistance to kanamycin (such as *nptII*, *neo* and *aphA-6*) can be used. The *codA* gene (see Section II.C.3) is also an effective negative selection marker (Serino and Maliga, 1997). Over the years other SMGs have been developed including the following:

- The *ASA2* gene coding for the feedback-insensitive anthranilate synthase (AS) alpha-subunit of tobacco (Barone *et al.*, 2009), allowing GM plants to grow on medium containing tryptophan analogs as the selection agents.
- The *Badh* gene from spinach allowing transformed plants to grow on medium containing betaine aldehyde, which is phytotoxic to many plant cells (Daniell *et al.*, 2001).
- The bacterial *cat* gene conferring resistance to chloramphenicol (Li *et al.*, 2011a).
- Genes encoding subunits of the photosynthetic complexes. These genes can be used as selectable markers by restoring photoautotrophy after transformation of mutant hosts (Day and Goldschmidt-Clermont, 2011).

Genes that confer resistance to herbicides, such as the *bar* and *epsps* genes described in Section II.B, were tested for selection of GM plants following chloroplast transformation. The *hppd* (4-hydroxyphenylpyruvate dioxygenase) gene from *Pseudomonas fluorescens* allowing transformants to acquire a resistance to the herbicides isoxaflutole, isoxazole and sulcotrione has also been tested successfully in tobacco and soybean (Dufourmantel *et al.*, 2007). The lethality of herbicide selection generally prevents the successful use of these markers in the initial round of selection because the genes must be established in a sufficient proportion of the plastomes to confer herbicide resistance. These genes are therefore used for secondary selection, for instance to mediate removal of an ARMG used in primary selection (Day and Goldschmidt-Clermont, 2011).

Reporter genes can be used for facilitating selection of chloroplast transformants, as reported for the *gfp* and *uidA* gene described previously (Rosellini, 2012). Chimeric genes containing the *aadA* gene (functioning as a conventional selectable marker) fused with a reporter gene have been used to optimize the identification of transplastomic plants (Khan and Maliga, 1999; Tungsuchat-Huang *et al.*, 2011).

Removal of SMGs from a transplastomic plant may be desirable for biosafety reasons, since the possibility of gene transfer to wild plants or microorganisms cannot be completely ruled out and the expression of plastid SMGs is governed by plastid regulatory elements of prokaryotic nature thereby potentially increasing the risk associated with marker gene transfer to bacteria (Pontiroli *et al.*, 2009; Pontiroli *et al.*, 2010). Other considerations include avoiding substantial metabolic load associated with heterologous gene expression (the presence of thousands of copies of the SMG per cell might use a significant amount of the host cell's resources, removing those resources away from host cell metabolism), or allowing multiple rounds of plastid transformation using the same SMG (Day and Goldschmidt-Clermont, 2011). To obtain marker-free transplastomic plants, some of the approaches described in Section D have been successfully applied (Puchta, 2003; Tian, 2007; Lutz and Maliga, 2007; Upadhyaya *et al.*, 2010; Manimaran *et al.*, 2011).

Marker removal can be achieved by homologous recombination via direct repeats flanking the SMG (Iamtham and Day, 2000; Kode *et al.*, 2006). This approach exploits the efficient native homologous recombination pathway in plastids, which excises any sequence between two directly oriented repeats. Excision of the SMG can be promoted by placing this gene inside a functional gene. Excision of the SMG concomitantly

restores the integrity of the functional gene allowing selection of marker-free plants through the gain of the corresponding function, such as herbicide resistance (Dufourmantel *et al.*, 2007). This approach has been implemented in soybean (Lestrade *et al.*, 2010). The recombination frequency depends on the length and number of direct repeats and the distance between them (Day and Goldschmidt-Clermont, 2011). Optimizing these factors is therefore key for the efficiency of the process and for obtaining rapidly homoplastomic GM cells in which chloroplast genomes are completely marker-free (for example, each tobacco cell contains approximately 100 chloroplasts harboring each up to 10,000 copies of their genome).

The site-specific Cre/*lox* system can be used for plastid marker gene removal (Corneille *et al.*, 2001; Lutz *et al.*, 2006a; Oey *et al.*, 2009). The SMG flanked with directly oriented *loxP* sites and the GOI are first stably introduced into plastids. Marker excision occurs in a second phase after expression of a nuclear-encoded plastid-targeted Cre recombinase introduced either by *Agrobacterium*-mediated retransformation or by crossing with Cre-expressing lines. The *cre* gene has to be removed from the nuclear genome by segregation once the plastid marker gene has been excised. Unintended plastid DNA rearrangements have been reported due to recombination of *lox*P sequences with fortuitous "pseudo-*lox* sites" in the plastid genome, or enhanced homologous recombination adjacent to *lox*P sites that resulted in the loss of plastidic DNA fragments between directly repeated sequences (Hajdukiewicz *et al.*, 2001; Corneille *et al.*, 2003). These undesirable effects can be (at least partly) avoided by transiently expressing the recombinase after agro-infiltration (Lutz *et al.*, 2006b). Marker removal through the use of the phiC31 site-specific recombinase has been proposed as a way to avoid unintended recombination events associated with the Cre/*lox* system (Kittiwongwattana *et al.*, 2007; Lutz *et al.*, 2007).

Co-transformation and segregation is used typically to generate marker-free plastid genomes when herbicide resistance is the trait of interest since, as mentioned above, primary selection is not possible with herbicide resistance SMG (Ye *et al.*, 2003). An ARMG and the GOI (herbicide resistance gene) are inserted in two different plasmids which after transformation give rise to heteroplasmic cells with both or either of the genes. Cells carrying both genes are first selected on antibiotic-containing media. Amongst these transformants ARMG-free segregants containing the GOI only can be isolated spontaneously when cultured on antibiotics-free media (Day and Goldschmidt-Clermont, 2011).

Klaus *et al.* (2004) have reported the generation of marker-free chloroplast transformants in tobacco using transient co-integration of the SMG. The SMG is inserted into the vector backbone outside of the flanking regions used for homologous recombination. In this configuration, integration of the SMG in the plastid genome is very unstable but sufficient to provide selection. A further round of recombination in the absence of selection results in the rapid loss of the SMG. Marker excision can be monitored through restoration of a wild-type phenotype (e.g., pigmentation) in the GM plant. The main advantage is that marker-free plants can be generated directly in the first generation (T0) without retransformation or crossing.

The chimeric *aadA* gene is by far the most common SMG used in biotechnological applications of plastid transformation. Some other markers have been used only sporadically (Scotti *et al.*, 2012; Ahmad and Mukhtar, 2013). Marker gene removal by homologous recombination is a promising approach and is presumed to suffer less technical and biosafety limitations than in nuclear transformation. However, the applicability and efficiency of marker removal approaches for the development of GM plants tested in the field or commercialized remains to be demonstrated.

F. Marker-less Transformation

Transforming without SMGs would be the ideal way to obtain marker-free GM plants. The successful recovery of GM plants without the use of SMGs has been reported for several plant species including *Arabidopsis*, potato, tobacco, lime, peanut, triticale, cassava, barley (reviewed by Manimaran *et al.*, 2011; Schaart *et al.*, 2011; Chong-Pérez and Angenon, 2013), alfalfa (Ferradini *et al.*, 2011b), apple (Malnoy *et al.*, 2010), orange (Ballester *et al.*, 2010), *Prunus* (Petri *et al.*, 2011), wheat (Liu *et al.*, 2011) and tomato (Xin and Guo, 2012). In most cases putative transformed plants were screened by Polymerase Chain Reaction (PCR) for the presence of the transgene. Selection of transformants may also be facilitated by expressing a screenable marker such as the *uidA* (GUS) or GFP gene, by challenging the putative transformants to a phenotypic property associated with the expression of the GOI (see e.g., Bai *et al.*, 2009), or based on the direct screening of GOI expression product (Rukavtsova *et al.*, 2013).

In some of the studies mentioned above, experimental refinements were applied to improve two important factors affecting the successful application of this approach, namely the efficiency of the DNA delivery method and the plant regeneration system. Such refinements include optimizing treatment conditions to promote *Agrobacterium*-mediated transformation, e.g., via vortex-mediated transformation of cold-treated seedlings (Rosellini and Veronesi, 2007; Weeks *et al.*, 2008), using *A. tumefaciens* strains exhibiting extremely high transformation efficiency (de Vetten *et al.*, 2003), or improving plant tissue culture. Some of these refinements exploit the progress made in recent years in identifying plant proteins and other factors involved in the transformation process and characterizing the precise molecular mechanism leading to successful integration of the T-DNA (Anand *et al.*, 2010; Barampuram and Zhang, 2011).

Marker-free transformation via the pollen-tube pathway (exogenous DNA is taken up by the egg cell or zygote after fertilization via pollen-tube pathway, and then integrated into the host genome at mitosis) is widely used in China and has been successfully applied in several crops including cotton, rice, wheat, maize (Yang *et al.*, 2009b), soybean (Yang *et al.*,

2011) and melon (Hao *et al.*, 2011). One marker-free *Bt* cotton produced by this method is available on the market. This method does not need tissue culture and allows the introduction into plants of minimal linear gene cassettes devoid of selectable markers and vector backbone sequences, although with low transformation frequency. Higher transformation frequencies have been obtained in maize using the ovary-drip method, a variant of the pollen-tube pathway method involving the complete removal of the styles and the subsequent application of a DNA solution directly to the ovaries (Yang *et al.*, 2009a). Aziz and Machray (2003) have suggested that marker-free generation of GM plants should be possible through the biolistic introduction of gene constructs into unicellular microspores, followed by *in vitro* development of these microspores to mature pollen subsequently used for pollination.

In spite of continued improvements, recovery range of transformed events without the use of SMGs remains very variable (between 1 and 25%) and at least two or three fold lower than the range obtained using ARMGs. Therefore, with the exception of a few specific cases (e.g., Bhatnagar *et al.* (2010) who developed marker-free GM peanut plants with 75% transformation efficiency), a large number of GM transformants is required to select lines that harbor only the GOI. Moreover, because of the lack of selection against non-GM tissue, chimeric plants that only partly consist of genetically modified tissue can be obtained (Li *et al.*, 2009; Joshi, 2010).

G. New Breeding Techniques

During the last decade several new breeding techniques (NBTs) have been developed including oligonucleotide-directed mutagenesis (ODM), RNA-dependent DNA methylation (RdDM), cisgenesis/intragenesis, grafting on GM rootstocks, reverse breeding, agroinfiltration and nuclease-based techniques (Lusser *et al.*, 2011; Lusser *et al.*, 2012; Curtin *et al.*, 2012). The NBTs can be discussed in terms of SMGs in two aspects. The first aspect considers the application of NBTs as a general way to minimize the presence of non-plant DNA sequences, particularly ARMGs, in the final plant/product released in the environment or commercialized. This occurs with reverse breeding, grafting and cisgenesis/intragenesis. Reverse breeding utilizes SMGs only at an intermediate step. Grafting allows avoiding SMGs in some parts of the plant such as fruit, seeds or flowers. Application of cisgenesis/intragenesis makes use of SMGs of plant origin (Sections II.A and II.C), or reduces the presence of SMGs through marker gene removal (Section II.D) or marker-free transformation (Section II.F). The second aspect considers the direct implementation of NBTs as a tool to avoid/eliminate heterologous marker genes in GM plants. This applies to ODM, RdDM and nuclease-based techniques. The latter includes zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), homing endonucleases (reviewed by Voytas, 2013) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). This aspect is illustrated by three main examples.

The first example refers to modified plant cells that are resistant to antibiotics without expression of heterologous antibiotic resistance genes. Aufsatz *et al.* (2009) have used RdDM technology to down-regulate a putative chloroplast transporter *At5g26820* gene that takes part in kanamycin transport into chloroplasts. *Arabidopsis* was transformed with an inverted repeat construct containing the predicted chloroplast transit sequence of the *At5g26820* gene. Plants with RNAi-mediated suppression of this gene displayed kanamycin resistance. Such transporter homologs exist in other plant species suggesting that this approach might be also useful for a broad range of plants. Potential risks associated with gene knockdown using RdDM technology have to be carefully assessed on a case-by-case basis since silencing effects are highly dependent on precise experimental conditions and unintended off-target effects cannot be excluded.

The second example refers to the selection of plants using specific traits, such as herbicide or disease resistance, conferred by endogenous plant genes mutated through ODM or nuclease-based techniques. The possibility of using chimeric oligonucleotides to induce site-specific mutations in plants was first shown by Beetham *et al.* (1999). An amino acid substitution was introduced at the codon encoding Pro-196 of acetolactate synthase (*ALS*) gene by bombardment of tobacco cells with self-complementary chimeric oligonucleotides. In this study regeneration of herbicide resistant plants was not achieved and alteration of the *ALS* gene was identified in cell lines growing on herbicide containing medium by sequencing analysis. Kochevenko and Willmitzer (2003) used an oligonucleotide-mediated strategy to create single point mutations at two different positions within the tobacco *ALS* gene. Stable transmission of chlorsulfuron resistance from cellular to the whole-plant level was confirmed by Mendelian inheritance of mutations in T1 progeny. ODM has been also applied to crop species. Imidazolinone resistant maize plants were engineered through oligonucleotide-mediated targeted modification of the acetohydroxyacid synthase (*AHAS*) gene (Zhu *et al.*, 2000). The modified *AHAS* gene was transmitted to the next generation and exhibited the expected phenotype. The feasibility of ODM technology to mutate an endogenous herbicide-sensitive gene product was also shown for rice (Okuzaki and Toriyama, 2004). Chimeric DNA/RNA nucleotides have been used to target the Pro-171, Trp-548 and Ser-627 codons of the rice *ALS* gene. ZFNs have been used to introduce herbicide resistance into the plant genome. Townsend *et al.* (2009) demonstrated that ZFN-stimulated targeting of tobacco acetohydroxyacid synthase (*SurA* and *SurB*) genes resulted in imidazolinone and sulfonylurea resistance phenotypes. Tobacco plants regenerated from herbicide resistant calli carried *surA* and *surB* mutations. TALENs offer also interesting perspectives in that respect (Mahfouz and Li, 2011). TALEN-based gene editing has been successfully applied to produce disease-resistant rice (Li *et al.*, 2012). Zhang *et al.* (2013) have recently developed a methodology to alter

endogenous *sur* loci using TALENs resulting in herbicide resistant tobacco cells.

The third example refers to the application of NBTs for marker gene elimination by homologous recombination. As mentioned in Section II.D.3, sequence-specific nucleases can be used to induce double-strand breaks (DSBs) in the genome (reviewed by Yau and Stewart, 2013). The SMG is flanked at the 5' and 3' ends by nuclease cleavage sites. The nuclease introduces two DSBs at the recognition sites. Following re-ligation of DSBs the SMG can be excised. Proof of concept for this approach was provided for ZFNs and homing endonucleases. The use of ZFNs to create DSBs to increase the frequency of homologous recombination was first demonstrated by Wright *et al.* (2005) in tobacco. Petolino *et al.* (2010) have also described a successful application of ZFN-mediated transgene deletion. Two types of transgenic tobacco plants harboring a target *gus* and ZFN constructs respectively have been designed, then *gus*-expressing plants were crossed with ZFN-expressing plants. The *gus* gene elimination was observed and confirmed by sequencing in T1 progeny. Siebert and Puchta (2002) showed that a SMG flanked by sites of the rare cutting endonuclease I-SceI could be excised efficiently from the tobacco plant genome with increased HR frequencies following expression of I-SceI in the plant. Antunes *et al.* (2012) gave another example of successful utilization of custom-made nucleases for marker gene removal. An engineered homing endonuclease derived from the I-CreI endonuclease excised a *bar* SMG flanked by enzyme recognition sequences for the enzyme from transgenic *Arabidopsis* plants. Application of designed nucleases for gene deletion would have several advantages in comparison to recombinase-mediated marker gene excision: (i) DNA excision does not require pre-insertion of target sites into the genome and (ii) no additional target sites are left behind in the genome. A novel class of genome engineering tools that might be useful for SMG elimination was recently discovered. It is based on the RNA-guided Cas9 nuclease from the Type II prokaryotic CRISPR system. CRISPR-mediated genome modification exploits cell DNA repair pathways that are initiated by the DSBs at specific sites. The Cas9 enzyme can be programmed to cleave DNA at any site defined by guide RNA sequence without the need to engineer a new protein for each DNA target sequence. The potential of the CRISPR-Cas9 system for genome editing and targeted gene mutation in plants has been reported in several recent papers (Jiang *et al.*, 2013; Li *et al.*, 2013b; Mao *et al.*, 2013; Miao *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013; Xie and Yang, 2013).

NBTs provide new approaches for the development of marker-free GM plants. However these techniques have some technical limitations. The mutation frequencies reported for ODM and nuclease-based techniques are usually low and depend on numeral factors such as plant species, design of the oligonucleotides (ODM), nucleases, target sequence, chromatin and DNA methylation, developmental stage of the plant cells, delivery of genetic material and selection methods (Kochevenko and Willmitzer, 2003; Okuzaki and Toriyama, 2004; Townsend *et al.*, 2009). An additional drawback of nuclease-based techniques is incomplete marker gene excision (Petolino *et al.*, 2010; Antunes *et al.*, 2012). Another limitation is a non-precise genome modification associated with unspecific base alterations or nuclease cleavage (Petolino *et al.*, 2010; Antunes *et al.*, 2012). Non-specific base alterations were also reported for ODM-mediated site-specific mutagenesis (Kochevenko and Willmitzer, 2003). ZFNs and TALENs can cleave DNA at genomic loci that share a certain degree of homology with the target site potentially leading to cytotoxic effects. Transient nuclease expression through the use of inducible promoters (Marton *et al.*, 2010; Mahfouz and Li, 2011), or direct delivery into cells of nuclease-encoding mRNA or the nuclease proteins themselves, has been presented as a way to reduce these unintended effects (Voytas, 2013). Outcrossing the mutant lines with wild-type plants is also a mean of segregating unwanted genomic alterations.

III. ISSUES ASSOCIATED WITH INTELLECTUAL PROPERTY RIGHTS

Intellectual property rights (IPRs) associated with plant transformation technologies represent a potential limitation for developing GM plants. Free use of a proprietary technology in non-commercial research is generally granted, but their use for commercial purposes is considered patent infringement (Chi-Ham *et al.*, 2012). This is also true for SMGs and techniques for removal of marker genes. IPRs from the perspective of developing or commercializing GM plants, including the impact on innovation and diffusion of technologies, have been reviewed and discussed by several authors (see, e.g., Mayer *et al.*, 2004; Dunwell, 2005; Roa-Rodriguez, 2003; Yin and Zhang, 2010; Rommens, 2010a; Dunwell, 2011; Chi-Ham *et al.*, 2012). In agricultural biotechnology the public sector represents a substantial source of IPRs (24% according to Graff *et al.*, 2003) characterized by a high degree of fragmentation of technology ownership across numerous institutions. In addition, an increasing shift toward patenting of research outputs has been observed in plant biotechnology research (Michiels and Koo, 2008). Although the role of patents in disseminating scientific knowledge remains a contentious issue, patents are now a significant source of information about R&D developments which is complementary to data published in "traditional" peer-reviewed journals.

Information about patents can be retrieved from freely available patent databases in Europe (http://www.epo.org/ and http://www.espacenet.com), the USA (http://www.uspto.gov/patft/index.html), the World International Patent Organization (http://patentscope.wipo.int/) and other international sites (e.g., http://www.google.com/patent; http://www.pat2pdf.org; http://www.freepatentsonline.com; http://www.patents.com/). A very useful site is the Patent Lens (http://www.patentlens.net/), a core activity of the CAMBIA's BIOS (Biological Innovation for Open Society) initiative. This initiative focuses on attempts to free the basic technology tools of biotech

for general use and promotes a protected commons license for use in this regard (Dunwell, 2011).

Reviewing patent data is complex, due to the diversity of patent offices and procedures, different routes to file for patent protection (national or international), and different (sometimes overlapping) scopes, status and dates in patent documents (grants, international phase, etc.). This paper does not aim at presenting a detailed review of all patent applications related to SMGs and approaches for their removal. It rather presents a few examples to illustrate that their use beyond academic research requires a complete and case-by-case assessment of an increasingly complex IPRs landscape.

The most widely used SMGs in plant genetic engineering to date, i.e., *nptII*, *hpt*, *epsps* and *pat/bar*, have been or are still largely protected by patents held by companies such as Monsanto, Syngenta or Bayer CropScience (Mayer *et al.*, 2004; Roa-Rodriguez, 2003; Chi-Ham *et al.*, 2012). The IPRs landscape is however changing all the time due to the expiration of key patents or the recording of new ones. For instance, Monsanto held until 2008 an extensive and international patent family for antibiotic-based resistance systems, but almost all Monsanto's patents in that field expired.

The use of the two most extensively described positive SMGs in the scientific literature is patented: (i) the PMI system, marketed by Syngenta (Positech® technology, Schiermeier, 2000), and (ii) the *ipt* gene (see e.g., Keller *et al.*, 2000), including its use in the MAT vectors (Nippon Paper Industries Co. Ltd, Sugita *et al.*, 2007). Other positive SMGs are patent-protected (see, e.g., http://www.patentlens.net/daisy/PositiveSelection/1787.html), including At-*WBC19* (Stewart and Ayalew, 2011), *xylA* (Morawala and Rajyashri, 2007), or *dhps* (Hildebrand and Rao, 2009). Searching patent databases also reveals positive SMGs not described in the scientific literature such as the *tmt* gene allowing selection in the presence of potassium thiocyanate or potassium iodide (Saini *et al.*, 2005), or esterase-encoding genes allowing selection in the presence of non-ionic, fatty acid ester detergents (Gabriel and Reddy, 2008). In contrast, a few marker genes are not patented, such as *Gsa* and *hemL* (Rosellini, 2012).

Patents also cover the use of reporter genes (Bidney *et al.*, 1998). Next to genes a number of selection approaches have been subject to patent protection, as illustrated by the following examples covering marker removal after co-transformation (Bidney and Scelonge, 2000), site-specific recombination (Sugita *et al.*, 2007) or transposition (Central Research Lab, 2005), the use of the phiC31 or other new recombinases (Ow *et al.*, 2004; Ow and Thomson, 2006), marker removal from the plastid genome based on Cre-*lox* (Maliga *et al.*, 2007) or homologous recombination (Lestrade *et al.*, 2010), or marker-less transformation (Wolters *et al.*, 2003).

In response to the growing significance and complexity of IPRs issues in plant biotechnology, a number of public sector initiatives have been developed to facilitate access to legal information and provide assistance in negotiating licenses (Stewart, 2005). Besides the CAMBIA's BIOS initiative mentioned above, the patent 'pool' approach of the non-profit organization PIPRA (Public Intellectual Property Resource for Agriculture) includes the development and broad availability of plant transformation platforms that integrate technical, regulatory and freedom-to-operate considerations (Boettiger and Bennett, 2007; Chi-Ham *et al.*, 2012). The first transformation platform was based on a standard T-DNA vector where the trait and *nptII* marker gene are in a single construct. Other platforms based on alternative SMGs or marker-free approaches such as transposon- or co-transformation-based methods, are also under development. The transformation platforms include license-options to support translational research for commercial deployment or humanitarian use for developing countries. "Freedom-to-operate" can also be granted on a case-by-case basis by patents owners for certain GM products. One of the well-known examples relates to the Golden Rice and the use of Syngenta's PMI system (Al-Babili and Beyer, 2005).

IV. CURRENT USE OF ARMGs AND ALTERNATIVE SELECTION APPROACHES IN GM PLANTS

The number of references in scientific papers and/or in patent databases for a specific selection approach (i.e., using a specific SMG, reporter gene, marker-removal method or no selectable marker) does not necessarily reflect its actual use in applied research or commercialized products. Indeed many approaches are still in a proof-of-concept phase and usable only for basic research. In order to get a better picture of the current use of selection approaches for *in vitro* selection of GM plants intended for field research or commercial introduction, we performed (i) a review of approaches based on some public databases listing information on GM plants authorized for commercialization and on field trials notifications, and (ii) a survey conducted through interviews with academics and companies actively involved in the development, testing and commercialization of GM plants.

A. GM Plants Authorized for Commercialization

To collect the most accurate information on GM plants approved for commercialization worldwide, we have consulted the CERA's database of safety information (http://ceragmc.org/), the Biosafety Clearing-House (http://bch.cbd.int/), the ISAAA database of biotech/GM crop approvals (http://www.isaaa.org/) and the OECD Biotrack products database (http://www2.oecd.org/biotech/default.aspx). From 1992 to 2012, a total of 319 GM events including 212 single and 107 stacked events have been identified, corresponding to 24 plant species (Table 2). Although stacked events usually do not involve the use of SMGs for their development, they have been included in the analysis because they still contain the marker genes used for selection of the corresponding single events. It is important to note that not all the events approved for commercialization have been placed on the market.

For each GM event, the selectable marker(s) present in the GMO or the marker-free selection approach used was identified

TABLE 2
GM plant species approved for commercialization (1992–2012)

Plant host	Number of events	% of events
Alfalfa (*Medicago sativa*)	3	0.9
Bean (*Phaseolus vulgaris*)	1	0.3
Carnation (*Dianthus caryophyllus*)	15	4.7
Chicory (*Cichorium intybus*)	3	0.9
Cotton (*Gossypium hirsutum*)	48	15.1
Creeping Bentgrass (*Agrostis stolonifera*)	1	0.3
Flax, Linseed (*Linum usitatissimum*)	1	0.3
Maize (*Zea mays*)	122	38.4
Melon (*Cucumis melo*)	2	0.6
Oilseed rape (*Brassica napus*)	30	9.5
Papaya (*Carica papaya*)	4	1.2
Plum (*Prunus domestica*)	1	0.3
Poplar (*Populus sp.*)	2	0.6
Potato (*Solanum tuberosum*)	31	9.8
Rapeseed (*Brassica rapa*)	4	1.2
Rice (*Oryza sativa*)	7	2.2
Rosa (*Rosa hybrida*)	2	0.6
Soybean (*Glycine max*)	22	6.9
Squash (*Cucurbita pepo*)	2	0.6
Sugar beet (*Beta vulgaris*)	3	0.9
Sweet pepper (*Capsicum annuum*)	1	0.3
Tobacco (*Nicotiana tabacum*)	2	0.6
Tomato (*Lycopersicon esculentum*)	11	3.5
Wheat (*Triticum aestivum*)	1	0.3
TOTAL	**319**	**100**

(Table 3). For stacked events, all SMGs present in the corresponding single events were considered.

As shown in Table 3, herbicide resistance SMGs are present in 61.4% of the GM events approved for commercialization, the *epsps* and *pat* gene being the most commonly used. In a huge majority of these events the SMG also serves as GOI. The *surB* gene is used mainly in GM carnation (15 single events). One third (106) of the GM events approved for commercialization contain an ARMG that was used for plant selection. Except for five of them, all contain the *nptII* gene. In addition, 37 events contain an ARMG that was used for bacterial selection in vector construction. Such ARMG are not found in single events commercialized since 2003 but are still present in stacked events commercialized after that time. Amongst the events containing an ARMG used for bacterial selection, 15 do not contain ARMGs used for plant selection. In total, ARMGs are therefore present in 121 (106+15) of the GM events (38%) approved for commercialization.

The *manA* gene (PMI system) is the only alternative SMG used. It is present in four single GM maize events, MIR162, MIR604, 3272 and 5307, and in several corresponding stacked events. The *uidA* (GUS) reporter gene is found in 14 events, including 9 single events (papaya, plum, soybean and sugar beet), where it was used in most of the cases in combination with the *nptII* gene. The reporter genes *DsRed2* and *nos* are found in maize and flax, respectively.

Marker-free GM plants account for only 2.8%. They correspond to nine events obtained using the following approaches:

- Co-transformation and segregation: Cotton COT67B, soybean MON87701, maize MON89034 and maize MON810. The latter has been developed by co-transformation with a vector containing the *cryIAb* and *nptII* genes, and a vector containing the *CP4 epsps*, *gox* and *nptII* genes. This second construct was lost through segregation during the crossing leading to line MON810. Although initially linked to the GOI, the *nptII* gene of the first plasmid is not found in MON810, which is therefore marker-free.
- Pollen-tube pathway: Cotton SGK321.
- Loss of the SMG during development of the GM event: Squash ZW20.
- No stable integration of the SMG in the genome: Potato SPBT02-5.
- Cre-*lox*-mediated site-specific marker gene elimination: Maize LY038.
- Stacked event not containing selectable marker: Maize MON810 × LY038.

Figure 1 shows that the type of SMG has evolved over the years. With regards to single GM events, ARMGs were the most commonly used SMGs in the 143 events approved for commercialization between 1992 and 2002 (43.4%), followed by herbicide resistance SMGs (41.3%). The use of ARMGs has decreased afterwards. Between 2003 and 2012, 23.2% of the 69 single events approved for commercialization contained an ARMG (Figure 1A): four cotton, two papaya, one plum, one poplar, two Rosa, three potato, two rice and maize MON87460. In contrast, 56.5% of the single events approved in the same period contained herbicide resistance genes. The four single GM maize events containing the *manA* gene were also approved during that period. In the last decade, the number of stacked events approved for commercialization has increased considerably (in particular GM maize): 143 single events *vs*. 6 stacked events in the period 1992-2002, and 69 single events *vs*. 101 stacked events in the period 2003-2012. As shown in Figure 1B and Table 3, these stacked events mostly contain herbicide resistance marker genes and to a lesser extent, the *manA* gene.

B. Field Trials with GM Plants in the EU and in the United States

Since concerns about the use of ARMGs in GM plants have been predominantly expressed in Europe, we checked

TABLE 3
Application of selection approaches in GM plants approved for commercialization

Selection approach	Number and percentage[1] of GM events	Number and percentage[2] of single events
Antibiotic resistance	**106 (33.2)**	**88 (41.5)**
nptII (kanamycin)	101 (31.7)	85 (40.1)
aph (hygromycin)	5 (1.6)	3 (1.4)
Herbicide resistance	**196 (61.4)**	**98 (46.2)**
epsps (glyphosate)	98 (30.7)	32 (15.1)
gat (glyphosate)	6 (1.9)	3 (1.4)
pat (glufosinate)	90 (28.2)	21 (9.9)
bar (glufosinate)	24 (7.5)	19 (9.0)
*surB/csr1-2/*modified ALS enzyme (sulfonylurea and imidazolinone herbicides)	21 (6.6)	20 (9.4)
bxn (bromoxynil)	2 (0.6)	2 (0.9)
aad-1 (2,4-D)	1 (0.3)	1 (0.5)
Other selectable markers	**46 (14.4)**	**4 (1.9)**
manA (PMI, mannose-based selection)	41 (12.9)	4 (1.9)
Reporter genes	**16 (5.0)**	**11 (5.2)**
uidA (GUS)	14 (4.4)	9 (4.2)
DsRed2	1 (0.3)	1 (0.5)
nos (nopaline synthase)	1 (0.3)	1 (0.5)
Marker-free	**9 (2.8)**	**8 (3.8)**
Co-transformation and segregation	4 (1.3)	4 (1.9)
Excision through site-specific recombination	1 (0.3)	1 (0.5)
Marker lost	2 (0.6)	2 (0.9)
Other	1 (0.3)	1 (0.5)
Stacked event without selectable marker	1 (0.3)	
Unknown selectable marker	**9 (2.8)**	**9 (4.2)**
Markers used for bacterial selection present in GM plant	**37 (11.6)**	**29 (13.7)**
aad (streptomycin/spectinomycin)	23 (7.2)	16 (7.5)
bla (ampicillin)	11 (3.4)	10 (4.7)
nptII (kanamycin)	4 (1.3)	4 (1.9)

Note. The second column refers to the absolute and relative number of GM events (single and stacked) where a specific marker gene is present or a marker-free selection approach was used. The third column refers to the absolute and relative number of single events.

[1]percentages of 319 total events.

[2]percentages of 212 total single events.

how far these concerns are reflected in the type of selection approach used in GMOs recently tested in the field in the EU. To this end we collected data from the GMO Register of the European Commission Joint Research Center (http://gmoinfo.jrc.ec.europa.eu/), the so-called "SNIF database." The database was analyzed for the period 2010 to 2012, covering a total of 169 notifications representing 16 different plant species. The large majority of the notifications referred to maize (47%), followed by sugar beet (14%), cotton (13%), potato (9%), and poplar (4%). Other species included flax and tobacco (four notifications each), barley (three notifications), soybean and wheat (two notifications each) and apple, *Arabidopsis*, crambe, plum, rice and triticale (one notification each). Notifications do not necessarily correspond to approved field trials or to completed field trials, and can also represent multi-year field trials.

Figure 2 shows that 73.4% of the notifications have been submitted by private companies (14 in total), Monsanto, Bayer CropScience, Pionneer Hi-Bred and Syngenta being the most important ones. The rest

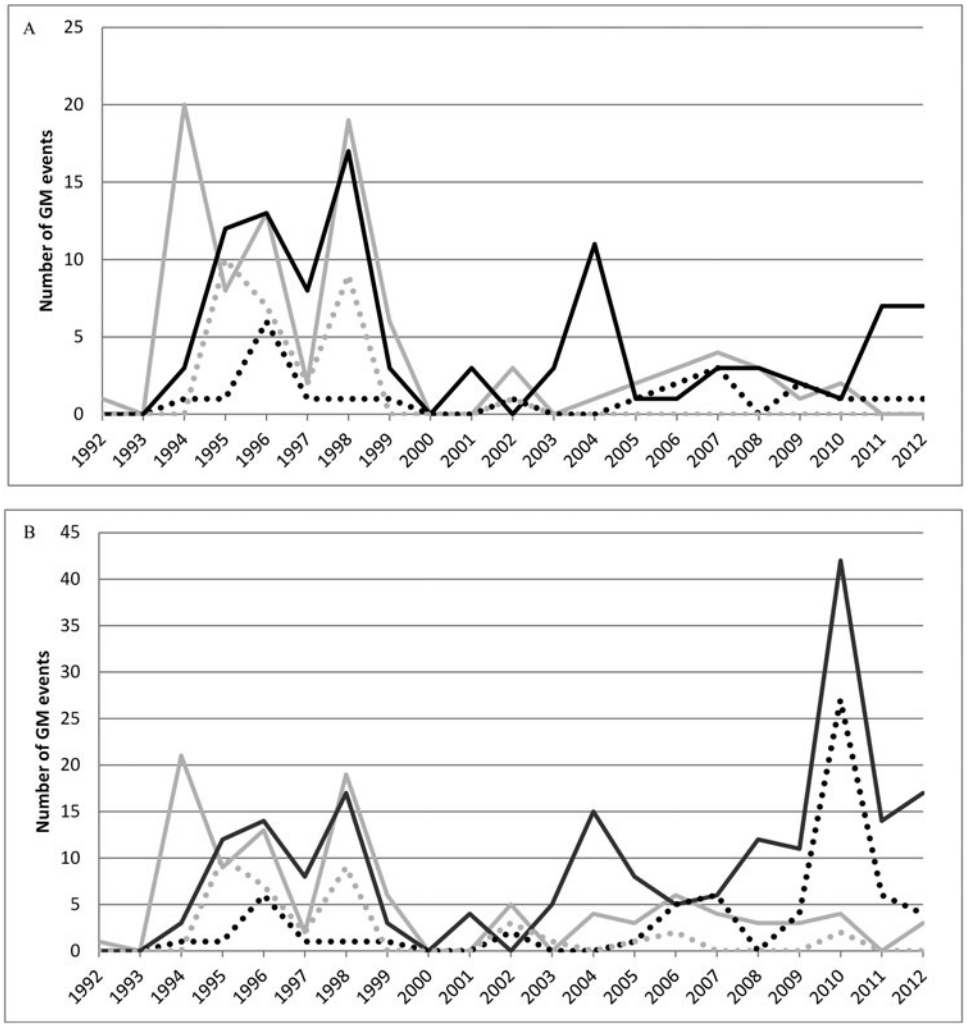


FIG. 1. Evolution of the use of SMGs or selection approaches between 1992 and 2012, in single events approved for commercialization (A), and in all GM events approved for commercialization (B). Grey filled lines: Events containing an ARMG for plant selection Grey dotted lines; Events containing an ARMG for bacterial selection; Black filled lines: Events containing an herbicide resistance SMG; Black dotted lines: Events containing another SMG; a reporter gene or marker-free.

falls on 28 different institutions from the academic sector.

For each notification, the selectable marker or marker-free selection approach used to develop the GMO was identified (Table 4). Only SMGs used for the selection of plant transformants and present in the final GMO were considered. Markers used for bacterial selection and still present in the GM plant were not considered since it was not always possible to clearly deduce their presence from the available data. A notification may include several GM plants and/or GM plants containing several selectable markers. When stacked events were involved, all SMGs used for the corresponding single events and present in the stacked event were considered. In consequence, one notification may correspond to more than one SMG and therefore the total number of SMGs considered in our analysis exceeds the number of notifications.

Of the field trials notified, 19.5% involve GM plants containing at least one ARMG. All the occurrences of the *nptI, nptII*, *hpt*, and *hyg* genes most probably correspond to different GM single events (30 in total), although it was sometimes difficult to unambiguously identify the event based on the available information. The three occurrences of the *aadA* gene correspond to two GM tobacco events where the SMG was used for plastid transformation. All these 32 single events correspond to GM plants not approved for commercialization. The event containing the *aph4* gene corresponds to the commercial GM cotton COT102. Looking at the distribution amongst institutions of the 33 notifications involving at least one ARMG, it appears that 23 of them (70%, all involving non-commercial events) have been applied by the academic sector, suggesting that ARMGs are still extensively used in this context.

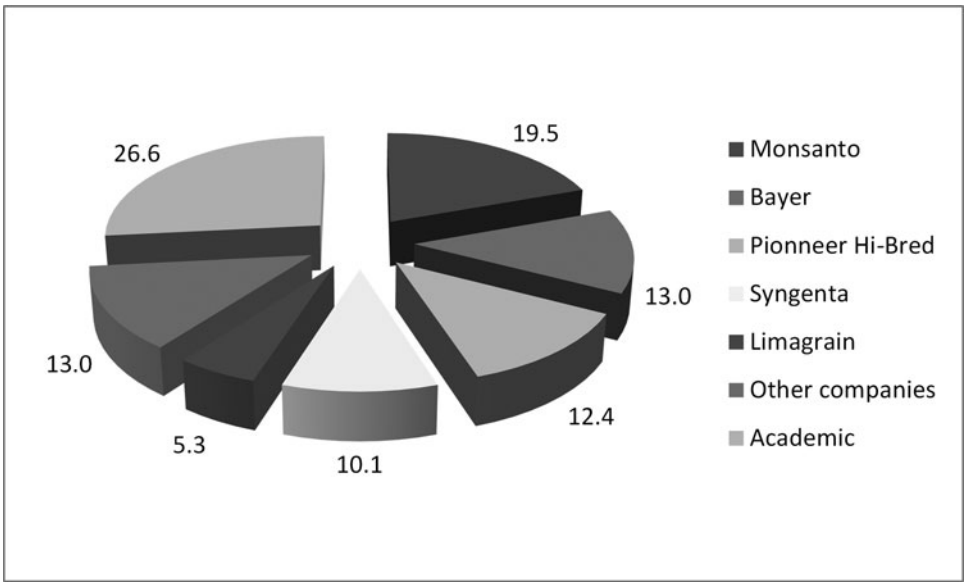


FIG. 2. Distribution (%) per institutions of the 169 field trial applications notified in the EU in the period 2010–2012.

TABLE 4
Selection approaches with and without SMGs in GM plants notified for field trials in the EU in the period 2010–2012

Selection approach	Number and corresponding percentage[1] of notifications	Number of corresponding single events
Antibiotic resistance	**33 (19.5)**	
nptI (kanamycin)	1 (0.6)	1
nptII (kanamycin)	21 (12.4)	21
hpt/hyg (hygromycin)	8 (4.7)	8
aph4 (hygromycin)	6 (3.6)	1
aadA (spectinomycin/streptomycin)	3 (1.8)	2
Herbicide resistance	**127 (75.1)**	
AHAS (imidazolinone)	3 (1.8)	1
bar (glufosinate)	23 (13.6)	11
pat (glufosinate)	24 (14.2)	4
epsps (glyphosate)	94 (55.6)	9
gat (glyphosate)	1 (0.6)	1
sul (sulfadiazine)	1 (0.6)	1
undefined gene	2 (1.2)	2
Other selectable markers	**12 (7.1)**	
manA (PMI, mannose-based selection)	12 (7.1)	3
Marker-free	**12 (7.1)**	
Co-transformation and segregation	3 (1.8)	3
Excision through site-specific recombination	1 (0.6)	1
Transformation without marker	3 (1.8)	1
MON810	2 (1.2)	1
Undefined selection method	3 (1.8)	3

Note. The second column refers to the absolute and relative number of notifications. The third column reports the number of single events for each SMG or marker-free approach.

[1]percentages of 169 total notifications.

Herbicide resistance SMGs are by far the most commonly used: 75.1% of the notifications involve GM plants containing one or several of these genes. This corresponds to a limited number of single events (29) since many of these notifications involve stacked events. Eleven of the single events are GM plants approved for commercialization in which the herbicide resistance gene also serves as GOI, i.e.,

- Cotton T304-40 and GHB119 harboring the *bar* gene.
- Maize TC1507, DAS-59122-7 and Bt11 harboring the *pat* gene.
- Maize NK603, MON88017 and GA21, sugar beet H7-1 and cotton GHB614 harboring *epsps* genes.
- Maize 98140 harboring the *gat* gene.

The 18 other single events containing herbicide resistance genes correspond to non-commercial events, suggesting that these SMGs are used much less frequently than ARMGs (18 *vs.* 32) in non-commercial GM single events tested in the field.

The *manA* gene (PMI system) is the only alternative SMG used in the notified GM plants. It is present in one rice event and two commercial events, sugar beet SBVR111 and maize MIR604.

Field trials involving marker-free GM plants account for 7.1% of the notifications. They correspond to a small number of single events:

- Cisgenic barley PAPhy07, GM flax and GM maize MON89034 developed by co-transformation followed by independent segregation of the SMG and the GOI.
- Cisgenic apple developed by site-specific recombination involving the R/*Rs* system (after initial selection by positive and negative SMGs).
- GM potato AV43-6-G7 developed without using any SMG (selection by PCR analysis).
- Maize MON810.
- Three marker-free GM potatoes for which it was not possible to determine the selection approach.

These quantitative data should be considered with caution given the limited number of events involved. However, they provide some insights on the current selection approaches used in GM plants tested in the field at the EU level. To get a broader picture, we have collected data from field trials with GM plants conducted in the United States, the major developer of agricultural GM plants. For this purpose, information was retrieved from the database of applications for permits for field testing of GMOs in the United States (http://www.isb.vt.edu/search-release-data.aspx), maintained jointly by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) and Virginia Tech. In order to get the largest view on the use of selection approaches in the R&D of GM plants, the database was analyzed for both applications notified and permits released in the period 2010–2012, independently of whether a field trial has actually been carried out or not. Applications that were denied, incomplete or withdrawn by the applicant were dropped from the analysis. In total 2113 field tests have been considered, involving 70 different plant species, mainly maize (39%), soybean (24%) and cotton (7%). Figure 3 shows that almost 80% of the applications have been submitted by private companies (46 in total), Monsanto having the lion's share. The rest falls into 52 different institutions from the public sector, representing mainly US universities but also non-profit institutions (e.g., PIPRA) and the USDA.

Given the high number of records, a detailed individual analysis of each application with regards the selection approach used was not performed. Information was extracted from the APHIS database by searching from the "gene" criteria, using relevant keywords listed in the database. In particular the database was analyzed for the most commonly used ARMGs in GM plants as well as all SMGs mentioned in Section II. When possible this information was complemented by an additional search using relevant keywords from the "phenotype" criteria. Results are presented in Table 5. They should be considered taking into account some limitations inherent to the APHIS database, in particular the limited information available for certain genes or traits, the occasional impossibility to identify whether a specific gene was used as SMG, to determine exactly which GM event(s) were involved per application, or to distinguish single and stacked events, and the absence of controlled vocabulary in the keywords of the search criteria (meaning that retrieving the global situation on the use of a specific SMG or selection approach implied using a combination of many different keywords).

15.7% of the applications involved GM plants containing at least one ARMG. Resistance to kanamycin or hygromycin accounts for most of the cases. This situation is comparable to that observed for field trials in the EU (Table 4). Looking at the distribution amongst institutions of applications involving ARMGs (Figure 4), it appears that 64% of them have been notified by the public sector, suggesting that ARMGs are still extensively used in this context. Once again, this figure is very similar to the one observed in the EU.

As for EU field trials, herbicide resistant GM plants are by far the most frequently present in field trial applications notified in the United States. 978 applications (46%) were retrieved from the database when using the "phenotype category" criteria and the "HT (Herbicide Tolerance)" keyword. Due to the limitations mentioned above, it was not possible to perform a quantitative analysis of the use of genes conferring resistance to herbicides as SMGs. In many cases information on HT genes is labeled confidential and/or insufficient to determine if a gene was actually used as SMG. The available information indicates that genes conferring resistance to glyphosate (*epsps*, *gat*), glufosinate (*bar*, *pat*), sulfonylurea/imidazolinone (*als*) or dicamba are the most commonly used. A few other genes are found in a limited number of applications, i.e., genes

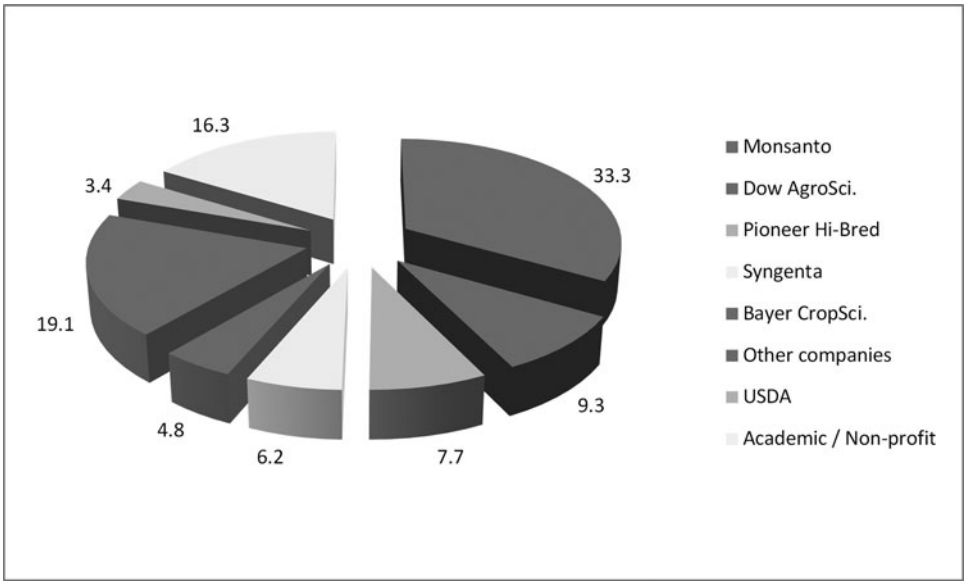


FIG. 3. Distribution (%) per institutions of the 2113 field trial applications notified in the USA in the period 2010–2012.

conferring resistance to isoxaflutole and isoxazole, *TUB1* (alpha-tubulin), *sul* (resistance to sulfonamides) and *PDS* (phytoene desaturase).

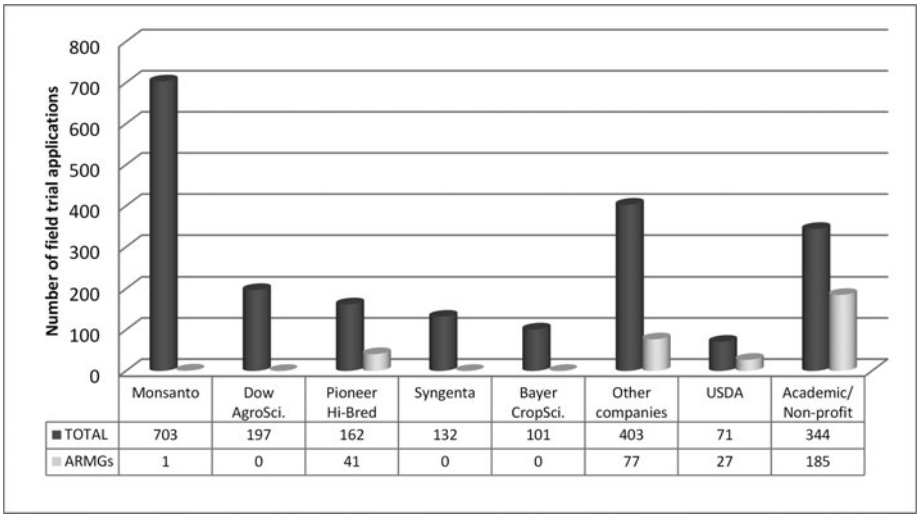
When the database was checked for the possible use of other genes as selectable markers (using all relevant keywords in the "gene" or "phenotype" criteria corresponding to the SMGs mentioned in Section II), only a few applications were recovered: 4 referring to the *manA* gene (PMI), 24 to the *ipt* gene, one to the *codA* gene and one to the *DREB2A* gene. It was impossible to clearly determine whether these genes have been used in the selection of transformed cells, considering that most of these applications also refer to the use of an ARMG. Although these results might be an underestimation of the real situation, they seem to indicate that very few alternative SMGs are used in GM plants tested in the field in the United States.

Nine applications (0.4%) were clearly identified as involving marker-free GM plants, obtained either using the Cre-*lox* system (three applications), the FLP/*FRT* system (one application), both systems (one application), the *Ac/Ds* transposition approach (one application) or an undefined approach (three applications).

TABLE 5
Selection approaches with and without SMGs in GM plants notified for field trials in the US in the period 2010–2012

Selection approach	Number of applications (*n*) and corresponding percentage (n*100/2113)
Antibiotic resistance	**331 (15.7)**
nptII, aph(3')II / Resistance to kanamycin	233 (11.0)
aph4, hpt, hyg / Resistance to hygromycin	109 (5.15)
Resistance to gentamycin	5 (0.25)
bla / Resistance to ampicillin	1 (0.05)
Resistance to streptothricin	1 (0.05)
Herbicide resistance	**Undetermined**
Other selectable markers	**Undetermined**
Marker-free	**9 (0.4)**
Excision through site-specific recombination	5 (0.25)
Excision through transposition	1 (0.05)
Undefined selection method	3 (0.15)

Note. The second column refers to the absolute and relative number of applications in the APHIS database where a specific SMG or selection approach was identified based on relevant search keywords.



	Monsanto	Dow AgroSci.	Pioneer Hi-Bred	Syngenta	Bayer CropSci.	Other companies	USDA	Academic/ Non-profit
TOTAL	703	197	162	132	101	403	71	344
ARMGs	1	0	41	0	0	77	27	185

FIG. 4. Number of field trial applications in the USA notified by public institutions and private companies between 2010 and 2012. The black columns refer to the total number, and the grey column to applications involving GM plants containing at least one ARMG.

C. Surveys of Current Approaches Used for *In Vitro* Selection of GM Plants

In order to substantiate the findings of the literature and database reviews and get more insight in the underlying practical, scientific and/or regulatory arguments supporting the choice of selection approaches, two surveys were carried out between September 2012 and January 2013. For the first survey participants were selected amongst applicants involved in a field trial with a GM plant in the EU between 2010 and 2012. For the second survey the main plant breeding/biotech companies and other GM plant developers were contacted (Table 6).

The surveys were conducted mainly through interviews by phone based on a questionnaire serving as a guide. The questionnaire partly contained prepared options/multiple choice and partly open questions. The interviews included questions related to the interviewed person and institution, the selection approach used, the considerations for using this approach or for not using other approaches (ease of use, effectiveness, cost-efficiency, IPRs, biosafety and regulatory constraints, public perception) and general questions about the use of ARMGs or alternative selection approaches in GM plants. Due to the limited number of interviewees, the information provided was analyzed only qualitatively. The results are presented in an aggregate form.

1. *Applicants involved in a field trial with a GM plant in the EU between 2010 and 2012*

Thirty-three organizations involved with field trial applications in the EU between 2010 and 2012 (see Section IV.B) were contacted. Twenty of them provided information, covering mainly public institutions (15 respondents) and to a lesser extent private companies (5 respondents). Although recent field trial notifications were selected, they do not always represent recently developed material. Therefore, for some of the GM events considered, the selection approach was reflecting considerations relevant at the development time and not necessarily today's developer policy.

The use of selection approaches amongst the interviewees was as follows: ARMG (10 interviewees, mainly using *nptII*), herbicide resistance SMG (8), screenable markers (4, always in combination with a SMG), PMI selection system (2), and marker-free GM plants (4, namely co-transformation, site-specific recombination and transformation without using any SMG).

The eighteen interviewees using ARMGs or herbicide resistance SMGs indicated the ease of use and effectiveness as important drivers (the main driver for thirteen of them) for the choice of these selection approaches. Cost efficiency was the next important consideration. In contrast, biosafety aspects, regulatory constraints, public perception and IPRs had less influence or even (for almost half the respondents) did not influence the choice at all. Two interviewees using SMG had considered initially not using any marker gene for selection. They finally opted for a SMG because identifying GM plants without selection was considered too time-consuming and not efficient enough. For the six interviewees using marker-free approaches or a SMG different from antibiotic or herbicide resistance marker genes, all the above-mentioned considerations had usually influenced the choice to a certain extent. Biosafety aspects and public perception were indicated more often as an important driver, followed by regulatory constraints and effectiveness. For most of the interviewees using model or non-food plants, public perception was not at all considered for choosing the selection approach.

The interviewees were also invited to indicate their views regarding general statements about the use of ARMGs or alternative selection approaches in GM plants. They had also the

TABLE 6
List of organizations contacted for surveys 1 and 2

Organization	Survey 1	Survey 2
Aarhus University	X	
ABBA Gaia, S.L.	X	
African Agriculture Technology Foundation		X
Agricultural Research Service - United States Department of Agriculture		X
Agritec, Research, Breeding & Services, Ltd.	X	
BASF	X	X
Bayer	X	X
Centro Nacional de Biotecnología-CSIC	X	
Commonwealth Scientific and Industrial Research Organisation (CSIRO)		X
Danforth Center		X
Dow AgroSciences		X
Empresa Brasileira de Pesquisa Agropecuária (Embrapa)		X
Florigene/ Suntory Holdings Limited		X
Fruit Research and Development Station Bistrita	X	
Idén Biotechnology S.L.	X	
Institute of Experimental Botany AS CR	X	
Instituto de Agrobiotecnología, Universidad Pública de Navarra/ Consejo Superior de Investigaciones Científicas	X	
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)	X	
International Potato Center (CIP)		X
International Rice Research Institute (IRRI)		X
KWS		X
Limagrain	X	X
Maharashtra Hybrid Seeds Company (Mahyco)		X
Monsanto	X	X
Pioneer Hi-Bred	X	X
Plant Breeding and Acclimatization Institute, National Research Institute	X	
Rothamsted Research	X	
Stichting Dienst Landbouwkundig Onderzoek (DLO)	X	
Swedish University of Agricultural Sciences	X	

(Continued on next page)

TABLE 6
List of organizations contacted for surveys 1 and 2 *(Continued)*

Organization	Survey 1	Survey 2
SweTree Technologies AB	X	
Syngenta	X	X
Teagasc	X	
The Sainsbury Laboratory	X	
Transactiva srl	X	
Umeå University	X	
Universidad de Málaga	X	
Universidad Politécnica de Madrid	X	
University of Ghent	X	
University of Leeds	X	
University of Lleida	X	
University of Rostock	X	
University of Wroclaw	X	
VIB (Vlaams Interuniversitair Instituut voor Biotechnologie)	X	
Warsaw University of Life Sciences	X	

possibility to provide open comments on these general issues. The results provided in Table 7 must be regarded as individual contributions and cannot serve to draw general conclusions. They provide however a useful snapshot of opinions, keeping also in mind that most of the interviewees belong to public institutions.

A large majority of the interviewees shared the opinion that there are no scientific reasons supporting the full prohibition of ARMGs as selectable marker in GM plants, although most of them could agree with the exclusion of ARMGs providing resistance to last-resource antibiotics. To substantiate these views, it was mentioned that ARMGs are present in microbes in nature, even in plasmids that can easily be mobilized. Moreover, horizontal gene transfer from plant to bacteria and subsequent stabilization is a very rare event, which makes the overall risk negligible. It was also noted that the safety of the use of ARMGs such as *nptII* had already been positively assessed by EFSA and other official bodies. Although it was recognized that potential risks associated with ARMGs should be assessed on a case-by-case basis taking into account the level of exposure (i.e., small-scale research trial *vs*. commercial cultivation), regulations should ensure that GM plants carrying ARMGs that have been safely assessed can be authorized. Broadly speaking potential health or environmental risks associated with ARMGs were not considered a main trigger to shift to alternative selection approaches.

With regards to regulatory aspects, a majority of the interviewees agreed that it was an important factor in deciding to use alternative selection approaches. It was mentioned that any limitations or restrictions on GM plants containing ARMGs in one jurisdiction should be consistent with policies of other countries, and consistent with WTO rules. This was felt particularly critical for the EU since it is highly dependent on imports on some GM commodities. It was also noted that prohibiting the use of ARMGs in GM plants might confer a competitive advantage for larger companies that would use patent rights to limit access to new selection approaches.

Public acceptance was perceived as trigger for not using ARMGs. However it was mentioned that regulatory decisions should be more driven by science-based consideration than by public perception. Moreover, the use of ARMGs was not seen as the main obstacle to public acceptance of GM crops in Europe.

Technical issues related to the species one is working with might limit the choice of selection approaches. In case it would be possible to use alternatives, half of the interviewees answered that they would choose the alternative. It was stressed that this should be considered case-by-case, depending on the available techniques for the particular plant at the time of event creation, the intended use and some of the considerations mentioned above. It was also mentioned that the use of marker removal approaches was difficult to envisage when several events are tested simultaneously in the field. Most of the respondents considered that even if alternative techniques were advanced, some stakeholders would also question their use.

2. *Main plant breeding/biotech companies and other GMO developers*

Seventeen companies and international research institutes were contacted for the survey. An effort was done to identify developers who had used selection approaches identified in the literature review as the most promising alternatives to the use of ARMGs. Seven of them provided information, covering three

TABLE 7
General statements about the application of ARMGs or alternative selection approaches in GM plants

Statement	Number of respondents		
	Fully agree	Neutral	Disagree
1. There are no scientific reasons to abandon the use of ARMGs as selectable marker in GM plants.	16	3	1
2. It is acceptable to exclude certain ARMGs, e.g., those providing resistance to last-resource antibiotics, from use as selectable marker in GM plants.	13	4	3
3. Potential health or environmental risks associated with the presence of ARMGs in GM plants is a good trigger to use alternative selection approaches.	1	9	9
4. Regulatory problems associated with the presence of ARMGs in GM plants is a good trigger to use alternative selection approaches.	11	3	5
5. Week public acceptance of the presence of ARMGs in GM plants is a good trigger to use alternative selection approaches.	8	6	5
6. I would use an alternative selection technique to ARMGs if this would be possible in the species that I work with.	9	9	1
7. As alternative techniques are advanced, some stakeholders will also question their use as well.	13	7	0

private companies and four public institutions. The interviewees indicated they were using ARMGs (5 respondents, *nptII* and/or *hptII*), herbicide resistance SMGs (4), as well as the PMI selection system (2). The most popular reporter genes, namely *gusA* and *gfp*, were also used by two respondents.

One interviewee from a private company declared to have a policy on the use of selection approaches, namely to have no ARMGs in commercial crops. When such markers were used for the initial selection of GM events, removal strategies were subsequently applied, either co-transformation followed by segregation of the marker gene from the GOI or marker excision via site-specific recombination.

Three public institutions considered not using a selectable or screenable marker, the reason being public perception (3) and regulatory constraints (2). They eventually declined the idea for reasons of easiness (3), effectiveness (3), use of SMGs for similar GM plants (3) and cost-efficiency (1). The same respondents also considered removing the SMG from the final product, mainly because of public perception. Two removal strategies were specified: co-transformation followed by segregation, and marker excision via inducible site-specific recombination. The SMG was eventually left in the GM plant either because the gene has a function in further product development (identification in back-crossing programs), or in the final product (herbicide resistance), because of time constraints or because of technical considerations.

Overall, reasons for choosing a selectable marker are effectiveness (6), ease of use (5), precedents in similar use with this approach (5), cost-efficiency (3), and freedom to operate (2). Only one of the respondents from a private company mentioned biosafety concerns, regulatory constraints and public perception as one of the elements driving their choice. Others indicated that the *nptII* gene is safe and has a history of regulatory approval throughout the world. One research institution explained that alternative, non-GM methods were deployed to incorporate the same traits as their previously developed GM crops, thereby avoiding the controversy.

In the course of this survey, a private company informed that, regarding the policy for using selection techniques in Europe, CropLife International member companies agreed to take active steps to limit the use of ARMGs and explore alternatives to ARMGs in new product developments. For products already on the market the phasing-out should follow the product's life cycle.

V. DISCUSSION AND PERSPECTIVES

ARMGs have been extensively used in the development of GM plants of the first generation. The *nptII* gene was present in the FLAVRSAVR tomato, the first GM plant commercialized in 1992, and in a large number of GM plants approved for commercialization afterwards, in particular until the end of the last century. From a technical perspective, the effectiveness and efficiency of ARMGs, in particular the *nptII* gene, have been demonstrated in a large number of plants species, by means of reproducible protocols. These advantages overcome some drawbacks, most of them being associated with all positive selection systems, such as the regeneration of escapes, growth retardation of the transformed cells owing to long-time exposure to stringent selection, or impaired regeneration of the transformed cells due to release of toxic substances by dying cells. As shown in our survey, ease of use and effectiveness are the main reasons

advanced by GMO developers for using ARMGs as selection system.

The use of ARMGs in GM plants has always been a controversial issue, particularly in the EU. Despites this controversy a few GM plants containing ARMGs have still been approved for commercialization between 2000 and 2012. ARMGs are also present in several GM plants recently tested in the field, including in the EU. Our survey seems to indicate that potential safety concerns raised with ARMGs like *nptII* do not impede their use as SMGs, at least in GM plants tested in the field. Several GMO developers and users consider that the safety of the *nptII* gene is well documented and assessed. Biosafety considerations are not regarded as a main trigger to shift to alternative selection approaches although they have influenced at least partly the people using such alternative approaches. This could reflect the fact that potential risks associated with the use of the *nptII* gene in GM plants are based on theoretical assumptions and that no substantiated cases of adverse impact on human health or the environment have been documented so far.

On the other hand, decisions for field trials or commercialization of GMOs, especially in the EU, are not based on scientific evidence and understanding only but also on political constraints, public concern and underlying emotions. For policy-makers risk perception is at least equally important than the risk itself. This could also influence GMO developers. Our survey seems to indicate that regulatory constraints and public perception associated with the use of ARMGs can be important factors in deciding to use alternative selection approaches. An illustration of this can be the *Golden Rice* Project. Although the initial selection of the transformants was made using an ARMG, the latest GM events have been developed free of a marker gene, using either co-transformation or the PMI selection system in order to address public perception reasons (http://goldenrice.org).

Against this background the European Commission has insisted to step up efforts to phase out ARMGs in GM plants. Our review shows that more than fifty other SMGs and dozens of protocols allowing the development of marker-free GM plants have been described in the literature. However, in practice, a few of them have been used for developing GM plants tested in the field or commercialized. This raises questions as to what extent these selection approaches represent feasible alternatives to ARMGs and what could be the trigger(s) for moving from ARMGs to alternative selection approaches.

Herbicide resistance marker genes represent the most commonly used alternative to ARMGs. They share most of the technical advantages of ARMGs and are widely used in GM plants. However this situation is due to the fact that they mostly also function as GOI. Potential safety and agronomic concerns mentioned in Section II.B might limit their use as alternatives to ARMGs for selecting GM plants where herbicide resistance is not the trait of interest.

The *manA* gene (PMI) is the only other SMG that has been used for developing commercialized GM plants so far. Although its potential as SMG has been demonstrated in many plant species and its safety positively assessed, it is found in a limited number of GM single events, most probably due to its patent protection. IPRs represent indeed a potential limitation for using alternatives to ARMGs in commercial applications. However researchers should be able to carry out scientific work on any SMG as long as it is not for direct commercial purposes. Most selection systems are patented and their availability in specific countries will depend whether patent coverage was sought or not. A few freely available SMGs exist and freedom-to-operate can be envisaged via initiatives of the public sector or licensing with patent holders.

Several other positive SMGs described in the literature offer interesting features. However none of them can compete with the ease of use, efficiency and versatility of ARMGs, at least in the short term. Successful transformation protocols are in most cases only available for model plant species such as tobacco and *Arabidopsis*. Their performances in terms of transformation and selection efficiency vary widely and do not always reach the efficiency of antibiotic- or herbicide-based selection systems. Transformation efficiencies using positive selection systems have been shown to depend strongly on the selection pressure, meaning that the most efficient concentration of the compound or condition used for selection should be determined case-by-case, a time consuming exercise. The wide use of some positive SMGs can also be hampered by the need to use expensive or highly toxic selection compounds (e.g., $HgCl_2$ used in combination with the *merA* and *merB* genes). Positive SMGs confering to plant cells the ability to autonomously produce growth regulators must be used in more elaborated selection approaches involving excision and/or transient expression of the SMG. In particular, the use of the *ipt* gene has been extensively reported in the literature, e.g., in the MAT vectors. Although this selection approach was developed more than 15 years ago and successfully applied in several plant species, it has never been used in GM plants intended for marketing.

One important aspect that could limit the use of other positive SMGs relates to the fact that for all of them except the PMI system, the safety assessment has not progressed yet to the level of the antibiotic resistance and herbicide resistance marker genes. Most of the positive SMGs are involved in endogenous metabolic pathways and detrimental effects on the host plant have already been reported in some cases. Depending on the intended use of the GM plants containing such markers, data still need to be collected regarding the safety of the introduced protein, the impact of its expression on the overall plant metabolism, nutritional, and compositional characteristics, the consequences on human or animal health, and the potential ecological impacts. In that respect, genes involved in one biosynthetic and non-regulatory step only, within a single metabolic pathway, might be considered as ideal SMG candidates. Using SMGs of plant origin has also been proposed as a way to bypass some biosafety concerns while being compatible with the development of cisgenic or intragenic plants.

Selection approaches based on removal of the SMG offer a powerful solution to obviate concerns associated with the use of ARMGs and to remove technical limitations for plant re-transformation. Amongst the several approaches described, co-transformation is the oldest and probably the simplest. It has been applied to date to four commercialized GM single events. Its successful application is tied with having an efficient transformation system, which is typically a difficulty for some crops, and with reaching high level of segregation. As illustrated in this review many variations of the co-transformation approach have been and are still developed to overcome the drawbacks. The use of site-specific recombinases is a more complicated approach, which was implemented only in one commercial GM event. In this case also technical limitations observed in initial studies are addressed through continuous new innovations.

Compared with established selection approaches like ARMGs, experimental protocols for marker removal are more complex and have to be adapted on a case-by-case basis, which increases the production time for marker-free GM plants. This might hamper their easy and routine application especially in R&D activities where many GM lines are developed within larger programs. For commercial applications, a crucial aspect in all marker-removal approaches is the appropriate point of time to remove the SMG, since it is generally useful to keep a SGM for further back-crossing during the selection process. Moreover, several of these approaches are typically applicable to sexually propagated plants, but much less efficient in species that are vegetatively propagated and/or have a long reproductive cycle, which includes many important crops like potato, apple, grapevine, strawberries, cassava, and banana, as well as poplar and eucalyptus. However, extensive efforts are being invested by researchers to develop approaches that are efficient, rapid, precise, applicable to many plant species and transformation methods, and not requiring sexual reproduction steps. This includes self-excision recombinase-based marker gene removal approaches and the application of site-specific nucleases.

Biosafety concerns might curb the shift to marker removal approaches, in particular those involving site-specific recombinases or nucleases. As illustrated in this review, the potential for chromosomal rearrangements or alterations could make the case-by-case risk assessment of the corresponding GM plants more complex than those containing a SMG with a safe profile.

Using no SMG at all is of course the simplest approach to generate marker-free GM plants and to minimize the presence of inserted nucleic acid(s) sequences as well as associated biosafety concerns. From the experimental viewpoint some of the protocols have proven successful, such as the production of commercial *Bt* cotton via the pollen-tube pathway method or GM potato AV43-6-G7 via *Agrobacterium*-mediated transformation. The main limitation of marker-free transformation is the very low recovery rate of transformants. It does not seem practical at a large-scale for introducing functional genes of agronomic interest in most agriculturally-important plant species, unless the efficiency of transformation protocols is much improved. It is probably more realistic to envisage a case-by-case application of this approach for plants that are recalcitrant to the classical transformation and selection processes, and for vegetatively propagated plants.

Some of the new breeding techniques described above, i.e., mutagenesis directed through oligonucleotides (ODM) or nucleases, are also inherently designed to avoid the use of SMGs. Their efficiency remains however low and possible negative side-effects associated with nuclease-based techniques are still a matter of discussion. Refinements are expected in the coming years to provide tools with high-level control of genetic modification.

Regulatory requirements in the European Union for phasing-out ARMGs in GMOs have undoubtedly contributed to stimulate further development and application of alternative selection approaches. This is particularly the case for companies and institutions involved in the commercial development of GM plants, for which the phasing-out of ARMGs may help to improve public confidence in the long term. This rationale may be less relevant for GM plant developers involved in R&D only who primarily want to use the "easiest/best/cheapest" selection approach. Our analysis of the applications for field trials in the EU and in the United States shows indeed that ARMGs are still extensively used in public institutions.

Shifting from ARMGs to other approaches is not a clear choice for GM plant developers. ARMGs remain a very attractive selection approach. For most of the alternatives, time and effort are still required to attain practical efficiency and their implementation in commercial GM plants seems difficult to envisage in the short term. It has been estimated that the time from the initiation of a GM plant development to commercial launch is 13.1 years on average (McDougall, 2011). Moreover, avoiding ARMGs will be almost impossible in GM plants consisting in stacks of previously approved products containing ARMGs. Limiting or restricting GM plants containing ARMGs in the EU will probably have little influence on GM plant development in the rest of the world since decisions on the design of commercial GM plants are often taken outside Europe and most of the research and development has now moved outside Europe.

In the end, since biosafety concerns are the main reason advocated for phasing-out ARMGs, they should also be the main trigger for performing further research and to guide the choice of selection approaches for the development of GM plants in the future. Replacement of selection approaches that make use of ARMGs such as *nptII* will make sense only if the new approaches have at least the same degree of scientific knowledge and safety confidence.

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