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INVITED REVIEW

Selectable marker genes from plants: reliability and potential

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Abstract Selectable marker genes (SMGs) are still useful to efficiently obtain transgenic plants, although marker-free techniques are available, but with limitations. The presence of SMGs, especially bacterial antibiotic resistance genes, in transgenic crops is criticized. Fortunately, several genes isolated from plants are available that can serve as SMGs. Here, I review the plant genes reported to have been used as SMGs. Some are wild-type genes that, when overexpressed, confer a selective advantage during *in vitro* plant regeneration, whereas some are mutated genes encoding enzymes resistant to inhibitory chemicals. Most of the genes have not yet been tested in a significant number of species. The effect of SMGs expression on the phenotype has often been superficially examined and should be better characterized. The sequence conservation of some SMGs could allow derivation of a SMGs from any plant species, if an intragenic or cisgenic approach to genetic engineering is preferred. I conclude that several promising SMGs have been isolated from plants, allowing avoidance of bacterial genes for transformation, transgene stacking, and intragenic or cisgenic engineering approaches. Nonetheless, further testing in more plant species would be useful to fully assess phenotypic neutrality, efficiency, and versatility. Patent rights restrict the immediate use of most plant SMGs for commercial applications, but freely available marker systems do exist.

Keywords Antibiotic resistance · Biotechnology acceptance · *In vitro* selection · Plant genetic engineering · Transformation efficiency

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Introduction

In plant genetic transformation, a small fraction of the cells exposed to the transformation treatment become stably transgenic, and, consequently, *in vitro* selection is necessary to efficiently produce transgenic plants. Selectable marker genes (SMGs) make it possible by conferring to the transformed plant cell a selective advantage with respect to the non-transgenic cells: either resistance to a phytotoxic substance, the ability to utilize an unconventional carbon source, or the capacity to regenerate in the absence of growth regulators (reviewed by Miki and McHugh 2004).

The conventional SMGs are antibiotic resistance genes from bacteria. They are efficient, and there is general agreement on their safety in transgenic plants and in food (Ramessar *et al*. 2007). In particular, the neomycin phosphotransferase II (*npt*II) gene, present in many commercially grown crops, has been thoroughly scrutinized for safety (EFSA 2007 and references therein). However, the acceptance of bacterial antibiotic resistance SMGs by the general public and the regulatory agencies is problematic (Ramessar *et al*. 2007).

Marker-free transgenic plants can be obtained in several ways. Some reports of 'marker-less' transformation, that is, introduction of only the useful gene(s), followed by regeneration without selection and screening of the regeneration events to find the transgenic ones, are available in the literature (de Vetten *et al*. 2003; Popelka *et al*. 2003; Doshi *et al*. 2007; Jia *et al*. 2007). Despite a few recent reports of efficient marker-less transformation protocols in some species (Weeks *et al*. 2008; Bhatnagar *et al*. 2010), it appears that marker-less transformation cannot be routinely applied due to the time and money required by large-scale screening of many regeneration events. For example, we

have obtained transformation efficiencies lower than 11% in alfalfa without selection (unpublished).

Marker elimination techniques allow the use of SMGs for efficient transformation, with subsequent removal of the SMG from the transgenic plants. Several methods are available (reviewed by Miki and McHugh 2004). These techniques require the preparation of relatively complex genetic constructs and rely on microbial recombination systems, which when expressed in the plant genome may cause chromosomal alterations and phenotypic aberrations (discussed in Srivastava and Gidoni 2010).

So called 'intragenic' and 'cisgenic' genetic engineering approaches have been proposed to improve public acceptance and ease the authorization process of Genetically Modified Plants. The first approach uses genetic elements from sexually compatible species that are assembled with conventional gene cloning methods (Nielsen 2003). The second approach is limited to the isolation of genomic fragments containing one or more genes with their native regulatory sequences and their insertion into sexually compatible species (Jacobsen and Schouten 2007). Both these techniques do not admit the use of conventional SMGs, but may employ plant-derived SMGs.

Several plant genes have just recently been tested as SMGs that may overcome the limitations discussed above. In this review, we describe and critically evaluate plant-derived SMGs. All of them are 'positive markers', that is, they confer a selective advantage to transgenic cells *in vitro*, either through resistance to a phytotoxic chemical or by replacing growth regulator supplementation of the culture media. Wild-type or mutated plant genes have been used as SMGs, and we will deal with these two categories separately. Tables 1 and 2 summarize the characteristics of all the SMGs. Plant genes that can serve as reporters but do not confer a selective advantage to transgenic cells (Simmonds *et al*. 2004; Kim *et al*. 2010) will not be reviewed here.

Wild-type Plant SMGs

The WBC*19* gene, encoding an ATP-binding cassette (ABC) transporter, was identified as a potential kanamycin resistance SMG during screening of *Arabidopsis thaliana* knock-out mutants that showed kanamycin susceptibility *in vitro* (Mentewab and Stewart 2005). It was expressed in tobacco under the CaMV35S promoter (hereafter named 35S). The maximum efficiency of the *WBC19* gene alone (about 40%), obtained at a kanamycin concentration of 100 mg L^{-1}, was significantly higher than with *npt*II alone (about 20%). The mechanism of resistance is probably based on active transport of kanamycin into the vacuole.

This gene was recently implemented in transformation of hybrid aspen (*Populus tremula x P. alba;* Kang *et al*. 2010). Although data were not replicated, efficiency and escape percentage appear to be comparable to those obtained with *npt*II. In aspen, but not tobacco, *WBC19* conferred resistance to other aminoglycoside antibiotics. A report on *Atwbc19*-based transformation of muskmelon appeared recently (Bombale *et al*. 2010).

In the Author's lab, a transformation experiment has been performed in alfalfa with the original *WBC19* vector (unpublished results), but no transgenic plants were obtained. It is possible that overexpression of *WBC19* has species-specific features that may prevent its wide use as an SMG, as also suggested by the narrow- and wide-resistance to aminoglycoside antibiotics that it confers to tobacco and aspen, respectively.

According to Burris *et al*. (2008), if horizontal gene transfer to bacteria were to occur from a transgenic plant, a low level of kanamycin resistance would result, so *WBC19* may be preferable to *npt*II. On the other hand, the use of this SMG may need to be restricted to non-food species, due to the non-specificity of ABC transporters, which belong to the large group of Multiple Drug Resistance (MDR) transporter proteins that can recognize multiple hydrophobic molecules (Conte and Lloyd 2011). It has been hypothesized that overexpression of *WBC19* may result in accumulation of heavy metals, herbicides, fungicides, or pesticides in vacuoles, depending on the concentration of such chemicals in the soil (Rommens 2006). The same caveat potentially applies to all the genes encoding MDR proteins, with the possible exception of apoplast secretion proteins. The use of these genes as SMGs can be problematic.

Nitrite reductase (NiR). Nitrate is the main source of nitrogen for plant tissue culture. It is first reduced to nitrite, and then to ammonium by the plant cells. Because nitrite can be toxic, the efficiency of nitrite reduction can influence the ability of the cells to proliferate and regenerate.

In rice, the *NiR* genes, encoding a ferredoxin nitrite reductase, were isolated from genotypes that show high and low regeneration capacity through map-based cloning (Nishimura *et al*. 2005; Ozawa and Kawahigashi 2006). Differences in both the coding and promoter sequences and in the expression levels were found, with much higher transcription in the highly regenerating genotypes.

The NiR genes of highly regenerating varieties were introduced into the agronomically valuable variety Koshihikari, which has very low regeneration capacity. Either the native or a constitutive promoter was used to drive the expression of NiR. In both cases, abundant regeneration was obtained in non-selective conditions, demonstrating the value of NiR as an SMG. The transformation efficiency was different in the two studies: Ozawa and Kawahigashi (2006), introducing the whole genomic fragment containing the gene, obtained low

Table 1. Plant-derived wild-type selectable marker genes

Gene	Source	Promoter	Selection	Plant species transformed	Transf. Efficiency%[a]	Escape%	Associated phenotype	References
BADH	*Spinacia oleracea*	Tobacco 16S rRNA	Betaine aldehyde	*N. tabacum* (Biolistic, plastid)	250[c](+)	27 (−)	None observed	Daniell *et al.* 2001
DEF2	*Arabidopsis thaliana*	MMV M24	Actinonin	*N. tabacum*	nr (=)	nr	nr	Hou *et al.* 2007
DREB2A	*Oryza sativa*	4ABRC	NaCl	*O. sativa*	13.5 (−)	22	nr	Zhu and Wu 2008
GPT	*A. thaliana*	35S	Tunicamycin	*A. thaliana*	1 [(b)]	0	None observed	Koizumi 2003
KN1	*Zea mais*	35S	Lack of cytokinin and auxin	*N. tabacum*	120–260 (+)	nr	nr	Luo *et al.* 2006
M6PR	*Apium graveolens*	35S	Sucrose-free, mannose medium	*A. thaliana*	0.68 (=)	21	nr	Song *et al.* 2010
NiR	*O. sativa*	Native/35S/OsActin1	None/$NaNO_2$	*O. sativa*	8 (−) 43	nr <20	Higher nitrite reductase activity	Nishimura *et al.* 2005 Ozawa and Kawahigashi 2006
PFLP	*Capsicum annuum*	35S	*Erwinia carotovora*	*Oncidium* sp.	4 (=); 3 (biolisitic)(=)	0	Soft rot resistance	You *et al.* 2003
SOS1	*A. thaliana*	4ABRC	NaCl	*O. sativa*	7.4 (−)	48	nr	Zhu and Wu 2008
TDC	*Catharanthus roseus*	35S	4-methyl tryptophan	*N. tabacum*	nr	20 (=)	Aminoacid profile change	Goddijn *et al.* 1993
TPS1	*A. thaliana*	35S	Glucose	*A. thaliana* *N. tabacum*	1[b] nr	20 6	None observed	Leyman *et al.* 2006
TSB1	*A. thaliana*	35S	5-methyltryptophan $CdCl_2$	*A. thaliana*	0.11–0.14[b]	0	Higher free tryptophan	Hsiao *et al.* 2007
WBC19	*A. thaliana*	35S	Kanamycin	*Nicotiana tabacum*	~ 40 (+)	27 (=)	None observed	Mentewab and Stewart 2005
				Populus canescens x P. grandidentata	7 (=)	80 (=)	nr	Kang *et al.* 2010
				Cucumis melo	20–91	nr	nr	Bombale *et al.* 2010

[a] Number of transgenic plants per 100 explants; where available, a comparison with the conventional antibiotic-based selection system is provided in parentheses as follows: +, −, =: better, worse, equal performance than conventional marker; *Agrobacterium*-mediated transformation unless otherwise indicated

[b] Flower dip method

[c] Number of regenerated plants per 100 bombarded plates

nr not reported, *na* not applicable, *HR* herbicide resistance

Table 2. Plant-derived selectable marker genes carrying mutations

Gene	Source	Promoter	Selection	Plant species transformed	Transf. efficiency%[a]	Escape%	Associated phenotype	References
ALS	*A. thaliana*	Native	Chlorsulfuron	*N. tabacum*	nr	nr	HR	Olszewski *et al.* 1988; Gabard *et al.* 1989
		Native/*35S*	Chlorsulfuron	*N. tabacum*	3–7 (−)	nr	HR	Charest *et al.* 1990
		Native	Chlorsulfuron	*Linum usitatissimum*	na	na	HR	McHughen 1989
		Native/Dual *35S*	Chlorsulfuron	*Populus tremula x P. alba*	22–77 (=)	0–12	HR	Brasileiro *et al.* 1992
		Native/nos	Imazamox	*Solanum tuberosum*	100	0–13	HR	Andersson *et al.* 2003
		Native/35S	Chlorsulfuron	*O. sativa*	na (protoplast transf.) (−)	nr	HR	Li *et al.* 1992
		35S	Chlorsulfuron, imazethapyr	*Brassica juncea*	4–10 (+)	0	HR	Ray *et al.* 2004
		35S	Chlorsulfuron	*Z. Mais* (biolistic)	nr	nr	HR	Fromm *et al.* 1990
					nr	0	HR	Howe *et al.* 2002
		35S	Chlorsulfuron	*Z. Mais*	nr	76–84	HR	Zhang *et al.* 2005
		35S	Chlorsulfuron	*Zea Mais* (Agro/Biolisitc)	nr	76–84	HR	Zhang *et al.* 2005
	O. sativa	Native	Bispyribac-sodium	*O. sativa*	nr	nr	HR	Osakabe *et al.* 2005
		Zea mais Ubiquitin	Bispyribac-sodium	*O. sativa*	nr	nr	HR	Okuzaki *et al.* 2007
		CSP	Pyriminobac	*O. sativa*	10–30	6–15	HR	Wakasa *et al.* 2007
		Native	Bispyribac-sodium	*T. aestivum* (Biolistic)	6–17	8–60	HR	Ogawa *et al.* 2008
		Dual 35S	Bispyribac-sodium	*Glycine max*	0.3–1.2	36–69	HR	Tougou *et al.* 2009
	N. tabacum	Native	Chlorsulfuron	*N. tabacum*	na (protoplast)	0–79	HR	Lee *et al.* 1988
		Zea mais Ubiquitin	Chlorsulfuron	*Musa acuminata*	na (cell suspension)	0	HR	Ganapathi *et al.* 2001
	Gossypium hirsutum	Native	Imazethapyr/imazaquin	*G. hirsutum*	6–12	0	HR	Rajasekaran *et al.* 1996
ASA	*N. tabacum*	35S	4-methylindole 7-methyl-DL-tryptophan	*N. tabacum*	14 7	79 78	Higher free tryptophan	Barone and Widholm 2008
	O. sativa	*Zea mays Ubiquitin*	5-methyl-tryptophan	*O. sativa*	0–7 (=)	0	Higher tryptophan; anthocyanin pigmentation *in vitro* (potato)	Yamada *et al.* 2004
		35S		*Solanum tuberosum*	25–70 (=)	0–63		Kawagishi-Kobayashi *et al.* 2005
EPSPS	*Z. mais*	Dual 35S	Glyphosate	*Z. mais*	nr	0	HR	Howe *et al.* 2002
	O. sativa	35S	Glyphosate	*O. sativa*	9 (=)	0	HR	Charng *et al.* 2008
GSA	*Medicago sativa*	Dual 35S	Gabaculine (3-amino-2,3-dihydrobenzoic acid)	*M. sativa* *N. tabacum*	43 47	6 0	None observed None observed	Ferradini *et al.* 2011
				Triticum durum	1 (=)	20 (+)	None observed	Gadaleta *et al.* unpublished

[a] Number of transgenic plants per 100 explants; where available, a comparison with the conventional antibiotic-based selection system is provided in parentheses as follows: +, −, =: better, worse, equal performance than conventional marker; *Agrobacterium*-mediated transformation unless otherwise indicated

HR herbicide resistance, *nr* not reported, *na* not applicable

efficiency (7.8% *vs* 75% with the conventional *hpt*/hygromycin selection); and Nishimura *et al*. (2005), overexpressing the gene, reported 54% regeneration frequency, with 80% of the regenerated plants expressing the reporter GUS. No visible phenotypic effect of the introduced NiR expression was reported.

The usefulness of *NiR* for transformation could be limited by the fact that it would not confer a selective advantage to highly regenerating genotypes that have high endogenous *NiR* activity. However, Nishimura *et al*. (2005) succeeded in using this gene as an SMG in transformation of the highly regenerating variety Nipponbare, using 500 mg L^{-1} $NaNO_2$ as a selective agent and overexpressing *NiR* with the rice *Actin1* gene promoter. A protocol for transformation of rice genotypes with different regeneration potential based on *NiR* was published (Nishimura *et al*. 2006).

NiR can certainly be useful for rice transformation, allowing efficient 'intragenic' transformation. To our knowledge, it has not yet been applied to other crop species, but it would be interesting to utilize this gene for transformation of recalcitrant cereal varieties. The influence of *NiR* overexpression on nitrogen assimilation and nitrogen content of transgenic plants should be carefully investigated to check for possible side effects.

Threalose-6-phosphate synthase. Glucose can be toxic to plant cells at high concentrations because it switches off the photosynthetic machinery. In *A. thaliana,* germinating seed *in vitro* in the presence of high glucose produces chlorotic plantlets that do not grow, whereas overexpression of threalose-6-phosphate synthase (*TPS1*) makes plants less sensitive to glucose (Avonce *et al*. 2004). In fact, the enzymes TPS1 and threalose-6-phosphate phosphatase, together, convert glucose into the non-toxic disaccharide threalose. The *TPS1* gene from *A. thaliana* was overexpressed in *A. thaliana* and tobacco, allowing *in vitro* selection of transgenic plants in the presence of 5–6% glucose (Leyman *et al*. 2006). No effect on the phenotype was detected.

Since *TPS1* is a regulator of glucose, ABA, and stress signaling (Avonce *et al*. 2004), the phenotypic neutrality of its overexpression should be carefully checked under a variety of growth and stress conditions. Callus-specific promoters may be used to avoid undesired effects of *TPS1* expression.

Peptide deformylase (DEF). Peptide deformylase catalyzes the hydrolysis of the *N*-formyl group from the initiating methionine in newly translated proteins and is essential for all subsequent N-terminal protein processing. In all plants species investigated to date, there are two peptide deformylase genes, *DEF1* and *DEF2*, and the encoded enzymes localize to the plastids and mitochondria. In particular, DEF2 exhibits a strong polypeptide sequence preference for the N terminus of the D1 polypeptide subunit of the photosystem II complex, and DEF inhibition seems to impair co-translational processing of the D1 polypeptide. Consequently, peptide deformylase inhibitors, such as actinonin (an antibiotic isolated from an actinomycete), are lethal to plants (Hou *et al*. 2006). Overexpression of *AtDEF2* in tobacco under the *Mirabilis* mosaic virus M24 promoter resulted in resistance to actinonin *in vitro*, and selection with actinonin allowed regeneration of transgenic plants with an efficiency similar to that of kanamycin (Hou *et al*. 2007). Because a large pool of chemicals with peptide deformylase inhibitory activity exists, these authors suggest that new, broad-spectrum herbicides may be developed and that DEF-mediated resistance introduced in crop plants. The phenotypic effects of DEF overexpression in plants remain to be investigated.

Tryptophan synthase beta 1 (TSB1). Tryptophan is at the base of the synthesis of all the compounds containing the indole ring, such as auxins, glucosinolates, nicotinic acid, phytoalexins, and alkaloids. A few genes of the tryptophan biosynthetic pathway have been tested as SMGs by exploiting the phytotoxic activity of tryptophan analogs.

Plant tryptophan synthase is made of two subunits: the α subunit catalyzes the formation of indoleglycerol phosphate to indole, whereas the β subunit synthesizes tryptophan from indole and serine. The *Arabidopsis TSB1* gene, encoding the β subunit and driven by the 35 S promoter, was introduced by floral dip into *Arabidopsis*, resulting in seedling resistance to the tryptophan analog 5′ methyl-tryptophan and to $CdCl_2$ (the mechanisms of tryptophan involvement in $CdCl_2$ resistance are not known; Hsiao *et al*. 2007). The plants accumulated free tryptophan up to 15-fold more than non-transgenic plants. Plant morphology was not apparently affected. The efficiency of a selection system employing *TSB1* with 5-methyl-tryptophan or $CdCl_2$ selection appeared to be comparable to that of hygromycin-based selection. Since the tryptophan biosynthetic pathway is hosted by the plastid, this selection system may be engineered for plastid transformation.

Tryptophan decarboxilase. In plants, the enzyme tryptophan decarboxilase (TDC) converts L-tryptophane into tryptamine, a precursor of terpenoid indole alkaloids; it can also decarboxylate phytotoxic derivatives of tryptophan. Goddijn *et al*. (1993) used the *Catharanthus roseus TDC* gene as an SMG in conjunction with 4-methyl tryptophan as the selective agent. They first transformed tobacco with a construct harboring *TDC* and *npt*II, both controlled by the 35S promoter, and applied kanamycin selection. The transgenic plants were tested for 4-methyl tryptophan resistance *in vitro*. Based on the results obtained in this screening, transformation with 0.1 mM 4-methyl tryptophan

selection was successfully performed. The efficiencies of 4-methyl tryptophan *vs* kanamycin selection were compared visually and reported to be similar, and 80% of the regenerated plants were found to express the *TDC* mRNA.

TDC has been tested as a means to manipulate amino acid balance in plants and, to our knowledge, this is the only paper on its use as an SMG. Transgenic tobacco seedlings overexpressing *TDC* displayed altered aromatic and non-aromatic amino acid pools and a root-curling phenotype associated with the depletion of the tryptophan pool (Guillet *et al*. 2000); similar alterations of amino acid levels occurred in potato tubers (Yao *et al*. 1995). Leaf necrotic lesions were associated with chloroplast-targeted *TDC* expression and consequent high tryptamine accumulation (Di Fiore *et al*. 2002). Because of unexpected effects of its expression on plant metabolism and/or phenotype, the use of *TDC* as an SMG is not recommended.

Thiocyanate detoxifying genes: HOL1 and TMT. Plant genes encoding enzymes able to detoxify thiocyanate (-SCN) compounds have been proposed as SMGs. *HARMLESS TO OZONE LAYER 1 (HOL1)* is an *A. thaliana* gene involved in metabolism of glucosinolate hydrolysis products, such as thiocyanate, and in emission of methyl halide. It has been shown that *HOL1* overexpression confers potassium thiocyanate resistance to *A. thaliana* seedlings, and the conditions for its use as an SMG were investigated (Midorikawa *et al*. 2009). Seven days in the dark during the initial seedling growth are required for efficient selection, probably due to suppression of endogenous HOL1 expression in these conditions. A similar system, patented by Saini *et al*. (2005), is based on overexpression of the *Brassica oleracea* thiol methyltransferase (*TMT*) gene and employs sodium thiocyanate or potassium iodide for selection.

UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine-1-P transferase (GPT). The *A. thaliana* gene encoding GPT was successfully employed for *Arabidopsis* transformation by Koizumi (2003). GPT catalyzes the initial reaction for the synthesis of asparagine-linked glycans, essential for correct protein folding. When overexpressed, *GPT* confers resistance to the antibiotic tunicamycin, a known inhibitor of asparagine-linked glycosylation. With respect to kanamycin, selection occurred at an earlier seedling germination stage. No phenotypic effects of *GPT* overexpression were observed.

Ferrodoxin-like protein (PFLP). The sweet pepper (*Capsicum annuum)* PFLP gene was used for orchid (*Oncidium*) transformation (You *et al*. 2003). This gene had previously been demonstrated to have antibacterial activity when overexpressed in transgenic plants. Selection was applied by *in vitro* infection with *Erwinia carotovora,* a dangerous orchid pathogen (that was then killed in culture with antibiotics). The efficiency of selection appeared to be slightly higher than that of hygromycin selection in both *Agrobacterium* and biolistic transformations. In addition, the transgenic plants were resistant to soft-rot disease, at least *in vitro*. No phenotypic effects of PFLP overexpression were observed. This selection system is attractive for the added value of bacterial resistance, and application to other plant species could be useful. The need for *Erwinia* infection complicates the transformation protocol, which may not be easily transferable to other species.

Mannose-6-phospate reductase (M6PR). The *Escherichia coli* gene encoding phosphomannose isomerase (PMI) has been used for plant transformation by replacing sucrose with mannose in the culture media (Joersbo *et al*. 1998). In fact, bacterial PMI isomerizes D-mannose (which many plants cannot metabolize), converting it into D-fructose-6-P, which can be metabolized.

Mannose-6-phosphate reductase (M6PR) can also allow plants to metabolize mannose, by converting mannose-6-P (formed from mannose by nonspecific hexokinases) into mannitol-1-P, which is then converted to fructose. M6PR has been associated with salt tolerance in celery (*Apium graveolens*) and in *A. thaliana* (references in Song *et al*. 2010).

In a recent work, the *M6PR* gene from celery was introduced as an SMG in *A. thaliana*, and a transformation efficiency of 0.68% was realized using culture media containing mannose instead of sucrose (Song *et al*. 2010). Some plants, like tobacco, are naturally capable of metabolizing mannose and cannot be transformed with this selection system. Interestingly, tobacco overexpressing *M6PR* is inhibited in shoot regeneration in the presence of mannose, probably due to a negative interaction between the endogenous mannose biosynthetic pathway and the introduced M6PR enzyme. This may provide a new negative selection marker for 'mannose tolerant' plant species (Song *et al*. 2010).

Salt tolerance genes: DREB2A and SOS1. Two salt tolerance genes were assessed as SMGs (Zhu and Wu 2008). The first is *DREB2A* from rice, coding for a dehydration-responsive element (DRE) binding protein involved in tolerance to dehydration, salt (NaCl) and other abiotic stress factors. The second gene is the *S*alt *O*verly *S*ensitive *1* (*SOS1*) gene of *A. thaliana,* encoding a putative Na^+/H^+ antiporter, also capable of conferring salt tolerance when overexpressed. The two genes have been tested as SMGs in rice *Agrobacterium*-mediated transformation

using NaCl as the selection agent. The stress-responsive promoter ABRC, repeated four times, was used to drive the expression of both genes.

The efficiency of selection, based on PCR analysis of the regenerated plants, was reported to be one third (*SOS1*) to half (*DREB2A*) that of the conventional antibiotic selection system, but sufficient for practical use. The regenerated plants were not tested for salt tolerance *in vivo* nor was salt concentration of plant tissues studied. The added value of stress tolerance of such SMGs makes them attractive, but further characterization and extension to other crops is needed.

Plant SMGs for the transformation of the plastid genome: plastidial antibiotic resistance genes and betaine aldehyde dehydrogenase (BADH). The transformation of the plastid genome was originally accomplished by using a mutated, antibiotic resistant plastid *16S* rRNA gene as the SMG. Numerous mutations conferring spectinomycin and/or streptomycin resistance have been described in the *16S* or *23S* rRNA genes and in the plastid ribosomal protein *rps12* gene. Some of them have been used as SMGs for plastid transformation (reviewed by Dix and Kavanagh 1995). Antibiotic resistance is conferred by the inability of the antibiotic to bind to the mutated plastid ribosome, so these recessive mutations are defined as 'binding type' mutations that need to be in the homoplastomic (homozygous) condition to allow for efficient selection. With these markers, the native ribosomal gene is replaced by the antibiotic resistant variant by homologous recombination, which occurs spontaneously in the plastid. As a result, the transplastomic plant is marker-free. Recently, the tobacco plastome was transformed efficiently by the combined use of a mutation in the plastid 16*S rRNA* gene conferring spectinomycin resistance and a mutation in the 3′ region of the plastid *rps12* gene conferring streptomycin resistance (Craig *et al*. 2008).

With a different approach, the nuclear betaine aldehyde dehydrogenase (BADH) gene, isolated from spinach, was used to obtain transplastomic tobacco (Daniell *et al*. 2001). This is a dominant SMG based on the conversion of phytotoxic betaine aldehyde into non-toxic glycine betaine. This substance is an osmoprotectant produced in chloroplasts of a few plant species adapted to dry and saline conditions.

In tobacco, a high selection efficiency 25-fold higher than that with conventional spectinomycin selection was obtained. No phenotype was associated with BADH expression.

No other reports of the use of this interesting selection system were found in the literature. We have tested betaine aldehyde as a selective agent in alfalfa, but it did not inhibit somatic embryogenesis. The same may occur in other species.

Regeneration-promoting and cell cycle genes. New potential SMGs have been identified thanks to investigations on plant cell cycle and differentiation. Many genes affecting the acquisition of totipotency, the entry into the S phase of the cell cycle (the transformation-competent phase), cell fate, and regeneration are known, mostly in *A. thaliana* (reviewed by Zuo *et al*. 2002; Arias *et al*. 2006). These genes are involved in phenomena such as chromatin remodeling, cytokinin biosynthesis, and signaling, and their manipulation can markedly improve the efficiency of transformation protocols, particularly in recalcitrant plant species. Their potentiality has been demonstrated in the model species *A. thaliana*, without advancing them to actual use in crops.

One of them, *Zea mays KN1*, has been assessed as an SMG in tobacco (Luo *et al*. 2006) This gene plays a critical role in shoot meristem initiation, and its overexpression allows regeneratation of many shoots in the absence of cytokinin, similarly to the widely studied cytokinin biosynthetic gene *ipt* from *A. tumefaciens*. However, its constitutive expression results in a 'shooty' or 'bushy' phenotype, which does not allow the regeneration of normal plants.

The use of regeneration-promoting and cell cycle genes as SMGs requires that their expression is limited to the transformation and/or regeneration phases, because their constitutive expression is detrimental. This can be realized either by post-transformation marker excision (see above), or by inducible gene expression. The best inducible expression systems available for plants to date make use of chimeric transcription factors composed of viral, bacterial, and eukaryotic elements (Zuo *et al*. 2000; Padidam 2003): this would make the approval of such selection system in food crops very unlikely.

Mutated Plant SMGs

Acetolactate synthase (ALS). Acetolactate synthase (ALS, also known as acetohydroxy acid synthase, AHAS) is an enzyme of the biosynthetic pathway of the branched-chain amino acids isoleucine, leucine, and valine. It is the target of several classes of herbicides: pyrimidinylcarboxylates, sulfonylureas, imidazolinones, triazolopyrimidine sulfonamides, sulfonylaminocarbonyltriazolinones, and pyrimidinyl oxybenzoates.

Single or double amino acid substitutions can make ALS herbicide-resistant, and some mutated *ALS* genes are in commercial use (Tan *et al*. 2005).

ALS has been tested as an SMG since the beginning of the plant genetic engineering era. A mutated *ALS* gene from herbicide resistant *A. thaliana* was introduced into tobacco,

but its SMG performance was either not estimated or found to be much lower than that of *npt*II (Olszewski *et al*. 1988; Gabard *et al*. 1989; Charest *et al*. 1990).

Li *et al*. (1992) employed this gene for rice protoplast transformation. At best, transformation efficiency was 64% of that obtained with the conventional hygromycin selection, but only when the 35S promoter was used, whereas the *A. thaliana* endogenous promoter was not sufficiently active. The same constructs were used for transformation of aspen (Brasileiro *et al*. 1992), with good efficiency. Flax was transformed with *A. thaliana ALS* and chlorsulfuron selection (McHughen 1989), but no details on efficiency were given.

More recently, *A. thaliana ALS*-based selection has been compared with *npt*II in transformation of potato (Andersson *et al*. 2003); in this work, two promoters (*A. thaliana ALS* and *Nos*) and four potato varieties were used, demonstrating very high efficiency of *ALS*-based selection that was better than *npt*II. Oilseed mustard (*Brassica juncea*) was also transformed with the same SMG, with a maximum transformation efficiency of 10% (Ray *et al*. 2004).

Zhang *et al*. (2005) adopted *A. thaliana ALS* and chlorsulfuron selection in maize transformation with different methods (pollen tube, biolistic or *Agrobacterium*). They did not present efficiency data but reported high percentages of escapes. A mutated maize *ALS* gene conferring chlorsulfuron resistance was used for maize transformation (Fromm *et al*. 1990; Howe *et al*. 2002).

A cotton *ALS* gene was isolated, mutated at each of the two residues, and reintroduced into several cotton varieties by both *Agrobacterium* and the biolistic technique (Rajasekaran *et al*. 1996). Selection with two herbicides showed slightly lower, but comparable efficiencies, in comparison with *npt*II-based selection (using the antibiotic G418). This work showed that cotton transformation can be achieved without foreign DNA.

Lee *et al*. (1988) isolated two mutant *ALS* genes from tobacco and used them in tobacco transformation, with low efficiency. Banana was transformed with one of these genes, controlled by the maize *UBI1* promoter with good results (Ganapathi *et al*. 2001).

Quite some work has been done on *Oryza sativa ALS* genes. A genomic DNA fragment containing the regulatory and coding sequences of the rice *ALS* gene was mutated to an herbicide-resistant form by introducing two point mutations (Osakabe *et al*. 2005). This was then agro-transformed back into rice, and transgenic plants were efficiently selected with the herbicide bispyribac-sodium salt. The authors analyzed the transcription pattern of the rice *ALS* gene promoter with the *Gus* reporter gene showing expression in aerial tissues, seedlings, and root tips. The use of a rice gene under the native control sequences may be preferable with respect to conventional selection tools.

Recently, Wakasa *et al*. (2007) used a callus-specific promoter (CSP) to drive a mutated rice *ALS* gene obtained through somaclonal variation. Considering that the variety Koshihikari used in this work is difficult to transform, the reported transformation efficiencies (up to 30%) are good.

Okuzaki *et al*. (2007) isolated another mutated rice *ALS* gene from cell culture that was resistant to the herbicide bispyribac-sodium. Using the same herbicide for selection and the mutated gene driven by the maize UBI promoter a few transgenic plants were obtained. The same research group isolated a second mutated *ALS* from another culture in the presence of the same herbicide, but did not use it for selection in rice transformation (Kawai *et al*. 2007).

Wheat was also transformed with rice *ALS* and herbicide selection (Ogawa *et al*. 2008) with reasonable efficiency. In the dicot soybean, rice *ALS* has been tested as an SMG through 35S mediated overexpression, but with very low efficiency and a high frequency of escapes (Tougou *et al*. 2009).

A modified version of a soybean *ALS* gene was made by introducing two amino acid changes known to confer herbicide tolerance to tobacco *ALS*. Expression by the soybean constitutive S-adenosyl-L-methionine synthetase (SAMS) promoter yielded transgenic soybean using herbicide selection (http://www.gmo-compass.org/pdf/regulation/soybean/305423_soybean_application_foo_feed.pdf). No data on the efficiency of this selection system were found.

Recently, *ALS* loci have been targeted efficiently by homologous recombination mediated by artificial zinc-finger nucleases in tobacco. Introduction of the herbicide resistance mutations into the donor sequences (Townsend *et al*. 2009) followed by *in vitro* selection of calli with the herbicides chlorsulfuron and imazaquin was successful in selecting events, although regeneration of transgenic plants is not reported.

ALS has no mammalian toxicity and has a favorable environmental profile. Being a herbicide resistance gene, low efficiency of *ALAS* as SMGs can be forgiven if herbicide resistance is the useful trait. However, since its overexpression likely alters the amino acid profile (for example, threonine and glutamic acid were slightly increased in soybean; http://www.cera-gmc.org/?action=gm_crop_database&mode=Submit&evidx=541), expression under a native promoter would be preferable to the use of a strong constitutive promoter. Regardless, the amino acid composition of the transgenic plant products should be carefully checked. Limiting the expression of *ALS* to the regeneration steps (Wakasa *et al*. 2007) would be advisable.

Anthranilate synthase α subunit (ASA). Plant anthranilate synthase is a heterotetrameric enzyme that catalyzes the first step of tryptophan biosynthesis by converting chorismate to anthranilate. The α subunit alone can synthesize anthranilate

using ammonia as the amino donor. It is feedback inhibited by tryptophan and analogs such as 5-methyltryptophan. From a 5-methyltryptophan-resistant tobacco cell suspension, a feedback insensitive *ASA (ASA2)* cDNA was isolated and assessed as an SMG in *A. rhizogenes* transformation of *Astragalus sinicus* (Cho *et al*. 2004). When *ASA2* was overexpressed under the 35S promoter, transgenic hairy roots were obtained with an efficiency comparable to that of kanamycin selection, and they showed strongly increased free tryptophan concentration. Expression with the native promoter was not effective.

Based on this results, *ASA2* was used for tobacco transformation (Barone and Widholm 2008). Nine tryptophan analogs were tested for inhibition or regeneration, and 4-methylindole and 7-methyl-DL-tryptophan found to be the most effective. A test of seedling growth inhibition in the T_1 progeny demonstrated the utility of this selection system for progeny screening. However, the frequency of escapes was high, and optimization of this marker system appears necessary for wide adoption.

Free tryptophan accumulation, in some cases >7-fold more than that of the control, can be a desirable trait in many crop plants for increased nutritional value, considering that tryptophan is an essential amino acid. However, its effect on amino acid composition should be carefully tested case by case. Perhaps surprisingly, no phenotypic effect of tryptophan accumulation was observed.

A point-mutated, 5-methyltriptophan-resistant rice *ASA* gene (*OASA1D*) was implemented as an SMG by Yamada *et al*. (2004). The gene was overexpressed in rice using the maize ubiquitin promoter and in potato using the 35S promoter. Transgenic plants were obtained in both species with an efficiency comparable to conventional antibiotic selection, and the optimal concentration of 5-methyltriptophan was established. However, the frequency of escapes was high in potato. The content of free tryptophan was increased up to 28-fold in transgenic potato tubers, without a consistent effect on total amino acid concentration. No data on tryptophan concentration in transgenic rice was presented.

This gene was also introduced into *A. thaliana* by floral dip, but selection was performed with hygromycin (Kawagishi-Kobayashi *et al*. 2005). Homozygous T_1 seeds containing the *OASA1D* gene were then mixed with non-transgenic seeds, and *in vitro* selection conditions were simulated. The results suggest that 5-methyltryptophan selection should be effective for *A. thaliana* transformation. Like *TSB1, ASA*-based selection systems could be adapted for plastid transformation.

Enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate resistance is the most widespread trait in commercial transgenic plants. However, the use of glyphosate resistance genes as SMGs has been limited, probably due to the high toxicity of the herbicide, even at low concentration. Howe *et al*. (2002) isolated a maize *EPSPS* gene and, by changing two amino acids, produced a glyphosate resistant form and placed it under the control of the Dual 35S promoter. Its introduction by the biolistic technique allowed *in vitro* selection and regeneration of many transgenic maize lines without escapes.

Similar results were obtained in rice by cloning and mutating the rice EPSPS genomic sequence and introducing it into rice by *Agrobacterium* (Charng *et al*. 2008). An efficiency comparable to that obtained with conventional hygromycin selection was observed.

Glutamate 1-semialdehyde aminotransferase (GSA). The plant GSA enzyme catalyzes the conversion of glutamate-1-semialdehyde into aminolevulinic acid, a step in the synthesis of tetrapyrrolic compounds, including chlorophyll and phytochromes. GSA is irreversibly inhibited by gabaculine (3-amino-2,3-dihydrobenzoic acid). A mutant *Synechococcus hemL* gene encoding GSA has been demonstrated to be an efficient SMG in tobacco (Gough *et al*. 2001) and alfalfa (Rosellini *et al*. 2007); in the latter, it performed better than *npt*II-based selection. The mutated enzyme is gabaculine insensitive due to both a methionine to isoleucine (M->I) substitution in the cofactor (pyridoxal phosphate) binding site and a three amino acid deletion close to the amino terminus.

Based on this evidence, we cloned and sequenced the *Medicago sativa GSA* cDNA and reproduced the M->I mutation by means of a G to T transversion at nucleotide position 858. This mutated gene (*MsGSAgr*) was assessed as an SMG in tobacco and alfalfa *Agrobacterium* transformation, in comparison with the wild type gene, both driven by the Dual 35 S promoter (Ferradini *et al*. 2011).

When the M->I GSA was introduced in tobacco, about 43% of the leaf explants produced green shoots, whereas in alfalfa 47% of the explants produced green embryos, in the presence of gabaculine. Escapes were absent in tobacco and only 6% in alfalfa, and no effect on the plant phenotype was noticed. Transformation with the wild type gene did not produce any regeneration, proving that gabaculine resistance was due to the introduced M-> I mutation and not just to overexpression. The mutant GSA appeared to perform better than the bacterial *hemL* gene and than *npt*II for alfalfa transformation.

The same *MsGSAgr* construct was tested for durum wheat transformation, with good results (Gadaleta *et al*. unpublished).

MsGSAgr can be considered a safe and widely acceptable SMG, because GSA is involved in one biosynthetic, non-regulatory step, within a single metabolic pathway; thus, unintended effects of its overexpression are highly

unlikely. Gabaculine is neurotoxic, but its use in the lab poses no significant risk due to the low concentrations used (25–30 μM).

Because GSA functions in the plastid, *MsGSAgr* can be tested for its suitability to plastid transformation, adding a new plant-derived SMG to the few available for this particular application. Due to its plant origin, efficacy and versatility, *MsGSAgr* shows potential for wide application. Finally, because of its high sequence conservation, it should be easy to develop a gabaculine-resistant GSA specific for any plant species of interest, if intragenic transformation is preferred. This selection system is not patented.

RTS3. Recently, it has been demonstrated that kanamycin resistance can be induced in *A. thaliana* by knocking out or transcriptionally silencing the *RTS3* gene, which encodes a putative chloroplast transporter protein (Aufsatz *et al.* 2009). The overexpression of an inverted repeat construct producing a hairpin RNA homologous to the 5′ region of this gene allowed selection of transgenic seedlings *in vitro* by kanamycin resistance.

This work contributes to the elucidation of the mechanism of kanamycin toxicity in plants and provides the first selection system based on RNA silencing of a plant gene. *RTS3*, like *WBC19,* encodes a non-specific, transmembrane transporter protein; however, in this case, antibiotic resistance is obtained by silencing, not by overexpression, so unpredictable accumulation of unwanted molecules in the plastids would not occur. In experimental conditions, no phenotypic effect of silencing was noticed, but the down-regulation of a chloroplast transit protein may have detrimental consequences that should be ruled out before such selection system is adopted in crop plants. No data on the efficiency of selection was given. It would be interesting to confirm this result by silencing orthologous genes in crop species.

Conclusions

Several plant genes have been demonstrated to function as SMGs. Wild-type SMGs need overexpression, because it is the excess concentration of the encoded protein that confers resistance to the inhibiting chemical or the growth regulator-independent regeneration. This requirement can be a limiting factor, because overexpression can alter the phenotype to the point of lethality, so that either post-transformation excision, inducible expression, or *in vitro*-specific expression are necessary. Over-expressed wild type SMGs are also not suitable for the cisgenic engineering approach.

On the contrary, mutated SMGs, although in most cases the demonstration of their activity has been obtained through overexpression, may be expressed by their native promoters, as demonstrated for ALS in some cases. They carry one or two point mutations that can occur spontaneously, which should pose minor obstacles to the authorization of their use in crop plants. The cisgenic approach may employ this type of SMG, if the genes are from sexually compatible species.

Versatility is an important feature for a selection system to become widely adopted. To demonstrate the usefulness of an SMG, several diverse plant species should be efficiently transformed. To date, this has been done for a handful of markers, so it would be useful, even though not exciting, to test the promising markers in other species.

Mutated *ALS* genes are by far the most widely tested plant SMGs. Their performances *in vitro* are not always comparable with that of standard SMGs, and this may explain why they have not been widely adopted, despite the fact that herbicide resistance is itself a useful trait and would allow convenient screening of advanced progenies of transgenic plants.

The efficiency of the SMGs with respect to established antibiotic-based selection systems varies, but examples of highly efficient plant SMGs exist. However, it should be kept in mind that, in most cases, the efficiency estimation is merely indicative because it derives from non-replicated experiments.

Finally, most selection systems are patented, and consequently not readily available for commercial applications.

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