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Review

Selectable marker genes in transgenic plants: applications, alternatives and biosafety

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Abstract

Approximately fifty marker genes used for transgenic and transplastomic plant research or crop development have been assessed for efficiency, biosafety, scientific applications and commercialization. Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates. Positive selectable marker genes are defined as those that promote the growth of transformed tissue whereas negative selectable marker genes result in the death of the transformed tissue.

The positive selectable marker genes that are conditional on the use of toxic agents, such as antibiotics, herbicides or drugs were the first to be developed and exploited. More recent developments include positive selectable marker genes that are conditional on non-toxic agents that may be substrates for growth or that induce growth and differentiation of the transformed tissues. Newer strategies include positive selectable marker genes which are not conditional on external substrates but which alter the physiological processes that govern plant development.

A valuable companion to the selectable marker genes are the reporter genes, which do not provide a cell with a selective advantage, but which can be used to monitor transgenic events and manually separate transgenic material from non-transformed material. They fall into two categories depending on whether they are conditional or non-conditional on the presence of external substrates. Some reporter genes can be adapted to function as selectable marker genes through the development of novel substrates.

Despite the large number of marker genes that exist for plants, only a few marker genes are used for most plant research and crop development. As the production of transgenic plants is labor intensive, expensive and difficult for most species, practical issues govern the choice of selectable marker genes that are used. Many of the genes have specific limitations or have not been sufficiently tested to merit their widespread use. For research, a variety of selection systems are essential as no single selectable marker gene was found to be sufficient for all circumstances. Although, no adverse biosafety effects have been reported for the marker genes that have been adopted for widespread use, biosafety concerns should help direct which markers will be chosen for future crop development. Common sense dictates that marker genes conferring resistance to significant therapeutic antibiotics should not be used.

An area of research that is growing rapidly but is still in its infancy is the development of strategies for eliminating selectable marker genes to generate marker-free plants. Among the several technologies described, two have emerged with significant potential. The simplest is the co-transformation of genes of interest with selectable marker genes followed by the segregation of the separate genes through conventional genetics. The more complicated strategy is the use of site-specific recombinases, under

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the control of inducible promoters, to excise the marker genes and excision machinery from the transgenic plant after selection has been achieved.

In this review each of the genes and processes will be examined to assess the alternatives that exist for producing transgenic plants.

Keywords: Selectable marker genes; Transgenic plants; Biosafety

1. Introduction

Breakthroughs in DNA cloning and sequencing technologies are yielding unprecedented amounts of information on the composition of genes and their regulatory elements as well as the structural elements that give organization to the genomes of different organisms. The most powerful experiments for assessing their function have used technologies for modifying cloned sequences and inserting them into genomes of diverse organisms to study the outcome on the transgenic organisms. This technology has made possible the construction of organisms with novel genes and regulatory sequences that are the products of experimental design rather than the products of evolutionary processes. Transgenic organisms allow scientists to cross the physical and genetic barriers that separate pools of genes among organisms. A sampling of the plant molecular biology literature in 2002 revealed that transgenic plants are used as an important research tool in about a half of the refereed publications (Table 1). The current economic growth in transgenic crops is reflected in the global rate of adoption for the major commodities in 2002. These are soybean (51%), cotton (20%), canola (12%) and corn (9%) (James, 2002). In 2002, 58.7 million hectares of transgenic crops were grown globally (James, 2002).

All transformation systems for creating transgenic plants require separate processes for introducing cloned DNA into living plant cells, for identifying or selecting those cells that have integrated the DNA into the appropriate plant genome (nuclear or plastid) and for regenerating or recovering fully developed plants from the transformed cell. Selectable marker genes have been pivotal to the development of plant transformation technologies because the marker genes allow scientists to identify or isolate the cells that are expressing the cloned DNA and to monitor and select for the transformed progeny. As only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Since the selectable marker gene is expected to function in a range of cell types, it is usually constructed as a chimeric gene using regulatory sequences that ensure constitutive expression throughout the plant. The selectable marker gene is usually co-transformed with a gene of interest. Once the transgenic plant has been generated, characterized and bred through conventional genetic crosses, the selectable marker gene generally no longer serves an essential purpose. If the selectable markers are to

Table 1
Utilization of transgenic plants and selectable marker genes in papers published in selected journals in 2002

Journals	*Plant Cell* (%)	*Plant Molecular Biology* (%)	*Molecular Breeding* (%)	*Transgenic Research*[a] (%)
Papers using transgenic plants	56	39	76	52
Kanamycin resistance	56	70	44	61
Hygromycin resistance	20	21	31	19
Phosphinothricin resistance	20	4	17	19
Other herbicide resistance (chlorsulfuron or glyphosate)	1	4	3	–
Other selection strategies	1	2	3	–

The papers did not include *Arabidopsis* T-DNA mutants. Approximately 450 papers were examined.
[a] Transgenic Research publishes in both plant and animal science.

remain expressed within the transgenic plant, it is important for both scientific and economic reasons that the selectable marker gene does not have broad pleiotropic effects. Consequently, the use of biological processes that are foreign to plants and that have a high level of enzyme specificity was initially adopted.

The questions that relate to the biosafety of the selectable marker genes are the same as those that relate to other genes associated with plants, humans and our environments: Do they code for toxic products or allergens? Will they create unwanted changes in the composition of the plant? Will they compromise the use of therapeutic drugs? Will there be horizontal gene transfer to relevant organisms and pathogens? Can gene transfer to other plants create new weeds or compromise the value of non-target crops? Clearly, there is no single answer and every gene has to be assessed individually. A variety of strategies are being developed to eliminate marker genes after the selection phase of plant production to create marker-free transgenic plants or to restrict pollen flow from transgenic plants. Once again the need for the adoption of these strategies depends on the gene of interest that is being co-transformed with the marker gene as well as the characteristics of the particular marker gene.

In this comprehensive review, we will examine the full range of selectable marker genes that have been developed for use in transformation systems for producing transgenic plants, what we know about their characteristics and their use in crop plants. We will review the information that is available on the biosafety of various selectable marker genes and examine the status of systems for creating marker-free transgenic plants. This information needs to be examined in order to assess the alternatives that are available or that must be developed for generating safe transgenic plants for research and commercialization.

2. Selectable marker gene systems

2.1. Background

As no single selection system is adequate for all purposes, there is a need for several systems. An examination of the scientific literature from the year 2002 appearing in the peer-reviewed journals *The Plant Cell*, *Plant Molecular Biology*, *Molecular Breeding and Transgenic Research*, revealed that three selection systems were employed in over 90% of the scientific publications. These were selection on the antibiotics kanamycin or hygromycin and the herbicide phosphinothricin (Table 1). An examination of the selectable marker genes used in commercial transgenic varieties showed that selectable markers that confer resistance to kanamycin or phosphinothricin were the most common (Table 2). In confined field trials the incidence of hygromycin selection was also very high (Table 3). As herbicide resistance provides a natural selectable marker system, herbicide-resistant lines and varieties can usually be produced without the need for other selectable marker genes (Tables 2 and 3). The popularity of these selection systems reflects the efficiency and general applicability of their use across a wide range of species and regenerable tissue culture systems. In a search for greater efficiency and freedom to operate, almost fifty different selection systems have been reported but few have reached practical application. For the sequential pyrimiding of transgenes into plants the use of a variety of efficient selectable marker genes is the easiest experimental approach for most research labs. Vectors have been developed for this purpose with different selectable marker genes (Barrell et al., 2002); however, a variety of other strategies are being developed which include co-transformation or marker gene excision and gene targeting (reviewed by Ow, 2002).

The terminology used in the plant literature to describe selection systems has been confusing and at times inconsistent with terminology used with other organisms. We have adopted the terminology of positive and negative, conditional and non-conditional selection systems to accurately describe the various systems for plants and to be consistent with the broader use of the terminology across organisms (Babwah and Waddell, 2000).

Positive selection systems are those that promote the growth of transformed cells. They may be divided into conditional-positive or non-conditional-positive selection systems. A conditional-positive selection system consists of a gene coding for a protein, usually an enzyme, that confers resistance to a specific substrate that is toxic to untransformed plant cells or that encourages growth and/or differentiation of the transformed cells. In plant conditional-positive selection systems the substrate may act in one of several ways.

Table 2
Selectable markers in genetically modified crops with approvals for commercial use (information extracted from AGBIOS, 2003)

Crop	Identifier	Phenotypic trait	Selectable markers[a] (gene–enzyme)
Beta vulgaris (sugar beet)	GTSB77 (InVigor™)	Glyphosate herbicide resistance	*uidA*–GUS
	T120-7	Phosphinothricin herbicide resistance, specifically glufosinate ammonium	*neo*–NPTII
Brassica napus (canola, oilseed rape)	23-18-17, 23-198	High laurate and myristate canola	*neo*–NPTII
	GT200 (Roundup Ready®)	Glyphosate herbicide resistance	CP4 *epsps*–EPSPS, *goxv247*–GOX
	GT73, RT73 (Roundup Ready®)	Glyphosate herbicide resistance	CP4 *epsps*–EPSPS, *goxv247*–GOX
	HCN10 (Liberty-Link™ Independence)	Phosphinothricin herbicide resistance, specifically glufosinate ammonium	*pat*–PAT
	HCN92 (Liberty Link™ Innovator)	Phosphinothricin herbicide resistance, specifically glufosinate ammonium	*pat*–PAT, *neo*–NPTII
	HCN28	Phosphinothricin herbicide resistance, specifically glufosinate ammonium	*pat*–PAT
	MS1, RF1 → PGS1	Male sterility, fertility restoration, pollination control, glufosinate herbicide resistance	*neo*–NPTII, *bar*–PAT
	MS1, RF2→PGS2	Male sterility, fertility restoration, pollination control, glufosinate herbicide resistance	*neo*–NPTII, *bar*–PAT
	MS8 X RF3	Male sterility, fertility restoration, pollination control, glufosinate herbicide resistance	*bar*–PAT
	OXY-235	Tolerance to herbicides bromoxynil and ioxynil	*bxn*–nitrilase
	PHY 14, PHY35	male sterility, fertility restoration, phosphinothricin herbicide resistance	*bar*–PAT
	PHY36	Male sterility, fertility restoration, phophinothricin herbicide resistance	*bar*–PAT
Carica papaya (papaya)	55-1/63-1	Papaya ringspot virus resistance	*uidA*–GUS, *neo*–NPTII
Cichorium intybus (chicory)	RM3-3, RM3-4, RM3-6	Male sterility, phosphinothricin herbicide tolerance, specifically glufosinate ammonium	*neo*–NPTII, *bar*–PAT
Cucumis melo (cantaloupe)	A, B	Delayed ripening	*neo*–NPTII
Cucurbita pepo (squash)	CZW-3	Resistance to cucumber mosaic virus, watermelon mosaic virus, zucchini yellow mosaic virus	*neo*–NPTII
	ZW20	Resistance to watermelon mosaic virus and zucchini yellow mosaic virus	*neo*[b]–NPTII
Dianthus caryophyllus (carnation)	4, 11, 15, 16	Modified flower colour, sulfonylurea herbicide resistance	*surB*–ALS
	66	Delayed senescence, sulfonylurea herbicide resistance	*surB*–ALS
	959A, 988A, 1226A, 1351A, 1363A,1400A	Modified flower colour, sulfonylurea herbicide resitance	*surB*–ALS

Table 2 (*Continued*)

Crop	Identifier	Phenotypic trait	Selectable markers[a] (gene–enzyme)
Glycine max L. (soybean)	A2704-12, A2704-21, A5547-35	Phosphinothricin herbicide tolerance, specifically glufosinate ammmonium	*pat*–PAT
	A5547-127	Phosphinothricin herbicide tolerance, specifically glufosinate ammmonium	*pat*–PAT, *bla*[c]
	GU262	Phosphinothricin herbicide tolerance, specifically glufosinate ammmonium	*pat*–PAT, *bla*[c]
	G94-1, G94-19, G168	Modified fatty acid content, specifically high oleic acid	*uidA*–GUS, *bla*[c]
	GTS 40-3-2 (Roundup Ready®)	Glyphosate herbicide tolerance	CP4 *epsps*–EPSPS
	W62, W98	Phosphinothricin herbicide tolerance	*uidA*–GUS
Gossypium hirsutum L. (cotton)	MON-15985-7 (Bollgard II®)	Resistance to lepidopteran insects	*neo*–NPTII, *uidA*–GUS, *aad*[c]
	19-51A	Sulfonylurea herbicide resistance	*als* (*S4-HrA*)–ALS
	31807/31808	Resistance to lepidopteran insects, oxynil herbicide resistance	*neo*–NPTII
	BXN	Oxynil herbicide tolerance	*neo*–NPTII
	MON 1445/1698 (Roundup Ready®)	Glyphosate herbicide tolerance	*neo*–NPTII, *aad*[c]
	MON 531/757/1076 (Bollgard®)	Resistance to lepidopteran insects	*neo*–NPTII, *aad*[c]
Linum usitatissimum L. (flax, linseed)	FP967	Sulfonylurea herbicide resistance	*neo*–NPTII, *nos*–NOS *bla*[c], *spc*[c]
Lycopersicon esculentum (tomato)	1345-4	Increased shelf life (delayed ripening)	*neo*–NPTII
	35 1 N	Delayed ripening	*neo*–NPTII
	5345	Resistance to lepidopteran insects	*neo*–NPTII, *aad*[c]
	8338	Delayed ripening	*neo*–NPTII
	B, Da, F	Delayed softening	*neo*–NPTII
	FLAVR SAVR	Delayed softening	*neo*–NPTII
Nicotiana tabacum (tobacco)	C/F/93/08-02	oxynil herbicide tolerance	*bxn*–nitrilase
Oryza sativa (rice)	LLRICE06, LLRICE62 (Liberty-Link™)	Phosphinothricin herbicide tolerance, specifically glufosinate ammmonium	*bar*–PAT
Solanum tuberosum (potato)	ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, ATBT04-36, SPBT02-5, SPBT02-7 (Atlantic and Superior NewLeaf®)	Resistance to colorado potato beetle	*neo*–NPTII
	BT6, BT10, BT12, BT16, BT17,BT18, BT23 (Russet Burbank NewLeaf®)	resistance to colorado potato beetle	*neo*–NPTII
	RBMT21-129, RBMT21-350, RBMT22-082 (Russet Burbank NewLeaf® Plus)	Resistance to colorado potato beetle, resistance to potato leafroll luteovirus	*neo*–NPTII, CP4 *epsps* (in RBMT22-82 only)
	RBMT15-101, SEMT15-02, SEMT15-15 (NewLeaf® Y)	Resistance to colorado potato beetle, resistance to potato virus Y	*neo*–NPTII, *aad*[c]
Zea mays (maize)	176 (NaturGard ™, Knockout™)	Resistance to european corn borer, phosphinothricin herbicide tolerance	*bar*–PAT, *bla*[c]

Table 2 (*Continued*)

Crop	Identifier	Phenotypic trait	Selectable markers[a] (gene–enzyme)
	676, 678, 680	Male sterility, phosphinothricin herbicide resistance	*pat*–PAT
	B16 (DLL25)	Phosphinothricin herbicide tolerance, specifically glufosinate ammmonium	*bar*–PAT, *bla*[c]
	BT11 (X4334CBR, X4734CBR)	Resistance to european corn borer, phosphinothricin herbicide tolerance	*pat*–PAT
	CBH-351 (StarLink™)	Resistance to european corn borer, phosphinothricin herbicide tolerance	*bar*–PAT, *bla*[c]
	DBT418 (Bt Xtra™)	Resistance to european corn borer, phosphinothricin herbicide tolerance	*bar*–PAT, *bla*[c]
	GA21 (Roundup Ready®)	Glyphosate herbicide resistance	*m-epsps*-modified maize EPSPS
	MON80100	Resistance to european corn borer	CP4 *epsps*–EPSPS, goxv247–GOX, *neo*[c]
	MON802 (Yeildgard®)	Resistance to european corn borer, glyphosate herbicide tolerance	CP4 *epsps*–EPSPS, goxv247–GOX, *neo*[c]
	MON809	Resistance to european corn borer	CP4 *epsps*–EPSPS, *goxv247*–GOX
	MON810 (Yeildgard®)	Resistance to european corn borer	*CP4 epsps*–EPSPS[b], *goxv247*–GOX[b]
	MON832	Glyphosate herbicide tolerance	CP4 *epsps*–EPSPS, *goxv247*–GOX, *neo*[c]
	MON863	Resistance to corn root worm	*neo*–NPTII
	MS3 (InVigor™)	Male sterility, phosphinothricin herbicide resistance specifically glufosinate ammonium	*bar*–PAT, *bla*[c]
	MS6 (InVigor™)	Male sterility, phosphinothricin herbicide resistance specifically glufosinate ammonium	*bar*–PAT, *bla*[c]
	NK603 (Roundup Ready®)	Glyphosate herbicide tolerance	CP4 *epsps*–EPSPS
	T14, T25 (Liberty-Link™)	Phosphinothricin herbicide resistance, specifically glufosinate ammonium	*pat*–PAT, *bla*[c]
	TC1507 (Herculex™ I)	Resistance to european corn borer, phosphinothricin herbicide tolerance	*pat*–PAT

[a] Abbreviations: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; GOX, glyphosate oxidoreductase; GUS, β-glucuronidase, NPTII, neomycin phosphtransferase II; NOS, nopaline synthase; PAT, phosphinothricin *N*-acetyl transferase.

[b] Marker was used for selection but was segregated away in the final product.

[c] *bla*, *aad*, and in certain cases *neo* are under the control of bacterial promoters and were used for bacterial selection. They are not expressed in plant cells.

It may be an antibiotic (Table 4), a herbicide (Table 5), a drug or metabolite analogue (Table 6) or a carbon supply or phytohormone precursor (Table 7). In each case the gene codes for an enzyme with specificity to a substrate to encourage the selective growth and proliferation of the transformed cells. The substrate may be toxic or non-toxic to the untransformed cells. The *npt*II gene, which confers kanamycin resistance by inhibiting protein synthesis (Table 4), is the classic example of a system that is toxic to untransformed cells. There is a concern that the transformation efficiencies are suboptimal with toxic substrates because dying untransformed cells may inhibit transformed cells from proliferating by secreting inhibitors or preventing transport of essential nutrients to the living transformed cells (Hardrup et al., 1998a). The *manA* gene, which codes for phosphomannose isomerase, is an example of a conditional-positive selection system where the selection substrate is not toxic (Table 7). In this system, the substrate mannose is unable to

Table 3
Marker genes listed in US field test notifications and release permits for the years 2001 and 2002 (data extracted from ISB, 2003)

Enzyme	Number of records in 2001 and 2002
Neomycin phosphotransferase II	949
Hygromycin B phosphotransferase	65
Phosphinothricin *N*-acetyltransferase	327
5-Enolpyruvylshikimate-3-phosphate synthase	507
Acetolactate synthase or acetohydroxyacid synthase	5
Nitrilase	0
Cyanamide hydratase	2
β-Glucuronidase	91
Luciferase	4
Green fluorescent protein	20

act as a carbon source for untransformed cells but it will promote the growth of cells transformed with *manA*. In the literature, the positive nature of this selection strategy, has been emphasized. Unfortunately, the erroneous implication is that systems, such as the *npt*II gene, are negative selection systems because toxic selective agents are used (Joersbo and Okkels, 1996).

Non-conditional-positive selection systems do not require external substrates yet promote the selective growth and differentiation of transformed material. An example is the *ipt* gene that enhances shoot development by modifying the plant hormone levels endogenously (Table 8). As these selectable markers often alter cell division and differentiation there is a significant alteration in the morphology, development and physiology of the transgenic plant. Strategies are therefore needed to limit the expression of the markers by using inducible promoters or by creating marker-free plants.

Negative selection systems have been described in plants for genes that result in the death of transformed cells. These are dominant selectable marker systems that may be described as conditional and non-conditional selection systems. When the

Table 4
Toxic antibiotics and selectable marker genes used for the conditional-positive selection of transgenic and transplastomic plants

Antibiotics	Genes	Enzymes	Sources	Genome	References
Neomycin Kanamycin Paramomycin, G418	*neo*, *npt*II *(aphA2)*, *nptI (aphA1)*	Neomycin Phosphotransferases	*Escherichia coli* Tn*5* *Escherichia coli* Tn*601*	Nuclear Plastid	Fraley et al., 1983 Carrer et al., 1993
Aminoglycosides[a]	*aaC3*	Aminoglycoside-*N*-acetyl transferases	*Serratia marcesens*	Nuclear	Hayford et al., 1988
	aaC4		*Klebsiella pneumoniae*		
	6′gat		*Shigella* sp.		Gossele et al., 1994
Spectinomycin	*aadA*	Aminoglycoside-3″-Adenyl transferase	*Shigella* sp.	Nuclear Plastid	Svab et al., 1990 Svab and Maliga, 1993
Spectinomycin Streptomycin	*SPT*	Streptomycin Phosphotransferase	Tn*5*	Nuclear	Maliga et al., 1988
Hygromycin B	*hph* (*aphIV*)	Hygromycin Phosphotransferase	*Escherichia coli*	Nuclear	Waldron et al., 1985
Bleomycin Phleomycin	*Ble*	Bleomycin resistance	*Escherichia coli* Tn*5* *Streptoalloteichus hindustanus*	Nuclear	Hille et al., 1986 Perez et al., 1989
Sulfonamides	*sul*I	Dihydropteroate synthase	*Escherichia coli* pR46	Nuclear	Guerineau et al., 1990
Streptothricin	*sat3*	Acetyl transferase	*Streptomyces* sp	Nuclear	Jelenska et al., 2000
Chloramphenicol	*cat*	Chloramphenicol acetyl transferase	*Escherichia coli* Tn5	Nuclear	DeBlock et al., 1984
			Phage p1cm	Plastid	DeBlock et al., 1985

[a] Aminoglycosides include kanamycin, neomycin, geneticin (G418), paramomycin gentamicin, tobramycin, apramycin, depending on the specificities of the enzymes.

Table 5
Toxic herbicides and selectable marker genes used for the conditional-positive selection of transgenic plants

Herbicides	Genes	Enzyme	Source	Genome	References
Phosphinothricin	*pat*, *bar*	Phosphinothricin acetyl transferase	*Streptomyces hygroscopicus*, *Streptomyces viridochromogenes* Tu494	Nuclear	DeBlock et al., 1989
Glyphosate	*EPSP synthase*	5-Enolpyruvylshikimate-3-phosphate synthase	*Petunia hybrida*, *Zea mays*	Nuclear	Zhou et al., 1995; Howe et al., 2002
	aroA		*Salmonella typhimurium*, *Escherichia coli*		Comai et al., 1988; della Cioppa et al., 1987
	cp4 epsps		*Agrobacterium tumefaciens*		Barry et al., 1992
	gox	Glyphosate oxidoreductase	*Ochrobactrum anthropi*		Barry et al., 1992
Sulfonylureas	*csr1-1*	Acetolactate synthase	*Arabidopsis thaliana*	Nuclear	Olszewski et al., 1988
Imidazolinones	*csr1-2*	Acetolactate synthase	*Arabidopsis thaliana*	Nuclear	Aragao et al., 2000
Oxynils	*bnx*	Bromoxynil nitrilase	*Klebsiella pneumoniae* subspecies *ozanaenae*	Nuclear	Freyssinet et al., 1996
Gabaculine	*hemL*	Glutamate-1-semialdehyde aminotransferase	*Synechococcus* PCC6301	Nuclear	Gough et al., 2001
Cyanamide	*cah*	Cyanamide hydratase	*Myrothecium verrucaria*	Nuclear	Damm, 1998; Weeks et al., 2000

Table 6
Toxic drugs, metabolite analogues and enzymes used for the conditional-positive selection of transgenic plants

Drugs and analogues	Genes	Enzymes	Sources	Genome	References
2-Deoxyglucose	*DOG*^{R}*1*	2-Deoxyglucose-6-phosphate phosphatase	*Saccharomyces cerevisiae*	Nuclear	Kunze et al., 2001
Betaine aldehyde	*BADH*	Betaine aldehyde dehydrogenase	*Spinacia oleracea*	Nuclear, plastid	Ursin, 1996; Daniell et al., 2001
S-Aminoethyl L-cysteine (AEC)	*DHPS* *ocs*	Dihydropicolinate synthase, Octopine synthase	*Escherichia coli*, *Agrobacterium tumefaciens*	Nuclear	Perl et al., 1993; Koziel et al., 1984
4-Methyltryptophan (4-mT)	TDC	Tryptophan decarboxylase	*Catharanthus roseus*	Nuclear	Goddijn et al., 1993
Methotrexate	DHFR	Dihydrofolate reductase	*Escherichia coli* mouse	Nuclear	Herrera-Estrella et al., 1983; Eichholtz et al., 1987
			Candida albicans	Nuclear	Irdani et al., 1998

selection system is not substrate dependent, it is a non-conditional-negative selection system (Babwah and Waddell, 2000). An example is the expression of a toxic protein, such as a ribonuclease to ablate specific cell types (Mariani et al., 1990).

When the action of the toxic gene requires a substrate to express toxicity, the system is a conditional

Table 7
Non-toxic agents and enzymes used for the conditional-positive selection of transgenic plants

Non-toxic agents	Genes	Enzymes	Sources	Genome	References
D-Xylose	*xylA*	Xylose isomerase	*Streptomyces rubignosus*, *Thermoanaerobacterium sulfurogenes*	Nuclear	Haldrup et al., 1998a; Haldrup et al., 1998b
D-Mannose	*manA* (*pmi*)	Phosphomannose isomerase	*Escherichia coli*	Nuclear	Joersbo et al., 1998
Benzyladenine-*N*-3-glucuronide	*uidA*(*gusA*)	β-Glucuronidase	*Escherichia coli*	Nuclear	Joersbo and Okkels, 1996

Table 8
Enzymes used for the non-conditional-positive selection of transgenic plants

Selective agent	Genes	Enzymes	Sources	Genomes	References
None	*ipt*, *pga 22*	Isopentyl transferases	*Agrobacterium tumefaciens*, *Arabidopsis thaliana*	Nuclear	Endo et al., 2001; Zuo et al., 2002a
None	*rol*	"Hairy root"phenotype	*Agrobacterium rhizogenes*	Nuclear	Ebinuma et al., 2001
None	*ESR1*	Transcription factor (enhancer of shoot regeneration 1)	*Arabidopsis thaliana*	Nuclear	Banno and Chua, 2002
None	*CKI1*	Histidine kinase (cytokinin-independent 1)	*Arabidopsis thaliana*	Nuclear	Zuo et al., 2002a

negative selection system (Babwah and Waddell, 2000). Some conditional-negative selection systems used in plants are described in Table 9. They include the bacterial *codA* gene, which codes for cytosine deaminase (Stougaard, 1993), the bacterial cytochrome P450 mono-oxygenase gene (Koprek et al., 1999), the bacterial haloalkane dehalogenase gene (Naested et al., 1999), or the *Arabidopsis* alcohol dehydrogenase gene (Lopez-Juez et al., 1998). Each of these converts non-toxic agents to toxic agents resulting in the death of the transformed cells. The *codA* gene has also been shown to be an effective dominant negative selection marker for chloroplast transformation (Serino and Maliga, 1997). The *Agrobacterium aux2* and *tms2* genes are interesting in that they can also be used in positive selection systems. Combinations of positive-negative selection systems may be invaluable for enriching certain kinds of events in plant cells, such as gene targeting (Thykjaer et al., 1997) and for screening against certain genetic events.

One cannot assume that plant resistance to a selective agent conferred by a specific gene will result in a good selectable marker gene system just because highly-resistant plants can be obtained. For example the bacterial gene *tfdA*, which codes for 2,4-dichlorophenoxyacetate mono-oxygenase (DPAM), confers high levels of resistance to the synthetic auxin 2,4-D but it is completely ineffective as a selectable marker gene in tobacco leaf disc transformation and for selection of transgenic seedlings in germination assays (Streber and Willmitzer, 1989). To be effective, a selectable marker gene system must encourage the selective growth and differentiation of the transformed tissue in addition to providing resistance to a substrate. It is commonly found that some conditional-positive selection systems will be more effective in certain plant species and regeneration systems than others. An example is the lower efficiency of kanamycin resistance as a selection system in cereals than in dicots.

Table 9
Chemicals and enzymes for the conditional-negative selection of transgenic tissues

Substrates	Genes	Enzymes	Sources	Genome	References
5-Fluorocytosine	*codA*	Cytosine deaminase	*Escherichia coli*	Nuclear, plastid	Stougaard, 1993; Serino and Maliga, 1997
Naphthalene acetamide	*aux2*	Amido hydrolase	*Agrobacterium rhizogenes*	Nuclear	Beclin et al., 1993
Indole-3-acetamide	*tms 2*	Indoleacetic acid hydrolase	*Agrobacterium tumefaciens*		Depicker et al., 1988
Dihaloalkanes	*dhlA*	Dehalogenase	*Xanthobacter autotrophicus*	Nuclear	Naested et al., 1999
Sulfonylurea R7402	*CYP105A*	Cytochrome P450 mono-oxygenase	*Streptomyces griseolus*	Nuclear	O'Keefe et al., 1994
Allyl alcohol	*cue*	Alcohol dehydrogenase	*Arabidopsis thaliana*	Nuclear	Lopez-Juez et al., 1998

2.2. Conditional-positive selection systems using antibiotics

All of the effective sources of antibiotic resistance that have been used to develop selectable marker genes for transgenic plants have been taken from bacterial sources (Table 4). The genes require regulatory sequences that are functional in plants and therefore all are chimeric structures. Some of the genes can act as selectable markers for both the nuclear and plastid genomes; however, they require separate regulatory sequences (Cheung et al., 1988). In plastids, the selectable marker genes are targeted to favourable sites within the plastid genome by homologous recombination (Svab and Maliga, 1993). In the nucleus, the insertions are random and therefore subject to position effects; however, technologies for targeted insertions are being developed (reviewed by Ow, 2002).

2.2.1. Aminoglycoside-modifying enzymes

The aminoglycoside antibiotics include a number of molecules (e.g. kanamycin, neomycin, gentamicin derivative G418, paromomycin) that are very toxic to plant, animal and fungal cells (reviewed by Nap et al., 1992). Kanamycin, which has played a prominent role in the development of plant transformation technologies, is produced by the soil actinomycete *Streptomyces kanamyceticus* as a trisaccharide composed of a deoxystreptamine and two glucosamines. Neomycin is a tetrasaccharide produced by another actinomycete, *Streptomyces fragdiae*. These antibiotics inhibit protein synthesis in bacteria by binding to the ribosomal subunits and similarly inhibit protein synthesis in eukaryote plastids and mitochondria.

A variety of aminoglycoside-modifying enzymes are commonly found among bacteria and antibiotic-producing actinomycetes and are usually encoded on extrachromasomal elements such as bacterial plasmids and transposons. Consequently, aminoglycoside resistance is prevalent among soil and enteric microbes (reviewed by Nap et al., 1992; Shaw et al., 1993; Davies and Wright, 1997). Three major classes of aminoglycoside-modifying enzymes have been used to create selection systems for plants; they confer resistance through ATP-dependent *O*-phosphorylation by phosphotransferases, acetyl CoA-dependent *N*-acetylation by acetyltransferases and ATP-dependent *O*-adenylation by nucleotidyltransferases.

2.2.1.1. Aminoglycoside-O-phosphotransferases.

Neomycin phosphotransferase. Bacterial aminoglycoside 3′-phosphotransferase II (APH [3′] II, E.C 2.7.1.95), also known as neomycin phosphotransferase II (NPTII), was shown to be effective as a selectable marker in mammalian and yeast cells, therefore it was the first to be tested in plants. Since that time it has become the most widely used selectable marker system in plants. NPTII catalyses the ATP-dependent phosphorylation of the 3′-hydroxyl group of the amino-hexose portion of certain aminoglycosides including neomycin, kanamycin, geneticin (G418), and paramomycin. The *npt*II (also known as *neo*) gene from *Escherichia coli* transposon Tn*5* was first used to construct chimeric genes for constitutive expression in plants by fusing it with the 5′ and 3′ regulatory sequences of the *A. tumefaciens* T-DNA gene nopaline synthase (*nos*). It was shown to be efficient in the selection of transformed petunia or tobacco cells on kanamycin or G418 (Fraley et al., 1983; Bevan et al., 1983; Herrera-Estrella et al., 1983). To a lesser extent the chimeric *nptI* gene from Tn*601* was also effective (Fraley et al., 1983). The *npt*II gene used in many plant selectable marker constructs, contained a mutation in the coding region that reduced the enzyme activity of NPTII (Yenofsky et al., 1990). This mutation has subsequently been corrected in some vectors (Datla et al., 1991). Research applications using *npt*II gene constructs have also diversified. For example, gene tagging experiments have been conducted in which promoterless *npt*II genes have been inserted randomly into *Nicotiana plumbaginifolia* and *Nicotiana tabacum*. Selection on kanamycin was used to recover insertions into expressed genes or gene regulatory elements to probe the plant genome for new and novel genes and regulatory elements that are not accessible through conventional cloning strategies (Andre et al., 1986; Teeri et al., 1986).

Regulation of *npt*II expression may be changed in various ways to alter the selection conditions. Elevation of transcription levels with strong promoters, like the cauliflower mosaic virus 35S promoter or the enhanced 35S promoter, raised the level of NPTII enzyme activity and tolerance to kanamycin without

creating instability in the expression of the *npt*II gene (Sanders et al., 1987; Kay et al., 1987). A potential problem with the 35S promoter is that, in addition to plants, it functions in bacteria, such as *E. coli* and *A. tumefaciens*. The same is true for the nopaline synthase promoter (nos) which was used in many early vector constructs. Furthermore, the 35S promoter is active in fungi and endophytic bacteria that colonize plants (discussed in Maas et al., 1997). There is a concern that expression in microorganisms may interfere with the study of the early events in transformation (Vancanneyt et al., 1990) and raises concerns about horizontal transfer of the *npt*II gene (Libiakova et al., 2001). The insertion of plant introns, such as intron 3 from the bean storage protein gene, phaseolin, into the *npt*II gene sequence has been shown to limit expression to the plant (Paszkowski et al., 1992). Furthermore, intron 2 from the potato ST-LS1 gene was found to limit *npt*II expression to dicots and monocots (Maas et al., 1997) without reducing tobacco or potato transformation efficiency (Libiakova et al., 2001). Other introns, such as intron 1 of the maize *Shrunken 1* (*Sh 1*) gene, limited expression selectively to monocots (Maas et al., 1997). These experiments demonstrate that the regulatory sequences fused to selectable marker genes are very important for maximizing efficiency for specific plants.

The *npt*II gene is the most frequently used selectable marker gene for generating transgenic plants for research purposes. An examination of research publications from the year 2002 appearing in the peer-reviewed journals, *The Plant Cell*, *Plant Molecular Biology*, *Molecular Breeding and Transgenic Research*, revealed that 44–77% of the studies that used transgenic plants used the *npt*II gene as the selectable marker (Table 1). The gene is very efficient in model research species such as *Arabidopsis* and tobacco, which represent 15–73% of the dicot species or rice and maize, which are the most common monocots used in published studies (4–33%). A review of field trial notifications and permits in the US in 2001 and 2002 shows that *npt*II is the most widely used selectable marker in transgenic crops (Table 3). It is found in many of the crops currently approved for commercial production (Table 2). International regulatory agencies have approved the commercial release of genetically modified oilseed rape, corn, potato, tomato, flax, chicory and cotton containing the *npt*II gene (Table 2).

There have been no reports of adverse effects of either NPTII or the *npt*II gene on humans, animals or the environment (Flavell et al., 1992; US FDA, 1998; European Federation of Biotechnology, 2001).

Generally, the amount of NPTII protein expressed in plants is low ranging, for example, from 0.00005 to 0.001% of the fresh weight of cotton seed, potato tuber or tomato fruit. To obtain enough protein for safety assessments, the protein was expressed in *E. coli* and purified (Fuchs et al., 1993a). Studies with mice revealed that NPTII degraded rapidly in simulated gastric and intestinal fluids suggesting that the use of aminoglycoside antibiotics would not be compromised and that allergic responses would be unlikely (Fuchs et al., 1993b). Furthermore, consumption of massive dosages of NPTII did not generate ill effects on the health of mice (Fuchs et al., 1993b). NPTII has been approved by the US Food and Drug Administration (FDA) as a food additive for tomato, cotton and oilseed rape (US FDA, 1994). Because of the relative toxicity of kanamycin and neomycin and the wide spread resistance to these antibiotics, they are rarely used for human therapy. A 1993 WHO workshop concluded that the use of the *npt*II marker gene in genetically modified plants posed no risks to human health (WHO, 1993).

An assessment of the ecological impact of the use of the *npt*II gene in crops has been discussed at length by Nap et al. (1992). It seems that the amount of free kanamycin accumulating in soils, through the action of microorganisms or animal feces, is restricted by absorption to soil components so that no direct selection pressure for kanamycin resistant plants can occur. Changes to the genotype of transgenic plants are limited and enhancement of physiological fitness resulting from pleiotropic effects of *npt*II expression has not been documented.

All of the above studies addressed *npt*II expression in the nuclear genome. Low levels of kanamycin can also be used to select for transformation of the chloroplast genome. The promoter P_{rrn}, which is the strong constitutive promoter of the rRNA operon, was fused transcriptionally to the 5′ untranslated region and the first five codons of the *rbc*L gene (Carrer et al., 1993). The efficiency of selection is about 3–20-fold lower than with the *aadA* gene (see below) as the toxicity of kanamycin to plant cells does not allow sufficient time for the transplastome to replicate and distribute over

several cell divisions (Carrer et al., 1993). Eventually, amplification of the inserted *npt*II gene will achieve 10,000 copies per cell and accumulate NPTII up to 1% of total soluble protein (Carrer et al., 1993).

Hygromycin phosphotransferase. Hygromycin B is an aminocyclitol antibiotic inhibitor of protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes. In plants, the antibiotic is very toxic. The *E. coli* gene *aphIV* (*hph*, *hpt*), coding for hygromycin B phosphotransferase (HPT, E.C. 2.7.1.119), confers resistance on bacteria, fungi, animal cells and plant cells (discussed in Waldron et al., 1985; van den Elzen et al., 1985) by detoxifying hygromycin B via an ATP-dependent phosphorylation of a 7″-hydroxyl group. Chimeric genes have been shown to be effective in selection with diverse plant species, including dicots, monocots and gymnosperms (Ortiz et al., 1996; Tian et al., 2000). This enzyme has been used as a selectable marker when *npt*II was not found to be effective (Twyman et al., 2002).

Hygromycin B is the second most frequently used antibiotic for selection after kanamycin; for instance, a sampling of publications in 2002 revealed that it was used in 19–31% of the papers in which transgenic plants were generated for research purposes (Table 1). Consistent with this observation is that HPT is the second most prevalent antibiotic selectable marker listed in the US field trials data base (Table 3, ISB, 2003).

Streptomycin phosphotransferase. The gene coding for streptomycin phosphotransferase (SPT, APH [3″], E.C. 2.7.1.87) comes from the bacterial transposon, Tn*5* (Mazodier et al., 1985). A mutant form of SPT, containing a two amino acid deletion near the carboxy-terminus of the protein, was placed under the control of the T-DNA transcript 2′ promoter and introduced into *N. tabacum*. Transformed calli were selected in the presence of streptomycin. As streptomycin causes bleaching rather than cell death, transformed tissue was recognized as green tissue. The efficiency of transformation using this streptomycin resistance marker was comparable to the *npt*II gene under control of the *nos* promoter (Maliga et al., 1988). This marker system has not been adopted for general use.

2.2.1.2. Aminoglycoside-N-acetyltransferases. The aminoglycoside-*N*-acetyl transferases (AAC) are another class of aminoglycoside-modifying enzyme with potential to act as plant selectable marker genes (reviewed by Nap et al., 1992). Two of these enzymes, AAC(3)-III and AAC(3)-IV, have been examined in petunia and *Arabidopsis* under the control of the 35S promoter and *nos* 3′ sequences (Hayford et al., 1988). These enzymes acetylate gentamicin, kanamycin, tobramycin, neomycin and paromomycin. AAC(3)-IV additionally modifies apramycin and G418. Both genes conferred high levels of resistance to gentamicin in petunia; however, the level of cross resistance to kanamycin by AAC(3)-IV was marginal (Hayford et al., 1988). The gene was effective in a variety of plants including *Brassica napus*, *Nicotiana tabacum* and tomato (Hayford et al., 1988).

Another enzyme that acetylates the 6′ amino group, aminoglycoside-6′-*N*-acetyltransferase (AAC(6′)) from *Shigella* sp., yielded efficient selection of transformed tobacco protoplasts on high levels of kanamycin (Gossele et al., 1994). The gene, *6′ gat*, under the control of the 35S promoter, is therefore a functional alternative to the *npt*II gene.

2.2.1.3. Aminoglycoside-O-nucleotidyltransferases. Aminoglycoside-*O*-nucleotidyltransferases represent the third class of enzymes that modify the aminoglycoside antibiotics that can be used as plant selectable marker genes (reviewed by Nap et al., 1992). The bacterial *aadA* gene codes for the enzyme aminoglycoside-3″-adenyltransferase. When driven by the 35S promoter, the *aadA* gene conferred resistance to spectinomycin and streptomycin in *N. tabacum*; however, the selection was for the contrast between green tissue and chlorotic tissue rather than for survival and growth (Svab et al., 1990). Similar results were obtained with white clover (Larkin et al., 1996) and with maize (Lowe et al., 1995). This gene has not been broadly adopted as a nuclear selectable marker gene for the production of transgenic plants. However, it is the most widely used selectable marker for plastid transformation. When combined with spectinomycin selection, plastid transformation frequencies in tobacco may approach the levels of nuclear transformation (Svab and Maliga, 1993).

The *aadA* gene is found in several transgenic lines approved for commercialization (Table 2) but it is

under the control of a bacterial promoter, not a plant promoter. It was used as a selectable marker during bacterial cloning and not for the selection of transgenic plants (AGBIOS, 2003)

2.2.2. Bleomycin resistance

Phleomycin and Bleomycin are novel antibiotics that belong to the bleomycin family of glycopeptides that act by site-specific, single- and double-stranded DNA cleavage (discussed in Hille et al., 1986; Perez et al., 1989). Interestingly, strand cleavage does not appear to generate mutations when applied to plants. Bleomycin interferes with tobacco plant regeneration through morphogenesis (Perez et al., 1989). Two sources of resistance have been described for plants: the resistance gene found on *E. coli* transposon Tn*5* and a chromosomal gene of *Streptoalloteichus hindustanus* (Hille et al., 1986; Perez et al., 1989). When expressed at high levels from the 35S promoter, both genes yield high levels of resistance to phleomycin and regeneration of tobacco plants (Perez et al., 1989). So far, this system does not appear to have been widely adopted.

2.2.3. Mutant dihydropteroate synthase

A large number of sulfonamides or sulfa drugs exist as antimicrobial compounds that inhibit the enzyme dihydropteroate synthase (DHPS, E.C. 2.5.1.15). DHPS catalyzes a rate limiting step for folic acid synthesis in bacteria and plants (discussed in Wallis et al., 1996; Guerineau et al., 1990). Resistance is encoded by *sul* genes on bacterial R plasmids (discussed in Guerineau et al., 1990). The resistance gene *sul*I from plasmid R46 codes for a mutant form of DHPS that is resistant to inhibition by the sulfonamides. To be effective in plants, the enzyme must be targeted to the chloroplast. For example, cleavage of the transit peptide sequence of the pea ribulose bisphosphate carboxylase/oxygenase gene fused to the *sul*I gene, results in the deposition of the enzyme into the chloroplast stroma. Effective selection and regeneration of tobacco were demonstrated when this construct was expressed using the 35S promoter. The selection system differs from the others described so far in that the mechanism is a mutation of the enzyme resulting in resistance rather than detoxification of the antibiotic by the enzyme. Interestingly, the chimeric *sul*I gene described above is one of the few alternatives to *npt*II for the transformation of potato cv Russet Burbank because of inefficiencies and abnormalities associated with other selection systems (Wallis et al., 1996). In the Mediterranean, where parasitic weeds such as broomrape (*Orobanche* spp.) are a constraint to production, resistance to the sulfonamide asulam may allow the use of sulfonamides as a herbicide (Surov et al., 1998).

2.2.4. Streptothricin acetyltransferase

Streptothricins produced by *Streptomyces* spp. are antimicrobial agents that consist of gulosamine, streptolidin and a peptide chain of 1–6 residues (reviewed in Jelenska et al., 2000). They inhibit protein synthesis by binding to the ribosomal small subunit. The *E. coli sat3* gene codes for an acetyl transferase activity that inactivates streptothricins. When controlled by the 35S promoter the *sat* gene acted as a selectable marker gene in a variety of dicot plant species (Jelenska et al., 2000).

2.2.5. Chloramphenicol acetyltransferase

Chloramphenicol acetyltransferase (E.C. 2.3.1.2, CAT) from *E. coli* Tn*9* has been used for the selection of tobacco transformants with the *cat* gene driven by the *nos* promoter (DeBlock et al., 1984). Selection on chloramphenicol was much less efficient than selection on kanamycin conferred by the *npt*II gene. The inefficiency has limited the use of the *cat* gene as a selectable marker; however, the sensitive assay for enzyme activity enhanced its use as a reporter gene for transformation events in early studies. This enzyme is no longer widely used as a reporter gene.

Only four occurrences of the CAT selectable marker in plants were found in the database of US field trial notifications (ISB, 2003). The most recent of these notifications was in 1992 indicating that this marker is no longer widely used. Three of the four notifications list NPTII as the selectable marker in addition to CAT.

The CAT gene controlled by the *nos* promoter has also been introduced in the tobacco chloroplast genome by *Agrobacterium*-mediated transformation under selection with chloramphenicol (DeBlock et al., 1985).

2.3. Conditional-positive selection systems using herbicides

Like antibiotics, herbicides act on a variety of specific target sites within plants. The sources of genes

used to achieve selection on herbicides range from bacterial to plant in origin (Table 5). Some of the plant genes code for enzymes in essential metabolic and biosynthetic pathways. At least two mechanisms are employed to achieve resistance. One mechanism uses the resistance found in natural isozymes or generated by enzyme mutagenesis, and the second involves detoxification of the herbicide by metabolic processes. Selection with antibiotics and herbicides is similar in that both categories of agents are toxic to non-transformed plant cells and transformed plant cells are provided with mechanisms that allow them to escape the toxicity.

2.3.1. Phosphinothricin N-acetyltransferase or bialophos resistance gene

The L-isomer of phosphinothricin (PPT; glufosinate ammonium) is the active ingredient of several commercial broad spectrum herbicide formulations (e.g. Basta™, Ignite™, Liberty™). An analogue of L-glutamic acid, PPT is a competitive inhibitor of glutamine synthetase (GS) which is the only enzyme that can catalyse the assimilation of ammonia into glutamic acid in plants. Inhibition of glutamine synthetase ultimately results in the accumulation of toxic ammonia levels resulting in plant cell death (OECD, 1999).

Two sources of resistance have been described. Elevation of GS expression levels using strong promoters will confer resistance to PPT (Eckes et al., 1989) but this approach has not been adopted for commercial applications. Secondly, bacterial acetyltransferases that confer resistance to bialophos (consisting of two L-alanine residues and PPT) have been used in plants to achieve resistance to herbicides that contain PPT.

Two genes (*pat* and *bar*) encoding the enzyme phosphinothricin *N*-acetyltransferase (PAT) have been used to confer tolerance to L-PPT in transgenic plants. The *bar* (bialophos resistance) gene from *S. hygroscopicus* (Thompson et al., 1987) and the *pat* gene from *S. viridochromogenes* (Wohlleben et al., 1988) are 87% similar at the nucleotide level. PAT uses acetyl CoA as a cofactor to catalyze the acetylation of the free amino group of L-PPT. The acetylated form of L-PPT is unable to bind to and inactivate glutamine synthetase. The *bar* gene driven by plant promoters was shown to be an effective selectable marker gene in *Brassica napus* and *Brassica oleracea* (DeBlock et al., 1989) and subsequently found to be an excellent selectable marker for many species including maize (Fromm et al., 1990; Gordon-Kamm et al., 1990), wheat (Vasil et al., 1992), rice (Rathore et al., 1993), legumes (Larkin et al., 1996) and conifers (Brukhin et al., 2000). The *bar* gene is particularly useful in plants, such as orchids, that are naturally tolerant to antibiotics (Knapp et al., 2000).

Expression of the *bar* gene in the tobacco plastid genome yielded field levels of resistance to PPT; however, direct selection for transplastomic plants using *bar* was not successful indicating that the compartment, in which PAT is located, is essential for selection on PPT (Lutz et al., 2001).

In samples of research papers published in 2002, the *bar* gene was the most extensively-used herbicide-resistance selectable marker gene (4–31%). The level of use was similar to that of the *hpt* gene, which confers resistance to the antibiotic hygromycin B (Table 1). L-PPT tolerance is also being extensively used in plants undergoing transgenic field trials. For example, in the years 2001 and 2002 alone, 327 records containing the enzyme PAT were listed in the US field trial database (Table 3; ISB, 2003). From the records in the database, it is evident that a variety of companies and researchers are using PAT in their studies.

L-PPT tolerant plants containing the *pat* or *bar* genes have been deemed safe by various international government regulatory agencies for unconfined release and food and livestock feed use (Table 2, AGBIOS, 2003). *B. napus* L. line HCN92, which contains the *pat* gene, was the first transgenic L-PPT tolerant plant to receive government approval (CFIA, 1995b). Since then other L-PPT tolerant lines including oilseed rape, maize, chicory and sugar beet lines have received approval for commercialization (Table 2).

2.3.2. 5-Enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidase

Glyphosate (*N*-[phosphonomethyl]glycine) is a broad-spectrum herbicide that is the active ingredient of the commercial Roundup® formulations. It acts as an inhibitor of the plastid enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, E.C. 2.5.1.19) which is essential in the shikimate pathway for the biosynthesis of the aromatic amino acids. A number of mechanisms for glyphosate

resistance have been described. Examples include the following: over expression of a petunia EPSP synthase gene using the 35S promoter generated glyphosate tolerance in transformed petunia (Shah et al., 1986); expression of mutant forms of the EPSP synthase gene *aroA* from *Salmonella typhimurium* (Comai et al., 1988) or *E. coli* (della-Cioppa et al., 1987) targeted to chloroplasts, conferred glyphosate resistance to tobacco; a naturally-glyphosate-resistant EPSP synthase gene from the *A. tumefaciens* strain CP4 (Barry et al., 1992) fused to the transit peptide sequence of *Arabidopsis* EPSP synthase for chloroplast targeting has conferred glyphosate resistance to several crop species (Table 2; AGBIOS, 2003); catabolism of glyphosate to glyoxylate and aminomethylphosphonic acid (AMPA) by bacterial glyphosate oxidoreductase (GOX) targeted to the chloroplast has conferred glyphosate resistance to several different plants (Barry et al., 1992; Howe et al., 2002).

The GOX gene from *Ochrobactrum anthropi* strain LBAA has been modified to improve expression in plants and fused to the transit peptide sequence of *Arabidopsis* ribulose bisphosphate carboxylase small subunit gene, SSU1A-CTP1 for transport to the chloroplast (Barry et al., 1992; Barry and Kishore, 1995; Monsanto, 2003). It has been used as a selectable marker in tobacco, *Arabidopsis*, potato and sugarbeet (Barry and Kishore, 1995). GOX was ineffective as a selectable marker in maize although the regenerated plant had resistance to glyphosate (Howe et al., 2002). GOX has been used as a selectable marker in conjunction with EPSPS that has been fused to the transit peptide sequence of *Arabidopsis* EPSP synthase for chloroplast targeting. In Roundup-Ready® canola, a variant of the GOX gene from *Ochrobactrum anthropi* strain LBAA (*goxv247*) and the *cp4 epsps* gene are linked on a single T-DNA to achieve glyphosate resistance (Monsanto 2003). Direct selection for glyphosate resistance using the *gox* and *cp4 epsps* genes have been demonstrated, for instance, in wheat (Zhou et al., 1995). The *cp4 epsps* gene alone has been shown to be effective in soybean (Clemente et al., 2000) and functional in maize (Armstong et al., 1995; Russell and Fromm, 1997). The maize EPSP synthase gene, altered by site-directed mutagenesis to increase tolerance to glyphosate, was shown to be very effective as a selectable marker gene for maize (Howe et al., 2002). The *cp4 epsps* gene also confers resistance to glyphosate when expressed in the chloroplast genome; however, the transplastomic plants were selected using antibiotic resistance (Ye et al., 2001). Generally, selection on glyphosate has not been adopted broadly for basic research involving transgenic plants (Table 1).

The use of EPSP synthase (and GOX) in transgenic plants has undergone extensive safety evaluations (Padgette et al., 1996; Monsanto, 2003). Transgenic plants, which contain glyphosate resistance as either an agronomic trait or a selectable marker, have received approval for commercialization (Table 2). These include Roundup Ready® canola, corn, soybean and cotton. The *goxv247* gene no longer appears to be used in crop development. Of the commercially grown Roundup Ready® crops, only Roundup Ready® canola contains both the *cp4 epsps* and *goxv 247* genes. A search of the information available on the US field trials database did not reveal any public records after 1998 containing GOX (ISB, 2003). However, the *epsps* gene is still widely used, mostly to confer glyphosate resistance. In 2001 and 2002, 507 records containing EPSPS were found in the US field trials database, which includes the use of the EPSP synthase to confer herbicide tolerance and/or as a selectable marker (Table 3). The overwhelming majority of these notifications were from Monsanto (ISB, 2003).

2.3.3. Acetolactate synthase or acetohydroxyacid synthase

Acetolactate synthase, also known as acetohydroxyacid synthase (ALS, AHAS: E.C. 4.1.8.13), is the target for several classes of herbicides including the sulfonylureas, imidazolinones, triazolopyrimidines and pyrimidinyl thiobenzoates (Singh and Shaner, 1995). ALS is a regulatory enzyme in the biosynthetic pathway to branched-chain amino acids in chloroplasts and it is encoded by a limited number of nuclear genes depending on the plant species. ALS genes are amenable to mutation and yield mutant enzymes that are resistant to one or more of the herbicides that act on ALS. Many of the specific sites have been mapped for ALS genes (Guttieri et al., 1996). Several plant mutants have been isolated directly through mutagenesis and selection strategies; for example, imidazolinone-resistant *B. napus*, which is in production in Canada (Swanson et al., 1989; CFIA, 1995a). In general, herbicide resistant forms of ALS differ by only one or two amino acids

from the native form. Selection for sulfonylurea and imidazolinone resistance is very efficient and was used to demonstrate targeted modifications of endogenous ALS from wild-type to herbicide resistance form using chimeric RNA/DNA oligonucleotides. This was achieved with tobacco (Beetham et al., 1999) and maize (Zhu et al., 2000), generating plants with targeted mutations that were not transgenic (i.e. foreign DNA sequences were not integrated into the plant genome).

It is therefore not surprising that mutant forms of plant ALS would act as effective selectable marker genes when combined with sulfonylurea or imidazolinone herbicides. Selection of transgenic tobacco plants on sulfonylureas in culture was shown with a mutant *Arabidopsis* gene, *csr 1-1* (Olszewski et al., 1988; Charest et al., 1990) and direct selection under greenhouse conditions was demonstrated for *B. napus* canola (Miki et al., 1990). A mutant form of the maize ALS gene was found to be very efficient in the selection of transgenic maize in culture from embryogenic cells (Fromm et al., 1990). A mutant *Arabidopsis* ALS gene that confers resistance to imidazolinones was used to recover transgenic soybean from cultured apical meristems, which accumulate the imidazolinone, Imazapyr (Aragao et al., 2000).

Several lines of genetically modified carnation approved for commercialization were developed using the ALS encoding mutant gene *surB* from tobacco as a selectable marker (AGBIOS, 2003). Five public records containing ALS or AHAS were listed in the US field trials database for the years 2001 and 2002 suggesting that this gene is not being widely adopted as a selectable marker system (Table 3; ISB, 2003).

2.3.4. Bromoxynil nitrilase

The oxynil herbicides, such as bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile), are inhibitors of photosystem II electron transport that are active in many plants but not in monocots. A nitrilase enzyme (3,5-dibromo-4-hydroxybenzonitrile aminohydrolase: E.C. 3.5.5.6), coded by the *bnx* gene from *Klebsiella pneumoniae* subspecies *ozanaenae*, hydrolyzes bromoxynil into 3,5-dibromo-4-dihydroxybenzoic acid and ammonia. The *bnx* gene has been shown to confer resistance to bromoxynil in tobacco (Stalker et al., 1988) and *B. napus* without using other selectable marker genes (Freyssinet et al., 1996). The gene is therefore another example of a herbicide-resistance selectable marker gene.

A complete safety assessment of the use of the *bxn* gene in transgenic plants has led to the regulatory approval for the commercialization of at least three transgenic lines containing the *bxn* gene. In canola line Oxy-235, bromoxynil was used as the only selective agent during transformation. This line was approved for environmental release and for food and livestock feed in Canada in 1997 (AGBIOS, 2003; CFIA, 1998; Health Canada, 1999). It is the parental line for commercial Navigator™ canola varieties (AGBIOS, 2003). Two cotton lines contain the *bnx* gene but the *npt*II gene was used as the selectable marker (Table 2). No public records for the nitrilase enzyme as a selectable marker were listed in the US field trials database in 2001 or 2002 suggesting that it is not widely used (ISB, 2003).

2.3.5. Glutamate-1-semialdehyde aminotransferase

Gabaculine (3-amino-2,3-dihydrobenzoic acid) is a bacterial phototoxin that inhibits a wide range of pyridoxal-5-phosphate-linked aminotransferases. A mutant form of glutamate-1-semialdehyde aminotransferase (GSA-AT, E.C. 5.4.3.8) encoded by the *hem*L gene, was discovered in a gabaculine-resistant cyanobacterium, *Synechococcus* PCC6301 strain GR6. The *hem*L gene, expressed at very high levels in tobacco using the double 35S promoter and targeted to chloroplasts with the transit peptide of the ribulose bisphosphate carboxylase small subunit, yielded green transformed tissue that could be distinguished from chlorotic untransformed tissue (Gough et al., 2001). Seedlings also segregated as green and white phenotypes (Gough et al., 2001). It was suggested that the system may be used to develop a chloroplast selection system but no experiments were presented.

2.3.6. Cyanamide hydratase

Cyanamide is a nitrile derivative that in its aqueous or calcium salt forms can be used as a fertilizer. It has the additional characteristic of acting as a non-persistent herbicide when applied prior to seed germination. The gene *cah* coding for the enzyme cyanamide hydratase (urea hydrolase; E.C. 4.2.1.69) has been isolated from the soil fungus *Myrothecium verrucaria* (Maier-Greiner et al., 1991a). Cyanamide

hydratase catalyzes the hydration of the nitrile group of cyanamide to form urea, which can be used for plant growth. The enzyme has an extremely narrow substrate specificity. The use of cyanamide hydratase as a selectable marker has been demonstrated in wheat (Weeks et al., 2000), tobacco (Maier-Greiner et al., 1991b), potato, tomato, rice and *Arabidopsis* (Damm, 1998). A search of the US field trials database shows that cyanamide hydratase has also been used in sorghum and soybean (Table 3; ISB, 2003).

2.4. Conditional-positive selection systems using toxic metabolic intermediates, analogues and drugs

Enzymes acting in a wide range of metabolic pathways in plants can be targets for inhibitors or drugs (Table 6). Furthermore, sources of resistance may be found in diverse organisms as discussed for the herbicides. The manipulation of metabolic and biosynthetic pathways can potentially alter the composition and form of the transgenic plants. This has been reported in some but not all cases. The research and assessment of these selectable marker genes has not progressed to the level of the major antibiotic and herbicide-resistance marker genes.

2.4.1. 2-Deoxyglucose-6-phosphate phosphatases

The glucose analogue, 2-deoxyglucose (2-DOG), is phosphorylated by hexokinase to form 2-DOG-6-phosphate. 2-DOG-6-phosphate competes with glucose-6-phosphate causing cell death through the inhibition of glycolysis. The yeast gene *DOG*^{R}*1*, coding for 2-deoxyglucose-6-phosphate phosphatase, was placed under the control of the 35S promoter. Use of this construct as a selectable marker gene resulted in the selection of transgenic tobacco plants at lower efficiencies than with the *npt*II gene and the selection of transgenic potato with comparable efficiencies (Kunze et al., 2001). The selection system was also demonstrated in pea (Sonnewald and Ebneth, 1998). Abnormalities were not observed in the plants presumably due to the narrow substrate specificity of the enzyme (Kunze et al., 2001).

2.4.2. Aldehyde dehydrogenase

Small aldehydes, such as betaine aldehyde, are phytotoxic to many plant cells. The spinach enzyme, betaine aldehyde dehydrogenase (BADH), is highly specific for betaine aldehyde and converts it to glycine betaine, which accumulates in a few crop species as an osmoprotectant. The enzyme is nuclear encoded but is transported to the chloroplast, which is the site of action. Expression in tomato allowed the direct selection and regeneration of plants in the presence of betaine aldehyde at efficiencies lower than that of the *npt*II gene (Ursin, 1996).

The enzyme is well suited as a chloroplast selectable marker gene. It is 25-fold more efficient than spectinomycin resistance conferred by the *aadA* gene and acts much faster (Daniell et al., 2001). Homoplasty was achieved in the transplastomic tobacco plants and they were morphologically normal. BADH appears to be a good alternative to the use of antibiotic resistance marker genes for the production of transplastomic plants.

2.4.3. Dihydrodipicolinate synthase and aspartate kinase

The aspartate family pathway, which leads to the biosynthesis of lysine, threonine, methionine and isoleucine, is regulated by a number of feedback loops. Key enzymes, such as aspartate kinase, are feedback-inhibited by lysine and threonine (LT). Dihydrodipicolinate synthase is inhibited by lysine or its toxic analogue S-aminoethyl L-cysteine (AEC), which competes with lysine in protein synthesis. Growth in the presence of lysine and threonine causes methionine starvation due to inhibition of the pathway and results in strong inhibition of growth. The enzymes from *E. coli* are less sensitive to feedback inhibition. When controlled by the 35S promoter, *E. coli* enzyme constructs yielded transgenic potato plants with very few escapes on selection with LT for aspartate kinase and AEC for dihydrodipicolinate synthase (Perl et al., 1993). One of the potential drawbacks is that the overproduction of lysine or threonine resulting from the modification of metabolism causes abnormalities in some plants (Perl et al., 1993).

2.4.4. Octopine synthase

Potential pathways for the detoxification of the lysine analogue, AEC, may involve the enzyme, octopine synthase or lysopine dehydrogenase. The gene for this enzyme is part of the T-DNA component of the *Agrobacterium tumefaciens* octopine Ti plasmids. The enzyme converts pyruvate and lysine into lysopine

and appears to metabolize AEC to carboxyethyl-AEC. Callus tissues that express the enzyme appear to be 20-fold more tolerant to AEC (Dahl and Tempe, 1983). Selective growth of callus on AEC was shown in preliminary experiments with petunia stem explants (Koziel et al., 1984).

2.4.5. Tryptophan decarboxylase

In *Catharanthus roseus*, tryptophan decarboxylase (TDC; E.C. 4.1.1.28) is an enzyme in the terpenoid indole alkaloid pathway that converts L-tryptophan into tryptamine. Another substrate of TDC, 4-methyltryptophan (4-mT), is toxic to plants that do not have TDC activity but will be converted to typtamine in those plants that do have it. When the *C. roseus* gene coding for TDC was placed under the control of the 35S promoter and introduced into tobacco, direct selection on 4-mT yielded transgenic plants with the same efficiency as the *npt*II gene (Goddijn et al., 1993). Although the specificity of the reaction was considered an advantage, a possible drawback could be the accumulation of tryptamine in the transformed plants (Goddijn et al., 1993).

2.4.6. Dihydrofolate reductase

Antifolate drugs, such as trimethoprim and methotrexate (Mtx), bind to the active site of the enzyme dihydrofolate reductase (DHFR, E.C. 1.5.1.3) resulting in impaired protein, RNA and DNA biosynthesis and subsequently cell death. Plant cells are generally very sensitive to low levels of Mtx. Sources of resistant DHFR have been found in the bacterium *E. coli* (Brisson and Hohn, 1984; Herrera-Estrella et al., 1983), the fungus *Candida albicans* (Irdani et al., 1998) and mutant mammalian cells (Eichholtz et al., 1987). Testing in transgenic tobacco and petunia confirmed that these genes could be used for selection of transgenic plants on Mtx. A novel and unexpected observation was the finding that the *C. albicans* gene provided resistance in plants when used with the endogenous fungal regulatory sequences (Irdani et al., 1998), suggesting that the level of expression required for resistance with this gene may be very low.

2.5. Conditional-positive selection systems using non-toxic metabolic intermediates

Conditional-positive selection systems with non-toxic metabolic intermediates are recent developments and limited in number as shown in Table 7. This category differs significantly from the previously discussed systems in that the external substrates are basically inert until they are converted into molecules that provide the transformed plant cells with a growth advantage. This approach appears to yield generally higher transformation frequencies and seems to be broadly applicable across a range of plant species making it is an area of major interest for crop plants. The systems described so far use bacterial genes as selectable markers that act on fundamental plant metabolic pathways. Currently, the information is not as extensive as that available for the major antibiotic and herbicide resistance genes.

2.5.1. Xylose isomerase

Plant cells from species such as tobacco, potato and tomato cannot use D-xylose as a sole carbon source. The enzyme xylose isomerase (D-xylose ketol-isomerase; E.C. 5.3.1.5) catalyzes the isomerization of xylose to D-xylulose, which can then be used as a carbon source. The *xylA* genes, coding for xylose isomerase from *Streptomyces rubiginosus* (Haldrup et al., 1998a) and *Thermoanaerobacterium thermosulfurogenes* (Haldrup et al., 1998b), have been fused to the enhanced 35S promoter and the Ω′ translational enhancer from tobacco mosaic virus for testing in transgenic tobacco, potato and tomato as selectable markers. The efficiency of selection was much greater than for the *npt*II gene and the regeneration of shoots was significantly faster. Furthermore, for at least some Solanaceous species, the overall efficiency of transformation was enhanced with both *xylA* genes. It was suggested that the enzyme from *S. rubiginosus* posed no biosafety issues as it is used in the food industry and considered safe (Haldrup et al., 1998a).

2.5.2. Phosphomannose isomerase

Mannose like xylose is not toxic to plant cells. However, mannose will prevent cell growth and development when mannose is converted by hexokinase to mannose-6-phosphate, which on accumulation inhibits glycolysis. Phosphomannose isomerase (PMI; E.C. 5.3.1.8) catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate, which allows mannose to become a carbon source. Although the enzyme is widely distributed in na-

ture, it is absent in many plants although leguminous plants such as soybean have PMI activity (Privalle et al., 2000). Using mannose as the selective agent, the *E. coli manA* (*pmi*) gene under the control of the 35S promoter was found to be an effective selectable marker. Using this selection system, 10-fold greater transformation frequencies were obtained in sugar beet (*Beta vulgaris* L.) compared with the frequencies obtained using the *npt*II gene and kanamycin as the selective agent (Joersbo et al., 1998). These dramatic results were followed by similar findings in maize, wheat, barley, watermelon (reviewed in Reed et al., 2001) and in rice (Lucca et al., 2001). In all cases significantly higher transformation frequencies were observed and very few escapes were found. It is believed that the arrest in cell growth of untransformed cells by starvation rather than the necrosis induced by toxic selective agents may contribute to the survival and growth of the transformed cells and the high transformation frequencies reported. In some species, such as cassava, the frequency of transformation was lower than that achieved with the *hpt* gene (Zhang and Puonti-Kaerlas, 2000).

The system is being marketed as the Positech™ selection technique by Syngenta. Safety assessments have been performed including allergenicity and toxicity studies (Privalle et al., 2000; Reed et al., 2001; Privalle, 2002). The enzyme was found to be completely digested in simulated mammalian gastric and intestinal fluids. PMI protein had no adverse effects on mice following acute oral toxicity studies. Furthermore, there appeared to be no changes in the glycoprotein profiles of transgenic maize or sugar beets. Field trials conducted on seven independent transformation events demonstrated that there were no differences in the agronomic performance or grain composition of transgenic maize compared to non-transgenic controls (Privalle et al., 2000; Reed et al., 2001).

2.5.3. β-Glucuronidase

The enzyme β-glucuronidase (GUS, E.C. 3.2.1.31), encoded by the *E. coli uidA* (*gusA*) gene, will be discussed later as a non-selectable marker or reporter gene. GUS catalyses the hydrolysis of β-D-glucuronides. The glucuronide substrate has been conjugated with the cytokinin, benzyladenine, to create benyladenine *N*-3-glucuronide which does not affect plant growth and differentiation. However, hydrolysis by GUS releases benzyladenine which will stimulate shoot regeneration. This process has been shown to be an effective conditional-positive selection strategy in tobacco (Joersbo and Okkels, 1996). The frequency of transformation scored by shoot regeneration was much greater than that achieved by the *npt*II gene in control experiments (Joersbo and Okkels, 1996). An added advantage is that the activity of GUS can be used as visual marker without the use of an additional gene or gene fusion.

2.6. Non-conditional-positive selection systems

Positive non-conditional selection systems include new strategies that promote plant regeneration without the use of selective agents. They provide novel opportunities to develop new selectable marker genes. An obstacle to the development of this technology is the lack of knowledge of the genetic and biochemical controls of plant regeneration through organogenesis and embryogenesis. Presently, information on the mechanisms governing shoot organogenesis and cytokinin signal transduction is greater than for embryogenesis. A number of genes that confer cytokinin-independent shoot formation have been discovered (reviewed by Zuo et al., 2002a). Some of these may also act as selectable markers as described in Table 8. They include genes encoded by the T-DNA region of *Agrobacterium* Ti and Ri plasmids as well as *Arabidopsis* genes coding for the putative cytokinin receptor, CKI1 (Takimoto, 1996; Zuo et al., 2002a), and the transcription factor, ESR1 (Banno and Chua, 2002).

The need for genes that control embryogenesis has been argued by Zuo et al. (2002a) as most crops regenerate through embryogenesis rather than organogenesis. Genes that act very early in embryogenesis have been discovered using a variety of experimental approaches but experiments to demonstrate their utility as selectable marker genes have not yet been published. Except for the SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (*SERK1*) gene, these genes code for transcription factors that are important in the control of development. In *Arabidopsis* the *AtSERK1* gene is expressed in the embryo sac prior to fertilization and throughout early embryo development. Ectopic expression of *AtSERK1* from the 35S promoter increased the efficiency of somatic embryogenesis from callus by 3–4-fold (Hecht et al.,

2001). The *Arabidopsis* transcription factor LEAFY COTYLEDON 1 (LEC1) is a CCAAT box-binding factor HAP3 subunit homolog that appears to play several roles in embryo development. When expressed ectopically, it will generate embryos from the vegetative leaf cells of germinated seedlings (Lotan et al., 1998). It therefore plays a central role in the induction of embryogenesis; however, plants with normal morphology were not recovered even with an inducible promoter system (Zuo et al., 2002a). LEC2, another B3 domain transcription factor, also induces somatic embryo development in transgenic *Arabidopsis* (Stone et al., 2001). The *B. napus* transcription factor BABYBOOM (BBM) is a member of the AP2-domain transcription factors that also plays a central role in embryogenesis. It was isolated from microspores undergoing the transition from the pollen to embryo developmental pathways. When expressed at high levels from the 35S promoter (Boutilier et al., 2002), BBM converted the vegetative cells of *Arabidopsis* and *B. napus* seedlings into somatic embryo-producing cells. Regenerated plants expressing very high levels of BBM possessed abnormal morphologies. The *Arabidopsis* homeodomain transcription factor, WUSCHEL (WUS) was a potent inducer of the vegetative-to-embryonic cell transition and is believed to be involved in embryonal stem cell formation (Zuo et al., 2002b). An interesting finding was that WUS appears to play an important role in both embryogenesis and the shoot apical meristem through separate developmental pathways (Zuo et al., 2002b). Further research on the genes that control plant embryogenesis may soon result in the development of new selectable marker strategies.

2.6.1. Isopentyl transferases

Organogenesis in vitro occurs in three phases: the acquisition of competence, determination of organ formation governed by phytohormone balance and morphogenesis (Sugiyama, 1999). For shoot formation in culture, high cytokinin:auxin ratios are required. Genes that promote this condition endogenously will enhance regeneration of shoots thus providing a novel non-conditional-positive selection strategy. The enzyme isopentyl transferase (IPT), which is encoded by the T-DNA of *A. tumefaciens* Ti plasmids, contributes to crown gall formation in infected plants. The enzyme catalyzes the synthesis of isopentyl-adenosine-5′-monophosphate which is the first step in cytokinin biosynthesis. When the *ipt* gene regulated by the 35S promoter is transferred to tobacco, the transformation efficiency measured by the regeneration of transformed shoots is 2.7-fold greater than that achieved by a 35S-*npt*II gene construct. Moreover, the effectiveness of the *npt*II gene as a selectable marker was enhanced when it was co-transformed with the 35S-*ipt* gene construct (Endo et al., 2001). The observation paralleled previously-discussed observations made with conditional-positive selection systems that avoid toxic selective agents. It appears that the *Arabidopsis* genome codes for a family of IPT genes that catalyze similar reactions and generate the same phenotype when expressed in transgenic plants (Takei et al., 2001; Sun et al., 2003). They may be effective substitutes for the *A. tumefaciens ipt* gene.

The difficulty with this system is that all of the regenerated shoots have abnormal morphologies resulting from the high endogenous cytokinin levels which include the loss of apical dominance and lack of roots (i.e. the shooty phenotype). The use of a β-estradiol-inducible, artificial promoter system to restrict expression of the *ipt* gene during the selection phase appeared to eliminate these morphological abnormalities in regenerated tobacco shoots and plantlets (Kunkel et al., 1999). A high frequency of escapes have been described. They are assumed to result from cytokinins produced in the transformed cells that migrate to non-transformed cells and induce shoot formation (Zuo et al., 2002a); however, this assumption is uncertain (Kunkel et al., 1999).

2.6.2. Histidine kinase homologue

Activation tagging of cytokinin-independent genes identified a potential cytokinin receptor, CKI1 (Kakimoto, 1996). When CKI1 was expressed in transgenic calli using the 35S promoter, typical cytokinin responses, such as shoot production and lack of roots, were observed without added cytokinin. Subsequent experiments using the β-estradiol-inducible promoter system to express the CKI1 gene in *Arabidopsis*, yielded calli that produced shoots in the absence of exogenous cytokinin and in the presence of the inducer β-estradiol to activate the promoter (Zuo et al., 2002a). On removal from non-inductive media the shoots developed into normal plants. Interestingly, no escapes were generated. This contrasts with

observations made with the *ipt* gene where cytokinin leakage could generate escapes from neighbouring cells. It was suggested that over-expressed CKI1 protein would not leak to neighbouring cells and the protein as a cytokinin receptor, somehow activated the downstream signal transduction pathway without cytokinin accumulation (Zuo et al., 2002a).

2.6.3. Hairy root-inducing genes

A. rhizogenes-mediated transformation generates plants with altered morphology (i.e. the hairy root phenotype) and the responsible *rol* genes have been used in certain plant transformation vectors as a selectable marker (reviewed by Ebinuma and Komamine, 2001). Generally, the selection system has not been extensively used except to monitor the transposition or excision or the marker genes in the development of marker-free technologies. This has been largely superceded by the use of the *ipt* gene (Ebinuma and Komamine, 2001).

3. Non-selectable maker gene systems—reporter genes

3.1. Background

Non-selectable marker genes or reporter genes (Table 10) have been very important as partners to selectable marker gene systems. They have been used in co-transformation experiments to confirm transgenic events where escapes may be common. Moreover, they have been used to improve transformation systems and the efficiency of recovering transgenic plants by allowing the visual detection of transformed tissues. This may permit the manual selection of transformed tissues prior to the application of selective agents to enrich the tissues in transformed cells.

Green fluorescent protein (GFP) has been particularly important in the development of these strategies as the assay is non-destructive and simple to apply (reviewed by Stewart, 2001). Furthermore, GFP has become a valuable tool for monitoring gene expression in field trials and for following pollen flow. Other genes that generate coloured tissues may also be useful markers (Trulson and Braun, 1997) and novel applications can extend their importance. They may be used for example, as visible markers for monitoring and identifying transgenic escapes or for generating sentinel plants for monitoring environmental contaminants. Reporter genes that can be detected through other senses, such as taste (e.g. ThaumatinII; Witty, 1989) or smell, may also be considered. Although destructive assays are needed to measure the activity of reporter genes such as GUS, they have been very important early tools for measuring the activity of gene regulatory elements in plants and for histochemical localization of marker gene expression (Jefferson, 1987). As a reporter, luciferase (LUC) can be monitored in living tissue but this requires specialized detection equipment (Ow et al., 1986). The use of fusion proteins where the coding region of a reporter gene is

Table 10
Non-selectable marker genes or reporter genes demonstrated in transgenic plants

External substrates	Genes	Enzymes	Sources	Genomes	References
ONPG, X-gal	*lacZ*	β-Galactosidase	*Escherichia coli*	Nuclear	Helmer et al., 1984
MUG, X-gluc	*uidA* (*gus*A)	β-Glucuronidase	*Escherichia coli*, *Bacillus* sp.	Nuclear, Plastid	Jefferson et al., 1987; Kilian et al., 1999; Daniell et al., 1991
Luciferin	*Luc*	Luciferase	*Photinus pyralis*	Nuclear	Ow et al., 1986
Decanal	*luxA, B luxF*		*Vibrio harveyi*		Koncz et al., 1987
None	*gfp*	Green fluroescent, protein (GFP)	*Aequorea victoria*	Nuclear, plastid	Ahlandsberg et al., 1999; Khan and Maliga, 1999
None		Phytoene synthase	*Erwinia herbicola*	Nuclear	Trulson and Braun, 1997
None	R,C1, B	Anthocyanin pathway regulatory factors	Maize	Nuclear	Ludwig et al., 1990; Bower et al., 1996
None		Thaumatin II	*Thaumatococcus danielli* Benth	Nuclear	Witty, 1989
Oxalic acid		Oxalate oxidase (OxO)	Wheat	Nuclear	Simmonds et al., 2003

fused in-frame with a second gene of interest has been particularly useful in these experiments.

In species where the transformation frequencies are very high or where novel cell systems are being investigated, transgenic plants may be generated and recovered without the use of selection systems (Aziz and Machray, 2003). Generally, this situation is rare. Non-selectable marker genes or reporter genes may aid in the identification of the transformed cells.

3.2. β-Galactosidase

The bacterial enzyme β-galactosidase (E.C. 3.2.1.23), which is coded by the *E. coli lacZ* gene, has been a useful marker gene in many cell systems because it can be easily assayed and can form N-terminal translational fusions with other proteins. Although some plants have background galactosidase activity, experiments with tobacco and sunflower showed that ectopic enzyme activity could be measured with the synthetic substrate *O*-nitro-phenyl-β-D-galacto pyranoside (ONPG) and tissues that express the enzyme will stain with 5-bromo-4-chloro-3-indoyl-β-D-galactosylpyranoside (X-Gal). The *lac Z* gene is therefore a conditional non-selectable marker gene. The protein does not appear to be toxic to plant cells. Since the initial report on the use of the marker gene in plants (Helmer et al., 1984) it has not been widely adopted.

3.3. β-Glucuronidase

The bacterial enzyme β-glucuronidase, which is coded by the *E. coli uidA* (*gusA*) gene is the most widely used reporter in plants. The enzyme utilizes the external substrates 4-methyl umbelliferyl glucuronide (MUG) for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histological localization (Jefferson, 1987). It is therefore a conditional non-selectable marker gene. GUS activity is found widely in microorganisms, vertebrates and invertebrates (Gilissen et al., 1998) but there is very little background activity in plants. The GUS enzyme is very stable within plants and is non-toxic when expressed at high levels. A secreted, codon optimized form of the *Bacillus* GUS enzyme, *Bo*GUS, has been developed which is very stable under denaturing conditions and with very high activity (Kilian et al., 1999). Histochemical localization of gene expression is detectable at the subcellular level, for instance, in plastids (Daniell et al., 1991). The major drawback with the use of GUS as a reporter is that the assays are destructive to the plant cells.

A useful feature of GUS is that it can be fused with other proteins (Jefferson et al., 1987). For example, GUS fusions with selectable marker genes such as *npt*II allow the visualization of transformation in addition to selection. The capacity to generate fusions with other proteins has extended the usefulness of GUS for gene tagging experiments and has resulted in the discovery of novel genomic elements such as cryptic gene regulatory elements (Fobert et al., 1994; Foster et al., 1999).

GUS is rapidly degraded under conditions found in the stomach (Fuchs and Astwood, 1996). Humans and animals are continuously exposed to GUS from bacteria residing in their intestinal tracts and from non-transgenic food sources without harmful effects; therefore, the low level of GUS protein from genetically modified plants is not a concern with regard to toxicity or allergenicity (Gilissen et al., 1998).

GUS genes have frequently been co-transformed with selectable marker genes, for example, the *bar* selectable marker gene, to facilitate the selection of transformed tissues (Vasil et al., 1992). GUS expression was used as a reporter to help detect transformation events in tissue culture during the production of a number of plant lines approved for commercialization. These lines include Bollgard II® cotton, the glyphosate resistant sugar beet line GTSB77 (variety InVigor™), papaya line 55-1, three soybean lines with modified fatty acid content (G94-1, G94-19, G168) and two PPT tolerant soybean lines (W62 and W68) (Table 2). With 91 records, GUS is the most frequently listed reporter gene in the US field trials database in 2001 and 2002 (Table 3; ISB, 2003).

3.4. Luciferase

Luciferase (LUC, E.C. 1.13.12.7), as a reporter, offers several advantages including the capability of monitoring gene expression patterns non-destructively in real time with great sensitivity (Ow et al., 1986; Millar et al., 1992). For example, this allows the continuous monitoring of gene activity during development (Verhees et al., 2002). The firefly (*Photinus*

pyralis) luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin. After the reaction occurs the luciferase is inactive until the oxyluciferin is released from the enzyme complex. This is a slow process and the LUC half life is very short; thus, it is believed that LUC activity more accurately reflects transcriptional activity than some other reporter genes that are more stable and accumulate over time (Millar et al., 1992; van Leeuwen et al., 2000). Bacterial sources of luciferase (LUX, E.C. 1.14.14.3) isolated from *Vibrio harveyi* have also been tested successfully in plants (Koncz et al., 1987). Luciferase is often used with other marker genes as an internal control and is also used as a visual marker of transformation for the manual selection of transgenic material undergoing selection (Chia et al., 1994; Lonsdale et al., 1998). Both *luc* and *lux* are conditional non-selectable marker genes.

Four public records containing luciferase were listed in the US field trials database for the years 2001 and 2002.

3.5. Green fluorescent protein

The green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) has become a powerful reporter gene to complement selectable marker genes and can be used to select for transformed material alone (Ahlandsberg et al., 1999; Jordan, 2000). A number of sequence variants have been generated by mutation or codon optimization to enhance activity, stability and detection (reviewed by Stewart, 2001). The great advantage of GFP as a non-conditional reporter is the direct visualization of GFP in living tissue in real time without invasive procedures such as the application or penetration of cells with substrate and products that may diffuse within or among cells. Both considerations provide a significant improvement over GUS and LUC as reporter genes. As GFP does not appear to have any cytotoxic effects on plant cells, it is possible to identify cells in which GFP is expressed shortly after transformation and to assess whether the cells are dividing (Harper et al., 1999). This is particularly important for species, such as the cereals, that have been difficult to transform. GFP allows the manual removal of the transformed tissues to enrich them prior to the application of selection pressure with herbicides or antibiotics. This increases the efficiency of transformation (Jordan, 2000) and reduces the time for producing transgenic plants (Vain et al., 1998). The strategy has been widely used for the nuclear transformation of dicots, gymnosperms and cereals (reviewed by Stewart, 2001). It has been adopted as a co-transforming gene (Sidorov et al., 1999) and as a gene fusion (Khan and Maliga, 1999) to enrich for chloroplast transformation which tends to be inefficient in most species.

GFP has not been extensively used as a reporter for studies in the regulation of gene expression or the study of regulatory elements; however, it has been a very useful tag for monitoring intracellular location and transport when fused to other proteins of interest. Fusions with genes of agronomic importance, such as the *cry1Ac* gene, have been introduced into canola (Halfhill et al., 2001). These studies showed that GFP did not impose a fitness cost to field-grown canola and provided a method to monitor pollen flow to non-target plants (Harper et al., 1999). The increased use of GFP as a reporter gene is evident from the US field trials database. Of the 41 reports listed in the database up to the end of 2002, twenty were in 2001 and 2002 (Table 3) and all have been since 1998.

3.6. Phytoene synthase

The bacterial gene coding for phytoene synthase from *Erwinia herbicola* can act as a non-conditional reporter gene by altering the carotenoid biosynthetic pathway in chloroplasts so that coloured carotenoids accumulate. The coloured tissues expressing the reporter gene can then be manually removed and cultured to generate transgenic plants. Phytoene synthase catalyses the synthesis of phytoene from geranylgeranyl pyrophosphate and phytoene is a precursor of lycopene, the carotenoid that imparts the red colour to tomato. *E. herbicola* phytoene synthase targeted to the chloroplast, generated transgenic orange callus as a visual marker for transgenic tissue at about 50% efficiency and may be used to monitor transgenic plants (Trulson and Braun, 1997).

3.7. Maize R, C1 and B transcription factors

The maize *R*, *C1*, *P1* and *B* transcription factor genes regulate the anthocyanin biosynthetic pathways in specific plant tissues. Ectopic expression of *R* or *B* initiated the non-selective accumulation of anthocyanins in plant cells raising the potential use of

the transcription factors as non-conditional reporter genes that do not require the application of external substrates or destructive assays (Ludwig et al., 1990; Radicella et al., 1992). Although the *R*, *C1* and *B* transcription factor genes showed promise as visible markers for optimizing transformation methods, expression of the genes was toxic to transformed cells (Bower et al., 1996) and expression was subject to environmental stimuli (Chawla et al., 1999). The system has therefore not been extensively adopted as a marker gene system.

3.8. Oxalate oxidase

Oxalate oxidase (OxO: E.C. 1.2.3.4) activity has a narrow range of expression in cereals and appears to be absent in dicots. The wheat gene coding for OxO can function as a conditional reporter gene for monocot and dicot species (Simmonds et al., 2003). The assay depends on the relatively inexpensive substrates, oxalic acid and 4-chloro-1-naphthol and permits rapid histochemical localization of enzyme activity. Quantitative measurements of OxO enzyme activity can also be performed.

4. Marker-free strategies

4.1. Background

The rationalization for creating marker-free transgenic plants has been discussed in detail in several reviews (Yoder and Goldsbrough, 1994; Ow, 2001; Hare and Chua, 2002). For commercialization of transgenic plants it would simplify the regulatory process and improve consumer acceptance to remove gene sequences that are not serving a purpose in the final plant variety. For scientific purposes, eliminating the marker genes from the final plant would permit the use of experimental marker genes that have not undergone extensive biosafety evaluations or that may generate negative pleiotropic effects in the plants. Furthermore, it would permit the recycling of useful marker genes for recurrent transformation of transgenic plants if they were eliminated prior to the next round of transformation.

Although a number of strategies have been described in the scientific literature for generating marker-free transgenic plants, all are more difficult to implement or are less efficient than procedures which leave the marker genes in the plant. Presently, sufficient data has been accumulated to indicate that co-transformation of non-selected genes with selectable marker genes followed by rounds of segregation will create marker-free plants. However, this process is labor intensive requiring the production of several fold more transgenic plants to isolate the plant of interest and further crossing steps after the initial transformation experiment. Furthermore, the strategy is not suitable for vegetatively-propagated species. For vegetatively-propagated species the use of transposons or homologous recombination to eliminate the marker genes may work but at very low efficiency. The use of transposons to reposition genes into a stable chromosomal location may provide an advantage for certain applications. Currently, the research area of greatest promise is the use of site-specific recombinases under the control of inducible promoters to excise the selectable marker genes and excision machinery once selection has been achieved (Ow, 2001). Concerns exist about pleiotropic effects induced by the action of recombinases on cryptic excision sites in the plant genomes, but the use of inducible promoters may limit the extent of damage. Presently, many of these processes are experimental and insufficient information is available to rate the commercial significance of the technologies.

4.2. Co-transformation and segregation of marker genes

Co-transformation involves the simultaneous delivery and integration of two or more separate genes. This may result in linkage of the genes at a single locus as often occurs with biolistic-mediated transformation or it may result in independently-segregating, unlinked loci, as often occurs with *Agrobacterium*-mediated transformation. Co-transformation provides unique advantages for the production of transgenic plants. It allows the simultaneous insertion of a large number of genes, independent of gene sequence, into a plant with a limited number of selectable marker genes. For example, in rice, two to thirteen transgenes have been simultaneously inserted using biolistics (Chen et al., 1998; Wu et al., 2002). The co-transformation frequencies were very high, for example, 85% in the R_0

generation for at least two genes (Chen et al., 1998). 17% of R_0 plants contained more than nine different transgenes (Chen et al., 1998). As the co-transformed genes integrated at a single locus they segregated together. Similar results were obtained in soybean (Hadi et al., 1996). The high incidence of linkage using biolistic-mediated transformation would be important for the manipulation of multi-genic traits using cloned genes but would be impractical for the elimination of marker genes from transgenic plants.

An advantage of *Agrobacterium*-mediated co-transformation technologies over biolistic transformation is that co-transformed genes often integrate into different loci in the plant genome. Unlinked selectable marker genes can then be segregated away from the genes of interest and allow the production of marker-free transgenic plants (reviewed by Ebinuma et al., 2001). This technology is not useful for plants that reproduce vegetatively as segregation is essential for the separation of the marker genes from the genes of interest.

4.2.1. Co-transformation with separate plasmids in one or two agrobacterium strains

*Agrobacteri*um-mediated co-transformation of non-selected genes with selectable marker genes has been demonstrated at relatively high frequencies in a variety of dicot and cereal species. This has been demonstrated in a number of ways. Two separate strains of *A. tumefaciens* (Depicker et al., 1985; McKnight et al., 1987) or *A. rhizogenes* (McKnight et al., 1987) have been shown to co-transform tobacco and/or tomato at frequencies of about 50% or better. The T-DNA insertions were generally unlinked; however, co-transformation of *B. napus* with nopaline strains of *A. tumefaciens* resulted in a higher than expected occurrence of linked insertions indicating that variations in plants and strains could alter linkage relationships (DeBlock and Debrouwer, 1991). The tendency towards multiple T-DNA insertions by nopaline strains may contribute to these observations although the mechanisms involved are unknown (DeBlock and Debrouwer, 1991).

Using a single octopine *A. tumefaciens* strain containing two separate binary vectors, co-transformation frequencies of >50% were obtained in tobacco and *B. napus* for the GUS gene and *npt*II selectable marker gene. Insertions at different loci allowed segregation of the genes and therefore the recovery of marker-free plants. When compared to methods that produce plants where the marker gene is linked to the gene of interest, this method requires about a four-fold greater production of transgenic lines to recover a comparable number of marker-free plants (Daley et al., 1998).

4.2.2. Co-transformation with single plasmids carrying multiple T-DNA regions

An alternative approach for co-transformation proposed by Komari et al. (1996) is the use of octopine strains with binary vectors that carry more than one T-DNA region. They demonstrated that this approach yields higher frequencies of co-transformation than mixtures of *A. tumefaciens* strains carrying separate vectors. In this study, the GUS and *hpt* genes co-transformed tobacco and rice with about 50% frequency at unlinked loci permitting segregation of the GUS gene from the *hpt* selectable marker to create marker-free plants. Although it is believed that the interaction between the bacterial and plant cells is the major factor influencing transformation efficiency (Depicker et al., 1985), it was recently found that the relative size of the co-transforming T-DNA has a major impact (McCormac et al., 2001). Co-transformation frequencies of 100% were achieved in tobacco when the selected T-DNA was two-fold larger than the non-selected T-DNA. The elevation of co-transformation efficiency to practical levels has been demonstrated (McCormac et al., 2001). In maize, co-transformation with an octopine strain carrying a binary vector with two T-DNAs yielded co-transformation frequencies of 93% for the *bar* and GUS genes in the R_0 generation. 64% of the R_1 progeny segregated as *bar*-free plants expressing GUS (Miller et al., 2002). This contrasted dramatically with the 11.7% co-transformation frequency with mixed *Agrobacterium* strains (Miller et al., 2002). In barley, a similar approach with more compact vectors yielded 66% co-transformation frequencies but only 24% of these segregated as marker-free plants perhaps because nopaline strains were required for barley transformation (Matthews et al., 2001). The studies clearly demonstrate that marker-free plants can be generated at varying efficiencies using *Agrobacterium*-mediated co-transformation followed by segregation of the genes in the subsequent sexual generations. This technology is not suitable for all

plant species and its efficiency is clearly dependent on a number of variables including the *Agrobacterium* strain used and the plant tissue being transformed.

4.3. Transposon-mediated repositioning of genes

4.3.1. Tranposition-mediated repositioning of the gene of interest

The maize *Ac*/*Ds* transposable element system has been used to create novel T-DNA vectors for separating genes that are linked together on the same T-DNA after insertion into plants. Once integrated into the plant genome, the expression of the *Ac* transposase from within the T-DNA can induce the transposition of the gene of interest from the T-DNA to another chromosomal location. This results in the separation of the gene of interest from the T-DNA and selectable marker gene. The system is functional in a wide range of plants. It only requires the activity of the *Ac* transposase which can be expressed from plant promoters to enhance activity (Honma et al., 1993) and the approximately 200 bp terminal repeat target sequences which must surround the gene to be transposed (Goldsbrough et al., 1993). Although the creation of marker-free transgenic plants is one outcome, the repositioning of the gene of interest within the genome can also result in favourable position effects that can enhance the expression profile of the gene of interest without creating more transformation events. In tomato, transposition of the GUS marker gene and the generation of *npt*II-free plants was demonstrated for plants with both single and multiple T-DNA insertions (Goldsbrough et al., 1993). In rice, a related approach was used to create *hpt*-free rice plants that expressed the *Bt* endotoxin coded by the *cry 1B* gene (Cotsaftis et al., 2002). In this study, the *cry1B* gene was placed in the leader sequence of a *gfp* marker gene so that transposition could be monitored by the activation of GFP activity. It was found that excision and reinsertion occurred at very high frequencies (37 and 25%) and plants were recovered with high levels of resistance to striped stem borer (Cotsaftis et al., 2002). The stability of the transposed gene seems to include a tendency to less gene silencing as shown for a transposed *bar* gene in barley (Koprek et al., 2001).

This technology relies on crossing plants to segregate the gene of interest from the marker gene and the transposase; therefore, this technology is of limited use in plants that are vegetatively propagated or have a long reproductive cycle. This technology also has limitations for pyramiding multiple genes because introduction of the transposase in subsequent rounds of transformation and marker gene removal may result in the transposition of the first transgene into another chromosomal location.

4.3.2. Tranposition-mediated elimination of the selectable marker gene

An alternative strategy for exploiting the *Ac*/Ds system is to transpose the genes coding for the selectable marker and the transposase from the T-DNA leaving only the gene of interest in the inserted copy of the T-DNA. This research generated the *ipt*-type MAT (multi-auto-transformation) vector system which uses the *ipt* gene as a selectable marker and is designed to remove the *ipt* gene after transformation by using the *Ac* transposable element. This vector system supports recurrent transformation for the pyrimiding of genes into plants (Ebinuma et al., 1997a,b).

Transgenic tobacco and hybrid aspen were transformed using the *ipt* gene as the selectable marker (Ebinuma et al., 1997a). The *ipt* gene was interesting in this study as it was used as both a negative and positive selectable marker. In the first positive selection step, transformed tissue proliferated as adventitious shooty material that was abnormal in morphology and could not regenerate due to the overproduction of cytokinin. In the second negative selection step, after several weeks or months in culture, normal shoots appeared (due to the elimination of the *ipt* and transposase genes by transposition) and regenerated into transformed marker-free plants. This occurred at a frequency of about 5%. As the system does not require a sexual reproduction step, it is an alternative for vegetatively propagated germplasm and plants with a long reproductive cycle (Ebinuma et al., 1997a).

4.4. Intrachromosomal homologous recombination to remove selectable marker genes

Studies on the use of homologous recombination to eliminate selectable marker genes after insertion are few and presently poorly understood. The 352 bp attachment P (attP) region of bacteriophage λ is the target for three specific proteins that mediate the integration and excision of the phage within the *E. coli*

genome. In tobacco the attP region appears to function without the proteins to effect excision of DNA sequences flanked by the attP repeats (Zubco et al., 2000). Transgenic tobacco shoots transformed with a T-DNA vector in which the gene of interest was separated from the region carrying the marker genes *npt*II, *gfp* and *tms 2* by attP repeats were examined in the presence of naphthalene acetamide (NAM). The *tms 2* gene from *A. tumefaciens* codes for an enzyme that converts NAM to the auxin NAA, which prevents root development and induces callus production (Table 9). The regeneration of roots under this counter selection strategy was indicative of marker gene elimination by intrachromosomal homologous recombination. This strategy is not always associated with homologous recombination and larger deletions may occur as a result of illegitimate recombination (Zubco et al., 2000).

4.5. Site-specific recombinase-mediated excision of marker genes

Several simple bacterial and fungal recombination systems have been described in which single enzymes (e.g. Cre, FLP, R) acting on specific target sequences (*lox*, *FRT*, *RS*, respectively) have been adapted for use in plants (reviewed by Ow, 2002; Ow and Medberry, 1995). Each of the target sites is similar in that short oligonucleotides surrounded by short inverted repeats determine the orientation of the target site. Recombinase-mediated DNA rearrangements can include site-specific excision, integration, inversion and interchromosomal recombination; therefore, the range of applications for this technology is very broad. Rapid progress has been made in the development of these technologies for generating marker-free transgenic plants. The technologies have implications for additional benefits such as the modification of copy number at insertions sites. For example, complex multicopy integration patterns generated by biolistics-mediated transformation of wheat were reduced to single-copies by Cre-mediated recombination of the outermost copies (Srivastava et al., 1999). A concern is that high levels of recombinase expression may result in genome rearrangements at cryptic-target sites in plants. Although such sites have not been described in nuclear genomes of plants, chloroplast cryptic *lox* sites have been described (Hajdukiewicz, 2001). Furthermore, the constitutive overexpression of Cre has been correlated with phenotypic aberrations in plants (Coppoolse et al., 2003). Solutions to this potential problem included the use of inducible promoters (reviewed by Hare and Chua, 2002) or transient expression strategies to limit expression of the recombinase (Vergunst et al., 2000) acting on nuclear genes. Selectable markers have also been successfully removed from plastids using the Cre–*lox* system (Corneille et al., 2001).

4.5.1. Cre–lox

The Cre–*lox* system from bacteriophage P1 was the first of the recombination systems shown to be effective in the generation of marker-free plants. The T-DNA vector carrying the gene of interest was constructed with *lox* sites flanking the *hpt* selectable marker gene and inserted into tobacco. The Cre recombinase was then introduced by a second round of transformation to achieve precise excision of the marker gene (Dale and Ow, 1991). This was subsequently confirmed with other plants and other marker genes. To avoid the introduction of marker genes along with the Cre gene, it was found that transient expression of the Cre-gene construct without selection was sufficient to yield enough Cre recombinase to create a small number of lines (0.25%) that were free of selectable markers and the Cre gene (Gleave et al., 1999). A significant refinement of the strategy was developed using the β-estradiol-inducible promoter system in which an artificial transcription factor, XVE was constructed for use in plants with its target promoter (Zuo et al., 2001). In this system, the gene of interest was separated from its promoter by a fragment containing the genes coding for the XVE transcription factor, the *npt*II selectable marker and the Cre recombinase (under the control of the inducible promoter) surrounded by *lox* sites. Transformation of *Arabidopsis* was achieved by selection for kanamycin resistance. Subsequent induction with β-estradiol resulted in the excision of the complete induction system along with the Cre recombinase and selectable marker genes. The final product was the reconstituted gene of interest, in this case GFP. In *Arabidopsis*, excision occurred in all of the plants with high efficiency in the germline cells (29–66%) using a single transformation (Zuo et al., 2001). This new strategy satisfies many of the criticisms

associated with the earlier applications of the technology as discussed in Section 4.5. Data with crop species is now needed to evaluate the full potential of the system for agriculture.

4.5.2. FLP–FRT

The FLP–*FRT* system derived from the *Saccharomyces cerevisiae* 2μ plasmid has also been tested in plants. In tobacco and *Arabidopsis*, plants transformed with the FLP recombinase were crossed with plants transformed with T-DNA in which the GUS coding region is separated from the 35S promoter by a *hpt* gene bracketed by *FRT* sites. This resulted in excision of the *hpt* gene and activation of the GUS gene in all cases (Kilby et al., 1995). Interestingly, the soybean *Gmhsp17.6L* heat shock promoter was used and performed as an inducible promoter in a subset of cells. In transgenic maize callus similar results were obtained and transient expression was shown to result in excision at a frequency of 2–3% (Lyznik et al., 1996).

4.5.3. R–RS

The *R*–RS system from *Zygosaccharomyces rouxii* has been used in the MAT vectors as an alternative to the *Ac* transposase-mediated transposition of the genes as described above (reviewed by Ebinuma and Komamine, 2001). Tobacco plants were transformed with T-DNA vectors in which the *ipt* selectable marker gene and the gene coding for the *R* recombinase were surrounded by RS sites. The *ipt* gene provided the initial selection for morphological abnormalities (i.e. the shooty phenotype). The *A. rhizogenes rol* genes (Table 8) which confer the rooty phenotype have also been used (Ebinuma et al., 1997b; Cui et al., 2001). Co-expression of the *R* recombinase, under the control of the 35S promotor, eventually excised the *ipt* and *R* genes resulting in the development of normal marker-free shoots at very high frequencies (39–70%; Sugita et al., 1999). 67% of marker-free transgenic tobacco plants had more than three T-DNA insertions. This was presumably due to the strong constitutive expression of the *R* gene by the 35S promoter, which resulted in the removal of the *ipt* gene in low-copy-number callus before transgenic shoots could be generated.

To control excision events, the 35S promoter controlling the R gene was replaced with the chemically inducible glutathione-*S*-transferase (GST-II-27) promoter from maize. By driving the *R* gene with the GST-II-27 promoter, the frequency of marker-free plants increased to 88%. Furthermore, 86% of these had single T-DNA insertions (Sugita et al., 2000). The GST-II-27 promoter was induced by the herbicide antidote ‘Safener R29148’ in tissue culture for 2 weeks after transfer of the *ipt*-induced shooty explants to hormone-free solid media. As sexual crossing was not required for the recovery of marker-free plants, the system was tested in hybrid aspen as a model for vegetatively propagated plants. Transgenic marker-free aspen were recovered with 21% efficiency (Matsunaga et al., 2002).

A potential criticism of the technology is the dependence on organogenesis whereas most economically-important crops are regenerated by embryogenesis. However, in rice the system has performed effectively (25% efficiency) in generating transgenic marker-free plants through organogenesis in a single step without forming *ipt*-shooty intermediates using the 35S-driven *R* gene (Endo et al., 2002).

5. Environmental risks of marker genes

The presence of selectable-marker genes in genetically modified (GM) plants has raised public concerns that they will be transferred to other organisms. In the case of antibiotic resistance markers, there is a fear that the presence of these markers in GM crops could lead to an increase in antibiotic resistant bacterial strains. In the case of herbicide-resistance markers, the concern is that the markers will contribute to the creation of new aggressive weeds. Before GM crops are released for field trials or commercialization, these issues are addressed as a fundamental part of the international regulatory process (MacKenzie, 2000; Nap et al., 2003).

5.1. Marker gene flow to crops and related species

The potential for GM crops to become weeds or to pass their transgenes to wild or weedy relatives is often cited as a potential risk in the commercialization of transgenic crops. The potential risks of GM plants to the environment have been extensively reviewed (Warwick et al., 1999; Wolfenbarger and Phifer, 2000;

Wilkensen, 2002; Dale et al., 2002; Conner et al., 2003).

Domestic crops have been grown near wild or weedy relatives over long periods of time. Gene flow to weedy relatives depends on whether hybridization and introgression are possible. Most of the world's major crops can hybridize to wild relatives somewhere where they are grown agriculturally (Ellstrand et al., 1999; Eastham and Sweet, 2002). Crop-to-weed gene flow may lead to significant changes in the recipient wild population, and has been of particular concern where areas of crop cultivation coincide with centres of crop origin or areas known for extensive genetic diversity (e.g., landraces, etc.); indeed hybridization has been implicated in the extinction of certain wild relatives (reviewed in Ellstrand et al., 1999).

The potential spread of herbicide resistance (HR) to wild species and non-transgenic crop plants has raised separate concerns. Pollen flow between canola cultivars with different herbicide-resistant traits is known to result in unintentional gene stacking. In 1998 and 1999, volunteer canola plants with multiple herbicide tolerances were identified in fields in Canada (Hall et al., 2000; Orson, 2002; Beckie et al., 2003; Warwick and Miki, in press). Canola has numerous wild relatives in Canada and worldwide (Warwick et al., 1999; Eastham and Sweet, 2002; Warwick et al., 2003) and is able to hybridize with several related weedy species (Scheffler and Dale, 1994; Eastham and Sweet, 2002; Warwick et al., 2003). A 3-yr gene flow study between *B. napus* and four related weedy species (*B. rapa*, *Raphanus raphanistrum*, *Erucastrum gallicum*, and *Sinapis arvensis*) in commercial HR canola fields has been conducted in Canada (Warwick et al., 2003). Gene flow from HR *B. napus* to natural wild populations of *B. rapa* was confirmed in two commercial HR canola fields in Québec; thus, representing the first documented occurrence of transgene escape from commercially released transgenic crops into a natural weed population. There was no evidence of gene flow in the other three species. A study commissioned by DEFRA in the UK monitored the agricultural releases of genetically modified oilseed rape from 1994 until the end of the year 2000 (Norris and Sweet, 2002). This study found that depending on the environmental, varietal and agronomic factors in natural field conditions, the degree of outcrossing of GM plants with neighbouring related varieties can give very different results. The report concludes that gene flow will occur between *B. rapa* and *B. napus* when they are grown in close proximity but they did not detect gene flow with any other close wild relative. The planting of barrier crops to act as "absorbers" of GM pollen or changes in isolation distances for cross-pollinating transgenic crops may help with containment (Eastham and Sweet, 2002).

5.1.1. Strategies for restricting gene flow

A number of molecular approaches are being developed to restrict gene flow from GM plants to other crops and wild plant populations. The development of transplastomic plants in which the transgenes are incorporated into the chloroplast genome is a promising technology being developed to reduce the probability of transgene transfer through pollen dispersal (Daniell et al., 2002). A unique feature of plastids of most plants is that they are maternally inherited, limiting the potential spread of transgenes through pollen. A study to assess the likelihood of future transplastomic *B. napus* to hybridize with *B. rapa* demonstrated maternal inheritance of chloroplasts in hybrids of *B. napus* and *B. rapa* and concluded that there was negligible pollen-mediated dispersal of chloroplasts from oilseed rape (Scott and Wilkinson, 1999). Although the authors felt that gene flow would be rare if plants were genetically engineered via the chloroplast genome, they could not entirely rule out the possibility that introgression of *B. rapa* could occur if *B. napus* acted as the female parent. So far, there have been no reports of transformation of *B. napus* chloroplasts. The transformation of plant chloroplasts is challenging and so far stable transplastomics have been identified only in tobacco, tomato and potato (Daniell, 2002; Daniell et al., 2002). Clearly, studies in other crop plants are required before this technology can be widely adopted.

A number of other approaches are being developed to restrict gene flow from GM plants to other crops and to wild plant populations. Like plastid transformation they are applicable to transgenes in general and not just limited to selectable marker genes. These strategies are designed to limit the spread of pollen, affect seed sterility or impose hybridization barriers. Most are still in early stages of development and have limitations. Detailed descriptions are beyond the scope of this review and have been reviewed elsewhere (Daniell, 2002).

5.1.2. Need for marker gene removal

The potential spread of GM traits into weedy or wild relatives has fuelled debate over the necessity of selectable marker genes in plants. Even if gene flow into other crops and natural plant populations does not pose an environmental or agricultural risk, it may still seriously reduce public acceptance of genetically modified plants. The selectable marker will only contribute to weediness if there is a selective advantage for the presence of the marker in the weedy plant. In future crop development selectable markers can be chosen that do not confer a potential competitive advantage. In the case of antibiotic resistance genes, there is no evidence that these genes will provide any selectable advantage. However, it may be more difficult to predict what impact individual selectable markers that alter plant metabolism may have if they become introgressed into wild species.

5.2. Horizontal gene transfer

The use of antibiotic resistance selectable marker genes in genetically modified crops have raised concerns about the potential transfer of these genes to gut and soil bacteria or to the cells of animals who eat these plants. This has been reviewed by a number of authors (Dröge et al., 1998; Neilsen et al., 1998; FAO/WHO, 2000; Smalla et al., 2000; Thompson, 2000) and the general conclusion from available evidence is that the transfer of DNA from genetically modified plants to other organisms would be an extremely rare occurrence.

5.2.1. Mechanisms of horizontal gene transfer and occurrence

Horizontal gene transfer between bacteria occurs by three general mechanisms: transduction (viral transfer of DNA), conjugation (cell to cell mediated transfer of genes on plasmids) and transformation (uptake of exogenous DNA by bacteria) (Neilsen et al., 1998). The most likely mechanism to contribute to the transfer of GM plant DNA to bacteria is called “natural transformation”(Neilsen et al., 1998; Bertolla and Simonet, 1999). There are a number of barriers that must be overcome for horizontal gene transfer to occur: the relevant gene must survive digestion in the intestinal tract or soil; the bacteria or mammalian cells must be competent to take up exogenous DNA; the DNA must survive restriction enzyme digestion by the host prior to incorporation into the genome by rare DNA repair or recombination events (Neilsen et al., 1998; FAO/WHO, 2000). Furthermore, if a gene transfer event did occur, considerable selective pressure would be required for the transfer event to become stabilized (Neilsen et al., 1998).

Studies have looked for horizontal gene transfer of antibiotic resistance genes from transgenic-plant nuclear DNA into native bacteria. No one has demonstrated that this can occur under natural conditions (Syvanen, 1999; Smalla et al., 2000). However, Kay et al. (2002b) recently showed that gene transfer can occur from transplastomic tobacco plants if the receiving microorganism contains sequences homologous to the chloroplast DNA. Transplastomic plants contain about 10,000 copies of the transgene per cell compared to a copy number of less than 10 in plants that have undergone genetic modification of the nuclear genome (Daniell et al., 1998). The increased copy number potentially increases the probability of gene transfer from plant DNA to bacterial cells. Kay et al. (2002b) conducted studies with transplastomic tobacco plants containing the *aadA* gene, conferring resistance to spectinomycin and streptomycin, to determine if gene transfer to bacteria could be detected. The soil bacterium *Acinetobacter* sp. strain BD413 was used to co-infect the transplastomic plants with the plant pathogen *Ralstonia solanacearum. Acinetobacter* sp.strain BD413 develops a competent state while actively colonizing plants infected with *R. solanacearum* (Kay et al., 2002a). To optimize the probability of gene transfer, the *Acinetobactor* sp. BD413(pBAB2) contained a plasmid with homology to the chloroplast genome. *Acinetobacter* sp. transformants containing the *aadA* gene were isolated from plants co-infected with *Acinetobacter* sp. BD413 (pBAB2) and *R. solanacearum*. However, no *Acinetobacter* transformants were obtained when homologous sequences were omitted or when experiments were conducted with nuclear transgenic plants. The increased gene copy number associated with chloroplast integration of the transgene, combined with DNA sequence homology, increased the frequency of transformation to a detectable level. These recent data raise the possibility that horizontal gene transfer may occur under optimal natural conditions from transplastomic plants when the bacterial genome contains sequences

with homology to the plant transgene (Kay et al., 2002b).

Until recently, the production of transplastomic plants in tobacco has relied almost totally on the use of the *aadA* gene as a selectable marker, however new technologies are being developed to replace the use of antibiotic resistance markers in plastids. Methods involving homologous recombination (Iamtham and Day, 2000) or the Cre–*lox* site-specific recombination system (Hajdukiewicz, 2001; Corneille et al., 2001) are being developed to remove the *aadA* gene after chloroplast transformation. Also, alternative markers for chloroplast transformation such as betaine aldehyde dehydrogenase (Table 6) are being developed (Daniell et al., 2001).

5.2.2. Biosafety and horizontal gene transfer

In recent years, growing public concern regarding the spread of antibiotic resistance has limited consumer acceptance of genetically modified plants, especially in Europe (European Federation of Biotechnology, 2001). Of particular public concern are the *bla*TEM1 and *aadA* genes, found in some GM plants, that are driven by bacterial promoters (Table 2). These genes were used for selectable markers in bacteria and are present in GM plants because of limitations in vector cloning technology available at the time of plant development. They are not expressed in the GM plants. These antibiotic resistance markers are widely distributed in nature and the possibility of increasing the reservoir of antibiotic resistance through horizontal gene transfer from plants is extremely remote (Thompson, 2000). Kurtland et al. (2003) suggest that genes transferred by horizontal gene transfer would be quickly eliminated from the genome particularly in the absence of selection pressure. Currently, available cloning technology and vector design eliminates the presence of residual bacterial selectable marker genes in future GM plants.

Although, the main cause for concern is the widespread overuse of antibiotics in human and veterinary medicine (Saylers, 1996), concerns about the potential spread of antibiotic resistance genes through horizontal gene transfer has led to the recommendation that antibiotics widely used for clinical or veterinary use, not be used as selectable markers in plants (US FDA, 1998). The antibiotic resistance marker genes that are currently widely used as plant selectable markers, such as *npt*II and *hpt*, are widely dispersed in nature and have limited therapeutic use (US FDA, 1998). Given the low probability of horizontal gene transfer from GM plants and the limited use of the antibiotics to which *npt*II and *hpt* confer resistance, these selectable markers would not contribute in any meaningful way to increased antibiotic resistance.

Although there is no evidence to suggest that the currently used antibiotic resistance markers, such as *npt*II, pose any risks to humans, animals or the environment, to alleviate public concerns recommendations have been made to eliminate all antibiotic resistance genes from GM plants as new technologies become available (FAO/WHO, 2000; EFB, 2001).

6. Concluding comments

Examination of the scientific literature revealed that a large number of selectable marker genes exist, but few have been adopted for wide use in the production of transgenic plants. The research needed to evaluate their effectiveness and biosafety is considerable and requires many years and substantial resources to complete. For commercialization, the need to conform with regulatory guidelines will often dictate whether new systems will be adopted because of the expenses that must be incurred to provide the data on the safety of the system. The major selectable markers (*npt*II, *hpt*, *bar*) that are most prominently used by the scientific community and for commercialization are among the first generation of selectable marker genes to be developed that worked efficiently in a variety of applications. They have proven to be effective for the development of the first generation of transgenic plants.

Experience is now accumulating that will dictate the parameters that will be needed for the next generation of selectable marker genes and a similar amount of time and effort will be required to develop them. Studies on horizontal gene flow and pollen flow to non-target organisms are just providing the important information needed to define some of these parameters. Progress has been made in extending the traditional approach of using a selective agent with high specificity for an enzyme that will encourage the growth of transformed cells. The bacterial phosphomannose isomerase gene, *manA*, is an example of such as gene. The use of mannose as a selective agent

is less toxic to untransformed cells than antibiotics, herbicides or drugs and therefore seems to yield greater transformation frequencies. Whether it will provide a greater margin of safety than the major selectable markers that are currently in use needs to be determined. The rationale for the development of new selectable markers appears to be public perception and acceptability.

Major conceptual steps have been made in the evaluation of genes that control development. Progress is being made in studying genes that control organogenesis and it has been demonstrated that they may function as selectable marker genes. In the future, genes that control embryogenesis will also prove useful. When modifying plant metabolism and development, pleiotropic effects are likely to occur and must be fully understood. The first generations of selectable markers were usually borrowed from bacterial systems and pleiotropic effects have not been seen in the field performance of the plants containing them. Generally, bacterial detoxification systems are distinct enough from plant processes that phenotypic interactions between the marker genes and the co-transforming genes are unlikely; however, the use of the newer selectable markers that alter plant metabolism and development may require more extensive testing. There is clearly a need for a variety of selectable marker genes for plants and each must be individually assessed and improved.

Generally, selectable marker genes are not required once the transgenic plants are regenerated and the genetic analyses completed. As they serve no purpose in the final plant, methods are being developed to create marker-free plants. In herbicide resistant crops, the herbicide resistance trait is often used as the selectable marker, eliminating the need for any additional marker. Presently, co-transformation of genes with selectable marker genes will allow the elimination of the marker gene by segregation in subsequent sexual generations. If *Agrobacterium*-mediated transformation is used and the species is not vegetatively propagated then it is likely that marker-free plants can be generated with sufficient time and effort. An exciting area that deserves attention at this time is the use of site-specific recombinases under the control of inducible promoters to excise the marker genes after the transgenic plants have been selected. Although in the early stages of research, these technologies have yielded encouraging results in model systems. As these technologies are still being developed, they may not be ready for general use for some time.

Judging from the use of transgenic plants in published research, the selectable marker genes in current use have served scientific discovery very well. Given the acreage of transgenic crops planted worldwide without any harm to health or environment, the selectable markers do not appear to be a significant risk. For the future, continued development of selectable marker gene systems is very important as scientists challenge the capacity of transgenic plants and determine more complex applications for their use.

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