



Advances in selectable marker genes for plant transformation

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Summary

Plant transformation systems for creating transgenics require separate process for introducing cloned DNA into living plant cells. Identification or selection of those cells that have integrated DNA into appropriate plant genome is a vital step to regenerate fully developed plants from the transformed cells. Selectable marker genes are pivotal for the development of plant transformation technologies because marker genes allow researchers to identify or isolate the cells that are expressing the cloned DNA, to monitor and select the transformed progeny. As only a very small portion 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. Advent of recombinant DNA technology and progress in plant molecular biology had led to a desire to introduce several genes into single transgenic plant line, necessitating the development of various types of selectable markers. This review article describes the developments made in the recent past on plant transformation systems using different selection methods adding a note on their importance as marker genes in transgenic crop plants.

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Introduction

The advent of recombinant DNA technology provided an immense potential in the field of plant transformation. In order to achieve high food productivity and to ensure nutritional quality, genetic engineering methods in generating transgenics with useful agronomic traits are assuming significance. Plant biotechnology is based on the delivery, integration and expression of defined genes into plant cells, which can be grown to generate transformed plants. Efficiency of stable gene transfer is not high even in the most successful transfer systems and only a fraction of the cells exposed integrate the DNA construct into their genomes. Therefore, systems to select the transformed cells, tissues or organisms from the non-transformed ones are indispensable and selectable marker genes are vital to the plant transformation process. From the development of first transgenic plants during early 1980s and subsequently after its commercialization worldwide over a decade, antibiotic and herbicide resistance selectable marker genes were one among the integral feature of plant genetic modification (Ramessar et al., 2007). Selectable marker genes are introduced into plant genome to express a protein generally with an enzymatic activity, which allows distinguishing transformed from non-transformed cells (Brasileiro and Dusi, 1999). In all plant transformation systems to

generate substantial number of non-chimeric transformants, genes conferring resistance to selective agents such as antibiotics or herbicides were widely employed to select transformants. These selectable marker genes enable the transformed cells to survive on medium containing the selective agent, while non-transformed cells and tissues die. The low efficiencies of most plant transformation methods necessitate the use of selectable marker genes to identify those cells that successfully integrate and express transferred DNA. Genes encoding resistance to specific antibiotics or herbicides have proved particularly effective for selection and provide a means of rapidly identifying transformed cells, tissues and regenerated shoots that have integrated foreign DNA and that express the selectable gene product and by inference, the gene(s) of interest (Goodwin et al., 2004). Different selectable marker genes were used for transgenic and transplastomic plant research or crop development. These marker genes were assessed for efficiency, biosafety, scientific applications as well as commercialization (Miki and McHugh, 2004). 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 in the presence of external substrates. Positive selectable marker genes are defined as those that allow the growth of transformed tissues, whereas negative selectable marker genes result in the

death of the transformed tissue (Joersbo et al., 1998; Miki and McHugh, 2004). Most commonly and recently developed selectable marker genes, selection agents, enzymes, source of those genes and their importance and applications in plant transformation have been discussed in this review.

Classical selectable marker genes

Most of the published scientific literature on transgenic crop plants revealed that antibiotics (kanamycin or hygromycin) and herbicide (phosphinothricin, PPT) was widely used as selection agent since the early days of plant transformation. The popularity of these selection systems reflects on the efficiency, availability and applicability of their use across a wide range of plant species and its regenerative efficacy in plant tissue culture sys-

tems. Positive selection systems are those that allow the growth of transformed plant cells. Based on their functionality the positive selection systems can be classified into conditional and non-conditional positive selection system. A conditional-positive selection system consists of a gene encoding for a protein, usually an enzyme that confers resistance to a specific substrate that may be toxic to the untransformed plant cells or that facilitates the growth as well as differentiation of the transformed cells alone. This system includes antibiotics, herbicides (Table 1a), toxic and non-toxic drugs or metabolite analog or a carbon source (Table 1b). Non-conditional-positive selection systems do not require external substrates but promote the selective growth and differentiation of transformed cells (Miki and McHugh, 2004). Almost all the antibiotic resistant selectable marker genes for use in transgenic plants have been identified from bacterial sources.

Table 1

Selection agent	Genes	Enzymes	Sources	References
<i>(a) Antibiotics and herbicides used as selection agent in plant transformation</i>				
<i>Antibiotics</i>				
Neomycin, kanamycin	<i>neo nptII</i>	Neomycin	<i>Escherichia coli</i> Tn5	Fraley et al. (1983) Bevan et al. (1983)
Kanamycin	<i>(aphA2), Atwbc19</i>	Phosphotransferases	<i>Escherichia coli</i> Tn601	Carrer et al. (1993)
		ATP-binding cassette	<i>Arabidopsis thaliana</i>	Mentewab and Stewart (2005)
Paramomycin, G418 Aminoglycosides (kanamycin, neomycin, geneticin, paramomycin, gentamycin, tobramycin, apramycin)	<i>nptI, (aphA1) aaC3</i>	Aminoglycoside-N- acetyltransferase	<i>Serratia marcescens</i>	Hayford et al. (1988)
			<i>Klebsiella pneumoniae</i> <i>Shigella</i> sp.	Gossele et al. (1994)
Spectinomycin	<i>aaC4 6'gat aadA</i>	Aminoglycoside-3''-adenyl transferase	<i>Shigella</i> sp.	Svab and Maliga (1993)
Streptomycin	<i>SPT</i>	Streptomycin phosphotransferase	Tn5	Maliga et al. (1988)
Hygromycin B	<i>hph, (aphIV)</i>	Hygromycin phosphotransferase	<i>Escherichia coli</i>	Waldron et al. (1985)
Bleomycin Phleomycin	<i>Ble</i>	Bleomycin resistance	<i>Escherichia coli</i> Tn5 <i>Streptoalloteichus</i> <i>hindustanus</i>	Hille et al. (1986) Perez et al. (1989)
			<i>Escherichia coli</i> pR46 <i>Streptomyces</i> sp.	Guerineau et al. (1990) Jelenska et al. (2000)
Sulphonamides	<i>sull</i>	Dihydropteroate synthase	<i>Escherichia coli</i> Tn5, Phage p1cm	De Block et al. (1984, 1985)
Streptothricin	<i>sat3</i>	Acetyl transferase		
Chloramphenicol	<i>cat</i>	Chloramphenicol acetyl transferase		

Table 1. (continued)

Selection agent	Genes	Enzymes	Sources	References
<i>Herbicides</i>				
Phosphinothricin	<i>pat, bar</i>	Phosphinothricin acetyl transferase	<i>Streptomyces hygroscopicus</i>	De Block et al. (1989)
Methionine sulfoximine		Phosphinothricin acetyl transferase		Chai et al. (2007)
Glyphosate	<i>EPSP synthase</i>	5-Enolpyruvylshikimate-3-phosphate synthase	<i>Petunia hybrida</i> ,	Zhou et al. 1995
	<i>aroA</i>		<i>Zea mays</i> <i>Salmonella typhimurium</i> <i>Escherichia coli</i>	Howe et al. (2002) Comai et al. (1988) Della-Cioppa et al. (1987)
	<i>cp4 epsps</i>		<i>Agrobacterium tumefaciens</i>	Barry et al. (1992)
	<i>gox</i>	Glyphosate oxidoreductase	<i>Ochrobactrum anthropi</i>	Barry et al. (1992)
Sulfonylueras	<i>csr1-1</i>	Acetolactate synthase	<i>Arabidopsis thaliana</i>	Olszewski et al. (1988)
Imidazolinones	<i>csr1-2</i>	Acetolactate synthase	<i>Arabidopsis thaliana</i>	Aragao et al. (2000)
Oxynils	<i>bnx</i>	Bromoxynil nitrilase	<i>Klebsiella pneumoniae</i> sub sp. <i>Ozanaenae</i>	Freyssinet et al. (1996)
Gabaculine	<i>hemL</i>	Glutamate-1-semialdehyde aminotransferase	<i>Synechococcus</i> PCC6301	Gough et al. (2001)
Cyanamide	<i>cah</i>	Cyanamide hydratase	<i>Myrothecium verrucaria</i>	Weeks et al. (2000)
Acetochlor, chlorpropham amiprofos-methyl, chlorsulfon, norflurazon and pendimethalin	P450	Cytochrome P450	Human	Inui et al. (2005)
Norflurazon and fluridone	<i>pds</i>	Phytoene desaturase	<i>Hydrilla verticillata</i>	Arias et al. (2006)
Trifluralin	<i>TUAm</i>	α -Tubulin	<i>Eleusine indica</i>	Yemets et al. (2008)
Butafenacil		Protoporphyrinogen oxidase	<i>Myxococcus xanthus</i>	Lee et al. (2007)
<i>(b) Toxic and non-toxic agents, drug and metabolite analogs used as selection agent in plant transformation</i>				
<i>Drugs and analogs</i>				
2-Deoxyglucose	<i>DOG 1</i>	2-Deoxyglucose-6-phosphate phosphatase	<i>Saccharomyces cerevisiae</i>	Kunze et al. (2001)
Betaine aldehyde	<i>BADH</i>	Betaine aldehyde dehydrogenase	<i>Spinacia oleracea</i>	Daniell et al. (2001)
S-Aminoethyl	<i>DHPS</i>	Dihydropicolinate synthase	<i>Escherichia coli</i>	Perl et al. (1993)
L-Cysteine (AEC)	<i>ocs</i>	Octopine synthase	<i>Agrobacterium tumefaciens</i>	Koziel et al. (1984)
4-Methyltryptophan (4-mT)	<i>TDC</i>	Tryptophan decarboxylase	<i>Catharanthus roseus</i>	Goddijn et al. (1993)
Methotrexate	<i>DHFR</i>	Dihydrofolate reductase	<i>Escherichia coli</i> , mouse, <i>Candida albicans</i>	Herrera-Estrella et al. (1983) Eichholtz et al. (1987) Irdani et al. (1998)
L-Methionine sulfoximine	<i>bar</i>	Phosphinothricin acetyl transferase	<i>Streptomyces hygroscopicus</i>	Chai et al. (2007)
5-Methyltryptophan	<i>ASA2</i>	Anthranilate synthase	Tobacco	Cho et al. (2004)
5-Methyltryptophan (5MT)	<i>OASA1D</i>	Mutant anthranilate synthase	Rice	Kobayashi et al. (2005)
5MT/Cadmium chloride	<i>TSB1</i>	Tryptophan synthase beta	<i>Arabidopsis thaliana</i>	Hsiao et al. (2007)
L-O-Methylthreonine	<i>ilvA</i> or <i>ilvA-466</i>	Threonine deaminase	<i>Escherichia coli</i>	Ebmeier et al. (2004)

Table 1. (continued)

Selection agent	Genes	Enzymes	Sources	References
<i>Non-toxic agents</i>				
5-Fluorocytosine (5-FC)	<i>codA</i>	Cytosine deaminase	<i>Escherichia coli</i>	Kobayashi et al. (1995)
D-Xylose	<i>xylA</i>	Xylose isomerase	<i>Streptomyces rubiginosus</i> <i>Thermoanaerobacterium sulfurogenes</i>	Haldrup et al. (1998b) Haldrup et al. (1998b)
D-Mannose	<i>manA (pmi)</i>	Phosphomannose isomerase	<i>Escherichia coli</i>	Joersbo et al. (1998)
Benzyladenine-N-3-glucuronide	<i>uidA (gusA)</i>	β -Glucuronidase	<i>Escherichia coli</i>	Joersbo and Okkels (1996)
None	<i>ipt</i>	Isopentyl transferase	<i>Agrobacterium tumefaciens</i>	Endo et al. (2001)
None	<i>iaaM, iaaH</i>	Indole acetic acid	<i>Agrobacterium tumefaciens</i>	Tuominen et al. (1995)
None	<i>rolC</i>	'Hairy root' phenotype	<i>Agrobacterium rhizogenes</i>	Ebinuma and Komamine, (2001)
D-Amino acids (D-alanine and D-serine)	<i>dao1</i>	D-Amino acid oxidase	<i>Rhodotorula gracilis</i>	Erikson et al. (2004)
Arabitol	<i>atID</i>	Arabitol dehydrogenase	<i>Escherichia coli</i> strain C	LaFayette et al. 2005
D-Serine	<i>dSDA</i>	D-Serine ammonia lyase	<i>Escherichia coli</i>	Erikson et al. (2005)
Glucose	<i>AtTPS1</i>	Trehalose-6-phosphate synthase	<i>Arabidopsis thaliana</i>	Leyman et al. (2006)
Hormone-free medium	<i>kn1</i>		Maize	Luo et al. (2006)

***Arabidopsis thaliana* ATP-binding cassette (ABC) transporter**

ABC proteins are ubiquitous proteins that share 30–40% identity between family members and are characterized by the presence of an ABC (Higgins, 1992). *Arabidopsis thaliana* ABC transporters have been classified on the basis of their domain organization and their homology to orthologous genes, but their functions remain largely unknown (Sanchez-Fernandez et al., 2001). The *AtWBC* family (White-Brown Complex homologs) is the largest group of all *Arabidopsis thaliana* ABC transporters, with 29 members. A plant gene, *Atwbc19* (2178 bp) that encodes an *Arabidopsis thaliana* ABC transporter was characterized and it confers antibiotic (kanamycin) resistance to transgenic plants. The mechanism of resistance is novel, and the levels of resistance achieved are comparable to those attained through expression of bacterial antibiotic-resistance genes in transgenic tobacco using CaMV 35S promoter. Because ABC transporters are endogenous to plants, the use of *Atwbc19* as a selectable marker in transgenic plants may provide a practical alternative to current bacterial marker genes in terms of the risk for horizontal transfer of resistance genes (Mentewab and Stewart, 2005). Hence this marker gene may be valuable for transformation of agriculturally important dicot species such as soybean, cotton, *Brassica* crops, tomato and other *Solanaceae* as

well as forest tree species including angiosperms (e.g., polar and elms) and gymnosperms (e.g., pine and spruce).

Neomycin phosphotransferase II (NPT II) (*nptII*)

The *nptII* (or *neo*) gene was isolated from the transposon Tn5 of *Escherichia coli* and it encodes NPT II (E.C. 2.7.1.95), also known as aminoglycoside 3'phosphotransferase II (Fraley et al., 1983; Herrera-Estrella et al., 1983). The active aminoglycoside antibiotic inhibits the protein synthesis in prokaryote cells, by binding to the 30S subunit of the ribosome, blocking the formation of initiation complexes and decreasing the fidelity of translation.

Hygromycin phosphotransferase (HPT) (*hpt*)

The *hpt* (or *aph IV*) gene of *Escherichia coli* codes for the enzyme HPT (E.C. 2.7.1.119) and confers resistance to the antibiotic hygromycin B (Waldron et al., 1985). When hygromycin occupies the ribosomal binding site of the elongation factor 2 (EF-2) in prokaryotic cells, consequently the elongation of polypeptide chain is inhibited and protein synthesis interrupted, causing the same symptoms described for the other aminoglycoside antibiotics. In plant cells, these antibiotics exert its effect on

mitochondria and chloroplast, acting in the same manner by impairing protein synthesis. These organelles have ribosomes that are similar to those found in bacteria and are also susceptible to aminoglycoside antibiotics. Therefore, in the presence of antibiotic, the plant tissue will show a chlorosis, caused by the lack of chlorophyll synthesis and inhibition of growth (Benveniste and Davies, 1973; Brasileiro, 1998).

Phosphinothricin *N*-acetyltransferase (PAT) (*pat* and *bar*)

Since 1987, the bialaphos resistance genes *bar* from *Streptomyces hygroscopicus* and *pat* from *Streptomyces viridochromogenes* encoding the enzyme PAT have been used as selectable marker in plants (De Block et al., 1989). The PPT (ammonium glufosinate) is analogous to glutamate, the substrate of glutamate synthetase (GS), and acts as a competitive inhibitor of GS. The enzyme GS catalyzes the conversion of glutamate to glutamine, removing the toxic ammonia from the cell. This enzyme plays an essential role in the regulation of nitrogen metabolism and ammonia assimilation. When the GS is inhibited, it results in ammonia accumulation and consequently disruption of chloroplast that leads to inhibition of photosynthesis and to death of the plant cell (Lindsey, 1992).

Methionine sulfoximine (MSO)

An alternative selection agent, L-MSO was used for selection of the *bar* marker, which also confers resistance to PPT. MSO like PPT is a glutamate analog that inhibits growth of dicotyledonous plant *Arabidopsis* due to its action on GS. Maughan and Cobbett (2003) demonstrated the utility of MSO as an effective and economical alternative to PPT for selection and regeneration of plants. MSO was 40-fold more potent, inhibits growth of wild-type plants and detoxified in the similar manner as PPT by PPT acetyl transferase. In a recent study, MSO was employed as a novel selection agent for genetic transformation of orchids. The successful application of MSO in orchid transformation opens up new avenues to study functional orchid genes as well as genetic engineering of orchids with commercially valuable traits. This readily available selection agent also has great potential for the transformation of economically important monocotyledonous plant species (Chai et al., 2007).

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (*epsps*)

The *aroA* (or *epsps*) gene was isolated from *Salmonella typhimurium* treated with mutagenic agent and selected for resistance to the herbicide glyphosate. The herbicide glyphosate inhibits, by competition, the enzyme EPSP synthase (E.C. 2.5.1.19), which is involved in the enzymatic pathway of aromatic amino acids biosynthesis in bacteria and plants. The inhibition of EPSPS results in shikimate accumulation, inhibition of synthesis of aromatic amino acids and secondary metabolites causing cell death. An excellent and comprehensive review on many other selectable marker genes in transgenic crop plants has been published (Miki and McHugh, 2004).

Cyanamide hydratase (*cah*)

The *cah* gene 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. Cyanamide is a nitrile derivative that in its aqueous or calcium salt forms can be used as a fertilizer. It has the additional characteristics of acting as a non-persistent herbicide when applied prior to seed germination. The enzyme has 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; Kirubakaran and Sakthivel, 2008), potato, tomato, rice, *Arabidopsis* (Damm, 1998) and soybean (Zhang et al., 2005; Ulanov and Widholm, 2007). In a recent study, the heterologous expression of *cah* marker gene was reported. Due to its innate ability to convert calcium cyanamide to urea and the broad-spectrum antimicrobial activity of cyanamide, the *cah* gene can also be used to facilitate plant growth promotion and biocontrol of phytopathogens (Kirubakaran and Sakthivel, 2008).

Cytochrome P450 monooxygenases

P450 monooxygenases are heme proteins that use electrons from NADPH to catalyze the activation of molecular oxygen. The catalyzed reaction is usually a mono-oxygenation, with the formation of a molecule of water and an oxygenated product. Mammalian P450 species show overlapping and broad substrate specificity and confer the ability

to metabolize a number of chemicals, including herbicide. Most classes of herbicides are aryl- or alkyl-hydroxylated or *N*-, *S*- or *O*-dealkylated by P450 species. The phenylurea herbicide chlortoluron is detoxified either via hydroxylation of the ring-methyl or via di-*N*-demethylation (Gonneau et al., 1988). The introduction of highly active mammalian P450 into plant species metabolizes herbicide thus providing not only herbicide tolerance, but also acts as a selectable marker. Human P450 species have been used to generate herbicide-tolerant tobacco, potato and rice plants (Inui et al., 1999, 2001). Interestingly, Kawahigashi et al. (2002) reported that the *CYP2B6* gene could also be used as a negative selectable marker gene, due to its ability to metabolize the herbicides benfuresate and ethofumesate to more toxic compounds. Human P450 species are much more effective at herbicide metabolism than plant P450 species (Ohkawa et al., 1998). In a study, the human P450 genes were employed for *Arabidopsis thaliana* by *Agrobacterium*-mediated transformation method. Herbicide-tolerant seedlings transformed with *CYP1A1*, *CYP2B6*, *CYP2C9* or *CYP2C19* were selected using acetochlor, amiprofos-methyl, chlorpropham, chlorsulfuon, norflurazon and pendimethalin. Results revealed that herbicide-tolerant plants transformed with *CYP1A1*, *CYP2B6* and *CYP2C19* expressed the corresponding P450 cDNAs in transgenic *Arabidopsis* plants coordinately as well as functioned as selectable marker because of active metabolism of the herbicides (Inui et al., 2005).

Phytoene desaturase (PDS)

The enzyme PDS has been the main target for herbicides that inhibit the carotenoid biosynthetic pathway. In plants, PDS converts phytoene to ζ -carotene. Carotenoids are essential components of the photosynthetic apparatus. They participate in light harvesting and protect the chloroplast from the harmful effect of singlet oxygen formed during photosynthesis (Sandmann and Böger, 1997). PDS-inhibiting herbicides prevent the formation of carotenoids, resulting in the degradation of chlorophyll and destruction of chloroplast membranes, which is characterized by the photobleaching of green tissues (Boger and Sandmann, 1998). Mutations of the cyanobacterium *Synechococcus* PDS have resulted in the herbicide-resistant microbial enzymes (Chamovitz et al., 1991) and have conferred herbicide resistance when expressed in transgenic tobacco plants (Wagner et al., 2002). Recently, naturally occurring mutations at amino

acid 304 of PDS in aquatic weed *Hydrilla verticillata* have been reported to impart herbicide resistance (Michel et al., 2004). Based on the aforementioned studies, Arias et al. (2005) has reported for the first time the evolved resistance to PDS inhibitors in higher plants. Factors that may have contributed to this unique case include: the unusual growth habits, multiple means of vegetative reproduction of *Hydrilla verticillata* as well as the methods adopted to control aquatic environments using fluridone. Arias et al. (2006) identified the potential use of this herbicide-resistant gene from higher plant as a selectable marker. The amino acid 304 of *Hydrilla verticillata* PDS was substituted with the other 19 amino acids and the activity of the enzymes was tested *in vitro* against fluridone. Four of these mutations (Threonine – Thr, Cysteine – Cys, Alanine – Ala and Glutamine – Gln), in addition to the wild-type PDS were selected for expression in *Escherichia coli* and *Arabidopsis thaliana* for further characterization. Results revealed that *Arabidopsis* plants transformed with *Hydrilla pds* containing mutations for Cys, Ser, His and Thr at position 304 showed increase in resistance to fluridone and norflurazon. *Hydrilla* PDS with Thr304 was proposed as a selectable marker based on two main advantages. Firstly, consumers may better accept a genetically modified (GM) food if transformed with a plant gene. Secondly, *Hydrilla* PDS Thr304 confers high resistance to a limited spectrum of herbicides (fluridone, norflurazon and flurtamone), whereas it renders the GM plants more susceptible than the wild-type to other PDS inhibitors, such as diflufenican, picolinafen and beflubutamid. These features could be beneficial to eliminate undesirable transgenic plants in the wild, rather than creating highly resistant weeds (Arias et al., 2006).

α -Tubulin gene

To facilitate the development of a new generation of plant transformation systems, naturally occurring variant of tubulin genes conferring antimicrotubule drug resistance may be appropriate (Baird et al., 2000). Recently, a mutant α -tubulin gene from goosegrass that confers resistance to dinitroaniline herbicide (trifluralin, TFL) was employed as selectable marker gene for transformation both in monocots and dicots (Yemets et al., 2008). In goosegrass (*Eleusine indica*), two alleles of α -tubulin 1 (each is the result of a single unique point mutation) have been identified, which confers either an intermediate or high level tolerance to a number of anti-microtubule herbicides such as

dinitroanilines (trifluralin) and phosphoramidates (amiprofos-methyl, APM) (Nyporko et al., 2002). Yemets et al. (2008) tested the possibility to use the mutant α -tubulin as a selective marker gene for transformation of finger millet, soybean, flax and tobacco using TFL as selective agent. The efficiency of transgenic plants selection using TFL was comparable with those using kanamycin or PPT. The anti-microtubule herbicides were used as selective agents for effective and successful selection of plant somatic hybrids after symmetric and asymmetric fusions using *Nicotiana plumbaginifolia* mutants resistant to TFL or APM. These findings have thus furthered the evidence for the effective use of TFL as a selection agent in plant transformation systems.

Protoporphyrinogen oxidase (PPO)

PPO is a key enzyme in the chlorophyll/heme biosynthetic pathway, catalyzing the oxidation of protoporphyrinogen IX to protoporphyrin IX (Smith et al., 1993). This is the last common step in the production of heme and chlorophyll. Heme is an essential cofactor in cytochromes, hemoglobin, oxygenases, peroxidases, and catalases, and, therefore, is a necessary product for all aerobic organisms. The production of chlorophyll, a light-harvesting pigment, is an essential process for all green photosynthetic organisms. This characteristic makes PPO an excellent gene target for herbicide development (Nandihalli and Duke, 1993). When PPO is inhibited by the PPO family of herbicides, protoporphyrin IX accumulates and causes light-dependent membrane damage (Lee et al., 1993). Peroxidizing herbicides target the enzyme PPO and have been used commercially since 1960 to control annual grasses and dicotyledonous weeds in soybean, peanut, cotton, rice and other crops (Matringe et al., 1989; Duke et al., 1991; Scalla and Matringe, 1994). Diphenyl ethers, triazolones, *N*-phenylpyrazoles, oxadiazoles, thiadiazoles, pyrimidindiones, *N*-phenyl-phthalimides and oxazolidinediones are major structural families of herbicidal PPO inhibitors. As partial structural analogs of protoporphyrinogen IX, these herbicides bind to and competitively inhibit PPO activity (Nandihalli et al., 1992). New potent PPO inhibitors continue to be developed and are reported to be effective peroxidizing herbicides (Hwang et al., 2004). The inhibition of PPO results in the massive production of singlet oxygen, which is followed by peroxidation of membrane lipids and subsequent cell death (Lee et al., 1993). Since the first report of a transgenic tobacco expressing an *Arabidopsis* PPO (Lermontova

and Grimm, 2000), many attempts have been made to develop transgenic crops resistant to peroxidizing herbicides through the ectopic expression of native or mutant forms of PPO. An *Arabidopsis* double mutant of PPO and a *Myxococcus xanthus* (Mx) PPO were shown to confer a high level of resistance to peroxidizing herbicides in transgenic maize and rice, respectively. Furthermore, the *Arabidopsis* double mutant of PPO was successfully used as a selectable marker gene, in combination with the herbicide butafenacil as a selection agent, for generating transgenic crops (Li et al., 2003; Jung et al., 2004). In a recent report, transgenic rice plants were developed using the Mx PPO gene as a selectable marker coupled with the herbicide butafenacil as a selection agent. The overexpression of Mx PPO confers a high level of herbicide resistance in rice. Among the peroxidizing herbicides, butafenacil (0.1 μ M) has an efficiency \sim 1000-fold that of oxadiazon, as judged by calli susceptibility tests upon herbicide treatment (Lee et al., 2007).

Anthranilate synthase (AS) (ASA2)

ASA2 gene was used as a selectable marker since it is naturally occurring gene of plant origin (*Nicotiana tabacum*, tobacco) involved in tryptophan (Trp) biosynthesis (Song et al., 1998) that should have no detrimental effects when transferred to other plants or microbes. AS catalyses the first reaction in the multi-step Trp biosynthesis pathway by converting chorismate to anthranilate. AS is feedback inhibited by the end product Trp, which binds to an allosteric site on the AS catalytic α -subunit. AS is the control point in the Trp branch in plant cells as indicated by pathway-intermediate feeding studies (Widholm, 1974). Feedback inhibition of the respective enzyme activities (Singh and Widholm, 1974) and 5-methyltryptophan (5MT) resistance yielded selection of lines with altered feedback-inhibited AS and higher free Trp (Wakasa and Widholm, 1987). Siehl et al. (1997) reported that AS is an effective herbicide target based on experimental evidence linking the herbicidal activity of 6-methylanthranilate or 4-methyltryptophan with inhibition of this enzyme. Cho et al. (2004) demonstrated that the tobacco feedback-insensitive ASA2 gene can be inserted into legume hairy roots (*Agrobacterium sinicus* and soybean) and expressed to produce feedback-insensitive AS activity, which leads to increase in free Trp levels and resistance to Trp analog 5MT. Direct selection with 5MT produced *Agrobacterium sinicus* hairy root lines that all expressed ASA2 mRNA while *nptII*

expression was variable. The expression of *ASA2* in plant tissues leads to increased levels of free Trp, which could be desirable since Trp is an essential amino acid required in the diets of humans and non-ruminant animals. Since soybean hairy roots expressing *ASA2* selected with Kan were also resistant to 5MT, direct selection will be effective in this case. Recently, rice *OASA1D* a mutant anthranilate synthase α subunit gene was used as selectable marker for effective transformation in *Arabidopsis thaliana* (Kobayashi et al., 2005), rice and potato (Yamada et al., 2005). As demonstrated, *ASA2* can be used as a selectable marker gene to select transformants in different plant species with the Trp analog 5MT as the selection agent.

Tryptophan (Trp) synthase beta 1 (TSB1)

Trp, one of the essential amino acids present in plants, is not synthesized by animals and is the major contributor of the indole ring for the synthesis of auxins, glucosinolates, nicotinic acid, phytoalexins and alkaloids (Pruitt and Last, 1993). Biosynthesis of Trp in plants is not constitutive and hence, it is produced when required, presumably under different stress conditions such as wounding. Although the Trp biosynthetic pathway is primarily derived from bacteria and fungi, the real biochemistry and mechanism of Trp synthesis and its regulation in plants became well understood only after the innovation of *Arabidopsis* mutants *trp1-1*, *trp2-1* (Last et al., 1991; Pruitt and Last, 1993), *yucca* (Zhao et al., 2001) and yeast cDNA screening (Hull et al., 2000). Two genes, *Arabidopsis thaliana* tryptophan synthase beta 1 (*AtTSB1*) and *AtTSB2*, encode the Trp beta subunit in *Arabidopsis*. Even though both are highly conserved, *AtTSB1* mRNA transcript is more abundant than that of *AtTSB2* in leaf tissues (Pruitt and Last, 1993). Hence, the *AtTSB1* gene in plants plays a key role in the Trp biosynthesis pathway in converting indole and serine into Trp (Last et al., 1991). Recently, Hsiao et al. (2007) explored the practical applicability of the *AtTSB1* gene as a novel non-antibiotic selection marker in plant transformation using a simple, efficient, 5MT or heavy metal-resistant selection procedure. Transgenic plants were efficiently selected on MS medium supplemented with 75 μ M 5MT or 300 μ M CdCl₂ devoid of antibiotics. Engineering the *AtTSB1* gene to overexpress and accumulate Trp for its greater availability and multi-flux distribution is an important step in metabolomics. Results demonstrate that *AtTSB1* overexpression in *Arabidopsis* increases Trp content and enhances Cd²⁺ tolerance. The high level of Trp

presumably may allow detoxification of excess Cd²⁺. These observations imply that accumulated levels of Trp in *AtTSB1* transgenic plants may restrict the uptake of Cd²⁺ and other heavy metals or may affect the activity of metal transporters. Therefore, the use of *AtTSB1* gene as selection marker in agronomically important crop species transformation has generated transgenic plants that may also be successfully cultivated in heavy metal polluted soils.

Threonine deaminase (TD)

TD is one of the key enzymes downstream in the aspartate family biosynthetic pathway, involved in the first step of isoleucine (Ile) biosynthesis. TD converts threonine to α -ketobutyrate, and itself is feedback-regulated by Ile. Inhibition of TD is herbicidal to plant cells, and the herbicidal activity of TD inhibition can be circumvented with the supplements of α -ketobutyrate and Ile itself (Szamosi et al., 1994), reflecting the depletion of Ile as the cause of cell death. An alternative approach to mimic Ile deprivation is the use of structural analog of the amino acid that can effectively compete during translation to induce cell death. One such example is *O*-methylthreonine (OMT) (Rabinovitz et al., 1955). As part of large mutant screening in *Arabidopsis* lines having altered biosynthesis of branched-chain amino acids, Mourad and King (1995) characterized a set of mutants resistant to OMT. The ability of these mutant lines to develop on OMT was attributed to a single nuclear gene *omr1*, a mutant allele of TD, which was mapped to chromosome 3. The OMT-tolerant *Arabidopsis* lines possessed substantially enhanced intracellular levels of Ile compared with wild-type plants. Moreover, there was a 50-fold increase in the Ile feedback insensitivity of TD activity in the OMT-tolerant lines compared with that in wild-type. The *omr1* allele of *Arabidopsis thaliana* may serve as an effective plant selectable marker gene, coupled with OMT, for use in plant genetic engineering studies (Mourad and King, 1995). Slater et al. (1999) introduced into *Arabidopsis thaliana* and *Brassica napus* with the *Escherichia coli* wild-type TD gene, *ilvA*, or a feedback-insensitive mutant *ilvA-466* transformant expressing either of these transgenes displayed elevated intracellular levels of Ile. Ebmeier et al. (2004) designed two binary plasmids that harbored an *nptII* cassette and either the wild-type *ilvA* or mutant *ilvA-466*. The *ilvA* gene was effectively utilized as a selectable marker gene to identify tobacco transformants when coupled with OMT as

the selection agent. However, the transformation efficiency was substantially lower than that observed with *nptII* using kanamycin as a selection agent. Moreover, in the subset of the *ilvA* transformants and in majority of *ilvA*-466 transgenic lines, a severe off-type was observed under greenhouse conditions that correlated with increased level expression of the *ilvA* transgene.

Cytosine deaminase (*codA*)

codA enzyme in bacteria catalyzes the conversion of cytosine to uracil. This enzyme is also capable of converting non-toxic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), a toxic compound for plant growth (Kobayashi et al., 1995). In plants, a chimeric *codA* gene under the control of CaMV 35S promoter was introduced into tobacco and *Lotus japonicus*. The results revealed that the *codA* marker gene is useful for substrate-dependent negative selection and segregates as a dominant marker. The lack of cytosine deaminase activity in many plants species including *Arabidopsis*, pea, barley, soyabean and sugar beet indicates the fact that this gene can be employed widely as negative selective marker (Stougaard, 1993; Koprek et al., 1999). Perera et al. (1993) also reported that *codA* gene works as a negative selectable marker in *Arabidopsis*. The effectiveness of the negative selection with the *codA* gene, however, would be dependent on the permeability and transport of the substrate 5-FC in plant tissues and on the intensity of expression, tissue specificity and temporal specificity of the promoter fused to the *codA* gene. Since CaMV 35 S promoter is sufficiently active in root cells (Benfey et al., 1989) and the root tissues can be in contact directly with the substrate *in vitro*, the P35S should be a suitable promoter in the *codA* negative selection system. Therefore, cytosine deaminase gene from *Escherichia coli* is functional and useful for negative selection in *Arabidopsis* and sensitivity to 5-FC, which makes *codA* gene a suitable conditional negative selectable marker gene in plants (Kobayashi et al., 1995).

Xylose isomerase (*xylA*)

The *xylA* genes were isolated from *Streptomyces rubiginosus* (Haldrup et al., 1998a) and *Thermoanaerobacterium thermosulfurogenes* (Haldrup et al., 1998b). Plant cells from species such as tobacco, potato and tomato cannot use D-xylose as a sole carbon source. This 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 carbon source. The efficiency of selection in this case was much greater than for the *nptII* gene and the regeneration of shoots was significantly faster. It was suggested that the enzyme from *Streptomyces rubiginosus* posed no biosafety issues as it is used in the food industry and is considered safe (Haldrup et al., 1998a).

Phosphomannose isomerase (PMI) (*manA*)

The *manA* gene codes for the enzyme PMI (E.C. 5.3.1.8) isolated from *Escherichia coli* (Miles and Guest, 1984). In the presence of mannose in transformed cells, the PMI converts mannose-6-phosphate into fructose-6-phosphate that can be immediately incorporated in the plant metabolic pathway (Privalle et al., 1999). Thus, the mannose can be used as sole source of carbohydrate for the transformed cells. This selection system is immediate and extremely efficient (Joersbo et al., 1998). Mannose cannot be usually metabolized by non-transformed cells and is converted into mannose-6-phosphate by endogenous hexokinase. Therefore, when mannose is added to the culture medium, it could minimize the plant growth due to mannose-6-phosphate accumulation. Even though most of the plant species are sensitive to mannose, some species, especially dicotyledonous have shown a considerable insensitivity to this sugar, including carrot, tobacco, sweet potato and leguminous crops. Other species are extremely sensitive and were successfully transformed using mannose as selective agent such as sugar beet (Joersbo et al., 1998), maize (Negrotto et al., 2000; Wang et al., 2000), wheat (Wright et al., 2001), rice (Lucca et al., 2001), sweet orange (Boscarior et al., 2003), oat, barley, tomato, potato, sunflower, oilseed rape and pea (Joersbo et al., 1999, 2000). The supporting principle in this approach is the inability of some plants to use mannose as carbon source. The major difference from selection based on antibiotics or herbicides, which kill the non-transformed cells, whereas the mannose selection system arrests the growth and development of non-transformed cells by carbohydrate starvation (Wang et al. 2000) but still survive (Haldrup et al., 1998a, b). Owing to this growth advantage of transformed cells, this strategy is called 'positive selection' (Joersbo and Okkels, 1996). Some plant transformation protocols that utilize the positive selection system with PMI have shown at least 10 times more efficiency than the traditional protocols based on the use of kanamycin as selection agent.

Isopentyl transferase (IPT) (*ipt*)

The enzyme IPT, which is encoded by the T-DNA of *Agrobacterium tumefaciens* Ti plasmids, contributes to crown gall formation in infected plants. This enzyme catalyzes the synthesis of isopentyladenosine-5'-monophosphate, which is the first step in cytokinin biosynthesis. Studies using the *ipt* gene under the control of constitutive promoters showed that *ipt* overexpression leads to elevated cytokinin levels in transgenic plants (Medford et al., 1989) resulting in poor internode elongation, altered leaf morphology and delayed leaf senescence. Moreover, in most of the transgenic regenerants, root growth is delayed or absent and the plants are often sterile (Smigocki and Owens, 1989; Ebinuma et al., 1997). The selection system using *ipt* gene is a positive selection that does not stress the transformants with a low-level expression of transgene. Endo et al. (2001) suggested that overexpression of *ipt* gene from *Agrobacterium tumefaciens* favors plant regeneration from transformed cells and the transformation frequency of the *ipt* plus kanamycin selection resulted in 1.6-fold higher transformation efficiency than kanamycin selection alone. The reason for the increase in transformation frequency was the increase in endogenous hormone with the introduction of *ipt* gene. Endogenous zeatin and zeatin riboside concentrations in transformed cells increased 100-fold compared with non-transformed cells (Smigocki and Owens, 1989). Several researchers have observed that *ipt* gene induces abnormal shoots and cell proliferation in the medium without a supplementary hormone in potato (Ooms et al., 1983), cucumber (Smigocki and Owens, 1988) and other *Nicotiana* species (Smigocki and Owens, 1989). These physiological abnormalities prevent the development of *ipt* as a selectable transformation marker.

Indole acetic acid (*iaaM* and *iaaH*) and hairy root inducing (*rolABC*)

These genes have been extensively employed in forest tree biotechnology for genetic transformation of tree species, including the possible applications for improving and introducing novel traits into forest tree-species. The use of the *iaaM* and *iaaH* auxin-biosynthetic genes from *Agrobacterium tumefaciens* significantly affected several wood characteristics in transgenic hybrid aspen (Tuominen et al., 1995). The transgenic trees were generally smaller than the controls, exhibiting reduced growth rate, leaf size and stem diameter. Although

these changes seem undesirable for the improvement of wood quality, other changes, such as a reduction in the number of side shoots following decapitation and changes in the xylem structure and composition show some potential for transgenic trees expressing these genes.

The *rolC* gene from *Agrobacterium rhizogenes* has also been shown to alter the growth and development of transgenic forest trees. Overexpression of this gene leads to dwarfing, breaking of apical dominance, reduced growth rate and decreased stem-internode length in transgenic hybrid aspen trees (Nilsson et al., 1996). It is hard to predict the specific silvicultural advantages of the *rolC* gene in woody plants from their phenotypic appearance, but transgenic aspen plants expressing the *rolABC* native fragment from *Agrobacterium rhizogenes* increased the growth rate *in vitro*, as well as giving improved rooting characteristics and stem:root production index (Tzfira et al., 1997). These unique characteristics can be related to the combination of several *rol* genes and their specific, low expression pattern, as they are driven by their native promoters. It should be noted that undesirable alterations, such as reduced apical dominance and the breaking of axillary shoot buds, were also observed. *Agrobacterium 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. In general, this selection system is not extensively used except to monitor the transposition or excision or marker genes in the development of marker-free transformation technologies (Ebinuma and Komamine, 2001).

D-Amino acid oxidase (DAAO) (*dao1*)

D-Amino acids are naturally present in many higher plant species including the dicots and monocots (Brückner and Hausch, 1989). The marker gene *dao1* encoding DAAO (E.C. 1.4.3.3) was used as a conditional marker in *Arabidopsis thaliana* and proven to be versatile allowing either positive or negative selection depending on the substrate (Erikson et al., 2004). DAAO catalyzes the oxidative deamination of a range of D-amino acids (Alonso et al. 1998). Selection is based on the toxicity of different D-amino acids and their metabolites to plants. Thus, D-alanine and D-serine are toxic to plants, but are metabolized by DAAO into non-toxic products, whereas D-isoleucine and D-valine have low toxicity, but are metabolized to toxic keto acids 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate, respectively. The toxicity of

some D-amino acids on organisms is not well known and is very rarely studied in plants (Gamburg and Rekoslavskaya, 1991). Erikson et al. (2004) apart from *Arabidopsis thaliana*, they have also tested the susceptibility D-serine in other plant species (tobacco, barley, maize, tomato and spruce) and found susceptible at millimolar range similar to that shown to be toxic for *Arabidopsis thaliana*. Therefore, this new plant selectable marker gene using the genes involved in D-amino acid metabolism of plants can be widely employed both as positive and negative selection in plants.

Arabitol dehydrogenase

Different *Escherichia coli* strains can use a multitude of carbohydrate sources, due to a series of operons that are located within the *Escherichia coli* genome (Reiner, 1975). One of these, in particular is the ability of *Escherichia coli* strain C, but not the laboratory K12 strains to grow on D-arabitol (Scangos and Reiner, 1978). Since most plants cannot metabolize most sugar alcohols, including D-arabitol (Stein et al., 1997), there is an opportunity to develop positive selection system based on sugar alcohols. In *Escherichia coli* strain C, the arabitol genes are located in the *atl* operon and include *atlT*, *atlD*, *atlK* and *atlR* (Reiner, 1975). *AtlD* encodes D-arabitol dehydrogenase (EC 1.1.1.11), which converts arabitol into xylulose. Xylulose is an intermediate of the oxidative pentose phosphate pathway (Kruger and Von Schaewen, 2003). Plants can grow on D-xylulose (Haldrup et al., 1998b), so if a plant cell could express arabitol dehydrogenase, then such a cell would be able to grow in a medium containing D-arabitol, whereas an untransformed plant cell would not proliferate. LaFayette et al. (2005) used *Escherichia coli* arabitol dehydrogenase gene as a plant selectable marker in rice crop. The enzyme converts the non-plant-metabolizable sugar arabitol into xylose, which is metabolized by plant cells. Selection on 2.75% arabitol and 0.25% sucrose yielded a transformation efficiency (9.3%) equal to that obtained with hygromycin (9.2%). Thus, arabitol could serve as an effective means for plant selection.

D-Serine ammonia lyase (DSD)

DSD (EC 4.2.1.14, formerly D-serine dehydratase), encoded by the *dsdA* gene from *Escherichia coli* is a pyridoxal 5'-phosphate-requiring enzyme, which catalyzes the deamination of the D-enantiomers of serine, threonine and allothreonine to the

corresponding keto acids, ammonium and water. D-Serine is toxic to *Escherichia coli* strains lacking *dsdA* as a result of the inhibition of L-serine and pantothenate synthesis, and the recognized function of DSD is detoxification of D-serine, which also enables the bacteria to use D-serine as a carbon and nitrogen source (Cosloy and McFall, 1973). Furthermore, the *dsdA* gene has proven useful as a selectable marker in transformation of *Escherichia coli*, by using strains naturally lacking DSD activity (Maas et al., 1995). Plants are sensitive to D-serine, but functional expression of the *dsdA* gene, encoding D-serine ammonia lyase from *Escherichia coli* can alleviate this toxicity. Plants in contrast to many other organisms lack the common pathway for oxidative deamination of D-amino acids. This difference in metabolism has major consequence for plant responses to D-amino acids, since several D-amino acids are toxic to plants even at relatively low concentrations. In a study, Erikson et al. (2005) has explored the use of *dsdA* gene encoding D-serine ammonia lyase from *Escherichia coli* as a selectable marker in the model plant *Arabidopsis thaliana*. D-Serine ammonia lyase catalyzes the deamination of D-serine into pyruvate, water and ammonium. *dsdA* transgenic seedlings can be clearly distinguished from wild type, having an unambiguous phenotype immediately following germination when selected on D-serine containing medium. The *dsdA* marker allows flexibility in application of selective agent, it can be applied in sterile plates, as foliar sprays or in liquid culture.

Trehalose-6-phosphate synthase

Trehalose is non-reducing disaccharide that is abundantly present as stress protectant in microorganisms and some desert plants such as *Selaginella lepidophylla*. In higher plants such as *Arabidopsis thaliana*, trehalose is only detected in small quantities (Vogel et al., 2001). In yeast *Saccharomyces cerevisiae*, trehalose is synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate (T6P) synthase (Tps1) and T6P phosphatase (Tps2). Deletion of *TPS1* eliminates growth on glucose because both Tps1 and T6P are indispensable for the regulation of glucose influx into glycolysis (Thevelein and Hohmann, 1995). The *Arabidopsis* genome encodes 11 *TPS1* homologs that fall into 2 subfamilies displaying most similarity either to yeast *TPS1* (Class I, *AtTPS1-4*) or *TPS2* (Class II, *AtTPS5-11*) (Leyman et al., 2001). *AtTPS1* complements the yeast *tps1* strain for its growth defect on glucose, which suggests a possible regulatory role for *AtTPS1* in

plant carbon metabolism (Van Dijck et al., 2002). Sugar homeostasis is tightly regulated in plants; plantlets germinated in the presence of high glucose concentrations remain white and petite because the external sugar switches off the photosynthetic machinery. Previous report states that *AtTPS1* plays a regulatory role in this phenomenon as ectopic expression of *AtTPS1* in *Arabidopsis* rendering plants significantly less sensitive to glucose compared with wild-type plants (Avonce et al., 2004). Based on this trait, researches started to exploit *AtTPS1* as a selection marker for plant transformation in combination with glucose as selection agent. In order to develop a purely plant-based and environment-friendly selection system Leyman et al. (2006) explored the usefulness of the trehalose-6-phosphate synthase gene *AtTPS1*. This novel selection system was based on enhancing the expression of plant intrinsic gene using a harmless selection agent. Overexpression of *AtTPS1* in tobacco allows selecting glucose-insensitive transgenic shoots. Selection takes advantage of the reduced glucose sensitivity of seedlings with enhanced expression of *AtTPS1*. As a result, transformants can be identified as developing green seedlings amongst the background of small, pale non-transformed plantlets on high glucose medium.

Knotted1 (kn1) homeobox gene

The maize homeobox gene *kn1* and its homologs from other plant species are expressed normally in shoot meristems (Smith et al., 1992; Jackson et al., 1994) and are essential for meristem initiation and maintenance (Long et al., 1996). Transgenic plants overexpressing *kn1* gene exhibit morphological alterations, including changes in leaf shape, loss of apical dominance and production of ectopic meristems on leaves (Tamaoki et al., 1997). Homeobox gene *knotted1 (kn1)* from maize was used as a selectable marker gene for plant transformation in *Nicotiana tabacum* cv. *Xanthi* via *Agrobacterium*-mediated transformation. Under non-selective conditions (without antibiotic selection) on a hormone-free Murashige Skoog (MS) medium, a large number of transgenic calli and shoots were obtained from explants that were infected with *Agrobacterium tumefaciens* LBA 4404 harboring the 35S::*kn1* gene. On the other hand, no calli or shoots were produced from explants that were infected with an *Agrobacterium* strain harboring pBI121 (*nptII* selection) or from uninfected controls cultured under identical conditions. Relative to kanamycin selection conferred by *nptII*, the use of *kn1* resulted in a 3-fold increase in

transformation efficiency. Based on the results, *kn1* gene could be used as an effective alternative selection marker with a potential to enhance plant transformation efficiency in many plant species. With *kn1* gene as a selection marker, no antibiotic-resistance or herbicide-resistance genes are needed, so that potential risks associated with the use of these traditional selection marker genes can be eliminated (Luo et al., 2006).

Future prospects and conclusion

Plant transformation technology offers an array of opportunities for basic scientific research and for genetic modification of crops. The generation of transgenic plants requires the use of different selectable marker genes that are introduced together with the exogenous gene of interest. Recent progress in plant molecular biology and genome research has lead to a desire to introduce several genes into a single transgenic plant, necessitating the importance of various types of selectable marker genes. Among the selectable marker genes applied to plant transformation, the most popular markers are bacterial genes that confer resistance to the antibiotics (kanamycin and hygromycin) or to the herbicide (glufosinate ammonium). As these have been the target of concern among environmental authorities, scientists have been encouraged to develop alternative selection strategies (ACNFP, 1994), although such concerns may prove unfounded (Flavell et al., 1992).

Development of environment-friendly marker-assisted selection system involving natural plant materials is gaining momentum. Novel selection procedures for orchid transformation have been developed by introducing a native plant gene, sweet pepper ferredoxin-like protein (*pflp*), as a selection marker into the *Oncidium* genome via *Agrobacterium*-mediated and particle bombardment. Putatively transformed plants were screened by use of the natural bacterial pathogen *Erwinia carotovora* as a selection agent (You et al., 2003; Chan et al., 2005). In the recent years, use of conditional-positive selection system using the non-toxic metabolic intermediates such as xylose and mannose has gained importance due to its ease and scientific merit. Lai et al. (2007) demonstrated the use of α -amino acids using *dsdA* (α -serine dehydratase) gene from *Escherichia coli* and the *dao1* (α -amino acid oxidase) gene from *Rhodotorula gracilis* as a new set of effective selectable markers for maize transformation. Utility of selectable markers in crop plants fulfils the demand for

alternative selection markers in the development of genetically modified organisms. Earlier studies have supported the safety of the use of marker gene systems in plant transformation (Ramessar et al. 2007).

In summary, selectable marker genes play a vital role to stimulate regeneration of transformed plant cells, thus supporting transgenic research of future plant biotechnologists and geneticists. Plant transformation works in monocot and dicot crops have proved that a wide range of selectable marker genes can be employed for successful genetic transformations. Selection systems do not pose any threat to mankind as evidenced by several experimental reports, thus leading to accelerated research towards the identification of new and novel marker gene systems from different sources.

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