Review

Current progress of biosafe selectable markers in plant transformation

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Accepted 4 January, 2012

Plant transformation plays a crucial role in modern plant breeding system, leading to the improvement of agronomical traits and fundamental studies of plant science. To develop an efficient and reliable transformation approach, selectable marker is the prerequisite and extremely useful in enabling successful transformation. A number of environmental safe and user-friendly selectable marker genes have been exploited in recent years and utilized to mitigate as a result of concerns for bio-security. Considering the different function of each of the marker genes, these are generally divided into four groups namely positive, visual, negative and pathogen-based, respectively, depending on their selection mechanism. This review describes the mechanism of each of these categories of selected markers and summarizes the current progress in genetic transformation, helping researchers to make better judgement on the choice of selectable markers and accelerate their potential application during plant genetic transformation.

Key words: Bio-safety, selectable marker, plant transformation.

INTRODUCTION

Since the first transgenic plant reported in 1983, plant transformation has been playing an important role in modern plant breeding. A number of plant varieties and relevant products from transgenic soybean and maize are available now in commercial channels (www.monsanto.com). Due to the very low efficiency and randomness of transgene integration into genome of host cells, a marker gene is always needed to distinguish the “true” transformed cells, tissues and regenerated shoots from non-transformed ones. At an early stage of plant transformation research, selectable markers were focused on genes encoding for resistance to specific antibiotics, like nptII gene (kanamycin and related aminoglycoside antibiotics) and (herbicide glufosinate) bar gene for effective selection of transformants (Herrera-Estrella et al., 1983; Bevan et al., 1983).

Once the transgenic plants are generated, selection markers become useless and are likely to have a negative impact on the metabolic activity of transgenic plants due to over-expression of marker gene-encoded proteins or enzymes. More importantly, the microorganism-derived marker genes, usually imparting antibiotic or herbicide resistance in the transformants, have been a source of problem for public acceptance and considered as a hindrance in their commercialization. To solve this problem, an array of bio-safe and environmentally friendly selectable markers found qualified were discovered for selection system and gradually replaced the traditional antibiotics. This paper summarizes the current progress of selectable markers, paving the road for readers to deeply understand the function of selectable markers in plant transformation. The selection strategies of these marker genes can largely be classified into four groups including positive, visual, negative and pathogen based selection,
respective.

SELECTION STRATEGIES

Positive selection

Compared to the conventional approach which usually relies on negative selection, these positively selectable marker genes allow for the transformed cells to be identified without causing lethal effect in the non-transformed counterparts. Such genes are divided into three types based on metabolic processes involved such as hormone biosynthesis, saccharide metabolism and amino acid metabolism.

Genes associated with hormone biosynthesis

The uidA gene

This gene is originally isolated from Escherichia coli, and encodes the β-glucuronidase enzyme (GUS). Due to its unambiguous performance, it has been extensively used as a reporter gene in plant transformation in which a glucuronide derivative of the cytokinin benzyladenine (benzyladenine N-3-glucuronide) is utilized as the selectable reagent to inactive cytokinin. However, upon hydrolysis by GUS, active cytokinin is released which will stimulate the transformed cells to propagate. Development of the non-transformed cells will be restrained due to the lack of GUS gene, thereby the selection of transformed versus non-transformed cells can be achieved. The application of this system in tobacco transformation yielded a 1.7-2.9 fold higher frequency than kanamycin selection (Joersbo and Okkels, 1996; Okkels et al., 1997).

The ipt gene

The isopentenyl transferase (IPT) is a key enzyme in cytokinin biosynthesis. The ipt gene isolated from Agrobacterium tumefaciens could be used as a marker for transformed cells without any additional selection reagent. In this system, the ipt transgenic cells can be regenerated into plants on the medium without cytokinin because IPT enhances the de novo biosynthesis of cytokinin. However, the non-transformed cells cannot survive due to the lack of the hormone. Although over-production of cytokinin was expected to cause morphological abnormalities in transgenic plants, a few plants like tobacco and lettuce were reported to exhibit normal morphology (Ebinuma et al., 1997; Kunkel et al., 1999). Actually, the ipt gene is now widely used as selection gene, together with the site-specific recombination system, to induce marker-free transgenic plants (Khan et al., 2011a, b).

The kn1 gene

The maize homeobox gene knotted1 (kn1) and its homologues from other plant species express normally in shoot meristems, and are essential for meristem initiation and maintenance. Transgenic plants over-expressing kn1 exhibit morphological abnormalities which are similar to the ipt over-expressing seedlings. The identification of allele gene of maize kn1, kn1-DL, suggested that kn1 establishes proximal/distal patterning when expressed in distal locations (Ramirez et al., 2009). There was three fold increases in transformation frequency by utilization of this gene as a selection marker in tobacco compared to the use of nptII (Luo et al., 2006).

SACCHARIDE METABOLISM PATHWAY RELATED GENES

The genes in this group encode particular enzymes in saccharide metabolism pathway and those rare saccharides (mostly monosaccharides) or their ramifications cannot be used as carbon sources by plants under normal conditions.

The pmi gene

The pmi gene from Escherichia coli encodes the phosphomannose isomerase (PMI), which catalyzes the reaction to convert mannose-6-phosphate into fructose-6-phosphate. Plant cells lacking this enzyme are not capable of surviving on the medium containing mannose as the main or sole carbon source. In pmi/mannose selection system, only the transformed cells genetically modified to express E. coli manA gene can utilize mannose and develop further while the non-transformed cells have negligible growth without appropriate energy source (Reed et al., 2001). The pmi gene is the most widely used alternative bio-safe selectable marker and has been successfully applied in transformation of many species such as wheat (Gadaleta et al., 2006) and sugarcane (Jain et al., 2007).

The transformation efficiency using pmi as a selectable marker for those plants were considerably higher compared with the antibiotics-based selection. For instance, the duration of regeneration time of transgenic orchid plants Oncidium Gower Ramsey was significantly shorter in mannose selection system (4 months) than in hygromycin selection system (8 months) (Thiruvengadam et al., 2011). More information about pmi gene is available in recently published review (Stoykova and Stoeva-Popova, 2011). It was reported that three genetically engineered corn events from Syngenta, Event
The xyIA gene

Xylose isomerase (XI), encoded by xyIA gene from Streptomyces rubiginosus and Thermoaerobacterium thermosulfurogenes, catalyses the formation of D-xylulose from D-xylose in bacteria and some fungi. Some plant species can utilize D-xylulose instead of D-xylose as carbon source. Thus, the cells expressing xyIA can grow and recover de novo plants while the wild-type cells are disabled when using xylose as the sole carbon source. This gene has been successfully used as a selectable marker in transformation of many plants like tobacco (Haldrup et al., 2001) and sunflower (Morawala and Rajyashri, 2007). Our group has isolated xyIA gene from E. coli and transformed it into crowtoe (Lotus corniculatus), and transformants were obtained on the medium containing xylose (unpublished data). Recently, some novel XI genes were isolated from soil metagenomic library (Parachin and Gorwa-Grauls, 2011), Opuntia (Ravikumar et al., 2011b) and Marine Bacterium (Umemoto et al., 2011), together with xyIA-like genes from Thermoanaerobacter ethanolicus (Fan et al., 2011) and xerophyte Cereus (Ravikumar et al., 2011a), offering more opportunities for choosing markers in plant transformation work.

The atlD gene

The atlD from E. coli strain C encodes the arabitol dehydrogenase, which converts arabitol into xylulose metabolized by plant cells. In this selection system, arabitol has a dual function as the main carbon source and the selection reagent. Only the transformed cells can grow and regenerate into plantlets. A transformation efficiency of 9.3% was obtained in rice by the application of this system, almost equal to that obtained by using hygromycin as the selection agent (9.2%) (LaFayette et al., 2005).

The AtTPS1 gene

The AtTPS1 gene is one of 11 homologues encoding trehalose-6-P synthase (TPS) in Arabidopsis thaliana and its orthologues have been found in many other plants. Because it is plant-intrinsinc, AtTPS1 is feasible to be used in plant transformation as another saccharide metabolism related gene. The transformed plant cells over-expressing AtTPS1 are able to grow normally in high osmotic medium and develop into green seedlings, whereas the non-transformed ones form small or even albino plantlets. High glucose insensitive transgenic tobacco shoots were obtained employing this gene as a selectable marker (Leyman et al., 2006). Besides being environmentally friendly marker, AtTPS1 was found to play an essential role in modifying embryo or flower development (Chary et al., 2008), as well as regulating the vegetative growth and sugar metabolism (Gómez et al., 2010).

AMINO ACID METABOLISM ASSOCIATED GENES

The AK and DHPS genes

AK gene encodes aspartate kinase (AK) and the DHPS encodes the dihydrodipicolinate synthase (DHPS). Both of them are two key enzymes in branched-chain amino acids (lysine, threonine, methionine and isoleucine) synthesis pathway from asparagine. In a wide range of plant species, the activity of these two enzymes is strongly feedback-inhibited by lysine. However the homologous enzymes from bacteria are less sensitive to lysine inhibition and can be used as selectable markers for plant transformation. In AK/lysine and DHPS/lysine selection system, the metabolism of endogenous AK and DHPS is feedback-inhibited by milli-molar of external lysine in non-transgenic cells, which grow slowly as a result of the lack of lysine and/or threonine. In contrast, the transformants can grow normally and regenerate de novo plants. These systems have been used in transformation of several plants successfully (Ufaz and Galili, 2008).

The DAO1 gene

DAO1 gene from yeast Rhodotorula gracilis encodes the D-amino acid oxidase (DAAO) which belongs to flavoprotein and is located in peroxisome with the function of detoxification of amino acids. As a conditional marker gene, it has dual role of both positive and negative selection in plants depending on the substrate (Erikson et al., 2004). This feature offers an opportunity to apply the negative selection after a positive selection using dao1 gene by changing D-alanine or D-serine to D-isoleucine or D-valine. The transformation system of Arabidopsis thaliana using dao1 as a selectable marker has been established successfully. It is versatile and enables selection immediately after germination. Moreover, hypothetically this gene can be used in the
removal of marker genes (Scheid, 2004).

**The TSB1 gene**

TSB1 gene encodes the *Arabidopsis* tryptophan synthase beta 1 (AtTSB1). It is a novel amino acid metabolism associated gene originally identified from *A. thaliana* and has been used in transformation of some plant species. In TSB1 selection system, 5-methyl-tryptophan (5MT, a tryptophan analog) and/or CdCl₂ are used as selection reagent(s). This system has been applied in floral dip transformation of *Arabidopsis* (Hsiao et al., 2007). More importantly, over-expression of AtTSB1 gene in *Arabidopsis* and tomato were proven to enhance tryptophan accumulation and improve cadmium tolerance (Sanjaya et al., 2008).

**The lyr gene**

The *lyr* gene encoding lysine recemase was isolated from a soil metagenome by using L-lysine as selection agent which is toxic to plants and can be converted by Lyr into D-lysine. Transgenic plants use D-lysine as nitrogen source and can survive among non-transformed plants. The selection efficiencies were 23 and 2.4% for transgenic tobacco and *Arabidopsis* plants, respectively and no pleiotropic effects were observed compared with wild-type plants (Chen et al., 2010). This selectable marker system is less controversial and inexpensive than the traditional ones.

**VISUAL SELECTION**

In this strategy, the marker gene was often used in co-transformation trials as the partner to monitor and identify transgenic events rather than selection. In general, these marker genes encode some proteins that directly emit fluorescent light, such as the green fluorescent protein (GFP). Some other genes produce enzymes that catalyze corresponding substrates to emit fluorescence (for example, *lacZ*), or enzymes catalyze some color reactions (for example, *lacZ*). Normally, the transformants can be picked up manually with or without the help of a fluorescent microscope.

**The lacZ gene**

The *lacZ* gene of *E.coli*, coding for β-galactosidase, is a reporter gene to study gene expression in microbial, animal and plants systems. In the experiments of plant transformation, positive transformants are identified by color development in the presence of X-Gal. This gene was applied in tobacco and sunflower transformation via *lacZ* gene fusion (Helmer et al., 1984). Since many plants had the background of endogenous activities, making the detection of its products in transgenic plant cells more complicated, the potential application of *lacZ* gene was hampered and not widely adopted (Miki and McHugh, 2004).

**The luc and lux genes**

These two genes, both encoding the luciferase, were isolated from firefly (*Photinus pyralis*) and *Vibrio harveyi*, respectively. Luciferase catalyzes the conversion of luciferin to oxyluciferin which emits fluorescence of 560 nm, thereby enabling the transformed cells to be picked up manually under a fluorescent microscope. This system has been applied in transformation of orchid, wheat and barley (Chía et al., 1994; Lonsdale et al., 1998; Harwood et al., 2002). Though theoretically the two genes are feasible being used as individual marker genes, they are often combined with other marker genes.

**The gfp gene**

GFP encoded by *gfp* gene from jellyfish (*Aequorea victoria*) is the most widely used and powerful reporter marker. With the advantage of direct *in vivo* and real-time visualization, it can be used to enrich the transformed tissues. This gene has been used as marker gene in the transformation of rice (Vain et al., 1998), barley (Ahlandsberg et al., 1999), wheat (Jordan, 2000), papaya (Zhu et al., 2004) and other plants. Several new fluorescent proteins, AmCyan, AsRed, DsRed, ZsGreen and ZsYellow, have been isolated from reef-coral organisms. These five proteins act like GFP. They do not require a substrate to emit fluorescence and can be tested *in vivo* without destruction of the tissues under study. Wenc’s group has evaluated them on a variety of plants of both monocots and dicots and their results indicated that these proteins were invaluable reporting tools for transformation of many different crop species (Wenc et al., 2003). Although, these genes have proven to exhibit very high selection efficiency, none of them is reportedly being used as single selection marker in plant transformation.

**The BGL1 gene**

Glucosidase belongs to the glucosyl hydrolase (GH) family 3 enzymes, which specifically hydrolyze terminal, nonreducing β-D-glucose residues. Combined with 4-methylumbelliferyl-β-D-glucopyranoside (MUGluc) as the substrate, the *Aspergillus niger* β-glucosidase (encoded by *BGL1*) can be expressed in the endoplasmic reticulum (ER) of plants as an efficient visual selection marker. This
The system was used to select the dry seeds without germination in Arabidopsis (Wei et al., 2004).

AtMYB12

MYB-type proteins belong to a super family of transcription factors regulating the developmental processes and defense responses in plants. One of them AtMYB12 was originally identified as transcriptional activator in A. thaliana and indispensable for the biosynthesis of flavonols. Transformants expressing AtMYB12 gene can be identified after 1 day of transformation without any environmental and health concerns. For wheat transformation with AtMYB12, the purple pigment was exhibited in the epidermal cells of developing coleoptiles of transformed wheat, making them very easy to be recognized. Similarly, this method of selection also can be applied on other explants like scutella (Gao et al., 2011).

NEGATIVE SELECTION

This strategy differs from positive selection in that the selection of transformed tissue is achieved at the cost of non-transformed tissue. There is a description of some bio-safe negative selectable genes.

The DOG\textsuperscript{R1} gene

This gene derives from baker’s yeast and encodes the 2-deoxyglucose (2-DOG) \textsuperscript{-6}-phosphate phosphatases, resulting in the resistance towards 2-DOG. Hexokinase catalyzes the conversion of 2-DOG into 2-DOG-6-phosphate, which can cause inhibition of respiration, impair cell wall formation and protein glycosylation and eventually leading to the cell death. However, the 2-DOG-6-phosphate phosphatase can detoxify 2-DOG-6-phosphate by converting it into 2-DOG through dephosphorylation reaction. In this selection system, only the transformed cells expressing DOG\textsuperscript{R1} gene can survive and develop into shoots. This system has been successfully demonstrated in tobacco, tomato and potato (Kunze et al., 2001). Our group also found that it works well in crowte (Lotus corniculatus) (Guo, 2007).

The galT gene

Galactose-1-phosphate uridylytransferase (galT) is an enzyme responsible for converting ingested galactose to glucose. It is well known that galactose is toxic to many plant species due to the accumulation of galactose-1-phosphate, caused by endogenous galactokinase. It has been demonstrated that this toxicity can be sufficiently alleviated by expression of galT gene, ensuring the transgenic plant to survive on the medium containing galactose as selection agent. This selection system has proven very effective in transformation of potato and oil seed rape, providing a new alternative being distinct from conventional positive selection system (Joersbo et al., 2003).

The badh gene

As an enzyme in the plant biosynthetic pathway, betaine aldehyde dehydrogenase (BADH) converts the toxic chemical betaine aldehyde (BA) to the non-toxic glycine betaine (GB), one of best-studied compatible solute protecting plants against various abiotic stresses (Chen and Murata, 2011). Recent research has identified the potential for badh as an antibiotic-free marker for selection of transgenic plants, with BA as the selection reagent. More importantly, expression of badh also improves plant to tolerant abiotic stress. Kumar et al. (2004) reported that the transformed chloroplast genome of carrot harboring spinach-derived badh gene showed highest lever of GB accumulation in transgenic plants to date, conferred strong tolerance to salt stress.

PATHOGEN BASED SELECTION STRATEGY

In this strategy, pathogens are used as selection reagents and the whole selection is carried out inside the host plants. Plant ferredoxin-like protein (PFLP) is a photosynthetic type ferredoxin originally isolated from sweet peper (Capsicum annuum L.). Although, this protein contains an N-terminal signal peptide responsible for chloroplast targeting, its expression can be detected not only in chloroplast, but also in cytoplasm (Lin et al., 2010), resulting in enhanced resistance to bacterial pathogen like Erwinia carotovora which causes soft rot disease. These findings not only provide a strategy for plant breeders to improve resistance of crops against bacterial pathogens, but also a useful tool for selection of transgenic events in plants. This system has been applied in several plant species (You et al., 2003; Huang et al., 2006; Yip et al., 2007).

PROSPECTIVES

Great progress has been made by scientists in developing new bio-safe marker genes to replace the conventional marker genes such as antibiotic and herbicide resistant genes. New challenges have emerged. Some of these markers are not feasible for transformation due to low selection efficiency and very limited applicability. For instance, the pmi gene can not be highly expressed in specific species like some
leguminous plants with high activity of endogenous PMI. Therefore, the pmr/man system cannot be chosen for transformation of these species. Although no adverse biosafety effects have been reported for the marker genes widespread used in current studies, public concerns push forward the removal of selectable markers from transformants. In recent years, marker-free strategy constitutes a fast growing new trend. So far, several methods have been developed to eliminate the marker genes including co-transformation, transposon-mediated repositioning, intrachromosomal homologous recombination and site-specific recombinase-mediated excision (Miki and McHugh, 2004).

However, most of the approaches still use antibiotic resistant genes as selection markers in the initial stages considering their efficacy for transformant screening. For instance, through site-specific recombination system cre/loxP, the selectable marker like nptII is finally deleted from the transformants. Besides the markers mentioned above, researchers also exploit new selectable marker (Ferradini et al., 2011), or even directly obtain marker-free transformants without selectable marker (Liu et al., 2011). Very recently, the versatile, ready-to-use platform, namely the Recombination-assisted Multifunctional deoxyribonucleic acid (DNA) Assembly Platform (RMDAP), is developed to provide many attractive features like multigene transformation, a wide range of flexible, marker-free vectors regulating gene over-expression or gene silencing (Ma et al., 2011). The following diagram describes the basic principle of how to construct the vector with gene stacking (Figure 1). This platform is confirmed by the experiments in Arabidopsis and tobacco plants and reveals to us the light of coordinating the regulation of metabolic pathway and molecular breeding.

ACKNOWLEDGEMENT

This work was supported by Science and Technology Developmental Program (No. 20072561) and postdoctoral fund (No.05211) of Jilin Provence.

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