Chapter 3
Concepts of Marker Genes for Plants

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3.1 Introduction

Historically, plant breeding has been based on trial and error. While environmental factors originally determined selection, pre-agricultural men eventually developed a more purposeful extension of this process. This meant that selection was mainly based on appearance, yield, vigorous growth, taste and smell. Especially the past century resulted in new breeding programs that led to exceptional increases in both the quality and quantity of crops. In recent years genetic transformation techniques have been developed which complement classic breeding as it represents an additional way of generating new genetic diversity. This new technology is based on the introduction of DNA into the plant cell, followed by regeneration of the transformed cells to an entire plant (see Chap. 1). Marker genes, more exactly named selectable marker genes, are absolutely essential for the production of such transgenic plants. Despite optimization of the transformation efficiency of many crops, it is a fact that (even after three decades of agricultural biotechnology, also in model plants like *Arabidopsis*) the insertion of genes is restricted to a few cells among thousands of untransformed ones. Marker genes are required to identify, to "mark" the introduced genes and finally to enable the selective growth of transformed cells. These genes are co-transformed with the gene of interest (GOI); they are linked to the GOI and therefore remain in the transformed cell. However, once transgenic cells have been identified und regenerated to whole plants, the marker genes are no longer needed. For this reason new concepts of marker genes are discussed with regard to the safety of genetically modified plants, both for the environment and the consumer. Therefore this chapter reviews the most important marker genes

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available for gene transfer to plants, focusing particularly on recent advances, and discusses new systems for marker gene-free transformation techniques as well as marker gene deletion.

3.2 Criteria for Choosing the Marker Gene System

The most common type of selectable marker genes used for the efficient transformation and regeneration of plant cells are antibiotic resistance genes or herbicide resistance genes (Miki and Hugh 2004). The criteria for choosing these genes are the efficiency of the systems, their applicability to a wide range of plant species and of course their availability for the scientific community. Nonetheless criteria have changed within recent years, especially for the development of new varieties and new traits for the market. Furthermore the marker gene systems have to fulfil the requirements of regulatory and market acceptance (see Fig. 3.1).

Over the years, general opinion has accepted that using conventional transformation methods, including conventional vectors, can cause problems. Extra copies of transgenes or residual selection marker genes and their regulatory elements can increase the frequency of homology-based post-transcriptional and transcriptional gene silencing (Que and Jorgensen 1998). That implies problems due to the variability and instability of transgene expression (Matzke et al. 2000). The extra gene copies can be a result of both multi-copy insertions (during the transformation process) and trait stacking. Herbicide tolerance and insect resistance (Bt) often are introduced simultaneously to a crop in one transformation event or the combined traits are a result of re-transformation or crossing two single events. For example the third most commonly grown transgenic crop was stacked insect-resistant/herbicide-tolerant maize. Combined herbicide and insect resistance was the fastest growing GM trait from 2004 to 2005, grown on over 6.5 million hectares in the United States and Canada and comprising 7% of the global biotech area (GMO-Compass 2007). For the 2008 planting season quad stacks have been announced that protect the corn crop against both corn borer and corn rootworm while providing tolerance to various herbicides. This second generation of traits and the upcoming third generation of "output trait" products provide multi-resistance to pests and several types of pathogens, providing new products from metabolic engineering, offering new benefits to farmers and consumers (Halpin 2005).

However, that also means that the complexity of the GMO will increase in total because this includes the interaction between numerous genes. This multiple stacking of traits, also called "pyramided" traits with potential new management requirements or possible negative synergistic effects, may evoke an additional environmental safety assessment (http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9408e.shtml). It will be necessary to control these multiple genes, by developing new technologies for the coordinated manipulation of such traits. This includes cutting-back the complexity of the GMO. The more complex, the longer

- **Simple efficient plant transformation system**
    - **Efficient method of plant transfer**
        - **Direct DNA transfer**
        - ***Agrobacterium*-mediated transfer**
    - **Optimal identification or selection system**
        - **Technically simple**
        - **Fast**
        - **Economical**
        - **Removable selection marker**
- **Optimum performance of the GMO comparable with standard varieties**
        - **Substantial equivalence**
- **Process of deregulation dependent on:**
    - **Acceptance by the responsible authorities**
    - **Biosafety**
    - **Complexity of the GMO**
        - **One or only a few insertions**
        - **No marker genes or completely harmless ones**
        - **No additional unnecessary sequences**
    - **Complete characterization: molecular analysis, biochemical analysis**
    - **Provision of quality control systems**

Fig. 3.1 Criteria for the development of new plant varieties by gene technology

the time for trait commercialization will be. In reality product development and deregulation will need a minimum of 10–15 years. Therefore, there is a demand both from the authorities and from the scientific community to improve the transformation systems, to avoid additional unnecessary sequences, to abstain from marker genes, to eliminate marker genes or to use only completely harmless ones.

3.3 Availability of Selectable Marker Gene Systems and Alternatives

There are different categories of selectable marker and alternative systems available, as shown in Fig. 3.2.

3.3.1 Positive Selection Marker

There are sometimes different and confusing definitions in using the terms "positive selection marker" and "negative selection marker". At present, positive selection systems are those that enable the growth of transformed cells, whereas negative selection systems kill the transformed cells (see Sect. 3.3.4).

3.3.1.1 Antibiotics

The most widely used selection marker systems are based on aminoglycoside-modifying enzymes. These amino glycoside-modifying enzymes confer resistances against antibiotics as kanamycin, neomycin, gentamycin, paramomycin, streptomycin and spectinomycin.

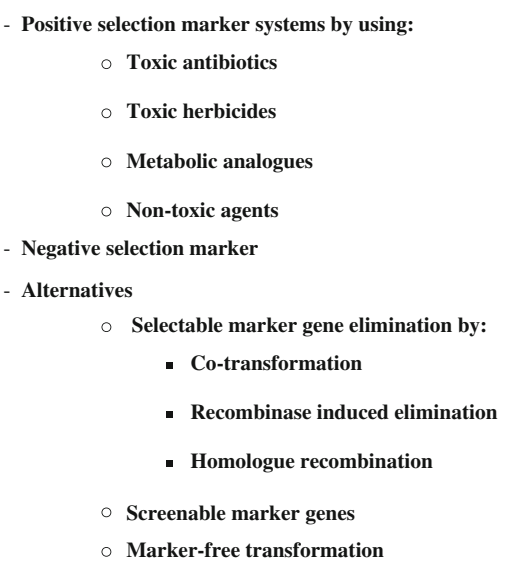

Fig. 3.2 Overview of selection systems

Neomycin Phosphotransferase II

Within the aminoglycoside-modifying enzymes the *neomycin phosphotransferase II* (*nptII*), originated from transposon Tn5 of *Escherichia coli* K12 (Garfinkel et al. 1981), is the most used selectable marker gene. There are many advantages in using *nptII* in comparison with other selectable marker genes:

- The gene confers resistance against different antibiotics: kanamycin, neomycin, paramomycin, geneticin.
- The gene is efficient in model plants such as Arabidopsis, Petunia or Nicotiana tabaccum but also in most of the cultivated plants, both in monocots and dicots, in legumes and Gramineae.
- Reproducible protocols are available for the transformation of most of these crops.
- NPTII is available in combination with various regulation sequences, e.g. promoters.
- There are mutated forms of the nptII gene that encode enzymes with reduced activity.
- By using an intron-containing nptII gene only eukaryotic organisms will be able to process the gene (Paszkowsky et al. 1992; Maas 1997; Libiakova 2001). Accordingly, the potential risk of horizontal gene flow of antibiotic resistance genes from transgenic plants to bacteria is eliminated.
- *NPTII* can be used not only as a selectable marker but also as a scorable marker, as a reporter gene for studying gene expression and regulation. In vitro assays (ELISA or use of radioisotopes) for quantitative or semi-quantitative analysis of the NPTII activity are available (McKenzie 2000; Ziemienowicz 2001).
- The patent on the *nptII* coding sequence combined with regulatory sequences will expire soon (König et al. 2003).

Most of the first-generation transgenic crops contain *nptII*, and to this date *nptII* is the best studied selectable marker with regard to safety. Already in 1994, the use of *nptII* as a marker and as a food additive for transgenic tomatoes, oilseed rape, and cotton was evaluated by the United States Food and Drug Administration (FDA). The FDA found the use of *nptII* as a selection marker safe (FDA 1994). The conclusion was based on data from Calgene (1993), Redenbaugh et al. (1994), Fuchs et al. (1993), Nap et al. (1992), Flavell et al. (1992), Kasid et al. (1990) and Blease et al. (1990), among others. But unattached, without reference of scientific evaluation, the presence of antibiotic resistance genes, mainly NPTII, increases public and consumer criticism and still is dogged by controversy.

Mainly the concerns about the potential spread of antibiotic resistance genes through horizontal gene transfer led to the final recommendation that antibiotics widely used for clinical or veterinary use may not be used as selectable markers in plants (Miki 2004; FDA 1998). Also in Europe the use of antibiotics as selection marker was acknowledged as a problem and resulted in Directive 2001/18/EC, which requires the step by step phasing out of antibiotic resistance genes which may have adverse affects on human health and environment by the end of 2004 (EFSA 2004). However the European GMO Panel came to the conclusion in 2004 that the

use of the *nptII* gene as selectable marker in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment. These safety assessments were confirmed by the EFSA in 2007 again in the light of all relevant reviews and expert consultations: Ramessar et al. (2007), Goldstein et al. (2005), Miki and McHugh (2004), Working Party of the British Society for Antimicrobial Chemotherapy (Bennett et al. 2004), FAO/WHO Consultation on Foods Derived from Biotechnology (FAO/WHO 2000), Scientific Steering Committee of the European Commission (SSC 1999), Zentrale Kommission für die Biologische Sicherheit, DE (ZKBS 1999), The Advisory Committee on Novel Foods and Processes, UK (ACNFP 1996), Nap et al. (1992).

But again, in contrast, in 2005 the WHO classified kanamycin and neomycin as critically important antibiotics (WHO 2005). To sum up there is no recommendation of a general ban of antibiotic markers, only a restricted use, but there are disagreements concerning the classification of antibiotics (mainly for kanamycin) whether they are of high, minor or no therapeutic relevance in human medicine.

Hygromycin Phosphotransferase

Cloning of the *hygromycin phosphotransferase* (*hph*) gene and fusion with eukaryotic promoters resulted in the development of vectors that permit selection for resistance to hygromycin B in both prokaryotic and eukaryotic cells (Elzen et al. 1985). Besides kanamycin, hygromycin B is the most frequently used antibiotic for selection. In comparison with kanamycin, hygromycin is more toxic and therefore kills sensitive cells faster. However, hygromycin is the preferred antibiotic resistance marker for the selection of monocotyledonous plants, although it is not user-friendly. Extreme care has to be taken when handling hygromycin B as it is very toxic by inhalation, in contact with skin and if swallowed.

Antibiotic Resistance Genes Beside *nptII* and *hph*

There are a lot of other marker genes, for example antibiotics like streptomycin (Maliga et al. 1988), spectinomycin (Svab and Maliga 1993), bleomycin (Hille et al. 1986) and chloramphenicol (de Block et al. 1984), which have been used in plant transformation experiences or are at least part of used transformation vectors. But most of them are under the control of a bacterial promoter and have been used for selection in bacteria not specified for selection in plants. In the end the genes are mostly integrated outside the left and right border regions of the used transformation vectors and therefore not part of the transgenic plants.

3.3.1.2 Herbicides

Millions of hectares are being planted with transgenic herbicide resistant plants (see also Chap. 9), meanwhile often “stacked” with insect resistance in the same seeds to

enhance their value. The database summary *Global status of approved genetically modified plants* of AGBIOS (2009) shows 80 records for the trait herbicide tolerance. So, by far, herbicide tolerance is still the most used selection criteria. The advantages of the systems are the usage of the herbicide tolerance both as a desired trait in the field and as a selection marker (Goldstein et al. 2005) during developmental period. The most used systems comprise *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*, resistance to glyphosate), *phosphinothricin acetyl transferase* (*bar*/*pat*, resistance to glufosinate) *acetolactate synthase* (*ALS*, resistance to chlorosulfuron) and *bromoxinil nitrilase* (*Bxn*, resistance to bromoxinil) in descending order of AGBIOS records. In 2006 glyphosate-resistant crops have grown to over 74 million hectares in five crop species in 13 countries (Dill et al. 2008).

Meanwhile new and improved glyphosate-resistant crops are being developed. These crops will confer greater crop safety to multiple glyphosate applications and these glyphosate-resistant plants are expected to continue to grow in number and hectares planted. But there is no guarantee that new molecular stacks conferring resistance to glyphosate and ALS-inhibiting herbicides or glyphosate with glufosinate will prevent the development of resistant weeds in the future. There are already several weed biotypes with confirmed resistance to glyphosate. So the question arises whether the presence of herbicide selection markers like glyphosate resistance in some years may be undesirable when the trait is no longer necessary or inapplicable for product function. Apparently, the same conclusion is valid for herbicide resistance marker genes as for other marker genes, needed in the first place but undesirable shortly afterwards.

There are alternatives to the most used herbicide resistance genes, for example selectable marker genes which mediate resistance against the herbicides cyanamide (Weeks et al. 2000), Butafenacil (Li et al. 2003; Lee et al. 2007), Norflurazon (Inui et al. 2005; Arias et al. 2006; Kawahigashi et al. 2007) or Gabaculine (Gough et al. 2001). However, by today, most of these alternatives have not been subjected to regulatory consideration for international approvals.

3.3.1.3 Metabolic Analogous, Toxic, Non-Toxic Agents

Many other new approaches comprise manipulating the plant's metabolic or biosynthetic pathways. This is done by using metabolic analogous, toxic agents, non-toxic elements such as phytohormones, or carbon supplies which are natural to the plant. There is a wide range of used genes. *XylA*, *dog*, *ipt*, *tps* and *manA* are only a choice of new genes which were used to develop additional selection systems.

2-Deoxyglucose-6-Phosphate Phosphatase

The deoxyglucose (DOG) system is based on the sugar 2-deoxyglucose (2-DOG) which is phosphorylated by hexokinase yielding 2-DOG-6-phosphate (2-DOG-6-P)

in plant cells. 2-DOG-6-P is toxic to plants, since it inhibits respiration and cell growth. Over-expression of the gene enzyme *2-deoxyglucose-6-phosphate phosphatase* (*dog^{R}1*) in plant cells results in resistant plants (Kunze et al. 2001). Transgenic potato plants have been tested under field conditions with the result that no differences were found between the transgenic plants and the control plants. Whether the system can be applied without safety concern in the future has to be investigated further (GMO-Safety 2005).

Xylose Isomerase

The *xylose isomerase* (*xylA*) system is based upon selection of transgenic plant cells expressing the *xylA* gene from *Streptomyces rubiginosus*, which encodes xylose isomerase, on medium containing xylose (Haldrup et al. 1998). In contrast to antibiotic or herbicide selection, the system is generally recognized as safe because it depends on an enzyme which is already being widely utilized in specific food processes, especially in the starch industry. But to this day selectable markers like *xylA* have not yet appeared in approved food plants.

Isopentenyl Transferase

The enzyme *isopentenyl transferase* (*ipt*) is a more often used selection marker. The gene, encoded by the T-DNA of *Agrobacterium tumefaciens*, catalyzes the synthesis of isopentyl-adenosine-5'monophosphate, which is a precursor of the phytohormone cytokinin. Over-expression of *ipt* by using the gene under the control of a constitutive promoter yields enhanced cytokinin levels in transgenic plants. Cytokinins stimulate organogenesis; therefore due to the enhanced cytokinin concentrations the regeneration of transformed shoots is promoted. The combination of the *ipt* gene together with the kanamycin selection system enhances the transformation efficiency(Ebinuma et al. 1997; Endo et al. 2001).The system, also called the MAT system, is usable as a visible selection system since the transformed shoots lose their apical dominance and the ability to root. These abnormal morphologies of the shoots, so-called "*extreme shooty phenotype*" (ESP) prevented the development of *ipt* as a selectable transformation marker in practice, because it is only usable in combination with inducible artificial promoter systems (Kunkel et al. 1999; Zuo et al. 2002) or with marker elimination systems (Ebinuma et al. 2000, 2001). The use and removal of *ipt* were demonstrated in different plant species but the efficiency of the system was low, therefore further optimization of the selection system is required. Recently new publications (Rommens et al. 2004, 2006; Bukovinszki et al. 2006; Richael et al. 2008) give hope for an improved system. New methods (e.g. "All-native DNA transformation") for the production of transgenic plants utilize *isopentenyl transferase* cytokinin genes in negative selection against backbone integration (e.g. see Sect. 3.4.3 and Chap. 4).

Phosphomannose Isomerase

The *manA* gene codes for the enzyme *pmi* (*phosphomannose isomerase*). Many plants are normally not able to use the sugar mannose as a source of carbohydrate. When plants are forced to grow on mannose as the only carbon source they first convert mannose to mannose-6-phosphate, which is no longer utilizable for the plants. Transformed with the *pmi* gene, the plant converts mannose-6-phosphate to fructose-6-phospate, which can be used in the plant metabolic pathway from there. Thus mannose can function as the only carbon source (Joersbo et al. 1998; Privalle et al. 1999). Species which have been successfully transformed using mannose as selective agent, among others, are sugar beet (Joersbo et al. 1998; Lennefors et al. 2006), sunflower, oilseed rape, pea, barley (Joersbo et al. 1999, 2000), sorghum (O'Kennedy et al. 2006), sugarcane (Jain et al. 2007), rice (Lucca et al. 2001; Ding 2006), tomato and potato (Bříza et al. 2008), apple (Degenhardt 2006), papaya (Zhu 2005), torenia (Li et al. 2007) and citrus (Ballester et al. 2008). Ballester and co-workers compared various selection systems with the same Citrus genotypes: *nptII*, *ipt* and *pmi* systems. The highest transformation rates were obtained with the *pmi*/mannose system, which indicates that this marker is also an excellent candidate for citrus transformation. So at the moment, beside the kanamycin and the glyphosate selection system, the *pmi* system is the most successful one. Regulatory approvals have been received for environment, food and feed with transgenic maize varieties in Mexico, Australia, Japan, Canada and the United States.

3.3.2 Alternative Systems

The rising demand both from the authorities and the public for genetically modified plants containing only foreign sequences needed for the immediate function encouraged the development of alternative systems, including:

- Subsequent elimination of marker genes by co-transformation techniques, transposon usage, specific recombination systems or homologous recombination
- Marker-free transformation without usage of any selection marker
- Combinations of different systems, e.g. usage of screenable marker, recombination systems and/or positive/negative selection in the same system

3.3.2.1 Selectable Marker Gene Elimination

Co-Transformation

Among the techniques developed to eliminate selectable marker genes, co-transformation is the simplest one. The method is based on the strategy to introduce the

marker gene and the gene of interest into plant genome as unlinked fragments. In the progeny the selectable marker gene is segregated from the gene of interest. The introduction of the genes can occur either by using two *Agrobacterium* strains (mixed strain method), each with a binary vector (one carrying the selectable marker gene, the other carrying the gene of interest; Framond et al. 1986; McKnight et al. 1987), or by using a single *Agrobacterium* strain with two plasmids (dual binary vector system or binary vector plus cointegrate vector; Komari et al. 1996; Sripriya et al. 2008), or by using a single *Agrobacterium* strain with one binary plasmid carrying on the plasmid two T-DNAs (two-border vector system; McCormac et al. 2001; Breitler et al. 2004), one with the selectable marker gene and the second with the gene of interest. There are some prerequisites to make co-transformation functional: the efficiency of the co-transformation should be high and in the progeny the segregation efficiency should be also high. Both requirements are dependent on each other and respectively are dependent from the used co-transformation system. Comparing the systems, the mixed strain method reduces the co-transformation efficiency but enhances the integration into separate loci whereas the two border system enhances the co-transformation efficiency and reduces significantly the segregation efficiency. Overall the predisposition of plant cells for the simultaneous integration of T-DNAs naturally supports the multicopy insertions and reduces the probability to identify single copy events. Additional factors which can affect the systems are the plant varieties which have to be transformed, the *Agrobacterium* strains (nopaline or octopine strains), the size of the Ti-plasmids or the ratio of amount of used Ti-plasmid with the selection marker to the amount of the Ti-plasmid with the gene of interest (Yoder and Goldsbrough 1994; de Block 1991; Mathews et al. 2001).

To overcome some of the problems, co-transformation systems are combined with the additional usage of screenable marker genes or negative selection marker. Combination of the kanamycin resistance gene with the negative selection marker *codA* (*cytosine deaminase*) on one T-DNA enables the automatic elimination of the unwanted plants after segregation by 5-fluorcytosine treatment (Park et al. 2004). Another interesting strategy to improve or to speed up co-transformation technology is the usage of androgenetic segregation. Plant breeders require homozygous plants to ensure that the traits are passed on to all progeny. Subsequent to co-transformation with uncoupled T-DNAs, unripe pollen is isolated from the regenerated plants, androgenetic development is induced and the cells of the pollen's haploid chromosome set can spontaneously divide, diploidize and finally regenerate to completely homozygous doubled haploid plants (Goedeke et al. 2007; GMO-Safety 2007). Co-transformation technology is not restricted to *Agrobacterium*-mediated transformation but can also be used for particle bombardment. Integration of vector backbone sequences or additional unnecessary vector sequences can be avoided by applying minimal constructs containing only the promoter, coding region and terminator.

This "clean DNA transformation" using two minimal gene cassettes, one with the selectable marker gene, one with the gene of interest, was successful in various

crops (Fu et al. 2000; Breitler et al. 2002; Romano et al. 2003; Vidal et al. 2006; Zhao et al. 2007).

The advantage of the co-transformation system is based on the simplicity of the technique, the possibility to use standard vectors and the fact that no additional genes or elements are needed. But the technology is not suitable for all plant species because segregation and recombination occurs only during sexual reproduction of the plants. Therefore, this method is not applicable for cultivated plants propagated by vegetative methods or for plants with extreme long generation times, like some trees.

Recombinase-Induced Elimination

Beside the co-transformation technology, the site-specific recombinase-mediated excision of marker genes is the most used method to get rid of marker genes. The strategy is based on the use of a two-component system comprising a specific enzyme and two short DNA sequences. The enzyme, which is a recombinase, recognizes the specific short DNA target sites and catalyzes the recombination/elimination of the sequence between the target sites. The most common systems used for the production of marker-gene-free transgenic plants are the bacteriophage P1 *Cre*/*loxP* system (Sternberg et al. 1981; Dale and Ow 1991), the *FLP*/*FRT* system from *Saccharomyces cerevisiae* (Cox 1983; Kilby et al. 1995) and the *R*/*RS* system from *Zygosaccharomyces rouxii* (Zhu et al. 1995; Sugita et al. 2000). All systems, reviewed by Ow (2002), Hare and Chua (2002) and Ebinuma and Komamine (2001), require the expression of the enzyme in transgenic plants. This expression can be achieved by crossing two transgenic plants: one allocates the recombinase, the other owns the gene of interest and the marker gene to excise. After segregation the next generation of recombinase- and marker-free plants are obtained (Hoa et al. 2002; Arumugam et al. 2007; Chakraborti et al. 2008). To improve and speed up the system new basic approaches were proposed, including transient expression of the recombinase and the introduction of the recombinase gene into transformed plants in combination with a negative selection marker, inducible promoters or germline-specific promoters.

Feasibility was proven for the expression of *Cre* under the control of chemical induction (Zuo et al. 2001; Sreekala et al. 2005) under the control of heat-shock promoters (Hoff et al. 2001; Zang et al. 2003; Cuellar et. al. 2006; Luo et al. 2008) and for the transient expression of *Cre recombinase* by *PVX-Cre* and *TMV-Cre* recombinant viruses (Kopertekh et al. 2004). Quite recently published results from various authors give hope that, with new modified site-specific recombination vectors, it appears to be possible not only to excise the selectable marker gene but also to get single copy insertion, backbone-free integration or even marker-gene-free and homozygous plants together in one step. Verweire (2007) presented an approach where it is possible to get marker-gene-free plants via genetically programmed auto-excision without any extra handling and in the same time frame, as compared to conventional transformation without marker gene elimination. The

basic idea of the approach is to control the *Cre recombinase* by a germ-line-specific promoter. As a consequence of auto-excision of the marker gene in the male and female gametes, the plants of the next generation are marker-free. The University of Connecticut (Luo et al. 2007) has also recently developed a new technology, called "gene deletor", or also called "GM gene deletor". The system functions through combination and interactions of the bacterial phage *Cre* and the *Saccharomyces cerevisiae FLP* recombinases with the flanking recognition sites *loxP* and *FRT* as it was proven already by Srivastava and Ow (2004). The technology could be used to remove selectable marker genes but also all transgenes from any organs of a transgenic plant when the functions of transgenes are no longer needed or their presence may cause concerns. Results obtained by Mlynarova et al. (2006) and Bai et al. (2008), by using microspore, pollen or seed-specific promoters, also demonstrated the function of these auto-excision vectors. One advantage of all these auto-excision systems is that all extraneous DNA and multi-copy insertions (e.g. flanked by *Lox*/*FRT* sites in direct orientation) is eliminated. Therewith complex transgene loci can be simplified. Similar results were reported by Kondrák et al. (2006), who removed marker genes by using a binary vector carrying only the right border (RB) of T-DNA, the *Zygosaccharomyces rouxii R*/*Rs* recombination system and a *codA-nptII* bi-functional, positive/negative selectable marker gene. In a first step the whole plasmid was inserted as one long T-DNA into the plant genome and, after positive and negative selection, it was shown that by recombinase enzyme activity both the bi-functional marker genes as well as the backbone of the binary vector have been eliminated.

Even though a lot of work has been invested to obtain marker-free plants by site-specific recombination, the practical suitability still leaves a lot to be desired, among others due to the complex and complicated systems, due to the inefficient inducer transport, due to insufficient promoter specificity or due to insufficient Cre expression, or expression at the wrong time or at the wrong place. But, in contrast to many other marker elimination systems, regulatory approval has already been received for such a system. The maize line LY038 with enhanced lysine level, from which the selectable marker was excised by using *Cre*/*Lox*, received approval for food and feed in the United States, Canada and Japan (AGBIOS 2008).

Transposon-Based Elimination

Some 25 years ago, Goldsborough et al. (1993) reported first about marker gene elimination by transposon usage. The most used system is the maize *Ac*/*Ds* transposable element system. The *Ac transposase* is able to reposition marker genes or the gene of interest located between *Ds* elements. When these plants are crossed with other plants, they produce progeny which, as a result of naturally occurring segregation processes, carries either only the target gene with minimal *Ds* sequences at both ends or the marker gene with the *Ac* gene. When the selectable marker gene is flanked with *Ds* elements, the system can also be used for

vegetatively propagated plants, because very often there is excision of the elements without subsequent reintegration (Ebinuma et al. 1993). Furthermore marker-free transgenic plants containing insect-resistant *Cry1b* gene between the Ds elements have been produced (Cotsaftis et al. 2002). Also in sugar beet it has been demonstrated in a research project that the *Ac/Ds* transposon system works in principle (GMO-Safety 2006). However, a few questions still need to be answered before a practical application of the system is possible. This includes questions concerning imprecise excisions, deletions/alterations in the DNA sequence because of many insertion and excision cycles, and low efficiency of the system.

Homologous Recombination

Various methods have been tested to increase the efficiency of gene targeting by homologous DNA recombination in plants. One possibility is to insert DNA at specific points with the help of targeted double-strand breaks in the plant itself. The double-strand breaks are generated by a rare cutting restriction enzyme, *I-SceI*, which can enhance homologous integration frequency at the target site (Puchta et al. 2002). By using cutting sites of the *I-SceI* enzyme in the transgene construct before and after the marker gene, it is possible to induce double-strand breaks on each side of the marker gene following expression of *I-SceI* in the plant. Thereby the marker gene can then be removed. Another approach for the future can be the use of zinc-finger nucleases to target specific DNA sequences for gene modification (Lloyd et al. 2005; Wright et al. 2005). These methods can be powerful tools to modify plants genetically, but today their efficiency in plants is still not high enough for routine applications.

3.3.3 Screenable Marker Genes

Screenable markers encode a protein which is detectable because it produces a visible pigment or because it fluoresces or modifies the phenotype elsewhere under appropriate conditions. Screenable markers include *galactosidase* (*lacZ*; Herrera-Estrella et al. 1983), *β-glucuronidase* (*GusA*; Jefferson 1987), *luciferase* (*luc*; Ow et al. 1986), *green fluorescent protein* (*gfp*; Haseloff and Amos 1995), *red fluorescent protein* (*rfp*; Campbell et al. 2002) and *isopentenyl transferase* (*ipt*; Ebinuma et al. 1997). Screenable marker genes can be used as independent genes or as fusion constructs. They cannot be used for positive selection but they can help to improve transformation efficiency, they can be used as visual marker of transformation and they allow the enrichment of transformed tissue and therefore speed up the whole transformation process. Screenable markers are usable within the T-DNA, outside T-DNA on the backbone or as part of the co-transformation vector. In the meanwhile the integration of screenable marker genes outside the T-DNA borders is an often used strategy to identify vector backbone sequences in order to limit the

production of events with superfluous DNA. In a similar manner the usage of *ipt* can serve as a visual screenable marker of backbone integration within the plant genome (Bukovinszki et al. 2006).

3.3.4 Negative Selection Marker

Comparable with screenable marker genes, so-called negative selection markers are used to optimize transformation efficiency. Thereby negative selection systems kill the transformed cells. This allows new strategies to limit the production of vector-backbone-containing plants by flanking the T-DNA with negative selection marker genes. The most used negative selection marker gene is the *codA* gene from *E. coli* encoding *cytosine deaminase*. The usefulness of *codA* as a conditional toxic gene was explored in different *Agrobacterium*-mediated transformation protocols (Koprek et al. 1995; Schlaman 1997). Plant cells which are transgenic for *codA* show sensitivity to 5-fluorocytosine (5-FC) at different developmental stages. The negative selection marker confers a lethal phenotype on the transformant and is therefore often part of co-transformation systems. Co-transformation with *codA* is a viable method for the production of easily distinguished, selectable marker gene-free transgenic plants (Park et al. 2004). As described by Verweire et al. (2007) the *cytosine deaminase* gene can be used as a counter-selectable marker. In this system *CodA* is a component of a germline-specific auto-excision vector, in which *codA* is present in tandem with the recombinase and the positive selection marker between *lox* sites. After auto-excision of the whole *Lox*-cassette, marker-free regenerates can be identified by growing on medium containing 5-fluorocytosine.

Often mentioned in literature concerning marker genes are the *tms2* and the *dao1* gene as negative selection marker genes (Upadhyaya et al. 2000; Erikson et al. 2004). The marker gene, *dao1*, encoding *D-amino acid oxidase* (*DAAO*) can be used for either positive or negative selection, depending on the substrate. 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 by *DAAO* into the toxic products respectively. Hence, both positive and negative selection is possible with the same marker gene.

3.3.5 Marker-Free Transformation Without Usage of Any Marker Gene

De Vetten et al. (2003) reported first about transformation of potatoes without the usage of any selectable marker. Transgenic plants were analyzed and identified exclusively by PCR. In another study marker-free tobacco transformants with efficiency up to 15% of the regenerants were produced by agroinfiltration

(Jia et al. 2007). Genetic transformation of apples was also achieved without using selectable marker genes (Malnoy et al. 2007).

In summary there is proof that marker-free plants can be produced by a single-step transformation without marker genes. Whether the methods are applicable to other crops has yet to be shown.

3.4 Conclusions and Perspective

There have been many excellent reviews concerning selectable marker genes and marker gene elimination including biosafety aspects in recent years (Miki and Hugh 2004; Darbani et al. 2007; Ramessar et al. 2007; Sundar et al. 2008). This report is only an extract of the most important usable techniques, the marker systems which are available, but as a result it manifests how difficult it is for the user to decide which system will be the right one. The criteria for choosing the marker gene system including the marker gene elimination systems have changed within recent years. There are new requirements from the users, from the authorities and from the consumers concerning technical, regulatory and biosafety aspects. To combine all this aspects within one marker system is one of most difficult problems. A variety of selection systems seem to be essential for different plant species because no single marker gene works well in all situations. Many marker genes exist and many new marker genes have been tested in recent years, but only a few of them are widely used and still fewer have received approval from the authorities for food and feed. To this day that is the case mainly for the genes *nptII*, *pat*, *Cp4epsps* and *gus*. In an overall picture there is a wide range of marker genes in theory but in reality only some of them are used and are accepted.

Regulatory requirements for selectable marker genes in the European Union was one of the main reasons to think about the removal of marker genes from the plants once the genes have done their job. Several strategies have been developed, including co-transformation systems, site-specific recombination systems and transposon-based elimination systems, often in combination with screenable marker or negative selection marker systems. Co-transformation is technically simple but needs high transformation efficiency and the technique is usually not suitable for use in vegetatively propagated crops. Many site-specific excision systems have been proved and show promising developments for the future. But very often the problem arises that complicated systems for the marker gene elimination are needed in order to achieve the goal to simplify the GMO. Whether all these new systems can be applied without safety concern in the future is to be investigated further. The development of additional new marker technologies, including marker gene elimination technologies, will continue to be important in the production of transgenic plants.

In the end, everyone has to keep an eye on the insight that the replacement of old technologies (e.g. the use of antibiotic-resistant marker genes, herbicide-resistant marker genes) by new systems only makes sense if these new technologies can at

least ensure the same degree of scientific knowledge and safety like the old technologies.

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