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RISK OF BIOTECHNOLOGY ON HUMAN HEALTH ASSOCIATED WITH THE USE OF SELECTABLE MARKERS AND RECENT METHODS USED TO REDUCE THE RISKS

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Abstract

One aspect of biotechnology is production of genetically modified organism by using genetic engineering technique by means of cutting and joining of the gene which carries specific function. To understand the success of effective transform of target gene to the organism needs marker genes which have the potential to distinguishes transformed cell from non transformed cell because of the presence of the marker gene is an indicator for the presence of the gene of interest in both type of marker gene such as selectable marker genes and reporter genes. but the use of selectable marker have their negative effect on ecosystem for example Selectable marker genes express protein products that confer antibiotic- or herbicide resistance traits, and typically reside in the end product of GM plants. The presence of these genes in GM plants, and subsequently in food, feed and the environment, are of concern and subject to special government regulation in many countries due to release of antibiotic resistance microbes, metabolic burden for the host, weedness and other non targeted effects in the human and animal health, and the environment so the main objective of this review paper are to identify the risk that are associate with selectable marker gene that affects our ecosystem in negative way and what are the possibility methodology that are used to reduce the risk of selectable marker so this review paper are shine light about the information regarding about selectable marker for the reader

Keywords: Genetic engineering, transgenic crop, selectable marker and risk management

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INTRODUCTION

Biotechnology specifically plant biotechnology is based on the insertion (delivery), integration and expression of single genes into plant cells which is a tot potency mean generate to large plant from single transformed cell and finally generate transformed plants but to achieve these smart technology needs systems to select the transformed cells, tissues or organisms from the non-transformed ones the transformation process are done and selectable marker genes are vital to the plant because of Selectable marker genes have been pivotal to the development of plant transformation technologies because the marker genes allow scientists to identify or isolate the cells that are expressing the cloned DNA and to monitor and select for the transformed progeny remain expressed within the transgenic plant, If the selectable markers are important for both scientific and economic reasons that the selectable marker gene does not have broad pleiotropic effects. Consequently, the use of biological processes that are foreign to plants and that have a high level of enzyme specificity was initially adopted (Pérez Massot etal., 2013, Miki & Mchugh, 2004).

Marker genes are used to help find the transformed cells, and ultimately produce GM plants, that contain the gene(s) of interest, conferring a desired trait or traits in the plant. For plant transformation, marker genes are often combined in the same piece of DNA as the gene of interest, such that they are transferred together. Marker genes may also be used on separate pieces of DNA, as often both types of introduced DNA are taken up and integrated into the genome in the same cells during the transformation process. Thus, the presence of the marker gene is an indirect indicator for the presence of the gene of interest. There are two types of marker genes: selectable marker genes which confer resistance to a selective agent (such as an antibiotic or herbicide); and reporter genes which produce products that can be detected visually or biochemical assays. When assessing potential risks to the health and safety of people and the environment that may be posed when dealing with GM plants, the gene technology regulator considers any introduced genetic material including any marker genes(Lee & Gelvin, 2008).

Having those crucial advantage of selectable marker for plant transformation there is the questions that relate to the biosafety rule of how we use biotechnology tool selectable marker genes are the same as those that relate to other genes associated with plants, humans and our environments so do they code for toxic products or allergens, Will they create unwanted changes in the composition of the plant, Will they compromise the use of therapeutic drugs? Will there be

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horizontal gene transfer to relevant organisms and pathogens, Can gene transfer to other plants create new weeds or compromise the value of non-target crops, definitely no single answer and every gene has to be assessed individually so those question needs answer for sustainable biotechnology product (Brian Miki., 2004).

To reduce the risk that scientist afraid caused by selectable marker gene are developed variety of strategies to eliminate marker genes after the selection phase of plant production to create marker-free transgenic plants or to restrict pollen flow from transgenic plants. Once again the need for the adoption of these strategies depends on the gene of interest that is being co-transformed with the marker gene as well as the characteristics of the particular marker gene (Brian Miki., 2004).

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

2. Risks of selectable markers

It is extremely important to evaluate the potential risks, as perceived by the consumer, associated with the marker genes in the transgenic crops prior to their field release. Recent evidence of horizontal flow of *bar* gene (Messeguer *et al,* 2004) by pollen dispersal from transgenic to cultivated rice is indeed a great concern, and strongly recommends removal of this gene from transgenic crops prior to their field release. Similar experimental designs using antibiotic resistance genes as tracer marker must be conducted to assess the escape of foreign genes through pollen dispersal from engineered plants to other cultivars of the same species or to weed relatives, before eliminating them from transgenic crops.

Although approximately 50 marker genes used for transgenic plant research or crop development have been assessed for efficiency, biosafety, scientific applications, and commercialization, only three selectable marker genes were used in more than 90% of the scientific reports (Miki and McHugh, 2004). These three genes are resistance to the antibiotics kanamycin and hygromycin and to the herbicide phosphinothricin.

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2.1 Risks of antibiotic Resistance gene in human and animal health

There are concerns that these genes might unexpectedly recombine with pathogenic bacteria in the environment or with naturally occurring bacteria in the gastrointestinal tract of mammals who consume genetically modified food, contributing to the growing public health risk associated with antibiotic resistance for infections that cannot be treated with traditional antibiotics. The presence of antibiotic resistance genes in foods might produce harmful effects. First, consumption of these genetically modified foods might reduce the effectiveness of antibiotics to fight bacterial diseases; antibiotic resistance genes produce enzymes that degrade antibiotics. Second, antibiotic resistance genes might be transferred to human or animal pathogens, making them resistant to antibiotics (Miki, B. and McHugh, S., 2004).

To date, given that there are no reports of DNA itself being toxic or allergenic, and the very long human history of DNA consumption from a wide variety of sources, it is concluded that such consumption poses no significant risk to human health, and that additional ingestion of recombinant DNA, which is chemically indistinguishable from non modified DNA, has no effect (Craig *et al*., 2008). The extent of the clinical antibiotic resistance problem depends on the presence of the antibiotic and resistance gene, the spread of resistant bacteria and the cell-to-cell spread of the resistance gene. Given the presence of both the antibiotic and a resistance gene, drug resistant bacteria will be selected and propagated (Levy, 1997).

The prevalence of antibiotic resistant bacteria and the number of antibiotics to which they are resistant are increasing because of the use of antibiotics. As a consequence the morbidity and mortality of previously treatable bacterial diseases is increasing. Essential lifesaving antibiotics are becoming less effective and there are fewer alternatives available for treatment (JETACAR, 1999). During the last decade the development of new antibiotics has become more difficult, expensive and uncommon. This contrasts with previous decades following the discovery of penicillin in 1940. Emergence of resistance and control of antibiotic resistant pathogens are now major challenges in both hospitals and the community. Multi-drug resistant pathogens such as penicillin-resistant pneumococci, vancomycin-resistant enterococci, and methicillin-resistant staphylococci have emerged as well as a variety of multi-resistant Gram-negative organisms (e.g. *Pseudomonas aeruginosa, Acinetobacter baumanii*) (Levy, 1998). Evaluation of the human health impact of antibiotic resistance depends on the importance of the antibiotic or antibiotic class in medicine and potential human exposure (direct and indirect) to resistant bacteria, in particular

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those that are human pathogens. However bacteria that are not usually pathogenic may cause infections in some people (e.g. those who are hospitalized or immune-compromised).

2.1.1 Consideration of potential risks from Ampicillin resistance gene

Ampicillin is an antibiotic that is used to treat a variety of bacterial infections in humans and animals. A genetically engineered Bt corn variety from Novartis includes an ampicillin resistance gene (Cannon, 1996). A number of European countries, including Britain, have refused to allow the Novartis Bt corn to be grown because of concern that the ampicillin resistance gene might be transferred from Bt corn to bacteria, making ampicillin far less effective antibiotic against bacterial infections (Bakshi, 2003). A report was released on genetic engineering that recommended the termination of the use of antibiotic resistance marker genes in engineered food products (Royal Society, 1998). According to one prediction, alternative types of marker genes would be developed in approximately 5 year and no new transgenic crops using antibiotic resistance marker genes would appear on the market (Henney, 2000). However, the contribution of the antibiotic resistance markers in genetically modified organisms to antibiotic resistance in bacteria in the gastrointestinal (GI) tract has not been studied; it is expected to be very small (Royal Society, 1998) for several reasons—efficient destruction of the resistance gene in the human gastrointestinal tract and the very low intrinsic rate of plant-microbe gene transfer. In addition, it should be noted that resistance genes occur widely in nature and the antibiotics involved are not widely prescribed by physicians (Society of Toxicology, 2002).

Bacteria have to compete in nature with other microorganisms for their survival, including other bacteria and fungi. They have developed very sophisticated mechanisms to produce antibiotics to eliminate competitors and assure their own survival. Simultaneously with their ability to produce such compounds, bacteria also need to have a defense against the compounds they produce to eliminate their competitors in the form of resistance genes. Over time, target bacteria counter the effects of antibiotics produced by other bacteria through resistance mechanisms (Ramessar *et al*, 2007). In addition to developing their own resistance mechanisms, bacteria frequently acquire antibiotic resistance genes that are already present in the bacterial pool surrounding them (EFB,2001). Bacteria have well-developed mechanisms to accomplish this (Bennett *et al*, 2004). The presence of an antibiotic confers an advantage to a resistant bacterium and as a consequence, the development of resistance and spread increases (EFB, 2001).Bennett *et al*, (2004) questioned whether the transfer of a number of commonly used antibiotic resistance genes in plant molecular

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biology, if it were to take place in bacteria, would pose a threat to public health. In addition, recent advances in genetic engineering do not employ the use of such selection markers (Koprek *et al,* 2000) and their use is likely to diminish.

2.1.2 Consideration of potential risks from nptll and hph gene

Although antibiotic resistance genes play no role in the desired phenotypes of the GM plants in the field, they usually remain in the plant genomes. In this context, they potentially pose two risks: (I) their protein products may directly or indirectly have a negative effect on people and/or animals that consume the plant material, and (II) plants possessing these genes may cause environmental harm *e.g.* have increased weedness leading to harm to the environment (Yau and Stewart,2013).

However, there is no evidence that the NPTII and HPH proteins are toxic or allergenic. Bioinformatics analyses have failed to find homology to any known toxins or allergens (EFSA 2009; Lu *et al,* 2007). Further, neither protein is known to be involved in the production of a toxic or allergenic compound. Toxicity experiments with animals (mainly mice and rats), often involving the feeding of exaggerated doses of these proteins, have failed to establish any deleterious effects of either NPTII (Fuchs *et al,* 1993) or HPH (Lu *et al,* 2007; Zhou *et al,* 2009). GM foods containing the NPTII or HPH proteins have been approved for sale in Australia (FSANZ 2004; FSANZ 2010). Dietary intake of the protein products of antibiotic selection genes could conceivably reduce the therapeutic efficacy of antibiotics taken orally. This is especially important in regards to the *riptIf* gene, as Neomycin has human and animal therapeutic importance (EFSA 2009). Hygromycin is not used with humans, but may be used in animals such as pigs and poultry. However, like most proteins, NPTII and HPH are rapidly inactivated in simulated mammalian gastric juice (FSANZ 2004, Fuchs 1993). Therefore, under normal digestion, it would be expected that any antibiotic resistance protein would be degraded before it could be inactivated the corresponding antibiotic, negating any possible interference with oral administration of the antibiotic (EFSA 2009). It has been suggested that the transfer of antibiotic resistance genes from GM plant material to intestinal bacteria or other microorganisms could lead to antibiotic resistant populations of these organisms (Flavell *et al,* 1992). However, evidence strongly suggests that such 'horizontal gene transfer' from plants to bacteria is extremely rare (Keese 2008). Most genetic material (DNA) is likely degraded in the stomach and intestines. The feeding of GM soybeans to humans has shown that although a small proportion of DNA may

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survive the stomach and small intestine, what remains is degraded in the large intestine (Netherwood *et al,* 2004). Similarly, experiments involving the feeding of GM plant material to animals such as chickens have suggested that most DNA fails to survive the digestion in the stomach (Chambers *et al,* 2002). In addition, these genes were originally isolated from bacteria which are widespread in the environment, including in the gastro-intestinal tract of people and animals. Transfer of these genes between bacteria is far more likely than transfer from GM plants to bacteria. No feasible pathway links a plant possessing either the *nptlI* or *hph* gene and environmental damage. Given that NPTII and HPH are naturally widespread in the environment, organisms in the environment are already exposed to these proteins. A GM plant with such a gene would only have a selective advantage and potentially become a weed in the presence of the antibiotic, and this is unlikely to occur in a natural situation (Nap *et al,* 1992). The use of the *nptII* and *hph* genes as selectable markers in GM plants and derived food or feed do not pose a risk to human or animal health or to the environment (EFSA,2009).

2.2 The potential effect of marker genes on the environment

2.2.1 Micro organism evolution

The presence of selectable marker genes in genetically modified (GM) crops has raised public concern that they will be transferred to other organisms. A potential risk is the development of multiple drug resistance bacteria, which has been analyzed in some detail and represents something of a paradigm for the roles of gene flow and DNA rearrangement in bacterial evolution. Transfer of DNA between microorganisms in a natural environment could occur by conjugation, transformation of released genetic material or even by bacteriophage-mediated transduction (Ow D.w., 2001). This issue has become more topical with the inclusion of antibiotic resistance genes in some transgenic plants intended for release into the food chain and this has fueled the debate about the existence of mechanisms by which DNA introduced into a transgenic plant might be acquired by bacteria. Antibiotic resistance genes have been introduced into transgenic plants as selection markers for their primary transformation. A significant factor is that antibiotic resistance is already widespread in bacteria and rare gene transfer from a GM food source is unlikely to be of practical consequence (Eastham and Sweet, 2002). Genes in this category include *bla, aad* and *npt*II. All these genes confer resistance to those antibiotics that have a greater clinical use than do kanamycin and neomycin. The most common reason for the presence of these genes in transgenic plants is the trait gene which was first engineered into a

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bacterial vector containing these antibiotic-resistance genes using cloning of *E. coli*. The complete vector was then used to deliver the transgene by biolistic or protoplast transformation. In addition, bacterial antibiotic resistance genes have been included in the T-DNA introduced into transgenic plants by the *Agro bacterium tumefaciens* binary vector system. These genes are not directly selectable in plants and there is no good reason for their presence in transgenic material destined for use as food (Chevre Am, 2000).

2.2.2. Weedness

The presence of herbicide resistance markers may affect non-target species, out-cross to produce uncontrollable weeds or have adverse effect on human health (Wolfenbarger and phifer, 2000). The potential for GM crops to become weeds or to transform its transgene to wild relatives is often considered as a potential risk in the commercialization of GM crops and has been extensively reviewed by Eastham and Sweet, (2002). Gene flow to weed species depends on whether hybridization and introgression are possible. Most agriculture crops can hybridize to wild relatives (Eastham and Sweet, 2002). Gene flow between wild and domesticated populations is a relevant process in shaping the structure of genetic diversity and in the evolution of crops and their wild relatives. Hybridization between introduced and native species can cause problems in conservation (Nap JP, 1992) and has become an important topic in the debate about the release of transgenic crops (Chevre Am, 2000). An extensive amount of literature exists concerning pollen movement in different crops (Raybould A.F. and Gray A., 1993). The mobility of pollen is determined by wind, insects and other agents, as well as by pollen longevity. Pollination distances of many kilometers have been recorded in some species, (Decosa B, 2001) which has caused the sexual compatibility of crops with weeds to be reviewed. In the United Kingdom, the probability of hybridization with weeds is considered minimal for wheat, low for oilseed rape and high for sugar beet (Ell strand NC, 2001). Oilseed rape can hybridize with heavy mustard and with wild radish (Ellstrand, 2001). The consequence of gene flow from a GM crop depends on the nature of the novel gene and the biology and ecology of the recipient weed species. The potential spread of herbicide resistance to wild relatives and non-transgenic crops has raised separate concerns. The transfer of herbicide-resistance gene to weed species could potentially give weed species a selective advantage. Sheffler and Dale (reviewed in Ramachandran *et al*, 1997) have proved this by using an insect-resistant transgenic oilseed rape in greenhouse experiments and field-plot

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studies. In another study Stewart *et al,* (2003) have shown that the increased fitness in oilseed rape varieties expressing.

3. Recent methods to reduce these risks

Once the transgenes have been transferred into the plant genome and the transformed shoots successfully generated from the explants, the selectable marker gene becomes unnecessary and undesirable (Zou, *et al,* 2013). Moreover, the presence of marker genes raises public concerns (such as food safety and the ecological risks of the nptII or bar genes and their production) regarding the field release of transgenic plants (Ramessar *et al,* 2007).Several methods have been developed to overcome these problems (Tuteja *et al,* 2012). For example, co transformation or transposon-mediated transgene repositioning were used for the removal of marker genes via segregation in crops (Daniell 2002), but this is not feasible in vegetatively propagated plants that possess a very long juvenile period. Chloroplast transformation, which has been applied to crop improvement (Daniell *et al,* 2005), is one of the more promising containment technologies, especially cytoplasmic sterility engineered via the chloroplast genome (Ruiz and Daniell 2005), which could provide a new tool for transgene containment in plants. Molecular containment strategies based on site- recombination offer methods to directly generate marker-free plants (Daniell 2002; Hare and Chua 2002). An advanced containment method is recombinase-mediated auto-excision, which was widely applied to avoid pollen-mediated transmission of transgenes and produce marker-free genetically modified (GM) plants (Mlyna´rova´ *et al,* 2006; Verweire *et al,* 2007).

3.1 Safer marker genes

3.1.1 Green fluorescent protein gene

The gene (udiA) from jellyfish (*Aquorea victoria*) possesses unique qualities that make it an ideal reporter marker gene for gene-expression analysis (Elliott,A.R., 1999). It emits fluorescence upon excitation with ultraviolet or blue light (Davis, S.J and Vierstra, R.D., 1999). No substrates or cofactors are required for GFP expression, and observations can be made repeatedly at any time on living cells without cell disruption or destruction. Also, modified forms of the GFP gene have been shown to be strongly expressed in plants (Kamate, 2000). The transformation events, formation of calli followed by the emergence of fluorescing shoots can all be observed successively in each step of transformation and during different phases of development by fluorescence microscopy. GFP-expressing cells and tissues can easily be distinguished from

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untransformed ones, without destroying the studied material (EL-shemy, H., 2004). The ratio between fluorescing and no fluorescing cells, shoots and various organs as a measure of transformation efficiency has been successfully used to improve the various stages and procedures in transformation protocols. It may help to identify and therefore to reduce negative events linked with plant transformation (e.g. gene silencing) by redesigned *gfp* to reduce H-NS-mediated transcription silencing and simultaneously improve translation *in vivo* without altering the amino acid sequence of the GFP protein because H-NS, which preferentially targets and silences A+T-rich genes, binds the ubiquitous reporter gene *gfp (60%AT)* and dramatically reduces local transcription, and also it assists for the successful recovery of transgenic plant tissues which stably express the gene of interest (Hraska M., *et al*, 2005). GFP selection system seems to be promising for tissue culture/transformation systems that are inefficient, for recalcitrant genotypes, and for plant species for which no system exists.

3.1.2 The gus gene (B-glucuronidase)

In the traditional selection system using antibiotics or herbicides, the transgenic cells convert the selective agent to a detoxified compound, but that may still exert a negative influence on plant cells. Further, the release of toxic metabolites by dying adjacent cells may also inhibit the growth of transformed cells.

In contrast, a new set of markers known as positive selection markers are being developed, which can overcome some of the limitations encountered by the traditional selection system. One such selection system has been established which uses gus gene from *E. coli* as a selectable gene. The gus gene codes for the β-glucuronidase enzyme (GUS; EC 3.2.1.31) and was isolated from Escherichia coli. This gene is widely used as a reporter gene in transgenic plants. In this system, the selective agent is a glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide), an inactive form of the plant hormone cytokinin. This glucuronide present in the selection medium can be hydrolysed by the GUS enzyme produced in the transformed cells, releasing active cytokinin (benzyladenine) in the medium. This cytokinin will be a stimulator for transformed cell regeneration while the non-transformed cell development is arrested. The selective agent (benzyladenine N-3-glucuronide) does not have any effect on the non-transformed cells because the cytokinin is in its inactive form. There are only few reports concerning the successful use of this system in the effective recovery of transgenic plants (Joersbo and Okkels, 1996 and Okkels et al, 1997).

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A widely used reporter gene in plants is the *udiA* or gusA gene that encodes the enzyme ß-glucuronidase (GUS).This enzyme can cleave the chromogenic substrate X-gluc (5-bromo-4-chloro-3-indolyI ß-D-glucuronic acid), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity. Moreover, Plant cells themselves do not contain any GUS activity, so the production of a blue color when stained with X-gluc in particular cells indicates the activity of the promoter that drives the transcription of the gusA gene in that particular cell (Pawar *et al*.,2010). The GUS assay is easy to perform, sensitive, relatively inexpensive, highly reliable, and safe, requires no specialized equipment, and is highly visual.

3.1.3 Rol genes

It is a unique transformation system that uses morphological changes caused by rhizogene (rol gene) which control the endogenous levels of plant hormones and the cell responses to plant growth regulators as the selection marker(Tuteja et al,2012). The *rol* genes derived from *A. rhizogenes* are responsible for the proliferation of hairy roots, which spontaneously regenerate into transgenic plants with abnormal phenotype such as wrinkled leaves, shortened internodes and reduced apical dominance. Such plants are easily identified, thus necessitating eviction of selectable markers (Edinuma, H., *et al,* 2000). The occurrence of abnormal ipt and rol phenotypes allowed the visual selection of transformants (Zelasco et al. 2007).

3.1.4 Betaine aldehyde dehydrogenase gene

Recently, Danielle *et al,* (2005) engineered chloroplast genome without the use of antibiotic selection. The betaine aldehyde dehydrogenase (*BADH*) gene from spinach was used as the selectable marker. This enzyme is present only in the chloroplast of a few plant species (members of Chenopodiaceae, Poaceae, etc.). Rapid regeneration of chloroplast transgenic plants was obtained under betaine aldehyde (BA). The selection process involves the conversion of the toxic BA by the chloroplast BADH enzyme to a nontoxic glycine betaine, which also serves as an osmoprotectant for enhancing drought and salt stress tolerance in plants (Depicker *et al,* 1985). Transgenic plants were morphologically indistinguishable from untransformed plants and the induced trait was stably inherited in the subsequent generations. Chloroplast transformation efficiency was 25-fold higher in BA selection than spectinomycin (Rathinasabapath *et al,* 1994), which is widely used for chloroplast transformation.

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3.1.5. No selectable marker or reporter gene (PCR ANALYSIS)

Direct introduction of DNA into a cell with the potential to develop into a whole organism is the best method of genetic modification for the release of organisms into the environment. Micro-injection has been applied to a wide range of higher organisms in particular animals, including fish, and some plant species, and avoids the use of markers (Harding, 1999). Direct DNA transfer via micro-injection of DNA into cells of microspore-derived embryoids may improve the efficiency of gene transfer to acceptable values. Cell finder system is a technique in which a computer-controlled microscope allows easy positioning and relocation of cultured cells and protoplasts in combination with improved gene delivery techniques and may also develop into a system that allows the identification and isolation of GM plants only carrying the desired gene (Metz and Nap, 1997)**.** Apple transformation technique was developed without use of any selectable marker gene by using RT-PCR analysis selection system (Malnoy, *et al,* 2010). Ballester, *et al,*(2010) have reported the direct production of selectable marker-free transgenic orange plants under non-selective conditions, using a ‘‘clean’’ binary vector carrying only the transgene of interest, and through the recovery of transformants by polymerase chain reaction (PCR) analysis of all regenerated shoots.

Currently it is practical to avoid the use of marker genes. Foreign DNA can be readily identified by PCR based assay systems and methods are available that require direct isolation of DNA, so large numbers of cells can be screened rapidly. However transformation efficiency is still very low. A single GM plant is also rarely enough; several GM plants are usually sought for each construct due to variation in transgene expression levels and stability (Langridge, 1997).

3.2 Elimination of the selectable marker

Except for herbicide resistance, selectable marker genes are not aimed at any change or improvement of the agronomic or other characteristics of the organism involved. Elimination of the gene is therefore an alternative strategy. These selectable markers are mostly based on genes conferring antibiotic or herbicide resistance. The presence of the marker gene will lead to unpredictable environmental hazards, so on the basis of economic incentives and safety concerns, several methods, such as site-specific recombination, homologous recombination and co-transformation, have been developed to eliminate these genes from the genome after successful transformation has been achieved. Gene transfer without the incorporation of an antibiotic-resistance marker or herbicide-resistance marker in the host genome should convince the public

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with regard to the field release of transgenic organisms (Natarajan and Turna, 2007).there is another so many elimination methods which is used differently as required based on the type of plant and transformation system. These are some examples of GM crop with elimination system.

GM plants	Elimination system	Reference
potato	Inactivation of SMGs**Gs**	De ventten et al,2003
Tobacco	>>	Schmulling&Rohrig,1995
Tobacco & Rice	Co transformation	Komari et al,1996
Tobacco	>>	RamananRao& Veluthambi, 2010
Tobacco	Site specific recombination(Cre/loxP)	Gidoni et al,2008
Citrus plant	>>	Zou et al,2013 Ballester et al,2007 YeuYau,2013
Maize	>>	Ow,D.W.,2007 Li et al,2010
Rice	>>	Chen et al,2004 Hu et al,2013
Tomato	>>	Zhang et al. 2006 Ma et al. (2009)
maize	Transposition mediated(Ac/Ds maize)	Metz & Nap,1997
Rice	>>	Li and Charng,2011, and Tai et al,2011
rice	Chemical inducible method	Lin et al. **2008**
potato	Heat inducible method	Cuellar et al. 2006
Tobacco	>>	Wang et al. 2005

3.2.1. Simple microbial recombinase based systems

Microbial recombinase method are one of the techniques that are used for marker gene removal by using microbial recombinase enzymes in plants to excise marker transgenes that were flanked by microbial recombination sequences, both the recombinase gene and marker gene must subsequently be separated from the desired trait gene by using recombinase enzyme, for this purpose the most utilized microbial recombinase enzyme are **loxP** and FLP because both of them have the potential to remove selectable marker (E.C. Dale *et al*, 199, P. Hare and N.H. Chua, 2002)

3.2.2. Transposable element-based systems

Plant transposons elements are used for the removal of marker genes, for example one resent study shows the maize Ac transposable element was engineered to contain the *ipt* gene, conferring

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a selectable extreme shooty phenotype. The Ac element encodes its own transposase and so its excision conveniently removes this gene along with the *ipt* marker gene. However, transposon based systems of marker gene removal suffer from a number of disadvantages. Their efficiency is low, partly due to the tendency of transposable elements to reinsert elsewhere in the genome. Excision of transposons is frequently imprecise, and repeated cycles of insertion and excision may lead to the generation of mutations at numerous unknown loci. The continued presence of heterologous transposons May be also lead to genomic instability in transgenic plants. For these reasons, transposon-based systems seem to be currently less favored as a means of the removal of marker genes(J. Yoder and Goldsbrough, 1994, H. Ebinuma, *et al* , 1997)

3.2.3. The CLX chemically inducible system

This selectable marker removable methodology are highly sophisticated approach to nuclear marker gene removal by using Cre-*lox* recombination system has been engineered to be chemically inducible. Antibiotic selection using the CLX vector system for plant transformation and marker gene removal is based on an *nptII* gene and driven by a constitutive promoter. This *nptII* gene is positioned adjacent to a *Cre*-recombinase gene driven by the hybrid, chemically inducible OLexA-46 promoter, and a hybrid XVE gene, encoding the binding protein necessary for the induction of *Cre* gene transcription(J.R. Zuo *et al*., 2000,J.R. Zuo *et al* ., 2001)

3.2.4. Inactivation of the selectable marker gene

Limiting the expression of the selectable marker gene to the stages at which selection for transformation is applied will result in GM plants in which the transgene is present but not the transgene encoded protein. It has been shown that the wound-inducible promoter AoPR1 isolated from asparagus, when fused to the kanamycin resistance gene, allows selection during transformation but results in very low levels of the transgene product in the mature plant (Metz and Nap, 1997).

Gene silencing may possibly be developed into a method to obtain plants without selectable marker gene activity (Metz and Nap, 1997). De Vetten *et al*, (2003) developed a silencing construct (pKGBA50mf-IR 1.1) and transformed in to potato (karnico) via highly virulent LBA4404 or AGL Agrobacterium-mediated transformation without the use of selection marker gene. They have developed a PCR based detection method. Schmulling and Rohrig, (1995) demonstrated that in GM tobacco hybrids that single genes could be selectively inactivated on T-DNAs harbouring several genes. They concluded that it is likely that several factors determine the

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probability of interaction between different loci and therefore influence the differences in the kinetics of inactivation and restoration of gene expression (e.g. accessibility of the loci for pairing and relative positions of the inserts in the genome).

3.2.5. Co transformation

The co transformation systems appear to be the simplest strategies to eliminate marker genes and have been widely used in direct transformation methods. In these systems the marker gene and desired gene are introduced into the plant genome on separate vectors. In this method selectable marker gene can be eliminated from the plant genome at the time of segregation and recombination that occurs during sexual reproduction by selecting on the transgene of interest and not the selectable marker gene in progeny (Tuteja *et al,* 2012). Another advantage of co transformation systems is that the construction of the separate molecules for the marker gene and desired gene is less tedious than creation of linked DNA fragments.

Komari *et al.,* (1996) obtained GM tobacco and rice plants free from selectable markers by a relatively simple procedure consisting of *Agro bacterium*-mediated co transformation and segregation of the progeny. Since the vector system functioned efficiently both in a dicotyledonous, tobacco, and a monocotyledon, rice, this system is potentially useful for a wide range of plant species. Ramana Rao and Veluthambi, (2010) reported efficient strategies to employ marker elimination and achieved marker-free transgenic tobacco in the first generation itself. The feasibility of co transformation is disputed but it has been suggested that the method would be suitable for transformations using *Agro bacterium* and cloned gene transfer strategies. Frequencies of co transformation are low and not potentially useful for species that are difficult to transform (ACNFP, 1994). Biolistics with independent DNA molecules may also result in unlinked co transformation (Metz and Nap, 1997).

3.2.6. Site-specific recombination

Site-specific recombination systems have advanced in diversity and applications in recent years. Applications include selectable marker gene removal. Site-specific recombination systems are common in prokaryotes and lower eukaryotes such as yeast and serve various biological functions (Grindley *et al*, 2006).A highly efficient Cre-mediated deletion system, offering a good alternative for producing marker-free transgenic plants that will relieve public concerns regarding GMOs, was first developed in citrus. This Cre/loxP mediated excision was highly efficient and precise in citrus and provides a reliable strategy for auto-deletion of selectable marker genes from

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transgenic citrus to produce marker-free transgenic plants (Zhou, *et al,* 2013).Site-specific recombination is the enzyme-mediated cleavage and ligation of two defined deoxy-nucleotide sequences, because once transformants with the desired gene are obtained the selectable marker gene is no longer necessary (YeuYau, 2013).This recombinase protein catalyzes recombination of DNA between two recognition sites. The outcome of the recombination can be site-specific excision, integration, inversion or translocation depending on the position and the relative orientation of the two recognition sites on the DNA molecules (either linear or circular form), and the type of reaction is dependent on enzyme type. Cre-*lox* site-specific recombination system was the first to be used for SMG excision in tobacco (Dale and Ow, 1991). Since then, many labs have successfully used Cre/*lox* or other later-identified site-specific recombination systems for SMG removal in plants (Gidoni *et al*, 2008).

Initial studies using site-specific recombination systems to remove selectable marker genes in plants used either a hybridization strategy, or a re-transformation strategy. For the hybridization strategy, the target plant is produced with a GOI and the SMG flanked by recognition sites. The recombinase-expressing transgenic plant is hybridized with the target plant so that hybrid F1 plants will have selectable marker genes excised. F1 plants containing both transgenes are used to screen for deletion events (selectable marker removal). Transgenic plants with recombination events are then backcrossed to wild type for obtaining offspring with germ line transmission of the final product of excision and absence of the recombinase gene. For the re-transformation strategy, after target line plants are produced, they are re-transformed with a recombinase-expressing cassette, for which a different selectable marker gene is needed. The retransformed lines are screened for the presence of the recombinase-expressing gene. Gleave *et al,* (1999) have developed a plant transformation vector incorporating the Cre/lox site-specific recombination system to facilitate the elimination of marker genes from GM plants and the cytosine deaminase gene (codA). Strategic placement of the recognition sites into the plant genome has permitted the deletion, inversion, integration and translocation of host and introduced DNA fragments. Recombinase-based strategies afford precise and predictable engineering of the plant genome (Ow and Medberry, 1995). It can be applied to a wide range of plants but is not feasible in vegetative propagated crops with low fertility and seed selection might scramble elite genomes in clonally propagated plants (Harding, 1999). Zubko *et al,* (2000) have developed a system to remove selectable marker genes from tobacco based on intra chromosomal homologous

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recombination between two homologous sequences using a vector containing nptII and attachment regions from bacteriophage. It does not require removal of recombinase by genetic segregation and is therefore less time consuming.

3.2.7. Transposition-mediated repositioning

Transposition-mediated repositioning requires the introduction of the selectable marker gene and the desired gene on a vector containing a transposase function. The selectable marker gene moves away from the gene or vice versa to become unlinked from the gene e.g. transformation system using the *Ac*/*Ds* maize transposable element (Metz and Nap, 1997). Large numbers of progeny plants are needed for selection and the construction of the vector has to allow transposition at a sufficient distance from the selectable marker gene to ensure that the selectable marker gene and desired gene are separated during recombination (ACNFP, 1994). Successful transgenic plants after screening with the selection agent could be obtained by induction of the transposition resulted in the "marker-off" transgenic progenies (Li and Charng, 2011, Tai, *et al*, 2011). An inducible transposon system truncate a selectable marker in transgenic plants by locating one end of the transposon in the intron of the marker gene e.g. glyphosate tolerant *epsps* gene (Tai *et al*, 2011).

3.2.8. Modulation of gene expression

Antisense RNA technology could be applied to marker genes. When an antisense gene is introduced into a cell it encodes antisense RNA that is complementary to the messenger RNA of the original marker gene and blocks its expression. Catalytic RNA such as ribozymes cleaves either itself or other RNA molecules. It has created new opportunities to repress the expression of selectable marker genes (Harding, 1999). Expression of a ribozyme gene directed towards the target mRNA of the *npt* marker gene in plants resulted in a reduction of the gene product expression (Steinbeck *et al*, 1992). Genetically modified plants containing selectable markers could be further modified with genes that encode antibodies against the gene product (Hiatt *et al*, 1989).

CONCLUSION

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