Australian Government
Department of Health
Office of the Gene Technology Regulator

December 2017

Risk Assessment Reference: Marker Genes in GM Plants

Introduction

Methods for generating genetically modified (GM) plants[1] are generally inefficient, and only a very small percentage of cells are successfully genetically modified (or 'transformed') with the gene(s) of interest. 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 (Miki and McHugh, 2004).

For plant transformation, marker genes are often combined in the same piece of DNA as the gene(s) of interest, such that they are transferred together (Miki and McHugh, 2004). 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 (Breyer et al., 2014). 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, either directly or following a biochemical assay (Breyer et al., 2014).

When assessing 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.

Information is provided here on the most commonly used antibiotic resistance selectable marker genes and reporter genes used in GM plants. The potential for these genes to cause harm to the health and safety of people and the environment is also addressed.

Herbicide tolerance genes are not covered here – these genes are assessed in the risk assessment and risk management plans prepared for relevant licence applications.

Antibiotic resistance marker genes expressed in GM plants

Genes encoding resistance to antibiotics are a common type of selectable marker in GM plants. Antibiotics are usually lethal to sensitive plant cells as they block specific metabolic processes (Padilla and Burgos, 2010). The presence of an introduced antibiotic resistance gene allows a transformed (i.e. GM) cell or plant to survive in the presence of the corresponding antibiotic (Miki and McHugh, 2004).

Generally, following the process for introduction of the new genes to the plant cells or tissues, the plant cells or tissues are placed on a synthetic growth medium containing the antibiotic. Only cells containing the antibiotic resistance gene can grow and, when large enough, these can be tested for the gene(s) of interest.

[1] Information on plant transformation methods can be found in the risk assessment reference document *Methods of Plant Genetic Modification* available on the OGTR website.

The most common antibiotic resistance genes for the selection of transformed plant cells are the *nptII* and *hph* genes (Breyer et al., 2014). These genes are considered in detail below.

In addition to antibiotic resistance genes used for selection in plants, other antibiotic resistance genes may also be present in some GM plants. These are antibiotic resistance genes that were used for selection of bacteria carrying the genes of interest prior to their introduction into plant cells (Breyer et al., 2014). When the expression of these genes is controlled by genetic regulatory elements that work only in bacteria, these genes are not expressed in the GM plants. The lack of expression of the bacterial antibiotic resistance genes in the GM plant means that no toxicity/allergenicity consideration of these bacterial antibiotic resistance proteins is required in a risk assessment for the GM plant.

nptII *gene*

The *nptII* gene, derived from *Escherichia coli* (*E. coli*) strain K12, codes for an aminoglycoside 3'-phosphotransferase II enzyme (APH(3')-IIa), also known as neomycin phosphototransferase II (NPTII). This enzyme inactivates kanamycin and structurally related antibiotics such as neomycin, paromomycin, ribostamycin, butirosin, gentamicin B, and geneticin (G418), which would normally inhibit protein synthesis (Beck et al., 1982; Zhang et al., 2001; EFSA, 2004; Padilla and Burgos, 2010).

hph *gene*

The *hph* (also abbreviated as *hpt*) genes code for hygromycin phosphotransferase (HPH or HPT) enzymes, which are members of the aminoglycoside phosphotransferase (APH) family. These enzymes confer resistance to the antibiotic hygromycin B. *Hph* genes have been isolated from *E. coli* (also referred to as the *aph(4*), *aph4* or *aphIV* gene) and *Streptomyces hygroscopicus* (*aph7"*) (Leboul and Davies, 1982; Rao et al., 1983; Berthold et al., 2002; Stogios et al., 2011). The encoded HPH enzymes inactivate hygromycin B via phosphorylation of different regions of the hygromycin B molecule, depending on the origin of the protein (Stogios et al., 2011). The gene from *E.coli* is used most often in GM plants.

Consideration of risks from *nptII* and *hph*

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 and express the encoded protein. In this context, they may 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 with these genes may cause environmental harm (e.g. have increased weediness leading to harm to the environment).

There is no evidence that the NPTII and HPH proteins are toxic or allergenic. Bioinformatic analyses have failed to find homology to any known allergens (Fuchs et al., 1993; Lu et al., 2007; EFSA, 2009). Toxicity experiments with animals (mainly mice and rats), often involving the feeding of exaggerated doses of these proteins by gavage (use of a small tubing to insert food), have failed to establish any deleterious effects of either NPTII (Flavell et al., 1992; Fuchs et al., 1993) or HPH (Lu et al., 2007; Zhuo et al., 2009). Food derived from GM canola, corn and cotton with the *nptII* gene and food derived from GM cotton with the *hph* gene has been approved for sale in Australia (FSANZ website, accessed 23 November 2017).

Dietary intake of the protein products of antibiotic selection genes could conceivably reduce the therapeutic efficacy of antibiotics taken orally (Nap et al., 1992). This is especially important with regard to the *nptII* gene, as kanamycin, neomycin, ribostamycin and gentamicin are listed by the WHO (2017) as Critically Important Antimicrobials for human and veterinary use. Hygromycin is not used for humans, but may be used in animals such as pigs and poultry (US FDA website, accessed 14 November 2017). However, like most proteins, NPTII and HPH are rapidly inactivated in simulated mammalian gastric juice (Fuchs et al., 1993; FSANZ, 2004; Lu et al., 2007). Therefore, under normal digestion, it would be expected that any antibiotic resistance protein would be degraded before it could inactivate

the corresponding antibiotic, negating any possible interference with oral administration of the antibiotic (EFSA, 2009).

No feasible pathway links a plant with either the *nptII* or *hph* gene and environmental damage. A GM plant with an antibiotic resistance gene would only have a selective advantage and become a weed in the presence of inhibitory concentrations of the antibiotic, and this is unlikely to occur in a natural situation (Nap et al., 1992; Woegerbauer et al., 2015). The European Food Safety Authority concluded that the use of the *nptII* and *hph* genes as selectable markers in GM plants (and derived food or feed) does not pose a risk to human or animal health or to the environment (EFSA, 2004, 2009).

Potential for transfer of GM antibiotic resistance genes from plants to other organisms

It has been suggested that the transfer of antibiotic resistance genes from GM plant material to intestinal or soil bacteria could lead to antibiotic resistant populations of these organisms (Woegerbauer et al., 2015).

Free DNA from plants and other organisms is present in soils. The ability of antibiotic resistance genes from free DNA in soil to be incorporated into the genomes of bacteria has been demonstrated in laboratory experiments (Poté et al., 2010); however, evidence strongly suggests that such 'horizontal gene transfer' from plants to bacteria is extremely rare in natural environments (Keese, 2008; Woegerbauer et al., 2015).

When food is ingested, most genetic material (DNA) is degraded in the stomach and intestines. Fragments of transgenic gene and promoter sequences from GM feeds are often found in the gastro-intestinal tract of animals, and have occasionally been found in animal blood and organs (Nadal et al., 2017). 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 (Keese, 2008).

There is no evidence that the widespread use of antibiotic resistance genes as markers for the selection of GM plants has led to a significant increase in clinical antibiotic resistance (Breyer et al., 2014).

Reporter genes expressed in GM plants

Reporter genes code for molecules that can be readily identified visually or by biochemical assays, allowing the identification of cells/tissue expressing the introduced protein (Breyer et al., 2014). These genes are commonly used as "reporters" of gene expression, by linking them to other genes or promoters in GM plants so that they are expressed in the same pattern as the linked gene or promoter (Miki and McHugh, 2004). They can also be used as selection markers in plant transformation. The proteins of reporter genes are non-toxic to plant tissues, enabling their constitutive or regulated expression (temporal and/or spatial) in plants. The *uidA* (or *gus*) and *gfp* genes are commonly used reporter genes.

uidA *gene*

The *uidA* (also abbreviated as *gusA* or *gus*) gene from *E.coli* encodes the enzyme β-glucuronidase (GUS), which enables *E.coli* to metabolise β-glucuronides as a source of carbon and energy (Gilissen et al., 1998). GUS expression from an introduced *uidA* gene can then be detected in GM plant tissue, in a process that kills the plant cells, using a substrate of the GUS enzyme which produces a coloured product when cleaved by GUS (Jefferson and Wilson, 1991). Using differing substrates enables either the measurement of the amount of protein present or visualisation of the expression pattern (i.e. distribution) in plant tissue (Gilissen et al., 1998).

The *uidA* gene, and its associated protein, is found in a wide range of organisms. In addition to *E. coli*, the *uidA* gene is found in many other bacteria, including other microorganisms of the digestive tract and many soil bacteria (Gilissen et al., 1998). GUS activity is very common in almost all tissues of

vertebrates, with high activity in the kidney, liver and spleen. GUS activity is also present in invertebrates such as molluscs, nematodes and insects (Gilissen et al. 1998). Low GUS-like activity has been detected in over 40 different plant species including a number of human food sources such as carrot, parsley and tomato (Hu et al., 1990).

gfp *gene*

The *gfp* gene, derived from the jellyfish *Aequorea victoria,* encodes the green fluorescent protein (GFP) (Elliott et al., 1999). GFP emits a green light when exposed to blue or ultraviolet light. Although its physiological role is unclear, GFP contributes to the bioluminescence of these jellyfish (Zimmer, 2002).

GFP is valuable as a marker of gene expression in both GM plant cells and GM animal cells (Elliott et al., 1999; Hoffman, 2015). Expression of GFP can be seen in living tissue through exposure to ultraviolet or blue light, avoiding the need to destroy the tissue. This makes it useful for observing the intracellular location and movement of linked proteins within living cells (Leffel et al., 1997; Kallal and Benovic, 2000; Hanson and Köhler, 2001). Mutation of the *gfp* gene sequence has resulted in the development of a number of variants with useful properties (Zimmer, 2002).

Consideration of risks from *uidA* and *gfp*

The GUS protein is not considered to be toxic or allergenic by the United States Environmental Protection Agency, which has exempted it from a requirement to establish a tolerance level (US EPA, 2001). The protein does not demonstrate any oral toxicity when administered at high doses to rodents and is rapidly degraded in gastric fluids (US EPA, 2001; FSANZ, 2003). The *uidA* gene was isolated from *E.coli*, which is found in the human digestive tract, as well as in soil and water ecosystems (Gilissen et al., 1998). Further, genes coding for GUS proteins are found in a range of vertebrate and invertebrates, including humans, and microorganisms other than *E.coli* (Gilissen et al., 1998; Pellock and Redinbo, 2017). Food derived from GM sugar beet and cotton with the *uidA* gene has been approved for sale in Australia (FSANZ website, accessed 23 November 2017).

Likewise, the GFP protein is not regarded as toxic or allergenic to humans or other organisms. Humans are not known to consume the jellyfish *A. victoria*, and as such people have not been exposed to the GFP protein through food. Feeding of the protein to rats failed to show any toxic or allergenic reaction (Richards et al., 2003). The protein was rapidly degraded in gastric digestion experiments, indicating that GFP is unlikely to be a food allergen.

The amino acid sequences of both the GUS and GFP proteins are not related to those of any known toxins or allergens, and the enzymatic activities of both proteins are not known to produce any toxic or allergenic compounds (FSANZ, 2003; Richards et al., 2003).

There are no reports of a GM plant expressing the GUS protein or GFP causing environmental harms associated with increased weediness. The reactions catalysed by the GUS and GFP proteins are not known to be associated with any biochemical process related to plant weediness and therefore expression of these proteins in GM plants is not expected to increase the weediness of these plants.

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