Australian Government
Department of Health and Aged Care
Office of the Gene Technology Regulator

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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 modified (or 'transformed') with the gene(s) of interest. Marker genes are DNA sequences used to identify the transformed cells and facilitate the production of GM plants carrying the gene(s) of interest, conferring a specific trait or traits in the plant (Miki and McHugh, 2004).

In plant transformation, marker genes are often co-located with the gene(s) of interest, within the same DNA fragment, such that both genes are transferred together (Miki and McHugh, 2004). Marker genes may also be present on a separate DNA fragment, as often both DNA fragments are taken up by the same cell and integrated into the cell genome 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.

The two main types of marker genes used in plants are selectable marker genes that confer resistance to a selective agent such as an antibiotic or herbicide, and reporter genes that 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.

This document discusses the most commonly used antibiotic resistance selectable marker genes and reporter genes used in GM plants and addresses the potential for these genes to cause harm to the health and safety of people and the environment. It also considers the likelihood of transfer of these genes from GM plants to other organisms in the environment.

Herbicide tolerance genes are not considered in this document. These genes confer traits that are regularly assessed in the risk assessment and risk management plans prepared by the Office of the Gene Technology Regulator for relevant licence applications.

Antibiotic resistance marker genes expressed in GM plants

Antibiotics are usually lethal to sensitive plant cells via mechanisms that block specific metabolic processes (Padilla and Burgos, 2010). The presence of an introduced antibiotic resistance gene allows a GM cell or plant to survive in the presence of the corresponding antibiotic (Miki and McHugh, 2004).

After introducing new genes to plant cells or tissues, including antibiotic resistance genes as selectable markers, the plant cells or tissues are placed on a synthetic growth medium containing the antibiotic.

[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.

Only cells containing the antibiotic resistance gene can grow and, when the plant is large enough, its tissue can be tested for the gene(s) of interest.

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

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 phosphotransferase 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 in susceptible bacteria (Beck et al., 1982; Zhang et al., 2001; EFSA, 2004; Padilla and Burgos, 2010).

hph *genes*

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 *hph* gene from *E.coli* is used most often in GM plants.

Consideration of risks from *nptII* and *hph*

After the selection of the GM plant, the antibiotic resistance marker genes play no role in the desired phenotypes of the GM plants in the field. However, these genes remain within the plant genome and express the encoded protein. When assessing the risks associated with introduced genes, including antibiotic resistance genes, there are two main considerations (i) the potential for the protein products to have a negative effect on people and/or animals that consume the plant material, and (ii) the possibility of environmental harm, such as increased weediness causing damage to the environment.

There is no evidence that the NPTII and HPH proteins are toxic or allergenic. Bioinformatic analyses have not found 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 administration of excessive doses of these proteins by gavage (use of a small tube to administer the test material), have not identified 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 have been approved for sale in Australia (Food Standards Australia New Zealand (FSANZ) website, accessed 13 December 2023).

Over the past decades, concerns have been raised over the dietary intake of the protein products of antibiotic resistance genes present in plants and their potential to reduce the therapeutic efficacy of antibiotics taken orally (Nap et al., 1992). This is especially important with regard to the *nptII* gene, as aminoglycoside antibiotics, including kanamycin, neomycin, ribostamycin and gentamicin are listed by the WHO (2019) 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, 2024). 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 plausible pathway links a plant containing either the *nptII* or *hph* gene to 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 environment (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).

Antibiotic resistance genes present in GM plants but not expressed

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.

Reporter genes expressed in GM plants

Reporter genes produce molecules that can be easily identified visually or by biochemical assays, allowing the selection of cells or 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). The proteins of reporter genes are non-toxic to plant tissues, enabling their constitutive or regulated expression (temporal and/or spatial) in plants (Miki and McHugh, 2004). The *uidA* 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 that produces a coloured product when cleaved by GUS (Jefferson and Wilson, 1991). The use of different substrates allows the measurement of the amount of protein present and how it is distributed in the 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 (US EPA), 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 13 December 2023).

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 did not result in any toxicity (Richards et al., 2003). The protein was rapidly degraded in gastric digestion experiments, adding to the weight-of-evidence 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; FSANZ, 2022).

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.

Potential for transfer of marker genes from plants to other organisms

The non-sexual transmission of genes between organisms is known as horizontal gene transfer (HGT) or lateral gene transfer. HGT events occur naturally and are considered to be an important evolutionary mechanism in bacteria. It has been also observed in a number of other organisms including fungi, plants, insects and humans (Burmeister, 2015).

In the context of gene technology risk analysis, HGT is assessed as a potential pathway for transfer of introduced genes from a GM organism to a non-GM organism, and whether or not this event can lead to harm to people or the environment (Phillips et al. 2022). The likelihood of a HGT event depends on a number of factors, including the availability of DNA in the environment, the integrity of the DNA molecule, and the presence of a recipient organism (Phillips et al. 2022).

The potential for HGT of GM plant DNA to other eukaryote organisms has been reviewed in the literature and considered as unlikely (Keese, 2008; Philips et al., 2022a). This will not be further discussed in this document.

Since the introduction of GM crops, concerns have been raised regarding the potential risks associated with the transfer of marker genes from GM plant material to intestinal or soil bacteria, particularly transfer of antibiotic resistant genes leading to antibiotic resistance in microbial populations (Woegerbauer et al., 2015).

HGT from plants to bacteria in the soil

Free DNA from plants and other organisms is present in soils. This includes naturally occurring antibiotic resistance genes derived from soil bacteria as well as bacteria present in animal manure (Radu et al., 2021). The ability of antibiotic resistance genes from free bacterial DNA in soil to be

incorporated into the genomes of other bacteria has been demonstrated in laboratory experiments (Poté et al., 2010). However, HGT from plants to bacteria is extremely rare in nature and there is no evidence of HGT from GM plants to soil bacteria (Keese, 2008; Woegerbauer et al., 2015; Philips et al., 2022b). For example, a study showed that cultivating GM grapevine containing an antibiotic resistance gene for 6 years did not impact the number of antibiotic resistant bacteria in the soil, and HGT from the plants to soil bacteria was not observed (Hily et al., 2018).

DNA in aquatic environments

DNA from pollen, leaves, and other plant debris of GM plants may enter aquatic environments. As in terrestrial settings, the integrity of DNA molecules is crucial for incorporation into the genome of aquatic microorganisms (Phillips et al. 2022). A study showed that naked plasmids and plant DNA degrade in groundwater and river water within 48-96 h. No DNA uptake was detected when these water samples were used to transform bacteria in laboratory settings. In addition, when mimicking natural conditions, no transformants were observed when bacterial DNA was incubated in groundwater or river water for up to 7 days (Zhu, 2006). These results emphasise the importance of DNA integrity for HGT and suggest that natural HGT from plants to bacteria is unlikely to occur in aquatic environments.

HGT from plants to bacteria in the gastrointestinal tract

As previously discussed, the availability and integrity of the DNA, along with the presence of a recipient organism, are key factors for HGT to occur. Daily ingestion of animal-, plant- or microorganism-derived food is a source of DNA in the diet of humans and animals (Jonas et al., 2001). Studies have estimated that the introduction of GM grains to the diet of adult humans or cows would result in ≤0.00009% of the total ingested DNA being derived from GM DNA (Beever and Phipps, 2001; Jonas et al., 2001). In addition, when food is ingested, most genetic material, including DNA, is degraded by enzymes in the stomach and intestines and only small fragments of transgenic gene and promoter sequences from GM feeds have been found in the gastrointestinal tract of animals, and occasionally in blood and organs of animals (Nadal et al., 2018). Studies conducted with birds and animals fed with GM grains or purified DNA plasmid did not detect HGT of GM DNA to bacteria in the gastrointestinal tract (Beever and Phipps, 2001; Nordgård et al., 2012; Sieradzki et al., 2013; Zhao et al., 2016). These findings suggest that the use of GM plants as human food or animal feed is unlikely to result in HGT of antibiotic resistance genes from GM plants to gut bacteria, given the low availability and poor integrity of the GM DNA in the gastrointestinal tract.

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), and a broad search of recent peer reviewed scientific literature indicated that this is still the case. As discussed previously, these antibiotic resistance genes were originally isolated from bacteria, which are widespread in the environment, including the gastrointestinal tract of people and animals. Transfer of these genes between bacteria is far more likely than transfer from GM plants to bacteria.

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