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Health aspects of marker genes in genetically modified plants

Report of a WHO Workshop

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1. Introduction

A WHO Workshop on Health Aspects of the Use of Marker Genes in Plants and Possibilities for their Use in Identification and Control of Genetically Modified Plants was held in Copenhagen, Denmark from 21-24 September 1993; the participants are listed in Annex 1.

The Workshop was opened by Dr. F. Käferstein, Chief, Food Safety on behalf of the Director-General of the World Health Organization.

In welcoming the participants, Dr. Käferstein remarked that the Workshop was to be seen as a continuation of the work started in 1990 with the Joint FAO/WHO Consultation, the report of which, "Strategies for Assessing the Safety of Foods Produced by Biotechnology", had been published by WHO in 1991.

Among the various recommendations of this Consultation it had been proposed that international organizations should promote a harmonized approach on the part of national governments to the safety assessment of foods produced by biotechnology. The Consultation had also recommended that timely expert advice on the impact of biotechnology on the safety assessment of foods is provided to Member States, the Codex Alimentarius Commission, to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). While the objective of the 1990 Consultation was to reach international consensus on broad strategies for assessing the safety of foods produced by biotechnology, this Workshop was going to address a much more narrow, detailed issue related to the health aspects of the use of marker genes in genetically modified plants. The Workshop also considered whether the use of marker genes might be feasible in the identification in food, of material derived from genetically modified plants.

As in the case of the 1990 Consultation, this Workshop would again not address environmental aspects related to the specific topic under consideration. It was therefore recalled that over and above any conditions this Workshop might agree upon regarding food safety aspects related to the use of marker genes in plant modifications, there may be additional conditions to be met which relate to environmental issues. In this context Dr. Käferstein was glad to welcome Dr. Kearns of OECD whose organization was likely to make the bridge from food safety to the environment.

The Workshop also recognized additional issues which were outside the remit of this Workshop, but which were nevertheless very important non-scientific issues, related to ethics, consumer perception and food labelling. In this context reference was again made to the 1990 Consultation which had recommended that consumers be provided sound scientifically based information on the application of biotechnology in food production and processing and on the safety issues. In its recently published policy document on Food Safety Towards and Beyond the Year 2000, the International Organisation of Consumers Unions (IOCU) devoted two full chapters to biotechnology in agriculture and to biotechnology as a product and a process. Dr. Käferstein expressed WHO's regret that IOCU was unable to be represented in this Workshop. He also regretted that no representative from the International Potato Center, Peru,

from the International Rice Research Institute, Philippines, and from the Chinese Academy of Science was participating. The unique know-how and experience of these institutions working in developing countries would be missed during this Workshop.

Dr. Käferstein concluded by placing on record WHO's deep appreciation to the National Food Agency of Denmark and to the Nordic Council of Ministers for their contributions towards this Workshop.

The Workshop elected Dr. Knudsen as Chairman, Dr. Hallikainen as Vice-chairman and Dr. Maryanski as Rapporteur. The deliberations of the Workshop were based on a number of working papers (Annex 2) and a case study submitted by Calgene, Inc. (Annex 3).

2. The role of marker genes in plant biotechnology

2.1 Introduction

The Workshop was convened to provide advice on the health aspects of marker genes in genetically modified plants and their potential use in food control. DNA sequences used to "tag" genetically modified plants whilst not expressing a product in the host plant, were considered by the Workshop not to present a health concern *per se*. The Workshop primarily addressed marker genes used in conjunction with the newer methods of genetically modifying plants, such as recombinant DNA techniques, and did not address in detail marker genes used in other breeding methods.

Marker genes are genes used to identify cells or plants during the selection of plant varieties. The use and development of markers is not a new concept in plant breeding. Wrinkled leaves and colouration of flowers are well known examples. Such phenotypic markers are followed during the breeding process because they may indicate the presence of other genes of interest such as those for yield, disease resistance, etc. which can not be directly assessed. These linkages are part of the traditional development of new varieties.

Traditional breeding depends upon the identification of observable markers that are linked to traits of interest. Recombinant DNA techniques permit the design of marker genes and the construction of linkages to genes controlling traits of interest. In certain cases the gene of interest may be the marker gene as well.

In plant transformation the marker gene may also facilitate selection of the rare cells and plants that contain the newly added linked traits. Marker genes of this type are referred to as selectable markers. A selectable marker gene usually allows a cell expressing that marker gene to grow in the presence of a selective agent that inhibits the growth of cells that do not contain the marker gene. The selectable marker gene often allows growth by inactivating or neutralizing the selective agent. In many cases the use of this selective agent is limited to the laboratory at early stages of the modification process. It may be noted that herbicide-resistance markers are widely used for field selection during backcrossing.

The frequency with which transformed cells are obtained varies from species to species and with various transformation methods but is often as low as 1 in 10,000 or 1 in 100,000 of the cells treated (Fraley *et al*, 1984). To find the cells and plants resulting from these rare events would be a daunting task in the absence of selectable marker genes and selective media to identify the transformed cells and eliminate all others.

The newly added trait usually does not provide an easily identifiable property to the cell carrying it. For example, a gene for altered ripening only shows its effect in the maturing fruit of regenerated plants many months after the transformation event. In view of the low transformation rates, it would be impractical and prohibitory to regenerate each cell from a transformation experiment to test the fruits of all the plants produced. By its linkage to the added gene on the same piece of introduced DNA, the

selectable marker gene permits the identification of cells with the added gene. Only cells that contain added DNA and the altered fruit ripening trait are regenerated.

2.2 Scope of the Workshop

The Workshop considered the following categories of marker genes used in recombinant DNA techniques: those that are selectable such as antibiotic resistance genes and herbicide tolerance genes, those that could be used in other selection systems and those that are screenable marker genes, such as colour markers. The Workshop also considered general principles for assessing the safety of marker genes, but did not evaluate the safety of any particular marker gene.

2.3 Categories of selectable marker genes

2.3.1 Antibiotic resistance marker genes

Table 1 contains a comprehensive list and brief description of the antibiotic resistance marker genes described in the literature and currently used to produce transgenic plants (Bowen, 1993). All but one confer resistance to aminoglycoside antibiotics that have extremely limited human and animal use. Aminoglycosides used therapeutically are toxic to specific tissues and as a class cause hearing loss and kidney damage. Managing this toxicity requires careful control of dosage to allow effective disease control without undue harm to the patient. Despite these cautions, certain aminoglycosides still have some clinical uses for treatment of selected infectious diseases (Siegenthaler *et al.*, 1986). Table 1 also contains information on the veterinary use of these antibiotics in the USA. It should be noted that both clinical and veterinary use vary from country to country.

Phleomycin is not an antimicrobial but causes breakdown of DNA leading to death of rapidly growing cells and has limited use in cancer therapy (Gatignol *et al.*, 1988) and is rarely used in plant transformation.

All of these aminoglycoside antibiotic resistance genes were obtained from bacteria that were isolated from nature or in clinical settings. These marker genes were isolated from the genome of the microorganism that produces the antibiotic or from DNAs of resistance factors present in bacteria. Resistance factors carrying these genes are common and can be found at high frequency in natural populations of bacteria (Calgene, 1990; Shaw *et al.*, 1993).

For example, the neomycin phosphotransferase II (*nptII*) gene coding for kanamycin resistance, was isolated from transposon Tn5 in kanamycin resistant bacteria. Kanamycin resistant bacteria are ubiquitous and although all are resistant to kanamycin, such resistance can be caused by many different genes. Thus, only a fraction of these contain the *nptII* gene and enzyme product. In one survey, 3 out of 184 kanamycin resistant bacterial isolates from three stream sites in South Carolina contained Tn5 *nptII* gene sequences (Leff *et al.*, 1993). In another survey of over 4200 clinical isolates resistant to one or more aminoglycoside antibiotics, 2.5% of the bacteria contained the *nptII* gene sequences (Shaw *et al.*, 1993). Most of the natural

microbial kanamycin resistance is caused by other bacterial resistance genes described by Shaw *et al* (1993).

Not all of the antibiotic resistance marker genes described in Table 1 are equally effective for selecting transformed cells due to the level of sensitivity of the particular plant species or variety or the ability of the marker gene to protect the transformed tissue from the effects of the selective agent. The review by Ritchie and Hodges (1993) of the regeneration of transformed plants provides a listing of 128 reports of dicot and monocot species that have been transformed and the markers used. All but a dozen of these papers (over 90%) describe the use of the *nptII* marker gene for kanamycin resistance selection.

One reason for this bias is that the *nptII* marker gene was one of the first markers to be developed and was available from many laboratories. Practically, the kanamycin - *nptII* kanamycin resistance marker gene system provides excellent selection because of the properties of the antibiotic and the resistance gene (Hinchee *et al*, 1993). The kanamycin antibiotic and other aminoglycosides inhibit protein synthesis and growth of non-transformed plant cells in intact tissues. This is achieved without causing these cells to produce compounds that poison surrounding cells in the tissues, including those that contain the *nptII* gene and protein. The kanamycin resistance marker gene acts at the individual cell level and does not allow adjacent, non-transformed cells to survive. The timing of cell death is also important. With kanamycin, cell death is slow enough to permit expression of the added marker gene and inactivation of the antibiotic to occur before cell death. With some selective agents, exposure to the selective treatment must be delayed to ensure that cells with the marker gene will survive. The consequence is that cells are left for a longer time in tissue culture, which is less desirable as somaclonal variation may occur. The hygromycin resistance marker and some of the herbicide resistance markers offer similar advantages to the *nptII* gene and their use is increasing.

Bacterial marker genes are sometimes present on vector DNAs introduced into plants. The ensure maintenance of the vector DNA during culture and can be shown not to express the gene product in the transformed plant or food from the transformed plant.

2.3.2 Herbicide tolerance marker genes

Inherent traits for herbicide tolerance are found in most food crops and protect them from the action of selective herbicides whilst the competing weeds are controlled. Thus, the introduction of herbicide tolerance traits through biotechnology expands on existing plant genetic traits.

Each herbicide tolerance gene provides protection from specific herbicides for which inherent traits did not exist in the non-transformed plant. They have been developed for their value in selective and efficient weed control. The tolerances are normally maintained *in vitro*, which provides excellent selectable markers. The applicability of the individual genes and selectivity of their products varies and of the six herbicide tolerance genes listed in Table 2, only a few are widely used. The mechanism of tolerance is modification and inactivation of the herbicide for all those listed, except for the *als* and *epsps* genes which code for altered target proteins resistant to the herbicide.

Because the herbicide tolerance marker genes can be used under field conditions at the whole plant level, they have also proven valuable as markers to facilitate selection of transformed plants in segregating populations and for environmental safety assessments. Glufosinate tolerance and glyphosate tolerance have been used to follow pollen dispersal (Dale, 1992), outcrossing to wild species (Kerlan *et al*, 1992) and competitiveness and survival in unmanaged habitats (Crawley *et al*, 1993). It is highly unlikely that any single herbicide tolerance will become the predominant "marker" in plant biotechnology as such use could interfere with the agronomic use of the herbicides, eg for the control of volunteer plants.

Genes for tolerance toward herbicides that are no longer available or permitted for agricultural use might still be useful as marker genes at the laboratory level.

2.3.3 Other selectable marker systems

As shown in Table 2, there is also a small group of other potential selectable marker genes that provide resistance to selective agents and that interfere with the metabolism of the transformed plant. These, some of which are described below, are not believed to have been used to date to develop new plants.

The original *Agrobacterium* "tumour" genes were shown to interfere with the phytohormone balances of the transformed tissue. Based on the partial introduction of the tumour specific auxin biosynthetic pathway, a selection procedure can be designed. The fine tuning may be very crop-specific and influence to a large extent the regeneration capacity and procedure.

Another phytohormone selection system depends upon conversion by the *gus* gene of an inactive form of the plant hormone cytokinin (a cytokinin-glucuronide) to a biologically active form. This gives the transgenic cells the competitive advantage and growth stimulation over the non-transgenic cells in the presence of the substrate. The hormone balance of the regenerated transgenic plant is therefore not affected.

Genes for enzymes of the amino acid biosynthetic pathways that are insensitive to feedback inhibitors can permit the growth of cells in the presence of these compounds and are being developed as selectable markers. Examples include the bacterial dihydrodipicolinate synthase and desensitised aspartate kinase genes (Perl *et al*, 1993).

Finally, genes for proteins that bind heavy metals provide survival of the transformed plant cells when treated with heavy metal ions such as cadmium.

2.3.4 Screenable marker genes

A distinction is made between selectable (eg antibiotic resistances or herbicide tolerances) and screenable marker genes (also referred to as scoreable or assayable), which only allow the identification of the presence of a gene through some kind of assessment. Disadvantages of using screenable marker genes to identify transformants compared to use of selectable markers include:

- The need for an additional step to separate transformed from non-transformed tissues/cells;
- The destructive nature of some assessment procedures: if applied in a development situation, the identification has to be postponed until sufficient material of the same cellular origin is available to take a sample for assessment. Until that time all individual potential lines (cellular or plants) have to be maintained; and
- Increased dependency of a biochemical assay on products and equipment.

Although the use of these screenable marker genes in detecting transformation events and assaying expression levels has become established, the actual incorporation in selection procedures is practically hindered by the low transformation efficiency.

2.4 Marker genes present in crops currently under development

As of September 1993, no food crops modified by recombinant DNA methods have been commercialised. A survey of the industry showed that 45 modified crops were intended for commercialization before the end of the decade and these are shown in Table 3. Information pertaining to some of these varieties, and food from them, have been presented to authorities for consideration.

Of these 45 crops, 60% used *nptII* or *hpt* as the marker gene and 40% used one of 4 herbicide tolerances (chlorsulfuron, glyphosate, glufosinate or bromoxynil). It should be emphasised that this list is incomplete and other products are in development so that the number of actual varieties which could soon be marketed will be much higher.

2.5 The use of marker genes in the identification of genetically modified plants.

The Workshop considered whether the use of marker genes (as defined in this report) could be used to identify food or material derived from genetically modified plants.

In principle, the presence of such material might be detected either through the use of nucleic acid probes or the direct assay of gene products derived from marker genes. However, a number of significant limitations were recognised:

- Detection techniques cannot be applied to highly processed foods in which DNA or gene products are absent or have been degraded;
- Plant material or food could contain indigenous microorganisms possessing the marker trait leading to false positive results;
- A plant variety could have acquired a marker gene through cross-pollination without acquiring the linked trait;
- Genetically modified plant varieties might lose their marker genes whilst retaining a functional gene(s) of interest introduced through

a modification event; this loss could occur either through natural processes (eg spontaneous mutation) or through the use of plant breeding techniques; and

- The marker gene may have limited expression (eg it may be tissue specific or inducible).

The Workshop concluded that the detection of a specific marker trait is only an indication of the presence of the specific marker gene. It is not confirmation of the presence of other sequences with which the marker may have been linked at specific modification event(s) in the past. Also, the failure to detect a given marker trait(s) is not proof that the food in question does not contain material derived originally from a genetically modified strain. Therefore, the results of detection techniques applied to marker genes and their products in food must be interpreted with caution.

3. Safety issues arising from the use of marker genes in plant biotechnology

3.1 Introduction

Strategic approaches to the safety assessment of foods derived from genetically modified sources have been discussed previously (WHO, 1991; OECD, 1993). Many of the general safety issues that also apply to marker genes have been addressed in these documents. These issues entail: the potential toxicity of the DNA, the toxicity and allergenicity of the gene product and possible secondary and pleiotropic effects of the insertion (WHO, 1991). One issue not addressed in detail in these documents, and therefore considered in more detail by the Workshop, was the potential for, and implications of, the transfer of genes from genetically modified food plants to gut microorganisms.

3.2 Safety issues common to all marker genes

The DNA from all living organisms is structurally similar. For this reason, the presence of transferred DNA in food products, in itself, poses no health risk to consumers (WHO, 1991). Therefore, no safety concerns are raised by the presence in food of marker gene DNA *per se*.

There are a large number of proteins of different structure and function in the human diet that have been safely consumed for thousands of years. Generally, these proteins are degraded through normal digestive processes. There are only a few proteins in nature that are known to be toxins and these are not components of food. They have been well characterized, eg bacterial toxins. Usually, proteins encoded by genes currently under consideration as markers in plant biotechnology do not belong to the classes of proteins known to be toxic. Therefore, the safety assessment of these marker gene proteins can be based primarily on their function rather than on their structure.

If a transferred marker gene is derived from a source that is known to cause food allergy, it will be necessary to consider the allergenic potential of the expressed protein in the modified plant. However, unless a marker gene is derived from a source known to cause food allergy, there is no reason to believe that marker gene proteins, *per se*, would cause allergenic reactions.

Thus, the presence in food of gene products, *per se*, expressed by plant marker genes is unlikely to constitute a new toxic or allergenic hazard to health and poses no additional food safety concerns in this respect.

The food safety implications of secondary and pleiotropic effects from the insertion of all genes, including marker genes, need consideration (WHO, 1991). However, there are no characteristics of marker genes or their products that suggest that their site of insertion into the plant genome will give rise to additional secondary and/or pleiotropic effects.

3.3 Horizontal gene transfer from plants to microorganisms

Horizontal gene transfer was not considered by the earlier Joint FAO/WHO Consultation (WHO, 1991). If it occurs, it may be of food safety concern if a gene from ingested plant material (whether or not it is expressed in the plant) is expressed in gut microorganisms.

The Workshop considered the probability of horizontal gene flow from plants to microorganisms to be vanishingly small for the following reasons:

- No mechanism for transfer of genes from plants to microorganisms is known and no cases of such transfer have been adequately documented.
- The introduced genes are stably incorporated into the plant genome (IFBC, 1990);
- Plants do not contain the necessary mechanisms for the transport of DNA into bacteria (IFBC, 1990). The Workshop noted that for bacterial transformation, the cells must undergo five steps in order to take up DNA: induction of competence; binding of DNA; penetration of the cell wall and translocation across the cell membrane; transfer to the host genome; and integration of the transferring DNA into the host genome; and
- Bacterial transformation requires a high frequency of homology between the donor DNA strand and the recipient DNA in the host. At least 20 base pairs in a complete homologous sequence is required for significant recombination (Watt et al, 1985).

There are a few examples of potential horizontal gene flow in the literature to be considered. Carlson and Chelm (1986) argued for an eukaryotic (plant) origin of glutamine synthetase II in bacteria, albeit over an evolutionary time period. They suggested that this was evidence that horizontal gene flow from plants to microorganisms had occurred at one point in evolution. However, their paper was directly refuted by Shatters and Kahn (1989) who concluded that "the glutamine synthase proteins are highly conserved and the divergence of these proteins is proportional to the phylogenetic divergence of the organisms from which the sequences were determined. No transfer of genes across large taxonomic gaps is needed to explain the presence of GSII in these bacteria." There are other reports that horizontal gene flow occurs from plants to microorganisms involving transient changes (non-heritable) such as transencapsidation of chloroplast DNA (Rochon and Siegel, 1984) or possibly endocytosis (Bryngelsson *et al*, 1988). Neither of these mechanisms have been shown to result in actual transfer of genes from plants to microorganisms. No mechanism by which plant DNA could be incorporated from plants into the genomes of the microorganisms has been proposed. For *Agrobacterium*-mediated transformation, Zambryski *et al*. (1982) provided evidence that once inserted DNA is integrated into the plant host genome, it cannot be remobilized even if acted on again by *vir* genes.

Much of the literature relating to horizontal gene transfer relates to transfer in environments other than the human gut. There are several differences between the gastrointestinal tract and other environments, eg in soil, which need to be taken into account in assessing possible food safety concerns:

- The free DNA for uptake is continuously degraded in the gastrointestinal tract;
- There are no authenticated reports of bacterial transformation in the environment of the human gastrointestinal tract;
- DNA degradation in the gastrointestinal tract begins well before the arrival of plant material at the critical sites in the gut for transformation (lower small intestine, caecum, and colon);
- The degradation of DNA sequences in the gastrointestinal tract rapidly fractionates the DNA to sequences smaller than needed for proper expression; and
- For effective uptake of plant DNA by bacteria in the gastrointestinal tract, the plant DNA should undergo recombination and be expressed in the recipient bacteria.

The Workshop concluded that there is no substantial evidence for the transfer of genes from ingested plant material to microorganisms in the gut. Furthermore, if transfer were to occur, the nature of the gene and its expression product and the conditions in the gastrointestinal tract will determine whether or not it is a food safety problem. This will need to be evaluated on a case by case basis.

3.4 Issues specific to marker genes

Since the issues common to all marker genes, outlined above, have been discussed in detail elsewhere, the Workshop agreed that its consideration of the safety of marker genes should focus on those concerns specific to such genes.

3.4.1 Antibiotic resistance markers

The potential for inactivation of an oral dose of a specific antibiotic in human antibiotic therapy for which an antibiotic resistance marker gene has been inserted into a food crop is a potential food safety issue. This situation could arise only if the marker gene protein inactivates an antibiotic used in human clinical medicine. For example, kanamycin is used medically whereas hygromycin is not. For there to be any safety concern, any co-factors required by the marker gene protein would need to be available and the gene protein would need to maintain its activity in the gastrointestinal tract.

If horizontal gene transfer to gut microorganisms could occur in the gut from an ingested food plant containing an antibiotic resistance marker gene, it might transfer the antibiotic resistance trait to gut microorganisms. If gene transfer does occur and if the transformed microorganisms survive, colonise the gut and express the protein (whether or not it is expressed in the plant), this could lead to an increase in the gastrointestinal tract of microorganisms resistant to the specific antibiotic. In turn, this might lead to an increased potential for the transfer of the antibiotic resistance marker gene to pathogenic microorganisms or result in the inactivation of an oral dose of the antibiotic. However, the possibility that all of these events will occur, resulting in a public health problem, is very unlikely.

Although not of direct concern for human safety, the Workshop noted the possible presence in animal feed of antibiotic resistance marker gene products from genetically modified plants. If this were the case, the effectiveness of the use of antibiotics in animal feed could be compromised and may need consideration.

3.4.2 Herbicide tolerance markers

The presence of herbicide tolerance genes in crop plants provides increased management options for weed control. Specific health concerns are related to potential new metabolites or residues of the herbicide that could occur as a result of the introduced tolerance and the interaction between the gene product and the herbicide.

Some herbicide tolerances are introduced only for use in product development as a selective agent in the initial stages to identify the transgenic cells, and commercial application of the corresponding herbicide is not envisaged. In such cases, direct exposure of the food crop to the herbicide does not occur, and the metabolites and/or residues of the herbicide are not a food safety concern.

The Workshop acknowledged that the presence of a herbicide-tolerance gene, even though included only for marker purposes, may encourage unauthorised use of the corresponding herbicide. However, the illegal use of a herbicide is not a unique consequence of biotechnology and is handled through existing laws and regulations.

3.4.3 Other marker genes

A range of activities, other than antibiotic resistance or herbicide tolerance, might be encoded by genes introduced into plants, in the future, as markers. Because of the diversity of functions involved, it was not possible for the Workshop to be specific about their possible health concerns. However, one category that gave rise to particular concern was metabolic markers.

Metabolic markers function by interfering, to a greater or lesser extent, with the metabolism of the host plant, eg its amino acid pathways. This may reduce the sensitivity of the plant to high concentrations of substances normally found in the plant. Because of the direct interference with the metabolism of the plant it is of concern that there could be changes in some important components of the plant such as its vitamin, specific amino acid, or natural toxin content.

The degree of interference to a metabolic pathway that might occur from the product of the inserted metabolic marker gene can be expected to vary considerably between different plant species. Thus it may not be possible to extrapolate from the results of a safety evaluation of a metabolic marker gene in one plant species to another species, eg from potatoes to rice.

4. Strategies for the food safety assessment of plants containing marker genes

4.1 Introduction

The complexity of whole foods and the wide range of modifications possible in whole foods derived from plants, require a wide-ranging safety assessment. This embraces both a general safety assessment and an assessment of the concerns arising from the use of a particular marker gene during the modification process. A general strategy for the safety assessment of plants generated by modern biotechnology was developed by the earlier Joint FAO/WHO Consultation (WHO, 1991). This takes into consideration the proposed use of the food including the preparation procedures as well as the potential intake.

4.2 Safety assessment of marker genes, primary effects

4.2.1 General aspects

The safety assessment of plant marker genes in relation to human health is based upon an integrated approach taking into account molecular, chemical and biological data. These data embrace:

- A description of the marker gene, its construction and expression in plants;
- The intended technical effect of the marker gene, eg antibiotic resistance, herbicide tolerance;
- Methods for analysing and quantifying the marker gene and its product in foods;
- Relevant data from studies to address potential toxicological and/or nutritional effects related to the function of the marker gene; and
- A consideration of the potential for horizontal gene transfer to gut microorganisms.

These data are applicable to the use of a specific marker gene construct in all plants. Such data, once created, should in principle cover use of that marker gene construct in any combination or linkage with any other functional genes to be inserted.

The function of the marker gene product will determine any potential effects of the gene protein on the host plant's metabolism. Such effects might be addressed by analytical means: demonstrating, in the food, the expected presence/absence or changed amounts of compound(s) specific for the marker gene product in question, eg metabolites or specific amino acids.

Information may also be needed regarding possible function of the specific marker gene product in the food when ingested. It may include information on anticipated levels of the marker gene protein in the food plant as ingested. In addition, the possibility that the marker gene enzyme might exert its specific effect in the human gut (eg interference with common human medical treatment such as oral intake of neomycin) may need to be considered.

Because the possibility of horizontal gene transfer is considered to be vanishingly small, data on such gene transfer will only be needed when the nature of the marker gene is such that, if transfer were to occur, it gives rise to a health concern.

In addition to the general aspects outlined above, specific concerns, identified in Section 3, will need to be addressed.

4.2.2 Antibiotic resistance marker genes

Procedures to determine whether inactivation of an oral dose of antibiotics could occur might include:

- Simulation of the human gastrointestinal environment to determine if the antibiotic resistance gene product is degraded;
- Determination of whether co-factors are required for the enzyme to function and, if so, whether they are present in the gut and at what levels;
- Identification of all oral uses of the specific antibiotic and whether it is likely to be used under atypical conditions (eg high stomach pH) which might affect degradation of the gene product; and
- Calculation of the level, if any, of potential inactivation of an oral dose of the antibiotic: such calculations should be based on the maximum intake of transgenic plant material which could be consumed during oral therapy.

Procedures to determine whether horizontal gene transfer could occur from ingested plant material to gastrointestinal microorganisms, thereby transferring the antibiotic resistance trait to them, might include:

- Calculation of any potential change in the number of gastrointestinal microorganisms that have antibiotic resistance; and
- Direct measurement of horizontal gene transfer, should it occur. Horizontal gene transfer is, however, extremely difficult to measure because of the low frequency of transfer and the high background of natural resistance in gut microorganisms.

Procedures to determine whether antibiotics in animal feed could be inactivated during storage as a result of the activity of the antibiotic resistance marker gene product, might include:

- Production of feed from the transgenic crop and its analysis to show that the antibiotic resistance gene product has enzyme activity in the feed; and
- Addition of the specific antibiotic to the feed and analysis of the feed for inactivation after storage under the usual conditions of storage of the feed.

4.2.3 Herbicide tolerance marker genes

The Workshop noted that the only specific food safety concern over the use of herbicide tolerance marker genes in food plants was contamination of the plant by the herbicide and/or its metabolic or breakdown products. Such aspects, which are not unique to plants produced by biotechnology, are addressed comprehensively during procedures for approving the use of specific herbicides on specific crops. As such, they are covered by existing legislation.

4.2.4 Metabolic marker genes and others

Strategies for assessing the safety of specific marker genes other than those encoding antibiotic resistance or herbicide tolerance, need to be addressed on a case by case basis. The particular concerns arising from the use of metabolic markers may or may not be predictable from their known function. If such markers are used, the food safety assessment of the modified plant should be conducted initially using the general procedures described elsewhere (WHO, 1991; OECD, 1993).

4.3 Safety assessment of marker genes, secondary effects

Most genetically modified plants intended for food use will be varieties derived from existing food plants. The most practical approach to the determination of safety is to consider whether they are *substantially equivalent* to the analogous conventional food (OECD, 1993).

Possible secondary changes in the food plant due to the insertional event, which may influence the overall safety of the food, need to be determined on a case by case basis. These changes might be due to mutation of the original genome by the insertion or to unexpected influences of the new gene product on the plant's metabolism. Actual approaches include measurement of any changes in key substances in that plant food including inherent nutrients and natural toxins. In principle, such information should enable a comparison to be made with the parent strain and facilitate the establishment of the substantial equivalence of the food plant in question to its parent (OECD, 1993).

4.4 The overall safety assessment

The overall evaluation procedure will need to balance the potential hazard and the possible exposure/intake by considering whether the marker gene presents a *de novo* situation or whether the gene and the gene product are known from other human exposures, eg as an antibiotic resistance gene normally present in the gut flora. Account should be taken of special groups at risk. In the overall safety assessment of a food plant carrying a marker gene, both the primary and secondary changes have to be assessed.

Assessment of the primary effects in a food plant of a marker gene may in part be based on general information concerning the marker gene itself and its products. The primary effects depend upon the products of the marker gene constructs. The Workshop concluded that if the concept of a positive list for marker genes is to be accepted, it has to be based upon scientific knowledge indicating that the primary effects of the marker gene in the list

are evaluated as being safe irrespective of the host plant in which it is inserted.

Secondary effects of a marker gene in a food plant are expected to be highly dependent on the host plant and on the site of insertion of the marker gene. Secondary and or pleiotropic effects need to be evaluated case by case independent of the marker gene construct used. Assessment of the secondary effects can be made by comparing key substances in order to establish substantial equivalence.

5. Conclusions and Recommendations

5.1 The Workshop noted that many genetically modified varieties of food plants were approaching commercialisation and that its consideration of the health aspects of marker genes used in plant biotechnology was most timely.

5.2 The Workshop recognised that there was a need for marker genes in plant biotechnology to facilitate identification and selection of modified varieties following a genetic modification process even though these genes may have no function in the final product. It was impractical at present to remove marker genes from modified plants after they had fulfilled their function.

5.3 The Workshop noted that although a number of different marker techniques had been investigated, the number of marker genes in varieties approaching commercialisation was restricted to two antibiotic resistance markers and to a few herbicide tolerance markers. This was because of the ease of availability of these marker systems and the level of understanding of their mode of action.

5.4 The Workshop concluded that the presence of marker genes *per se* in food would not constitute a safety concern.

5.5 The Workshop concluded that in assessing the safety of the proteins expressed by marker genes used in plant biotechnology, the focus of the assessment should be on the function of the expressed protein rather than its structure.

5.6 The Workshop concluded that there is no reason to suppose that marker gene proteins pose a particular allergenic concern. However, if the genes are obtained from a source known to cause food allergy, the allergenicity of the gene product will need to be investigated.

5.7 The Workshop concluded that there are no characteristics of marker genes or their products that suggest that their site of insertion into the plant genome will give rise to additional secondary and/or pleiotropic effects.

5.8 The Workshop concluded that there is no recorded evidence for the transfer of genes from plants to microorganisms in the gut. If transfer did occur, any health concern would depend on many factors, including the ability of the transformed microorganisms to replicate in the gut and to express the gene product. Unless the transferred gene was under the control of bacterial promoters (and thus not expressed in the plant) there was no mechanism for expression in gut bacteria.

5.9 The Workshop concluded that the general safety assessment strategies elaborated by FAO/WHO and OECD should be applied to the safety assessment of plant varieties containing marker genes. The Workshop noted the development of metabolic markers for use in plant biotechnology and that the use of these markers could introduce effects of possible health concern. Safety data relating to the use of any such marker in one crop might not be applicable to other crops.

5.10 The Workshop concluded that specific strategies would need to be applied to different categories of marker genes, such as antibiotic resistance marker genes, herbicide tolerance marker genes, and metabolic marker genes.

5.11 The Workshop concluded that, at the present time, it was not possible to develop a positive list of plant marker genes which did not cause food safety concerns. Never-the-less, such a list is considered to be valuable and, once the data become available to enable such a list to be constructed, this should be done under the auspices of an international health agency like WHO.

5.12 The workshop concluded that the presence or absence of marker genes in food could not be correlated reliably with the presence or absence of recombinant DNA. Hence, while the detection of marker genes might provide a possible screening test for the presence of genetically modified plants in food, the results of such tests would need to be interpreted with caution.

5.13 The Workshop considered that, generally, the principles established for assessing the human food safety of genetically modified plants would also be applicable for the assessment of the safety of genetically modified plants used for animal feed. However, the Workshop felt that there may be issues specific to the use of marker genes in animal feed that are beyond the scope and expertise of this Workshop and should be brought to the attention of the relevant organizations, such as FAO.

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Table 1. Sources of selectable antibiotic resistance marker genes and current use of antibiotics

<u>Marker Gene</u>	<u>Source and Mechanism</u>	<u>Antibiotic(s)</u>	<u>Clinical/Veterinary^a Use</u>	<u>Ref.</u>
kanamycin resistance	Tn601 from <i>E. coli</i> aph 3'-I, nptII neomycin phosphotransferase I	kanamycin	little clinical use high ototoxicity and nephrotoxicity	Fraley et al., 1983
hygromycin resistance	Tn5 from <i>E. coli</i> aph 3'-II, nptII neomycin phosphotransferase II	kanamycin paromomycin geneticin (G418)	little clinical use high ototoxicity and nephrotoxicity ^b some veterinary use	Fraley et al., 1983; Nap et al., 1992
streptomycin spectinomycin resistance	<i>Streptomyces hygroscopicus</i> aphIV, hpt hygromycin phosphotransferase	hygromycin	no clinical use veterinary use: swine/poultry	Waldron et al., 1985
gentamicin resistance	<i>E. coli</i> aadA(3'') adenyltransferase aph 3'', spt phosphotransferase	streptomycin spectinomycin	some clinical use despite high ototoxicity and nephrotoxicity	Svab et al., 1990; Maliga et al., 1988
phleomycin resistance	<i>E. coli</i> aacIII/IV gentamicin-3-N-acetyltransferase AAC(3)III/IV	gentamicin	used clinically despite ototoxicity and nephrotoxicity veterinary use?	Hayford et al., 1988
	<i>Streptoalloteichus hindustanus</i> , Tn5 from <i>E. coli</i> drug binding protein	phleomycin bleomycin	limited used tumor therapy no anti- microbial use	Hille et al., 1986

^a Complete Handbook of Approved New Animal Drug Applications in the United States. (1991).

^b The limited uses of these antibiotics for humans have been replaced by more effective aminoglycoside antibiotics (e.g. amikacin and netilmycin) that are not substrates for the neomycin phosphotransferase II protein (Nap et al., 1992).

Table 2. Additional marker systems for plant transformation.

Type	Gene	Selective agent or screened activity	Reference
Selectable Marker - Herbicide tolerance	<i>bar</i>	glufosinate	De Block <i>et al.</i> , 1987
	<i>als</i>	sulfonylurea	Lee <i>et al.</i> , 1988
	<i>epsps</i>	glyphosate	US patent n° 4940835 US patent n° 5094945 Comai <i>et al.</i> , 1985 PCT patent application n° WO9200377
- Phytohormone production	<i>gox</i>	glyphosate	Streber & Willmitzer, 1989
	<i>ifdA</i>	2,4 D	Stalter <i>et al.</i> , 1988
	<i>bxn</i>	bromoxynil	Depicker <i>et al.</i> , 1988 PCT Patent application n° WO93/05163 Goddijn <i>et al.</i> , 1993
- Aminoacid metabolism	T-DNA gene2 <i>gus</i>	LAM (auxin) cytokinin-glucuronide	
	plant tryptophane decarboxylase	tryptophane analogues	Perl <i>et al.</i> , 1993
	dihydropicolinate synthase	lysine analogues	Perl <i>et al.</i> , 1993
- Heavy metal tolerance	aspartate kinase	lysine/threonine	Lefebvre <i>et al.</i> , 1987
	mammalian metallothionine	heavy metals	
	<i>rcn</i>	anthocyanin production	Ludwig <i>et al.</i> , 1990
Screenable marker - Phenotype	<i>luc</i>	luciferase	Ow <i>et al.</i> , 1986
	<i>gus</i>	glucuronidase	Jefferson <i>et al.</i> , 1987
	<i>lacZ</i>	galactosidase	Matsumoto <i>et al.</i> , 1988
- Biochemical	<i>ocs, nos</i>	opinesynthase	Zambryski <i>et al.</i> , 1983

Table 3. Selectable marker genes in some food and other crops under development.

Marker	Trait and Crop	Planned Commercialization
Kan ^R	Virus resistant tobacco	1993
Kan ^R	Delayed softening tomato	1993-94
Kan ^R	Virus resistant tomato	1994
Cls ^R	Delayed ripening tomato	1994-95
None	Virus (I) resistant squash	1994-95
Kan ^R	Delayed softening tomato	1994-95
Kan ^R	Virus (II) resistant squash	1995-96
Kan ^R	Virus resistant cantaloupe	1995-96
Kan ^R	Bromoxynil tol. cotton	1995-96
Glp ^R	Glyphosate tol. soybean	1995-96
Kan ^R	Insect resist. potato	1995-96
Kan ^R	Insect resist. cotton	1995-96
Glp ^R	Glyphosate tol. canola	1995-96
Kan ^R	Delayed ripening tomato	1995-96
Kan ^R	Canola with modified oils	1995-96
Cls ^R	Flavor enhanced pepper	1995-97
Kan ^R	Insect resistant corn	1996-97
Kan ^R	Virus resistant tomatoes	1996-98
Glu ^R	Insect resistant corn	1997-98
Herb ^R	Improved corn	1997-98
Kan ^R	Fungal resistant tomato	1996-97
Kan ^R	Fungal resistant potato	1997-98
Kan ^R	Nematode resistant potato	1997-98
Kan ^R /Glp ^R	Glyphosate tol. sugar beet	1998-99
Kan ^R	Virus resistant sugar beet	1999-00
Kan ^R	Fungal resistant sugar beet	1999-00
Kan ^R	Virus resistant tomato	(1992)
Hyg ^R	Virus resistant rice	(1994)
Hyg ^R	Virus resistant rice	(1994)
Kan ^R	Virus resistant melon	(1994)
Hyg ^R	Improved oil soybean	unknown
Cls ^R	Sulfonyleurea tol. cotton	"
Glu ^R	SeedLink™ hybrid canola	"
Kan ^R	Virus resistant alfalfa	"
Glu ^R	Virus resistant corn	"
Kan ^R	Nutrition. improved soybean	"
Bxn ^R	Bromoxynil tol. tobacco	"
Glu ^R	Glufosinate tol. canola	"
Glu ^R	Glufosinate tol. corn	"
Glu ^R	Virus resistant melon	"
Kan ^R	Virus resistant tobacco	"
Hyg ^R	Low amylose rice	"
Hyg ^R	Low protein rice	"
Hyg ^R	Low allergen rice	"
Kan ^R	Virus resistant potato	"

Kan^R - kanamycin resistance, Cls^R - chlorsulfuron resistance, Glp^R - glyphosate resistance, Glu^R - glufosinate resistance, Herb^R - resistant to a specific, unnamed herbicide, Hyg^R - hygromycin resistance; Bxn^R - bromoxynil resistance. The information in the table is compiled from published reports or directly from company representatives and duplications reflect different genes and/or different companies.

() indicates completion of field trials.

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Annex 2

LIST OF WORKING PAPERS

Title	Author
Current Use and Future Needs for Antibiotic Resistance Marker Genes	Dr. Stephen G. Rogers, Senior Fellow Monsanto Agriculture Group 700 Chesterfield Pkwy North St. Louis, MO 63198 USA
Status for the Use of Herbicide Resistance Genes and other Marker Genes (exclusive antibiotic resistance genes) and Future Needs	Dr. Patrick Rüdelsheim Director for field operations Plant Genetic System Josef Plateau Straat 22 B-900 Gent (Ghent) Belgium
Do the Authorities Need marker Genes for Control?	Dr. Sirpa Kärenlampi, Assistant Professor, University of Kuopio Dep. of Biochemistry and Biotechnology P.O.Box 1627 SF-70211 Kuopio Finland
Food Safety Implications of the Use of Antibiotic Resistance Markers in Genetically Modified Plants	Ms Ranjini Rasaiah Biotechnology & Novel Foods Branch MAFF-Food Science Division II Room 208, Ergon House, c/o Nobel House 17 Smith Square London, SW1P 3JR UK
Gene Flow from Transgenic Plants into Microorganisms - a Potential Risk?	Dr. Folmer D. Eriksen Institute of Toxicology National Food Agency Mørkhøj Bygade 19 DK-2860 Søborg
Aminoglycoside 3'-Phosphotransferase II (APH(3')II): Safety and use in the Production of Genetically Engineered Plants	Dr. Keith Redenbaugh, Manager Regulatory Affairs - Calgene Fresh Calgene, Inc. 1920 Fifth Street Davis, CA 95616 USA

- Calculations based on Pao *et al.*, (1982) survey of a three-day consumption period;
- Stoichiometric reaction of 100% of the ATP in ingested food with orally administered neomycin (this is highly unlikely);
- Administration of neomycin simultaneously with consumption of a transgenic food containing APH(3')II and other fruits or vegetables rich in ATP;
- Presence of intact, functional APH(3')II enzyme, which requires a buffered stomach environment (Ph 7); and
- Stability of ATP in the stomach environment (buffered stomach).

The *kanr* gene has the potential, if not degraded, if active, if in the presence of sufficient ATP, and if under suitable environmental conditions, to inactivate neomycin sulfate added to animal feed. A bioassay experiment demonstrated the stability of antimicrobial activity of neomycin sulfate in cottonseed meal and rapeseed meal prepared from transgenic lines containing the *kanr* gene and stored for 8 weeks at 37°C. Meal samples, assayed for the presence of active APH(3')II enzyme, showed no enzyme activity.