

Antibiotic Resistance Markers in Genetically Modified (GM) Crops

- *What are they and why are they used?*
- *The regulatory framework for the safety assessment of GM crops*
- *Risk assessment of antibiotic resistance genes in GM crops*
- *Are there alternatives and can they be removed?*

The use of marker genes for resistance to certain antibiotics in the development of genetically modified (GM) crops has given rise to considerable public concern. This briefing paper reviews what they are and why they are used, how the safety of GM crops is regulated and the possible alternatives to their use. The overall aim is to provide balanced information and advance public debate. This paper results from the combined contributions of scientists, industrialists, and governmental and public interest organisations across Europe. It is intended to provide information and does not represent the views or policy of the European Federation of Biotechnology or any other body.

Introduction

The combination of antibiotic resistance genes and antibiotic is an important tool in genetic engineering in general and in plant biotechnology in particular. A key task in genetic engineering is the identification and selection of cells into which a new gene has been introduced. Antibiotic resistance genes have the ability to selectively inactivate certain antibiotics and consequently protect cells against these antibiotics. An antibiotic resistance gene can thus be used to "tag" a gene carrying a trait or characteristic of interest. In practice the antibiotic gene is linked to a gene carrying the trait of interest prior to its introduction into the recipient cells, whether of bacterial, yeast, plant or animal origin. These cells are then incubated in the presence of the antibiotic. The only cells which multiply under these conditions are the ones that have incorporated the antibiotic resistance gene together with the trait of interest.

The identification of the "transgenic cells" would be extremely tedious, and even impossible, without this selection procedure since only a very small fraction of the cell population incorporates the introduced genes (one out of several thousands of cells). A selection procedure is a necessity in genetic engineering, and this is the reason why antibiotic resistance genes have been so widely used in many different areas of biotechnology for a number of years.

Recent approvals for the commercialisation of GM crops containing antibiotic resistance markers have raised concern in Europe about the risk of spreading antibiotic resistance genes to previously unsusceptible microbes and hence making them resistant to the antibiotics used. Public concern about this topic is widespread and the issue is repeatedly raised in the media. As a consequence governments in the European Union have recommended "phasing out" GM crops containing antibiotic resistance markers contrary to the recommendations of several national and European scientific committees.

What are they and why are they used?

Antibiotics and antibiotic resistance in nature

Bacteria are microbes that are present everywhere in the environment and in plants and animals. We are permanently exposed to them, for example, ingesting them with our food. Microbes occupying the same habitat compete for nutrients and for their own survival some have evolved naturally to produce antibiotics to eliminate their competitors. Antibiotics inhibit a cell's growth by blocking some of its essential metabolic processes. Bacterial strains producing a given antibiotic therefore have to carry resistance to inactivate the corresponding antibiotic and thus prevent its own self-destruction. In the evolutionary race between microbes the production of new antibiotics is usually countered through the development of resistance mechanisms both by producing- and target-organism. There is in nature a wide range of antibiotic and corresponding antibiotic genes. However, rather than developing their own resistance mechanisms, targeted bacteria will in general acquire antibiotic resistance genes which are already present in the bacterial pool surrounding them. This is facilitated due to the fact that bacteria are generally quite promiscuous about exchanging genetic material between each other. The presence of an antibiotic confers an advantage to a resistant bacterium and so under these conditions resistance development and spread increases.

The discovery of antibiotic action against disease-causing agents at beginning of the 20th

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century had a major impact on medicine. However, it has been used without sufficient limitation. Man's use of antibiotics has drastically increased their global distribution and consequently promoted the spread of resistant microbes. Increased use of antibiotics in clinical and also veterinary medicine is the major cause of the increasing incidence of antibiotic resistance in bacteria. In addition, antibiotics have been and still are used extensively as animal feed additives which has brought about the selection of resistant bacteria in healthy animals. Antibiotics are also widely sprayed on crop plants, orchards, vines, etc to protect against pathogens.

Today resistance to antibiotics is so widespread that some of the first generation of antibiotics are of no use anymore. Multiple antibiotic resistance in pathogenic strains of *Staphylococcus* and *Mycobacterium tuberculosis*, especially in hospitals, is of particular concern to the medical community. Concern about the use of antibiotic resistance markers in relation to overall antibiotic use management is expressed, for example, in the following quotation from the Belgium Biosafety web-site: "*Such a scenario does twist the overall picture of antibiotic misuses by pinpointing a very small aspect of the whole problematics of antibiotics uses management. Furthermore, such a fashionable scenario is ultimately dangerous for Public Health management because it does promote the picture of a tree masking the forest, at least from the public viewpoint. On the other side, it is rather politically and technically easier to ban the use of these antibiotics resistance markers in transgenic plants on the basis of the precautionary principle than to regulate the feed market and to control its upstream agricultural practices*". (http://biosafety.ihe.be/ARGMO/GMO_Plants.html)

Antibiotic resistance markers as tools in plant biotechnology

Since the mid-1980s modern biotechnology methods have been developed to improve agricultural crops through the introduction of genetic material that confers advantageous characteristics.

There are two types of antibiotic resistant marker (ARM) genes used in transgenic plants:

1 genes driven by bacterial promoters. These genes have been used during the initial stages of the assembly of the pieces of DNA intended for transfer into the plant cells. The purpose of these genes was to select for the amplification of the pieces of constructed DNA in the receiving bacteria. The gene providing resistance to ampicillin belongs to this category. Genetically modified plants containing those genes are from the earliest generation of technologies and present technologies only allow the removal of these genes before initiating the plant transformation process.

2 genes which allow the selection of plant cells which have up taken the piece of DNA carrying the trait or characteristic of interest. The insertion of a gene into a plant cell by transformation is a very inefficient process since only a few thousand cells of the many millions used take up the desired gene. The transfer of an antibiotic resistance marker gene together with the gene of interest allows these very few cells to be selected as only those cells that have taken up both genes will survive and multiply in the presence of the corresponding antibiotic in the growth medium. A genetically modified plant is then grown from these modified cells and the marker is no longer needed.

The time needed for the development of a genetically modified crop with a new trait usually exceeds 10 years. Safety aspects are taken into consideration at each step of product development. This starts at the selection of appropriate proteins and genes to insert into the plant, followed by experiments designed to assess potential impacts on human health from the consumption of the GM crop and then several years of field trials with the genetically modified crop to assess potential environmental safety impacts.

Antibiotic resistance markers used in the development of genetically modified crops have been selected by scientists according to various safety criteria. These include that the marker genes occur frequently in natural microbial populations (most have been isolated from common bacteria in the human gut) and that they confer resistance to a narrow range of specific antibiotics with limited application in human and veterinarian medicine.

The most widely used antibiotic resistance marker for the selection of transformed plant cells is the *nptII* gene, also called *aph(3')-II*, which confers resistance to the antibiotics neomycin and kanamycin. This gene is present in ten of the fourteen GM plants containing antibiotic resistant marker gene submitted for marketing in the EU. For example, it has been used to develop the delayed ripening tomato, herbicide-tolerant and insect-protected corn and cotton varieties. The choice of using this antibiotic resistant marker gene has been driven by the fact that the antibiotics kanamycin and neomycin are not important in medical treatment and that, on average, 20 to 40% of the bacteria that occur naturally in human or animal digestive tracts are already resistant to kanamycin. Kanamycin/neomycin resistant bacteria are ubiquitous in nature, their prevalence being dependent upon the source of the bacteria isolated, the highest level being found in pig manure.

Regulatory framework and regulatory decisions in the US and the EU

Before a genetically modified crop can be placed on the market in the European Union several approvals have to be obtained under national and European legislation. The two most important pieces of legislation for commercialisation are the Directive 90/220/EEC on the environmental release and

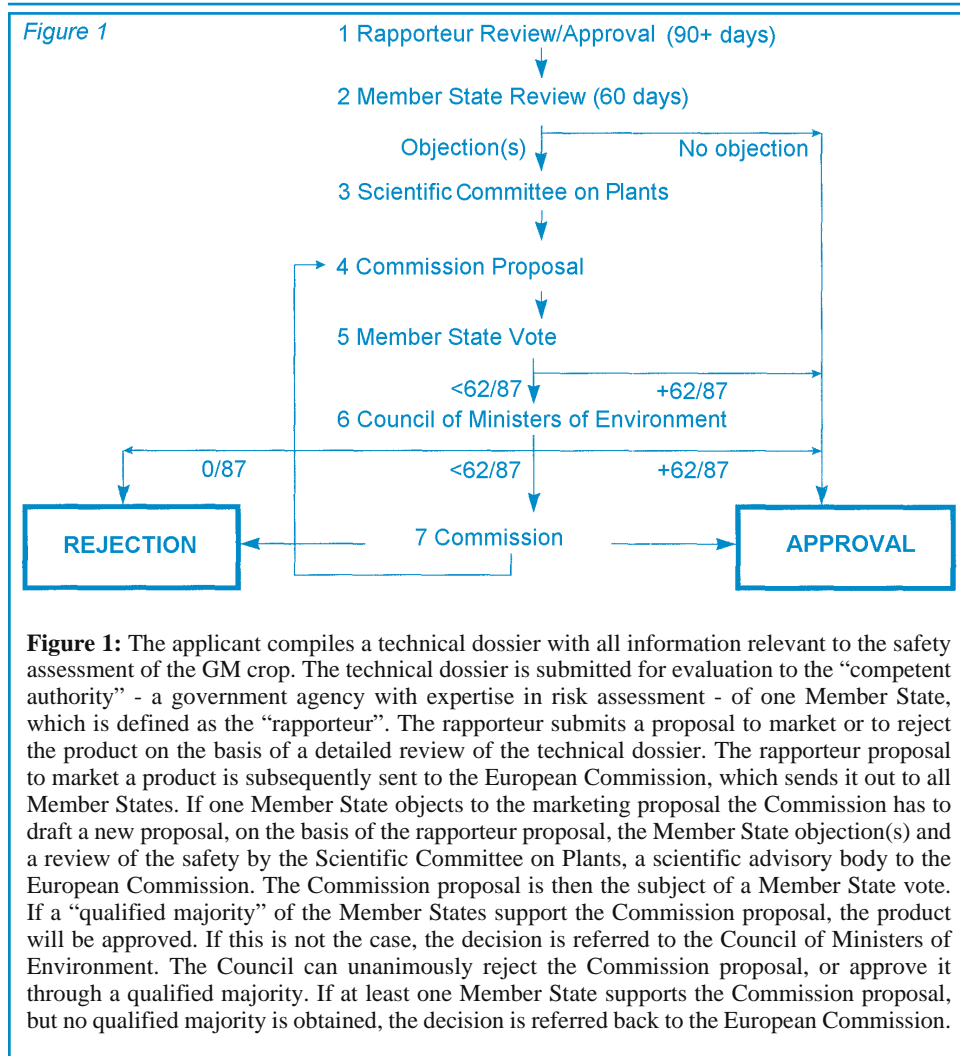
placing on the market of GMOs and the Regulation 258/97 on Novel Foods and Food Ingredients. The main decision-making procedures under both sets of legislation are very similar. The decision-making procedure on GM crops under Directive 90/220/EEC is set out in Figure 1.

The debate on the safety of antibiotic resistance markers in the EU began in 1996 during the approval of the Novartis maize, CG176, containing a gene for ampicillin resistance. The U K Advisory Committee on Novel Foods and Processes (ACNFP) played a major role in sustaining arguments against approval of this product because the gene, when expressed in bacteria, confers resistance to the clinically important antibiotic ampicillin. Based on the concerns voiced in the UK, the Commission proposal to market this product did not obtain a qualified majority of Member States and at the Council of Ministers of Environment level. As Member States failed to take a decision, the Commission, authorised the product to be placed on the market after additional reviews by three Commission Scientific Committees. Each of the three Scientific Committees concluded that there were no significant food, feed or environmental concerns about the commercial release of this product. The French government approved the cultivation of the product under the condition of monitoring for increased insect-resistance and potential transfer of the ampicillin resistance gene to microorganisms. Monitoring attempts so far failed as ampicillin resistance occurs in up to 10% of bacteria in the environment and a gene that would be transferred potentially from the GM maize to an environmental bacterium cannot be distinguished from the gene that is already wide-spread in the bacterial population. Due to the lack of market acceptance of GM crops, the GM maize has not been cultivated on more than 500 hectares in France.

Regulatory decisions based on the Directive 90/220/EEC, to be replaced in October 2002 by Directive 2001/18/EEC, have approved for marketing GM maize crops that contain the *nptII* antibiotic resistance marker. Four other recent proposals also containing *nptII* did not receive the qualified majority support needed and since then three applications have been deferred. Member States and the Commission shall ensure that GMOs which contain genes expressing resistance to antibiotics in use for medical or veterinary treatment are taken into particular consideration when carrying out an environmental risk assessment, with a view to identifying and phasing out antibiotic resistance markers in GMOs which may have adverse effects on human health and the environment. Marker genes of clinical importance will be phased out by 2005.

Safety assessment of antibiotic resistance in GM crops

The safety assessment of crops containing antibiotic resistance markers has been thoroughly reviewed by experts from internationally recognised scientific bodies and scientific committees in the European Union and governmental experts from Canada, Japan,



Switzerland and the United States among other countries. The experts evaluated the safety of crops containing the *nptII* marker gene conferring resistance to the antibiotics kanamycin and neomycin, the *aad* gene conferring resistance to streptomycin and spectinomycin and the *bla* gene conferring resistance to ampicillin. They concluded that the risk of gene transfer from transgenic crops containing these genes to the microbial population is negligible and, if it were to occur, there would be no impact on the present high frequency of occurrence of antibiotic resistance in microbial populations. The European Commission Scientific Committee on Plants provided an opinion on the safety of a Bt insect-protected maize product containing the *nptII* gene.

Their conclusions are based on the following observations:

1 The antibiotic resistance markers used in plant biotechnology were obtained from naturally occurring bacteria. The *nptII* gene in particular was isolated from bacteria in the human gut. The markers in approved products are already widespread because effective natural transfer mechanisms exist to shuttle the genes directly between bacterial cells. This process confers advantages to bacteria. Some microbial populations can hence act as reservoirs for certain antibiotic resistance genes that then

are rapidly spread in response to selective pressures.

- 2 The antibiotics inactivated by *nptII*, kanamycin and neomycin are rarely used in human therapy since they have been replaced by less toxic antibiotics with greater efficacy.
- 3 There is no known mechanism for the transfer of genes from plant cells to bacteria. Gene transfer to soil micro-organisms could in theory occur in fields where GM crops are cultivated. However, such events have not yet been detected. Both bacterial and plant DNA have been shown to persist in soil for weeks or months by binding to the surfaces of soil particles. The availability over time of plant DNA, an important factor in determining likelihood of gene transfer, is therefore likely to be higher in the soil than in the gut. However, while certain bacteria have the ability to take up naked DNA spontaneously, numerous barriers would need to be overcome before intact genes from a plant could be taken up from a natural environment, and be functional and maintained in bacteria; such an event has never been shown to occur under natural conditions. Even if transfer were to occur at a very low frequency, this would not add significantly to the number of antibiotic resistant bacteria that already exist in nature.

4 The rate of DNA transfer between bacteria under optimal conditions in nature is in the range of 10^{-2} to 10^{-5} . The estimated frequency of uptake of the *nptII* gene marker from transgenic plants to bacteria under optimal conditions is 10^{-17} and thus considered to be insignificantly small. The *nptII* gene corresponds to only 0.00004% of the total maize genome and would compete with the rest of the DNA for uptake by the bacterium. The availability of free GM plant-derived DNA in the rumen or the gastro-intestinal tract is further limited due to rapid digestion by pancreatic fluids and acid saliva. The participation of *nptII* gene from transgenic plants to the overall pool of kanamycin resistant bacteria appears even more insignificant when compared to the large pool of genes providing resistance towards kanamycin that already exists in bacteria and the high level of gene transfer that is constantly occurring in bacterial populations. Therefore a bacterium is several orders of magnitude more likely to acquire a resistance gene from another bacterium rather than from the DNA of the GM crop.

5 Antibiotic resistance markers only confer resistance against specific antibiotics. Antibiotic resistance markers do not result in antibiotic production. There are therefore no antibiotics present in food produced from plants produced using biotechnology.

Are there alternatives to antibiotic resistance markers and can they be removed?

Alternatives to antibiotic resistance markers

Alternative selectable markers for plants fall into two categories. Some markers confer resistance to chemicals other than antibiotics that kill plant cells such as herbicides and lethal concentrations of the amino acids lysine and threonine. The enzyme that confers resistance to high concentrations of lysine and threonine can interfere with amino acid biosynthesis and if expressed at high levels cause abnormal plant development. The relevant genes are therefore not suitable as marker systems. The presence of herbicide-tolerance markers may be undesirable. Glyphosate is the most effective herbicide to control potato seed from previous years in temperate regions with mild winters and therefore glyphosate-tolerant potatoes would not be suitable in these regions. Furthermore, plants containing herbicide-tolerance marker genes which are not registered as herbicide-tolerant crops may tempt the misuse of herbicides.

Other alternative marker systems rely on the growth of plant cells in the presence of unusual nutrients, including cytokinin, glucuronides, xylose or mannose, which will not allow non-transformed plant cells to grow. For example, plant cells usually do not use mannose as a source of sugar. The delivery of a gene allowing mannose to be metabolised in plant cells and the subsequent cultivation of those cells in a medium containing mannose as the sole source of sugar would allow only those cells which have taken up the gene to grow.

When these systems, which are still in their development phase, work reliably on a large scale in a wide range of different environments risk assessments will have to be conducted to assess the potential ecological impacts of plants that can grow on a new substrate, the impact on the overall plant metabolism and the consequences on human or animal diet from increased levels of metabolites in these crops that might not be present in the conventional counterpart.

Removal of Markers

It is not possible to remove marker genes once they are integrated into a plant genome unless a particular mechanism for removal is incorporated along with the marker gene and the gene of interest at the time of the transformation. As was mentioned above, it is possible to avoid introducing into plant cells antibiotic resistant marker genes which are only used for the assembly and amplification of the DNA constructs in bacteria, and therefore are not necessary during the plant step of the transformation procedure.

The removal prior to commercialisation of marker genes which are driven by plant promoters and are used for selection of plant cells has become the aim of both consumers and industry. Extensive research with this aim is being carried out both by industry and academic institutions. Among the technologies being assessed are:

- 1 The use of meganucleases (e.g.: Cre/lox system). These are enzymes which can specifically recognise long DNA sequences. These recognition sequences are introduced on both sides of the antibiotic resistant marker gene to be introduced into the plant cell. Once the transformed cells have been selected on the corresponding antibiotic, the meganuclease is introduced into the plant cell, and will allow the excision of the antibiotic resistant marker gene. This technology has proven to be very efficient in certain plants, but difficult to handle in others possibly because the meganuclease recognises sites in the plant genome itself.
- 2 The presence of homologous DNA sequences on both side of the antibiotic resistant marker gene may allow for random recombination and elimination of the gene. This process of homologous recombination occurs at low frequency and may be plant specific.
- 3 It is possible to introduce the trait of interest and the antibiotic resistant marker on different DNA constructs. Following transformation, each molecule integrates on a different chromosome. In this case it is possible to segregate the trait of interest from the marker gene at the next generation. Frequencies of integration on separate chromosomes can be quite low when compared to integration at the same locus. Factors involved in regulating these frequencies are not yet understood.

Extensive effort is being invested by researchers to develop these technologies. Some products using one or other of these strategies are already in the pipeline. But today none of these approaches can routinely be used for every crop. The development of these technologies will probably be limited in the first place to laboratories having a strong infrastructure. These laboratories will have to be able to afford to develop a greater number of transgenic plant lines containing the antibiotic marker gene than will be needed for the subsequent screening for the elimination of the marker gene.

Modification of regulatory elements of marker genes

The concern about antibiotic resistance marker genes is predominantly about their transfer and expression in bacterial cells and a technology which would prevent such expression might have to be considered. Already the genes used for selection in plants are controlled by plant promoter sequences which render them unlikely to be sufficiently expressed in bacteria. The introduction of an intron sequence in the marker gene would restrict its expression to plant cells and definitively prevent any expression in bacteria. Introns are sequences of DNA that naturally interrupt the coding sequences of animal and plant cells. These are equipped with mechanisms allowing their removal during the transcription process while bacteria are not equipped to do this and therefore would be unable to read a gene containing introns.

Antibiotic resistance marker genes such as the *npII* gene which provides resistance towards kanamycin are very well researched in all the relevant aspects such as their functioning, biochemical properties and prevalence in the bacterial community. Their safety has been well examined and assessed. Under these conditions it is likely that achieving the same level of confidence as has been established for *npII*

with another selection system may be long and difficult. Any remaining concerns attached to such genes could be removed by the addition of an intron.

Conclusions

Scientific experts agree that the main cause for the spread of antibiotic resistance is the overuse of antibiotics in human and veterinarian medicine. Public concerns on the use of GM crops with antibiotic resistance markers are, however, widespread, especially since this issue was repeatedly raised in the media. Several governments in the European Union have recommended the phasing out GM crops containing any antibiotic resistance markers. However, the risk of compromising the efficacy of antibiotics is considered to be vanishingly small. Most alternatives are still in their development phase, are not widely available and will be difficult to implement in a less developed country.

Even if antibiotic resistance markers or other markers are not proven to be harmful, as is the case in general, it would be preferable in the long run if transgenic crops carried only those genes necessary for the crop's performance and not the selectable markers.

Alternative markers and marker removal systems are being investigated in response to public concerns and to expand the number of tools available in plant molecular biology. Since the time for development of new alternative methods varies between different crops, it will be necessary to allow for a gradual transition to such technologies. It will also be critical to conduct safety assessments on new systems before they are used in products that are to be commercialised. Replacement of the technology which makes use of antibiotic resistant marker gene such as *npII* will be desirable when the new technologies have ensured at least the same degree of scientific knowledge and confidence regarding their use as *npII* gene and products containing it.

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