# Safety of Genetically Engineered Crops

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**Editor:**
René Custers
regulatory affairs manager, VIB

**This report can be obtained from:**
VIB
Rijvisschestraat 120
B-9052 Zwijnaarde, Belgium
tel.: ++32 (0)9 244 66 11
fax.: ++32 (0)9 244 66 10
e-mail: vib@vib.be
web: http://www.vib.be

**Responsible publisher:**
Jo Bury, VIB
Rijvisschestraat 120
9052 Zwijnaarde

# Contents

**A. Introduction** ..... **4**

1. Setting the scene ..... 4
2. What is the debate on genetically engineered crops all about? ..... 4
3. What does this report do and what does it not do? ..... 5

**B. Some information on breeding, genetic engineering and agricultural practices** ..... **6**

1. Conventional breeding and selection ..... 6
2. Genetic engineering of plants ..... 7
3. Changes in plants obtained by genetic engineering or by conventional breeding ..... 8
4. Differences between conventional breeding and genetic engineering ..... 11
5. Environmental effects of current agricultural practices ..... 12

**C. Summary** ..... **13**

**D. Discussion** ..... **20**

**E. Safety aspects of genetically engineered crops** ..... **26**

1. Toxicity and Food Safety of Genetically Engineered Crops ..... 27
   *Jan Pedersen, Folmer D. Eriksen, Ib Knudsen*
2. Safety of Genetically Engineered Plants: an Ecological Risk Assessment of Vertical Gene Flow ..... 60
   *Klaus Ammann, Yolande Jacot, Pia Rufener Al Mazyad*
3. Effects on Non-target Organisms of the Release of Genetically Modified Crops into the Environment ..... 88
   *Julian Kinderlerer*
4. Allergenicity of Foods derived from Genetically Modified Organisms ..... 108
   *André Penninks, Leon Knippels, Geert Houben*
5. The Biosafety of Antibiotic Resistance Markers in Plant Transformation and the Dissemination of Genes through Horizontal Gene Flow ..... 135
   *Philippe Gay*

**Annex 1: Overview of referees** ..... **160**

# A. Introduction

## 1. Setting the scene

Genetically engineered crops have been and still are a subject of public controversy. Debate started in 1986 when the first field trials were initiated. The debate has taken a second and more profound round after the first ships with genetically engineered soybeans arrived in Europe in the autumn of 1996. In February 1997, the worldwide presentation of Dolly the cloned sheep has furthered the awareness of the impact of biotechnology among the general public. Especially in Europe the debate on genetically engineered crops is now very intense, but also in the US new rounds of discussion can be seen.

## 2. What is the debate on genetically engineered crops all about?

The issues that are subject of the debate on genetically engineered crops can be divided into the following categories:

**1. Biological questions**

Are genetically engineered crops dangerous for human health? What about food safety? Are there risks for our environment and the organisms living in our countryside? Do they pose a threat to biological diversity? How can they help attain sustainability? All these questions are repeatedly asked and perhaps they are not adequately answered or perhaps also new questions keep arising as the technology develops further and is applied to an ever increasing scale.

**2. Societal questions**

What do genetically engineered crops mean for the power- and dependence relationships in the food chain? What about patents and ownership? How important is freedom of choice and how can it be guaranteed? What do genetically engineered crops mean for (our relationship with) the developing countries? What about the economics of genetically engineered crops?

Underlying the two debates described above there are questions relating to the direction in which our society wants to develop. What are the goals our society strives for? What are the problems that we face today in our agriculture and environment and how can these problems be solved or circumvented? What developments or technologies can seriously contribute to the solving of these problems? And what criteria do we use to evaluate and weigh these different developments against each other? Often these goals and criteria are not made explicit, but implicitly they play an important role in the debate, because they determine the attitudes of different stakeholders in the debate.

## 3. What does this report do and what does it not do?

This report wants to contribute to the discussion on some of the biological risks of genetically engineered crops, namely those that are subject of the different environmental and food regulations that apply to genetically modified food crops. In the debate about these crops the following issues come up very frequently:

1. Toxicity and food safety of genetically engineered crops
2. Vertical gene flow of genetically engineered crops
3. The effects of genetically engineered crops on non-target organisms
4. Allergenicity of foods derived from genetically engineered crops
5. The biosafety of antibiotic resistance markers in plant transformation and the dissemination of genes through horizontal gene flow

This report contains for each of these topics a state-of-the-art review that has been written by a respected scientist with expertise in the issues involved (part E of this report). The reviews have been refereed by a number of peers, also experts in the subjects involved (see annex 1). These reviews are a summary of the current scientific knowledge on the safety aspects of genetically engineered crops, show what is known about the current genetically engineered crops that are on the market, and make some recommendations for future research and safety evaluations.

The reviews do not represent an opinion of VIB as an institute, but should be seen as useful material gathered by VIB for further discussions. VIB has the ambition to disseminate this information very broadly, so it can be used by as many persons and organizations as possible that want to participate in the debate.

This report does not touch upon all aspects of the discussion on the biological risks of genetically engineered crops, nor does it touch upon the issues catalogued above as ‘societal questions’ or ‘goals society strives for’. VIB acknowledges the importance of these issues, but has chosen in this instance to limit the scope of the report to the risk assessment aspect.

# B. Some Information on Breeding, Genetic Engineering and Agricultural Practices

## 1. Conventional breeding and selection

The crops that are used in our current agriculture have been derived from wild plants during a long process of domestication and selective breeding. Selective breeding is the improvement or alteration of crops by selecting only those individual plants that have certain desired properties. Selective breeding has been intensified dramatically during the 20th century and has become a highly specialized profession. Sexually compatible individual crop plants with different characteristics are crossed to generate offspring combining these characteristics, resulting in plants with possibly interesting phenotypes. Whether the desired characteristics are actually combined is a matter of chance. Many individual crosses have to be made to generate one new variety. During the last century the number of techniques for generating new varieties has been widely enlarged. Important techniques are:

- Hybridization (to generate 'hybrid vigour'[1]).
- Alteration of Ploidy[2] (haploids, tetraploids, etc.).
- Induction of mutations through irradiation or the use of chemical mutagenic agents.

  Seeds are treated with chemical agents or irradiation resulting in mutations (alterations) in the genetic make up of the plant. Individual plants with different mutations in their genome are grown, and individual plants with interesting phenotypes are selected and screened. The mutations result in a greater genetic variation.
- Somaclonal variation.

  In-vitro multiplication of plants is used to generate individuals with different phenotypes. These differences are thought to be the result of genome instability in the in-vitro stages which results in plants (clones) with different phenotypes from one and the same parent plant.
- Embryo rescue

  Embryo rescue is the rescue of embryo's of a cross between two plants that normally – in nature - would not result in offspring, thus enabling certain 'wide crosses' (crosses between plants that in nature are not sexually compatible). Such crosses have only been made with plants within one family.
- Anther culture

  Anthers are grown in-vitro and hormones are used to regenerate plants from the anthers.
- Marker assisted breeding.

  The use of molecular markers[3] associated with certain phenotypic characteristics to add more precision to breeding and to speed the selection.

[1]Hybrid vigour is the result of the combination of two different inbred lines, where the result hybrid shows a far better performance than could be expected from the performance of two parent lines (1/2+1/2>1).
[2]The ploidy number shows how many times one chromosome is present in the genome of an individual. Haploids have only one of each chromosome (1N), diploids have two of each chromosome (2N), and tetraploids have four of each chromosome (4N).
[3] A molecular marker is a genetic marker (a piece of DNA) that co-segregates with a particular trait.

Many of our currently used food crops do not resemble their wild ancestors anymore (see for instance maize and its wild ancestor teosinte). Others are still more close to their wild relatives (like for instance certain grasses). And even other plants would never have existed without human intervention (like for instance nectarines, or triticale).

Table 1: examples of crops in which 'modern' breeding techniques have been used

| Type of breeding technique | Examples of crops where the technique is used frequently |
|---|---|
| Hybridization | Maize, onions, carrots, oilseed rape, chicory, sunflower, rice |
| Polyploidy induction | Different types of grasses, red clover, sugarbeets |
| Mutation through irradiation or chemical mutagenic agents | Barley, Triticale |
| Somaclonal variation | Potato, ornamentals |
| Embryo rescue | In crosses between plants of different species, for instance melon x cucumber (a recently made 'wide cross') |
| Anther culture | Barley |
| Marker assisted breeding | Many different crops |

All these techniques are frequently used in daily practice and are all covered under the umbrella of what today is called 'conventional breeding'. Some of these techniques – and especially techniques like mutagenesis and embryo rescue – can have distinct effects on the genome level. But these alterations on the genome level are often not exactly known. This is also true for the effects of in-vitro multiplication of plants. The multiplication of one individual plant through in-vitro culture results in many individual plants that show sometimes aberrant, but in any case different phenotypes. These phenomena are thought to be the result of genome instability in the in-vitro stages, but the exact changes on the DNA level – if any – are not exactly known.

## 2. Genetic engineering of plants

A new, not conventional technique is genetic engineering of plants. Genetic engineering is the introduction by man of a piece of genetic material into a plant in a way that is not possible using breeding or natural recombination. This technique has been developed in the beginning of the nineteen-eighties. The most important methods to introduce specific genetic material into a plant are:

**1. *Agrobacterium* based transformation**

**2. The biolistic method (the 'gene gun')**

The bacterium *Agrobacterium tumefaciens* possesses the natural ability to transfer part of its genetic information on the 'Ti-plasmid' (a circular genetic element in this bacterium) to plant cells. Infection of the plant cells results in the transfer of genetic material from the bacterium to the plant genome. In nature this leads to a tumor formation on the plant in which the bacterium lives and feeds. For genetic engineering of plants the oncogenic sequences of the Ti-plasmid are replaced by the gene-of-interest. Plant cells that have taken up the genetic material can be regenerated to whole plants, using the already well-established in-vitro culture techniques. To be able to select only those plants that have taken up the genetic construct, a selection marker is present in the construct. Until now, antibiotic resistance markers have often been used for this purpose. *Agrobacterium* mediated transformation does not work in all types of plants.

In the biolostic method genetic constructs coated on small gold particles are shot into the plant tissue. In some cases this results in integration of the genetic material into the genome of the plant tissue. The further process to regenerate transgenic plants is identical to other in-vitro regeneration methods. The current crops like Bt-maize have been made using this technique.

Like in conventional breeding not just one new genetically engineered plant is made, but preferably several hundreds of plants using the same genetic construct. Of these many individual transformants only those plants are maintained that have a preferred genetic constitution (only one insert instead of more, stable expression over several generations, etc.) and which show the desired phenotypical behaviour (elite events). It takes many years of testing before one new genetically engineered variety can be launched.

The constructs that are brought into the plants using genetic engineering consist mostly of the following:

1. **The gene(s) of interest**
   Under the control of regulatory sequences. Depending on the specific regulatory sequences, expression of the gene can be constitutive, time or tissue specific or triggered by external factors.
2. **A selection marker**
   Also under the control of regulatory sequences that are responsible for expression of the marker gene in the plant tissue. This marker is necessary to be able to recognize plants that have taken up gene of interest together with the marker.
3. **Some non-coding sequences surrounding the genes present in the construct**
   In the case of *Agrobacterium* mediated transformation also the so-called 'T-DNA borders' will be present in the plant. In some cases other DNA sequences like 'Scaffold Attachment Regions' (SAR's) are also present. SAR's are known to stabilize the expression of the neighbouring genes.

It is known that *Agrobacterium* transformation does not always lead to 'clean' insertions. In some cases not only the T-DNA insert is present in one copy, but also small repetitions, or even the whole plasmid is inserted into the genome. When whole plasmids are present in the plant, also bacterial sequences like origin of replication and bacterial marker genes (often an antibiotic resistance marker) will be present in the plant.

Both methods result in the random integration of the sequences somewhere in the genome of the plant. *Agrobacterium* mediated transformation leads to the insertion of one or a few copies of the sequences. The biolistic method often results in the integration of multiple copies of the sequences. Molecular characterization of the selected transgenic plants determines what sequences have been actually transferred to the plant. The sequences are most often present in the transciptionally active parts of the genome of the plant.

## 3. Changes in plants obtained by genetic engineering or by conventional breeding

Often conventional breeding, when compared to genetic engineering, is referred to as a 'black box' exercise. But what does really happen when a plant is modified, either through genetic engineering or through conventional breeding? In the following box an example of plant modification is given where the goal is to change the flower color. All names and data in this example are fictitious, and situations are oversimplified for the purpose of easy understanding.

**Box 1: The example of the changing of flower color**

The starting point is a pink flowering plant, where the pink color is the result of a mixture of red and white pigments that are the end result of a branched biochemical pathway. The goal is to produce a red flowering plant. In theory this can be achieved by blocking the pathway that produces the white pigment, resulting in only red pigment being made.

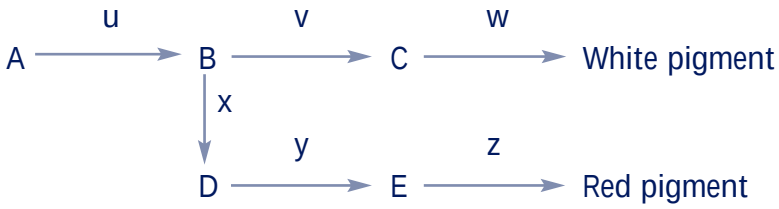



Figure a: The biochemical pathway leading to white and red pigment via the chemical components A, B, C, D and E, with the help of the enzymes u, v, w, x, y and z. In the starting plant the pathway is thus regulated that the same amounts of red and white pigment are formed.

Genetic engineering of the pink plant

With genetic engineering a gene is introduced into the pink plant which produces a protein 'm' that is known to inactivate the enzyme 'w', thereby inhibiting the formation of white pigment. Although the principle is simple, in practice it is often a very though job to find a protein 'm' that specifically binds to the enzyme 'w' and results in inactivation of this enzyme. The search for this protein may be very time consuming and costly.

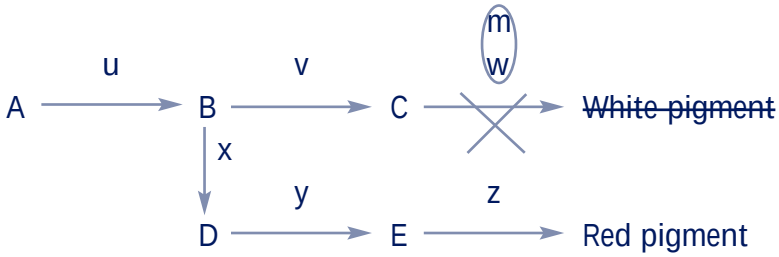



Figure b: As a result of the interaction of protein 'm' with enzyme 'w', the formation of white pigment is decreasing, resulting in a red flower.

The protein 'm' can in principle originate from any organism. After the introduction of the protein 'm' it can turn out that the inactivation is not 100% so there still is some white pigment being formed, or that the protein does not bind specifically enough. Also other changes to the plant – other than a changed flower color – could result from the possible insertion of the gene on an existing active plant gene, thereby possibly disrupting its function. If there is an insertion effect, the consequences of it cannot be easily predicted. There is also a possibility that the protein 'm' could have a pleiotropic effect. Pleiotropic means 'resulting in a second changed characteristic'. This could in this case be the result of the protein 'm' interacting also with other enzymes in other pathways. The chances of the occurrence of pleiotropic effects can be significantly lowered if the protein 'm' and its characteristics are fully known in detail. Additional testing of the interaction of protein 'm' with structural analogues of the enzyme 'w' will give a better idea of any possible pleiotropic effects.

**Conventional crossings of the pink plant**

To obtain a red plant using conventional breeding, a large number of crosses (perhaps 'wide crosses') is made with plants of the same family. These plants are likely to be quite different from the pink plant, in phenotype (differences in appearance and behaviour), as well as in genotype (differences in the genetic make-up of the plant). They can also have a different flower color. Of the many crosses that are made, a few plants may show red flowers. In these plants the changing of the flower color is the result of some new interaction that was not present in the parent pink plant. It is not known what actually causes the flowers to be red. One can try to find the cause using markers or other methods, but this can be quite difficult and laborious. It can be caused by many things, but somewhere in the flower color pathway a change is introduced: It can be that a new component is now present in the plant that interacts somewhere in the pigment pathway, with the enzymes 'v' or 'w' for instance. It can also be that there are factors that break down the protein 'C', or the white pigment itself. It could also be that the red pigment branch of the pathway is upregulated. Another possibility is that there is a mutation introduced somewhere in the genes that control the pathway. It is also possible that through conventional breeding no red flowering plants can be obtained, which makes the technique unsuitable for this purpose.

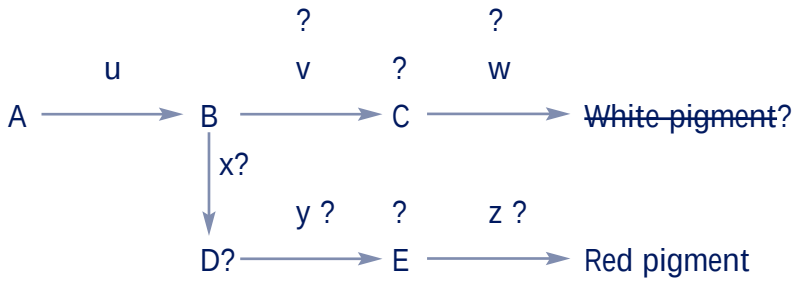



Figure c: In the conventional cross there are many possibilities that result in a red flower color. The actual cause will not be known.

In the conventional cross, many other changes are introduced in addition to the desired changes in the flower color pathway. The larger the genetic distance between the two parent plants, the larger the chances will be to find a red flowering plant, but also the larger the introduced genetic changes. Changes like the one in the flower color pathway may be introduced in other metabolic and signal transduction pathways. Not all of these changes can be seen in the morphology and the behaviour of the plant. To get rid of any unwanted changes as much as possible, plant breeders make many backcrosses with the pink parent plant (while selecting only plants with the red flower color). This means that the plant is crossed a number of times with the pink flowering parent plant, but every time selecting for only the red flowering descendents. After a number of backcrosses most of the genetic material will then be from the pink flowering plant.

## 4. Differences between conventional breeding and genetic engineering

To conclude it can be stated that the major differences between conventional breeding and genetic engineering of crop plants are twofold:

1. With genetic engineering it has become possible to transfer a single gene (or a specific number of genes) into a crop while in conventional breeding large parts of the plant genome are changed. Genetic engineering enables breeders to selectively introduce the characteristic(s) they are interested in, and to avoid the introduction of other less interesting or even undesired characteristics.

2. Conventional breeding is limited to breeding within plant families. Genetic engineering is not limited to species barriers. This is possible because DNA is the universal carrier of genetic information in all organisms. This means that genes found in bacteria, or in any other organism, can be engineered into a crop plant. In contrast to 'wide crosses' - where individual plants still need to have a close evolutionary relationship - the genetic background of the gene does not have to relate to the plant in evolutionary terms at all.

Table 1: Differences and similarities between conventional breeding and genetic engineering

| | | **Evolutionary relationship between host and donor** | **Precision** (in terms of numbers of genes) | **Effects on the genome level** |
|---|---|---|---|---|
| **Conventional** | Within one variety (mutation breeding) | - | No genes added, but many genes can be affected | Can result in many changes, ranging from small mutations to large rearrangements |
| | Intraspecies* | Very high | Low (many unknown, but related genes introduced) | Normal 'fit' of genomes, with only rare, spontaneous recombinations and rearrangements |
| | Interspecies** (for instance using embryo rescue | High | Low (many unknown genes introduced) | Often improper 'fit' where rearrangements are necessary to stabilize the genome |
| **GMO** | | Can be very low | High (one or a few (non-related) genes introduced) | Random insertion somewhere in the genome, leaving other parts of the genome intact |

* = within species

** = between species

# 5. Environmental effects of current agricultural practices

Agriculture is a major type of land use throughout the world. Agriculture - in its amount of land use - has to compete with the surface used by housing, traffic, industrial activities and nature and wildlife conservation. Because of its scale the impact of agriculture on the environment, nature and biodiversity is considerable.
Throughout the ages, agriculture has changed the landscapes and influenced plants and other rural inhabitants. The intensification of agriculture in the 20th century by introducing chemical fertilizers, chemical pesticides and the scale increase has reached its goals of food security in many areas, but it has also had drawbacks. The first chemical pesticides were not that environmentally friendly. Some of these chemicals were even highly toxic and accumulated in the fat tissue of birds, resulting in a dramatic decline of birds of prey in the sixties and seventies. These and other environmental impacts resulted in more stringent procedures for the authorization of the use of chemicals.
A second example of a drawback is that in some countries there are problems with the quality of ground and drinking water as a result of the extensive use of chemical fertilizers and manure. A last example of the fact that agriculture is not neutral in terms of environmental changes is the profound shift in agricultural land use as a result of the only recent large-scale cultivation of maize in the north-western parts of Europe. Such large shifts in crops cannot be realized without environmental changes.

In the last few decades the attention to biodiversity has grown worldwide. This has led among other things to the Convention on Biological Diversity in 1992 that has been signed by many countries all over the world. As a result of this convention there is a more explicit attention to biodiversity and many countries have reported to the permanent secretariat of the convention on the status of the biodiversity in their home country. There are not that many quantitative data on the changes of biodiversity during the past century. There are figures for instance on birds pointing at a decline in the number of species and at an increase of the number of birds of certain species living in the countryside. But for plants, insects, and even more for micro-organisms, the figures are less clear. It is even more difficult to link changes in biodiversity directly to a specific cause. Often, more than one factor or even a complex combined action of different factors will be responsible. In addition to the biodiversity surrounding the crops and present outside the fields, the biodiversity of the crops themselves can be looked at. The more different varieties are used, the higher the biodiversity. The increasing scale of agriculture has led to a reduction in the number of varieties that are grown and thus of biodiversity and in many areas large monocultures exist.

Current agriculture is still dependant on rather large chemical inputs, but more integrated ways of growing crops are on the increase. Also the current types of chemical pesticides are less polluting than the ones used 30 years ago. There are of course still large differences in the toxicity of the current chemical pesticides. There is a strong incentive towards sustainability and there is more attention to conservation of biodiversity on the farm level.
Together with this trend towards greater sustainability in conventional agricultural practices, there is the growing attention to organic agriculture. Organic agriculture does not use (synthetic) chemical inputs and the agriculture is often small scale and makes use of intensive crop rotation systems. In the last 10 to 20 years, the acreage of organic farming has steadily grown. In most western countries the percentage of organic farming is now somewhere between 0.5 to 2%. There are examples where this percentage is much higher: in Austria for example 8% of the agriculture is organic. Predictions are that the percentage of organic agriculture will grow further in the coming years. Organic agriculture has earned its place as an alternative to the conventional practices using chemical inputs. Organic agriculture does not use genetically modified organisms.

# C. Summary

## 1. Toxicity and Food Safety of Genetically Engineered Crops

**Food, toxicity and genetic engineering**

Man has learned by trial and error to avoid poisonous plants. But our current food is still not without toxic substances. Some plants even have to be cooked before they can be eaten safely. Traditional breeding can lead and has led to changes in the levels of these substances leading to their present levels. There is no formal food safety assessment required for these traditional foods because we have a 'history of safe use' with these types of foods. This history of safe use is however, not backed up by empirical evidence.

Like traditional breeding, genetic engineering has the potential to alter the toxicity of foods. A newly introduced protein can be toxic, but also random changes could occur resulting from insertion or pleiotropy, leading to changes that are relevant from a toxicological perspective. The phenomenon of insertion is not new, because it is also known from some traditional breeding techniques. In contrast to traditional crops, for genetically engineered crops we do require a formal food safety assessment, and for the reason that we do not have a history of safe use of these genetically engineered foods.

**Genetically engineered crops and a new paradigm in food safety assessment**

The food safety assessment of genetically engineered foods should determine whether the modified food is as safe as its traditional counterpart. As a starting point for the safety assessment the concept of 'substantial equivalence' was introduced as a means of establishing a benchmark of safe food. The food safety assessment of genetically engineered foods is now considered to consist of four parts: (1) a molecular characterization of the insert, (2) determination of any unwanted direct toxicological effects as can be predicted from the nature of the inserted sequences, (3) determination of any unwanted indirect toxicological consequences resulting from the modification, and (4) a morphological and behavioral analysis of the plant under relevant field conditions. The introduction of modified foods has led to a shift in the food safety assessment towards a greater need for whole food safety assessment.

**Direct toxicological effects as can be predicted from the transgene**

Standardized in-vitro and in-vivo toxicity tests are used to test the direct toxicity of the products introduced by genetic engineering. In addition, also homology searches in databases of known toxicants can be helpful in the safety assessment. The approaches used by companies to determine the toxicity of the introduced products have differed somewhat. Not in all cases exactly the same analyses have been performed. But in all cases the data provided for the crops currently on the market, were accepted by the authorities and considered adequate. The approach of assessing the direct toxicity as can be predicted from the nature of the transgene can differ depending on the type of modification. The simplest is when only one gene product is added, and no interactions with other components in the plant are expected. The situation becomes more complicated when multiple genes are involved and when the modification results in changes in one or more pathways. Knowledge on the involved gene products and the eventual involved pathways are then necessary to design the proper analysis and tests.

**Substantial equivalence**

To determine whether there are unwanted indirect effects resulting from the modification, the concept of substantial equivalence is used as a principle to guide the assessment. To test for such effects it has been determined that a comparison should be made between the modified crop and its non-modified counterpart. This is a rather indirect method looking only for symptoms. The comparison looks at substances that are relevant from a toxicological, nutritional, or wholesomeness point of view. There are no standardized lists yet of what substances to compare. For the crops currently on the market, such analyses have been made and showed no significant changes. But, if significant changes would be found, this would indicate that an unwanted effect has taken place resulting either from (1) pleiotropy, (2) insertion, or (3) somaclonal variation. The finding of such a significant change triggers further analysis to find the actual cause, but this may proof difficult. Probably, in not all cases an explanation will be found. In future perhaps new methods (proteomics, metabolomics, micro-array) will be available that are more able to make the comparison between the modified and the non-modified.

**Improving the food safety assessment**

To further improve the food safety assessment a proposal is done for an overall safety testing procedure (for details, see part E1 of this report).This procedure starting from the four points mentioned above should give a more explicit overall approach to the analysis. It is a two-step procedure leading to a judgment of the wholesomeness of the modified food. It should also make more explicit in what cases a history of safe use is enough to establish safety.

## 2. Safety of Genetically Engineered Plants: an Ecological Risk Assessment of Vertical Gene Flow

**Gene flow and hybridisation**

Sexual reproduction among crops, weeds and wild plants is possible on the conditions that (1) the crop and the weed or the wild relative are within a distance that pollination can occur, and (2) the plants are sexually compatible. Sexual compatibility is hampered by the existence of external and internal reproductive isolation barriers which determine whether or not viable hybrids are formed. In practice this means for a number of crops like maize, potato and tomato that there are no wild relatives in Europe in which successful hybridisation could occur. But it should be realized that genetic engineering, like classical breeding, has the potential to change reproductive isolation barriers. The experience with transgenic plants like oilseed rape shows that gene flow from these plants does take place as predicted from the knowledge on the sexual reproduction of these plants. Even though isolation barriers can limit the chances of hybridisation, in many cases chances of hybridisation are not zero and for risk assessment purposes the chances then have to be considered to be one.

**Codes to help risk assessment**

Gene flow indices (Dpdf) consisting of the three factors: (1) dispersal of pollen (Dp), (2) dispersal of diasporas (Dd), and (3) frequency of distribution (Df), can help in the risk assessment of genetically engineered crops, especially in the case of field trials. Through the combination of the three codes a risk classification of crops in a certain region can be made. The higher the risk class, the more stringent safety measures will have to be taken to prevent outcrossing. What currently is missing is a sort of risk factor for genes: a measure for the hazard

posed by a particular gene. If such a factor would exist it would truly help risk assessment. It is however difficult to foresee such a measure because a gene may be hazardous in one plant but harmless in another.

**Weediness**

Genetic engineering, like conventional breeding, is able to alter characteristics of crops resulting in a crop or its wild relative to become more weedy. In many crops, and especially the ones that have been domesticated to such an extent that they are no longer able to compete with wild species in natural habitats, the addition of a few genes is very unlikely to turn the crop into a weed. In crops that are still very close to their wild and sometimes weedy variants, the addition of one gene might be enough to trigger weediness.

Herbicide tolerance is not a major concern from the viewpoint of weediness. Only during application of the herbicide there is a selective advantage for the plants possessing this characteristic. However, to prevent acceleration of the selection of tolerant weed and possible future problems with (herbicide tolerant) volunteers it will be important to apply the necessary crop- and herbicide rotation.

**Selective advantage and improved fitness**

If a trait provides a selective advantage this means that after the outcrossing of the trait to a wild relative it has a good chance of being accumulated in the wild population. Selection results in the trait being preferentially attained. The fitness of a plant is commonly measured by the number of successful offspring compared to the offspring of other plants and it is always defined in relation to environmental variables. The concepts of selective advantage and fitness are used in risk assessment to help to determine the risks associated with a transgene. Traits related to the success of gene flow, resistance to biotic or abiotic stress might result in selective advantages or serious fitness improvements, if the absence of the trait in nature is an important determing factor in the existing ecological balances. Whether there is a real risk in specific cases can only be determined through thoughtful analysis and experimentation. It should be kept in mind that many of the above mentioned types of traits can also be obtained through classical breeding methods and that their possible ecological effects should be assessed in the same way.

Experience from conventional breeding and the introduction of exotic genes and genomes forms the proof of the fact that the introduction of new varieties and new crops has the potential to influence the natural flora. However, reports of fitness advancement for hybrids in natural ecosystems are rare. In opposite, gene flow from domesticated crops has mostly made the wild plants less competitive. The experience from non-transgenic practice directs us to take great care.

**Ecological view and consequences for risk assessment**

For the future risk assessment a rational stepwise approach is necessary taking into account the knowledge of the crop and its wild relatives, knowledge on the biogeographical situation, and knowledge on the transgene. The testing of the transgenic crop should follow a step-by-step procedure evaluating data of a first step before stepping into a next phase. The more risky the crop and/or the transgene, the more stringent the testing scheme will have to be before the transgenic crop can be allowed to be grown commercially on a large scale. But in the end one dilemma will remain: even after the most careful risk assessment process, only a mass release will bring all effects to the surface. The small-scale field trials do not allow to investigate the ecological risks of widespread commercialization. Therefore in order to achieve sustainability in cultivating transgenic crops, the focus should be on long term monitoring of several years in the same field where the transgenic crop was planted.

# 3. Effects on Non-target Organisms of the Release of Genetically Modified Crops into the Environment

**Non-target effects**

Non-target effects are defined as the unwanted (negative) effects of crops or their accompanying farming practice on organisms living in or around the agricultural field that are not intended to be hurt. Although the topic here is the impact of transgenic organisms on non-target organisms, the impact of new modified varieties produced using traditional breeding may be as significant, if not more so.

Non-target effects could arise from a number of factors: (1) the gene product affects organisms for which the plant was not designed, (2) there may be effects as a result of changed agricultural practice.

Farming, especially in Europe, is known to have profound effects on the environment since large portions of the countryside are farmed. The intensification of agriculture has led to changes in the farmed environment. The use of chemicals also has had a clear effect on the organisms living in the farmed environment. There are for instance examples known of the use of certain chemicals leading to a decline in bird life.

**Genetically modified crops**

Only a limited amount of genetically modified traits have reached the market and are now being grown on a large scale. Non-target effects are not limited to these transgenic plants, but they may exacerbate the problem as they are specifically designed to modify farming practice. Changes could be both positive or negative. Changes can also be transferred to the natural environment if the transgenic trait is transported to wild relatives through cross-pollination.

An impact analysis for examining the likely effects on non-target organisms should consider: (1) those species reliant on the crop itself, whether through using it for food or shelter, (2) those plants and animals that live within the field and might be damaged if changes are made to the crop that modifies their habitat or their ability to survive, (3) plants and animals living in the field margin or hedges and walls, if the management of the crop modifies the size, extent or susceptibility to herbicides and pesticides of this field area, (4) that soil and soil organisms may be affected by the changes in plant variety or management.

Here, we do not give specific recommendations of what species to consider, but in determining them, the following is relevant: (1) how far should one go in testing the effects on non-target organisms? Should we test all organisms, or only a limited number that is considered useful or relevant from a biodiversity point of view? (2) Specific species and their number may differ from region to region. (3) Tests performed should be relevant and validated.

**Insect resistant crops versus insecticides: non-target effects**

Insecticides can have a significant effect on non-target organisms depending on the insecticide, the time and way of application. Not all insecticides are as selective as one would hope for.

Bt crops are the most well known genetically modified insecticidal crops. Bt is highly selective against certain classes of insects. The action of one particular Bt protein can be against one or a few insects and the possibility exists that the Bt crops hits sensitive insects that live in or around the crop. An intensive discussion has emerged after it was shown that larvae of the Monarch butterfly were sensitive to the Bt protein present in Bt maize. A number of laboratory and field studies have been performed to test whether the Bt protein would actually pose a serious risk to the Monarch. The results of these studies point towards a limited risk.

At this moment there is no comparison made between the non-target effects of the modified versus the non-modified practice. It will be difficult to do so, but it would probably be a fair way of weighing the risks versus the benefits. When comparing the two practices the following may be relevant to consider for both practices: (1) the insecticides used, (2) its type of use, (3) direct toxicity to relevant organisms, (4) total amount of active ingredient, (5) type of exposure to non-target organisms, (6) biodegradability, and (7) effectiveness.

**Non-target effects of herbicide tolerant plants**

Herbicide tolerant plants may have an effect on the type and number of weeds present in a field. This shift could have an effect on non-target organisms. On the other hand the genetically modified practice replaces a practice in which often more and different herbicides are used. In that case the number of weeds may be greater, but on the other hand the herbicides themselves could have an unwanted effect. Like for insect-tolerant plants the comparison between the two practices is not explicitly made.

**Other modifications and their effects on non-target organisms**

In the future many other types of modifications will be introduced ranging from plants with changed nutritional characters to plants that resist abiotic stress like drought. All of these crops can have an impact on non-target organisms and they should be considered in the manner proposed above. Especially the introduction of a genetically engineered crop into a region where this crop was never able to grow before, like the introduction of a conventionally bred crop into a new habitat, will have an impact on the organisms living there.

The experience with the current genetically engineered crops, engineered to replace conventional agricultural practices, has not revealed indications of dramatic effects on non-target organisms of the growing of these new crops.

## 4. Allergenicity of Foods derived from Genetically Modified Organisms

**Food allergy and food allergens**

Food allergy can be defined as a food hypersensitivity – an adverse reaction to food – in which the reactions are primarily immunologically mediated. A sensitizing food or food constituent, when eaten, is partly degraded, absorbed and triggers a reaction in the immune system. IgE antibodies play an important role. Food allergy associated clinical symptoms involve the oral cavity, the gastrointestinal tract, the skin, the respiratory tract and the circulatory system. The symptoms may range from mild to very severe anaphylactic shock.

Food allergy is present in about 1.5-5% of the general population, corresponding to 8-10% in the paediatric population, and around 1% in adults. Many children 'outgrow' their allergies. Most (major) food allergens have a number of features in common: they are glycoproteins with a molecular weight between 10-70 kD, are often relatively stable to acid- and heat-treatment, and relatively resistant to digestive breakdown. Of the worldwide documented food allergies over 90% is caused by 8 food or food groups: peanuts, milk, eggs, soy-bean, tree nuts, fish, crustacea and wheat.

**Allergenic potential of foods derived from genetically modified organisms**

Since only a limited number of traditional foods or food groups are known to cause allergies there is, from a scientific viewpoint, no serious indication to expect that new transgenic foods will more frequently result in

allergies, but there still may be changes with allergenic consequences that have to be considered in a safety assessment.
In case new genes are added to a food crop, the source of the gene is of ultimate importance, for it is possible that genes coding for allergens could be transferred. If a source has an unknown history of safe use in food production one has to be cautious as well. Also random changes could occur that may have consequences that are relevant from the viewpoint of allergenicity. On the other hand, genetic engineering can also be used to eliminate allergens from foods.

**Assessment of the potential allergenicity of (new) food proteins**

Current strategies to assess the potential allergenicity of genetically engineered foods are based on a case-by-case screening using a decision tree as suggested by IFBC/ILSI. The strategy starts from looking at the source of a particular gene. In the approach different immunochemical, in vivo, and physico-chemical analyses are used to assess the potential allergenicity. What tests are actually performed depends on the source of the genes involved and the results of particular tests. The approach has a rather good positive predictive value in case of (known) allergens for which sera of allergic patients exist. Its positive and negative predictive value in case of potential allergens for which no sera of allergic patients exist is less good, but it is generally considered to result in a *reasonable certainty of no evidence of allergenicity*. Questions with regard to its positive and negative predictive value are based upon concerns regarding some statistical aspects in cases where sera of allergic patients are very difficult to obtain, and on the fact that the physico-chemical analysis in the 'right hand arm' of the decision tree is mainly based upon some general characteristics that most major allergens have in common. The fact that there are exemptions in both ways (there are proteins that possess these characteristics that are not allergens, and there are proteins that do not possess these characteristics that are allergens) undermines its predictive value. This is why recently an FAO/WHO expert consultation has proposed alterations to the original decision tree to take into account the latest knowledge and techniques. Important alterations are that the endpoints of the assessment now is an estimate of the likelihood of allergenicity and that additional targeted serum screening and animal models have been taken up in the assessment. Wether the alterations indeed lead to a greater positive and negative predictive value will depend on the actual performance of the animal models.

**From assessment of potential allergenicity towards allergy risk assessment**

Another concern with the current approach is that it is focused mainly on the allergenic potency. It does only in a minimal sense take into account that there are differences between minor and major allergens, in some cases there even do not have to be relevant risks on health effects. Also, even though a certain protein is found to have allergic potency, it does not have to result in the sensitization of consumers as a result of too low concentrations in the food or factors in the food processing. From the current approach mainly based on hazard identification it is therefore proposed to go towards a real risk assessment and risk management approach. In such an approach data on the allergenic potency relative to known allergenic potencies of existing allergens and on the expected exposure would be included. In such an approach perhaps a distinction could be made between minor and manageable risks and major risks that could better be avoided.

## 5. The Biosafety of Antibiotic Resistance Markers in Plant Transformation and the Dissemination of Genes through Horizontal Gene Flow

**Microbes and genes**

Microbes and especially bacteria are an integral part of human life. For instance one gram of human faeces contains approximately 100 billion bacteria of more than 50 genera. Bacteria, but also bacterial genes are present everywhere in our environment. Also antibiotic resistance genes are present in large amounts. These genes are

required for the self-protection of microbes that produce antibiotic substances themselves.
Antibiotic resistance genes have become key tools in genetic engineering to enable researchers to select transformed clones that have taken up the fragment of DNA under study. This fragment and the antibiotic resistance marker gene are physically linked for this purpose.

**The antibiotic resistance marker genes used in plant genetic engineering**

Also in plant genetic engineering antibiotic resistance marker genes are widely used to be able to select the plants that have taken up the desired DNA fragment. The most important ones that are used are *bla* TEM1 (ampicillin and amoxicillin resistance), *aad* (streptomycin/spectinomycin resistance), *npt-II* (kanamycin/neomycin resistance), *npt-III* (kanamycin/neomycin/amikacin resistance), *hpt* (hygromycin resistance), and cat (chloramphenicol resistance). The *npt-II* marker gene is the one that is mostly used in the plants on the market and in field trials, but also *bla* TEM1 appears in a marketed crop.

**The use of antibiotics versus natural antibiotic resistance**

Kanamycin and neomycin are rather toxic antibiotics that are of no clinical importance. Ampicillin and amoxycillin are widely used in human and animal chemotherapy but their use is declining. Resistance to all these four antibiotics is widespread in nature. The spread of the *bla* TEM1 resistance gene through horizontal gene transfer to different species of bacteria is well documented. The antibiotic resistance genes are present in large resistance reservoirs from which the gene can be easily picked up by bacteria when there is a need to do so.

**Transgenic plants as a potential contributor to the spread of (antibiotic resistance) genes**

There is no dedicated mechanism of horizontal gene transfer from plants to bacteria. For bacteria to be able to pick up a plant gene a number of requirements have to be met: (1) plant cells (i.e. of decaying plants) should release DNA fragment in the environment of at least the size of an average gene, (2) the DNA should persist in the environment for a longer period of time, (3) bacteria should be able to take up the released DNA, (4) the DNA should be stably established in the recipient cells, and (5) the establishment should at least be neutral so that the transformed cells are not counter-selected. The uptake and expression of plant DNA by bacteria is therefore a multiple step process in which each step can be a limiting factor, making it a very improbable event. It has never been found under natural conditions. Laboratory experiments show that the probability of the event is below $10^{-11}$.
Calculations show that the number of *bla* TEM1 antibiotic resistance genes present in soil is larger than the number of genes present in for instance transgenic maize that contain these genes. The genes in the soil are also present on highly transmissible plasmids, where in the plants they are very much less transmissible.
It can be concluded that it is many factors more likely that a bacterium acquires an *npt-II*, *bla* TEM1, or *hpt* gene from a plasmid present in the different resistance reservoirs than from a transgenic plant.

**Horizontal transfer of DNA ingested by mammals**

Mammals daily eat DNA in large amounts and there is large history of safe intake of DNA. Nonetheless one has tried to determine the fate of DNA in the intestinal tract. M13 DNA was fed to mice to test this. No M13 phages could be found in the faeces of the treated animals, but surprisingly M13 DNA fragments could be found in the faeces, in the blood stream and even integrated in some mice cells including cells of the foetuses borne by the pregnant female mice. There still are many questions surrounding this finding and no further results have been reported. It opens up the question of the potential mutagenic role of ingested DNA.

# D. Discussion

## 1. Differences and similarities between genetically engineered and traditional crops

There are differences as well as similarities between genetically engineered and conventionally bred crops. The differences lie in the fact that through genetic engineering species barriers no longer exist. Genes of any organism can be introduced into a plant. In traditional breeding different species have been crossed that do not cross in nature (known as 'wide crosses'), but crossed species still belong to the same plant family. Another difference is that in genetic engineering only one or a few known genes are transferred, whereas in classical breeding about half of the genome is being transferred, without knowing the exact properties of the products of these genes and their effects on the plant.

One similarity between genetic engineering and conventional breeding is that undesired 'insertion effects' can be present in both (see chapter 1 in part E). These effects can be the result of knocking in a gene somewhere in the genome (genetic engineering), or of (wide) crosses in which pieces of genomes are put together in a rather rough manner, but also of transposon activity or irradiation (conventional crops). Small changes to plants resulting from somaclonal variation are also already known from classical breeding where they are the result of in vitro propagation methods. They deliberately make use of this phenomena to create additional genetic variation.

**Conventional varieties and conventional agricultural practices do have undesired effects, and they do not represent a zero risk scenario. In dealing with transgenic crops it is only fair to take this into account.**

Other similarities can be found in the final result of genetic engineering and classical breeding. The introduction of herbicide tolerance through genetic engineering or through classical breeding for instance: the effects on the use of the specific herbicide, the effects on farming practice, but also the effects on the environment can be comparable. In the reviews in part E of this report it is shown that many effects of genetic engineering also result from classical breeding:

- A conventional pest resistance can outcross to wild compatible species as well.
- Any piece of (plant) DNA can be taken up by bacteria – this phenomena is not restricted to antibiotic resistance markers – although there is no dedicated transfer mechanism and chances are extremely low. In the end selection pressure will play an important role in whether sequences will be maintained or not.
- The impact on non-target organisms of new varieties using traditional breeding techniques may be as significant as the impact of transgenic crops, if not more so.
- It is not excluded that a traditional variety can be toxic or allergenic. Allergenicity will come to the surface in practice – see the kiwi example -, but toxicity, and especially chronic toxicity, is very difficult to notice because food is a very complex mixture of compounds that can sometimes interact. And even if a minor effect is seen, it will be very difficult to attribute it to one particular source.

These arguments are only given to point out that in discussing transgenic crops the effects of current practices should not be forgotten.

Both with genetic engineering and conventional breeding it is possible to create plants with undesired or even hazardous properties. So there is good reason to analyze them carefully, before introducing them into the field or into the food chain.

There is however still good reason to look very seriously at genetically engineered crops, because there are differences with classical breeding, and we are able to do things we have never been able to do before. Genetic engineering can generate plants with undesired or even hazardous properties, and the health of humans as well as the environment should be well protected. A second reason to look at them carefully is that transgenic crops have no history of safe use.

Because existing (species) boundaries have been broken with genetic engineering and there is no history of safe use, a close and careful look at genetically engineered crops is necessary.

## 2. The applied safety standard

When the regulations were developed for assessing the safety of genetically engineered crops, especially with regard to their food safety, it was determined that the endpoint of the safety assessment should be the safety level of conventionally bred crops. A GMO should be as safe as a conventional variety. It is important to notice that conventional varieties do not represent a zero risk baseline, neither for food safety, nor for environmental safety.

It can be questioned whether the practice that is being established for assessing the food safety of gm crops still takes conventional crops as a baseline reference. The most important argument is that many of the effects that can be present in gm crops also can be present in conventional crops, while for these crops almost no tests are legally required. Secondly, the amount of safety tests is increasing and the tests are becoming more precise. The safety level increases as a result of these developments, or at least the level of uncertainty about the absence of unwanted effects decreases. The conventional crops reference baseline is also hampered by the fact that conventional crops are too much a sort of black box. Safety of these crops is assumed by their history of safe use, and does not result from required safety testing. The net result might be that the actual safety level of gm crops will ultimately be higher than the level attained by conventional varieties. As food safety is the number one priority for European consumers (as a result of BSE and dioxin food scares), such improved safety levels for gm crops will be appreciated. But it also opens the discussion on what the food safety standard for new conventional varieties should be. It should also be realized that increasing the safety standard literally will have its price.

Because of improving knowledge and improving safety testing the safety level of genetically engineered crops is increasing. Ultimately they might reach a safety level that is higher than that of conventional varieties.

For the environmental safety, especially in Europe, conventionally bred varieties are not taken as the baseline reference. The environmental safety of genetically engineered crops is dealt with as such. There is no cost-benefit analysis in which the environmental effects of genetically engineered crops are weighed against the effects of current agricultural practices. This leaves much room for discussion and the question is what kind of effects on the environment can be accepted and to what extent. Somehow it should be possible to get a better definition of undesired environmental effects or a set of broadly defined criteria.

But even after agreement on the baseline or reference point to which genetically engineered crops should be compared, one point of discussion may still be open: Should genetically engineered crops be an improvement from an environmental point of view when compared to existing technology, or is it good enough that they reach the same level as conventional crops. Some people argue that these new genetically engineered crops should only be allowed when they represent an actual environmental improvement. The discussion on this topic, however, might be circumvented by defining unwanted environmental effects or a set of workable safety criteria.

A better definition of what types of environmental effects are acceptable, and how much uncertainty about the absence of unwanted environmental effects is acceptable can greatly help the risk assessment of genetically engineered crops. This would even be more so if this understanding would be translated into a set of broadly defined criteria for these crops.

With regard to the safety of transgenic crops in the end one dilemma will remain. One can do the best of safety testing and the best of risk assessment (case-by-case and step-by-step in a multi-year effort), but only a mass release over a longer period of time will bring all possible risks and negative effects to the surface. Exactly this is the greatest concern of those who are critical on genetic engineering: even if testing does not bring to the surface any negative effects, could it not be possible that in the longer term negative effects will turn up? Perhaps this is the same for the introduction of any new technology, and to a certain extent, this may also be true for the classical breeding.

In the end unexpected long-term effects of the large-scale application of genetically engineered crops remain the most important concern.

## 3. Current knowledge and lacunas

The introduction of transgenic plants has led to an intensification of risk assessment research. Crucial in risk assessments is knowledge on the conventional systems as a baseline reference: knowledge on ecosystems, knowledge on effects of current practices, on plants, on birds, on insects, etc. This is because familiarity is the major concept on which the risk assessment of transgenic crops builds. Familiarity provides data on the behaviour of non-modified crops as a baseline. But it also provides information that is necessary to be able to design relevant risk assessment experiments that have a good predictive value.

Knowledge of natural phenomena is crucial to be able to perform risk assessments for both conventional and transgenic crops. Not only as a baseline, but also as necessary information to be able to design experiments which have a good predictive value. The risk assessment of transgenic crops will work as a catalyst in the understanding of natural phenomena.

The debate and research on the safety of transgenic crops has led to an increased understanding of what we know and especially on what we don't know of natural phenomena, and especially on what we don't know on the effects of conventional varieties. For instance, it is admitted that very little is known about the potential long-term effects of any food. There is however a wealth of knowledge on all kinds of natural phenomena which should be used to its full potential, but for the risk assessment of transgenic crops specific required data can be missing. For risk assessment of transgenic disease resistant plants, which can hybridise with wild relatives, it may be necessary to gather more information on the presence of the disease in wild habitats. For effects on non-target organisms, and especially the indirect effects on these organisms, specific information may be necessary on the organisms that live and rely on the crops and the interactions that they have.

The increase in knowledge on natural phenomena will contribute to a better understanding of the behaviour and effects of both transgenic and conventional crops. It will not lead to zero risk, but the certainty of no harm will improve. Such an improved level of certainty of no harm will then undoubtedly become the standard.

## 4. Conclusions with regard to current crops on the market

The approved marketing dossiers show that there is (or at least was) no standardized way of doing a risk assessment. Companies have on their own initiative provided a set of data to the competent authorities, taking into account the information requirements and risk assessment guidelines. But these requirements are not always clear in terms of what they mean for necessary data to be provided. This has led to dossiers where competent authorities have asked additional questions and to companies performing additional tests and providing additional information. To a certain extent differences in provided data are logical, because it often concerns GMO's with different properties, but on the other hand there are examples of comparable GMO's where the provided data and tests differ.

The current transgenic crops on the market are with the first to have gone through the regulatory approval process. Sometimes there are differences in the type of data present in the dossiers and the data that experts would ideally see as the results of a risk assessment process. But even with these start-up problems all these transgenic crops are considered to be as safe as their conventional counterparts.

Sometimes also the type of data that is present in the dossiers does not totally meet the type of data that experts would ideally want to see as the result of the risk assessment process. But even with that taken into

account the experts seem to see no indication of the transgenic crops currently on the market, to present any danger for human health or the environment. For this we refer to the cases that are presented in the reviews in part E of this report.

## 5. Future directions for the risk assessment of (genetically engineered) crops

The modifications that have been seen so far have been relatively simple, herbicide tolerance being the type example. In the future, modifications are expected to become more complex. First examples can already be seen, as for instance the 'golden rice', where multiple genes were introduced to incorporate complete pathways. Especially for the food safety assessment this will have consequences. Whole food assessments are likely to be needed in more cases. It is exactly in this area that the current food safety tests have the least predictive value and will have to be further improved.

Also the modifications until now have not yet really altered the fitness of the crops. This is however expected to change in the future and the focus will have to turn even more on the environmental effects of the transgenes themselves.

The current approach for the testing of allergenicity is well suited for identifying existing allergens that have been transferred as a result of the modification. When it concerns minor, new allergens, the predictive value of the allergenicity decision tree is less good. It does result in a strong reduction of the likelihood to cause harm, but it does not mean that a certain protein cannot turn out to be allergenic to some extent. Improvements to this decision tree have been proposed by a FAO/WHO expert consultation. Animal models have been introduced to improve the predictive value.

Until now, the transgenic crops have only replaced existing conventional varieties of the same crop and have not yet led to the introduction of crops into areas where they have never been grown before. With regard to the environmental effects it is exactly these kinds of introductions that have to be done very carefully, as the risks of undesired effects are likely to be much higher. The introduction of the crop itself already will have to be considered to be a significant change to which the ecosystem will have to adapt.

## 6. Overall conclusion

The goal of this report was to provide relevant information on the biological questions that are most frequently asked about genetically engineered crops. It is shown that quite some information on these questions is available. It is also shown that improvements are still possible in the risk assessment process and that we probably will keep on improving it as our knowledge increases. However, to better guide the risk assessment we urgently need a better understanding of the baseline to which genetically engineered crops are assessed. If this baseline is our conventional practices then it is shown these do not present a zero risk baseline.

In the end it is important that the assessment of both genetically engineered crops and conventional crops is

fair, and that effects are prevented on human health and the environment that we have agreed upon that should not take place.

This report is neither a beginning nor the end of the debate on the safety of genetically engineered crops, but we hope that it is a useful contribution to this debate. The floor is now open for further discussion and we invite all parties to step in.

# E. Safety Aspects of Genetically Engineered Crops

**Toxicity and Food / Feed safety of Genetically Engineered Crops**
*Jan Pedersen, Folmer D. Eriksen, Ib Knudsen*

**Safety of Genetically Engineered Plants: an Ecological Risk Assessment of Vertical Gene Flow**
*Klaus Ammann, Yolande Yacot, Pia Rufener Al Mazyad*

**Effects on Non-target Organisms of the Release of Genetically Engineered Crops into the Environment**
*Julian Kinderlerer*

**Allergenicity of Foods derived from Genetically Modified Crops**
*André Penninks, Leon Knippels, Geert Houben*

**The Biosafety of Antibiotic Resistance Markers in Plant Transformation and the Dissemination of Genes through Horizontal Gene Flow**
*Philippe Gay*

# Toxicity and Food Safety of Genetically Engineered Crops

Authors: **Jan Pedersen, Folmer D. Eriksen, Ib Knudsen**

**Danish Veterinary and Food Administration**

**Institute of Food Safety and Toxicology**

**Morkhoj Bygade 19**

**DK-2860 Soborg, Denmark**

1. Food, toxicity and genetic engineering ..... 28
1.1 Learning by trial and error ..... 28
1.2 Understanding the toxicity of current foods ..... 28
1.3 Traditional breeding and toxicity ..... 29
1.4 Safety assessment of traditional food crops ..... 30
1.5 Genetic engineering and toxicity ..... 30
1.6 Differences between genetic engineering and traditional breeding from a toxicological point of view ..... 31
1.7 Formal food safety assessment of genetically engineered foods ..... 32
2. The food safety assessment of genetically engineered plants; the appearance of a new paradigm ..... 33
3. Evaluation of possible direct toxicological effects resulting from the introduction of a sequence ..... 35
3.1 Introduction ..... 35
3.2 The product of the inserted gene is known in relation to food ..... 35
3.3 The product of the inserted gene is not known in relation to food ..... 38
3.4 The genetic engineering results in reduction of the expression or knockout of a gene ..... 39
3.5 The inserted gene(s) is (are) involved in change of a pathway or introduction of a new pathway ..... 39
3.6 Conclusion on direct toxicity testing ..... 40
4. Determining substantial equivalence ..... 41
4.1 How to determine substantial equivalence ..... 41
4.2 Genetically engineered crops on the market and determination of their substantial equivalence ..... 44
4.3 Non-substantial equivalence ..... 45
4.4 Expected future developments in the food safety assessment of genetically engineered food ..... 46
4.5 Conclusion ..... 47
5. Improving the overall food safety assessment procedure of GM crops ..... 48
5.1 The current food safety assessment procedure of GM crops ..... 48
5.2 A proposal for an overall food safety assessment procedure of GM crops ..... 49
6. Discussion ..... 52
7. Cases ..... 54
7.1 Tomatoes with extended shelf life (Zeneca) ..... 54
7.2 Bt herbicide tolerant maize (Novartis, event 176) ..... 56
8. References ..... 59

# 1. Food, Toxicity and Genetic Engineering

## 1.1 Learning by trial and error

Since the beginning of history, man has eaten what nature has provided for. In this, plants have always been and still are an important source of food. By trial and error man has learned that only some plants can be safely eaten. Many other plants proved to be toxic. During domestication man has started to farm only those crops that were known to be safe to eat. Some of them, they learned, could only be safely eaten after being cooked or treated otherwise. Knowledge on the safe use of food crops was passed from generation to generation. A relatively recent example on how man learned about the toxicity of food by trial and error is the introduction of potato in Europe. People died as a result of eating the poisonous berries of the potato plant. Later they found that the tuber could be safely eaten after being cooked (the anecdote being that they ate a potato that was burned in a fire). Now it is a major source of carbohydrates.

## 1.2 Understanding the toxicity of current foods

The toxicity of food always has been a point of attention, and with good reason. During the last century, and especially in the last 50 years, man has learned more about what constitutes the toxicity of many plants as a result of chemical analyses and toxicity testing. By trying to find the reason why some plants could only be eaten after treatment, it was found that many of our current food crops contain certain levels of toxic substances, many of which are destroyed after the right treatment such as for instance cooking. Table 1 shows some of the most well known natural toxicants in a number of common crops.

Food as such proves to be a very complex mixture of many different chemicals that provide man and animals with essential macro- and micronutrients and energy. As already stated some of these components proved to be toxic, but others are thought to have disease-preventive or health-promoting properties. Some food compounds will display both beneficial and toxic properties depending on the amounts ingested. It is this tenet of toxicology that the dose differentiates between a poison and a remedy and it is therefore the intake level that is critical in determining the position of the balance between health effects and toxicity.

Table 1: Natural toxicants in selected crop plants: (Data compiled from Holm et al. 1998)

| Crop plant | Toxic constituent | Organ of expression |
|---|---|---|
| Wheat | Dhurrin | Young vegetative plant parts |
| | Lectins | |
| | Proteinase inhibitors | Grain |
| Rice | Trypsin inhibitor | Grain |
| Maize | Cyanogenic glycoside | Young vegetative plant parts |
| | Trypsin inhibitor | Grain, Cob |
| Barley | Epiheterodendrin | Young vegetative plant parts |
| | Trypsin inhibitor | Grain |

| Crop plant | Toxic constituent | Organ of expression |
|---|---|---|
| Soybean | Saponins | |
| | Lectins | |
| | Coumestrol | |
| | Daidzein | Seeds |
| | Genistein | Seeds |
| | Proteinase inhibitors | |
| Sorghum | Dhurrin | Cyanogenic glycosides in unripe plants |
| Cassava | Linamarin* | Tubers, total cyanogenic glycosides |
| | Lotaustralin* | contents (preliminary limit 10 mg/kg, JECFA) |
| | Proteinase inhibitors | Tubers |
| Potato | α-chaconine* | Tubers, Total glycoalkaloid content (limit at 200 mg/kg in old varieties and 100 mg/kg in new varieties, Nordic Working Group) |
| | α-solanine* | |
| Tomato | α-tomatine | |
| | Nicotine | |
| | Lectins | |
| Rapeseed | Glucosinolates | 9 different compounds |
| | Erucic acid* | (limit 5% for food consumption in DK) |
| | Saponins | |
| | S-methyl-L-cysteine sulfoxide | (toxic amino acid) |
| Chicory | Lectins | |
| | Lactucin | Leaves, root |
| | Lactucopicrin | Leaves, root |
| | 8-deoxylactucin | Leaves, root |

*Toxic components in plants with limit level for food consumption, recommended not to be exceeded

## 1.3 Traditional breeding and toxicity

Man has been selecting and breeding plants for many, many years. Plants have been selected with the purpose of obtaining plants with desirable properties, for instance higher yield or better taste. As a result of this domestication and plant breeding, plants have been changed dramatically, both in their morphology and behavior. Many methods have been used to introduce changes: crosses within species, mutation breeding[1], or methods for wide crosses[2] where it is possible to increase the gene pool and to include new resistance genes (Goodman et al 1987). Other methods to increase the variation in the genome are polyploidy induction[3], chromosome manipulations, and somaclonal variation[4]. These methods all result in the introduction of changes at the genome level, for instance point mutations (either spontaneous[5] or induced), recombinations,

[1] More than 460 commercial varieties of food plants have been produced following induced mutations using chemicals or irradiation, primarily in barley (Lindsey and Jones 1989). In mutation breeding, mutations are created at random in the plant genome and plants with desirable phenotype are selected.

[2] Wide crosses are crosses between plant species that do not cross breed in nature, for instance cucumber and melon. Special laboratory techniques have to be used to obtain fertile offspring.

[3] Polyploidy induction is the induction of plants with a higher copy number of chromosomes. In normal plants the polyploidy number is 2N (two copies of each chromosome). In polyploidy induction the copy number can be altered for instance to be 4N.

[4] In tissue culture a new plant is derived from one single somatic plant cell. It was expected that tissue cultured plants derived from one and the same parent plant would all be the same. In practice these plants show variation, which is called: somatic variation. This variation is thought to be the result of the callus phase which is associated with chromosome instability. In tissue culture the new plant is derived from one single somatic plant cell.

[5] The plant genome is a dynamic biological element with high capacity for changes. Spontaneous mutations followed by recombination are the major source for genetic variability. Gene mutations occur naturally at a low frequency, in the order of 1 in $10^4$ to $10^6$ alleles for individual loci (Maessen and Derksen 1998). These changes are used in traditional plant breeding where most of the breeding methods are based on recombination or other changes to the plant genome.

chromosome rearrangements, transposon insertion[6], and/or addition of whole chromosomes, or parts of chromosomes from other species (for instance Triticale). These alterations make that genes are switched on or off, genes are added, which can result in changes in levels of proteins, in signal transduction pathways, metabolic pathways, etc.

It is obvious from this that plant breeding can result in plants with higher or lower levels of natural toxicants. The level of natural toxicants in crop plants as it is today, is the result of all those years of domestication and breeding. It can be argued that the current level of toxicants is lower than in the crops' wild ancestors. This is because breeding has been directed towards increasing the yield of crop plants in well-controlled agricultural systems. In these systems plants are provided with chemical fertilizers for growth and chemicals for protection from weed competition, plant diseases, and insect infestation, thereby partly substituting the plants own heritable protection mechanisms, leaving room for allocation of energy towards higher yield. The amount of natural resistance genes or their level of expression has been lowered as a result. Most of the plant compounds, produced by natural resistance genes, are secondary metabolites. It is among these secondary metabolites that most of the compounds are found that are potentially toxic. Saponins, for instance, are known toxic secondary metabolites involved in resistance (Papadopoulou et al 1999). Re-incorporation of natural resistance genes from wild plants by conventional breeding techniques can potentially contribute to increasing the toxicity of crop plants.

## 1.4 Safety assessment of traditional food crops

Most national authorities accept that new varieties of already marketed food crop plants can be introduced without any formal toxicological assessment provided that the levels of a few specific toxic compounds in some crop plants are not exceeded, cf. table 1. For instance, in the Nordic countries (Nordic Council 1998) it is recommended, that the total alkaloid level in potato ($\alpha$-solanine and $\alpha$-chaconine) is less than 200 mg/kg for varieties on the market and less than 100 mg/kg for new varieties (Slanina 1990). There are however no legal requirements for these levels.

The rationale behind this acceptance without formal assessment, is a "history of safe use", not backed up by empirical evidence, but built basically on anecdotal evidence of no harm. The validity of this approach from a safety point of view can be questioned in a world of fast changing eating habits. Also many new studies of the positive and negative health effects of food plant constituents are now being published going far beyond the few well-known toxic compounds. It is even admitted that very little is known on the potential long term effects of food (WHO, 2000).

## 1.5 Genetic engineering and toxicity

Genetic engineering of plants[7] results in the introduction of one or a few known genes or sequences that either express one or more new constituents in the plant, or change the expression of existing constituents, either positively or negatively. This newly introduced DNA can result directly in the production of toxic

[6] Transposons are mobile genetic elements that can initiate recombination events leading to genome rearrangements. Insertion in a structural gene usually results in dysfunction of the gene and insertion into regulatory elements can change the expression. If the transposon contains promoters and/or enhancers, the gene can become subject to an entirely new regulator regime (McDonald 1995). Active transposons are repressed in most cells by RNA interference (Tabara et al. 1999) and methylation of their DNA sequences. Other mobile elements such as retrotransposons are also common in plants (Schmidt 1999). In maize 50% of the genome are repetitive sequences of the retrotransposon type (SanMiguel et al. 1996).

[7] The preferred methods of genetic engineering of plants at this moment are *Agrobacterium tumefaciens* T-DNA mediated transformation and biolistic (micropojectile bombardment) transformation.

constituents, or, in case the expression of toxic constituents in the plant is changed, to a decrease or increase of the amount of toxic constituent that is present.

Genetic engineering can however, also have indirect effects that lead to the production or accumulation of toxic constituents. Such side effects can be the result from either insertion, pleiotropy[8] or somaclonal variation. Insertion of a transgene into a host plant is, by the methods available today, a random process, which can turn the regulation of other genes, e.g. for toxicological compounds, both up and down (Gelvin 1998). For instance if the transgene inserts into a gene involved in the regulation of a toxic constituent, this insertion may lead to changed levels of this constituent being present in the plant.

At this moment there are no alternative transformation methods in which such possible side effects, like insertion side effects, can be prevented. However, technology is evolving and there is work being done to develop methods of homologous recombination in plants. One recent example with only limited applicability is the method employed by Pioneer Hi-bred International using a chimeric RNA-DNA oligonucleotide to introduce a one-nucleotide change in a acetohydroxy-acid synthase (AHAS) gene resulting in resistance to the herbicide 'Lightning' (a mixture of imazethapyr and imazapyr) (Zhu et al. 1999, 2000).

## 1.6 Differences between genetic engineering and traditional breeding from a toxicological point of view

Both the introduction of transgenic gene constructs and traditional breeding techniques are able to cause unpredictable (and/or undesired) changes of the expression of host genes. It is not possible in a general way to predict the toxicological aspects of randomly placed genetic modifications. On the other hand, there is already experience with insertion effects from traditional breeding as caused by the introduction of foreign DNA through genetic engineering, from traditional breeding. The insertion of a transposon for instance causes after all similar effects. Also, breeding mechanisms such as natural mutations, induced mutations, transposon activity and somaclonal variation generate the same type of random changes as insertion by genetic engineering.

The principal difference between traditional plant breeding and genetic engineering from a toxicological point of view first appears by expression of an inserted gene(s) that normally would not be present in that plant in form of a transcription product (RNA) and in most cases also in form of a protein. The crossing of species barriers makes it possible to introduce proteins that are not known to the plant and not known with respect to exposure to humans.

In traditional breeding a 50/50 mixture of the genetic material of the two parent plants is formed after a first crossing. Mostly one is only interested in the addition of a limited amount of genetic material from one plant to the other, and in the crossing many (indirect) unwanted properties can be present as a result of the 50/50 mixture. This is why in traditional breeding an extensive selection program in many generations is performed. The practice of consecutive backcrossing[9] is also a common procedure used to eliminate unintended effects.

[8] Pleiotropic effects are in this paper defined as the not intended and not expected changes caused by the expression of a transgene other than the intended effect of the newly introduced gene product. If the new product is an enzyme expected to work in a specific metabolic pathway and if this enzyme also shows (unknown) activity in another metabolic pathway, this derived activity is named a pleiotropic effect.

[9] Backcrossing is the crossing of the hybrid plant with one of its parents. So if the plant AAAA is crossed with the plant BBBB thereby creating the hybrid AABB, then backcrossing is the crossing of AABB with the parent plant AAAA, thereby creating the plant AAAB. Consecutive backcrossing leads to the reduction of the amount of 'foreign' genetic material from 50% to very small percentages (5% or less, depending on the number of backcrosses).

The combination of selection and backcrossing in traditional breeding removes unwanted effects to such a degree that new varieties are in general considered as safe as the genetic starting material.

In genetic engineering a 999/1 (often even far less) mixture of the genetic material is formed after the transformation: only one or a few genes are added to the existing plant genes. Backcrosses to eliminate unwanted genetic material are not necessary. But like for conventionally bred crops, also for genetic engineered plants an extensive selection process takes place starting with many independent transformants from which well-performing lines are selected for further research. In this way, like in traditional breeding lines with marked unwanted effects are eliminated. When finally a desired transgenic line is obtained it will be used to cross the new trait into the desired varieties.
With regard to the possible direct toxic effects of the introduction of a foreign gene into a crop plant, genetic engineering has a higher predictability than for instance the introduction of foreign genes from other plant species using wide crosses.

## 1.7 Formal food safety assessment of genetically engineered foods

For genetically engineered crops a formal food safety assessment is required by the European Novel Food Regulation 258/97. As already stated in paragraph 1.4, such a requirement does not exist for the traditionally bred crops. There are two reasons why this formal safety assessment for genetically engineered foods does exist:
(1) The fact that the product of the transgenic plant can be completely new for the crop plant and thus never eaten before by the consumer, for instance the gene coding for green fluorescent protein (GFP) from jellyfish. In such cases it is important to look at the eventual toxic properties of the GFP protein, before allowing it to be consumed. And
(2) The fact that there is no history of safe use of the technique of genetic engineering in plants (when compared to traditional breeding).

So, especially the possible direct toxicological consequences of the introduction of a gene are a concern from a toxicological point of view. But in addition also the possibility of interference of the new product with other factors in the host plant (pleiotropic effects) forms an additional concern from a toxicological point of view. This is why in the required food safety assessment not only the possible direct effects of the inserted genes have to be taken into account, but also the possible indirect effects. However, these pleiotropic effects, like insertion effects or effects of somaclonal variation, cannot be predicted from the nature of the inserted genes, and this makes that there are no direct means of measuring them. In the following chapters of this review it is explained how both possible direct and indirect toxicological effects of genetic engineering of plants can be measured or estimated.

# 2. The Food Safety Assessment of Genetically Engineered Plants; The Appearance of a New Paradigm

At the end of the 1980s the food safety assessment of genetically engineered foods was for the first time discussed at a larger international level. It was determined that the endpoint of the safety assessment should be to determine whether the modified food is as safe as its traditional counterpart. In these discussions the concept of 'substantial equivalence' was introduced as a means of establishing a benchmark definition of safe food. This concept was introduced by the OECD's group of National Experts on Safety in Biotechnology as an approach for assessing the food safety of GMOs and has been further elaborated by other groups (OECD 1993, Nordic Council 1998). The determination of substantial equivalence is not the endpoint of a safety assessment, but rather a practical approach that guides the safety assessment process (see chapter 4 and 5).

In 1997, Europe introduced the Novel Food regulation 258/97 which specifies the procedure to obtain market approval for the introduction of a novel food or a novel food ingredient. In this regulation, the requirements for the safety assessment of foods from transgenic plants have been specified in a general way, without specifying all details.

The food safety assessment process of genetically engineered crops is now considered to consist of four parts:

1. A molecular characterisation of the insert;
2. Determination of any unwanted direct toxicological effects as can be predicted from the nature of the inserted sequences;
3. Determination of any unwanted indirect toxicological consequences resulting from the modification.
4. A morphological and behavioural analysis of the plant under field conditions.

The molecular characterisation of the insert may give rise to the need for further analysis. For instance when the DNA is inserted in such a way that it might cause transcription and translation of DNA that normally would not be expected to be translated e.g. plant DNA at the border of the insert.

The determination of the direct toxicological effects can be rather straightforward. For instance, if the GM plant is transformed to produce a protein, it should obviously be analysed for the presence of the protein and the direct toxicity of this protein should be assessed. This is done using the classical toxicological tests that are also performed on substances like additives, flavourings, or residues of pesticides, such as in-vitro toxicity studies or animal feeding studies. But the situation can also be more complex when for instance multiple genes are introduced that interfere in metabolic pathways in the plant. These different situations and examples of how the tests are performed, are described in chapter 3 of this paper.

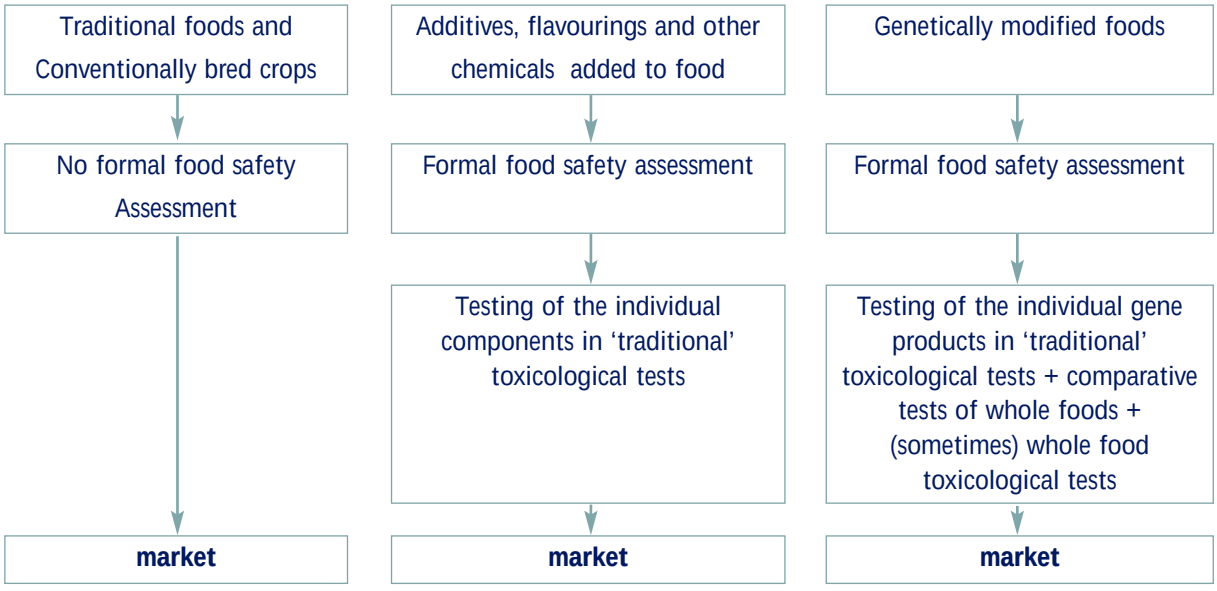
The assessment of possible indirect toxicological consequences is less simple, because it concerns effects one cannot know, predict or expect. This also makes it difficult to design direct means of measuring them. Guided by the concept of substantial equivalence, the current assessment takes a comparative approach where the GMO and its counterpart – the non-GMO – are compared and any differences are further evaluated. If the comparison does not reveal any substantial differences – other than those expected to result from the inserted sequences – the genetically modified plant can be considered to be substantially equivalent and thus as safe

as the traditional plant. If the comparison reveals qualitative or quantitative differences, these differences will be subject to further evaluation and may demand further analyses. The need for toxicological tests and the type of tests that might be demanded will depend on the nature of the established differences. How substantial equivalence is established and what tests are performed, is described in chapter 4 of this paper.

The analysis of morphology and behaviour of the plant under field conditions is to see whether the plants behave as one would expect to behave, taking into account the genetic modification. Unexpected phenotypical behaviour may be an indication of the occurrence of unwanted indirect effects of the genetic modification. Are there no such indications, then this analysis can be used to back-up the results of the analysis of substantial equivalence.

The assessment of possible indirect toxicological effects resulting from the genetic engineering has led to a shift in focus in toxicological risk assessment of food. Where traditional foods were not tested at all (at the most there is a recommended maximum level of toxicants), and later introduced chemical components like additives or pesticide residues were only tested in traditional toxicological tests as such (and not part of the whole food), for genetically modified foods there more often is a (legal) necessity to test whole foods, especially when comparison between the GMO and its counterpart reveals substantial differences that cannot be explained by the nature of the inserted transgene.

Figure 1: a shift towards the assessment of whole foods as a result of the introduction of genetically modified foods




*Note: First there were only traditional foods, later additives and flavourings (either natural or chemically synthesized) were added to foods, but also pesticides or residues of pesticides could be present. Genetically modified foods have been introduced the most recently. For traditional foods and substances like flavourings whole food assessments have never been that important. The introduction of gm foods has shifted attention towards the testing of whole foods in addition to testing the newly introduced (gene)product.*

The following chapters will describe how the evaluation of the food safety of genetically modified foods is done in practice (including examples and data), and give some suggestions on how to improve the current assessment approach by providing an explicit overall assessment procedure.

# 3. Evaluation of Possible Direct Toxicological Effects Resulting from the Introduction of a Sequence

## 3.1 Introduction

The introduction of one or a few known genes or sequences results in the expression of one or more new constituents in the plant, or in the changing of the expression of existing constituents, either positively or negatively. In certain cases this will only have limited effects (for instance the presence of one new protein with no further interactions in the plant), in other cases this can result in a chain of alterations in the plant. For the assessment of the possible direct toxicological effects as they can be predicted from the nature of the inserted sequences, the specific changes in genetically engineered plants can be grouped into four categories:

1. The product from the inserted gene is known in relation to food.
2. The product from the inserted gene is not known in relation to food.
3. The inserted gene results in a reduction in the expression of a gene in the host.
4. The inserted gene(s) is (are) involved in change of a pathway including introduction of a new pathway.

The result of the genetic engineering of a plant may include one or more of these four categories at the same time. They may also involve both non-toxic components as well as toxic components. The first two categories are closely related to the protein produced from the inserted gene whereas the third category is related to the lack or reduced expression of a protein. The fourth category represents the more complex situation where the genetic engineering has caused changes in one or more pathways. These four categories are described below.

## 3.2 The product of the inserted gene is known in relation to food

GM plants can contain inserted genes coding for proteins that are already known in relation to food consumption and digestion, and there are already examples on the market.

For instance, the Flavr Savr tomato from Calgene (Redenbaugh et al. 1993) which was the first transgenic plant on the market in the USA, contains the marker gene coding for the enzyme neomycin phosphotransferase II (NPTII) giving resistance to the antibiotic kanamycin. Several micro-organisms commonly present in the gut flora produce this enzyme (Flavell et al. 1992) and therefore it is not new in relation to humans. Other examples are transgenic tomato expressing the coat protein from the tomato yellow leaf curl virus, which gives the tomato resistance to that virus (Kunik et al.1994) and several maize varieties with inserted cryIA(b) gene from the *Bacillus thuringiensis* coding for insect toxin (Bt-toxin gene). The virus protein is present in traditional tomatoes infected with the virus and the Bt-toxin is widely used as a biological insecticide on plants and is found on food plants. Both are consumed by people.

Plant genes coding for insect resistance have been transferred to other plants. The genes of interest are two major groups of plant genes, namely inhibitors of digestive enzymes (proteinase and amylase inhibitors) and lectins (Schuler et al. 1998). Some of these proteins such as some of the lectins, which naturally occur in certain food plants, are already known to be more or less toxic to humans (see also table 1).

When a GM food plant is engineered to produce a substance known from other organisms used for food production or known in relation to food intake as illustrated above, the first question is whether such substances can be accepted just because they are known from other food plants or whether additional toxicological data are needed. This means that the history of safe use should be taken into consideration. The above mentioned examples of NPTII, plant virus protein and Bt-toxin do have their own history of intake, which is considered to be safe.

The intake history of a gene product in other traditional food crops can only be used as a decisive element in the safety evaluation, if there is a long history of safe food use of the donor organisms and if the total intake level of these gene products after their introduction in the genetically modified food plants will not exceed the level related to the previous history of safe use. Furthermore, a history of safe use means that information and exact data from previous exposure, including intake level of the substance of interest, exists and can be used for a scientifically based toxicological assessment of the substances. However, this may only be the case for very few substances. Furthermore, the quality and the quantity of toxicological information for inherent plant toxicants are often insufficient for a stringent safety assessment.

As an example of the difficulties of defining a safe history of use, the cultivated common mushroom *Agaricus bisporus* may be considered to have a history of safe use. On the other hand, toxic substances (hydrazines) have been found in the mushroom and animal tests indicate that these substances may induce cancer (Toth 1995). This example demonstrates that a history of safe use can be difficult to establish based on human dietary records, and it cannot always form a solid basis for clearing all substances in a plant (or mushroom) as safe for human consumption. This is because effects of individual substances can be extremely difficult to identify or rule out due to the "noise" from variations in the overall food intake, the lifestyle and environment which may influence the health of the individual. It may therefore be more conceivable that the history of the intake of the known product will be used only as an element in the risk assessment rather than as the basis or the endpoint for this assessment.

If the inserted gene codes for a toxin known from other food plants some questions need to be answered. The stability and variation of the level of the known, (toxic) substance in the GM plant needs to be established by years of measurements. This will include data of the GM plants grown under different conditions (soils, climate, crop management etc.) representative of the future use of the genetically modified plant. If the donor of the inserted gene is another food plant, the data should be compared to similar data of this plant. Some data already exist about the level of different plant substances in traditional food plants that might be useful for comparison.

If there is a history of safe use, and the overall intake level of a known substance is not expected to increase by the introduction on the market of a GM plant, this new crop plant should not pose any new or additional health risk and might be immediately acceptable from a toxicological point of view. This might especially be the case if the inserted genes are not expressed or only expressed at a very low level in the edible part of the plant. On the other hand, if the history of safe use cannot be established, and/or the overall intake level of the new substance in a GM plant is expected to increase, additional toxicological tests might be necessary before the GM plant is accepted for food use. Toxicological tests have in fact been performed on proteins of inserted genes for most of the GM crops evaluated so far in EU for market approval (see table 2 and 3). These toxicological tests have been performed regardless of the fact that it can be argued that some of them have a

safe history of use e.g. the Bt-toxin and the NPTII. For these and other proteins introduced into GM plants, a number of *in-vitro* and *in-vivo* studies have been performed like the mouse acute gavage study (*in-vivo*) and simulated mammalian digestive fate studies (*in-vitro*) (WHO 1995, Fuchs et al. 1993). The acute gavage studies cover doses from 100 to ~5200 mg/kg. In gavage studies the protein produced by the transgene is added to the diet of the animal in a single or multiple dose and the animals are monitored to see whether there are any undesirable effects. After a certain period the animal is killed and dissected to evaluate the state of different organs.

The data in table 2 and 3 show that the companies have included results of subacute to subchronic[10] animal studies in their applications. Both for the evaluation of maize, soybean and tomato these data have been considered sufficient by the SCF on a case-by-case basis. The studies showed no subacute or subchronic toxicity of the tested foods or food components.

Table 2. Testing of the direct toxicity of gene products: four examples of GM crops

| Plant* | Gene | Protein test in animal. Single dose subacute gavage study | Source B=Bacteria P= Plant | Digestibility of protein** | Subchronic animal test of plant material*** | Search in database for homology**** |
|---|---|---|---|---|---|---|
| Maize | PAT | Animal: mouse<br>Dose: 5050 mg/kg<br>Days: 14 | B | No data given | | No signficant homology |
| | CryIA(b) | Animal: mouse<br>Dose: 4000 mg/kg<br>Days: 14 | B (E.coli) | No data given | | no significant homology |
| Maize 176 | CryIA(b) | Animal: mouse<br>Dose: 5050 mg/kg<br>Days: 14 | P | rapidly digested | 38 days broilers | no significant homology |
| | CryIA(b) | Animal: mouse<br>Dose: 5050 mg/kg<br>Days: 14 | B (Bacillus) | rapidly digested | | |
| | PAT | Animal: mouse<br>Dose: 5050 mg/kg<br>Days: 14 | B | rapidly digested | | |
| Maize GA21 | MEPSPS | Animal: mouse<br>Dose: 50 mg/kg<br>Days: 13 | B | rapidly digested | 38 days broilers | no significant homology |
| Soybean | CP4-EPSPS | Animal: mouse<br>Dose: 572 mg/kg<br>Days: not given | B (E.coli) | rapidly digested | 6 week broiler<br>4 week cow<br>4 week rat<br>10 week catfish<br>5 day quail | No data given |
| | NPTII***** | Animal: mouse<br>Dose: 5000 mg/kg<br>Days: 8-9 | B (E.coli) | | | |

*All the given maize and soybean plants have been or are in review for marketing in the EU

**The digestibility of the protein is important for evaluating the allergenic potential of a protein. The proteins have been tested in-vitro for their digestibility.

***Plant material has been fed to the animals during longer periods of time. The results of such a feeding test does not allow to conclude on the direct toxicity of the introduced transgene product. Because any ill effects can be caused by any factor present in the plant material. Such tests are more indicative on the chronic toxicity of the GM crop as a whole. Any found toxicity could just as good be caused by indirect effects of the genetic modification.

****The amino acid sequence of the proteins were compared to that of known toxicants, and it was proven that there is no significant homology with these known toxicants

***** The NPTII protein was tested in relation to the use of the gene in different plants (Fuchs et al. 1993).

PAT = Phosphinotrycine acetyl transferase, CryIA(b) = a crystalline toxin produced by Bacillus thuringiensis, MEPSPS = modified 5-enolpyruvaat-3-phosphoshikic-acid-synthase, CP4 EPSPS = EPSPS gene from the CP4 Agrobacterium strain

[10] Toxicity studies can either look at the acute, subacute, subchronic or chronic toxicity of a substance. The acute toxicity tells something about the toxicity of a single dose. Chronic toxicity is about the toxicity of a substance when it is administered in small amount regularly over longer periods of time. Subacute and subchronic are intermediates.

Table 3. The direct toxicity testing of Bt tomato

| Plant | Gene | Protein test in animal. Subchronic gavage study | Source B=Bacteria P= Plant | Digestibility Of protein | Subchronic animal test of plant material | Search in database for homology |
|---|---|---|---|---|---|---|
| Bt-Tomato* | CryIA(b) | Animal: mouse<br>Dose: 0,45 mg/kg daily<br>Days: 28 | B (E.coli) | No data given | 90 day rat | No data given |
| | | Animal: rabbit<br>Dose: 0,06 mg/kg<br>Days: 31 | | | | |

*The data presented for the tomato were not part of a marketing approval dossier, but are derived from a general safety assessment of the GM tomato; looking at the protein *cryIA(b)* and the *NPTII* (Noteborn 1995).

## 3.3 The product of the inserted gene is not known in relation to food

In the evaluation of new proteins not known in relation to food, sequence homology can be useful in indicating likely toxicity or allergenicity if a high match with known toxicants or allergens is obtained. Some toxic proteins have primary structures in common which might help to identify them as potential toxins. For instance, the heat stable enterotoxin of some *Escherichia coli* strains shares some amino acid sequences with enterotoxins of *Yersinia sp.*, *Vibrio sp*, and *Citrobacter sp.* (Hirayama and Wada 1995). Toxicity or allergenicity is often caused by relatively short sequences of amino acids. Homology therefore makes a protein suspect with regard to toxicity (or allergenicity). But unfortunately, the absence of homology cannot be taken as evidence of safety and additional testing will have to be done to evaluate the potential toxicity of the protein.

Many of the genetically modified plants have genes inserted that produce proteins of micro-organisms that are normally not found in food. For example, the genes providing tolerance to the herbicides glyphosate or glufosinate-ammonium are taken from an *Agrobacterium* strain and a *Streptomyces* strain respectively. The genes coding for male sterility and fertility restorer are derived from *Bacillus amyloliquefaciens* (Mariani et al. 1990, 1992). These gene products have no history of safe use in the food supply and should, as a starting point, be evaluated from their known function in the donors. Homology searches can be helpful. The modified maize EPSPS protein in the GA21 maize has been checked for homology to toxins in databases but no homology was found. However, this can only serve as a starting point for the safety evaluation and in order to do a real risk assessment of such new proteins there is a need for data of animal tests.

In the case of transgenic hybrid rapeseed the genes coding for male sterility (barnase gene producing RNase) and fertility restorer (barstar gene) are only expressed in pollen precursor cells and not in seeds or leaves. Cells in which barnase is expressed will be killed and ensure that this product cannot (or only in small amount) be present in the plant part for food. In this case, toxicological animal tests are not necessary.

Many of the new proteins in genetically modified plants presently on the market have been tested in animals. For instance the genetically modified tomato of Monsanto (Finn et al. 1996, Reed et al. 1996) modified by the insertion of a gene coding for a 1-aminocyclopropane-1-carboxylic acid deaminase (ACCd, involved in the pathway for ethylene production resulting in delayed ripening of the tomatoes) has been tested. This ACCd has been purified both of the plant and the micro-organism for a detailed comparison, which demonstrates identity. The protein of the micro-organism was then used for animal toxicity testing. This procedure has also been used in several other cases (see table 2).

## 3.4 The genetic engineering results in reduction of the expression or knockout of a gene

The knockout of a gene that is not involved in a pathway, will pose no toxicological risk unless the gene product itself is an inhibitor or modifier of other toxic compounds e.g. does act as an anti-carcinogenic compound or an antioxidant. This is because toxicological problems are considered to be an aspect of the substances being present and not as missing. A decrease of nutritional value including reduced contents of vitamins is not considered to be a toxicological effect but must be considered in the overall assessment of the health risk of the GM plant.

In cases where the genetic engineering – or any modification - leads to a lower level of a toxic substance in a plant and where this may form the basis for acceptance of the plant for food use, it will also be very important that the stability of the new trait is established. The testing of the stability of a new trait is routinely performed over a number of generations.

## 3.5 The inserted gene(s) is (are) involved in change of a pathway or introduction of a new pathway

Regulation of a pathway should be assessed based on the knowledge about the pathway and the components/substances involved and both upstream and downstream changes should be analysed. If changes in a pathway lead to one of the above-mentioned situations – new components and /or regulation of the formation of components in the plant - these changes can be handled as described above. However, future genetic changes of pathways may involve many genes, that will probably give rise to many differences between the genetically modified plant and its counterpart. In this situation there may be a need for assessing the new food plant not only by the testing of the individual changes but also by testing the whole food item e.g. in a battery of animal tests, in vitro tests and chemical/analytical studies (see chapter 6). There are different examples of modifications resulting in changes in pathways.

**Changing metabolic pathways through silencing**

Examples are the introduction of the granule bound starch synthase gene to change the amylose/amylopectin ratio in potatoes (Visser et al. 1991) or the *GmFad 2-1* gene increasing the oleic acid content in soybean (Kinney 1998). In these cases the antisense/sense technique is used to reduce or prevent expression of a specific gene in the plant. The inserted genes result only in the production of RNA. RNA - like DNA - is not considered to be toxic. However, there is a small possibility that the inserted gene will be translated into a protein. This possibility should be ruled out by analysing the inserted gene for open reading frames, ribosome-binding sites and by analysis to detect the expected protein in the GM-plant. Without such an analysis the novel-food dossier will most probably not be approved in the EU.

Insertion of a sense or antisense sequence of a target gene in the pathway results in downregulation of the target gene but this can cause both up and down regulation depending on the role of the target gene product in the pathway. Up regulation can be the result of blocking one branch of a pathway and thereby increasing the other branch of the pathway competing for the same substrate. An example is the formation of the high oleic acid content in soybean through silencing of the gene for production of the enzyme ω-6 desaturase. By this silencing, the conversion of oleoyl-CoA to linoleoyl-CoA acid is down regulated resulting in a large

increase in the oleic acid content of the triacylglycerol with a corresponding reduction in polyunsaturated fatty acid content (Kinney 1998). GM-plants that have been regulated in a pathway should therefore be analysed for substances both upstream and downstream to the point of interference in the pathway. Any changes should be subject to further safety assessments.

**Knocking in a new enzyme for metabolic pathway engineering**

Another way to change a metabolic pathway is the insertion of a gene, whose product (an enzyme) can take part in a metabolic pathway and redirect the metabolic flow to a new product. The protein of the gene should be evaluated as other new proteins, while further attention should be paid to the changed pathway. An example is the insertion and expression of a gene of *Cuphea hookeriana* making the C8:0 /C10:0 specific thioesterase in rapeseed (Dehesh et al. 1996). Another example is the insertion of the enzyme acyl carrier protein thioesterase in rapeseed (Yuan et. al 1995) to give high level of the fatty acid laurate. Oilseed rape does not produce these medium length fatty acids. Therefore it is necessary in these cases to consider both the new enzymes and the new fatty acids (caprylic acid and capric acid or laurate) as well as the overall change in output of fatty acids of the metabolic pathway.

Another aspect relevant in the detection of differences is the fact that enzymes can have more than one substrate such as the enzyme *6-desaturase producing both *-linolenic acid and octadecatetraenoic acid in a transgenic tobacco (Reddy and Thomas 1996). Testing the enzyme specificity is one way to characterise new enzymes. In the application of the insect and glufosinate resistant maize of the company Northrup King the enzyme phosphinothricin acetyl transferase of the *pat* gene has been tested for its specificity. Because the substrate, L-phosphinothricin, is an amino acid, the substrate specificity of this enzyme was tested for the 22 most common amino acids in plants. No affinity was found to the 22 amino acids.

## 3.6 Conclusion on direct toxicity testing

The direct toxicity testing of the transgene products in the GM crops now on the market has been relatively easy. Standardised *in-vitro* and *in-vivo* toxicity tests have been performed on the concerned proteins, either isolated from bacteria or isolated from the GM crop. The approaches by the different companies differ from crop to crop. Not all have performed homology searches in databases of toxins; not all have performed plant material feeding trials, and the companies who did, involved sometimes more, or different animals. Such feeding trials involving whole plant material cannot be conclusive on the direct toxicity of a new protein in the plant, because any effect can be the result of other, not known alterations in the plant, perhaps caused by some indirect effects of the modification.
The data presented by the companies on the direct toxicity of the involved gene products showed no indications of direct toxicity. These data have been accepted by the authorities.

The approach for assessing the direct toxicity as can be predicted from the nature of the transgene can differ depending on the type of modification. The simplest situation is when only one gene product is added, like for instance NPTII, and no interactions with other components in the plant are expected. The situation becomes more complicated when multiple genes are involved and when the modification results in changes in one or more pathways. Knowledge on the involved gene products and the eventual involved pathways are necessary to design the proper analyses and tests. It is expected that the future genetically engineered crops will involve more complicated modifications, where the predictability of the effects in the plants may be more difficult than for the first generation of GM crops.

# 4. Determining Substantial Equivalence

## 4.1 How to determine substantial equivalence

To diminish the likelihood of any unwanted indirect toxicological consequences resulting from the genetic modification, the legislation requires that a comparison is made between the genetic engineered crop and its non-modified counterpart. The goal is to determine whether these crops are substantially equivalent or whether they are different. But what to compare? Based on the familiarity with traditional foods and on knowledge about their toxicity, it was determined that the comparison should in first instance take into account the toxicologically relevant substances of the crop, but also look at constituents that are relevant from a nutritional or wholesomeness point of view. This however, also implies that the more familiarity we have with a crop, the better we will be able to focus the assessment on relevant aspects.

For the comparison, the traditional counterpart should be the most related non-transgenic plant. However, different breeding techniques, such as F1 hybrids, outcrossings (e.g. maize) and cloning (e.g. potatoes) make a direct comparison difficult. In addition, strong selection for specific traits in the transgenic plant can also enlarge the variation between the transgenic plant and non-transgenic counterpart. In these cases closely related varieties should be included as a baseline for the evaluation of substantial equivalence. It is also of importance that the plants used for the comparison are grown under conditions that represent normal practice for the crop plant e.g. the analyses of herbicide tolerant plants should include plants sprayed with the herbicide. If the conditions used do not represent normal practice, the test data are not likely to be accepted by the authorities.

There is no international standard yet as to which constituents for which crop should be subject to the comparison. In an attempt to come closer to such an international standard, the Nordic Council (1998) has suggested which types of chemicals should be analysed in the case of maize, oilseed rape, tomato, cotton and soybean. Table 4 shows which chemicals are suggested to be analysed for these five crop plants. This approach needs to be further elaborated and expanded to other crop plants. Databases or lists with international accepted key substances for each crop plant including accepted levels and variations of the substances in food plants are needed and a necessary pre-requisite for the future work in this area.

Table 4: Recommended minimum lists for compositional analysis of specific crop plants (Nordic Council 1998).

| | Cotton | Oilseed rape | Maize | Soybean | Tomato |
|---|---|---|---|---|---|
| **Proximate analysis** | | | | | |
| Protein | X | X | X | X | X |
| Fat | X | X | X | X | X |
| Ash | X | X | X | X | X |
| Moisture | X | X | X | X | X |
| **Carbohydrates** | | | | | |
| Fibre | X | X | X | X | X |
| Starch | | | X | | |

| | Cotton | Oilseed rape | Maize | Soybean | Tomato |
|---|---|---|---|---|---|
| **Fatty acid profile** | 8[a] | 8[a] | 5[a] | 5[a] | |
| **Amino acid profile** | | | 16[b] | 16[b] | |
| **Toxicants/antinutrients allergenic sub.** | | | | | |
| Total gossypol | X | | | | |
| Free gossypol | X | | | | |
| Sterculic acid | X | | | | |
| Malvalic acid | X | | | | |
| Dihydrosterculic acid | X | | | | |
| Glucosinolates | | 4[c] | | | |
| Erucic acid (C22:1) | | X | | | |
| Phytic acid | | | | X | |
| Soy lectin | | | | X | |
| Glycinin | | | | X | |
| β-conglycinin | | | | X | |
| Trypsin inhibitor (Kunitz) | | | | X | |
| Diadzein | | | | X | |
| Genistein | | | | X | |
| Genistin | | | | X | |
| α-tomatine | | | | | X |
| **Nutrients, Vitamins, Minerals** | | | | | |
| Sodium | | | | | X |
| Potassium | | | | | X |
| Calcium | | | | | X |
| Magnesium | | | | | X |
| Phosphorus | | | | | X |
| Iron | | | | | X |
| Vitamin A | | | | | X |
| Vitamin B1 | | | X | | X |
| Vitamin B2 | | | X | | X |
| Vitamin B6 | | | | | X |
| Vitamin C | | | | | X |
| Folic acid | | | X | | X |
| Tocopherols | X | X | X | | |

Notes to table 4:

[a] *Fatty acid profile. For oil plants the fatty acid profile is a minimum requirement. The numbers indicate how many fatty acids must be analysed as a minimum. These fatty acids should be the quantitatively most important ones in the specific oil plant. The requirement of measurements of eight fatty acids in cotton and oilseed rape versus five fatty acids in maize and soybean reflects the larger number of fatty acids in cotton and oilseed rape.*

[b] *Amino acid profile. In plants grown for protein or meal for human consumption, the amino acid profile is a minimum requirement. The numbers indicate how many amino acids must be analysed as a minimum. Living organisms make use of 20 different amino acids. These amino acids should be the quantitatively most important ones in the specific crop plant.*

[c] *Glucosinolates. In oilseed rape the minimum requirement is comparison of the four main alkylglucosinolates, i.e. 3-butenyl (gluconapin), 4-pentenyl (glucobrassicanapin), 2-OH-3-butenyl (progoitrin) and 2-OH-4-pentenyl (napoleiferin) which are indicated by the number 4.*

From a toxicological point of view, the comparison should also take into account the type of use of the plant. E.g. a genetically modified maize plant that might be used for oil and starch production but also can be eaten as boiled cob, should be analysed for differences in the oil and starch content and composition. Table 5 shows the most important food products that are derived from soybeans, oilseed rape, cotton, tomato and maize. By

using this approach, the toxicological risk assessment will comprise any traditional food product made from the maize, or fraction of the maize kernel. For applications covering only one specified product of a plant, such as oil from transgenic maize or oilseed rape plants, the analysis might be narrowed. Such oil in which there are both a substantial equivalent fatty acid profile to comparable non-transgenic plants and no detectable new protein of the inserted genes, should be handled as substantial equivalent and no further studies are required independent of the inserted genes.

It is also important that the comparison is done with a sufficient variety and number of samples determined by reliable analytical methods and relevant statistical calculations. If small differences can be explained by comparison of factors as growth conditions, subspecies (variety) / species variation, genetic distance to counterpart etc., then plants still will be considered equivalent. But if the average value of these parameters differs significantly from the value of the non-modified counterpart, an explanation should be sought. In a Nordic report a difference of more than 20% is suggested to be significant (Nordic Council 1998). Plants often already show a relatively broad range of natural levels of constituents, so only relatively large differences are taken to be significant. The number of parameters to be examined will depend on the plant food product in question. To establish a reliable substantial equivalence assessment and to predict unexpected changes more than 10 parameters representing different groups of substances should be analysed (Nordic Council 1998).

If the comparison shows significant changes in the toxicologically and nutritionally relevant substances, this should be seen as indirect symptoms indicating that some unwanted effects have occurred in the plant, resulting from either pleiotropy, insertion or somaclonal variation. If not, then the plant is said to be substantially equivalent, and it is estimated that the likelihood of any relevant toxicologically unwanted effects has been thus reduced, that the plant is considered to be (at least) as safe as its non-modified counterpart.

Table 5: The products derived from a number of crops, and their use.

| Crop plant | Derived food and feed products | Use |
|---|---|---|
| Cotton | Cotton oil | Margarines and other oil containing foods |
| | Protein | Processed foods |
| | (Carboxy)methylcellullose | Food additive (thickener) |
| | Press cake | Animal feed |
| Oilseed rape | Oil and fats | Margarine, cooking fat, vegetable oil |
| Maize | Kernels (sweet corn) | Consumed as such |
| | Maize starch | Binder for puddings and sauces |
| | Maize flour | Bakery products |
| | Maize glucose syryp | Processed foods |
| | Maize oil | Baking, bakery products |
| | Maize dextrose | Processed foods |
| | Maize | Animal feed and silage |
| Soybean | Soybeans | Tofu, tempeh, and other fermented products |
| | Soya oil and fat | Bakery products, processed foods, margarines, oils, etc |
| | Soya flour | Bakery products |
| | Soya lecithin | Emulsifier |
| | Soya scrap | Animal feed |
| Tomato | Tomato fruit | Fresh food |
| | Peeled tomato and paste | As such, and in processed foods |

## 4.2 Genetically engineered crops on the market and determination of their substantial equivalence

For all of the GM plants applied for marketing approval in the EU a comparative chemical analysis has been performed to establish substantial equivalence (see table 6). The analysed chemicals cover micro- and macronutrients as well as key substances. There are major differences in the approaches in different food safety dossiers, even within the same plant species. One GM-rapeseed showed a lower concentration of glucosinolates when compared to its non-GM counterpart. It is possible that this was the result of the traditional selection and breeding procedures after the transformation. Further testing should make this clear or determine that this particular GM rapeseed is safe to eat (see 4.3). For all the other genetically engineered plants it was shown that all the components tested were present in amounts that were within the natural fluctuations, and it was concluded that they were substantially equivalent.

These overall results for several GM plants showing their chemical substantial equivalence, do indicate that the selection and back-cross procedures applied to GM plants lines are able to eliminate undesirable effects, just like the current practice in traditional breeding do.

Table 6: Examples of GM plants and comparative compositional tests to determine substantial equivalence or difference.

| **Plant*** | Maize T25 | Maize MON | Maize Bt11 | Maize MON | Maize GA21 | Maize 176 | Rapeseed HCN92 | Rapeseed GT73 | Rapeseed | Soybean | Tomato |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene | PAT | CryIA(b) | CryIA(b) PAT | CryIA(b) CP4-EPSPS | MEPSPS | CryIA(b) PAT | PAT NPTII | CP4-EPSPS Gox | Bar Barstar Barnase NptII | CP4-EPSPS | |
| Company | Aventis | Monsanto | Northrup-King | Pioneer Hi-Bred | Monsanto | Ciba-Geigy | Aventis | Monsanto | PGS | Monsanto | Zeneca |
| Morphology and [1]phenotypical behaviour | X** | X | X | X | X | X | X | X | X | X | X |
| Protein | X | X | X | X | X | X | X | X | X | X | X |
| Fat | X | X | X | X | X | X | X | X | X | X | X |
| Amino acids[2] | X | X | X | X | X | X | X | X | | X | |
| Fatty acids[3] | X | X | X | X | X | X | X | X | X | X | |
| Carbohydrates | X | X | X | X | X | X | | X | | | X |
| Natural toxicants | | | | | | | glucosino-lates, Erucic acid | glucosino-lates, Erucic acid | glucosino-lates, Erucic acid | Lectins | Glycoalkaloids, biogenic amines, histamine, nicotine, lectin |
| Nutrients (minerals) | | | X | | | | | X | | | |
| Vitamins | | | B1, B2 | | | B1 | E | E | | | A+E+C |
| Antinutrients | Phytate | | | | | | | phytate | | Phytic acid Trypsin inhibitor | |
| Misc. Components | | | Xantho-phyll | | | Xantho-phyll | sterols chloro-phyll | sinapine | | Isofla-vones | |

[1] Covering a wide range of different measurements and observations such as detailed morphological description, yield, resistance to pests, normal agronomic obervation, pollen flow, etc.
[2] With only few exceptions 18 amino acids are included in the analysis
[3] The analysis covering different fatty acids
*All the maize, rapeseed and soybean plants have been or are in review in the EU for marketing.
**An "X" indicates at least one measurement in one year

## 4.3 Non-substantial equivalence

When substantial equivalence is tested on the level of the plant, one would expect (and hope) in most cases that the plant would turn out to be substantially equivalent, except for the changes as can be predicted from the modification. When looked at a different level, for instance at the level of tubers or seeds, or the oil of seeds, in quite some cases these parts can turn out to be totally substantially equivalent to their non-modified counterparts. But what if the GM plant or one of its components turns out to be significantly different and the difference, at first sight, cannot be explained by the modification? As already indicated such unexpected differences could arise from the following:

1. Pleiotropy: the transgene does not only result in the expected new trait, but also results in another change in the plant. There are many different mechanisms possible that result in the unexpected change: (a) the transgene product can interact unexpectedly with other components in the plant, (b) the pathway in which the transgene product has its function is (unexpectedly) cross linked with other pathways, (c) changed expression level of an enzyme may trigger other pathways, or a downregulation respons.
2. Insertion: the transgene has landed somewhere in a gene thereby disrupting this gene's function and resulting in changes in the plant's constituents.
3. Somaclonal variation: in the *in-vitro* regeneration of the transgenic plants, due to chromosomal instability, changes have appeared in the plant, resulting in changes in morphology, behaviour, or macro- or micro-constituents. What actually happens during *in-vitro* regeneration, and what changes occur to the genome of the plant, is not yet understood.

Non-substantial equivalence results in a need for further analysis. There are no general guidelines yet on what the analysis should be. It will also depend very much on the difference(s) found. But a first logical step before starting laborious testing, would be to search literature and other sources of knowledge on the concerned plant and try to find some clues. If one is lucky, one will find indications on where to look and what type of analysis to do. If pleiotropy is suspected, analysis of the pathway(s) surrounding the new gene product may be relevant. If this does not give any results, one cannot consider this to be proof for the absence of pleiotropy, but it may be appropriate to consider insertion or somaclonal variation. Whether insertion is in play can be researched by sequencing the DNA outside the insertion, analysing the presence of a disrupted gene, and searching databases to see whether the involved gene is known. If so, and the function of this gene is known, then the consequences of interrupting the function of this gene can be analysed. If not, then the analysis becomes laborious and difficult, and perhaps even impossible, and it may be appropriate to consider the possibility of somaclonal variation. Whether an unexpected change is the result of somaclonal variation is at this moment impossible to determine. So if non-equivalence is found, then there will be a number of cases where no explanation can be found.

Because of the difficulties of finding the exact explanation for specific cases of non-substantial equivalence it can be discussed how much energy should be put into this. Therefore, a second additional or parallel approach becomes imminent: this is to analyse whether the change is relevant from a toxicological point of view. If not, one could argue that the food (crop) should be allowed into the food chain. So, not all cases of non-equivalence - and also cases that where changes are not explained - have to result in the food being withheld from the market.

There is at this moment only one type of assessment available for the food safety testing of non-equivalent genetically modified foods, and this is whole food assessment. One of the major current approaches of whole food assessment is to feed the genetically engineered plant material over longer periods of time to different kinds of animals. If no adverse effects are found, the risk of toxicity becomes quite low. However, the negative predictive value of whole food assessment, especially with regard to (minor) chronic toxicity, is problematic.

There is only very limited experience with cases of non-equivalence, and there will be quite some discussion among the authorities if a specific case would be put to them in the form of a novel food dossier. It will probably take a number of cases before a general framework will be worked out. An attempt to design such a framework is given in chapter 5 of this review. On the other hand, the number of cases of non-equivalence without explanation will be limited, because companies will tend to eliminate such plants, and only market the food that is shown to be substantially equivalent, or where any non-equivalence can be explained.

## 4.4 Expected future developments in the food safety assessment of genetically engineered food

**Assessment of direct toxicity as can be predicted by the genetic modification**

Future genetic modifications are likely to become more complex. Multiple genes possibly involved in different pathways will be transferred to plant. The assessment of the direct toxicity will probably become more complex as it will become more difficult to predict all the changes that are directly caused by the new transgene. On the other hand it is also possible that our knowledge on plant genetics and plant physiology grows simultaneously to our needs and helps to improve the predictability of changes. It is also very well possible that the only way to assess these complex modification is by doing whole food assessment. Whole food assessment is now being done (see feeding of plant material trials in table 2), but the negative predictive value of such test may not meet the required standards.

**Assessment of substantial equivalence**

There still is discussion on how the equivalence of a crop should best be tested. Like for assessment of the direct toxicity this will be especially the case for the more complicated modifications that are to be expected for the future. It could be argued that in case of the introduction of complex modifications involving many genes and effecting different pathways, there is a greater chance of the occurrence of pleiotropic effects. There is also still critique on the fact that only a limited number of indirect symptoms are measured, only resulting in a strong reduction of the likelihood of the presence of any toxicologically unwanted effects. On the other hand it can be argued that there is no need to further increase the safety levels because the secondary effects as they can result from insertion are already known from traditional breeding where no safety analysis is required.

**Novel methods**

Methods such as biochips (microarray), mRNA profiling, proteome analysis etc. are being looked at to see whether they can improve the comparison between GM crops and their non-modified counterparts and also improve the whole food assessment, because instead of looking at individual components (toxicants, nutrients) these methods tend to look at the whole food. At this moment the results of these measurements are still very difficult to interpret. Often it is not yet known what a difference in a profile actually means in the

plant. There is an urgent need for further research within this area before any conclusion can be drawn about the sensitivity, specificity and predictive value of these techniques. These methods will be valuable if they can distinguish between essential and non-essential differences from a wholesomeness point of view.

## 4.5 Conclusion

For the determination of any indirect effects of the genetic modification, the concept of substantial equivalence is important. It guides the assessment process and takes a comparative approach in which substances of the GM crop are compared to those of its non-modified counterpart. Chosen substances are either relevant from a toxicological point of view, or relevant for nutritional value or wholesomeness. Any differences should be seen as symptoms that any unwanted effects have occurred in the plant, either resulting from pleiotropy, insertion or somaclonal variation. To be able to choose the right components for the comparison - and thereby making the comparison relevant – it is crucial to have knowledge on the involved plants, its behaviour and its constituents.

Direct measurement of any effects of the genetic modification is impossible, because any effects resulting from pleiotropy, insertion, or somaclonal variation cannot be predicted. One would not know what to look for. The proposed indirect measurements using the substantial equivalence approach therefore do not result in 100% certainty on the absence of any toxicologically unwanted effects, but in a strong reduction of the likelihood that such unwanted effects have occurred. The fact that such effects can also occur in conventionally bred crops, makes that if no substantial differences are found, the modified crop is considered to be (at least) as safe as the conventionally bred varieties.

Substantial equivalence has been determined for the crops currently on the market or currently in the approval process. The approaches of the companies have differed from crop to crop. Different constituents have been included in the comparison. There is a need for further standardization, although the authorities have accepted the presented data despite of the differences. Substantial equivalence can be determined at different levels: at the level of the whole plant, or on the level of a derived product. The comparison is only relevant if the comparison takes the closest non-modified counterpart and plants are grown under comparable and relevant conditions.

Non-substantial equivalence will trigger further analysis: either to determine the reason for the non-equivalence, or to determine the safety of the non-equivalent whole food. It is not yet clear what data will be acceptable to the authorities at this moment, and further discussion on this subject will be needed. This is also true to prepare the food safety assessment for the more complex modifications that will make up the next generations of genetically engineered crops. It will probably be necessary to improve the negative predictive value of the whole food assessments and alternative new methods, based on micro-array or any other new technology, will have to be evaluated for their suitability and robustness.

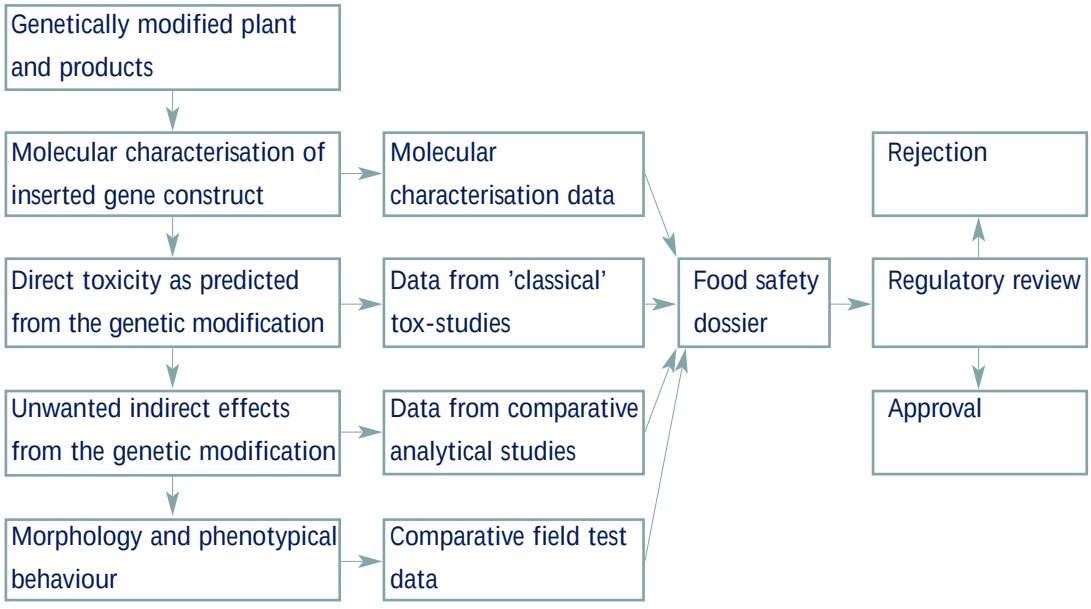
# 5. Improving the Overall Food Safety Assessment Procedure of GM Crops

## 5.1 The current food safety assessment procedure of GM crops

The European Novel Food regulation 258/97 concerning the placing on the market of novel foods and novel food ingredients is a procedural regulation that lays down the different steps in the authorisation procedure. It does not state explicit criteria for the novel foods other than that the food must not present a danger to the consumer, nor does it state what type of testing has to be done. It requires that the authorities are provided with information that substantiates the safety for the consumer. A European Commission recommendation has been published (commission recommendation 97/618/EC) concerning the information necessary to support applications for the placing on the market of novel foods and novel food ingredients. In this recommendation a number of decision trees is given that help to determine what kind of information should be provided.

In practice, companies have based their food safety assessments on the given information requirements. They have tried to answer the questions asked. These questions have become more detailed and explicit over the years, as a result of growing experience. As already stated in chapter two the questions in the food safety assessment relate to four aspects: (1) molecular characterisation, (2) direct toxicological effects as predicted from the genetic modification, (3) unwanted indirect toxicological effects, and (4) phenotype (see fig. 2). In trying to answer the questions related to these aspects the different companies have all developed their own format for the food safety testing. As shown in the former chapters these formats are not always identical.

Figure 2: The current approach for the safety assessment of genetically engineered food



## 5.2 A proposal for an overall food safety assessment procedure of GM crops

Based on the considerations in the former chapters and in an attempt to improve the uniformity of the safety assessment, a proposal is given for the overall safety testing and assessment procedure for GM food (see fig. 3). It is important to notice that the proposed procedure takes the concept of chemical substantial equivalence as the guiding principle of the assessment procedure, both in its approach to determine what type of testing has to be done, and in the formulation of the final result. Substantial equivalence is regarded the central concept based on international agreement on this principle. Like in the current approach, there is no direct measurement of possible toxicologically unwanted indirect effects of the genetic modification. The goal of this proposal is to make the food safety assessment and the choice of testing more uniform.

The proposed procedure is to be seen as a 2-step procedure. The first step is to determine whether the GM food is (i) substantial equivalent, or (ii) non-substantial equivalent to its traditional counterpart. This first assessment of equivalence/non-equivalence is based on information regarding the inserted gene construct, quantitative information on key substances, measurement of the presence of new or altered gene products as can be predicted from the genetic modification, and qualitative information on morphological appearance and behaviour. The quantitative information on key substances is crop specific and should be based on standardised lists per crop of substances that should be analysed (as proposed in chapter 4 of this paper). It is important to keep in mind that the term substantial equivalence is defined under the assumption that only substantial differences that may raise essential questions regarding the safety of the food should be taken into consideration. Morphological and phenotypic changes will only qualify in this respect if they raise doubt about the equivalence from a wholesomeness point of view of the GM plant when compared to the traditional counterparts.

If **substantial equivalence** for a GM food plant or a derived food item is established, approval is granted based on history of safe intake and the general assumption that the substantially equivalent food item is simply intended to replace the traditional food item. Feeding studies to confirm substantial equivalence should not be necessary.

If **non-substantial equivalence** is established, **and the differences are the logical result of the introduction of one or more transgenes**, then the safety of the new or altered substances need to be examined in compliance with the categories discussed in chapter 3:

- The new gene product is well known from traditional food intake. If there is a history of safe intake and no increased intake of the gene product from the transgenic plant is expected, there is no need for further toxicological studies, except in the following cases; 1) The gene product and derived compound, although there is a history of safe intake, is not desirable in foodstuffs based on present toxicological or allergenicity data that question the safety status of the compound, 2) if the history of safe use is connected to a specific preparation of the traditional food, which may not be applicable to the new GM food item.
- If the inserted trait includes a gene product (protein) that is not known from usual food intake, further studies are needed to assess safety.
- If a non-protein product not known from human food intake is produced due to the function of the inserted gene, e.g. when the function (enzyme activity) of the inserted gene modifies a metabolic pathway to make new non-protein products, this will trigger further testing.
- The fourth category includes testing due to a cascade of new products due to GM enzymes, which influence a range of metabolic pathways. Actually this may become a whole food safety assessment.

These products may be either proteins, or a combination of proteins and non-proteins. For new proteins in the new GM plant the structure-activity-relationships, demonstration of biochemical identity, digestibility and acute toxicity tests in animals need to be performed, if identical proteins have not already been evaluated. Sometimes, e.g. high levels of new proteins without history of intake, also subacute/chronic animal feeding studies may need to be performed.

Normally the acceptance of new proteins will be expressed in terms of an *Acceptable Daily Intake (ADI)* or an *ADI not specified*[11] . For the non-proteins the traditional toxicity test package for food additives will need to be applied on a case-by-case basis depending on the history of intake. Here acceptability will normally be expressed in terms of an *ADI*.

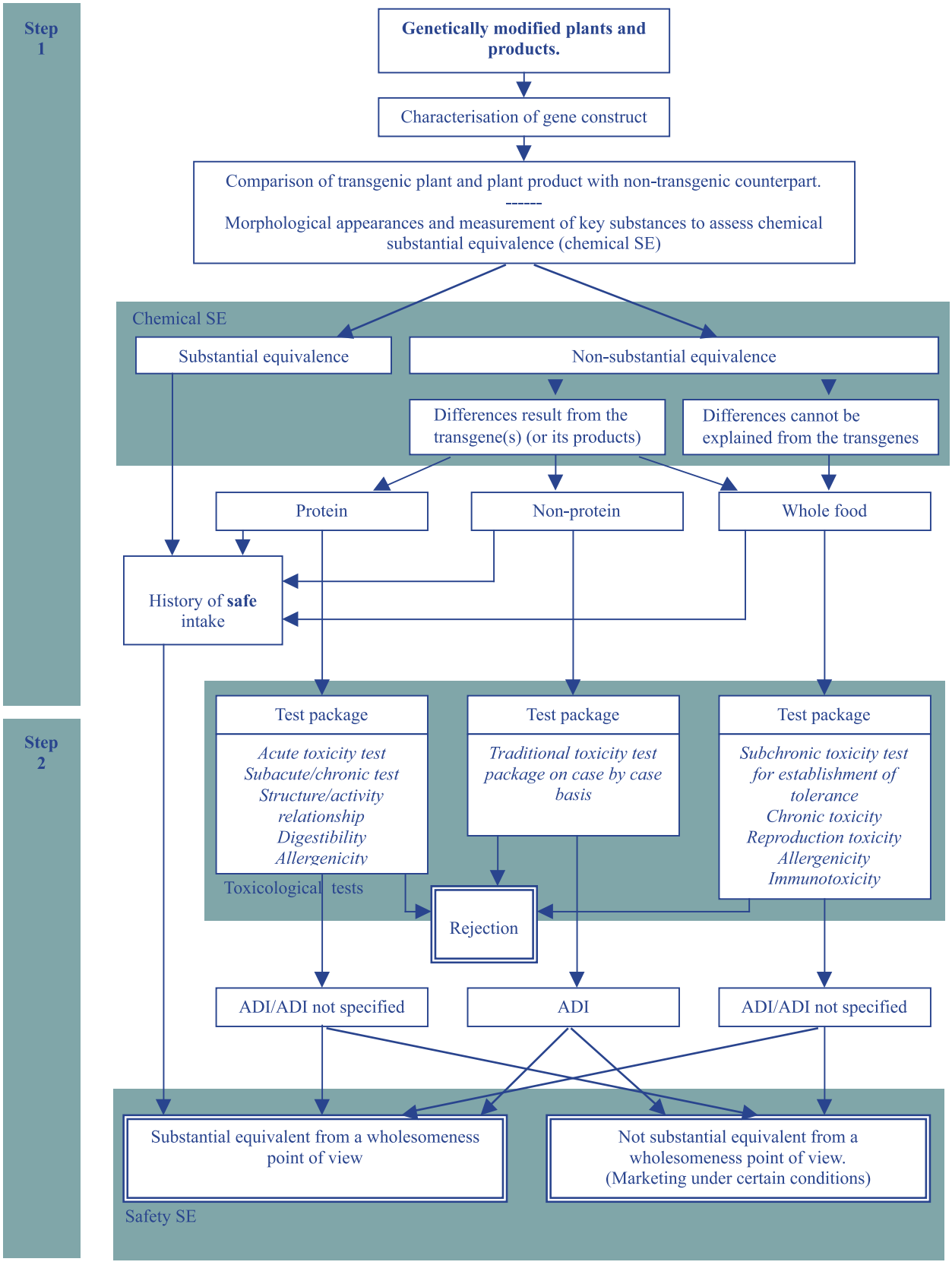
If non-substantial equivalence on a chemical compositional basis is established, and the differences cannot be explained by the newly introduced transgenes, then the safety of the whole food may be established based on the history of intake. Otherwise specific safety studies are needed. Whole food testing in experimental animals is unable to ensure the application of the usual large safety factors due to the distortion of the animal diet by inclusion of too large proportions of the novel food item in the diet. Therefore animal studies on whole food items without history of food intake are mostly suited to establish margins of tolerance for the novel food. An acceptance will possibly be expressed as an ADI *not specified*. For some of the novel GM plant foods it may be relevant in addition to the whole food testing to address the safety of specific new proteins or non-proteins or specific inherent proteins or non-proteins due to substantially higher amounts of those than in the traditional counterpart. Here an ADI may need to be established. The latter becomes even more important if significantly higher intake of the novel GM plant food than the traditional counterpart can be foreseen.

The end of step 1 of the assessment procedure may lead to the determination of a history of safe intake of the protein, non-protein and the whole food. Thereby, substantial equivalence of the GM food from a wholesomeness point of view is established.

If history of safe intake cannot be established, step 2 of the procedure is initiated. Step 2 may lead to the establishment of ADI's or ADI's not specified, and if not, the products are rejected due to toxicity, scientific uncertainty regarding the toxicity or due to lack of data. If an **ADI** or an **ADI not specified is established** the product may either be regarded as **substantial equivalent from a wholesomeness point of view** or it may be regarded as **not substantial equivalent from a wholesomeness point of view**. In the latter case the food is safe as such, but here the specific conditions for use will need to be established on a case-by-case basis.

[11] An ADI is normally given as mg/kg body weight/day throughout lifetime. An "ADI not specified" means that no health problems are expected from intake limited by the physiological need for food from a variety of sources for normal growth, maintenance and reproduction.

Figure 3: Proposal for the overall safety testing procedure for GM food plants and their products



# 6. Discussion

The introduction of genetic engineering has been met with a demand of consumers for a high safety level standard for the new foods. This demand for a high safety level has been complied with by setting up guidance for evaluation and/or development of pre-market approval systems of these new products that include extensive documentation, analysis and tests. The argument for this high safety level, which seems to be much higher than the safety level for traditional food plants, is the present limitation of experience (lack of history of safe intake) with genetically engineered foods.

The current approach of food safety assessment of genetically engineered food is based on the concept of substantial equivalence and the endpoint of the assessment should be to determine whether the genetically modified food is as safe as traditional food. In practice food safety dossiers are comprised of data regarding: (1) molecular characterisation of the insert, (2) direct toxicological consequences as can be predicted from the nature of the transgene, (3) possible unwanted and indirect toxicological consequences of the genetic modification, and (4) morphology and phenotypical behaviour. Especially the data concerning (3) and (4) are guided by the concept of equivalence, as there are no direct means of measuring any unwanted effects resulting either from pleiotropy, insertion or somaclonal variation. On the other hand, it are just these kinds of effects that are already known from traditional breeding, where wide crosses, mutagenesis (for instance through irradiation), and other methods result in qualitatively similar effects.

Although all companies have included data on the four above mentioned aspects in their food safety dossiers of genetically engineered crops, there are differences in the type and amount of data, and differences in the tests employed. In all these individual cases the data have been accepted by the authorities to be sufficient, but the analyses show that there is a clear need in further standardisation of the food safety testing. This could start with the determination of a standard list of plant constituents per crop to be analysed to substantiate substantial equivalence (an example is given in table 4). Also it should become more clear in what cases to do which test package of toxicological studies. Also the role of history of safe intake should play a more explicit role to prevent sometimes unnecessary and laborious animal studies.

With regard to the safety of traditional food plants, there is considerable knowledge on individual toxic substances, but there is a serious lack of knowledge about the combined effects of different compounds in a complex matrix like a plant food item. The knowledge about the combination effects of different compounds with opposite effects such as carcinogenic and anti-carcinogenic compounds in a complex food is still in an early phase. Therefore, the toxicological assessment should not be seen as an exact scientific evaluation that will stand forever, but as an assessment based on today's knowledge and therefore may be changed over time. The accumulation of knowledge and experiences both in traditional crops and in genetically engineered crops can lead to either a further increase in the demand for documentation, analyses and tests for genetically engineered crops, or to a decrease in this demand. But the latter only when experience shows that there is no need to screen for any possible toxicologically unwanted indirect effects resulting from the modification. For traditional foods the demand for testing is only likely to increase.

To meet this possible higher demand, the validation and development of new multi-factorial methods such as proteome analysis, differential display and DNA microarray, which might give a broad covering of relevant compounds in a plant, might be helpful. These methods will only be valuable if they are able to differentiate between differences that are relevant from a toxicological point of view and those that are not. At this moment none of these methods qualifies for these demands. But if such methods can be developed that more directly and in a validated way can detect relevant differences, then this would be a positive development for the food safety of both genetically modified and traditional crops.

The overall testing procedure to screen GM plants or plant products for insertional, pleiotropic and somaclonal effects, and the focus on specific new products or the whole GM plant, rely on laborious animal studies. Animal studies for detection of adverse effects of GM plants and their products is today a suitable tool for the assessment of wholesomeness. On the other hand these types of tests have a rather poor negative predictive value when it comes to possible chronic toxicological consequences. In the future, however, it is expected that some of the multi-factorial techniques should at least partially replace some of the animal test screening methods. These methods may lead to more detailed, informative, faster and cheaper results. The regimen for the overall testing protocols and the assessment procedure should not at the present state of the art be too rigid, but be kept flexible to absorb new developments in science and overall experiences with past and present assessments. Also it is foreseen that acceptance of certain gene products through ADI or "ADI not specified" may be linked to certain conditions for harvesting, processing and labelling. E.g. plants with lectins might need to be soaked and cooked before eaten.

This report has not specified feed safety assessment in a direct manner. The rationale is that genetically modified plants for feed production might follow the same guidelines as for food evaluation considering the fact that feed can be tested on the animal and the potential influence of the feed on the animal as food item such as milk and meat.

# 7. Cases

## 7.1 Tomatoes with extended shelf life (Zeneca)

**Description of the genetic modification**

Polygalacturonase (PG) degrades pectin in tomatoes, causing softening of the fruit. Zeneca developed a tomato for processed food (line TGT-7F) in which the first 731 basepairs of the tomato PG-gene are inserted. This is only a small fragment of the native PG gene (Bird et al 1988). This PG-gene fragment (in sense direction) is regulated by a constitutive promoter resulting in the continuous production of small RNA fragments that interfere with the expression of the native PG gene. The RNA fragments result in a decreased synthesis of the PG-enzyme through a mechanism of messenger breakdown. This results in a prolonged shelf life. The transgenic tomatoes also contain the *nptII* antibiotics resistance marker gene which is originally isolated from the bacterial transposon Tn5. The *nptII* gene allows the selection of modified cells after the transformation (for more information see the review on antibiotic resistance markers). It is widely used, has been well-characterised (Nap et al. 1992) and codes for kanamycin resistance.

Genetic and compositional analyses showed that only one copy of the gene had been inserted into the tomato genome and that no other part of the vector was present. Also open reading frame and promoter DNA motif analysis were carried out, concluding that none of the open reading frames was associated with potential transcription and translation control sequences. The results of these analyses demonstrate that the inserted PG-gene fragment does not lead to the production of protein itself, that it reduces the amount of native PG-protein present in the plant, and that the only new protein in the tomato is the NPTII protein.

**The new protein from the inserted nptII gene**

It can well be argued that the NPTII protein from the nptII marker gene is not new for the consumer, because the protein is already present in microorganisms in the human gastro-intestinal tract making them naturally resistant to kanamycin. The NPTII protein was however new to the transgenic tomato and for this reason it was assessed as a new product. The NPTII protein is not expected to interfere with the plant cell metabolism, because it is known to be highly specific for a few antibiotics of the aminoglycoside-group. These antibiotics are not present in the plant.

The tomatoes were only to be used as processed tomatoes (tomato paste). The processing involves different steps including heating in which proteins can be denatured. The NPTII protein is known to be heat labile, therefore no active protein was expected to be present in the tomato paste. The following discussion of the new protein however covers both use of fresh and processed tomato material to show the principles.

The amount of NPTII protein was determined in fresh tomatoes by ELISA (enzyme linked immunosorbant assay) and was shown to be present in low amounts. In processed tomato (paste) the NPTII concentration was much lower and could hardly be detected. With regard to the toxicity of the NPTII protein it was already known from earlier studies that the protein is not inherently toxic (Flavell et al. 1992, Nap et al. 1992).

Besides this the NPTII protein was shown to degrade very rapidly in the gastro-intestinal tract (<10 sec. in simulated gastric fluid) (Fuchs et al. 1993), and behaves as a normal food protein. The protein as such was therefore not considered to be toxic.

On top of this analysis of the possible inherent toxicity of the NPTII protein possible other unwanted consequences for human health of the presence of this protein were considered. As nptII is an antibiotic resistance gene it was considered important to assess the potential for compromising the therapeutic efficiency of other aminoglycoside antibiotics, when administered orally. It should be recognised however that this is not a direct food safety aspect. To compromise the activity of such antibiotics, the NPTII protein should be able to phosphorylate them in the acidic environment of the stomach. The enzymatic reaction of NPTII to modify kanamycin requires ATP (Adenosine 5'-triphosphate) as co-factor, which is unstable in acid environments such as the stomach. The ATP concentration in the gastro-intestinal tract is lower than required for catalytic activity (Nap et al. 1992). Calgene has estimated a potential inactivation based on a worst case scenario, such as simultaneous ingestion of high ATP content fruit and vegetables together with transgenic tomatoes and neomycin through a neutral pH stomach (Redenbaugh et al. 1995). Their estimation shows that ingestion of tomatoes with NPTII protein will not compromise the efficacy of orally taken aminoglycoside antibiotics.

Both assessment of allergenicity of the NPTII protein and the discussion regarding possible horizontal gene transfer from plants to micro-organisms are discussed in the reviews on allergenicity and horizontal gene transfer. In this review it is concluded that the NPTII protein is not considered to be allergenic, and that horizontal gene transfer of the *nptII* gene is highly unlikely, but even in case such an event would take place, make no significant contribution to the pool of resistance genes already existing in nature.

**No protein from the PG gene fragment**

The partial sense PG gene fragment does not itself produce a protein, but is expected to change the PG level. The level of PG enzyme should be measured as an indicator of the change in pectin degradation, but there is no basis for doing a specific toxicological test since there is no product to test.

The level of PG in transgenic plants was measured in many field trials (different environments) over ten generations showing that the silencing effect is stable.

**Possible insertional or pleiotropic effects**

To make sure that the insertion of the genes does not cause other effects in the transgenic plant it must be screened for secondary disturbances. The principle of substantial equivalence is the tool for the comparison between the transgenic tomato and the comparable non-transgenic tomato. Substances that are relevant from a toxicological point of view, or relevant for their nutritional value or wholesomeness should be looked at (see chapter 4 of this review).

Tomato is known to accumulate the glycoalkaloid α-tomatine. Measurements of both transgenic and comparable non-transgenic tomatoes at fresh fruit level and as paste documented no difference in the concentration of α-tomatine.

The applicant has also measured other natural toxicants including the biogenic amines (tyramine, tryptamine and serotonin), histamine, nicotine, and lectins. Most of the results show amounts under the detection level of

the methods used. When the measurements were above the detection level, there were no differences between the transgenic tomato and the comparable non-transgenic tomato. These results demonstrated on the one hand that there are no differences for a number of compounds, but on the other hand that it is important to establish guidelines for the analytical testing of compounds. It is not useful to test for substances that most probably will be under the detection level and methods used should be as sensitive as possible.
In addition the applicant measured a wide range of general nutritional parameters: different carbohydrates and fibres, minerals, vitamins, amino acid profiles, and acids as malic- and citric acid. The applicant also compared the lycopene content, (lycopene is expected to be an important anti-carcinogenic compound) and contaminants such as the toxic metals arsenic, cadmium, lead and mercury. All these measurements were done both with fresh fruits and tomato paste. In all these comparisons there were no significant difference between the transgenic tomato and the comparable non-transgenic tomato.

**Conclusion**

The results for comparison of the transgenic tomato with the comparable non-transgenic tomato plant do not show any significant differences except the new gene product NPTII and the reduced expression of polygalacturonase. The insertion of the new gene construct has not introduced a new unexpected effect to a degree that it can be detected by this screening. From a toxicological point of view the new tomato is assessed as having the same degree of safety as its non-transgenic tomato counterpart when used for the same purpose.

## 7.2 Bt herbicide tolerant maize (Novartis, event 176)

**Description of the genetic modifiction**

Novartis has made maize plants resistant to the larvae of the European corn borer by inserting a *Bacillus thuringiensis* endotoxin gene *(cry1A(b))*. In the transgenic maize (event 176) a gene construct with two *cry1A(b)* genes is inserted, one with a promoter for expression in the male flowers and one with a promoter for expression in the green parts of the plant. The product of the *cry1A(b)* is an endotoxin which is toxic to larvae of the *Lepidoptera*[12] species. Two other genes are also present in the plant: (1) the *bar* gene, whose product phosphinotrycin acetyl transferase (PAT) increases tolerance to the herbicide glufosinate ammonium (phosphinotrycin). The *bar* gene is inserted for early selection of transgenic cells and plants, and (2) the bacterial *bla* TEM1 gene providing resistance to the antiobiotics ampicillin and amoxycillin. This gene is not expressed in the maize plant as it is under control of a bacterial regulation system. The ampicillin resistance gene is used for bacterial selection before insertion of DNA (plasmids) into maize. So even though three genes are inserted, only two new gene products are made in the transgenic maize plant: the CRY1A(b) protein and the PAT protein. Both proteins are not expected to interact with the host plant metabolism, because they are both known to specifically interact with substances that are not present in the plant (phosphinotrycin and an insect midgut receptor).

The genes were inserted in the maize using the biolistic method which is known to often result in the insertion of more than one copy of the inserts. For technical reasons two different plasmids were used in this procedure, one with the two *cry1A(b)* genes and the ampicillin resistance gene and one with the *bar* gene and

[12] Lepidoptera is a family of winged insects including moths, and butterflies.

the ampicillin resistance gene. In event 176 two to five copies of both types of plasmids are inserted in the same insertion site at chromosome 1. Molecular analysis has shown that the inserted sequences are inserted intact and show no unwanted breaks or rearrangements.

**The new proteins from the inserted transgene.**

The CRY1A(b) protein is well-known from biological pesticides. Farmers have sprayed plants with the Bt toxin-producing bacteria as protection against insects for the last 40 years. The applicant has demonstrated in different studies that the active Bt toxin product from the transgenic maize is equivalent to the product from the bacteria. It is well documented that the Bt toxin is expressed in the green part of the plant and in the male flowers (tassels), and only trace amounts are present in maize kernels.

The transgenic maize plant produces insufficient amounts of PAT protein to protect the maize plant against glufosinate ammonium in field conditions. Its tolerance to glufosinate ammonium however was high enough to be able to serve as a selective marker during transformation. In the approved plant the PAT protein concentration is under detection level in maize kernels. In leaves and stem trace amounts can be detected.

The toxicological assessment of the two new proteins focuses on the issues; are the proteins inherently toxic and is there any effect of ingestion of the proteins?

Both CRY1A(b) and PAT proteins have been assessed for acute toxicity by feeding mice with purified protein in high doses. Proteins for such tests cannot be extracted from plant material in sufficient amounts, but must be produced in bacteria. The bacterium produced material is then compared with the plant-produced material to verify that the pure product is the same. In the acute toxicity studies, doses of new protein at more than 1000 times the human exposure with normal intake of maize kernels, show no adverse effects in mice. In addition feeding studies with mice, broiler chicken and quails fed with maize kernels and maize protein showed no indications of (sub)chronic toxicity. There were also no differences found in growth and behaviour between animals fed with transgenic maize and non-transgenic maize. So neither CRY1A(b) nor PAT protein show signs of inherent toxicity for humans and higher animals.

The effect of ingestion is analysed both in *in-vitro* degradation studies of CRY1A(b) and PAT protein and in simulated gastric fluid. Both proteins are degraded very rapidly (< 1 min). From these studies it is expected that the CRY1A(b) and PAT proteins after intake will be degraded at the same time/place as most food proteins. Thus no derived effects from ingestion of CRY1A(b) or PAT protein would be expected.

**Possible insertional or pleiotropic effects**

Comparison between transgenic maize and non-transgenic maize can be difficult in hybrid plant breeding, because there are no direct comparable non-transgenic plants. In these cases without direct comparable mother material, the comparison should be done with the closest relative plant material, and the assessment of the comparison data should take this into account. In practice the substantial equivalence assessment is documented further by use of a range of close conventional plant varieties to illustrate the variation.

In order to screen for unknown effects such as insertional effects and pleiotropic effects, a number of morphological parameters have been examined together with yield and one of maize natural protectants

towards European corn borer 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). There was no significant difference in morphology, yield or concentration of DIMBOA.

In table 1 cyanogenic glycosides and trypsin inhibitor are noted as toxic compounds. Cyanogenic glycosides are only expressed in the maize plant in the plantlet stage. Trypsin inhibitors is a group of compounds, found in most if not all plants, of which the anti-nutritional effects should be elaborated in more detail. In maize there are no significant toxic compounds, which must be measured for the purpose of substantial equivalence assessment. It could be argued that comparison of trypsin inhibitor content should be included in substantial equivalence assessment. The applicant presented measurements of a range of general nutritional parameters: starch, vitamin B1, amino acid profiles and fatty acid profiles. In addition the applicant compared the xanthophyll and b-carotene content. These measurements were done on grain samples and all values for the grain from genetically modified lines fall within the range of values observed in the cited literature.

**Conclusion**

The comparison of the transgenic maize with comparable non-transgenic maize plant shows no significant differences except the new gene products CRY1A(b) and PAT. The insertion of the new gene constructs has not made new unknown effects to a degree that it could be detected by this screening. From a toxicological point of view the new transgenic maize has been assessed as having the same degree of safety as the comparable non-transgenic maize.

# 8. References


Dehesh K, Edwards P, Hayes T, Cranmer AM, Fillatti J 1996: Production of high levels of 8:0 and 10:0 fatty acids in transgenic canola by overexpression of *Ch FatB2*, a thioesterase cDNA from *Cuphea hookeriana*. Plant Physiol. **110**:203-210

Finn RF, Leimgruber RM, Boyle DM, Jennings MG, Kimack NM, Smith CE, Bishop BF, Frazier RB, Magin KM, Fuchs RL, Reed AJ 1996: Purification and biochemical comparison of 1-aminocyclopropane-1-carboxylic acid deaminase proteins expressed in delayed ripening tomato and *Escherichia coli*: studies for a safety assessment. J. Agric. Food Chem **44**:381-387

Flavell RB, Dart E, Fuchs RL, Fraley RT 1992: Selectable marker genes: safe for plants? Biotechnology **10**:141-144

Fuchs RL, Ream JE, Hammond BG, Naylor MW, Leimgruber RM, Berberica SA 1993: Safety assessment of the neomycin phosphotransferase II (NPTII) protein. Biotechnology **11**:1543-1547

Gelvin SB 1998: The introduction and expression of transgenes in plants. Curr. Opin. Biotech. **9**:227-232

Goodman RM, Hauptli H, Crossway A, Knauf VC 1987: Gene transfer in crop movement. Science **236**:48-54

Hirayama T, Wada A 1995: Heat-stable enterotoxin of enterotoxigenic Escherichia coli. In: Molecular Approaches to food safety (Eklund M, Richard JL, Mise K eds.) pp. 513-524. Alaken, Inc.; Fort Collins.

Holm S, Alexander J, Andersson C, Aune T, Gry J, Phu Lich N, Schlatter TJ, Strigl A 1998: Nettox list of food plants prioritised for inclusion in a future European database. (Report no.6) Danish Vet. Food Adm.

Kinney AJ 1998: Manipulating flux through plant metabolic pathways. Current Opinion in Plant Biology **1**:173-178

Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, Gafni, Y, Czosnek H 1994: Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. Biotechnol. **12**:500-504

Lindsey K, Jones MGK 1989: Plant Biotechnology in Agriculture. Open University Press: Milton Keynes.

Maessen GDF, Derksen J 1998: Mutation and transposition frequencies in maize, petunia and potato. Report for the Dutch Ministry of Economic Affairs (CCRO report)

Mariani C, De Beuckeleer M, Truettner J, Leemans J, Goldberg RB 1990: Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature **347**:737-741

Mariani C, Gossele V, De Beuckeleer M, De Block M, Goldberg, RB, De Greef W, Leemans J 1992: A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. Nature **357**:384-387

McDonald JF 1995: Transposable elements: possible catalysts of organismic evolution. Trends Ecol. Evol. **10**:123-126

Nap, J-P, Bijvoet J, Stiekema WJ 1992: Biosafety of kanamycin-resistant transgenic plants. Transgenic Res. **1**:239-249

Nordic Council of Ministers, Nordic Working Group on Food Toxicology and Risk Evaluation 1998: Safety Assessment of Novel Food Plants. Tema Nord **591**

Noteborn HPJM, Bienenmann-Ploum ME, van den Berg JHJ, Alink GM, Zolla L, Reynaerts A, Pensa M, Kuiper HA 1995: Safety assessment of the Bacillus thuringiensis insecticidal crystal protein CRYIA(b) expressed in transgenic tomatoes In: Genetically Modified Foods. (Engel K-H, Takeoka GR, Teranishi R, eds.) ACS symposium series **605**:134-147

OECD 1993: *Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles*, (Organisation for Economic Co-operation and Development), Paris

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE 1999: Compromised disease resistance in saponin-deficient plants. Proc Natl Acad Sci USA **96**:12923-12928

Reddy AS, Thomas TL 1996: Expression of a cyanobacterial D6-desaturase gene results in g-linolenic acid production in transgenic plants Nature Biotechnol. **14**:639-642

Redenbaugh K, Berner T, Emlay D, Frankos B, Hiatt W, Houck C, Kramer M, Malyj L, Martineau B, Rachman N, Rudenko L, Sanders R, Sheehy R, Wixtrom R 1993: Regulatory issues for commercialization of tomatoes with an antisense polygalacturonase gene. In vitro Cell Dev. Biol. **29**:17-26

Redenbaugh K, Hiatt W, Martineau B, Emlay D 1995: Determination of the safety of genetically engineered crops. In: Genetically Modified Foods. (Engel K-H, Takeoka GR, Teranishi R, eds.) ACS symposium series **605**:72-87

Reed AJ, Kretzmer KA, Naylor MW, Finn MW, Magin KM, Hammond BG, Leimgruber RM, Rogers SG, Fuchs RL 1996: Safety assessment of 1-aminocyclopropane-1-carboxylic acid deaminase protein expressed in delayed ripening tomato. J. Agric. Food Chem. **44**:388-394

SanMiguel P, Tikhonov A, Jin Y-K, Motchoulskaia N, Zakharov D, Melake-Berhan A, Springer PS, Edwards KJ, Lee M, Avramova Z and Bennetzen JL 1996: Nested retrotransposons in the intergenic regions of the maize genome. Science **274**:765-768

Schmidt T 1999: LINEs, SINEs and repetitive DNA: non-LRT retrotransposons in plant genomes. Plant Molec. Biol. **40**:903-910

Schuler TH, Poppy GM, Kerry BR, Denholm I 1998: Insect-resistant transgenic plants. Trends Biotech. **16**:168-175

Slanina P. 1990: Assessment of Health-risks related to glucoalkaloids (" Solanine") in potatoes: A Nordic View. Vår Föda **43**: supl. 1.

Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC 1999: The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell **99**:123-132

Toth B 1995: Mushroom toxins and cancer (Review). Internat. J. Oncol. **6**:137-145

Visser RGF, Somhorst I, Kuipers GJ, Ruys NJ, Feenstra EJ, Jacobsen E 1991: Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs. Mol. Gen. Genet. **225**:289-296

WHO 1995: Application of the Principles of Substantial Equivalence to the Safety Evaluation of Foods or Food Components from Plants Derived by Modern Biotechnology, Report of a WHO Workshop, Food Safety Unit, World Health Organization, Geneva

WHO 2000: Safety aspects of genetically modified foods of plant origin, Report of a joint FAO/WHO expert consultation on foods derived from biotechnology, WHO headquarters, Geneva, Switzerland, 29 May – 2 June 2000

Yuan L, Voelker TA, Hawkins DJ 1995: Modification of the substrate specificity of an acyl-acyl carrier protein thioesterase by protein engineering. Proc. Natl. Acad. Sci. USA 92:10639-10643

Zhu T, Peterson DJ, Tagliani L, St.Clair G, Baszczynski CL, Bowen B 1999: Targeted manipulation of maize genes in vivo using chimeric RNA/DNA oligonucleotides. Proc. Natl. Acad. Sci. USA 96:8768-8773

Zhu T, Peterson, DJ, Tagliani L, Baszczynski CL 2000 : Engineering herbidicide-resistant maize using chimeric RNA/DNA oligonucleotides. Nature Biotechnology 18:555-558.

# Safety of Genetically Engineered Plants: an Ecological Risk Assessment of Vertical Gene Flow

Authors: **Klaus Ammann, Yolande Jacot, Pia Rufener Al Mazyad**
**University of Bern, Botanical Garden**
**Altenbergrain 21, CH - 3013 BERN**

**1. Introductory remarks ..... 61**
**2. Gene flow and hybridisation ..... 62**
2.1. Definition ..... 62
2.2. Factors of hybridisation ..... 62
2.3. Hybridisation of crops with their wild relatives ..... 63
2.4. Hybridisation with wild relatives in Europe ..... 63
2.5. Genetic engineering and effects on hybridisation and reproduction ..... 64
2.6. Gene flow from transgenic plants ..... 64
2.7. Conclusion ..... 65
**3. Codes to help the evaluation of risk regarding field release of transgenic crops ..... 66**
3.1. Gene flow indices as a help in risk assessment ..... 66
3.2. Classification by combination of the three codes ..... 67
3.3. Probability of gene dispersal versus gene effects ..... 68
**4. Weediness of transgenic crops ..... 69**
4.1. A definition of weeds ..... 69
4.2. Weed characteristics ..... 69
4.3. Weediness in relation to other concepts in risk assessment ..... 70
4.4. Genetic engineering of crops and weediness ..... 70
4.5. A particular case: transgenic herbicide tolerant crops and weediness ..... 72
4.6. Conclusion ..... 73
**5. Selective advantage and improved fitness of transgenic crops ..... 74**
5.1. Definition of selective advantage and fitness ..... 74
5.2. Risky transgenes? ..... 74
5.3. Lessons learned from traditional breeding ..... 77
5.4. Lessons from exotic species and exotic genomes ..... 78
5.5. Conclusion ..... 79
**6. Ecological view and consequences for risk assessment ..... 80**
6.1. Ecology and safety research ..... 80
6.2. Consequences for risk assessment ..... 80
6.3. Testing scheme for risky crops ..... 81
**7. Discussion and Conclusion ..... 84**
**8. References ..... 86**

# 1. Introductory Remarks

Genetically engineered plants are a hot topic in the public debate in Europe. One concern that gives rise to controversy is the potential longterm environmental risk of the large scale application of genetically engineered crops.

The environmental risks of genetically engineered crops have been categorised as follows (Journal of Molecular Ecology, vol.3, 1994):

- Invasiveness of the transgenic crop (in the agricultural system as a weed or in natural habitats).
- Invasiveness of transgene itself (vertical gene flow[1] through hybridisation with wild relatives).
- Environmental side effects of the transgenic products (for instance effects on non-target organisms).

This study will only deal with the first two environmental risk categories of the use of transgenic crops. The third category is dealt with elsewhere in this report in the paper by Julian Kinderlerer. This study will describe what factors determine the successful hybridisation with wild relatives and it will describe some concepts that are thought to be important for determining the invasiveness of the transgenic crop or the transgene itself. These are the concepts of weediness, fitness and selective advantage. Wherever possible it will be tried to make a healthy comparison with facts known from conventional plant breeding, without jumping to preliminary conclusions or generalisations.

The risk assessment process is very important in determining the possible and actual ecological consequences of the use of transgenic crops. This study will try to gather relevant risk assessment concepts and it will give the example of gene flow indexes as a means of categorising crops into risk classes.

[1] **Vertical gene flow** is the flow of genetic material from parent plants to their descendants, where these descendants are either clones of their parental plants (for instance a potato plant resulting from a tuber), or where these descendants are the result of mating between sexually compatible organisms. This is in contrast to **horizontal gene flow** which is defined as the transfer of genetic material from one organism to another non-sexually compatible organism (for instance from a potato to a bacterium), and where the organism that has taken up the genetic material is not a descendant of the donor of the genetic material. Horizontal gene transfer is dealt with elsewhere in this report in the paper by Philippe Gay.

# 2. Gene Flow and Hybridisation

## 2.1. Definition

The vertical flow of transgenes from genetically engineered crops to wild relatives is a major concern in the environmental risk assessment of genetically engineered crops. Vertical gene flow is a well-known phenomenon in population genetics. It is by no means something new and it is not connected solely to transgenic plants. Vertical gene flow from transgenic plants to wild relatives requires successful hybridisation to take place between the transgenic crop and a wild relative. "Hybridisation" can be defined as the cross-breeding of different individual plants. These individuals may be genetically similar, differ in a few or in numerous genes or be very different genetically. They may belong to various populations or races of the same taxonomic species (intraspecific or interpopulational hybridisation) or to different species (interspecific hybridisation). From an agronomic point of view hybridisation is very important as the major mechanism for generating new varieties of crops through cross-breeding. Most of the current varieties are the result of hybridisation between different existing varieties or hybridisation with wild relatives.

## 2.2. Factors of hybridisation

The first and major requirement for hybridisation to occur between two individual plants is that they have to be sexually compatible. But even if two individual plants are sexually compatible this does not mean that the dispersal of pollen in nature will lead to hybridisation in every case. The probability of successful pollination depends on a great number of interrelated factors, such as:

- Level of pollen production of (transgenic) plants.
- Rate of self- and cross-fertilisation of receptor plants.
- Rate of dispersion of donor pollen.
- Properties of pollinating agents[2] , where plants sometimes use highly specialised insect pollinators and have highly adapted flowers, resulting in a very effective genetic isolation (mechanical isolation).
- The existence of spatial distance between the pollen donor and the wild recipient population (spatial isolation). How large the distance is within which pollination can occur depends on factors like: wind turbulence, speed and direction and/or the flying range of insects and the time during which pollen is viable.
- Local density of recipient population.
- A difference in flowering season between the crop and the wild population (phenological isolation).

In addition to these factors a number of other factors are sometimes responsible for the fact that even though plants are sexually compatible, they do not hybridise in practice, or that the formed hybrid is not viable (Hadley and Openshaw, 1980). These are prevention of fertilization, hybrid weakness or inviability, hybrid sterility, and hybrid breakdown.

[2] Many plants make use of insects to transport the fertile pollen to another flower. In some cases there is a highly specialised relationship between the plant and the pollinating agent.

## 2.3. Hybridisation of crops with their wild relatives

Even though the barriers to hybridisation exist in nature as described above, many economically important crops show low hybridisation barriers. Such low hybridisation barriers have been important for agriculture because it has enabled plant breeders to develop new varieties using cross-breeding. It has been shown that hybridisation was a common technique in the breeding history of 31 out of 39 plant families, and in 70 out of 90 plant genera, i.e. 75-80 percent of the cases (Simmonds, 1976). To make new varieties plant breeders often use wild plant material as a source of genetic variation. The cultivation area of a particular crop in many cases overlaps the area in which these wild, sexually compatible relatives occur, and sometimes even the centre of origin of the crop is within that overlap. Ellstrand et al. (1999) demonstrated that 12 of the 13 worldwide most important crops hybridise with wild relatives somewhere in their cultivation area (Table 1). So if one of these crops is genetically modified and grown worldwide, the newly incorporated transgene will most probably be transferred to a wild relative somewhere in the world.

Table 1: Hybridisation of crops with wild relatives. Sugar beets are added to this list of Ellstrand et al. (1999) due to their European importance.

| Crop | Relative(s) | Crop | Relative(s) |
|---|---|---|---|
| 1. WHEAT | Wild *Triticum turgidum* subspecies, some *Aegilops* species | 8. MILLETS | *Eleusine coracana* ssp. Africana, Wild *Pennisetum* species |
| 2. RICE | Wild *Oryza* species | 9. BEANS | Wild *Phaseolus* species |
| 3. MAIZE | Wild *Zea mays* subspecies | 10. OILSEED RAPE | Some wild *Brassicaea* species |
| 4. SOYBEAN | *Glycine gracilis, Glycine Soya* | 11. PEANUT | No report |
| 5. BARLEY | *Hordeum spontaneum* | 12. SUNFLOWER | Wild *Helianthus annuus* |
| 6. COTTON | Wild *Gossypium* species | 13. SUGARCANE | Wild *Saccharum* species |
| 7. SORGHUM | Wild *Sorghum* species | 14. SUGARBEET | Wild *Beta* species |

## 2.4. Hybridisation with wild relatives in Europe

On a smaller scale, the perspectives for cross to hybridise with wild relatives may change dramatically. In Europe for instance, not all crops have wild relatives with which they can hybridise successfully. Table 2 gives an indication of the hybridisation possibilities for a number of crops in Europe. But even within Europe there are still large regional differences, where in one area crops can hybridise with wild relatives and in other regions it cannot, for instance sugarbeet.

Table 2: Possibilities for successful hybridisation of some European crops with wild relatives

| Crop | Wild relative(s) within Europe | Crop | Wild relative(s) within Europe |
|---|---|---|---|
| 1. POTATO | None | 8. SUGARBEET | Wild Beta species |
| 2. SOYBEAN | None | 9. SUNFLOWER | None |
| 3. MAIZE | None | 10. CARROT | Wild Carota species |
| 4. BRASSICA SPP. | Wild Brassica species | 11. GRASSES | Wild grasses |
| 5. TOMATO | None | 12. WHEAT | None |
| 6. RICE | None | 13. COTTON | None |
| 7. BARLEY | Hordeum spontaneum | 14. BEANS (Phaseolus) | None |

Table 2 shows that in Europe in the risk assessment for crops like potato, tomato, soybean, sunflower, rice, wheat, cotton, beans and maize, the invasiveness of the transgene is not a concern, but only the possible invasiveness of the crop itself. This of course starts from the assumption that the genetic modification itself does not alter the possibilities of the crop to hybridise in a positive sense.

## 2.5. Genetic engineering and effects on hybridisation and reproduction

The possibilities for crops to hybridise with wild relatives as described in the former paragraphs are not a static situation. Reproductive isolation barriers can break down or be built up. Many examples of changes in reproductive isolation barriers resulting from classical breeding are known, especially in compositae, grasses, orchids and other ornamentals. Genetic engineering, like classical breeding, has the potential to change characteristics of a plant resulting in changes in the possibilities for successful hybridisation.

The changing of flower shape could for instance have consequences in the case that mechanical isolation is the actual reproductive isolation barrier, resulting in hybridisation between plants that did not occur before. The changing of the flowering season could result in new types of hybridisation where phenological isolation forms an effective isolation barrier. Flowering period is a multigenic trait and up to now, transformation has not resulted in alterations in flowering period. There are other examples. Research on for instance flower morphology is being done and genes involved in the development of flowers are being isolated. There are no transgenic plants in field trials in which a reproductive isolation barrier has been broken down.

Even more research is done to introduce new reproductive isolation barriers into crop plants. The improvement of the biosafety of transgenic crops is one of the driving forces. Examples are male sterility, apomixis, or gene switch technologies that require the addition of an external factor to allow germination. The latter is also often referred to as 'terminator technology' and is being abandonned by many companies because of societal pressure as a consequence of negative socio-economic and ethical considerations.

## 2.6. Gene flow from transgenic plants

In relation to gene flow from transgenic plants there is a true flood of smaller and more extensive review papers produced (Abbott 1994a, 1994b, Ammann 1995, Bazin et al. 1995, Bevan et al. 1995, Ellstrand 1992, Gregorius et al. 1993, Raybould et al. 1993, 1994, Snow et al. 1995, Tiedje et al. 1989, van der Meer 1993). Lots of ingenious pollen release experiments have been performed, over 1600 field trials have been counted until 1994 according to extracts from GIBiP Database on field trials 1986 - 1994 (Green Industry Biotechnology Platform 1995).

**Transgene spread from oilseed rape**

Oilseed rape has been the first major transgenic crop for which gene flow to wild plants is a genuine concern. Much attention has gone in determining the actual gene flow from transgenic oilseed rape. The minimum average safety distance between transgenic and non-transgenic oilseed rape in order to avoid unwelcome gene flow is determined by different groups and differs from 70 to 400m (Dale et al. 1993, Scheffler et al. 1993, Sinemus 1994). Such isolation distances are already known from traditional breeding, in particular for the growing of certified seed that must have a high degree of purity. In the case of oilseed rape, isolation distances of several hundred meters are known to give a seed purity of 99% or more. Still, there is a possibility, that the partially entomophilous oilseed rape can spread its pollen over distances of several kilometers, but successful pollination and fertilization over such big distances must be considered as very rare events (Eckert 1933).

Recent publications by Mikkelsen et al. (1996) and Timmons et al. (1996) show the evidence of transgene spread in the case of oilseed rape. The results suggest a rapid spread of transgenes through interspecific backcrossing under field conditions. The occurrence of fertile, transgenic weed-like plants after just two generations of hybridization should be taken into account when considering the consequences of transferring new traits to oilseed rape. The wild species mentioned in this article is *Brassica rapa ssp. campestris* (often referred to as *B.campestris*). The authors themselves give a rather pragmatic interpretation of their own results: it depends strictly on the nature of the transgene whether there will be a rapid spread of the new transgenic weed or not.

## 2.7. Conclusion

Sexual reproduction among crops, weeds and wild plants is possible on the condition that (1) the crop and the weed or the wild relative are within a distance that pollination can occur, and (2) the plants are sexually compatible. Sexual compatibility is hampered by the existence of external and internal reproductive isolation barriers which determine whether or not viable hybrids are formed. In practice this means for a number of crops like maize, potato and tomato that there are no wild relatives in Europe with which successful hybridisation could occur. But it should be realised that genetic engineering, like classical breeding, has the potential to change reproductive isolation barriers. The experience with transgenic plants like transgenic oilseed rape shows that gene flow from these plants does take place as predicted from the knowledge on the sexual reproduction of these plants. Even though isolation barriers can limit the chances of hybridisation, in many cases chances of hybridisation are not zero and for risk assessment purposes they then have to be considered to be one.

# 3. Codes to Help the Evaluation of Risk Regarding Field Release of Transgenic Crops

## 3.1. Gene flow indices as a help in risk assessment

To be able to evaluate the risk of the field release of a genetically engineered crop it is important to have knowledge on the possibilities of gene flow for a certain crop in a certain region. Frietema De Vries ([Frietema] De Vries et al. 1994 and 1996) has introduced gene flow indices that give an indication of the possibilities of a certain plant to successfully hybridise with wild relatives and the impact this may have. These gene flow indices were not specifically developed for transgenic crops, but apply to them just as well. These indices can help in the risk assessment, in particular the risk assessment for field trials. The higher the gene flow indices, the more containment measures one will have to take if one wants to prevent outcrossing to wild relatives. For the risk assessment in case of the marketing of a genetically engineered crop these gene flow indices are less relevant, because for this particular risk assessment it is more a black-and-white situation: either plants are able to hybridise with wild relatives (even if chances are low), or they are not. This is because after the marketing of a particular genetically engineered crop, when it is grown on a very large scale, it will be very difficult or even impossible to prevent outcrossing with wild relatives.

The gene flow indices are built up from data for a particular plant on its possibilities for dispersal of pollen, dispersal of diaspora[3], and the presence and density of wild relatives in a given region. These data are classified into three different codes: the Dp-code (dispersal of pollen), the Dd-code (dispersal of diaspora) and the Df code (frequency of distribution of wild relatives). Together they are a measure for the possibilities of gene flow of a certain crop in a certain region and a measure of how widespread this gene flow may be. The Dpdf gene flow indices are always applicable for a given region, and this region therefore has to be mentioned in all cases where gene flow indices are used.

Table 3: The Dpdf gene flow indices after Frietema De Vries (1994, 1996), adapted to European needs as a whole

*Classification of the codes of dispersal of pollen (Dp)*

Dispersal of pollen hybridisation potential, including a differentiation of possible negative ecological effects of the inserted gene itself. Categories 0 (lowest risk) to 5 (highest risk) and U (unknown).

**Dp 0:** No wild relatives in the area (country) under consideration

**Dp 1:** No compatible wild relatives in the area (country) under consideration

**Dp 2:** No records of spontaneous hybrids in the area (country) under consideration

**Dp 3:** Occasional natural hybridisation, no backcrosses observed in the area (country) under consideration

**Dp 4:** Natural hybridisation occurs and hybrids are fertile and do backcross

**Dp 5:** Natural hybridisation occurs fairly often, hybrids are fertile and do backcross frequently

**Dp U:** Data too scanty or lacking at all, no evaluation possible.

[3] Diaspora are reproductive plant parts, such as seeds, fruit, or spores, that are modified for dispersal

*Classification of the codes for the dispersal of diaspores (Dd)*

**Dd 0:** No chance for diaspore dispersal (seeds are sterile or deficient)

**Dd 1:** Diaspore dispersal possible occasionally under very favourable and exceptional conditions

**Dd 2:** Diaspore dispersal possible under favourable conditions

**Dd 3:** Diaspore dispersal occurs, fruiting is usually undesirable and is normally suppressed by various methods

**Dd 4:** Diaspore dispersal is important, fruiting occurs normally during cultivation

**Dd 5:** Diaspore dispersal is the rule, fruiting occurs very frequently and is very abundant

**Dd U:** Data too scanty or lacking at all, no evaluation possible.

*Classification of the codes for Df (frequency of distribution)*

**Df 0:** Wild relatives not known in the wild or as feral populations in the area (country) under consideration

**Df 1:** Wild relatives extremely rare in the wild and do not occur as feral populations in the area (country) under consideration

**Df 2:** Wild relatives very rare in the wild and/or they occur sporadically as feral populations in the area (country) under consideration

**Df 3:** Wild relatives and/or their feral populations not very common in the wild in the area (country) under consideration

**Df 4:** Wild relatives and/or their feral populations not frequent in the wild but well distributed over the whole plateau in the area under consideration

**Df 5:** Wild relatives and/or their feral populations common in the wild and well distributed over the whole area (country) under consideration

**Df U:** Data too scanty or lacking at all, no evaluation possible.

## 3.2. Classification by combination of the three codes

The codes have been translated into a risk classification of gene dispersal probability from transgenic crop to the wild flora. After an evaluation of the three single factors (see above, dispersal codes), the combination of these codes enables us to estimate the impact of gene flow on the wild flora. Five categories of risk probability have been developed, which give guidance to the safe execution of field trials:

**1. No gene flow effect**

- No related species or no compatible related species of the crop are known in a given region. Field releases of species belonging to this category are possible without any containment or short term monitoring.
- Certain transgenes have to be tested in medium term field experiments regarding their secondary effects on ecosystems: Sustainable resistance must be achieved. To reach this goal a long term monitoring is required.

**2. Minimal gene flow effects**

- No records of spontaneous hybridization between the crop and the wild relatives are known in a given region. Field releases are possible after a thorough clarification of the biogeographical situation. Short term monitoring in confinements should be done prior to large scale field releases.
- Certain transgenes have to be tested in medium term field experiments regarding their secondary effects on ecosystems (pest and insect resistance genes).

**3. Low but local gene flow effects**

- Gene flow occurs towards wild or feral species existing also outside agricultural environment and control. Release experiments should first be done in confinements and afterwards in small scale releases closely monitored.
- This statement is restricted to transgenes not causing enhanced competitiveness outside agricultural environment, such as herbicide tolerance. Any other transgenes should be carefully tested in confinements.

**4. Substantial but local gene flow effects**

- Gene flow is high and substantial, but still locally controllable.
- Field releases could be done within strict confinements. A case by case analysis including the potential effects of the transgene is required before any field releases are done.
- Long term monitoring of field releases under strict biological or geographical confinement conditions is necessary in order to study competitiveness of the transgenic crop. Risky transgenes have to be avoided.

**5. Substantial and wide-spread gene flow effects**

- Gene flow is high, substantial, and widespread and will not be controllable by any means.
- No field releases of species belonging to this fifth category are possible.
- Medium term monitoring under strict confinement conditions is necessary in order to find out about competitiveness of the transgenic varieties.
- Experiments with less risky crop varieties (e.g. with male sterility) having the same favourable effect desired.

In Europe, crops like tomato, maize or potato would fall into the 'no gene flow effect' category. Crops like carrot would probably fall into the fourth or fifth category (substantial effects), which means that field trials with carrot should be performed with extreme care, especially when the newly introduced trait is considered to be risky.

## 3.3. Probability of gene dispersal versus gene effects

The gene flow indices give a good idea of the probability of gene dispersal from a particular transgenic crop to the wild flora, but they do not tell what will happen if a gene is dispersed to the wild flora. Will the gene be maintained in the wild flora or not? Will it have detrimental effects on the wild plant? Will it give the plant a selective advantage? This will all depend on the particular gene and the properties it confers to the plants containing it. The system of gene flow indices could perhaps be extended with a factor that could be called the **Dg-code** (the 'gene-factor', named in line with the other three codes). Distinctions could perhaps be made between 'risky' genes and less risky genes. Whether such a factor could work in practice remains to be seen, because genes that can be risky in one particular crop do not have to pose a risk in another crop at all. With regard to the risk of transgenes themselves the concepts of weediness, selective advantage, and fitness are thought to be relevant for the estimation of the level of risk. These concepts will be dealt with in the following two chapters.

# 4. Weediness of Transgenic Crops

## 4.1. A definition of weeds

There are various concepts defining weeds and there is no classical approach generally accepted. Lambelet-Haueter (1990) divides weed definitions into popular, economical and ecological concepts whereas Holzner (1982) groups them similarly into subjective and ecological ones. *Popular* as well as *subjective concepts* define weeds as plants of *any* kind growing in the wrong place, causing damage, being of no benefit and suppressing cultivated plant species. *Economical concepts* reflect the view of agronomists who concentrate on the reduction of yield, thereby stressing the damage aspect. A weed problem is solved as soon as the plant no longer creates considerable damage in the fields, a state which is reached by means of adjusted weed control (crop rotation, tillage, herbicide application).

In contrast to the previous concept, *ecological definitions* include habitats outside agrosystems colonised by weeds. The usual preference of weeds for habitats disturbed by man is stressed, like cultivated fields, gardens, disturbed areas on road sides, recently built artificial slopes, etc.. An aggressive weed can cause damage not only in agrosystems but also in (semi-)natural plant communities by outcompeting weak species.

Following Holzner (1982), it is sometimes difficult to call a plant a weed because one and the same species may be considered in some parts of its area as a harmless component of natural vegetation, in others as a weed and again in others, even as a useful plant species. Williamson (1988) pays attention to the fact that 17 out of 18 most feared „World's Worst Weeds" (Holm et al. 1977) are also cultivated.

## 4.2. Weed characteristics

Weeds are well adapted to life conditions in areas disturbed by man, which means that surviving strategies are so variable that any list of weed characteristics remains incomplete, even the famous one from Baker (1967, 1974):

1. Germination requirements fulfilled in a broad range of habitats
2. Discontinuous germination (internally controlled) and great longevity of seeds
3. Rapid growth through vegetative phase to flowering
4. Continuous seed production for as long as growing conditions permit
5. Self-compatible but not completely autogamous[4] or apomictic[5]
6. When cross-pollinated, unspecialized visitors or wind utilised
7. Very high seed output in favourable environmental circumstances
8. Produces some seed in wide range of environmental conditions; tolerant and plastic
9. Adaptations for short- and long-distance dispersal
10. If a perennial[6] , vigorous vegetative reproduction or regeneration from fragments
11. If a perennial, brittleness[7] , so not easily drawn from ground
12. Has ability to compete interspecifically by special means (rosette, choking growth, allelochemics).

[4] Autogamous plants are plants where all seeds are always the result of selfing.
[5] Apomicts are plants that require pollination to trigger embryo formation, but there is no actual fusion of gametes. The embryo is a clone from the mother plant.
[6] Perennial plants are overwintering plants.
[7] Brittle plants are vulnerable plants, that, when drawn from the ground, break in such a way that the roots remain in the soil.

A weed never possesses all these characteristics, therefore we have to speak of a weed-syndrome with even additional characteristics not mentioned here. The only attribute which all weeds might have in common is a marked plasticity enabling adaptation to continuous environmental changes.

## 4.3. Weediness in relation to other concepts in risk assessment

From the above it is clear that the concept of weediness can be used either very broadly or within more limited terms. In the public debate the term 'superweed' is sometimes used for transgenic plants that have become very noxious and have a strong ability to overgrow other plants. In this same public debate, distinctions between effects within the field or outside the field in natural habitats are not very often made. In this paper we want to use the term weediness next to the terms 'selective advantage' and 'fitness', and use the term weediness starting from a basically economical perspective. Without unwanted effects in the field, a particular transgenic crop is not considered to be weedy. If a transgenic crop only has unwanted effects outside the field we will use the concepts of selective advantage and fitness in the risk assessment of this transgenic crop.

## 4.4. Genetic engineering of crops and weediness

It is known that crops can give rise to weeds in three different ways, and this applies to transgenic crops as well (Zoldan, 1993 and Rauber, 1977):

1. Hybrids between the crop and wild relatives evolve into weeds.
2. Crops evolve into weeds.
3. Crops appear as unwanted volunteers in the subsequent culture where they are considered to be weeds.

**Weeds resulting from hybridisation between crops and wild species**

Gene flow from conventional crops to weeds has had important practical and economic consequences since it promotes the evolution of more aggressive weeds (e.g. Anderson 1949, Barrett 1983). There are many recorded examples of crops becoming weeds after hybridisation with wild relatives, for instance:

- Annual weedy beets resulting from hybridisation between sugarbeet and wild beet in Western Europe. These beets can be very difficult to control in fields of sugarbeet (Boudry et al. 1993). The weedy variant differs only in one single allele from the cultivar *Beta vulgaris ssp. vulgaris* (Hoffmann et al 1970);
- *Secale cereale* in California, where a weedy rye probably derived from a cross between *S. cereale* and *S. montanum* is leading to the abandonment of rye cultivation;
- Squash *(Cucurbita pepo)* - in the Southern United States,
- "Hybrid Grain Sorghum" and others (NRC Report on Field Testing, 1989 and NAS Report 2000 p. 81 ff).

In crops that are known to hybridise with wild species to form a weedy variant, the addition of a transgenic trait will in many cases result in the transfer of this trait to the weedy variant. If this trait provides a selective advantage to the weedy variant, or lead to an improved fitness of the weedy variant, the weedy variant may become more difficult to control.

For Western Europe herbicide tolerant sugarbeet is a relevant case to look at in more detail. Herbicide tolerant beets will most probably be the first transgenic beets to come on the market. For these sugarbeets the

following is a likely scenario. Introduction of glyfosate tolerance (only used as an example here) into sugarbeet will lead to the outcrossing of the glyphosate tolerance to the weedy variant, but only if transgenic bolters[8] are not controlled. This glyphosate tolerant weedy variant would then no longer be controllable in fields with glyphosate tolerant sugarbeets by means of application of the herbicide. This would mean a return to the original situation where weedy beets can only be controlled by mechanical means and a specific combination of herbicides. Therefore without the strict control of transgenic bolters the introduction of herbicide tolerance in sugarbeet in regions where there are many weedy beets will only lead to a temporary improvement of the situation until the moment that this herbicide tolerance has successfully outcrossed to the weedy beets. On the other hand if transgenic bolters can be controlled – and this would require quite a commitment from the farmer –, transgenic herbicide tolerant sugarbeet can be helpful in controlling the weedy variant.

In contrast to sugarbeet, which is not meant to flower, the outcrossing of transgenic traits from crops like rye or squash to their weedy variants cannot be prevented. These crops are meant to flower to produce grain or fruit. Other means like male sterility and apomixes would have to be introduced to prevent outcrossing of a transgenic trait to the weedy variant.

**(Transgenic) crops evolving into weeds**

Domesticated crops, such as wheat, maize and soybean, have been modified in traditional breeding to such an extent that they can no longer compete effectively with wild species in natural ecosystems. These crops are unlikely to revert to a weedy condition upon further genetic modification. Weediness is mostly considered to be a multicharacter attribute (Lupi, 1995, and Baker, 1974), which means that it is unlikely that the addition of a few genes will turn these crops into a weed problem. Some crops that have a low degree of domestication such as forage grasses and canola[9] are more likely to revert to a weedy condition (NRC report on Field Testing, 1989). One mutation may cause the weedy form of the crop that then successfully spreads (Sukopp and Sukopp, 1994 and Bartsch et al., 1993). The loss of spikelet spindle toughness of cereals, for example, is sufficient for regaining the ability to spread diaspores.

**Volunteers**

The third way in which crops can become a weed is when the crop appears as a volunteer in the subsequent culture after remaining in the field by harvest loss. Almost all crops are able to appear as volunteers in the subsequent culture, but it will depend very much on local conditions whether they will do so (Schlink, 1994). In Northwestern Europe for instance maize is very unlikely to appear as a volunteer, while on the other hand in Mexico it is very likely to do so. In Europe especially Brassica napus and Brassica rapa are problematic in terms of volunteers. These crops make large amounts of seeds of which quite some are lost during harvest and these seeds are able to stay viable in the soil for many, many years. Changes to crops, whether the result of classical breeding or genetic engineering, can lead to volunteers that are more persistent or more difficult to control. For instance if a potato tuber becomes more resistant to cold, it is likely that more potato volunteers will appear in the subsequent culture in regions where the winters are not very cold.

[8] Bolters are the flowering plants of perennial plants that normally do not flower in the first year of growth. Vernalisation – exposure to a cold temperature for a certain period – is normally necessary to trigger flower formation in these plants.
[9] Canola is the originally Canadian brandname of oilseed rape lacking eruric acid and glucosinolates, which is comparable to the European 0/0 oilseed rape.

## 4.5. A particular case: transgenic herbicide tolerant crops and weediness

Herbicide tolerance is currently the most widely used transgenic trait with 78% of all transgenic crops worldwide in 1999 carrying a herbicide tolerance gene (ISAAA, 2000). In the public debate on transgenic crops there is often a link made between herbicide tolerance and weediness. To determine whether herbicide tolerance is problematic from a weediness point of view, the following aspects are relevant:

1. Does the outcrossing of herbicide tolerance turn wild plants into weeds?
2. Does the herbicide tolerant crop itself turn into a weed?
3. Do herbicide tolerant crops lead to problems in weed control in any other way?

**Outcrossing of herbicide tolerance to wild plants**

Herbicide tolerance can occur in wild species by cross-pollination with herbicide tolerant crops. Of course not all crops are able to cross-pollinate successfully with wild species (see chapter 2). This outcrossing will only turn the wild plant into a weed if it is present in places where one wants to control it with the herbicide to which the plant is tolerant. With the use of another herbicide this situation can be overcome in a simple manner. Examples of this principle are known from conventional practices. One example is the emergence of triazine tolerant *Brachypodium distachyon* in Israel in triazine herbicide treated roadsides. The use of other herbicides easily decimated the *Brachypodium* and has made the original situation return where the plant lives in the sandstone hills. In this particular case the herbicide tolerant *Brachypodium* was not the result of outcrossing of the herbicide tolerance. It was the result of selection of herbicide tolerant individuals through mutation. But this does not make a difference to show the principle that there is little risk of a wild species remaining a weed for long periods, as long as agricultural practices can adapt to the new situation. In (semi-)natural habitats a herbicide tolerance does not provide an advantage to a wild relative, because there is no selection pressure in favour of herbicide resistance in natural habitats (Crawley et al, 1993). It is also unlikely that the herbicide tolerance alters the competitive ability and growth behaviour of the wild crop. A hybrid between seabeet *(Beta maritima)* and transgenic sugarbeet *(Beta vulgaris)* with a glyphosate tolerance, did not grow better or have a better competitive ability when compared to a non-transgenic parental type or a non-transgenic hybrid between sugarbeet and seabeet if the herbicide was not applied (Madsen, 1994). Similar data are available by the experiments of Bartsch et al. (1996), Brown (1999) and Pohl-Orf et al. (in press). This suggests that only during herbicide application the selection pressure privileges the herbicide tolerant types.

**Herbicide tolerance and the weediness of the crop itself**

Herbicide tolerance is unlikely to turn the crop itself into a weed. However, if the crop is known to form volunteers in the subsequent culture, as for instance oilseed rape does, and this following crop carries the same herbicide tolerance (for instance basta tolerant oilseed rape followed by basta tolerant sugarbeets), then these volunteers cannot be controlled by the herbicide. To prevent this from happening it will be important to apply both crop- and herbicide rotation.

**Herbicide tolerant plants and problems in weed control**

Herbicide tolerant plants have been made to make weed control more effective, easier, and also more environmentally friendly. The introduction of transgenic herbicide tolerant crops has led to the replacement of combinations of different herbicides by the application of only one active ingredient. Some are worried that

through the use of only one active ingredient the emergence of tolerant weeds will be accelerated. The rate of development of tolerance in weeds can be estimated from the following. To date, resulting from the application of glyphosate (Roundup) during 26 years, two cases of glyphosate resistant weeds have been documented: annual rigid ryegrass *(Lolium rigidum)* and goosegrass *(Eleusine indica)* (Hartzler, 1998 and 1999). Glyphosate resistant ryegrass has been confirmed in Australia and California (wheat production), and resistant goosegrass was observed in Malaysia (oil palm production). In both cases, resistance occurred after 10-15 years of intensive glyphosate use (>2 applications per site per season).

It is expected that the use of these broadspectrum herbicides like glyphosate in first instance will lead to a shift towards weeds with limited leafsurface. The examples of ryegrass and goosegrass show that monocultures of only one type of herbicide year after year will promote the emergence of tolerant weeds. To prevent a backlash to the 'pre-herbicide tolerant crop situation' with the use of different, sometimes environmentally more problematic herbicides, it will be important to apply herbicide rotation. At this moment two transgenic herbicide tolerances are dominant: tolerance to glyphosate (Roundup) and to glufosinate (Basta, Liberty, Finale). It will be important to rotate these herbicides. Herbicide rotation will become easier if there is enough supply of different herbicide tolerant crops. It is expected that in the near future plants with tolerances to herbicides other than glyphosate and glufonisate will be introduced on a fairly regular basis.

## 4.6. Conclusion

Genetic engineering, like conventional breeding, is able to alter characteristics of crops resulting in a crop or its wild relative to become more weedy. In many crops, and especially the ones that have been domesticated to such an extent that they are no longer able to compete with wild species in natural habitats, the addition of a few genes is very unlikely to turn the crop into a weed. In crops that are still very close to their wild and sometimes weedy variants, the addition of one gene might be enough to trigger weediness. In such cases the risk assessment should be performed with great care. Experimental approaches will be needed to determine the actual risks of unwanted effects.

Herbicide tolerance is not a major concern from the viewpoint of weediness. Only during application of the herbicide there is a selective advantage for plants possessing this characteristic. However, to prevent acceleration of the selection of tolerant weeds and possible future problems with (herbicide tolerant) volunteers it will be important to apply the necessary crop- and herbicide rotation.

But, since there is no long term monitoring on transgenic crops existing which concentrates on weediness in all aspects, scenarios must remain speculative.

# 5. Selective Advantage and Improved Fitness of Transgenic Crops

## 5.1. Definition of selective advantage and fitness

**Selective advantage**

In contrast to where weediness points to the undesired – sometimes economical - effects of the presence of a plant in (mostly) the agronomic environment or in habitats disturbed by man, the term selective advantage is used in risk-assessment to point to a qualitative characteristic of a newly introduced trait. A selective advantage means that after the outcrossing of the trait to a wild relative it has a good chance of being accumulated in the wild population. Selection results in the trait being preferentially attained. If a trait has no selective advantage, or also a selective disadvantage, then the outcrossing does not result in the accumulation of the new trait. It will disappear or diminish to a very low level. The term selective advantage is therefore more than the term weediness linked to the ecological effects of outcrossing of transgenes to plants in natural habitats.

**Fitness**

The fitness of a genotype is commonly measured by the number of successful offspring compared to the number of offspring of other genotypes (i.e., relative fitness). To this end, fitness of outcrossing species is necessarily connected to hybridisation with sexual compatible relatives. Fitness should always be defined in relation to environmental variables, e.g. habitat and climate.

The concepts of selective advantage and fitness are therefore mostly used to help to determine the risks connected to a transgene. This transgene is either part of the crop and can play a role in determining the invasiveness of the crop itself, or it is transferred to sexually compatible wild species and can play a role in determining the population size in natural habitats.

## 5.2. Risky transgenes?

There are many different transgenes transferred to plants using genetic engineering. The genetically engineered plants now on the market and in field trials, contain genes that provide the following characteristics:

1. Tolerance to herbicides.
2. Resistance to biotic stress (virus, fungus, insect, parasite diseases)
3. Resistance to abiotic stress (salt tolerance, drought resistance, tolerance to heavy metals etc.)
4. Quality characters (better processing, improved starch quality, improved oil profile, improved nutritional value, improved vase life, etc.)
5. Altered flower colour
6. Male sterility and restorer
7. Molecular f(ph)arming (production of vaccines, pharmaceuticals, biopolymers, etc.)

In laboratory research many other types of traits are being used, not only with the goal to develop a genetically engineered crop, even more with the goal of understanding plant growth and behaviour.

The question is whether the genes responsible for the abovementioned characteristics are risky and whether or not they provide a selective advantage or improve the fitness of the transgenic plants containing these characteristics. Although escaped transgenes are basically not retrievable, it should be noted that there are also considerable chances that an escaped gene will not persist in nature beyond several decades as a result of outcompeting and backcrossing. Escaped transgenes will only survive in nature for long periods of time under special circumstances (Harrison 1993). Below some considerations are given with regard to the risks of a number of categories of transgenes in terms of selective advantage or improved fitness.

**Tolerance to herbicides**

Herbicide tolerance provides a selective advantage only when the particular herbicide is used. In other circumstances this trait does not provide an advantage. It is also very unlikely to alter the fitness of the plants containing this type of alterations in a positive sense (see chapter 4 for more detail).

**Traits related to biotic stress**

Resistance to bacterial, viral or fungal disease and insect infestation are very important traits, especially for crops grown in the tropical and subtropical regions. Virus and insect resistance are already available on the market (virus resistant papaya, Bt maize), bacterial and fungal resistance are being developed. Resistance to biotic stress is thought to be able to lead to a selective or a fitness advantage, but only in the cases where in the wild population the viral disease or the insect infestation is a determining limiting factor in the population size. If disease plays an important role in the wild populations, then this should be taken into account very seriously in the risk assessment. However, often the wild plants are more disease resistant than the related crops. For virus resistant sugarbeets it is known that the virus is not present in the European wild beet population. Virus resistance in sugarbeets is therefore not expected to lead to a selective advantage of the beets.

**Traits related to abiotic stress**

Resistance to drought, salt or other stress can imply that crops can be introduced into areas where they were never able to be grown before. When it is possible to introduce traits that really have significant effects, then these crops could turn out to be able to invade new habitats outside the fields that they have never inhabited before. General conclusions are however impossible, and it will depend very much on the crop, the newly introduced trait, and its competitiveness compared to the natural flora in these areas. At this moment there are almost no empirical data on the selective advantage or fitness of these types of crops. Most of them still are in fundamental research phases, tested in laboratories or greenhouses. One of the few examples ready to be tested in the field are aluminum tolerant plants in Mexico. These plants are able to grow in areas polluted with aluminum. There are no data available yet on the question whether these plants have a selective advantage or an improved fitness in areas not polluted with aluminum.

**Quality characters**

There are many examples of traits related to quality that are being engineered. Examples of plants already on the market are tomatoes with an improved shelflife and carnations with improved vase life. In both cases the

introduced traits are not thought to be problematic in terms of selective advantage or fitness. In the case of the tomato in Europe there are no wild relatives with which the transgenic tomato could hybridise. The „gene-question" (what will happen **if** the gene gets into the wild population) is therefore not relevant for tomato, and therefore also the question with regard to selective advantage and fitness.

There are no general predictions possible on what the effects are in terms of selective advantage and fitness of genes related to quality characters. The types of changes are too diverse. One might however speculate that there might be a difference between crops that are very much domesticated and no longer able to compete with the wild plants in natural habitats, and crops that are still very close to their wild relatives. In the latter case the addition of a few genes could perhaps easier lead to detrimental effects. It is therefore not only the question whether a particular trait will result in a selective advantage or an improvement of fitness, but even more on whether this selective advantage or improved fitness would lead to unwanted effects in the natural population. To illustrate this it can be imagined that the introduction of a certain trait in a very domesticated crop like potato can improve its fitness (it is stronger, grows more tubers, etc.), but that this will not lead to detrimental effects in the natural population. Although its fitness may be improved, the crop does not have to become invasive. To be able to have a better idea on the effects additional experience with transgenic crops will have to be gained. Especially for crops that are close to their wild relatives, or that have wild relatives in their growing area with which they can successfully hybridise, data on their behaviour will have to be carefully assessed.

**Molecular f(ph)arming**

In cases of molecular farming the traits that are added to crops are not put into plants with the goal of improving their fitness or behaviour. Transgenic plants are only used as a means to produce a certain compound. It can be speculated that such traits are more likely to be a selective disadvantage to the plant, rather than a selective advantage. Still we think it will be better to work in physically or biologically confined situations.

**Traits related to hybridisation**

Genetic modification may affect hybrid formation either by changing frequency with which it occurs, or by altering the range of species with which the crop is sexually compatible. In case of an enhancement of reproduction characteristics, this could lead to selective advantages. But, increasingly the evidence suggests that modification has little impact on either factor, except for a laboratory study of Bergelson et. al. (1998), where a transgenic Arabidopsis showed an increased pollen fertility, full data are not revealed yet, so the case remains open and needs more discussion.

**Traits related to effects on pollinators**

Another important question is whether or not the relation between pollinators and plants will be modified by transformation. First the new proteins synthesized by the transgenic plant should not be toxic for bees. The non-toxicity of chitinase has been proven (Pham-Delègue et al. 1992). Could the character of a transgenic plant modify the activity of the insect? The same group demonstrated that the foraging time was shorter on a transgenic plant than on a non-transgenic one. However, Pham-Delègue et al. concluded that transgenic oilseed rape has no negative effects on foraging bees under controlled conditions. They need to repeat the experiment in the field (Grallien et al., 1995). Similarly, Paul et al. (1991) found that there are no differences in the range of animals and the frequency of visits between modified and non-modified tobacco plants.

Insects like bees are attracted by light of a wavelength between 300 nm and 650 nm (Dumas 1984). This includes part of the ultraviolet light but excludes the long-waved red light. A change in the flower colour could disturb the attractiveness of the flower for the insects and change cross-pollination rate for insect-pollinated plants. This would imply that the changing of flower colour resulting in a lower attractiveness for pollinating insects would not result in the trait being selectively attained in a wild population and vice versa, but only when the finding of the flowers by the insects would be a limiting factor for hybridisation in nature.

On the other hand, genetic engineering may be favourable to the hybridisation process. Studies regarding alfalfa (Medicago sativa) treated by a pesticide (dimethoate) clearly demonstrated that this pesticide is found in the pollen and the nectar at a very low level but even at a low level this pesticide is toxic for the bees (Dumas 1984). This has an effect on the entomophilous pollination. Plants resistant to diseases will not be treated with pesticides, so that the efficiency of pollination for entomophilous plants could be enhanced.

**Traits related to seed production**

Seed production may also be influenced by transformation. In the case of an experiment comparing mutant and transgenic herbicide tolerant Arabidopsis, Purrington et al. (1997) found that herbicide-resistant individuals produced 26% fewer seeds than their susceptible counterparts. In another of many more experiments Linder (1998) results suggest that high-laurate wild-crop hybrids lack germination cueing mechanisms and will germinate primarily at inappropriate times. It is becoming gradually clear, that fitness differences produced by transgenic traits up to now are readily comparable with differences of traits produced by classic breeding methods.

**Experience from field trials**

In various review papers the impacts of gene transfer have been evaluated (Ahl Goy et al. 1994, 1995 and 1996, see also the regular accounts of Clive James from ISAAA). Reviews of hundreds of field trials with male sterility in oilseed rape, herbicide tolerance in oilseed rape, sugarbeet and maize and insect resistance in potato and maize has not revealed any hints that one of these transgenes would enhance competitiveness and therefore cause invasions of crop plants into natural habitats, resulting in negative effects (Crawley 1992, Crawley et al. 1993, Fredshavn and Poulsen, 1993, Crawley, personal communication). Crops like oilseed rape, potatoes and maize with these kinds of traits have the same competitiveness outside agrosystems as the non-transgenic counterparts. They hardly can persist more than one generation. In no case sexual reproduction has been observed in natural ecosystems (Crawley personal communication and Crawley et al. 1993, Crawley et al, 2001).

From the experience with transgenic crops it is postulated by some ecologists that selective advantage, fitness, but also weediness through addition of a certain characteristic should be opposed to the additional 'genetic load' accompanied by the addition of that characteristic. It is believed that in many cases this additional genetic load will be the cause that the characteristic will disappear from a population.

## 5.3. Lessons learned from traditional breeding

Many of the above mentioned traits, like for instance tolerances to (a)biotic stress, quality characters, and others can also be achieved through conventional breeding, although it may require more time or they may be altered to a somewhat lesser extent. But the fact that the traits can be similar in a qualitative sense makes that experience from conventional breeding is relevant for a number of transgenic crops.

Traditional breeding has so far focussed on yield improvement. In comparison to wild relatives, cultivars are in general genetically less diverse and therefore less adapted to natural environments. Reports of fitness advancement for hybrids in natural ecosystems are rare. In opposite, hybridisation and escape of genes of conventional cultivars has lead to disadvantages for wild plant populations in some documented cases. Hybridisation with domesticated species has also been implicated in the extinction of certain wild crop relatives (e.g. Ellstrand & Elam 1993, Small 1984). This proves that the exchange of genetic material between crops and wild plants can influence the natural flora. It also suggests that the pressure from (genes from) domesticated crops until now mostly has had an impact in the sense that the outcrossed domesticated genes have made the wild plant less competitive. This is also confirmed by recent experiments by Keller et. al. (2000), discussed in Moore, P.D. (2000). In these experiments individual plants of the same species but from different regions in Europe were cross-hybridised. The first generation offspring showed greater biomass, but in the second generation the plants showed a decreased biomass yield, survivorship and seed mass. The overall message of these experiments is that introduction of genes of distant plant populations is more likely to do harm to the native flora of an area than to have positive effects in terms of biodiversity.

Pressure of gene flow from a crop to a wild relative does not necessarily result in a decrease in the genetic diversity of the wild plant, as shown by Bartsch et. al. (1999) in their study of gene flow from traditionally bred beets into the wild sea beet populations of north-eastern Italy. In this case the cultivated beets were far less diverse and outnumbered the wild relatives by the factor 10.000 to 1.

There is also a number of examples of cultivars or genes of cultivars escaped into natural ecosystems (Williamson 1993, Bartsch et al. 1993, Bartsch and Ellstrand 1999), one being carrot *(Daucus carota)* where some wild Northwest European *Daucus* populations have almost certain been derived from once cultivated populations. Some of the changes caused by man have been regretted, other changes like in the case of carrot not. These new carrot populations are now appreciated as added value to the biodiversity. Oilseed rape escaped to ruderal places all over the world, but does not take over in natural habitats.

## 5.4. Lessons from exotic species and exotic genomes

According to Sukopp and Sukopp (1993) there are hundreds if not thousands of new and foreign genomes introduced with trees, shrubs, herbs, microbes and higher and lower animals each year. Many of those survive and can, after years and even many decades of adaptation, begin to be invasive. This trend, overlooked by most and realised and judged to be a true hazard by only a few ecologists, is not evident, since it works slowly but steadily all over the world thanks to human activity in transport and tourism, which so often go hand in hand with the destruction of habitats. The dynamics of this trend is not yet sufficiently known. There are already many cases known where virulent new weeds invade ecosystems. Insular ecosystems are especially fragile and need to get much more attention regarding introduced new genomes in the future. Examples are Guava on Mauritius and *Pittosporum undulatum or Goldfussia (Acanthaceae)* in Jamaica.

The knowledge of the introduction of wild genomes is often referred to as the *„exotic species model"* and therefore a close comparison has its pitfalls as is pointed out by Scholz (1993), because all the examples in the exotic species model are wild species or cultivated wild species, and not crops. Crop plants, even in the phase of escaping from their agrosystems are not wild species and cultigeneous species cannot be compared in their genotype and phenotype with truly wild species.

## 5.5. Conclusion

Selective advantage and fitness are important concepts that can help in the determination of the risks of genetically engineered crops. They provide a measure for the hazards posed by a genetically engineered crop containing a certain genetically modified trait. But if a genetically engineered crop has obtained a certain selective or fitness advantage through genetic engineering, one cannot conclude that the newly introduced gene will have a selective or fitness advantage in all plants. It is always the combination of the plant, the trait and the environment together that determine a selective or fitness advantage. Information on the selective advantage of a certain trait in a certain crop can therefore not be transferred to other plants. Selective or fitness advantage will have to be determined on a case-by-case basis.

For the transgenic crops on the market now like herbicide tolerant maize there are no indications that these crops have an improved competitiveness or that gene flow from these organisms has a negative impact on the wild flora. But it should be stated that many of the field trials with transgenic plants have not been monitored as they could have been, and many of them lack a longterm perspective.
Classical breeding, and the introduction of foreign genomes where gene flow has been implicated in negative events like the extinction of certain wild crop relatives (e.g. Ellstrand & Elam 1993, Small 1984). This evidence of conventional crops and of the introduction of foreign genes through the introduction of foreign plants shows that negative effects of gene flow from transgenic crops are a realistic possibility. For future releases of transgenic crops the risks should not be underestimated, because possibilities of gene flow, as proven by the experience with conventional crops, are rather realistic for crops like carrot, sugarbeets, oilseed rape or wild grasses (Ammann, 1995). Introductions of such crops should be done with great care.

# 6. Ecological View and Consequences for Risk Assessment

## 6.1. Ecology and safety research

In considering the ecological risks of a crop it should be avoided to focus strictly only on transgenic crops. Many genes (or combinations of genes) responsible for various kinds of traits including pest resistances have been brought back to crops by classic breeding methods and subsequently released in large number into the field.

On the other hand we should not jump to preliminary conclusions or generalisations which are based on a relatively short experience with transgenic crops when compared to non-transgenic crops (Gabriel 1993, Regal, 1994). In genetic engineering only *genes* are moved in contrast to classical breeding where usually *alleles* are moved around. Still Regal (1994) does conclude that many transgenic crops *will* be non-competitive because, (1) the parent organisms were highly modified forms such as extensively domesticated maize to begin with, (2) there may be cases in which the genetic engineering process itself does demonstrably incapacitate the transgenic form ecologically and (3) if the host is the sort of foreign wild species that simply cannot persist without human help under local conditions of inappropriate weather, soil, etc., biotechnology is unlikely to turn it into a locally ecologically vigorous organism. Still, domestication should not be the miracle key word for safety of any transgenic crop.

The sound use of existing knowledge from plant ecology and from the use of conventional crops, together with carefully planned and monitored field trials should give scientific data good enough for taking good risk management decisions. For determining gene flow use can be made of harmless tracer genes which can be screened on their pathway. Bartsch et al. (1993) postulate experiments in confined and open systems within the framework of long term monitoring on the basis of scientific criteria. Also from ecological monitoring one can learn a lot, even though findings often remain inconclusive. Andow (1994) suggests that mathematical models of resource competition might be useful for identifying categories of plants that either are unlikely to alter community structure or that have the potential for altering community structure.

## 6.2. Consequences for risk assessment

When we take the knowledge and experience into account from classical breeding, from the introduction of foreign genomes, and from the transgenic crops in the field until now, a rational approach to the risk assessment of genetically engineered crops would have to:

- list up all related species having possible gene flow with the transgenic cultivar,
- to define a given region (and thus a given biogeographical situation),
- to deal with specific transgenes,
- follow a step by step procedure.

One example of a scheme that can help to structure this step-by-step procedure is the one proposed by Rissler and Mellon (1993) (cited in Snow et al. 1995). The disadvantage of such procedures is that they suggest it is

always ‘either’ ‘or’. Bearing the complex interactions between fitness factors in mind, it is often not that simple. This means that the scheme is an aid, but results from one step will have to be looked at carefully before stepping blind into a next step of a procedure.

## 6.3. Testing scheme for risky crops

For all transgenic crops the approach mentioned above should be followed. The first two steps could be made easier when for all relevant regions listings were given of the Dpdf-codes for the different crops and that on the basis of these codes the crops were divided into one of the five risk categories as given in chapter three (ranging from ‘no gene flow effect’ to ‘substantial and widespread effect’). The question then is what type of testing to require for risky crops. It is postulated here that for all crops and related wild species, which belong to the two highest risk categories (substantial but local effect, substantial and widespread effect), the following testing scheme should be performed in a medium to long term monitoring using an experimental approach.

Crops belonging to the highest category of risk should be treated according to a test procedure proposed by Fredshavn et al. 1993, which can be carried through in greenhouses. Competition experiments in confinements will reveal data in the influence of plant size on competitiveness, on substitution rates as a measure of competitiveness and on the interaction between habitat and gene expression. But even with these preliminary greenhouse experiments it will be impossible to predict the exact ecological consequences of a release. It is, however, possible to test a transgenic plant in a confinement in critical phases of the life cycle and compare it with a range of non-transformed well-known varieties, and thus detect any principal changes in growth behavior. A set of standard growth conditions is proposed. If these experiments do not reveal any major change in the competitiveness of the transgenic crop, then the field experiment procedures according to Rissler and Mellon (1993) can be started.

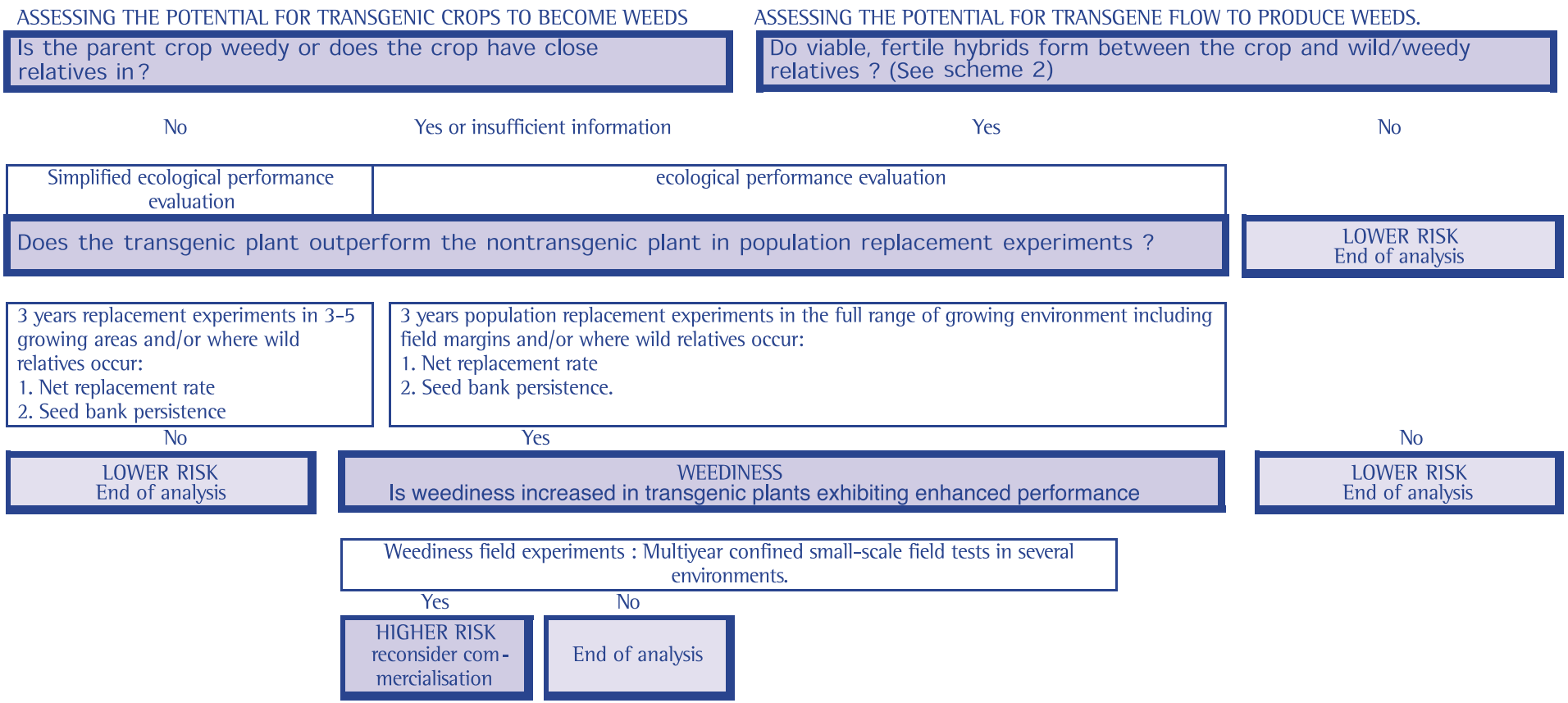
This approach involves a threestep analysis to evaluate both crop weediness and gene flow. The steps are designed to identify non-risky plants early in the analysis and to require extensive field testing only for plants that appear to pose substantial risks. The evaluation proceeds under the assumption that crops on the lower end of the spectrum of weediness potential are sufficiently unlikely to be converted to weeds by the addition of transgenes that they can be subject to simplified population replacement experiments.

The first step assesses:

1. The potential for weediness. It separates crops into two risk categories. The lower-risk category contains crops that are not weedy and do not have close weedy relatives in Switzerland. By contrast, the higher-risk group is weedy or has close weedy relatives. The higher-risk crops are subject to a standard set of experiments, while the ones with lower-risk undergo an abbreviated procedure.
2. Experimental assessment of the potential for transgene flow determines whether transgenic hybrids will form between transgenic crops and their wild/weedy relatives. Where hybrids are not formed, the transgenic crop is deemed to pose low risk in terms of gene flow and no further tests are required. Where hybrids are formed, the analysis moves to step 2. Once gene transfer occurs, the assessment of potential adverse impacts is the same as for the transgenic crop itself.

The second step analysis relies on relatively simple experiments, which can be conducted along with efficacy tests, to evaluate the performance of transgenic crops relative to non transgenic ones.

The third step analysis allows developers an opportunity to demonstrate that transgenic crops that outperform non-transgenics in the ecological performance tests do not pose risks as weeds under conditions of commercial use.




There are a number of ways to evaluate the replacement capacity of a genetic type in a population of plants. Option A describes one alternative. N.Ellstrand, J.Hancock, P.Kareiva, R.Linder, R.Manasse and M.L.Roush were especially helpful in developing this experimental approach given by Rissler and Mellon (1993). Here we cite it from Snow et al. (1995), appendix 3:

Do viable fertile hybrids form between the crop and wild/weedy relatives? Does the crop reproduce sexually?

Yes or insufficient information

No → LOWER RISK End of analysis

Does the crop have outbreeding potential with relatives in the region where the plants will be introduced ?

Yes or insufficient information

No → LOWER RISK End of analysis

Do the crop-relative breeding systems permit gene flow in and out ?

Yes or insufficient information

No → LOWER RISK End of analysis

Does the flowering period of the crop and weed/weedy relatives overlap, or nearly so ?

Yes or insufficient information

No → LOWER RISK End of analysis

Do crop and wild/weedy relatives share the same means of pollination ?

Yes or insufficient information

No → LOWER RISK End of analysis

Do crop and wild/weedy relatives naturally cross-pollinate, fertilize, and set viable , fertile seeds under field condition ?

Yes or insufficient information

No → LOWER RISK End of analysis

Go to the ecological performance of transgenic wild/weedy plants. (go to scheme 1)

# 7. Discussion and Conclusion

The risks associated with vertical gene flow from genetically engineered plants form a major concern in the discussions on the safe use of transgenic crops. How invasive are transgenic crops themselves and how invasive are the transgenes that are present in these crops? Transgenes can only invade wild populations if wild sexually compatible relatives are present. But what will be the effect of such gene flow? In this paper the concepts of weediness, selective advantage and fitness have been discussed to see whether they can help in determining the risks associated with particular transgenes.

From this paper it will be clear that sexual reproduction among crops, weeds and wild plants can occur but depends on the specific crop and the region where the crop is cultivated. Maize, potato, rice, wheat, beans (Phaseolus) and tomato for instance have no wild relatives in Europe with which successful hybridisation can occur. For these crops only the invasiveness of the crop itself is a concern. Succesful hybridisation is determined by many factors, some of which can also be altered using breeding methods or genetic engineering. The alteration of crops can therefore also lead to the alteration of the success with which a particular crop can hybridise with its wild relatives. There is quite some knowledge on the sexual compatibility of crops with wild relatives, but on a worldwide scale additional studies are necessary to determine whether escaped transgenes are likely to persist in wild populations.

To give a measure to the possibilities of gene flow to wild relatives and to how widespread the effects of gene flow will be, gene flow indices have been developed which are based on data concerning: (1) the dispersal of pollen, (2) the dispersal of diaspora, and (3) the frequency of distribution of wild relatives in a given region. These gene flow indices can be a tool to divide crops into gene flow risk categories. The higher the risk class, the more careful one should be with introducing 'risky' genes. However, it should be beared in mind the gene flow indices are not completely static measures, but that genetic engineering, like classical breeding has the potential to change reproductive isolation barriers thereby changing the success of gene flow. Changing these types of properties of plants also changes the gene flow codes and the risk classes. The risk classes may be important in two different ways: (1) by guiding the measures necessary to be able to perform field trials in a safe manner, and (2) by guiding the testing procedure for the marketing of a crop; the higher the risk class the more stringent testing schemes will be necessary.

Even more important than determining the chances of gene flow to wild relatives is the question how risky the transgene itself is, especially in those cases where the crop is expected to be easily reverted or transformed into a weedy form. The closer a crop is to weedy or wild variant, the higher the chances that the addition of one or a few genes can make a crop invasive. Crops that are very much domesticated, and no longer capable of competing with wild plants in natural habitats, are less likely to become invasive after the addition of one or a few genes.

The addition of genes, either through conventional breeding or genetic engineering has the potential to make crops more weedy. It can also make wild plants to which the trait has been transferred more weedy. If genetic

exchange between transgenic crops and wild relatives has weediness potential, there should nearly always be evidence of this process with non-transgenic crop/weed complexes. Weediness as such is a multiform characteristic that will not be easily obtained by the addition of one transgene, or only in cases where the crop is close to the weed. Herbicide tolerance is - based on the experience from traditional breeding - from a weediness point of view not a concern, if there are enough possibilities for crop and herbicide rotation. In the case of transgenic herbicide tolerant beets, this herbicide tolerance can help to control weedy beets, but only in the case that transgenic bolters are controlled. If not, it can be expected that the herbicide tolerance will be transferred to the weedy beets, making them again as difficult to control as they are today.

Selective advantage and fitness are important concepts for specifying the risk of a transgene. It is very difficult to give general conclusions, but for instance traits related to the success of gene flow, resistance to biotic or abiotic stress might result in selective advantages or serious fitness improvements, if the absence of the trait in nature is an important determing factor in the existing ecological balances. Whether in specific cases there is a real risk can only be determined through thoughtful analysis and experimentation. It should be kept in mind that many of the above mentioned types of traits can also be obtained through classical breeding methods and that their possible ecological effects should be assessed in the same way. Further research is also needed to predict how escaped transgenes are likely to affect the abundance and invasiveness of the transgenic hybrids.

Experience from conventional breeding and the introduction of exotic genes and genomes forms the proof of the fact that the introduction of new varieties and new crops has the potential to influence the natural flora. However, reports of fitness advancement for hybrids in natural ecosystems are rare. In opposite, gene flow from domesticated crops has mostly made the wild plants less competitive. There are few recorded examples of the opposite, for instance in the case of carrot where some wild populations have most certainly been derived from once cultivated variants. In contrast to the known effects of these conventional crops, there are no indications that the transgenic crops currently on the market have such unwanted effects. However, it should be stated that these crops have not been monitored as they could have been. This is also no proof whatsoever for the transgenic crops now being developed. The experience from non-transgenic practice directs us to take great care.

For the future risk assessment a rational stepwise approach is necessary taking into account the knowledge of the crop and its wild relatives, knowledge on the biogeographical situation, and knowledge on the transgene. The testing of the transgenic crop should follow a step-by-step procedure evaluating data from a first step before stepping into a next phase. The more risky the crop and/or the transgene, the more stringent the testing scheme will have to be before the transgenic crop can be allowed to be grown commercially on a large scale. But in the end one dilemma will remain: even after the most careful risk assessment process, only a mass release will bring to the surface all effects. The small-scale field trials do not allow to investigate the ecological risks of widespread commercialization. Therefore in order to achieve sustainability in cultivating transgenic crops, the focus should be on long term monitoring of several years in the same field where the transgenic crop was planted.

# 8. References


Abbott R. 1994a. Ecological risks of transgenic crops. TREE 9, 8 August, 280-281

Abbott R. 1994b. Reply to Thacker J.R.M. Transgenic plants. TREE 9, 12 December, 486

Ahl Goy P., Chasseray E. and Duesing J. 1994. Field trials of transgenic plants: an overview. Agro-Industry hi-tech March/April, 10-15.

Ahl Goy P. and Duesing J. 1995. From Plots to Plots: Genetically Modified Plants on Trial. Biotechnology 13, May, 454-458.

Ahl Goy P. and Duesing J. 1996. Assessing the Environmental Impact of Gene Transfer to Wild Relatives. Biotechnology 14, January, 39-40.

Ammann K. 1995. Gentechnisch veränderte Kulturpflanzen in der Umwelt: Chancen und Probleme- Die Suche nach gangbaren Wegen. In: Gentechnik und Ernährung. Erbersdobler H.F., Hammes W. and Jany K.-D., Wissenschaftliche Verlagsgesellschaft mbH Stuttgart, 161-195.

Ammann K. 1995. Die ökologischen Risiken der Gentechnologie und wie wir damit umgehen können. In: Behrens, M., S.Meyer-Strumberg und G.Simonis (Hrsg), Gentechnik und Nahrungsmittelindustrie. Mensch undTechnik, Sozialverträgliche Technikgestaltung. p.177-190.

Ammann K., Felber F., Keller [Senften] J., Jacot Y., Küpfer Ph., Rufener [Al Mazyad] P. and Savova D. 1994: Dynamic biogeography and natural hybridization of selected weedy species in Switzerland. Symposium "Gene Transfer: Are Wild Species in Danger?", Le Louverain, Switzerland, Nov.1994. Environmental Documentation, Federal Office of Environment, Forests and Landscape (FOEFL), 12: 36-39.

Anderson E. 1949. Introgressive hybridisation. John Wiley & Sons, Inc., New York, Chapman & Hall, Limited, London.

Andow D.A. 1994. Community response to transgenic plant release: using mathematical theory to predict effects of transgenic plants. Molecular Ecology 3, 65-70.

Baker H.G. 1967. Characteristics and modes of origin of weeds. In : The genetics of colonizing species. Baker H.G. and Stebbins G.L. (eds.). Academic Press. New York and London,147-172.

Baker H.G. 1974. The evolution of weeds. Ann. Rev. of Ecology and Systematics 5: 1-24

Barrett S.C.H. 1983. Crop mimicry in weeds. Econ. Bot., 37 (3), 255-282.

Bartsch D, Sukopp H. and Sukopp U. 1993. Introduction of plants with special regard to cultigens running wild. In: Transgenic organisms. Wöhrmann, K. and Tomiuk, J. (eds), Birkhäuser Verlag Basel/Switzerland, 135-151.

Bartsch D and Schmidt M (1997) Influence of sugar beet breeding on populations of Beta vulgaris ssp. maritima in Italy. Journal of Vegetation Science 8(1), 81-84.

Bazin M.J. and Lynch J.M. 1995. Environmental Gene Release. Models, Experiments and Risk Assessment. Chapman & Hall, New York, 166p, ISBN 0-412-54630-2.

Bergelson J, Purrington CB, Wichmann G., 1998. Promiscuity in transgenic plants Nature 395: (6697) 25-25 SEP 3

Bevan M.W., Harrison B.D. and Leaver C.J. 1995. The Production and Uses of Genetically Transformed Plants, Chapman & Hall, New York.

Boudry P., Mörchen M., Samitov-Laprade P., Vernet P. and Van Dijk H. 1993. The origin and evolution of weed beets: consequences for the breeding and release of herbicide-resistant transgenic sugar beets. Theor. Appl. Genet., 87: 471-478.

Crawley M.J. 1992. The comparative ecology of transgenic and conventional crops. In: 2nd international symposium on the biosafety results of field tests of genetically modified plants and microorganisms. May 11-14, 1992, Goslar, Germany, 43-52.

Crawley M.J., Halls R.S., Rees M., Kohn D. and Buxton J. 1993. Ecology of transgenic oilseed rape in natural habitats. Nature, 363(6430): 620-623. See also comments of Peter Kareiva in same Vol. p. 580. (1993).

Crawley M.J., Brown S.L., Hails R.S., Kohn D.D., Rees, M. 2001. Transgenic crops in natural habitats. Nature 409: 682-683.

Dale P.J., R.Parkinson and J.A. Scheffler 1993. Dispersal of genes by pollen - the PROSAMO-Project. British Crop Protection Council Monograph No. 55, Cambridge, 133-143.

Dumas C. 1984. Ecologie florale et pollinisation. In : Pollinisation et production végétales. Pesson P. et Louveaux J. (eds.). INRA, Paris, 31-46

Eckert J.E. 1933. The flight range of honeybee. J. Agric. Res. 47, 257-285.

Ellstrand N. 1992. Gene flow by pollen : implications for plant conservation genetics. Oikos, 63: 77-86.

Fredshavn J. and Poulsen G.S. 1993. Growth behaviour and competitive ability of transgenic crops. Agric. Ecosystems Environ.

Frietema De Vries F.T. 1996. Cultivated plants and the wild flora. Effect analysis by dispersal codes. Thesis. Rijksherbarium, Hortus Botanicus, Leiden, 100p.

Frietema De Vries F.T., Van der Meidjen R. And Bradenburg W.A. 1994. Botanical files on lettuce (Lactuca sativa L.) for gene flow between wild and cultivated lettuce (Lactuca sativa L. Including L. Serriola L., Compositae) and the generalized implications for risk assessment on Genetically Modified Plants. Gorteria, suppl. 2, 44p.

Gabriel W. 1993. Technologically modified genes in natural populations: some skeptical remarks on risk assessment from the view of population genetics. Transgenic Organisms. K. Wöhrmann and J. Tomiuk (eds.). Birkhäuser, Basel 1993, 109-116.

Grallien E., Marilleau R., Pham-Delègue M.H. and Picard-Nizou A.L. 1995. Impact of pest insect resistant oilseed rape on honeybees. In :Rapeseed today and tomorow, 9th International rapeseed congress, Cambridge, UK, 4-7 july 1995, 3: 784-786.

Gregorius H.-R. and Steiner W. 1993. Gene transfer in plants as a potential agent of introgression, In: Transgenic Organisms. K. Wöhrmann and J. Tomiuk (eds.), Birkhäuser, Basel 1993, 109-116.

Hadley H.H. and Openshaw S.J. 1980. Interspecific and intergeneric hybridization. In . Hybridization of crop plants. Fehr. W.R. and Hadley H.H. (eds.). American Society of Agronomy-Crop Science Society of America, Madison, 133-159.

Harrison Richart G. 1993. Hybrid Zones and the Evolutioinary Process. Oxford University Press, 364 p.

Hartzler B, Roundup resistant ryegrass. Iowa State University Weed Science Online, http://www.weeds iastat.edu/weednews/rigidryegrass.htm, 1998.

Hartzler, B, Glyphosate resistance updates. Iowa State University Weed Science Online, http://www.weeds.iastate.edu/mgmt/qtr99-1/glyresistance.htm, 1999.

Holm L.G. et al. 1977. The world's worst weeds. Distribution and biology. University Press of Hawaii, Honolulu.

Holzner W. 1982. Concepts, categories and characteristics of weeds. In: Holzner, W. and Numata, N. (eds), Biology and ecology of weeds, Dr. W. Junk Publishers, The Hague, 3-20.
ISAAA, ISAAA brief 17, 2000, The global status of commercial transgenic crops, 1999, by Clive James.
Keller, M, Kollmann, J, & Edwards, P.J.J., Appl. Ecol. 37, 647-659, 2000.
Lambelet-Haueter C., 1990. Mauvaises herbes et flore anthropogène. I. Définitions, concepts et caractéristiques écologiques. Saussurea 21: 47-73.
Linder CR. 1998. Potential persistence of transgenes: Seed performance of transgenic canola and wild x canola hybrids. Ecological Applications, 8: (4) 1180-1195
Lupi C. 1995. Genetic engineering for plant protection - Methods, state of the art and applications. Technology assessment of pest resistant transgenic plants, report 1. BATS, 60p.
Madsen K.H 1994. Weed management and impact on ecology of growing glyphosate tolerant sugarbeets (Beta vulgaris L.). Ph.D.-Thesis. The Royal Veterinary and Agricultural University, Weed Science, Copenhagen, Denmark and MARIBO Seed, Holeby, Denmark, 61p.
Meer Van der P.J. 1993. Proceedings (working dokument) Pan-European conference on the potential long-term ecological impacts of the release of genetically modified organisms. Council of Europe, Strasbourg 1993.
Mikkelsen T.R., Andersen B. and Jörgensen R.B. 1996. The risk of crop transgene spread. Nature 380, 7 March, 31.
Mizukami Y. and Ma H. 1992. Cell 71, 119-131.
Moore, P.D., Nature, vol 407, 12 october 2000, 683-685,
NAS Report 2000 Prepublication copy: Genetically Modified Pest-Protected Plants: Science and Regulation. Committee on Genetically Modified Pest-Protected Plants. Board on Agriculture and Natural Resources, National Research Council. National Academy Press, Washington DC., www.nap.edu/books/0309069300/html/
NRC Report on Field Testing 1989. National Research Concil of the National Academy of Sciences. Committee on scientific evaluation of the introduction of genetically modified micro-organisms and plants into the environment. Field testing genetically modified organisms: Framework for decisions. National Academy Press, Washington, D.C., 170 p, ISBN 0-309-04076-0.
Paul E.M., Lewis G.b. and dunwell J.M. 1991. The pollination of genetically modified plants. Acta Horticulturae, 288, 425-429.
Pham-Délègue M.H., Picard-Nizou A.L., Arnorld G. Grison R., Toppan A., Olsen L. and Masson C. 1992. Impact of genetically modified rapeseed on insect pollinators (honeybees). In : Proceedings of the 2nd International Symposium on „the biosafety results of field tests of genetically modified plants and micro-organisms", May 11-14, 1992, Goslar, Germany, Casper R. and Landsmann J. (eds.).
Pickersgill B. 1981. Biosystematics of crop-weed complexes. Kulturpflanze, XXIX: 377-388.
Purrington CB, Bergelson J. 1997. Fitness consequences of genetically engineered herbicide and antibiotic resistance in Arabidopsis thaliana, Genetics 145: (3) 807-814
Rauber R., 1977. Evolution von Unkräutern. Ergebnisse der 1. Deutschen Arbeitsbesprechung über Fragen der Unkrautbiologie und -bekämpfung, 1977, Stuttgart, Hohenheim. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz, Sonderheft 8: 37-55.
Raybould A. and Gray A. 1993. Genetically modified crops and hybridization with wild relatives: A UK perspective. Journal of Applied Ecology 30, 199-219
Raybould A. and Gray A. 1994. Will hybrids of genetically modified crops invade natural communities ? TREE , 9(3): 85-88.
Regal P.J. 1994. Scientific principles for ecologically based risk assessment of transgenic organisms Molecular Ecology (1994) 3, 5-13.
Rissler J. and M.Mellon 1993. Perils amidst the promise: ecological risks of transgenic crops in a global market. Union of Concerned Scientists, Cambridge, M.A.
Scheffler J., R.Parkinson and P.Dale 1993. Frequency and distance of pollen dispersal from transgenic oilseed rape (Brassica napus) . Transg. Res. 2, 356-364.
Schlink S. 1994. Ökologie der Keimung und Dormanz von Körnerraps (Brassica napus L.) und ihre Bedeutung für eine Überdauerung der Samen im Boden. Dissertationes Botanicae Band 222. Cramer, Berlin, Stuttgart, 194p, ISBN 3-443-64134-2.
Scholz H. 1993. Kommentar zur Studie: „Ökologische Langzeit-Effekte der Verwilderung von Kulturpflanzen". Gutachten erstellt im Auftrag der Abteilung „Normbildung und Umwelt", Forschungsschwerpunkt Technik-Arbeit-Umwelt, des Wissenschaftszentrums Berlin für Sozialforschung (WZB), Berlin.
Sinemus K. 1994 Biologische Risikoanalyse gentechnisch hergestellter herbizidresistenter Nutzpflanzen. Erarbeitung eines Modells zur Risikominimierung bei der Freisetzung transgener Nutzpflanzen. Verlag Mainz, ISBN 3-930911-95-7, 223 S.
Small E. 1984. Hybridization in the domesticated weed-wild complex. In : Plant Biosystematics. Ed. Grant W.F., Academic Press, Toronto, 195-210.
Snow A.A. and P.Moran-Palma 1995. Ecological risks of cultivating transgenic plants. Article submitted to the US Environmental Agency. Dept. of Plant Biology Ohio State University, September 1, final version, 1-
Sukopp U. and Sukopp H. 1993. Das Modell der Einführung und Einbürgerung nicht einheimischer Arten - Ein Beitrag zur Diskussion über die Freisetzung gentechnisch veränderter Arten. Gaia 2(5): 267-288.
Sukopp U. and Sukopp H. 1994. Ökologische Lang-Zeiteffekte der Verwilderung von Kulturpflanzen. Verfahren zur Technikfolgenabschätzung des Anbaus von Kulturpflanzen mit gentechnisch erzeugter Herbizidresistenz. Heft 4. TU Berlin, 144p.
Summermatter Kathrin 1996. Systemisch induzierte Resistenz bei Arabidopsis thaliana. Inaugural-Dissertation, Université de Fribourg, 49 pp.
Tiedje J.M., Colwell R.K., Grossmann Y.L., Hodson R.E., Lenski R.E., Mack R.N. and Regal Ph.J. 1989. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70,2, 298-315.
Timmons A.M., Charters Y.M., Crawford J.W., Burn D., Scott S.E., Dubbels S.J., Wilson N.J., Robertson A., O'Brien E.T., Squire G.R., Weber
Williamson M.H. 1988. Potential effects of recombinant DNA organisms on ecosystems and their components. TREE, 3: 32-35.
Williamson M.H. 1993. Invaders, weeds and the risk from genetically manipulated organisms. Experientia, 49: 219-224.
Williamson M., Perrins J. and Fitter A. 1990. Releasing genetically engineered plants: Present proposals and possible hazards. Trends in Ecology and Evolution 5: 417-419
Zoldan J. 1993. Ackerunkräuter - ein Spiegel der landwirtschaftlichen Entwicklung. Spektrum der Wissenschaft, Juli 1993, 92-108

# Effects on non-target organisms of the release of genetically modified crops into the environment

Authors: **Julian Kinderlerer**
**Sheffield Institute of Biotechnological Law & Ethics**
**The University of Sheffield**
**Sheffield, S10 2TN, United Kingdom**

**1. Introduction** ..... **89**
1.1. Current modern day crops ..... 89
1.2. A definition of non-target effects ..... 89
1.3. The agricultural environment ..... 90
**2. Genetically modified crops** ..... **92**
2.1. Genetically modified crops on the market ..... 92
2.2. Genetically modified crops: introduced changes ..... 93
2.3. Non-target effects of genetically modified crops ..... 93
2.4. Impact analysis ..... 94
**3. Insect resistant crops versus insecticides: non target effects** ..... **96**
3.1. Introduction ..... 96
3.2. Non-target effects of insecticides ..... 96
3.3. Non-target effects of Bt-crops ..... 96
**4. Non-target effects of herbicide tolerant plants** ..... **100**
4.1. Introduction ..... 100
4.2. Non-target effects of herbicide use ..... 100
4.3. Herbicide tolerant plants versus herbicides ..... 101
**5. Other modifications and their effects on non-target organisms** ..... **103**
5.1. Plants designed to express changed chemical or nutritional qualities ..... 103
5.2. Plants designed for phytoremediation or detoxification ..... 103
5.3. Plants designed to resist abiotic stress ..... 104
**6. Conclusions** ..... **105**
**7. References** ..... **107**

# 1. Introduction

## 1.1. Current modern day crops

Genetically modified crops are produced using techniques of molecular biology that allow the introduction of specific genes into the plant. At present it is possible to specifically introduce genes or DNA sequences derived from other organisms into plants, but not to modify genes already present in the cell so that they resemble those from other organisms nor to delete specific genes. The processes currently available allow for the expression of a gene in a particular tissue, and as knowledge increases, will allow the expression to be controlled in time (relative to the life-cycle or to special types of trauma, such as insect attack of the plant).

Scientists have long been modifying crops using 'traditional techniques' that primarily have involved selection processes, but also using techniques that allow the crossing of organisms that, in the absence of 'scientific' intervention, could not cross-fertilise. Commodity crops are the product of many centuries of selection in order to enhance yield, disease resistance and agronomic performance. Selection has been used to substantially modify crop characteristics, but requires much investment both in time and money to achieve an effect similar to that that may be achieved by insertion of a gene from an unrelated organism. In some cases the result of the modification using traditional methods may be similar to those using modern biotechnology, although the process of modern biotechnology may be more specific and is less likely to involve multiple changes in the characteristics of the plant. Plant breeding techniques have become increasingly sophisticated since 1900 and have routinely employed techniques such as cell fusion (since 1909), mutation via X rays (since 1927) and embryo rescue (since the 1960s) (Royal Society, London, 1998).

## 1.2. A definition of non-target effects

Non-target effects are defined as the unwanted (negative) effects of crops or their accompanying farming practice on organisms living in or around the agricultural field that are not intended to be hurt. Some crops or practices are intended to hurt specific pests or plagues: both genetically modified crops and insecticides are meant to kill certain insects. These are target effects. But they are not meant to kill beneficial insects or others organisms living in and around the field. If they do, they are said to have non-target effects. Such non-target effects can be problematic in terms of negative effects on biodiversity.

Although this essay deals with the impact of transgenic crops on non-target organisms, the impact of new modified varieties produced using traditional technologies may be as significant, if not more so. The introduction of herbicide tolerance using mutation, for example, is not different in essence from that introduced using genetic modification, and indirect effects are likely to be identical.

It should not be presumed that the impact on non-target organisms due to the introduction of transgenic plants is deleterious or beneficial. Changes might result in an increase or decrease in range, diversity or

number of non-target animals, birds and insects, modifying both natural and agricultural biodiversity. These changes in biodiversity could be considered either to be beneficial in that they allow threatened species to survive, or could so modify the environment as to be actually or perceived to be harmful.

**Non-target effects could arise from a number of factors:**

● The gene product affects organisms for which the plant was not designed. This may be in a direct way by killing the organisms that eat the gene product, or in an indirect way by killing organisms (like predators) that eat the organisms that have taken up the gene product. If predators are beneficial insects, the use of the new crop could impact on other crops grown in the vicinity. Examples of these types of non-target effects are addressed later in this text, but might include the impact of toxins introduced into plants in order to affect specific insects. Performance in one particular geographic locality may (or may not) provide direct evidence of behaviour in a different environment.

● There may be effects on non-target organisms as a result of changed agricultural practices, whether through changes in chemical usage or in the manner in which the land is managed. Although these changes cannot by themselves be thought to be due to the manner in which the new product is produced, their introduction changes the way in which the plant interacts with its environment, and hence cannot be excluded from any process of impact assessment.

## 1.3. The agricultural environment

A very large proportion of the European countryside is farmed; some 50 to 75%. In North America there are vast tracts of land used for farming, but there are very large areas not used for agriculture or effectively set aside for nature conservation. The proportion used for farming is very much lower although there are parts of Europe where climatic conditions dictate a lower usage of land for agricultural production. The potential effects of GM crops on biodiversity in North America and Europe should therefore not be compared too closely to one another. The way Europe uses its land has to a large extent shaped the fauna and flora and farms play a crucial role in sustaining the wildlife diversity and abundance currently observed, with chemicals and pesticides acting to modify both diversity and abundance. Farming must therefore co-exist with nature. In Europe the agricultural environment is perceived to be the natural environment. In addition, the way farming is done in much of Europe has changed dramatically since the Second World War, primarily to increase yields in both crop production and the rearing of animals. Farms have become much bigger, and hedges and walls have often disappeared[1]. These changes have affected habitat types and reductions in the abundance and diversity of wildlife have been observed. Changes that occurred before the introduction of new 'modified crops' include moves away from mixed farming, mechanical land management, including deep ploughing, and a very large increase in the use of chemicals, particularly insecticides and herbicides. The use of chemicals (chemical fertilizers and chemical pesticides) in agriculture worldwide is still rising, and (for example) chemical usage in Europe is much greater than that in the United States, thought primarily to be due to the intensity of farming. More changes may likely occur as new varieties of plants become available (howsoever produced), as farming techniques will once again change to accommodate the new characteristics of modified plants. It is

[1] Between 1984 and 1990 there was a net loss of 23% of hedges in the U.K. (about 130,000 km in Great Britain)

believed that the impact on the 'wider' environment of genetically modified plants will be significantly different from that observed in the last fifty years due to the 'green revolution'. The manner in which change will occur cannot be predicted, for there is greater control over the genetic material that is inserted into new varieties of plants.

**Effects of the use of chemicals in the agricultural environment**

The effects on birds of the use of the modern pesticides used in farming have been a recognised problem since their widespread adoption in the 1950's and 1960's (Campbell LH *et al.*, 1997). Use of insecticides and even fungicides leads to a reduction in the amount of insects or fungi that serve as the food source for birds. This is likely to lead to reduced breeding success, poor fledgling survival and possibly, increased adult mortality (Campbell LH *et al.*,1997). Current pesticides are less toxic that the ones introduced in the 1950's and 1960's, but there are still products on the market (especially in the developing world) with undesired effects on non-target organisms. Toxicity can differ very much from pesticide to pesticide. Herbicide use reduces weeds that could both be hosts for insects or (as seeds) be food for bird life. There has been great concern that the changes in chemical farming practice, and in particular, in the use of modified organisms that express insecticide throughout the life of the plant rather than sporadic spraying, will so modify the environment as to modify the prevalence of bird populations, and perhaps lead to changes in abundance and diversity. English Nature, a statutory organisation responsible for nature conservation in England has argued that the use of herbicide tolerant or insecticide containing plants are likely to harm the countryside. They argue that herbicide tolerant cropsmight harm the diversity of farmland by a further reduction of weeds through the use of more effective broad-spectrum herbicies, tereby reducing the number and diversity of plants upon which insects and birds can feed and find shelter. The decline in bird and butterfly populations, it is argued, is a direct result of the change in farming practice, and, they argue, this will be exacerbated by the introduction of genetically modified varieties. The thesis depends on an argument that more efficient herbicides are of special concern, and that the introduction of herbicide tolerant crops would increase the use of these herbicides (Johnson B, 1999). There are others who believe the introduction of herbicide tolerant crops may well have the opposite effect. Since this generally represents a shift to more environmentally benign herbicides and the total chemical load on the environment is lower (Della Cioppa G *et al.*,2000).

The purpose of this review is to provide facts, figures and thoughts on the actual and possible non-target effects of genetically engineered crops and put these into perspective with the conventionally bred crops and current agricultural practices.

# 2. Genetically Modified Crops

## 2.1. Genetically modified crops on the market

A very small number of GM crops have been granted approval for placing on the market in the EC. These approvals cover the importation of bulk commodity crops such as soybeans and maize for processing, or the planting of some varieties of maize and oil seed rape. GM maize resistant to the European corn borer is being grown commercially in Spain and France[2]. The development of modified varieties of major arable crops such as wheat and barley is still at a relatively early stage as these crops have apparently been less easy to modify (ACRE, 1999). The area of land used for genetically modified crops in Europe is as yet virtually non-existent when compared to that under cultivation in the United States, Canada, Argentina and China.

Most countries, including the European Union have chosen to regulate the release of genetically modified organisms into the environment, indicating concerns at the possible impact on the environment due to the commercial exploitation of these organisms with novel traits. Historically the regulatory process required the consideration of changes to the organism or in sexually compatible organisms with an emphasis on unwanted effects on the environment, human and/or animal health, resulting from the modification. Therefore, a risk assessment is made based on the use of the organism with regards to its use in food or feed or the potential to be pathogenic in the environment. These risk assessments questioned both the possible impacts on the general environment (including humans) and the possible impact on human and animal health where these organisms are used as food or feed or might be pathogenic when released into the environment. Applicants for permits to release a modified organism have been required to identify the likely impact of the plant when released, and concern about 'super-weeds' and introgression into related species formed part of the assessment. Largely because of the types of plants considered at the early stages in field-testing and commercialisation of the new technology, the indirect effects might not have been considered as carefully. In Europe the possible indirect effects will be taken up in the revision of the 90/220/EC directive, which is expected to be published somewhere in 2001. By far the most field trials of modified organisms have been in the United States of America, where the responsible authority has been the US Department of Agriculture (USDA). The USDA is responsible, under the broad terms of the National Environment Protection Act (NEPA) for making an environmental assessment prior to commercialisation of modified organisms, and in the event of finding adverse consequences, performing a detailed environmental impact assessment. Through the terms of Directive 90/220 (EC Directive, 1990), the European Union Countries regulate both experimental release and commercial release of modified organisms. Risk assessments need to consider the management of the GM crop in comparison to the equivalent non-modified crop. The European approach is precautionary (European Commission, 2000), unlike that of the US, but both require comparative studies and a consideration of impact on the more general environment. Formal comparisons with non-modified crops grown under similar conditions are neither required in the European Legislation, nor in the US legislation.

[2] *'Placing on the market'* includes importing for processing or for animal feed, growing seed for multiplication, and growing in commercial agriculture.

## 2.2. Genetically modified crops: introduced changes

The introduction of new genes into a plant is not precise. The genes may insert in many different sites in the plant genome, and may disrupt genes found therein. If these genes are important in its lifecycle, the plant will not survive the transformation. If in an area of the genome 'not used', the changes will be minimal, and may be limited to the desired characteristic introduced. If incorporation occurs at sites in the genome only normally expressed when the plant is under stress, the impact – if any - may not be observed until that particular stress phenomenon is experienced. Through genotypic and phenotypic testing over several generations and under different conditions, plants with noticeable undesired behaviour are discarded. But it is possible that minor changes stay unnoticed. For instance, the secondary metabolism of a plant may change, providing a minute change in growth characteristics or palatability of the plant to those organisms that eat it. This is a change that may be even more important in conventionally produced varieties. A reduction in the number, type or range of organisms using the plant tissue may start a chain reaction that modifies the environment in a significant manner.

Most crop plants that have been modified so far have had traits introduced for herbicide tolerance, pesticidal action or virus resistance. Changes to nutritional quality, other agronomic characters or tolerance of new environments are likely to appear as the understanding of plant genomics and identification of factors that modify behaviour (phenotype) increases.

The presence of new genes in specific varieties of a particular crop means that these new characteristics will almost certainly be transferred to other varieties of the same plant, if the plant cross pollinates, or to sexually compatible relatives. This transfer of genes has been discussed in the paper by Klaus Ammann, elsewhere in this report, and will not be discussed in this paper.

## 2.3. Non-target effects of genetically modified crops

**Introduction**

The natural environment in Europe is a farmed environment. Any changes made to the plants grown (whether conventionally bred or genetically modified), or the management techniques used on the farm may cause changes in the wildlife that surrounds a particular crop. Absence of spraying, for example, may encourage inconsequential weeds within a field, and the introduction of herbicide tolerant plants may result in less overall spraying and therefore, less of an impact on the uncultivated borders of fields. The result could be a change in the organisms that are found within and around a field when compared to traditional agricultural practice for the same crop. These are non-target effects. Appearance and/or disappearance of a range of wildlife could follow a change in the type of crop grown in a particular area, or in the management techniques that are used to ensure adequate growth of the crop.

**Changes in farming practice and changes in non-target effects**

Non-target effects are not limited to transgenic plants, but they may exacerbate the problem as they are specifically designed to modify farming practice. There is an urgent need to modify current chemically polluting agricultural practice in order to increase sustainability. Changes in non-target effects resulting from the introduction of transgenic plants can be both positive or negative. The same is true for changes in chemical usage could have a similar effect. An example of change that has an immediate effect is changes in ploughing practice. In the United States the new varieties of soybean have been grown using either 'no-till' or

reduced tillage. Traditionally ploughing has been used to control weeds and to prepare seedbeds, but this often results in soil erosion by both wind and water. Plants will be developed that are sown into less disturbed soil. "No till" techniques were first introduced in the United Kingdom in the early 1960's and have started to be used for many other crops throughout the world. This approach, encouraged by the ability to use genetic modification to modify crop plants so that they are more resistant to disease and therefore more able to be used under these conditions, or are tolerant to a broad-spectrum herbicide which makes ploughing for weed control unnecessary, is likely to change the agricultural environment, and therefore the impact on non-target organisms.

The changes introduced into new varieties of crops, however they are produced, may result in major changes to the insect and bird life that 'surrounds' them. For example, the introduction of the novel characteristic could result in unintentional changes to other characteristics of the new variety. "For example, the palatability of the crop to herbivores may be unintentionally reduced if the balance of substances which influence palatability of the plant's tissues is altered. This may be particularly important if the novel gene is transferred, through pollination, to closely related native species, giving them a selective advantage over other native plants due to decreased herbivore feeding" (ACRE, 1999). Genetically modified crop plants that express insecticide characteristics might result in very different insecticide use on the crop as a whole, and this may have a significant effect on wildlife. "Although significant efforts have been focused on improving pest control concepts and technologies in the past 30 years, we are often unable to control insect and mite pests in and environmentally benign manner" (Entomological Society of America, 1999). The Entomological Society of America (1999) believes that "Historically, the use of pest-resistant plant varieties has decreased pesticide use and has been a key component of many integrated pest management programs because of its compatibility with biological and cultural control tactics. New biotechnologies have expanded our capabilities to introduce pest resistant traits into plants and offer the potential to reduce pesticide use further". The Entomological Society also states that many non-target effects are eliminated when using pest resistant plants as pesticide drift both onto weeds within the crop and to non-crop areas of the field is eliminated. They are concerned, however, that some non-target herbivores and detrivores may be affected by the insecticidal action of the modified plants, and, as for new pesticides, the impact on the environment of the gene products and their breakdown constituents needs to be considered. The significantly different process of application also needs careful impact analysis.

Impacts on non-target organisms of plants growing in an environment in which they have not previously been found or using however little water there is in a drought environment need to be carefully considered before introduction. Changes to introduce stress-tolerance will have a significant effect on the environment, as the plants will be grown in regions and areas where they could not be previously grown. This in turn will change the wildlife normally found in the new environment, which may in turn change the environment in unpredictable ways. There is likely to be a conservative or buffering effect, which means that changes could be slight or gradual for a long period of time, which may be followed by an unexpected large step change at unpredictable time in the future catalysed by some minor change in the environment.

## 2.4. Impact analysis

An impact analysis for examining the likely effects on non-target organisms should consider:

1. Those species reliant on the crop itself, whether through using it for food, or shelter.

2. Those plants and animals that live within the field and might be damaged if changes are made to the crop that modifies their habitat or their ability to survive.

3. Plants and animals living in the field margin or hedges and walls, if the management of the crop modifies the size, extent or susceptibility to herbicides and pesticides of this field area.

4. That soil and soil organisms may be affected by the changes in plant variety or management.

The impact may be indirect, and the direct effect of a particular protein on organisms in or around the periphery of a field may not provide information for definitive assessment of risk to the environment.
In a following step the impacts of the transgenic crop should be compared with the impacts of the equivalent non-modified crop.

Here, no specific recommendations are given for the different species that should be considered. However, in determining such recommended lists of species to be considered, the following considerations are relevant:

1. How far should one go in testing the effects on non-target organisms? Is it necessary to test all organisms that are present? Most probably the only rational way of dealing with this is to test a limited number of key organisms that are either considered useful, or relevant from a biodiversity point of view.

2. Specific types and the number of species that is tested can differ from region to region. For instance bird and insect life can show large differences over Europe.

3. Tests performed should be relevant and validated. Especially laboratory tests should be performed in such a way that they have a true predictive value for the situation in the field.

The impact analysis as proposed above is not performed for traditional crops. Such traditional crops are introduced without consideration of their impact on non-target organisms. For the accompanying pesticides the situation is different. Here an impact analysis is performed on a limited amount of non-target organisms that can be exposed to the pesticide. This analysis is an integral part of the registration of pesticides process in Europe and most other countries. Data have to be provided on the effects on organisms like worms, insects, a number of marine organisms, etc.

For genetically engineered crops an approach similar to the classical approach for pesticides is followed, both in the US and in Europe, especially when the genetically engineered crop is considered to have pesticidal effects. This is for instance the case for Bt-crops. The concern for effects on non-target organisms however goes broader than only the transgenic crops with pesticidal effects. The European precautionary approach has led to also consider non-target organisms for other types of transgenic plants. The extent of testing for these other types of plants may be limited.

In the following chapters the most relevant types of genetically modified crops will be analyzed for their possible impact on non-target organisms providing data on their effect as far as available. These possible impacts will be compared to the impact of the traditional farming practices that are meant to be replaced by these transgenic crops.

# 3. Insect Resistant Crops Versus Insecticides: Non Target Effects

## 3.1. Introduction

Insect damage to crops leads to enormous losses in yield. The development of insect tolerant crops has been one of the major successful uses of traditional plant breeding, but genetic modification provides an opportunity to use genes identified in a wide variety of plant and micro-organism species to protect crops. The impact of insecticides on non-target species is an integral part of the registration of pesticides process in Europe and most other countries (Department of the Environment, Transport and the Regions, UK, 1998). There will be concerns if beneficial insects are reduced in number, either through the direct toxic effect of the pesticide (as this may cause secondary pest outbreaks which beneficial insects could help control), or through a decrease in insects upon which they prey. A reduction in the number or range of beneficial insects may be important because of their impact on insect numbers affecting other crops in the vicinity.

## 3.2. Non-target effects of insecticides

There may be a significant difference in the effects of pesticides depending on the time of application in the growing season. Pesticides may be applied at a time of year when non-target insects are essentially absent (e.g. treatment of winter cereals to control the aphid vectors of Barley Yellow Dwarf Virus) (Department of the Environment, Transport and the Regions, UK, 1998). The use of the same insecticide during summer months may severely reduce the numbers of beneficial insects. The specificity of the insecticide or pesticide used in modified crops as well as the tissues in which they are expressed may moderate their impact on non-target insects. Currently, farmers are aware of the impact of spraying at the wrong time and often moderate their use of insecticides. 'Insurance spraying' is however sometimes used, where the only consideration is yield of the particular crops without consideration for the impact on other crops; or on the general environment. In addition to a direct impact on the crop plants in the field, spraying may have an effect on organisms living in nearby vegetation either within the field itself or on the edges of fields. Field-side vegetation is important for beneficial insects and other wildlife and contamination is beginning to be avoided.

Pesticides applied to soil are primarily broken down by soil microbial activity. When introducing specific pesticides within genetically modified plants efforts should be made to ensure that their stability outside the plant is as low as possible. Currently, pesticides are usually dispersible (or soluble) in water to allow their application. This makes their leaching from the soil and into potable water likely, but transgenic plants may be designed to contain pesticides that are less easily mobilised in water, and therefore less likely to impact on organisms other than those for which the modification is designed.

## 3.3. Non-target effects of Bt-crops

The major insecticide introduced into transgenic plants has been the group of toxins produced by Bacillus thuringiensis (Bt). Under normal circumstances this protein is produced by the bacterium during sporulation in

a crystal form toxic to specific insects (depending on the particular variety of toxin). There are many strains of the bacteriu, each producing toxins with different (but often overlapping) specificity. Bt has been used commercially for many years and has been used by organic farmers and home gardeners for over forty years. It is known as an environmentally friendly insecticide because of its target specificity and its decomposition to non-toxic compounds when exposed to environmental factors (Bhatia, J. et al., 1999). The protein used in transgenic plants is truncated. The wild type protein is larger and is only activated in the gut of the insects after being enzymatically cleaved. The major targets of modified crops (so far) have been the European corn borer (ECB), the tobacco budworm, the cotton bollworm and the Colorado potato beetle.

Table 1: Bt crops and their target pests

| Crop | Target pest | Bt gene |
|---|---|---|
| potato | Colorado potato beetle | Cry3A |
| maize | European Corn Borer | CryIAb |
| cotton | Tobacco budworm<br>Cotton bollworm | <br>CryIAc |

Alstad (1997) has suggested that the protection offered against the European corn borer will have a significant effect on other diseases or pests that affect the crop. Less damage to the crop for instance leads to less growth on the crop of mycotoxin producing fungi.But the impact on organisms that depend on the corn borers predation could be significant as a result of effectively. *Bacillus thuringiensis* is highly selective against lepidoptera in particular, attacking only the stomach lining (with a specific pH) of susceptible insects. If this is the case, "it is safe to the environment and non target insects and animals, including other predators found in cornfields, humans, birds, fish or bees" (Bhatia *et al*,1999). There is no accumulation in insects or other pests of the toxin that could then affect predators of organisms accumulating the toxin. Bhatia also reports that extensive safety trials have been conducted in the laboratory, greenhouse and field which attempted to identify the probable direct effects of Bt corn on lady beetles, green lacewing larvae, spiders, minute pirate bugs and parasitic wasps. Although there may be indirect impacts on natural predators of the lepidoptera, as the decline in numbers will affect their number, the effect is likely to be far less than that due to the use of chemical insecticides. The specificity of the treatment, and the toxin's continuous presence, in comparison to other spraying regimes, will alter the balance between insect species. In addition, allergenicity to Bt spores themselves has been demonstrated (Bhatia *et al* 1999) — but there are no Bt spores produced by the transgenic plants.

When Bt maize was first approved in the United States and Canada the regulators assumed that all Bt proteins would kill Monarch butterfly larvae and other more rare lepidoptera, but that the exposure to the insecticide via pollen would be minimal. Furthermore, milkweed, the normal food for the Monarch larvae, is seldom found in cornfields. Extra-cellular Bt toxin is rapidly degraded by UV light as is likely for the toxic protein in pollen. A comparison of the normal spraying regimes in comparison to the impact of pollen containing Bt toxin suggested that the risk due to the pesticidal plants was small. In May 1999 Losey et al published a report indicating that the toxin expressed in pollen from maize plants could kill Monarch butterfly caterpillars in laboratory tests (Losey JJ. *et al*, 1999). Losey reported that "larvae of the Monarch butterfly reared on milkweed leaves dusted with pollen from Bt corn ate less, grew more slowly and suffered higher mortality than larvae reared on leaves dusted with untransformed corn pollen or leaves without pollen". The implications of

the paper are difficult to identify, for the presence of the pollen could have altered the feeding of the larvae and the amount of pollen loaded on the milkweed leaves were not measured or reported, so that the experiment cannot be repeated or verified. Results from pollen deposition studies in cornfields found that on average, the level of pollen was about one to 2 orders of magnitude below the estimated LD50 of Bt pollen for Monarch butterfly (Pleasants J. *et al.*, 1999). This would mean that even though Monarch larvae are susceptible to CryIAb protein, the concentration in the field will not be high enough to kill them. Stepping just 2 meters outside of the cornfield further reduces the pollen deposition by at least another order of magnitude. Another important point is timing. Pollen is usually produced by maize during mid-July through early August; Monarch butterfly larvae actively feed during June. However, if it is the Bt toxin that is affecting the larvae rather than pollen, then the ubiquitous presence of the gene product if included in many other products may present problems. In previous tests (Hansen and Obrycki, 1999) it had been shown that Bt corn pollen on milkweed near to modified corn was not as extensive a problem as identified by Losey (Hansen L. *et al.*, 1999), (Shelton AM *et al.*, 1999). Shelton and Roush (1999) assert that "[w]e believe that few entomologists or weed scientists familiar with butterflies or corn production (and the control of milkweed) give credence" to the view that Bt corn pollen poses a serious risk to the Monarch butterfly.

**Novartis study**

The effects of Bt Maize on a variety of non-target insects was been examined by Novartis at the end of 1999. Many different studies are reported in the compilation, including the toxicity of pollen to a variety of organisms, e.g. water fleas - *Daphnia magna*, lady beetle- *Coleomegilla maculata* (in particular larval development), and honey bees – *Apis mellifera L.* The paper reports on the impact of the Novartis maize on Diptera, Hymenoptera, Coleoptera and Homopterans. Little or no differences were observed between controls and those in which the Bt toxin was expressed. Also reported were the impact of their Bt maize on digestibility and acute oral toxicity in rodents and small mammals and no significant differences between control studies and those including the Bt gene product. Direct feeding of the Bobwhite quail again showed no significant differences.

**Conclusions**

It is undoubtedly true that pesticides introduced into plants will have a direct effect on insects that come into contact with the pesticide and are susceptible to it. The probability of coming into contact with the insecticidal protein must be properly calculated, and the impact of traditional agricultural chemicals used in non-transgenic environments on the same organisms needs be compared under normal commercial conditions in a particular environment. The argument that the Bt protein is expressed continuously (possibly at a low level) whereas spraying is only transient is a strong argument, but not when pollen is the vector for the insecticidal protein, that is, if the spread of pollen is the only way by which non-target organisms can come into contact with the Cry-protein. It is not true for the organisms that actually feed on the maize. They can be in contact with the Cry-protein during the whole season. Chemical treatment on the other hand, is sporadic and transient, but the chemical treatment may persist, both on the plant and in the soil (dependent on the insecticides used).

Much work has been done on the possible non-target effects of Bt crops (see above), but direct comparisons between all possible non-target effects of the conventional agricultural practices using chemical insecticides and the new gmo practices using Bt crops have not been done. It is very difficult to make general comparisons, because the results will depend on the type of insect one wants to kill, the types of insecticides

are used (if any), but perhaps also on other circumstances like for instance climatic conditions. Bt use in maize to kill ECB will be different from Bt use in cotton to kill cotton bollworm. In current non-gmo maize cultivation the amount of insecticide used to kill ECB is limited, while the amount of insecticide used to kill cotton bollworm is large. This is because ECB feeds inside the stem of the maize plant and is fairly well protected against insecticides. Spraying is not very effective. Also the insecticides used in maize and cotton can be very different both in their ecotoxicity and their persistence. When comparing the two practices, perhaps the following factors could give helpful indications. Both for the chemical insecticide use, and the Bt genetically modified crop an estimate should be made on how they score on these criteria:

- The insecticides used (Bt protein versus the chemical insecticides)
- Its type of use (constitutional or inducible expression of the Bt gene, versus single or multiple applications of chemical insecticides
- Direct toxicity to relevant worms, insects, birds and animals
- Total amount of active ingredient needed
- Type of exposure of non-target organisms
- Biodegradability
- Effectiveness (both in the short and long term)

From such a comparison it will be difficult to make exact predictions. Perhaps in particular cases there will be a strong case for one type of practice performing better or worse in terms of effects on non-target organisms. But in many cases direct comparisons in the field will be the only way to really make quantitative statements to determine what type of practice is the most environmentally friendly.

If the described comparison would show that the unwanted impact of a genetically modified crop on non-target organisms is similar or less than the impact of the conventional insecticidal practice, than there is no reason why the use of the genetically modified crop should not be allowed. From a sustainability point of view, however, there is a strong case for only allowing those crops or practices that prove to be an improvement of the currently available technology: to only allow genetically modified crops if they have less impact on non-target organisms.

Chemical insecticides are not without impact on non-target organisms and therefore non-chemical alternatives for pest control are important to improve sustainability (Pimentel, 2000). The introduction of resistance factors, either through conventional breeding or genetic engineering will be important to lower the use of these chemical insecticides. The genes involved in the production of toxins isolated from *Bacillus thuringiensis* are not the only resistance factors being considered for use in transgenic organisms. Insecticidal lectins - natural toxins produced in many plants - have been engineered into plants in order to observe their effect on susceptible insects with a view to using them as internal natural insecticides. Snowdrop lectin, (GNA) is another protein that may be toxic to specific lepidopteran pests when available within a plant's tissues. Predators of these lepidoptera could be affected by the lectin in their diets. It has been shown that the use of "GNA in transgenic crops to confer resistance to lepidopteran pests does not adversely affect the ectoparasitoid *E. pennicornis* to utilise the pest species as a host" (Bell HA *et al.*, 1999). More work needs to be done to ensure that the impact of such an insecticide does not adversely affect insects that may be important in the survival of nearby crop plants.

# 4. Non-Target Effects of Herbicide Tolerant Plants

## 4.1. Introduction

By far the greatest area of transgenic plants currently produced commercially contains variants of enzymes to allow them to tolerate the presence of systemic herbicides (in 1999, >50% of the world acreage of transgenic crops was herbicide tolerant). In practice, herbicide tolerance in transgenic crops is limited to glyphosate[3] and phosphinotricin[4], both of which are considered to be relatively benign herbicides (Tomlin, 1994). Glyphosate tolerance has been pioneered by Monsanto as *Roundup*. Tolerance to phosphinotricin has been marketed by Aventis CropScience (formerly AgrEvo) under the names *Liberty*, *Finale* or *Basta*. Both of these are broad-spectrum herbicides that do not distinguish between crop plants or weeds, and are very widely used. Tolerant plants make the use of broad-spectrum herbicides in agriculture possible, for all unwanted plants are removed. The removal of all weeds – resulting in a single plant variety field may have a considerable impact on wild life. But this impact may be less than the impact of traditional herbicide spray regimes, where there may be more weeds, but a greater impact as a result of the use of perhaps less environmentally friendly herbicides.

## 4.2. Non-target effects of herbicide use

The use of herbicide tolerant plants will ultimately increase the use of the accompanying systemic herbicides, with less of other herbicides being used until such time as herbicide tolerance is developed in major weed species, but before the appearance of herbicide toerlant weeds it is likely that there is a shift towards weeds that have limited leaf surfaces. Such weeds are more likely to survive spraying with the herbicide because they will be more difficult to be hit by the herbicide. As a result, pre-emergence herbicides may still need to be used, but it is argued that there will be far less use of other products after emergence of the crop carrying a particular tolerance. With a likely increase of the use of glyphosate and phosphinotricin, questions regarding the safety of these herbicide for organisms other than those targeted, weeds, are pertinent. Many studies have been conducted. Plants grown in the presence of these herbicides will contain traces of them, and this obviously depends on whether, when and how much herbicide has been used to maintain the supremacy of the crop plant relative to other plants (weeds) competing in the field.

There have been few reports of the toxicity of glyphosate itself to insects, animals or humans, and a large number of reports suggesting it is safe for most organisms other than those targeted[5]. Glyphosate has been used for over 25 years. Undesirable effects could occur from the presence of the proteins produced by the plants expressing tolerance, their enzymatic activity or any other products that might arise because of their presence. Using a variety of criteria, no allergenicity or toxicity of phosphinotricin or any of its metabolites have been reported.

[3] Glyphosate is a tertiary amine that inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that is found in most plants. EPSPS plays a role in the synthesis of aromatic aminoacids. Without this enzyme these aminoacids are not formed resulting in the death of the plant. The gene for an enzyme that performs the same action, but is not inhibited by glyphosate is used as a means of providing tolerance. Alternatively, and enzyme may be introduced to catalyse the removal of glyphosate — glyphosate oxidoreductase.

[4] Phosphinotricin is often called glufosinate or glufosinate ammonium. It inhibits glutamine synthetase, which is responsible for glutamate to be turned into glutamine. The absence of this reaction leads to the build-up of ammonia which eventually kills the plant. *Streptomyces* species produce an enzyme that deactivates the inhibition by acetylating the enzyme. The acetyl transferase which achieves this deactivation is termed PAT (phosphinotricin acetyl transferase, and the gene coding for the enzyme is termed the bar gene.

[5] Examples are Monsanto Company Reports — Glyphosate and beneficial arthropods" see http://www.biotech-info.net/glyphosate.html.

## 4.3. Herbicide tolerant plants versus herbicides

In the same manner as that identified for insect resistant crops, direct comparison of the biodiversity of conventional practices using chemical herbicides versus the cultivation of herbicide tolerant crops using a broad spectrum herbicide are difficult to make, and can differ according to the crop, region, or climatic conditions. But filling in the criteria like in table 2 may provide some indications. Actual field comparisons however are the only source of quantitative conclusions.

Table 2: An example of a comparison between a conventional and a genetically modified practice

| | Conventional maize | Phosphinotricin tolerant maize |
|---|---|---|
| **Herbicides used** | Different herbicides for pre- and post-emergence applications, for instance Atrazin, Bentazon, propachlorine, metolachlorine | Basta |
| **Type of use** | Pre- and post emergence | Mostly only post-emergence |
| **Total amount of active ingredient needed** | The greater number of applications makes that the amounts needed are relatively large | Relatively small amounts because of the limited number of applications (one or maybe two applications) |
| **Biodegradability** | Can differ greatly and will depend on the specific herbicide | Good (when compared to other herbicides) |
| **Effectiveness** | The effectiveness can vary greatly, depending on the presence of 'problem' weeds | Weed control is very good. In the long term there may be shifts toward weeds with minimal leaf surface and there may be a greater risk of tolerant weeds to emerge |
| **Direct toxicity to 'non target' organisms like worms, insects, birds and animals** | Can differ greatly and will depend on the specific herbicide | Low (when compared to other herbicides) |
| **Indirect effects on 'non target' organisms like worms, insects, birds and animals** | There might be a greater number and diversity of weeds in the field upon which insects, birds, and other organisms can feed and find shelter, but too large amounts of weeds can lower yields or negatively influence the quality of the harvest | There might be a somewhat lesser number and diversity of weeds in the field upon which insects, birds, and other organisms can feed and shelter |

Herbicide tolerance can both be obtained by many means, including conventional breeding and genetic engineering, meaning that the results in terms of effects of non-target organisms can be comparable. With regard to the specific examples of genetically modified herbicide tolerant crops that are used now (Roundup and Phosphinotricin tolerant crops), two trends can be identified that are relevant for effects on non-target organisms of these transgenic herbicide tolerant crops. On the one hand the trend towards lesser applications of herbicide and use of more environmentally friendly herbicides (which is a positive development). This may have the effect of lesser direct toxicity to all kinds of organisms. On the other hand the trend that weed control will be more effective. This is positive from the perspective of the farmer (easier weed control, higher quality of products), but it may result in a greater indirect effect on non-target organisms in the case weeds are diminished to such a level that it will influence the number and the diversity of organisms living in the crop. It will depend on the specific crop, the normally used herbicides and other factors, in what direction the balance will turn. Either positive for genetically modified crops, or for the conventional practice.

# 5. Other Modifications and their Effects on Non-Target Organisms

## 5.1. Plants designed to express changed chemical or nutritional qualities

In the near future there are likely to be many crops with changed nutritional characteristics. An example close to commercialisation is rice modified to produce much larger quantities of Vitamin A. Safety is a prime concern, and the new varieties will need careful safety testing before being introduced, particularly into societies where the rice could form the staple diet. Any changed characteristics (howsoever produced) will undoubtedly alter the palatability of the crop to all organisms, and could modify ecosystems occupied by the new varieties of plants significantly. It is not yet possible to identify the changes, and both small-scale field trials and large-scale trials will have to monitored carefully for the impact on wildlife. A change in oil structure is also likely to modify all sorts of characteristics. Different oil quality or different viscosity will have an effect on bacterial and mould growth, insect and ultimately bird life. If multiple variants of the same crop are produced, each variant having new nutritional characteristics, the impact may well be an increase in biodiversity, smaller fields with each variant biologically of physically isolated from others. This is particularly important where pharmacologically active compounds are to be produced in crop plants. In most cases, high value active chemicals, if produced in plants, will require careful management to ensure that the pharmacological activity is isolated from susceptible organisms. None of these changes is new, for every new variety of a particular crop, or a change in the crop grown (or rotation) would have similar effects. Just because they are produced using 'modern biotechnology' should not become the criterion for assuming that damage is inevitable.

## 5.2. Plants designed for phytoremediation or detoxification

It is likely that plants will be modified so as to grow on heavily polluted land, and they could sequester heavy metal. Conventional farming practices apply metals to soils through addition of fertiliser (principally cadmium and animal feedstuff contributions to farm manure contents, especially copper and zinc). The zinc and copper loadings per unit land area receiving inputs decrease in the order sewage sludge>pig or poultry manure > cattle manure > atmospheric deposition > fertilisers or lime (Department of the Environment, Transport and the Regions, UK, 1998). Plants containing high quantities of these and other metals may well pose a significant threat to grazing animals or insects. In addition, phytoremediation is likely to be become an important tool in the detoxification of polluted land, and it is likely that genetically modified plants will be used for this purpose. Care will need to be taken with the resulting plant material so that polluting substances are not simply diluted in the environment, posing threats to many organisms, including birds, but that metals may be recovered from plants and organic substance mineralised.

## 5.3. Plants designed to resist abiotic stress

Plants are being engineered to resist stressing conditions like drought or saline conditions. The goal is to be able to grow plants on very marginal land under harsh conditions, where plant growth until now has been limited to som highly adapted species. The image of turning de desert into an oasis may be overestimating the possibilities of genetic engineering, but it is estimated that to some extend it will be possible to introduce crops to areas where they have never been grown before. The introduction of such a genetically engineered crop, like the introduction of a conventionally bred crop into a new habitat, will have an impact on the organisms living in that habitat. Probably such an introduction will have a greater impact on non-target organisms, than the introduction of a new variety into an existing agricultural practice, or the replacement of a conventionally bred crop by a genetically modified variety. Instead of introducing a new characteristic, which may or may not significantly change agricultural practice, it is the introduction of a completely new organism. Te existing ecosystem, whether rich or poor in biodiversity, can be dramatically changed, and it will depend on the actual changes, whether these are perceived to be either good or bad. Even if a certain habitat is poor in biodiversity, it does not mean that a change resulting in greater biodiversity will always be seen as positive, for instance when the changes in biodiversity result in a threat for certain rare species. Rare species are often vulnerable.

In cases of introduction of genetically modified varieties into a new habitat, negative effects on non-target organisms and biodiversity – if any – will most probably (have to) be weighed against socio-economic arguments like labour, income, food security, and other.

# 6. Conclusions

The introduction into agriculture of this new range of transgenic crops has caused much debate, a great deal of heat, and very little light. Any discussion of the impact of these organisms on either the agricultural environment or the natural environment should take into account current practice. It may be that any change in use of agricultural land will have an impact on non-target organisms that is unacceptable to those living close to farms, and that decisions about the use of these crops need be considered in a wider context than a scientific risk analysis.

The debate about the introduction of transgenic crops in Europe seems to be based on ensuring that non-target effects are not to be tolerated; the effects of current practice are not taken into account. Perhaps it is helpful to first determine what type of biodiversity we want to protect and/or stimulate (beneficial micro-organisms, beneficial insects, birds, rodents?). In a second step we should determine how these biodiversity goals should be achieved. It may be that this will mean a number of biodiversity criteria for the cultivation of crops, either conventional or genetically modified. On the other hand it may direct us towards the stimulation of biodiversity near the agricultural field by planting and growing trees, hedgerows, and by setting land aside for nature and wildlife. If genetic engineering is able to contribute to a further increase in yield, this may be an incentive to decrease the amounts of farmland and increase the amounts of non-farmland.

From a biodiversity point of view it is important to reverse some of the trends of numbers and spectrum of organisms living in our countryside. Genetically modified crops are both presenting a perspective and in some cases a risk. A perspective because it can contribute to the lowering of the use of potential detrimental pesticides, which can have effects on all kinds of organisms. A risk because it might in certain cases modified crops could have direct or indirect effects on non-target organisms.

Most regulatory systems only consider and assess hazard and risk when attempting to minimise harm to both the agricultural and natural environment. It would perhaps be better to consider both risk and benefits to the environment, and include the risks and benefits of either continuing current practice or changing (say, to organic agriculture) without the use of genetically modified organisms. A discussion of these issues is without the terms of reference of this document.

The impact of transgenic organisms on non-target organisms cannot be simply predicted. Each individual case has to be considered. It is not the transgenic organism itself that may have a substantial impact on other organisms, or on biodiversity, but rather the management system into which the crop is placed compared to the complete management system for the crops it replaces. In this document it is shown that even for the cases of insect resistance and herbicide tolerance – the cases already most well known from practice – it will depend on the specific crop and the current (pesticidal) practices, how the balance for effects on non-target organisms will turn; either pro genetically engineered or pro conventional. Organic agriculture is not taken into account in this document because genetically engineered crops are not intended to replace organic crops.

In a wider perspective the performance of organic agriculture in terms of effects on non-target organisms is of course also relevant, but this is beyond the scope of this paper.

The impact of genetically engineered crops, like that of conventional crops, can be considerable when the crops are introduced into new habitats. With genetic engineering, however, it may be possible to alter the drought-, stress, or other resistance to abiotic stress to such an extend that it may be possible to grow the crops in areas where it was never possible to cultivate them before. It is important to perform an impact analysis in such cases, where the possible changes can be different than the changes as a result of the change of a farming practice in existing agriculture.

The experience with the current genetically engineered crops, engineered to replace conventional agricultural practices, has not revealed indications of dramatic effects on non-target organisms of the growing of these new crops.

# 7. References


A discussion paper prepared for the Advisory Committee on Releases to the Environment in the UK "The Commercial Use of Genetically Modified Crops in the United Kingdom: the Potential Wider Impact on Farmland Wildlife", 1999, (http://www.environment.detr.gov.uk/acre/wildlife/index.htm).

Alstad DN et al, (1997). BT-corn and European Corn Borer – Long term success through resistance management. *University of Minnesota*. http://www.extension.umn.edu/Documents/D/C/DC7055.html.

Bell HA et al (1999). The effect of snowdrop lectin (GNA) delivered via artificial diet and transgenic crops on Eupholus pennicornis (Hymenoptera : Eulophidae), a parasitoid of the tomato moth Lacanobia olereacea (Lepidoptera : Noctuidae). *Journal of Insect Physiology*, 45, 983-991.

Bhatia J, Grant SE, Powell D.A. (1999). Backgrounder : Genetically-engineered Bt containing field corn. *Technical Report* 11, July 21, 1999 on http://www.oac.uoguelph.ca/riskcomm/plant-ag/bt-survey/bt-backgrounder.htm.

Campbell LH, Avery MI, Donald P, Evans AD, Green RE and Wilson JD (1997). A review of the indirect effects of Pesticides on Birds *JNCC Report* No. 227, Joint Nature Conservation Committee, Peterborough UK (ISSN 0963-8091).

Campbell LH, Avery MI, Donald P, Evans AD, Green RE and Wilson JD (1997). A review of the indirect effects of Pesticides on Birds *JNCC Report* No.227, Joint Nature Conservation Committee, Peterborough UK (ISSN 0963-8091) Section 1.2, page 2.

Della Cioppa G and Callan M. Sex lies and herbicides. *Nature Biotechnology*, 18, 241, 2000.

Department of the Environment, Transport and the Regions, UK (1998). Environmental Effects of Agriculture, Final Report, pages 72-76.

Department of the Environment, Transport and the Regions, UK (1998). Environmental Effects of Agriculture, Final Report, pages 104-106.

de Zoeten G (1995). Virus resistant plants : heterologous encapsidation and recombination environment. Proceedings of the Workshop on Key Biosafety Aspects of Genetically Modified Organisms, Braunschweig, Germany – http://www.bba.de/gentech/worksh02.htm.

*Entomological Society of America*. Position Statement on Appropriate Uses of Genetic Engineering in the Production of Crops (1999) – http://www.isb.vt.edu/rarm/esa.html or http://www.entsoc.org/about_esa/statements/transgenic.html.

EC Directive 90/220/EEC (1990) on the Deliberate Release into the Environment of Genetically Modified Organisms. Official Journal L117 (8 May 1990) p15-27.

Communication from the European Commission on the Precautionary Principle (2000), Com (2000)1.Brussels February 2000.

Hansen L and Obrycki J. (1999). Non-target effects of Bt corn pollen on the Monarch butterfly (Lepidoptera : Donaidae). 54th Compiled Proceedings, Annual Meeting, North Central Branch of the Entomological Society of America – http://www.ent.iastate.edu/entsoc/ncb99/prog/abs/D81.html.

Johnson Brian, 1999. Birds, bugs and biotechnology, where do we go from here, *Agbiotech* '99, Nov 14-16, 1999, London, United Kingdom.

Losey JJ, Raynor L and Cater ME (1999). Transgenic pollen harms Monarch larvae. *Nature*, 399, 214.

Nap JP (1999). A transgene-centred approach to the Biosafety Assessment of Transgenic herbicide tolerant crops. *Biotechnology and Development Monitor* 38, 7-11.

Novartis (1999). Bt-Maize and non-target organisms – compilation of available data, Basel, October 1999.
Pimental, D.S., and Raven, P.H. (2000). Bt corn pollen impacts on nontarget Lepitoptera: assessment of effects in nature, PNAS, juli 18, 2000, vol 97, no. 15, 8198-8199.

Pleasants J, Hellmich R, Lewis L (1999). Pollen deposition on milkweed leaves under natural conditions and assessment of risk to Monarch butterfly larvae from Bt pollen. Nov 2, 1999, Monarch Symposium, Chicago, IL.

Royal Society, London : Statement September 1998 'Genetically modified plants for food use'.

Shelton AM and Roush RT (1999). False Reports and the ears of men. *Nature Biotechnology* 17, 832.

Tomlin, C., The Pesticide Manual, a world compendium. Tenth edition. British Crop Protection Council, Surrey, UK.

# Allergenicity Of Foods Derived from Genetically Modified Organisms

**Authors: André Penninks, Leon Knippels, Geert Houben**
**TNO Nutrition and Food Research Institute**
**P.O.Box 360**
**3700 AJ Zeist, The Netherlands**

**1. Introduction . . . . . 109**
**2. Food allergy and food allergens . . . . . 110**
2.1 Adverse reactions to foods . . . . . 110
2.2 Mechanism and clinical manifestations of IgE mediated food allergy . . . . . 111
2.3 Prevalence of IgE mediated food allergy . . . . . 111
2.4 Food allergens . . . . . 111
**3. Food derived from Genetically Modified Organisms and their possible impact with respect to food allergy . . . . . 113**
3.1 Aim of the changes . . . . . 113
3.2 Type of biotechnological modifications . . . . . 113
3.3 Relevance of type of biotechnological modifications for allergenicity . . . . . 114
3.4 History of safe use . . . . . 114
**4. Assessment of the potential allergenicity of (new) food proteins . . . . . 116**
4.1 Current strategies . . . . . 116
4.1.1 The source of the protein of interest is a known food allergen . . . . . 118
4.1.2 The source of the protein of interest has no history of dietary exposure or an unknown history in terms of allergenicity . . . . . 118
4.2 Points of concern with regard to the proposed assessment strategies . . . . . 119
4.3 False negatives versus false positives . . . . . 121
4.4 Animal models in research on allergenicity of food proteins . . . . . 121
4.5 Levels of allergens in food . . . . . 122
4.6 Conclusion . . . . . 122
**5. Case studies . . . . . 123**
5.1 Methionine enriched soybeans . . . . . 123
5.2 Glyphosate-tolerant soybeans . . . . . 124
5.3 Transgenic hypo-allergenic rice . . . . . 125
5.4 Conventional kiwi . . . . . 126
5.5 Conclusion . . . . . 126
**6. From assessment of potential allergenicity towards allergy risk assessment . . . . . 127**
6.1 Hazard Characterization: the allergenic potency . . . . . 128
6.2 Exposure Assessment . . . . . 129
6.3 Risk Characterization . . . . . 130
6.4 Decision making and risk management . . . . . 130
**7. Conclusion . . . . . 131**
**8. References . . . . . 133**

# 1. Introduction

Modern techniques enable the production of new generations of food and food ingredients. Particularly the use of genetically modified organisms in food production has gained quite some attention during the past few years. New food products or new food ingredients may only be introduced on the market after their safety has successfully been demonstrated. As a matter of fact, the data required for the evaluation of novel foods and novel food ingredients are for an important part focussed on consumer safety and should address aspects of genetic modification, nutritional aspects, micro-biological aspects, and toxicological and allergenicity aspects.

A toxicological evaluation and risk assessment will in most cases be major items in the safety evaluation of novel foods and novel food ingredients. In addition to a "classical" toxicological evaluation, the potential of novel foods and novel food ingredients, especially those consisting of or containing proteins, of inducing allergic reactions in consumers should have special attention. Particularly for novel foods or novel food ingredients consisting of or derived from genetically modified organisms, it is suggested that allergenicity may pose a concern.

In the present paper, the issue of allergenicity of foods derived from genetically modified organisms (GMOs) is addressed. In Chapter 2, an overview is given on what food allergy is and some essential background information on involved mechanisms and epidemiological aspects is provided. Furthermore, some relevant scientific knowledge on interactions between food proteins and the immune system and on food allergens is described. Chapter 3 addresses possible (foreseen) new types of food products of GMO origin (types of modifications) and their possible impact with respect to food allergy. Also the usefulness and limitations of the history of safe exposure concept is addressed. Issues that are relevant in this respect include the relevance of new combinations of ingredients, the role of a possible new physico-chemical phenotype of a protein after transfer and expression, and new exposure levels and conditions. Chapter 4 is dedicated to methods and strategies for allergenicity assessment, their possibilities and limitations, and new and future developments in this respect. This is followed by a description of some specific cases in Chapter 5. Finally, Chapter 6 discusses allergenicity of food proteins from a risk assessment and risk management point of view.

# 2. Food Allergy and Food Allergens

## 2.1 Adverse reactions to foods

Adverse reactions to foods can be distinguished between toxic, nontoxic reactions, and aversion (Fig. 1). In food aversion, the food is not tolerated for psychological reasons. An example is that someone who has had a bad experience as a result of eating contaminated fish may, due to psychosomatic mechanisms, show allergy like symptoms upon renewed exposure to fish. Toxic reactions to food will occur in any exposed individual provided that the dose is high enough. An example of toxicity is vomiting after having eaten poisonous mushrooms. The toxicity of genetically engineered food is discussed in the paper by Knudsen and Pedersen, elsewhere in this report. Nontoxic adverse reactions to foods, often also referred to as food hypersensitivity, may be defined as a qualitatively and/or quantitatively extremely different reaction to food, which is not so much caused by the food itself, but rather by a specific trait of the person who takes the food. These reactions can be divided into reactions due to food allergy and food intolerance. Food allergy may be defined as a food hypersensitivity in which the reactions are primarily immunologically mediated, while non-immunologically mediated mechanisms play the major role in food intolerance. An example of food intolerance is lactose-intolerance: the inability to digest lactose present in sweet milk.

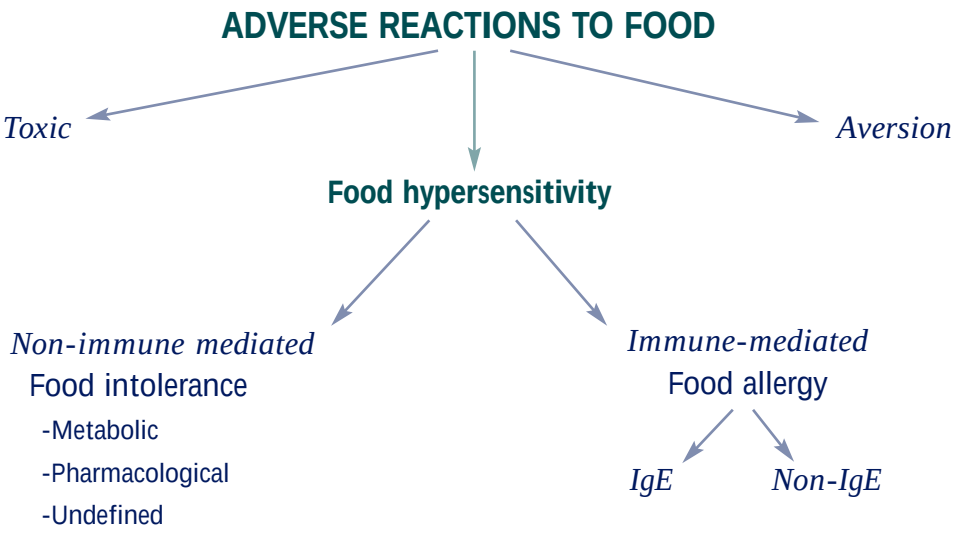



Figure 1. Schematic presentation of adverse reactions to food

In most known cases of food allergy, the immune system reacts with the production of a certain type of immunoglobulin, Immunoglobulin E (IgE), against "harmless" proteins present in food and is referred to as Type I or immediate type hypersensitivity (Bruijnzeel-Koomen et al., 1995). IgE-mediated (food) allergy often occurs as a part of the so called atopic syndrome. People with atopy are considered to have a hereditary trait (the atopic constitution) associated with a greater risk of development of IgE-mediated allergies. However, up to 10% of the children of healthy, non-atopic parents was also calculated to develop atopic diseases (Saarinen *et al.*, 1995). Although, genetic factors play a major role in the development of allergic diseases, other factors,

like the introduction of new allergens and air pollution, are also thought to be responsible for the increase in the prevalence of allergic diseases (Popp *et al.*, 1989). In this paper IgE-mediated food allergy will be discussed as introduction of new proteins in our food that may result especially in this type of allergy.

## 2.2 Mechanism and clinical manifestations of IgE mediated food allergy

When first ingested, the allergen(s), i.e. the sensitizing food or food constituent, is to some extend degraded by digestive enzymes, absorbed by the gut mucosa, processed in immunopotent cells and then presented to the immune system which will result in the production of food protein-specific IgE. The food-allergen specific IgE antibodies circulate in the body and will bind to mast cells throughout the body tissues and basophils in the blood. Upon renewed contact with the food-allergen, the allergen binds to the IgE antibodies present on the mast cells and basophils. Binding of the food-allergen triggers the cells. This will result in degranulation of the mast cells and basophils and production of several mediators (Fig. 2). These mediators induce a variety of food allergy associated clinical symptoms involving the oral cavity, the gastrointestinal tract, the skin, the respiratory tract and the circulatory system (Table 1). The symptoms may occur within minutes to days after ingestion of the offending food and may even result in an anaphylactic shock and sometimes death.

Table 1. Clinical aspects of food allergy

| | |
|---|---|
| Oral cavity | Oral allergy syndrome (itching and swelling of lips, mouth or throat) |
| Gastrointestinal tract | Vomiting, cramps, diarrhea, abdominal pain, angioedema |
| Skin | Urticaria, atopic dermatitis (eczema), angioedema |
| Respiratory tract | Asthma, rhinitis, bronchospasm, wheezing, angioedema |
| Cardio-vascular | Decrease in blood pressure, anaphylaxis |

## 2.3 Prevalence of IgE mediated food allergy

The prevalence of food allergy is estimated to be about 1.5-5% of the general population, corresponding to about 8-10% of the paediatric population, and around 1% in adults (Bruijnzeel-Koomen *et al.*, 1995). Food allergy in children usually appears to be a transient phenomenon. Over 75% of food allergic children has "outgrown" their respective reactivities within 5 to 9 years after the onset of clinical symptoms. However, some food allergies, like those for peanuts, are more persistent and often do not diminish or disappear while growing up. The decreased incidence of food allergy with age suggests that immaturity of the immune system may be an important factor in the pathophysiology of the disease.

## 2.4 Food allergens

Most food allergens are glycoproteins with a molecular weight between 10 and 70 kD (Taylor, S, and Lehrer, S, 1996). In theory, every food (glyco)protein may act as a food allergen. Factors that determine the allergenicity of food proteins are poorly known, but an important factor may be the digestibility of the protein in the gastro-intestinal tract since it is known that food allergens are relatively stable to acid- and heat-treatment, and relatively resistant to digestive breakdown (Taylor *et al.*, 1992; Astwood *et al.*, 1996). In addition, digestive processes may increase allergenicity by unmasking epitopes or formation of new epitopes as was shown for milk-proteins (Wal, 1998; Maynard *et al.*, 1997). Moreover, even small molecules are known

to cause sensitization either directly or via the hapten-carrier mechanism. It is also known that carbohydrate structures on proteins in part determine or influence the allergenicity of proteins (Aalberse, 1997). In particular with respect to B-cell epitopes, since carbohydrate structures may for an important part determine the secondary and tertiary structure of proteins and as such may strongly determine the conformational B-cell epitopes. The allergenicity not only differs between proteins from different food products, but also between proteins from one product. For instance, cow's milk contains proteins that only play a minor role in allergic reactions, while other milk proteins demonstrate strong allergenic properties. Proteins for which many patients are sensitized are often referred to as "major allergens". Of the world wide documented food allergies over 90% is caused by 8 foods or food groups viz. peanuts, milk, eggs, soybean, tree nuts, fish, crustacea, and wheat (Metcalfe, D. et.al., 1996).

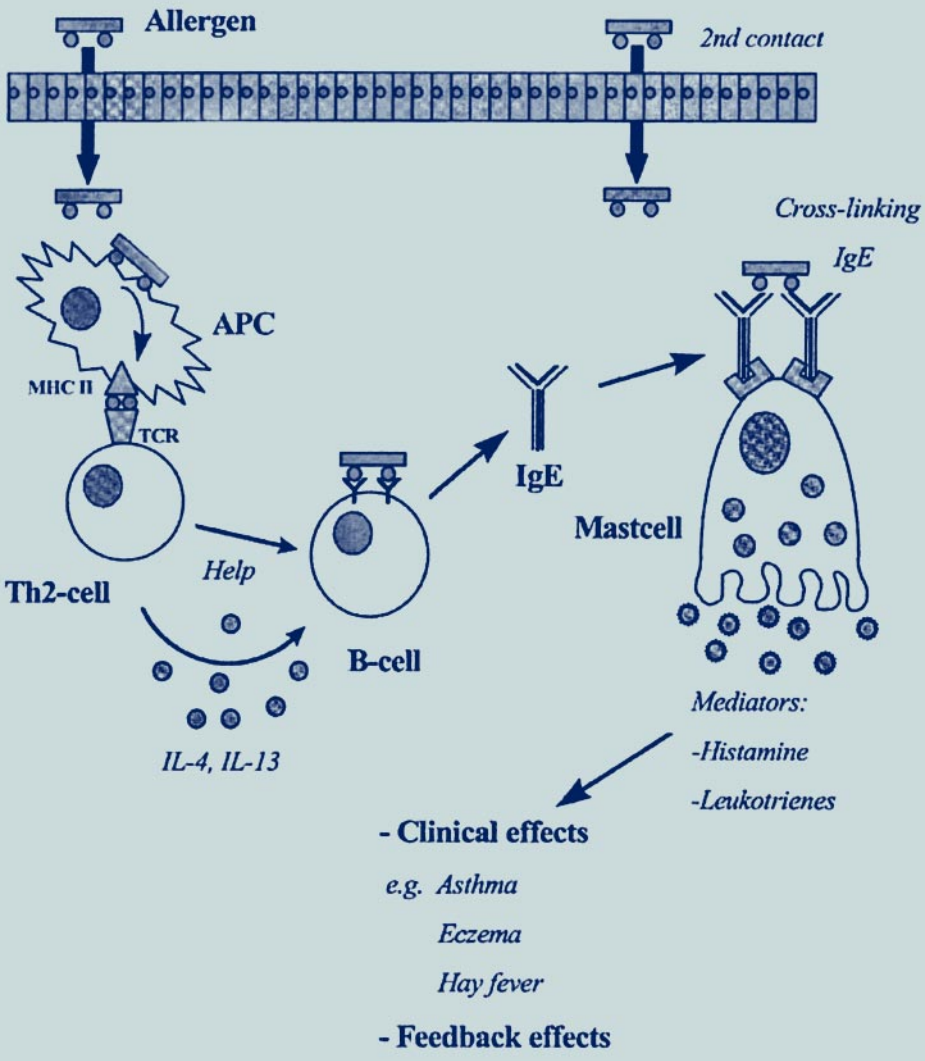



Fig 2: Schematic presentation of the mechanisms in *type I, IgE mediated*, hypersensitivity reactions

Legend to figure 2: APC = antigen presenting cell, MHC = major histocompatibility complex, TCR = T-cell receptor, Th2 = T-cell helper cell, B-cell = specific type of white blood cell, IL = interleukin

# 3. Food derived from genetically modified organisms and their possible impact with respect to food allergy

## 3.1 Aim of the changes

A large number of different plant species, including the most economically important crops, have been successfully genetically engineered in the past few years, but only a few have reached the marketplace. It is expected that in the coming years a further market introduction of these genetically engineered crops and food products derived from them will follow. Although the first products introduced were mainly technology driven with almost exclusively agronomical advantages, products with more consumer-oriented advantages, like foods with health improving ingredients, are in development now. Traits that are mainly used up to now are those introducing herbicide tolerance in different crops, introducing novel mechanisms for disease and pest control like viral, fungal and insect resistance and other possibilities that improve the quality of the product like delayed ripening and softening and changes in the content and type of proteins, (pro)vitamins contents, oils and starch (Day *et al.*, 1996).

## 3.2 Type of biotechnological modifications

The biotechnological techniques available nowadays can be used to inhibit the activity of genes (reduced production of proteins), to activate existing genes (initiate production of protein), to replicate existing genes (increased production of specific proteins), to change existing genes (changes in or elimination of proteins) or to introduce new genes from other species (introduction of species-foreign proteins). As any food that contains protein may have the potential to cause allergic reactions in some individuals, this also implies that the introduction of new proteins, any chance in the type of existing proteins, or their level in foods may affect the potential allergenicity of the derived foods. We should be aware, however, that plants contain millions of proteins of which only a few hundreds are known as allergens. Since only a limited number of traditional foods or food groups are known to cause allergies, there is from a scientific viewpoint no serious indication to expect that new transgenic foods will more frequently result in allergies. However, we should be careful as apart from nutritional and environmental aspects, these changes still may have toxicological and/or allergenic consequences that have to be considered in the safety assessment and approval procedure.

The genetically engineered food products marketed up to now have all been tested on the potential allergenicity of their proteins. They all passed successfully the currently recommended test strategy that mainly uses the source(s) of the transferred gene(s) as a starting point (see chapter 4). We should, however, be cautious and recognize that the discussions related to the adequacy of the currently used strategies to assess the potential allergenicity of transgenic crops are still ongoing. One example of a genetically engineered food crop that did not pass the test strategy is Cry9C maize (SAP, 2000). The fact that the Cry9C protein is not digested very rapidly has led the competent authorities to ask for additional information with regards to its potential allergenicity. In 1998 this maize was only allowed to be grown for feed and industrial purposes. But in 2000 it was shown that it had also ended up in products for human consumption, in particular taco shells. The likelihood that the Cry9C protein will cause an allergic reaction is still being discussed.

## 3.3 Relevance of type of biotechnological modifications for allergenicity

Factors that may affect the potential allergenicity of a new transgenic crop will largely depend on the introduction of, or changes in, gene(s) encoding for new or changed proteins. In case new genes are introduced the source of the gene is of ultimate importance. If the genes are derived from major or minor allergenic sources special attention should be paid to transfer of allergens from the allergenic source. In case the gene is transferred from a source with unknown history of safe use in food production one has to be cautious that the new protein may be a potential allergen.

Any change in the expression of a certain gene, the introduction of a new gene or the elimination of an existing gene may also affect the composition of other plant proteins or constituents. Random changes may occur in existing proteins, neighbouring genes at the site of integration of the foreign DNA may be turned off or turned on, and following point mutations in a gene new epitopes may become expressed on the translated protein. All these changes may affect the potential allergenicity of the commodities as result of the introduction of new allergens, the increase in the concentration of existing allergens or an increase in the concentration of a protein that was not recognized as an allergen before but reaches threshold levels of sensitization. However, the possibility of the occurrence of the indicated alterations is considered to be quite low. It should also be kept in mind that such alterations can be the result of conventional breeding methods. In addition, if accumulation of a protein occurs in a certain cell compartment, where it is better protected for digestion, it may subsequently still come in contact with the immune system and result in sensitization. Also the site of expression of the protein(s) in the plant (like in the whole plant or only in the seed, leaves, roots or pollen) will be of importance in respect to potential sensitization. The site of expression of the gene will not only affect possible exposure but may also affect possible sensitization either by the oral route in case the gene is expressed in the plant part that is eaten, or by inhalation if expressed in pollen.

By using genetic modification techniques it will also be possible to eliminate allergens from allergenic food sources. Normally, DNA transcription occurs only in one direction from the 5' to the 3' end along the molecule. If a coding sequence is inserted in the DNA in an inverted order, transcription occurs from the antisense strand of the DNA molecule. This will result in the production of an antisense form of mRNA. If this will bind to the sense form of mRNA this interaction may prevent normal levels of translation into an amino acid sequence. This may result in a greatly reduced level of gene product and an altered phenotype. This antisense RNA technique has been reported to be successfully used to reduce the amount of the main 16-kDa allergen in rice. Similar antisense approaches could be used to decrease the levels of other allergenic proteins in foods. Moreover, sense expression also works to lower the amount of allergenic proteins produced by a mechanism of messenger breakdown.

## 3.4 History of safe use

In the safety evaluation of GMO products the history of safe use concept often is used as a starting point. However, caution is needed when using this concept and the assessment should be focused on the whole of new circumstances. For instance it may be possible that in products of GMO origin, or after consumption of these products, new interactions occur between constituents that can be of major importance. Tomatoes contain tomatines that are considered to affect the permeability of the gastro-intestinal tract that subsequently may affect the absorption of (allergenic) proteins.

Glycosylation of proteins is also known to affect its allergenicity, as it increases both stability and solubility of proteins which are well recognized features of allergens. Although, most glycoproteins are not allergens, attention should still be paid to genes transferred from bacterial origin in plants as the protein expressed may become glycosylated in the eukaryotic cell. Moreover, glycosylation of plant proteins may be changed upon transfer to another plant. Differences in glycosylation are also an important drawback to the production in bacteria (for safety study purposes) of proteins intended to be introduced in eukaryotic systems.

The prevalence of allergic sensitivities to specific food varies from one country to another depending on the frequency and levels with which the food is eaten in that country at the typical age at its introduction in the diet. This has resulted in the current situation that shows for instance that in the USA peanut, in Japan rice and soybean and in Scandinavia codfish are the main food allergens. Keeping this in mind this also indicates that if a certain minor consumed allergenic food will become more attractive for consumption, the prevalence of its allergy may increase, which may become even more problematic if also the starting age of consumption changes.

# 4. Assessment of the Potential Allergenicity of (New) Food Proteins

## 4.1 Current strategies

There is no universal, reliable and relevant test for evaluation of the allergenic potency of food products and a case-by-case approach is required (Wal, 1999). For the assessment of the allergenic potential of foods derived from genetically engineered plants (GM-foods) one of the well known proposals is a careful step-wise process using a decision tree strategy as suggested by the IFBC/ILSI (Metcalfe *et al.*, 1996; Fig. 3) and which is also adopted by the OECD. The first critical point to consider in this assessment of potential allergenicity is the source from which the gene is derived.

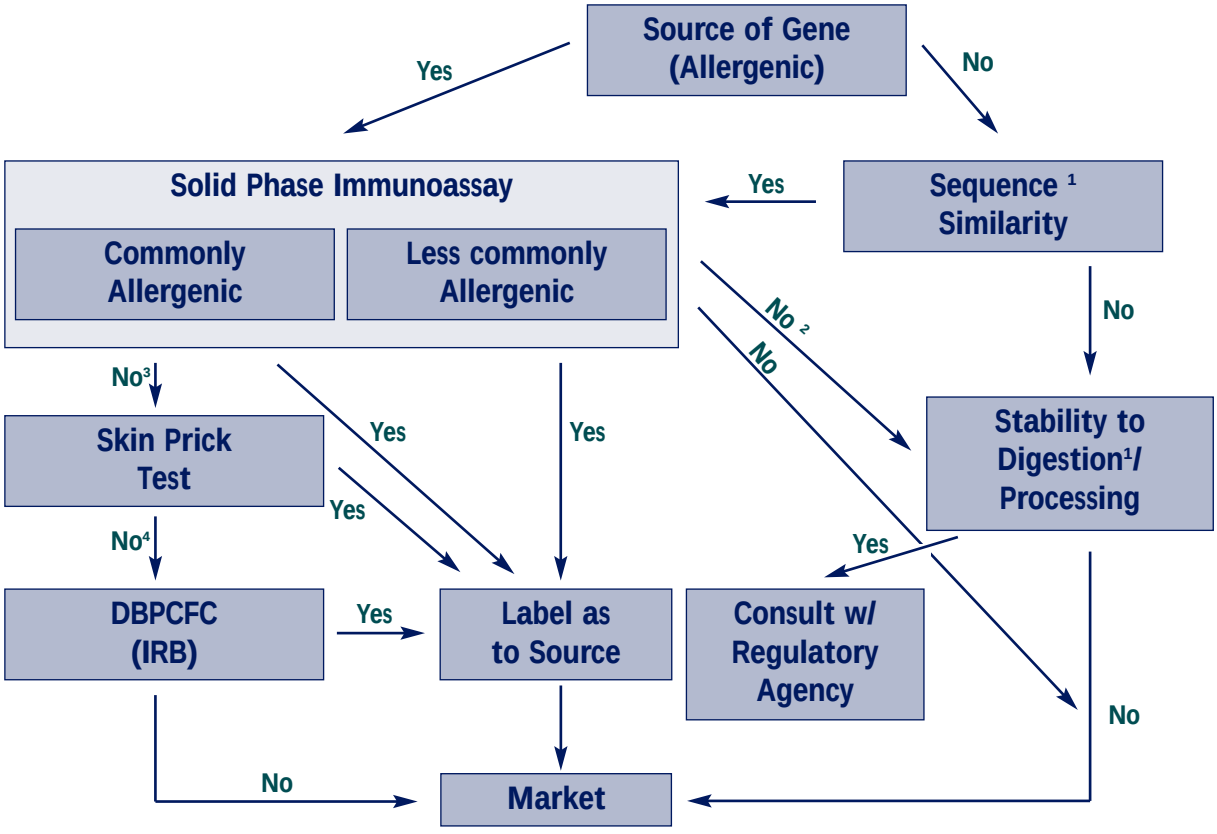



Figure 3. Assessment of the allergenic potential of foods derived from genetically engineered crops. Adapted from Metcalfe *et al.* (1996).

Recently a number of FAO/WHO expert consultations have resulted in the development of a revised decision tree strategy: as is shown in see fig. 4 (FAO/WHO, 2000 and 2001). A first important difference with the original IFBC/ILSI decision tree is that the endpoint of the assessment is no longer focussed on "whether or not to market with or witout labeling". In the new decision tree the endpoints give now an indication of the likelihood of the food being an allergen, ranging from 'likely allergenic' to 'low to high probability of allergenicity'. Other changes include the introduction of additional targeted serum screening and animal models in the assessment.

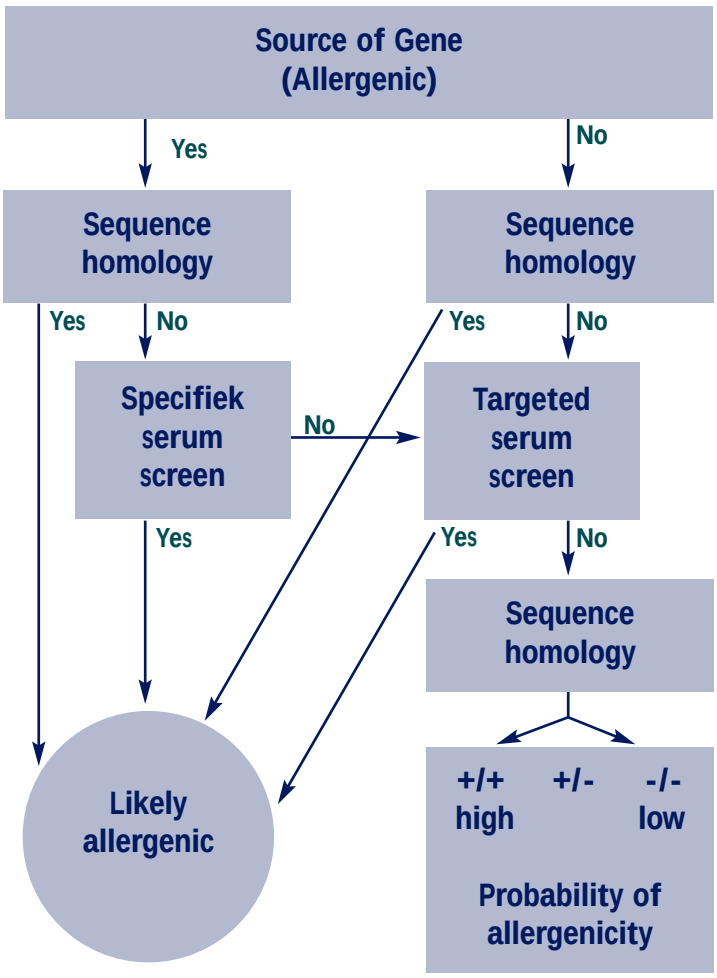



**Fig.4: Decision tree for the assessment of the allergenic potential of foods derived from biotechnology (FAO/WHO 2001)**

The adaptations made in the original IFBC/ILSI decision tree intend to meet the criticism related to certain of the criteria used. The major goal was the improvement of the predictive value of the outcome of the assessment, especially for the right hand arm of the decision tree, and the alterations reflect the latest knowledge and techniques. Although the original IFBC/ILSI decision tree was quite good in tracing known allergens (the left hand arm of the decision tree), the predictive value to assess the allergenic potential of new proteins or proteins that have never been eaten before was less good. However, it was generally recognized that although a negative result in this assessment was not conclusive it equalled a ´reasonable certainty of no evidence of allergenicity'.

In the following paragraphs the assessment strategies are discussed starting from the fact whether the source of the protein of interest is a known allergen or not.

### 4.1.1 The source of the protein of interest is a known food allergen

If a gene is obtained from a known allergenic source and the protein encoded is expressed in a food component of the new plant variety, data should be generated to assure that the gene does not code for an allergen (see fig. 3 and 4). In case the protein of interest shows sequence homology with a known allergen, it is considered that this protein is likely allergenic. The further in vivo testing in patients, viz. skin prick testing and oral challenges as reflected in the ILSI/IFBC decision tree, are not included anymore in the FAO/WHO 2001 decision tree. As these procedures will require approval from Ethics Committees they will not easily be approved in all countries, but can be considered in selected cases. Sequence homology is considered to be significant if a stretch of 6 or more amino acids is identical or when there is an overall amino acid sequence homology of at least 35%. Specific serum screens, using blood samples of patients known to be allergic to the source material, are used to verify the allergic status of the protein of interest. For this specific serum screening no distinction is made anymore between commonly and less commonly allergenic source materials in the revised FAO/WHO 2001 decision tree. If a protein of interest has no significant sequence homology with known allergens and the specific serum screens using blood samples from patients known to be allergic to the allergenic source are negative, further testing should follow the right hand arm of the decision tree starting with additional targeted serum screens followed by pepsine resistance and animal model testing (see fig. 4). Targeted serum screens should use serum samples from patients with high levels of IgE antibodies to the category of organisms to which the source of the protein of interest belongs like for instance derived from a monocot, dicot, a mould or an (in)vertebrate. This means that if a gene is derived from a mould, serum samples with high levels of IgE antibodies to moulds, yeasts and fungi should be used, etc. It is recommended to use 25 individual serum samples with high levels of IgE to the selected group of airborne allergens and (if applicable) 25 with IgE to the selected group of food allergens. The serum screens may include the various solid-phase immunoassays such as the RAST or RAST inhibition assay, the enzyme-linked immunosorbent assay (ELISA) or additional immunoblotting assays. If negative, assessment of pepsine-stability and animal models should follow.

### 4.1.2 The source of the protein of interest has no history of dietary exposure or an unknown history in terms of allergenicity

In many cases the source of the gene is not known to cause (food)allergies, either by having no history of dietary exposure, or by having an unknown history in terms of allergenicity. The evaluation of the allergenic potency of the molecule introduced or expressed in the food is then a vital but complex question. The first step is to perform a sequence homology search, and if this is negative possible cross-reactivity is tested in targeted serum screens, in which serum samples are used that contain high levels of IgE antibodies with a specificity to the gene source. If these serum screens are negative, additional analyses will have to be performed. The next steps proposed are tests to measure the ability of the protein to resist digestive breakdown (pepsin resistance) and testing of the protein in relevant animal models for antigenicity/allergenicity. If the results from these last tests are negative, the protein of interest has a very low probability of causing an allergic reaction.

## 4.2 Points of concern with regard to the proposed assessment strategies

**The IFBC/ILSI decision tree**

The right hand arm of the IFBC/ILSI decision tree is mainly based upon a number of general characteristics that most food allergens have in common. Important physical and chemical characteristics associated with food allergens are a molecular weight between 10 and 70 kD, heat stability, resistance to digestion, proteolysis and hydrolysis, water solubility, and stability to acids. Some food allergens only posses one or two of these characteristics, some possess many. But on the other hand there are also exemptions: some food allergenic proteins are smaller than 10 kD, some are quite heat labile, some are not very stable to digestion in the gut, not all allergens are water soluble. It can also be the other way around: some proteins that do possess one or more of these characteristics are not an allergen.

These facts make that both the positive and negative predictive value of the right hand arm of the decision tree are not as good as we perhaps would like them to be. Therefore, if the analysis of a certain protein using the right arm gives no indication of allergenicity, this is not a 100% certainty, but a *reasonable certainty of no evidence of allergenicity*. On the other hand, if the analysis using the right arm does result in the indication of any characteristic that is associated with allergenic proteins, then this does not prove that the protein will cause an allergic reaction. But it is in any case a trigger to perform additional analyses. These concerns have been the main driving force for updating and further improvement of the decision tree as now proposed by the FAO/WHO expert consultation (FAO/WHO 2001), with still no full assurance that the protein is or is not an allergen.

**Sequence homologies as an indication of allergenicity**

The determination of sequence homologies with known allergens is meant to quickly identify constructs that may pose a hazard. It is based on the principle that specific epitopes of allergic proteins interact with components of the immune system resulting in an allergic reaction. If a protein possesses the same epitope as a known allergen, then it might be possible that this protein interacts with the components of the immune system in the same way as the known allergen does. The presence of such homologies does not prove that the protein will cause allergic reactions, and therefore additional testing has to be done (see fig. 3 and 4). On the other hand, the absence of common or similar sequences does not strictly guarantee safety, because the available information in databanks is limited to a fraction of allergens. Moreover, these criteria are very restrictive and are limited to linear epitopes. Besides, sequence identity analysis requires a minimal number of contiguous identical amino acids to be considered indicative for an allergenic potential. This number has untill now referred to be eight. However, as demonstrated for example for Ara h 1, the major peanut allergen, its minimal epitope size was determined as 6 consecutive amino acids (Burks *et al.*, 1997). Moreover, cross-reactivity studies may serve as an example that also at the T-cell level stretches consisting of less than 8 identical amino acids can be immunologically relevant (Ferreira *et al.*, 1996). In addition, folding of the molecule could result in the formation of conformational epitopes (particularly relevant for B-cells and antibodies) which could mediate allergenic reactivity which will not be detected by comparison of sequence homologies with known allergens. This is why the FAO/WHO 2001 expert consultation now proposes to use two criteria instead of one, being: (1) an identical sequence of 6 contiguous amino acids, and (2) an overall amino acid sequence homology of 35%. When stating that failure to find a match of at least six identical contiguous amino acids or an overall amino acid sequence homology of 35% suggests that there is little

probability that the introduced protein could possess a shared epitope with known allergens, one has to be aware that at both the B-cell and T-cell level, relevant epitopes may still be missed. Therefore a negative outcome in the search for sequence similarities can never be used as the sole indicator of the absence of allergenicity, but always has to be considered in conjunction with other tests like stability to digestion and processing and animal testing.

**Immunochemical and in vivo assays**

Immunochemical analyses are meant to test in a direct manner whether a protein produced from a gene derived from a potential allergenic food indeed reacts with serum from patients allergic to the source material. These tests are to be used when the source of the gene is a known allergenic product or when sequence similarity with a known allergen has been found. The tests are based upon identification of an the interaction between the potential allergen and components of the immune system in serum (IgE antibodies), normally leading to an allergic reaction. Such an immunochemical analysis requires the use of sera of patients who have a well-documented allergy to the source material/allergen under investigation. In a specific serum screen according the ILSI/IFBC document (Metcalf et al, 1996), the use of 14 sera should enable detection of a major allergen (i.e. with which over 50% of the sensitive individuals react) with a probability greater than 99%, and detection of a minor allergen (with which 20% of the sensitive individuals react) with a probability greater than 95% (Metcalfe et al., 1996). However, the statistical power strongly decreases with lower response ratios for minor allergens (below 20% responders). As the possible responses to minor allergens may still be as severe as observed for major allergens, this strongly reduced statistical power remains a relevant concern. In the FAO/WHO 2001 decision tree the above mentioned figures have been adapted somewhat. Moreover, in this new decision tree no distinction is being made anymore between commonly and less commonly allergenic source materials with respect to specific serum testing. In case of a negative outcome a targeted serum screen in which the protein of interest is tested against serum samples from patients with high levels of IgE antibodies to the category of organisms to which the source of the protein of interest belongs. But in case the protein of interest is derived from for instance a bacterium, no general screen using targeted sera is currently available (FAO/WHO, 2001). In such a case serum screens are not possible and the assessment of potential allergenicity will rely on the outcome of subsequent pepsin resistance testing and the testing in validated animal models.

**Physico-chemical parameters**

The assessment of physico-chemical parameters is meant to determine whether a potential allergen for which no sera of allergic patients are available, possesses one or more of the physico-chemical characteristics of common allergens. Because not all known allergens possess these characteristics and some known non-allergens do possess such characteristics, the identification of such a characteristic is a trigger for performing additional analyses.

The first critical point in the assessment is the strict chemical identity of the foreign protein which is present in the food and of the test protein which is used for allergy assessment experiments. In GM-foods the foreign protein is generally expressed at relatively low levels and the amount of material that is necessary to perform the experiments precludes its extraction from the plant which is a practical problem. Therefore larger quantities of the foreign protein may be produced in genetically modified micro-organisms. However, this may result in slight structural differences of the protein (e.g. glycosylation) that may affect the outcome of the tests performed with this protein. Carbohydrate structures are known to determine or influence the allergenicity of proteins, especially with regard to B-cell epitopes (Aalberse, 1997).

Since for many food allergens a relative resistance to digestion was demonstrated (Taylor et al., 1992) it is

hypothesized that a protein that is largely stable to proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa thereby increasing the chance for sensitization. Besides resistance to digestion, relative stability of an allergen to conditions encountered during processing operations used for specific food products (e.g. heat denaturation) is also an important property of many food allergens. For these reasons, digestibility and stability during processing are considered when assessing the potential allergenicity of a protein introduced into a given food using an in vitro simulated gastric model. Applied to the main known food allergens, tests show that major allergens in general are stable for more than 8 times longer time periods in comparison to common food proteins with no history of allergenicity (Astwood et al., 1996). However, these criteria are pertinent but not absolute, particularly as the test conditions are harsh and do not correspond to the real conditions of digestion, like the influence of the food matrix and its composition on protein (allergen) digestion, or individual variability, distribution and regulation in pH values and of kinetics of secretion of proteolytic enzymes and release of products. Moreover, it is now well established that peptide fragments resulting from digestion conserve some of whole protein's allergenicity and the allergic reaction therefore not solely depends on a major fraction of the protein. In addition, digestive processes may increase allergenicity by unmasking epitopes or formation of new epitopes (Maynard et al., 1997). Stability testing during processing and digestion can supply useful information, but extended experiments are required, both for gaining more certainty of the potential of a protein to do harm, but also to eliminate false positives. This should be done by looking at the results of the assessment of the physico-chemical properties in conjunction with the results of other analyses such as sequence similarity with known allergic epitopes, and perhaps in the future by testing these proteins in validated animal models for allergenicity.

## 4.3 False negatives versus false positives

Especially the indirect test methods for potential allergens for which no sera of allergic patients are available can give rise to false negatives, but also to false positives. This is due to the fact that the indirect methods are based upon common characteristics of allergens to which there are exemptions to the rule.

With regard to the false negatives a number of concerns have been formulated in the paragraphs above. Similar considerations are valid for the possibility to come up with false positives. From a consumer health point-of-view especially the false negatives are a concern, from a business perspective both the false negatives *and* the false positives are a concern.

Like for the false negatives, for the false positives the only possibilities to limit the chances of this happening are to always consider results of one type of indirect analysis with the results of other types of analyses, in this way strengthening the burden of proof. The only other option is to try to develop new and better methods for the determination of the allergenicity of a certain food, that have less chances of false negatives or false positives. Any false negatives that have been introduced onto the market will eventually come to the surface, especially when the more stringent monitoring and tracebility requirements come into force as foreseen by the new European directive on the deliberate release of genetically engineered organisms. False positives will most probably not be allowed to be introduced on to the market, and this may in some cases prevent a producer, consumer or environmental benefit to be introduced.

## 4.4 Animal models in research on allergenicity of food proteins

Although still in development animal models have now been introduced in the assessment procedure proposed by the FAO/WHO 2001 expert consultation (see fig. 4) in an attempt to increase both the positive and the negative predictive value of the testing scheme for allergenicity of (new) proteins. As a basic preliminary experiment, immunogenicity of new food proteins could be tested by immunizing animals and animal antisera

could be applied to identify immunoreactive peptides in digests. But also for these experiments one would have to be aware of false negative or positive results. Validated animal models from which the results can be extrapolated to humans are, however, not yet available. Still much research is devoted in developing suitable animal models that mimic the antibody response, or even the symptoms, observed in human allergic patients. In the past several oral sensitization assays have been developed mainly in guinea pigs (Piacentini et al., 1994), and nowadays more in mice (Ito et al., 1997, Li et al., 1999) or rats (Atkinson et al., 1994, Knippels et al., 1998, 1999a, 1999b). -It was recently shown that upon oral exposure of rats to different food proteins, without the use of an adjuvant, the induced immune responses demonstrated a high correlation with those established in food allergic patients (Knippels et al., 2000). Moreover, in the same model it was shown that an oral challenge in previously orally sensitized animals induced clinical effects comparable to those seen in food allergic patients (Knippels et al., 1999c). Altogether, these findings suggest that this rat model might provide a suitable animal model to study the allergenicity of food proteins although further validation of the model is needed and underway. Other models in development include intraperitoneal administration in murine models (Dearman et al, 2000). The FAO/WHO 2001 expert consultation is of the opinion that these models in development can provide informative data, but validation should be critically followed.

## 4.5 Levels of allergens in food

In addition to the factors mentioned above, the amount of protein present in a food is considered to be another important factor to be related with allergenicity. Known main food allergens represent an important fraction of the food's composition. Therefore, it has been proposed that if the amount of novel foreign protein is below a threshold concentration of 1% of the total protein content this should not trigger a concern for allergenicity. However, for some food allergens it is known that the main allergens represent less than 1% of the total protein content of the food indicating that this assumption is not always true. It has to be mentioned also that stimulation of T-cells by low dose and not high dose of an allergen is required for the switch towards an IgE response (Björksten *et al.*, 1996). Moreover, the amount of protein which is ingested not only depends on the levels of these proteins in products but also on consumption patterns, frequency of consumption, and the age of the consumer. Altogether these points indicate that expression levels or abundance of proteins in foods should be re-evaluated as a criterion for allergenicity.

## 4.6 Conclusion

The methods and strategies currently used or proposed to be applied in the assessment of the possible allergenicity of food proteins may be expected to have an acceptable positive predictive value. However, as substantiated in the previous sections, especially with the IFBC/ILSI decision tree some concern remains regarding both the negative and the positive predictive value. Future experience will have to show whether the alterations to the decision tree as proposed by the FAO/WHO 2001 expert consultation actually will lead to an improvement of especially the negative predictive value. Especially experience with the use of the proposed animal models will determine whether this actually will be achieved. An important advantage of animal models may turn out to be the possibility to obtain information on the potential allergenicity of proteins in addition to information on its potency relative to well-known allergens. In addition post marketing surveillance programs may provide an important tool in establishing whether foods from GMO origin are well tolerated and whether the assessment strategies are adequate. However, the feasibility of certain aspects of its implementation still needs further investigation and its implementation is recommended to be studied further (FAO/WHO, 2001).

# 5. Case Studies

## 5.1 Methionine enriched soybeans

Due to a relative deficiency of methionine in the protein fraction of the seeds of various legumes, including soybeans, their nutritional quality for both humans and animals is compromised. To ensure adequate intake of methionine, domestic animals fed with diets based on soybean meal have to be fortified with methionine or protein sources that contain this essential amino acid. Human vegetarians also need to balance their diets to ensure an adequate intake of methionine. By means of traditional plant breeding techniques several attempts were performed in order to modify the balance of essential amino acids. However, the improvements in nutritional quality achieved were often associated with reduced agronomic properties such as yield and grain quality. In order to improve the methionine content of soybean, the introduction of genes encoding sulfur-rich proteins from other plants was considered as a promising strategy for improving its nutritional quality without adversely affecting the agronomical performance. As the 2S albumin from the seeds of the Brazil nut contains 18 % of methionine and 8 % of the other sulfur-containing amino acid cysteine, it was considered an ideal protein for gene-transfer.

Since it was known that Brazil nut causes anaphylactic reactions in a small number of individuals (Gillespie *et al.*, 1976; Arhad *et al.*, 1991), the potential allergenicity of the transgenic soybean was subsequently assessed using the decision tree approach developed by the IFBC/ILSI (fig.3). Sera of allergic patients were collected for performance of some appropriate *in vitro* tests. The ability of proteins from transgenic and non-transgenic soybeans, from Brazil nuts, and purified 2S albumin to bind to IgE in the serum from Brazil nuts allergic patients was determined by a Radio Allergo Sorbent test (RAST). SDS-PAGE gel electrophoresis and immunoblotting with autoradiography was used as an additional *in vitro* test to demonstrate possible interaction of serum IgE from Brazil nut allergic patients with the various protein extracts. A positive RAST was observed with pooled sera of 4 Brazil nut sensitive individuals and on immunoblotting, serum IgE from 8 of 9 subjects bound to purified 2S albumin from the Brazil nut and to proteins of similar molecular weight in the Brazil nut and the transgenic soybean (Nordlee *et al.*, 1996). Also Skin Prick Testing was performed on 3 Brazil nut allergic patients which all had positive reactions to extracts of Brazil nut and transgenic soybean and showed negative reactions to soybean extract. These results clearly showed that the gene obtained from Brazil nut probably encoded for a Brazil nut allergen. Moreover, the obtained results demonstrate the value and effectiveness of the *in vitro* assays and *in vivo* assays in the left-hand arm of the IFBC/ILSI decision tree (see figure 3) to identify the transfer of an allergenic protein from a known allergenic species by genetic engineering (positive predictive value). As Brazil nut was a known allergenic food, the potential allergenicity of the 2S albumin was also assessed in an oral feeding study with mice using a Passive Cutaneous Anaphylaxis assay (PCA) as a read out system (Melo *et al.*, 1994). This study reported that the 2S albumin protein did not elicit an IgE response in the mouse strains used under specific conditions. Since it is known that oral antigen exposure to mice most easily results in tolerance induction (Ngan and Kind, 1978; Mowat, 1987; Stokes *et al.*, 1983; Melamed and Friedman, 1994; Friedman *et al.*, 1994), the mouse was possibly not the most appropriate animal model. Although speculative, a more appropriate animal model, like possibly the described BN rat model (Knippels *et al.*, 1998), might have predicted the allergenicity more accurately. Because the producer

was afraid that the Brazil nut 2S albumine enriched soybean intended for animal feed only might be mixed up with soybean intended for human consumption, the product was not further developed. For the same reason the regulatory authorities would most probably not have allowed the product to be marketed.

## 5.2 Glyphosate-tolerant soybeans

Glyphosate (N-phosphonomethyl-glycine) is the active ingredient in the popular non-selective broad-spectrum herbicide Roundup. However, the sensitivity of crops to glyphosate prevented the in-season use of this herbicide over-the-top on crops. This could be overcome by the advances achieved in plant biotechnology that made it possible to insert a gene into soybeans to provide tolerance specifically to the non-selective herbicide. The mechanism of glyphosate tolerance in glyphosate tolerant soybean (GTS) is based on the fact that the only physiological target of glyphosate is an enzyme, viz. 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS), involved in the synthesis of aromatic amino acids essential for the protein synthesis of plants. This EPSPS is present in all plants, bacteria, and fungi, but not in animals as they do not make their own aromatic amino acids and receive them from plant, microbial, or animal foods. By introducing a glyphosate-tolerant EPSPS enzyme in soybean the GTS plant remains unaffected upon treatment with glyphosate, as it will still be able to fulfill the plant's need for aromatic amino acids. The EPSPS enzyme from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) was selected from a screen of microorganisms as having the most favorable glyphosate tolerance kinetic parameters. The gene encoding for this CP4 EPSPS was finally transferred into soybean in order to confer glyphosate tolerance.

As there are up to now no predictive assays available to clearly assess the allergic potential of (new) proteins from a source with no history of allergenicity several, mainly physico-chemical parameters of the new protein were assessed and compared with those of well known allergic proteins. Therefore, as is also depicted in the right-hand arm of the IFBC/ILSI decision tree approach (see figure 3), the possible amino acid homology with known protein allergens, the process- and digestive stability, the molecular weight, the glycosylation and the level of the CP4 EPSP synthase protein were the main characteristics studied to assess the allergenic potential of GTS (Harrison *et al.*, 1996). Using the current main protein databases (GenBank, PIR protein, and SwissProt), CP4 EPSPS did not show significant homology to any known protein allergen. Furthermore, it was found that although the molecular weight of CP4 EPSPS of 47.6 kDa fits with the mass criterion of 10-70 kDa of most food allergens, as in fact do most proteins, it did not possess any of the other characteristics common to protein allergens. Moreover, after processing and toasting procedures CP4 EPSPS was found to be heat labile (Padgette *et al.*, 1996) and upon using simulated gastric- and intestinal fluids, CP4 EPSPS was found to be easily degraded by both acidity and proteolytic activity. When purified from GTS seed, CP4 EPSPS was also found not to be glycosylated, which is in contrast with most allergenic proteins. In addition, unlike most allergenic foods, in which allergens are frequently present in major amounts, CP4 EPSPS is present only in minor amounts in the GTS. Levels of CP4 EPSPS protein in GTS are approximately 0.08% of the total protein content or 0.03% of the fresh weight. Because of the low level of expression this protein will be less likely able to sensitize. However, although a low concentration of the transferred protein may be of importance with respect to its allergic potential it cannot be a conclusive criterion, as based on consumption patterns the expected daily intake may still result in considerable exposure levels reaching threshold levels for sensitization. As soybeans are well known allergenic food products it was also studied whether the genetic transfer of the CP4 EPSPS itself affected qualitatively and/or quantitatively the endogenous allergens in soybean (Burks *et al.*, 1995). This effect was studied by preparation of protein extracts from soy flour derived from two GTS lines, a parental variety and three commercially available classical soy preparations. On the

Coomassie blue-stained SDS-PAGE gels the separated protein bands of the glyphosate-tolerant varieties, the parental variety and the three commercial soybean preparations were comparable. Upon blotting of these SDS-PAGE gels onto nitrocellulose membranes the specific detection of the allergenic proteins of the different soy varieties was assessed using a serum pool containing soybean-specific IgE antibodies of 5 soy allergic patients. Both the presence and the relative levels (cannot be adequately assessed by this technique) of the endogenous allergenic proteins in all of these soybean preparations were comparable, demonstrating that the endogenous allergenic proteins were not altered in the glyphosate-tolerant soy. Based on the above described results, obtained with the methodologies available today, it was concluded that there is no reason up to now to believe that the transferred CP4 EPSPS protein in soy will pose a significant allergenic risk. However, it goes without saying that upon the introduction of such a new crop a post marketing surveillance would be of value in order to confirm and validate the safety evaluation-strategy used. Until now, after some 4 years of large scale application, no cases of allergenicity to glyphosate-tolerant soybeans have been reported.

## 5.3 Transgenic hypo-allergenic rice

The prevalence of allergic sensitivities to specific foods varies from one country to another depending on the frequency and amounts by which the food is eaten in that country. Therefore, food allergy to rice appears to be rare in Western countries but is more common in Asian countries like Japan, where people experience more rice allergies than in other cultures. From studies of Matsuda *et al*. (1988) it was concluded that a 16 kDa globuline protein, tentatively designated RP16 kD, was the major rice allergenic protein as was tested with IgE containing sera of rice allergic patients. There are, however, two other major allergens a 15.5-kDa and a 19-kDa protein. In several ways one has screened for or tried to produce rice with reduced allergenicity. Using antibodies raised to this 16 kDa allergenic protein approximately 150 rice strains were screened for the content of this protein (Adachi *et al*., 1995). It was observed that all of the screened Japanese cultivars contained nearly the same amount of this protein, whereas some cultivars and wild-type species from other Asian countries contained little or no detectable levels of this protein. Some of these cultivars might be useful for rice-allergic patients in Japan as substitutes for japonica rice, although no or low allergenicity still has to be confirmed by clinical tests. As the wild-type species have found to contain poor endosperm this might hamper their eating quality and therefore improvement of the seed protein of these wild-type rice species will be needed. To reduce the allergenic protein content of rice the use of proteolytic enzymes was established by Watenabe *et al*. (1990). Although this hypo-allergenic rice may be an improvement for rice-allergic patients its price is rather high owing to the use of expensive proteolytic enzymes. Finally, Tada *et al*. (1996) made genetically modified rice plants by using the antisense gene strategy to reduce the 14-16 kDa allergenic proteins after the amino-acid sequence of the 16 kDa protein and its cDNA was cloned (Nakamura and Matsuda, 1996). A 5 fold reduction of the allergenic protein content in the seeds the second generation of these genetically modified rice plants was assessed by immunoblot analysis and by competitive ELISA's using monoclonal antibodies.

From these studies it is clear that the antisense RNA method can effectively repress the allergen synthesis in rice. However, it is still uncertain whether the hypo-allergenic rice seeds obtained from the genetically modified rice plants will be tolerated by rice allergenic patients, as threshold values for allergic reactions in rice allergic patients are not known and even very small amounts of residual allergens may elicit an allergic reaction in patients. Moreover, not all rice allergic patients will react to the same allergens and therefore some patients may be allergic to some other proteins of rice than the reduced 16 kDa proteins in the genetically modified rice. Further studies are needed in particular to assess the efficacy of the hypo-allergenic genetically modified rice in patients.

## 5.4 Conventional kiwi

For natural food products like kiwi it was not required to study their potential allergenicity. The introduction of kiwi in the Western society has resulted in the development of kiwi-allergic patients that did not exist before. Extensive research has shown now that kiwi contains several allergens, of which a 30 kDa protein is the major allergen (Pastorello *et al.*, 1996; Voitenko *et al.*, 1997). Moreover, these studies have also demonstrated that kiwi-fruit shows cross-reactivity with birch-pollen allergens. If kiwi-fruit was introduced nowadays it probably would have been considered a novel food. This would have meant that the kiwi-fruit would have to be tested according to the current guidelines on novel food before marketing. According to the EU novel food recommendations attention should have been given than to allergenicity aspects, although it is not indicated how and which tests are recommended. It can only be speculated what would have happened. But if the kiwi-fruit allergens possess some of the characteristics of common food allergens, like resistance to digestion, the kiwi-fruit would have become suspect. And the question is then whether one would have allowed the kiwi-fruit to be put on the market.

## 5.5 Conclusion

The cases above show that both genetic engineering and conventional breeding can alter the allergenicity of foods, making them either less or more allergic. It also illustrates that the current strategy to test allergenicity of genetically engineered foods is able to successfully trace allergens in GM crops from known allergenic sources. In the case of proteins not formally present in food (the CP4 EPSPS) the total of the negative results of the indirect test methods sums up to a reasonable certainty of no harm. When a novel food is considered to pose a risk in terms of allergenicity it is very unlikely to enter the market, either by a decision of the company or institution not to develop this novel food any further, or by a decision of a regulatory authority not to allow the novel food to be marketed. This is not the case for new varieties of crops obtained through conventional breeding methods that are not considered to be a novel food. For these types of crops no formal allergenicity testing is required.

# 6. From Assessment of Potential Allergenicity towards Allergy Risk Assessment

With respect to the pre-marketing assessment of food safety, current effort in the field of allergenicity is almost exclusively put into the development of tools for and research on the establishment of the potential allergenicity (the ability to sensitize and/or to evoke allergy symptoms in sensitized individuals) of substances: the hazard identification. In chapter 4 this hazard identification and some of its problems are described. Also the frequently used IFBC/ILSI decision tree is only dedicated to hazard identification, and so does the FAO/WHO 2001 proposed approach. However, the identification of a hazard (i.e., with respect to allergenicity, the recognition that a substance may act as an allergen) does not necessarily imply the identification of an unacceptable risk. Once a substance is identified as a potential allergen, an intrinsic property of the substance is established. The allergenicity may however, in practice, become manifest only under specific or exceptional circumstances, e.g. after injection or inhalation of the substance or upon repeated high dose consumption. Under normal circumstances, the risk for sensitization or induction of allergy symptoms may be very (and perhaps acceptably) low. Therefore, knowledge on the potential allergenicity not always provides sufficient information for sound decision making and we should go more towards a risk assessment and risk management like approach of food allergenicity. To allow a science-based decision making based on acceptability of (minor) risks, additional information is needed to interpret the hazard identification results in a proper perspective. In this chapter, allergenicity of food proteins is discussed in a perspective of toxicological risk assessment. It should be noted that when discussing risks of allergens, distinction needs to be made between risks for sensitization (of relevance in terms of prevalence of allergies) and risks for effects elicitation (of relevance in terms of incidences of occurrence of possible adverse health effects). It should also be realised that the considerations in this chapter concerning the allergy risk assessment do not only count for foods resulting from genetic engineering, they are relevant for *any* food containing new proteins, even if obtained through classical breeding.

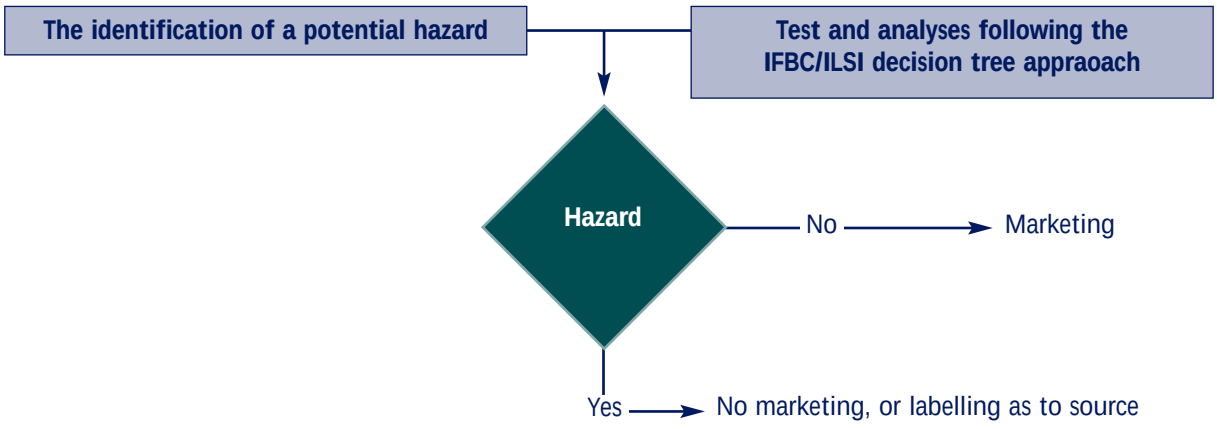



**Figure 5: The assessment approach used until now with regard to food allergy risk assessment**

## 6.1 Hazard Characterization: the allergenic potency

Following the hazard identification for a substance (i.e. the identification of the intrinsic property of a substance to cause harm; see for allergenicity testing a.o. chapter 4 of this paper, Houben et al., 1997, and Metcalfe *et al.*, 1996), information on the potency to cause such harm (the allergenic potency for allergens) is of major relevance. It is well known that some (glyco)proteins have a strong potential to cause sensitization and health complaints, while others only relatively infrequently, seldom, or virtually never cause sensitization or health effects, even in individuals with a predisposition to develop IgE mediated allergies (the atopic constitution) (see a.o. FAO, 1995). With respect to the effect elicitation phase, it should further be recognized that some allergens rather frequently induce severe allergy reactions, including life threatening effects, while others predominantly induce less severe effects (Bruijnzeel-Koomen *et al.*, 1995). To characterise the allergenic hazard of a substance, it is of major importance to find out whether or not the substance under consideration possesses characteristics that contribute to the allergenic potency and whether it therefore may be expected to behave like a major and/or dangerous allergen or like a minor or virtually non-allergen.

At present, we do not have sufficient knowledge and validated tools for the establishment of the allergenic potency of a food protein as an absolute measure. The IFBC/ILSI or FAO/WHO 2001 decision tree approaches, for instance, may give an indication on the potential to cause an allergic reaction, but not how severe such a reaction would be. However, efforts are being made to generate knowledge and to develop tools to establish, at pre-marketing stages, the allergenic potency of intended new food proteins relative to the (more or less known) allergenic potencies of existing food proteins (see a.o. Kimber *et al.*, 1999). With respect to the sensitization phase, some of the research methods described and discussed in Chapter 4 of this paper, particularly the digestion and processing stability testing methods and animal models, are the starting point for most of these efforts. Studies indicated that major allergens in general tend to be rather stable to processing and digestion (see a.o. Metcalfe *et al.*, 1996). Furthermore, recent results obtained with an animal model developed by Knippels *et al.* (2000) demonstrated a high correlation between immune responses to various food proteins as established in the model and those established in food allergy patients. Combinations of such model approaches together with epidemiological knowledge may result in applicable methods and strategies to predict whether a certain protein may have a sensitizing potency comparable to a known strong sensitizing allergen or to proteins that less frequently or virtually never cause sensitization. However, further validation programs are needed to establish the predictive value of such approaches. Studies on sensitization in humans will of course be possible, yet only to a limited extend, in controlled prospective trials in some cases or in retrospect (post-marketing surveillance). Besides, post-marketing surveillance programs may contribute to a further validation and refinement of model-based hazard assessment strategies, although many practical limitations will hamper the feasibility of post-marketing programs.

Although with respect to GM foods the whole hazard assessment strategy must be focussed primarily on prevention of sensitization and not on the effect elicitation phase, it is of importance for risk evaluation and management for known allergens (and in the future new allergens) to improve our knowledge on the potency of the allergen in the effect elicitation phase.

With respect to the effect elicitation phase, pre-marketing assessment of the allergenic potency of intended new food proteins is troublesome. Successful attempts have been made to induce clinical effects as noted in allergy patients also in sensitized rats upon oral application of known food proteins (Knippels *et al.*, 1999). However, a high inter- and intra-species variability as well as a high time- and circumstances-determined variability within patients may be expected for effect elicitation. Moreover, a further variability may be expected because the clinical symptoms may vary between patients and even within patients from day-to-day

or through the years. Therefore, a protein-specific pre-marketing assessment of the potency in effect elicitation seems hardly possible. Generation of data on trigger thresholds for humans sensitized for existing food allergens would be a most practical alternative approach to give input for improved knowledge on risk assessment. These efforts may be focussed on generating information on trigger thresholds in general (distributions for various allergens) or, from a safety point of view preferably, on trigger thresholds for worst case known allergens (i.e. distributions for allergens that are known to induce effects at rather low doses). Such distributions may in theory be established per type of clinical response or clinical response pattern. Some studies on trigger thresholds have been reported in recent years (e.g. Hourihane *et al.*, 1997; Moneret-Vautrin *et al.*, 1998). Further information on exposure levels at which patients start to react to food allergens (and thus also on exposure levels at which no effects have been noted) will be of major importance in the assessment of risks for allergen exposure.

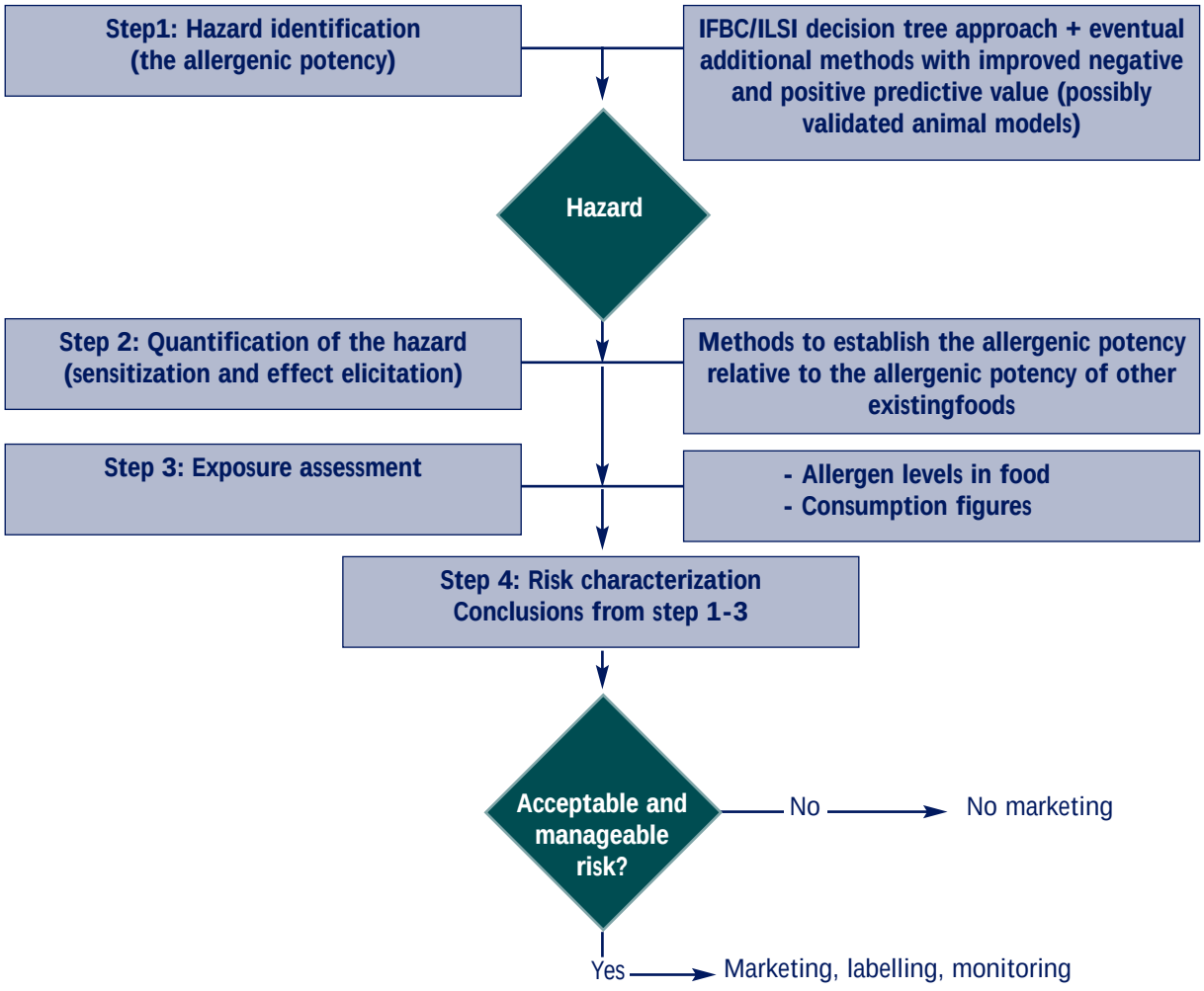



**Figure 6: Proposed food allergy risk assessment approach**

## 6.2 Exposure Assessment

The ultimate risk of an allergen is not determined by its allergenic potency as such but by the allergenic potency in sensitization and, particularly, effect elicitation together with the susceptibility of exposed groups, the levels of exposure, the exposure patterns, and the conditions of exposure. To give further input for an assessment of risks, information on these issues needs to be available and evaluated. This assessment would

need to include at least studies on and an evaluation of possible allergen levels in the final food products and applicable consumption figures.

## 6.3 Risk Characterization

When discussing risks of allergens, distinction needs to be made between risks for sensitization (of relevance in terms of prevalences of allergies) and risks for effect elicitation (of relevance in terms of incidences of occurrence of possible adverse health effects). At present, insufficient knowledge and tools are available for a reliable and full characterization of risks of allergen exposure. Additional knowledge and validated tools and strategies need to be generated in future to allow a quantitative risk assessment. Information on the (relative) sensitizing potency of substances together with information on the target consumers populations, exposure levels, patterns, and conditions, and epidemiological knowledge may ultimately provide the most important input for a (quantitative) assessment of the risk for sensitization and thus the prevalence of sensitization to be expected. The risk for adverse health effects and possible incidences of adverse health effects may be assessed on the basis of the expected or established prevalence of sensitization for the substance under consideration or cross-reacting allergens, the potency in inducing effects on challenge, the types of food products involved, the allergen levels in these food products, and the consumption figures. When applicable, the risks or incidences may be assessed or estimated per type of effect (distinction based on nature and severity of the effect).

At present, several research groups are putting effort in generating knowledge and tools that may in future give input for risk assessment approaches for allergen exposure. However, even if only part of the ideally needed information is available, conclusions regarding health risks may be possible to a certain extend. For instance, when reliable data become available to estimate a lowest trigger threshold or to calculate trigger threshold distributions for groups of patients and groups of allergens, conclusions on possible risks of low concentration levels of allergens in food products may be feasible.

## 6.4 Decision making and risk management

The risk assessment process should ideally give an indication or estimate of the probability that a person becomes sensitized for a food protein (or is already sensitized due to previous exposure to the protein or to cross-reacting allergens) and subsequently eats such a combination and/or such amounts of food products containing the allergen under consideration at such levels and under such circumstances that an adverse event (of a certain type) should be expected to occur. A science-based decision making based on acceptability of risks could than be based on the expected probability or incidence and severity of adverse events and the possibilities for monitoring and adequately managing the risks, taking into account possible benefits of the GMO and/or products thereof. Quality and hygiene programs such as HACCP or GMP approaches, analytical monitoring programs, and labelling may provide important tools for managing possible risks (see a.o. Deibel et al.,1997). Risks assessors may thus in future, but also at present, contribute to a quantitative characterization of the risks and to an estimation of the probability/likely incidence of occurrence of adverse events (either or not specified for types of effects) or the evaluation of risks in a perspective to risks of known (existing) food allergens. Furthermore, risk assessors may contribute to the evaluation of monitoring and risk assessment programs. However, for a final decision making, positions have to be taken regarding the acceptability of risks of allergens. Of course, there is an important role for (self) regulatory bodies in the establishment of standards in this respect.

# 7. Conclusion

Food allergenicity is a serious problem with a prevalence of 1.5-5% in the general population. Within this population reactions can vary greatly from very minor to severe health effects. Food allergies are caused by the interaction of proteins present in food with components of the immune system. Factors that play a role in the severity of the effects are the type of allergen, the constitution of the person involved, and the circumstances of the consumption.

Any new protein that is introduced into the food chain is a potential new allergen. However, from the experience with current foods the chances of such a new protein actually being an allergen is very small. Only a minor fraction of the proteins in food appears to cause allergic reaction in practice, the vast majority does not. Most of the major food allergens have a number of characteristics in common, like a molecular weight between 10 and 70 kD, heat stability, resistance to digestion, proteolysis and hydrolysis, water solubility, and stability to acids, but there are quite some exemptions to the rule.

Genetic engineering is one method that may result in the introduction of proteins that have never been consumed before. Genetic engineering may also result in the transfer of genes coding for an allergen from one food to another. Also, random changes resulting from genetic engineering may occur leading to changes in existing proteins. On the other hand genetic engineering may also result in knock out of known allergens from foods. The possibilities for introducing changes to foods or food crops through genetic engineering are major. This is why introductions of changes to foods with this technique have to be assessed carefully with regard to possible changes of allergenicity. From a scientific point of view any other technique leading to the introduction of new proteins into foods, or leading to changes in existing proteins should also be assessed with the same care.

The current assessment procedure for determining the potential allergenicity of (new) proteins or (new) protein containing foods is mainly based on the IFBC/ISLI decision tree approach (see chapter 4) that takes the source of the genes as the major starting point. Alterations to this decision tree have very recently been proposed by a FAO/WHO 2001 expert consultation with the goal of improving the negative predictive value of the right hand side of the IFBC/ILSI decision tree. In these approaches different immunochemical, in vivo, and physico-chemical analyses are used to assess the potential allergenicity. In the proposal of the FAO/WHO 2001 expert consultation targeted serum screens and animal models have been included as additional steps. What tests are actually performed depends on the source of the genes involved and the results of particular tests. Both approaches have a rather good positive predictive value in case of (known) allergens for which sera of allergic patients exist. Its positive and negative predictive value in case of potential allergens for which no sera of allergic patients exist is less good, but even for the IFBC/ILSI approach it is generally considered to result in a reasonable certainty of no evidence of allergenicity. The approach proposed by the FAO/WHO 2001 expert consultation should be an improvement, but this will depend on the actual performance of the proposed

animal models. It should be recognised that these models are still in development and further validation is needed.

A concern with the current approaches is that it they are focused mainly on the allergenic potency. They do only in a minimal sense take into account that there are differences between minor and major allergens, in some cases there even do not have to be relevant risks on health effects. Also, even though it may be found that a certain protein has an allergic potency, this does not have to result in the sensitization of consumers as a result of too low concentrations in the food or factors in the food processing. From the current approach mainly based on hazard identification it is therefore proposed to go towards a real risk assessment and risk management approach. In such an approach data on the allergenic potency relative to known allergenic potencies of existing allergens, and on the expected exposure would be included. In such an approach perhaps a distinction could be made between minor and manageable risks and major risks that could better be avoided.

# 8. References


Aalberse R.C. Food Allergens. Env. Toxicol. Pharmacol, 1997, 4:55.

Adashi T., Alvarex A., Aoki N., Nakamura R., Garcia V., Matsuda T. Screening of rice strains deficient in 16-kDa allergenic protein. Biosci. Biotechnol. Biochem., 1995, 59:1377.

Arhad S. H., Malmberg E., Kraft K., Hide D. W. Clinical and immunological characteristics of Brazil nut allergy. Clin. Exp. Allergy, 1991, 21:373.

Astwood J. D., Leach J.N., Fuchs R. L. Stability of food allergens to digestion in vitro. Nat. Biotechnol., 1996, 14:1269.

Atkinson H. A. C., Johnson I. T., Gee J. M., Grigoriadou F., Miller K. Brown Norway rat model of food allergy: Effect of plant com-po-nents on the develop-ment of oral sensi-ti-za-tion. Fd. Chem. Toxic., 1996, 34:27.

Björksten B., Holt B. J., Baron-Hay M. J., Munir A.K., Holt P. Low-level exposure to houst dust mites stimulates T-cell responses during early childhood independent of atopy. Clin. Exp. Allergy, 1996, 26:775.

Bruijnzeel-Koomen C., Ortolani C., Aaas K., Bindslev-Jensen C., Bjorksten B., Moneret-Vautrin D.A., Wuthrich B. Adverse reactions to food. Allergy, 1995, 50:623.

Burks A. W. and Fuchs R. L. Assessment of the endogenous allergens in glyphosate-tolerant and commercial soybean varieties. J. Allergy Clin. Immunol., 1995, 96:1008.

Burks A. W., Shin D., Cockrell G., Stanley J. S., Helm R. M., Bannon G. A. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and major alergen in peanut hypersensitivity. Eur. J. Biochem., 1997, 245:334.

Day P. R. Genetic modification of proteins in food. Crit. Rev. Fd . Sci and Nutrition, 1996, 36 (S): S49.
Deibel K., Trautman T., DeBoom T., Sveum W. H., Dunaif G., Scott V. N., Bernard D. T. A Comprehensive approach to reducing the risk of allergens in foods. J. Fd. Protection, 1997, 60:436.

Dearman R.J., et al. Divergent antibody isotope responses induced in mice by systemic exposure to proteins: a comparison of ovalbumin with bovine serum albumin. *Food Chem Toxicol*, 2000, 38: 351-360

De Jong E. C., Van Zijverden M., Spanhaak S., Koppelman S. J., Pellegrom H., Penninks A. H. Identification and partial characterization of multiple major allergens in peanut proteins. Clin. Exp. Allergy, 1998, 28:743.

FAO: Report of the FAO Technical Consultation on Food Allergies, Food and Agricultural Organization of the United Nations, Rome, Italy, 13-14 November, 1995.

FAO/WHO Expert Consultation on “Safety aspects of genetically modified foods of plant origin”, Food and Agricultural Organization of the United Nations, Geneva, Switzerland, may 29-June 2, 2000

FAO/WHO expert consultation on “Evaluation of allergenicity of genetically modified foods”, Food and Agricultural Organization of the United Nations, Rome, Italy, 22-25 January, 2001

Ferreira F., Kramer B., Casari G. Scheiner O., Breitenbach M., Ebner C., Rheinberger H. J. Amino acid positions influencing IgE binding to the major birch pollen allergen Bet v 1: analysis by site-directed mutagenesis. Allergy Suppl. 52, 1996, (suppl 39):30.

Friedman A., Al-Sabbagh A., Santos L. M. B, Fishman-Lobell J., Polanski M., Prabhu Das M., Khoury S. J., Weiner H. L. Oral tolerance: a biologically relevant pathway to generate peripheral tolerance against external and self antigens. Chem. Immunol., 1994; 58:259.

Gillespie D. N., Nakajima M. D. S., Gleich M. D. Detection of allergy to nuts by the radioallergosorbent tests. J. Allergy Clin. Immunol., 1976, 57:302.

Harrison L.A., Baily M. R., Naylor M. W., Ream J. E., Hammond B.G., Nida D. L., Burnett B. L., Nickson T. E., Mitsky T. A.,Taylor M. L., Fuchs R. L., Padgette S. R. The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from Agrobacetrium sp. Strain CP4, is rapidly digested in vitro and is not toxic to acutely gavaged mice. J. Nutr., 1996, 126:728.

Houben G. F., Knippels L. M. J., Penninks A.H. Food allergy: predictive testing of food products. Env. Toxicol. Pharmacol., 1997, 4:127.

Hourihane J. O’ B., Kilburn S. A., Nordlee J. A., Hefle S.L., Taylor S. L., Warned J. O. An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: A randomized, double-blind, placebo-controlled food challenge study. J. Allergy Clin. Immunol., 1997, 100:596.

Ito K., Inagaki-Ohara K., Murosaki S., Nishimura H., Shimokata T., Torii S., Matsuda T., Yoshikai Y. Murine model of IgE production with predominant Th2-response by feeding protein antigen without adjuvants. Eur. J. Immunol., 1997, 27:3427.

Kimber I., Kerkvliet N .I., Taylor S. L., Astwood J. D., Sarlo K., Dearman R .J. Toxicology of protein allergenicity: Prediction and characterisation. Toxicol. Sci., 1999, 48:157.

Knippels L. M. J., Penninks A. H., Spanhaak S., Houben G. F. Oral sensitization to food proteins: a Brown Norway rat model. Clin. Exp. Allergy, 1998, 28:368.

Knippels L. M. J., Penninks A. H., Spanhaak S., Houben G. F. An oral sensitization model in Brown Norway rats to screen for potential allergenicity of food proteins. Methods, 1999a, 19:78.

Knippels L. M. J., Penninks A. H., van Meeteren M., Houben G. F. Humoral and cellular immune responses in different rat strains upon oral exposure to ovalbumin. Fd. Chem. Toxicol., 1999b, 37:881.

Knippels L. M. J., Penninks A. H., Smit J. J., Houben G. F. Immune-mediated effects upon oral challenge of ovalbumin sensitized Brown Norway rats; further characterization of a rat food allergy model. Tox. Appl. Pharm., 1999c, 156:161.

Knippels L. M. J., Felius A. A., van der Kleij H. P. M. , Penninks A. H., Koppelman S., Houben G. F. Comparison of antibody responses to hen's egg and cow's milk proteins in orally sensitized rats and food-allergic patients. Allergy, 2000, 55:1.

Li X. M., Schofield B. H., Huang C. K., Kleiner G. I., Sampson H. A. A murine model of IgE-mediated cow's milk hypersensitivity. J. Allergy Clin. Immunol., 1999, 103:206.

Matsuda T., Sugiyama M., Nakamura R., Toril S. Purification and properties of an allergenic protein in rice grain. Agric. Biol. Chem., 1988, 52:1465.

Maynard F., Jost R., Wal J. M. Human IgE binding capacity of tryptic peptides from bovine alpha-lactalbumin. Int. Arch. Allergy Immunol., 1997, 113:478.

Melamed D., Friedman A. In vivo tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. Eur. J. Immunol., 1994, 24:1974.

Melo V. M .M., Xavier-Filho J., Lima M. S., Prouvost-Danon A. Allergenicity and tolerance to proteins from Brazil nut (Bertholletia excelsa H.B.K.). Fd. Agric. Immunol., 1994, 6:185.

Metcalf D. D., Astwood J. D., Townsend R., Sampson H. A., Taylor S. L., Fuchs R. L. Assessment of the allergenic potential of foods derived from genetically engenered crop plants. Crit. Rev. Fd. Science Nutr., 1996, 36, suppl., S165.

Moneret-Vautrin D .A., Rance F., Kanny G., Olsewski A., Gueant J. L., Dutau G., Guerin L. Food allergy to peanuts in France - evaluation of 142 observations. Clin. Exp. Allergy, 1998, 28:1113.
---
Mowat AM. The regulation of the immune responses to dietary protein antigens. Immunol. Today, 1987, 8:93.

Nakamura R. and Matsuda T. Rice allergenic protein and molecular-genetic approach for hypoallergenic rice. Biosci. Biotechnol. Biochem., 1996, 60:1215.

Ngan J., Kind L.S. Suppressor T cells for IgE in Peyer's patches of mice made tolerant by the oral administration of ovalbumin. J. Immunol., 1978, 120:861.

Nordlee J .A., Taylor S.L., Townsend J. A., Thomas L.A., and Bush R.K. Identification of a Brazil-nut allergen in transgenic soybeans. N. Engl. J. Med., 1996, 334:688.

Padgette S. R., Taylor N.B., Nida D.L., Baily M. R., MacDonald J., Holden L. R., and Fuchs R.L. The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. J. Nutr., 1996, 126: 702.

Pastorello E. A., Pravettoni V., Ispano M., Farioli L., Rotondo F., Incorvaia C., Asman I., Bengtsson A., Ortolani C. Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timithy and bircg pollen. J. Allergy Clin. Immunol., 1996, 98:601.

Piacentini G. L., Bertolini A., Spezia E., Piscione T., Boner A. L. Ability of a new infant formula prepared from partially hydrolyzed whey to induce anaphylactic sensitiza-tion; evaluation in a guinea pig model. Allergy, 1994, 49:361.

Popp W., Zwick H., Streyrer K., Rauscher H., Wanke T. Sensitization to aero-allergens depends on environmental factors. Allergy 1989, 44:572.

Saarinen U. M., Kajosaari M. Breastfeeding as prophylaxis against atopic disease-; prospective follow-up study until 17 years old. Lancet, 1995, 346:1065.

SAP, SAP report no. 2000-06, December 2000, Report on a FIFRA Scientific Advisory Panel Meeting entitled: a set of scientific issues being considered by the Environmental Protection Agency regarding "Assessment of Scientific Information concerning Starlink corn", Novermber 28, 2000.

Stokes C.R., Newby T.L., Bourne F. J. The influence of oral immunization on local and systemic immune responses to heterologous antigens. Clin. Exp.Immunol., 1983, 52:399.

Tada Y., Nakase M., Adachi T., Nakamura R., Shimada H., Takahashi M., Fujimura T., Matsuda T. Reduction of 14-16 kDa allergenic proteins in transgenic rice plants by antisense gene. FEBBS Lett.,1996, 391:341.

Taylor S.L. Chemistry and detection of food allergens. Food Technol., 1992, 39:146.

Taylor, S, and Lehrer, S, Principles and characteristics of food allergens. In Critical reviews in food Science and Nutrition,1996, 36, S91-S118.

Voitenko V., Poulsen L. K., Nielsen L., Norgard A., Bindslev-Jensen C., Skov P. S. Allergenic properties of kiwi-extract: Cross-reactivity between kiwi-fruit and birch-pollen allergens. Allergy, 1997, 52: 136.

Wal J. M. Assessment of allergic potential of (novel) foods. Nahrung, 1999, 43:168.

Watenabe M., Miyakawa J. Ikezawa Z., Suzuki Y., Hirao T., Yoshizawa T., Arai S. Production of hypoallergenic rice by enzymatic decomposition of constituent proteins. J. Food Sci., 1990, 55: 781.

Ye X., Al-Babili S., Kloti A., Zhang J., Lucca P., Beyer P., Potrykus I. Engeneering the provitamin A ( Beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science, 2000, 287:303.

# The Biosafety of Antibiotic Resistance Markers in Plant Transformation and the Dissemination of Genes through horizontal Gene Flow

Authors: **Philippe Gay**
**GMOs consultants**
**119 bis rue de Colombes**
**F-92600 Asnières, France**

**1. Introduction ..... 136**
1.1. Microbes as part of human life ..... 136
1.2. Antibiotic resistance marker genes ..... 137
**2. The antibiotic resistance marker genes used in plant genetic engineering ..... 139**
2.1. The need for marker genes in plant genetic engineering ..... 139
2.2. The antibiotic resistance marker genes used in plant genetic engineering ..... 140
2.3. Conclusion ..... 142
**3. The use of antibiotics versus natural antibiotic resistance ..... 143**
3.1. The use of antibiotics and the occurrence of resistance ..... 143
3.2. Reservoirs ..... 144
3.3. Conclusion ..... 146
**4. Transgenic plants as a potential contributor to the spread of antibiotic resistance genes ..... 147**
4.1. Requirements for horizontal gene transfer from plants to bacteria ..... 147
4.2. Fate of the DNA released in the environment ..... 147
4.3. Bacterial transformation ..... 149
4.4. Approaches for the evaluation of DNA mediated transformation frequencies ..... 151
4.5. Conclusion ..... 152
**5. Horizontal transfer of DNA ingested by mammals ..... 153**
**6. General Discussion ..... 154**
**7. Cases ..... 156**
**8. References ..... 157**

# 1. Introduction

## 1.1. Microbes as part of human life

Microbes and especially bacteria are an integral part of human life, though most people only become aware of their existence when they cause a health problem. Bacteria are key partners of our digestive track. One gram of faeces of an adult human contains approximately 100 billion bacteria belonging to more than 50 genera. The most well known of them: *Escherichia coli*, is among the less represented in this habitat, approx. 100 millions per gram. The mouth, the upper respiratory track, the skin and the vagina are colonized by several tenths of commensal bacterial species (Stanier et al, 1987). Bacteria are present in our food, either as contaminants or commensals of raw food ingredients, especially milk and vegetables, as contaminants of stored food or as basic constituents of fermented food. Bacteria are everywhere in our environment. The number of bacteria in soils ranges from millions to billions per gram (Foster 1988). Millions of bacteria currently colonize each $cm^2$ of vegetable leave (Nguyen-the and Carlin 1994).

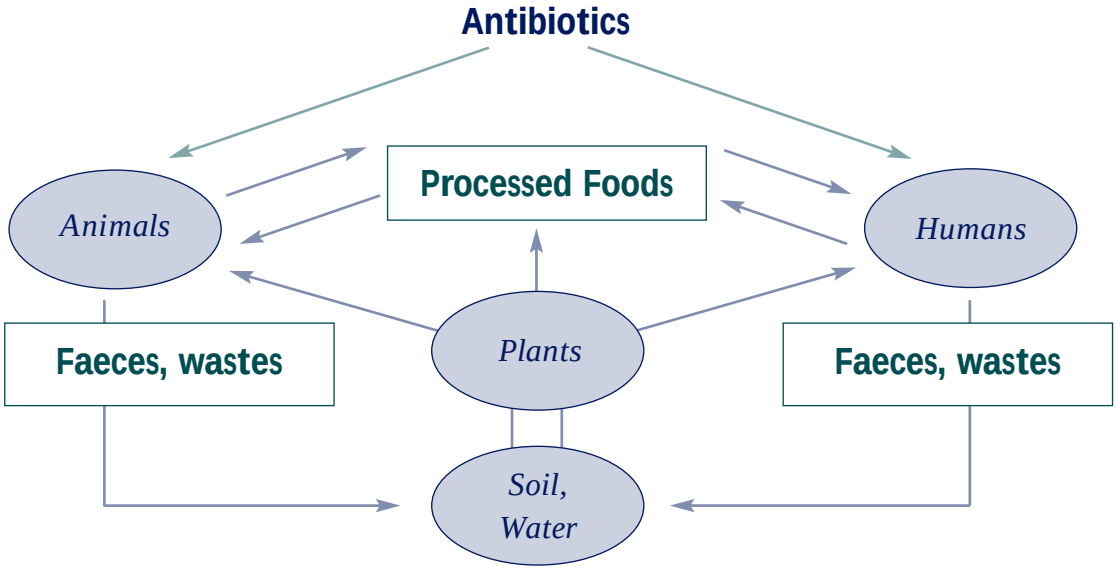
Figure 1 depicts the dissemination of bacteria and bacterial genes in our environment. Though various species have highly specialized or at least preferred habitats, human and animal activities result in an intermingling of bacterial species. These exchanges between various biotopes/reservoirs are however quite strongly counter-selected, meaning that exchanges result only in some cases to presence of bacteria in another niche, something that is not represented in the figure. What is true for bacteria is also true for the DNA which they release either as the result of physiological secretion mechanisms or as the result of their death and decay. It is noteworthy that the food industry destroys the bacteria present in the raw matters as well as most of the DNA present. The exception of course are raw fermented products.

Some bacteria cause infectious diseases which, until the appearance of antibacterial drugs, were by far the major cause of human and animal mortality. Courvalin and Acar, (1998) have called the last decades of the 20th century "the golden age of antibiotics", when nearly all bacterial diseases could be controlled by these drugs, of which the penicillins are the most well known. Like the penicillins, most antibiotics are substances that are produced by different micro-organisms naturally. This golden age may come to an end as a result of the generalized spread of antibiotic resistance among most bacteria and the lack of discovery of new antibiotics based on novel working mechanisms. Antibiotic resistance is currently considered one of the biggest threats to public health (American Society for Microbiology report 1995; Kumin C.M. 1993.)

Antibiotic resistance is caused by the expression of antibiotic resistance genes resulting in different physiological mechanisms such as detoxification of the antibiotic, mutation of the target of the antibiotic resulting in insensitivity, or pumping the antibiotic out of the cell. These antibiotic resistance genes are widely found in nature. They are required for the self-protection of microbes that produce antibiotic substances themselves (Davies 1994). Their existence is also assumed to be the result of the co-evolution of soil micro-

organisms that required protection against other microbes that produce antibiotics in the same habitats. The spread of antibiotic resistance genes is the result of the combination of both the selection of resistant clones in the presence of antibiotics and of an active inter-generic transmission of the resistance genes, also called **horizontal gene transfer** (Mazodier and Davies, 1991; Lorenz and Wackernagel, 1994). It is generally recognized (see http://www.cdc.gov/ncidod/dbmd/antibioticresistance/) that the widespread use of antibiotics in human and animal therapy as well as in animal farming (feed additives) over the last decades has predominantly contributed to an important increase of the frequency of resistance genes in bacteria. It has been proven that modifying antibiotic prescription practice can result in significant decrease of the frequencies of resistant pathogens (see http://www.apic.org/html/educ/gccfinal.html).

Figure 1: The dissemination of bacteria and bacterial genes in the environment




*Circles represent various biotopes. Blue arrows indicate a flux of bacteria and/or bacterial genes between the different biotopes. Boxes represent intermediate reservoirs that play a role in the flux of bacteria and/or bacterial genes between different biotopes. Brown arrows indicate a selection by the use of antibiotics in human and animal therapy and in the animal industry which influences the presence and flow of antibiotic resistance genes into different biotopes. Industrial food processing results in a strong diminution of the bacterial and of the DNA content originally present in raw matters. Processed food can be however contaminated by accident, and more frequently during domestic storage and use.*

## 1.2. Antibiotic resistance marker genes

Some of the antibiotic resistance genes are key tools in genetic engineering where genes are transferred (added) to cells of organisms. When the genes are transferred, one somehow has to identify the cells that have taken up the additional genetic material. For this one uses marker genes. These are genes that are responsible for a trait that can be easily identified/selected. It is physically attached to the fragment of DNA under study, so if the marker gene is present, the fragment of DNA under study will also be present. The cells that have actually taken up the DNA can be identified either through:

1. Selection: cells are being grown in such circumstances that only cells that have taken up the DNA can survive. For this one often uses antibiotic resistance genes.
2. Screening: cells that have taken up the marker gene are easily identified (for instance visually) and can be picked out.

Selection is the most used method, because it requires less effort. The ampr, kanr, tetr genes, respectively responsible for resistance to the antibiotics ampicillin, kanamycin and tetracycline, are used daily as selective markers by tens of thousands of scientists. Since the beginning of the eighties marker genes, and mostly antibiotic resistance genes, have been used by biotechnologists to help to create genetically engineered plants. Most of the genetically engineered plants that have been grown on millions of hectares in North and South America since 1996 include antibiotic resistance marker genes.

*Whether the presence of antibiotic resistance marker genes in transgenic crops could contribute to the spread of resistance and thus be a threat to public health by diminishing the possibilities to treat infectious diseases has become the subject of intense public debate in Europe over the last years. It is this question that will be addressed in this paper.*

The issue is already covered by an abundant scientific literature including the opinions of the EU scientific committees, (http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html), the Advisory Committee for Novel Food and Processes (ACNFP) in the UK (http://www.maff.gov.uk/), recent reviews by Nielsen et al (1998) and Bertolla and Simonet (1999), and opinions of other well known experts such as Salyers (http://www.healthsci.tufts.edu/apua/biblio.htm#7) and a number of scientific reports to which we will refer below.

It might however be of interest to revisit the issue in the light of the latest developments of the scientific knowledge and of the argumentation opposed to the dissemination of crops bearing antibiotic resistance marker genes (ACNFP reports, Courvalin 1998). In this paper this latest scientific knowledge is presented together with as many facts and figures as possible.

# 2.The Antibiotic Resistance Marker Genes Used in Plant Genetic Engineering

## 2.1. The need for marker genes in plant genetic engineering

The engineering of plants is a process that includes two major steps:

**a. The transforming DNA (bacterial selective markers)**

For the preparation of DNA to transform a plant, small circular genetic elements – plasmids – are used. Plasmids occur naturally in bacteria. Plasmids can be easily purified from bacterial cultures, engineered *in vitro*, and re-introduced in bacteria where they multiply. The genes to be transferred in plants are pasted *in vitro* into these bacterial plasmids. The resulting engineered plasmids, or transgene vectors, are re-introduced in bacteria which can either serve directly as gene donors to plants (*Agrobacterium* mediated transformation), or which are used to generate large numbers of copies of the engineered plasmids in order to obtain enough DNA to carry out direct transformation of plant cells by using for instance the Biolistic® method (the 'gene gun') or electroporation.

Bacterial plasmids used in genetic engineering can hardly be maintained in bacteria unless forced. For this purpose an antibiotic resistance gene is included in the plasmid and the relevant antibiotic is added to the culture medium so that only the bacteria bearing the engineered plasmid can survive. By far, the marker gene most frequently used for maintaining plasmids in the bacterium *Escherichia coli* is the amp$^r$ gene also termed *bla* TEM1, that allows bacteria to resist to ampicillin, an antibiotic of the β-lactam family.

A consequence of this process is that when a whole plasmid is transferred to the plant, the resulting transgenic plant will include a bacterial antibiotic resistance marker gene. This is the case in the Novartis 176 maize. This bacterial antibiotic resistance marker gene cannot be expressed in the plant, because it is preceded by a bacterial promotor. When submitted to the regulation of a bacterial promoter the genes are not expressed in plants, and vice versa.

**b. The plant transformation step (plant selective markers)**

The transformation of plant cells by either Agrobacterium mediated conjugation, or direct gene transfer, is a rather rare event. In order to select the few transformed cells that will allow the regeneration of a transformed plant, a marker is required that is expressed in the plant. Therefore another marker gene is added to the vector which is put under the control of a plant promotor. This will allow the transformed cells to be selected or identified. Such markers include herbicide resistance genes or genes allowing the resistance to antibiotics that are toxic for plants cells such as kanamycin or hygromycin.

**Remarks**

The bacterial selective marker used in step a. is not required in principle in the plant transformation when using direct gene transfer techniques such as Biolistics®. However in the early nineties, the state of the art

plant transformation techniques used entire plasmids as donor DNA, resulting frequently in the integration in the plant of the *bla* TEM1 gene, or part of it. This is the case for the following plants that were approved for growing and processing in the EU: Ciba/Novartis maize (EU application number C/F/94/11-03) and AgrEvo maize (EU application number C/F/95/12-07). A plant selective marker is presently still an absolute requirement to ensure successful DNA-transfer to plant cells. AgrEvo Oilseed rape (EU application number C/UK/95/M5/1), includes two selective markers, the herbicide resistance gene *pat* and *nptII* antibiotic resistance marker. For more details see http://biosafety.ihe.be/AR/ARmenu.html.

## 2.2. The antibiotic resistance marker genes used in plant genetic engineering

The antibiotic resistance genes used in plant genetic engineering are listed in table 1. They all originate from bacterial transposons (small naturally 'jumping' genetic elements).

Table 1: Main antibiotic resistance genes used in plant biotechnology[1]

| **Antibiotic resistancegene** | **Bacterial phenotype*** | **Plant phenotype **** | **Main use** |
|---|---|---|---|
| amp$^r$<br>*bla* TEM1 | Ampicillin and amoxicillin resistance | None | Bacterial selective marker |
| *aad*<br>or<br>*ant (3'')-Ia3* | Streptomycin and spectinomycin resistance | None | Bacterial selective marker |
| *aph(3')-II*<br>or<br>*nptII* | Kanamycin,neomycin resistance | Kanamycin resistance | Plant selective marker |
| *aph(3')-III*<br>or<br>*npt III* | Kanamycin, neomycin, amikacin resistance | Kanamycin resistance | Plant selective marker |
| *hgh*<br>or<br>*hpt* | Hygromycin resistance | Hygromycin resistance | Plant and fungi selective marker |
| cm$^r$<br>or<br>*cat* | Chloramphenicol resistance | Expression of chloramphenicol acetyl transferase | Reporter gene |

* only when the gene is expressed under the regulation of a bacterial promoter

** only when the gene is expressed under the regulation of a plant promoter

The relative importance of these markers can be evaluated according to their presence either in plants approved for cultivation both in the EU and US, or plants for which the marketing dossiers are currently reviewed in the EU and plants tested in the fields in the US, as shown in table 2 and 3.

[1] Abbreviations: bla = Beta-lactamase
aad = aminoglycoside adenyltransferase
ant = aminoglycoside nucleotidetransferase
aph = aminoglycoside phosphotransferase
npt = neomycine phosphotransferase
hpt = hygromycine phosphotransferase
cat = chloramphenicol acetyltransferase

Table 2: Antibiotic resistance genes in approved transgenic crops (see sources below)

| Gene | EU * | USA** |
|---|---|---|
| *bla* TEM1 | Corn | Corn |
| *nptII* | Corn | Corn |
| *nptII* | Oilseed rape | Oilseed rape |
| *nptII* | Chicory | Chicory |
| *nptII, aad, cat* | | Tomato |
| *nptII* | | Cotton |
| *nptII* | | Potato |
| *nptII* | | Squash |
| *nptII* | | Flax |
| *nptII* | | Papaya |

* for more details see URL http://biosafety.ihe.be/ARGMO/GMO_Plants.html

** for more details see APHIS at URL http://www.nbiap.vt.edu/

Table 3: Antibiotic resistance genes in plants tested in the fields in the US (total number of records 5769, source APHIS, http://www.nbiap.vt.edu/)[1]

| Marker gene | Number of trials applications total | Number of trials applications since Jan 1998 |
|---|---|---|
| *Cat* | 4 | 0 |
| *Hpt* | 185 | 95 |
| *nptII* | 2295 | 845 |
| *Ant* | 1 | 0 |
| *Bla* | Statistics not available* | Most likely 0 *** |
| *npt III* | Statistics not available** | Statistics not available** |

* the bla gene is not considered a plant marker gene in the statistics,

** marker not listed, either covered by confidential business information, or more likely not used.

*** see explanations below

From the above given tables it can be deduced that the *nptII* kanamycin resistance gene still is the most important selective marker in plant genetic engineering. About 90% of the plants in R&D contain this marker gene. Although not given in the tables above, herbicide tolerance is more important than *hpt* and comes in second. Many plants on the market or in R&D phases are genetically modified to be herbicide tolerant and in a number of cases the herbicide tolerance is combined with another trait, for instance insect resistance or male sterility. There seems to be a trend towards an increased use of the hygromycin resistance gene *hpt* though it is not yet present in a registered transgenic crop. In contrast to many other antibiotics, hygromycin is in practice not used as an anti-microbial drug. Another trend is to develop new selective markers to replace the antibiotic resistance markers (Kunkel *et al.*1999; Stein and Hansen 1999). It will however take time to have a full assessment of the usefulness and the harmlessness of such new markers. Recent examples are the cytokin

[1] For field tests in the EU comparable statistics are not available

based system by Chua *et al*. (1999) and the mannose based selective marker developed by Novartis (Privalle, *et al*, 2000). An increased efficacy of the techniques of gene transfer to plants might lead in the near future to a situation where selective markers are no longer necessary.

Though *nptIII* does not appear in the statistics to which we had access, it is noteworthy that it is present in potatoes for which a marketing application had been filed in the EU by Avebe (application C/NL/96/10). This *nptIII* gene was present in the plasmid used in the *Agrobacterium*-mediated transformation. In this particular case the whole plasmid was transferred to the plant including the *nptIII* gene. This is a kind of textbook case since the issue of horizontal gene transfer has been raised in connection with the fact that the gene causes resistance to amikacin which is an antibiotic of value to fight nosocomial infections (http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html). Avebe has withdrawn its application, when it was clear that the presence of this *npt-III* gene led to serious objections to the request to put the potatoes on the market.

Two remaining remarks are first that the *bla* gene is by far the most frequently used marker in the laboratory for gene cloning purpose in bacteria. It is however not to be expected that this gene will be present in plants that will be marketed in the future. And secondly that a streptomycin resistance gene was integrated in a cotton line from Monsanto company.

## 2.3. Conclusion

In the immediate and mid-term future, the issue of the presence of antibiotic resistance marker genes in genetically engineered plants will be restricted to the following genes:
a) The *bla* TEM1 gene. This gene is present in Novartis 176 maize commercialized in the US and Canada, and registered in EU (Commission Decision 97/98/EC). A partial copy is present in the AgrEvo T25 maize which is also registered for growing and processing in the EU (Commission Decision 98/293/EC). It will not be present in plants to be marketed in the near future.
b) The *nptII* gene present in several plants registered in the US, and especially in AgrEvo Topaz 19/2 oilseed rape authorized in the EU ( Commission Decision98/293/EC). These crops are grown on millions of hectares. The *npt-II* gene is also present in plants in very large amounts (thousands) of field trials. Many transgenic crops that will be marketed in the near future will therefore contain this marker gene.
c) The *hpt* gene that is not yet present in a registered transgenic crop, but this may be different in the future, because there is a trend toward an increased use of hygromycine. About 10% of the plants in field trials contain this marker gene.

In the following we will particularly focus on risks that could be associated with the antibiotic resistance genes present in commercialized transgenic crops.

# 3. The Use of Antibiotics Versus Natural Antibiotic Resistance

## 3.1. The use of antibiotics and the occurrence of resistance

Publicly available statistics on the use of antibiotics are scarce. The search of scientific literature and the web, and the interviewing of a number of specialists in infectious diseases, including practitioners, microbiologists, epidemiologists, managers in the public health insurance system, did not result in precise figures on the evolution of the use of specific antibiotics. However some trends are unanimously acknowledged.

**1. Kanamycin, neomycin**

Kanamycin and neomycin are rather toxic antibiotics of the aminoglycoside-group. They are of no clinical importance. Resistance to kanamycin and neomycin is not considered to be a threat for public health. Their use has been nearly abandoned in the treatment of infectious diseases, because of their toxicity In clinical practice today their use is limited to some applications of external use. Kanamycin appears only in one ointment formula in the French *Vidal* dictionary of pharmaceutical specialities and neomycin only appears in OTC topical formulations for the treatment of dermatitis, and superficial burns and wounds.

Borne by the Tn5 transposon detected in *Escherichia coli*, the *nptII* gene, rendering resistance to kanamycin and neomycin is abundant in nature (Leff et al. 1993; Smalla, 1993, De Vries and Wackernagel 1998).

**2. Ampicillin, amoxicillin**

Ampicillin and amoxicillin are antibiotics of the β-lactam group that are widely used in human and animal chemotherapy. In human therapy they are used against numerous bacterial diseases such as ear infections, infections of the respiratory tract, of the urinary and digestive tracts, etc.. Though this does not appear in literature, the specialists interviewed indicated that the use of ampicillin is significantly declining. However this decline does not extend to the use of amoxicillin. There are alternatives to cure the diseases that were previously treated by both ampicillin and amoxicillin.

Resistance to ampicillin and amoxicillin is ubiquitous, likely due to the massive use of ampicillin over the last 25 years. Resistance to ampicillin also results in resistance to amoxicillin. Figures vary from country to country but it is clear that resistance is found in a large spectrum of bacteria infecting humans (Calva, 1996), farm animals (DANMAP Report. 1997), and is present in soils and ground waters (McKeon, D. 1995). In hospitals, about 50% of the Enterobacteriaceae are resistant to ampicillin, and more than 80% of these resistances are caused by the *bla* TEM1 gene (Livermore 1995). The *bla* TEM 1 gene codes for a β-lactamase that cuts the ampicillin molecule thereby inactivating the antibiotic.

Since the *bla* TEM1 gene was first identified in the early 1960s, a number of β-lactam antibiotic resistance genes - to-date more than sixty - have been identified. They encode β-lactamases that provide resistance to most β-lactam antibiotics, including the third generation cephalosporins (Medeiros, A. A. 1997). Resistance has build up to such an extend that a safe use of ampicillin or amoxicillin is to complement them with a β-lactamase inhibitor, clavulanic acid, though resistance also has build up against this mixture (Nordmann P et al. 1994).

**3. Hygromycin**

The antibiotic hygromycin is not used in human medicine because of its very high toxicity. Only in farm animals it is used as an anti-helmintic (= anti-worm). (http://www.fda.gov/cvm).

The *hpt* gene produces the hygromycin phosphotransferase protein that inactivates the hygromycin molecule by phosphorylation. It is widely present in bacteria in soil and Enterobacteriaceae (ZKBS, 1999).

**4. Streptomycin**

The use of streptomycin has been nearly abandoned in human medicine except for the treatment of some cases of gonorrhea. Its use might be revived for the treatment of severe endocarditis. (Courvalin 1998).
No streptomycin resistance gene is present in a crop reaching the human food chain.

The frequency of occurrence of the above mentioned resistance genes in the reservoirs is so far not limited by the availability of the genes and of horizontal gene transfer mechanisms but rather by the selection exerted by the use of antibiotics in human and animal therapy and likely by the use of antibiotics as additives in animal feed.

## 3.2. Reservoirs

It is generally agreed that antibiotic resistance genes have appeared over millions of years of bacterial evolution as a result of a) the need for antibiotic producing bacteria to protect themselves against their own poisons (Davies, 1994), b) the co-evolution of soil bacteria and various antibiotic producer microbes.

The resistance genes present in resistant bacteria in a relevant biotope form resistance reservoirs. These reservoirs consist of pre-existing genes. Soil has likely been the initial reservoir of antibiotic resistance genes. From there, resistant bacteria migrated into new biotopes, including animals, where resistance genes have invaded numerous species, including humans, thanks to natural horizontal gene transfer mechanisms (HGT). HGT is the transfer of genetic material from one individual organism to the other without the involvement of mechanisms of reproduction. The transfer of genetic material from a parent to a daughter organism involving mechanisms of reproduction is called *vertical* gene transfer.

Humans, especially their intestinal tracts, also present a resistance reservoir. Contributions to this reservoir occur among other things through direct contamination, for instance, thanks to: a) bacteria present in the food chain such as the bacteria on raw vegetables (Corbet D. 1988), raw fermented products, and stored food, and b) direct contact with other humans and their effluents (this is particularly the source of nosocomial infections), with animals and their effluents and with soil.

**The spread of bla and npt-II in the natural environment through horizontal gene transfer**

Both the *bla* TEM1 and *nptII* genes have been initially identified in bacteria on transposons (Tn3 and Tn5 respectively). Transposons are small genetic elements that can be transposed or "jump" from place to place in chromosomes or plasmids. Plasmids are mini-chromosomes that can be infectious. This means that they can be naturally transmitted from one bacterium to the other. These bacteria do not have to belong to the same

species (Courvalin, 1994). This natural mechanism of HGT is called conjugation. Tn3 and Tn5 can be found on several plasmids widely disseminated across bacterial species by conjugation (bacterial mating) (Mazodier and Davies 1991, Amabile-Cuevas and Chicurell 1992, Nielsen et al 1998).
The spread of the *bla* TEM1 gene in human pathogens resulted from the transfer by conjugation of plasmids that invaded several pathogenic species, starting initially with enterobacteriaceae in the mid sixties, then *Pseudomonas aeruginosa* in 1970, followed by *Haemophilus influenzae* in 1974, *Neisseria gonorrhoeae* in 1976, *Acinetobacter* in 1978, *Vibrio cholerae* in 1979, then *Neisseria meningitidis* in 1983, (Arlet and Philippon, 1997). This evolution clearly correlates with the explosion of the use of ampicillin in human and veterinary medicine. The extensive use of ampicillin has been responsible for a strong selection pressure favouring the bacteria with antibiotic resistance. This illustrates that the evolution of the ampicillin resistance reservoir depends on selection in as much as genes and horizontal transfer mechanisms exist. This key role of the selection depending on the heavy use of antibiotics is emphasized in several reports (Betts et al. 1984; Pear et al. 1994; Seppala et al. 1997; Austin et al. 1999). It is actually the case that humans, their direct environment and livestock have now become the main reservoirs of resistance, in their turn feeding the soil and plant biotopes with resistant bacteria.

In addition to the mechanisms of HGT, spontaneous mutations play a role in the formation of resistance reservoirs. Like any other gene, antibiotic resistance genes are subject to spontaneous mutations. It is generally agreed that the spontaneous mutation rate of a given gene is approximately $10^{-6}$ per generation. This means that the gene in one in 1 million bacteria is hit by one mutation. In microbes such rates can have consequences in a short time frame, given the size of microbial populations and their multiplication rates. It is estimated that a healthy human being disseminates daily between 500 millions and 5 billions bacteria carrying an ampicillin resistance gene of the *bla* TEM family through his faeces. This means that even without selection pressure, such a bacterial population already includes a significant number of mutants, and some of them will carry a mutation in the ampicillin resistance gene, perhaps even resulting in an extended spectrum of resistance to β-lactams. This means that the mutation rate is of such an order that it is not a limiting factor for the evolutions of resistance reservoirs. The amount of selection pressure will determine the evolution in the resistance reservoirs of extended resistances.

Ampicillin was given here as an example of how also mutations can effect changes in antibiotic resistances. But this is also true for kanamycin resistance. Even more so, in the case of the *npt-II* gene objections were raised against the use of *nptII* in plants (Courvalin 1998) arguing that a single mutation would transform *nptII* in a *nptIII* like gene causing amikacin resistance (Kocabiyik, 1992). Resistance to amikacin is a serious issue since this compound is a reserve antibiotic used to fight nosocomial infections (Opinion of the EU Scientific Committee for plants, 2 October 1998). However it should be noted that amikacin resistance, is caused by different genes, including *aac (6')-I* (Miller et al. 1995) and *aph(3')-III* (Trieu Cuot and Courvalin 1983) that are already rather abundant in nature.

As a résumé it can be stated that resistance reservoirs are determined by the following:

- The availability of resistance genes.
- The dissemination of the resistant bacteria and of their resistance genes within a biotope and across biotopes.
- The variations and mutations that affect these genes.
- The selection pressure that regulates the frequency of the genes within the bacterial community in a biotope.

## 3.3. Conclusion

The antibiotics kanamycin and hygromycin are of no clinical importance. Ampicillin and amoxicillin are still abundantly used in human medicine though the use is of ampicillin is considerably declining. Resistance to all of these antibiotics is abundant in nature and the resistance genes are readily available in resistance reservoirs. One way to get round resistance in order to further benefit from these antibiotics is to add the β-lactamase inhibitor clavulanic acid to amoxicillin. However, resistance to this mixture is already rather high.

# 4. Transgenic Plants as a Potential Contributor to the Spread of Antibiotic Resistance Genes

Can transgenic plants bearing bacterial ampicillin, hygromycin or kanamycin resistance genes be a new factor influencing the frequency of these genes in resistance reservoirs? Can these genes when they are present in foodstuffs, be transferred to the bacteria in the gut of individuals and pose an unacceptable risk? These are the two main issues raised by the presence of antibiotic resistance marker genes in crops, already grown on millions of hectares.

To our knowledge, as well as in literature, there has so far not been a report of a direct observation of any transfer of genes from plants to bacteria in the natural environment. In order to investigate whether and how such a horizontal gene transfer would be possible, the likelihood of each of the requirements to be fulfilled should be considered and if necessary subjected to an experimental approach.

## 4.1. Requirements for horizontal gene transfer from plants to bacteria

There is no known dedicated mechanism for horizontal gene transfer from plants to other organisms. Therefore horizontal gene flow requires the direct transfer of plant DNA to bacterial cells, i.e. DNA mediated transformation. This requires the following:

a) Plants cells, i.e. decaying or ingested plant parts should release in the environment DNA fragments of at least the average size of a (resistance) gene (fragments should be in the kilobase range and more).

b) After release, DNA should persist in an (aggressive) environment for a longer period of time (to allow it to be taken up by bacteria).

c) Bacteria should be able to take up the released DNA.

d) DNA taken up should be stably established in the recipient cells.

e) This establishment should at least be neutral so that the transformed cells are not counter-selected.

## 4.2. Fate of the DNA released in the environment

**Some orders of magnitude**

- Novartis 176 maize, includes one functional copy of the *bla* TEM1 gene together with the origin of replication of the pUC 18 plasmid.
- The number of cells of fresh maize is in the range of $10^7$ to $10^8$ per gram.
- In mid-summer, the number of maize cells per ha is thus in the range of $10^{15}$ meaning as many copies of the maize *bla* TEM1 gene.
- In the upper layer (ploughed) of agricultural land, each gram of soil includes on average $10^7$ bacterial colony forming units (cfu) including approx.$10^5$ ampicillin resistant bacteria bearing the *bla* TEM1 (Simonet, personal communication). These $10^7$ cfu refer to the organisms that can be cultivated in the laboratory from soil. It is estimated that the species that can be cultivated from soil represent only 1% of the actually present species in soil. Therefore these cfu represent only a small proportion of the actual soil bacterial population.

This majority of bacteria that cannot be cultivated in the laboratory might also include amp$^r$ (*bla*) bacteria.

- The top 10 cm of a hectare of agricultural land can currently include about $10^{16}$ cfu, among which $10^{14}$ cfu each bearing 10 copies of *bla* TEM1[2]. This means that it includes at least $10^{15}$ copies of the *bla* TEM1 gene, borne by highly transmissible plasmids.

For the *npt-II* and *hpt* antibiotic resistance genes similar calculations can be made, because these genes, like the *bla* TEM1 gene are widely present in soil- and Enterobacteria (ZKBS, 1999).

**The fate of linear plant DNA fragments released in soil**

For most crops at least parts of the crop remain on the field after harvest. This remaining plant material is often ploughed into the soil where the plant material decays and the plant cells break down thereby releasing the cell content including the DNA. The plant cell content itself is a threat for DNA. The destruction of the cell infrastructure releases nucleases that attack the DNA when released from the nucleus. In all plant DNA extraction protocols in the laboratory chelators are added to the extraction medium before grinding the cells otherwise no DNA can be recovered.

In addition to the degradation by the plant cell content itself, DNA molecules released in the environment are sensitive to mechanical shearing and to the nucleases released in the environment by other organisms, especially bacteria (Blum et al., 1997), both resulting in the breakdown into smaller fragments. However, in soil,

a) DNA fragments of a size of few kilobases - that is of the size of some bacterial antibiotic resistance genes - are remarkably resistant to mechanical shearing.
b) DNA molecules can be protected against nuclease action in aquatic environments (Paul et al. 1989) and in soil (Lorenz and Wackernagel, 1992; Romanowski et al., 1993; Paget et al., 1992), so they could persist long enough in this apparently hostile environment to allow a possible uptake by acceptor cells.

**The fate of linear plant DNA fragments in the intestinal tract of mammals**

Schubbert et al, (1994, 1997) have shown that DNA ingested by mice is not totally degraded in the intestinal tract and that fragments of the size of an antibiotic resistance gene could resist degradation and become available for horizontal transfer. Such large fragments represent less than 4% of the ingested plant DNA, but this still is a significant amount.

To our knowledge, the fate of DNA released in the intestinal tract of ruminants, like cows and sheep has not been documented. Ruminants have three stomachs in their intestinal tract. The DNA is released in the first stomach, the rumen, which is a fermentation vessel of more than 100 liters, that includes approximately 10 to 100 billions bacteria per ml. It is assumed to be an extremely hostile environment for free DNA given the abundance of nucleases secreted by the bacteria and protozoa that degrade the ingested plant material. However, most of the feed present in rumen is under a solid phase, i.e. involves microenvironments where DNA might be protected like in soil and further made available for horizontal gene transfer. The content of the rumen is however digested downstream in the abomasum, the second stomach of ruminants (in an acidic medium similar to the stomach of monogastric animals) .

[2] Resistant bacteria contain multiple copies of the *bla* TEM1 gene. The average is about 10 copies per bacterium.

**The fate of plant DNA: possibilities for reconstitution of plasmids**

Plant DNA is released as linear fragments. In some transgenic plants however, like for instance the Novartis 176 maize, the bacterial colE1 origin of replication is present, which means that fragments that are released from these plants can include both the bla TEM1 gene and the colE1 origin of replication i.e. the material required to reconstitute a functional plasmid. Both linear fragments and plasmids can be taken up by bacteria, but functional plasmids are more likely to be attained by bacteria than linear fragments of DNA. If the locus contains internal duplications, then the chance that functional plasmids are formed is enlarged, because loops can be formed (duplications are sticky) that can be spontaneously excised from the chromosome thereby releasing a circular plasmid. Such a case had never been encountered at Novartis, where, in the early nineties, the DNA of more than 5000 plants was analyzed (Gay P. unpublished data). Moreover, in an attempt to rescue such plasmids from Novartis maize DNA, A.Kahn (1996) showed that transgenic maize DNA treated with a restriction enzyme and further circularised by ligase treatment (deliberately *making* circular genetic element which could contain the colE1 origin), allows the rescue of a functional plasmid by electroporation of *E.coli* cells at a frequency of $10^{-9}$, while untreated DNA did not. These experiments did neither allow any rescue by exposure of competent *E.coli* to maize DNA. So even though the formation of plasmids in this case was identified as a possibility which would increase the chances of successful transfer of genes from the transgenic plant to bacteria, these data show that the chances of this happening in practice are extremely low.

Conclusions on the fate of DNA in the environment

Whether released in water, soil, in animal or human intestine, the proportion of plant DNA escaping degradation after release is small but most likely not insignificant. DNA degradation results in a decrease of the absolute amount of fragments bearing intact antibiotic resistance genes to an extent that the instant concentration of this DNA might become limiting to allow successful contact with transformable bacteria, in soil or in the digestive tract of animals and humans.

It should be noted that the same environments include micro-organisms that are sources of the same antibiotic resistance genes present on circular plasmids that are by far better candidates for horizontal gene transfer than transgenic plant DNA. This means that if the use of antibiotics leads to the selection of resistant bacteria, the antibiotic resistance genes will be more likely to be acquired from these plasmid sources and not from horizontal gene transfer from the plant material to the bacteria.

Transgenic plants carrying bacterial ampicillin or kanamycin resistance genes are therefore, based on the data given above, not a factor that significantly influences the frequency of these genes in resistance reservoirs.

## 4.3. Bacterial transformation

For bacteria to become antibiotic resistant two requirements have to be fulfilled: (1) antibiotic resistance genes should be available, and (2) bacteria should be able to absorb these genes and express them. In the paragraphs above it is already shown that the npt-II, bla TEM1, and hpt genes present in transgenic crops present only a minor contribution to the frequency of these genes in resistance reservoirs. In the following the second requirement is being discussed to determine the chances that the antibiotic resistance genes from transgenic plants are actually being taken up and expressed by bacteria in soil or in other biotopes.

Bacterial transformation is crucial for the uptake and expression of DNA from the environment. Not all bacteria are known to be transformable. Lorenz and Wackernagel (1994) have listed more than 40 naturally transformable bacterial species. New species e.g. *Escherichia coli* and *Ralstonia solanacearum* (Bertolla et al., 1997), have since been added to this list and more will be added in the future.

Transformation of bacteria involves the following steps.

**a) Competence:**

Competence is a state that allows bacteria to bind DNA with which they make contact and initiate the transfer of the DNA molecule into the cell. Competence is generally a developmentally regulated state. In the laboratory, the development of competence in several organisms is shown to be inducible under specific conditions, composition of the medium, population size, growth conditions etc. which seem to be hardly achievable under natural conditions. However laboratory experiments cannot pretend to mimic the broad variety of environments in which bacteria can develop, especially growth on solid supports, such as soil, plants or food particles. For example the founding paper of Griffith, F. (1928) shows that competence of *Streptococcus pneumoniae* can occur in the blood stream of a mouse. Also *Ralstonia solanacearum* can reach spontaneously a competent stage in the infected plants.

Some bacterial species do not develop the competent state in nature though they can be transformed following some laboratory treatments that allow extra-cellular DNA to penetrate the cellmembranes. This is for instance the case for *E.coli*.

**b) DNA uptake:**

DNA binding by competent bacteria is followed by its uptake. In some cases, such as *Haemophilus influenzae* and *Neisseria sp*., DNA uptake involves the recognition of specific sequences. In other cases, including *Bacillus subtilis* or *Streptococcus pneumoniae* DNA of any origin can be taken up. DNA is taken up as single stranded DNA (Steward and Carlson 1986).

The uptake of a given DNA sequence or gene will depend on the relative concentration of this gene or sequence among the DNA molecules surrounding the bacteria. In other terms DNA entry is subject to a competition between the molecules available in the environment of the cell. Antibiotic resistance marker genes are expected to be borne by fragments of a size of few kilobase pairs representing approximately a millionth of the plant genome and a smaller proportion of the overall DNA potentially available in the relevant environments. The dilution of the antibiotic resistance marker genes is thus likely to be a hurdle for their horizontal transfer.

**c) Stabilization of transforming DNA.**

Maintaining the incoming genes in the cell genome is likely to be the most critical step in bacterial DNA mediated transformation. It requires either the integration of the incoming single stranded DNA in the chromosome, in a resident plasmid or the reconstitution of an autonomous replicon.

The integration of the incoming DNA in chromosomes or resident plasmids requires the existence of sequence homologies between the donor and the recipient cell DNA (de Vries and Wackernagel, 1998, Gebhard and Smalla 1998).

The reconstitution of an autonomous replicon, e.g. a plasmid, in a recipient cell has more requirements. Firstly a strand complementary to the incoming single strand DNA-molecule should be synthesized. This requires a priming by a partial duplex. Therefore more than one DNA fragment originating from the transgenes has to be taken up. When this is achieved, this duplex sequence should be further replicated. This requires the inclusion

of an origin of replication that is recognized as such by the recipient bacterium. In the case of Novartis 176 Maize, the colE1 origin of replication would limit the replication of a reconstituted plasmid to Enterobacteriaceae, i.e. genera in which attempts to transfer plant antibiotic resistance marker genes by exposure of the cells to DNA actually failed (A. Kahn, 1996); Novartis application C/F/94/11-03; Schlüter et al., 1995).

The uptake and expression of DNA by bacteria therefore involves a multiple step process in which each step can be a limiting factor in the actual success of the bacterium obtaining an antibiotic resistance. In the following an estimation will be given of the frequencies with which such transformation can succeed.

## 4.4. Approaches for the evaluation of DNA mediated transformation frequencies

Smalla and coworkers quoted by Nielsen et al (1998) as well as Bertolla and Simonet (1997), have failed to detect any transfer of antibiotic resistance markers in soil bacteria, following the cultivation of either transgenic sugar beets or transgenic tobacco, while the genes could be isolated from soil.

Plant DNA can be available for transformation in two different ways: as fragments of linear DNA, but perhaps in some exceptional cases also as plasmids. In the case of the Novartis 176 maize for instance the colE1 origin of replication is present, which means that a functional plasmid can be formed if the linear DNA containing the origin would circularize and close. Plasmids have far higher chance of transforming bacteria than linear DNA. This is why Novartis researchers have attempted to rescue a pUC like functional plasmid from 176 maize DNA in competent *Escherichiacoli* cells. The attempt has failed. The results indicated that the probability of rescuing such a plasmid was at least $10^{10}$ times lower than rescuing a plasmid extracted from a bacterial culture. The experiments were reproduced by Kahn (1996) with the same result. Kahn also digested maize DNA with the restriction enzyme Xho1 that makes no cut in the 176 maize transgene and re-ligated the DNA. The mixture was electroporated in *E.coli* and one $amp^r$ transformant colony was obtained which, as shown by the analysis of the plasmid, undoubtedly derived from the maize DNA. Electroporation of native or sheared maize DNA, did not allow any rescue of an $amp^r$ plasmid. This means that in reality the formation of a closed plasmid in this case is the limiting factor.

Schlüter et al (1995) attempted unsuccessfully to transfer an ampr gene (*bla* TEM1) present in transgenic potato in the enterobacterium *Erwinia chrysanthemii*. From an extrapolation of their data, they considered that the probability of such a transfer in nature is below $2x10^{-17}$.
De Vries and Wackernagel (1998) as well as Gebhard and Smalla (1998) have designed constructs in *Acinetobacter* BD413 that allow the rescue of the *nptII* gene whether present in the DNA of transgenic crops, or in soil. The system is optimized in that sense that a) the strains include a large homology sequence with the *nptII* donor DNA, b) high levels of competence are achieved in vitro. Both publications report a rescue of the *nptII* gene present in transgenic plant DNA with transformation rates in the range of $10^{-8}$. However, neither author could obtain any horizontal transfer of plant nptII genes in the original strain of *Acinetobacter* BD413. Interestingly De Vries and Wackernagel (1998) show that their technique allows a titration of any selectable gene in a natural extract, with an efficiency of approximately one transformant per $10^4$ copies of the gene to be detected, that is an efficiency only a thousand fold lower than PCR, while less prone to artifacts. More

recently, Wackernagel and De Vries (2000) showed that if the *Acinetobacter* acceptor culture does not include any homology with the donor DNA, no transformation can be detected meaning that the transformation frequency is less than $10^{-13}$ despite the high level of competence of the culture.

**The case of silage**

Silage is an important way to store fresh maize and other forage crops. The whole plants are harvested prior to maturity, crushed and stored under anaerobic conditions. The ensiled material is subject to a fermentation mainly mediated by lactic bacteria resulting in few days in a drop of the pH that prevents the development of other bacteria and allows the conservation of the forage for the whole season. Novartis has shown that in ensiled maize DNA is heavily degraded likely due to the low pH. Preliminary studies by J. Heritage (personal communication) indicate that during the early stage of the fermentation process no stable transfer of the *bla* gene of Novartis maize could be detected in the streptococci that are responsible for the lactic fermentation of silage. Again if the *bla* gene is transferred, the frequency of the phenomenon is below the detection limits.

**Transformation rates**

Thanks to systems artificially designed to detect a transfer of plant antibiotic resistance genes in bacteria, the experiments of De Vries and Wackernagel (1998) and Gebhard and Smalla (1998) have shown that such a transfer is not impossible. The systems employed are however designed in such a way that the chances of transfer are several orders of magnitudes higher and do not represent the natural situations. The search for horizontal transfer of antibiotic resistance marker gene in soil bacteria under natural conditions has failed so far. It could not be found either in laboratory simulations of natural conditions, although in the laboratory, each of the steps required for DNA mediated transformation has been successfully achieved.
The probability of the event to happen in nature is thus below the detection limits, that is below at least $10^{-11}$ to $10^{-13}$ per bacterium exposed to transforming DNA.

## 4.5. Conclusion

Horizontal gene transfer of plant DNA to bacteria is very unlikely and has not been observed in nature. To be able to transform bacteria, the plant DNA first has to become available after decay of the plant material. It is proven that fragments of plant DNA can persist in the soil over longer periods of time and that fragments can be large enough to contain intact resistance genes. The antibiotic resistance genes that are released from the transgenic plants and persist in the soil only just add up to the enormous number of these genes that are already present in the resistance reservoir. For *npt-II*, *bla* TEM1, and *hpt* these resistance reservoirs are quite large. An important difference between the resistance genes in the reservoir and the ones from the transgenic plants is that the genes in the reservoir are carried by easily transmissible plasmids, while the plant resistance genes are present on broken linear fragments. Plasmids can far more easily transform bacteria than the linear plant DNA. Estimates on the successful transformation of bacteria with linear plant DNA, which requires three different steps to be taken successfully are below $10^{-11}$.

It can be concluded that it is many factors more likely that a bacterium acquires an *npt-II*, *bla* TEM1, or *hpt* gene from a plasmid present in the different resistance reservoirs than the chances of acquiring such a gene from a transgenic plant.

# 5. Horizontal Transfer of DNA Ingested by Mammals

Based on the observations in the former chapter one cannot exclude that horizontal transfer of either $amp^r$ or $kan^r$ genes could occur in the rumen microflora. Large enough DNA fragments may be present and the steps necessary to achieve bacterial transformation are not impossible. The rumen environment is so complex that it can hardly be simulated in the laboratory, and even less subjected to direct investigations. It should be noted that in order to produce a number of $amp^r$ bacteria as high as the number already present in faeces, transformation rates in the overall population should be in the range of one in a hundred, a figure that cannot be seriously envisaged, because this would mean a factor of $10^6$ better than the best transformation rates that can be achieved under optimized laboratory conditions.

But nonetheless one has tried to determine the fate of DNA in the intestinal tract, among other things to see whether this DNA would transform the bacteria that are present in the intestinal tract. Schubbert, Doerfler and coworkers (1994, 1997, 1998) have fed mice with large amounts of double stranded DNA of the bacteriophage M13. Despite the fact that bacteriophage infection is an outstandingly powerful amplification system that could probably signal any single transformation event that would occur in Enterobacteriaceae, no M13 phages could be found in the faeces of the treated animals. This indicates that no potential M13 host present in the mice microflora had been transformed. From this we can infer that the ingestion of Novartis 176 maize is extremely unlikely to result in the transformation of gut commensal bacteria in man. This is even more so because, unlike in Schubbert et al. experiments, the maize derived ingredients in food have been subjected to various industrial processes that are expected to destroy most of the DNA. Nevertheless, transgenic crops like fruits or vegetables can also be eaten unprocessed and raw.

Schubbert's and coworkers'experiments however gave rather unexpected results. The authors found that M13 phage DNA fragments (most fragmenst between 200 and 400 basepairs) could be recovered in the mice faeces, in the blood stream and even integrated in some mice cells including cells of the foetuses borne by the pregnant female mice. Such fragments of course are too small to constitute functional genes like for instance antibiotic resistance genes. But, this finding opens a new and fascinating field of investigations regarding the potential mutagenic role of ingested DNA, and especially its potential carcinogenic role. Therefore the issue raised by the discoveries of Schubbert, Doerfler and collaborators is more relevant to determine the consequences of a random integration of the DNA fragments originating from the gut contents rather than the nature of the genes themselves.

# 6. General Discussion

The *npt-II* and *bla* TEM1 genes, corresponding to resistance to the antibiotics kanamycin/neomycin and ampicillin/amoxicillin respectively, are the most important antibiotic resistance markers used in plant genetic engineering. The antibiotics kanamycin and neomycin are of no clinical importance. Ampicillin and amoxicillin are still used but the trend is downwards. Both resistance genes are present in very large amounts in natural resistance gene pools. The use of these genes in plant genetic engineering does not significantly contribute to these reservoirs of resistance genes.

The horizontal gene transfer from plants to bacteria has never been observed under natural conditions. Also in the laboratory, providing better circumstances for the observation, no horizontal gene transfer has been observed. Only when the conditions of the experiment were manipulated with the goal to provoke transfer, horizontal gene transfer from plants to bacteria has been observed. Gebhard et Smalla, (1998) and De Vries et Wackernagel, (1998) have shown that plant DNA can actually transform cells of bacteria (in this case *Acinetobactebacter sp.*) provided the recipient strain has been engineered so it can rescue the *nptII* gene present in plant DNA. In the experimental system there was sequence homology between the plant DNA and bacterial DNA and there was selection pressure to favour uptake of the antibiotic resistance gene. When such homology and selection pressure is not present the chances of transfer are so low that they have not been observed. The data show especially that DNA of prokaryotic origin can transform bacteria irrespective of the fact that it is inserted in plant DNA. The arguments often heard in the debate referring to a specific status of plant DNA are therefore not valid and this also makes obsolete all considerations relating to the scarcity of horizontal gene transfer from plants to bacteria in the evolution.

The transfer of plant (antibiotic resistance marker) genes to bacteria in the soil, in silage or in the digestive track of herbivores (including mankind) has to overcome a series of hurdles. Experimental approaches have demonstrated that each of the steps required by horizontal gene transfer can be achieved in nature. It is quite clear that the realization of each of them is the exception rather than the rule, so that their combination/succession can be regarded as a very highly improbable event. Unless a true case of spontaneous transfer of antibiotic markers genes from a plant to a bacterium was identified, the evaluation of the probability of such an event should rely on the orders of magnitudes indicated by negative data.

Evaluations of the probability of the transfer of the plant resistance genes vary according to the authors. Avoiding extrapolations, we can safely state that the transformation frequencies in the absence of DNA homologies are below the experimental detection levels, i.e. below $10^{-11}$, per bacterium exposed to transforming DNA. If the transfer of the maize *bla* TEM1 gene of Novartis maize would occur with this frequency in soil, it would account for one billionth of the resistant bacteria already living in this environment, meaning that it would have strictly no impact on this important reservoir of ampicillin resistance.

Though the information about natural transformation of bacteria in the digestive track of mammals are scarce, (Schubbert et al. 1994, 1997, 1998; Mercer et al. 1999a, 1999b), based on the data described above, one can assume that the frequencies of exchange are in the same range as in soil, that is incommensurate with the importance of the already existing reservoirs of resistance. That the number of resistant bacteria that could be produced by horizontal transfer in the human gut could match the number of ampicillin and kanamycin resistant bacteria ingested with raw vegetables such as single salad (Corpet, 1988) is unthinkable.

We have seen that the build-up of reservoirs requires horizontal transfer systems. While horizontal transfer by DNA mediated transformation is at least unlikely, bacteria are unfortunately well equipped to transfer plasmid borne resistance genes very efficiently by conjugation (Courvalin 1994). Unlike transformation, natural conjugation was found wherever it was looked for. No doubt that this is the origin of the dissemination of the *bla* TEM based ampicillin resistance in numerous pathogens as shown above. However we have also emphasized that the dissemination was made possible because of the existence of a strong selection by the use of the antibiotic.

As stated above the horizontal transfer of antibiotic resistance genes present in transgenic crops is an issue that so far can be restricted to two cases, *nptII* and *bla* TEM1. Although the risks of the use of these antibiotic resistance markers in plant genetic engineering is extremely low, the discussions in the public arena, where often no distinction was made between the differences in risk levels of different antibiotic resistance markers, has led to development of alternative marker genes. It is likely that the use of antibiotic resistance markers in transgenic plants meant for commercial growth will be phased out. For laboratory purposes antibiotic resistance markers will probably stay important for a longer period. Alternatives to antibiotic resistance markers are the inducible isopentenyl transferase system developed by Chua (1999) and the mannose based system developed by Novartis. The latter is already in plants that are grown in field trials. Also systems are discussed in which it is possible to remove the marker after the successful transformation of the plant, for instance by using the cre-lox system (Koenig, 2000). But like for antibiotic resistance markers it is important that the safety of these alternatives is properly investigated. What could for instance the effects of cre-lox be when it outcrosses to the wild flora?

The chances that these alternative markers – or in fact **any plant gene** - are transferred to bacteria or to herbivores (including humans) are just as high as for antibiotic resistance markers. Many different steps have to be taken and the chances are extremely low. The difference between antibiotic resistance markers and other marker might however be that antibiotic resistance markers are more likely to be attained, as a result of selection pressure caused by the use of the antibiotic.

# 7. Cases

**A. Case of the nptII gene**

The *nptII* gene is present in registered varieties of oil seed rape. It is likely to be present in further transgenic crops. This gene is responsible for the resistance to kanamycin and neomycin which are neither antibiotics of any clinical importance.

As pointed out by Courvalin (1998) mutations could occur in the *nptII* gene borne by canola that would transform it in a *nptIII* like gene i.e. an amikacin resistance gene. The probability of a natural transfer of the plant genes to bacteria is so low that it cannot be measured. Therefore the impact of the mutations occurring in the plant *nptII* gene on the reservoir of amikacin resistance genes will be insignificant compared to the impact of the mutations of the *nptII* genes already present in bacteria which themselves have a lesser impact than *nptIII* genes. Therefore it is considered that horizontal transfer of the *nptII* gene from transgenic plants to bacteria does not cause any harm to mankind. The presence of this antibiotic resistance gene in transgenic crops has never been invoked by any expert group against the release of transgenic crops.

**B. Case of the bla TEM1 gene**

The *bla* TEM1 gene raises more passion since unlike aminoglycosides, ampicillin and β-lactams in general are most popular and symbolize the victory of modern medicine against diseases (see prior remarks). We have confirmed the analysis of the EU experts as well as other experts (see *Antibiotic resistance via the food chain: Fiction or reality? (J. Shuman, ed.) Foundation for Nutritional Advancement, Boston MA*) showing that the probability of horizontal transfer of the *bla TEM1* gene present in maize cannot influence the frequency of the gene in existing reservoirs in a measurable proportion.

Again the issue of the mutation of the maize *bla* marker gene giving rise to alleles that would cause resistance to the last generation of β-lactams is irrelevant, since those mutations are already abundant in the existing reservoirs, and since the possible contribution of maize to this evolution is limited to the horizontal transfer rate, meaning it is a billion times lower than the actual contribution of the already existing ampicillin resistant microflora.

# 8. References


Amabile-Cuevas, C. F., and Chicurel M. E.. 1992. Bacterial plasmids and gene flux. Cell 70:189-199.

American Society for Microbiology 1995. report of the ASM Task force on antibiotic resistance: Antimicrob. Agent Chemother (suppl.) 1-23

Arlet, G., and Philippon A., 1997. Les ß-lactamases : une évolution sous la contrainte. Médecine Thérapeutique HS1:66-76.

Austin D. J., Kristinsson K. G., Anderson R. (1999) The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance PNAS; 96: 1152-1156.

Berche P. Les plantes transgéniques et la résistance aux antibiotiques. Médecine Thérapeutique 4 (9) :709-719

Bertolla and Simonet (1997) Potentialities for DNA transfer from plants to soil microorganisms. Nordic Seminar on antibiotic resistance marker genes and transgenic plants. June 12-13, 1997.

Bertolla, F., Van Gijsegem, F., Nesme, X. and Simonet, P. 1997. Conditions for natural transformation of Ralstonia solanacearum. Appl. Environ. Microbiol. 63(12) : 4965-4968

Bertolla F, Simonet P 1999. Horizontal gene transfers in the environment: natural transformation as a putative process for gene transfers between transgenic plants and microorganisms. Res Microbiol 150(6):375-84

Betts R.F., Valenti W.M., Chapman S.W., Chonmaitree T., Mowrer G., Pincus P., et al. (1984). Five-year surveillance of aminoglycoside usage in a university hospital. Annals of Internal Medicine; 100: 219-222.

Calva, J. J., Sifuentes-Osornio, J. and Ceron. C. 1996. Antimicrobial resistance in fecal flora: Longitudinal community-based surveillance of children from urban Mexico. Antimicrob. Agents Chemother. 40: 1699-1702.

Chua, et al., 1999, Inducible isopentenyl transferase as a high-efficiency marker for plant transformation, Nature Biotechnology, 17, sept 1999 : 916

Corpet D.E. 1988 Antibiotic resistance from food. N Engl J Med 5;318(18):1206-7

Courvalin P. 1994. Transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 38: 1447-1451.

Courvalin P. 1998. Plantes transgéniques et antibiotiques. La Recherche 309 : 36-41

Courvalin P. and Acar J. (1998) : La fin de l'âge d'or des antibiotiques, La Recherche, 1998, 314:50-52)

DANMAP Report. 1997. Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Danish Zoonosis Centre, Bulowsvej 27, DK-1790, Copenhagen V, Denmark.

Davies J 1994 Inactivation of antibiotics and the dissemination of resistance genes, Science 264 (5157):375-82

De Vries, J. and Wackernagel, W. 1998. Detection of npt II (kanamycin resistance) gene in genomes of transgenic plants by marker-rescue transformation. Mol. Gen. genet. 257 : 606-613.

Foster, R.C. 1988. Microenvironments of soil microorganisms. Biology and fertility of soils. 6: 189-203.

Gebhard, F.and Smalla, K. 1998. Transformation of Acinetobacter sp. BD413 by transgenic sugar beet DNA. Appl. Env. Microbiol. 64(4) : 1550-1554.

Griffith, F. 1928. The significance of pneumococcal types. J. Hyg. 27 : 113-159.

Kahn A. 1996 (data presented at the joint SCAN/SCF meeting, Brussels, December 6, 1996)

Kocabiyik, S., and. Perlin M. H. 1992. Altered substrate specificity by substitutions at Tyr218 in bacterial aminoglycoside 3' -phosphotransferase-II. FEMS Microbiol. Lett. 93:199-202

Koenig, A, 2000, Development and biosafety aspects of transgene excision methods, In: Proceedings of the 6th International Symposium on The Biosafety of Genetically Modified Organisms, July 2000, Saskatoon, Canada.

Kumin C.M. 1993. Resistance to anti-microbial drugs. A worldwide calamity. Annals Intl Med 118:557-561)

Kunkel T., Niu QW, Chan YS and Chua NH 1999. Inducible isopentenyl transferase as a high-efficiency marker for plant transformation. Nature Biotechnology 17:916-919

Leff, L. G., J. R. Dana, J. V. McArthur, and L. J. Shimkets. 1993. Detection of Tn5-like sequences in kanamycin-resistant stream bacteria and environmental DNA. Appl. Environ. Microbiol. 59:417-421

Livermore DM, 1995 _ lactamase in laboratory and clinical resistance, Clin. Microbiol. Rev., 8 557-584).

Lorenz, M. G. and Wackernagel, W 1992. DNA binding to various clay minerals and retarded enzymatic degradation of DANN in sand/clay microcosm, p 103-113, in M.J. Gauthier (ed.) Gene transfers and environment. Springer Verlag KG. Berlin.

Lorenz, M. G. and Wackernagel, W. 1994. Bacterial gene transfer by genetic transformation in the environment. Microbiol. Rev. 58 : 563-602

Mazodier P, Davies J 1991. Gene transfer between distantly related bacteria. Annu Rev Genet;25:147-71

McKeon, D. M., J.P.Calabrese, and G. K. Bissonnette. 1995. Antibiotic resistant gram-negative bacteria in rural groundwater supplies. Water Res. 29: 1902-1908).

Medeiros, A. A. 1997. Evolution and dissemination of ß-lactamases accelerated by generations of ß-lactam antibiotics. Clin. Infect. Dis. 24: S19-45.. 1-4

Mercer DK, Scott KP, Bruce-Johnson WA, Glover LA, Flint HJ, 1999a. Fate of free DNA and transformation of the oral bacterium Streptococcus gordonii DL1 by plasmid DNA in human saliva Appl Environ Microbiol ;65(1):6-10

Mercer DK, Melville CM, Scott KP, Flint HJ, 1999b. Natural genetic transformation in the rumen bacterium Streptococcus bovis JB1. FEMS Microbiol Lett 15;179(2):485-90

Miller GH, Sabatelli FJ, Hare RS, Glupczynski Y, Mackey P, Shlaes D, Shimizu K, Shaw KJ 1997. The most frequent aminoglycoside resistance mechanisms--changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups Clin Infect Dis ;24 Suppl 1:S46-62

Nguyen-the C;, Carlin F. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. 34: 371-401

Nielsen KM, Bones AM, Smalla K, van Elsas JD 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria--a rare event? FEMS Microbiol Rev 22(2):79-103

Nordmann P, Naas T, Labia R., 1994. New trends in enterobacterial ß-lactamases. APUA Newslett.; 12: 1-4

Paget, E., Jocteur Monrozier, L. and Simonet, P. 1992. Adsorption of DNA on clay minerals : protection against DNase I and influence on gene transfer. FEMS Microbiol. Lett. 97 : 31-40.

Paul, W.J., Jeffrey, W. H., David, A. W., De Flaun, M. F. and Cazares, L. H. 1989. Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of Southwest Florida. Appl. Environ. Microbiol. 55 : 1823-1828

Pear S.M., Williamson T.H., Bettin K.M., Gerding D.N, Galgiani J.N. (1994).Decrease in nosocomial Clostridium difficile-associated diarrhea by restricting clindamycin use. Annals of Internal Medicine ; 120: 272-277.

Privalle L.S, et al, (2000), Phosphomannose isomerase – a novel system for plant selection, in: the proceedings of the 6th symposium on the biosafety of genetically modified organisms, July 2000, Saskatoon, Canada.

Romanovski, G., Lorenz, M.G. and Wackernagel, W. 1993. Plasmid DNA in a groundwater aquifer microcosm-adsorption, DNase resistant and natural genetic of Bacillus subtilis. Mol. Biol. Ecol. 2 : 171-181

Schlüter, K., Fütterer, J. and Potrycus, I. 1995. Horizontal gene transfer from transgenic potatoe line to a bacterial pathogen (Erwinia chrysanthemi) occurs-if at all- at an extremely low frequency. Biotechnology. 13 : 1094-1098.

Schubbert R., Lettmann C. and Doerfler W. (1994). Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Mol. Gen. Genet. 242,495-504.
Schubbert R., Renz D., Schmitz B. and Doerfler W. (1997). Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. Proc. Natl. Acad. Sci.USA 94, 961-966.

Schubbert R, Hohlweg U, Renz D, Doerfler W 1998. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the foetus. Mol Gen Genet 259(6):569-76

Seppala H., Klaukka T., Vuopio-Vakila J., Muotiala A., Helenius H., Lage K., Huovinen P. (1997). The effects of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. New England Journal of Medicine; 337: 441-446.

Smalla, K., L. S. van Overbeek, R. Pukall, and J. D. van Elsas. (1993). Prevalence of nptII and Tn5 in kanamycin resistant bacteria from different environments. FEMS Microbiol. Ecol. 13:47-58.

Stein JC and Hansen G (1999) Mannose induces an endonuclease responsible for DNA ladering in plant. Plant physiol, 121: 71-80

Stewart G.J. and Carlson C.A. (1986) The biology of natural transformation. Ann. Rev. Microbiol. 40: 211-235

Stanier RY, Ingraham JL, Wheelis ML, Painter PR, 1987 , General Microbiology, MacMillan Education LTD).

Syvanen M, 1999. In search of horizontal gene transfer Nature Biotechnology 17:833

Trieu-Cuot P, Courvalin P 1983 Nucleotide sequence of the Streptococcus faecalis plasmid gene encoding the 3'5"-aminoglycoside phosphotransferase type III. Gene;23(3):331-41

ZKBS, 1999. Stellungnahme der ZKBS zur Biologischen Sicherheit von Antibiotika-Resistenzgenen im Genom gentechnisch veränderten Pflanzen, Juli 1999.

# Annex1: Referees

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Andrew Chesson, Rowett Research Institute, Aberdeen, Scotland
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Mike Syvanen, Medical Microbiology and Immunology, University of California, Davis, USA
Steve Taylor, University of Nebraska, Dept. of Food Science & Technology, Lincoln, Nebraska, USA
Wilfried Wackernagel, Oldenburg University, Oldenburg, Germany